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Molecular epidemiology of invasive isolates of *Salmonella enterica* serovar Typhimurium in Gauteng, South Africa, 2006-2008

Sarika Dwarika

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2009

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 Enteric Diseases Reference Unit (EDRU), National Institute for Communicable Diseases (NICD) and University of the Witwatersrand, Johannesburg.

I declare that this dissertation is my own, unaided work. It is being submitted for the fulfilment for the degree of Master of Science to the Faculty of Science, University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

(Signature of candidate)

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ABSTRACT

The clonality of 840 invasive human *Salmonella enterica* serotype Typhimurium isolates isolated in Gauteng Province during January 2006 to May 2008 was investigated. PFGE analysis revealed 38 clusters: three (clusters 3, 5 and 11) were primary clusters. Most isolates originated from Hospital 2 and were isolated from patients in the age-range of 15-64 years. Ninety-two percent (256/277) of patients with known HIV status were HIV-positive. Antibiotic susceptibility testing showed the most commonly expressed antibiotic resistance profiles were ACSSuNa (13%; 90/671) and ACSSuTNa (12%; 82/671). Thirty-five nosocomial isolates were identified in 12 clusters, of which most isolates came from two of our three major clusters: cluster 3 (31%; 11/35) and cluster 5 (23%; 8/35). In South Africa, *Salmonella* Typhimurium remains an important opportunistic infection of HIV-positive patients and may circulate as a nosocomial pathogen over prolonged periods within the hospital environment.

The study included characterization of 47 *Salmonella* isolates recovered from a 150 chicken specimens purchased in Gauteng Province during September 2007 to April 2008. *Salmonella* Heidelberg (34%; 16/47) and *Salmonella* Infantis (34%; 16/47) were the most common serotypes isolated from chickens. PFGE analysis showed *Salmonella* Heidelberg and *Salmonella* Hadar isolates were similar in PFGE profile to equivalent human serotypes, indicating that for these two serotypes some chicken and human isolates may be related. PFGE analysis and MLVA showed that some

chicken and human *Salmonella* Typhimurium isolates were similar in molecular profile, indicating a relationship between these isolates. An epidemiological relationship between chicken and human isolates could not be confirmed; however results suggest that *Salmonella* strains with similar molecular profiles circulate in the animal and human communities, supporting the suggestion of animal-to-human transmission or possibly human-to-animal transmission. Further work is required to confirm this theory.

DEDICATION

To my father

Pravin Dwarika

and

my mother

Danwathie Dwarika

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LIST OF SYMBOLS

%	Percent
<	less than
>	greater than
≤	less than and equal to
≥	greater than or equal to
+ve	positive
-ve	negative
µl	microlitre
°	degrees
°C	degrees Celsius
®	copyright
™	Trade Mark

LIST OF ABREVIATIONS

A	ampicillin
AIDS	Acquired Immune Deficiency Syndrome
bp	base pair
C	chloramphenicol
CDC	Centers for Diseases Control
CLSI	Clinical Laboratory Standards Institute
DCA	Deoxycholate
DMP	Diagnostic Media Products
DNA	Deoxyribose Nucleic Acid
dNTPs	deoxynucleoside triphosphate
Dr.	Doctor
EDRU	Enteric Diseases Reference Unit
EDTA	EthyleneDiamineTetra-Acetate acid
ESBL	extended-spectrum β-lactamase
et al.	and others
Etc.	et cetera
Foodnet	Foodborne Disease Active Surveillance Network
g	grams
GERMS-SA	Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa
H	flagellar antigen

HIV	Human Immunodeficiency Virus
i.e.	that is
kb	kilobase
kDa	kilodalton
kV	kilo volts
L	Liters
LDC	lysine decarboxylase
Ltd.	Limited
mg/ml	milligrams per milliliter
mM	Millimolar
ml	milliliter
MAC	MacConkey
MLCB	mannitol lysine crystal violet brilliant green
MDa	megadalton
MLST	multiple-locus sequence typing
MLVA	multiple-locus variable-number tandem-repeats analysis
N	sample size
Na	nalidixic acid
NHLS	National Health Laboratory Service
NICD	National Institute of Communicable Diseases
NMSU	National Microbiology Surveillance Unit
NNIS	National Nosocomial Infections Surveillance System Report

NSRL	National *Salmonella* Reference Laboratory
nt	nucleotide
NTS	non-typhoidal *Salmonella*
O	somatic antigen
ODC	ornithine decarboxylase
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pH	percentage hydrogen
PI	Pathogenicity Island
pmol	pico molar
RNA	ribonucleic acid
RNase	ribonuclease
Pty.	Proprietary Limited Company
rpm	revolutions per minute
RSA	Republic of South Africa
RV	Rappaport Vassiliadis
s	seconds
S	streptomycin
SC	selenite cystine
SDS	sodium-dodecyl sulphate
SPI	*Salmonella* pathogenicity island
SPI-1	*Salmonella* pathogenicity island 1

SPI-2	*Salmonella* pathogenicity island 2
Su	Sulfamethoxazole
T	tetracycline
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEM	Temoniera
Tris	Tris-(hydroxymethyl)-aminomethane
UPGMA	unweighted pair group method with arithmetic averages
UK	United Kingdom
USA	United States of America
UV	ultraviolet
VNTR	variable number tandem repeats
v	version
v/cm	volts per centimetre
Vi	capsular antigen
vol/vol	volume per volume
WHO	World Health Organization
X1	pulsed-field electrophoresis banding patterns
XLD	xylose-lysine-deoxycholate

CHAPTER 1

Salmonellosis

1.1 Infectious Diseases

Intestinal infections cause a great deal of morbidity and mortality in the world and rank as the third most common infectious disease (WHO, World Health Report, 2004), particularly amongst human patients belonging to extreme age-ranges (Brenner *et al.,* 2000; Ikumapayi *et al.,* 2007) and immunocompromised patients such as HIV-infected patients (Bick, 2004). Salmonellae are a common cause of foodborne illnesses (Farmer and Kelly, 1991). These organisms are frequently transmitted through the food chain and may even be transmitted from person-to-person via the faecal-oral route (Hanes, 2003).

1.2 Characteristics of Salmonellae

In 1880, salmonellae were described for the first time by Eberth (Darwin and Miller, 1999). Salmonellae are gram-negative bacteria which are usually motile via peritrichous flagella. They are aerobic but can be facultatively anaerobic and do not form spores (Bopp *et al.*, 1999). Characteristics shared by most of the *Salmonella* species are that they ferment glucose, maltose and mannitol and forms gas and acid; cannot ferment lactose, salicin and sucrose; can use citrate as the sole carbon source and furthermore, they produce H_2S but cannot produce indole (Farmer and Kelly, 1991; Hanes, 2003). Salmonellae can survive for a long time in soil and water as well as other environments with the pH ranging from four to nine and the temperatures

ranging from 8°C to 45°C (Hanes, 2003). Salmonellae can be killed through the process of milk pasteurization and adequate cooking of food at temperatures of 70°C and above (Hanes, 2003).

The bacterial genus *Salmonella* belongs to the family Enterobacteriaceae (Bopp *et al.,* 1999). The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori* (Figure 1.1). *Salmonella enterica* can be further subdivided into six subspecies (See figure 1.1) (Brenner *et al.,* 2000). The usual habitat of *Salmonella enterica* subspecies *enterica* is warm-blooded animals and the common habitat for the remaining *Salmonella enterica* subspecies (II, IIIa, IIIb, IV, VI) as well as *Salmonella bongori* (V) are cold-blooded animals and the natural environment (Brenner *et al.*, 2000). These species are further classified at a sub-species level into serovars or serotypes based on their cell surface antigens (Baron, 1996). O and H surface antigens are bacterial surface structures that are used to differentiate between different serotypes of salmonellae, according to the Kauffmann-White scheme (Popoff *et al*., 1998; Popoff, 2001; and Keddy *et al.,* 2005). The O antigen defines the serogroup and the H antigen defines the serovar (Popoff, 2001). *Salmonella enterica* subspecies *enterica* is further clinically subdivided into: *Salmonella* Typhi and non-typhoidal *Salmonella* (NTS). *S. enterica* serotype Enteritidis (*Salmonella* Enteritidis) and *S. enterica* serotype Typhimurium (*Salmonella* Typhimurium) are the most dominant among the NTS serotypes affecting humans and animals on a global scale (Lim *et al.,* 2003, Palmgren *et al.,* 2006, Sheehan and van Oort, 2006). To date, there are approximately 1454 serotypes within the *S. enterica* subspecies *enterica* and in

total there are over 2400 serotypes of salmonellae that are capable of causing disease in humans and animals (Brenner *et al.*, 2000). Salmonellae can be further subtyped into different strains. “A strain is a descriptive subdivision of a species” and is defined as “an isolate or group of isolates”. These strains can be distinguished from other isolates of the same genus and species by phenotypic or genotypic methods (Tenover *et al.*, 1995).

1.3 *Salmonella* disease

Salmonella infections are a major cause of gastroenteritis (Hanes, 2003). Susceptibility to *Salmonella* infection, onset of infection and severity of salmonellosis depends on several of the host’s physiological factors such as the host’s age, fitness level and nutritional state etc. Other determining factors are the *Salmonella* serotype and the number of organisms consumed by the host (Darwin and Miller, 1999). The average incubation time of gastroenteritis is about six to 72 hours after the consumption of food or water which are contaminated with salmonellae. Generally an infectious dose of 100-1000 salmonellae are required to initiate an infection, however for certain *Salmonella* serotypes only as little as 15 to 20 cells are required (Clark, 2008). These pathogens reside in the host’s gastrointestinal tract where they cause gastrointestinal complications (Hanes, 2003). Some studies show that the distal part of the small intestine is the primary site of infection in humans (Hughes and Galán, 2002). Gastroenteritis usually only lasts for about a week and the clinical symptoms of salmonellosis include diarrhoea, abdominal cramps and a fever of 38°C to 39°C (Bopp *et al.*, 1999).

In immunocompromised patients, salmonellosis may be prolonged and symptoms may be more severe (Weinstein, 1998; Wenzel and Edmond, 2001; Hanes, 2003). As a result of a compromised immune system, salmonellae may proliferate at a high rate and become invasive by breaking through the mucosa of the small bowel and entering the blood stream causing bacteraemia. Prolonged illness is often accompanied by low-grade fever, bloody diarrhoea and loss of weight (Hanes, 2003). Previous studies showed that children in the age-range of five years and younger living in tropical Africa are more susceptible to bacteraemia caused by NTS (Vaagland *et al.,* 2004). HIV-positive patients are more predisposed to recurring bacteraemia caused by salmonellae compared to immunocompetent patients (Bick, 2004).

Salmonellae can progress to any anatomical site in the human body via the circulating blood (Spraycar, 1995) and may cause local infections and produce abscesses (Govinden *et al.,* 2008). The most common sites are the lungs, connective tissue, joints, long bones and meninges (Kuppermann, 1999; Kramer and Shapiro, 1997). Meningitis caused by salmonellae are a common local infection seen in infants (Vaagland *et al.,* 2004). In addition, immunosuppressed patients, who do not receive treatment (Kuppermann, 1999; Kramer and Shapiro, 1997), have a 5% to 10% chance of bacteraemia progressing into local and systemic infections (Peques *et al.,* 2005).

1.4 Diagnosis

Diagnosis of foodborne diseases is made by the isolation of the infecting organism from suspected food and patient stool samples during the acute phase of salmonellosis (Hanes, 2003). The detection of salmonellae in stool cultures is time consuming and often medical practitioners have to treat patients based on their symptoms (Owen and Warren, 2008). Little or no *Salmonellae* can be isolated from stool cultures after about a week of salmonellosis and antibiotic treatment, however laboratory findings have shown that salmonellae carriers test positive for these causative organisms in stool cultures months after being infected (Hanes, 2003; Chambers *et al.*, 2008). Most commonly patients in the age-range of five years and younger are reported to be asymptomatic carriers of salmonellae for longer periods than older patients (Buchwald and Blaser, 1984). Patients become chronic carriers of salmonellae if the infecting organism colonizes the gall bladder (Hanes, 2003). Chronic carriers of salmonellae however do not suffer with the symptoms of salmonellosis (Owen and Warren, 2008).

In addition to the culture sensitivity tests, PCR evaluation can also be performed to detect *Salmonella* species (Owen and Warren, 2008). Primer sets which are specific for *Salmonella* species are selected (Malorny *et al*., 2003). PCR is performed on sample DNA using these primer sets (Malorny *et al*., 2003). Amplified DNA is electrophoresed in an agarose gel containing ethidium bromide, after which DNA fragments in the gel are visualized under UV light (Rahn *et al*., 1992; Malorny *et al*.,

2003; Malorny *et al*., 2004). The sensitivity of PCR tests on blood, urine and faeces are 85%, 69% and 47%, respectively (Hatta and Smits, 2007).

1.5 Isolation and identification

1.5.1 Preenrichment

The test sample is put into a preenrichment broth which allows almost all of the microorganisms present in the test sample to grow. Peptone water, mannitol purple sugar broth as well as lactose and brilliant green are examples of preenrichment broths (Hanes, 2003).

1.5.2 Enrichment

This step is performed to provide a favourable environment for the infective organism of interest to grow while suppressing the growth of other microorganisms present in the test sample. Examples of selective enrichment broths which are specific for enhancing the growth of salmonellae are selenite cystine (SC) broth and Rappaport Vassiliadis (RV) broth (Hanes, 2003). This step is followed by plating out some of the test sample from the selective broth onto highly differential media. For *Salmonella* species, MacConkey (MAC) agar, Deoxycholate (DCA) agar and Xylose-lysine-deoxycholate (XLD) agar are commonly used. Thereafter a series of biochemical tests are performed on all suspected *Salmonella* colonies (Hanes, 2003).

1.5.3 Biochemical identification

Biochemical tests include fermentation of glucose, lysine decarboxylation, ornithine decarboxylation, dulcite fermentation, H_2S production, motility, indole, mannitol and inability for the organism to ferment sucrose or lactose (Table 1.1).

1.6 Serotyping

O and H surface antigens are bacterial surface structures that are used to differentiate between different serotypes of salmonellae, according to the Kauffmann-White scheme (Popoff, 2001). The O antigen defines the serogroup and the H antigen defines the serotype (Tenover *et al.*, 1995). *Salmonella* Typhi also has a virulence (Vi) or capsular antigen (Popoff, 2001; Koneman, 2006). Salmonellae O, H and Vi antibodies are commercially available for *Salmonella* serotyping (Hanes, 2003). *Salmonella* serotyping is performed by first determining the O phase (Popoff, 2001; Hanes, 2003; Koneman, 2006) which is then followed by determining the H phase. Many *Salmonella* species contain more than one H phase and sometimes may only express one of the H phases. This is overcome by blocking the recognised H phase for that O phase with the H monovalent antibody, which then allows the organism to express the other H phase or H phases (Popoff, 2001; Koneman, 2006). *Salmonella* serotypes in *S. enterica* subspecies *enterica* (I) are identified and named according to the O and H antigens expressed on their bacterial surfaces, whereas unnamed *Salmonella* serotypes in *S. enterica* subspecies *salamae* (II) through *S. enterica* subspecies *indica* (VI) are identified by their antigenic formula (Brenner *et al.*, 2000).

1.7 Molecular epidemiology

Pulsed-field gel electrophoresis (PFGE) (Baggesen *et al.,* 2000), multiple-locus variable-number tandem-repeats analysis (MLVA) (Lindstedt *et al.,* 2003), multiple-locus sequence typing (MLST) (Fakhr *et al.,* 2005) and DNA miccroarrays (Boyd *et al.,* 2001) are a few common techniques used to characterize salmonellae at a molecular level (Pelludat *et al.,* 2005).

PFGE is a molecular technique which is used to characterize and determine strain relatedness among bacterial isolates. In contrast to conventional gel electrophoresis, PFGE is able to separate large DNA fragments greater than 50000 base pairs (bp) as a result of periodic changes in the direction of the electric field during electrophoresis. To execute this method, bacterial cells are first immobilized in agarose plugs for protection against any accidental breakage of genomic DNA during this procedure. This is followed by the lysis step. Agarose plugs which contain bacterial genomic DNA are incubated in lysis buffer containing proteinase-K, N-lauroylsarcosine sodium salt and ethylenediaminetetra-acetic acid (EDTA) to release and protect genomic DNA. A slice of the agarose plug is then put into a solution containing restriction enzyme which digests genomic DNA into several large fragments. The number of fragments produced depends on the restriction enzyme chosen for digestion. Following the digestion step, agarose slices are placed on a comb and set in an agarose of 0.8% gel. The agarose gel is then subjected to PFGE which allows for the separation of large DNA fragments. Periodic changes in the direction of the electric field during electrophoresis allows DNA molecules to enter and move

through the pores of the agarose matrix in a snake-like fashion to produce fingerprint patterns. Fingerprint patterns generated by PFGE analysis is visualized using UV light after staining the agarose gel with ethidium bromide (Tenover *et al.,* 1995; Gautom, 1997). PFGE analysis is the "gold standard" for the subtyping of salmonellae. Fakhr and colleagues (Fakhr *et al.,* 2005) revealed that compared to MLST, PFGE analysis has a high discriminatory power for the subtyping of *Salmonella* Typhimurium (Fakhr *et al.,* 2005). Another study done by Weill and co-workers on approximately 4000 *S. enterica* serotype Typhimurium phage type DT104 (*Salmonella* Typhimurium DT104) isolates showed a high incidence of PFGE profiles X1. However, another method is required for discriminatory subtyping of such isolates (Weill *et al.,* 2006). Research done by Torpdahl and co-workers showed that even though PFGE analysis is the standard method for the subtyping of salmonellae, it sometimes fails to differentiate between certain related and unrelated strains and within definite phage types due to the lack of discriminatory abilities (Torpdahl *et al.,* 2006).

Variable number tandem repeats (VNTR's) are DNA motif repeats found in the genome sequence. These DNA motif repeats fall into different classes. The 'direct class repeats' are called minisatellites, which can contain genetic polymorphisms. Minisatellites are found in pathogenic bacteria and humans. Minisatellites are 'hypermutable'. Hypermutations occur during DNA replication as a result of slipped strand mispairing. Since VNTR's play a role in the expression of genes and often the coding of surface exposed proteins, mutations in the DNA sequence result in changes

in the mechanism of function and proteins made. Due to these changes, the host's immune system finds it difficult to recognize bacteria. This enables bacteria to multiply rapidly and have a high survival rate in the host (Lindstedt *et al.,* 2005). VNTR analysis also known as MLVA is used to screen genomic DNA for repeat motifs. MLVA is applied by screening approximately three to eight VNTR loci. Specific PCR primer pairs are used to amplify each locus and usually, all primer pairs are multiplexed into a single PCR. PCR products are then subjected to electrophoresis which separates these PCR products by size to generate DNA fingerprint patterns. Unique DNA fingerprint patterns are produced by each individual strain of bacteria. MLVA is traditionally performed manually, however due to technological advancements over recent years, MLVA has progressively become automated. The traditional method of MLVA is carried out by using conventional gel electrophoresis, after which DNA fingerprint patterns can be visually examined using UV light. Automated MLVA is performed using capillary electrophoresis. This method is executed by using primer pairs which are labelled with distinctive fluorescent dyes (fluorophores). Each VNTR locus can be labelled with a unique fluorophore; this subsequently allows the combination of all primer pairs into a single multiplex-PCR. Capillary electrophoresis is performed using a genetic analyzer. PCR products are moved past a laser beam which excites fluorophores causing it to emit light of distinctive colours. The light is detected and translated into detection of PCR products. An internal size standard is used to size PCR products. These sized PCR products are reported as a MLVA allele profile (Lindstedt *et al.,* 2004; Torpdahl *et al.,* 2007). For example, if five loci were analyzed by MLVA, the MLVA allele

profile representing the size of PCR products in base pairs could be reported as '160-248-314-385-525'. MLVA is useful in discriminating within common phage types (Torpdahl *et al.,* 2007). Torpdahl *et al.,* (2007) demonstrated in their study that MLVA is superior to PFGE analysis for surveillance and outbreak investigations (Torpdahl *et al.,* 2007). An earlier study showed that MLVA is a quick, appropriate method particularly for outbreak situations and to monitor different phage types (Torpdahl *et al.,* 2006). Lindstedt and co-workers used MLVA to show a high level of discrimination among 78 strains of *Salmonella* Typhimurium (Lindstedt *et al.,* 2003; Lindstedt, 2005).

1.8 Treatment and prevention

Fluid and electrolyte replacement is necessary for the correction of dehydration caused by diarrhoea (Hanes, 2003). Antibiotics are not suggested for uncomplicated diarrhoea and chronic carriers. Studies show that antibiotics given to chronic carriers prolong the duration of salmonellae excreted in the stool (Sirinavin and Garner, 2000). Antibiotic therapy is however necessary for immunocompromised patients. Different countries address health issues differently: the choice of antibiotic treatment, antibiotic usage, dosage and duration of antibiotics recommended to patients depend on local antibiotic resistance profiles expressed by salmonellae (Yates and Amyes, 2005). Surveillance of salmonellae are routinely conducted in several countries worldwide; to detect emerging multidrug-resistant salmonellae that may pose a risk to future antibiotic treatment and as a result allows scientists to use

this information to make educated decisions about the choice of treatment that can be given to patients (Yates and Amyes, 2005).

Nonfoodborne transmission of *Salmonella* is particularly common among slaughterhouse workers, farmers, chefs and veterinarians if exposed to *Salmonella* infected animals. The spread of *Salmonella* in the above mentioned fields of work can be prevented by proper hygienic practice such as adequate hand washing, avoiding contact with animal faeces, removal of all soiled work clothes before going indoors as well as reducing exposure of soiled work clothes to household members (Hendriksen *et al.*, 2004).

Pasteurization of milk and thorough cooking of food at temperatures of 70°C and above will greatly reduce foodborne transmission of *Salmonella* (Hanes, 2003). Post-contamination of food can be prevented by covering cooked food, thorough hand washing, washing of utensils and decontaminating food-preparation areas such as table tops with disinfectants (Mosupye and von Holy, 1999).

Typhoid vaccines are available for the prevention of typhoid fever; however there are no vaccines available for the prevention of human salmonellosis caused by NTS (Mastroeni and Ménager, 2003).

1.9 Epidemiology

Salmonella can be isolated from the intestinal tract of a wide range of hosts which include humans, domestic and non-domestic animals (Hanes, 2003). Statistical studies show that asymptomatic carriers of *Salmonella* can spread these causative organisms to 0.2% of the common population (Hanes, 2003). *Salmonella* may be transmitted via several routes which include foodborne routes of *Salmonella* such as consumption of food contaminated by *Salmonella* infected animals or people (Hanes, 2003); nonfoodborne routes of *Salmonella* such as direct contact with humans or animals infected with *Salmonella* (Hendriksen *et al.,* 2004); as well as human-to-human transmission via the faecal-oral route (Hanes, 2003).

Salmonella is most commonly transmitted via foodborne routes (Butaye *et al.,* 2006). In the USA, approximately one million people get infected with *Salmonella* each year, of which 95% or more of all the cases are owed to the ingestion of contaminated food and water (Butaye *et al.,* 2006; Linam and Gerber, 2007). Water, milk, dairy products, beef, poultry, eggs, vegetables and fruit are all common reservoirs for *Salmonella* (Braden, 2006; Linam and Gerber, 2007). Food can be contaminated during different stages of preparation. Studies show that about 1% to 50% of the meat that is sold at commercial markets is contaminated with *Salmonella* during the slaughtering and processing of food animals (Hanes, 2003). Post-cooking contamination is often attributed to poor hygiene practiced by people handling food,

the use of contaminated cooking utensils and exposure of cooked food to contaminated surface areas and dust containing *Salmonella* (Mosupye and von Holy, 1999; Hanes, 2003).

Salmonellae can be carried by various domestic and non-domestic animals and exposure to these animals is thus a potential risk factor for human salmonellosis. The infection rate of salmonellae in animals fluctuates from less than 1% to greater than 20% (Hanes, 2003). *Salmonella* serotypes can be isolated from the gastrointestinal tract of approximately 90% of reptiles and amphibians (Linam and Gerber, 2007). There is a 6% chance of acquiring an infection via direct contact with reptiles and amphibians (Linam and Gerber, 2007). Children are more at risk of getting infected with salmonellae from playing with their pet animals such as dogs, cats, turtles and snakes etc. (Hanes, 2003). In addition to animal-to-human transmission of salmonellae, salmonellae can also be carried by humans and transmitted through direct or indirect contact with animals such as pet birds (Anonymous, 2008), zoo animals (Jang *et al.*, 2008) and bovine animals (CDC, 2000).

Animal feed produced for domestic and food animals is also frequently contaminated with salmonellae at food packing industries (Hanes, 2003). Handling of contaminated animal feed or contact with animals who have consumed these contaminated animal

feeds, pose a potential risk of *Salmonella* infection to humans (Hanes, 2003; White *et al*., 2003; Hendriksen *et al.,* 2004).

Person-to-person contact is another route through which salmonellae can be transmitted. An example of this is nosocomially acquired infections. Nosocomial infections are infections acquired at long-term medical care facilities such as hospitals (Edelstein *et al*., 2004) and nursing homes etc. (Olsen *et al.,* 2001). Hospital acquired infections are common in developing countries but are less common in first world countries such as in the USA (Olsen *et al.,* 2001). Nosocomial infections are more severe in very young patients, immunosuppressed patients and patients receiving a cocktail of antibiotics (Olsen *et al.,* 2001).

Reporting inaccuracies are due to underreporting and underdiagnosing of salmonellosis and as a result, only a certain fraction of the true number of *Salmonella* cases are reported (Hanes, 2003; Thomas *et al.,* 2006). Large outbreaks of salmonellosis tend to draw the attention of health-care professionals and the general public and thus are further investigated and reported whereas random cases of salmonellosis are often underreported for various reasons (Hanes, 2003). Underreporting and underdiagnosing of *Salmonella* cases is a potential limitation to surveillance studies (Thomas *et al.,* 2006). Underreporting makes it difficult to evaluate the true trend patterns of *Salmonella* infections in humans. The following are

a few possible reasons as to why a large number of *Salmonella* cases go underreported (Hanes, 2003; Thomas *et al.,* 2006):

- Outbreaks may be represented by a small number of people with little or no severity of illness. This may not alarm health-care professionals or other individuals in the community as a public health hazard and as a result may go unreported (Hanes, 2003).
- Health care professionals may not be aware of the outbreak or they may lack the motivation to report an outbreak (D'Ortenzio *et al.,* 2008).
- There may be few or no resources available for conducting active surveillance studies due to numerous circumstances (Klovning, 2007).
- Foodborne outbreaks in cafés and restaurants etc. may also be underreported by owners or workers of these eating places, as a scheme to protect the reputation of their businesses or jobs, respectively (Klovning, 2007).
- Unavailability of medical services to *Salmonella* infected people, such as people living in rural areas (Klovning, 2007).

1.10 Pathogenicity

Salmonellae are transmitted to the host via several routes but most commonly through the consumption of contaminated food and water (Butaye *et al.,* 2006; Linam and Gerber, 2007). The initiation of infection depends on the *Salmonella* serotype, the number of infecting organisms in the food, the type of food consumed by the host, as well as the host's physiological factors (Owen and Warren, 2008). Some studies show

that there should be a large number of infecting organisms to survive the host's gastrointestinal tract's first line of defence (Darwin and Miller, 1999), which is the gastric acid in the stomach and the normal bacterial flora in the small intestine (Hanes, 2003; Bick, 2004). The pH of the food consumed also influences susceptibility of an individual to infection (Darwin and Miller, 1999; Owen and Warren, 2008). For example, if the food consumed has a high pH, it will neutralize the gastric acid once it reaches the stomach and will make the individual more susceptible to infection (Darwin and Miller, 1999). Reduced peristalsis allows salmonellae to flourish in the small intestine and hence prolongs infection (Bick, 2004). Individuals who have a low immunity, gastric acid with a high pH, decreased number of normal bacterial flora in the small bowel and decreased peristalsis (bowel movement) are more susceptible to *Salmonella* infection (Bick, 2004; Owen and Warren, 2008). The following are a few examples of individuals who are more predisposed to *Salmonella* infection:

- Young patients who have immature immune systems and bacterial flora in their gastrointestinal tract (Hanes, 2003; Owen and Warren, 2008).
- Older patients who have declining immune systems and gastric acid with a high pH (Hanes, 2003; Owen and Warren, 2008).
- Patients taking antacids and acid suppression drugs have decreased levels of gastric acid in their stomach (Bick, 2004; Owen and Warren, 2008).
- Patients receiving a cocktail of different types of antibiotic treatment or purgatives as well as patients who have undergone bowel surgery have

decreased levels of normal bacterial flora in their intestinal tract (Olsen *et al.*, 2001; Bick, 2004; Owen and Warren, 2008).

- Patients taking opiate drugs have decreased intestinal movement (Bick, 2004; Owen and Warren, 2008).

Once salmonellae have overcome the host's protective mechanisms such as the gastric acid in the stomach and the normal bacterial flora, it then attaches itself to intestinal cells via fimbriae or pili (Darwin and Miller, 1999; Hanes, 2003; Owen and Warren, 2008). The interaction of salmonellae with the mucosa of the host's intestinal tract has being previously described by using various animal models such as the rhesus monkey (Darwin and Miller, 1999), the guinea pig (Takeuchi and Sprinz, 1967) and mice (Darwin and Miller, 1999; Hanes, 2003). Mice are currently the primary choice of animal model for molecular research to enhance our understanding on the subject of the interaction of salmonellae with the human intestinal mucosa (Darwin and Miller, 1999). Despite various studies that have been carried out using different animal models (Francis *et al*., 1993; Darwin and Miller, 1999; Wallis and Galyov, 2000; Mishra *et al*., 2000; Hanes, 2003; Owen and Warren, 2008), these studies are common in their findings that salmonellae first attach to and then invade the host's intestinal mucosa. Invasion of the intestinal mucosa triggers off the host's proinflammatory response to salmonellae and hence causes inflammation of the lymph nodes and lamina propria of the intestinal tract (Owen and Warren, 2008). Owing to these studies, it is hypothesized that a similar host-pathogen interaction occurs when humans get infected with salmonellae (Darwin and Miller, 1999).

Salmonella Typhimurium has four different types of fimbriae called type 1 fimbriae, long polar fimbriae, plasmid-encoded fimbriae and thin aggregative fimbriae. Fimbriae are associated with adhesion, virulence and may also be associated with direct or indirect recruitment of neutrophils (immune cells) (Darwin and Miller, 1999; Owen and Warren, 2008). Despite various studies that have been performed on type I fimbriae, their function with regards to adhesion and virulence is still poorly understood (Darwin and Miller, 1999). Studies performed on a mouse model have shown that long polar fimbriae are responsible for the attachment of salmonellae to the Peyer's patches of the small intestine which makes it easier for salmonellae to break through the host tissue. Another study which was performed using the small intestinal tissue of a mouse model under tissue culture conditions showed that plasmid-encoded fimbriae have a high affinity to the host's villous intestines. Thin aggregative fimbriae may be involved in adhesion of salmonellae to the host's cell epithelium however, attachment to specific cell types is not yet known. Thin aggregative fimbriae have a propensity to aggregate, hence the name. Scientists proposed that this aggregative behaviour increases the chance of salmonellae surviving the host's protective mechanisms such as the gastric acid in the stomach (Darwin and Miller, 1999; Owen and Warren, 2008). Studies using mice as animal models show that about 25% of salmonellae colonize the Peyer's patches in the small intestine (Darwin and Miller, 1999; Hanes, 2003). Salmonellae initially and most exclusively invade mice mucosal M cells and at a later stage invade mice eukaryotic cells such as the intestinal absorptive cells (Darwin and Miller, 1999; Hanes, 2003; Owen and Warren, 2008). M cells are present in specialized epithelium which covers

the Peyer's patches in the small intestine (Owen and Warren, 2008). M cells play a role in gastrointestinal immunity by sampling antigens from the lumen and transporting them to antigen presenting cells (Mishra *et al*., 2000). Once salmonellae are attached to the host's M cells, they send bacterial signals which cause cytoskeleton rearrangements in the host's intestinal cells and hence initiate membrane ruffling (Darwin and Miller, 1999; Hanes, 2003). Membrane ruffling of M cells causes subsequent pinocytosis of salmonellae (Francis *et al*., 1993; Hanes, 2003). Pinocytosis is the internalization of bacteria by columns of polymerized actin which encircle and engulf bacteria (Francis *et al*., 1993; Wallis and Galyov, 2000; Hanes, 2003). M cells containing salmonellae then break off from the host's intestinal mucosa, leaving damaged mucosal tissue exposed to the salmonellae in the lumen. This allows salmonellae to enter underlying mucosal tissue and the lymphatic system (Hanes, 2003).

A number of plasmids are common in the majority of *Salmonella* serotypes (Hanes, 2003). Pathogenicity islands can be localized to *Salmonella* chromosomes or to plasmids (Hanes, 2003) and are shown to be responsible for mucosal invasion and virulence (Hanes, 2003). Previous studies have identified three systems that are localized to the *Salmonella* Typhimurium chromosome: *Salmonella* pathogenicity island 1 (SPI-1), a system for invasion and virulence; *Salmonella* pathogenicity island 2 (SPI-2), a type III secretion system for the survival in macrophages; and a flagellar assembly system for motility (Darwin and Miller, 1999). SPI-1 has numerous characteristics that are suggestive of horizontal gene transfer from another bacterial

species during its evolution (Wallis and Galyov, 2000; Hanes, 2003). SPI-1, a 40kb region, contains over 30 genes which encode for different proteins such as secreted effector proteins, regulatory proteins and components of type III secretion system (Darwin and Miller, 1999; Hanes, 2003). The gene responsible for invasion (apart from the invasion system that is encoded at centisome 63) is the SPI-1 genes, *invABC(D)* (Darwin and Miller, 1999). Salmonellae invasion depend on bacterial attachment to the host's epithelial cell receptors (Wallis and Galyov, 2000). SPI-1 has three proteins that play a role in mucosal invasion. These proteins contribute to a supramolecular structure which secretes effector proteins and hence send bacterial signals to the host's eukaryotic cells (such as the enterocytes and dendritic cells). These signals cause rearrangements of the eukaryotic cytoskeleton which initiates membrane ruffling (Darwin and Miller, 1999; Hanes, 2003). The ruffling mechanism causes eruption of the host's cell membrane and subsequent internalization of salmonellae (Francis *et al*., 1993; Hanes, 2003). Salmonellae that overcome the host's immune response will enter into the blood stream from the lymphatic system via the thoracic duct and cause bacteraemia (Hanes, 2003).

1.11 *Salmonella* infections in HIV/AIDS patients

The lymphoid tissue in the intestinal tract represents about 70% of the human body's immune system (Anitei, 2008). HIV destroys the intestinal immune cells called T helper cells which play a role in warning other immune cells in the body regarding the site of infection (Anitei, 2008). Studies show that HIV persists and multiplies in the tissue macrophages of the reticuloendothelial system in the gut mucosa. HIV

causes mass destruction of intestinal immune cells and subsequent decrease in the host's immune response (Gordon *et al.,* 2002; Anitei, 2008). The decrease in the host's immune response allows opportunistic pathogens such as salmonellae to invade the intestinal mucosal barrier and enter into the blood stream (Gordon *et al.,* 2002). In addition, research shows that pathogens are able to survive for a long time in monocyte and macrophage type immune cells which further prolong infection in HIV-positive patients (Gordon *et al.,* 2002). Disease caused by salmonellae is a common opportunistic infection associated with HIV disease (Ikumapayi *et al.,* 2007). Symptoms include difficulty in swallowing, loss of weight, prolonged diarrhoea, low CD4 lymphocyte counts, high incidence of *Salmonella* bacteraemia and *Salmonella* septicaemia (Bick, 2004). HIV/AIDS patients are about 20 times more prone to *Salmonella* infection and about a 100 times more prone to bacteraemia caused by salmonellae than patients with competent immune systems (Fernandez Guerrero *et al.,* 1997). Studies also show that about 25% of HIV-positive patients with salmonellosis suffer from focal infections (Fernandez Guerrero *et al.,* 1997). Salmonellosis may be difficult to diagnose in HIV-infected patients, however if they are diagnosed and treated during the early stages of *Salmonella* infection, this may improve their lives considerably (Bick, 2004). *Salmonella* infections are more likely to relapse in HIV/AIDS patients after receiving treatment (Bick, 2004). Recurrent salmonellosis is regarded as an AIDS-defining illness (CDC, 1992). Studies show that salmonellosis in HIV-positive patients is associated with high morbidity and mortality rates (Fernandez Guerrero *et al.,* 1997).

According to statistical studies there were about 33 million people worldwide that were living with HIV/AIDS at the end of 2007, of which there were two million people that died in 2007. Twenty-two million people living with HIV/AIDS were from sub-Saharan Africa which represented the majority (67%) of HIV/AIDS patients, worldwide (UNAIDS, 2008). Of the remaining HIV/AIDS patients, 15% were living in Asia, 6% in North America, Western and Central Europe, 5% in Latin America, 5% in Eastern Europe and Central Asia, 1% in North Africa and Middle East, 0.7% in Caribbean and 2.0% in Oceania (UNAIDS, 2008).

In North America, about 30% to 70% of HIV-positive patients suffer from diarrhoeal diseases (May *et al.*, 1993). An Asian study carried out in the year 2000 by Barraclough showed that musculoskeletal infections in HIV-infected patients caused by salmonellae were commonly seen in Chinese participants, but were not frequently seen in people from Thailand where salmonellosis is common (Barraclough, 2000). In Africa, about 90% of HIV/AIDS patients suffer from diarrhoea caused by salmonellae and other diarrhoeagenic enteropathogens (Obi *et al.*, 2007). AIDS patients in sub-Saharan Africa infected with NTS bacteraemia have a mortality rate of 35% to 60%. Of the HIV-positive patients who survive, 25% to 45% suffer from recurrent NTS bacteraemia about one to six months after the first non-typhoidal infection (Kankwatira *et al*., 2004; Obi *et al*., 2007). In Blantyre, Malawi, recurrent salmonellosis and bacteraemia caused by NTS is common in HIV-infected adults and accountable for a high death rate seen amongst many individuals (Gordon *et al.*, 2002). In Kenya, a study carried out by Gilks *et al.*, (1990) showed that 11% of HIV-

infected patients suffered from hospital acquired salmonellosis and *Salmonella* bacteraemia.

The burden of HIV disease in South Africa is high (Pembrey, 2008). In South Africa in 1998 through to 1999, multidrug-resistant *Salmonella* Typhimurium DT104 strains, isolated from HIV-positive patients from the Chris Hani Baragwanath Hospital in the Gauteng Province (one of the nine provinces in South Africa) were investigated and reported in 2000 by Crewe-Brown and colleagues (Crewe-Brown *et al.*, 2000). Annual reports for the years 2006 and 2007 showed that approximately 1.42 million people in the Gauteng Province of South Africa were infected with HIV (ASSA, 2008).

Studies show that *Salmonella* infections in HIV/AIDS patients are particularly high in developing countries such as South Africa especially due to limited potable water and poor sanitation in rural areas (Obi *et al.*, 2007). HIV-positive patients living under these adverse conditions may be faced with a number of predicaments. For example, woman living with HIV/AIDS may deny their infant children breast milk as a measure taken to prevent mother-to-child transmission of HIV and they may alternatively resort to using untreated water contaminated with salmonellae for the preparation of food for their children. This may consequently lead to high morbidity and mortality rates among these children as a result of their immature immunity (Obi *et al.*, 2007; Dunne *et al.*, 2001).

In addition, many people living in the developing countries may not publicize the death of their family members or friends out of fear and shame of being discriminated and rejected by society (Engelbrecht, 1998). They may consequently resort to secret and inappropriate burying of corpses at or near water supply points and as a result salmonellae from dead bodies may permeate into water resources increasing the risk of *Salmonella* infection to the whole community (Obi *et al.*, 2007).

1.12 Nosocomial Salmonellosis

One of the contributing factors to human-to-human transmission is nosocomial infections. This is a secondary infection that a patient acquires after being admitted to a long-term medical care facility such as a hospital or a nursing home etc. (Olsen *et al.*, 2001; Edelstein *et al.*, 2004). Nosocomial outbreaks caused by salmonellae were common worldwide during the 1970's, however after the implementation of control measures, nosocomial outbreaks have been less common in developed countries. In the last ten years however, there has been an alarming increase of nosocomial outbreaks (Edelstein *et al.*, 2004) reported in Russia and Belarus in the 1990's and 1994 through to 2003 (Edelstein *et al.*, 2004), the USA in 1996 to 1998 (Caratolli *et al.*, 2002), Italy in 1998 to 2000 (Mammina *et al.*, 2002), Spain in 1999 to 2000 (Navarro *et al.*, 2001) and Romania in 2002 (Miriagou *et al.*, 2002). Reports showed that nosocomial infections accounted for 10% to 30% of all *Salmonella* cases in the USA (Black *et al.*, 2004). According to the National Nosocomial Infections Surveillance (NNIS) System Report for January 1992 through to June 2004, most of the nosocomial infections reported in the USA occurred in major teaching hospitals

particularly in surgical, medical and paediatric intensive care units (NNIS, 2004). Nosocomial outbreaks due to salmonellae are common in developing countries such as Brazil (Olsen *et al*., 2001), India (Olsen *et al*., 2001), Tanzania (Vaagland *et al.,* 2004) and South Africa (Kruger *et al.,* 2004).

Most common causes of nosocomial infections are due to direct or indirect contact with *Salmonella* infected patients, hospital personnel, contaminated hospital environments and consumption of contaminated medication or food (Black *et al.*, 2004). Nosocomial infections are most severe in extremely young patients, patients receiving multiple antibiotic treatments, immunocompromised patients and patients in burn units (Riggle and Kumamato, 2000; Hanes, 2003).

Studies show that nosocomial outbreaks caused by salmonellae are common in extremely young patients receiving parenteral nutrition as well as patients with central intravascular catheters (Cartolano *et al.,* 2003). Statistical analysis shows that approximately 50% of all nosocomial outbreaks that occur in the USA are due to nosocomial infections that occur at paediatric wards (Black *et al.*, 2004). Cartolano *et al.,* (2003) described person-to-person transmission of *Salmonella* Brandenburg from an infected family member to a newborn baby in a hospital in France.

In a hospital setting, antibiotic resistance is largely due to the misuse of antibiotics which exert selective pressure on resistance in salmonellae (Olsen *et al.,* 2001).

Nosocomial outbreaks caused by salmonellae in Russia and Belarus in 1994 to 2003 were due to cefotaxime-resistant *Salmonella* Typhimurium isolates (Edelstein *et al.,* 2004). Another nosocomial outbreak in young patients was caused by CTX-M-14-type extended-spectrum β-lactamase (ESBL) producing strains of *S. enterica* serotype London (*Salmonella* London) was reported by Yong *et al.,* (2005). Olsen *et al.,* (2001) described in their study the first nosocomial outbreak of fluoroquinolone resistant salmonellae isolated from 11 elderly patients in Oregon in the USA. In 2006, Wadula and colleagues (Wadula *et al.,*2006) described a nosocomial outbreak caused by *S. enterica* serotype Isangi (*Salmonella* Isangi) producing ESBL in a tertiary hospital in Gauteng, South Africa. A later study carried out in 2008, showed a nosocomial outbreak caused by ESBL producing salmonellae in Durban, South Africa (Govinden *et al.,* 2008).

1.13 *Salmonella enterica* subspecies *enterica* serotype Typhimurium

Salmonella Typhimurium is a predominant serotype isolated from both humans and animals (Casin *et al.,* 1999; Lim *et al.,* 2003, Palmgren *et al.,* 2006, Sheehan and van Oort, 2006). Studies show that apart from *Salmonella* Typhimurium causing a significantly high number of infections in both humans and animals worldwide, it raises a cause of concern because it shows the highest prevalence of antibiotic resistance compared to other *Salmonella* serotypes (Casin *et al.,* 1999). Multidrug-resistant *Salmonella* Typhimurium DT104 was first isolated in 1984 from humans living in the UK (Ribot *et al.,* 2002). Studies show that *Salmonella* Typhimurium DT104 is a global concern as it is an emergent multidrug-resistant *Salmonella* phage

type in Canada, in the USA and in many European countries since the 1990's (Baggesen *et al*, 2000; Hanes, 2003). Research shows that salmonellosis caused by multidrug-resistant *Salmonella* Typhimurium correlates with a high patient admission rate to medical care facilities and high patient mortality rates (Boyd *et al.,* 2001). Numerous reports have been documented for *Salmonella* Typhimurium isolates recovered from humans living in North America, Central America and European countries, however very little data exists for human *Salmonella* Typhimurium isolates recovered from developing countries such as South Africa.

1.14

1.14.1 Global Scale

Europe

Belgium

Studies show that *Salmonella* Typhimurium DT104 caused a major outbreak of *Salmonella* infection in Belgian livestock in 1998 (Baggesen *et al*., 2000).

Denmark

Salmonella Typhimurium together with *Salmonella* Enteritidis represents more than 50% of most commonly isolated serotypes in Denmark (Torpdahl *et al.,* 2005). A study performed in Denmark in 1998 showed that multidrug-resistant *Salmonella* Typhimurium DT104 caused *Salmonella* infection in pig herds (Baggessen *et al.*, 2000).

France

In 1993 to 1994 and in 1998 to 2003, *Salmonella* Typhimurium accounted for the second most frequently isolated *Salmonella* serotype, however during the time period of 1995 to 1997 *Salmonella* Typhimurium was the first most commonly isolated serotype in France (Weill *et al.,* 2006).

Germany

Salmonella Typhimurium DT104 was isolated from humans in Germany in 1997 (Baggesen *et al*., 2000).

Greece

A study performed by Markogiannakis *et al.,* (2000) showed that since 1991 through to 1996, *Salmonella* Typhimurium DT104 was commonly isolated from chicken, humans, pigs and pigeons.

Italy

Multidrug-resistant *Salmonella* Typhimurium DT104 was a major cause for human salmonellosis in 1998 in Sardinia, Italy (Baggesen *et al*., 2000; Weill *et al.,* 2006).

Ireland

An annual report of the National *Salmonella* Reference Laboratory of Ireland for 2006 (NSRL Report 2006) showed that *Salmonella* Typhimurium represented 23% of all the NTS cases.

Netherlands

Hendriksen *et al.,* (2004) described in their study nonfoodborne animal-to-human transmission of salmonellae. *Salmonella* Typhimurium DT104A variant was transmitted to a pig, a calf and a child living on a farm.

United Kingdom (UK)

Salmonella Typhimurium DT104 was first isolated from humans living in the UK in 1984 (Ribot *et al.*, 2002).

Sweden

Palmgren *et al.*, (2006) compared the genetic relatedness between *Salmonella* Typhimurium isolates recovered from gulls with those *Salmonella* Typhimurium isolates recovered from humans and domestic animals. The results from their study suggested that gull *Salmonella* Typhimurium isolates were genetically similar to human and domestic animal *Salmonella* Typhimurium isolates and further theorized that gulls may play a role in spreading *Salmonella* Typhimurium to humans and domestic animals in Sweden.

North America and Central America

Canada

Multidrug-resistant *Salmonella* Typhimurium DT104 was isolated from poultry in Canada in 1996 (Baggesen *et al*., 2000).

United States of America (USA)

Multidrug-resistant *Salmonella* Typhimurium DT104 was first isolated in 1985 from humans living in the USA, a year after it was first recovered from humans living in the UK (Ribot *et al.*, 2002). It is hypothesized that *Salmonella* Typhimurium DT104 was first introduced into the human population through an animal source (Ribot *et al.*, 2002). During 1985 to 1998 *Salmonella* Typhimurium ranked the most frequently isolated serotype in the USA (Rabatsky-Ehr *et al*., 2004). Multidrug-resistant

Salmonella Typhimurium represented less than 1% of human salmonellosis in 1980 and represented greater than 35% in 1996 (Rabatsky-Ehr *et al.,* 2004). In 2003 through to 2005, *Salmonella* Typhimurium showed an incidence of 51% in the USA (Bopp *et al.,* 1999).

Asia

India

Saha *et al.,* (2001) studied nosocomial isolates recovered from children who suffered from diarrhoeal diseases in Culcatta, India, during the time period of August 1993 to September 1996. Results showed that out of approximately 1000 children who suffered from diarrhoea, 16% (157/1000) of the cases were caused by four different *S. enterica* serotypes. Of these *Salmonella* Typhimurium accounted for the majority (70%; 110/157) of all the *Salmonella* serotypes.

Israel

Studies show that multidrug-resistant *Salmonella* Typhimurium DT104 was a cause of human salmonellosis in Israel (Baggesen *et al*., 2000).

Africa

Kenya

Invasive multidrug-resistant *Salmonella* Typhimurium was isolated from blood specimens taken from children aged less than seven years in Nairobi, Kenya during a time period of 2002 through to 2004 (Kariuki *et al*., 2006). *Salmonella* Typhimurium represented the majority (55%; 106/193) of all the NTS isolates isolated from these

children. In addition, studies showed that *Salmonella* Typhimurium is a common cause of bacteraemia in children living in Kenya (Kariuki *et al*., 2006).

Rwanda

In 1990, multidrug-resistant *Salmonella* Typhimurium was responsible for a severe outbreak which occurred among 246 young patients living in Rwanda. Eleven percent (26/246) of these patients had metastatic focal infections, of which 46% (12/26) had meningitis caused by *Salmonella* Typhimurium (Lepage *et al.,* 1990).

Zaire

In 1997 in Zaire, Cheesbrough *et al.,* (1997) reported that invasive *Salmonella* Typhimurium was responsible for an outbreak in five of the 120 children.

1.14.2 South Africa

In South Africa in 1998 to 1999, multidrug-resistant *Salmonella* Typhimurium DT104 isolated from HIV-positive patients from the Chris Hani Baragwanath Hospital in the Gauteng Province were investigated and reported in 2000 by Crewe-Brown and colleagues (Crewe-Brown *et al.,* 2000). Swe Swe *et al.,* (2006) reported a case of meningitis caused by *Salmonella* Typhimurium in an adult patient living with AIDS. Govinden *et al.,* (2008) reported a nosocomial outbreak among paediatric patients caused by *Salmonella* species at a tertiary hospital in Durban, South Africa. Seventeen of the 41 *Salmonella* serotypes isolated from these patients were *Salmonella* Typhimurium. Data recorded in the Group for Enteric, Respiratory and Meningeal Surveillance in South Africa (GERMS-SA) surveillance database 'Epi Info software' (version 6.04d, CDC, Atlanta, USA) for the years 2003 to 2005

showed that a total of 7974 *Salmonella* isolates were received at the Enteric Diseases Reference Unit (EDRU), of which 28% (2270/7974) were serotyped *Salmonella* Typhimurium (Keddy, unpublished work).

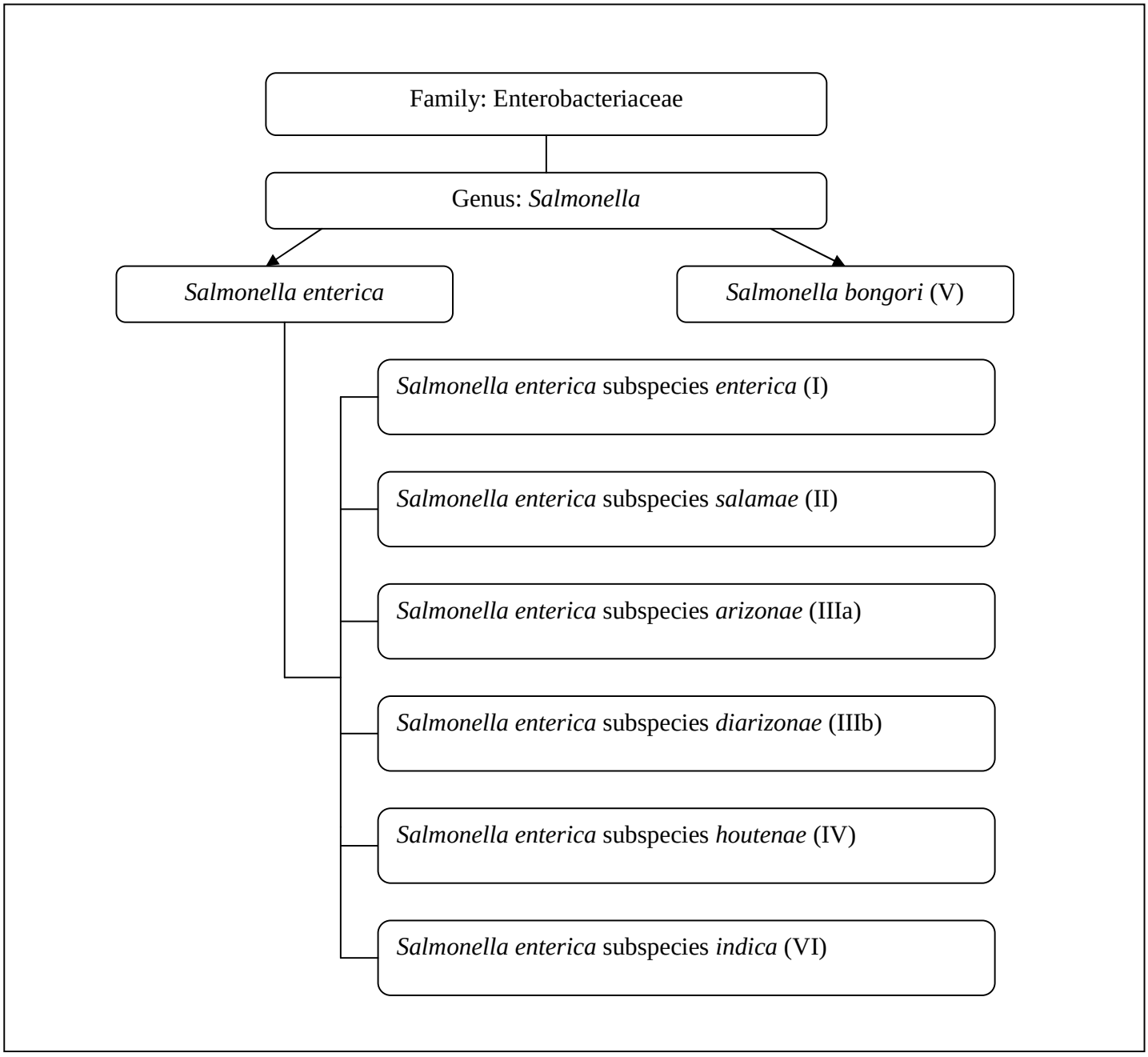


Figure 1.1 *Salmonella* nomenclature

(Brenner *et al.*, 2000)

Table 1.1 Biochemical reactivity of *Salmonella*

Biochemical tests for *Salmonella* species	
Test or substrate	Reactivity
Glucose	+ve [a]
ODC	+ve [c]
LDC	+ve [b]
Dulcite	+ve [d]
H_2S	+ve [e]
Motility test	+ve [e]
Indole test	-ve
Sucrose	-ve
Mannitol	+ve [a]

[a] +ve, 100% positive
[b] +ve , 98% or more positive
[c] +ve , 97% or more positive
[d] +ve , 96% or more positive
[e] +ve , 95% or more positive

(Murray *et al.*, 1999)

1.15 Aims and objectives of the study

The aim of the research presented in this dissertation was to describe the characterization of invasive human isolates of *Salmonella* Typhimurium in Gauteng, South Africa, 2006 through to 2008 and the characterization of salmonellae isolated from chickens in Gauteng, South Africa, 2007 through to 2008. The objectives were as follows:

- To investigate strain relatedness and cluster formation of invasive human *Salmonella* Typhimurium isolates using the technique of PFGE analysis with restriction enzyme *Xba*I.
- To employ MLVA as a second genotypic method to further analyze representative isolates of invasive human *Salmonella* Typhimurium isolates.
- To enhance our understanding of the nosocomial nature of *Salmonella* Typhimurium in Gauteng.
- To characterize chicken isolates isolated from chicken meat.
- To investigate strain relatedness and cluster formation of human *Salmonella* isolates with chicken *Salmonella* isolates by PFGE analysis incorporating restriction enzymes *Xba*I and *Not*I.
- To employ MLVA as a second technique to further analyse representative human and chicken *Salmonella* Typhimurium isolates.

CHAPTER 2

Molecular epidemiology of invasive isolates of *Salmonella enterica* serotype Typhimurium in Gauteng, South Africa, 2006-2008

2.1 Introduction

Salmonellae on a global scale are responsible for foodborne enteric infections in humans and animals (Bopp *et al.*, 1999). *Salmonella* Typhimurium is among the most common *Salmonella* serotypes causing *Salmonella* infections, internationally (Lim *et al.*, 2003; Weill *et al.*, 2006). Since 1984, *Salmonella* Typhimurium DT104 was frequently isolated from humans and animals and still continues to pose a global health concern (Kariuki *et al*., 2006; Lepage *et al.*, 1990; Baggesen *et al*., 2000). In Europe in 1998 to 2003, *Salmonella* Typhimurium was the second most common serotype of all the other NTS serotypes responsible for most of the nosocomial outbreaks (Edelstein *et al.*, 2004; Weill *et al.*, 2006). Studies have also shown that *Salmonella* Typhimurium is a common NTS serotype responsible for severe infections in HIV/AIDS patients (Obi *et al.*, 2007; Crewe-Brown *et al.*, 2000).

Very little epidemiological data exists for *Salmonella* Typhimurium isolates recovered from human patients who suffered from nosocomial acquired *Salmonella* infections and from *Salmonella* infected HIV-positive patients in South Africa (Crewe-Brown *et al.*, 2000; Govinden *et al.*, 2008; Swe Swe *et al.*, 2006).

The aim of the current study was to clarify:

- The molecular epidemiology of *Salmonella* Typhimurium isolates in Gauteng, South Africa, for the years 2006 through to May 2008, by using PFGE analysis and MLVA.
- To enhance our understanding of the nosocomial nature of these organisms.
- To better understand the presentation and outcome of HIV-positive patients (living in Gauteng) infected with *Salmonella* Typhimurium.
- To identify epidemiological clusters that may assist in the interventions to stop further spread of disease.

2.2 Methods

2.2.1 Case definition

The Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA) is a national laboratory-based surveillance system which monitors bacterial and fungal diseases. This program is coordinated by staff members who are based at the National Institute of Communicable Diseases (NICD), a branch of the National Health Laboratory Services (NHLS), Johannesburg. EDRU is a part of the GERMS-SA. As a part of routine surveillance, *Salmonella* isolates are sent from national participating laboratories in South Africa to the EDRU of the NICD for further phenotypic and genotypic characterization of *Salmonella* isolates. *Salmonella* Typhimurium isolates from all normally sterile body sites in human patients were included.

Ethical clearance was obtained through the Human Research Ethics Committee (Medical), Wits University, Johannesburg (Protocol number M060449).

2.2.2 Enhanced surveillance

Surveillance officers are appointed to the four enhanced sites in Gauteng, South Africa (Govender *et al.*, 2006). Surveillance officers complete basic patient information by interviewing patients or reviewing patient records. Information is recorded in the GERMS-SA surveillance database 'Epi Info software' (version 6.04d, CDC, Atlanta, USA) (Keddy, unpublished work).

2.2.3 Selection of isolates for analysis

Analysis of isolates was restricted to the Gauteng Province in South Africa from January 2006 through to May 2008. A total of 3310 *Salmonella* isolates that were isolated from human patients was received at the EDRU, of which 36% (1194/3310) were serotyped *Salmonella* Typhimurium. Of the 1194 isolates, 840 (70%) were invasive *Salmonella* Typhimurium isolates, accounting for 25% (840/3310) of all *Salmonella* isolates. In the current study, all invasive isolates were analysed. The isolates were stored at -75°C in a tryptic soy broth with 10% (vol/vol) glycerol (Diagnostic Media Products (DMP), Sandringham, South Africa).

2.2.4 Bacterial identification and phenotypic characterization

Bacterial isolates were identified using standard microbiological techniques (See section 1.5 of chapter 1). Specific anti-sera (Statens Serum Institut, Copenhagen, Denmark; Remel Europe Ltd, Dartford, Kent, UK; and BioMérieux, Marcy-I'Ètoile, France) were used to serotype *Salmonella* Typhimurium isolates (Appendix A). This was performed according to the Kauffman-White scheme.

2.2.5 Antimicrobial susceptibility testing

Antibiotic susceptibility was determined using Etests (AB Biodisk, Solna, Sweden) according to the breakpoint guidelines as published in the Clinical and Laboratory Standards Institute's protocol (Appendix B) (Table 2.1). The following antibiotics were tested: chloramphenicol (C), streptomycin (S), tetracycline (T), nalidixic acid (Na), ciprofloxacin (CI), cotrimoxazole (TS), trimethoprim (TR), sulfamethoxazole (Su), kanamycin (KM), ampicillin (A), augmentin (XL), imipenem (IP), ceftriaxone (TX), cefepime (PM) and ceftazidime (TZ). For the present study, we particularly focused on the following six antibiotics: ampicillin (A), chloramphenicol (C), streptomycin (S), sulfamethoxazole (Su), tetracycline (T) and nalidixic acid (Na).

2.2.6 Nosocomial isolates

For the purpose of the current study, patients were identified as having acquired a nosocomial infection according to the following criteria: Salmonellosis was not detected on the patient's admission to hospital and that the specimen (confirming salmonellosis) was taken from the patient after two or more days following hospital admission.

2.2.7 Incomplete patient data

Patient data corresponding to patients and patient isolates were received from microbiological laboratories in Gauteng. Patient demographic information such as

HIV status and age etc. may have been incomplete for some patients due to undefined reasons. Incomplete patient information was presented as unknown values in table 2.3 and table 2.4. Missing information may have skewed results and for that reason may have been a drawback to the current study. However, patient data were complete for patients from enhanced sites.

2.2.8 Pulsed-field gel electrophoresis (PFGE) analysis of isolates

PFGE analysis was performed on isolates as previously described (Ribot *et al.*, 2006). The specimen was inoculated on a 5% blood agar plate (DMP) and incubated at 37°C overnight. Bacterial cells were collected from the blood agar plate and were resuspended into 800 µl of cell suspension buffer (Appendix C). The cell concentration of the bacterial suspension was measured using a turbidity meter (Dade Behring, Randjesfontein) and turbidity readings were adjusted to approximately 0.70. Two hundred microlitres of the bacterial suspension was then transferred to another tube to which 20 µl of proteinase-K (Roche Diagnostics GmbH, Mannheim, Germany) (Appendix C), 28 µl of 10% SDS (Appendix C) and 275 µl of 1% agarose (SeaKem® Gold Agarose, Rockland, USA) (Appendix C) were added. The mixture (300 µl) was pipetted into moulds (Bio-Rad Laboratories, California, USA) and allowed to solidify for ten minutes. To lyse bacterial cells, agarose plugs containing genomic DNA were incubated in a shaking water bath held at 55°C, in 50 ml tubes containing cell lysis buffer (Appendix C). This step was performed to release genomic DNA. Following the lysis step, the plugs were washed once with 15 ml of deionized water for 15 minutes and then washed for four times with 15 ml of Tris-

EDTA (TE) buffer for 15 minutes each (Appendix C). Bacterial DNA was digested by placing a slice of the agarose plug into a 150 µl solution containing 30 units of restriction enzyme, *Xba*I (Roche Diagnostics GmbH). Thereafter the slices were put on a comb and set in a 1% agarose gel (SeaKem® Gold Agarose) (Appendix C). Electrophoresis was performed using the CHEF-DR electrophoresis systems (Bio-Rad Laboratories Inc.) in 0.5x Tris-borate EDTA (TBE) buffer (Appendix C). The following run parameters were used: A voltage of 6 volts, at a run temperature of 14°C, a run time of 21 hours, an initial switch time of 2.2 seconds and a final switch time of 63.8 seconds. These patterns were then visualized by the UV illumination after staining the agarose gels with ethidium bromide (Appendix C). Fingerprint patterns were analysed using the BioNumerics® (version 5.1) software (Applied Maths, Sint-Martens-Latem, Belgium). Patterns were normalized against the reference pattern *S. enterica* serotype Braenderup (strain H9812). Dendrograms were produced by using the unweighted pair group method with arithmetic means. Analysis of the band patterns was performed with dice-coefficient at an optimization setting and position tolerance setting of 0.5% and 1.5%, respectively. For the purpose of the current study three or more isolates with a similarity value of ≥90% was defined as a PFGE cluster. The clusters were numbered 1, 2, 3, etc. for referral purposes (Please see figure 2.1 which shows an example of a typical dendrogram of PFGE patterns). Figure 2.1 shows isolates representing three clusters (1, 2 and 3) however the remaining isolates did not belong to any clusters due to the criteria described above.

2.2.9 Preparation of crude bacteria DNA

Bacterial cells were suspended in 400 µl of TE buffer (Appendix C) and boiled at 95°C for 25 minutes. Thereafter the bacterial suspension was centrifuged at 3500 rpm for three minutes. The supernatant was used directly in the PCR reactions.

2.2.10 Multiple-locus variable-number tandem-repeats analysis (MLVA)

MLVA was based on five VNTR gene loci (STTR3, STTR5, STTR6, STTR9 and STTR10pl) as previously described by Lindstedt *et al.,* (2004). The PCR primers used to amplify VNTR loci are seen in table 2.2. The forward primer for each locus was labelled with a distinctive fluorescent dye (Qiagen, Venlo, Netherlands). The Qiagen PCR multiplex kit (Qiagen) was used to multiplex these five primer pairs into two solutions. Solution one was made up as follows: 12.5 µl of PCR master mix, 9.5 µl of RNAnase free water, 2.5 µl of primer mix which comprised of 10 pmol each of the STTR3 and STTR6 primer pairs and 0.5 µl of crude bacterial DNA, all of which made up a total volume of 25 µl. For solution two, the same volume of PCR master mix, RNAnase free water, crude bacterial DNA and primer mix were added, except the primer mix comprised of 10 pmol each of the STTR5, STTR9 and STTR10pl primer pairs. PCR was performed using the Bio-Rad Thermal i-Cyler (Bio-Rad Laboratories Inc.). The PCR run conditions were as follows: 95°C for 15 minutes; 25 cycles of 94°C for 30 seconds, 63°C for 90 seconds and 72°C for 90 seconds; and a hold at 72°C for ten minutes. The two resultant PCR solutions were pooled as follows: 10 µl of solution one + 2.5 µl of solution two. This pooled mixture was then diluted 1:7 in deionised water. One microlitre of this diluted mixture was then mixed

with 1 µl of GeneScan™ 600 LIZ® size standard (Applied Biosystems, Foster city, USA) and 12 µl of Hi-Di™ formamide (Applied Biosystems). This 14 µl mixture was then incubated at 95°C for three minutes and cooled to room temperature before being subjected to capillary electrophoresis using an Applied Biosystems 3130 Genetic Analyzer. Electrophoresis was performed through POP-7 polymer (Applied Biosystems) with an injection voltage of 15 kV for five seconds at a temperature of 60°C. Raw data were captured and analyzed using GeneMapper® (version 4.0) software (Applied Biosystems). VNTR locus was identified by its distinctive colour (fluorescence) and the gene product was sized by comparison to the internal size standard. Data (fragment lengths) were then entered into BioNumerics® v5.1 (Applied Maths). Dendrograms were produced by using Euclidian distance and Ward algorithm. Isolates were considered identical if they were identical at all loci. Isolates were considered closely related if they were polymorphic at only one locus. Isolates were considered unrelated if they were polymorphic at two or more loci.

2.2.11 Statistical analysis

In addition to descriptive analysis, univariate logistic regression was performed to determine which individual explanatory variables were significantly associated with the outcome variables – HIV status and nosocomial infections – by calculation of unadjusted odds ratios and 95% confidence intervals.

2.3 Results and Discussion

2.3.1 PFGE analysis and clusters of isolates

PFGE analysis was performed on 840 invasive human *Salmonella* Typhimurium isolates collected from different referral hospitals in the Gauteng Province in South Africa from January 2006 through to May 2008. Of the 840 invasive human isolates, 80% (671/840) of the isolates could be grouped into distinctive clusters. For the remainder of this chapter, presentation and discussion of data was focused on these 671 isolates. These data are summarized in table 2.3. For January 2006 through to May 2008, a total of 38 clusters (1 to 38) were identified amongst these 671 isolates. The smallest cluster was represented by three isolates and the largest cluster was represented by 160 isolates. Of the 38 clusters identified, three clusters (3, 5 and 11) were primary clusters represented by 116 (17%), 160 (24%) and 116 (17%) isolates, respectively. Three secondary clusters (7, 10 and 20) were categorized into the isolate number range of 29 (4%) to 43 (6%) of 671. Small numbers of isolates ($n \leq 12$ isolates) made up the remaining 32 clusters.

2.3.2 Hospital of origin

Human isolates were sourced from 21 hospitals in the Gauteng Province. Seven hospitals accounted for 92% (616/671) of all isolates (Table 2.3). Most isolates (48%; 324/671) originated from Hospital 2, a major public and teaching hospital located in Gauteng (van de Heever, 2008). For three hospitals (H1, H2 and H49) (14%; 3/21), some evidence was found for nosocomial acquisition of isolates, of which Hospital 2 accounted for most nosocomial isolates. Research has shown that *Salmonella*

infections are more common in a hospital environment due to overcrowding of patients and insufficient medical staff (Bouallègue-Godet *et al.,* 2003). In addition, *Salmonella* infections in hospitals are usually associated with the use of contaminated medical equipment and the consumption of contaminated meals served at hospitals. Food may be contaminated as a result of poor hand hygiene and inadequate cooking of meals by kitchen staff (Bouallègue-Godet *et al.,* 2003). The above mentioned examples may account for possible routes by which the majority of the clusters could have circulated in Hospital 2. Cluster 5 was a predominant cluster (24%; 160/671) in all 21 hospitals in Gauteng from January 2006 to May 2008 and was thus responsible for most of the infections caused by *Salmonella* Typhimurium during these years.

2.3.3 Age-range of patients

Most of the isolates (56%; 376/671) came from patients between 15 and 64 years of age (Table 2.3). Surveillance studies performed in South Africa for the years 2006 and 2007 have also shown that salmonellae are more frequently isolated from patients in this age-range (Govender *et al.,* 2006; Govender *et al.,* 2007). Furthermore, the majority (61%; 229/376) of the isolates from patients in this age-range were represented in clusters 3 (20%), 5 (24%) and 11 (17%). Twenty-nine percent (195/671) of the isolates came from patients aged four years and younger (Table 2.3). Young patients have an immature immune system and are more prone to *Salmonella* infections and hence this finding is not unexpected (Olesen *et al.,* 2005; Ikumapayi *et al.,* 2007). The majority (58%; 113/195) of the isolates from this young age-range were represented in clusters 3 (10%), 5 (24%) and 11 (24%). Of the 671 isolates, only

three isolates were recovered from patients aged 65 years and older (Table 2.3). Older patients are usually more predisposed to acquiring salmonellosis as a secondary infection as a result of their weakened immunity (Hanes, 2003). Conversely, the results in the current study suggest that salmonellosis in elderly patients in Gauteng for 2006 to 2008 occurred relatively less frequently. These results could possibly be accounted for by the underreporting of *Salmonella* cases in older patients due to numerous undefined reasons (Hanes, 2003) or overwhelming salmonellosis identified in patients of other age-ranges (less than 65 years) due to the high burden of HIV infection in these age-ranges (Cheesbrough *et al.*, 1997; Kuppermann, 1999; Kankwatira *et al.*, 2004; Ikumapayi *et al.*, 2007).

2.3.4 HIV disease

In the current study, the HIV status of patients was only known for 41% (277/671) of the patients, of which 92% (256/277) were HIV-positive (Table 2.3). Patients in the 15-64 year age-range were 12.3 times more likely to be HIV-positive than patients in the 0-4 age-range ($P<0.001$; 95% CI [3.4-43.8]). The majority (61%; 156/256) of the isolates from HIV-positive patients fell in the clusters 3 (21%), 5 (27%) and 11 (14%). HIV is a life-threatening epidemic in South Africa and accounts for up to 1000 deaths of AIDS patients, daily (Pembrey, 2008). Statistical reports have shown that in South Africa at the end of 2007, approximately 5.7 million people were living with HIV (Pembrey, 2008). HIV destroys the immune cells in the gut and predisposes HIV-positive patients to NTS: infection is 20 times more likely compared to immunocompetent patients (Hanes, 2003; Fernandez Guerrero *et al.*, 1997). Despite

the unknown HIV status for 59% (394/671) of the patients in the current study, the results still suggests that *Salmonella* Typhimurium may be responsible for extensive co-morbidity suffered by HIV-positive patients living in the Gauteng Province of South Africa.

2.3.5 Antibiotic resistance profiles

Antibiotic resistance profiles of *Salmonella* isolates are shown in Table 2.3. The most commonly expressed antibiotic resistance profiles of isolates from all the PFGE clusters were: ACSSuTNa (12%; 82/671) and ACSSuNa (13%; 90/671). Previous studies have shown that the penta-resistant ACSSuT pattern is frequently reported in *Salmonella* Typhimurium strains isolated in the UK, France and North America (Casin *et al.*, 1999; Helms *et al.*, 2005). An increase in resistance to the quinolone class of antimicrobials (nalidixic acid) in addition to the penta-resistant pattern has also been documented in an international survey performed on representative isolates of *Salmonella* Typhimurium for the years 1992 to 2001 (Helms *et al.*, 2005). For the current study, antibiotic resistance in the primary clusters (3, 5 and 11) are as follows: For cluster 3, 91% (105/116) of the isolates were resistant to three or more antibiotics. The most distinguishing features for the majority of the isolates in cluster 3 were that they expressed the following multidrug-resistant patterns: ACSSuTNa (18%), ACSuTNa (10%), ASSuTNa (9%), ASSuT (15%), ASuNa (9%) and ASu (9%). For cluster 5, 91% (145/160) of the isolates were resistant to three or more antibiotics. The most common antibiotic resistance profiles were ACSSuTNa (21%), ACSSuNa (39%) and ACSSu (14%). In cluster 3 and 5, the majority of the isolates

were multidrug-resistant isolates and this suggests that treatment given to patients with *Salmonella* Typhimurium may be compromised resulting in longer morbidity periods and possibly a higher death rate amongst patients. Cluster 11 (n=116) included our largest group of antibiotic susceptible isolates. Cluster 11 included 45 isolates showing susceptibility to all six antibiotics and 26 isolates showing resistance to sulfamethoxazole only. In addition, 21 isolates represented in cluster 11 expressed the resistance profile, SuT. For this cluster of isolates, the treatment given to patients may be uncomplicated and morbidity may possibly be less severe.

2.3.6 Nosocomial isolates

Of the 671 isolates, only 383 had data which allowed us to draw conclusions as to possible nosocomial acquisition of the isolate. Of these 383 isolates, 35 (9%) were identified as nosocomial isolates: these isolates fell into 12 clusters (Table 2.4). Ninety-four percent (33/35) of the nosocomial isolates were recovered from patients with a known HIV status, of which the most distinctive feature was that the majority of the isolates came from HIV-positive patients (97%; 32/33) and from patients in the age-range of 15 to 64 years (66%; 23/35). All the nosocomial isolates were sourced from only three hospitals (H1, H2 and H49), with patients from Hospital 49 being 18.8 times more likely than patients in Hospital 1 to have nosocomial infections ($P<0.004$; 95% CI [2.7-129.7]) and 4.8 times more likely than patients in Hospital 2 to have nosocomial infections ($P<0.02$; 95% CI [1.2-19.0]). In addition, patients from Hospital 2 were 3.9 times more likely than patients from Hospital 1 to have nosocomial infections ($P<0.1$; 95% CI [0.9-17.0]). Hospital 2 accounted for the

majority (83%; 29/35) of the nosocomial isolates: these isolates belonged to multiple clusters. Isolates in cluster 3 were 6.2 times more likely than those in cluster 5 to be nosocomial. P<0.001; 95% CI [2.3-16.8] and 8.1 times more likely than those in cluster 11 to be nosocomial. P<0.003; 95% CI [2.1-31.2]). Most of our 35 nosocomial isolates were encompassed in clusters 3 (31%; 11/35) and 5 (23%; 8/35). Further discussion will be focussed only on these two clusters.

Cluster 3

A total of 11 nosocomial isolates fell in cluster 3, all of which were isolated from patients hospitalized at Hospital 2 (Table 2.4). Hospital 2 is a large academic hospital which accommodates not only patients living in the surrounding areas in the Gauteng Province, but also serves as a referral hospital for a large part of South Africa and neighbouring African countries (van de Heever, 2008). Past studies have shown that nosocomial infections occurred most commonly when there was an over population of patients and relatively fewer health care workers. There tends to be a reduction in infection control practiced by health care professionals to comply with the demand of seeing and treating an increased number of patients (Bouallègue-Godet *et al.,* 2003) which could possibly account for the high number of nosocomial isolates in Hospital 2. Infection in Hospital 2 could also have been acquired through direct contact (person-to-person) or through contact with common contaminated surfaces in the hospital environment (Hanes, 2003). Eight of the 11 (73%) nosocomial isolates were from patients aged 15 to 64 years (Table 2.4). These results were similar to the findings reported by Govender *et al.,* (2006; 2007). Nine of the ten (90%) nosocomial

isolates which came from patients with a known HIV status were HIV-positive. These results support the observation of previous studies, that immunocompromised patients such as HIV-positive patients are more susceptible to nosocomial infections (Hanes, 2003). Ten of the 11 (91%) nosocomial isolates were resistant to three or more antibiotics. The earliest isolate was sourced in February 2006 and the most recent isolate was sourced in August 2007, suggesting that this nosocomial cluster has been circulating in Hospital 2 for at least 19 months.

Cluster 5

Eight nosocomial isolates fell within cluster 5 (Table 2.4). Seven (88%) isolates were from Hospital 2 and one (13%) isolate was received from Hospital 49. All the isolates were from patients who were HIV-positive as found in other studies (Hanes, 2003). Five (63%) of the isolates were from patients aged 15 to 64 years, while three (38%) of the isolates were from patients aged four years and younger. All seven nosocomial isolates from Hospital 2 were resistant to four or more antibiotics. Nosocomial outbreaks caused by multidrug-resistant *Salmonella* Typhimurium are commonly reported (Olsen *et al.*, 2001; Govinden *et al.*, 2008). The earliest isolate was sourced in January 2006 and the most recent isolate was sourced in July 2007, suggesting that this nosocomial cluster has been circulating in Hospital 2 for at least 19 months.

2.3.7 Evaluation of multiple-locus variable-number tandem-repeats analysis (MLVA) for genotyping South African isolates of *Salmonella* Typhimurium

MLVA was performed as per the method described by Lindstedt *et al.,* (2004). The MLVA profile of an isolate constitutes a report of the size of five gene loci and is reported in the following genetic order: STTR9-STTR5-STTR6-STTR10pl-STTR3. MLVA was performed on 21 isolates which all were found to have a 100% identical PFGE profile. MLVA separated these 21 isolates into 11 distinct MLVA types (3, 15, 28, 29, 30, 31, 32, 33, 34, 35 and 36) (Figure 2.2). Most isolates (n=10) showed MLVA type 3 (160-216-342-370-450). MLVA type 3 differed to MLVA type 30 (160-216-356-370-450) at only one locus, so these MLVA types were very similar. Isolates (n=2) showing MLVA type 30 were considered to be closely related to the ten isolates showing MLVA type 3. A second example of very similar MLVA types were MLVA type 29 (160-216-328-325-450) and MLVA type 35 (160-216-314-325-450), of which isolates showing these MLVA types were considered to be closely related. Furthermore, this MLVA investigation has shown that the STTR5, STTR6 and STTR 10pl loci, displayed high degrees of polymorphisms for most isolates and thus suggesting that these loci are the best loci for high discriminative subtyping of South African isolates of *Salmonella* Typhimurium. Similar findings were reported by Lindstedt *et al.,* (2004) who showed that the same three loci (STTR5, STTR6 and STTR10pl) were polymorphic in allele distribution for most *Salmonella* Typhimurium isolates included in their study. In this current study, MLVA was able to discriminate *Salmonella* Typhimurium isolates that have the same PFGE profiles. Similar findings were shown in a study carried out by Witonski and colleagues

(Witonski *et al.*, 2006). *Salmonella* Typhimurium isolates recovered from human patients showed identical PFGE patterns. When MLVA was performed on these isolates, results showed that some of the isolates were distinguishable from one another whereas other isolates had identical MLVA profiles to each other (Witonski *et al.*, 2006).

Table 2.1 Antibiotics tested and breakpoints of antibiotics.

Antibiotic	CLSI[a] breakpoints	
	S	R
Ampicillin	< 8	>32
Chloramphenicol	≤ 8	≥ 16
Streptomycin	0	≥ 64
Sulfamethoxazole	≤ 256	≥ 512
Tetracycline	≤ 4	≥ 8
Nalidixic acid	≤ 16	≥ 32

[a](CLSI, 2005)

Table 2.2 Primers selected for PCR

MLVA primer name	Dye sequences (5'-3')
STTR3-F	**6FAM**-CCCCCTAAGCCCGATAATGG
STTR3-R	TGACGCCGTTGCTGAAGGTAATAA
STTR5-F	**VIC**-ATGGCGAGGCGAGCAGCAGT
STTR5-R	GGTCAGGCCGAATAGCAGGAT
STTR6-F	**NED**-TCGGGCATGCGTTGAAA
STTR6-R	CTGGTGGGGAGAATGACTGG
STTR9-F	**6FAM**-AGAGGCGCTGCGATTGACGATA
STTR9-R	CATTTTCCACAGCGGCAGTTTTTC
STTR10pl-F	**PET**-CGGGCGCGGCTGGAGTATTTG
STTR10pl-R	GAAGGGGCCGGGCAGAGACAGC

(Lindstedt *et al.*, 2004)

Table 2.3 Division of *Salmonella* Typhimurium isolates into PFGE clusters followed by further subdivision by: hospital of origin, age- range of patients, HIV status of patients and antibiotic resistance profile (actual numbers of isolates are shown)

		PFGE cluster							
		3 (n=116)	5 (n=160)	7 (n=43)	10 (n=34)	11 (n=116)	20 (n=29)	Other[a] (n=173)	Total (n=671)
Hospital of origin[b]	H1	13	24	2	2	17	4	20	82
	H2	70	77	14	12	48	19	84	324
	H17	8	23	11	7	17	3	30	99
	H18	7	3	5	2	7	1	9	34
	H30	2	5	0	6	8	0	8	29
	H46	6	15	0	1	5	0	5	32
	H49	1	2	7	2	3	0	1	16
	Other[c]	9	11	4	2	11	2	16	55
Age-range of patients	≤4 years	20	47	12	8	46	13	49	195
	5-14 years	4	4	2	2	1	0	9	22
	15-64 years	75	91	25	22	63	11	89	376
	≥65 years	0	1	1	0	1	0	0	3
	unknown	17	17	3	2	5	5	26	75
HIV status of patients	+ve	53	68	14	9	35	10	67	256
	-ve	4	1	2	0	5	1	8	21
	unknown	59	91	27	25	76	18	98	394
Antibiotic resistance profile[d]	ACSSuTNa	21	34	1	1	2	3	20	82
	ACSSuT	2	5	3	0	2	4	10	26
	ACSSuNa	7	63	8	0	2	0	10	90
	ACSuTNa	12	2	2	0	1	1	7	25
	ACSSu	0	23	4	0	3	0	8	38
	ACSNa	0	0	1	0	0	0	0	1
	ACSuT	2	0	0	0	0	8	2	12
	ACSuNa	2	3	0	0	0	0	0	5
	ASSuTNa	11	0	2	0	0	1	2	16
	ASSuT	17	3	2	2	0	2	5	31
	ASSuNa	8	5	5	0	0	0	2	20
	ASTNa	1	0	0	0	0	0	0	1
	ACT	0	0	0	1	0	0	1	2
	ASSu	2	2	2	1	0	1	6	14
	ASuT	5	0	0	0	1	0	3	9
	ASuTNa	4	0	1	0	0	0	3	8
	ASuNa	11	5	1	1	0	0	9	27
	SSuT	0	0	0	0	2	0	6	8
	CSuT	0	0	0	0	0	0	1	1
	ASu	11	3	4	1	0	2	12	33

	AT	0	1	0	0	0	0	0	1
	CSu	0	0	0	0	0	0	1	1
	SSu	0	0	0	0	0	0	1	1
	SuT	0	1	1	5	21	0	10	38
	SuNa	0	0	1	0	0	0	2	3
	Su	0	3	2	10	26	3	21	65
	TNa	0	1	0	0	0	0	0	1
	A	0	1	0	0	0	0	1	2
	C	0	0	0	1	0	0	0	1
	T	0	1	0	1	8	1	4	15
	Na	0	0	0	0	2	0	3	5
	susceptible	0	3	3	10	45	4	24	89

[a] These represent the remaining 32 clusters
[b] H1, Hospital 1; H2, Hospital 2; H17, Hospital 17; H18, Hospital 18; H30, Hospital 30; H46, Hospital 46; H49, Hospital 49
[c] These represent the remaining 14 hospitals
[d] A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole, T, Tetracycline; Na, Nalidixic acid; susceptible, susceptible to all 6 of the former mentioned antibiotics

Table 2.4 Attributes of nosocomial isolates of *Salmonella* Typhimurium

Isolate number	PFGE cluster	Hospital of origin[a]	Month/year of isolation	Antibiotic resistance profile[b]	Age-range of patients (years)	HIV Status of patients
N600	3	H2	February 2006	ASSuTNa	5-14	-ve
N637	3	H2	February 2006	ASSuT	≤4	+ve
N503	3	H2	April 2006	ACSuTNa	15-64	+ve
N282	3	H2	May 2006	ACSSuTNa	15-64	+ve
N283	3	H2	May 2006	ACSSuTNa	15-64	+ve
N312	3	H2	May 2006	ASSuT	15-64	+ve
N093	3	H2	June 2006	ACSSuTNa	15-64	unknown
N196	3	H2	June 2006	ACSSuTNa	15-64	+ve
N591	3	H2	August 2006	ASuNa	15-64	+ve
N039	3	H2	January 2007	ASu	≤4	+ve
N636	3	H2	August 2007	ACSuTNa	15-64	+ve
N367	5	H2	January 2006	ACSuTNa	15-64	+ve
N200	5	H2	March 2006	ACSSuNa	15-64	+ve
N038	5	H2	February 2007	ACSSuNa	15-64	+ve
N859	5	H2	February 2007	ACSSuTNa	≤4	+ve
N810	5	H49	February 2007	ASu	≤4	+ve
N767	5	H2	May 2007	ACSSuTNa	≤4	+ve
N520	5	H2	July 2007	ASSuT	15-64	+ve
N537	5	H2	July 2007	ASSuT	15-64	+ve
N801	7	H49	June 2006	Su	≤4	+ve
N487	7	H2	January 2008	SuT	15-64	+ve
N956	10	H49	March 2008	susceptible	15-64	+ve
N402	10	H1	November 2007	Su	15-64	unknown
N599	11	H2	May 2006	susceptible	15-64	+ve
N511	11	H2	July 2007	Su	≤4	+ve
N213	11	H49	October 2007	Su	15-64	+ve
N012	18	H1	August 2006	ACSuTNa	15-64	+ve
N787	21	H2	June 2006	susceptible	≤4	+ve
N197	22	H2	June 2006	SSu	≤4	+ve
N771	22	H2	June 2006	ASSu	≤4	+ve
N520	25	H2	July 2007	ASSuT	15-64	+ve
N511	25	H2	July 2007	Su	≤4	+ve
N196	29	H2	June 2006	ACSSuTNa	15-64	+ve
N624	31	H2	April 2008	SuT	15-64	+ve
N708	36	H2	September 2006	ASuNa	15-64	+ve

[a] H1, Hospital 1; H2, Hospital 2; H49, Hospital 49
[b] A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole, T, Tetracycline; Na, Nalidixic acid; susceptible, susceptible to all 6 of the former mentioned antibiotics

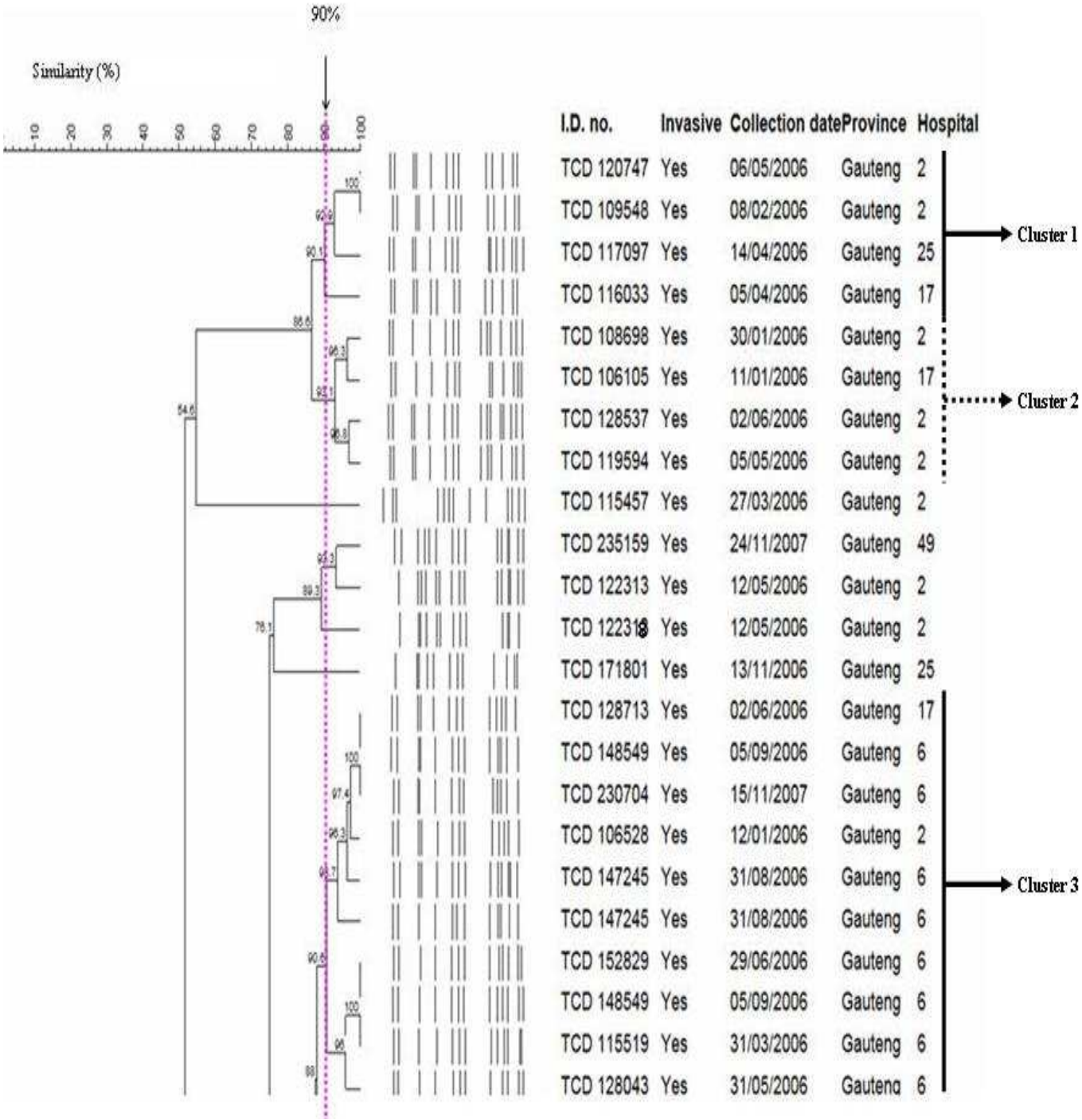


Figure 2.1 Dendrogram analysis of *Xba*I-digested DNA of *Salmonella* Typhimurium isolates. A percentage scale of similarity is shown above the dendrogram. The purple dotted line indicates the 90% similarity value.

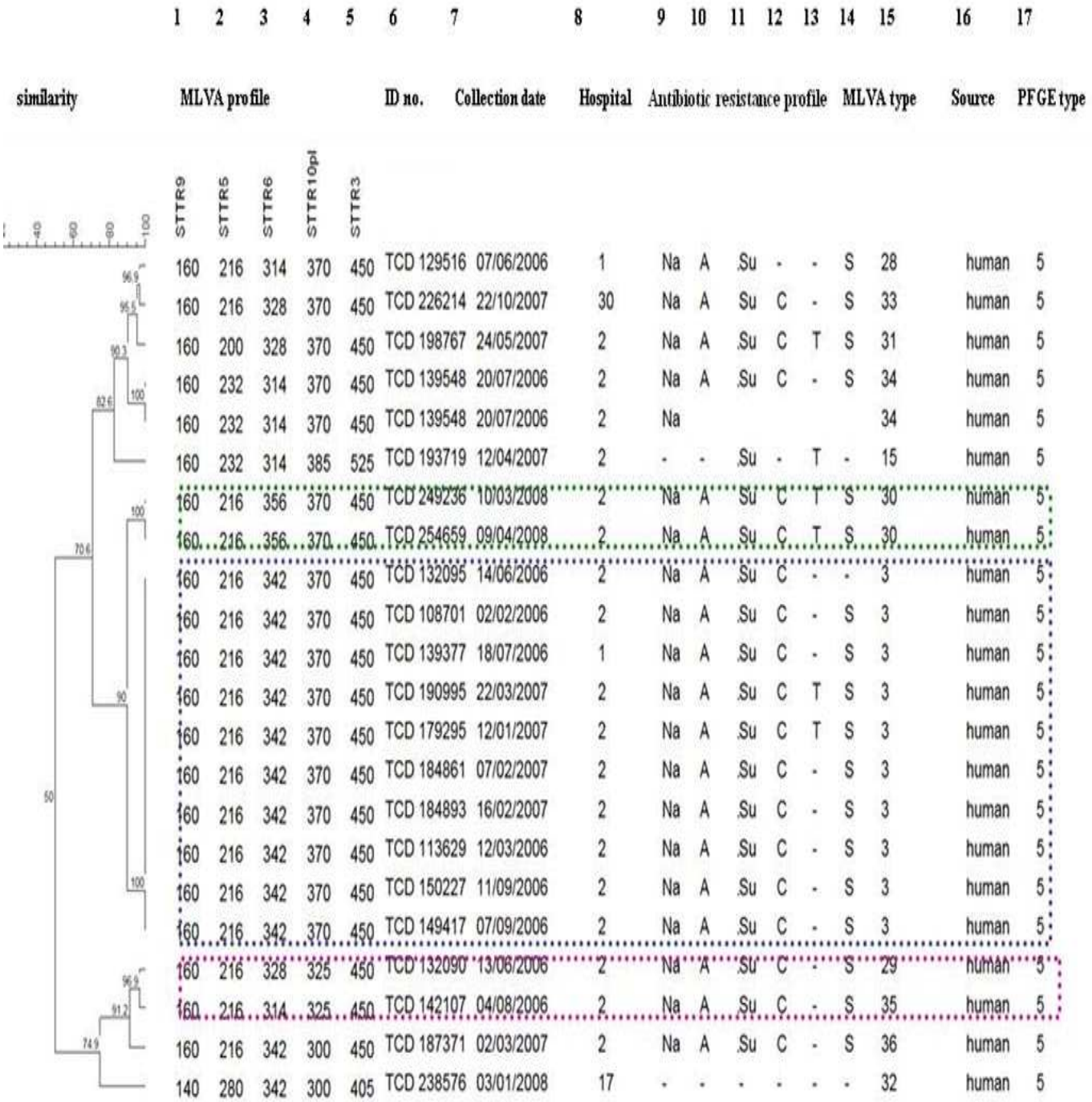


Figure 2.2 Dendrogram of representative MLVA-typed *Salmonella* Typhimurium isolates in the current study. Lanes 1-5: MLVA profile in the order: STTR9-STTR5-STTR6-STTR10pl-STTR3. Lanes 9-14 (Former mentioned antibiotics): Na, Nalidixic acid; A, Ampicillin; Su, Sulfamethoxazole, C, Chloramphenicol; T, Tetracycline; S, Streptomycin; -, susceptible.

CHAPTER 3

Characterization of *Salmonella* isolated from chickens in Gauteng, South Africa, 2007-2008; and comparison to invasive human isolates

3.1 Introduction

Salmonellae are a frequently isolated foodborne zoonotic pathogen in humans and animals. Salmonellosis is mostly caused by the consumption of food and water that are contaminated with salmonellae (Bopp *et al.,* 1999). Contamination of food with salmonellae can occur during and after food preparation (Mosupye and von Holy, 1999). Studies show that about 1% to 50% of meat sold at commercial markets is contaminated with bacterial pathogens during slaughtering and processing of food animals (Hanes, 2003). Cross-contamination of cooked food is often due to poor hygiene practiced during food preparation (Mosupye and von Holy, 1999). Furthermore, the use of antimicrobials in food animals by food production industries has led to an increase in the development of bacterial resistance to multiple antibiotics and consequently further complicates treatment for humans (Velge *et al.,* 2005). Previous reports showed that salmonellae is more frequently isolated from poultry and poultry products in comparison to any other animal species and is a major source of salmonellosis in humans (Carli *et al*., 2001; Cardinale *et al.,* 2003; Myint, 2004; Giannatale *et al.,* 2008). *Salmonellae* are a significant burden to the economy worldwide, particularly to the poultry industry due to productivity losses (Sarwari *et al*., 2001; Nógrády *et al.*, 2007)**.** Worldwide data for the years 1950 to 1970 showed that *Salmonella* Typhimurium was the most common *Salmonella* serotype isolated from poultry (Poppe, 2000). However, studies performed in 1984 through to 1999

showed that *Salmonella* Enteritidis replaced *Salmonella* Typhimurium as the most prevalent serotype isolated from poultry and eggs (Altekruse *et al.*, 2006) and correlated with the increase in the number of *Salmonella* Enteritidis isolates recovered from humans (Lee, 1974; Rabsch *et al.*, 2000). In the late 1990's, this problem was addressed by the implementation of quality assurance programs by poultry and egg production industries. This led to a 50% reduction in the number of cases of human salmonellosis caused by *Salmonella* Enteritidis (Poppe, 2000; Altekruse *et al.*, 2006).

The current study was performed to investigate the prevalence of salmonellae in chickens sold at formal and informal outlets in the Gauteng Province of South Africa and to determine whether a genetic relationship exists between these chicken isolates and human isolates in Gauteng.

3.2 Materials and Methods

3.2.1 Origin of *Salmonella* isolates

Chicken isolates

Chicken *Salmonella* isolates were isolated from chicken specimens which were purchased from retail and informal outlets such as formal and informal shops, street vendors and home-based abattoirs from various regions in Gauteng during the time period of September 2007 through to April 2008. These isolates were stored at -75°C in a tryptic soy broth with 10% (vol/vol) glycerol (DMP).

Human isolates

Chicken isolates were compared to human isolates. Human isolates were restricted to those which were isolated from patients from different referral hospitals in the Gauteng Province. These isolates were received at the EDRU for routine surveillance. Chicken isolates of various serotypes were compared to equivalent serotypes of representative human isolates which were received during the time period of January 2007 through to May 2008 with the exception of human *Salmonella* Typhimurium isolates. Chicken *Salmonella* Typhimurium isolates were compared to invasive human *Salmonella* Typhimurium isolates that were received from different referral hospitals in the Gauteng Province during the time period of January 2006 to May 2008.

3.2.2 Isolation and identification of *Salmonella* from chicken meat

Salmonellae were isolated from chicken meat using standard microbiological procedures (Appendix D).

3.2.3 Bacterial identification and phenotypic characterization

Bacterial identification and phenotypic characterization was performed on human and chicken *Salmonella* isolates. This method has already been described (See section 2.2.4 of chapter 2).

3.2.4 Antimicrobial susceptibility testing

Antibiotic susceptibility testing was determined using the Etests on all human and chicken *Salmonella* isolates in the current study. This method has already been described (See section 2.2.5 of chapter 2).

3.2.5 Pulsed-field gel electrophoresis (PFGE) analysis of isolates

PFGE analysis was performed on all human and chicken *Salmonella* isolates as previously described (See section 2.2.8 of chapter 2). In addition to this, secondary PFGE analysis was performed on human and chicken *Salmonella* isolates (excluding

Salmonella Typhimurium isolates) using restriction enzyme *Not*I (Inqaba Biotechnical Industries (Pty.) Ltd., Pretoria, South Africa).

3.2.6 Multiple-locus variable-number tandem-repeats analysis (MLVA)

MLVA was performed for secondary genotypic analysis of *Salmonella* Typhimurium isolates. This method has already been described (See section 2.2.9 and 2.2.10 of chapter 2).

3.2.7 Interpretation of genotypic data and determining relationships between chicken and human isolates

Chicken and human isolates of the same serotype were considered to be closely related only when two different genotyping methods both revealed significant similarity between the isolates.

For *Salmonella* Typhimurium, the two genotyping methods were PFGE analysis (*Xba*I digestion) and MLVA. Two isolates of *Salmonella* Typhimurium were considered to be closely related when their PFGE patterns were at least 90% similar and their MLVA profiles differed at no more than one locus.

For all other *Salmonella* serotypes, the two genotyping methods were PFGE analysis (*Xba*I digestion) and PFGE analysis (*Not*I digestion). Two isolates of the same *Salmonella* serotype were considered to be closely related when their *Xba*I PFGE

patterns were at least 90% similar and their *Not*I PFGE patterns were at least 90% similar.

3.3 Results and Discussion

3.3.1 *Salmonella* serotypes isolated from chicken meat

One-hundred and fifty chickens were processed and 47 S*almonella* strains were isolated. *S. enterica* serotype Heidelberg (*Salmonella* Heidelberg) (34%; 16/47) and *S. enterica* serotype Infantis (*Salmonella* Infantis) (34%; 16/47) were the most common *Salmonella* serotypes isolated from chickens; followed by *S. enterica* serotype Hadar (*Salmonella* Hadar) (11%; 5/47), *Salmonella* Enteritidis (11%; 5/47) and *Salmonella* Typhimurium (11%; 5/47). *Salmonella* Enteritidis (Lee, 1974; Poppe, 2000; Rabsch *et al.*, 2000; Altekruse *et al.*, 2006), *Salmonella* Infantis (Poppe, 2000; Kudaka *et al.*, 2006; Nógrády *et al.*, 2007), *Salmonella* Heidelberg (Poppe, 2000; Nayak and Kenney, 2002; Myint, 2004), *Salmonella* Hadar (Poppe, 2000; Cruchaga *et al.*, 2001; Sarwari *et al*., 2001; Giannatale *et al.*, 2008) and *Salmonella* Typhimurium (Poppe, 2000; Heuzenroeder *et al.*, 2004; Wedel *et al.*, 2005; Zaidi *et al.*, 2007; Harnett *et al.*, 1998) are common serotypes recovered from poultry and poultry products.

3.3.2 *Salmonella* Infantis

Salmonella Infantis was one of the most (34%; 16/47) isolated serotypes from chicken meat. PFGE analysis with *Xba*I digestion and with *Not*I digestion grouped 16 chicken isolates into four distinct clusters (Figure 3.1 and Figure 3.2). Cluster 1 was the largest cluster which is comprised of eight (50%; 8/16) chicken isolates which were isolated from chickens that were purchased from two different areas in the Johannesburg metropolitan (Soweto) (Figure 3.1 and Figure 3.2). All eight chicken

isolates from cluster 1 were susceptible to all six former mentioned (ACSSuTNa) antibiotics (Figure 3.1 and Figure 3.2). Cluster 2 shown in figure 3.1 and figure 3.2 was the smallest cluster and was represented by a single chicken isolate (TCD F56) which was resistant to tetracycline. Cluster 3 (Figure 3.1 and Figure 3.2) was represented by two (13%; 2/16) chicken isolates (TCD F54 and TCD F58) which were isolated from chickens that were purchased from two different areas in the Johannesburg metropolitan (Soweto). One of the two chicken isolates (TCD F54) showed susceptibly to all 6 (ACSSuTNa) antibiotics, whereas the remaining isolate (F58) was resistant to tetracycline. Cluster 4 (Figure 3.1 and Figure 3.2) was represented by five chicken isolates (31%; 5/16) which were isolated from chicken meat purchased from the same area in the Johannesburg metropolitan (Soweto). Four of the five chicken isolates (80%) were resistant to chloramphenicol, streptomycin, sulfamethoxazole and tetracycline, while the remaining isolate was resistant to streptomycin and tetracycline. PFGE analysis with *Xba*I digestion (Figure 3.1) and *Not*I digestion (Figure 3.2) showed that the PFGE patterns of chicken isolates were completely different to all PFGE patterns of human isolates, implying that these chicken isolates were unrelated to our human isolates. A study performed by Pelkonen *et al.,* (1994) in Finland showed that human *Salmonella* Infantis isolates for the years 1980 and 1989 compared to chicken *Salmonella* Infantis isolates collected from five broiler chicken companies for the years 1986 to 1991 were not related by molecular subtyping (Pelkonen *et al.,* 1994). Similar results were found between human and chicken *Salmonella* Infantis isolates by PFGE analysis in the current study. In contrast to the current study, a study performed by Nógrády *et al.,* (2007) in

Hungary, showed that broiler chicken and human *Salmonella* Infantis isolates were related by *Xba*I PFGE fingerprinting.

3.3.3 *Salmonella* Enteritidis

Salmonella Enteritidis was one of the least frequently isolated serotypes (11%; 5/47) from chicken meat (Figure 3.3 and Figure 3.4). Chicken isolates were recovered from chicken meat which were purchased from the Ekurhuleni metropolitan (Tembisa) and Johannesburg metropolitan (Soweto) in Gauteng. The majority of the isolates (80%; 4/5) were susceptible to all six antibiotics. PFGE analysis with *Xba*I digestion (Figure 3.3) and *Not*I digestion (Figure 3.4) showed that the PFGE patterns of chicken isolates were completely different to all PFGE patterns of human isolates, implying that chicken isolates were unrelated to our human isolates. Data from other countries showed that an increase in the number of *Salmonella* Enteritidis isolated from chicken meat and eggs, correlated with an increase in *Salmonella* Enteritidis isolates from humans (Lee, 1974; Poppe, 2000; Rabsch *et al.*, 2000). In New York in the USA in 1987, the consumption of contaminated poultry and poultry products prepared at acute and long term care hospitals led to the largest hospital outbreak of human salmonellosis caused by *Salmonella* Enteritidis (Poppe, 2000). A case study carried out in the late 1980s in England and more recent studies carried out in the years 2004 and 2006 in the USA showed that consumption of chicken meat contaminated with *Salmonella* Enteritidis predisposed humans to sporadic cases of salmonellosis (Altekruse *et al.*, 2006). However, unlike the results outlined in the

above mentioned studies, the results found in our study exhibited no link between human and chicken *Salmonella* Enteritidis isolates in Gauteng, South Africa.

3.3.4 *Salmonella* Heidelberg

Salmonella Heidelberg was one of the most frequently isolated serotypes (34%; 16/47) from chicken meat (Figure 3.5 and Figure 3.6). These chicken isolates were isolated from chicken meat which were purchased from various areas in the Johannesburg metropolitan (Soweto), the Ekurhuleni metropolitan (Tembisa) and the Tshwane metropolitan (Pretoria) in Gauteng. The majority (63%; 10/16) of the chicken isolates were resistant to chloramphenicol, sulfamethoxazole and tetracycline. PFGE analysis with *Xba*I digestion (Figure 3.5) and PFGE analysis with *Not*I digestion (Figure 3.6) showed that three chicken *Salmonella* Heidelberg isolates (TCD F18, TCD F19 and TCD F20) and two human *Salmonella* Heidelberg isolates (TCD 210902 and TCD 194954) had similar PFGE patterns. Human isolates were collected from patients in April and September 2007 from Hospital 174 which is located in the Tshwane metropolitan and from an unknown clinic in Gauteng, respectively. Three of the chicken *Salmonella* Heidelberg isolates (TCD F18, TCD F19, TCD F20) came from chickens purchased in November 2007 from the Tshwane metropolitan (Pretoria) and the Johannesburg metropolitan (Soweto). The current results suggest that these chicken and human *Salmonella* Heidelberg isolates (TCD F18, TCD F19, TCD F20, TCD 210902 and TCD 194954) were highly related based on our criteria for the determination of significant similarity between human and chicken isolates (See section 3.2.7). Chickens contaminated with this clone of

Salmonella Heidelberg may have been a food vehicle for human salmonellosis in Gauteng since April 2007 through to November 2007. Results shown in the current study are typical of those results previously reported in other studies which show a link between human and poultry *Salmonella* Heidelberg isolates (Berrang *et al.,* 2006; Chittick *et al.,* 2006; Andrysiak *et al.,* 2008). Berrang *et al.,* (2006) showed in their study that retail poultry is a common vehicle for spreading antibiotic resistant *Salmonella* Heidelberg to humans throughout Italy. Another study carried out in Canada by Andrysiak *et al.,* (2008) showed that *Salmonella* Heidelberg was rarely isolated from other food sources such as pigs and cows; however a large number of *Salmonella* Heidelberg isolates were recovered from retail chickens and were implicated as food vehicles in human illness. Chittick and colleagues (Chittick *et al.,* 2006) showed in their study that poultry and egg-related food products were common food vehicles for *Salmonella* Heidelberg causing several outbreaks of human salmonellosis in the USA since 1973 through to 2001.

3.3.5 *Salmonella* Hadar

Salmonella Hadar was one of the least frequently isolated serotypes (11%; 5/47) from chicken meat (Figure 3.7 and Figure 3.8). These chicken isolates were isolated from chicken meat which was purchased from two different areas in the Tshwane metropolitan (Pretoria). All the chicken isolates were resistant to tetracycline. PFGE analysis with *Xba*I digestion (Figure 3.7) and PFGE analysis with *Not*I digestion (Figure 3.8) showed that four chicken *Salmonella* Hadar isolates (TCD F13, TCD F14, TCD F15 and TCD F16) and one human *Salmonella* Hadar isolate (TCD

193687) had similar PFGE patterns. The human isolate was received in April 2007 from Hospital 46 which is located in the Ekurhuleni metropolitan. All four chicken specimens were purchased in November 2007 from two different areas in the Tshwane metropolitan (Pretoria). Our data suggest that the four chicken isolates (TCD F13, TCD F14, TCD F15, TCD F16) and one human *Salmonella* Hadar isolate (TCD 193687) are related based on our criteria for the determination of significant similarity between human and chicken isolates (See section 3.2.7). This strain of *Salmonella* Hadar may have been circulating in chickens from April 2007 to November 2007. Chicken flocks contaminated with *Salmonella* Hadar in the USA, were a major cause of human salmonellosis in 1988 (Sarwari *et al*., 2001). A decrease in *Salmonella* Hadar recovered from broiler chickens during the time period of 1990 to 1995 correlated with the decrease in *Salmonella* Hadar isolates recovered from humans in the USA. These results further suggested that consumption of broiler chickens contaminated with *Salmonella* Hadar may have been responsible for the majority of the human *Salmonella* outbreaks which occurred during this time period (Sarwari *et al.,* 2001). Previous studies were also able to show an epidemiological link between poultry and human *Salmonella* Hadar isolates using molecular techniques and hypothesized that poultry contaminated with *Salmonella* Hadar may have been a primary food vehicle for human salmonellosis in Spain in 1998 (Cruchaga *et al.,* 2001) and 2005 (Giannatale *et al.,* 2008) and in most European countries from 2000 to 2001 (Giannatale *et al.,* 2008). Results in our study are typical of those reported in the above mentioned studies (Cruchaga *et al.,* 2001; Sarwari *et al.,* 2001; Giannatale *et al.,* 2008).

3.3.6 *Salmonella* Typhimurium

Salmonella Typhimurium was one of the least frequently isolated serotypes (11%; 5/47) from chicken meat (Table 3.1). Chicken *Salmonella* Typhimurium isolates were isolated from chickens purchased from various regions in the Johannesburg metropolitan (Soweto) and the Ekurhuleni metropolitan (Tembisa). Two of the isolates were resistant to sulfamethoxazole and tetracycline, two were only resistant to sulfamethoxazole and the remaining isolate was susceptible to all six (ACSSuTNa) antibiotics.

PFGE analysis with *Xba*I digestion showed that two (TCD F3 and TCD F22) of the five chicken *Salmonella* Typhimurium isolates were not related to any human *Salmonella* Typhimurium isolate (Data not shown). Conversely, PFGE analysis with *Xba*I digestion showed that one of the five chicken isolates (TCD F23) was a 100% identical in PFGE profile to eight human *Salmonella* Typhimurium isolates (Data not shown). MLVA separated these eight human *Salmonella* Typhimurium isolates and one chicken *Salmonella* Typhimurium isolate into eight distinct MLVA types (10, 14, 21, 22, 23, 24, 25 and 26) (Figure 3.9). MLVA showed that chicken *Salmonella* Typhimurium isolate TCD F23 is different from all eight human *Salmonella* Typhimurium isolates at two or more loci. Therefore the chicken isolate was not related to any human isolate, as defined by our criteria for the determination of significant similarity between human and chicken isolates (See section 3.2.7).

For the remaining two chicken isolates (TCD F21 and TCD F34), their PFGE profiles were a 100% identical to the PFGE profiles of ten human isolates (Data not shown). MLVA was performed on all of the above isolates. MLVA separated these 12 isolates into four distinct MLVA types (14, 15, 20 and 27) (Figure 3.10). Two chicken *Salmonella* Typhimurium isolates (TCD F21 and TCD F34) and one human *Salmonella* Typhimurium isolate (TCD 226213) showed an identical MLVA type 20 (160-248-314-370-525). Chicken isolate TCD F21 was isolated from a chicken specimen purchased in February 2008 from the Ekurhuleni metropolitan (Tembisa) and chicken isolate TCD F34 was isolated from a chicken specimen purchased in April 2008 from the Johannesburg metropolitan (Soweto). The human isolate TCD 226213 was received in October 2007 from Hospital 49 which is located in the Tshwane metropolitan. These results suggest that the human and chicken *Salmonella* Typhimurium isolates are related and that this strain of *Salmonella* Typhimurium may have been circulating in chickens from October 2007 to April 2008. Previous studies performed in the USA (Wedel *et al.*, 2005), in the UK (Harnett *et al.*, 1998), in Canada (Zaidi *et al.*, 2007) and in Australia (Heuzenroeder *et al.*, 2004) have showed a link between human and poultry *Salmonella* Typhimurium isolates using molecular techniques and have implicated poultry as being a potential reservoir for human salmonellosis caused by *Salmonella* Typhimurium.



Figure 3.1 Dendrogram analysis of *Xba*I-digested DNA from 16 food *Salmonella* Infantis isolates and 6 human *Salmonella* Infantis isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: (Former mentioned antibiotics): A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible

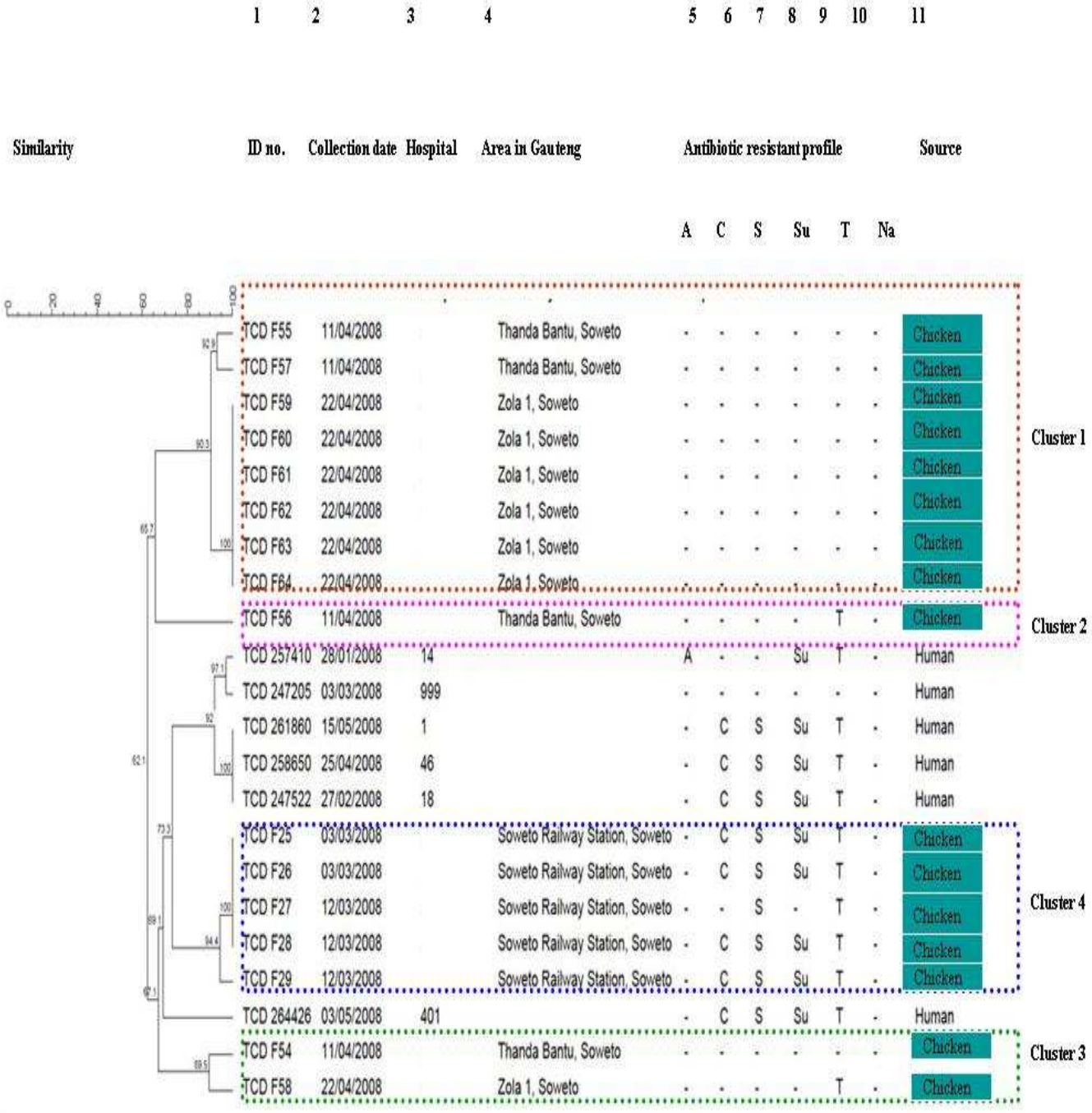


Figure 3.2 Dendrogram analysis of *Not*I-digested DNA from 16 food *Salmonella* Infantis isolates and 6 human *Salmonella* Infantis isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible

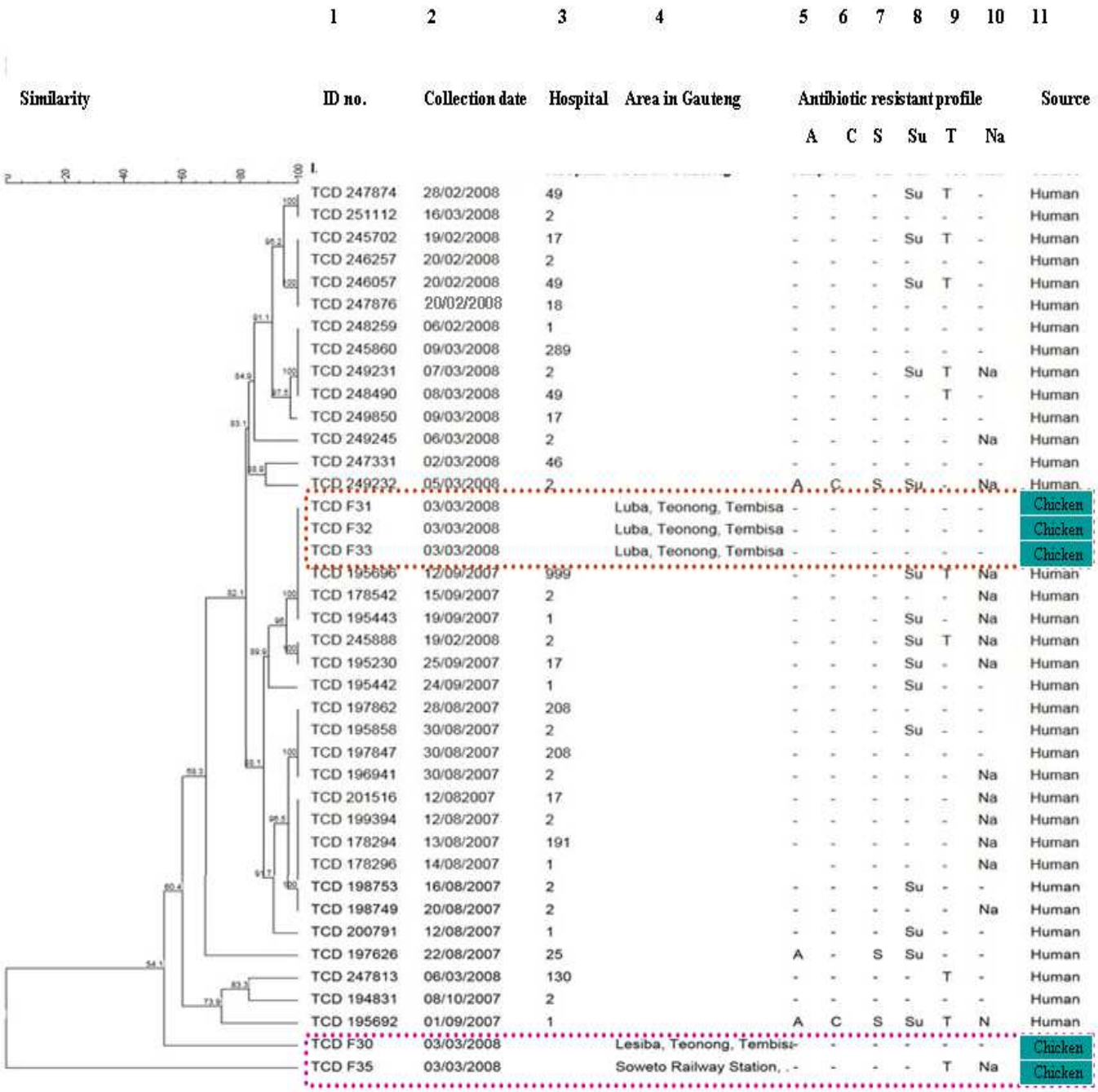


Figure 3.3 Dendrogram analysis of *Xba*I-digested DNA from 5 food *Salmonella* Enteritidis isolates and 35 human *Salmonella* Enteritidis isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible

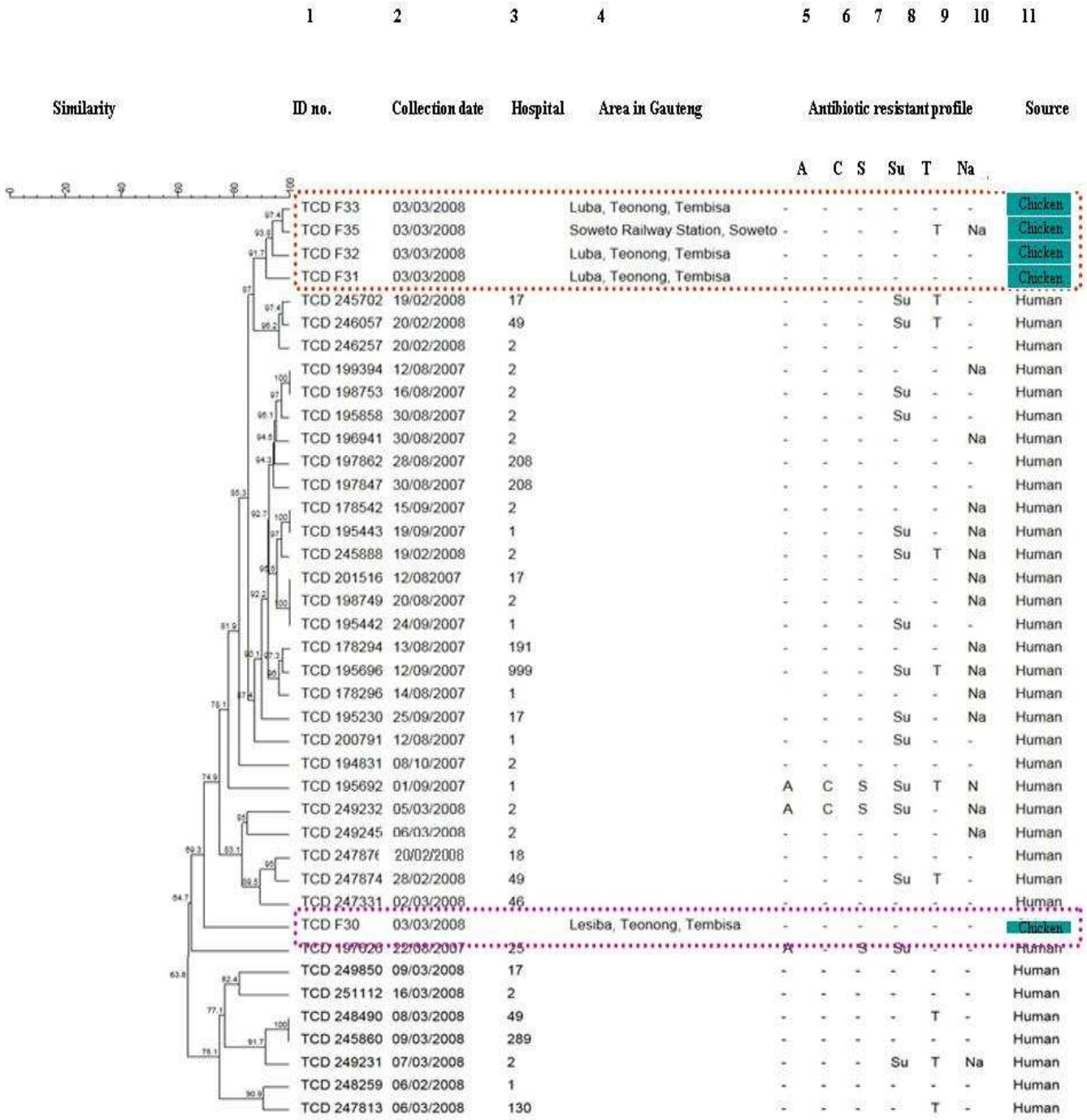


Figure 3.4 Dendrogram analysis of *Not*I-digested DNA from 5 food *Salmonella* Enteritidis isolates and 35 human *Salmonella* Enteritidis isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible.

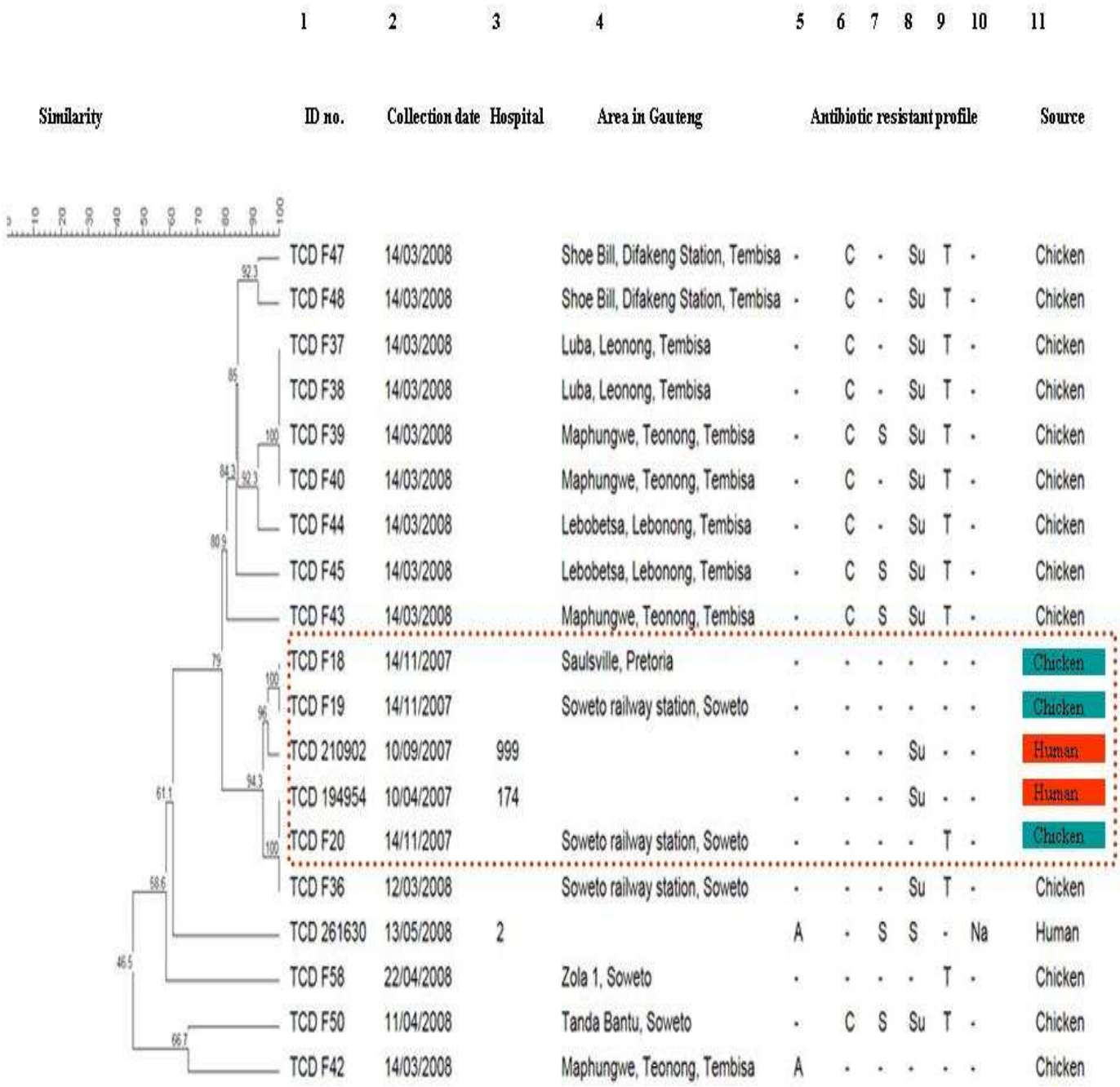


Figure 3.5 Dendrogram analysis of *Xba*I-digested DNA from 16 food *Salmonella* Heidelberg isolates and 3 human *Salmonella* Heidelberg isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible



Figure 3.6 Dendrogram analysis of *Not*I-digested DNA from 16 food *Salmonella* Heidelberg isolates and 3 human *Salmonella* Heidelberg isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible

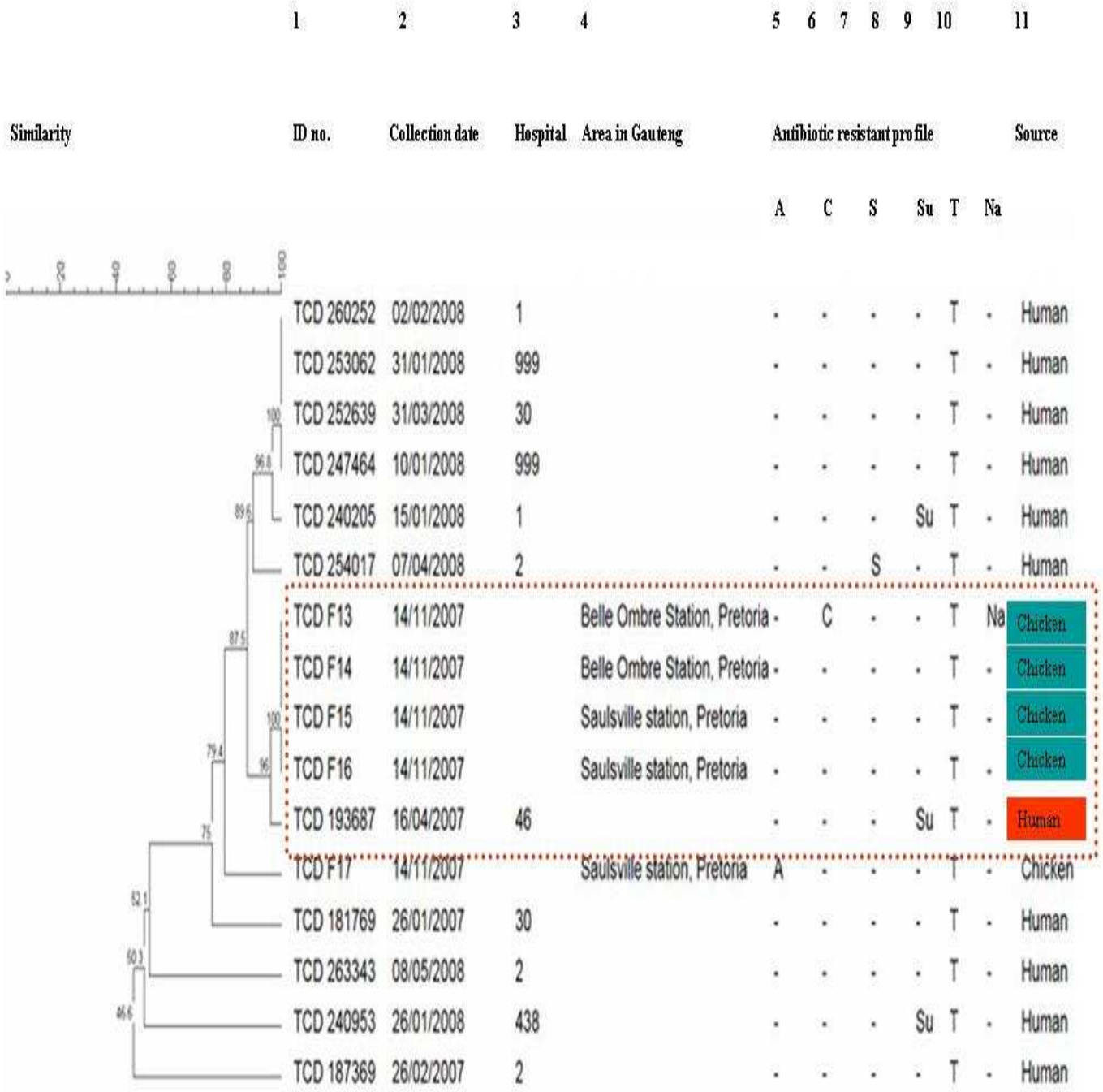


Figure 3.7 Dendrogram analysis of *Xba*I-digested DNA from 5 food *Salmonella* Hadar isolates and 11 human *Salmonella* Hadar isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible

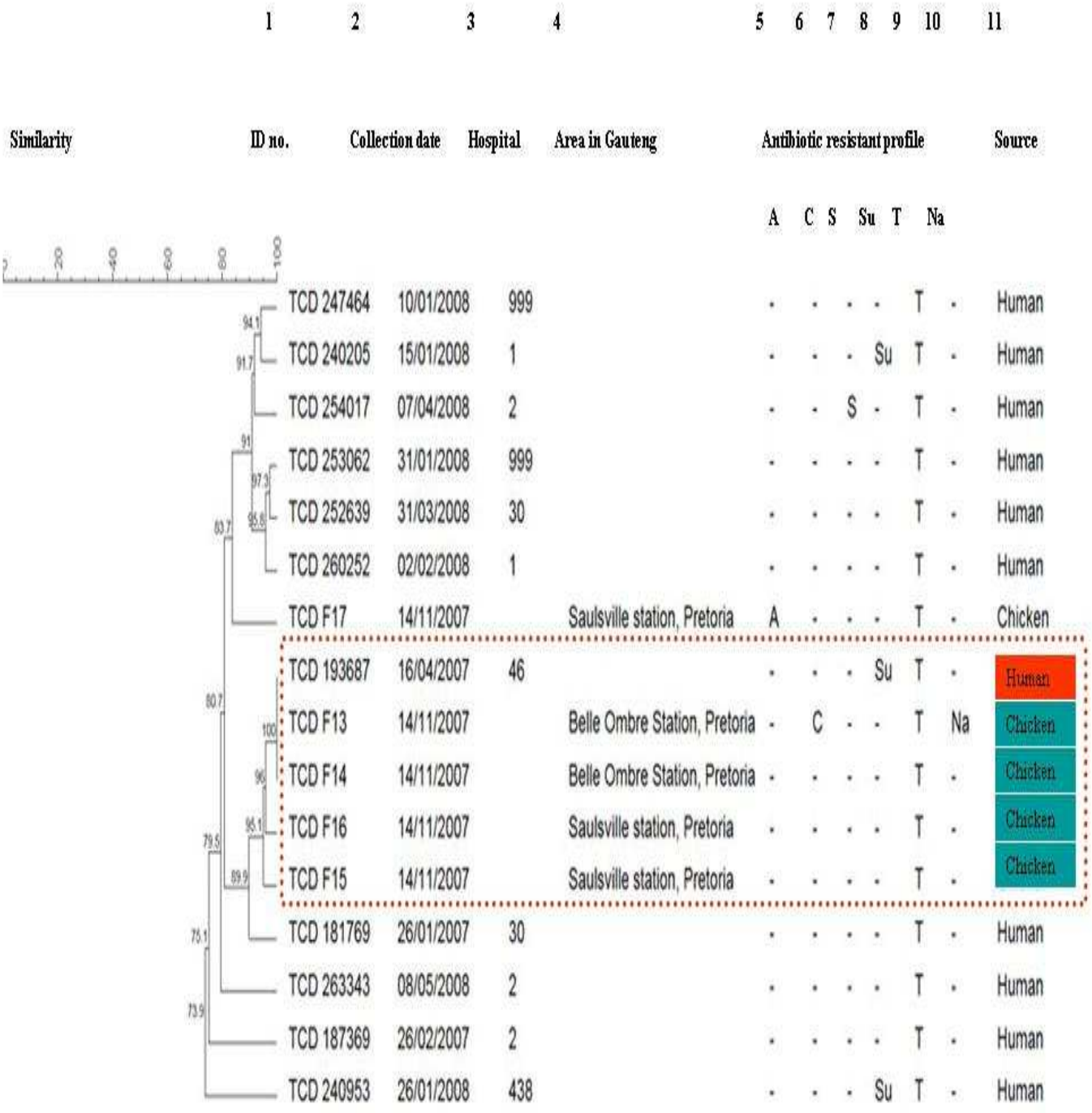


Figure 3.8 Dendrogram analysis of *Not*I-digested DNA from 5 food *Salmonella* Hadar isolates and 11 human *Salmonella* Hadar isolates separated by PFGE. Lanes 1: ID no, identification number. Lanes 5-10: A, Ampicillin; C, Chloramphenicol; S, Streptomycin; Su, Sulfamethoxazole; T, Tetracycline; Na, Nalidixic acid; -, susceptible

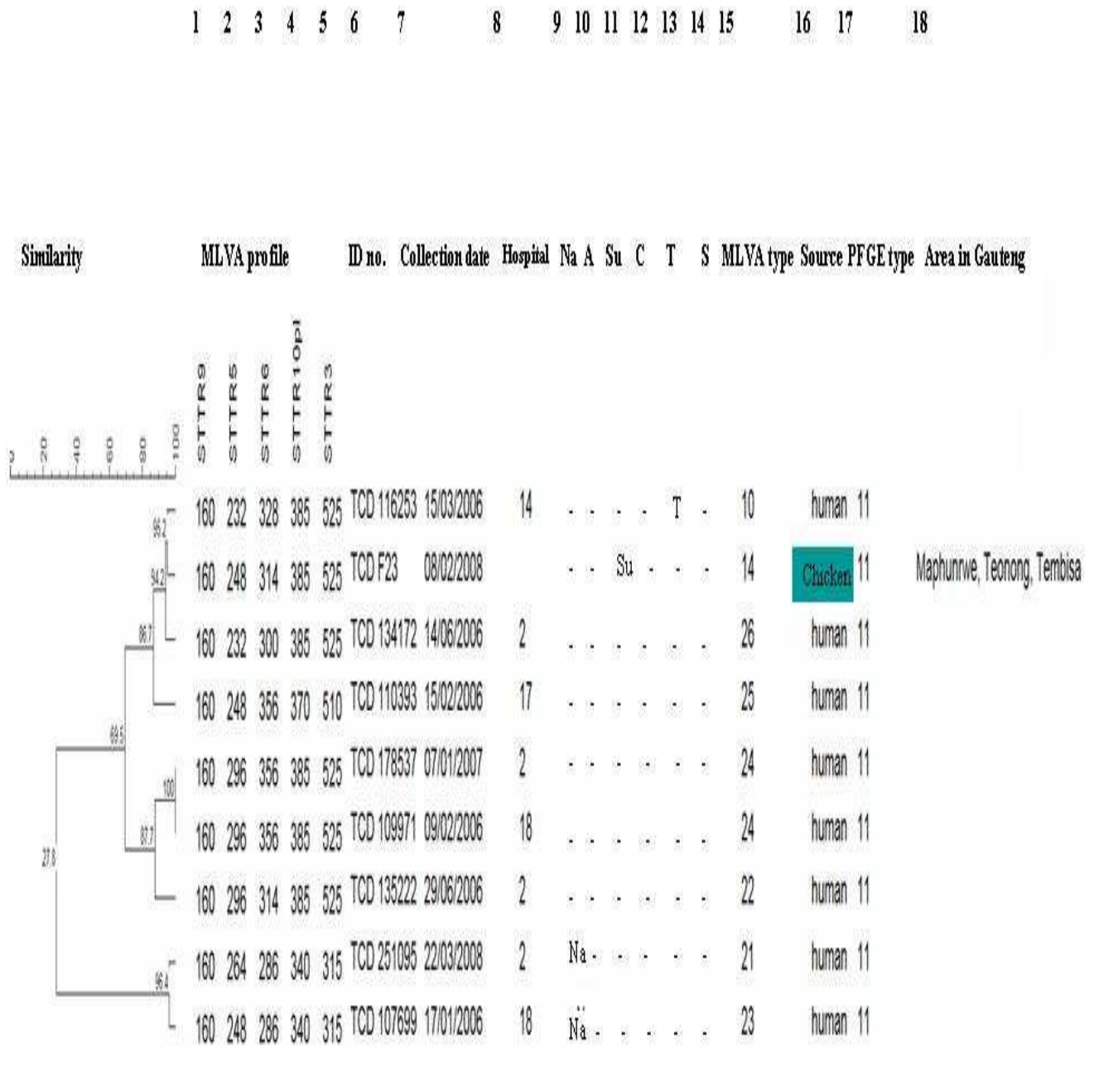


Figure 3.9 Dendrogram analysis of multiple-locus variable-number tandem-repeats (MLVA) of 8 human *Salmonella* Typhimurium isolates and 1 chicken *Salmonella* Typhimurium isolate. Lanes 1-5: MLVA profile in the order: STTR9-STTR5-STTR6-STTR10pl-STTR3. Lanes 6: ID no, identification number. Lanes 9-14: Na, Nalidixic acid; A, Ampicillin; Su, Sulfamethoxazole, C, Chloramphenicol; T, Tetracycline; S, Streptomycin; -, susceptible

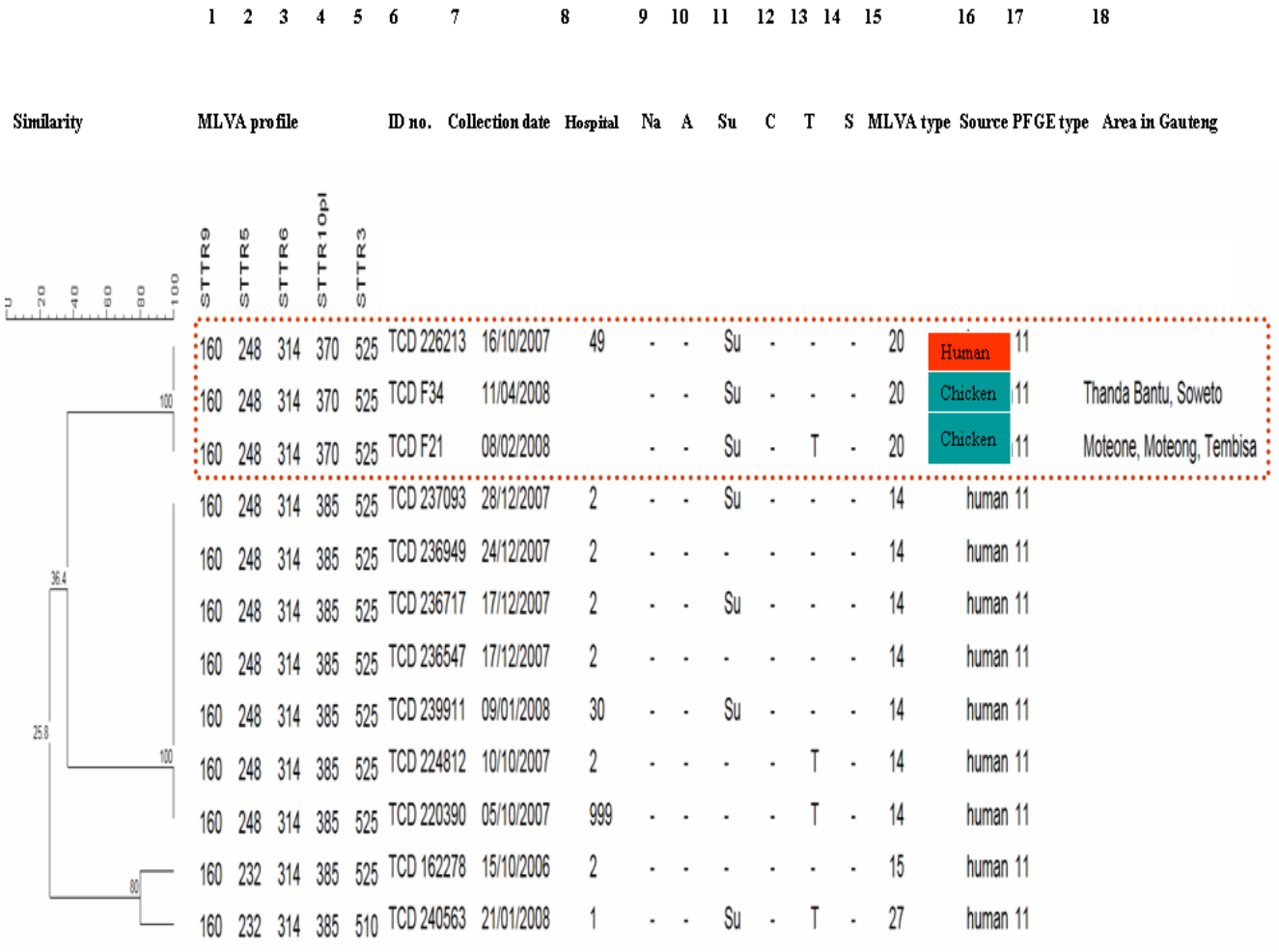


Figure 3.10 Dendrogram analysis of multiple-locus variable-number tandem-repeats (MLVA) of 10 human *Salmonella* Typhimurium isolates and 2 chicken *Salmonella* Typhimurium isolate. Lanes 1-5: MLVA profile in the order: STTR9-STTR5-STTR6-STTR10pl-STTR3. Lanes 6: ID no, identification number. Lanes 9-14: Na, Nalidixic acid; A, Ampicillin; Su, Sulfamethoxazole, C, Chloramphenicol; T, Tetracycline; S, Streptomycin; -, susceptible

Table 3.1 Characteristics of 5 *Salmonella* Typhimurium strains isolated from chicken meat

Identification number	Collection date	Area in Gauteng	Antibiotic resistance profile[a]	Related to any human isolates (PFGE analysis)	Related to any human isolate (MLVA)
TCD F3	16/10/2007	Inhlazane railway station, Soweto	susceptible	No	Not done
TCD F22	08/02/2008	Koti Street, Moteong, Tembisa	SuT	No	Not done
TCD F23	08/02/2008	Maphunwe, Teonong, Tembisa	Su	Yes	No
TCD F21	08/02/2008	Moteone, Moteong, Tembisa	SuT	Yes	Yes
TCD F34	11/04/2008	Thanda Bantu, Soweto	Su	Yes	Yes

[a] Su, Sulfamethoxazole; T, Tetracycline

CHAPTER 4

Conclusions

4.1 Molecular epidemiology of invasive isolates of *Salmonella enterica* serotype Typhimurium in Gauteng, South Africa, 2006-2008

The current study describes the epidemiology of invasive *Salmonella* Typhimurium isolates in the Gauteng Province of South Africa from January 2006 through to May 2008. The bacteria demonstrated an extensive genetic diversity as shown by PFGE analysis which was able to segregate 671 isolates into 38 clusters. MLVA was useful in distinguishing 21 invasive *Salmonella* Typhimurium isolates (that shared an indistinguishable PFGE pattern) into 11 distinct MLVA types. PFGE was useful for subtyping a large number of isolates and provided an overview of clonal relatedness between *Salmonella* Typhimurium isolates in the current study. Conversely, apart from PFGE analysis being a highly discriminatory method for the subtyping of salmonellae, there were a few potential drawbacks of this method. These limitations included the generation of ambiguous results due to gel variation and difficulty experienced during comparison of DNA fingerprint patterns as a result of differing gel quality and the subjective nature of band identification in a PFGE pattern. MLVA in comparison to PFGE analysis was a more discriminative subtyping method which was faster and data generated by MLVA was unambiguous, easier to analyze and interpret.

Most isolates showed resistance to multiple antibiotics. Most isolates were from patients aged between 15 and 64 years, while patients were mostly HIV-positive. The predominance of certain clusters (clusters 3, 5 and 11) within different hospitals in the Gauteng Province supports the premise that either patients or staff may have moved between hospitals over the period preceding or during the study. The occurrence of certain clusters over a prolonged period of time is cause for serious concern: it suggests that appropriate infection control measures have been lacking in those hospitals for extended periods or are only intermittently followed. In Gauteng, South Africa, invasive *Salmonella* Typhimurium remains an important opportunistic infection particularly associated with HIV-positive patients and is associated with nosocomial transmission. It is therefore necessary to improve primary antiretroviral treatment and subsequent prophylaxis for HIV-infected patients as well as the prevention and treatment of HIV related diseases which include salmonellosis. In addition, the current study necessitates for re-evaluation of infection-control procedures practiced by hospital staff in the Gauteng Province as well as the implementation of appropriate changes and conducting of further surveillance studies to prevent future nosocomial outbreaks caused by *Salmonella* Typhimurium in Gauteng.

4.2 Characterization of *Salmonella* isolates from chicken meat in Gauteng, South Africa, 2007-2008

The current study describes the characterization of salmonellae isolated from chicken meat in Gauteng, South Africa for January 2007 through to May 2008. PFGE analysis using restriction enzymes *Xba*I and *Not*I, suggest that *Salmonella* Infantis and *Salmonella* Enteritidis isolates recovered from humans are unrelated to chicken isolates. Conversely, PFGE analysis using the above mentioned restriction enzymes suggests that a strong correlation exists between some strains of human *Salmonella* Heidelberg and *Salmonella* Hadar isolates with those isolates sourced from chickens. Furthermore, PFGE analysis using restriction enzyme *Xba*I and MLVA suggests a strong correlation between some human and chicken *Salmonella* Typhimurium strains. MLVA was useful in distinguishing human and chicken *Salmonella* Typhimurium isolates that shared a common PFGE pattern. It is advisable that PFGE analysis be applied with a secondary genotypic method for obtaining more discriminatory results for future surveillance and control of salmonellosis in both humans and chickens in Gauteng, South Africa.

There were two potential limitations to the current study which include the fact that chicken samples were purchased from various regions in Gauteng from September 2007 through to April 2008 and therefore these results do not reflect a true number of *Salmonella* serotypes isolated from chicken meat during this time period. The other limitation was that no epidemiological investigations were conducted to substantiate an epidemiological link between human salmonellosis and foodborne salmonellae or

to estimate the contribution of chicken meat to human salmonellosis in Gauteng. Although the work conducted in the current study could not confirm that there is a link between foodborne salmonellae and human salmonellosis, it does show that *Salmonella* strains with comparable molecular profiles circulate in the animal and human communities, supporting the suggestion of either animal-to-human transmission or possibly human-to-animal transmission. Furthermore, foodborne diseases are historically not fully investigated in South Africa. The current study indicates that further investigation and improvement of the epidemiological techniques linking human infection and bacterial contamination of foodstuffs is warranted to confirm this theory.

APPENDICES

Appendices

Appendix A

Determining the O antigen group of a *Salmonella* species

- A single colony is inoculated onto a tryptose slope (Diagnostics Media Products) and incubated at 37°C overnight for 18 to 24 hours.
- The next day sterile normal saline (Diagnostics Media Products) is added to the tryptose slope and homogenized by vortexing.
- A loopful of this bacterial suspension is placed on a clean glass slide and examined for auto-agglutination.
- If auto-agglutination occurs then some of the *Salmonella* cells must be resuspended in saline and boiled for 20 to 30 minutes after which this suspension should be allowed to cool.
- Thereafter, serotyping should be repeated.
- If no agglutination occurs then add one drop of O polyvalent anti-sera (Statens Serum Institut and BioMérieux) to the bacterial suspension and mix by tilting the glass slide back and forth and observe for agglutination.
- If agglutination occurs in O polyvalent anti-sera then the bacteria are further tested with the appropriate O monovalent anti-sera (Statens Serum Institut and BioMérieux).

Determining the H antigen group of a *Salmonella* species

- A loopful of bacteria from the tryptose slope is inoculated onto the centre of a swarm agar plate (Diagnostics Media Products) which is then incubated at 37°C in an upright position for 18 to 24 hours.
- The next day a loopful of bacteria are picked up from the edge of bacterial growth on the swarm agar plate and placed on a clean glass slide, to which one drop of H polyvalent anti-sera is added (Statens Serum Institut and BioMérieux).
- Mix by tilting the glass slide back and forth to observe for agglutination.
- If agglutination occurs in H polyvalent anti-sera then the bacteria are further tested with the appropriate H monovalent anti-sera (Statens Serum Institut and BioMérieux).
- If agglutination occurs in H polyvalent anti-sera but no agglutination occurs in H monovalent anti-sera then it can be concluded that the *Salmonella* organism being tested contains more than one H phase.
- Swarm agar is autoclaved and cooled.
- A drop of the known H monovalent anti-sera is added to some cooled swarm agar in a petri-dish (Diagnostics Media Products) and mixed together by swirling the mixture in an “S” shape. This is done to block the known H-phase and allows the expression of the other H phase or H phases.

- The swarm agar mixture is then allowed to solidify. Once the gel is set, a loopful of bacteria from the tryptose slope is added to the centre of the swarm agar plate.
- The plate is then incubated at 37°C in an upright position overnight for 18 to 24 hours.
- Serotyping should be performed as described above.

Appendix B

Etests

Preparing an inoculum of *Salmonella* bacteria

- Subculture isolates on 5% blood agar plates (Diagnostics Media Products) and incubate the plates overnight at 37°C for 18 to 24 hours.
- The next day, pick three to five colonies from the incubated blood agar plate and resuspend in sterile saline (Diagnostics Media Products).
- The density of the culture should be adjusted to the turbidity reading that equals to the reading obtained by a 0.5 McFarland standard (Diagnostics Media Products).

Inoculating agar plates

- Dip a cotton wool applicator into the inoculum and remove excess fluid from cotton wool applicator.
- Mueller Hinton agar plates (Diagnostics Media Products) must be left to reach room temperature and must not have any moisture on agar surface before use.
- Streak the Mueller Hinton agar plates three times at 60° with the cotton wool applicator (containing the inoculum) by rotating the agar plate with the use of an AB Biodisk disk rotator (AB Biodisk).
- Leave the agar plates aside for ten to 15 minutes to allow for moisture to be absorbed.

- Use forceps to place Etest strips (AB Biodisk) on the surface of inoculated Mueller Hinton agar plates.
- Incubate inoculated plates at 37 °C for 16 to 20 hours.
- Breakpoints were read accordingly (Table 2.1).

Appendix C

Reagents and buffers

- Lysis buffer

  0.5 M EDTA (Sigma-Aldrich, Inc.), pH8

  1% N-lauroylsarcosine sodium salt (Sigma-Aldrich, Inc.)

  0.1 mg/ml proteinase-K (Roche Diagnostics GmbH)

- Suspension buffer

  100 mM Tris (Merck Chemical Ltd., Nottingham, England and Wales)

  100 mM EDTA (Sigma-Alrich, Inc.)

  pH 8

- Proteinase-K

  10 mg proteinase-K (Roche Diagnostics Gmbh)

  1 ml of TE buffer

- 10% SDS

  10 g sodium dodecyl sulphate (Merck Chemical Ltd.)

  100 ml deionized water

- 1% agarose gel (plugs)

  0.2 g agarose (SeaKem® Gold Agarose)

  20 ml TE buffer

  Boil to dissolve agarose, hold on a heating block set at 55°C

- 1% agarose gel

  1.5 g agarose (SeaKem® Gold Agarose)

  150 ml 0.5 x TBE buffer

  Boil to dissolve agarose, cool slightly and pour the gel into the agarose gel casting unit.

- 0.5x TBE buffer

  50 ml of 10x TBE buffer

  950 ml of deionized water

- Ethidium bromide staining solution

  250 ml of 0.5x TBE buffer (Merck Chemical Ltd.).

  25 µl of 10 mg/ml ethidium bromide (Merck Chemical Ltd).

- TE buffer

  10 mM Tris (Merck Chemical Ltd.)

  1 mM EDTA (Sigma-Alrich, Inc.)

  pH8

Appendix D

Isolation of salmonellae from chicken meat

Preenrichment

- Twenty-five grams of chicken meat was weighed out and placed in a sterile plastic bag.
- A small volume of sterile peptone water (Oxoid, Ltd., Basingstoke, Hampshire, England) was taken from a bottle containing 225 ml of sterile peptone water and added to the weighed test sample.
- The mixture was then homogenized for 30 seconds using a stomacher (Labotec, Italy) and then poured into the remaining buffered peptone water to make up a 1:10 dilution suspension preparation and thereafter it was incubated at 36°C for 24 hours.

Enrichment

- One milliliter of the incubated peptone water was added to 10 ml of SC broth (Diagnostics Media Products).
- One hundred microlitres of the incubated peptone water and 100 µl of 2 mg/ml of novobiocin stock (Sigma-Aldrich® Chemical Co., St. Louis, Missouri, USA) were added to RV broth (Diagnostics Media Products).
- SC broth and RV broth were then incubated for 24 hours at 36°C and 42°C, respectively.

- Thereafter a loopful of culture from SC broth and RV broth each were inoculated on XLD (Diagnostics Media Products) and mannitol lysine crystal violet brilliant green (MLCB) agar plates (Diagnostics Media Products).
- XLD and MLCB agar plates were incubated at 36°C for 24 hours.

Biochemical identification

- All presumptive *Salmonella* colonies that were oxidase negative were inoculated into Singer's broth and incubated at 36°C for four to five hours.
- If the singer's broth turned a green or yellow colour, the isolate was further tested using conventional stool sugars (Diagnostics Media Products).

Conventional stool sugars

- Organisms were identified as *Salmonella* species if their biochemical reactivity (when subjected to a series of biochemical tests) correlated with the biochemical reactions shown in Table 1.1.

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