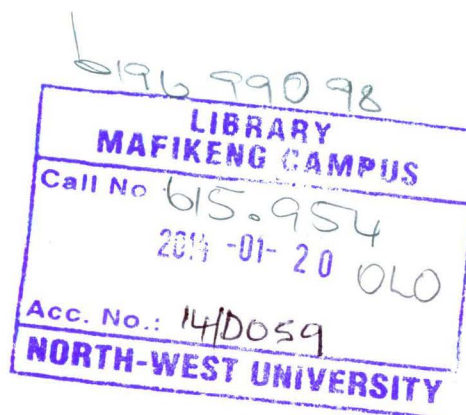


**SCREENING AND CHARACTERIZATION OF NON-TYPHOIDAL *SALMONELLA*
AND OTHER COLIFORMS ISOLATED FROM BROILER PRODUCTS IN THE
NORTH WEST PROVINCE OF SOUTH AFRICA**

by



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SUBMITTED: NOVEMBER, 2012



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Mafikeng Campus Library

DEDICATION

This work is dedicated to the Almighty God for His faithfulness and love towards me and my family.

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I'm grateful to the Lord God Almighty, the giver of wisdom and understanding, for His grace and protection upon me and my family throughout the period of this program. My gratitude also goes to the following persons:

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LIST OF ABBREVIATIONS

ACMSF	Advisory Committee on Microbiological Safety of Food
ASP	Acid shock proteins
ATCC	American Type Culture Collection
ATR	Acid tolerance response
bp	basepair
BPW	Buffered peptone water
cAMP	Cyclic adenosine monophosphate
CFU	Colony forming unit
ClpXP	Caseinolytic protease ATPases
CP	Chicken parts
CXC	Chemokines with the two N-terminal cysteines separated by an amino acid designated as 'X'
DNA	Deoxyribose nucleic acid
ESBL	Extended-spectrum β -lactamase
Etc	et cetera
EU	European Union
GERM-SA	Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa
H	Flagellar antigen
HACCP	Hazard analysis critical control point
HIV	Human immunodeficiency virus
HSP	Heat shock proteins
MAR	Multiple antibiotic resistance
NA	Nutrient agar
NB	Nutrient broth
NICD	National institute for communicable diseases
NTS	Non-typhoidal <i>Salmonella</i>
NWP	North West Province
O	Somatic antigen

OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Percentage hydrogen
RAPD	Random amplification of polymorphic DNA
Rev	Revolutions
RNA	Ribonucleic acid
RTE	Ready-to-eat
RV	Rappaport Vassiliadis
SCFA	Short chain fatty acids
SCV	<i>Salmonella</i> containing vacuole
SDS	Sodium-dodecyl sulphate
TSI	Triple sugar iron
TTSS	Type III secretion system
UPGMA	Unweighted pair group method with arithmetic averages
WBC	Whole broiler carcass
WHO	World health organisation
XLD	Xylose-lysine-desoxycholate

ABSTRACT

Non-typhoidal *Salmonella* (NTS) organisms are an important causes of food borne infections worldwide, with highest prevalence in the sub-Saharan Africa. Contaminated meat and poultry products, among many other factors, have been documented as the major sources of infection. The disease burden is further compounded by the prevalence of multi-drug resistant strains of the organisms, resulting in failures in empirical antimicrobial treatments. Other members of *Enterobacteriaceae* family (pathogenic or not) have also been indicated to contribute significantly to the spread of antimicrobial resistance genes, particularly within the family. It has therefore become imperative to control the contamination of meat and poultry products by NTS serotypes and other coliforms. The effectiveness of this process however, depends on a proper definition of the risk analysis framework, beginning with risk assessment. In the North West Province (NWP) of South Africa, there have been reports of food borne illness outbreaks that were associated with consumption of poultry products, and in which cases NTS have been isolated. adequate data on the prevalence and characteristics of NTS serotypes in poultry products is lacking. Studies were therefore conducted, to evaluate the incidence of NTS serotypes and other coliform contaminants in raw and ready-to-eat (RTE) broiler products in the NWP of South Africa, and to characterize the isolates based on antimicrobial susceptibility and stress response.

In the first study, raw and RTE broiler products, obtained from the NWP of South Africa, were screened for NTS contamination. A total of 120 raw broiler samples and 60 samples of polonies and smoked viennas were obtained from retail points in major cities and towns of the province. Samples were subjected to aerobic plate count and later screened for the presence of NTS using phenotypic and genotypic techniques. Average bacterial count in raw products was 3.1×10^5 cfu/g whereas bacterial contamination of RTE products was 1.8×10^3 cfu/g. The rate of contamination in both products is however, within the recommended safety limits for some developed countries. Samples obtained from butcherries had higher bacterial counts (2.2×10^5 cfu/g) than samples obtained from supermarkets (1.4×10^4 cfu/g). Similarly, raw chicken portions were more contaminated (7.6×10^5 cfu/g) than whole chicken carcasses (2.5×10^5 cfu/g). The average recovery rate of NTS species from raw broiler products was 12.5% and the

serotypes identified were *S. typhimurium* (46.4%), *S. enteritidis* (30.9%) and *S. newport* (22.9%). No NTS was recovered from the RTE products. Significant difference in the rate of *Salmonella* isolation was noticed between locations ($P = 0.009$), with Mafikeng having the highest isolation rate (18.2%). Furthermore, incidence of *Salmonella* contamination was higher in butchery (32%) than supermarket samples (18%).

The rate of contamination of whole broiler carcasses was higher (30%) than that of chicken portions (20%). There were significant differences in the rate of isolation of the various serotypes from product types ($P = 0.05$). *S. typhimurium* was the predominant serotype in whole carcasses whereas *S. enteritidis* and *S. newport* were prevalent in chicken portions. Out of the 160 presumptive *Salmonella* isolates screened by PCR, 140 (87.5%) were confirmed for the presence of *Salmonella*-specific *invA* gene. In addition, 115 (82.4%) of the confirmed isolates harboured the plasmid *spvC* gene. RAPD fingerprinting of isolates using RAPD 1 and RAPD 3 primers revealed some inter- and intra-serotype genetic diversity among isolates, suggesting varying sources of contamination. No NTS was recovered from the RTE products. This study indicates that raw broiler products from the NWP are contaminated with NTS serotypes and therefore could be a potential vehicle of NTS food-borne infections.

In the second study, 60 representative isolates (20 isolates from each serotype) were evaluated for antimicrobial resistance by disk diffusion method, using a panel of ten antibiotics. The antimicrobials used were ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), trimethoprim-sulphamethoxazole (25 µg), tetracycline (30 µg), cefotaxime (30 µg), meropenem (10 µg), gentamicin (10 µg) and erythromycin (15 µg). Isolates that were resistant to ampicillin ($n = 48$), tetracycline ($n = 60$) and chloramphenicol ($n = 12$), common antibiotics used for treatment of invasive salmonellosis were further screened by PCR for antibiotic resistance genes, targeting the *bla*TEM, *tet* and *cat* genes. All the strains tested were resistant to two or more antibiotics. However, all isolates were susceptible to cefotaxime, meropenem, gentamycin and amikacin whereas all were resistant to tetracycline. Resistance to trimethoprim-sulphamethoxazole, ciprofloxacin and chloramphenicol was low, being 8.3%, 13.3% and 20% respectively. Multi-drug resistance was discovered only in nine *S. typhimurium* strains, representing 15% of the tested isolates. Analysis of multiple antibiotic resistance (MAR),

revealed seven phenotypes with resistance to ampicillin, tetracycline and erythromycin as the most common pattern. In addition, the blaTEM gene fragment was identified in 15 (31.3%) of the isolates screened, whereas the *tet* and *cat* genes were found in 12 (20%) and 6 (50%) of tetracycline and chloramphenicol-resistant isolates respectively. The observations of this study indicate that NTS strains isolated from broiler products are resistant to multiple antibiotics, including quinolones. This may constitute significant public health problem.

Furthermore, the ability of NTS serotypes to survive stress conditions, such as those encountered during food preparation, is an attribute of direct influence on both survival and virulence of the organisms. Therefore, in the third study, *S. typhimurium*, *S. enteritidis* and *S. newport* isolates obtained from the raw broiler products, were evaluated for response to thermal, acidic and osmotic stresses. The generation time of the isolates was initially determined. Thereafter, isolates were exposed to thermal stress (50°C, 55°C and 60°C), rosemary vinegar (6% acidity, used to adjust growth media to pH of 2.0, 3.0, and 4.0) and NaCl (1M and 2M). The maximum period of exposure was 60 min during the log phase of growth. The average generation time obtained for the isolates was 38 min. Rosemary vinegar at pH 3.0 reduced colony counts by 44.5% after 30 min of acid exposure whereas counts were reduced to zero at pH 2.0 during the same period. Exposure of isolates to a temperature of 50°C showed significant decline of 25.3% in colony counts in 10 min. This was followed by 9.2% ($P = 0.6$) and 30.5% ($P = 0.03$) increase in counts at 20 and 30 min respectively. However, when isolates were exposed to 55°C or 60°C for 20 or 10 min, colony counts were reduced to zero. Adjustment of growth media with 2M NaCl showed a reduction of 3.3% in log cfu of isolates. This *in-vitro* study indicates that the NTS serotypes isolated from the NWP can survive treatment with NaCl, and are only sensitive to 6% rosemary vinegar (adjusted to pH 2.0) and heating at 55 or 60°C for 20 or 10 min respectively. Thus, the latter two treatments may be employed as effective strategies to control NTS in contaminated raw chicken products from slaughterhouse to table.

In the fourth study, both raw and RTE broiler products (a total of 180 samples) were screened for the presence of other members of the family *Enterobacteriaceae* (coliforms). Coliform species were identified by correlating cultural characteristics on MacConkey and XLD agars and biochemical reactions on Enteropluri-test®. Identified isolates were then tested for antibiotic

resistance by disk diffusion method using ampicillin (10 µg), ciprofloxacin (5 µg), amikacin (30 µg), trimethoprim-sulphamethoxazole (25 µg), cefotaxime (30 µg), meropenem (10 µg), gentamicin (10 µg) and erythromycin (15 µg). Results obtained showed that 75% (n = 90) of raw and 25% (n = 15) of RTE products were contaminated with various genera of coliforms. In raw products, the genera identified include *Enterobacter* (n = 34), *Klebsiella* (n = 13), *Citrobacter* (n = 6), *Serratia* (n = 14), *Hafnia* (n = 9), *Escherichia* (n = 11), *Yersinia* (n = 1) and *Proteus* (n = 2) whereas *Klebsiella* (n = 3), *Serratia* (n = 11) and *Stenotrophomonas* (n = 1) were recovered from RTE products. In general, two to four antimicrobial resistance phenotypes were observed among the isolates. A high frequency of resistance was manifested to ampicillin (93.6%) and erythromycin (97.9%) whereas resistance to other antibiotics tested was moderate to low (27.7% and 6.4% respectively). However, *Yersinia pseudotuberculosis* (from raw chicken) and *Stenotrophomonas maltophilia* (from RTE) were both resistant to seven out of the eight antibiotics tested, and were only susceptible to ciprofloxacin and trimethoprim-sulphamethoxazole respectively. This study indicates that both raw and ready-to-eat poultry products are contaminated with various coliform species, some of which are resistant to easily accessible antibiotics used in the treatment of human infections. There is therefore a need to establish effective strategies to control these resistant pathogens particularly in RTE products, to which no further preparation is applied before consumption.

These studies therefore represent the first reports on the incidence, stress response and antimicrobial resistance patterns of NTS strains isolated from raw chicken products in the NWP of South Africa. It is also the first report on the isolation of coliforms, particularly *Stenotrophomonas maltophilia* from RTE products in the province. However, observations of this study may be a reflection of poor hygienic conditions in the processing environments, or probably as a result of some post-processing contaminations of products. Thus the levels of distribution and consumption of poultry products in the NWP somewhat calls for establishment of good hygiene practices from farm to fork, in order to prevent the spread of resistant pathogens to the human community through foods of animal origin.

Key words: Non-typhoidal *Salmonella*, stress, antimicrobial, coliforms, chicken, NWP

PREFACE

For the purpose of clarity, the four sections of research included in this thesis are presented in article format. The four study units are amended with general introduction, literature review of related works and conclusion and recommendation chapters.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General background

Chicken meat remains the most affordable protein source relative to other meat protein sources, with a growing trend towards processed chicken meat particularly in South Africa (Vanquelin, 2009). In recent years, South Africa has experienced a tremendous growing demand of broiler meat, such that total potential broiler production for the year 2008 was 920.4 million units. The nation's poultry industry thus accounts for an estimated 16% of the total gross value of primary agriculture (GAIN, 2007). Part of the rise in chicken meat consumption seen in the last few years results from South Africa's steady economic growth and rising standards of living. These factors have equally fuelled the growth of foodservice markets with the establishment of quicker service restaurants as more consumers are pushed towards convenience. Thus demand factors have driven the growth of poultry industry, making it a big business in South Africa (GAIN, 2007). However, poultry meat and eggs have been rated globally as one of the major food items associated with food-borne illnesses, particularly salmonellosis (Rodrigo *et al.*, 2006).

Salmonellosis, which is characterized mainly by acute gastroenteritis with occasional complications of bacteremia, is caused by different serovars of *Salmonella* species. Some of the *Salmonella* serotypes are host-adaptive (e.g. *S. gallinarum* and *S. pullorum* in birds and *S. typhi* in humans) whereas others (non-typhoidal species) affect a wide variety of hosts. Although *Salmonella* is ubiquitous, the intestinal tract of animals remains the primary reservoir of non-typhoidal serotypes, particularly with the advent of intensive animal production systems which favour colonisation (Antunes *et al.*, 2003; Cooke *et al.*, 2007). The most commonly implicated source of non-typhoidal salmonellosis in humans is handling of raw poultry or consumption of undercooked poultry and poultry products (Foley *et al.*, 2008). Reports from previous studies indicate the presence of non-typhoidal *Salmonella* (NTS) infections in South Africa. For instance, *Salmonella heidelberg* was incriminated in a food-borne illness outbreak that was reported in Mpumalanga Province in August 2010 (NICD, 2010). Similarly, an autopsy study

carried out on 50 patients who died with a pre-mortem clinical diagnosis of tuberculosis in South Africa showed that about 94% of the patients were HIV-positive and 23% of the HIV-positive patients were harbouring splenic NTS (Martison *et al.*, 2007). Kidanemariam *et al.* (2010) recently identified *S. heidelberg* as the 6th most common NTS serotype isolated from food animals in South Africa with poultry accounting for 99% of the isolates. These reports indicate that NTS is present in South Africa and may constitute a public health burden. Given the rate of processing and consumption of poultry products in the country, and the level of risk associated with such activities, adequate knowledge of prevalence of *Salmonella* in chicken products has become very necessary. This calls for defining a risk analysis framework which begins with risk assessment. A few investigations have been conducted in South Africa to ascertain the contamination of chicken meat from retail stores with various pathogenic bacteria including *Salmonella* (van Nierop *et al.*, 2005). However, despite the fact that the NWP is one of the provinces with the largest production and distribution of broilers in the country, the incidence of NTS in its broiler products has not been documented. . This study therefore constitutes a valuable tool in risk analysis and strategic planning for the control of human salmonellosis in the NWP.

1.2 Literature Review

1.2.1 Introduction

Poultry meat and eggs are still one of the cheapest sources of animal protein for the majority of South Africans (Gericke, 2009). Consequently, the broiler industry continues to dominate the agricultural sector as the main supplier of animal protein in South Africa, with the largest distribution (25%) in the NWP (SAPA, 2010). The local consumption of poultry meat in 2009 accounted for approximately 56% of total meat consumption in South Africa, out of which 15% consisted of broiler imports (TAS, 2010). Furthermore, *per capita* consumption of commercially produced chicken meat in South Africa increased from 21.7 kg/person in year 1997 to 31 kg/person in year 2010 (Mons, 2010). Figs. 1.1 and 1.2 show *per capita* meat consumption in South Africa and the provincial distribution of broilers. Recognized as an important public health problem, salmonellosis is one of the most frequent food-borne diseases in almost all

industrialized countries, with reports of significant increase in its incidence in the last few decades (D'Aoust, 1997; Antunes *et al.*, 2003).

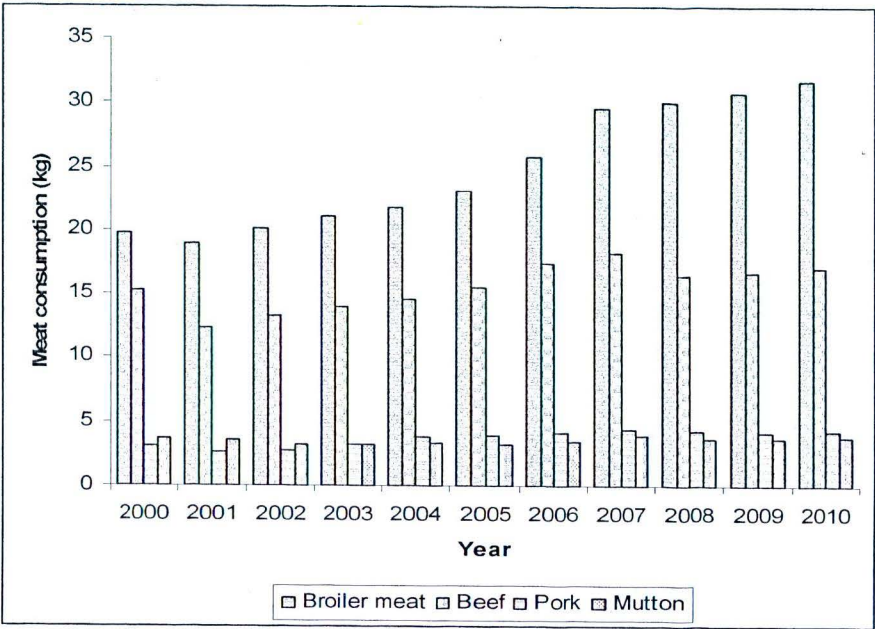


Figure 1.1: *Per capita* consumption of meat (kg) in South Africa over a ten year period (Adapted from Mons, 2010)

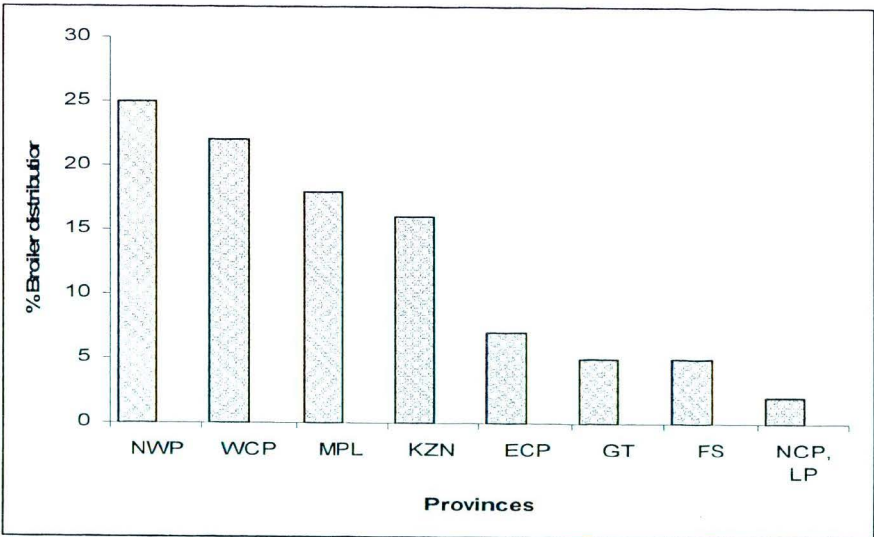


Figure 1.2: Provincial distribution of broilers in South Africa (SAPA, 2010). NWP = North West Province, WCP = Western Cape Province, MPL = Mpumalanga, KZN = KwaZulu-Natal, ECP = Eastern Cape Province, GT = Gauteng, FS = Free State, NCP, LP = Northern Cape Province, Limpopo

1.2.2 *Salmonella*

Salmonella organisms are Gram-negative rod-shaped bacteria belonging to the family *Enterobacteriaceae* and genus *Salmonella* (Grimont *et al.*, 2000). They are non-encapsulated bacteria and are either obligate aerobes or facultative anaerobes. Their optimum temperature for growth is 35 to 43°C while the optimum pH is 6.6 to 8.2 with a water activity above 0.94 (Hanes, 2003). Most strains of *Salmonella* species are motile with the aid of flagella (Fig. 1.3) except a few host-specific serotypes such as *S. gallinarum* and *S. pullorum* (Grimont *et al.*, 2000). *Salmonella* organisms are usually catalase positive and oxidase negative. They are also urease and indole negative, glucose and dulcitol fermenters and produce hydrogen sulphide gas (Grimont *et al.*, 2000). Most non-host specific strains of *Salmonella* are prototrophic whereas the host-adapted and/or mutant strains are usually auxotrophic particularly for aromatic compounds (Grimont *et al.*, 2000; Pounikar and Dawande, 2010).

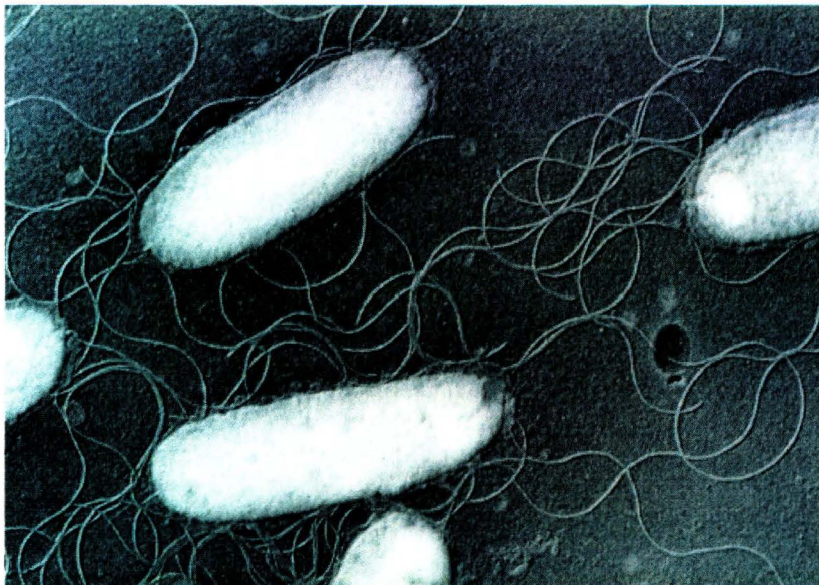


Figure 1.3: Flagellated *Salmonella* species (Adapted from Rao *et al.*, 2004)

1.2.3 History and taxonomy of *Salmonella*

In 1880, Eberth first observed typhoid bacillus in spleen sections and mesenteric lymph nodes of a patient who died of typhoid and in 1884, Graffky cultivated the typhoid bacillus (Le Minor, 1994). In 1886, the organism, then known as *Salmonella choleraesuis* was isolated from pigs by

Salmon and Smith when it was suspected to be the cause of swine fever (Grimont *et al.*, 2000). At this early stage, strains of *Salmonella* isolated from different clinical cases were thought to be different species and were thus given different names. However, the close DNA-relatedness among the *Salmonella* serotypes has been revealed through recent studies. Thus the existence of a common ancestor of the genus, about 25 – 40 million years ago, can be estimated (Baumler *et al.*, 1998). Later, *Salmonella* serovars were named on the basis of animal species affected and then on geographical location (Tindall *et al.*, 2005). However, Kauffmann in 1966, grouped the genus *Salmonella* into four subgenera viz: *S. kauffmannii* (group I), *S. salamae* (group II), *S. arizonae* (group III) and *S. houtanae* (group IV) based on biochemical characterization (Popoff *et al.*, 2003). This grouping was somewhat limited by the genetic relatedness among the four groups.

Thereafter, Le Minor and Popoff (1987) proposed the name *S. enterica* for the single *Salmonella* species, with about seven subspecies viz: *S. enterica* subsp *enterica*, *S. enterica* subsp *salamae*, *S. enterica* subsp *arizonae*, *S. enterica* subsp *diarizonae*, *S. enterica* subsp *houtanae*, *S. enterica* subsp *bongori* and *S. enterica* subsp *indica*. The proposal requested an opinion which has not been awarded by the Judicial Commission of the International Committee of Systematic Bacteriology (Tindall *et al.*, 2005). Currently, two species of the genus *Salmonella* are recognized, namely *S. enterica* and *S. bongori*, based on taxonomic definition of genomic species (Reeves *et al.*, 1989; Hohmann, 2001). In this nomenclature, serovar names are no longer written as species names. For example, *S. typhimurium* is written as *S. enterica* subsp *enterica* serovar Typhimurium (abbreviated as *S. Typhimurium*). Antigenic factors (O-somatic, Vi-capsular and H-flagellar antigens) enabled further subdivision of *S. enterica* into six subspecies with more than 2,500 serotypes now identified within the species (Bennasar *et al.*, 2000; Callaway *et al.*, 2008).

1.2.4 *Salmonella* detection methods

For decades, detection of *Salmonella* in various types of samples has been by phenotypic identification based on cultural isolation on different types of media and biochemical reactions (Andrews, 1996). The results were often based on colony morphology and reactions with

different sugars. This method is usually time consuming and takes about 4 - 7 days for results to be obtained (van der Zee and Huis in't Veld, 2000). The results were also sometimes confusing as some members of the family *Enterobacteriaceae* exhibit morphology and biochemical reactions that are similar to those of *Salmonella* (Waltman, 2000). Regardless of the source of samples, steps involved in *Salmonella* isolation include i) direct culture particularly from clinical disease situations ii) non-selective pre-enrichment in media such as buffered peptone water iii) selective enrichment in Rappaport Vassiliadis broth iv) inoculation on plating media such as Xylose-lysine-desoxycholate and v) biochemical and serological characterization (Waltman, 2000). Serotyping however is the method commonly used to differentiate strains of *Salmonella* using *Salmonella* polyvalent somatic (O) and flagellar (H) antisera (Yan et al., 2003). The conventional serotyping was later found to have some limitations, such as limitations in detection of Vi antigens (Wain *et al.*, 2005) and strains which are not typeable (Rasschaert *et al.*, 2005). Alternative methods were therefore introduced with the aims of reducing the analytical time, savings in staff time and media requirements and improving overall performance of the *Salmonella* detection procedure. Some of the alternative methods include immunological and nucleic acid-based (PCR) assays. The latter, combined with automated instrumental techniques form the basis of current *Salmonella* monitoring systems (van der Zee and Huis in't Veld, 2000)

1.2.5 Host specificity of *Salmonella*

Although most *Salmonella* serotypes infect both man and animals, they can be divided into host-specific, host-restricted and generalist serotypes based on the degree of host adaptation (Hoelzer *et al.*, 2011). Host-specific serotypes cause disease in one host species (e.g. *S. typhi* in human) whereas the host-restricted serotypes predominantly cause disease in one species but can also infect other species (e.g. *S. dublin* in cattle). Infections with either host-specific or host-restricted serotypes are usually systemic and marked with low morbidity and high mortality. On the contrary, the generalist serotypes (e.g. *S. typhimurium*) usually cause disease in a wide range of hosts (Uzzau *et al.*, 2000), with gastroenteritis as the predominant symptom. Morbidity is usually high with low mortality.

1.2.6 Salmonellosis

Salmonellosis is a bacterial infection of both humans and animals caused by various strains of *Salmonella* species. In chickens, the disease occurs in two forms viz: true avian salmonellosis (fowl typhoid and pullorum) caused by host-specific *Salmonella gallinarum* and paratyphoid or non-typhoidal salmonellosis caused by various serotypes of *Salmonella spp* (Breytenbach, 2004). The latter form is of both economic and public health importance. Similarly, human typhoidal salmonellosis is caused by host-specific *S. typhi* and *S. paratyphi* with enteritis as the major clinical symptom. The major route of transmission of human typhoidal salmonellosis is fecal-oral via contaminated food and water (Cooke *et al.*, 2007). The World Health Organisation estimates global incidence of typhoid fever to be 22 million cases resulting in 200,000 deaths annually (Crump *et al.*, 2004). Non-typhoidal salmonellosis may occur subclinically in birds, but may also affect animal performance and provoke morbidity and mortality particularly in chicks (Callaway *et al.*, 2008). Most importantly, non-typhoidal *Salmonella spp* are able to colonise the gastrointestinal tract of chickens, leading to carcass contamination. The end result is onward transmission of the pathogens to human consumers through the contaminated carcasses, resulting in human non-typhoidal salmonellosis (Berchieri *et al.*, 2000). In the United States, approximately 95% of cases of human salmonellosis are associated with the consumption of contaminated products such as poultry meat (Foley and Lynne, 2008).

Non-typhoidal *Salmonella* (NTS) infections cause four clinical syndromes in humans which include diarrhoeal disease (gastroenteritis), invasive bacteremic illness, focal suppurative infection and asymptomatic carrier in the stool (Gordon, 2008). In immunocompetent individuals, the disease may be restricted to self-limiting diarrhea that does not require hospitalization. However, immunocompromised patients may present primary bacteremia or other extraintestinal forms with high morbidity and mortality (Dhanoa and Kia, 2009). The gastroenteritic disease is usually characterized by fever, abdominal cramps, nausea, vomiting and watery diarrhoea which can sometimes contain blood and mucus. Clinical manifestations of the disease can set in as early as 6 to 72 hours post exposure to *Salmonella*, and can last for 4 to 7 days (Zand *et al.*, 2003).

1.2.7 Epidemiology of human salmonellosis

Salmonella infections continue to have a serious impact on human and animal health globally, causing significant morbidity and mortality with serious socioeconomic effects (Cooke *et al.*, 2007). Human NTS infections thus constitute a major public health problem and represent a huge cost for many countries. The World Health Organisation (WHO) estimated an annual incidence of 1.3 billion cases of diarrhea due to NTS, resulting in about 3 million deaths (Maripandi and Al-Salamah, 2010). An annual estimate of 1.4 million cases of human NTS infections resulting in 582 deaths have been reported in the U.S. (McDermott, 2006). A comparative study in U.K. revealed an annual estimate of 41,616 cases resulting in 119 deaths (Adak *et al.*, 2002). A total of 154,099 cases of human salmonellosis were reported by the 25 EU Member States in 2004 (EFSA, 2009), with the major sources being egg and poultry meat.

The endemicity of invasive NTS infections to the sub-Saharan Africa has been established by several studies (Morpeth *et al.*, 2009). For instance, NTS infection case-fatality estimates of 4.4% – 27% for children and 22% – 47% for adults among hospitalized patients in Africa have been documented (Gordon *et al.*, 2002; Brent *et al.*, 2006). Non-typhoidal *Salmonella* have been isolated from up to 35% of HIV-infected African adults with documented bloodstream infections, and mortality rate of 35 – 60% have been reported in sub-Saharan Africa (Hohmann, 2001; Kankwatira *et al.*, 2004). Furthermore, Keddy *et al.* (2009) reported *S. typhimurium* as a major opportunistic pathogen particularly of HIV-positive individuals. In Kenya, the incidence of 88 cases of NTS bacteremia per 100,000 persons/year has been reported in children under the age of 5 years (Morpeth *et al.*, 2009). Similarly, an estimate of 7,500 NTS bacteremia cases per 100,000 persons/year has been reported among adults with CD4 cell counts of less than 200 cells/mm³ in Uganda (Gilks, 1998). In Mozambique, children aged 15 years and below have recorded childhood bacteremia cases as high as 120 per 100,000 persons/year (Sigauque *et al.*, 2009). Despite the fact that NTS cases are usually underreported, 1,318 cases of infective NTS were microbiologically confirmed in South Africa between years 2003 and 2004, with *S. typhimurium* and *S. enteritidis* as the most common serovars (Feasay *et al.*, 2010). Two different outbreaks of food-borne illness were reported in Mpumalanga Province of South Africa during years 2006 and 2010, incriminating *S. virchow* and *S. heidelberg* as the respective causes (Smith

et al., 2007; NICD, 2010). This is an indication that NTS is endemic in South Africa as illustrated by the recent provincial numbers of NTS cases reported to GERM-SA in 2011 (Fig. 1.4).

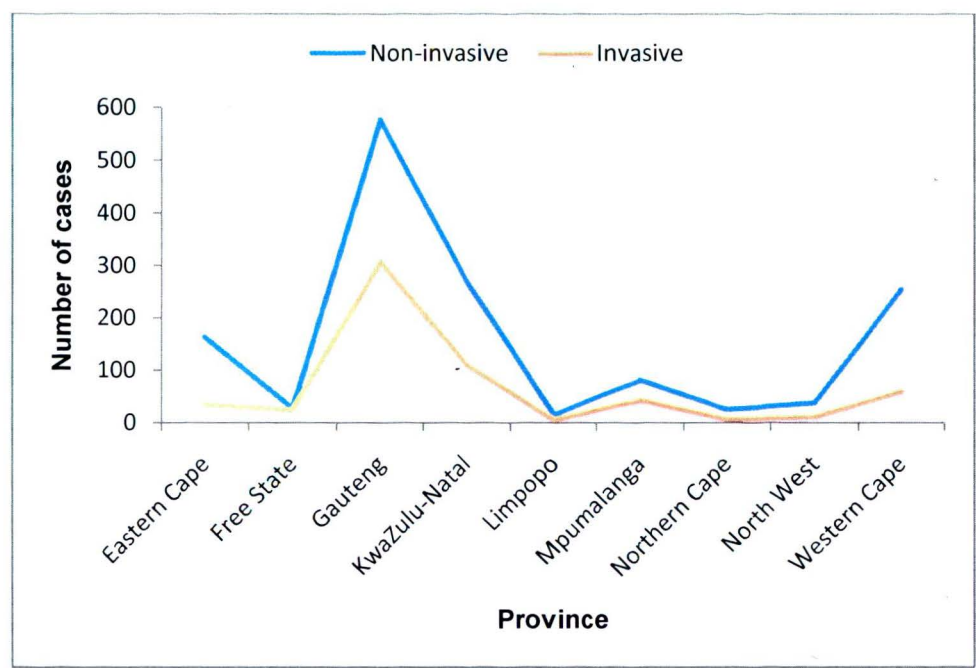


Figure 1.4: Number of NTS cases by province reported to GERM-SA in 2011 (Crowther-Gibson *et al.*, 2011).

1.2.8 Risk factors of *Salmonella* infection

Risk factors for NTS infections in Africa have not been properly characterized. However, studies from developed countries provide guidelines for identification of the risk factors in developing countries. The risk factors can be categorized into environmental and host factors. The environmental factors include food/water, nosocomial infections, direct/indirect contact with animals, and transmission between humans (Morpeth *et al.*, 2009). Host factors however, include age, malnutrition, disease conditions (e. g. sickle cell, malaria, schistosomiasis and HIV infections) and recent antibiotic use (Pavia *et al.*, 1990; Berkley *et al.*, 2005; Enwere *et al.*, 2006). NTS infections appear to be age specific with higher prevalence in children less than 3 years old and adults above 50 years of age (Sigauque *et al.*, 2009). Medications that reduce gastric acidity also tend to increase the risk of gastroenteritis (Leonard *et al.*, 2007). Reports from surveillance studies have indicated the association of NTS infections with yearly

seasonality, with highest incidence recorded during the summer months (Cho *et al.*, 2008). Similarly, recent antibiotic use coupled with malnutrition can destabilize normal intestinal flora and compromise mucosal integrity thereby increasing the risk of NTS gastroenteritis (Morpeth *et al.*, 2009). Individuals with severe clinical immunosuppression are more prone to NTS infections and usually present primary bacteraemia, leukopenia and opportunistic infections without gastroenteritis (Dhanao and Fatt, 2009).

1.2.9 Impact of HIV/AIDS on salmonellosis

Patients with AIDS and other syndromes with T-cell defects are at risk for severe infections with *Salmonella* (Jacobs *et al.*, 1985). HIV-infected persons have an estimated 20- to 100-fold increased risk of salmonellosis compared with the general population (Celum *et al.*, 1987). The clinical course of *Salmonella* infections in patients with AIDS is different from that seen in non-AIDS persons, one major difference being the incidence of bacteraemia (Jacobs *et al.*, 1985; Wolday and Erge, 1998). Among HIVinfected persons in Africa, including South Africa, *Salmonella* species are one of the most common causes of bacteraemia, are often multi-drug resistant and associated with high mortality (24% - 80%) and recurrence rates (43%) (Berkowitz, 1984; Gordon *et al.*, 2002). South Africa has the highest number of HIVinfected people in the world, with an estimated 5 million infected by the virus (Fassin and Schneider, 2003). Yet, many HIV/AIDS patients in South Africa are residents of rural areas, which lack adequate sanitation, proper hygienic practices, and efficient supply of potable water (Grabow, 1996; Von Schirnding, 1993). This further aggravates the risk of exposure to bacterial pathogens including *Salmonella*.

1.2.10 NTS infection in chickens

It is very common for poultry to be infected with various serovars of *Salmonella*. The severity of infection is influenced by many factors which include age of birds, breed of chicken, route of infection, infectious dose and invasiveness of the serovar involved. In the domestic fowl, sources of *Salmonella* infection may be via horizontal or vertical transmission. Horizontal transmission may be by contaminated litter, faeces, feed, water, equipments and other fomites or by contact with rodents, pets, wild birds and personnel infected with *Salmonella* (Poppe *et al.*, 1991; Kinde

et al., 1996). Infection of the hen's follicles in the ovary or the developing eggs in the oviduct leads to vertical transmission. However, many of the factors influencing both horizontal and vertical transmissions are interrelated (Poppe, 2000). *Salmonella* infection in chickens is usually confined to the gastrointestinal tract, in which case infected birds shed the organism in their faeces (Cooke *et al.*, 2007). Studies have however shown that the bacteria can multiply in the liver and spleen following invasion, thereby resulting in systemic infection (Hinton *et al.*, 1990). Invasiveness of the organism is the major determinant of virulence although the incidence of invasion decreases with the age of birds (Poppe, 2000).

1.2.11 Prevalence of NTS in poultry flocks

The prevalence of *Salmonella* in poultry, particularly in broiler flocks in most countries has been well investigated (Harrison *et al.*, 2001; Chadfield *et al.*, 2001). A report from EFSA published in 2007 indicated an average of 23.7% of broiler farms in European Union that were positive for *Salmonella*. The Commission Regulation however set a community target of reducing the prevalence of *S. enteritidis* and *S. typhimurium* in broiler flocks to a maximum of 1% by December, 2011 (EFSA, 2007). In other developed countries, the prevalence varies. For instance, in the United Kingdom, the flock prevalence of *Salmonella* in poultry was found to range between 25 – 29% (Harrison *et al.*, 2001), in Canada, 76.9% (Chambers *et al.*, 1998), in Denmark, 25% (Chadfield *et al.*, 2001), in Turkey, 41.3% (Carli *et al.*, 2001), in France, 69.8% (Rose *et al.*, 1999) and in Brazil, 21.7% (Tavechio *et al.*, 2002). Sasipreeyajan *et al.* (1996) investigated *Salmonella* contamination in 35 different poultry farms comprising of broiler, breeder and layer flocks in Thailand. Samples from litter, feed and drinking water were also analyzed along with cloacal swabs from the birds. All the flocks except two layer farms were positive for *Salmonella*. Similarly, Boonprasert *et al.* (2009) screened the prevalence of *Salmonella* in two broiler breeder farms in Northern Thailand using samples from flocks of different ages. Their report revealed *Salmonella* prevalence of 82.8% which tends to increase with age. In Africa however, very little information is available on flock prevalence of *Salmonella*.

1.2.12 Prevalence of *Salmonella* in chicken carcasses

Oftentimes, poultry products have been established as vehicles of *Salmonella* transmission (Dhilon *et al.*, 2001; Smith *et al.*, 2007). Indeed, it has been estimated that approximately 10% of salmonellosis cases are caused by poultry meat (Oliveira *et al.*, 2006). Jerngklinchani *et al.* (1994) analyzed 705 chicken meat samples obtained from different retail outlets and processing plants in Bangkok and discovered that 66% of the samples were positive for *Salmonella*. Similar surveys undertaken by Boonmar *et al.* (1998) and Tangkawattana *et al.* (2009) in the same province revealed 72% and 42% levels of *Salmonella* contamination in the chicken meat samples respectively. Investigations in Korea revealed the prevalence of *S. enteritidis* in chicken and eggs from retail outlets to be 18.5% (Chang *et al.*, 2000). Prevalence of 15.91% has been recorded for *S. enteritidis* in retail chicken meat in India (Maripandi and Al-Salamah, 2010). Hang'ombe *et al.* (1999) reported a prevalence of 4.7% for *S. enteritidis* in market-ready chicken carcasses in Zambia whereas a prevalence of 18.1% has been reported from Khartoum state in Sudan (El Hussein *et al.*, 2010). The level of contamination of chicken carcasses by *Salmonella*, *Campylobacter* and *Listeria* in Gauteng, South Africa, was assessed by van Nierop *et al.* (2005). They observed that 19.2% of the carcasses harbour *Salmonella* and that significantly higher fresh carcasses from butcheries than other outlets were contaminated with *Salmonella*. A review of studies looking at the location of pathogens in food products revealed the average prevalence of *Salmonella* on the surface of poultry meat to be 22.6%. Furthermore, the prevalence of *Salmonella* on the surface of poultry meat was reported to be about 6 times higher than its prevalence inside the meat (Luber, 2009).

1.2.13 Routes of transmission of *Salmonella*

Salmonella is ubiquitous in nature and can be found in soil and water environment. However, intestinal tracts of both domestic and wild animals remain the primary reservoirs of NTS (Hendriksen *et al.*, 2011). During slaughter, *Salmonella* is passed from the intestinal tract of the host, through fecal contamination, to meat products. Similarly, some *Salmonella* serovars particularly *S. enteritidis* may colonise chicken ovaries and can be transmitted through eggs. Thus infection in humans may be acquired from contaminated meat or eggs, particularly when

undercooked. This seems to be the predominant situation in most developed countries (Morpeth *et al.*, 2009; Hendriksen *et al.*, 2011). Fruits and vegetables may also be contaminated with waste water from animal reservoirs, resulting in human infections (Hanning *et al.*, 2009). Other additional routes of human infection by NTS has been postulated in developing countries and these include hospital acquired infection, direct and indirect animal contact and transmission between humans (Morpeth *et al.*, 2009). Fig. 1.5 shows the various routes of *Salmonella* transmission between reservoirs and the human population.

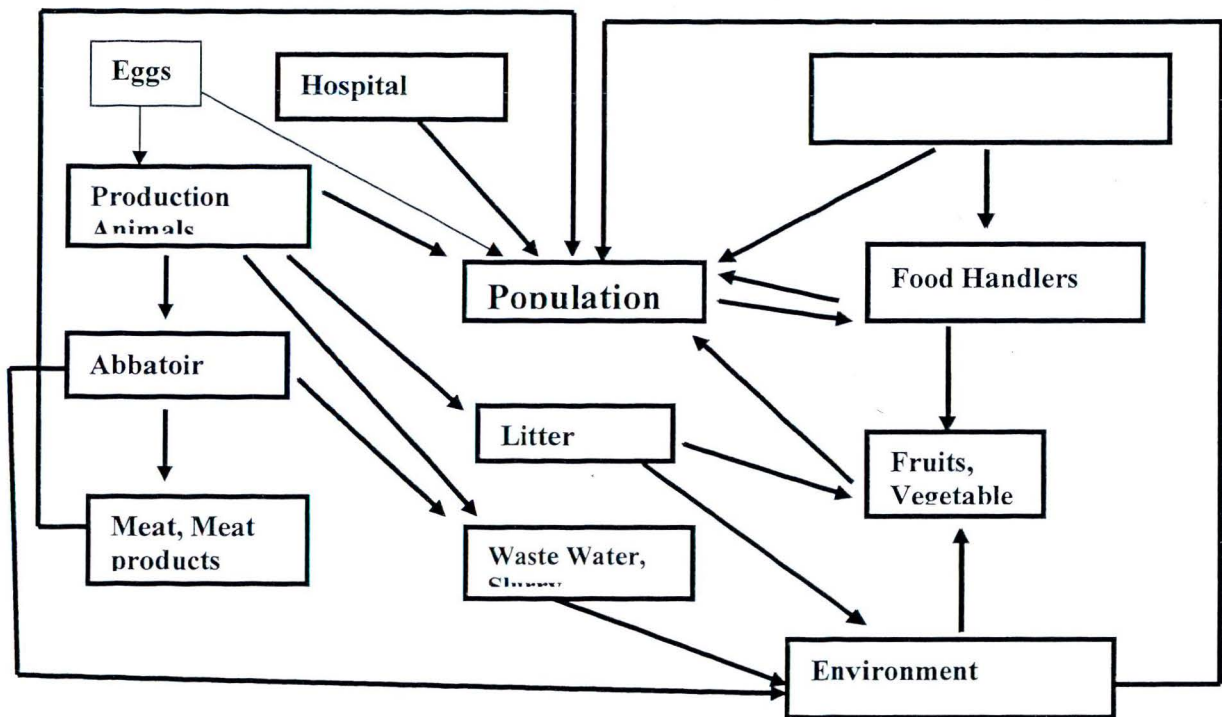


Figure 1.5: Routes of transmission of *Salmonella* between reservoirs and human population (Adapted from Hendriksen *et al.*, 2011)

1.2.14 Pathogenesis of human salmonellosis

Following ingestion of contaminated food/water, *Salmonella* passes through the mucosa of the intestine and attaches to intestinal epithelia (Cooke *et al.*, 2007). This is followed by invasion and intracellular replication of *Salmonella*, resulting in *Salmonella* containing vacuole (SCV). The SCV then contains large numbers of the bacteria within 6 hours post-infection (Finlay and

Falkow, 1990). *Salmonella typhimurium*, as revealed by structural analysis, prefers to invade M-cells in the follicle-associated epithelium of Peyer's patches and also the epithelial cells at the tips of absorptive villi (Santos *et al.*, 2002). Baumler *et al.* (1996) speculated that adhesins may be involved in targeting cell types for invasion. During invasion of epithelial cells, as demonstrated in bovine ligated ileal loop, *Salmonella* transits rapidly from apical location to basolateral location within 1 hour post-infection. This is followed by translocation into the lamina propria 1 – 2 hours after infection (Reis *et al.*, 2003).

During this process, *Salmonella* also translocates a number of effector proteins (SipA, SopA, SopB, SopD, SopE and SopE₂) into the host cell through Type III secretion system (TTSS-1). The TTSS-1 effectors trigger rearrangement of the cytoskeleton of the host cell and cause the formation of membrane ruffles coupled with bacterial internalization by macropinocytosis (Frances *et al.*, 1993). Furthermore, the effector proteins are necessary for induction of chemoattractants such as CXC chemokines. These trigger neutrophil influx leading to transepithelial migration of the neutrophils into the intestinal lumen (Lee *et al.*, 2000; Zhang *et al.*, 2003). The neutrophil influx in turn, stimulates chloride secretion in intestinal epithelial cells (Madara *et al.*, 1993), followed by sodium and water to balance charge and osmolarity respectively. This leads to diarrhea accompanied by severe dehydration. The TTSS-1 components (Fig. 1.6) are encoded by Salmonella Pathogenicity Island 1 (SPI1), a genomic region which is present in *Salmonella* serovars but absent in closely related bacteria (Li *et al.*, 1995). Fig. 1.7 shows *Salmonella* invasion, multiplication and dissemination in the host.

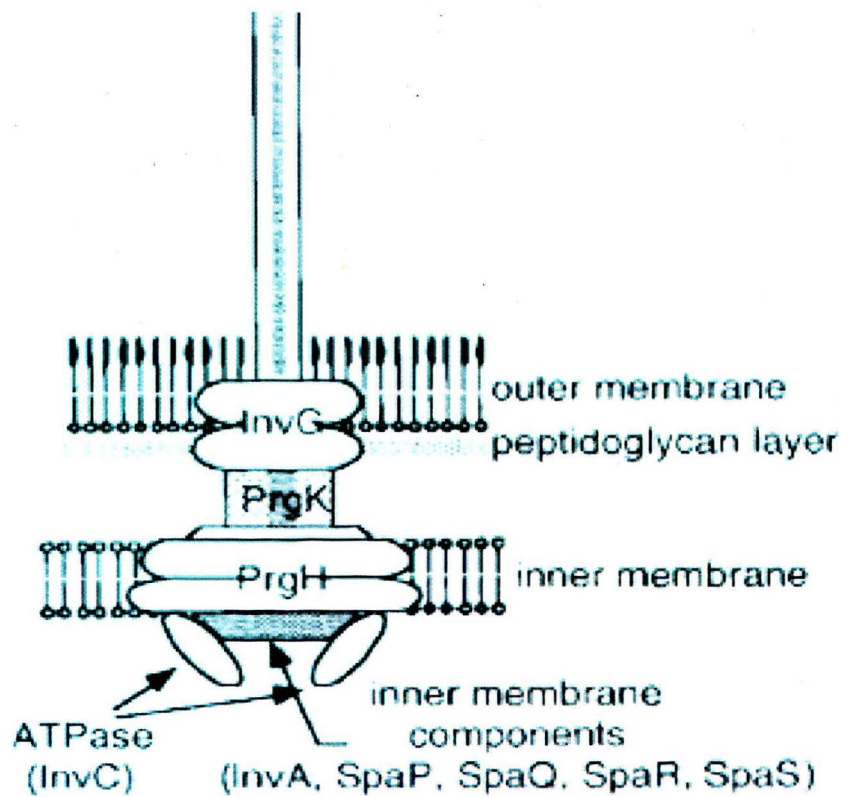


Figure 1.6: Diagram showing Type III secretion system of *Salmonella* (Adapted from Rao *et al.*, 2004)

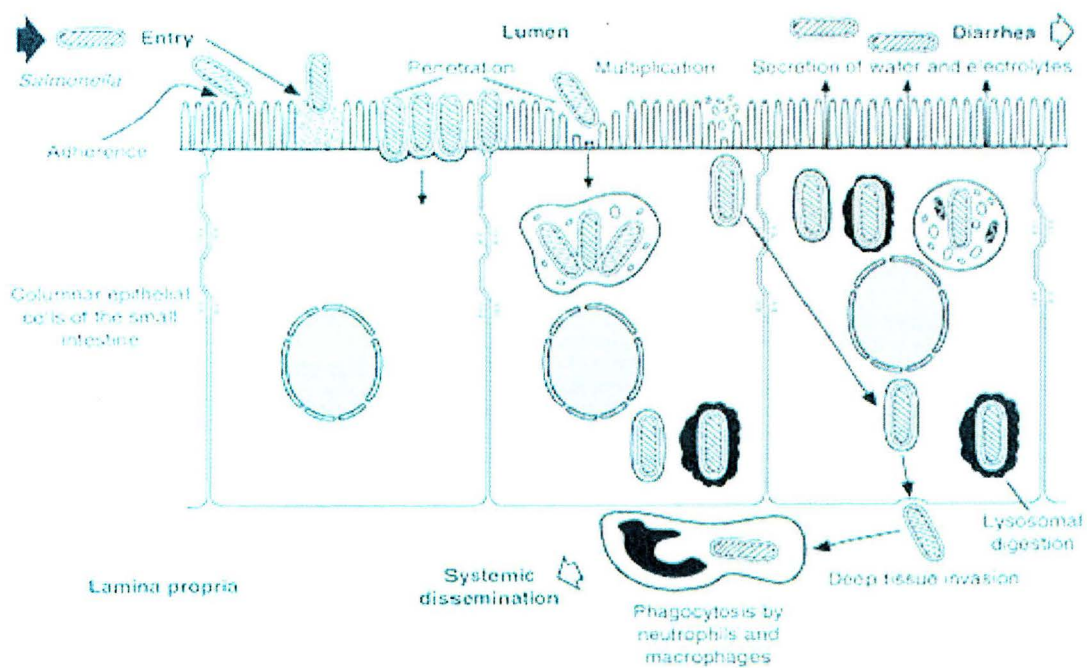


Figure 1.7: Schematic diagram of *Salmonella* invasion, multiplication and dissemination in the host (Rao *et al.*, 2004)

1.2.15 Virulence of *Salmonella*

Virulence factors that are responsible for pathogenicity in enteric bacteria are often encoded by plasmids. Virulence plasmids have only been found in *Salmonella* belonging to subspecies 1. In warm-blooded animals, the *Salmonella* virulence plasmid is necessary for bacterial multiplication in the reticulo-endothelial system (Rotger and Casadesus, 1999), thus indicating its importance in systemic infection (Baumler *et al.*, 2000). The importance of the virulence plasmid in systemic diseases caused by NTS serovars is evidenced by the presence of the *spv* (7.8 kb region of *Salmonella* virulence plasmid) operons in strains that are more associated with disseminated infections e.g. *S. typhimurium* and *S. enteritidis*. Gulig and Doyle (1993) reported increased ability of *S. typhimurium* to infect liver and spleen of mice through the aid of *spv* gene, which increased the rate of bacterial replication within the host cell. The *spv* region of *Salmonella* subspecies I harbors five genes organized as a regulon viz; *spvRABCD*. Entry of *Salmonella* into macrophages/epithelial cells induces the expression of *rpoS*, which in turn regulates the expression of *spvR*. The latter (*spvR*) exerts the regulation of *spvABCD* (Coynault *et al.*, 1992; Marshall *et al.*, 1999). Control of the expression of *spv* operon may also be by PhoP-PhoQ, the two-component regulatory system (Matsui *et al.*, 2000). Furthermore, lipopolysaccharide (LPS), which is a unique constituent of the outer membrane of Gram-negative bacteria, is a major determinant of virulence in *Salmonella* (Thomsen *et al.*, 2002). Location and size of the components of *spv* operon are shown in Table 1.1 below.

Table 1.1: Location and size of *spv* structural genes

<i>Spv</i> gene	Localization	Size (kDa)
<i>SpvR</i>	-	33
<i>SpvA</i>	Outer membrane	28
<i>SpvB</i>	Cytoplasm, Inner membrane	66
<i>SpvC</i>	Cytoplasm	28
<i>SpvD</i>	Outside the cell, cellular fractions	25

Adapted from Rotger and Casadesus (1999)

1.2.16 Antimicrobial resistance in *Salmonella*

NTS gastrointestinal infections in immunocompetent individuals usually do not require treatment as the disease is often self-limiting. However, treatment with antimicrobials is necessary in patients with severe infections, bacteremia or immunosuppression (Hohmann, 2001). Drugs of choice for treatment of salmonellosis include ampicillin, chloramphenicol, trimethoprim + sulphamethoxazole, fluoroquinolones and 3rd generation cephalosporins (McDermott, 2006). However, high levels of antimicrobial-resistant NTS have been reported in African countries (Lee *et al.*, 2009). Antimicrobial resistance increases the morbidity, mortality and costs associated with disease and therefore have serious social and economic consequences in both human and veterinary medicine (Helmuth, 2000).

Since the 1950s, antimicrobial agents have been used in livestock and poultry production to improve growth and feed efficiency as well as to treat infections (Angulo *et al.*, 2004). This practice has however, created a selective pressure that has resulted in the emergence and dissemination of antimicrobial-resistant bacteria in both human and animals (van den Bogaard and Stobberingh, 1999; Salisbury *et al.*, 2002). This happened as a result of frequent exposure of large numbers of animals to subtherapeutic concentrations of the antibiotics, irrespective of their health status (Franco *et al.*, 1990). Over the years, *Salmonella* has evolved several virulence and antimicrobial resistance mechanisms that has resulted in unending public health challenges. Many of the genes that encode resistance are located on transmissible elements such as plasmids which enable potential transfer of resistance among strains (Foley and Lynne, 2008). White *et al.* (2007) tested for *Salmonella* in meat, poultry and egg products in the U.S. through three U.S. regulatory testing programs. They observed that 10 out of the 148 *S. enteritidis* isolates identified were resistant to two or more antimicrobials. Similarly, in South Brazil, *Salmonella enteritidis* isolates from broiler carcasses, food, human and poultry-related samples were screened for antimicrobial resistance. About 90.1% of the strains identified showed resistance to at least one antimicrobial agent (de Oliveira *et al.*, 2005). This was further confirmed recently by the investigation of Duarte *et al.* (2009) who discovered that 94.7% of the 19 *Salmonella* isolates found in broiler carcasses were resistant to at least one antimicrobial agent. In Portugal, 75% of *Salmonella* isolates obtained from poultry products were resistant to one or more antibiotics,

with eight resistant profiles recorded (Antunes *et al.*, 2003). Similarly, 68.8% prevalence of multi-drug resistant strains of *Salmonella* has been reported in Turkey (Dogru *et al.*, 2010).

The close relationship among strains of *S. enteritidis* and the protracted spread of a single clone over a large geographical area was confirmed by Laconcha *et al.* (2000) when they investigated its phage types from three European countries. They discovered that many of the PT1, PT4 and PT6 strains from the three countries shared the same PFGE combination profiles. Similar characteristics have also been noted earlier in strains of *S. typhimurium* definitive phage type 104 (DT104), in which the multi-drug resistance has been linked to the spread of a single strain through food animals and humans (Glynn *et al.*, 1998; Threlfall, 2000). Indeed, it is believed that DT104 which possesses chromosome resistance to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline, increased antimicrobial resistance dissemination (Gross *et al.*, 1998). Furthermore, Ridley and Threlfall (1998) demonstrated resistance genes in DT104 to be in integrons. Briggs and Fratamico (1999) concluded that the resistance genes of DT104, both integron- and transposon-mediated are encoded by a discrete segment of DNA which appears to be related to plasmids of *Vibrio anguillarum* and *Pasteurella piscicida*

Thus, antimicrobial-resistant pathogens can either be transmitted directly to humans through food animals (van Hoek *et al.*, 2005) or transfer their genetic determinants for resistance to human pathogens of related species. This causes adverse consequences on human health and increases the potential for treatment failures (Angulo *et al.*, 2004). An example of such treatment failures was reported by Molbak *et al.* (1999) when two patients died from an outbreak of multi-drug resistant *S. typhimurium* DT104 in Denmark, sequelae to treatment with fluoroquinolone. The outbreak was attributed to contaminated pork and the *Salmonella* isolates from both human and pork samples were less susceptible to fluoroquinolones. This was corroborated by the result of a matched cohort study undertaken by Helms *et al.* (2003) in a Danish population. They discovered that particular risk was associated with quinolone resistance in food-borne *S. typhimurium*. They attributed the excess mortality among patients infected with multi-drug resistant *S. typhimurium* to additional quinolone resistance in many of the multi-drug resistant isolates.

In an epidemiological study, Delicato *et al.* (2004) analysed the profile of antimicrobial resistance among strains of *Salmonella* isolated from patients with gastroenteritis symptoms. They noted high resistance in *S. typhimurium* and *S. enterica* subsp.*enterica* serotype 4,5,12:i:- to the majority of antimicrobials tested. Identification and molecular characterization of bovine, porcine, and human multidrug-resistant *Salmonella* isolates that are resistant to extended-spectrum cephalosporins and cephamycins have also been reported (Miriagou *et al.*, 2004). A plasmid-mediated CMY-2 AmpC-like gene was identified in all the animal and human isolates (Winokur *et al.*, 2001). In Kenya, Kariuki *et al.* (2005) reported a rise in multidrug-resistant NTS from 31% in 1994 to 42% in 2005. In 1998 through to 1999 in South Africa, multi-drug resistant *Salmonella typhimurium* phage type DT104 strains were isolated from HIV-positive patients at the Chris Hani Baragwanath Hospital in Gauteng Province (Crewe-Brown *et al.*, 2000). Kruger *et al.* (2004) described the genetic basis for antibiotic resistance in *S. typhimurium* and *S. isangi*. They observed that most strains of the *Salmonella* serotypes produced either TEM 63 or TEM 131. This is a significant public health problem in South Africa, with particular reference to treatment of serious non-typhoidal *Salmonella* infections in children.

1.2.17 Mechanisms of antibiotic resistance in *Salmonella*

Three major mechanisms of antibiotic resistance have been identified viz:

- a) Modification or destruction of an antibiotic to render it inactive against the target microorganism. This is more common against lactam antibiotics e.g. TEM-type and PSE-type usually found in *Salmonella* (Medeiros, 1997).
- b) Prevention of access of an antibiotic into the cell or target site e.g. tetracycline resistance (Roberts, 1996).
- c) Modification of a target site to render it insensitive to antibiotics e.g. trimethoprim and fluoroquinolone resistance (Hooper and Wolfson, 1993).

Although resistance to antibiotics can be natural, acquired resistance is of greater public health significance. The latter is accomplished through acquisition of resistance genes by horizontal gene transfer (Carattoli, 2003). Resistance genes can either be located on extrachromosomal elements called plasmids or on segments that originate from another genome and inserted within the chromosome. In the latter case, acquisition of the new gene may occur by genetic

transformation or transduction. However, when resistance genes are located on plasmids, they can be mobilized by conjugative transfer at a very high frequency and efficiency and may involve simultaneous acquisition of several resistance genes (Waters, 1999). Transformation involves the uptake of naked DNA by cells (e.g. from dead bacterium), and is more efficient when the cells are in a state of competence whereas transduction is the process of delivering a gene by a virus or a bacteriophage into a new host cell (Helmuth, 2000). Conjugation on the other hand, is a parasexual, horizontal, gene-transfer process in which plasmids (self-replicating extra-chromosomal DNA molecules) are exchanged by a donor and recipient through formation of mating aggregates (Fig. 1.8).

Resistance gene-borne plasmids are known as R-factors consisting of several genetic units, including genes that are essential for the initiation and control of replication (Helmuth, 2000; Carattoli, 2003). However, the self-transferable R-factors basically carry the resistance transfer factor (RTF) which provides fertility, and the resistance determinant (R) which provides resistance phenotype. Two plasmids may be related and share common replication controls thus making it impossible for both plasmids to propagate in the same cell line (Couturier *et al.*, 1988). Based on this fact, plasmids are classified into incompatibility groups, of which four groups have been identified. These are Ti, IncF, IncP, and IncI plasmid groups respectively (Waters, 1999). Furthermore, other genetic structures such as transposons and integrons may be carried on R-factors and other DNA molecules. Transposons are usually the location sites for resistance genes encoded by plasmids whereas integrons are the DNA elements responsible for capturing and expression of resistance genes. Integrons encode the integrase (site-specific recombinase) which promotes the acquisition of exogenous genes (Hall and Collis, 1995).

Thus, integrase-mediated site-specific recombination is an efficient genetic mechanism by which bacteria acquire resistance genes (Carattoli, 2003). Four classes of integrons have been described viz:

- a) Class 1 integron carrying the integrase gene (*Int1*) and commonly found in bacterial pathogens (Carattoli, 2001).
- b) Class 2 integrons carrying the defective integrase gene (*Int2*) and associated with Tn7 family of transposons (Radstrom *et al.*, 1994).

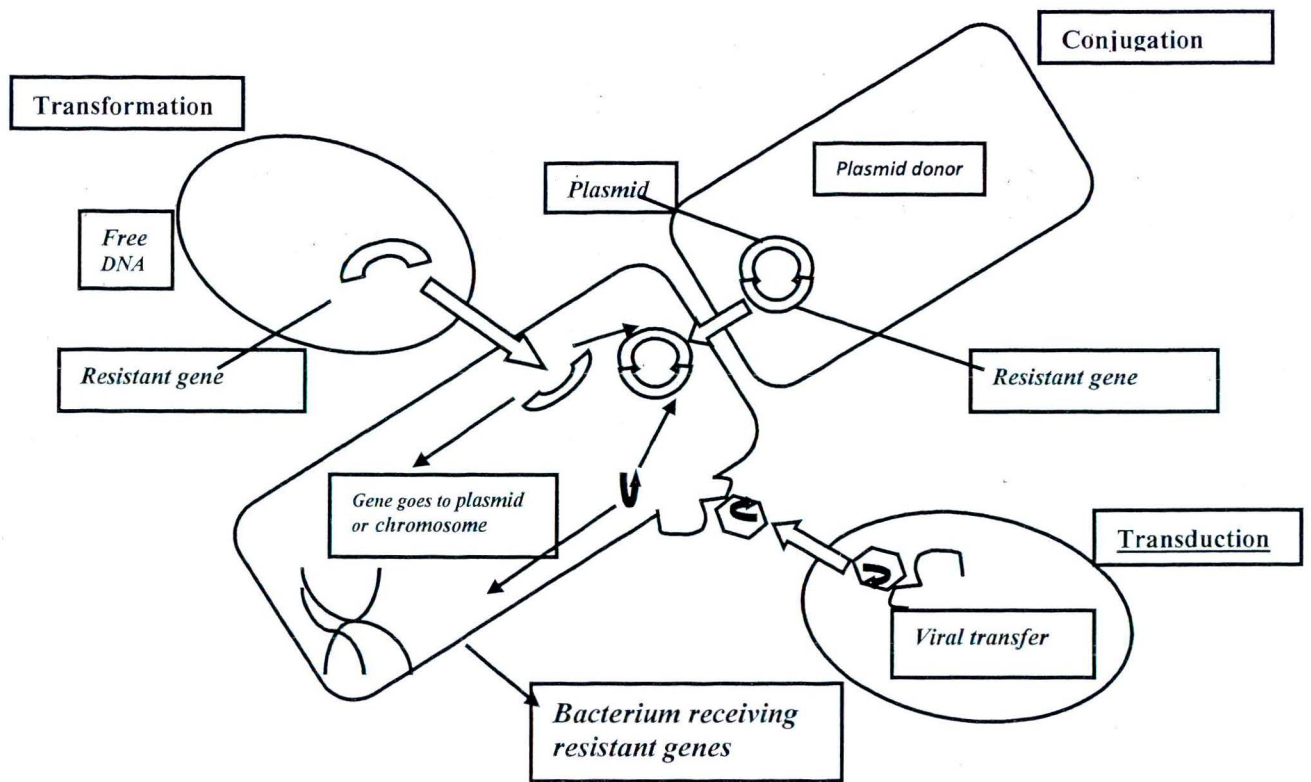


Figure 1.8: Diagrammatic representations of the various methods of acquisition of antibiotic resistance genes in *Salmonella* (Adapted from Henriques *et al.*, 2012)

- c) Class 3 integron containing *bla_{IMP}* gene cassette that confers resistance to carbapenems (Arakawa *et al.*, 1995).
- d) Class 4 integrons described in the chromosome of *Vibrio cholera* and associated with virulence genes (Rowe-Magnus *et al.*, 1999).

Class 1 integrons possess two conserved segments namely 5'-CS and 3'-CS, flanking a variable region (Hall and Collis, 1995). The 5'-CS contains a promoter (allows the expression of the gene cassette), the genes for the site-specific integration, the *Int1* and the attachment site (*attI1*) (Fig. 1.9). The 3'-CS however contains genes for resistance to disinfectants (*qacEΔ1*) and sulphonamides. The gene cassette, which is a discrete unit that consists of one complete open reading frame (ORF) and a recombination site called 59-base element, is integrated between the 5'-CS and 3'-CS (Bissonette and Roy, 1992; Recchia and Hall, 1995).

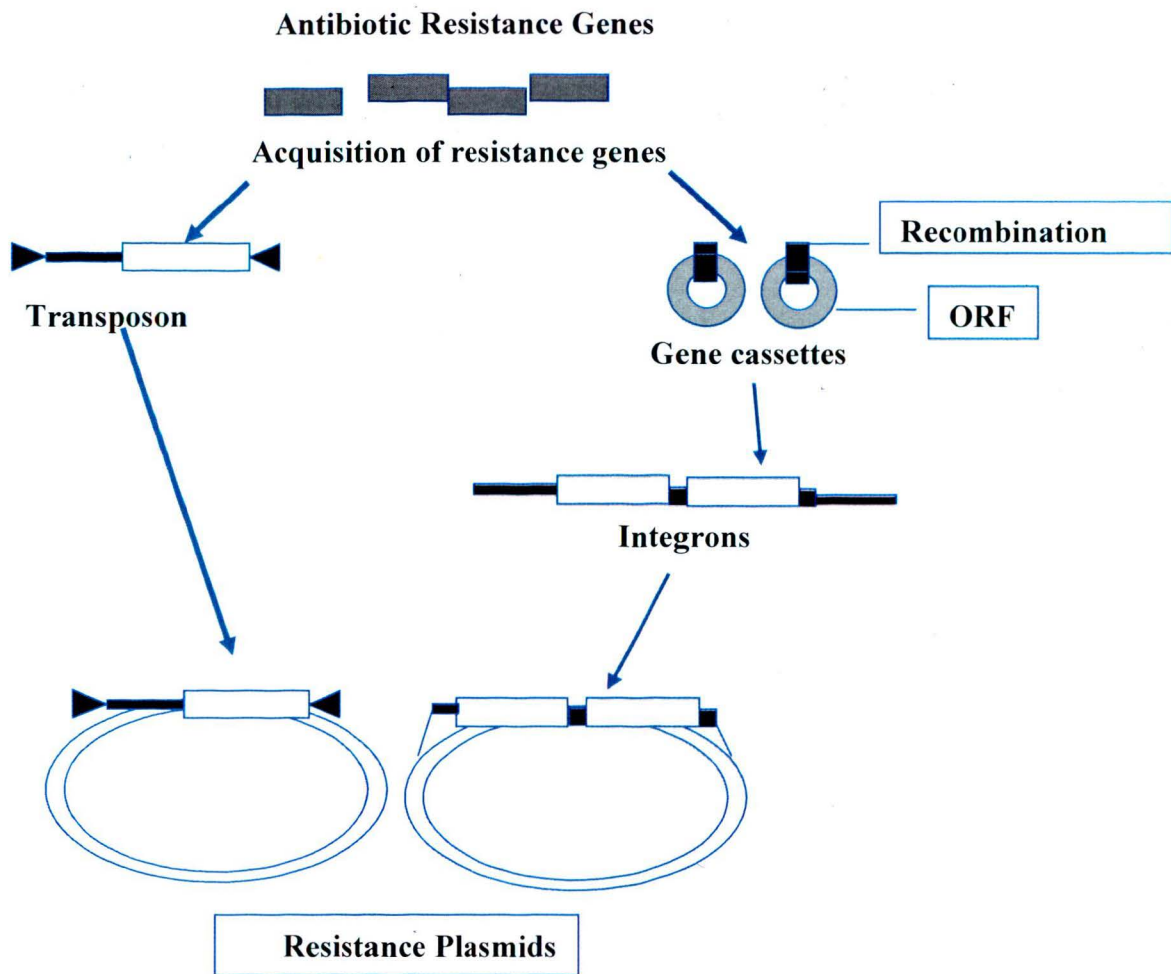


Figure 1.9: Schematic diagram of plasmid-mediated horizontal transfer of antibiotic resistance (Adapted from Carattoli, 2003)

1.2.18 Stress response in *Salmonella*

The fact that bacteria generally are able to respond or adapt to changes in their immediate environment is becoming a recognized challenge in the food industry. *Salmonella spp.*, which is no exception, encounters various environmental stresses within food, gastrointestinal tract and host immune system. Such stresses include temperature change, nutrient deprivation, osmotic shock, pH extremes and DNA damage (Foster and Spector, 1995). Depending on the duration and severity of exposure, the stresses may inhibit the growth/survival and subsequently influence the virulence of the organism (Kwon *et al.*, 1998).

It is therefore essential for *Salmonella* to be able to adapt rapidly to various environmental changes for survival. Response of *Salmonella* to stress is particularly manifested in three major areas which include response to:

- a) host defense systems during infection (Rouquette *et al.*, 1998).
- b) lethal or sub-lethal effects of physical/chemical treatments.
- c) conditions in preserved and minimally processed foods.

Various typical genes can be induced under stress conditions, coding mainly for transcriptional stress regulator proteins. These proteins can activate other genes that are necessary for adaptation/survival of such stress conditions (Kenyon *et al.*, 2002). Changes in gene expression, triggered by environmental signals, occur through various mechanisms including transcriptional repressors, alternative sigma factors (σ^S), etc (Shen and Fang, 2011) (Table 1.2). However, the σ^S is referred to as general stress regulator because of its ability to regulate a large number of genes (>500 genes) in response to stress (Hengge, 2009). This ability makes it an effective means for the cell to rapidly effect major changes in gene expression (Shen and Fang, 2011). Furthermore, response of bacteria to one stress can cross-protect the organism against other stress factors and cause changes in some aspects of the bacterial metabolism resulting in persistent, more resistant and dangerous pathogens (Shah, 2011). This is because both stress response and virulence genes are expressed during response to changes in environmental conditions. The various stress factors experienced by *Salmonella* during colonization in the host and the regulons induced are shown in Table 1.3 below.

Table 1.2: Different environmental stresses experienced by *Salmonella*

Stress factors	Regulators
C-starvation	σ^S ; σ^E ; cAMP-CPR
Oxidative	σ^H ; σ^S ; σ^E ; DkSA; LexA; Fur
Acid	σ^S ; σ^E ; AdiY; DkSA; Fur; ompR-EnvZ; Pho-PQ; RecA
Heat	σ^H ; σ^S ; σ^E ; cAMP-CPR
Osmotic	σ^S ; ompR-EnvZ

Adapted from Spector and Kenyon, 2012

1.2.19 Response of *Salmonella* to acid stress

Salmonellae must survive the acidic conditions of the stomach in order to gain entrance into the small intestine and cause disease. This, the organisms achieve by protecting themselves through certain pH homeostatic mechanisms such as:

- prevention of protons from entering the cell.
- pumping of protons out of the cell.
- repair of damaged macromolecules.
- manufacture of key enzymes isoforms with optimum pH in acid range (Foster, 2001).

Salmonella typhimurium is able to grow in minimal media with pH as low as 5.0 but at pH below 4.0, the cells undergo rapid death due to inability to maintain internal pH suitable enough for viability (Foster and Hall, 1991). Ngwai *et al.* (2007) reported a better survival of *S. typhimurium* and *S. enteritidis* at pH 4.0 compared with pH 2.6. It has however been shown that prior adaptation to mild or sub-lethal acidic conditions can enhance the survival of *S. typhimurium* in extremely harsh acidic conditions (Foster, 1991). For instance, Foster and Hall (1990) demonstrated improved survival of acid-adapted *S. typhimurium* in chicken meat.

Table 1.3: Different environmental stresses experienced by *Salmonella* during colonization of a susceptible host

Environment	Stress Factor	Regulons induced
Outside of host (1)	Low temperature, low nutrients	<i>rpoS, csp</i>
Stomach	Extreme acid pH	<i>rpoS, fur, ompR, phoP</i>
Duodenum	Bile	<i>phoP</i>
Ileum	Decreased oxygen supply, SCFA, competitive flora	<i>Fur, arcA</i> <i>rpoS</i> <i>sdiA, luxS</i>
Epithelium	CAMP	<i>phoP</i>
Outside of host (2)	Low temperature, aerobiosis	<i>rpoS, fur, csp, arcA, oxyR, soxRS</i>

Adapted from Rychlik and Barrow, 2005

Similarly, Foster (2001) noticed that exposure of exponential phase cells of *Salmonella* to pH 5.8 or 4.5 for 30 or 60 mins protected the cells when subsequently exposed to pH 3.0. This is due to the fact that pre-incubation of *Salmonella* cells at pH near 6.0 (optimum pH 5.8) triggers the acid tolerance response (ATR) as well as synthesis of acid shock proteins (ASPs). Proteome analysis revealed 60 and 45 ASPs produced during the adaptation of exponential phase and stationary phase cells of *Salmonella* respectively (Foster, 2001). The ASPs are induced by transcriptional regulators which are coded for by genes induced during exposure to acid stress (Hermans, 2007).

Log phase ATR is regulated by iron regulatory protein *Fur*, the two component regulatory system PhoPQ and the alternative RNA polymerase sigma factor σ^s (encoded by *rpoS* gene) (Bearson *et al.*, 1998; Hall and Foster, 1996; Lee *et al.*, 1995). Stationary phase ATR, on the other hand, is regulated by the classic response regulator OmpR (Bang *et al.*, 2000). Mutants of *S. typhimurium* lacking the regulators were found to be acid sensitive (Foster, 2001). Although the importance of the sigma factor σ^s is usually associated with stationary phase cells, it has been discovered that its level increases dramatically in log phase cells that are subjected to brief acid shock (Lee *et al.*, 1995). This is because the σ^s , which is rapidly degraded by ClpXP in log phase cells, becomes resistant to degradation following acid shock (Webb *et al.*, 1999; Foster, 2001). ClpXP is a protease complex that is important for degradation of misfolded proteins generated during stress (Thomsen *et al.*, 2002) Fig. 1.10 below shows the processes of transcription, translation and proteolysis of the sigma factor σ^s by various regulatory and environmental stress factors.

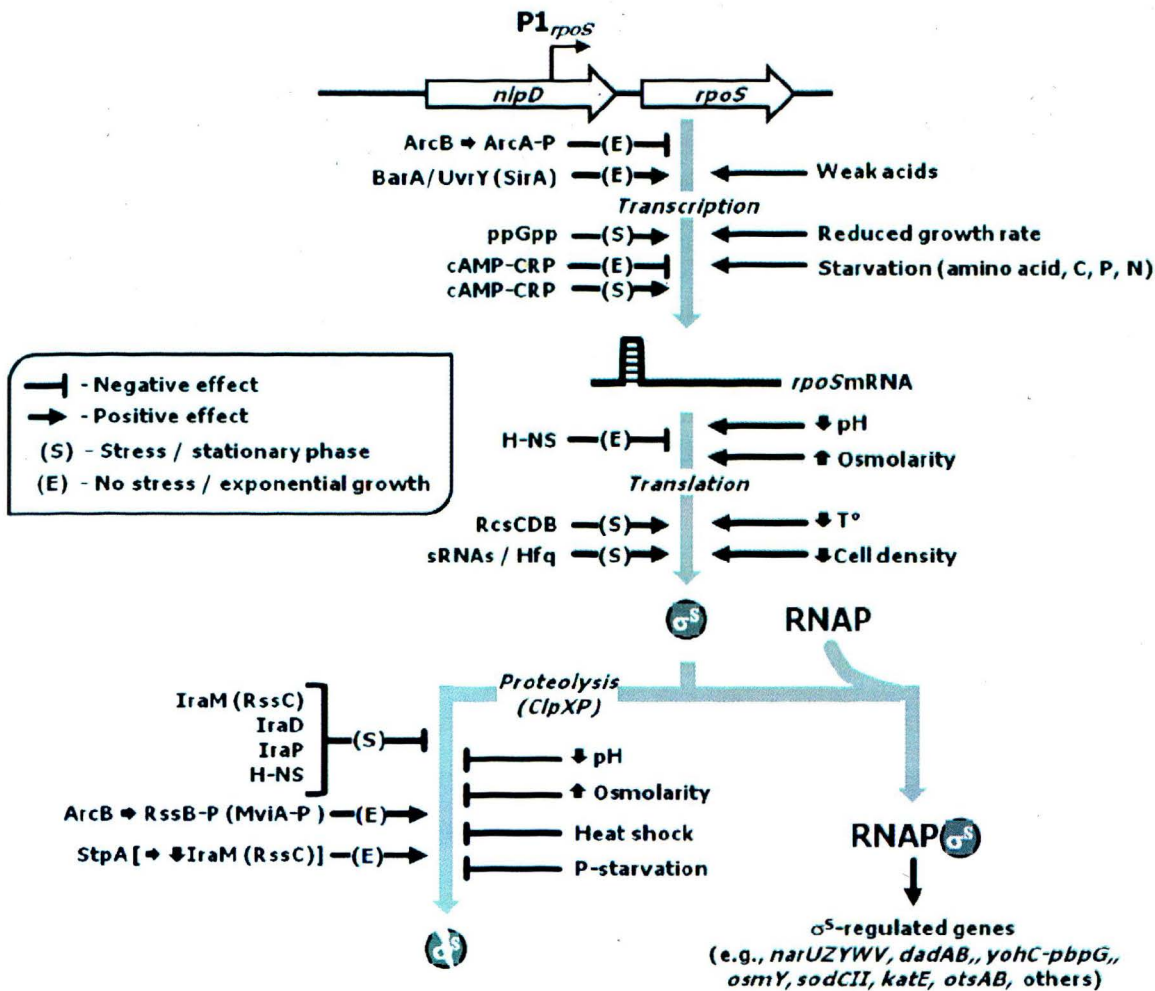


Figure 1.10: Flow diagram of the regulation of *rpoS* gene transcription, *rpoS* mRNA translation and σ^S proteolysis by various regulatory factors and environmental stress conditions (Spector and Kenyon, 2012)

1.2.20 Response of *Salmonella* to heat stress

Cooking or heating at high temperature is a primary method of food treatment to protect against food-borne diseases. However, increase in heat resistance post exposure to sub-lethal temperature has been reported in *Salmonella* spp. (Humphrey *et al.*, 1993). Although Ngwai *et al.* (2007) observed significant reduction in survival of *S. enteritidis* and *S. typhimurium* exposed to 52°C, Kumar and Kumar (2003) reported a better survival of *S. seftenberg* at 53 and 55°C after heat shocking at 50°C for 30 mins. The phenomenon of increase in heat resistance exhibited by cells grown at slightly higher temperature than optimum is attributed to production of heat shock

proteins (HSPs) (Ananthan *et al.* 1986). Response of *Salmonella* to heat stress is divided into cytoplasmic heat shock and extracytoplasmic response. The former is controlled by the heat-shock sigma factor σ^H whereas the latter is regulated by sigma factor σ^E (Nonaka *et al.*, 2006; Ades, 2008). Although the two factors are activated through different molecular mechanisms, both mechanisms allow for a quick response to thermal stress (Spector and Kenyon, 2012). Canovas *et al.* (2001) further discovered that trehalose, a disaccharide, is crucial for the growth of *Salmonella* at high temperature.

1.2.21 Response of *Salmonella* to osmotic stress

Salmonella spp rapidly responds to osmotic changes encountered in various host and non-host environments (Winfield and Groisman, 2003). The bacterial cells often attempt to maintain turgor pressure in response to either hypotonic or hypertonic environment. It does this by either increasing or decreasing the concentration of the solutes in the cytosol and periplasm, depending on the prevailing osmotic condition (Wood, 2006). However, the responses to osmotic stress in *Salmonella* (either physiological or genetic) usually involve an immediate temporary measure to counter the imbalance in osmotic pressure. The temporary measures are later replaced by long-term readjustments to the osmotic challenge (Spector and Kenyon, 2012). For instance, increase in osmotic pressure leads to loss of water from the cytoplasm. This results in the induction of an operon (kdpABC) encoding a high-affinity K^+ transport system causing rapid accumulation of intracellular K^+ (Balaji *et al.*, 2005). This is a temporary solution that is later replaced by accumulation of compatible solutes such as proline and trehalose (Empadinhas and da Costa, 2008).

1.3 Aim and objectives of this study

To establish the level of prevalence of non-typhoidal *Salmonella* spp in broiler products in the North West Province and to investigate their rate of antimicrobial and stress resistance using established conventional methods of identification, serological characterization and molecular techniques.

1.3.1 Hypothesis

Raw and RTE broiler products sold in NWP of South Africa are contaminated with antimicrobial and stress-resistant strains of NTS.

1.3.2 Objectives

1. Screen raw and ready-to-eat broiler products obtained from the NWP of South Africa for incidence of NTS contamination.
2. Investigate the resistance of NTS species isolated from broiler products in the Province to some common antimicrobials, and to screen the isolates for the presence of antibiotic resistance genes.
3. Evaluate the resistance of NTS spp. isolated from broiler products obtained from the NWP of South Africa, to thermal, acidic and osmotic stress.
4. Evaluate the incidence and antibiotic resistance of coliforms in raw and RTE broiler products obtained from the NWP of South Africa.

CHAPTER 2

INCIDENCE OF NON-TYPHOIDAL *SALMONELLA* STRAINS IN RAW AND READY-TO-EAT BROILER PRODUCTS IN THE NORTH WEST PROVINCE OF SOUTH AFRICA

2.1 Introduction

Non-typhoidal salmonellosis (NTS) is an important public health problem worldwide particularly in sub-Saharan Africa where it commonly manifests as gastroenteritis and/or bloodstream infections in both children and adults (Morpeth *et al.*, 2009). The gastroenteritis form, which may sometimes be self-limiting, is commonly found in industrialised countries. Immunocompromised individuals including patients with HIV, cancer or diabetes, are at higher risks of NTS bacteremia and often develop focal infections such as meningitis, septic arthritis, pneumonia and osteomyelitis (Hohmann, 2001). Although more than 2,500 serovars of *Salmonella enterica* have been reported, *S. typhimurium* and *S. enteritidis* are identified as the commonest causes of human infection (Humphrey, 2000; Herikstad *et al.*, 2002). A retrospective study in one hospital in the Democratic Republic of Congo from 2002 to 2006, revealed that NTS caused 59% of bacteremia in children. *Salmonella typhimurium* and *Salmonella enteritidis* were responsible for 82% of the cases (Vandenberg *et al.*, 2010). In Mozambique, NTS were reported to account for 120 cases of childhood bacteremia per 100,000 persons/year (Sigauque *et al.*, 2009). Contaminated poultry meat and eggs, among other factors, have been implicated as vehicles of transmission of these hardy pathogens (Dogru *et al.*, 2010). For these reasons, there have been numerous studies focusing on assessing the incidence/prevalence of non-typhoidal *Salmonella* strains in chicken carcasses and other meat products (Antunes *et al.*, 2003; Dogru *et al.*, 2010; El Hussein *et al.*, 2010).

In South Africa, the past few years have witnessed tremendous increase in chicken meat consumption (Mons, 2010). Concurrently, the Enteric Disease Reference Unit of the National Institute for Communicable Diseases has noted an increasing number of non-typhoidal *Salmonella* isolates despite the fact that human salmonellosis cases are rarely reported. This is

evidenced by recent outbreaks of food-borne illnesses in Mpumalanga Province of South Africa, incriminating NTS serotypes. One of the outbreaks involved the consumption of meals prepared with poultry products (Smith *et al.*, 2007; NICD, 2010). These outbreaks indicate the presence of NTS in South Africa, which may be an issue of public health concern. A few investigations have been conducted in South Africa to ascertain the contamination of chicken carcasses and ready-to-eat foods from retail stores, with various pathogenic bacteria including *Salmonella* (van Nierop *et al.*, 2005; Christison *et al.*, 2008). However, the incidence of NTS in broiler products in the North West Province (NWP) has not been established despite the fact that the NWP is one of the provinces with the largest production and distribution of broilers in the country. The objective of this study therefore is to screen raw and ready-to-eat broiler products obtained from the NWP of South Africa for incidence of NTS contamination.

2.2 Materials and Methods

2.2.1 Geographical description of the study area

The geographical area for screening is the NWP of South Africa. The province, which is located on latitude 25.8°S and longitude 25.5°E offers an almost year-round sunshine, with average rainfall of 300 – 700 mm annually. The province is also marked by two distinct seasons of summer and winter months, with summer temperature ranging between 22 - 34°C. Average winter temperature is 16°C though it may range between 2 - 20°C in a single day.

2.2.2 Sampling Design

The study design was cross-sectional in which sampling was done based on two shop types and three product groups. The towns and shops that were visited were randomly selected. Shops included butcheries and supermarkets, whereas product groups included whole broiler carcasses, chicken parts and ready-to-eat broiler products such as sausages. The sampling, which lasted for 6 months, commenced in October 2010 and ended in March 2011. This period corresponds to the

summer months in the study area, when conditions are expected to favour the thriving of many bacteria.

2.2.3 Determination of sample size and sampling procedure

Primary population size (shops) that was sampled was determined according to the formula of Magnani (1997):

$$\left(\frac{Z_{\alpha} * SD}{L} \right)^2 \text{ Where } SD = [p(1 - p)]^{1/2}; P = \text{Expected prevalence}; L = \text{Accepted error and } Z_{\alpha} = \text{value for normally distributed data at confidence level } \alpha$$

Based on expected prevalence of 40% and confidence level of 90%, the total number of shops that were sampled for analysis was 57 (comprising of 37 supermarkets and 20 butcheries). A total number of 180 samples were obtained for analysis based on purchase of a minimum of three product groups from each shop, and keeping 5% above the actual sample size as risk in preventing sample loss during analysis. The temperature of samples at point of purchase was recorded and it ranged from -20 to 4°C. All samples purchased were within the expiration date for consumption. Each sample was placed in a separate sterile plastic bag, labelled according to area of collection and product type, and transported to the laboratory on ice (Reid, 2009). Samples were maintained at 4°C until processing which was within 24 hrs after collection. The product information for each sample which included product name, brand, weight, name of store and location where product was obtained were recorded. Each sample was then assigned a working number for easy identification. For all the procedures involved in the analysis, *Salmonella typhimurium* ATCC 14028 was used as a positive control strain whereas *E. coli* ATCC 25922 was used as a negative control. For all culture, isolation and biochemical screening techniques were carried out using the MFHPB-20 procedure (Reid, 2009) with appropriate modifications. Figs. 2.1 and 2.2 below show the samples and various locations within the province where samples were obtained respectively. Similarly, Tables 2.1 and 2.2 indicate the number of samples obtained from specific locations and samples obtained by product and shop types respectively.



Figure 2.1A: Sample of whole chicken carcass (raw product)

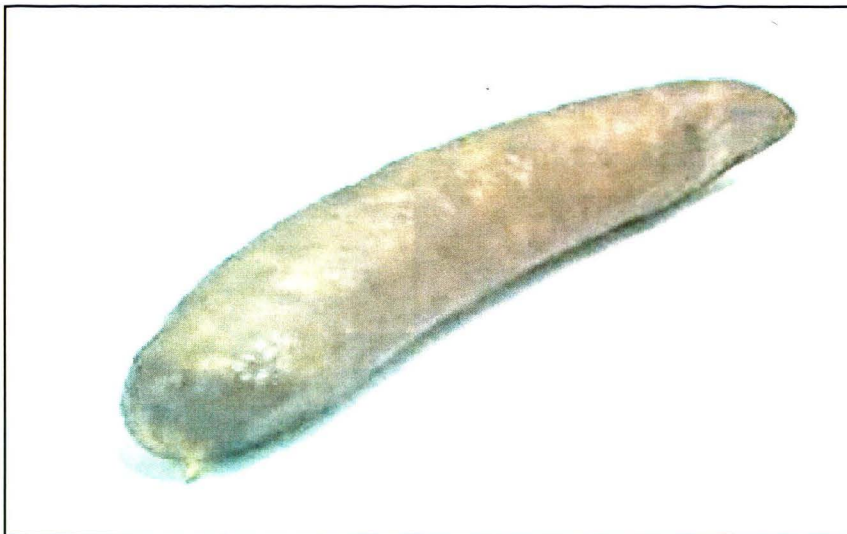


Figure 2.1B: Sample of chicken Vienna (ready-to-eat product)

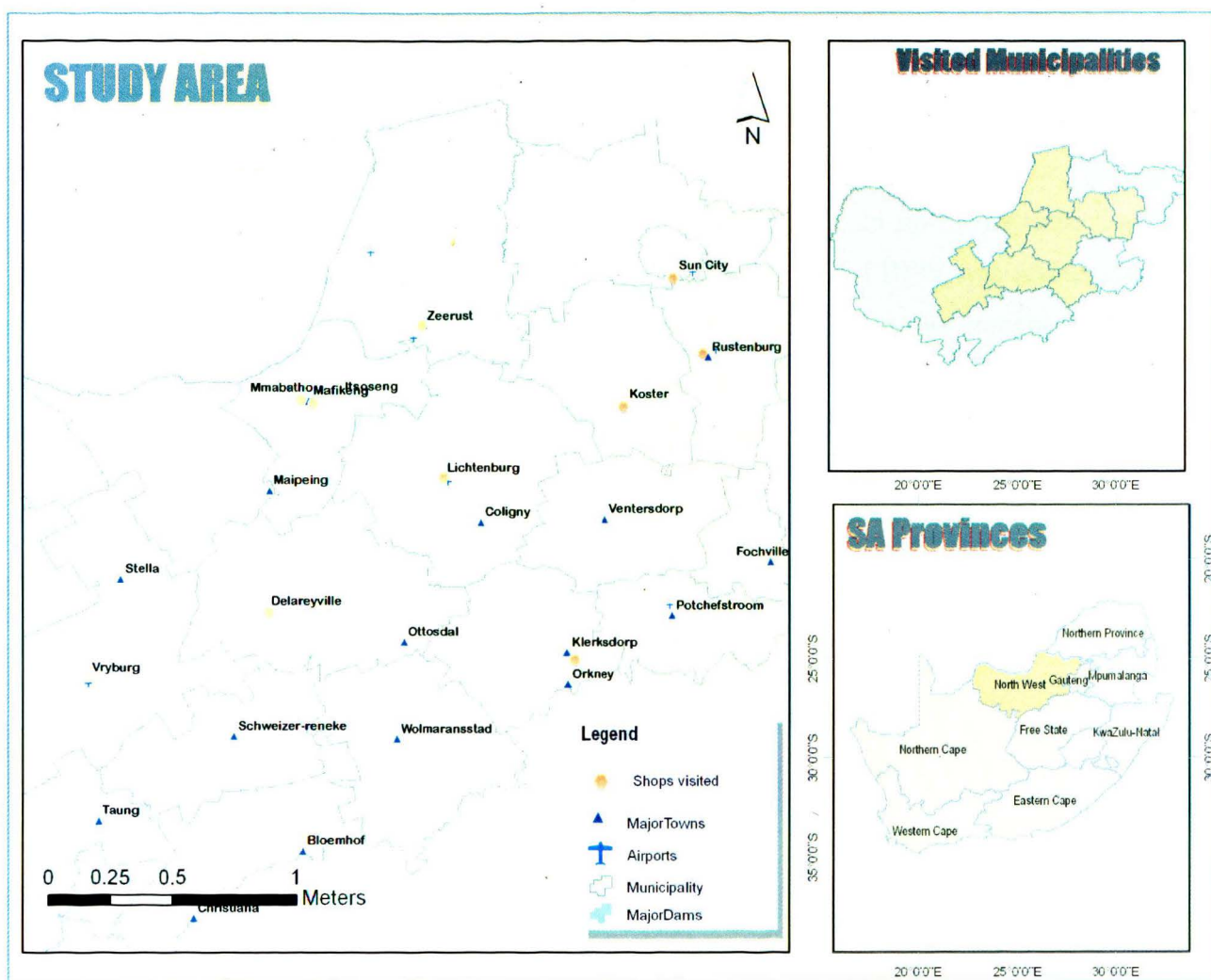


Figure 2.2: Map of the North West Province of South Africa showing locations where samples were obtained.

2.2.4 Determination of total bacterial counts in samples

Briefly, 25 g of each sample was aseptically removed and homogenized with 225 ml of 2% buffered peptone water (BPW) in a stomacher bag (Nasco, USA). Then, 1 ml of the homogenate was transferred to Eppendorf tubes after which serial dilutions were made using 2% BPW. Aliquots (0.1 ml) of each dilution were plated on plate count agar (Merck, SA) by spreading, and incubated at 37°C for 24 hours after which colonies were counted and recorded as cfu/g sample.

2.2.5 Isolation of *Salmonella* species from broiler carcasses and sausages

2.2.5.1 Broiler carcasses: Each sample was aseptically removed from the package and transferred to a sterile plastic bag. Then 150 ml of BPW was added to each bag and the mixture was shaken constantly for 2 mins to obtain carcass rinse. Thereafter, 25 ml of the rinse liquid was transferred to a Whirl-pak bag (Nasco, USA) and another 75 ml of BPW was added. Each bag was sealed and incubated at 37°C for 18 hours. Following incubation, the contents of each Whirl-pak bag (Nasco, USA) were homogenized and 0.1 ml aliquots of the homogenate were transferred to 10 ml of Rappaport-Vassiliadis (RV) broth (Merck, SA) for selective enrichment of *Salmonella*. The broth was incubated at 42°C for 24 hours.

Table 2.1: Number of shops^a visited and number of samples^b purchased per location

Sampling area	No. of shops visited	No. of samples obtained
Suncity	2	6
Koster	3	10
Delareyville	5	16
Vryburg	4	12
Mmabatho	3	10
Mafikeng	10	33
Zeerust	6	19
Rustenburg	6	18
Lichtenburg	5	16
Klerksdorp	5	15
Potchefsrroom	8	25
Total	57	180

^aDepended on number of shops available within a particular location; ^bminimum of three samples comprising of the three sample types were purchased from each shop.

2.2.5.2 Chicken sausages: Twenty-five grammes of each sample was aseptically removed and homogenized with 225 ml of full strength BPW in a stomacher bag (Nasco, USA). The mixture was incubated at 37°C for 18 hours. Thereafter, the mixture was homogenized and 0.1 ml aliquot of the homogenate was transferred to 10 ml of RV broth and incubated at 42°C for 24 hours.

Table 2.2: Number of products obtained by product and shop type

Product type	Shop type	
	Supermarket	Butchery
Whole broiler carcasses (WBC)	30	15
Chicken parts (CP)	50	25
Ready-to-eat (RTE)	40	20

Following incubation of inoculated RV broths for all samples, the broths were kept at room temperature for 24 hours (Waltman, 2000). Then a loopful of each broth sample was streaked on Xylose Lysine Desoxycholate (XLD) agar (Merck, SA) and *Salmonella Shigella* agar (Merck, SA) respectively and incubated at 37°C for 24 – 36 hrs. Presumptive isolates were further purified by streaking on XLD agar (Merck, SA) and thereafter preserved on nutrient agar (Merck, SA) slants for further analysis.

2.2.6 Biochemical screening

All presumptive isolates from the XLD agar culture were inoculated on Triple Sugar Iron (TSI) agar (Merck SA) slants and incubated at 37°C for 24 hours. Presumptive *Salmonella* isolates from TSI plates were further confirmed using API-20E test (bioMerieux, Inc., South Africa) performed according to manufacturer's instructions. Briefly, a plastic strip holding twenty mini-test tubes was inoculated with a saline suspension of a presumptive *Salmonella* culture. A few of the tubes were completely filled (for CIT, VP and GEL) while some tubes were overlaid with mineral oil such that anaerobic reactions can be carried out (for ADH, LDC, ODC, H₂S and URE). Oxidase reaction was carried out separately. Following incubation of inoculated strips at 37°C for 18 - 24 hours, the colour reactions were read and the reactions were converted to a seven-digit code. This was fed into the manufacturer's database for generic and species identification.

2.2.7 Serological identification

Isolates showing typical *Salmonella* biochemical reactions were serotyped by slide agglutination test using *Salmonella* specific polyvalent antisera (Davies diagnostics, South Africa) for O and H antigens. The test was performed according to manufacturer's instructions. Briefly, 10 µl volumes of sterile 0.85% saline solution were placed on sterile microscope slides in duplicate. Single colonies were picked and emulsified on both drops to obtain uniform turbidity. Then 10 µl aliquots of each polyvalent antiserum was added to one of the emulsions while a drop of saline was added to the other emulsion (control). The slides were rocked gently for 1 min after which they were observed for formation of precipitate.

2.2.8 Molecular characterization of presumptive *Salmonella* isolates

All isolates were screened for the presence of the invasion (*invA*) and virulence (*spvC*) genes by PCR.

2.2.8.1 DNA extraction

Genomic DNA was extracted using Zymo® kit (Inqaba biotec, SA), for amplification of bacterial 16S rRNA gene fragment as the internal control for all isolates and for fingerprinting by RAPD-PCR. The extraction was carried out according to manufacturer's protocol. Plasmid DNA was then extracted for effective amplification of *invA* and *spvC* genes. The extraction was done by alkaline lysis using the method of Sambrook *et al.* (1989) with modifications. Briefly, a single colony of presumptive *Salmonella* isolate was inoculated in 10 ml of nutrient broth (Merck, SA) and incubated overnight at 37°C with shaking at 120 rpm. Aliquots (1.5 ml) of the culture were then transferred to 2 ml Eppendorf tubes and centrifuged at 13,500 rpm (Biofuge Haereus, Germany) for 5 mins. The supernatant was decanted after which 100 µl of solution 1 (50 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM ethylene diamine tetraacetic acid) was added to the tube followed by vortexing to dissociate bacterial pellets. The tubes were iced for 10 mins. Thereafter, 200 µl of lysis solution (200 mM NaOH, 1% SDS) was added to the tube and the contents mixed by inverting the tube several times. The tubes were again iced for 10 mins. Then 150 µl of solution III (5 M Potassium acetate, glacial acetic acid) was added to the tube and the

contents mixed by inverting. Ten microlitres each of Lysozyme (1%) and Proteinase K (1%) were added to the mixture to facilitate cell wall lysis and denaturation of proteins respectively. The tubes were allowed to stand for 5 mins at room temperature after which the tubes were spun at 13, 500 rpm (Biofuge Haereus, Germany) for 5 mins. The supernatant was removed and transferred to a new sterile Eppendorf tube, after which 450 μ l of phenol and chloroform: isoamylalcohol (24:1) was added. The tubes were spun at 13,500 rpm for 1 min. The supernatants were decanted and the pellets were washed by adding 1 ml of absolute ethanol. The tubes were vortexed briefly and then spun at 13,500 rpm for 1 min. The ethanol was then thoroughly decanted and the tubes were left to air dry on the bench for 5 mins. Thereafter, 100 μ l of sterile distilled water was added to the tubes and the pellets were resuspended by incubating the tubes in a water bath at 65°C for 1 hour. The isolated DNA was then stored at -20°C till use.

2.2.8.2 Determination of the quantity and purity of extracted DNA

Quantification of DNA extracted from all isolates (including control strains) was done using UV Visible Spectrophotometer (model S-22, Boeco, Germany). A 1:10 dilution of the extracted DNA was prepared in glass cuvettes (Sigma, SA) using sterile distilled nuclease-free water, which was also used as a blank. The optical density (OD) of the diluted samples at wavelengths of 260 nm was used to determine the concentration of the DNA in solution, using the standard that an OD of 1 at 260 nm corresponds to approximately 50 μ g/ml of doublestranded DNA. The purity of the extracted DNA was estimated using the ratio of OD at a wavelength of 260 nm versus OD at a wavelength of 280 nm as described by following the method of Sambrook *et al.* (1989). The readings of extracted DNA in the current study were between 1.8 and 2.0. Thereafter, 5 μ l of the purified DNA was analysed by standard submarine gel electrophoresis using 0.8% (w/v) agarose at 5 Vcm⁻¹ for 2 hours, to check the integrity of the DNA.

2.2.8.3 Polymerase chain reaction (PCR) of extracted DNA

The 16S rRNA gene fragments of all isolates were initially amplified as an internal control (Muyzer *et al.*, 1995). Thereafter, presumptive *Salmonella* isolates were screened for the presence of invasion (*invA*) and virulence (*spvC*) genes for identity confirmation (Khan *et al.*,

2000). All the oligonucleotide primers used for PCR reactions were obtained from Inqaba biotech, South Africa, and details of the sequences are shown in Table 2.3. Cycling conditions for amplification of 16S rRNA gene include preincubation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 1 min. Final extension was carried out at 72°C for 5 min after the last cycle. For *invA* and *spvC* genes, preincubation was at 94°C for 90 sec followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 90 sec. After the last cycle, final strand extension was done at 72°C for 3 min. All PCR reactions were prepared in 25 µl volumes consisting of 1 µg/µl of the template DNA, 50 pmol of each oligonucleotide primer, 1X PCR master mix, 1U Taq DNA polymerase and nuclease free distilled water.

Table 2.3: Primers used for amplification of target genes

Primer	Sequence (5' - 3')	Target gene	Amplicon size (bp)	Reference
27F	AGAGTTTGATCCTGGCTCAG	16S	1450	Muyzer <i>et al.</i> , 1995
1492R	GGTTACCTTGTTACGACTT	ribosomal gene		
InvF	CGCGGCCCGATTTTCTCTGGA	<i>invA</i>	321	Khan <i>et al.</i> , 2007
invR	AATGCGGGGATCTGGGCGACAAG			
VirF	GGGGCGGAAATACCATCTACA	<i>spvC</i>	392	Khan <i>et al.</i> , 2007
VirR	GCGCCCAGGCTAACACG			

Amplifications were performed using a Peltier Thermal Cycler (model-PTC-220 DYAD™ DNA ENGINE). A reaction blank containing all the components of the reaction mixture except the template DNA was included in each PCR procedure to check for contamination. Thereafter, 10 µl of the PCR amplicons was analysed by standard submarine gel electrophoresis using 1% (w/v) agarose at 60 V for 6 hours. Gene ruler™ 100 bp (Fermentas, USA)/Lambda marker were used as molecular weight markers in all the electrophoretic analyses. Electrophoresis was conducted

in a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia biotech, Sweden) using 1x TAE running buffer (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid, pH 8.0). The gels were stained in ethidium bromide (0.1 mg/ml) for 15 min and amplicons were visualised under UV light (Sambrook *et al.*, 1989). A Gene Genius Bio imaging system (Syngene, Synoptics; UK) was used to capture the image using GeneSnap (version 6.00.22) software. Images were analysed using GeneTools (version 3.07.01) software. Amplicons which showed clear bands for 16S rRNA, *invA* and *spvC* genes were purified and sequenced (Inqaba Biotech, SA) for strain identification of the isolates. The search for amino acid homology of the sequence results was done using the NCBI Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.2.8.4 Random amplification of polymorphic DNA (RAPD)-PCR

Confirmed *Salmonella* isolates identified through the expression of *Salmonella*-specific *invA* gene were further subjected to fingerprinting by RAPD-PCR to evaluate the genetic diversity among the isolates. Oligonucleotides of short sequence were used (Table 2.4) and the PCR reactions were prepared as before. For RAPD 1, the cycling conditions included an initial denaturation step at 94°C for 5 mins followed by 42 cycles of denaturation at 94°C for 1 minute, annealing at 27°C for 45 seconds and extension at 72°C for 90 seconds. The last cycle was followed by final extension at 72°C for 5 mins (Habtamu-Taddele *et al.*, 2011). Initial denaturation step for RAPD 2 and 3 was at 94°C for 1 minute followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute and extension at 72°C for 2 mins. Final extension was carried out at 72°C for 10 mins (Quintaes *et al.*, 2004). Aliquots (10 µl) of the resulting amplicons were characterised on 1.2% agarose gel electrophoresed at 60 V for 6 hours. Isolates were later scored for the presence or absence (1 or 0) of each band on agarose gel (Habtamu-Taddele *et al.*, 2011).

Table 2.4 RAPD primers used for fingerprinting of isolates

Primer	Sequence (5' – 3')	Reference
RAPD 1	AGCGTCACTC	Quintaes <i>et al.</i> , 2004
RAPD 2	GCGGAAATAG	
RAPD 3	AACGCGCAAC	

2.2.9 Data analysis

To obtain the cfu/g of sample for total bacterial counts, colonies on plate count agar were counted and compared with the dilution factor as follows (McLandsborough, 2004):

$$\text{Cfu/g sample} = \frac{\text{No. of Colonies}}{\text{Dilution factor} \times \text{Weight of sample}}$$

All *Salmonella* populations were transformed to base 10 logarithms before analysis. Recovery rate of *Salmonella* isolates was calculated using the following equation as described by Charimba *et al.* (2010).

$$\text{Recovery rate} = \frac{\text{No of positive samples}}{\text{Total no of samples}} \times 100$$

Means in recovery rates between locations, shop and product types were compared using Student-t Test of SPSS (SPSS 10.0 for windows, SPSS Chicago, IL, USA). Differences were considered significant at $P \leq 0.05$. For cluster analysis, the distance matrices, means and standard deviations were calculated using the STATISTICA software and the result was used to construct a phylogenetic relationship among isolates by the neighbour-joining method of Saitou and Nei (1987).

2.3 Results and Discussion

2.3.1 Total bacterial count of samples

On the average, total bacterial counts of butchery samples (2.2×10^5 cfu/g) was higher than that of supermarket samples (1.4×10^4 cfu/g). Similarly, raw chicken parts were more contaminated (7.6×10^5 cfu/g) than whole chicken carcasses (2.5×10^5 cfu/g). Ready-to-eat products were the least contaminated (1.8×10^3 cfu/g). However, the level of microbial contamination of all analyzed products was within the recommended range for some developed countries such as the United Kingdom (10^4 - 10^5 cfu/g) and Australia (10^6 cfu/g) (Dahal, 2007).

2.3.1 Incidence of NTS in broiler products

Out of eleven locations sampled, *Salmonella* was recovered from products obtained from six (54.5%) of the locations (Table 2.5). Difference in *Salmonella* isolation rates between positive locations was significant ($P = 0.009$) with Mafikeng having the highest rate. However, *Salmonella spp* was not recovered from RTE products whereas the average recovery rate in raw broiler products was 12.5%. This rate is lower than 19.5% reported by van Nierop *et al.* (2005) in Gauteng Province of South Africa, 20% in the US (White *et al.*, 2001), 30% in Canada (Bohaychuk *et al.*, 2006), 39.7% in Mexico (Zaidi *et al.*, 2006) and 60% in Portugal (Antunes *et al.*, 2003). Other countries such as Zambia, Saudi Arabia, Turkey and Sudan, however, recorded lower incidences of NTS (4.7%, 5.92%, 8% and 9.2% respectively) in broiler products than noted in the current study (Hang'ombe *et al.*, 1999; Moussa *et al.*, 2010; Dogru *et al.*, 2010; El Hussein *et al.*, 2010). Incidence of *Salmonella* contamination was higher in whole chicken carcasses than chicken parts in most of the towns except Mmabatho and Mafikeng (Figs. 2.3 and 2.5). The former observation is contrary to the reports of Chaïba *et al.* (2008) and Moussa *et al.* (2010) who noted higher incidences of NTS in chicken cuts in Morocco and Sudan respectively. It may be worthy to note that the presence and distribution of NTS serovars vary from region to region, the sampling plan and detection limits of the methodology employed (Uyttendaele *et al.*, 1998; Dominguez *et al.*, 2002).

Butchery products were more contaminated with NTS than supermarket products particularly in Mafikeng (Figs. 2.3 and 2.4). This result is in agreement with the findings of Chaiba *et al.* (2008), and could be due to better hygienic standards maintained in supermarkets than butcheries. Frozen products had higher NTS recovery rate (10.4%) than fresh products (8.8%). This may suggest initial high contamination of the frozen products with NTS, in which case, the freezing temperature range was not sufficient to inactivate the pathogens. It has been documented that freezing may not be regarded as a means of destroying food borne microorganisms. This is because low freezing temperature of about - 20°C are less harmful to the pathogens than the median temperature ranges (Jay *et al.*, 2005).

Table 2.5: Number of *Salmonella* positive samples obtained from different locations

Sampling area	No. of samples obtained	No. positive samples	% positive samples
Suncity	6	0	0
Koster	8	1	12.5
Delareyville	16	0	0
Vryburg	12	0	0
Mmabatho	12	2	16.7
Mafikeng	33	6	18.2
Zeerust	19	0	0
Rustenburg	18	2	11.1
Lichtenburg	16	2	12.5
Klerksdorp	15	0	0
Potchefstroom	25	2	8

2.3.2 Molecular characterization of NTS isolates obtained from broiler products

A total of 160 presumptive *Salmonella* isolates obtained from analyzed broiler products were screened for the presence of invasion and virulence genes. All the isolates showed positive amplification of the 16S rRNA fragment (Fig. 2.6). Out of the 160 screened isolates, *invA* gene

was detected in 140 (87.5%) of the isolates (Fig. 2.7). The *invA* gene, located on the *Salmonella* pathogenicity island 1 (SPI 1), is highly conserved in *Salmonella* species and encodes a type III secretion system (TTSS) that exports proteins in response to bacterial contact with epithelial cells (Galan *et al.*, 1992; Rahn *et al.*, 1992). The *invA* gene operon is essential for full virulence in *Salmonella* (Torpdahl *et al.*, 2005). Torpdahl *et al.* (2005) and Khan *et al.* (2007) similarly detected *invA* gene in all *Salmonella* isolates recovered from poultry products and orange juice respectively.

Analysis of the sequenced isolates in the current study revealed three NTS serotypes viz: *S. typhimurium* (46.4%; n = 65), *S. enteritidis* (30.7%; n = 43) and *S. newport* (22.9%; n = 32). The rate of isolation of the different NTS serotypes is in agreement with the reports from previous researches (Chaiba *et al.*, 2008; Moussa *et al.*, 2010). All the identified serotypes were isolated from both supermarket and butchery samples (Fig. 2.8), and also from whole carcasses and chicken parts at different rates (Fig. 2.9). Furthermore, some of the products were found to be contaminated with multiple NTS serotypes. However, *S. enteritidis* was the only serotype isolated from free range products. This observation agrees with the work of Esteban *et al.* (2008) who reported *S. enteritidis* as the only *Salmonella* serotype isolated from free range chickens. This may be due to the potential for increased bacterial contamination in the free range poultry as a result of easy access to transmitting vectors such as rodents, wild animals, etc (Foley *et al.*, 2011).

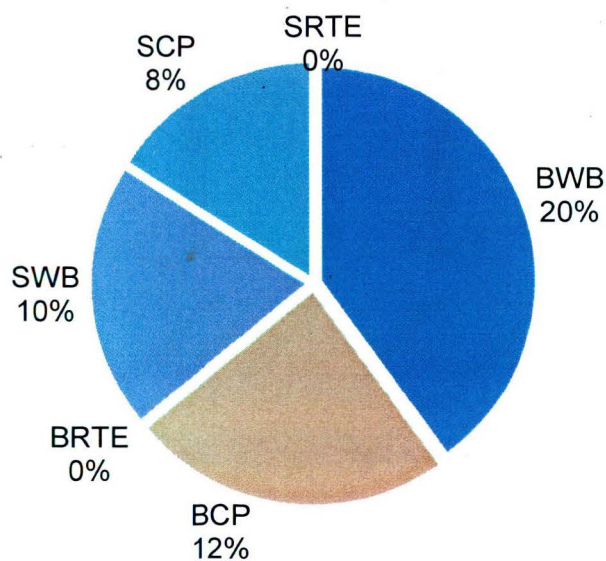


Figure 2.3: Percentage incidence of NTS in samples based on shop and product type. BWB = butchery whole broilers; BCP = butchery chicken parts; BRTE = butchery ready-to-eat; SWB = supermarket whole broilers; SCP = supermarket chicken parts; SRTE = supermarket ready-to-eat.



Figure 2.4: Percentage incidence of NTS in different locations by shop type.

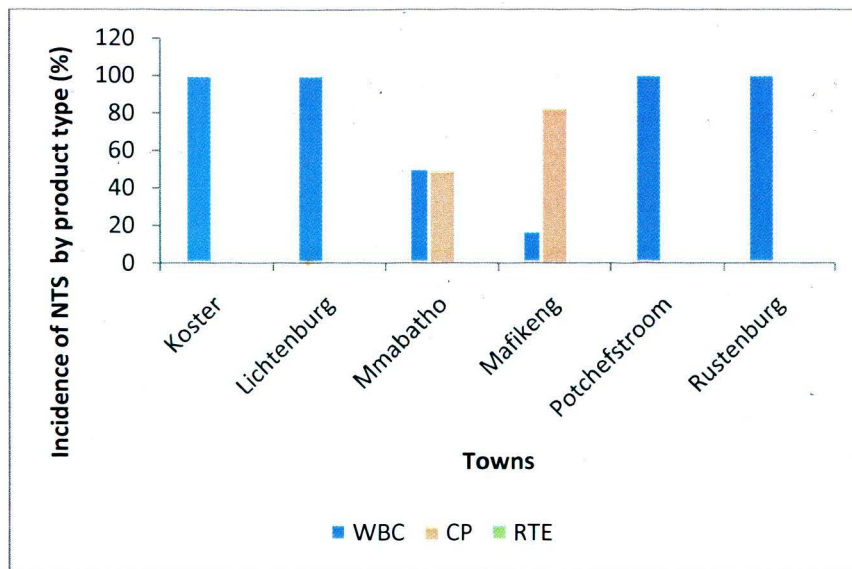


Figure 2.5: Percentage incidence of NTS in different locations by product type. WBC = whole broiler carcasses; CP = chicken parts; RTE = ready-to-eat

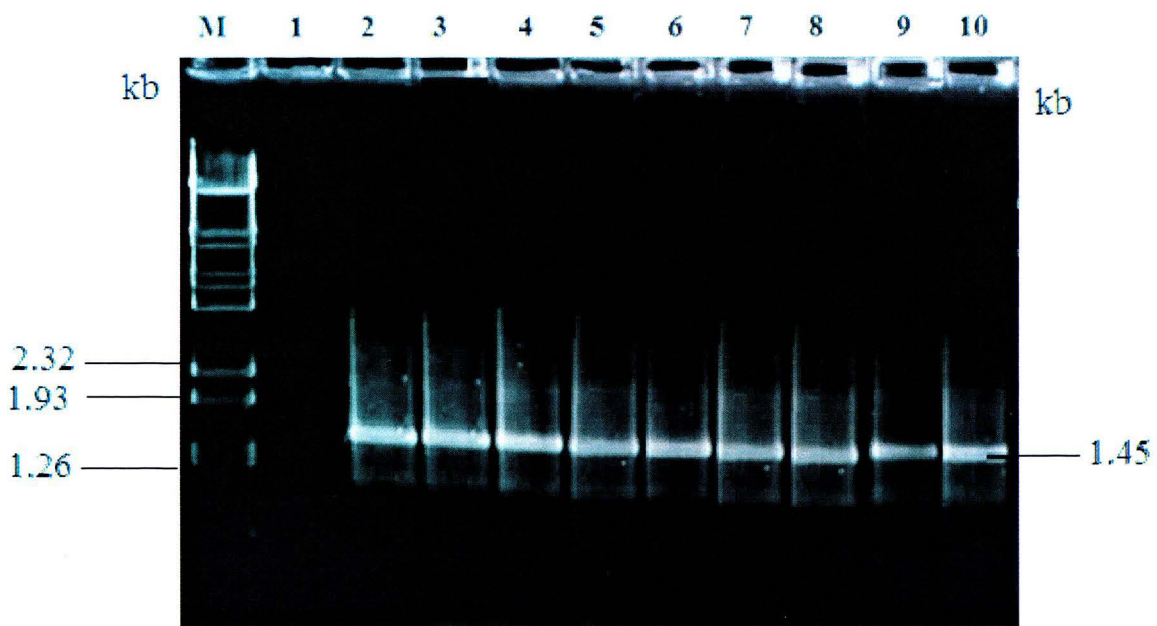


Figure 2.6: Agarose gel (1%) electrophoresis showing 16S rRNA gene fragments amplified from *Salmonella* spp. Lane 1 = No template (control); lane 2 = *S. typhimurium* ATCC 14028; lanes 3 - 10 = positive amplification of 16S rRNA gene fragments of *Salmonella* spp isolated from samples; lane M = DNA marker (Lambda).

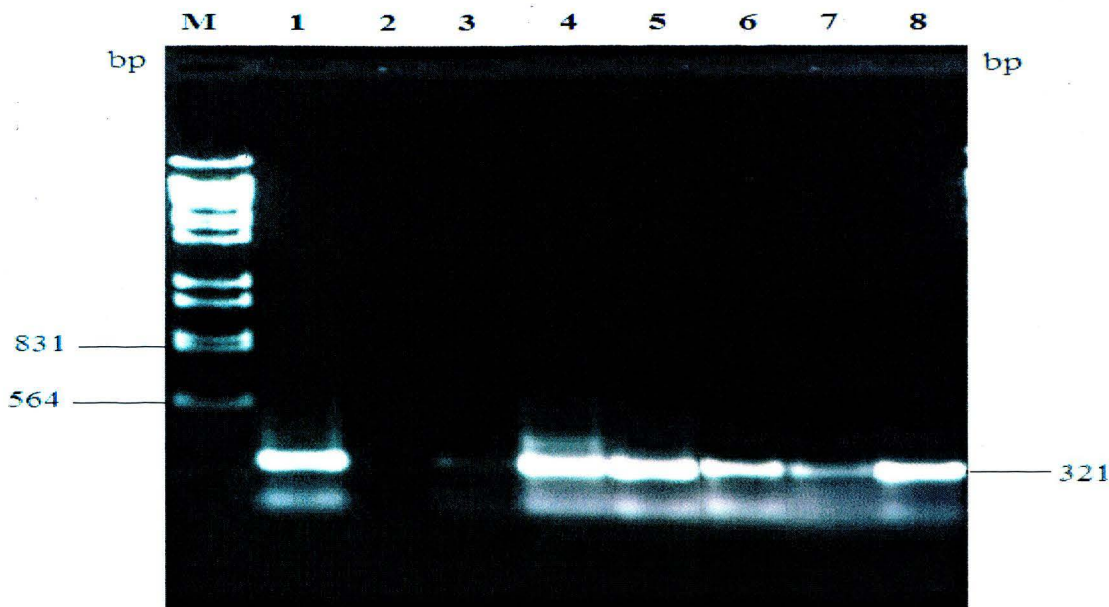


Figure 2.7: Agarose gel (1%) electrophoresis showing amplification of 321 bp fragments of *invA* genes of *Salmonella*. Lane 1 = *S. typhimurium* ATCC 14028; lane 2 = negative control; lanes 3 to 8 showing positive amplification of 321 bp fragments of *Salmonella* spp; lane M = DNA marker (Lambda).

Out of the 140 isolates confirmed for the presence of *invA* gene, 115 (82.4%) isolates were found to harbour the *spvC* plasmid gene (Fig. 2.10). This represents 71.9% of all the screened isolates. The prevalence of the *spvC* gene fragment was higher in isolates from butchery samples (71.4%) than supermarket samples (25.9%) and isolates from chicken parts (57.1%) than whole broiler carcasses (42.9%). The detection rate of the *spvC* gene in the current study agrees with the findings of Bolton *et al.* (1999) and Khan *et al.* (2000) who detected the gene fragment in 97% and 88% of *Salmonella typhimurium* DT104 strains respectively. In contrary however, Khan *et al.* (2007) did not detect *spvC* gene in any of the *Salmonella* serotypes subjected to multiplex PCR. It is worthy to note that the recovery rate of *S. typhimurium* in this study is higher than that of other serotypes (*S. enteritidis* and *S. newport*), as opposed to the report of Khan *et al.* (2007) who did not recover *S. typhimurium* isolates in the juice samples analysed. Thus the disparity in the detection of *spvC* gene fragment could possibly be due to prevalence of the gene in *S. typhimurium* serotype. The *spvC* gene in *Salmonella* spp. interacts with the host immune system and is responsible for an increased growth rate in host cells (Khan *et al.*, 2007).

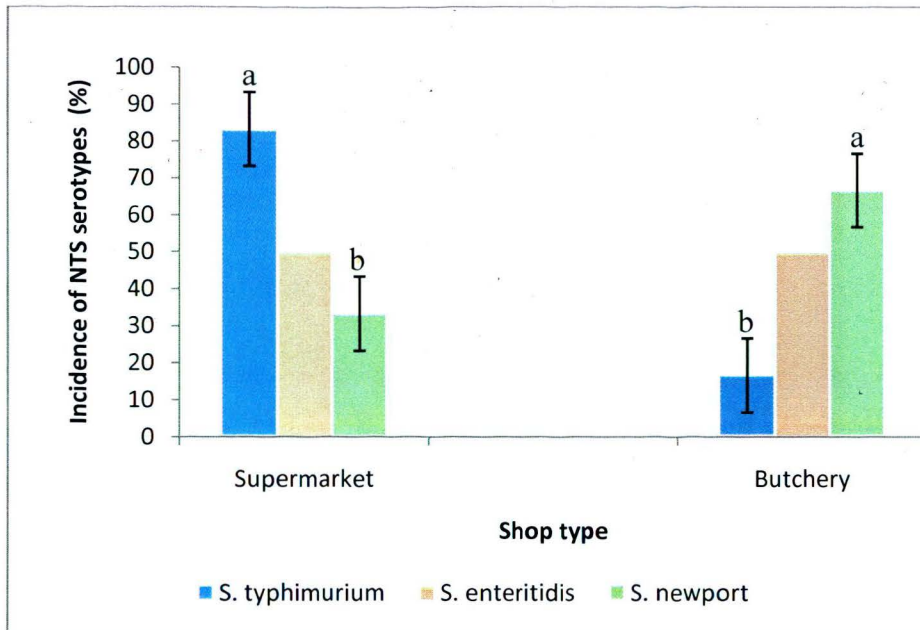


Figure 2.8: Percentage incidence of different NTS serotypes by shop type. Bars with different labels are significantly different ($P < 0.05$).

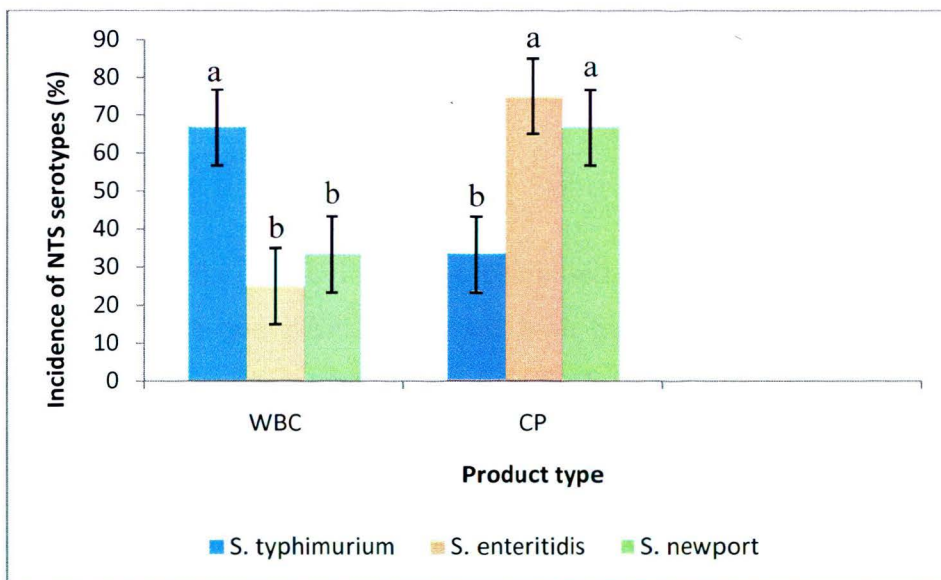


Figure 2.9: Percentage incidence of NTS serotypes by product type. WBC = whole broiler carcasses; CP = chicken parts; RTE = ready-to-eat. Bars with different labels are significantly different ($P < 0.05$).

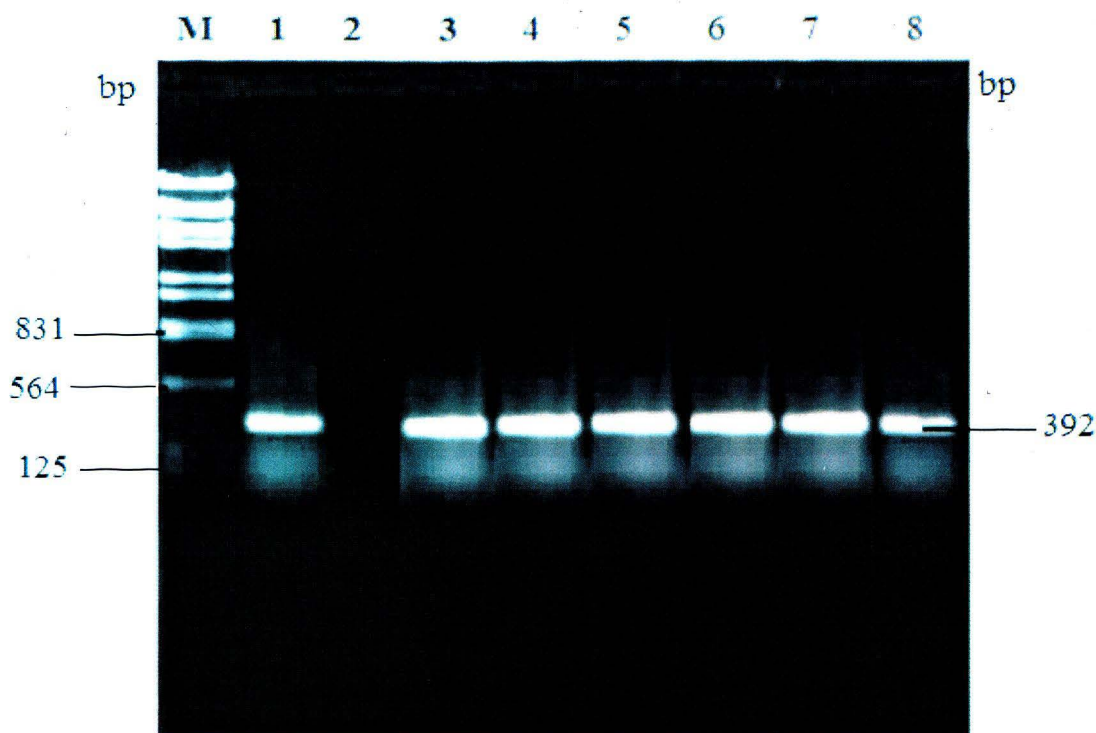


Figure 2.10: Agarose gel (1%) electrophoresis showing amplification of 392 bp fragments of *spvC* genes of *Salmonella*. Lane 1 = *S. typhimurium* ATCC 14028; lane 2 = negative control; lanes 3 to 8 showing positive amplification of 392 bp fragments of *Salmonella* spp; lane M = DNA marker (Lambda).

2.3.3 Analysis of RAPD-PCR

The profiles generated by the RAPD-PCR were composed of 1 – 5 bands ranging between 0.25 and 1.4 kb. Both RAPD 1 and RAPD 3 primers each generated four banding profiles for *S. typhimurium*, three for *S. newport* and two profiles for *S. enteritidis* (Figs. 2.11 and 2.12). One strain of *S. newport* (SN1), isolated from chicken parts obtained from the butchery, showed no bands with both RAPD 1 and RAPD 3 primers. Similarly, a strain of *S. typhimurium* (ST2) isolated from supermarket whole broiler carcasses, showed three bands with RAPD 1 but no bands with RAPD 3 primer. However, both strains produced one band of about 0.45 kb as did most of the isolates with RAPD 2 primer although the primer had very poor discriminatory power and was therefore not included in the analysis. Furthermore, cluster analysis of the RAPD 1 profiles revealed three different RAPD types for *S. newport* and *S. enteritidis* whereas *S. typhimurium* was classified into four types (Fig. 2.13). RAPD 3 primer on the other hand,

grouped *S. typhimurium* into four, *S. newport* into three and *S. enteritidis* into two RAPD types (Fig. 2.14). Although a few isolates of the same serotype showed either similar or same banding patterns, some level of heterogeneity was generally noticed in the isolates, irrespective of the sources (i.e. geographical location, shop or product type). Interestingly, a particular strain of *S. typhimurium*, isolated from Mafikeng product, was closely clustered with the positive control strain by both RAPD 1 and RAPD 3 primers. In spite of the heterogeneity manifested by the isolates, it may still be safely concluded that most of the isolates have the same clonal origin. However, the genetic diversity noticed among isolates of even the same serotype may indicate varying sources of product contamination (e.g. live birds, water, environment or product handler). Furthermore, changes in bacterial genome may occur in the same serovar through plasmid acquisition or other genetic mutations without manifesting phenotype alterations (Baggesen and Christensen, 1997). RAPD fingerprinting has been suggested to be the most convenient and sensitive technique, capable of revealing inter- and intraserotype-specific differences among *Salmonella* isolates (Tikoo *et al.*, 2001).

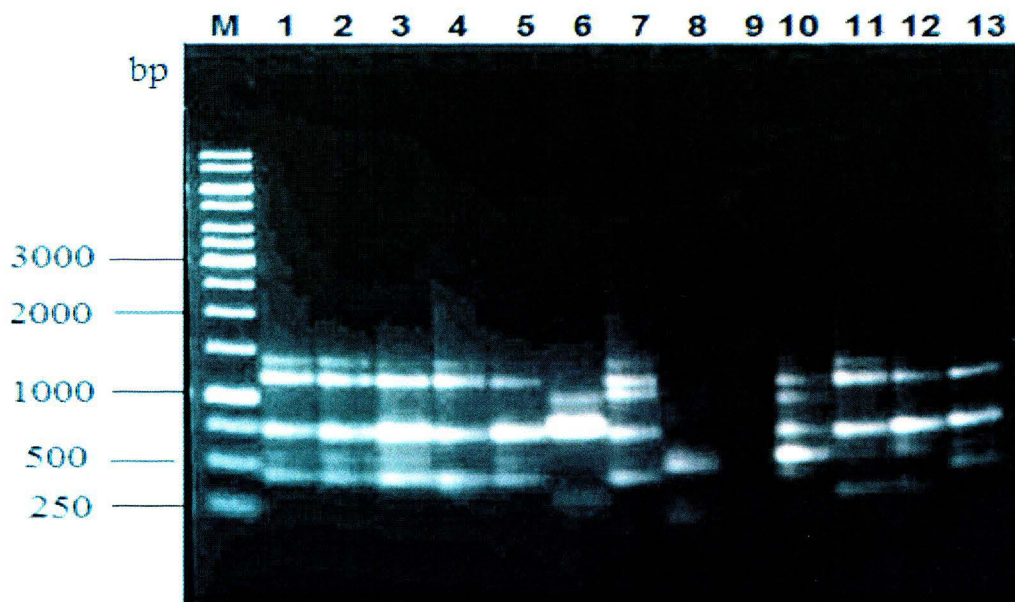


Figure 2.11: Agarose gel (1.2%) electrophoresis showing DNA fingerprinting of NTS serotypes generated using RAPD 1 primer. Lane M = DNA marker (1 kb); lane 1 = SE1; lane 2 = SE2; lane 3 = SE3; lane 4 = SE4; lane 5 = ST1; lane 6 = ST2; lane 7 = ST3; lane 8 = ST4; lane 9 = SN1; lane 10 = SN2; lane 11 = SN3; lane 12 = SN4; lane 13 = *S. typhimurium* ATCC 14208 (C).



Figure 2.12: Agarose gel (1.2%) electrophoresis showing DNA fingerprinting of NTS serotypes generated using RAPD 3 primer. Lane M = DNA marker (1 kb); lane 1 = SE1; lane 2 = SE2; lane 3 = SE3; lane 4 = SE4; lane 5 = ST1; lane 6 = ST2; lane 7 = ST3; lane 8 = ST4; lane 9 = SN1; lane 10 = SN2; lane 11 = SN3; lane 12 = ST4; lane 13 = *S. typhimurium* ATCC 14208 (C)

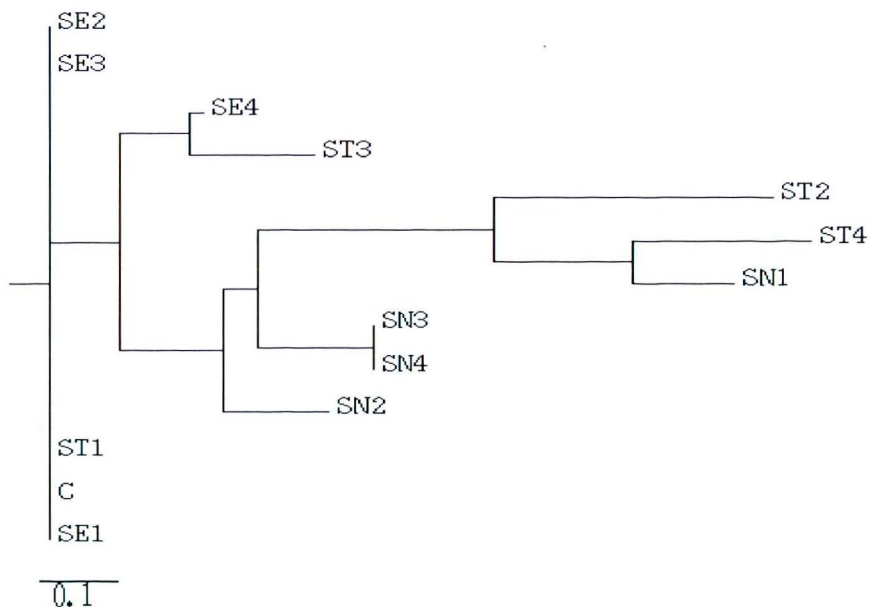


Figure 2.13: Dendrogram generated based on the RAPD analysis of NTS serotypes using RAPD 1 primer. SE = *S. enteritidis*; ST = *S. typhimurium*; SN = *S. newport*; C = control (*S. typhimurium* ATCC 14208). All serovars within a serotype showing the same band type are grouped as a sub-serotype (e.g. ST1)

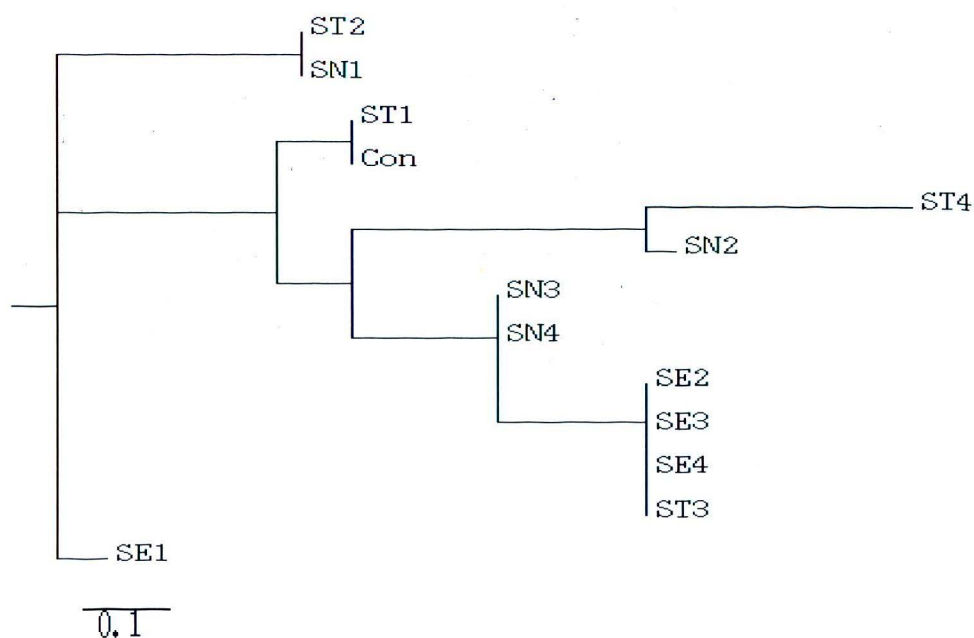


Figure 2.14: Dendrogram generated based on RAPD analysis of NTS serotypes using RAPD 3 primer

SE = *S. enteritidis*; ST = *S. typhimurium*; SN = *S. newport*; Con = control (*S. typhimurium* ATCC 14208). All serovars within a serotype showing the same band type are grouped as a sub-serotype (e.g. ST1)

In conclusion, it can be firmly stated that raw broiler products obtained from the NWP of South Africa were found to be contaminated with three major serotypes of non-typhoidal *Salmonella*, with an average incidence rate of 12.5%. This implies that consumers must exercise caution in handling and processing of raw broiler products. Storage of the products by freezing at -20°C must not be considered as a means of eliminating *Salmonella* organisms from contaminated products. The products must therefore be subjected to adequate processing prior to consumption to avoid infection.

CHAPTER 3

ANTIBIOTIC RESISTANCE OF NON-TYPHOIDAL SALMONELLA STRAINS ISOLATED FROM BROILER PRODUCTS OBTAINED FROM THE NORTH WEST PROVINCE OF SOUTH AFRICA.

3.1 Introduction

In recent years, occurrence of antimicrobial resistance among *S. enterica* serovars has been reported in many countries (Lee *et al.*, 2009; Fashae *et al.*, 2010; Wannaprasat *et al.*, 2011). Antimicrobial resistance is a major global health problem in both human and veterinary medicine, with serious socioeconomic consequences. The problem is however more pressing in developing countries where disease burden is high and use of newer antimicrobials is constrained by cost (Okeke *et al.*, 2005). β -lactam antibiotics such as ampicillin, tetracycline, chloramphenicol and trimethoprim-sulphamethoxazole are readily available antimicrobial agents for the treatment of invasive salmonellosis. Resistance to these drugs has however, been reported with increasing frequency in many countries (Ruiz *et al.*, 1999; Kariuki *et al.*, 2005). For instance, resistance of *Salmonella* isolates, from foods of animal origin, to ampicillin, tetracycline, sulphonamides and chloramphenicol was high in Malaysia (22 – 49%), Thailand (41 – 92%) and Vietnam (17 – 68%) (Benacer *et al.*, 2010; Vo *et al.*, 2010; Wannaprasat *et al.*, 2011). Zewdu and Cornelius (2009) similarly reported high antimicrobial resistance (62.1%) in *Salmonella* isolates from chicken carcasses in Ethiopia.

Concurrently, resistance of human NTS isolates to the commonly used antibiotics (ampicillin, tetracycline, etc) is high in developing countries, suggesting the extensive use or abuse of the antimicrobials in veterinary practice and human therapy. Sequel to this development, the fluoroquinolones became drugs of first choice in the treatment of invasive and systemic salmonellosis in both humans and animals (Yates and Amyes, 2005; Giraud *et al.*, 2006). However, *Salmonella* strains with decreased susceptibility to fluorinated quinolones have been reported in many countries and are now considered widespread. Fashae *et al.* (2010) reported a high frequency (up to 87%) of reduced susceptibility to ciprofloxacin in NTS isolates from

chickens in Nigeria. Similarly, Ellerbroek *et al.* (2010) observed that more than half of NTS spp. isolated from pigs were resistant to ciprofloxacin. Reduced susceptibility of clinical *Salmonella* isolates to ciprofloxacin has also been reported in Philippines (15%) and Singapore (25%). Whereas the production of β -lactamase is the primary cause of resistance to β -lactam antibiotics in *Enterobacteriaceae*, resistance to quinolones is believed to result from chromosomal mutations in genes that encode target enzymes or affect drug accumulation (Arlet *et al.*, 2006; Strahilevitz *et al.*, 2009). TEM and SHV penicillinases seem to be the most widely spread extended-spectrum beta lactamases (ESBLs), and nosocomial strains of *Klebsiella pneumoniae* and *Escherichia coli* are believed to be main reservoirs of ESBL genes (Miriagou *et al.*, 2004). However, hospital NTS isolates producing TEM, SHV, CMY and CTX-type ESBLs have been reported in South Africa (Kruger *et al.*, 2004; Keddy *et al.*, 2009; Usha *et al.*, 2008). Similarly, the first plasmid-mediated quinolone resistance in *Salmonella* from South Africa was observed in *S. typhimurium* isolated from the blood of an HIV-seropositive patient (Govender *et al.*, 2009). Antimicrobial resistance of NTS isolates from contaminated poultry products in the NWP of South Africa has not been elucidated. This study therefore aims at investigating the resistance of NTS species isolated from broiler products in the Province to some common antimicrobials, and to screen the isolates for the presence of antibiotic resistance genes.

3.2 Materials and Methods

3.2.1 Bacterial isolation and identification

Sampling, isolation and identification was done as described in Chapter 2.

3.2.2 Antimicrobial susceptibility testing of isolates

A total of sixty NTS isolates (*S. typhimurium* = 20; *S. enteritidis* = 20; *S. newport* = 20) identified by phenotypic and genotypic methods were subjected to antibiotic susceptibility testing using the Kirby-Bauer disc-diffusion method as previously described (Ndip *et al.*, 2005). This method conforms to the recommended standard of the Clinical and Laboratory Standards Institute (CLSI). *E. coli* ATCC 25922 was used as a control strain for the susceptibility testing.

Briefly, a small inoculum of each pure *Salmonella* isolate, taken from a fresh culture on nutrient agar, was emulsified in 3 ml of sterile normal saline in Bijou bottles. The density of the emulsion was compared with a barium chloride standard (0.5 McFarland) to ensure appropriate turbidity. A sterile cotton swab was then dipped into the standardized suspension of bacterial cultures and used to evenly inoculate Mueller-Hinton agar plates (Mercks, South Africa). The plates were allowed to dry. Antibiotic discs (Davies diagnostics, SA) with the following drug contents: ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), trimethoprim-sulphamethoxazole (25 µg), tetracycline (30 µg) cefotaxime (30 µg), meropenem (10 µg), gentamicin (10 µg) and erythromycin (15 µg) were placed at least 15 mm apart and from the edge of the plates to prevent overlapping of the inhibition zones. Plates were incubated at 37°C for 18 - 24 hours. The diameters of zones of inhibition were compared with recorded diameters of the control organism to determine the susceptibility or resistance of isolates to the various drugs. Results were recorded as susceptible, intermediate or resistant depending on the recommended diameter sizes for each antibiotic (CLSI). Multiple antibiotic resistance (MAR) phenotypes were generated for all isolates that showed resistance to two or more antibiotics. Index for antibiotic resistance phenotypes was calculated using the following equation (Singh *et al.*, 2010):

$$\text{MAR index} = \frac{\text{Number of resistance x antibiotics}}{\text{Total number of antibiotics tested}}$$

Based on antibiotic resistance patterns of the isolates, a dendrogram was constructed to show the relationship among the NTS isolates using the root-mean-square deviation (rmsd) coefficient, Unweighted Pair Group Method with arithmetic mean (UPGMA) (Saitou and Nei, 1987).

3.2.3 Molecular characterization of resistant isolates

Overnight cultures of the isolates that showed resistance to ampicillin, tetracycline and chloramphenicol were prepared in 10 ml of nutrient broth, from which the plasmid DNA of the isolates were extracted. The extracted DNA was subjected to PCR amplification, targeting *bla*TEM, *tet* and *cat* genes (Türkyilmaz *et al.*, 2009). The details of oligonucleotide sequences used and the PCR cycling conditions are shown in Table 3.1. DNA extraction and PCR was done as described in Chapter 2. Amplicons which showed clear bands for *bla*TEM, *tet* and *cat* genes

were purified and sequenced (Inqaba Biotech, SA) for gene identification. The search for amino acid homology of the sequence results was done as described before.

Table 3.1: Oligonucleotide sequences and PCR conditions used for amplification

Primer	Sequence (5' - 3')	Target gene	Amplicon size (bp)	PCR conditions
TEM1 F	ATGAGTATTCAACATTTCCGTG	<i>bla</i> TEM	793	95°C, 3 mins; 30 cyc. of 95°C, 1 min, 55°C, 1min, 72°C, 1min, ext. at 72°C, 5 mins
TEM2 R	TTACCAATGCTTAATCAGTGAG			
TET F	GCACTTGTCTCCTGTTTACTCCCC	<i>tet</i>	659	94°C, 2 mins; 35 cyc. of 94°C, 20 s, 53°C, 10 s, 65°C, 45 s, ext. at 65°C, 4 mins
TET R	CCTTGTGGTTATGTTTTGGTTCCG			
CAT F	TCCCAATGGCATCGTAAAGAAC	<i>cat</i>	310	95°C, 5 mins; 35 cyc. of 94°C, 30 s, 55°C, 30 s, 72°C, 30 s, ext. at 72°C, 10 mins
CAT R	TCGTGGTATTCACTCGAGAGCG			

Cyc = cycle; ext = extension

3.3 Results and Discussion

3.3.1 Antimicrobial susceptibility profiles

All the strains tested were resistant to two or more antibiotics, however, multi-drug resistance was discovered only in nine *S. typhimurium* isolates, representing 15% of all the tested isolates. The *S. typhimurium* isolates were resistant to ampicillin, tetracycline, chloramphenicol, ciprofloxacin, trimethoprim-sulphamethoxazole and erythromycin. Dogru *et al.* (2010) and Maripandi and Al-Salamah (2010) also reported resistance to multiple antimicrobials in *Salmonella* isolated from chicken carcasses. However, all isolates were susceptible to cefotaxime, meropenem, gentamycin and amikacin whereas all were resistant to tetracycline. High resistance of isolates to ampicillin and tetracycline observed in the current study is in

agreement with previous research findings (Antunes *et al.*, 2003; Cheong *et al.*, 2007; Fashae *et al.*, 2010). A low frequency of resistance was found towards trimethoprim-sulphamethoxazole (8.3%), ciprofloxacin (13.3%) and chloramphenicol (20%). *Salmonella typhimurium* showed the highest resistance to both chloramphenicol and ampicillin (35% and 100% respectively) and this is in agreement with the report of Soler *et al.* (2006). Figure 3.1 and Table 3.2 indicate the frequency of resistance to individual drugs and distribution of antimicrobial resistance in isolates respectively. Tetracycline and the β -lactams such as ampicillin are among the antibiotics used frequently in poultry production (Moussa *et al.*, 2007). Thus, uncontrolled use of these antimicrobials in the live birds may have contributed to the observed resistance patterns. Reduced susceptibility of NTS isolates to ciprofloxacin noted in this study is in accordance with the observation of Fashae *et al.* (2010) in NTS isolates from Nigeria. In contrary, Salam *et al.* (2003) and Zaidi *et al.* (2006) reported 100% susceptibility of NTS isolates to ciprofloxacin in Bangladesh and Mexico respectively. Thus, the source of isolates with respect to geographical location and the use of different antibiotics in different places may possibly be responsible for the disparity in observations noticed in this case. However, resistance to ciprofloxacin (a fluoroquinolone) noticed in the current study was found mainly in *S. typhimurium* which seems to be the prevalent serotype in the study area. This may be a cause for public health concern, particularly in the treatment of invasive salmonellosis.

MAR analysis revealed seven resistance phenotypes, with resistance to ampicillin, tetracycline and erythromycin being the most common pattern (Table 3.3). The highest frequency of this resistance pattern was found in *S. typhimurium*. In addition, four other resistance patterns were shown by *S. typhimurium*, including resistance to ciprofloxacin, ampicillin, chloramphenicol and tetracycline. This observation agrees with the report of Soler *et al.* (2006) who noted resistance to ampicillin, chloramphenicol, sulphonamide and tetracycline as the most frequent resistant patterns of *S. typhimurium*, particularly the DT104 strain. *S. newport* showed three resistance patterns including resistance to ampicillin, trimethoprim-sulphamethoxazole and tetracycline, whereas *S. enteritidis* showed only two resistance phenotypes.

3.3.2 Cluster analysis

Cluster analysis of all isolates based on antimicrobial resistance patterns revealed six major clusters (Fig. 3.2). However, *S. typhimurium* strains belonged to four distinct clusters whereas *S. enteritidis* and *S. newport* strains were grouped into three and two clusters respectively. Most of the NTS isolates tested showed MAR, irrespective of their sources. Therefore, the clonal diversity observed among the strains in the current study is an indication of possible circulation of antimicrobial resistance genes in *Salmonella* strains within the environment. This may significantly add to the health care burden.

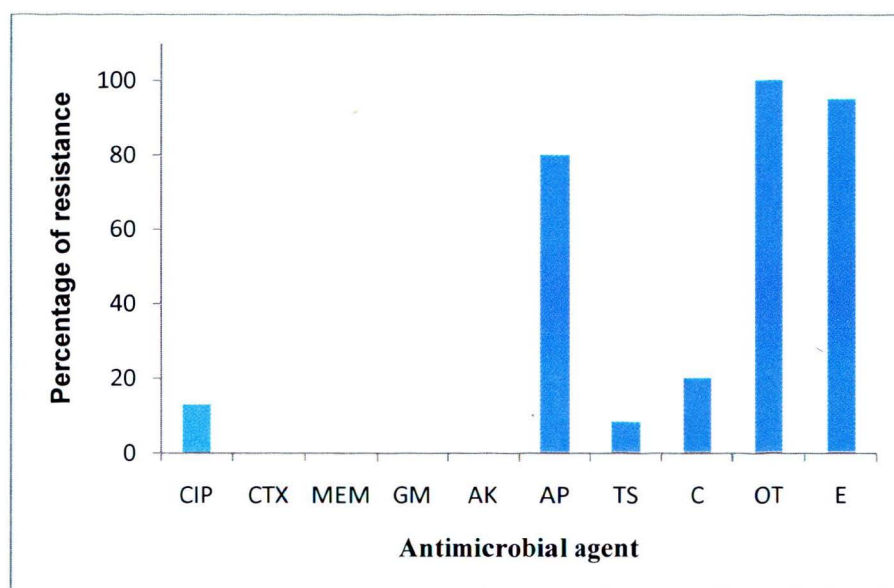


Figure 3.1: Frequency of resistance to individual antimicrobial agent. CIP = ciprofloxacin; CTX = cefotaxime; MEM = meropenem; GM = gentamycin; AK = amikacin; AP = ampicillin; C = chloramphenicol; OT = tetracycline; TS = trimethoprim-sulphamethoxazole; E = erythromycin

3.3.3 Antimicrobial resistance genes

Occurrence of resistance genes among antimicrobial-resistant isolates is shown in Table 3.4. Out of the 48 ampicillin-resistant isolates that were screened, 15 (31.3%) carried the blaTEM gene which is an ESBL gene (Fig. 3.3). This observation concurs with the report of Hamidian *et al.* (2009) who investigated the presence of ESBLs in *Salmonella* species obtained from patients in Tehran hospitals. Turkyilmaz *et al.* (2009) similarly noted that three out of five ampicillin-

resistant *Salmonella* species tested carried the blaTEM gene. The presence of TEM-63, TEM-116 and TEM-131 in NTS isolates have also been reported in Durban, South Africa (Usha *et al.*, 2008). The TEM type β -lactamase thus appear to be the most common gene that mediates resistance to ampicillin in *Salmonella* (Turkyilmaz *et al.*, 2009). Similarly, 12 (20%) out of 60 tetracycline-resistant isolates were found to harbour the *tetB* gene (Fig. 3.4), which is in agreement with the observations of Roberts (2005) and Turkyilmaz *et al.* (2009). In addition, *cat3* (chloramphenicol resistance) gene was found in 5 (42%) out of twelve chloramphenicol-resistant NTS isolates whereas one isolate (8%) haboured both *cat1* and *cat3* genes (Fig. 3.5).

Table 3.2: Percentage distribution of antimicrobial resistance among NTS isolates

Antimicrobial agent	<i>S. enteritidis</i> (n = 20)	<i>S. typhimurium</i> (n = 20)	<i>S. newport</i> (n = 20)
CIP (5)	25	15	0
CTX (30)	0	0	0
MEM (10)	0	0	0
GM (10)	0	0	0
AK (30)	0	0	0
AP (10)	75	100	65
TS (25)	0	15	10
C (30)	25	35	0
OT (30)	100	100	100
E (15)	100	85	100

CIP = ciprofloxacin; CTX = cefotaxime; MEM = meropenem; GM = gentamycin; AK = amikacin; AP = ampicillin; C = chloramphenicol; OT = tetracycline; TS = trimethoprim-sulphamethoxasole; E = erythromycin

Table 3.3: MAR patterns of obtained isolates

MAR phenotypes	<i>S. typhimurium</i>	<i>S. enteritidis</i>	<i>S. newport</i>	Total no. observed	MAR index
Ap-Ot-E	15	5	12	32	9.6
Cip-Ap-Ot-E	3	0	0	3	1.2
Cip-Ap-C-Ot-E	3	0	0	3	1.5
Ot-E	0	3	10	13	0.8
Ts-Ot-E	0	0	3	3	0.9
Ap-Ts-C-Ot-E	3	0	0	3	1.5
Ap-C-Ot	3	0	0	3	0.9
C-Ot-E	0	3	0	3	0.9

Cip = ciprofloxacin; Ap = ampicillin; C = chloramphenicol; Ot = tetracycline; Ts = trimethoprim-sulphamethoxasole; E = erythromycin

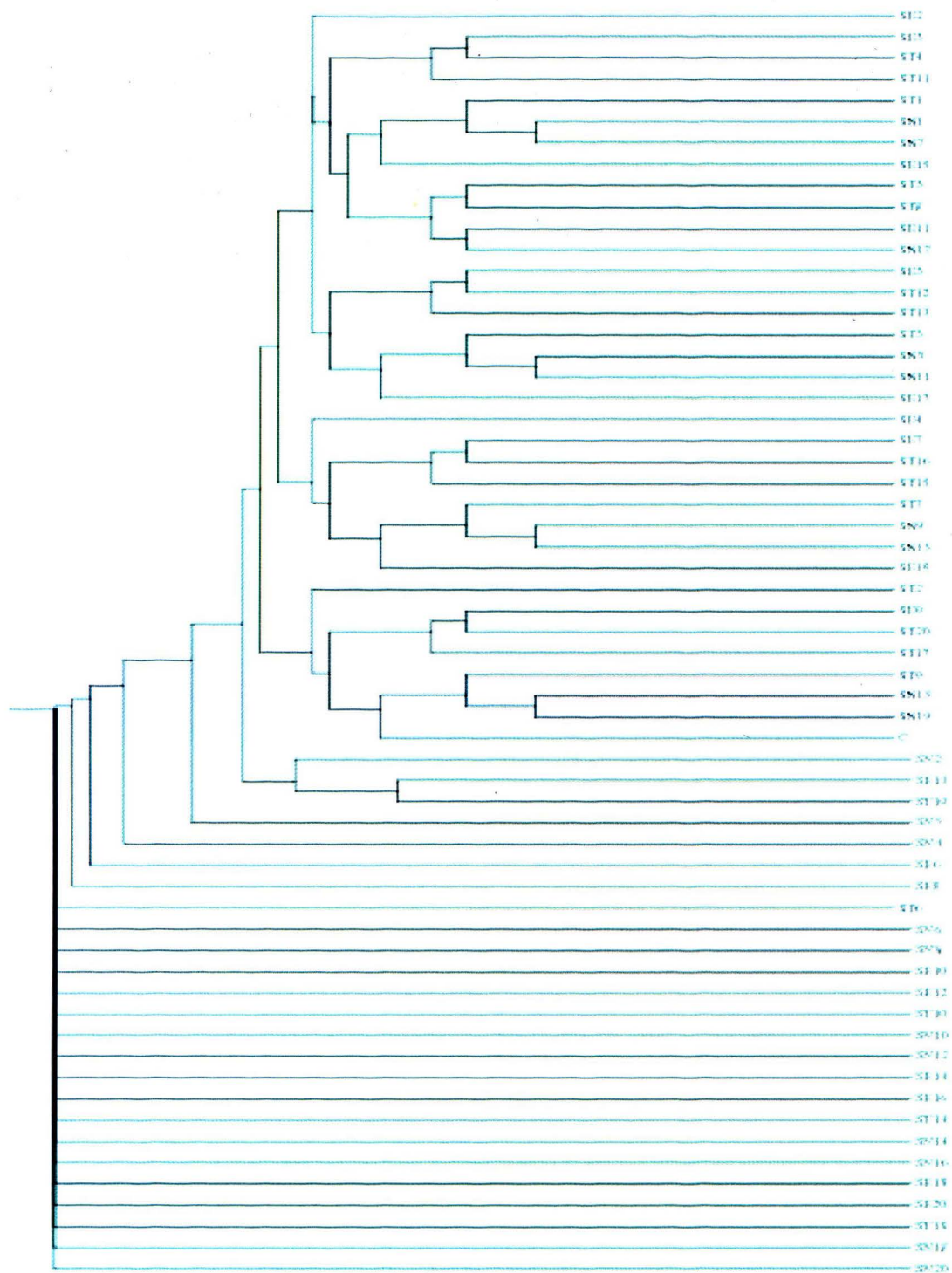


Figure 3.2: Dendrogram showing the relationship between NTS isolates obtained from raw broiler samples based on antibiotic resistance patterns. SE = *Salmonella enteritidis*; ST = *Salmonella typhimurium*; SN = *Salmonella newport*; C = control.

Table 3.4: Occurrence of resistance genes among antimicrobial-resistant NTS isolates

Antimicrobial agent	No. of resistant isolates	Genes tested	No. of positive isolates (%)
Ampicillin	48	<i>bla</i> TEM	15(31.3)
Tetracycline	60	<i>tet</i> B	12(20)
Chloramphenicol	12	<i>cat</i>	6(50)

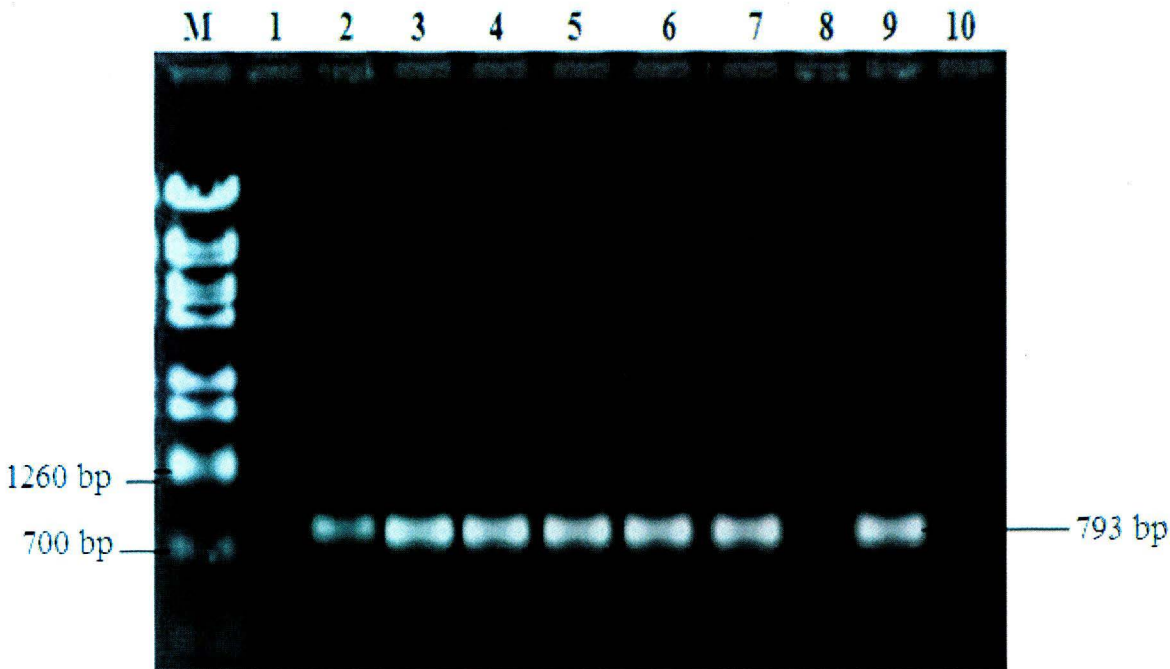


Figure 3.3: Agarose gel (1%) electrophoresis showing amplification of 793 bp fragments of *bla*TEM genes in ampicillin-resistant NTS isolates. Lane 1 = No template; lanes 2 to 7 and 9 = positive amplification of *bla*TEM gene; lanes 8 and 10 = negative; lane M = DNA marker (Lambda)



Figure 3.4: Agarose gel (1%) electrophoresis showing amplification of 659 bp fragments of *tetB* genes in tetracycline-resistant NTS isolates. Lane 1 = No template; lanes 3 and 9 = positive amplification of *tetB* fragment; lanes 2, 4, 5, 6, 7, 8, 10 and 11 = negative; lane M = DNA marker (100 bp)



Figure 3.5: Agarose gel (1%) electrophoresis showing amplification of 310 bp and 491 bp fragments of *cat3* and *cat1* genes in chloramphenicol-resistant NTS isolates. Lane M = DNA marker (100 bp); lanes 2, 4, 6, 8 and 9 = positive amplification of *cat3* fragment; lane 7 = positive amplification of *cat1* and *cat3* fragments

Chuanchuen and Padungtod (2009) similarly reported the expression of *bla*TEM, *tet* and *cmlA* genes in antibiotic-resistant NTS isolates that were obtained from live poultry. The isolates were found to be resistant to ampicillin, tetracycline and chloramphenicol.

In conclusion, the observations of this study indicate that NTS strains isolated from broiler products in the NWP of South Africa are resistant to multiple antibiotics, including quinolones. This may constitute significant public health problems and therefore calls for proper monitoring to reduce the risk of human exposure to the pathogens. This is because the *Salmonella* organisms that harbour antibiotic-resistance genes can easily transfer the genes to other pathogens of either the same or different genera or species thereby complicating treatments of human bacterial infections.

CHAPTER 4

STRESS RESPONSE OF NON-TYPHOIDAL *SALMONELLA* STRAINS ISOLATED FROM BROILER PRODUCTS IN THE NORTH WEST PROVINCE OF SOUTH AFRICA

4.1 Introduction

Non-typhoidal *Salmonella* species are frequently incriminated in food-borne illnesses involving consumption of contaminated livestock products, particularly poultry (Cox *et al.*, 2000). Many serotypes have been reported to be responsible for gastroenteritis and may cause invasive disease predominantly in HIV-positive patients (Kumar and Kumar, 2003; Keddy *et al.*, 2009). An annual estimate of 1.4 million cases of human NTS infections resulting in 582 deaths have been reported in the U.S. (McDermott, 2006). Despite the fact that clinical NTS cases are usually under-reported in the sub-Saharan Africa, about 2,244 cases were recorded by GERM-SA, South Africa in the year 2010 (CDS, 2011). For many years, exposure to heat and treatment with organic acids have been utilized as preservative/decontamination methods for poultry products in order to prevent outbreaks of salmonellosis due to consumption of such products (Mani-Lopez *et al.*, 2011). However, various serotypes of *Salmonella* have been shown to demonstrate increased resistance to heat after previous exposure to sub-lethally high temperatures (Humphrey *et al.*, 1993). Similarly, induction of acid tolerance response (ATR) in *Salmonella* following exposure to sub-lethal acidic conditions increases the ability of the organism to survive in high acidic foods as well as in the acidic conditions of the gastrointestinal tract (Yuk and Schneider, 2006). These recent attributes, which have a direct influence not only on the survival but also on the virulence of the organism, represent a great concern to food safety. The present study therefore, was aimed at evaluating the resistance of non-typhoidal *Salmonella* spp. isolated from broiler products obtained from the North West Province (NWP) of South Africa, to thermal, acidic and osmotic stress.

4.2. Materials and Methods

4.2.1 Bacterial culture and isolation

Refer to chapter 2 for details of culture and isolation protocols of the NTS isolates used in this study.

4.2.2 Identification of suspect isolates by PCR

Protocols for DNA extraction by alkaline lysis and the confirmation of both the quantity and purity of the extracted DNA were carried out using the procedure of Sambrook *et al.* (1989). Confirmatory identification of *Salmonella* isolates was by the amplification of *Salmonella*-specific *invA* gene fragment. Refer to chapter 2 for details of oligonucleotides used and the PCR procedure.

4.2.3 Determination of in-vitro generation time for *Salmonella* isolates

A cocktail of non-typhoidal *Salmonella* was prepared from field isolates identified as *S. typhimurum*, *S. enteritidis* and *S. newport* using the procedure of Payne *et al.* (2007) with modifications. Briefly, a single colony of each isolate, preserved on nutrient agar, was inoculated in 10 ml of nutrient broth (NB) and incubated for 24 hours at 37°C. Thereafter, 0.1 ml aliquots of each culture were transferred to fresh 10 ml NB and incubated overnight at 37°C in a shaking incubator. The cultures were standardized to an OD₆₀₀ of 0.3 after which 0.33 ml from each culture was used to inoculate 100 ml of fresh NB to a final concentration of approximately 10⁵ cells / ml in 250 ml Erlenmeyer flasks. The culture was incubated at 37°C for 96 hours with agitation at 177 rpm. At 2 hour intervals during the incubation, 1 ml aliquots of the suspensions were removed and the numbers of viable cells enumerated from 10-fold serial dilutions prepared from 2% buffered peptone water. Plating was done in duplicates on XLD agar and plates were incubated at 37°C for 24 hours. Colonies were then counted and expressed as log₁₀ cfu/ml. *Salmonella typhimurium* ATCC 14208 was used as a control strain. Generation time was calculated from the log phase of growth using the following equation (Todar, 2005):

$G = \frac{t}{n}$, where G = generation time; t = time interval (hours); n (number of generations) = $3.3 \log b/B$; B = number of cells at beginning of time interval; b = number of cells at end of time interval.

4.2.4 Heat stress assay

Heat stress assay was performed according to the procedure of Humphrey *et al.* (1993) with modifications. Briefly, an isolated colony of *Salmonella* was inoculated in 10 ml of NB and incubated overnight at 37°C with shaking. Then 0.1 ml aliquots of the culture were transferred to 10 ml of fresh NB, incubated for 3 hours at 37°C (log phase culture) and then shifted to 50, 55 and 60°C in a water bath. Samples were taken at time 0, 10, 20 and 30 mins to enumerate the number of viable cells on XLD agar. Plates inoculated from serial dilutions of the cultures were incubated at 37°C for 24 hours. Colonies were counted and expressed as log₁₀ cfu/ml. Experiments were performed in duplicates and growth of isolates at 37°C served as a control.

4.2.5 Acid stress assay

Evaluation of resistance to acid stress was studied by the method of Leyer and Johnson (1993). Aliquots from overnight cultures were inoculated in 10 ml of fresh NB and incubated for 3 hrs (approx. OD₆₀₀ = 0.2) at 37°C. Cultures were acidified to pH 2.0, 3.0 and 4.0 without previous adaptation using 6% rosemary vinegar and re-incubated at 37°C for a maximum period of 60 min. Samples were assessed for viable cell counts at time 0, 30 and 60 mins by plating on XLD agar as described for heat stress assay. Experiments for each acid level were performed in duplicates and growth of isolates at pH 7.0 was used as control.

4.2.6 Assay of salt stress

Log phase cultures were prepared as described above after which aliquots from the samples were inoculated in sterile NB that had been adjusted with either 1M or 2M NaCl following the method of Ngwai *et al.* (2007). Inoculated broths were incubated at 37°C for 60 mins. Viable cell counts were enumerated from samples at times 0, 30 and 60 mins as described for heat and stress assays.

4.2.7 Statistical analysis

All resulting *Salmonella* populations were converted to log₁₀ numbers of CFU/ml before statistical analysis. Analysis of survival data for the stress treatments was carried out by one-way ANOVA using the GLM procedure of SAS (SAS, 2001). Results were considered significant if $P \leq 0.05$.

4.3 Results and Discussion

4.3.1 In-vitro generation time for *Salmonella typhimurium* ATCC 14208 and *Salmonella* field isolates

Evaluation of the growth of field *Salmonella* isolates showed an average generation time of 38 mins whereas the obtained generation time for the control strain was 50 mins (Fig. 4.1). Both isolates, however, formed micro-colonies when stationary growth phase was extended beyond 36 hours. The shorter generation time observed for the field isolates in this study is in agreement with the findings of Smith (1985) when the growth of *Salmonella typhimurium* was evaluated on sheep carcasses at a temperature of 35°C. This is also an indication of the potential for the organisms to rapidly multiply under favourable conditions.

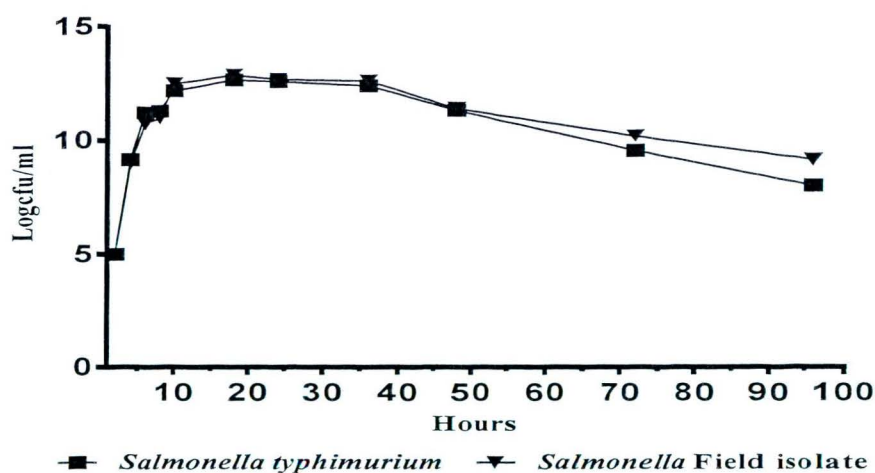


Figure 4.1: Growth of *Salmonella typhimurium* (ATCC 14208) and *Salmonella* field isolate over a period of 96 hours.

4.3.2 Response of *Salmonella* field isolates to heat stress

When isolates were subjected to heat stress at 50°C for 10 min, a significant decline of 25.3% in viable cell count was noticed, followed by an increase of 9.2% ($P = 0.6$) and 30.5% ($P = 0.03$) at 20 and 30 min respectively. However, exposure of isolates to a temperature of 55 or 60°C for 20 and 10 min respectively reduced colony counts to zero (Fig. 4.2). This response of *Salmonella* isolates to heat stress is in agreement with the observations of Humphrey *et al.* (1990) who reported D-value ranges of 2.3 – 6.4 min at 55°C and 0.2 – 0.4 min at 60°C for *S. enteritidis* and *S. typhimurium* in homogenized whole egg. Ngwai *et al.* (2007) similarly noted a significant reduction in the survival of both strains when isolates were exposed to a temperature of 52°C. An initial decrease followed by increase in viable cell count at 50°C heat exposure may be an indication that 50°C is sub-lethal to the isolates thereby enabling their adaptation to the temperature. This observation can be related to previous research findings in which *S. seftenberg* isolates were heat-shocked at 50°C for 30 min. The heat-shocked isolates were found to survive better at 53 and 55°C than the non-adapted isolates (Kumar and Kumar, 2003). The implication of the finding in this study, is that target cooking temperatures must be rapidly achieved to avoid pre-adaptation of NTS organisms in contaminated products.

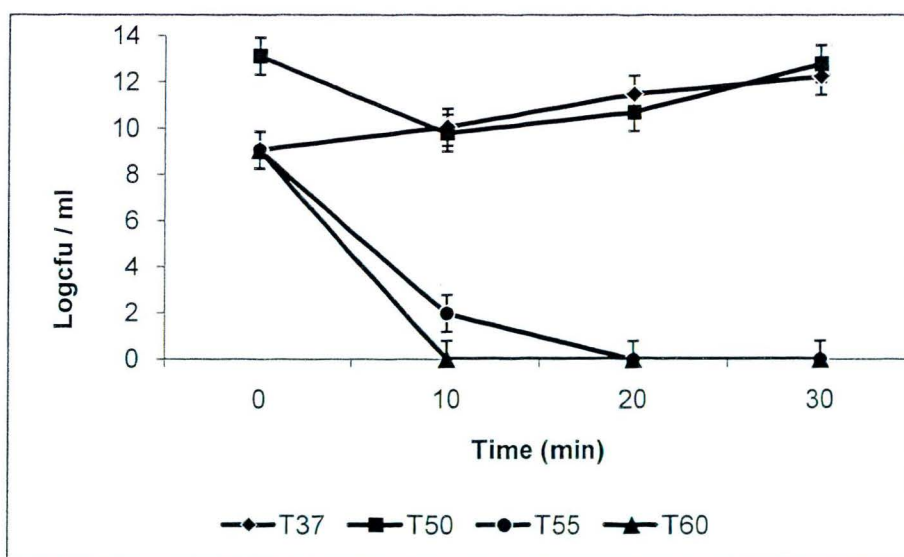


Figure 4.2: Survival curve for *Salmonella* isolates exposed to heat stress without prior adaptation

4.3.3 Response of *Salmonella* field isolates to acid stress

Exposure of isolates to acid stress at pH 4.0 and pH 3.0 for 60 min resulted in reduction of viable cell counts by 21 and 24% respectively ($P = 0.0004$ and 0.0001). Counts were reduced to zero ($P = 0.0001$) when isolates were exposed to pH 2.0 for the same period (Fig. 4.3). Similar responses of *S. typhimurium* and *S. enteritidis* to acid stress using 2N HCl have been reported (Ngwai *et al.*, 2007). Furthermore, Foster (2001) noted that *Salmonella* cells grown to exponential phase at pH 7.7 and shifted directly to pH 3.0 had less than 0.01% survival chances at 60 min of exposure. On the contrary, exposing log phase cells of *Salmonella* to mild acid stress for 30 to 60 min protected cells undergoing a subsequent transition to pH 3.0 (Foster, 2001; Kumar and Kumar, 2003). This shows that if *Salmonella* is not pre-exposed to sub-lethal acid stress during food processing, then it is not likely to survive the low pH of human gastrointestinal tract which ranges between 1.5 and 2.0.

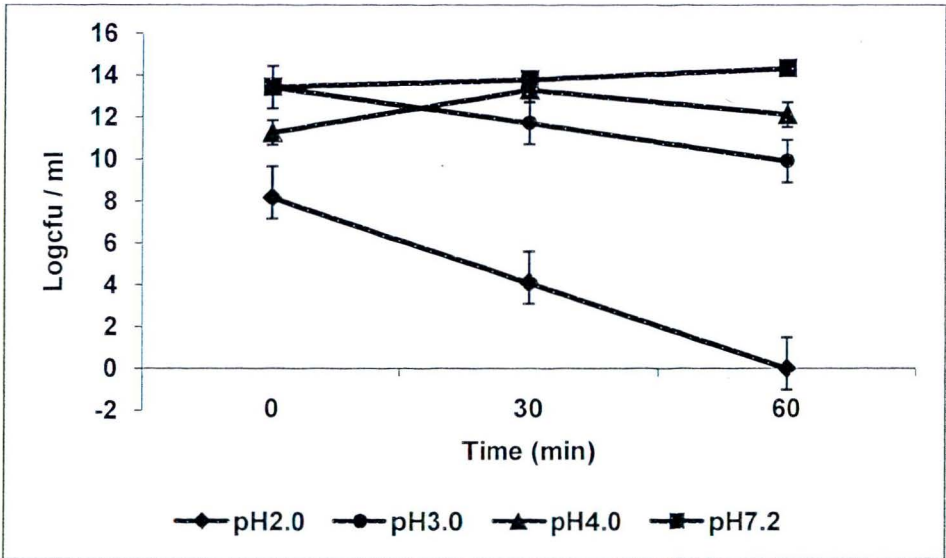


Figure 4.3: Survival curve for *Salmonella* isolates exposed to Rosemary vinegar acid stress.

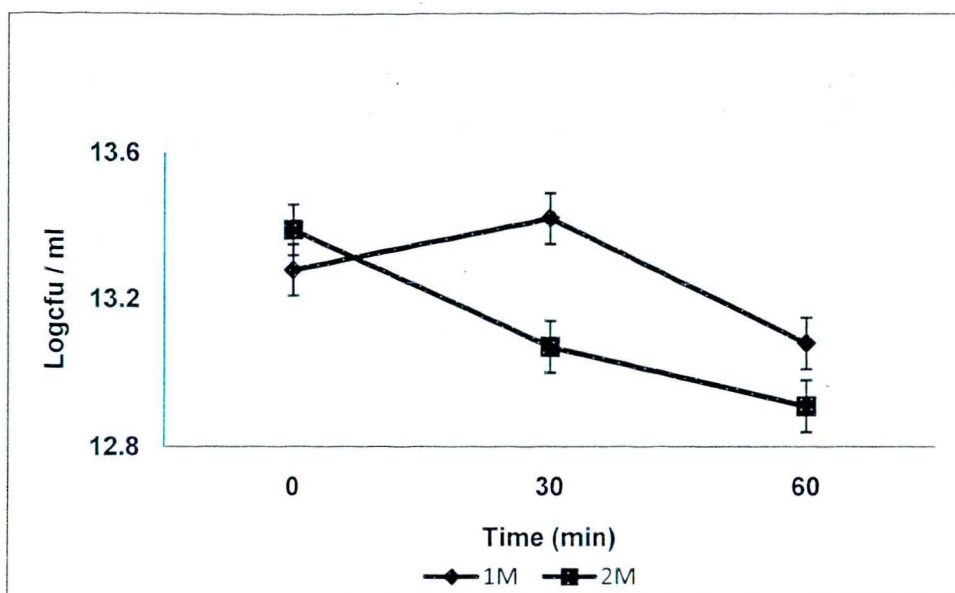


Figure 4.4: Survival curve of *Salmonella* isolates exposed to NaCl stress.

4.3.4 Response of *Salmonella* field isolates to salt stress

Adjustment of growth media with 2M NaCl steadily reduced colony count by 3.3% after 60 min of incubation whereas isolates continued to multiply in the presence of 1M NaCl for the first 30 min followed by 1.4% decline in counts at 60 mins (Fig. 4.4). Ca'Novas *et al.* (2001) noted that exposure to mild osmolarity can stimulate the growth of *Salmonella* isolates at upper limit growth temperatures. This may partly explain the initial increase in viable cell counts of *Salmonella* isolates in the presence of 1M NaCl observed in this study. Furthermore, in acid treatment, only the effect of pH on survival of isolates was found to be significant ($P = 0.0001$) rather than the interaction of pH and time ($P = 0.11$). However, in temperature and salt treatments, both the treatments and the interaction between treatment and time had significant effects ($P = 0.001$) on the survival of isolates. This shows that time duration is an important factor to be considered when decontaminating poultry products using either salt or heat treatments.

This study indicates that NTS strains isolated from poultry products obtained from the NWP of South Africa are sensitive to 6% rosemary vinegar (adjusted to pH 2.0) and heating at 55 or 60°C for 20 or 10 mins respectively. These two treatments may thus be employed as strategies to

control NTS in contaminated raw chicken products from slaughterhouse to table. Further studies are however needed to evaluate the *in-vivo* response of isolates to the various stress conditions employed in this study as the behaviour of the NTS organisms in food matrix may be different from the response on laboratory media.

CHAPTER 5

INCIDENCE AND ANTIBIOTIC RESISTANCE OF COLIFORMS IN RAW AND READY-TO-EAT BROILER PRODUCTS OBTAINED FROM THE NORTH WEST PROVINCE OF SOUTH AFRICA.

5.1 Introduction

Chicken meat contains essential micro- and macroelements and is therefore, high in nutritive value (Saikia and Joshi, 2010). It thus constitutes a part of balanced diet, with high consumption rate among South Africans (TAS, 2011). However, studies have shown that poultry meat is often contaminated with pathogens such as *Campylobacter* and pathogenic *E. coli* (Todd, 1997). This is because the high nutritive value of the meat creates an ideal culture medium for the growth of many organisms (Al-Mutairi, 2011). The fact that many of the contaminating organisms are associated with food borne illnesses in humans makes their potential presence in meat products an issue of public health concern (Fratamico *et al.*, 2005). Indeed, food borne microbial pathogens have been reported to cause an annual 6.5 to 33 million cases of human illness, resulting in 9000 deaths (CAST, 1994). Recent changes in eating habits, mass catering services and poor sanitary practices have been identified as contributing factors to this plight, particularly in developing countries (Hedberg *et al.*, 1992).

Sources of poultry meat contamination with pathogenic microbes include contents of intestinal tract of slaughtered birds, fecal material on feet and feathers, meat handlers, product packing and marketing (Saikia and Joshi, 2010; Al-Mutairi, 2011). Globally, the most challenging contaminants of raw and processed meat products are members of the family *Enterobacteriaceae* (mainly coliforms), particularly *Salmonella*, *Escherichia*, *Proteus* and *Klebsiella* species (Al-Mutairi, 2011). Most genera of *Enterobacteriaceae*, which further include *Enterobacter*, *Serratia*, *Citrobacter* and *Yersinia* are overt and opportunistic pathogens, many of which have been isolated from hospital settings where they are strongly associated with nosocomial infections (Micozzi *et al.*, 2000; Rostoff *et al.*, 2010). The disease burden caused by the pathogens is further increased by high levels of antibiotic resistance leading to complications in

the treatment of clinical cases. In addition to causing diseases in humans, species of *Proteus*, *Salmonella*, *Yersinia* and *Citrobacter* have been incriminated in spoilage of refrigerated meat and poultry products (Jay *et al.*, 2005). Previous reports showed annual wastage of approximately 3.5 billion kg of meat products at retailer and consumer levels, due mainly to microbial spoilage (Kantor *et al.*, 1997). The burden of disease and losses from product wastage as a result of microbial contamination calls for proper monitoring of the pathogens in meat and poultry products, for the development of effective control strategies. A few studies have been conducted in South Africa to assess microbial contamination of ready-to-eat (RTE) broiler street-vended foods and associated food preparation surfaces (Mosupye and van Holy, 1999; Christison *et al.*, 2008; Nyenje *et al.*, 2012). However, microbial contamination of raw and RTE poultry products has not been investigated. The present study therefore was aimed at evaluating the incidence and antibiotic resistance of coliforms in raw and RTE broiler products obtained from the North West Province (NWP) of South Africa.

5.2 Materials and Methods

5.2.1 Sampling, bacterial culture and isolation

For details of sampling, bacterial culture and isolation, please refer to chapter 2. However, MacConkey and XLD agars were used as selective media for isolation of the coliforms. Colonies were purified on XLD agar. The various coliform species were identified by correlating colony morphology, Gram stain, oxidase reactions and biochemical characteristics.

5.2.2 Biochemical screening

Suspected colonies for the various coliform species on XLD agar were subjected to Enteropluri-test[®] (Davies diagnostics, SA), performed according to manufacturer's instructions. Briefly, each Enteropluri-test[®] system was labelled with sample number and test date. Thereafter, the tip of inoculating needle, placed under the system cap, was used (without flaming) to pick a well isolated colony from the XLD agar. This was used to inoculate the Enteropluri-test, turning and withdrawing the needle throughout the sectors of the system. The needle, which was reinserted with a turning movement until the breakage notch, was broken, folding it in correspondence with

the notch. The portion of the needle remaining in the user's hand was used to punch the plastic film in sectors of the system corresponding to adonitol, lactose, arabinose, sorbitol, VP, dulcitol/PA, urea and citrate. This was done to support aerobic growth. The portion of the needle that remained inside the system was left to keep anaerobic conditions necessary for reactions of the glucose, lysine and ornithine sectors. The inoculated system was incubated at 36°C for 18 – 24 hrs. Thereafter, the colour reactions were read and converted to five-digit codes. The genera and species of the coliforms were identified by comparing the codes with manufacturer's database supplied in a codebook.

5.2.3 Antimicrobial susceptibility testing

Isolates identified by biochemical characterization were subjected to antimicrobial susceptibility test using a panel of eight antibiotics. These included ampicillin (10 µg), ciprofloxacin (5 µg), amikacin (30 µg), trimethoprim-sulphamethoxazole (25 µg), cefotaxime (30 µg), meropenem (10 µg), gentamicin (10 µg) and erythromycin (15 µg). The antibiotic sensitivity test was performed as described in Chapter 3.

5.3 Results and Discussion

5.3.1 Coliform bacterial species in raw broiler products

Coliforms were identified in 90 (75%) raw broiler products analyzed. A total of eight genera of coliforms belonging to the family *Enterobacteriaceae* were isolated from raw products. These include four species of *Enterobacter* (n = 34), three species of *Klebsiella* (n = 13), two species of *Citrobacter* (n = 6) and one specie each of *Serratia* (n = 14), *Hafnia* (n = 9), *Escherichia* (n = 11), *Yersinia* (n = 1), and *Proteus* (n = 2). In some instances, there were multiple contaminations of one sample with two genera/species although this was rare and usually involved less pathogenic strains. This may indicate multiple sources of product contamination. Table 5.1 shows details of coliform bacterial species recovered from raw products. Javadi and Safarmashaei (2011) similarly reported 100% incidence of coliforms in broiler meat marketed in Iran. The recovery rate, and variety of coliforms observed in this study, may either be a

reflection of poor hygienic conditions in the processing environment or post-processing contamination by personnel involved in packaging. An average of 50% in the incidence of coliforms belonging mostly to genera identified in this study has been reported in vegetables (Falomir *et al.*, 2010). Similarly, a high incidence of *Enterobacter aerogenes* (100%), *Escherichia coli* (98%), *Klebsiella pneumoniae* (98%), *Proteus* (49%) and *Citrobacter* (52%) species was noted in raw chicken products in North East India (Saikia and Joshi, 2010).

Furthermore, analysis of microbial contamination of raw meat products in Abakaliki, Nigeria revealed *Escherichia coli* and *Klebsiella pneumoniae* as highest occurring pathogens in the products (Iroha *et al.*, 2011). Members of the genus *Klebsiella* have also been recovered from free-range chicken samples in Western Cape Province of South Africa (Fielding *et al.*, 2012). The incidence of *Enterobacter cloacae* (26.7%) (Table 5.1) observed in the current study, is higher than the 9% incidence reported in street foods in Malaysia (Haryani *et al.*, 2008). In addition, *E. cloacae* and *E. sakazakii* have been isolated in infant formula (13.3 - 20%) and crushed wheat (33.3%) respectively (Shaker *et al.*, 2007). *Enterobacter* species are important nosocomial pathogens, and are capable of causing various community-acquired infections (Guentzel, 1996). Although only a few genera of bacteria identified in the current study have been recognised as common pathogens in humans and animals, all the genera can become pathogenic under special circumstances.

For instance, strains of *E. coli* are commonly associated with enterovirulence, urinary tract infections and meningitis (Guentzel, 1996). Similarly, although the pathogenic roles of *Serratia* and *Hafnia* species are yet to be defined, they are opportunistic pathogens that have been incriminated in nosocomial infections (Ramos and Damasos, 2000; Grimont and Grimont, 2006). Furthermore, *Klebsiella pneumoniae*, a nosocomial pathogen associated primarily with respiratory and urinary tract infections, was reported as an enteroinvasive pathogen in 1998 (Sabota *et al.*, 1998). In the United States, *Yersinia pseudotuberculosis* has been identified as a cause of gastroenteritis and lymphadenitis with a fairly high case-fatality rate (Long *et al.*, 2010). Therefore, the observations in this study are of serious public health significance. This is in view of the fact that when undercooked contaminated products are consumed, the immunocompromised persons are the most affected, usually with high fatality rates (Doyle and

Erickson, 2006). However, in addition to the public health significance, the *Enterobacteriaceae* family of bacteria are generally associated with spoilage of meat products (Jay *et al.*, 2005).

5.3.2 Coliform bacterial species in RTE chicken products

Coliforms were isolated from 15 (25%) of RTE products and three genera of coliforms were identified viz: two species of *Klebsiella* (n = 3) and one specie each of *Serratia* (n = 11) and *Stenotrophomonas* (n = 1) (Table 5.2). The recovery rate of coliforms in RTE products observed in this study is lower than the incidence of 42% and 75% reported for similar products in Korea and Taiwan respectively (Fang *et al.*, 2003; Cho *et al.*, 2011). *Klebsiella* species were also found to contaminate curry and tandoori chickens purchased from restaurants in Parbhani city, India (Rindhe *et al.*, 2008). Ammar (2005) similarly reported a 24% incidence of *Klebsiella oxytoca* in sausages whereas Al-Mutairi (2011) noted a much lower incidence of *Klebsiella* specie (4%) in similar products. Furthermore, the incidence of *Stenotrophomonas maltophilia* (6.7%) in the current study is lower than the 78% reported by Qureshi *et al.* (2005) in RTE salads in the United Kingdom. Isolation of coliforms in RTE products in the current study may suggest improper processing of contaminated raw poultry or post processing contamination of the products. In human clinical infections, all the isolates recovered in this study are associated with nosocomial infections and have been reported to cause bacteremia, particularly in immunocompromised individuals (Guentzel, 1996; Micozzi *et al.*, 2000). In addition, *Klebsiella pneumoniae* is strongly associated with pneumonia whereas *S. maltophilia* has been incriminated in cases of meningitis and pacemaker endocarditis (Yemisen *et al.*, 2008; Rostoff *et al.*, 2010). This study represents the first report on the incidence *S. maltophilia* in RTE foods in the NWP of South Africa.

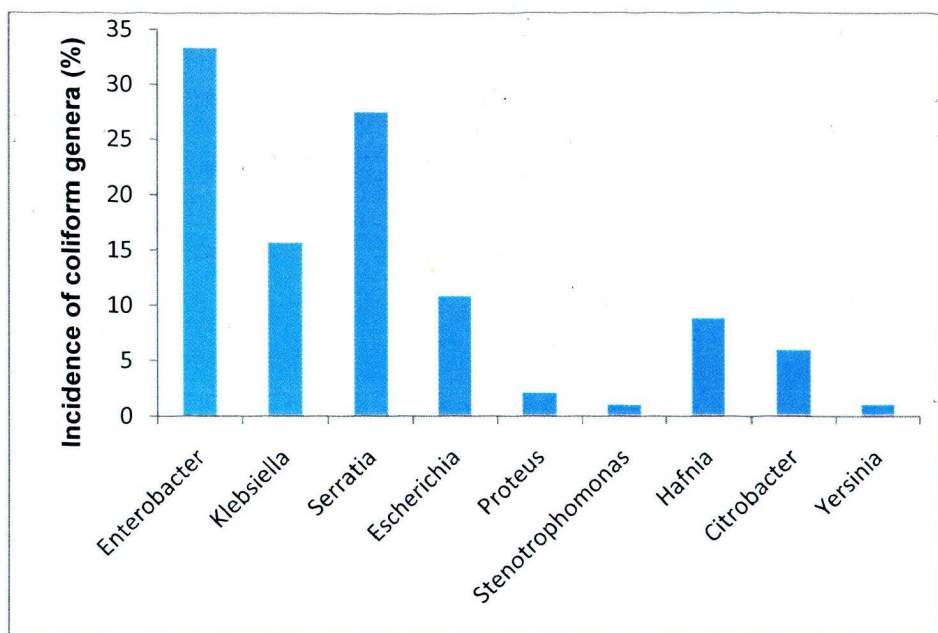


Figure 5.1: Percentage incidences of coliform species isolated from raw and RTE poultry products

Table 5.1: Incidence of coliform bacterial species in raw broiler products

Coliform species	No. of Isolates	Percentage of Isolates
<i>Enterobacter cloacae</i>	24	26.7
<i>Enterobacter sakazakii</i>	4	4.4
<i>Enterobacter aerogenes</i>	4	4.4
<i>Enterobacter amnigenus</i> 2	2	2.2
<i>Serratia liquefaciens</i>	14	15.6
<i>Klebsiella pneumoniae</i>	11	12.2
<i>Klebsiella ornitholytica</i>	2	2.2
<i>Hafnia alvei</i>	9	10
<i>Citrobacter freundii</i>	4	4.4
<i>Citrobacter braakii</i>	2	2.2
<i>Escherichia coli</i>	11	12.2
<i>Yersinia pseudotuberculosis</i>	1	1.1
<i>Proteus mirabilis</i>	2	2.2

Table 5.2: Incidence of coliform bacterial species in RTE poultry products

Coliform species	No. of isolates	Percentage of isolates
<i>Serratia liquefaciens</i>	11	73.3
<i>Klebsiella pneumoniae</i>	1	6.7
<i>Klebsiella ozonae</i>	2	13.3
<i>Stenotrophomonas maltophilia</i>	1	6.7

5.3.3 Antibiotic resistance patterns

Strains of *Enterobacter cloacae* showed four resistance patterns whereas *Klebsiella pneumoniae* showed three patterns. *Serratia liquefaciens*, *Hafnia alvei* and *E. coli* had two resistance patterns each (Table 5.3). However, all isolates were resistant to ampicillin with the exception of *Enterobacter sakazakii*, *Klebsiella ozonae* and a strain of *E. coli*. Similarly, resistance to erythromycin was found in all isolates except a strain of *Enterobacter cloacae*. Furthermore, *Yersinia pseudotuberculosis* and *Stenotrophomonas maltophilia* were both resistant to seven out of the eight antibiotics tested, and were only susceptible to ciprofloxacin and trimethoprim-sulphamethoxazole respectively. Susceptibility of *S. maltophilia* to trimethoprim-sulphamethoxazole is in agreement with the report of Qureshi *et al.* (2005). However, a contrary observation was reported by Rostoff *et al.* (2010) in a patient who died of pacemaker endocarditis caused by *S. maltophilia*. Resistance of *S. maltophilia* to β -lactams, aminoglycosides and ciprofloxacin accords with the observations of Micozzi *et al.* (2000) in haematology patients with *S. maltophilia* bacteremia. Resistance to more than one antibiotic noted in *Enterobacter cloacae* agrees with the observation of Haryani *et al.* (2008) who noted six resistance patterns in *E. cloacae* isolated from street foods in Malaysia.

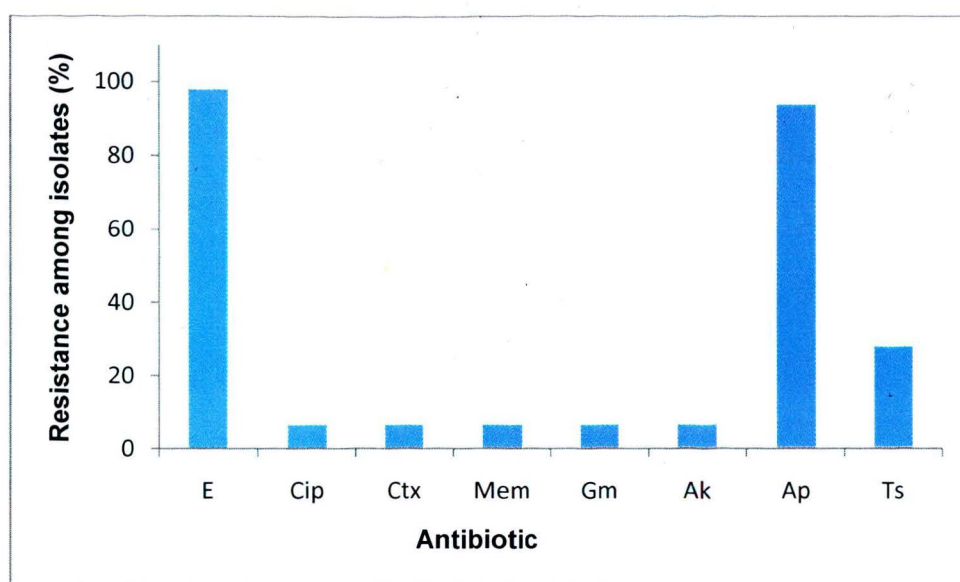


Figure 5.2: Percentage resistance of coliform isolates to individual antibiotics. Cip = ciprofloxacin; Ctx = cefotaxime; Mem = meropenem; Gm = gentamycin; Ak = amikacin; Ap = ampicillin; Ts = trimethoprim-sulphamethoxazole; E = erythromycin

Multiple antibiotic resistance was similarly reported in large proportions of *Klebsiella* species and *E. coli* isolated from slaughtered chickens in Italy (Turtura *et al.*, 1990). Indeed, members of *Enterobacteriaceae* family are commonly associated with production of ESBLs (Extended spectrum β -lactamases). For instance, production of TEM-63 was reported in *Klebsiella pneumoniae* and strains of *Enterobacter cloacae*, *E. coli* and *Proteus* in South Africa (Essack *et al.*, 2001; Gray *et al.*, 2006). Similarly, Tzelepi *et al.* (2000) studied coliform clinical isolates in Greece and reported transferable ESBLs in 23 and 58% of *Enterobacter cloacae* and *Enterobacter aerogenes* respectively. They further noted that the most frequent ESBL type identified in the aforementioned *Enterobacter* species is widely spread among *K. pneumoniae* and *E. coli* isolates, indicating horizontal gene transfer among *Enterobacteriaceae* family.

Table 5.3: Antibiotic resistance patterns of coliform species isolated from raw and RTE broiler products

Coliform species	Resistance patterns
<i>Enterobacter cloacae</i>	Cip-Ap-Ts-E; Ap-Ts-E; Mem-Ap-Ts; Ap-E
<i>Enterobacter sakazakii</i>	Cip-Ts-E
<i>Enterobacter aerogenes</i>	Gm-Ak-Ap-Ts-E
<i>Enterobacter amnigenus</i> 2	Ap-E
<i>Serratia liquefaciens</i>	Ap-Ts-E; Ap-E
<i>Klebsiella pneumoniae</i>	Ctx-Mem-Ap-Ts-E; Ap-Ts-E; Ap-E
<i>Klebsiella ozonae</i>	E
<i>Klebsiella ornitholytica</i>	Ap-E
<i>Hafnia alvei</i>	Ap-Ts-E; Ap-E
<i>Citrobacter freundii</i>	Ap-E
<i>Citrobacter braakii</i>	Ap-E
<i>Stenotrophomonas maltophilia</i>	Cip-Mem-Ctx-Gm-Ak-Ap-E
<i>Escherichia coli</i>	Gm-E; Ap-E
<i>Yersinia pseudotuberculosis</i>	Ctx-Mem-Gm-Ak-Ap-Ts-E
<i>Proteus mirabilis</i>	Ap-Ts-E

Cip = ciprofloxacin; Ctx = cefotaxime; Mem = meropenem; Gm = gentamycin; Ak = amikacin; Ap = ampicillin; TS = trimethoprim-sulphamethoxazole; E = erythromycin

Although previous studies reported low frequency of resistance transfer among coliforms isolated from retail poultry (Bensink and Botham, 1983), the resistance patterns expressed by some species in the current study calls for serious public health intervention. This is in view of the fact that similar resistance patterns were also noted in those same species when they were isolated in clinical cases (Micozzi *et al.*, 2000).

This study indicates that both raw and RTE poultry products in the NWP of South Africa are contaminated with various species of coliforms, some of which are highly resistant to most routine antibiotics used in treatment of human infections.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Existing data in NICD gives information on outbreaks of foodborne salmonellosis in South Africa and laboratory isolation of NTS serotypes from such outbreaks (NICD, 2010). However, despite the fact that such outbreaks have been associated with consumption of poultry products (Smith *et al.*, 2007), there is no adequate data on the prevalence of NTS in poultry products in South Africa, and particularly the North West Province (NWP). Raw broiler products obtained from the NWP of South Africa were found to be contaminated with three major serotypes of non-typhoidal *Salmonella*, with an average incidence rate of 12.5%. The strains identified were *S. typhimurium*, *S. enteritidis* and *S. newport*. The results of this study therefore represent the first report on the incidence of NTS in chicken products in the NWP, and the prevalent serotypes. It is however, worthy to note that the three NTS serotypes isolated in this study were also the same serotypes identified in 49 NTS cases reported from the NWP to GERM-SA in 2011 (Crowther-Gibson *et al.*, 2011). This incriminates raw chicken products as major possible vehicles contributing to foodborne salmonellosis in the NWP. Furthermore, multiple contamination of one sample by different serotypes, and the genetic diversity among isolates of the same or different serotypes (as expressed by RAPD fingerprinting) suggest varying sources of product contamination. This is a cause for public health concern. It therefore, calls for adequate monitoring and establishment of effective strategies to control contamination along the production/supply chain, in order to safeguard public health. No NTS was recovered from polonies and viennas (RTE products), and this could be attributed to the effectiveness of employed RTE processing techniques in eliminating *Salmonella* contaminants from raw products. This report should provide confidence and reassurance to both RTE producers and consumers. However, as discovered in this study, chances of *Salmonella* recovery from chicken samples could be greatly enhanced by leaving the inoculated RV enrichment broth (post-incubation) for 24 hrs at room temperature before plating on selective media. Growth on selective media, particularly XLD agar, could be further improved by incubating the inoculated plates at 37°C for 36 hrs instead of the routine 24 hrs.

Furthermore, the observations of this study indicate that NTS strains isolated from broiler products in the NWP of South Africa are resistant to multiple antibiotics, including quinolones. Resistance to ampicillin, tetracycline and erythromycin (common drugs used in poultry production) was the most frequent phenotype observed. Reports from GERM-SA on antimicrobial resistance patterns of *S. typhimurium*, *S. enteritidis* and *S. newport* strains isolated from NTS cases in the NWP showed lower resistance to ampicillin, tetracycline and chloramphenicol than observed in the current study. Results of this study therefore, incriminate the slaughtered broiler chickens as the most probable source of product contamination. This infers that there is either absence of, or lack of adherence to proper regulations regarding prudent use of antimicrobial agents in poultry production in the NWP. Indeed, widespread use of antimicrobial agents in food animals, particularly animal feeds, has been indicated as a major source of the spread of antimicrobial resistance in pathogens (Su *et al.*, 2004). In South Africa, data regarding the epidemiological situations of antimicrobial resistance in NTS, particularly in foods of animal origin, is lacking. The results of this study therefore provide useful information regarding the current status of antimicrobial resistance patterns of NTS serovars isolated from raw broiler products in the NWP. Furthermore, antimicrobial resistant bacteria are considered as hazards in antimicrobial resistance risk assessment. Therefore, the information obtained from this study could be used as part of risk analysis of the distribution of antimicrobial resistance in the NWP of South Africa. It is however, recommended that guidelines on the prudent use of antimicrobials in animal production in South Africa should be developed and adherence to such guidelines by poultry producers should be ensured. This is to prevent the potential risk of the spread of antimicrobial resistant pathogens from animal products to humans.

However, the NTS serotypes identified in this study were inactivated by 6% rosemary vinegar (adjusted to pH 2.0) and heating at 55 or 60°C for 20 or 10 mins respectively. Although 6% rosemary vinegar, which has a natural pH of 1.5, has been used for salad dressing and meat marination (Marianski *et al.*, 2009), there is no documentation about its direct effect on food microbial contaminants. This study therefore serves as the first report on the efficacy of rosemary vinegar in inactivating meat microbial contaminants. The current study also indicates that the use of 6% rosemary vinegar in the chicken meat processing chain may reduce the risk of exposure of consumers to NTS pathogens. Furthermore, the NTS serotypes isolated in the

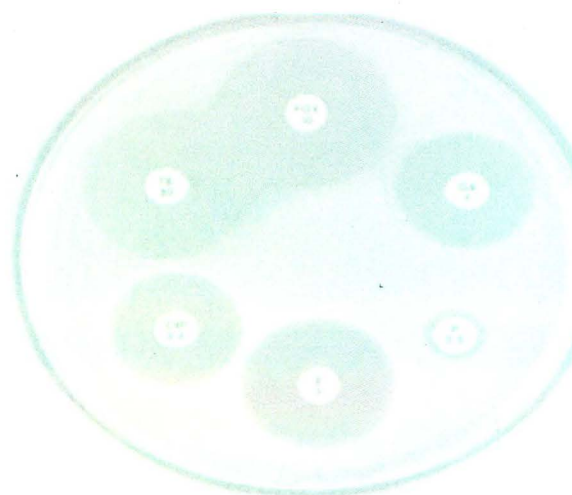
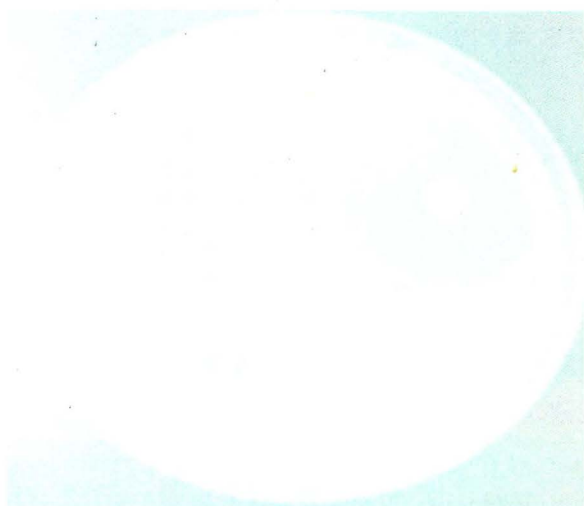
current study have the potential for rapid multiplication, with a generation time of 38 mins at 37°C. However, incubation at 55 or 60°C for 20 or 10 mins in the laboratory media was sufficient to inactivate them. In South Africa, outbreaks of NTS foodborne illnesses, associated with mass catering services are frequently reported (Smith *et al.*, 2007; Niehaus *et al.*, 2011). However no official temperature/time recommendations exist in law to guide the cooking of food, particularly meat/poultry products. This may be due to scarcity of data on heat resistance characteristics of pathogens. Thus the current study, which is a premier report in the NWP, on response of NTS isolates to heat stress, may serve as a guide in official temperature/time recommendations for cooking poultry meat. In the meantime, consumers should be encouraged to adopt the recommendations of Advisory Committee on Microbiological Safety of Food (ACMSF) on heating chicken portions/whole chickens to achieve a temperature of 85°C (Anon, 2006f). This is to give allowance for wide safety margin, as the behaviour of the NTS organisms in food matrix may be different from the response on laboratory media. Further studies are however needed to evaluate the *in-vivo* response of isolates to the various stress conditions employed in this study.

Both raw and RTE broiler products were contaminated with members of the family *Enterobacteriaceae* (coliforms), many of which have been associated with meat spoilage as well as human infections. Some of the coliforms were also highly resistant to routine antibiotics used in human infections. In recent times, test for *Enterobacteriaceae* in foodstuffs is used as indicator of hygiene and post-processing contamination. However, the emerging antibiotic resistance in the *Enterobacteriaceae* family, particularly to β -lactams, makes their presence in foods an issue of major public health concern. The isolation of various genera of *Enterobacteriaceae* from both raw and RTE products in the present study suggests lack of strict hygiene control measures in the food chain. Of particular significance is the isolation of multidrug-resistant *Stenotrophomonas maltophilia* from chicken polony, which is the first report on isolation of the organism from food in the NWP of South Africa. *Stenotrophomonas maltophilia*, which is emerging as an important nosocomial pathogen particularly in immunocompromised persons, sometimes resides as flora on the intact skin and nasal passages of man and farm animals (Qureshi *et al.*, 2005; Adegoke and Okoh, 2012). This suggests post-processing contamination of the contaminated RTE products analysed in this study. Although

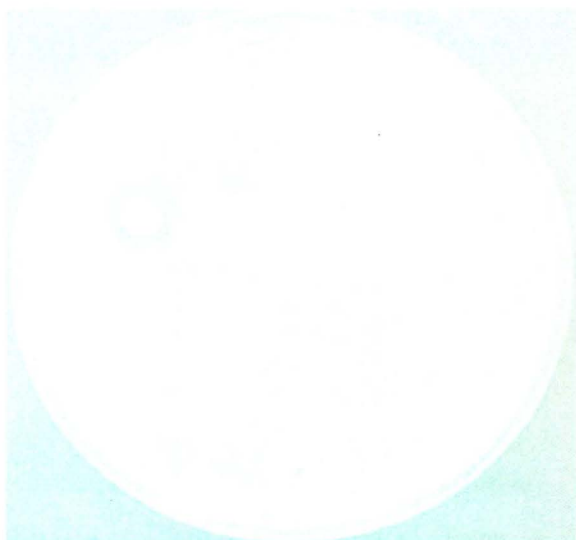
infections caused by *S. maltophilia* in healthy individuals are uncommon, its phenotypic display of antibiotic resistance, including to new generation antibiotics, places it as a high risk organism (Frere, 1995; Adegoke and Okoh, 2012). This is because of the organism's potential to transfer antibiotic resistance genes to other members of the *Enterobacteriaceae* family, some of which could cause more serious infections (Blahova *et al.*, 1997). RTE products are usually consumed without further preparation. The results of this study therefore indicate that the ever-increasing consumption of RTE poultry products can contribute to the spread of antibiotic resistant organisms. This calls for establishment of good hygienic practices in the food production chain, and adequate processing of RTE foods in order to curtail the dissemination of antibiotic resistance genes in the community.

In summary, the presence of multidrug resistant *S. typhimurium* and *Stenotrophomonas maltophilia* in the chicken products poses serious public health risks, particularly to immunocompromised persons. Hazard Analysis Critical Control Point (HACCP) procedures should be emphasized at all levels of poultry processing in order to reduce the spread of antibiotic-resistant pathogens from animals to humans. A minimum of four articles will be published from this study, out of which two articles have been submitted to peer-reviewed accredited journals for publication.

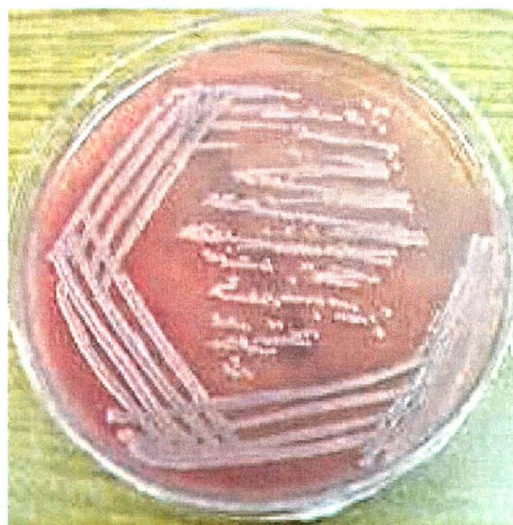
ANNEXURES



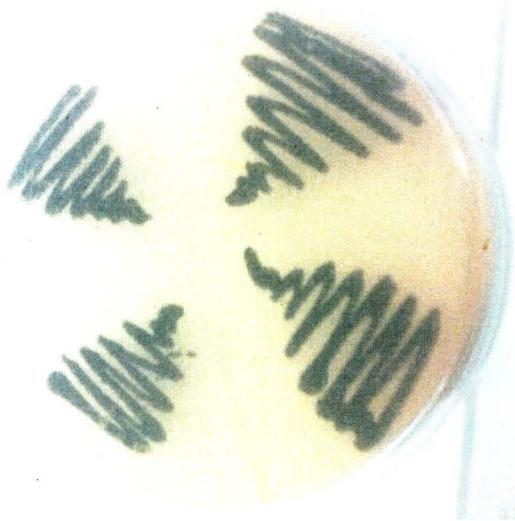
Pix 1 and 2: Antimicrobial susceptibility test showing susceptible isolates



Pix 3: Antimicrobial susceptibility test showing multidrug-resistant isolates



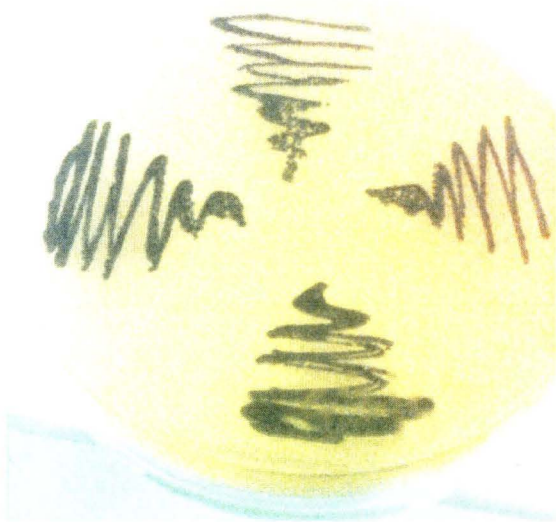
Pix 4: Growth of *E.coli* on MacConkey agar



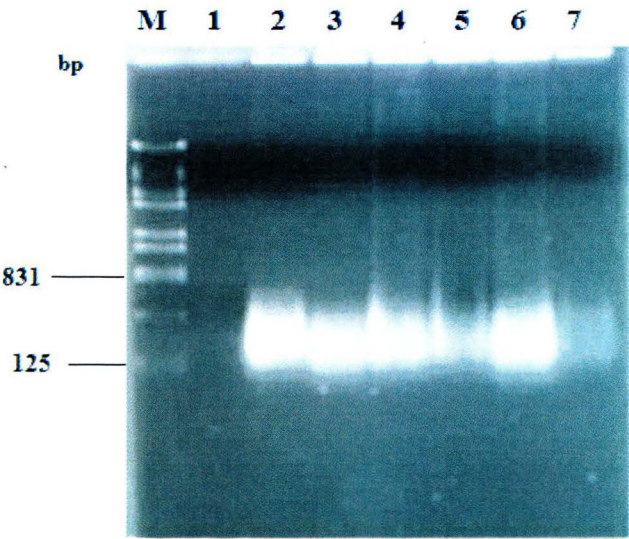
Pix 5: Growth of *S. enteritidis* on XLD agar



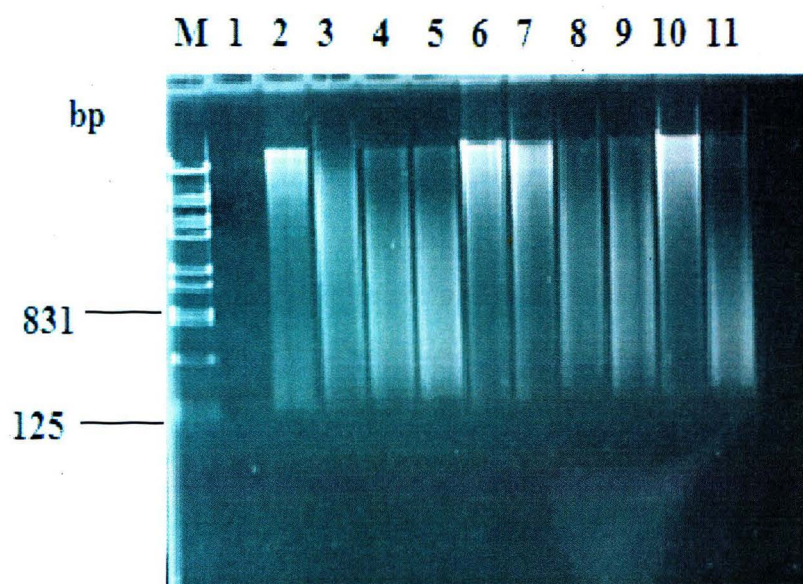
Pix 6: Growth of *S. typhimurium* on XLD



Pix 7: Growth of *S. Newport* on XLD agar



Pix 8: Agarose gel (0.8%) electrophoresis of extracted plasmid DNA



Pix 9: Agarose gel (0.8%) electrophoresis of extracted genomic DNA

BIBLIOGRAPHY

- Adak, G.K., Long, S.M., O'Brien, S.J., 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut* 51, 832-841.
- Adegoke, A.A., Okoh, A.I., 2012. Commensal *Staphylococcus* spp, *Acinetobacter* spp and *Stenotrophomonas maltophilia* as reservoirs of antibiotic resistance genes. *African J. Biotechnol.* 11, 12429-12435.
- Ades, S.E., 2008. Regulation by destruction: design of the sigmaE envelope stress response. *Current Opinion in Microbiol.* 11, 535-540.
- Al-Mutairi, M.F., 2011. The Incidence of *Enterobacteriaceae* causing food poisoning in some meat products. *Adv. J. Food Sci. and Technol.* 3, 116-121.
- Ammar, M.A.M., 2005. Spoilage and pathogenic microorganisms in traditional meat products in Assuit. MVS Thesis, Assiut University, Egypt.
- Ananthan, J., Goldberg, A.L., Voellmy, R., 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Sci.* 232, 522-524.
- Andrews, W.H., 1996. Evolution of methods for the detection of *Salmonella* in foods. *J. AOAC Int.* 79, 4-12.
- Angulo, F.J., Baker, N.L., Olsen, S.J., Anderson, A., Barrett, T.J., 2004. Antimicrobial use in agriculture: controlling the transfer of antimicrobial resistance to humans. *Seminars in Ped. Inf. Dis.* 15, 78 - 85.
- Anon, 2006f. Barbecuing food safety tips, preventing foodborne illness. Canadian Food Inspection Agency, Canada.
- Antunes, P., Réu, C., Sousa, J.C., Peixe, L., Pestana, N., 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. *Int. J. Food Microbiol.* 82, 97 - 103.
- Arakawa, Y., Murakami, M., Suzuki, K., Ito, H., Wacharotayankun, R., Ohsuka, S., Kato, N., Ohta, M., 1995. A novel integron-like element carrying the metallo-beta-lactamase gene blaIMP. *Antimicrob. Agents Chemother.* 39, 1612-1615.
- Arlet, G., Barrett, T.J., Butaye, P., Cloeckert, A., Mulvey, M.R., White, D.G., 2006. *Salmonella* resistant to extended-spectrum cephalosporins: prevalence and epidemiology. *Microbes Infect.* 8, 1945-1954.

- Baggesen, D.L., Christensen, J., 1997. Distribution of *Salmonella enterica* serotypes and phage types in Danish pig herds. In: Second International Symposium on Epidemiology and Control of *Salmonella* in Pork. Bech-Nielsen, S., Nielsen, J.P. (eds), Copenhagen, Denmark, 107-109.
- Balaji, B., O'Conner, K., Lucas, J.R., Anderson, J.M., Csonka, L.N., 2005. Timing of induction of osmotically controlled genes in *Salmonella enterica* serovar Typhimurium, determined with quantitative real-time reverse transcription-PCR. *Applied and Environ. Microbiol.* 71, 8273–8283.
- Bang, I.S., Kim, B.H., Foster, J.W., Park, Y.K., 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* 182, 2245–2252.
- Bäumler, A.J., Hargis, B.M., Tsois, R.M., 2000. Tracing the origins of *Salmonella* outbreaks. *Sci.* 287, 50 - 52.
- Baumler, A.J., Tsois, R.M., Ficht, T.A., Adams, L.G., 1998. Evolution of host adaptation in *Salmonella enterica*. *Infect. Immun.* 66, 4579–4587.
- Bäumler, A.J., Tsois, R.M., Heffron, F., 1996. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. *Infect. Immun.* 64, 1862–1865.
- Bearson, B.L., Wilson, L., Foster, J.W., 1998. A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J. Bacteriol.* 180, 2409–2417.
- Benacer, D., Kwai lin, T., Haruo, W., Savithri, D.P., 2010. Characterization of drug-resistant *Salmonella enterica* serotype Typhimurium by antibiograms, plasmids, integrons, resistance genes, and PFGE. *J. Microbiol. Biotechnol.* 20, 1042-1052.
- Bennasar, A., de Luna, G., Cabrer, B., Lalucat, J., 2000. Rapid identification of *Salmonella Typhimurium*, *S. Enteritidis* and *S. Virchow* isolates by polymerase chain reaction based on fingerprinting methods. *Int. Microbiol.* 3, 31 – 38.
- Bensink, J.C., Botham, F.P., 1983. Antibiotic resistant coliform bacilli, isolated from freshly slaughtered poultry and from chilled poultry at retail outlets. *Australian Vet. J.* 60, 80-83.

- Berchieri, A.J., de Oliveira, G.H., Pinheiro, L.A.S., Barrow, P.A., 2000. Experimental *Salmonella gallinarum* infection in light laying hen lines. *Brazilian J. Microbiol.* 31, 50-52.
- Berkley, J.A., Lowe, B.S., Mwangi, I., Williams, T., Bauni, E., Mwarumba, S., Ngetsa, C., Slack, M.P., Njenga, S., Hart, C.A., Maitland, K., English, M., Marsh, K., Scott, J.A., 2005. Bacteremia among children admitted to a rural hospital in Kenya. *N. Engl. J. Med.* 352, 39-47.
- Berkowitz, F.E., 1984. Bacteremia in black South African children: a one year study emphasizing nosocomial bacteremia and bacteremia in severely malnourished children. *Ame. J. Dis. in Children* 138, 55-56.
- Bissonnette, L., Roy, P.H., 1992. Characterization of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. *J. Bacteriol.* 174, 1248-1257.
- Blahová, J., Kralíková, K., Krcmery Sr, V., Toršová, V., 1997. Transferable antibiotic resistance in nosocomial *Stenotrophomonas maltophilia* strain. *Diagn. Microbiol. Infect. Dis.* 29, 129-132.
- Bohaychuk, V.M., Gensler, G.E., King, R.K., Manninen, K.I., Sorensen, O., Wu, J.T., Stiles, M.E., McMullen, L.M., 2006. Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. *J. Food Protec.* 69, 2176 – 2182.
- Bolton, L.F., Kelley, L.C., Lee, M.D., Fedorka-Cray, P.J., Maurer, J.J., 1999. Detection of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 based on a gene, which confers cross-resistance to florfenicol and chloramphenicol. *J. Clin. Microbiol.* 37, 1348-1351.
- Boonmar, S., Bangtrakulnonth, A., Pornrunangwong, S., Marnrim, N., Kaneko, K., Ogawa, M., 1998. *Salmonella* in broiler chickens in Thailand with special reference to contamination of retail meat with *Salmonella enteritidis*. *J. Vet. Med. Sci.* 60, 1233-1236.
- Boonprasert, N., Patchanee, P., Hafez, H.M., Zessin, K.H., 2009. Prevalence of *Salmonella* species in broiler breeding flocks during production in a poultry compartment in Northern Thailand (abstract). In Food Safety and Zoonoses Symposium for Asia Pacific, Chiang Mai, Thailand. 13.

- Brent, A.J., Oundo, J.O., Mwangi, I., Ochola, L., Lowe, B., Berkley, J.A., 2006. *Salmonella* bacteremia in Kenyan children. *Pediatr. Infect. Dis. J.* 25, 230-236.
- Breytenbach, J.H., 2004. *Salmonella* control in poultry. In Intervet international b.v
- Briggs, C.E., Fratamico, P.M., 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob. Agents and Chemother.* 43, 846–849.
- Ca'Novas, D., Fletcher, S.A., Hayashi, M., Csonka, L.N., 2001. Role of trehalose in growth at high temperature of *Salmonella enterica* Serovar Typhimurium. *J. Bacteriol.* 183, 3365–3371.
- Callaway, T.R., Edrington, T.S., Anderson, R.C., Byrd, J.A., Nisbet, D.J., 2008. Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella*. *J. Anim. Sci* 86, 163 – 172.
- Carattoli, A., 2001. Importance of integrons in the diffusion of resistance. *Vet. Res.* 32, 243-259.
- Carattoli, A., 2003. Plasmid-mediated antimicrobial resistance in *Salmonella enterica*. *Curr. Issues Mol. Biol.* 5, 113-122.
- Carli, K.T., Eyigor, A., Caner, V., 2001. Prevalence of *Salmonella* serovars in chicken and Turkey. *J. Food Prot.* 64, 1832 – 1835.
- CAST (Council for Agricultural Science and Technology), 1994. Foodborne pathogens: risk and consequences. Council for Agricultural Science and Technology, Ames, Iowa, USA.
- CDS (Communicable Diseases Surveillance), 2011. Non-typhoidal *Salmonella* species. *Communicable Diseases Surveillance Bulletin* 9, 36–38.
- Celum, C.L., Chaisson, R.E., Rutherford, G.W., Barnhart J.L., Echenberg, D.F., 1987. Incidence of salmonellosis in patients with AIDS. *J. Infect. Dis.* 156, 998-1002.
- Chadfield, M., Skov, M., Christensen, J., Madsen, M., Bisgaard, M., 2001. An epidemiological study of *Salmonella enterica* serovar 4, 12:b: in broiler chicken in Denmark. *Vet. Microbiol.* 82, 233 – 247.
- Chaiba, A., Fouzia, R.F., Abdelkader, C., Rachida, S.B., Mouloud, Z., 2008. Occurrence of *Salmonella* in chicken carcasses and giblets in Meknès-Morocco. *Pak. J. Nutr.* 7, 231-233.

- Chambers, J.R., Bisailon, J.R., Labbe, Y., Poppe, C., Langford, C.F., 1998. *Salmonella* prevalence in crops of Ontario and Quebec broiler chicken at slaughter. *Poult. Sci.* 77, 1497–1501.
- Chang, Y.Y., Eichel, J., Cronan, J. E. Jr., 2000. Metabolic instability of *Escherichia coli* cyclopropane fatty acid synthase is due to RpoH-dependent proteolysis. *J. Bacteriol.* 182, 4288–4294.
- Charimba, G., Hugo, C.J., Hugo, A., 2010. The growth, survival and thermal inactivation of *Escherichia coli* O157:H7 in a traditional South African sausage. *Meat Sci.* 85, 89–95.
- Cheong, H.J., Lee, Y.J., Hwang, I.S., Kee, S.Y., Cheong, H.W., Song, J.Y., Kim, J.M., Park, Y.H., Jung, J., Kim, W.J., 2007. Characteristics of non-typhoidal *Salmonella* Isolates from human and broiler-chickens in Southwestern Seoul, Korea. *J. Korean Med. Sci.* 22, 773–778.
- Cho, J., Cheung, C., Lee, S., Ko, S., Kim, K., Hwang, I., Kim, S., Cho, S., Lim, C., Lee, K., Kim, K., Ha, S., 2011. Assessment of microbial contamination levels of street-vended foods in Korea. *J. Food Safety* 31, 41–47.
- Cho, S.H., Shin, H.H., Choi, Y.H., Park, M.S., Lee, B.K., 2008. Enteric bacteria isolated from acute diarrheal patients in the Republic of Korea between the year 2004 and 2006. *J. Microbiol.* 46, 325–330.
- Christison, C.A., Lindsay, D., Von Holy, A., 2008. Microbiological survey of ready-to-eat foods and associated preparation surfaces in retail delicatessens, Johannesburg, South Africa. *Food Control* 19, 727–733.
- Chuanchuen, R., Padungtod, P., 2009. Antimicrobial resistance genes in *Salmonella enterica* isolates from poultry and swine in Thailand. *J. Vet. Med. Sci* 71, 1349–1355.
- Cooke, F.J., Wain, J., Fookes, M., Ivens, A., Thomson, N., Brown, D.J., Threlfall, E.J., Gunn, G., Foster, G., Dougan, G., 2007. Prophage sequences defining hot spots of genome variation in *Salmonella enterica* serovar Typhimurium can be used to discriminate between field isolates. *J. Clin. Microbiol.* 45, 2590–2598.
- Couturier, M., Bex, F., Berquist, L., Maas, W.K., 1988. Identification and classification of bacterial plasmids. *Microbiol. Rev.* 52, 375–395.

- Cox, N.A., Stern, N.J., Craven, S.E., Berrang, M.E., Musgrove, M.T., 2000. Prevalence of *Campylobacter* and *Salmonella* in the cecal droppings of turkeys during production. J. Appl. Poult. Res. 9, 542–545.
- Coynault, C., Robbe-Saule, V., Popoff, M.Y., Norel, F., 1992. Growth phase and SpvR regulation of transcription of *Salmonella typhimurium* spvABC virulence genes. Microb. Pathog. 13, 133–143.
- Crewe-Brown, H.H., Karstaedt, A.S., Khoosal, M., Keddy, K., Ward, L., 2000. *Salmonella typhimurium* DT104 bacteraemia at Chris Hani Baragwanath Hospital, South Africa. Interscience Conference on Antimicrob. Agents and Chemother. 40, 465.
- Crowther-Gibson, P., Govender, N., Keddy, K., Perovic, O., Quan, V., Von Gottberg, A., 2011. Non-typhoidal *Salmonella enterica* (NTS). GERMS-SA Annual Report 2011, 12 -14.
- Crump, J.A., Luby, S.P., Mintz, E.D., 2004. The global burden of typhoid fever. Bull. World Health Org. 82, 346–353.
- Dahal, N., 2007. Prevalence and antimicrobial resistance of *Salmonella* in imported chicken carcasses in Bhutan. MSc Thesis, Chiang Mai University and Freie University, Berlin.
- D'Aoust, J.Y. 1997. *Salmonella* Species, In: Food Microbiology: fundamentals and frontiers. American Society for Microbiology, Washington, D.C. U.S.A., 138 - 139
- de Oliveira, S.D., Flores, F.S., dos Santos, L.R., Brandelli, A., 2005. Antimicrobial resistance in *Salmonella Enteritidis* strains isolated from broiler carcasses, food, human and poultry-related samples. Int. J. Food Microbiol. 97, 297 – 305.
- Delicato, E.R., Mikcha, J.M.G., Fernandes, S.A., Pelayo, J.S., 2004. Resistance profile to antimicrobials of *Salmonella* spp. isolated from human infections. Braz. Arch. Biol. Technol. 47, 1 – 6.
- Dhanoa, A., Fatt, Q.K. , 2009. Non-typhoidal *Salmonella* bacteraemia epidemiology, clinical characteristics and its' association with severe immunosuppression. Annals of Clin. Microbiol. and Antimicrob. 8, 15.
- Dhilon, A.S., Shivaprasad, H.L, Roy, P., Alisantosa, B., Schaberg, D., Johnson, S., 2001. Pathogenicity of environmental origin *Salmonella* in specific pathogen – free chicks. Poul. Sci. 80, 1323–1328.

- Dogru, A.K., Ayaz, N.D., Gencay, Y.E., 2010. Serotype identification and antimicrobial resistance profiles of *Salmonella* spp. isolated from chicken carcasses. Trop. Anim. Health. Prod. 42, 893–897.
- Dominguez, C., Gomez, I., Zumalacarregui, J., 2002. Prevalence of *Salmonella* and *Campylobacter* in retail chicken meat in Spain. Int. J. Food Microbiol. 72, 165–168.
- Doyle, M.P., Erickson, M.C., 2006. Emerging microbiological food safety issues related to meat. Meat Sci. 74, 98–112.
- Duarte, D.A.M., Ribeiro, A.R., Vasconcelos, A.M.M., Santos, S.B., Silva, J.V.D., Arruda de Andrade, P.L., Pereira da Costa de Arruda Falcão L.C., 2009. Occurrence of *Salmonella* species in broiler chicken carcasses and their susceptibility to antimicrobial agents. Braz. J. Microbiol. 40, 569–573.
- EFSA (European Food Safety Agency), 2007. Report of the task force on zoonoses data collection on the analysis of the baseline survey on the prevalence of *Salmonella* in broiler flocks of Gallus gallus, Part A. The EFSA Journal 98, 1 – 85.
- EFSA (European Food Safety Agency), 2009. The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. The EFSA Journal 223, 16 – 250.
- El Hussein, A.A., Mayha, M., Elmadiena, N., Elsaid, S.M., Mohammed, A.M.S., Muckle, C.A., Cole, L., Wilkie, E., Mistry, K., 2010. Prevalence of *Salmonella enterica* subspecies *enterica* serovars in Khartoum State, Sudan. Res. J. Microbiol. 5, 966 – 973.
- Ellerbroek, L., Narapati, D., Phu Tai, N., Poosaran, N., Pinthong, R., Sirimalaisuwan, A., Tshering, P., Fries, R., Zessin, K.H., Baumann, M., Schroeter, A., 2010. Antibiotic resistance in *Salmonella* isolates from imported chicken carcasses in Bhutan and from Pig Carcasses in Vietnam. J. Food Protec. 73, 376–379.
- Empadinhas, N., da Costa, M.S., 2008. Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. Int. Microbiol. 11, 151–161.
- Enwere, G., Biney, E., Cheung, Y.B., Zaman, S.M., Okoko, B., Oluwalana, C., Vaughan, A., Greenwood, B., Adegbola, R., Cutts, F.T., 2006. Epidemiologic and clinical characteristics of community acquired invasive bacterial infections in children aged 2–29 months in The Gambia. Pediatr. Infect. Dis. J. 25, 700–705.

- Essack, S.Y., Hall, L.M., Pillay, D.G., McFadyen, M.L., Livermore, D.M., 2001. Complexity and diversity of *Klebsiella pneumoniae* strains with extended spectrum β -lactamases isolated in 1994 and 1996 at a teaching hospital in Durban South Africa. *Antimicrob. Agents Chemother.* 45, 88–95.
- Esteban, J.I., Oporto, B., Aduriz, G., Juste, R.A., Hurtado, A., 2008. A survey of food-borne pathogens in free-range poultry farms. *Int. J. Food Microbiol.* 123, 177–182.
- Falomir, M.P., Gozalbo, D., Rico, H., 2010. Coliform bacteria in fresh vegetables: from cultivated lands to consumers. In: Mendez-Vilas, A. (ed) *Current research, technology and education topics in applied microbiology and microbial biotechnology. Microbiology Series*, 22, 1175–1181.
- Fang, T.J., Wei, Q., Liao, C., Hung, M., Wang, T., 2003. Microbiological quality of 18°C ready-to-eat food products sold in Taiwan. *Int. J. Food Microbiol.* 80, 241–250.
- Fashae, K., Ogunsola, F., Aarestrup, F.M., Rene, S., Hendriksen, R.S., 2010. Antimicrobial susceptibility and serovars of *Salmonella* from chickens and humans in Ibadan, Nigeria. *J. Infect. Dev. Ctries.* 4, 484–494.
- Fassin, D., Schneider, H., 2003. The politics of AIDS in South Africa: beyond the controversies. *Bri. Med. J.* 326, 495–497.
- Feasey, N.A., Archer, B.N., Heyderman, R.S., Sooka, A., Dennis, B., Gordon, M.A., Keddy, K.H., 2010. Typhoid Fever and invasive nontyphoid salmonellosis, Malawi and South Africa. *Emerging Infect. Dis.* 16, 1448 – 1451.
- Fielding, B.C., Mnabisa, A., Gouws, P.A., Morris, T., 2012. Antimicrobial-resistant *Klebsiella* species isolated from free-range chicken samples in an informal settlement. *Arch. Med. Sci.* 1, 39–43.
- Finlay, B.B., Falkow, S., 1990. *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. *J. Infect. Dis.* 162, 1096–1106.
- Foley, S.L., Lynne, A.M., 2008. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *J. Anim. Sci.* 86, 173 – 187.
- Foley, S.L., Lynne, A.M., Nayak, R., 2008. *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *J. Anim. Sci.* 86, 149–162.

- Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Steven, J.H., Ricke, C., 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Appl. Environ. Microbiol.* 77, 4273–4279.
- Foster, J.W., 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. *J. Bacteriol.* 173, 6896–6902.
- Foster, J.W., 2001. Acid stress responses of *Salmonella* and *E. coli*: survival mechanisms, regulation, and implications for pathogenesis. *J. Microbiol.* 39, 89-94.
- Foster, J.W., Hall, H.K., 1990. Adaptive acidification response of *Salmonella typhimurium*. *J. Bacteriol.* 172, 771–778.
- Foster, J.W., Hall, H.K., 1991. Inducible pH-homeostasis and the acid tolerance response of *Salmonella typhimurium*. *J. Bacteriol.* 173, 5129–5135.
- Foster, J.W., Spector, M.P., 1995. How *Salmonella* survive against the odds. *Annu. Rev. Microbiol.* 49, 145-174.
- Frances, C.L., Ryan, T.A., Jones, B.D., Smith, S.J., Falkow, S., 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature* 364, 639–642.
- Franco, D.A., Webb, J., Taylor, C.E., 1990. Antibiotic and sulfonamide residues in meat: implication for human health. *J. Food Prot.* 53, 178-185.
- Fratamico, P.M., Debroy, C., Strobaugh Jr, T.P., Chen, C.Y., 2005. DNA sequence of the *Escherichia coli* O103 O antigen gene cluster and detection of enterohemorrhagic *E. coli* O103 by PCR amplification of the *wzx* and *wzy* genes. *Canadian J. Microbiol.* 51, 515-522
- Frere, J.M., 1995. β -lactamases and bacterial resistance to antibiotics. *Mel. Microbial.* 16, 385-395.
- GAIN (Global Agriculture Information Network), 2007. Republic of South Africa poultry and products voluntary report 2007, 1 – 9.
- Galán, J.E., Ginocchio, C., Costeas, P., 1992. Molecular and functional characterization of the *Salmonella typhimurium* invasion gene *invA* : homology of *InvA* to members of a new protein family. *J. Bacteriol.* 17, 4338–4349.
- Gericke, M., 2009. Report of the chick producers organization committee for 2008 In Congress and Exhibition 2009 brochure, South African Poultry Association, 27 – 34.
- Gilks, C.F., 1998. Acute bacterial infections and HIV disease. *Br. Med. Bull.* 54, 383–393.

- Giraud, E., Baucheron, S., Cloeckaert, A., 2006. Resistance to fluoroquinolones in *Salmonella*: emerging mechanisms and resistance prevention strategies. *Microbes and Infect.* 8, 1937–1944.
- Glynn, M.K., Bopp, C., Dewitt, W., Dabney, P., Mohktar, M., Angulo, F.J., 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *N. Engl. J. Med.* 338, 1333–1338.
- Gordon, M.A., 2008. *Salmonella* infections in immunocompromised adults. *J. Infect.* 56, 413–422.
- Gordon, M.A., Banda, H.T., Gondwe, M., Gordon, S.B., Boeree, M.J., Walsh, A.L., Corkill, J.E., Hart, C.A., Gilks, C.F., Molyneux, M.E., 2002. Non-typhoidal *Salmonella* bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. *AIDS* 16, 1633–1641.
- Govender, N., Smith, A.M., Karstaedt, A.S., Keddy, K.H., 2009. Plasmid-mediated quinolone resistance in *Salmonella* from South Africa. *J. Med. Microbiol.* 58, 1393–1394.
- Grabow, W.O.K., 1996. Waterborne diseases: update on water quality assessment and control. *Water South Africa (Pretoria)* 22, 193–210.
- Gray, K.J., Wilson, K.W., Phiri, A., Corkill, J.E., French, N., Hart, A., 2006. Identification and characterization of ceftriaxone resistance and extended-spectrum β -lactamases in Malawian bacteraemic *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 57, 661–665.
- Grimont, F., Grimont, P.A.D., 2006. The Genus *Serratia*. *Prokaryotes* 6, 219–244.
- Grimont, P.A.D., Grimont, F., Bouvet, P., 2000 Taxonomy of the Genus *Salmonella*. In: Wray, C., Wray, A. (Ed.) *Salmonella* in domestic animals. CABI Publishing, Wallingford, United Kingdom, 1–17.
- Gross, U., Tschape, H., Bednarek, I., Frosch, M., 1998. Antibiotic resistance in *Salmonella enterica* serotype Typhimurium. *European J. Clin. Microbiol. and Infect. Dis.* 17, 385–387.
- Guentzel, N.M. 1996. *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, and *Proteus*, In: Baron, S. (Ed.) *Medical microbiology*. University of texas, Gavelstone, USA, 1–19.
- Gulig, P.A., Doyle, T.J., 1993. The *Salmonella typhimurium* virulence plasmid increases the growth rate of salmonellae in mice. *Infect. Immun.* 61, 504–511.

- Habtamu-Taddele, M., Rathore, R., Dhama, K., Agarwal, R.K., 2011. Epidemiological characterization of *Salmonella gallinarum* isolates of poultry origin in India, employing two PCR based typing methods of RAPD-PCR and PCR-RFLP. *Asian J. Anim. Vet. Adv.* 6, 1037–1051.
- Hall, H.K., Foster, J.W., 1996. The role of fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. *J. Bacteriol.* 178, 5683–5691.
- Hall, R.M., Collis, C.M., 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol. Microbiol.* 15, 593–600.
- Hamidian, M., Tajbakhsh, M., Walther-Rasmussen, J., Zali, M.R., 2009. Emergence of extended-spectrum beta-lactamases in clinical isolates of *Salmonella enterica* in Tehran, Iran. *Jpn. J. Infect. Dis.* 62, 368–371.
- Hanes, D., 2003. Nontyphoid *Salmonella*. In: Miliotis, M.D. (Ed.) *International Handbook of Foodborne Pathogens*. Marcel Dekker, New York, USA, 137 - 150.
- Hang'ombe, B.M., Sharma, R.N., Skjerve, E., Tuchili, L.M., 1999. Occurrence of *Salmonella enteritidis* in pooled table eggs and market-ready chicken carcasses in Zambia. *Avian Dis.* 43, 597-599.
- Hanning, I.B., Nutt, J.D., Ricke, S.C., 2009. Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. *Foodborne Pathog. Dis.* 6, 635-648.
- Harrison, W.A., Griffith, C.J., Tennant, D., Peters, A.C., 2001. Incidence of *Campylobacter* and *Salmonella* isolated from retail chicken and associated packaging in South Wales. *Lett. Appl. Microbiol.* 33, 450–454.
- Haryani, Y., Tunung, R., Chai, L.C., Lee, H.Y., Tang, S.Y., Son, R., 2008. Characterization of *Enterobacter cloacae* isolated from street foods. *ASEAN Food J.* 15, 57-64
- Hedberg, C.W., Levine, W.C., White, K.E., Carlson, R.H., Winsor, D.K., Cameron, D.N., MacDonald, K.L., Osterholm, M.T., 1992. An international foodborne outbreak of shigellosis associated with a commercial airline. *JAMA* 268, 3208-3212.
- Helms, M., Vastrup, P., Gerner-Smidt, P., Molbak, K., 2003. Excess mortality associated with antibiotic resistant *Salmonella typhimurium*. *Ugeskr Laeger* 165, 235 – 239.

- Helmuth, R., 2000. Antibiotic resistance in *Salmonella*. In: *Salmonella* in Domestic Animals. CABI publishing, Wallingford, United Kingdom, 89-106.
- Hendriksen, R.S., Vieira, A.R., Karlsmose, S., Lo Fo Wong, D.M., Jensen, A.B., Wegener, H.C., Aarestrup, F.M., 2011. Global monitoring of *Salmonella* serovar distribution from the world health organization global foodborne infections network country data bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathog. and Dis.* 8, 887–900.
- Hengge, R., 2009. Proteolysis of sigmaS (RpoS) and the general stress response in *Escherichia coli*. *Res. in Microbiol.* 160, 667–676.
- Henriques, I.S., Alves, A., Saavedra, M.J., Mark, H.M., Montforts, M., Correia, A. 2012. Environmental antibiotic resistome: new insights from culture-independent approaches, In: Keen, P.L., mark, H.M., Montforts, M. (Ed.) *Antimicrobial Resistance in the Environment*. Wiley Blackwell, New Jersey, USA, 123-148.
- Herikstad, H., Motarjemi, Y., Tauxe, R.V., 2002. *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiol. Infect.* 129, 1-8.
- Hermans, A.P.M.H., 2007. Stress response and virulence in *Salmonella typhimurium*: a genomics approach. PhD Thesis, Wageningen University, Wageningen, Netherlands.
- Hinton, A.J., Corrier, D.E., Spates, G.E., Norman, J.O., Ziprin, R.L., Beier, R.C., DeLoach, J.R., 1990. Biological control of *Salmonella typhimurium* in young chickens. *Avian Dis.* 34, 626–633.
- Hoelzer, K., Isabel, A., Switt, M., Wiedmann, M., 2011. Animal contact as a source of human non-typhoidal salmonellosis. *Vet. Res.* 42, 34.
- Hohmann, E.L., 2001. Nontyphoidal salmonellosis. *Clin. Infect. Dis.* 32, 263-269.
- Hooper, D.C., Wolfson, J.S., 1993. Mechanisms of quinolone action and bacterial killing, In: Hooper, D.C., Wolfson, J.S. (Ed.) *Quinolone antimicrobial agents*. American Society for Microbiology, Washington, D.C., USA, 53–75.
- Humphrey, P.A., Chapman, T.J., Rowe, B., Gilbert, J., 1990. A comparative study of the heat resistance of salmonellas in homogenized whole egg, egg yolk or albumen. *Epidemiol. Infect.* 104, 237-241.
- Humphrey, T.J., 2000. Public-health aspects of *Salmonella* infections. In: Wray, C., Wray, A. (Ed.) *Salmonella* in domestic animals. CABI Publishing, Wallingford, England.

- Humphrey, T.J., Richardson, N.P., Statton, K.M., Rowbury, R.J., 1993. Effects of temperature shift on acid and heat tolerance in *Salmonella enteritidis* PT4. Appl. and Environ. Microbiol. 59, 1842–1847. .
- Iroha, I.R., Ugbo, E.C., Ilang, D.C., Oji, A.E., Ayogu, T.E., 2011. Bacteria contamination of raw meat sold in Abakaliki, Ebonyi State Nigeria. J. Pub. Health and Epidemiol. 3, 49-53.
- Jacobs, J.L., Gold, J.W.M., Murray, H.W., Roberts, R.B., Armstrong, D., 1985. *Salmonella* infections in patients with the acquired immunodeficiency syndrome. Ann. Inter. Med. 102,186-188.
- Javadi, A., Safarmashaei, S., 2011. Microbial profile of marketed broiler meat. Middle-East J. Sci. Res. 9, 652-656.
- Jay, J.M., Loessner, M.L., Golden, D.A., 2005. Protection of foods with low temperature. In: Modern Food Microbiology. Springer Science and Business Media Inc., New York, USA. 395-409
- Jerngklinchani, J., Koowatananukuv, C., Daengprom, K., Saitanu, K., 1994. Occurrence of *Salmonellae* in raw broilers and their products in Thailand. Food Protec. J. 57, 808 - 813.
- Kankwatira, A.M., Mwafulirwa, G.A., Gordon, M.A., 2004. Non typhoidal *Salmonella* bacteremia an underrecognized feature of AIDS in African adults. Trop. Doct. 34, 198-200.
- Kantor, L.S., Lipton, K., Manchester, A., Oliveira, V., 1997. Estimating and addressing America's food losses. Food. Rev. 20, 3-11.
- Kariuki, S., Revathi, G., Muyodi, J., Mwituria, J., Munyalo, A., Kagendo, D., Murungi, L., Hart, C.A., 2005. Increasing prevalence of multidrug-resistant non-typhoidal salmonellae, Kenya, 1994–2003. Int. J. Antimicrob. Agents 25, 39–45.
- Keddy, K.H., Dwarika, S., Crowther, P., Perovic, O., Wadula, J., Hoosen, A., Sooka, A., Crewe-Brown, H.H., Smith, A.M., 2009. Genotypic and demographic characterization of invasive isolates of *Salmonella typhimurium* in HIV co-infected patients in South Africa. J. Infect. Dev. Ctries. 3, 585-592.
- Kenyon, W.J., Sayers, D.G., Humphreys, S., Roberts, M., Spector, M. P., 2002. The starvation-stress response of *Salmonella enterica* serovar Typhimurium requires σ E-, but not CpxR-regulated extracytoplasmic functions. Microbiol.and Mol. Biol. Rev. 148, 113–122.

- Khan, A.A., Melvin, C.D., Dagdag, E.B., 2007. Identification and molecular characterization of *Salmonella* spp. from unpasteurized orange juices and identification of new serotype *Salmonella* strain *S. enterica* serovar Tempe. Food Microbiol. 24, 539–543.
- Khan, M.A.H., Chowdhury, A.H., Mosaddik M.A., Shajahan, M., 2000. Multidrug resistant gene (S) harboring on a 20 Kb plasmid in *Salmonella typhi* that causes typhoid-enteric fever. Pak. J. Biol. Sci. 3, 911-914.
- Kidanemariam, A., Engelbrecht, M., Picard, J., 2010. Retrospective study on the incidence of *Salmonella* isolations in animals in South Africa, 1996 to 2006. J. S. Afr. Vet. Assoc. 81, 37-44.
- Kinde, H., Read, D.H., Chin, R.P., Bickford, A.A., Walker, R.L., Ardans, A., Breitmeyer, R.E., Willoughby, D., Little, H.E., Kerr, D., Gardner, I.A., 1996. *Salmonella enteritidis*, phage type 4 infection in a commercial layer flock in Southern California: Bacteriological and epidemiologic findings. Avian Dis. 40, 665–671.
- Kruger, T., Szabo, D., Keddy, K.H., Deeley, K., Marsh, J.W., Hujer, A.M., Bonomo, R.A., Paterson, D.L., 2004. Infections with nontyphoidal *Salmonella* spp producing TEM-63 or a Novel TEM enzyme, TEM-131, in South Africa. Antimicrob. Agents and Chemother. 48, 4263 – 4270.
- Kumar, A., Kumar, S., 2003. Survival kinetics of *Salmonella enterica* serotype Senftenberg after heat and acid stress. World J. Microbiol. and Biotech. 19, 985–987.
- Kwon, Y.M., Ha, S.D., Ricke, S.C., 1998. Growth response of a *Salmonella typhimurium* poultry isolate to propionic acid in aerobic and anaerobic growth conditions. J. Food Safety 18, 139-149.
- Laconcha, I., Baggesen, D.L., Rementeria, A., Garaizar, J., 2000. Genotypic characterization by PFGE of *Salmonella enterica* serotype Enteritidis phage types 1, 4, 6, and 8 isolated from animal and human sources in three European countries. Vet. Microbiol. 75, 155 – 165.
- Le Minor, L., 1994. The genus *Salmonella*. In: Ballows, A., Trüper, H.G., Dworkin, M., Harber, W., Scheiffer, K.H. (Ed.) The Prokaryotes. Springer, New York, USA, 2760–2774.
- Le Minor, L., Popoff, M., 1987. Designation of *Salmonella enterica* sp. nov. as the type and only species of the genus *Salmonella*. Int. J. System. Bacteriol. 37, 465-468.

- Lee, D.J., Cox, D., Li, J., Greenberg, S., 2000. Rac1 and Cdc42 are required for phagocytosis, but not NF-kappaB-dependent gene expression, in macrophages challenged with *Pseudomonas aeruginosa*. *J. Biol. Chem.* 275, 141-146.
- Lee, H.Y., Su, L.H., Tsai, M.H., Kim, S.W., Chang, H.H., Jung, S.I., Park, K.H., Perera, J., Carlos, C., Tan, B.H., Kumarasinghe, G., So, T., Chongthaleong, A., Hsueh, P.R., Liu, J.W., Song, J.H., Chiu, C.H., 2009. High rate of reduced susceptibility to ciprofloxacin and ceftriaxone among nontyphoid *Salmonella* clinical isolates in Asia. *Antimicrob. Agents Chemother.* 53, 2696-2699.
- Lee, I.S., Lin, J., Hall, H.K., Bearson, B., Foster, J.W., 1995. The stationary-phase sigma factor (RpoS) is required for sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol. Microbiol.* 17, 155-167.
- Leonard, J., Marshall, J.K., Moayyedi, P., 2007. Systematic review of the risk of enteric infection in patients taking acid suppression. *Am. J. Gastroenterol.* 102, 2047-2056.
- Leyer, G.J., Johnson, E.A., 1993. Acid adaptation induces cross-protection against environmental stress in *Salmonella typhimurium*. *Appl. and Environ. Microbiol.* 59, 1842-1847.
- Li, J., Ochman, H., Groisman, E.A., Boyd, E.F., Solomon, F., Nelson, K., Selander, R.K., 1995. Relationship between evolutionary rate and cellular location among the Inv/Spa invasion proteins of *Salmonella enterica*. *Proc. Natl. Acad. Sci.* 92, 7252-7256.
- Long, C., Jones, T.F., Vugia, D.J., Scheftel, J., Strockbine, N., Ryan, P., Shiferaw, B., Tauxe, R.V., Gould, L.H., 2010. *Y. pseudotuberculosis* and *Y. enterocolitica* Infections FoodNet, 1996-2007 *Emerg. Infect. Dis.* 16, 566-567.
- Luber, P., 2009. Cross-contamination versus undercooking of poultry meat or eggs — which risks need to be managed first? *Int. J. Food Microbiol.* 134, 21 – 28.
- Madara, J.L., Patapoff, T.W., Gillece-Castro, B., Colgan, S.P., Parkos, C.A., Delp, C., Mrsny, R.J., 1993. 5'-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J. Clin. Invest.* 91, 2320-2325.
- Magnani, R., 1997. Sampling guide. Food and Nutrition Technical Assistance Project (FANTA) Washington, DC, USA. 8-22.

- Mani-López, E., García, H.S., López-Malo, A., 2011 Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Res. Int.* 45, 713–721.
- Maripandi, A., Al-Salamah, A.A., 2010. Analysis of *Salmonella enteritidis* outer membrane proteins and lipopolysaccharide profiles with the detection of immune dominant proteins. *American J. Immunol.* 6, 1-6.
- Marianski, S., Marianski, A., Marianski, R., 2009. Cooking poultry. In: *Meat smoking and Smokehouse Design*. Bookmagic, LLC., USA, 75-78.
- Marshall, D.G., Sheehan, B.J., Dorman, C.J., 1999. A role for the leucine-responsive regulatory protein and integration host factor in the regulation of the *Salmonella* plasmid virulence (spv) locus in *Salmonella typhimurium*. *Mol. Microbiol.* 34, 134-145.
- Martinson, N.A., Karstaedt, A., Venter, W.D., Omar, T., King, P., Mbengo, T., Marais, E., McIntyre, J., Chaisson, R.E., Hale, M., 2007. Causes of death in hospitalized adults with a premortem diagnosis of tuberculosis: an autopsy study. *AIDS* 21, 2043–2050.
- Matsui, H., Eguchi, M., Kikuchi, Y., 2000. Use of confocal microscopy to detect *Salmonella typhimurium* within host cells associated with Spv-mediated intracellular proliferation. *Microb. Pathog.* 29, 53–59.
- McDermott, P.F., 2006. Antimicrobial resistance in nontyphoidal Salmonellae, In: Aarestrup, F.M. (Ed.) *Antimicrobial Resistance in Bacteria of Animal Origin*. Danish Institute for Food and Veterinary Research, Copenhagen, Denmark, 293-314.
- McLandsborough, L.A., 2004. *Food Microbiology Laboratory*. CRC Press, Boca Raton.
- Medeiros, A.A., 1997. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. *Clin. Infec. Dis.* 24, 19–45.
- Micozzi, A., Venditti, M., Monaco, M., Friedrich, A., Taglietti, F., Santilli, S., Martino, P., 2000. Bacteremia due to *Stenotrophomonas maltophilia* in patients with hematologic malignancies. *Clin. Infec. Dis.* 31, 705–711.
- Miriagou, V., Tassios, P.T., Legakis, N.J., Tzouveleakis, L.S., 2004. Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. *Int. J. Antimicrob. Agents* 23, 547–555.
- Molbak, K., Baggesen, D.L., Aarestrup, F.M., Ebbesen, J.M., Engberg, J., Frydendahl, K., Gerner-Smidt, P., Petersen, A.M., Wegener, H.C., 1999. An outbreak of multidrug-

- resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. The New Engl. J. Med. 341, 1420–1425.
- Mons, G., 2010. Economy and imports pose biggest threats to African poultry industry. World Poult. 26, 6-7
- Morpeth, S.C., Ramadhani, H.O., Crump, J.A., 2009. Invasive non-typhi *Salmonella* disease in Africa. Clin. Infect. Dis. 49, 606-611.
- Mosupye, F.M., Alexanderson, V., 1999. Microbiological quality and safety of ready-to-eat street-vended foods in Johannesburg, South Africa. J. Food Protec. 62, 1278-1284.
- Moussa, I.M., Gassem, M.A., Al-Doss, A.A., Mahmoud, W.A.S., Abdel-Mawgood, A.L., 2010. Using molecular techniques for rapid detection of *Salmonella* serovars in frozen chicken and chicken products collected from Riyadh, Saudi Arabia. Afr. J. Biotechnol. 9, 612-619.
- Moussa, S.D., Fred, G.S., Fatoumata, D., Jane, P., Luke, M., Roland, B., Claudie, B., Pascal, D., Susan, B., Brent, J. S., Edward, T., 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, *Clostridium perfringens* and *Enterococcus counts*, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in *Escherichia coli* isolates. Appl. Environ. Microbiol. 73, 6566–6576.
- Muyzer, G., Teske, A., Wirsén, C.O., Jannasch, H.W., 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164, 165–172.
- Ndip, R.N., Dlonga, H.M., Ndip, L.M., Akoachere, J.F.K., Nkuo-Akenji, T., 2005. *Pseudomonas aeruginosa* isolates recovered from clinical and environmental samples in Buea, Cameroon: current status on biotyping and antibiogram. Trop. Med. and Int. Health 10, 74 – 81.
- Ngwai, Y.B., Wambebe, C., Adachi, Y., 2007. Survivability of *Salmonella typhimurium* L1388 and *Salmonella enteritidis* L1225 under stressful growth conditions. Online J. Health Allied Sci. 2, 4.

- NICD (National Institute for Communicable Diseases), 2010. *Salmonella* Virchow foodborne illness outbreak. In: Communicable Diseases Communiqué, Johannesburg, South Africa, 3.
- Niehaus, J.A., Apalata, T., Coovadia, M.Y., Smith, M.A., Moodley, P., 2011. An outbreak of foodborne Salmonellosis in rural Kwazulu-Natal South Africa. *Foodborne Pathog. Dis.* 8, 693–697.
- Nonaka, G., Blankschien, M., Herman, C., Gross, C.A., Rhodius, V.A., 2006. Regulon and promoter analysis of the *E. coli* heat-shock factor, σ_{32} , reveals a multifaceted cellular response to heat stress. *Genes and Dev.* 20, 1776–1789.
- Nyenje, M.E., Odjadjare, C.E., Tanih, N.F., Green, E., Ndip, R.N., 2012. Foodborne pathogens recovered from ready-to-eat foods from roadside cafeterias and retail outlets in Alice, Eastern Cape Province, South Africa: public health implications. *Int. J. Environ. Res. Pub. Health* 9, 2608–2619.
- Okeke, N.I., Laxminarayan, R., Bhutta, Z.A., Duse, A.G., Jenkins, P., O'Brien, T.F., Pablos-Mendez, A., Klugman, K.P., 2005. Antimicrobial resistance in developing countries. Part I: recent trends and current status. *Lancet Infect. Dis.* 5, 481–493.
- Oliveira, W.F., Cardoso, W.M., Salles, R.P.R., Romão, J.M., Teixeira, R.S.C., Câmara, S.R., Siqueira, A.A., Marques, L.C.L., 2006. Initial identification and sensitivity to antimicrobial agents of *Salmonella* sp. isolated from poultry products in the State of Ceara, Brazil. *Brazilian J. Poult. Sci.* 8, 193 – 199.
- Pavia, A.T., Shipman, L.D., Wells, J.G., Puh, N.D., Smith, J.D., McKinley, T.W., Tauxe, R.V., 1990. Epidemiologic evidence that prior antimicrobial exposure decreases resistance to infection by antimicrobial-sensitive *Salmonella*. *J. Infect. Dis.* 161, 255–260.
- Payne, J.B., Osborne, J.A., Jenkins, P.K., Sheldon, B.W., 2007. Modeling the growth and death kinetics of *Salmonella* in poultry litter as a function of pH and water activity. *Poult. Sci.* 86, 191–201.
- Popoff, M.Y., Bockemuhl, J., Gheesling, L.L., 2003. Supplement 2001 (no. 45) to the Kauffmann–White scheme. *Res. Microbiol.* 154, 173–174.
- Poppe, C., 2000. *Salmonella* infections in the domestic fowl, In: Wray, C., Wray, A. (Ed.) *Salmonella* in Domestic Animals. CABI Publishings, Wallingford, United Kingdom, 107 – 132.

- Poppe, C., Irwin, R.J., Messier, S., Finley, G.G., Oggel, J., 1991. The prevalence of *Salmonella enteritidis* and other *Salmonella* sp. among Canadian registered commercial chicken broiler flocks. *Epidemiol. Infect.* 107, 201–211.
- Pounikar, R., Dawande, A.Y., 2010. Detection of potential carcinogens by Ames test. *Asiatic J. Biotech. Res.* 01, 57–64.
- Quintaes, B.R., Leal, N.C., Reis, E.M., Hofer, E., 2004. Optimization of randomly amplified polymorphic DNA-polymerase chain reaction for molecular typing of *Salmonella enterica* serovar Typhi. *Rev. Soc. Bras. Med. Trop.* 37, 143–147.
- Qureshi, A., Mooney, L., Denton, M., Kerr, K.G., 2005. *Stenotrophomonas maltophilia* in salad. *Emerging Infect. Dis.* 11, 1157–1158.
- Radstrom, P., Skold, O., Swedberg, G., Flensburg, J., Roy, P.H.I., Sundstrom, L., 1994. Transposon Tn5090 of plasmid R751, which carries an integron, is related to Tn7, Mu, and the retroelements. *J. Bacteriol.* 176, 3257–3268.
- Rahn, K., Degrandis, S.A., Clarke, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtiss, R., Gyles, C.L., 1992. Amplification on an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes* 6, 271–279.
- Ramos, A., Dámaso, D., 2000. Extraintestinal infection due to *Hafnia alvei*. *Eur. J. Clin. Microbiol. Infect. Dis.* 19 708–710.
- Rao, P.S., Yamada, Y., Tan, Y.P., Leung, K.Y., 2004. Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol. Microbiol.* 53, 573–586.
- Rasschaert, G., Houf, K., Imberechts, H., Grijspeerdt, K., De Zutter, L., Heyndrickx, M., 2005. Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. *J. Clin. Microbiol.* 43, 3615–3623.
- Recchia, G.D., Hall, R.M., 1995. Gene cassettes: a new class of mobile element. *Microbiol.* 141, 3015–3027.
- Reeves, M.W., Evins, G.M., Heiba, A.A., Plikaytis, B.D., Farmer, J.J. III, 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. *J. Clin. Microbiol.* 27, 313–320.

- Reid, A., 2009. Isolation and identification of *Salmonella* from food and environmental samples. MFHPB20, 1 – 14.
- Reis, B.P., Zhang, S., Tsois, R.M., Baümle, A.J., Adams, L.G., Santos, R.L., 2003. The attenuated *sopB* mutant of *Salmonella enterica* serovar Typhimurium has the same tissue distribution and host chemokine response as the wild type in bovine Peyer's patches. Vet. Microbiol. 97, 269–277.
- Ridley, A., Threlfall, E.J., 1998. Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella* Typhimurium DT104. Microb. Drug. Resist. 4, 113–118.
- Rindhe, S.N., Zanjad, P.N., Doifode, V.K., Siddique, A., Mendhe, M.S., 2008. Assessment of microbial contamination of chicken products sold in Parbhani city. Vet. World 1, 208–210.
- Roberts, M.C., 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19, 1–24.
- Roberts, M.C., 2005. Update on acquired tetracycline resistance genes. FEMS Microbiol. Lett. 245, 195–203.
- Rodrigo, S., Adesiyun, A., Asgarali, Z., Swanston, W., 2006. Occurrence of selected foodborne pathogens on poultry and poultry giblets from small retail processing operations in Trinidad. J. Food. Prot.. 69, 1096 – 1105.
- Rose, N., Beaudeau, F., Drouin, P., Toux, J.Y., Rose, V., Colin, P., 1999. Risk factors for *Salmonella enterica* subsp. *enterica* contamination in French broiler-chicken flocks at the rearing period. Prev. Vet. Med. 39, 265 – 277.
- Rostoff, P., Paradowski, A., Gackowski, A., Konduracka, E., El Massri, N., Gajos, G., Pfitzner, R., Drwila, R., Sadowski, J., Piwowarska, W., 2010. *Stenotrophomonas maltophilia* pacemaker endocarditis in a patient with d-transposition of the great arteries after atrial switch procedure. Int. J. Cardiol. 145, 92–95.
- Rotger, R., Casadesús, J., 1999. The virulence plasmids of *Salmonella*. Int. Microbiol. 2, 177–184.
- Rouquette, C., de Chastellier, C., Nair, S., Berche, P., 1998. The ClpC ATPase of *Listeria monocytogenes* is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. Mol. Microbiol. 27, 1235–1245.

- Rowe-Magnus, D.A., Gueront, A.M., Mazel, D., 1999. Super-integrans. Res. Microbiol. 150, 641-651.
- Ruiz, J., Capitano, L., Núñez, L., Castro, D., Sierra, J.M., Hatha, M., Borrego, J.J., Vila, J., 1999. Mechanisms of resistance to ampicillin, chloramphenicol and quinolones in multiresistant *Salmonella typhimurium* strains isolated from fish. J. Antimicrob. Chemother. 43, 699-702.
- Rychlik, I., Barrow, P.A., 2005. *Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS Microbiol. Rev. 29, 1021-1040.
- Sabota, J.M., Hoppes, W.L., Ziegler Jr, J.R., 1998. A new variant of food poisoning: enteroinvasive *Klebsiella pneumoniae* and *Escherichia coli* sepsis from a contaminated hamburger. The Am. J. Gastroenterol. 93, 118-119.
- Saikia, P., Joshi, S.R., 2010. Retail market poultry meats of North-east India : a microbiological survey for pathogenic microorganisms. Res. J. Microbiol. 5, 36-43.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. and Evol. 4, 406-425.
- Salam, M.A., Saha, D., Khan, W.A., Bennish, M.L., 2003. Non-typhoidal *Salmonella* infection and their antimicrobial resistance in Bangladesh. In Interscience Conference on Antimicrobial Agents and Chemotherapy, Dhaka, Bangladesh.
- Salisbury, J.G., Nicholis, T.J., Lammerding, A.M., Turnidge, J., Nuun, M.J., 2002. A risk analysis framework for the long-term management of antibiotic resistance in food-producing animals. Int. J. Antimicrob. Agents 20, 153 – 164.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning 2nd ed. Edition. Cold Spring Harbour Laboratory Press.
- Santos, R.L., Zhang, S., Tsolis, R.M., Bäumler, A.J., Adams, L.G., 2002. Morphologic and molecular characterization of *Salmonella typhimurium* infection in neonatal calves. Vet. Pathol. 39, 20-215.
- SAPA (South African Poultry Association), 2010. Broiler industry. South African Poultry Industry Profile for 2009, 22 -29.
- SAS., 2001. SAS/STAT User's Guide Version 8.2, Statistics Edition. SAS Institute Inc, Cary, NC.

- Sasipreeyajan, J., Jerngklinchan, J., Koowatananukul, C., Saitanu, K., 1996. Prevalence of salmonellae in broiler, layer and breeder flocks in Thailand. *Trop. Anim. Health Prod.* 28, 174 - 180.
- Shah, J.D., 2011. Stress response and pathogenesis of *Salmonella enterica* serovar Typhimurium. PhD Thesis, Utah State University, All Graduate Theses and Dissertations, Logan, Utah.
- Shaker, R., Osaili, T., Al-Omary, W., Jaradat, Z., Al-Zuby, M., 2007. Isolation of *Enterobacter sakazakii* and other *Enterobacter* sp. from food and food production environments. *Food Control* 18, 1241–1245.
- Shen, S., Fang, F.C., 2011. Integrated stress responses in *Salmonella*. *Int. J. Food Microbiol.* 152, 75-81.
- Sigauque, B., Roca, A., Mandomando, I., Morais, L., Quinto, L., Sacarlal, J., Macete, E., Nhamposha, T., Machevo, S., Aide, P., Bassat, Q., Bardaji, A., Nhalungo, D., Soriano-Gabarro, M., Flannery, B., Menendez, C., Levine, M.M., Alonso, P.L., 2009. Community-acquired bacteremia among children admitted to a rural hospital in Mozambique. *Pediatr. Infect. Dis. J.* 28, 108-113.
- Singh, S., Yadav, A.S., Singh, S.M., Bharti, P., 2010. Prevalence of *Salmonella* in chicken eggs collected from poultry farms and marketing channels and their antimicrobial resistance. *Food Res. Int.* 43, 2027-2030.
- Smith, A.M., Gouws, A., Hoyland, G., Sooka, A., Keddy, K.H., 2007. Outbreaks of food-borne disease – a common occurrence but rarely reported. *S. Afr. Med. J.* 97, 1272.
- Smith, M.G., 1985. The generation time, lag time and minimum temperature of growth of coliform organisms on meat, and the implications for codes of practice in the abattoirs. *J. Hyg. Camb.* 94, 289-300.
- Soler, P., Gonzalez-Sanz, R., Bleda, M.J., Hernandez, G., Echeita, A., Usera, M.A., 2006. Antimicrobial resistance in non-typhoidal *Salmonella* from human sources, Spain, 2001–2003. *J. Antimicrob. Chemother.* 58, 310–314.
- Spector, M.P., Kenyon, W.J., 2012. Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Res. Int.* 45, 455–481.
- Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin. Microbiol. Rev.* 22, 664–689.

- Su, L., Chiu, C., Chu, C., Ou, J.T., 2004. Antimicrobial resistance in nontyphoid *Salmonella* serotypes: a global challenge. Clin. Infect. Dis. 39, 546–551.
- Tangkawattana, P., Angkititrakul, S., Kanistanon, K., 2009. Contamination of *Salmonella* in chicken meat and liver sold at the fresh markets in five upper Northeastern Provinces of Thailand. In Food Safety and Zoonoses Symposium for Asia Pacific, Chiang Mai, Thailand, 24.
- TAS (Trends in Agricultural Sector), 2011. Animal production. In Economic review for the 12 months that ended 30 June 2010, Pretoria, South Africa, 60-61.
- Tavechio, A.T., Ghilardi, A.C., Peresi, J.T., Fuzihara, T.O., Yonamine, E.K., Jakabi, M., Fernandes, S.A., 2002. *Salmonella* serotypes isolated from nonhuman sources in São Paulo, Brazil, from 1996 through 2000. J. Food Prot. 65, 1041 – 1044.
- Thomsen, L.E., Olsen, J.E., Foster, J.W., Ingmer, H., 2002. ClpP is involved in the stress response and degradation of misfolded proteins in *Salmonella enterica* serovar Typhimurium. Microbiol. 148, 2727–2733.
- Threlfall, E.J., 2000. Epidemic *Salmonella typhimurium* DT 104—a truly international epidemic clone. J. Antimicrob. Chemother. 46, 7–10.
- Tikoo, A., Tripathi, A.K., Verma, S.C., Agrawal, N., Nath, G., 2001. Application of PCR fingerprinting techniques for identification and discrimination of *Salmonella* isolates. Curr. Sci. 80, 1049-1052.
- Tindall, B.J., Grimont, P.A.D., Garrity, G.M., Euzéby, J.P., 2005. Nomenclature and taxonomy of the genus *Salmonella*. Int. J. Syst. and Evol. Microbiol. 55, 521–524.
- Todar, K., 2005. *Salmonella* and Salmonellosis. Department of bacteriology, Kenneth Todar University of Wisconsin-Madison USA.
- Todd, E.C., 1997. Epidemiology of foodborne diseases: a worldwide review. World Health Stat. Q 50, 30–50.
- Torpdahl, M., Skov, M.N., Sandvang, D., Baggesen, D.L., 2005. Genotypic characterization of *Salmonella* by multilocus sequence typing, pulsed-field gel electrophoresis and amplified fragment length polymorphism. J. Microbiol. Methods 63, 173–184.
- Türkyılmaz, S., Hazımoğlu, Ş., Bozdoğan, B., 2009. Antimicrobial susceptibility and resistance genes in *Salmonella enterica* serovar Enteritidis isolated from turkeys. Israel J. Vet. Med. 64 72-77.

- Turtura, G.C., Massa, S., Ghazvinizadeh, H., 1990. Antibiotic resistance among coliform bacteria isolated from carcasses of commercially slaughtered chickens. *Int. J. Food Microbiol.* 11, 351-354.
- Tzelepi, E., Giakkoupi, P., Sofianou, D., Loukova, V., Kemeroglou, A., Tsakris, A., 2000. Detection of extended-spectrum beta-lactamase in clinical isolates of *Enterobacter cloacae* and *Enterobacter aerogenes*. *J. Clin. Microbiol.* 38, 542-546.
- Usha, G., Chunderika, M., Prashini, M., Willem, S.A., Yusuf, E.S., 2008. Characterization of extended-spectrum beta-lactamases in *Salmonella* spp. at a tertiary hospital in Durban, South Africa. *Diagn. Microbiol. Infect. Dis.* 62, 86-91.
- Uyttendaele, M.R., Debevere, J.M., Lips, R.M., Neyts, K.D., 1998. Prevalence of *Salmonella* in poultry carcasses and their products in Belgium. *Int. J. Food Microbiol.* 40, 1-8.
- Uzzau, S., Brown, D.J., Wallis, T., Rubino, S., Leori, G., Bernard, S., Casadesus, J., Platt, D.J., Olsen, J.E., 2000. Host adapted serotypes of *Salmonella enterica*. *Epidemiol. Infect.* 125, 229-255.
- van Den Bogaard, A.E., Stobberingh, E.E., 1999. Antibiotic usage in animals: impact on bacterial resistance and public health. *Drugs* 58, 589 - 607.
- van der Zee, H., Huis in't Veld, J.H.J., 2000. Methods for the rapid detection of *Salmonella*. In: Wray, C., Wray, A. (Ed.) *Salmonella in Domestic Animals*. Cabi Publishing, Wallingford, Oxon, United Kingdom, 373-391.
- van Hoek, A.H., Scholtens, I.M., Cloeckaert, A., Aarts, H.J., 2005. Detection of antibiotic resistance genes in different *Salmonella* serovars by oligonucleotide microarray analysis. *J. Microbiol. Methods* 62, 13-23.
- van Nierop, W., Dusé, A.G., Marais, E., Aithma, N., Thothobolo, N., Kassel, M., Stewart, R., Potgieter, A., Fernandes, B., Galpin, J.S., Bloomfield, S.F., 2005. Contamination of chicken carcasses in Gauteng, South Africa by *Salmonella*, *Listeria monocytogenes* and *Campylobacter*. *Int. J. Food Microbiol.* 99, 1-6.
- Vandenberg, O., Nyarukweba, D.Z., Ndeba, P.M., Hendriksen, R.S., Barzilay, E.J., Schirvel, C., Bisimwa, B.B., Collard, J.M., Aidara, K.A., Aarestrup, F.M., 2010. Microbiologic and Clinical Features of *Salmonella* species isolated from bacteremic children in Eastern Democratic Republic of Congo. *Pediatr. Infect. Dis. J.* 29, 504-510.
- Vanquelin C., 2009. Broiler industry. *South African Poultry Industry Profile for 2008*, 43-51.

- Vo, A.T., van Duijkeren, E., Gastra, W., Fluit, A.C., 2010. Antimicrobial resistance, class 1 integrons, and genomic island 1 in *Salmonella* isolates from Vietnam. PLoS ONE 5, e9440.
- Von Schirnding, I., Yeah, D., Mathee, A., 1993. Health aspects of sanitation with special reference to South Africa. J. Comp. Health 4, 73-79.
- Wain, J., House, D., Zafar, A., Baker, S., Nair, S., Kidgell, C., Bhutta, Z., Dougan, G., Hasan, R., 2005. Vi antigen expression in *Salmonella enterica* serovar Typhi clinical isolates from Pakistan. J. Clin. Microbiol. 43, 1158-1165.
- Waltman, W.D. 2000. Methods for the cultural isolation of *Salmonella*, In: Wray, C., Wray, A. (Ed.) *Salmonella* in Domestic Animals. Cabi Publishing, Wallingford, Oxon, United Kingdom, 355–372.
- Wannaprasat, W., Padungtod, P., Chuanchuen, R., 2011. Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. Int. J. of Antimicrob. Agents 37, 457–461.
- Waters, V.L., 1999. Conjugative transfer in the dissemination of beta-lactam and aminoglycoside resistance. Frontiers in Bioscience 4, 416-439.
- Webb, C., Moreno, M., Wilmes-Riesenberg, M., Curtiss III, R., Foster, J.W., 1999. Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium*. Mol. Microbiol. 34, 112–123.
- White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, S., Waner, D.D., Meng, J., 2001. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. New Engl. J. Med. 345, 1147–1154.
- White, P.L., Naugle, A.L., Jackson, C.R., Fedorka-Cray, P.J., Rose, B.E., Pritchard, K.M., Levine, P., Saini, P.K., Schroeder, C.M., Dreyfuss, M.S., Tan, R., Holt, K.G., Harman, J., Buchanan, S., 2007. *Salmonella enteritidis* in meat, poultry, and pasteurized egg products regulated by the U.S. Food Safety and Inspection Service, 1998 through 2003. J. Food Prot. 70, 582–591.
- Winfield, M.D., Groisman, E.A., 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. Applied and Environ. Microbiol. 69, 3687–3694.
- Winokur, P.L., Vonstein, D.L., Hoffman, L.J., Uhlenhopp, E.K., Doern, G.V., 2001. Evidence for transfer of CMY-2 AmpC b-lactamase plasmids between *Escherichia coli* and

- Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. 45, 2716-2722.
- Wolday, D., Erge, E., 1998. Antimicrobial sensitivity pattern of *Salmonella*: comparison of isolates from HIV-infected and HIV-uninfected patients. Trop. Doc. 28, 139-141.
- Wood, J.M., 2006. Osmosensing by bacteria. Sci STKE 357, 43.
- Yan, S.S., Pandrak, M.L., Abela-Rider, B., Punderson, J.W., Fedorko, D.P., Foley, S.L., 2003. An overview of *Salmonella* typing public health perspectives. Clin. Appl. Immunol. Rev. 4, 189-204.
- Yates, C., Amyes, S., 2005. Extended-spectrum β -lactamases in non-typhoidal *Salmonella* spp. isolated in the UK are now a reality: why the late arrival? J. Animicrob. Chemother. 56, 262–264.
- Yemisen, M., Mete, B., Tunali, Y., Yentur, E., Ozturk, R., 2008. A meningitis case due to *Stenotrophomonas maltophilia* and review of the literature. Int. J. Infec. Dis. 12, 125-127.
- Yuk, H.G., Schneider, K.R., 2006. Adaptation of *Salmonella* spp. in juice stored under refrigerated and room temperature enhances acid resistance to simulated gastric fluid. Food Microbiol. 23, 694–700.
- Zaidi, M.B., McDermott, P.F., Fedorka-Cray, P., Leon, V., Canche, C., Hubert, S.K., Abbott, J., Leo'n, M., Zhao, S., Headrick, M., Linda Tollefson, L., 2006. Nontyphoidal *Salmonella* from human clinical cases, asymptomatic children, and raw retail meats in Yucatan, Mexico. Clin. Infec. Dis. 42, 21–28.
- Zand, J., Rountree, R., Walton, R. 2003. Symptoms of *Salmonella* poisoning, In: Smart Medicine for a Healthier Child: A Practical A- to -Z Reference to Natural and Conventional Treatments for Infants and Children. Penguin Group Inc., New York, USA, 301 – 305.
- Zewdu, E., Cornelius, P., 2009. Antimicrobial resistance pattern of *Salmonella* serotypes isolated from food items and personnel in Addis Ababa, Ethiopia. Trop. Anim. Health Prod. 41, 241–249.
- Zhang, S., Kingsley, R.A., Santos, R.I., Andrews -Polymenis, H., Raffatellu, M., Figueiredo, J., Nunes, J., Tsois, R.M., Adams, L.G., Andreas, J., Ba"umler, A.J., 2003. Molecular pathogenesis of *Salmonella* enterica serotype Typhimurium-induced diarrhea. Infec. and Immun. 71, 1–12.