MOLECULAR EPIDEMIOLOGY, RISK FACTORS AND ASSESSMENT OF ANTIBIOTIC RESISTANCE OF SALMONELLAE IN COMMERCIAL LAYER FARMS IN SIX SELECTED STATES IN NIGERIA

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE.

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

AUGUST, 2016

DECLARATION

I declare that the work in this Thesis entitled **"Molecular Epidemiology, Risk Factors and Assessment of Antibiotic Resistance of Salmonellae in Commercial Layer Farms in Six Selected States in Nigeria"** has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria. The information derived from literature has been duely acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Idowu Oluwabumi FAGBAMILA	……..…	……. ……..
Student	Signature	Date

CERTIFICATION

This Thesis titled **"Molecular Epidemiology, Risk Factors and Assessment of Antibiotic Resistance of Salmonellae in Commercial Layer Farms in Six Selected States in Nigeria"** meets the regulations governing the award of Doctor of Philosophy (Veterinary Public Health and Preventive Medicine) of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty, my wife, Mary and my three children.

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ABSTRACT

Poultry salmonellosis remains a major constraint to poultry production in all parts of Nigeria with farmers still experiencing great losses caused by *Salmonella* infection despite huge amounts of resources expended on vaccination and medication. Relatively few African countries report surveillance data on *Salmonella* and as such, very limited information is available on *Salmonella* isolation for this continent. A cross sectional study was carried out to determine the prevalence, risk factors and the circulating *Salmonella* serovars in commercial layer farms in Nigeria. The study also identified the different antibiotic resistance patterns seen in the *Salmonella* species and assessed the mechanisms of their resistance and determined the degree of relatedness within the different serovars isolated. In all, 358 farms were visited in the six selected States (Imo, Edo, Ogun, Kaduna, Plateau and Bauchi) and 1790 samples comprising of litter, dust, faeces, feed and water were collected and tested for *Salmonella* according to ISO 6579:2002; Annex D protocol. Samples were cultured for *Salmonella* after pre-enriching in buffered peptone water, enriched in Rappaport Vassiliadis, Mueller Kauffmann tetrathionate broth and Modified semi solid Rappaport Vassiliadis before plating on Brilliance *Salmonella* agar, Bismuth sulphite agar, and Xylose lysine deoxycholate agar. Biochemical tests were conducted and suspected isolates serotyped and phage-typed. Minimum inhibitory concentration (MIC) was conducted on 200 isolates using commercial Sensititre plate. Polymerase chain reaction (PCR) and sequencing were conducted on the resistant isolates to detect resistance genes and point mutation (s). Pulsed field gel electrophoresis (PFGE) was conducted on 110 isolates comprising 8 different serovars while Multilocus variable number tandem repeat analysis (MLVA) was conducted only on 5 *Salmonella* Typhimurium isolates. The study established a *Salmonella* farm prevalence of 43.6% and sample prevalence of 13.4% with the highest

isolation rate of 16.8% from faecal samples. Sixty three serovars were isolated with *S.* Kentucky being the most prevalent (16.73%) and widely distributed in 5 of the 6 selected states (83.3%) with Ogun State having the highest isolation rate (65.5% and 21.8% for farm and sample prevalence respectively). Using multivariable logistic regression model, type of housing (AOR = 2.84; 95% CI: 1.00 - 8.03), age of farmer (Adjusted odd ratio (AOR) = 2.79; 95% CI 1.33 - 5.86), crate type (AOR = 1.74; 95% CI 1.05 - 2.88) and poultry association of Nigeria (PAN) membership (AOR = 1.67; 95% CI 1.07 - 2.62) were found to increase risk of *Salmonella* occurrence on farms. Farm fencing (AOR = 0.46; 95% CI: 0.29 - 0.73) and staff having protective clothing on the farm (AOR = 0.54; 95% CI: 0.33 - 0.89) reduced the risk of *Salmonella*. Of the 200 isolates for which the MIC of 14 antimicrobial agents were determined, 95 (47.5%) had various resistance profiles. The predominant resistance pattern was GenSulTetCipNal. Resistance to conventional antibiotics (AMP =19.6%, GEN =47.4%, TET =75.3% and SUL =75.3%) was high but all the isolates were susceptible to Cefotaxime, Ceftazidime and Meropenem. Resistance to ciprofloxacin and Nalidixic acid was also high (89.7% and 77.3%) in most of the isolates and all the *S.* Kentucky were resistant to both antibiotics. Resistance genes detected included *tetA* (tetracycline), *sul1, sul2, sul3* (sulphamethoxazole), *catA1, cmlA1, floR* (chloramphenicol), *dfrA5-14* (Trimethoprim), *aac(3)-le* (gentamicin), *tem1, tem30, tem57* (ampicillin) and *qnrA, qnrB, qnrS* (quinolones). Mutations were detected on the *gyrA* gene (Ser83→Phe, Asp87→Gly, Ser83→Tyr, Leu55→Phe), *parC* gene (Thr57→Ser) and *pmrA* gene (Thr89→Ser, Val93→Gly). The comparison of PFGE profiles obtained from 110 isolates identified PFGE cluster patterns for each of the eight serovars investigated with significant ranges of genetic similarities. The MLVA result showed that two *S.* Typhimurium isolates had the same allelic genes STTR9 and STTR3 but differed in the rest of the allelic genes

while the other three isolates which were 100% genetically related had identical allelic genes STTR9, STTR5 and STTR3 but lack allelic genes STTR6 and STTR10. This study found *S.* Kentucky, *S.* Poona and *S.* Larochelle as major serovars associated with poultry and environmental contamination in Nigeria. In addition, this study reported the occurrence of rare/ infrequent serovars (e.g. *S.* Elizabethville, *S.* Larochelle, *S.* Carno and *S.* Schwarzengrund) which can be of global importance because of travels, transhumance, and trade in animals and food products. This study also highlighted occurrence of resistance to important antimicrobials like fluoroquinolones. As antimicrobials are easily obtainable over the counter, there is need for authorities in Nigeria to regulate antimicrobial use in agriculture and in clinics to limit the spread of multidrug-resistant *Salmonella* among humans and animals.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
AOR	Adjusted odds ratio
bp	Base pair
BS	Bismuth Sulphite Agar
BSA	Brilliance *Salmonella* Agar
CAT	Chloramphenicol acetyltransferase
CDC	Center for disease control
CI	Confidence interval
CLBW	Clinical laboratory reagent water
CLED	Cystine lactose electrolyte-deficient agar
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroic acid synthase
DIASALM	Diagnostic semi solid *Salmonella* medium
DNA	Deoxyribonucleic acid
DT	Definitive typing
EDTA	Ethylene diamine tetraacetic acid
ESBL	Extended spectrum β-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
FQ	Fluoroquinolones
JCICSP	Judicial commission of the International Committee on the Systemics of prokaryotes
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
MKTTn	Muller-Kaufmann tetrathionate broth
MLVA	Multilocus Variable tandem repeat analysis

MRSV	Medium Semi solid Rappaport-Vassiliadis
NT	Non-typeable
NTS	Non Typhoidal *Salmonella*
OIE	World organization for animal health
OR	Odds Ratio
PAN	Poultry association of Nigeria
PBPs	Penicillin binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PHLs	Public Health Laboratories
PMQR	Plasmid Mediated Quinolone Resistance
PT	Partial typing
QRDR	Quinolone Resistance Determining Region
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RV	Rappaport-Vassiliadis
SAPE	Streptavidin-R-phycoerythrin
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecyl sulphate
SNPs	Single nucleotide polymorphisms
SPI	*Salmonella* Pathogenicity island
STTR	*Salmonella* Typhimurium Tandem Repeat
TEM	Temoxicillinase
THFA	Tetrahydrofolic acid
TTSS	Type three secretion system
UPGMA	Unweighted pair group method with arithmetic mean

WHO	World Health Organization
XLD	Xylose Lysine Desoxycholate

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Nigeria is the largest country in sub-Saharan Africa with an estimated population of 174.5 million people in 2013 and a population growth rate estimated at three per cent annually. The estimated gross domestic product (GDP) in 2012 was $450.5 billion, ranking the country 31st globally and agriculture accounts for over 38% of non-oil foreign earnings and 70% of the labour force (FAO, 2006a). The poultry industry in Nigeria is rapidly expanding despite being faced with many problems such as avian influenza, global financial crisis and inadequate credit (FAO, 2008). Nigerian poultry industry increased from 150,700 million birds in 2005 to 192,313 million birds in 2010 (FAO, 2015). Across the different regions of the country, the poultry sector is characterized by a low level of production, specialization and generally with biosecurity measures at a very weak level (FAO, 2008). Bigger farms generally raise layers whereas the smaller producers prefer broilers (FAO, 2008).

In 2011, Nigerian hen egg production totalled 636,000 metric tonnes (MT) and was valued at $527.49 million, ranking 19th in world hen egg production and the top producer in Africa (FAO, 2015). Both big and small egg farms are scattered all over the country, although they are generally concentrated around the major urban centres (FAO, 2008). Poultry meat is one of the major sources of animal protein in Nigeria like in many developing countries because of its affordability and acceptability (Bettridge *et al.*, 2014). This source is however being threatened by diseases such as salmonellosis, Newcastle disease and avian influenza (FAO, 2006a).

Salmonella is the most commonly isolated genus of food borne pathogens in the United States (Crim *et al.,* 2014). It is an important cause of food illness and a public health concern in many parts of the world (WHO, 2005; EFSA, 2009) and it is thought to be linked to the consumption of eggs and poultry products (Rabsch, 2001; De jong and Ekdahl, 2006). A reduction in the burden of disease in humans involves the elimination of infection from livestock and the maintenance of high levels of hygiene throughout the food production chain. This may be achieved through the understanding of the risk factors associated with the spread of *Salmonella* (Snow *et al.,* 2010).

Many epidemiological studies have reported the wide variety of routes (risk factors) by which *Salmonella* can be disseminated within integrated poultry farms and across geographical areas in different countries at different times (Norgrady *et al.,* 2003; Namata *et al.,* 2008). Infection with *Salmonella* can occur through inadequate cleaning and disinfection of poultry houses, presence of carriers especially rodents and insects, contaminated litter, water, dust, equipment and feed (Carrique-Mas *et al.,* 2008; Marin and Lainez, 2009). They can also be transmitted by wild birds, other animals and personnel. Infection in day-old flocks could be vertical from infected breeder flocks or horizontal during hatching, loading and transport to the farm (Chriel *et al.,* 1999). Analytical epidemiological studies have also identified housing systems, flock size and the presence of birds of different ages on a site to be important factors in determining the likelihood of *Salmonella* being present in a flock (Snow *et al.,* 2010). Studies conducted in the UK, Belgium, Finland and Italy on the prevalence and distribution of *Salmonella* in poultry production chain and poultry products indicated that the major cause of human salmonellosis arising from the consumption of poultry and eggs were *S.* Enteritidis and *S.* Typhimurium. These studies have led to the development of a European Union-wide *Salmonella* monitoring programme to reduce the risk of infection

at meat and egg consumer level (Snow et al., 2007; Fisher and Threlfall, 2005). Very little information is available on the risk factors for *Salmonella* infections in Africa. In a study conducted in Senegal (Cardinale *et al*., 2004), it was reported that 28.6% of broiler flocks were infected with *Salmonella* and the risk of infection increased when no antibiotics were used during the production cycle and when no detergents were used for decontamination.

There are quite some few published studies on the prevalence of *Salmonella* in commercial poultry in Nigeria but study conducted by Muhammad *et al*. (2009) indicated that the most prevalent serotypes in chicks in the Jos area were *S*. Kentucky and *S*. Hadar. The same study also reported widespread multiple antimicrobial resistance amongst Salmonellae isolated from day-old chicks. Another study (Muhammad *et al*., 2010) reported widespread misuse of antimicrobials amongst commercial poultry producers.

1.2 Statement of the Research Problem

Salmonellosis is a bacterial disease affecting both humans and animals worldwide and Nigeria is not an exception. Majority of the 2,659 recognized serotypes of *Salmonella* cause infections in both humans and animals worldwide (Guibourdenche *et al.,* 2010; Scallan *et al.,* 2011; Issenhuth-Jeanjean *et al.,* 2014) with mild gastroenteritis. Life-threatening disseminated infections are also common especially among high risk categories (Raufu *et al.,* 2013). Invasive non-typhoidal *Salmonella* commonly cause infection among infants, children, the elderly and immunocompromised individuals worldwide and especially in African countries, where these diseases are driven in part by co-infection with malaria or HIV (Ao *et al.,* 2015). Sources and modes of transmission of non-typhoidal *Salmonella* are still poorly understood in Africa due to

lack of coordinated national epidemiological surveillance systems (Kagambèga *et al.*, 2013). *Salmonella* food poisoning has significant economic impact due to high medical costs, loss of working time and the costs of control. In the United States, there are approximately 1.4 million cases annually of food-borne salmonellosis and according to some studies up to 47% of these cases result from the consumption of contaminated eggs (Buzby, 2002).

Most of *Salmonella* infections are self-limiting especially in humans; however, antimicrobial treatment is required, especially for invasive infections or severe diarrhoea. Fluoroquinolones (FQs) and extended-spectrum β-lactams (especially extended spectrum cephalosporins) are often the antimicrobials of choice for treating salmonellosis. Normally, FQs are not recommended in the treatment of infections in paediatric patients, because of their potential to cause arthropathy. However, FQs are still considered one of the last treatment options for life-threatening *Salmonella* infections, caused by multi-drug resistance (MDR) isolates (Cui *et al.*, 2009). Massive use of antimicrobial agents in livestock production and also in human favoured the survival of MDR pathogens and resistant *Salmonella* isolates recovered from different sources have been frequently reported worldwide (Afema *et al*., 2014; Mihaiu *et al.*, 2014).

The increasing prevalence of MDR among *Salmonella* species, not only against the first-line antimicrobials such as Trimethoprim/Sulphamethoxazole, Chloramphenicol and Ampicillin but also, against antimicrobial agents classified by the World Health Organisation (WHO) as critically important for human medicine, such as FQs and third generation cephalosporins (which have been extensively used in Human and Veterinary

Medicine), is an important emerging public health problem (WHO, 2011; Lunguya *et al*., 2013).

In food-producing animals, *Salmonella* is one of the leading causes of infection especially in poultry and this has a direct impact on the global marketing of the respective food-producing animals and animal-derived food products (Magwedere *et al.*, 2015). Poultry salmonellosis remains a major constraint to poultry production in all parts of Nigeria (Osiyemi, 1976; Onunkwo and Onoviran, 1978: Mbuko *et al.*, 2009). Farmers still experience great losses (due to mortality, morbidity and drop in egg production) caused by *Salmonella* infection despite huge amounts spent on vaccination and medication. In early life, *S.* Pullorum causes extremely high mortality of both broilers and commercial laying birds (OIE, 2012). Similarly, older birds succumb heavily to other serovars of *Salmonella* and it is assumed that *Salmonella* infections of this category of birds are mainly due to *S.* Gallinarum (Shivaprasad, 1997; FAO, 2009; Mamman *et al.*, 2014).

With increasing urbanization and the spread of the fast food industry in Nigeria, commercial poultry farmers stand the chance of entering this market and improving their economic well-being. In order to mitigate the impact of mortality associated with *Salmonella* on their farms, farmers resort to the use of antimicrobials without sound diagnostic advice thereby resulting in the development of resistant isolates (Raufu *et al.*, 2014) and presence of the residue of the drug in the tissue of such animals (Kabir *et al.*, 2004; Fagbamila *et al.*, 2010; Omeiza *et al.*, 2012). Information about the genotypic resistance of *Salmonella* to these antibiotics is scanty in Nigeria.

1.3 Justification of the Study

The epidemiology of animal and human salmonellosis in a particular locality or country is important for the effective control of *Salmonella* (OIE, 2015). Epidemiological studies are necessary to develop an understanding of the factors involved in food safety, provide scientific data for sound policy decisions and to develop control programmes (Snow *et al*., 2010). In order to set a target for the reduction of contamination of poultry and poultry products, standardized prevalence surveys are necessary to provide baseline information of the most prevalent serotypes of *Salmonella*. The improvement of poultry health, increasing the productivity of farmers and combating zoonoses can be achieved by reducing the prevalence of salmonellosis. International trade in live poultry and poultry products may take place only if flocks are certified as *Salmonella*–free (Snow *et al*., 2010). Legislation will need to be put in place for the establishment of statutory testing programmes to reduce the risk of infection in breeding flocks and in chickens. This will provide assurances of the absence of *Salmonella* of public health significance to enable farmers' access to international markets.

Relatively few African countries report surveillance data on *Salmonella* and as such, very limited information is available on *Salmonella* isolation for this continent (Barbour *et al.,* 2015). This is also the case in Nigeria, where the few studies conducted so far show different drawbacks such as the limited number of samples considered, the lack of representativeness of the samples selected, or the restricted geographical coverage. Raufu *et al.* (2013), collected samples from three poultry slaughterhouses and five intensively managed poultry farms in a circumscribed area (Maiduguri) of Nigeria and reported a *Salmonella* prevalence ranging from 2 to 16%. Moreover, a study conducted in three commercial hatcheries in Jos reported a prevalence of 9%, with *S*. Kentucky and *S*. Hadar as the most frequent serovars (Muhammad *et al.*, 2010). Similarly, a study

involving five farms in Awka reported a *Salmonella* prevalence of 38.3% in poultry droppings and *S.* Paratyphi A, *S.* Gallinarum and S. Typhimurium as the most common serovars (Orji *et al.*, 2005).

In order to fully study the *Salmonella* serotypes circulating presently within poultry in Nigeria, serotype detection and strain determination by molecular tools is necessary. Furthermore, relationships between circulating strains must be determined by molecular methods such as Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Variable Number Tandem Repeat analysis (MLVA). The risk factors associated with *Salmonella* infection are still not well understood.

1.4 Aim of the Study

The aim of the study was to provide data on the prevalence and risk factors for the occurrence of *Salmonella* in commercial layer farms as well as assessing the mechanisms of antibiotic resistance and the molecular characteristic of the commonly circulating serovars with a view of developing a *Salmonella* control programme for the pathogenic serovars.

1.5 Objectives of the Study

The objectives of the study were to

1. Isolate, identify and determine the prevalence of *Salmonella* species in commercial layer farms in six selected States in Nigeria.
2. Determine the circulating serovars of *Salmonella* species in commercial layer farms in six selected States in Nigeria.
3. Determine the risk factors associated with *Salmonella* infection in the commercial layer farms in six selected States in Nigeria.

4. Determine the antimicrobial susceptibility patterns of the *Salmonella* strains isolated.
5. Detect antimicrobial resistance target genes using monoplex (simplex) and multiplex PCR methods.
6. Detect point mutations in the target resistance genes using sequence analysis.
7. Determine the genetic diversity/relatedness of the *Salmonella* serovars isolated using PFGE and MLVA.

1.6 Research Questions

1. What is the prevalence of *Salmonella* in commercial layer farms in the six selected States in Nigeria?
2. What serovars of *Salmonella* are present in commercial poultry in the six selected States in Nigeria?
3. What are the factors responsible for the occurrence of *Salmonella* infection in commercial layer farms in the six selected States in Nigeria?
4. What is the antimicrobial susceptibility profiles of the Salmonellae isolated from commercial layer farms in the six selected States Nigeria?
5. Are the genes mediating antimicrobial resistance harboured by the isolates that are multi-drug resistant?
6. Are there nucleotide and amino acid mutations in the sequenced genes of some of the isolates?
7. Is there any genetic diversity/relatedness amongst the different poultry *Salmonella* strains from different parts of Nigeria?

CHAPTER TWO

LITERATURE REVIEW

2.1 History of *Salmonella*

A German named Gaffky in 1884 cultivated the typhoid bacillus - *S. enterica* serovar Typhi (Kauffmann, 1978) that was first observed by Eberth in 1880 from the spleen and mesenteric lymph nodes of infected patients (Le Minor, 1994). Later in 1885, two American veterinarians, Salmon and Smith isolated the bacterium causing hog cholera (*Salmonella choleraesuis*) from infected pigs (Salmon and Smith, 1886). The name *Salmonella* was subsequently adopted in honour of Dr. Salmon (Meštrović, 2015). Over the decades following the pioneering work of Salmon and Smith, many other *Salmonella* were isolated from both animals and humans. 'Serodiagnostic' work was also carried out by scientists to demonstrate that convalescent sera from typhoid fever patients caused the organism to stick together in large balls and lose their motility (Widal, 1896). Also in 1896, two isolates were recovered from patients with clinical symptoms of typhoid but showed negative Widal serodiagnosis. The organism was called 'bacille paratyphique' (Achard and Bensuade, 1896).

2.2 The Aetiology of *Salmonella*

Salmonella is a Gram-negative facultative anaerobic rod-shaped bacterium in the same proteobacterial family as *Escherichia coli*, the family *Enterobacteriaceae*, trivially known as "enteric" bacteria. Salmonellae live in the intestinal tracts of warm and cold blooded animals. Some species are ubiquitous. Other species are specifically adapted to a particular host. In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection (Todar,

2015). The genus is composed of bacteria related to each other both phenotypically and genotypically and are differentiated by their serotyping results. *Salmonella* DNA base composition is 50-52 mol% G+C, similar to that of *Escherichia, Shigella,* and *Citrobacter*. The bacteria of the genus *Salmonella* are also related to each other by DNA sequence. Like other members of the family *Enterobacteriaceae*, they produce acid on glucose fermentation; reduce nitrates to nitrite and do not produce cytochrome oxidase (Farmer, 1995). Most organisms except *S.* Gallinarum–Pullorum are motile by peritrichous flagella. The differential metabolism of sugars can be used to distinguish some *Salmonella* serovars, e.g., most do not ferment lactose. *S.* Typhi is the only organism that does not produce gas in sugar fermentation. Salmonellae are non-capsulated except *S.* Typhi, *S.* Paratyphi C and some strain of *S*. Dublin (WHO, 2003).

2.3 Taxonomy of *Salmonella*

Recent advances in taxonomy have shown that the genus *Salmonella* consists of only two major species: *Salmonella enterica* and *Salmonella bongori* (Grimont and Weill, 2007). The third species called *Salmonella subterranea*, isolated from a single unusual environmental strain (Shelobolina *et al.*, 2004) does not belong to the genus *Salmonella* based on recent unpublished data (Grimont and Weill, 2007). *Salmonella enterica* is divided into six subspecies, which are distinguishable by certain biochemical characteristics, genetic relatedness and susceptibility to lysis by bacteriophage Felix O1 (Brenner *et al*., 2000). These subspecies are: *S. enterica, S. salamae S. arizonae, S. diarizonae, S. houtenae,* and *S. indica.* The majority of the known serovars that cause disease in warm-blooded animals are found in *Salmonella enterica* subspecies *enterica* (Langridge *et al*., 2008). The serovars of other species are more likely to be found in

poikilothermic (cold blooded) animals and in the environment, but are occasionally associated with human diseases (OIE, 2010).

The expression of somatic lipopolysaccharide ‘O’ antigen and flagellar ‘H’ antigens is the basis for the Kauffmann–White scheme classification of *Salmonella* strains into different serovars (Grimont and Weill, 2007). The O groups first defined were designated by capital letters A to Z and those discovered later by the number (51-67) of the characteristics O antigen (Old, 1996). It is now considered more correct to designate each O group by its characteristic O factor, i.e. to abandon the letters A-Z used to designate early O groups. Hence, O groups become: O2 (A), O4 (B), O7 (C1), O8 (C2), and O9,12 (D1), etc. (Old, 1996). Groups O2 to 03, 10 (A-E) contain nearly all the Salmonellae that are important pathogens in man and animals (Brenner *et al*., 2000). Newly described serovars are listed in regular updates of the Kauffman and White scheme.

2.4 Nomenclature of *Salmonella*

There was a suggestion by Achtman *et al.* (2012) to replace the traditional *Salmonella* serotyping method with a more reliable method using the multi locus sequence typing technique. This is yet to gain general acceptance by the OIE and WHO collaborating Centres for salmonellosis. This suggestion was based on the controversies associated with the traditional *Salmonella* nomenclature where names were given according to clinical considerations (e.g., *Salmonella typhi, Salmonella cholerae-suis and Salmonella abortus-ovis*) rather than the genetic relatedness (Tindall *et al.*, 2005)**.** When serological analysis was adopted into the Kauffmann-White scheme in 1946, a *Salmonella* species was defined as "a group of related fermentative phage-types" with the result that each *Salmonella* serovar was considered as species. Since the host-specificity suggested by

some of these earlier names does not exist (e.g., *S. typhi-murium, S. cholerae-suis* are in fact ubiquitous), names derived from the geographical origin of the first isolated strain of the newly discovered serovars were next chosen, e.g., *S. london, S. panama, S. Stanleyville.* Subsequently it was found that all *Salmonella* serovars form a single DNA hybridization group, i.e., a single species composed of seven subspecies, and the nomenclature had to be adapted. To avoid confusion with the familiar names of serovars, the species name *Salmonella enterica* was proposed with the following names for the subspecies:

Original subgenera		Current nomenclature
• Subspecies I	=	subspecies *enterica*
• Subspecies II	=	subspecies *salamae*
• Subspecies IIIa	=	subspecies *arizonae*
• Subspecies IIIb	=	subspecies *diarizonae*
• Subspecies IV	=	subspecies *houtenae*
[• Subspecies V	=	subspecies *Bongori*]
• Subspecies VI	=	subspecies *indica*

Each subspecies contains various serovars defined by a characteristic antigenic formula. Since this formal Latin nomenclature may not be clearly understood by physicians and epidemiologists, who are the most familiar with the names given to the most common serovars, the common serovar names are kept for subspecies I strains, which represent more than 99.5% of the *Salmonella* strains isolated from humans and other warm-blooded animals (Grimont and Weill, 2007).

To avoid confusion between serotypes and species, the serotype name is not italicized and starts with a capital letter. When cited at the first time in a report, the genus name is

given followed by the word “serotype” (or the abbreviation “ser.”) and then the serotype name, e.g., *Salmonella* serotype or ser. Choleraesuis, and *Salmonella* serotype or ser. Typhi. Afterward the name may be shortened with the genus name followed directly by the serotype name, e.g., *Salmonella* Choleraesuis or *S.* Choleraesuis, and *Salmonella* Typhi or *S.* Typhi (Grimont and Weill, 2007). Because the type species name, *enterica*, has not been approved before 2005, serotype names are used directly after the genus name without the mention of the species. Following the official approval of “*enterica*” as the type species name from January 2005 (J.C.I.C.S.P, 2005), further amendment to include species name in the *Salmonella* nomenclature by the Center for Disease Control and Prevention (CDC) may be expected. For those designated by their antigenic formulae, the subspecies name is written in Roman letters (not italicized) followed by their antigenic formulae, including O (somatic) antigens, H (flagellar) antigens (phase 1), and H antigens (phase 2, if present). A colon is used in between each antigen, e.g., *Salmonella* serotype II 39:z10: z6. For serotypes in *S. bongori* (previously belongs to subgenous V), V is still used for consistency, e.g., *S.* V 13,22:z35:- (Grimont and Weill, 2007).

The terms “serotype” and “serovar” are both frequently used, but according to the rules of the *Bacteriological Code* (1990 Revision) established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes (JCICSP), the term serovar is preferred to the term serotype. Thus “serovar” is used in the Kauffmann-White scheme. *Salmonella* nomenclature is complex and still evolving. Currently, the nomenclature system used at the CDC for the genus *Salmonella* is based on recommendations from the WHO Collaborating Centre. According to the CDC system, the genus *Salmonella* contains two species, *S. enterica*, the type species, and *S. bongori*.

The antigenic classification system of various *Salmonella* serovars used today is an accumulation of the results of many years of studies on antibody interactions with surface antigens of *Salmonella* organisms established by Kauffman and White almost a century ago. All antigenic formulae of recognized *Salmonella* serotypes are listed in a document called the Kauffmann-White scheme also known as *Salmonella* antigenic formula (Popoff and Le Minor, 2001). Every year newly recognized serotypes are reported in the *Research in Microbiology Journal*. In the last published scheme, Grimont and Weill (2007) reported a total of 2,579 serovars of the genus *Salmonella.* There have been two supplements to this scheme since then (Guibourdenche *et al.*, 2010; Issenhuth-Jeanjean *et al.*, 2014), which now brings the total number of serovars to 2,659. The number of serovars in each species and subspecies are as follows:-

S. enterica	2,637
S. enterica subspp *enterica*	1,586
S. enterica subspp *salamae*	522
S. enterica subspp *diarizonae*	338
S. enterica subspp *arizonae*	102
S. enterica subspp *houtenae*	76
S. enterica subspp *indica*	13
S. bongori	22
Total	2659

(Adapted from Issenhuth-Jeanjean *et al.*, 2014)

2.5 Antigenic Diversity

Classically, there are three antigens recognized for *Salmonella*: somatic (O), flagellar (H) and (mostly for serovar Typhi) surface or capsular (Vi) antigens (Popoff *et al.*, 2004). In addition, other bacterial antigens are also available. These include the mucus (M), and the fimbrial (F) antigens (Huckstep, 1962; Old, 1996). Analysis of O and H antigens, initiated by White (1926) and extended by Kauffmann (1941), resulted in the description of a great number of serovars.

2.5.1 O (somatic) antigens

Somatic antigens represent the side-chains of repeating sugar units projecting outwards from the lipopolysaccharide layer on the surface of the bacterial cell wall. Typing the O antigen denotes the serogroup. Sixty-seven (67) different O antigens have been recognized and they are designated by Arabic numerals. The O antigens are heat stable, being unaffected by heating for 2.5 hours at 100^{O}C, and alcohol stable withstanding treatment in 96% ethanol at 37^{O}C (Old, 1996; Lewis, 1998).

2.5.2 H (flagellar) antigens

The H antigens are carried by flagella. These *Salmonella* flagellar filaments are polymers of a highly antigenic protein, termed flagellin. The flagellar antigens are heat-labile proteins that endow the organism with motility (Smith and Selander, 1991).

Salmonella is unique among the *Enterobacteriaceae* in that it commonly has two distinct H antigens, the phase 1 (protein product of the *fli*C gene) and phase 2 (protein product of the *fljB* gene) flagellar antigens, that are coordinately regulated such that only one flagellar antigen is expressed at a time in a single cell (Smith and Selander, 1991). Some H antigens are composed of multiple antigens, termed factors; for example, H:

e,n,x is the designation for a flagellar antigen that consists of three separate factors, e, n, and x, that occur together in one flagellum. These factors represent different epitopes on the flagellar protein (Smith and Selander, 1991).

The 114 H antigens are composed of combinations of 99 distinct antigenic factors. Flagellar antigens that are immunologically related are known as complexes. For example, the G complex includes all flagellar antigen types that contain antigenic factor g (e.g., g,m; f,g; g,z51), plus flagellar antigen m,t. Flagellar antigen types that include antigen H:z4 are considered the Z4 complex (Smith and Selander, 1991). The flagellar antigens of phase I are labeled with lower case letters (a to z and z1 z2 z3, etc.) and phase 2 with a mixture of lower case letters and Arabic numerals.

The antigenic structure of any *Salmonella* is expressed as an antigenic formula which has three parts, describing the O antigens, the phase I and II flagellar antigens respectively (Lewis, 1998). Phase II used to be termed "the group" or non-specific antigens because numerous serotypes of *Salmonella*e share the same antigens when it is this phase. The presumptive identification of the serovars, therefore mainly depends on the identification of the H antigens in phase I, which are relatively 'specific' (Old, 1996). As an example, the antigenic formula for the serovar Typhimurium is 1, 4, 5, 12 : i : 1, 2 where 1, 4, 5 and 12 are O antigens, i is phase 1 H antigen and 1 and 2 are phase 2 H antigens (Kauffmann, 1950).

2.5.3 Vi (capsular or surface or virulence) antigens

Surface antigens in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with O antisera. Surface antigens, commonly observed in other genera of enteric bacteria (e.g., *Escherichia coli* and *Klebsiella),* may be found in some

Salmonella serovar. Vi antigens are linear homopolymers of 2-acetamido-2-deoxy-D-galacturonic acid linked by α (1-4) bonds. Only serovars Typhi, Paratyphi C and Dublin possesses this capsular polysaccharide which covers the cell wall (WHO, 2003). Strains of these three serovars may or may not have the Vi antigen. Loci *via*A, *via*B and *omp*B are involved in the genetic control of Vi production. Loci *via*A and *omp*B are not limited to Vi producing species and genera and are involved in the regulation of Vi synthesis. Locus *via*B is found only in strains able to produce Vi antigen. It contains genes coding for proteins involved in polysaccharide synthesis (*wcd*ABCD), polysaccharide exportation (*wza, wzm, wzt, wzf*) and anchoring to the bacterial surface (*wcd*E). The Vi antigen is the receptor of phages ViI, ViII and ViIV (Craigie and Yen, 1938a; 1938b).

2.6 Genome of *Salmonella*

Because of the presence of large number of serovars in *Salmonella*, the genome sequence projects concentrated on serovars that are either of importance to human disease or a representative of a particular branch of the *Salmonella* (Mastroeni, 2006). Genome information can be used to gain insights into the evolution of the *Salmonella* genus, to identify stable regions conserved between different *Salmonella* species and serovars, and to identify regions that appear to be specific for individual serovars (Mastroeni, 2006). A DNA sequence comparison between the genome of *S.* Typhimurium LT2 and *S.* Typhi CT8 show a median homology of 98% (McClelland *et al*., 2001). Comparison of the genes required for DNA replication, transcription, translation and central metabolism (‘housekeeping’ genes) of the *Salmonella* serovars indicates that they are extremely similar at the DNA level. Pair wise comparisons between any of the sequenced *Salmonella* genomes indicate that the similarity between

housekeeping genes ranges from 97.6% to 99.5% at the DNA level (Edwards *et al*., 2002).

Despite their overall similarity, pair wise comparisons of the *Salmonella* genomes reveal that each serovar has many insertions ('islands') and deletions ('atolls') relative to the other serovars. Approximately 500–600 kb of chromosomal DNA seems to be unique to each serovar, representing 10% to 12% of their 5 Mb genomes. The unique regions are distributed to many sites on the chromosome and range in size from <1 to >50 kb. These unique regions of the genome are likely to encode gene products responsible for the different abilities of the serovars to infect a variety of hosts, to cause a range of symptoms and to survive a diversity of immune responses (Mastroeni, 2006). The complete genome sequence was determined for a multidrug-resistant strain of *S. enterica* serotype Typhi (CT18). The CT18 genome harbours 4,809,037 base pairs with an estimated 4599 coding sequences. Significant homology has been seen among genomes of *S*. Typhi-CT18, *S*. Typhimurium LT2 (McClelland *et al*., 2001) and *E. coli* K12 (Blattner *et al*., 1997) genomes, indicating a common evolutionary origin. *S*. Typhimurium is also a member of sub-species I and is the leading cause of gastroenteritis in humans and unlike *S*. Typhi can infect mice and cause a typhoid-like disease. The genome size of *S*. Typhimurium LT2 is 4,857 Kbp (McClelland *et al*., 2001) and sequence comparison between these two organisms revealed 89% of *S*. Typhimurium LT2 CDSs were homologous to *S*. Typhi CT18 at the nucleotide level (McClelland *et al*., 2001). Although genetically similar to the *E. coli* K12 genome, both *S*. Typhi and *S*. Typhimurium have acquired large regions of extraneous DNA by horizontal transfer known as pathogenicity islands, which offer a selective pathogenic advantage to the organisms (Marcus *et al*., 2000).

Salmonella enterica strains contain plasmids which vary in size from 2 to more than 200 kb. There are different types of plasmids in *Salmonella* and the best described groups of plasmids are the virulence plasmids (50–100 kb in size) present in serovars Enteritidis, Typhimurium, Dublin, Cholerae-suis, Gallinarum, Pullorum and Abortus-ovis. The virulence plasmids have a common 8kb DNA region encoding the *spv (Salmonella* plasmid virulence) gene involved in intracellular macrophage survival of *Salmonella* (Gulig *et al*., 1993). Depending on serovar these plasmids code for additional virulence associated genes such as *rck* (resistance to complement killing), *pef* (plasmid encoded fimbriae), *srgA* (SdiA-regulated gene, putative disulphide bond oxidoreductase) or *mig-5* (macrophage-inducible) gene coding for putative carbonic anhydrase) (Rychlik *et al*., 2006).

Another group of high molecular weight plasmids are responsible for antibiotic resistance. The antibiotic resistance genes are often located within transposons which can transpose from plasmids to chromosome, and vice versa. Resistance genes can also be found in a form of gene cassettes incorporated into integrons (Hall and Collis, 1995). A gene cassette consists of one complete open reading frame followed by a recombination site. Gene cassettes exist free as covalently closed circular molecules generated by the integrase and the circular intermediate participates in the integrase-mediated process of insertion. As many as seven different gene cassettes have been described within a single integron (Carattoli, 2003). Small ColE1-type plasmids (pC) of 3-5.6kb have been found in *S. enterica serovar* Enteritidis and one of these plasmids carries an active restriction modification system, which could explain the high resistance of pC-carrying *S. enterica* serovar Enteritidis strains to phage infections (Gregorova *et al*., 2002). *Salmonella* genomes also contain a variable set of transposable elements and many phage genome sequences (Edwards *et al*., 2002).

2.7 Salmonellosis

2.7.1 Pathogenesis and immunology

Salmonellosis in both human and animal host is generally associated with *Salmonella enterica* subspecies *enterica* (also termed subspecies I) and acute infections can present in one of four ways: enteric fever, gastro-enteritis, bacteraemia, and extraintestinal (EI) focal infection. As with other infectious diseases the course and outcome of the infection are dependent upon a variety of factors including inoculating dose, immune status of the host and genetic background of both host and infecting organism (Cammie and Miller, 2000).

Broadly speaking the *Salmonella enterica* infections can be subdivided in to two groups: the enteric fever (typhoidal) group and non-typhoidal *Salmonella* (NTS), which typically cause gastro-enteritis but also can cause invasive disease under certain conditions. There are five serotypes of *Salmonella* associated with enteric fever: *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi), *S.* Paratyphi A, *S.* Paratyphi B, *S.* Paratyphi C and *S.* Sendai. *S.* Typhi forms a genetically homogenous group as do *S.* Paratyphi A and Sendai together, whereas *S.* Paratyphi B and C are heterogeneous (Selander *et al*., 1990).

All *Salmonella* infections begin with the ingestion of organisms in contaminated food or water. After leaving the stomach, *Salmonella* must traverse the mucosal layer overlaying the epithelium of the small intestine. After crossing the mucosal layer overlaying the intestinal epithelium, *Salmonella* interacts with both enterocytes and microfolds cells (M cells) (Francis *et al*., 1992). The organisms are rapidly internalized and transported into submucosal lymphoid tissue where they may enter into systemic circulation. *Salmonella* also have the ability to induce non phagocytic epithelial cells by a process known as

bacterial mediated endocytosis. This process involves the formation of large membrane ruffles around the organism and cytoskeleton rearrangement (Francis *et al*., 1992). *Salmonella* is then internalized within bound vacuoles through which organisms' trancytose from the apical to the basolateral surface (Rathman *et al*., 1997). Once it crosses the intestinal epithelium, *Salmonella* serotypes that cause systemic infections enter macrophages, and migration of infected macrophages to other organs of reticuloendothelial systems probably facilitates the dissemination of bacteria in the host (Rathman *et al*., 1997).

Salmonella Pathogenicity Island 1 (SPI1) function is required for the initial stages of salmonellosis, i.e. the entry of *Salmonella* into nonphagocytic cells by triggering invasion and the penetration of the gastrointestinal epithelium. Furthermore, SPI1 function is required for the onset of diarrhoeal symptoms during localized gastrointestinal infections. The function of SPI2 is required for later stages of the infection, i.e. systemic spread and the colonization of host organs. The role of SPI2 for survival and replication in host phagocytes appears to be essential for this phase of pathogenesis (Hansen-Wester and Hensel, 2001).

Gastroenteritis due to NTS may persist with fever, nausea, vomiting, abdominal pain and symptoms may continue for over a week. In contrast, the early symptoms of enteric fever are often vague, and may include a dry cough, severe headache, anorexia, fever and a tendency to constipation rather than diarrhoea (Parry *et al*., 2002). If enteric fever is not treated on time, serious complications like haemorrhage from ulcers can occur during the third week of illness or perforation of the Peyer's patches (PP) can cause generalized peritonitis and septicemias; these are the commonest cause of death in

typhoid fever. With the introduction of early and appropriate antibiotic therapy, the average case fatality rates for typhoid are less than 1% (Everest *et al*., 2001).

2.7.2 Virulence factors

The outcome of a *Salmonella* infection is determined by the status of the host and status of the bacterium. The status of the bacterium is determined by the so called virulence factors (van Asten and van Dijk, 2005).

2.7.2.1 Salmonella pathogenicity islands (SPIs)

The majority of virulence genes of *Salmonella* are clustered in regions distributed over the chromosome called *Salmonella* pathogenicity islands (McClelland *et al*., 2001). The SPIs are of major importance for the virulence of *S. enterica*. Hallmarks of *Salmonella* virulence, such as cell invasion, intracellular survival and the production of Vi antigen capsules are encoded by SPIs. Until recently more than 10 SPIs have been identified on the *Salmonella* chromosome, but SPI1 and SPI2 are the central ones for pathogenesis of *Salmonella* infections (Hansen-Wester and Hensel, 2001). All types of *S. enterica* have two large clusters of genes known as *Salmonella* Pathogenicity Island 1 and 2. *Salmonella* Pathogenicity Island 1 encodes genes necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory response (Galyov *et al*., 1997). *Salmonella* lacking a functional SPI1 Type III secretion system are unable to invade epithelial cells and induce cytokine synthesis (Hobbie *et al*., 1997). During invasion of the gut, the SPI1 encoded SipB protein triggers the activation of intracellular Caspase-1 within resident macrophages that induces apoptosis in the infected macrophages resulting in escape of *Salmonella* from these cells (Hersh *et al*., 1999). SPI1 also encodes an effector protein SopB which is an inositol phosphate phosphatase

and its enzymatic activity results in activation of chloride channel in the membrane of epithelial target cells leading to the secretion of chloride and loss of fluid into the intestinal lumen (Norris *et al*., 1998).

Salmonella Pathogenicity Island 2 encodes genes essential for intracellular replication and necessary for establishment of systemic infection beyond the intestinal epithelium (Hensel, 2006). The function of the SPI2 encoded Type III secretion system is required to protect the pathogens within the *Salmonella* containing vacuole (SCV) against the effector functions of innate immunity. It has been reported that SPI2 prevents colocalization of the phagocyte oxidase (Vazquez-Torres *et al*., 2000) and the inducible nitric oxide synthtases to the SCV (Chakravortty *et al*., 2002). As a consequence, intracellular *Salmonella* are protected against damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Boonmar *et al*., 1998) and against a potent antimicrobial activity of peroxynitrite, which is generated by reaction of RNS and ROS (Chakravortty *et al*., 2002). SPI2 genes enabled *S. enterica* lineages to establish a new niche as an intracellular pathogen in the intestinal mucosal and systemic tissue (Chakravortty *et al*., 2002).

2.7.2.2 . Type III secretion systems

Central to the pathogenesis of *S. enterica* is the function of specialized protein secretion systems, known as Type III secretion system (TTSS). The TTSS are specialized virulence devices that have evolved indirect translocation of bacterial virulence proteins into the host cell cytoplasm. Type III secretion systems are composed of several proteins that form a remarkable needle-like organelle in the bacterial envelope (Galan, 1998). So far the presence of two SPIs (SPI1 and SPI2) each encoding a TTSS, have been described for *Salmonella* species and may reflect the flexibility of this highly successful

pathogen in causing different forms of diseases. SPI1 is not present in *E. coli* isolates, which suggests that the acquisition of SPI1 by *Salmonella* was a fundamental step in the divergence of these two genera (Fierer and Guiney, 2001).

SPI1 and SPI2 both encode TTSSs and each TTSS has a distinct set of secreted substrate proteins. Furthermore, both SPIs encode specific regulators (R, DNA-binding regulator; S, sensor) that control gene expression. The function of each system is required for different forms of interaction with host cells at different stages of pathogenesis of salmonellosis (Hansen-Wester and Hensel, 2001).

2.7.2.3 *Regulatory proteins, toxins, plasmids and Vi antigens*

Regulatory proteins that control the synthesis of multiple proteins at the level of gene transcription are also essential to *Salmonella* pathogenesis. The best studied example is PhoP/PhoQ, which regulates genes important for survival within macrophages, resistance to cationic antimicrobial proteins and acid pH, and invasion of epithelial cells (Behlau and Miller, 1993). The PhoP/PhoQ regulated genes encode an acid phosphatase, cation transporters, outer membrane proteins, and genes important for the modification of lipopolysaccharide. These modifications promote resistance to antimicrobial cationic peptides and alter the ability of lipid A to stimulate tumour necrosis factor-α secretion by macrophage (Guo *et al*., 1997).

Other regulatory genes implicated in pathogenesis include *ompR/envZ*, the regulator of porin gene transcription; *katF*, an alternative bacterial sigma-factor that regulates catalase production; and *ssrAB*, which regulates genes in SPI2 that are important for systemic pathogenesis (Lindberg, 1980). Non-typhoidal *Salmonella* also carry a variety of virulence plasmids which might play a role in multiplication inside the cell,

destabilizing the cytoskeleton of the eukaryotic cell and also might be involved in resistance of *Salmonella* species to the bacteriolytic activity of serum. Enterotoxin may also play a role in *Salmonella* gastroenteritis. An enterotoxin antigenically similar to Cholera toxin also has been identified (Aguero *et al*., 1991). Flagella phase variation that is exploited by the majority of flagellated *Salmonella* might be related to escaping the host defense system (van Asten and van Dijk, 2005). The Vi antigen of *S.* Typhi prevents antibody mediated opsonization, increases resistance to peroxide and confers resistance to complement activation by the alternative pathway and to complement mediated lysis (Looney and Steigbigel, 1986).

2.7.3 Epidemiology of salmonellosis

2.7.3.1 Typhoidal salmonellosis

Typhoidal *Salmonella* are host-specific *Salmonella* affecting only humans but occasionally may be seen in other animals. Typhoid fever is a global problem and its real impact is difficult to estimate because the clinical picture is similar with those of many other febrile infections. The WHO estimated that globally there are more than 22 million cases of typhoid fever each year with more than 200,000 deaths (WHO, 2003).

S. Typhi have high host specificity for humans and most often, acquisition of the organisms occurs by ingestion of food or water contaminated with human excreta and associated with poor sanitation and hygiene (Miller *et al*., 2000). Laboratory accidents have also resulted in typhoid fever transmission to laboratory workers (Blaser *et al*., 1980). Typhoidal salmonellosis is endemic in many developing countries, particularly in Asia, Africa, Latin America and Caribbean regions (Crump *et al*., 2004). These countries share several characteristics including rapid population growth, increased urbanization, inadequate human waste treatment, limited water supply, and over-

burdened health care systems. In developed countries, typhoid fever is predominantly related to traveling to the previously mentioned regions (Le Hello *et al.*, 2011).

2.7.3.2 Host adapted salmonellosis (S. Gallinarium and *S.* Pullorum*)*

Fowl typhoid and pullorum disease (bacillary white diarrhoea), caused by *Salmonella enterica* subspecies *enterica* serovar Gallinarum biovars Gallinarum and Pullorum, respectively are widely distributed throughout the world especially in countries with less developed poultry industries (Shivaprasad, 2000; OIE, 2012). In the United States and the United Kingdom (UK) the serovar is referred to as Pullorum (Hitchner, 2004), even though the strains are now considered to be the same serovar that is derived from *S.* Enteritidis by gene deletion events (Thomson *et al.*, 2008).

Salmonella Pullorum and *S.* Gallinarum belong to the Kauffmann–White scheme serogroup D, along with *S.* Enteritidis, which is closely related (Grimont and Weill, 2007). The organisms are Gram negative non-sporogenic rods 1.0–2.5 μm in length and 0.3–1.5 μm in width. They are considered to be non-motile under normal conditions but inducement of flagellar proteins and motility has been shown in some strains of *S.* Pullorum when grown in special media (Holt and Chaubal, 1997). *Salmonella* Gallinarum and *S.* Pullorum are host adapted to avian species (Eswarappa *et al.*, 2009) and are considered to pose a minimal zoonotic risk (Shivaprasad, 2000), although the genome is continually evolving, which could theoretically widen the host range in future (Liu *et al*., 2002).

Salmonella Gallinarum causes fowl typhoid in chickens which results in huge economic losses as infected chickens have a high mortality rate, succumbing to septicaemia, enteritis, and haemolytic anaemia (Wigley *et al*., 2005). Fowl typhoid can occur in

young as well as older birds (OIE, 2012). In its acute form, pullorum disease is almost exclusively a septicaemic disease of young chickens of 2-3 weeks of age (Wigley *et al*., 2005). However, the organism may also be associated with disease in turkey poults and may be carried subclinically or lead to reduced egg production and hatchability plus a range of atypical signs in older birds (Wigley *et al.,* 2005). Red mites may be involved in the transmission of the disease and persistence in poultry houses (OIE, 2012). Ovarian transmission is a major route by which the organism can spread (Shivaprasad, 2000: Barrow, 2007).

In most countries of Europe and in North America and Australia, fowl typhoid has almost disappeared as a result of improved surveillance and slaughter practices (Christensen *et al.,* 1994; Baumler *et al.,* 2000). However, fowl typhoid has recently been detected on two commercial poultry premises in Northern Ireland after almost 30 years without cases, and was also reported in Great Britain during 2005/2006 following a gap of 20 years (AHVLA, 2012). Fowl typhoid and pullorum disease are serious threats to the poultry industry in Africa, Asia, and tropical Latin America (Baumler *et al.,* 2000). Of all the bacterial diseases that plaque the poultry industry in Nigeria, fowl typhoid and pullorum disease constitute the most important ones (Osiyemi, 1976; Onunkwo and Onoviran, 1978; Mbuko *et al.,* 2009).

2.7.3.3 Non-typhoidal Salmonella (paratypoid Salmonella)

Non-typhoidal *Salmonella* (NTS) species are important food borne pathogens with acute gastroenteritis being the most common clinical manifestation (Kariuki *et al*., 2006). However, invasion beyond the gastrointestinal tract occurs in approximately 5% of the patients with NTS gastroenteritis resulting in bacteraemia (Hohmann, 2001). Certain serovars of *Salmonella* show a much higher predilection for causing bacteraemia and

these serovars differ in different countries (Gordon, *et al.,* 2008; Kiratisin, 2008). Non-typhoid salmonellosis is acquired from multiple animal reservoirs. The main mode of transmission is from food products contaminated with animal products or wastes, most commonly eggs and poultry products (Miller *et al*., 2000).

In USA according to CDC estimates in 2001, there were 2 million cases annually with 500 to 2,000 deaths per year and the majority of the reported cases were caused by *S.* Typhimurium or *S.* Enteritidis (Cammie and Miller, 2000). In developing countries NTS is also an important cause of invasive disease, particularly in tropical regions of Africa, where *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) are consistently the most common causes of childhood bacteraemia, as well as important causes of meningitis, septic arthritis and pneumonia (Kariuki *et al*., 2006). According to the 2002 WHO Global *Salmonella* Surveillance report on the distribution of *Salmonella* serotypes from 2000 to 2002, during the 3-year period, *Salmonella enterica* serovar Enteritidis was by far the most common serotype reported from human isolates globally (Galanis *et al*., 2006). In 2002, it accounted for 65% of all isolates, followed by *S.* Typhimurium at 12% and *S.* Newport at 4%. Among nonhuman isolates, *S.* Typhimurium was the most commonly reported serotype in all 3 years, accounting for 17% of isolates in 2002. It was followed by *S.* Heidelberg (11%) and *S.* Enteritidis (9%). In Africa in 2002, *S.* Enteritidis and *S.* Typhimurium were each reported from approximately one fourth of isolates from humans (Galanis *et al*., 2006).

Very little is known regarding the source and transmission of NTS in developing countries. It is likely that animal-human transmission via the food chain is less responsible than is human-human transmission, with contaminated water an important

vehicle in communities with poor hygiene, sanitation and overcrowding (Graham, 2002; Kariuki *et al*., 2006). A major and worrying development that has global significance is the increasing and often rapid emergence of multidrug resistant (MDR) strains for all the above serovars (Hohmann, 2001; Graham, 2002). Studies from tropical Africa have consistently shown that NTS bacteraemia is more common during the rainy season. This may be the result of a seasonal increase of intestinal salmonellosis which has been reported from other (non-malarious) tropical regions. It may also reflect the seasonal pattern of diseases such as malnutrition and malaria, which may confer an increased risk of *Salmonella* infection (Graham, 2002).

2.7.3.4 Risk factors for the occurrence of Salmonella

Although several descriptive studies had already identified potential vectors and sources of *Salmonella* contamination in broiler and egg productions (Davies and Wray, 1995; Chadfield *et al.*, 2001; Shirota *et al.*, 2001), few quantitative epidemiological studies had been carried out in laying hen farms. The risk factors reported in these studies were: (i) a large flock size (Mollenhorst *et al.*, 2005; Namata *et al.*, 2006); (ii) an old or molted laying hen flock (Castellan *et al.*, 2004; Garber *et al*., 2003) or the presence of hens of different ages on the farm (Mollenhorst *et al.*, 2005); (iii) the housing system and the related poultry house characteristics. According to Mollenhorst *et al.* (2005), cage systems with wet manure reduced the risk of infection in contrast to cage systems with dry manure or on-floor systems. Garber *et al.* (2003) found that rearing pullets on the floor instead of in cages increased the risk of infection, whereas Namata *et al.* (2008) reported that the on-floor housing of laying hens appeared to have a protective effect; (iv) the absence of cleaning and disinfection of the poultry-house between flocks

(Garber *et al.,* 2003); and (v) an unusually high mortality rate during the laying period reported by the farmer (Castellan *et al.,* 2004).

The increased risk of contamination associated with trucks passing in close vicinity to the poultry-house has been reported in a study of risk factors for *Salmonella* contamination of broilers (Rose *et al.,* 1999; Huneau-Salau¨n *et al.,* 2009). It is thus possible that laying hen flocks might be contaminated by the mechanical carriage of *Salmonella* on the wheels of vehicles or on human footwear from areas of access to inside the premises despite the biosafety measures implemented at poultry-house entrances (Davies *et al.,* 1997). *Salmonella* contamination could be prevented by improving farm hygiene management on farms, while also considering housing system specificities (Huneau-Salau¨n *et al.,* 2009).

2.8 Isolation and Identification of *Salmonella* Isolates

In developing countries, salmonellosis is frequently diagnosed solely on clinical grounds. However, isolation of the causative organism is necessary for a definitive diagnosis, for performing antimicrobial susceptibility testing and for further characterization (OIE, 2010)

2.8.1 Culture

Various enrichment and selective media are used to isolate Salmonellae from different clinical specimens. Non-typhoidal *Salmonella* gastroenteritis is commonly diagnosed from stool culture. In cases where there is concern about bacteraemia, blood culture is indicated (WHO, 2003). *S.* Typhi and other typhoidal *Salmonella* are frequently isolated from blood during the first weeks of illness and usually positive stool cultures occur during the second and third weeks of disease (Cheesbrough, 2000). *S.* Typhi can also be

isolated from bone marrow, rose spots and, infrequently, from urine cultures (Khan *et al*., 1998). Occasionally *Salmonella* may be cultured from other samples such as joint aspirates, cerebrospinal fluid or endocarditic heart valves. Specialist environmental laboratories may look for *Salmonella* in food or water samples, either as routine or in outbreak situations. The best recovery of *Salmonella* species from faecal samples can be achieved by the use of direct plating and inoculating in standard enrichment broths. Many selective agar plates are available for *Salmonella*. Most laboratories use one medium with low selectivity, such as MacConkey, Deoxycholate agar (DCA) or Cystine Lactose Electrolyte-deficient (CLED) agar, and one with higher selectivity, such as Xylose Lysine Deoxycholate agar (XLD) (Cheesbrough, 2000). *Salmonella* enrichment broths (e.g. selenite broth and tetrathionate broth) may help to recover low numbers of organisms (OIE, 2010).

Culturing of faecal and environmental samples involves pre-enriching in buffered peptone water in a 1:10 sample to broth ratio at 37^0C for 24 h. A 1.0 ml of the pre-enriched broth is used to inoculate 10 ml of Mueller-Kaufmann tetrathionate broth (MKTTn) while 0.1 ml for Rappaport-Vassiliadis (RV) both simultaneously for enrichment. Also, 100 µl (3 drops) of the pre-enrichment broths is inoculated onto Medium Semi solid Rappaport-Vassiliadis (MRSV) agar, and incubated at 42^0C and 41.5^0C respectively for 24 h. Cultures from MKTTn, RV, and MRSV are then plated simultaneously onto Brilliance *Salmonella* Agar (BSA) or Bismuth Sulphite Agar (BS) and Xylose Lysine Desoxycholate (XLD) medium. The plates are then incubated at 37^0C for 24 h (ISO, 2002).

2.8.1.1 Pre-enrichment media

The number of Salmonellae in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low and it is usually necessary to use pre-enrichment media, such as buffered peptone water or universal pre-enrichment broth, to assist isolation. This may allow the small numbers of Salmonellae, which may otherwise be killed by the toxic effect of selective enrichment media, to multiply, or it may help to resuscitate Salmonellae that have been sub-lethally damaged, e.g. by freezing, heating, and exposure to biocides, organic acids, bacteriocins, phage or desiccation. Pre-enrichment may not be the best method for isolating less vigorous *Salmonella* strains, such as the host-adapted strains, from faeces because of overgrowth by competing organisms during non-selective pre-enrichment (ISO, 2002).

2.8.1.2 Enrichment media

Enrichment media are liquid or semi-solid agar media that contain additives that selectively permit Salmonellae to grow while inhibiting the growth of other bacteria. Some, however, are also relatively toxic to certain serovars of *Salmonella*, e.g. selenite inhibits *S.* Choleraesuis, and brilliant green is toxic to many strains of *S.* Dublin. Elevated temperatures have also been used to increase the selectivity of enrichment medium, and a temperature of 43°C is used in some laboratories, although this may be inhibitory with some media, e.g. tetrathionate and Rappaport–Vassiliadis at 43°C inhibit temperature-sensitive strains, especially *S.* Dublin and 41.5°C is now recommended for incubation of Rappaport–Vassiliadis broth-based media (ISO, 2002; OIE, 2010).

Selective motility enrichment may also be used to increase the sensitivity of *Salmonella* isolation and semisolid enrichment media, e.g. MSRV or diagnostic semi-solid *Salmonella* medium (DIASALM), may provide greater sensitivity (Voogt *et al*., 2001).

The formulation of the medium, which may vary between suppliers, or even between batches in some cases, temperature and duration of incubation and the volume of the samples used to inoculate the medium, may all serve to influence the isolation rate, and these variables should always be taken into account. Examples of selective enrichment media are sodium tetrathionate, as in Müller–Kaufman broth, selenite F, selenite cysteine, brilliant green broth and Rappaport–Vassiliadis broths, or semi-solid Rappaport–Vassiliadis medium. In some cases it may be advantageous to use more than one selective broth or to culture by both pre-enrichment and direct selective enrichment/direct plating, although often the benefit does not justify the extra cost (ISO, 2002; OIE, 2010). Additions such as Ferrioxamine E may be added to selective media to enhance isolation of *Salmonella* from iron or nutrient-limited samples such as eggs, water or soil (Reissbrodt, 1995) or antibiotics such as novobiocin may be added to suppress most Gram-positive organisms or other Gram-negative bacteria such as *Proteus*. Specific antibiotics can be added to enhance the isolation of antimicrobial resistant *Salmonella* strains (OIE, 2010).

2.8.1.3 Selective plating media

Selective media are solid, selective agars that permit differential growth to varying degrees. They inhibit growth of bacteria other than *Salmonella* and give information on some of the principal differential biochemical characteristics – usually non-lactose fermentation and hydrogen sulphide (H_2S) production. The results are read after 24 and 48 h of culture at 37°C. Salmonellae form characteristic colonies on such media that are usually distinguishable from the colonies of other bacteria on the plate, with the possible exceptions of *Proteus, Pseudomonas, Citrobacter* and *Hafnia*. Lactose-fermenting Salmonellae may occasionally be isolated and H_2S production may be variable. Such

atypical strains may be more effectively detected when semi-solid selective media are used. The DIASALM medium is particularly useful in this respect as presumptive confirmation by slide agglutination testing using polyvalent O, H or specific antisera can be carried out on liquid from the growth zone in the plate and plates such as desoxycholate-citrate agar (DCA) or bismuth sulphite agar (BS) can be used, but these are subject to a higher frequency of false-positive colonies (OIE, 2010).

Salmonella Abortusovis is a slow-growing serovar and it is usual to incubate plates for up to 72 h and to use the non-selective blood agar. Examples of selective media are brilliant green agar, xylose lysine desoxycholate agar, deoxycholate/citrate agar, and bismuth sulphite agar, though many more will be found in the literature and media catalogues. A wide range of chromogenic agars such as Rambach agar, *Salmonella* detection and identification medium (SMID), Aes Labooratoire *Salmonella* agar plate (ASAP), are now available. Many of these may aid differentiation of suspect colonies, but must be validated for the sample matrices, culture systems and serovar range used as sensitivity can be poor in some circumstances (OIE, 2010).

2.8.2 Biochemical tests

Salmonella species are motile (with a few exceptions), facultative anaerobic, produce acid from glucose usually with the production of gas, and are oxidase negative. Most produce hydrogen sulphide except *Salmonella* Paratyphi A and *Salmonella* Typhi, which is a weak producer. Most *Salmonella* species do not ferment lactose. However, approximately 1% of the organism is able to ferment this sugar and thus may not be detected by clinical laboratories that use MacConkey agar and most laboratories use XLD agar or similar selection media to detect lactose fermenter *Salmonella* isolates (Miller *et al*., 2000). Urease production and indole production are negative and most

NTS produce citrate and H_2S (Cheessbrough, 2000). Typical *Salmonella* colonies is confirmed by biochemical assays including glucose fermentation, lactose oxidation, gas and H_2S production and the lack of β-galactosidase (ONPG) and lysine decaboxylation. Biochemically confirmed isolates are then subjected to serotyping (Cheessbrough, 2000).

Many rapid confirmation and identification methods have been developed for *Salmonella* and a large number have been developed into commercial products. Standard culture methods are excessively time-consuming and thus many attempts have been made to maximize their efficiency by introducing new technologies, making reliability of detection more convenient, user friendly, as well as by reducing the costs of materials and labour (Weenk, 1992; de Boer and Beumer 1999). Biochemical confirmatory tests are being replaced by commercial identification kits such as the API 20E (BioMérieux) or other commercially available bacterial identification kits (de Boer and Beumer, 1999).

2.8.3 Serology

Rapid immunological identification and confirmation tests based on latex agglutination, enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) have been developed for *Salmonella*, and simple-to-use lateral flow test strips using immunochromatographic technology have also been developed into commercial products by a number of manufacturers (Koivunen and Krogsrud, 2006).

2.9 Mechanisms of Drug Resistance by *Salmonella* Species

Efforts aimed at identifying new antibiotics were once a top research and development priority among pharmaceutical companies. The potent broad spectrum drugs that emerged from these endeavors provided extraordinary clinical efficacy. Success, however, has been compromised. Researchers are now faced with a long list of microbes that have found ways to circumvent different structural classes of drugs and are no longer susceptible to most, if not all, therapeutic regimens (Alekshun and Levy, 2007). Resistance to various classes of antimicrobial agents has been encountered in many bacteria of medical and veterinary relevance. Over the years, various studies have reported the presence of genes and mutations conferring resistance to antimicrobial agents in zoonotic bacteria such as *Salmonella* (Michael *et al*., 2006).

There are three major mechanisms by which all bacteria (including *Salmonella*) have become resistant to antimicrobial agents: enzymatic inactivation; reduced intracellular accumulation of the antimicrobials; protection, alteration or replacement of the cellular target sites (Schwarz and Chaslus-Dancla, 2001).

2.9.1 Chloramphenicol

Chloramphenicol (CHL) is a broad-spectrum bacteriostatic agent and the antibacterial action of chloramphenicol is mediated by inhibiting protein synthesis after binding to the 50S subunit of the bacterial ribosome and thus preventing the transfer of the new amino acid from its tRNA to the growing peptide chain. This binding is achieved by molecular mimicry of the peptidyl adenyl terminus of the tRNA molecule (Schwarz *et al*., 2004). The first and still most frequently encountered mechanism of bacterial resistance to CHL is enzymatic inactivation by acetylation of the drug via different types

of chloramphenicol acetyltransferases (CATs) (Murray and Shaw, 1997). However, there are also reports on other mechanisms of CHL resistance, such as efflux systems, inactivation by phosphotransferases, mutations of the target site and permeability barriers (Murray and Shaw, 1997).

There are two defined types of CATs which distinctly differ in their structure: CAT-A and CAT-B enzymes. In *Salmonella,* enzymatic inactivation by type A or type B chloramphenicol acetyltransferases (CATs) as well as the export of chloramphenicol or chloramphenicol/florfenicol by specific efflux proteins is the dominant resistance mechanisms (Michael *et al*., 2006). Two different types of CAT-A proteins, encoded by the genes *catA1* and *catA2*, have so far been detected in *Salmonella* isolates. While the Tn9- borne resistance gene *catA1* has been found in several serovars, including Typhimurium (Chen *et al*., 2004), the gene *catA2* was detected on a multiresistance plasmid from *S*. Enteritidis, and *S*. Typhimurium (Randall *et al*., 2004).

Three different types of *catB* genes, *catB2*, *catB3* and *catB8*, are known to occur in *Salmonella*. All these *catB* genes are located on gene cassettes and have mainly been identified in class 1 multi-resistance integrons in *S*. Typhimurium (Nogrady *et al*., 2005). The chloramphenicol exporter gene *cmlA* is also a cassette borne gene which has been found in plasmid-located class 1 integrons in *S*. Typhimurium (Nogrady *et al*., 2005).

2.9.2 Co-trimoxazole

A combination of trimethoprim and sulphamethoxazole, known as co-trimoxazole has been successfully used to treat *Salmonella* infections. Since these two antimicrobials inhibit sequential stages in tetrahydrofolic acid (THFA) synthesis, it was believed that

administration of a combination therapy would have a selective advantage over a single administration (Nogrady *et al*., 2005). Sulfonamides and trimethoprim block different enzymatic steps in tetrahydrofolate biosynthesis. Sulfonamides are structural analogues of *p*-aminobenzoic acid and competitively inhibit the enzyme dihydropteroic acid synthase (DHPS) while trimethoprim competitively inhibits the enzyme dihydrofolate reductase (DHFR) (Sköld, 2001).

Chromosomal, plasmid and transposon mediated resistance have all been reported for these antimicrobials. Chromosomal mutations involve the over production of the dihydrofolate reductase (DHFR), which leads to trimethoprim resistance involving the need for a higher inhibitor concentration of drug inside the cell to decrease the residual enzyme activity. The commonest mechanism of trimethoprim resistance is associated with the production of an additional non-susceptible form of DHFR encoded by genes located on self-transmissible or mobile plasmids and transposons (Huang *et al*., 2004). More than 30 different trimethoprim resistance mediating dihydrofolate reductase (*dfr)* genes have been identified (Huang *et al*., 2004). These are subdivided on the basis of their structure into two major types 1 and 2, which are currently referred to as *dfrA* and *dfrB*. While *dfrB* genes have not yet been identified in *Salmonella*, a total of 13 different *dfrA* genes-most of which are cassette-borne genes located in class 1 or class 2 integrons have been sequenced from various *Salmonella enterica* serovars (Michael *et al*., 2006).

2.9.3 Fluoroquinolones

Fluoroquinolones (FQ) represent the second, third and fourth generations of quinolone development. The first generation of this group of compounds is represented by agents such as nalidixic acid and pipemidic acid and is characterized by limited activity against Gram-negative bacteria (Bager and Helmuth, 2001). The second generation quinolones

(namely ciprofloxin, norfloxacin, ofloxacin) have increased potency and antibacterial spectrum by modifying the original two-ring quinolone nucleus with different side chain substitutions and introducing fluorine at the 6th position. Their use now accounts for about 11% of overall prescriptions of antimicrobials in human medicine and one of them, ciprofloxacin, is the most used antibiotic in the world (Acar and Goldstein, 1997). Fluoroquinolones mechanism of action in *Salmonella* include inhibition of tertiary super coiling of bacterial DNA, primarily by inhibiting the action of DNA gyrase, a Type II topoisomerase, which consists of two GyrA and two GyrB subunits encoded by *gyrA* and *gyrB*, respectively(Anderson, 2007). A single point mutation in *gyrA* between amino acids 67 and 106 (known as the quinolone resistance-determining region or QRDR) can give rise to nalidixic acid (a first-generation quinolone) resistance among isolates of *Salmonella*. This resistance is usually accompanied by a reduction in the susceptibility (MIC 0.125-1.0 mg/L) of these isolates to ciprofloxacin (Murray *et al*., 2005).

A study of over1, 000 stored *Salmonella* isolates from Finland has confirmed or shown that resistance to nalidixic acid by means of disk diffusion is a sensitive and specific way of screening *Salmonella* isolates for reduced susceptibility to fluoroquinolones (Hakanen *et al*., 1999). Resistance to nalidixic acid appears to be a predictor of clinical "quinolone hyporesponsiveness," and it is a harbinger of bona fide resistance to the clinically useful fluoroquinolones. Mutation in *gry*B, which encodes the B subunit of gyrase, was identified at a much lower frequency than *gyr*A mutations. As in *E. coli* and other Gram-negative bacteria, topoisomerase IV, whose *parC* and *parE* genes are, respectively, homologous to *gyrA* and *gyrB*, is considered a secondary target for quinolones in *Salmonella*. This means that mutational modifications of this enzyme are expected to occur only in strains which already possess a mutated gyrase. Quinolone-resistance mutations in *parC* generally occur at codons Ser80 or less frequently at codon

Glu84, which are homologous, respectively, to the Ser83 and Asp87 codons of gyrase (Casin *et al*., 2003). It is assumed therefore that the mode of action of quinolones against both enzymes is similar. In Gram negative bacteria, for many fluoroquinolones, DNA gyrase is the primary target and topoisomerase IV may be a secondary target associated with high level resistance. However, for many fluoroquinolones in Gram positive bacteria, such as *Staphylococcus aureus,* topoisomerase IV is the primary target (Wain, 2000). Fluoroquinolone resistance mechanisms also include decreased accumulation due to active efflux and possibly decreased outer membrane permeability. Very recently, the presence of a plasmid-borne *qnr*-like gene conferring low-level quinolone resistance has been reported in clinical isolates of *S. enterica* serovar Enteritidis (Cheung *et al*., 2005). Thus, FQ resistance as observed in *Salmonella* isolates is the endpoint result of the accumulation of several, sometimes cooperating, and biochemical mechanisms, themselves resulting from various genetic events.

2.9.4 *β*-lactam antibiotics

The mode of action of beta lactam antibiotics is via penicillin binding proteins (PBPs) and inactivation of transpeptidases. The final stage in the synthesis of bacterial cell wall peptidoglycan is the transpeptidation of the free peptide strands of the cell wall peptide-glycan sub-units. This is achieved by acylation of the terminal amine group of one peptide chain unto the D-alanine residue of the next peptide chain (Wain, 2000).

Resistance to β-lactam antibiotics is mainly mediated by a large number of β-lactamases which differ in their abilities to hydrolyse the various beta-lactam antibiotics (Livermore, 1995). These enzymes can bind β-lactam molecules thus protecting the penicillin binding proteins in bacterial cell walls. Once bound, the acylation of a serine residue at the active site of the β-lactamase results in cleavage of the β-lactam ring of

the antibiotic and regeneration of the β-lactamase. Other resistance mechanisms include the acquisition of penicillin binding proteins with reduced affinity to β-lactams, mutations in the PBPs (Schwarz *et al*., 2004). Reduced β-lactam uptake due to alterations in the outer membrane of gram negative bacteria or export by multi-drug transporters have been also reported (Paulsen *et al*., 1996). Resistance against β-lactam antibiotics (penicillin and cephalosporins) in *Salmonella* is mainly mediated by β-lactamase enzymes, which inactivate the antibiotics (Bush *et al*., 1995). The β-lactamases so far detected in *Salmonella* constitute a diverse group of enzymes encoded by a considerable number of genes. At least 10 different subgroups of β-lactamase genes (*bla*) coding for TEM-, SHV-, PSE-, OXA-, PER-, CTX-M-, CMY-, ACC-, DHA-, or KPC-type β-lactamases have been identified (Michael *et al*., 2006; Burke *et al*., 2014).

2.9.5 Ampicillin

Bacterial resistance to ampicillin is mediated most commonly by a β-lactamase enzyme and the main family of β-lactamases responsible for ampicillin resistance is the temoxicillinase (TEM) β-lactamases. The gene for this enzyme has been shown to be present in resistant isolates of *S.* Typhi from Mexico and South East Asia (Taylor and Brose, 1985).

2.9.6 Cephalosporins

The cephalosporin nucleus is synthesized with a β-lactam ring attached to a six membered dihydrothiazine ring and based on cephalosporin C. Their mode of action is similar to the penicillin and unlike the penicillin nucleus, the cephalosporin nucleus is much more resistant to β-lactamase. Cephalosporins are classified into four groups or subdivided into four generations based on the spectrum of their activity (Livermoore and

Williams, 1996). First-generation cephalosporins (cefalotin, cefaloridin, cephalexin, cephapirin, cefazolin, cefadroxil, cephradine, and others) possess high biological activity with respect to Staphylococci, Streptococci, Pneumococci, and many types of enteric bacteria. Second-generation cephalosporins (cefuroxime, cefamandole, cefoxitin, cefotetan, cefaclor, and others) are characterized by high activity with respect to Gram-positive microorganisms that are resistant to β-lactamase action. They do not have a noticeable effect on enterococci (Parry *et al*., 2002). Third-generation cephalosporins (cefotaxime, ceftizoxime, ceftriaxone, ceftazidime, cefoperazone, and many others) differ in the highly antimicrobial activity against *Enterobacteriaceae* and are effective in treating typhoid fever. The fourth generation cephalosporin (Cefepime) is effective against Gram-positive (including methicillin susceptible *S. aureus*, α haemolytic streptococci, and some coagulase negative staphylococci) and Gram-negative organisms, including *P. aeruginosa* (Nathisuwan *et al*., 2001). Resistance to the broad spectrum cephalosporins is mainly due to the production of an enzyme called the extended spectrum β –lactamase (ESBL). The ESBL enzymes are plasmid - mediated which is capable of hydrolyzing and inactivating a wide variety of β lactams, including third generation cephalosporins, penicillins and aztreonam. These enzymes are the result of mutations of TEM-1 and TEM-2 and SHV-I. All of these β-lactamase enzymes are commonly found in the *Enterobacteriaceae* family. Widespread use of third generation cephalosporins and aztreonam is believed to be the major cause of the mutations in these enzymes that has led to the emergence of the ESBLs (Paterson and Bonomo, 2005). These enzymes mediate resistance to cefotaxime, ceftazidime and other broad spectrum cephalosporins and to monobactams such as aztreonam, but have no detectable activity against cephamycins and imipenem. Because, of their greatly extended substrate range these enzymes were called extended spectrum β-lactamases (Cheung *et al*., 2005).

Widespread fluoroquinolone use in children has been discouraged because of the potential adverse effects on cartilage development. Therefore, extended-spectrum cephalosporins (especially cefotaxime or ceftriaxone) are the mainstay of treatment of serious infections due to *Salmonella* species in children. The production of ESBLs or AmpC β-lactamases consequently has considerable implications for clinical microbiology laboratories and physicians in areas in which infections with *Salmonella* species are common (Kruger *et al*., 2004).

Although reports of ESBLs associated with *Salmonella* spp. are relatively rare compared to those for other species in the family *Enterobacteriaceae*, the number of reported cases in this organism has been increasing in recent years. *Salmonella* have been found to express a wide variety of ESBL types, including TEM, SHV, PER, OXA, and CTX-M enzymes (Winokur *et al*., 2001). The genes encoding these extended-spectrum cephalosporinases are carried by conjugative plasmids, transposons or integrons. These mobile genetic elements could spread horizontally between enteric organisms. Thus, not only can antimicrobial resistance in Salmonellae emerge because of the selection pressure derived from inappropriate antimicrobial use in food animals, but drug-susceptible Salmonellae can also become resistant via the *in-vivo* acquisition of drug resistance plasmids from other enteric pathogens in the intestinal tract of patients (Su *et al*., 2003).

The ESBLs are typically encoded on large, 80-kb to 300-kb plasmids that can be exchanged between bacterial species. In many cases, these plasmids also encode other antimicrobial resistance genes. Therefore, it is common for organisms expressing an ESBL to express co-resistances to aminoglycosides, trimethoprim-sulfamethoxazole and tetracyclines (Jacoby and Medeiros, 1991). *Salmonella* isolates that demonstrate reduced

susceptibility to one or more of ceftazidime, cefuroxime, cefotaxime, ceftriaxone, cefpodoxime or aztreonam (third generation cephalosporins) but remain susceptible to cefoxitin or cefotetan (second generation cephalosporins) are considered as potential producers of ESBLs (Sturenburg and Mack, 2003). Expanded-spectrum cephalosporins (ESCs) such as ceftriaxone, together with fluorinated quinolones, are the antibiotics of choice in the treatment of invasive *Salmonella* infections. Resistance to ESCs among non-typhoid *Salmonella* has been recognized since the late 1980s. Currently, ESC-resistant *Salmonella* strains are reported world-wide and in some areas their incidence is significant. Resistance is mainly due to acquisition of multiresistant plasmids encoding a variety of extended-spectrum and AmpC-type –lactamases (Miriagou *et al*., 2004).

2.10 Trends of Drug Resistance in *Salmonella*

Since the beginning of the 1990s, strains of *Salmonella* which are resistant to a range of antimicrobials, including first-choice agents for the treatment of humans, have emerged and are threatening to become a serious public health problem. This resistance results from the use of antimicrobials both in humans and animal husbandry. Multi-drug resistance to critically important antimicrobials are compounding the problems (Parry, 2003). Surveillance data demonstrated an obvious increase in overall antimicrobial resistance among *Salmonella* from 20%-30% in the early 1990s to as high as 70% in some countries at the turn of the century. The resistance rates, however, vary with different serotypes, antibiotics and in different geographical areas of the world (Su *et al*., 2004).

2.10.1 *Salmonella enterica* serovar Typhi

The presence of multi-drug resistant (MDR) *S.* Typhi, the casual agent of typhoid fever, now poses a major threat to the effective treatment of the disease (Mirza *et al*., 2000). In developing countries, the antibiotics most readily available for treatment of typhoid are: chloramphenicol, ampicillin and co-trimoxazole. Since 1948, chloramphenicol became the standard antibiotic for treating typhoid, but plasmid encoded chloramphenicol resistance emerged first in the early 1970's (Mirza *et al*., 2000). Although slightly less effective than chloramphenicol, ampicillin was used both for therapy and for elimination of the carrier state, again plasmid–encoded resistance soon developed (Wain and Kidgell, 2004). Finally, co-trimoxazole was introduced in 1980, and plasmid encoded resistance to trimethoprim and sulfonamides were observed shortly afterwards (Datta *et al*., 1981). Towards the end of 1980s and the 1990s, *S.* Typhi developed resistance simultaneously to all the drugs that were then used as first line treatment (chloramphenicol, trimethoprim, sulfamethoxazole and ampicillin) (Parry *et al*., 2002). Outbreaks of infections with these strains occurred in India, Pakistan, Bangladesh, Vietnam, the Middle East, and Africa (Pang *et al*., 1995). These multi-drug resistant strains also carried the 100,000 to 120,000 KD IncHI self-transmissible plasmids that encoded the resistance genes. Multi-drug resistant *S. enterica* serovar Typhi are less prevalent in Africa compared to Asia, but have been present in South Africa, Ghana (Mills-Rovertson *et al*., 2002) and Kenya (Kariuki *et al*., 2002). In recent years, however, there have been reports of declining resistance levels. At a major hospital in Mumbai, India, for example, the proportion of MDR strains declined from 46% in 2000 to 17% in 2002 (Rodrigues *et al*., 2003). In an infectious diseases hospital in Cairo, the isolation of multiple drug resistant isolates of *S.* Typhi declined from 100% in 1993 to 5% in 2000 (Wafsy *et al*., 2002). The authors of both reports suggest that this decline in

the levels of resistance may allow drugs such as chloramphenicol to be used again as first line therapy for enteric fever. Since the appearance of MDR to the commonly used drugs, ciprofloxacin and the third generation of cephalosporin (namely ceftriaxone) have become the first line of treatment for typhoid fever. Since 1993 however, there has been a global epidemic of nalidixic acid resistant *S. enterica* serovar Typhi. These strains exhibit decreased response to fluoroquinolones compared to those infected with nalidixic acid susceptible strains (Wain *et al*., 1997). In 1999 ceftriaxone resistant *S.* Typhi was detected in Bangladesh (Asna *et al*., 2003). Although it is threatening that resistance to ciprofloxacin is now emerging, it remains the drug of choice for the treatment of MDR typhoid fever (Rowe *et al*., 1995).

2.10.2 Non-typhoidal *Salmonella enterica* serovars

In the last two decades, the emergence and spread of antimicrobial-resistant pathogens, among the non-typhoidal *Salmonella*, has become a serious health hazard worldwide. The routine practice of giving antimicrobial agents to domestic livestock as a means of preventing and treating diseases, as well as promoting growth, is an important factor in the emergence of antibiotic-resistant bacteria that are subsequently transferred to humans by the food chain (Angulo *et al*., 2004). Multi-drug resistance in the NTS is defined as resistance to four or more drugs (Cooke and Wain, 2006).

In the developed world, the most common serotypes isolated from humans are *S.* Typhimurium, *S.* Enteritidis, *S*. Virchow and *S*. Hadar, which are zoonotic in origin and are often resistant to many commonly used antibiotics. The situation is different in the developing world, where the above serotypes, together with *S.* Dublin and *S.* Choleraesuis, tend to cause more invasive disease, particularly in the immunosuppressed, neonates, children and the elderly (Gordon *et al*., 2002). In the

1980s, NTS were fairly "sensitive" organisms. A remarkable bacterial "success story" of the l990s was the emergence and worldwide spread of *S.* Typhimurium definitive phage type 104 (DT104), having resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT R-type) was a major concern worldwide (Threlfall, 2002). This penta-resistance was found to be mediated by the antibiotic resistance gene cluster in *Salmonella* genomic island 1 (SGI1), and SGI1 has been identified in other *Salmonella* Typhimurium phage types and several other serotypes (Doublet *et al*., 2005). Multiple drug resistance is a problem in other Typhimurium phage types and *Salmonella* serotypes. A strain of Typhimurium DT204b resistant to ampicilin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulphonamides and tetracyclines and with low-level ciprofloxacin resistance caused an outbreak in five European countries in 2000 with more than 350 confirmed cases (Lindsay *et al*., 2002). In a European surveillance study in 2000, 36% of *S*. Virchow and 37% of *S*. Hadar isolates were resistant to four or more antimicrobials. Antimicrobial resistance has generally been less of a problem in *S*. Enteritidis. In Europe in 2000, 2% of isolates were resistant to four or more antimicrobials (Threlfall *et al*., 2003).

In some Africans countries NTS, in particular MDR *Salmonella enterica* serotype Typhimurium is the causative agent of serious outbreaks. For example in Zaire (Cheesbrough *et al*., 1997), Rwanda (Lepage *et al*., 1990) and Kenya (Kariuki *et al*., 2000) multidrug-resistant *S.* Typhimurium was the predominant cause of bacteraemic illness. A ten-year (1994-2003) retrospective study in Kenya indicated a steady increase in the proportion of multidrug resistance among NTS isolated from adult patients with bacteraemia. The prevalence of NTS with multiple resistances to commonly available drugs including ampicillin, streptomycin, co-trimoxazole, chloramphenicol and tetracycline rose from 31% in 1994 to 42% at 2003, with concomitantly higher

minimum inhibition concentrations (MICs) of each drug. Resistance was found to be encoded on large self-transferable 100–110 kb plasmids (Kariuki *et al*., 2005). Because of the increased resistance to conventional antibiotics, extended-spectrum cephalosporins and fluoroquinolones have become the drugs of choice for the treatment of infections caused by multi-drug resistant *Salmonella* serotypes. However, since 1991, many countries have been reporting outbreaks or cases of infections due to salmonellae that were resistant to extended-spectrum cephalosporins (Dunne *et al*., 2000).

Resistance to other antibiotics notably, resistance to quinolones (e.g., nalidixic acid) and their derivatives, such as fluoroquinolones (e.g., ciprofloxacin) has also begun to emerge. Of particular concern is an outbreak of infection with quinolone-resistant *S.* Typhimurium DT104 that has spread from animals to humans and has caused mortality (Molbak *et al*., 1999). It is now known that nalidixic acid the prototypic quinolone is a good predictor for the reduced susceptibility to fluoroquinolones in salmonellae (Hakanen *et al*., 1999). The increase in nalidixic acid resistance, in some way, may reflect the emergence of fluoroquinolone resistance. Perhaps the most alarming report of resistance from a clinical view point is the combined resistance to fluoroquinolones, associated with double mutations in topoisomerases, and broad spectrum cephalosporins caused by β-lactamase in *Salmonella enterica* serovars Choleraesuis. This serovar was observed in Taiwan and it was found that it is highly invasive and simultaneously resistant to ceftriaxone and ciprofloxacin in 2002 (Chiu *et al*., 2004). The emergence of such a resistance trait in *S.* Choleraesuis poses a serious threat to human health and should be monitored closely.

2.11 Typing of *Salmonella*

Typing is the splitting of organisms into useful groupings; if those grouping are based on genetic differences then the typing scheme is also a classification. Such information may be of clinical or epidemiological value. Clonally related organisms are members of the same species that share virulence factors, biochemical traits and genomic characteristics. However, there is sufficient diversity at the species level that organisms isolated from different sources at different times and in different geographical regions may be differentiated or classified into subtypes or strains (Olive and Bean, 1999). Typing is necessary when trying to determine the distribution of the widespread organism, association of type with disease, exclusion of sources, identification of carriers, determination of route of infection and assessment of the efficiency of preventive measures (Struelens, 1998). Methods for *Salmonella* species typing fall into three broad categories, phenotyping, genotypic and sequence-based typing techniques (Maslow *et al*., 1993).

2.11.1 Phenotyping

Phenotypic methods of typing are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, and antigen or protein types present on cell surface and antimicrobial susceptibility profiles are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth condition, growth phase and spontaneous mutation (Arbeit, 2000). Phenotypic methods are associated with problems such as low discriminatory power, poor reproducibility and less typeability properties (Maslow *et al*., 1993).

2.11.1.1 Serotyping

Salmonella serotyping is a surveillance tool that detects widespread outbreaks, identifies outbreak sources, monitors trends over time and attributes human disease to various food and animal sources. Serological analysis usually remains the first step in an epidemiological investigation of *Salmonella* and may be sufficient for epidemiological investigations associated with uncommon serotypes (Winokur, 2003).

Serotyping of *Salmonella* strains is carried out by identification of surface antigens (LPS, O-antigens) and flagella antigens (H-antigens) by the antigen-antibody reaction. Detection of the O and H- antigens are performed using mono-and polyvalent antisera on a fresh culture of the isolate. Most commonly, strains of *Salmonella* express two phases of H- antigens but aphasic, monophasic and triphasic variants are known and only one flagellar phase is expressed at a time. The definition of the serotypes is based on the antigen combination given in the "Kauffmann-White scheme" (Grimont and Weill, 2007).

2.11.1.2. Phage typing

In phage typing technique, *Salmonella* isolates are characterized by their susceptibility or resistance to lysis by each member of a panel of bacteriophages (Maslow *et al*., 1993). Phage typing is a commonly used method for epidemiological surveillance of *Salmonella* Enteritidis and *Salmonella* Typhimurium infection; however it requires specialized reagents and laboratory equipment and does not always yield sufficient information for epidemiological purposes (Kobayashi *et al.*, 2014). The power of resolution is limited, because several distinct strains of *S.* Typhimurium can belong to the same phage type and the technique is largely empirical and does not reflect true evolutionary classification (Schmieger, 1999). Phage susceptibility may be relatively

unpredictable and susceptibility changes can occur rapidly. Within six weeks, *S.* Enteritidis isolated from one patient changed from DT4 to DT7 and DT9a (Powell *et al*., 1995). Phage typing is often an important early step in an investigation, but needs to be supplemented with other techniques. Phage typing is technically demanding and requires the maintenance of stocks of biologically active phage and control strains are conditions that relegate this technique to reference laboratories (Maslow *et al*., 1993).

2.11.1.3 Biotyping

Biotyping refers to analysis of cellular metabolic enzymes. Some diseases often cluster among a few serotypes, so other methods like biotyping have been used to further subdivide a particular serotype (Winokur, 2003). Biotyping like most phenotyping methods has only modest reproducibility because the organism can alter unpredictable expression of many cellular products (Tenover *et al*., 1995).

2.11.1.4 Antimicrobial susceptibility pattern

Antimicrobial susceptibility technique is used to group *Salmonella* serotypes according to resistance profiles or R-types (Tenover *et al*., 1997). Antibiotic resistance in many *Salmonella* serotypes is increasing with a number of isolates showing multi-drug resistance (Winokur, 2003). Therefore, recognition of new or unusual antibiotic resistance among isolates has often instigated epidemiological investigations. Antimicrobial susceptibility patterns also have relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institutions and often is associated with mobile genetic elements (e.g., transposons and plasmids) which can be lost or acquired over short periods of time (Winokur, 2003).

Thus, antimicrobial resistance is not one of the most stable epidemiological markers for outbreak analysis.

2.11.2 Genotyping

Molecular genotyping of *Salmonella* strains is fundamental in tracking disease associated and drug-resistant strains in various populations. Genotypic methods are those that are based on analysis of the genetic structure of an organism and include polymorphism in DNA restriction patterns based on cleavage of the chromosomes (DNA) into hundreds of fragments (frequent cutters), or into 10 to 30 fragments (infrequent cutters), and the presence and absence of extra-chromosomal DNA. They are less subjected to natural variation, although DNA changes (insertions, deletions and random mutations) can have an effect on resulting fingerprinting (Arbeit, 2000). Molecular techniques which are commonly used in typing bacteria include: pulsed field gel electrophoresis (PFGE), ribotyping, restriction enzyme analysis of plasmid DNA, amplified fragment polymorphism, arbitrary primed PCR (AP-PCR) and repetitive sequence PCR (Winokur, 2003). For many bacterial species, combination of different methods or selection of the most discriminative methods is usually required in identifying a particular strain. For typing of *Salmonella* species including *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis, a combination of PFGE, ribotyping and plasmid analysis are used by most researchers/laboratories (Tsen *et al*., 2002).

2.11.2.1 Polymerase Chain Reaction Method, PCR (General Procedure for Conventional and Multiplex PCR)

The PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilobase pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size

(Cheng *et al.,* 1994). The quantity of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses (Carr and Moore, 2012). The steps in DNA extraction include lyses of the cell, degradation of cellular protein, precipitation and washing of DNA and centrifugation (Soumet *et al.*, 1994; Fadl *et al.*, 1995).

DNA primers

Molecular identification of *Salmonella* serovars involves the detection of invasion gene (*inv*A) common in all *Salmonella* serotypes, which can be amplified by PCR with a pair of primers (Del Cerro *et al.,* 2003). The identification and characterization of some serotypes can be carried out using Multiplex PCR which involves the use of *inv*A gene sequence (primer) alongside some virulence genes sequence specific to the serotype (Chiu and Ou., 1996). The primers sequences specific for different *Salmonella* species have been described (Rahn *et al*., 1992; Lim *et al*., 2003; Mirzaie *et al.,* 2010; Taghi *et al.,* 2012). A basic PCR set up requires several components and reagents which include DNA template (target/washed organism) to be amplified, Two primers, Taq polymerase, Deoxynucleoside triphosphates (dNTPs), Divalent cations (magnesium or manganese), Monovalent cation (potassium ions), and Buffer solution (Sambrook and Russel, 2001; Pavlov *et al.,* 2004).

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three. The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA

synthesis, the concentration of divalent ions and dNTPs in the reaction and the melting temperature of the primers (Rychlik *et al.,* 1990). The PCR steps include Initialization, Denaturation, Annealing, Extension/elongation, Final elongation and Final hold (Chien, *et al.*, 1976: Lawyer *et al.*, 1993; Sharkey *et al.*, 1994).

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size (s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known sizes, run on the gel alongside the PCR products.

2.11.2.2 Plasmid profile

Plasmid profile was among the earliest DNA based techniques applied to epidemiologic studies (Tenover *et al*., 1997). In the most basic system, plasmids are isolated from each isolate and then separated electrophoretically in agarose gel to determine their number and sizes. Additional information can be obtained by digesting the plasmid with a restriction endonuclease and then comparing the number and sizes of the resulting restriction fragments (Maslow *et al*., 1993). This procedure often referred to as restriction enzyme analysis of plasmids, is now the method of choice for plasmid studies. It is technically simple, requires only modest specialized equipment, and can be performed relatively quickly (Arbeit, 2000). Typing systems based on plasmid analysis suffer from significant limitations inherent in the fact that plasmids are mobile extra chromosomal genotypes that define the host strain. Plasmids can be spontaneously lost from or readily acquired by a host strain; consequently, epidemiologically related isolates can exhibit different plasmid profiles**.** Some studies regard the presence of a single, identical plasmid as sufficient proof that isolates are identical and therefore

epidemiologically related (Liebana, 2002). Other studies suggest that numerous plasmids must be present, and regard the presence of a single plasmid as insufficient representative of a clone (Maslow *et al*., 1993). Open circular or linear plasmid forms display different electrophoretic migration patterns to confuse the interpretation of banding patterns (Liebana, 2002). Despite this limitation, plasmid pattern determination has proven to be a useful epidemiological tool in outbreaks of *Salmonella* serotypes (Winokur, 2003). Plasmid profiles are most useful when they are combined with other methods for screening or typing (Arbeit, 2000).

2.11.2.3 Pulsed field gel electrophoresis (PFGE) of chromosomal DNA

The PFGE is often considered the "gold standard" of molecular typing methods. The PFGE is an agarose gel electrophoresis that permits analysis of bacterial DNA fragments over an order of magnitude larger than that of conventional restriction enzyme analysis (REA). Chromosomal DNA is digested with restriction enzymes that have few restriction sites, yielding 5-50 fragments ranging from 10 kb to 800 kb in length (Struelens, 1998; Olive and Bean, 1999). A major limitation of REA with enzymes with relatively frequent recognition sites is the difficulty of analyzing the resulting patterns composed of large numbers of overlapping, poorly resolved restriction fragments (Arbeit, 2000). The PFGE is a variation of agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically (pulsed) rather than being kept constant as in conventional agarose gel electrophoresis used for the REA and southern blot (Tenover *et al*., 1995; Thong *et al*., 1996). Theoretically, all bacterial isolates are typable by PFGE. The PFGE has proved to be one of the most discriminative methods for the subtyping of *Salmonella* strains (Rivera *et al*., 1991). Thong *et al*. (1995) used the PFGE method for the sub typing of *S.* Typhi strains

isolated from several Southeast Asian countries and found that PFGE was a powerful technique for the analysis of *S.* Typhi strains. Thong and his colleagues (1996) also found that although considerable genetic diversity existed among *S.* Typhi strains, some PFGE patterns might be shared between isolates obtained from different countries, for example, Malaysia, Indonesia, and Thailand.

A study in Vietnam also showed that PFGE is both reproducible and discriminatory and can be used to analyze multiple drug-resistant *S.* Typhi strains in regions where typhoid is endemic (Wain *et al*., 1999). In Malaysia, analysis of *S.* Typhi using PFGE indicated that an individual outbreak was associated with closely related strains, whereas isolates of *S.* Typhi from sporadic cases were very diverse (Thong *et al*., 1994). In South East Asia, PFGE studies revealed that multiple genetic variants of *S.* Typhi were present simultaneously and are associated with sporadic cases of typhoid fever and occasional outbreaks (Thong *et al*., 1995). The PFGE uses expensive equipment and software is needed for comparative results, so the technique is limited to reference and research laboratories. It samples a small proportion of sequence variation in *Salmonella* genome. Many authors (Harbottle *et al.,* 2006; Barco *et al.,* 2014; Lettini *et al.,* 2014) have concluded that when PFGE is used in combination with other methods (e.g. MLST, MLVA, Antibiogram) it seems to give the best discrimination for epidemiological purpose.

The Theory of PFGE Steps

The bacterial suspension is combined with molten agarose and mixed with a protease and with sodium dodecyl sulphate (SDS). The enzyme-detergent mixture denatures the cell membrane proteins thus forming holes in the cell through which the chromosomal DNA is released. The agarose keeps the DNA embedded in its gel matrix. Next, the plug

is washed several times to remove cell debris and proteases. These diffused out of the agarose gel matrix more easily than the large DNA molecules. It is important to remove the proteases so as not to harm the restriction enzymes that cleave the DNA in the next step of the process. A small piece of the plug is cut off and added to a restriction endonuclease (s) mixture which cleaves DNA at a specific sequence resulting in 10-30 DNA fragments ranging from 0.5 to 1,000 kb. The large DNA fragments are then separated based on size by pulsed-field gel electrophoresis. The PFGE facilitates the migration of the large DNA fragments (>600 kb) through the agarose gel by regularly changing the direction of the electrical field during electrophoresis, allowing the fragments to maneuver through the agarose. The smaller DNA fragments move faster through the agarose than the larger fragments and the result is a pattern of DNA fragments. Migration distances are compared to reference standards of known molecular weight and a profile for each strain/isolate is obtained. The PFGE pattern from one isolate can be compared to other patterns to determine whether the samples may have originated from a common source. The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye such as ethidium bromide. Gel results can be photographed and the data can be stored by using one of the commercially available digital systems. Data analysis can be accomplished by using any of a number of commercially available software packages (Tenover *et al.,* 1994; 1995; 1997; Olive and Bean, 1999). Use of PFGE has been greatly facilitated by the incorporation of standard methods of analysis suggested by Tenover *et al.* (1995), which has led to its widespread adoption. In their scheme, bacterial isolates yielding the same PFGE pattern are considered the same strain. Bacterial isolates differing by a single genetic event, reflected as a difference of one to three bands, are closely related. Isolates differing by four to six bands, representing two independent genetic changes, are possibly related.

Bacterial isolates containing six or more band differences, representative of three or more genetic changes, are considered unrelated. These criteria are applicable to small, local studies in which genetic variability is presumed to be limited. The PFGE is one of the most reproducible and highly discriminatory typing techniques available and is the most reliable technique for analysis of variety of foodborne pathogens: *Escherichia coli* O157:H7 (Arbeit, 1995), *Salmonella* Typhimurium (Tsen *et al.,* 2002) and *Salmonella* Enteritidis (Thong *et al.,* 1995).

2.11.2.4 Integron analysis

Horizontal gene transfer increases genetic diversity in prokaryotes to a degree not allowed by the limitations of reproduction by binary fission (Michael *et al*., 2004). The integron/cassette is one of the most recently characterized examples of a system that facilitates horizontal gene transfer (Michael *et al*., 2004).

An efficient route of acquisition and vertical and horizontal dissemination of resistance determinants is through mobile elements including plasmids, transposons and gene cassettes in integrons (Guerra *et al*., 2000). There are at least three classes of integrons (based upon the type of integrase gene they possess) and class 1 integrons are the most frequent in clinical strains, being found in many different organisms (Collis *et al*., 1998). Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination. Integrons have an intergase gene *(int),* a nearby recombination site (*att*I) and a promoter (Michael *et al*., 2004). Class 1 integrons have been examined most extensively and found that they consist of a variable region bordered by 5' and 3' conserved regions. The 5' region is made up of the *int* gene, *attI*, and the promoter which drives transcription of genes within the variable region. The 3' region consists of an ethidium bromide resistance

locus (qacED1), a sulfonamide resistance gene (*sulI*) and an open reading frame (Collis *et al*., 1998).

Class 1 integrons are mobile DNA elements that often encode one or more antimicrobial resistance genes (Winokur, 2003). At the present time, about 60 different cassettes associated with resistance genes have been identified and the same cassettes can be found in different classes of integrons (Guerra *et al*., 2000). Primers have been designed that amplify the variable cassette regions where these genes are inserted (Winokur, 2003). Integron PCR has been used for identification and to complement other typing techniques in studies of *S.* Virchow, the variability of the inserted integrons cassette was used to differentiate strains. These locus specific PCR technique show good reproducibility within and between laboratories, but care must be taken with integron analysis performed over time since they are mobile DNA elements (Martin MC, 2001).

2.11.2.5 Arbitrary Primed Polymerase Chain Reaction

Arbitrary Primed PCR (AP PCR), also referred to as randomly amplified polymorphic DNA assay, is a DNA fingerprinting technique that uses short (typically 9-15 bp) random sequence primers that hybridize at multiple random chromosomal sites (Winokur, 2003). It is a variation of the PCR technique employing a single short primer that is not targeted to amplify any specific bacterial DNA sequence. Rather, at low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis (Tenover *et al*., 1997). If one copy of the primer binds to one strand of DNA, and another copy of the primer binds on the opposite strand of DNA but in proximity of the first primer, a DNA fragment will be synthesized and amplification of that fragment will occur (Fadl *et al*., 1995; Tenover *et al*., 1995; Lin *et al*., 1996). The number and location of these random primer sites vary for different

strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, patterns of bands, which in theory, are characteristic of the particular bacterial strain results (Olive and Bean, 1999). All isolates are typeable and no prior knowledge of target genome sequences is necessary and the material costs and labour are low. However, identification of appropriate primers is arbitrary and requires significant test development time. Lin and his colleagues (1996) screened 65 primers before selecting six primers that resulted in multiple AP PCR banding patterns from *S.* Enteritidis isolates. After this laborious primer selection process, AP PCR did perform better than phage typing, ribotyping and PFGE. In a study conducted in Brazil, to type 30 strains of *S.* Typhi, it was found that conventional phenotyping methods as well as the DNA plasmid analysis, presented non-significant discriminatory power, however, RAPD-PCR analysis showed discriminatory power, reproducibility, easy interpretation and performance over phage types and plasmid DNA analysis, three phage types, four plasmid profiles and 8 genotypic patterns were detected (Quintaes *et al*., 2002).

Fadal and his colleague (1995) proved the superiority of RAPD over phage in typing *S.* Enteritidis isolates (three phages types and AP-PCR generated seven distinct random amplified DNA pattern were detected), while in certain serotypes, such as *S.* Dublin, ribotyping or other techniques have been found to be superior (Fadl *et al*., 1995; Keriuanton *et al*., 1999). Even though AP-PCR is rapid and relatively inexpensive, the reproducibility and discriminatory power of this technique is a subject of active discussion and investigation and has several draw backs. Banding patterns can vary with pH, magnesium and DNA concentrations and the source of DNA polymerase (Tenover *et al*., 1997). The banding patterns, too, are typically comprised of several dominant and several less intense bands. The intensity of these faint bands can vary from gel to gel and can complicate computer based gel analysis. This technique has poor inter-

laboratory reproducibility and is best used to evaluate a set of isolates analyzed in a single amplification reaction separated on a single gel (Tenover *et al*., 1997).

2.11.3 Sequence-based typing

Typing schemes based on variation in particular DNA sequences have the advantage of being digital in nature. This means that the same results should be achieved wherever the test is performed and any comparison of results is simple, quantitative and absolute in nature. Sequence-based typing schemes can also be considered as classification schemes and so genetic and evolutionary inferences can be made (Cooke *et al*., 2006).

Current DNA-sequence-based typing methods include the detection of DNA repeats and single nucleotide polymorphisms (SNPs). Variable Number of Tandem Repeat variation (VNTR) does not penetrate into the actual DNA sequence but produces data on the copy number of short repetitive sequences of individual isolates, by determining the size of the PCR product (Lindstedt, 2005). To date, VNTR seems to differentiate reliably between isolates of *S.* Typhimurium, but requires modification to be applied successfully to *S.* Enteritidis isolates. The availability of a large amount of genomic sequence from different strains of *Salmonella* in combination with microarray technology opens yet another perspective for typing of *Salmonella*. Microarrays containing all known sequences from *S. enterica* serotypes have already been prepared and used to interrogate the gene content of different serotypes. Although not currently suitable for diagnostic laboratories these methods may find utility in reference laboratoties for defining some aspects of genetic diversity such as antibiotic resistance. Other sequence-based methods that have been used include analysis of gene profiles coding for antibiotic resistance and pathogenicity markers e.g. use of real-time PCR to

distinguish mutations in the *sopE* virulence and *fliC* gene in *S.* Typhi (Mortimer *et al*., 2004).

2.11.3.1 Multilocus variable-number tandem-repeat analysis

The PFGE has proven to be useful and reliable in outbreak investigations due to its good discriminatory power (Lukinmaa *et al.*, 1999; Schmidetal, 2006). However, the method is plagued by some significant pitfalls, mainly the complex and time-consuming procedure and reduced comparability of results between different laboratories. Additionally, the discriminatory power of PFGE has proven to be insufficient for epidemiologically related and unrelated strains (Gatto *et al.*, 2006; Lukinmaa *et al.*, 2006).

Multilocus variable-number tandem-repeat analysis (MLVA) has been used for subtyping a variety of bacteria. Many bacterial genes or intergenic regions contain loci of repetitive DNA, the number of repeat units of which may vary among strains (van Belkum, 2007). Multiple loci of these variable number tandem repeats (VNTRs) have been identified in many bacterial species and have been successfully used for strain discrimination (Chang *et al.*, 2007). The MLVA is performed by amplification and fragment size analysis of polymorphic regions of DNA containing the VNTRs (van Belkum, 2007). The MLVA has also been reported to be very useful for subtyping other *Salmonella* serovars (Lindstedt *et al.*, 2004; Boxrud *et al.*, 2007; Davis *et al.*, 2009).

2.11.3.2 Multilocus sequence typing

Nucleotide sequence-based methods for bacterial typing (multilocus sequence typing; MLST) allow rapid and global comparisons between results from different laboratories. Combining this advantage with the reduced cost of high throughput sequencing,

increasing automation and the amenability of sequence data for evolutionary analysis, it seems inevitable that sequence based typing will eventually predominate over phenotypic methods like serotyping (Kidgell *et al*., 2002; Ikumapayi *et al*., 2007). Multilocus sequence typing (MLST) was developed by Maiden *et al*. (1998), for the naturally transformable Gram-negative pathogen *Neisserria meningitidis* but has since been applied to many pathogenic species and Ikumapayi *et al.* (2007) used MLST to characterize 62 invasive NTS isolates among children aged 2-29 months in rural Gambia and obtained ten different sequence types (STs). For example, in the case of *S*. Typhi for MLST analysis seven housekeeping genes were selected from the genome of *S.* Typhi CT18 on the basis that they are scattered around the chromosome, are flanked by genes of known function and that neither the gene chosen for sequencing nor the flanking genes are likely to be under diversifying selection (Kidgell *et al*., 2002).

The genes used were *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *hisD* (histidinol dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase) and *thrA* (aspartokinase+homoserine dehydrogenase) (Kidgell *et al*., 2002). The procedure is essentially an updated version of multilocus enzyme electrophoresis (MLEE), except that allelic types are determined by the sequence of housekeeping genes rather than by the electrophoretic mobilities of the enzyme they encode (Maiden, 2006). A serious drawback of MLEE and other gel-based methods such as pulsed-field gelectrophoresis (PFGE), is that it is often difficult to compare results between laboratories (Cooper and Feil, 2004). The MLST utilizes variability in the sequences of particular genes, due to mutation or recombination events, to determine the relatedness of bacteria. With MLST, multiple genes with conserved sequences are compared for nucleotide base changes. Housekeeping genes (genes required for basic

cellular functions) are most often sequenced because they are present in all isolates and are not subject to strong selective pressures that can lead to relatively rapid sequence changes (Foley *et al*., 2006). The MLST is used for long-term epidemiology and for the identification of lineages that have an increased propensity to cause disease (Urwin and Maiden, 2003). Compared to other molecular techniques, MLST has more discriminatory power among *Salmonella* isolates (Foley *et al*., 2006). Among the 128 *Salmonella* isolates tested, Foley and his colleagues (2006) were able to observe 84 Rep-PCR profiles, 86 PFGE patterns, 89 MLST patterns, 36 plasmid profiles, and 38 susceptibility profiles. Even though MLST originally was defined as a sequencing-based subtyping approach that includes the sequencing of 450 to 600 nucleotide fragments for six to seven housekeeping genes (Urwin and Maiden, 2003; Sukhnanand *et al*., 2005; Foley *et al*., 2006), *Salmonella* MLST schemes described in the literature have sometimes used sequencing of three to four genes (Sukhnanand *et al*., 2005). Some of these MLST schemes also included the sequencing of virulence or virulence-associated genes, e.g., *spaM, fim*A, *manB* and *mdh* which were shown to provide discriminatory power similar to that of a seven-gene MLST (Sukhnanand *et al*., 2005).

In a study conducted in the USA on population based comparisons of human and cow associated *Salmonella* subtypes and to evaluate the potential for cow-associated subtypes to be transmitted to humans, a collection of 179 human and 156 bovine clinical *Salmonella* isolates obtained from across New York state over the course of 1 year was characterized using serotyping and a multilocus sequence typing scheme based on the sequencing of three genes (*fimA*, *manB*, and *mdh*). The 335 isolates were differentiated into 52 serotypes and 72 sequence types (Alcaine *et al*., 2006). Therefore, the above experimental results indicate that MLST may be a good molecular epidemiological option to replace serotyping.

2.11.3.3 Sequences of the gene coding for phase 1 flagellin

Salmonella is unique among the members of the family *Enterobacteriaceae*, as it commonly has two distinct flagellar antigens, phase 1 and phase 2, that are co-ordinately regulated, so that only one flagellar antigen is expressed at any time (Kilger and Grimont, 1993). Serotyping by the traditional Kauffmann-White scheme is time consuming, requires well-trained technicians and uses large amounts of high-quality sera. For these reasons, the use of DNA methods, such as the multiplex PCR for H-antigen identification, is an attractive alternative to the more traditional techniques (Herrera-Leon *et al*., 2004). The *fliC* and *fljB* genes encode the phase-1 and phase-2 flagellins, respectively. These genes are coordinately expressed by a phase-variation mechanism. *fliC* is located in one of the flagellar biosynthesis operons, is present in all *Salmonella* and has a homologue in *Escherichia coli*. The *fljB* is located in a region of the genome that is unique to *Salmonella* and is present in four of the six subspecies. Isolates of *S. bongori* have been reported to have a gene homologous to *fljB*, although this species is typically monophasic. A triphasic isolate was genetically described that possessed the third flagellin gene, *flpA*, on a plasmid (McQuiston *et al*., 2004; Jacob and Jenabian, 2005).

The *fliC* and *fljB* genes are found at two different locations on the chromosome. Comparison of the amino acid sequences of *Salmonella* flagellins has led to the definition of eight variable regions. The amino and carboxy-terminal sequences (regions I and II and region VIII respectively) are conserved particularly at the 5' and 3', across most bacterial species and are thought to be important for polymerization and transportation. The central region, which comprises regions IV, V and VI, is highly variable in both sequence and length between flagellar antigen genes and, encodes the surface-exposed and antigenically variable portion of the filament. The central region

corresponds approximately to amino acids 181 to 390 (Herrera-Leon *et al*., 2004). *Salmonella* exhibits 70 serologically distinct flagellins, used internationally to diagnose and track infections. The terminal sequences of flagellin protein subunits are conserved in a range of bacteria and are used as evolutionary markers to reveal how new serotypes arise (Mortimer *et al*., 2007). The remarkable feature of *fliC* alleles is the sequence conservation of distal parts of the gene, thus making the gene of any serotype suitable for easy amplification. Jacob and Jenabian (2005) sequenced the variable region of the flagellin gene, *fliC*, from 96 *Salmonella* strains representing 51 different phase 1 H antigens and they found unique sequences for 45 of the 51 different antigens. This study showed that sequence-based typing of the phase 1 H antigen of *Salmonella* is a good alternative to serotyping when strains are non-typable by serological methods.

2.12 Treatment of Salmonellosis

2.12.1 Typhoidal salmonellosis

In areas of endemic disease, more than 60 to 90 per cent of cases of typhoid fever are managed at home with antibiotics and bed rest. For the hospitalized patient, effective antibiotics, good nursing care, adequate nutrition, careful attention to fluid and electrolyte balance and prompt recognition and treatment of complications are necessary to avert death (Parry *et al*., 2002). The fluoroquinolones are widely regarded as optimal for the treatment of typhoid fever. They are well tolerated and more rapidly and reliably effective than the former first-line drugs, viz. chloramphenicol, ampicillin, amoxicillin and trimethoprim-sulfamethoxazole (WHO, 2003). The fluoroquinolones attain excellent tissue penetration, kill *S.* Typhi in its intracellular attaining stage in monocytes/macrophages and achieve higher active drug levels in the gall bladder than other drugs. They produce a rapid therapeutic response, i.e. clearance of fever and

symptoms in three to five days and very low rates of post-treatment carriage (Cristiano *et al*., 1995). Concern has been expressed about three main issues regarding the use of fluroquinolones in the treatment of typhoid fever: the potential for toxic effects in children, the cost and the potential emergence of resistance (Bethell *et al*., 1996). Chloramphenicol, amoxicillin and trimethoprim-sulfamethoxazole remain appropriate for the treatment of typhoid fever in areas of the world where the bacterium is still fully susceptible to these drugs and where the fluoroquinolones are not available or affordable (Cammie and Miller, 2000). The disadvantage of using chloramphenicol includes a relatively high rate of relapse (5-7%), long treatment courses (14 days) may cause bone marrow depression and the frequent development of a carrier state in adults (Bhutta *et al*., 1991). The third generation cephalosporins (ceftriaxone, cefixime, cefotaxime and cefoperazone) and azithromycin are also effective drugs for typhoid. In general, in areas with high prevalence of multi-drug resistant *Salmonella* infection, all patients suspected of having typhoid fever should be treated with a quinolone or third-generation cephalosporin until the results of culture sensitivity studies become available (Miller *et al*., 2000).

2.12.2 Non-typhoidal salmonellosis

Gastroenteritis caused by *Salmonella* is usually a self-limiting disease and therapy should be directed primarily to the replacement of fluid and electrolyte losses. Therefore, antimicrobials should not be used routinely to treat uncomplicated non-typhoidal *Salmonella* gastroenteritis or to reduce convalescent stool excretion (Richards *et al*., 1993). However, antimicrobial therapy should be considered for any systemic infection (Parry *et al*., 2002). For susceptible organisms, treatment with an oral quinolone, trimethoprim-sulfamethoxazole, or amoxicillin is adequate. Because of the

increasing prevalence of antimicrobial resistance, empirical therapy for life threatening bacteraemia or local infection suspected to be caused by non-typhoidal *Salmonella* should include a third generation cephalosporin and a quinolone until susceptibility patterns are known. Amoxicillin and trimethoprim-sulfamethoxazole are effective in eradication of long-term carriage. The high concentration of amoxicillin and quinolone in bile and the superior intracellular penetration of quinolone are theoretical advantages over trimethoprim-sulfamethoxazole (WHO, 2003).

2.13 Prevention and Control of Salmonellosis

Theoretically, it is possible to eliminate Salmonellae that cause enteric fever since the bacteria survive only in human hosts and are spread by contaminated food and water. The control and near elimination of typhoid fever in developed countries has been achieved largely because of improved sanitation, surveillance, contact tracing and successful therapy; this is also supported with vaccination. In developing countries reducing the number of cases in the general population requires the provision of safe drinking water, effective sewage disposal and hygienic food preparation (Mastroeni, 2006). In areas where the epidemic is high, mass immunization has been used successfully. Currently three vaccine alternatives are available: 1) a heat–killed, phenol extracted, whole cell vaccine, 2) Ty21a, an attenuated *S.* Typhi vaccine, 3) Vi vaccine, consisting of purified Vi polysaccharide from the bacterial capsule (Cammie and Miller, 2000). In developed countries, most cases are the result of travel to endemic areas. Travelers in such areas need to take particular care with water and food (Parry *et al.*, 2002).

Non-typhoidal *S. enterica* infections are a major public health problem world-wide and reduction of these diseases presents a serious and challenging problem. These diseases

have several animal reservoirs. In additions, the fact that a large number of different *S. enterica* serovars cause gastroenteritis in humans probably makes vaccines very difficult to realize and/or use commercially (Strugnell and Wijburg, 2006). The incidence of nontyphoidal salmonellosis continues to rise along with rates of emergence of antibiotic resistant strains and increased centralization of food production. Thus, it is important to monitor every step of food production, from handling of raw products to preparation of finished foods. In particular, with the increasing prevalence of *S.* Enteritidis in egg-laying hens, it is recommended that pasteurized eggs should be substituted for bulk-pooled eggs. The prudent use of antimicrobial agents in both humans and animals is necessary to minimize the further emergence of antibiotic resistant strains (Cammie and Miller, 2000).

The control of host-adapted *Salmonella* infections should primarily focus on the farm since most of the risk factors are on the farm. Some ways of control include preventing contact with contaminated feed and visitors, wearing house-specific clothing, thorough cleaning and disinfection of the houses, vaccination (9R *S.* Gallinarum) and rodent control programmes (Namata *et al.,* 2008). Others are vehicular and equipment control, routine screening of flocks for *Salmonella,* sourcing of chicks from farms that are *Salmonella*-free, enforcing biosecurity measures on farms and feed mills, avoiding indiscriminate medication so as to prevent the development of drug resistance both in animals and man, carrying out routine surveillance on farms so as to detect the infection as early as possible, public enlightenment of the general public and farmers particularly on the zoonotic nature of salmonellosis and also as it affects their birds and their finances, routine surveillance of antimicrobial resistance and optimization of the intestinal flora using probiotics and vaccinations to maximize the protective mechanisms of the individual birds (Namata *et al.,* 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study area included six selected states (one from each geopolitical region) of Nigeria based on information obtained from the Federal Department of Livestock and Veterinary Services (FDL&VS). The states include Ogun, Imo and Edo in the southern part of Nigeria and Plateau, Kaduna and Bauchi in the Northern part of Nigeria.

3.1.1 Ogun State

Ogun State is located in the Southwest region and shares borders with Lagos State to the south, Oyo and Osun States to the north and Ondo State to the east. Its border with the Republic of Benin, to the west, makes it an access route to the expansive market of the Economic Community of West African States (ECOWAS). To the South, it is bordered by the Atlantic Ocean and by Lagos State, the former Capital of Nigeria which still remains the commercial nerve centre of the country and indeed the West Africa Sub-region. It has a land area of 16,409.26 square kilometres and a coordinate of 7^{o}00'N and 3^{o}35'E (Ogun State, 2016).

The climate in Ogun State is also conducive for business and industrial growth with its evergreen forest vegetation and soil in the Southern part that is most suitable for the cultivation of cash and food crops like oil palm, rice, kolanut, cocoa, cotton, cassava, cocoyam and vegetables. While in the northern part of the state, is a vast grazing savannah land that is suitable for animal husbandry.

3.1.2 Plateau State

Plateau State is located between latitude 80°24'N and longitude 80°32' and 100°38' East in the North Central geopolitical zone of Nigeria with a land area of 26,899 square kilometres. Its shares boundaries with Kaduna State in the North and West, Bauchi Sate in the East, Nassarawa in the South and Taraba in the South East. The state has a tropical climate with a near temperate temperature in the northern part. It lies within the Guinea savannah vegetation zone of Nigeria with an average annual rainfall of 1200 mm. The weather and rocky terrain are very conducive for livestock and arable crop production (Plateau State, 2016).

3.1.3 Edo State

Edo State is an inland state in South South of Nigeria with a land area of 17,802 square kilometres and a coordinate of 6º30'N and 6º00'E. It is bounded in the north and east by Kogi State, in the south by Delta State and in the west by Ondo State. Agriculture is the predominant occupation of people in this State. The major cash crops produced are rubber, cocoa and palm produce. In addition, the state produces crops as yams, cassava, rice, plantains, guinea-corn and different types of fruits and vegetables (Edo State, 2016).

3.1.4 Imo State

Imo State is located in the South-Eastern region of Nigeria with an area of 5,100 square kilometres. It occupies the area between the lower River Niger and the upper and middle Imo River and its bordered by Abia State on the East, River Niger and Delta State to the West, Anambra State on the North and Rivers State to the South. The State lies within latitudes 4°45'N and 7°15'N, and longitude 6°50'E and 7°25'E with an annual rainfall

varying from 1,500 mm to 2,200 mm (60 to 80 inches). The state has an average annual temperature of 20 C (68.0 °F) and an annual relative humidity of 75%, with humidity reaching 90% in the rainy season. The dry season experiences two months of Harmattan (Imo State, 2016).

The economy of Imo State depends primarily on agriculture and commerce. The chief occupation of the people is farming with economically exploitable flora such as iroko, mahogany, obeche, bamboo, rubber tree and oil palm predominating. Other natural resources found in the state are white clay, fine sand and limestone. The state is also rich in natural resources including crude oil, natural gas, lead and zinc (Imo State, 2016).

3.1.5 Bauchi State

Bauchi State occupies a total land area of 49,119 square kilometers representing about 5.3% of Nigeria's total land mass and is located between latitudes 9° 3' and 12° 3' North and longitudes 8° 50' and 11° East. The state is bordered by seven states, Kano and Jigawa to the north, Taraba and Plateau to the south, Gombe and Yobe to the east and Kaduna to the west (Bauchi State, 2016).

Bauchi State is one of the states in the northern part of Nigeria that span two distinctive vegetation zones, namely, the Sudan savannah and the Sahel savannah. The Sudan savannah type of vegetation covers the southern part of the state. Here, the vegetation gets richer and richer towards the south, especially along water sources or rivers, but generally the vegetation is less uniform and grasses are shorter than what grows even further south, that is, in the forest zone of the middle belt (Bauchi State, 2016).

3.1.6 Kaduna State

Kaduna State is a State in Northwest region of Nigeria located on Latitude 10°31′ 23″ N, Longitude 7° 26′ 25″ E and with a land area of 46,053 square kilometres. Kaduna State is located at the centre of Northern Nigeria. It has a political significance as the former administrative headquarters of the North during the colonial era. The State shares boundaries with Niger State to the West, Zamfara, Katsina and Kano States to the north, Bauchi and Plateau States to the East and federal capital territory (FCT) Abuja and Nassarawa State to the South. Zaria is one of the major towns in Kaduna State and is very rich in history (Kaduna State, 2016).

Agriculture is the main stay of the economy of Kaduna State with about 80% of the people actively engaged in farming. Cash and food crops are cultivated and the produce includes: yam, cotton, groundnut, tobacco, maize, beans, guinea corn, millet, ginger, rice and cassava. Over 180,000 tonnes of groundnut are produced in the state annually. The major cash crop is cotton which the state has a comparative advantage in as it is the leading producer in the country. Another major occupation of the people is animal rearing and poultry farming. The animals reared include cattle, sheep, goats and pigs. Kaduna State is blessed with minerals which include clay, serpentine, asbestos, amethyst, kyannite, gold, graphite and siltimanite graphite. Kaduna State is a metropolitan as well as a cosmopolitan industrialized State with over 80 commercial and manufacturing industries. Goods ranging from carpets, textiles, reinforced concrete materials, bicycle assembly, toiletries and cigarettes are produced in the State (Kaduna State, 2016).

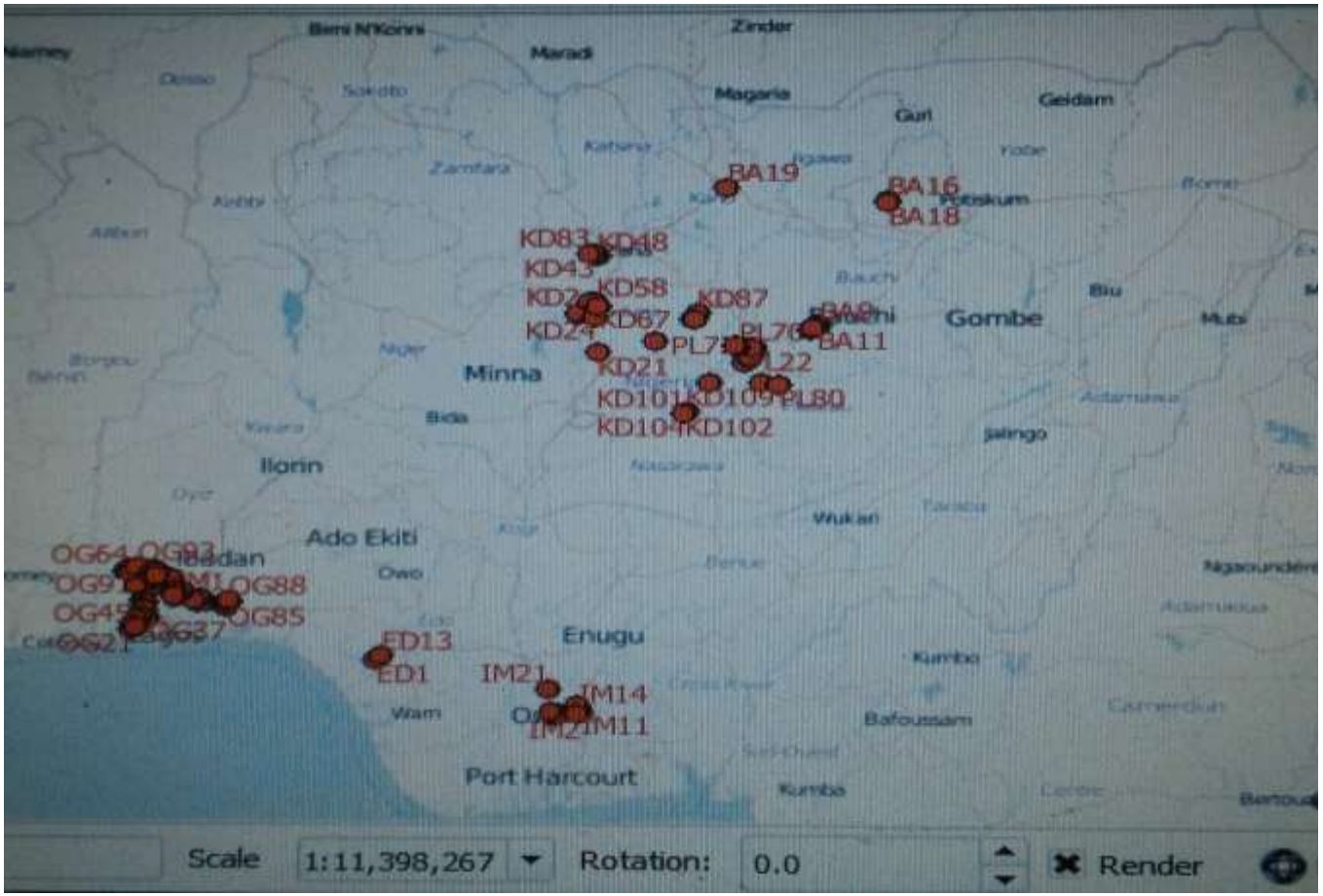


Figure 1: Map of Nigeria showing the six selected States where samples were collected from commercial poultry farms.

3.2 Sample Size

Sample size was calculated using the formula by Thrusfield, 2007.

$$\mathbf{N} = \frac{Z^2 P(1-P)}{e^2} \quad \text{where:}$$

Prevalence: 16% from environmental samples (Raufu *et al.,* 2013)

- Desired confidence level: 95%
- Allowable error (e): 4%
- N=sample size per sample type
- Z=1.96 (constant)

$$N = \frac{1.96^2 \times 0.16 \times 0.84}{0.04^2} = 322 \text{ samples}$$

The final sample size was arrived at by multiplying N with 1/(1-nr) where nr is the non response rate. $= N \times \frac{1}{(1-nr)} = \frac{N \times 1}{(1-0.1)} = \frac{322}{0.9} = 358$

Note: 358 samples of each of the five sample types (1790 samples) were analysed from 358 farms (one of each sample type per farm).

3.3 Sampling Frame

3.3.1 Criteria for selection of study areas, farms and samples

The criteria for the selection of study areas, farms and samples included the following:-

a. States were selected based on the level of commercial poultry production. Samples were collected from six selected states with the largest commercial poultry farms (one from each geopolitical zone) in Nigeria based on data obtained from Federal Department of Livestock and Veterinary Services (FDL&VS) on farm populations in the states. The states included three Southern States of Ogun, Imo, Edo and three Northern States of Plateau, Kaduna and Bauchi.

b. The sampling frame consisted primarily of farms with at least 500 laying hens. In States where the number of farms with at least 500 hens is smaller than the sample

size calculated, smaller farms were included, focusing preferably on farms with more than 200 hens.

c. Only one flock per farm was sampled. In cases of multiple age flocks, sampling focused on the oldest laying hens.

d. Selection of farms took into account the risk that certain initially selected farms may not be sampled (e.g. because of early slaughtering) and therefore a slightly bigger number initially was considered.

e. A multi-stage sampling method was adopted for farm and sample selections. The selected farms were based on a list of registered farms provided by the FLD&VS in the selected states and on the willingness of farmers to participate in the study.

f. A flock was considered to be infected and/or contaminated when at least one sample type was positive for *Salmonella*.

3.4 Sampling Procedure

3.4.1 Farm selection

The total number of farms surveyed in selected States and the farm category were calculated proportionately based on farm population in the states. In each State, the population of laying hens was stratified according to farm size. The stratification of farms was performed according to the FAO categorization of farm structures (FAO, 2006b)

Poultry keepers	Backyard or village poultry farm	1-199 birds
	Semi commercial farms	200-999 birds
Commercial Farmers	Small Scale farms	1,000-4,999 birds
	Medium Scale farms	5,000-9,999 birds
	Large Scale farms	≥10,000 birds

Note: The backyard or village poultry farms were not included in this study

3.4.2 Sample collection

Five different sample types were collected. They included dust, litters, faeces, feeds and water. Sterile tongue depressors were used to collect dust, feed and faecal samples into a sterile polyethene bag. Dust samples were collected from different places in the farm house (walls, nest boxes and cages). Litter was collected using boot swabs worn to a sterile polyethene socking on a wellington boot. Five fresh faecal droppings from the birds were collected to make one sample from each farm. Approximately 200-300 g of feed was collected from feed bags and feeding troughs at different points on the farm. Approximately 200 ml of unmedicated water samples were also collected from water tanks at different points.

All samples were immediately transported on ice to the nearest NVRI outstation laboratory for storage at 4^0C and subsequently transferred to NVRI Head Quarters in Vom, Plateau State within 24 h.

3.4.3 Risk factors determination

The determination of risk factors for *Salmonella* infections involved the administration of structured questionnaires to 358 different farmers to assess the association between management practices and general hygiene vis-a-vis *Salmonella* infections amongst commercial poultry flocks in the selected states. Consent was sought before administering the questionnaire and was administered to farm managers or the person who sees to the day-to-day management of the selected farm.

Demographic and management data collected included farm house characteristics, breed and source of day-old chicks, management of ill birds, rodent control, presence of other domestic animals on farm, feeding and watering practices, farm staff and visitors,

cleaning and disinfection procedures. The questionnaire was designed and pretested on 20 farms in and around Jos and questions adjusted as was necessary. However, data from the pretesting was not included in the final analysis (Appendix I).

3.5 Isolation and Characterization of *Salmonella* isolates using ISO 6579:2002 Method

Faecal and environmental samples were pre-enriched in buffered peptone water in a 1:10 sample to broth ratio at 37^0C for 24 h. Ten millilitres (10 ml) of Muller-Kaufmann tetrathionate broth, MKTTn (Oxoid, UK) and Rappaport-Vassiliadis, RV (Oxoid, UK) were respectively inoculated with 1.0 ml and 0.1 ml of the pre-enriched broth and incubated at 37^OC for 24 h. Also, 100 µl (about 3 drops) of the pre-enrichment broths were inoculated onto Medium Semi solid Rappaport-Vassiliadis, MRSV (Oxoid, UK) agar, and incubated at 42^0C for 24 h. Cultures from MKTTn, RV, and MRSV were plated simultaneously onto Brilliance *Salmonella* Agar, BSA (Oxoid, UK), Bismuth Sulphite Agar, BS (Oxoid, UK) and Xylose Lysine Desoxycholate, XLD (Oxoid, UK) medium. The plates were incubated at 37^OC for 24 h (ISO 6579, 2002 Annex D).

Typical *Salmonella* colonies were confirmed by biochemical assays including oxidase and indole test, gas and hydrogen sulphide (H_2S) production, citrate and urease utilization, sugar fermentation (glucose, lactose, sucrose, maltose, dulcitol, mannitol, inositol, ramnose, sorbitol, mannose, arabinose, malonate, and trehalose), lack of β-galactosidase (ONPG) and lysine decarboxylation. Duplicate samples were further analysed at the OIE *Salmonella* Reference Laboratory in Padova, Italy for confirmatory serotyping and molecular typing. Biochemically confirmed isolates were subjected to further analysis (such as serotyping, Bioplex bead assay, phage typing, MIC, PCR,

sequencing, PFGE and MLVA) at the OIE National Reference Laboratory for *Salmonella*, Instituto Zooprofilattico Sperimentale delle Venezie (IZSVe).

3.6 Serotyping

Serotyping of the *Salmonella* strains was carried out by identification of surface (LPS, O-antigens) antigens and flagella (proteins, H-antigens) antigens (Global Salm-Surv, 2004). Pure culture of *Salmonella* species previously confirmed as specified by ISO 6579 were inoculated into a tube of non selective Tryptose Agar (TA) and incubated at 37 for 18-24 h.

3.6.1 Microtitre plate method for *Salmonella* serotyping

By means of a sterile inoculating loop, test strain of *Salmonella* was inoculated into two falcon tubes containing 3 ml and 4 ml of Tryptone soy broth (TSB) respectively. The tube containing 3 ml of TSB (for detection of flagellar antigens) was incubated at 37^0C for 18-24 h while the tube containing 4 ml of TSB (for detection of somatic antigens) was incubated at 37^0C for 3 h.

3.6.1.1 Somatic antigens

At the end of incubation period at 37°C for 3 h, *Salmonella* strains within tubes containing 4 ml of TSB were inactivated by boiling for 1 h. Strains were then tested in a 96 wells round bottomed micro-titre plate using diluted sera for the detection of the related somatic antigens.

In each well of the plate 25 µl of each antisera were poured vertically at the defined dilution by using a multi pipette except for the wells of the last column which contain 25 µl of saline solution (SF) for the detection of possible auto-agglutination of the strains

(Table 3.1). Thereafter, 25 µl of the broth culture to be tested were added horizontally (1 strain per line) and Incubated at 50°C for 2 h.

Positive somatic agglutination appears as a spot more or less visibly adhered to the bottom of the well. In the case of negative reaction a white button (unagglutinated dead cells) is formed. Strains agglutinate only one of the monovalent antisera present in the plate, with the exception of the monovalent antisera 1 that is not a group specific antigen. In the case of positive results with polyvalent antisera OMB and ONNI, also concomitant reactions could be evidenced with other monovalent antisera, since the antisera OMB includes the following somatic antisera: 6, 7, 8, 11, 13, 14, 20, 22, 23, 24, 25; while the antisera ONNI agglutinates all somatic antigens from the Group A to 67.

At the end of incubation period at 37°C for 18-24 h, the tubes containing *Salmonella* bacteria cultures in 3 ml of TSB were inactivated by adding an equal volume of 1% formalin solution (diluted in saline solution). Then the tubes were kept at room temperature for 1 h. Four different polyvalent antisera (Pool 1, 2, 3, 4), called Spicer Edwards were used for this procedure. Combining the results of the agglutination seen in these four sera, it is possible to identify 35 different flagellar antigens. Table 3.2 shows the schematic interpretation of the H-antigen results. Some other antigens can be detected through the use of the polyvalent antisera HL, H1 and HE (Table 3.3). The following scheme includes all the flagellar antigens identified with the use of the polyvalent antisera.

Strains were tested in a 48-well micro-titre plate with diluted antisera for the detection of the flagellar antigens (Pool 1, 2, 3, 4, H1, HE and HL). This procedure allows for the identification of 90% of the flagellar antigens of *Salmonella* strains. The agglutination was toward the first phase, the second phase is not always evidenced in microtitre,

Table 3.1: Schematic table for the *Salmonella* somatic antigens.

B	7	8	9	10	19	15	1	20	OMB	ONNI	SF

Table 3.2: Schematic table for *Salmonella* flagella antigen detection based on agglutination reaction in four pools of antigens.

H-Antigen	Pool 1	Pool 2	Pool 3	Pool 4
a	+	+	+	-
b	+	+	-	+
c	+	+	-	-
d	+	-	+	+
e, h	+	-	+	-
G complex	+	-	-	+
i	+	-	-	-
k	-	+	+	+
r	-	+	-	+
y	-	+	-	-
z	-	-	+	+
z4 complex	-	-	+	-
z10	-	-	-	+
z29	-	+	+	-

Table 3.3: Schematic table used in the detection of some polyvalent *Salmonella* flagella antigens.

Pool	H Antigen recognized
HL	l,v; l,w; l, z13; l, z28, l, z40
H1	1,2; 1,5; 1,6; 1,7;
HE	e, h; e,n,x; e, n, z15

Table 3.4: Schematic table showing the distribution of antisera into each well

Pool 1	Pool 2	Pool 3	Pool 4	H1	HE	HL	SF

therefore it was necessary to use the phase inversion in order to block the dominant flagellar phase and allow expression of the other phase.

In each well of the plate, 250 µl of each antisera were added vertically at the defined dilution by using a multi channel pipette except for the wells of the last column which contained 250 µl of saline solution for the detection of possible self-agglutination of the strains (Table 3.4). Thereafter, 250 µl of the broth culture to be tested were added horizontally (1 strain per line) into the plate, sealed and incubated at 50°C for 1 h. Plates were kept for 1 h in a chemical cabinet before reading the results of the flagellar agglutination.

The flagellar agglutination results in whitish, irregular agglomerations in a clear suspension in the well. While in the case of negative reaction, the suspension in the well is cloudy.

3.6.2 Traditional (slide agglutination) serotyping

3.6.2.1 Somatic – O antigens typing

Detection of the O-antigen was performed by slide agglutination. A drop of Poly O antisera was placed on a slide and a small quantity of growth from the Tryptose Agar (TA) tube was mixed with the antisera using the wire loop. The mixture on the slide was then rocked gently for a maximum of 2 min for detection of clumping (positive reaction) whilst a homogenous suspension indicated a negative reaction. First, the strains were tested in the Poly O-sera-pools. Afterwards, they were then tested in the individual O-sera represented in the positive O-pool. The positively reacted (agglutination) samples were then tested for flagella antigens.

3.6.2.2 Flagellar – H antigens typing

First phase

Detection of the H-antigens was performed by slide agglutination. The identification of first phase was performed by using the same procedure described for somatic antigens but using Poly H antisera. Flagella agglutinations are less "floccular" in appearance than somatic agglutinations and sometimes form only around the edge of the drop. The strains were first tested in the Poly H-antisera-pools. Afterwards, they were then tested in the individual H-antisera represented in the positive H-pool (Global Salm-Surv, 2004).

Second phase

After identifying the first phase, the detection of the second phase was then carried out by "phase inversion", using specific antisera inhibiting the identified first phase and allowing the expression of second phase. About 5 ml of molten Swen Gard medium was poured into a small sterile Petri dish and allowed to solidify. Two drops of antiserum against the detected H- antigen was added onto the already solidified Swen Gard medium and allowed to spread over the surface of the medium. This was to block the first phase and allow the expression of the second phase. The strain was then inoculated in one spot at the centre of the Swen Gard plate and Incubated overnight at 37° C. The following day, a drop of Poly H- antigen was then placed on a glass slide and mixed with loops full of growth from the edge of the motility zone on Swen Gard medium. The slide was then rocked gently for a maximum of 2 min for appearance of agglutination. The strains were first tested in the Poly H-antisera-pools. Afterwards, they were then tested in the individual H-antisera represented in the positive H-pool (Global Salm-Surv, 2004).

For the controls, a loop full growth of the strain was mixed with saline on a glass slide. When lumps appears, the culture is autoagglutinating and no further typing was carried out, but when there was an opaque suspension, the stain was not autoagglutinating.

3.6.2.3 Serotype identification

The O- and H reactions were combined to identify the specific type (the serovar) in the "Kauffmann-White scheme". In some instances the scheme indicated other possibilities than the *S. enterica* subspp *enterica*. Therefore, in order to distinguish between the possible *Salmonella* subspecies, the necessary biochemical tests were performed as shown in the Table 3.5.

Table 3.5: Table showing the different biochemical characteristics of *Salmonella* species and subspecies.

Species	***S. enterica***						***S. bongori***
Subspecies	***enterica***	***salamae***	***arizonae***	***diarizonae***	***houtenae***	***indica***	
Characters							
Dulcitol	+	+	-	-	-	d	+
ONPG (2 h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Salicin	-	-	-	-	+	-	-
Lactose	-	-	- (75%)	+ (75%)	-	d	-

+ = 90 % or more positive reactions.
– = 90 % or more negative reactions.
d = different reactions given by different serovars.

3.6.3 Molecular determination of *Salmonella* serotypes using a Multiplex, Bead-Based Suspension Array (Bioplex)

3.6.3.1 Assay Procedure Extract and Quantify Isolate DNA

Bio-Rad InstaGene matrix was vortexed to resuspend it and 20 µl of InstaGene matrix added to each 0.2 ml PCR tube. A 1 µl loopful of fresh isolate was added to the corresponding 0.2 ml PCR tube. The tubes containing the mixture were vortexed for 15 sec and spinned in a PicoFuge for 5 sec to remove material from the tube lid. The PCR tubes were then placed in a thermocycler that has been programmed with the following conditions: 56°C for 10 min, 100°C for 5 min and 4°C until required. A 100 µl of molecular-grade water was added to each PCR tube and the PCR tubes vortexed for 15 seconds before placing in a microcentrifuge and spinned for 5 minutes. 50 µl of the supernatant (which contains the DNA) was carefully remove from each PCR tube and place into a new tube. The DNA was diluted to 100 ng/µl using molecular grade water.

3.6.3.2 Preparation and running of PCR

Three separate PCR master mixes, one for the O antigen (Table 3.6a), one for the H antigen (Table 3.6b), and one for the AT assay (Table 3.6c) were prepared for each sample. Sufficient reagents (overage) to cover for 2 additional samples for every 8 samples to be tested are also added in the calculation. A no-template control with each master mix (i.e. one for the O antigen, one for the H antigen, and one for the AT assay) was run alongside the test samples. For each sample, 23 µl of each master mix was dispensed into a separate PCR tube with each sample requiring three PCR tubes, one for each master mix. A 2 µl of the diluted DNA (100 ng/µL) was added to each PCR tube except the no-template control which was substituted with 2 µl of molecular grade water. The PCR tubes were centrifuged for 5 s to ensure all reaction components were at the bottom of the PCR tube before placing in a thermocycler using Table 3.7 conditions.

Table 3.6a: Polymerase Chain Reaction (PCR) master mix for O GROUP

Reagent	Volume per Sample	Number of Samples	Overage	Final
Qiagen HotStar Taq Master Mix	12.5 µl	8	2	125 µl
O-group Primer Pool	2.5 µl	8	2	25 µl
Molecular Grade Water	8 µl	8	2	80 µl

Table 3.6b: Polymerase Chain Reaction (PCR) master mix for H GROUP

Reagent	Volume per Sample	Number of Samples	Overage	Final
Qiagen HotStar Taq Master Mix	12.5 µl	8	2	125 µl
H-group Primer Pool	2.5 µl	8	2	25 µl
Molecular Grade Water	8 µl	8	2	80 µl

Table 3.6c: Polymerase Chain Reaction (PCR) master mix for AT.

Reagent	Volume per Sample	Number of Samples	Overage	Final
Qiagen HotStar Taq Master Mix	12.5 µl	8	2	125 µl
AT-group Primer Pool	2.5 µl	8	2	25 µl
Molecular Grade Water	8 µl	8	2	80 µl

Table 3.7: Polymerase Chain Reaction (PCR) conditions for the Bead multiplex microarray:

Cycles	Temperature	Time	Purpose
1	95°C	15 minutes	Taq polymerase activation
30	94°C 48°C 72°C	30 seconds 90 seconds 90 seconds	DNA denaturation Annealing Elongation
1	72°C	10 minutes	Final elongation

3.6.3.3 Hybridization and running of the Sample

The bead mixes were vortexed and sonicated for 10 sec to resuspend the microspheres. The O, H, and AT bead mixes supplied at 3.75X concentration to a 1X bead mix solution were diluted individually using Assay Buffer. Hence, 120 µl of stock beads was added to 330 µl of Assay Buffer to obtain 450 µl of 1X bead mix. For each sample, 45 µl of each 1X bead mixture was dispensed into a separate well on a 96-well PCR plate. Each sample requiring three wells, one for each microsphere mixture. Then 5µl of each PCR product was added to the wells that contain the corresponding microsphere mix in the PCR plate and sealed with a microseal 'A' film seal. The PCR plate was then transferred onto the thermocycler and incubated with the following conditions: 95°C for 5 min, 52°C for 30 min. The heat block on the Luminex 100/200 was then set at 52°C before preparing a SAPE solution of 6 µg/ml from the stock concentration of 1000 µg/ml using the Assay Buffer. Hence, 8.4 µl of stock SAPE was added to 1391.6 µl of Assay Buffer to obtain 1400 µl of working SAPE mix. After incubation in the thermocycler was completed, the PCR plate was removed and transferred onto the preheated brass block in the Luminex 100/200 and 50 µl of the SAPE working-concentration (6 µg/ml) solution was added to each well in the PCR plate. This was done while the PCR plate was in the preheated brass block (at 52°C) in the Luminex analyzer. The contents of each well was then pipetted up and down several times to mix and retract the PCR plate into the Luminex 100/200 after which the PCR plate was incubated on the heat block at 52°C for 10 min and the Luminex 100/200 ran to analyze the contents of the PCR plate (Luminex®, 2012; Fitzgerald *et al.,* 2007).

3.7 Phage Typing for *S.* Enteritidis and *S.* Typhimurium

One *S.* Enteritidis and five *S.* Typhimurium strains were inoculated on a Tryptose Agar (TA) plate and incubated at 37°C for 18 h to obtain isolated colonies

3.7.1 Phage for *S.* Enteritidis

Ten (10 µl) of each of the 17 stock phages were added to 990 µl of Terreno Difco Nutrient broth single strength (SSNB) in a clean 5ml glass tube. In this step each phage is diluted 1:100. Again, 100 µl of the previous dilutions were added to 900 µl of SSNB in a clean 5ml glass tube. The dilution so obtained was used for the test. 200 µl of each phage was then transferred into a 96 microtitre round bottom well plate as shown in Table 3.8.

3.7.2 Phage for *S.* Typhimurium

Ten (10 µl) of each of the 37 stock phages were added to 990 µl of SSNB in a clean 5 ml glass tube to obtain a dilution of each phage equal to 1:100. 200 µl of each phage was then transferred into a 96 microtitre round bottom well plate as shown in Table 3.9.

3.7.3 Phage inoculation

Colonies grown on TA plate were inoculated into 4 ml of Terreno Difco Nutrient Broth Double Strength (DSNB) and incubated at 37°C for 2 h (*S.* Enteritidis) and for 1.5 h (*S.* Typhimurium). The bacterial culture were then spread uniformly on Difco Nutrient Agar (DNA) plates using one plate for *S.* Enteritidis and two plates for *S.* Typhimurium. The plates were allowed to air dry for 5 min and 10 µl of each phage was then transferred onto a DNA plate using a multichannel pipette (Figure 3.1). For *S.* Typhimurium, additional (third) plate panel (c) was used. This panel was used if the first two plates gave a negative result.

Table 3.8: Schematic table showing *S. Enteritidis* phage inoculation into a microtitre plate.

1		2		3		4	
5		6		7		8	
9		10		11		12	
13		14		15		16	
17							

A 96-well microtitre plate

Table 3.9: Schematic table showing *S. Typhimurium* phage inoculation into a microtitre plate.

1		2		3		4	
5		6		7		8	
10		11		12		13	
14		15		16			
17		18		19		20	
21		22		23		24	
25		26		27		28	
29		32		35			
Additional Phages							
1		2		3		10	
10 var2		10 var3		18			

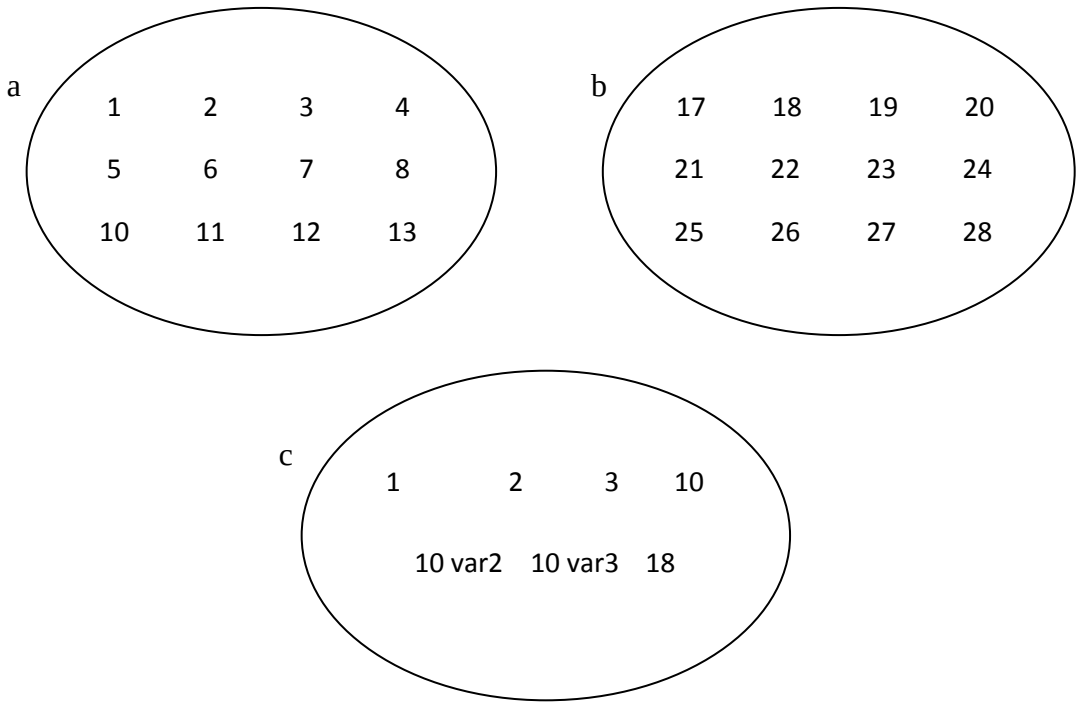


Figure 3.2: Schematic diagram of phage inoculation using three plate panels. Additional phage panel for *S.* Typhimurium (1, 2, 3, 10, 10 var2, 10 var3 and 18) was used if the first two plates give a negative result.

The plates were then incubated at 37°C for 18 h and the results read against a dark background and with incident light with the cover of the plate opened. The profiles of the results were then compared with the PHLs schemes to identify the phage type.

3.8 Antimicrobial Susceptibility Profiling

The antimicrobial susceptibility patterns of the *Salmonella* isolates were determined using the European Union Committee on Antimicrobial Sensitivity Testing (EUCAST) minimum inhibitory concentration (MIC) method (EUCAST, 2015). The isolates were tested with a panel of 14 different antimicrobials commonly used in veterinary and human medicine to treat bacterial infections, namely Sulfamethoxazole SMX (8-1024 ug/ml); Tetracycline TET (2-64 ug/ml); Azithromycin AZI (2-64 ug/ml); Trimethoprim TMP (0.25-32 ug/ml); Chloramphenicol CHL (8-128 ug/ml); Meropenem MERO (0.03-16 ug/ml); Ciprofloxacin CIP (0.015-8 ug/ml); Nalidixic acid NAL (4-128 ug/ml); Gentamicin GEN (0.5-32 ug/ml); Ampicillin AMP (1-64 ug/ml); Tigecycline TGC (0.25-8 ug/ml); Colistin COL (1-16 ug/ml); Cefotaxime FOT (0.25-4 ug/ml) and Ceftazidime TAZ (0.5-8 ug/ml).

The *Salmonella* strains were inoculated onto Tryptose agar (TA) plate and incubated at 37°C for 18-24 h. The following day, 3-4 colonies from the TA were transferred into 4ml of sterile distilled water and emulsified using a vortex. The turbidity of the inocula were adjusted to 0.5 McFarland standard (625 nm absorbance and between 0.08 and 0.13 of optical density) using a spectrophotometer. Ten microlitres (10 µl) of each culture suspension was then added to 10 mls of Mueller Hinton broth (commercial broth) and 50 µl of each mixture was then distributed into the MIC plates using an automatic Sensititre dispenser. An adhesive film was used to cover the plates and incubated at 37°C for 18 h. The plates were read the following day for bacterial growth

in each well of the plate using an automatic Sensititre plate reader. *E. coli* reference strain ATCC 25922 was used for quality control.

Interpretation of the growth inhibition and classification of isolates as sensitive, intermediate or resistant was carried out following the guidelines of the European Union Committee on Antimicrobial Sensitivity testing (EUCAST, 2015).

3.9 Detection of Antimicrobial Resistance Genes

3.9.1 Procedures for DNA extraction

A loop full of an overnight culture of each test isolate grown on TA was mixed with 300 µl of sterilized water in 1.5 ml DNAse free tubes. The mixtures were vortex and heated in the thermal heating block for 15 min before centrifuging at 12,000 rpm for 5 min to separate the supernatant (DNA) from the cell debris. The DNA was then gently aspirated into another labelled 1.5 ml DNAse free tube. The DNA was stored at -20^{o}C when analysis is to be performed the next day.

Both simplex and multiplex PCR to detect the plasmid-mediated quinolone resistance (PMQR) genes (*qnrA, qnrB, qnrS*), tetracycline resistance genes (*tetA, tetB*), chloramphenicol acetyltransferase genes (*catA1, cmlA1, floR*), sulphonamide resistance genes (*sul1, sul2, sul3*), dihydrofolate reductase genes (*dfrA10, dfrA12, dfrA5-14, dfrA7-17*), aminoglycoside modifying enzymes (*aac(3)-le, aacC4, aacC2, aadB, armA*) were carried out. The PCR procedures, master mix formula, PCR conditions and primers sequence for each of the PCR analysis are described in Appendix II. A known positive sample was used as the positive control while water was used as negative control. The PCR products (amplicons) were mixed with a loading buffer before loading unto a 2% agarose gel and ran in a gel electrophoresis chamber (110V, 2 Amp and 55 min) and

stained with 1% ethidium bromide solution. A known molecular weight marker was used to determine the molecular sizes of the amplicons. Observation and images was carried out using a UV light source. Also PCR (Appendix II) was carried out on Quinolone resistance determining region (QRDR) genes encoding for gyrase subunit A (*gyrA*) and topoisomerase IV subunit C (*parC*) and the Polymyxin reductase genes (*pmrA, pmrB*) as well as the temoxicillinase gene bla_{TEM}.

3.10 Sequencing of *gyrA, parC, pmrA* and *tem* Genes

Amplicons from the PCR of these target genes were sequenced using a sequencer. The DNA sequences were then blasted (analysed by a computer software) and compared with a reference strain to determine point mutations.

3.11 Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis (PFGE) was performed according to the European Food Safety Authority (EFSA, 2014a) protocol for typing *Salmonella* isolates. Briefly, *Salmonella* isolates were grown overnight on Tryptose agar slants and a small quantity (1 µl loop full) suspended in a cell suspension buffer (100 mM Tris; 100 mM EDTA, pH 8.0) using a disposable loop. The cell suspension was adjusted to a turbidity of 0.5-1.0 and an optical density of 600 nm using a spectrophotometer (Dade Microscan Turbidity Meter). To prepare the agarose plugs, 10 µl of Proteinase K (20 mg/ml stock) was added to 200 µl of the adjusted cell suspension. Then 200 µl of melted 1% SeaKem Gold: 1% SDS agarose was added to the 200 µl cell suspension/10 µl Proteinase K mixture and mixed gently. The mixture was immediately dispensed into wells of disposable plug molds. The bacterial cells in the agarose plugs were then lysed by treatment with a lysis

solution containing 25 µl Proteinase K (20 mg/ml stock), 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, and 1% N-lauroylsarcosine, for 2 h at 55 °C.

The plugs were then washed twice with pre-heated sterile Clinical Laboratory Reagent Water (CLRW) and four times with pre-heated TE buffer (10 mM Tris:1 mM EDTA, pH 8.0). A small slice (about 2 mm) of each plug was then digested with 50 U/sample of restriction enzyme XbaI for 2 h at 37 °C. Digested fragments were resolved in a 1% SeaKem Gold agarose gel in 0.5× Tris-Borate-EDTA (TBE) buffer using a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF Mapper, Bio-Rad Laboratories, Richmond, CA). Electrophoresis was performed at 30 kb (low MW) and 700 kb (high MW) with initial switch time of 2.16s and final switch time of 63.8 s for 22 h. Gels were cooled at 11 °C throughout the run and then stained with ethidium bromide and destained with distilled water. Banding patterns was visualized by UV and photographed. *Salmonella* Braenderup strain H9812 PulseNet standard was used as a molecular weight marker (Plate I).

According to InfoQuest^{TM} FP Software User Guide Version 5 (Biorad, 2008), fingerprinting profiles was examined by using the BioNumerics software with Dice coefficient and clustering based on the unweighted pair group method with arithmetic mean (UPGMA) to generate a dendrogram which describes the gentic correlation values as following:-

1. Dice coeff. = 100% → undistinguishable pulsotypes, were considered to be identical;
2. 99% < Dice coeff. < 85% → strictly related
3. 84% < Dice coeff. < 75% → posssibly related
4. Dice coeff. < 74% → unrelated

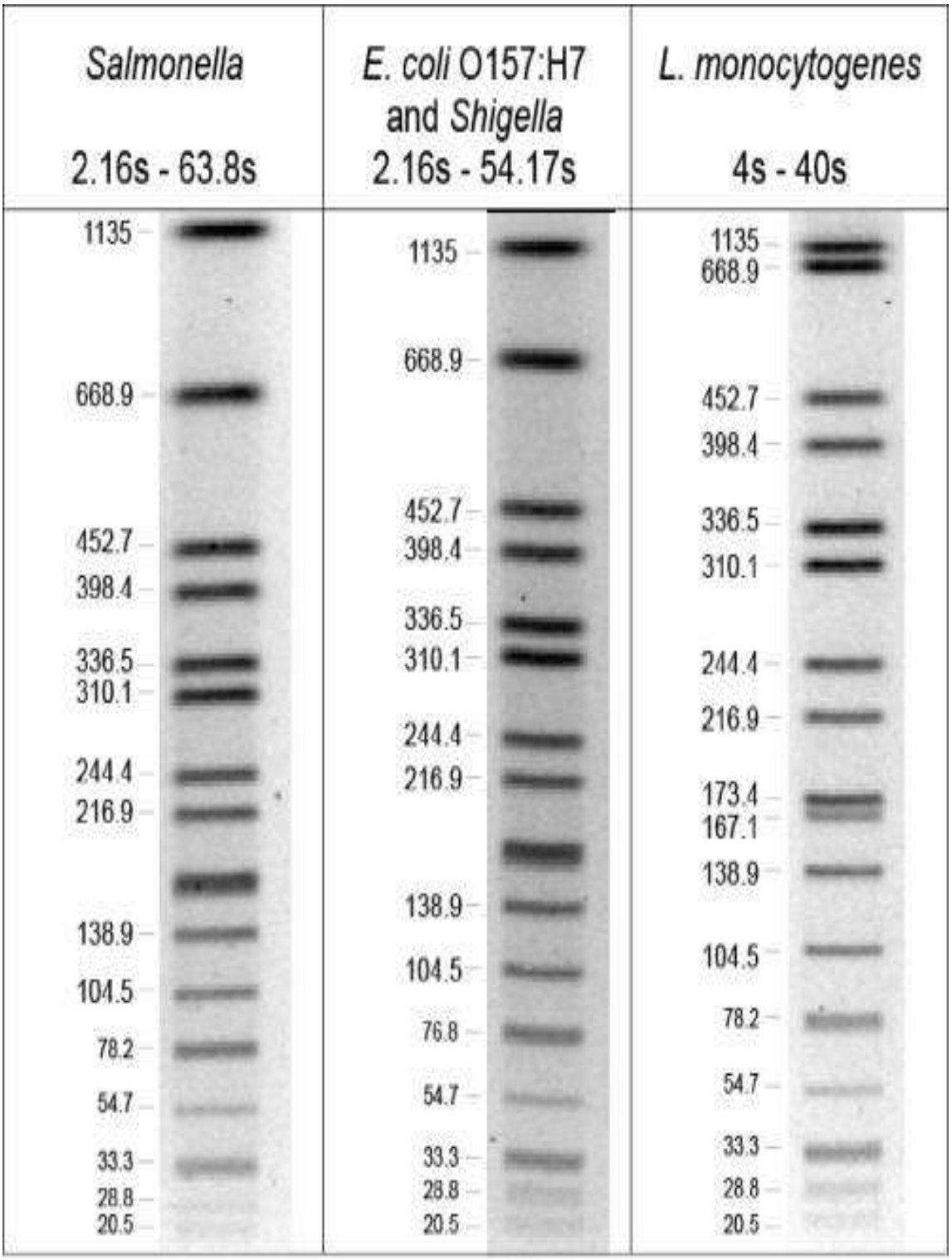


Plate I: Approximate band sizes in kilobases of the S*almonella* serotype Braenderup reference standard (H9812) restricted with XbaI and run under the PulseNet standardized electrophoresis conditions specific for each organism.

3.12 Multilocus Variable-Number Tandem-Repeat Analysis

Multilocus Variable-Number Tandem-Repeat Analysis (MLVA) was conducted by amplification of 5 loci (STTR-3, STTR-5, STTR-6, STTR-9, STTR-10) of five (5) S. Typhimurium isolates which were epidemiologically unrelated. It was performed according to the European Food Safety Authority (EFSA, 2014a) protocol for typing *Salmonella* isolates. Loop full of an overnight culture of *S.* Typhimurium grown on TA was inoculated into 200 µl of sterile water in a 1.5 ml microcentrifuge tube using a sterile disposable 1 µl loop. The tubes were capped and vortexed for 10–15 sec to disperse any clump and heated to 100°C in a heating block for 10 min. The tubes were centrifuged for 10 minutes at 10 000 rpm and the supernatant (containing the DNA) transferred into a new 1.5 ml microcentrifuge tube. Two separate primer mixes (forward and reverse) in 0.5 ml amber-coloured tubes were prepared in a sterile DNA free hood. The volume of the PCR master mix was calculated for all samples and 25 µl of the PCR master mix was then dispensed into a 96-well v-bottom PCR plate kept on PCR cooling block. Thereafter, 1 µl DNA lysate was added to each tube/well and covered with caps to avoid evaporation during PCR amplification. The plates were then spun down shortly to remove air bubbles and master mix attached to the side of the tube before running in a thermal cycler.

For each sample, the PCR product was diluted 1:85 by adding 170 µl RNase free, molecular biology grade water to the required number of PCR wells in a 96-well plate, before subsequently adding 2 µl of the PCR products. The fragment analysis master mix was then prepared. The fragment analysis master mix for one sample contains 1 µl size standard marker and 12 µl sterile RNase-free-water. For each sample, 13 µl fragment analysis master mix was aliquotted to the required number of wells in a 96-well v-bottom PCR plate. By using the 8-channel pipette, 1 µl of 1:85 diluted PCR products

was added to the appropriate columns and covered with a sealing tape in order to avoid evaporation. The sample plate was then denatured at 95°C for 3 min and cooled to 94°C on ice block for 1 min. The sample plate was then placed in a plate base and sealed carefully with the rubber septa before carrying out capillary electrophoresis (CE).

The CE analysis was prepared according to the procedures specified by Applied Biosystems. The buffer and POP7 polymer were checked to be sure they were not expired and that they were enough for the number of runs that were to be carried out. A run protocol was then created according to the machines' instruction and the machine started with the data automatically saved as .fsa files. The fragment sizes were determined by importing the .fsa files into the PeakScanner software as a new project. The correct size marker and the analysis method (sizing Default) were chosen for this project. A good quality score indication in the <Score> column was confirmed before proceeding. The correct sizes of the peaks were marked and read from the bottom pane before they were transferred to the analysis excel file 'STm MLVA allele assigning template.

In order to get a proper normalisation of the MLVA raw data, we determine the offset from the true fragment lengths that our specific hardware setup produces. This was done via a calibration run of the 33 standardisation strains. The results from the standardisation set were used to produce a correction table that showed how much each locus was compensated for in every possible fragment length. The results from the 33 standardisation strains was then inserted into the Excel document 'STm MLVA creating compensation table while the sample data was inserted in the yellow field in the sheet 'Original data'. A second sheet 'Allele data' for the five allelic genes was generated which contains the calculations that uses a floating average while a third sheet generated

is called the Correction table'. The new corrected size was then submitted to the gene bank to generate the new nomenclature for the alleles

3.13 Data Analyses

The data generated were entered into Excel 2007 (Microsoft) data base. Farm and sample prevalence of *Salmonella* in the six selected states of Nigeria were calculated. Prevalence of *Salmonella* based on sample types in the six selected states was also determined. The frequency of each of the *Salmonella* serovars isolated as well as the frequency of *Salmonella* serovars from the different sample type was determined. QGIS and Google mapping were used to determine the location and distribution of the *Salmonella* positive farms using geographical coordinates of each farm.

3.14 Statistical Analyses for Determining Risk Factors

All the data from questionnaire and laboratory test results were collated and entered into an Excel 2007 (Microsoft) data base. Possible data entry errors were cross-checked against the original questionnaire. Data were then analysed using Epi info version 7 to determine frequencies, proportions, prevalence ratios, odds ratios and chi square at 95% confidence interval. A bivariate analysis was conducted to identify risk factors associated with *Salmonella*. Any variable significant at $p < 0.05$ was included into the multivariate logistic regression model in a forward and backward step wise fashion. Variables were included or excluded from the model on the basis of the adjusted Wald test statistics, and only variables with $p < 0.05$ were retained. For variables with multiple levels, at least one variable needed to be significantly different from the baseline (reference) for the variable to be retained. Possible confounders were controlled for by including these variables into the logistic regression model.

CHAPTER FOUR

RESULTS

4.1 Results of Isolation and Prevalence of *Salmonella* based on Farm, Sample and Sample Types in Six Selected States of Nigeria

Three hundred and fifty-eight (358) farms were surveyed in six (6) states. A total of 156 farms out of the 358 farms were positive for *Salmonella*, which gives a farm prevalence of 43.6% while 240 (13.4%) samples tested positive for *Salmonella* from a total 1790 samples collected (Table 4.1). The farm to sample type isolation showed faecal samples recording the highest prevalence (16.8%). It was closely followed by litter (14.3%) and feed (13.7%). Water recorded the lowest prevalence (9.5%).

4.2 Results of Circulating *Salmonella* Serovars in Commercial Layer Farms in the Six Selected States in Nigeria

A breakdown of the 63 different serotypes isolated from the five different sample types and from the six selected states is shown in Table 4.2. *Salmonella* Kentucky was the most prevalent (16.73%) and widely distributed (83.3%) in 5 of the 6 selected states (Figure 4.1a and 4.1b) with Ogun recording the highest isolation rate (Table 4.1). Based on sample types, isolation of *S.* Kentucky was highest in faecal samples while *S.* Poona was highest in water samples (Figure 4.2). Of the 269 isolates serotyped, 267 (99.3%) belong to *S. enterica* subsp. *enterica*, 1 (0.4%) was *S. enterica* subsp. *diarizonae* and 1 (0.4%) was one was *S. bongori*. Also, out of the 267 *S. enterica* subsp. *enterica* isolates, 254 (95.1%) were serotyped in detail, while the remaining 13 isolates could not be assigned serogroups. Phage typing carried out on five of the *S.* Typhimurium revealed that they were DT1, DT161 (Plate II) and NT, while phage type of the only *S.* Enteritidis was PT1.

Table 4.1: Distribution of *Salmonella* isolates based on farm, sample types and serovars.

S/N	STATE	No. of farms sampled	No. of farms infected	No. of samples collected	No. of samples positive	Distribution of isolates based on sample type						Distribution of serovars based on sample type.					
						Litter	Dust	Faecal	Feed	Water	Total	Litter	Dust	Faecal	Feed	Water	Total
1	Ogun	110	72	550	120	24	19	31	25	21	120	30	21	35	29	21	136
3	Edo	18	3	90	3	1	1	0	1	0	3	1	1	0	1	0	3
6	Imo	26	10	130	14	1	5	2	4	2	14	1	5	2	5	2	15
8	Bauchi	21	12	105	19	5	5	5	2	2	19	5	5	6	2	2	20
9	Plateau	74	26	370	36	7	5	10	8	6	36	7	6	11	9	7	40
10	Kaduna	109	33	545	48	13	11	12	9	3	48	13	12	15	11	4	55
Total		**358**	**156 (43.6%)**	**1790**	**240 (13.4%)**	**51 (14.3%)**	**46 (12.9%)**	**60 (16.8%)**	**49 (13.7%)**	**34 (9.5%)**	**240**	**57**	**50**	**69**	**57**	**36**	**269**

Table 4.2: A breakdown of the Sixty three (63) different serotypes isolated from five different sample types and from six selected Nigerian states.

S/N	Serovars	Sample types to sample isolate identified					States to samples isolates identified						Total (%)
		Litter	Dust	Faecal	Feed	Water	Bauchi	Edo	Imo	Kaduna	Ogun	Plateau	
1.	*S.* Kentucky	8	11	13	5	8	5	0	1	12	14	13	45 (16.73%)
2.	*S.* Poona	4	2	4	4	6	0	0	1	8	11	0	20 (7.43%)
3.	*S. ent.* subsp *enterica*	3	5	2	3	0	2	0	0	0	10	1	13 (4.83%)
4.	*S.* Larochelle	3	2	4	0	3	1	0	0	5	5	1	12 (4.46%)
5.	*S.* Agama	3		5	3	0	0	0	1	0	10	0	11 (4.09%)
6.	*S.* Elisabethville	4	1	1	3	1	0			2	8		10 (3.72%)
7.	*S.* Saintpaul	0	4	4	1	0				7		2	9 (3.35%)
8.	*S.* Isangi	2	1	2	2	2					5	4	9 (3.35%)
9.	*S.* Virchow	1	3	2	2	0			2		6		8 (2.97%)
10.	*S.* Kingston	1	1	2	2	1					6	1	7 (2.60%)
11.	*S.* Liverpool	1	2	3	1	7					4	3	7 (2.60%)
12.	*S.* Berlin	1	1		3	1					6		6 (2.23%)
13.	*S.* Give		3	3			1		2		3		6 (2.23%)
14.	*S.* Telelkebir	2		3		5		2	1		2		5 (1.86%)
15.	*S.* Typhimurium	2		1	2	5	1			2	1	1	5 (1.86%)
16.	*S.* Nigeria	1	1	1	2	5			3	2			5 (1.86%)
17.	*S.* Dugbe	1	1	2							3	1	4 (1.49%)
18.	*S.* Goldcoast	2		2						3	1		4 (1.49%)
19.	*S.* Weltevreden	1	1		1	1					3	1	4 (1.49%)
20.	*S.* Jangwani	1	1	1	1	4	2			2			4 (1.49%)
21.	*S.* Muenster	1	1	1		1			1			3	4 (1.49%)
22.	*S.* Chomedey		1	2	1	4					4		4 (1.49%)
23.	*S.* Carno			1	2					3			3 (1.12%)
24.	*S.* Gaminara	1	1	1			3						3 (1.12%)
25.	*S.* Yaba			2	1						3		3 (1.12%)
26.	*S.* Namoda	1			2					3			3 (1.12%)
27.	*S.* Hadar	2		1			3						3 (1.12%)
28.	*S.* Sanktmarx	1		1	1						3		3 (1.12%)
29.	*S.* Schwarzengrund	1	1	1		3					3		3 (1.12%)
30.	*S.* Rubislaw	2		1			1			2			3 (1.12%)
31.	*S.* Kibi	1		1		1					3		3 (1.12%)
32.	*S.* Hull			1		1						2	2 (0.74%)

33.	*S.* Alachua	1	1						1		1		2 (0.74%)
34.	*S.* Ridge	1			1						2		2 (0.74%)
35.	*S.* Madjorio		1	1		2						2	2 (0.74%)
36.	*S.* Hato	1			1						1	1	2 (0.74%)
37.	*S.* Chichester	1	1									2	2 (0.74%)
38.	*S.* Corvallis	1	1							2			2 (0.74%)
39.	*S.* Agbeni				2	2					2		2 (0.74%)
40.	*S.* Canada	1							1				1 (0.37%)
41.	*S.* Bonariensis		1			1				1			1 (0.37%)
42.	*S.* Be		1								1		1 (0.37%)
43.	*S.* Mbandaka	1				1					1		1 (0.37%)
44.	*S.* Gege		1							1			1 (0.37%)
45.	*S.* Miami	1									1		1 (0.37%)
46.	*S.* Stanleyville	1									1		1 (0.37%)
47.	*S.* Millesi	1					1						1 (0.37%)
48.	*S.* Plymouth			1		1					1		1 (0.37%)
49.	*S.* Muenchen			1		1					1		1 (0.37%)
50.	*S.* Benfica		1			1					1		1 (0.37%)
51.	*S.* Urbana	1									1		1 (0.37%)
52.	*S.* Bongori		1			1					1		1 (0.37%)
53.	*S.* Durham	1						1					1 (0.37%)
54.	*S.* Livingstone		1			1					1		1 (0.37%)
55.	*S.* Lattenkamp			1		1					1		1 (0.37%)
56.	*S.* Lomita	1				1						1	1 (0.37%)
57.	*S.* Enteritidis	1										1	1 (0.37%)
58.	*S.* Mapo			1							1		1 (0.37%)
59.	*S.* Offa				1						1		1 (0.37%)
60.	*S.* Amba	1									1		1 (0.37%)
61.	*S. ent*.subsp.*diarizonae*	1				1					1		1 (0.37%)
62.	*S.* Deversoir		1			1			1				1 (0.37%)
63.	*S.* Nieukerk	1									1		1 (0.37%)
	Grand Total	**57**	**50**	**69**	**57**	**36**	**20**	**3**	**15**	**55**	**136**	**40**	**269 (100%)**

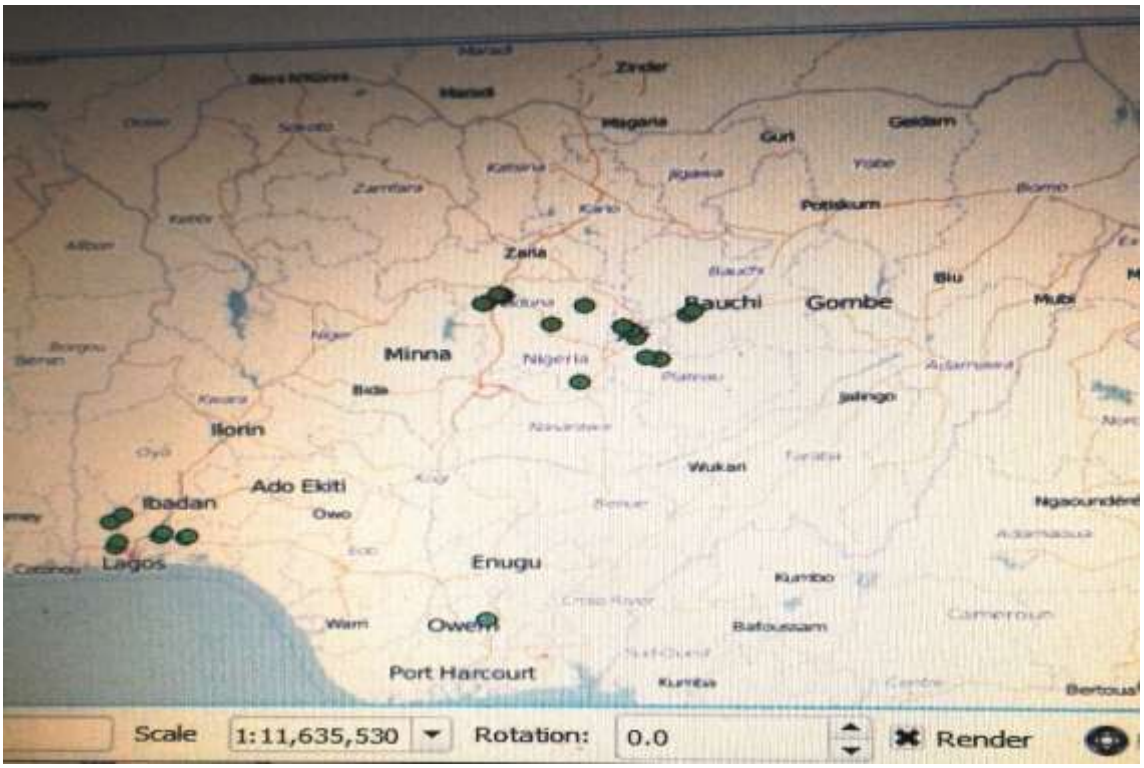


Figure 4.1a: Distribution of *Salmonella* Kentucky in six selected states of Nigeria

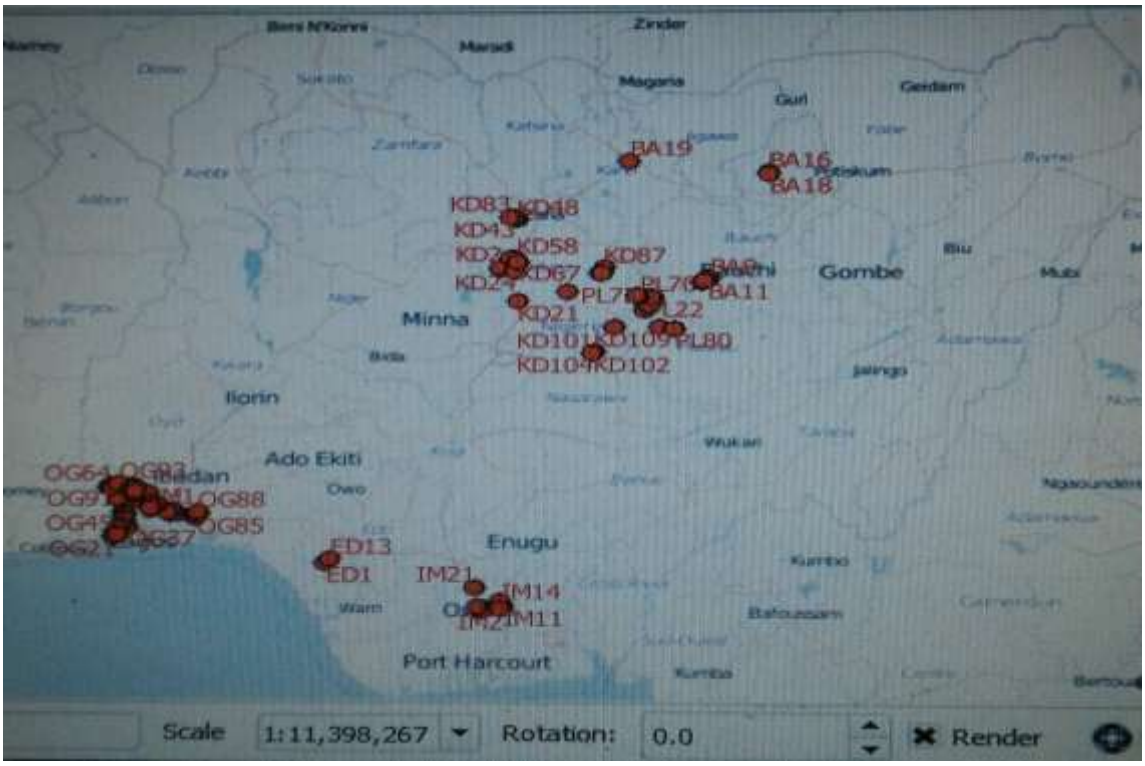


Figure 4.1b: Distribution of *Salmonella* serovars isolated in six selected states in Nigeria

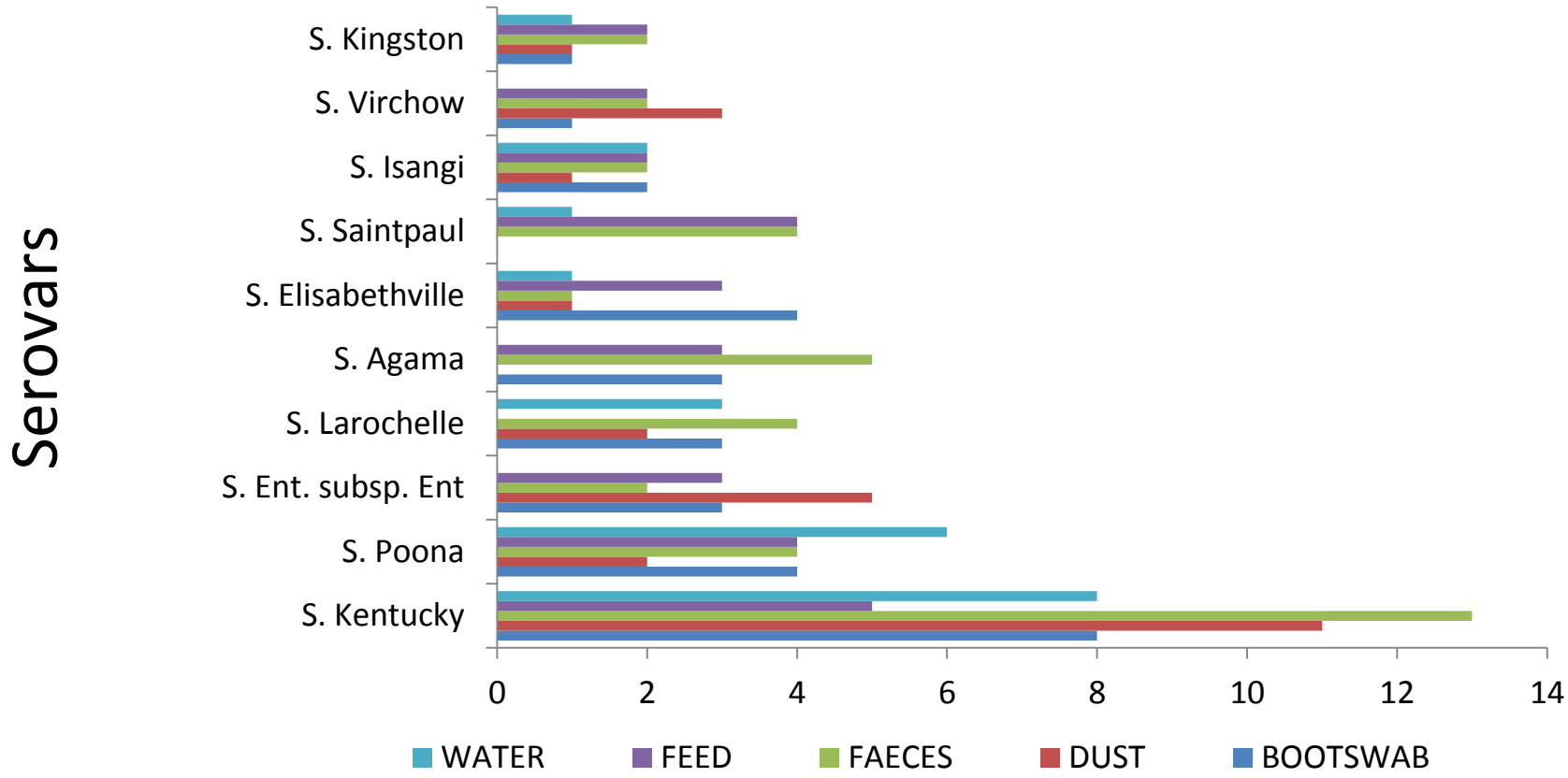


Figure 4.2: Frequencies of isolation of 10 most prevalent *Salmonella* serovars based on sample type isolated from six selected states in Nigeria.

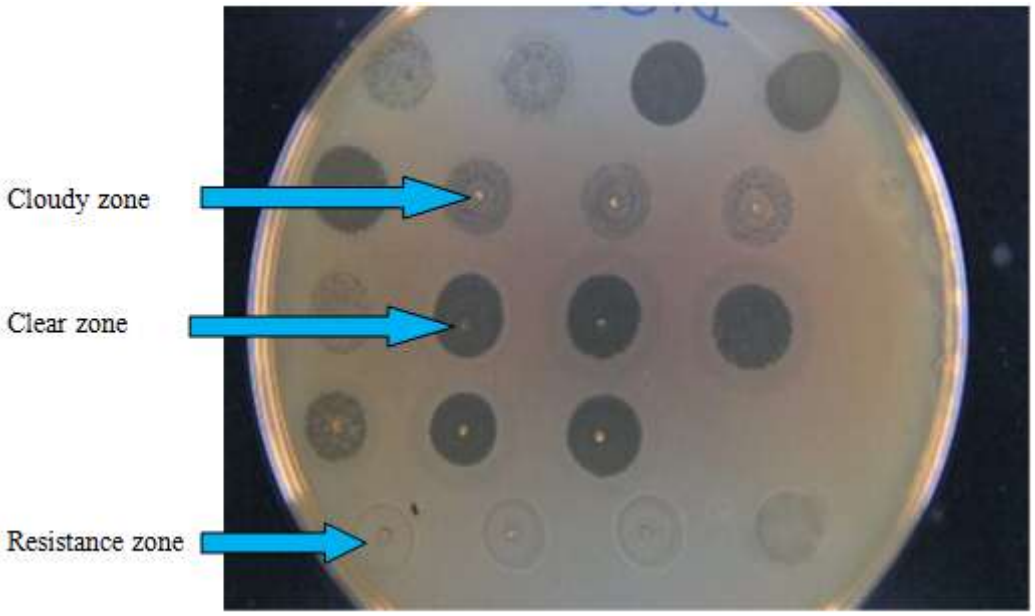


Plate II: Phage typing of *S*. Typhimurium showing some resistance zones and some clearly and cloudy opaque zones of inhibition to the different phages.

4.3 Results of the Risk Factors Associated with *Salmonella* Infection in Commercial Layer Farms in Nigeria

The results of univariate analysis on socio-demographic characteristics of poultry farmers in Nigeria (Table 4.3a), general awareness of *Salmonella* infection by farmers (Table 4.3b), sample stock details (Table 4.3c), disease management (Table 4.3d), farm biosecurity (Table 4.3e), personnel and sanitation (Table 4.3f), feed and water management (Table 4.3g) showed the frequencies and 95% CI of each variable tested.

The results of bivariate analysis on socio- demographic characteristics of poultry farmers in Nigeria (Table 4.4a), general awareness of *Salmonella* infection by farmers (Table 4.4b), sample stock details (Table 4.4c), disease management (Table 4.4d), farm biosecurity (Table 4.4e), personnel and sanitation (Table 4.4f), feed and water management (Table 4.4g) showed variables that were significantly associated ($P<0.05$) with *Salmonella* infection/contamination (boldly written). Factors found to increase the risk of *Salmonella* species occurrence were type of housing (Odds Ratio (OR) = 1.96 and 4.10; 95% CI: 1.24-3.10 and 1.52-11.07 for battery cage and deep litter/battery cage respectively), source of water (OR= 8.43; 95% CI: 1.79-39.65), additives in feed (OR = 2.11; 95% CI: 1.31-3.39), coccidiostat in feed (OR =2.18; 95% CI: 1.09-4.33), staff working in more than one farm (OR = 3.00; 95% CI: 1.19-7.55), wire meshed windows only (OR = 1.67; 95% CI: 1.03-2.71), Flock size (OR = 2.97; 95% CI: 1.18-7.46), Crate type (OR = 2.23 and 2.95; 95% CI: 1.43-3.49 and 1.26-6.94 for plastic and plastic/carton respectively), PAN membership (OR = 1.87; 95% CI: 1.22-2.87), losses suffered (OR = 2.71; 95% CI: 1.12-6.57), wearing footwear (OR = 1.87; 95% CI: 1.15-3.00) and the farmer's age (OR = 2.51 and 2.02; 95% CI: 1.41-4.45 and 1.16-3.52 for ages 31-40 and 41-50 respectively). Factors found to reduce the risk of *Salmonella*

Table 4.3a: Univariate analysis of the socio-demographic characteristics of poultry farmers in six selected states in Nigeria.

Characteristics	Frequency	Percentage	95% CI
Age group (years)			
20-30	45	12.57%	9.41%, 16.56%
31-40	96	26.82%	22.36%, 31.78%
41-50	110	30.73%	26.04%, 35.83%
41-50	1	0.28%	0.01%, 1.79%
Above 50	106	29.61%	24.98%, 34.68%
Duration in farming (Yrs)			
Less than 1 yr	37	10.34%	7.47%-14.08%
More than 10	142	39.66%	34.60%-44.96%
Up to 10	60	16.76%	13.12%-21.13%
Up to 5	119	33.24%	28.43%-38.42%
Gender			
Female	109	30.45%	25.78%-35.54%
Male	249	69.55%	64.46%-74.22%
Marital status			
Married	299	83.52%	79.17%-87.13%
Single	59	16.48%	12.87%-20.83%
PAN membership			
No	213	59.50%	54.20%-64.59%
Yes	145	40.50%	35.41%-45.80%
Primary profession			
Civil servant	59	16.48%	12.87%-20.83%
Others	12	3.35%	1.83%-5.94%
Poultry farmer	249	69.55%	64.46%-74.22%
Private sector	38	10.61%	7.71%-14.39%
Level of education			
Formal Education	348	97.21%	94.76%-98.57%
Informal/Adult Education	10	2.79%	1.43%-5.24%
Contact with animal health worker			
No	54	15.08%	11.62%-19.31%
Yes	304	84.92%	80.69%-88.38%
Age of farms			
0-5 years	169	47.21%	41.96%-52.52%
11-15 years	49	13.69%	10.39%-17.79%
15-20 years	20	5.59%	3.53%-8.64%
21-25 years	11	3.07%	1.62%-5.59%
26-30 years	14	3.91%	2.24%-6.63%
31-35 years	3	0.84%	0.22%-2.64%
6-10 years	92	25.70%	21.32%-30.61%

Table 4.3b: Univariate analysis of the general awareness of *Salmonella* infection by poultry farmers in six selected states in Nigeria.

Characteristics	Frequency	Percentage	95% CI
Awareness of fowl typhoid (Salmonellosis)			
No	63	17.60%	13.88%-22.03%
Yes	295	82.40%	77.97%-86.12%
Episode of *Salmonella* within 2 yrs			
More than twice	34	9.50%	6.76%-13.14%
None	31	8.66%	6.05%-12.19%
Not applicable	180	50.28%	44.99%-55.57%
Once	64	17.88%	14.13%-22.33%
Twice	49	13.69%	10.39%-17.79%
Disease outbreak during rearing			
No	205	57.26%	51.95%-62.42%
Yes	153	42.74%	37.58%-48.05%
Losses suffered			
Culling	7	1.96%	0.86%-4.16%
Drop in egg production	5	1.40%	0.52%-3.42%
Drop in egg production and culling	5	1.40%	0.52%-3.42%
Drop in egg production and mortality	9	2.51%	1.23%-4.89%
Drop in egg production, mortality and culling	118	32.96%	28.16%-38.13%
Mortality	7	1.96%	0.86%-4.16%
Mortality and culling	25	6.98%	4.66%-10.27%
Not applicable	182	50.84%	45.54%-56.12%
Awareness of clinical signs			
Knowledge of one sign	26	7.26%	4.89%-10.59%
Knowledge of two signs	61	17.04%	13.37%-21.43%
Knowledge of more than two signs	89	24.86%	20.54%-29.73%
Not applicable	182	50.84%	45.54%-56.12%

Table 4.3c: Univariate analysis of the sample stock details of poultry farms in six selected states in Nigeria.

Characteristics	Frequency	Percentage	95%CL
Age of birds on farms (in weeks)			
0-30	113	31.56%	26.83%-36.70%
31-60	158	44.13%	38.94%-49.45%
61-90	70	19.55%	15.65%-24.12%
91-120	17	4.75%	2.88%-7.64%
Crate type			
Carton	190	53.07%	47.76%-58.32%
Carton and Plastic	25	6.98%	4.66%-10.27%
Plastic	143	39.94%	34.87%-45.24%
Egg gathering system			
Automatic	2	0.56%	0.10%-2.23%
Manual	356	99.44%	97.77%-99.90%
Flock size			
200-999 birds	156	43.58%	38.40%-48.89%
1,000-4,999 birds	142	39.66%	34.60%-44.96%
5,000-9,999 birds	23	6.42%	4.21%-9.62%
10,000 birds and above	37	10.34%	7.47%-14.08%
Number of egg gathering per day			
More than twice	121	33.80%	28.96%-38.99%
Once	20	5.59%	3.53%-8.64%
Twice	217	60.61%	55.32%-65.67%
System of production			
Battery cage	125	34.92%	30.03%-40.13%
Deep litter	232	64.80%	59.58%-69.70%
Free range	1	0.28%	0.01%-1.79%

Table 4.3d: Univariate analysis of disease management by poultry farmers in six selected states in Nigeria.

Characteristics	Frequency	Percentage	95% CI
Use of antimicrobials in disease mgt			
No	148	41.34%	36.22%-46.65%
Not applicable	1	0.28%	0.01%-1.79%
Yes	209	58.38%	53.07%-63.51%
Disposal of dead birds			
Off-farm	190	53.07%	47.76%-58.32%
On-farm	168	46.93%	41.68%-52.24%
General vaccination			
No	12	3.35%	1.83%-5.94%
Yes	346	96.65%	94.06%-98.17%
Fowl typhoid vaccination			
No	228	63.69%	58.44%-68.63%
Yes	130	36.31%	31.37%-41.56%
Treatment of sick birds			
Others Vet Assist	10	2.79%	1.43%-5.24%
Self	68	18.99%	15.14%-23.53%
Vet	280	78.21%	73.50%-82.31%

Table 4.3e: Univariate analysis of farm biosecurity by poultry farmers in six selected states in Nigeria.

Characteristics	Frequency	Percent	95% CI
By product disposal			
Off-farm	218	60.89%	55.61%-65.94%
On-farm	140	39.11%	34.06%-44.39%
Changing dip			
Every month	5	1.40%	0.52%-3.42%
Every week	43	12.01%	8.92%-15.94%
Not applicable	247	68.99%	63.88%-73.70%
Others	63	17.60%	13.88%-22.03%
Changing room			
No	115	32.12%	27.36%-37.27%
Yes	243	67.88%	62.73%-72.64%
Farms within 1 km			
No	58	16.20%	12.62%-20.52%
Yes	300	83.80%	79.48%-87.38%
Fencing			
No	116	32.40%	27.63%-37.56%
Yes	242	67.60%	62.44%-72.37%
Floor type			
Concrete	351	98.04%	95.84%-99.14%
Others	7	1.96%	0.86%-4.16%
Foot dip			
No	249	69.55%	64.46%-74.22%
Yes	109	30.45%	25.78%-35.54%
Lavatory			
No	152	42.46%	37.31%-47.77%
Yes	206	57.54%	52.23%-62.69%
Other livestock on farm			
No	189	52.79%	47.48%-58.04%
Yes	169	47.21%	41.96%-52.52%
Presence of other livestock			
No	94	26.26%	21.84%-31.20%
Yes	264	73.74%	68.80%-78.16%
Rodent control			
No	72	20.11%	16.16%-24.72%
Yes	286	79.89%	75.28%-83.84%
Staff sharing farm			
No	336	93.85%	90.71%-96.02%
Yes	22	6.15%	3.98%-9.29%
Waste disposal			
Off-farm	224	62.57%	57.31%-67.56%
On-farm	134	37.43%	32.44%-42.69%
Wild bird access			
No	127	35.47%	30.56%-40.70%
Yes	231	64.53%	59.30%-69.44%
Wire mesh			
All openings	246	68.72%	63.59%-73.43%
Doors and windows	1	0.28%	0.01%-1.79%
Doors only	4	1.12%	0.36%-3.03%
No opening is wire-meshed	16	4.47%	2.66%-7.30%
Windows only	91	25.42%	21.06%-30.32%

Table 4.3f: Univariate analysis of personnel and sanitation of poultry farms in six selected states in Nigeria.

Characteristics	Frequency	Percentage	95% CI
Awareness of personal hygiene			
No	42	11.73%	8.68%-15.63%
Yes	316	88.27%	84.37%-91.32%
Sharing of work wears			
Always	184	51.40%	46.09%-56.67%
Never	69	19.27%	15.40%-23.83%
Sometimes	105	29.33%	24.72%-34.39%
Unit work assignment			
Always	198	55.31%	49.99%-60.51%
Never	78	21.79%	17.69%-26.50%
Sometimes	82	22.91%	18.72%-27.68%
Wearing boot when working			
Always	82	22.91%	18.72%-27.68%
Never	143	39.94%	34.87%-45.24%
Sometimes	133	37.15%	32.17%-42.41%
Work clothing			
Always	168	46.93%	41.68%-52.24%
Never	75	20.95%	16.93%-25.61%
Sometimes	115	32.12%	27.36%-37.27%
Work footwear			
Always	172	48.04%	42.78%-53.35%
Never	69	19.27%	15.40%-23.83%
Sometimes	117	32.68%	27.90%-37.85%

Table 4.3g: Univariate analysis of feed and water management by poultry farmers in six selected states in Nigeria.

Characteristics	Frequency	Percentage	95% CI
Coccidiostats in feed			
No	317	88.55%	84.68%-91.57%
Not applicable	3	0.84%	0.22%-2.64%
Yes	38	10.61%	7.71%-14.39%
Drinkers type			
Metal	56	15.64%	12.12%-19.92%
Metal and Plastic	13	3.63%	2.03%-6.28%
Plastic	289	80.73%	76.17%-84.60%
Drinkers maintenance			
Once a week	70	19.55%	15.65%-24.12%
Others	190	53.07%	47.76%-58.32%
Twice a week	98	27.37%	22.88%-32.36%
Feed additives			
No	260	72.63%	67.64%-77.12%
Not applicable	3	0.84%	0.22%-2.64%
Yes	95	26.54%	22.10%-31.49%
Feeders maintenance			
Once a week	114	31.84%	27.10%-36.98%
Others	175	48.88%	43.61%-54.18%
Twice a week	69	19.27%	15.40%-23.83%
Feeding frequency			
Once a day	31	8.66%	6.05%-12.19%
Others	9	2.51%	1.23%-4.89%
Three times a day	60	16.76%	13.12%-21.13%
Twice a day	258	72.07%	67.06%-76.59%
Source of feed			
Commercial feed	237	66.20%	61.01%-71.04%
Self compounded feed	121	33.80%	28.96%-38.99%
Water source			
Bore hole	180	50.28%	44.99%-55.57%
Bore hole and well	1	0.28%	0.01%-1.79%
Others River	12	3.35%	1.83%-5.94%
Public	15	4.19%	2.45%-6.97%
Public and bore hole	1	0.28%	0.01%-1.79%
Public and well	4	1.12%	0.36%-3.03%
Well	145	40.50%	35.41%-45.80%

Table 4.4a: Bivariate analysis of the socio- demographic characteristics of poultry farmers as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Farmer's age (years)						
20-30	18	27	1.48	0.75	0.39	0.72-3.04
31-40	51	45	**2.51**	**9.15**	**0.003**	**1.41-4.45**
41-50	53	58	**2.02**	**5.58**	**0.02**	**1.16-3.52**
Above 50	33	73	1.00 ref	0.02	0.88	0.56-1.79
Gender						
Male	110	139	1.13	0.15	0.69	0.71-1.78
Female	45	64				
Marital status						
Single	23	36	0.81	0.35	0.56	0.46-1.43
Married	132	167				
Level of education						
Formal	152	196	0.55	0.29	0.59	0.14-2.17
Informal	3	7				
Primary profession						
Civil servant	26	33	1.03	0.002	0.96	0.58-1.82
Poultry farmer	108	141	1.00 ref	0.008	0.93	0.70-1.43
Private sector	15	23	0.85	0.08	0.78	0.42-1.71
Others	6	6	1.31	.024	0.88	0.41-4.16
PAN membership						
Yes	76	69	**1.87**	**7.64**	**0.006**	**1.22-2.87**
No	79	134				
Contact with animal health and extension worker						
Yes	138	166	1.81	3.07	0.08	0.98-3.35
No	17	37				
Duration of farming						
Less than 1 year	16	21	1.04	0.006	0.94	0.50-2.16
Up to 5 years	49	70	0.96	0.003	0.96	0.58-1.57
Up to 10 years	30	30	1.37	0.74	0.39	0.75-2.50
More than 10 years	60	82	1.00 ref	0.01	0.90	0.62-1.60
Age of farms (years)						
0-5	73	96	1.00 ref	0.01	0.91	0.65-1.54
6-10	26	23	1.49	1.12	0.29	0.79-2.82
11-15	8	12	0.88	0.001	0.97	0.34-2.26
16-20	4	7	0.75	0.02	0.90	0.21-2.66
21-30	6	8	0.99	0.07	0.80	0.33-2.97
31-35	1	2	0.66	0.06	0.81	0.06-7.39

Table 4.4b: Bivariate analysis of the general awareness of *Salmonella* by poultry farmers as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Awareness on *Salmonella*						
Yes						
No	123	172	0.69	1.40	0.24	0.40-1.20
	32	31				
Farm experience						
Yes	65	105	0.67	3.00	0.08	0.44-1.03
No	90	98				
Awareness of clinical signs						
Knowledge of one sign	13	13	1.41	0.29	0.59	0.58-3.38
Knowledge of two signs	20	41	0.69	0.84	0.36	0.35-1.35
Knowledge of more than two signs	37	52	1.00 ref	0.02	0.88	0.55-1.82
Not applicable	85	97	1.23	0.45	0.50	0.74-2.06
Losses suffered						
Culling	4	3	2.41	0.56	0.46	0.52-11.30
Drop in egg production	1	4	0.45	0.06	0.81	0.05-4.18
Mortality	4	3	2.41	0.56	0.46	0.52-11.30
Culling and mortality	15	10	**2.71**	**4.16**	**0.04**	**1.12-6.57**
Drop in egg production and mortality	3	6	0.91	0.05	0.82	0.22-3.80
Drop in egg production and culling	1	4	0.45	0.06	0.81	0.05-4.18
Culling, drop in egg production and mortality	42	76	1.00 ref	0.02	0.89	0.59-1.70
Not applicable	85	97	1.59	3.18	0.07	0.99-2.55
Episode within 2 years						
Once	22	42	1.00 ref	0.03	0.85	0.48-2.07
Twice	18	31	1.11	0.004	0.95	0.51-2.41
More than twice	16	18	1.70	1.02	0.31	0.73-3.96
None	15	16	1.80	1.19	0.28	0.75-4.29
Not applicable	84	96	1.67	2.42	0.12	0.92-3.02
Disease outbreak during rearing period						
Yes	64	89	0.90	0.14	0.71	0.59-1.38
No	91	114				

Table 4.4c: Bivariate analysis of sample stock details as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Age of birds (in weeks)						
0-30	55	58	1.16	0.23	0.63	0.72-1.89
31-60	71	87	1.00 ref	0.01	0.91	0.64-1.56
61-90	24	46	0.64	1.85	0.17	0.36-1.15
91-120	5	12	0.51	0.94	0.33	0.17-1.52
Type of housing						
Battery cage	59	53	**1.96**	7.62	**0.006**	**1.24-3.10**
Deep litter	82	144	1.00 ref	0.01	0.92	0.68-1.47
Deep litter and battery cage	14	6	**4.10**	7.42	**0.007**	**1.52-11.07**
Egg gathering system						
Automatic	1	1	0.76	0.27	0.60	0.05-12.29
Manual	154	202				
Crate type						
Carton (paper)	64	126	1.00 ref	0.01	0.91	0.65-1.53
Plastic	76	67	**2.23**	**11.90**	**0.0006**	**1.43-3.49**
Plastic and Carton	15	10	**2.95**	**5.50**	**0.02**	**1.26-6.94**
Flock size						
200-999	69	87	1.25	0.71	0.40	0.79-1.99
1,000-4,999	55	87	1.00 ref	0.01	0.90	0.62-1.61
5,000-9,999	15	8	**2.97**	**4.65**	**0.03**	**1.18-7.46**
10,000 and above	16	21	1.21	0.10	0.76	0.58-2.51
Type of production						
Breeders	3	0	Undef	1.90	0.17	Undefined
Broilers	14	21	0.87	0,04	0.82	0.43-1.77
Cockerels	0	2	0.00	0.27	0.60	Undefined
Layers	136	177	1.00 ref	0.007	0.94	0.73-1.37
Layers and broilers	2	3	0.87	0.09	0.76	0.14-5.27
Daily gathering of eggs						
Once	7	13	0.54	1.06	0.30	0.21-1.41
Twice	108	109	1.00 ref	0.009	0.92	0.69-1.46
More than twice	40	81	**0.50**	**8.15**	**0.004**	**0.31-0.79**
System of production						
Battery cage	68	57	**1.99**	**8.77**	**0.003**	**1.28-3.09**
Deep litter	87	145	1.00 ref	0.009	0.92	0.67-1.46
Free range	0	1	0.00	0.07	0.79	Undefined

Table 4.4d: Bivariate analysis of disease management as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Vaccination						
Yes	147	199	0.37	1.87	0.17	0.11-1.25
No	8	4				
Fowl typhoid vaccination						
Yes	51	79	0.77	1.13	0.29	0.50-1.19
No	104	124				
Antimicrobials in disease management						
Yes	97	112	1.36	1.69	1.19	0.89-2.08
No	58	91				
Who treated						
Veterinarian	119	161	1.00 ref	0.007	0.93	0.72-1.40
Vet Assistant	4	6	0.90	0.03	0.87	0.25-3.27
Self	32	36	1.20	0.30	0.59	0.71-2.05
Disposal of dead birds						
On-farm	75	93	1.11	0.14	0.71	0.73-1.69
Off-farm	80	110				

Table 4.4e: Bivariate analysis of farm biosecurity by poultry farmers as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Farm fencing						
Yes	91	151	**0.49**	**9.16**	**0.003**	**0.31-0.77**
No	64	52				
Floor type						
Concrete	151	200	1.77	0.13	0.72	0.39-8.01
Others	4	3				
Poultry waste Mgt						
On-farm	66	68	1.47	2.72	0.10	0.96-2.27
Off-farm	89	135				
Rodent control						
Yes	121	165	0.82	0.38	0.54	0.49-1.38
No	34	38				
Presence of other livestock on farms						
Yes	77	92	1.19	0.51	0.48	0.78-1.81
No	78	111				
Presence of other poultry farms with 1km radius						
Yes	109	155	0.73	1.35	0.24	0.48-1.18
No	46	48				
Wild bird access						
Yes	104	127	1.22	0.60	0.44	0.79-1.89
No	51	76				
Presence of foot-dips						
Yes	46	63	0.94	0.03	0.87	0.60-1.48
No	109	140				
Presence of changing rooms						
Yes	101	142	0.80	0.72	0.40	0.51-1.26
No	54	61				
Lavatory on farms						
Yes	82	124	0.72	2.08	0.15	0.47-1.09
No	73	79				
Staff serving in more than one farm						
Yes	15	7	**3.00**	**4.88**	**0.03**	**1.19-7.55**
No	140	196				
Wire mesh						
Doors only	2	2	1.56	0.005	0.94	0.22-11.28
Windows only	47	44	**1.67**	**3.83**	**0.05**	**1.03-2.71**
Doors and Window	1	0	Undef	0.05	0.83	Undefined
All openings	96	150	1.00 ref	0.009	0.93	0.70-1.44
None	9	7	2.01	1.21	0.27	0.72-5.57

Table 4.4f: Bivariate analysis of personnel and sanitation of poultry farms as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Depopulation cleaning						
Disinfection only	2	1	2.57	0.04	0.83	0.23-28.64
Do not wash	0	1	0.00	0.02	0.90	Undefined
Fumigation only	0	8	0.00	4.44	0.04	Undefined
Fumigation & washing	0	2	0.00	0.28	0.60	Undefined
Fumigation,Disinfection & Washing	26	35	0.95	0.0006	0.98	0.54-1.67
Washing & Disinfection	120	154	1.00 ref	0.007	0.93	0.71-1.40
Washing only	7	2	4.49	2.81	0.09	0.91-22.01
Work clothing						
Always	85	83	1.00 ref	0.01	0.91	0.65-1.53
Sometimes	41	74	**0.54**	**5.58**	**0.02**	**0.33-0.88**
Never	31	38	0.62	2.50	0.11	0.35-1.07
Unit work assigned						
Always	91	107	1.00 ref	0.01	0.92	0.67-1.48
Sometimes	32	50	0.75	0.87	0.35	0.45-1.27
Never	32	46	0.82	0.37	0.54	0.48-1.39
Work footwear						
Always	78	94	1.00 ref	0.01	0.91	0.65-1.53
Sometimes	71	46	**1.86**	**5.96**	**0.02**	**1.15-3.00**
Never	31	38	0.98	0.007	0.93	0.56-1.72
Sharing of work wears						
Always	84	100	1.00 ref	0.01	0.92	0.66-1.51
Sometimes	41	64	0.76	0.93	0.33	0.47-1.24
Never	30	39	0.92	0.03	0.87	0.52-1.60
Wearing boot when working						
Always	36	46	0.99	0.01	0.91	0.58-1.72
Sometimes	56	77	1.00 ref	0.04	0.84	0.57-1.49
Never	63	80	0.92	0.01	0.91	0.63-1.60

Table 4.4g: Bivariate analysis of feed and water management as a factor associated with *Salmonella* infection on poultry farms in six selected states in Nigeria.

Variables	Positive	Negative	Odds ratio	Chi Square	P-value	95% CI
Additives in feed						
Yes	54	41	**2.11**	**8.84**	**0.003**	**1.31-3.39**
No	100	160	1.00 ref	0.008	0.93	0.70-1.42
Not applicable	1	2	0.80	0.17	0.68	0.07-8.94
Coccidiostats in feed						
Yes	23	15	**2.18**	**4.34**	**0.04**	**1.09-4.33**
No	131	186	1.00 ref	0.007	0.94	0.73-1.37
Not applicable	1	2	0.71	0.10	0.76	0.06-7.91
Water Source						
Bore hole	67	113	1.00 ref	0.01	0.91	0.65-1.53
Bore hole and Well	0	1	0.000	0.07	0.79	Undefined
Rivers	10	2	**8.43**	**8.13**	**0.004**	**1.79-39.65**
Public	8	7	1.93	0.91	0.34	0.67-5.55
Public and Bore hole	1	0	Undef	0.07	0.80	Undefined
Public and Well	2	2	1.69	0.00	1.00	0.23-12.25
Well	67	78	1.45	2.32	0.13	0.93-2.26
Source of feed						
Commercial feed	96	141	1.40	1.90	0.17	0.90-2.17
Compound own feed	59	62				
Drinkers type						
Metal	23	33	0.93	0.01	0.92	0.52-1.66
Plastic	124	165	1.00 ref	0.007	0.93	0.72-1.39
Metal and Plastic	8	5	2.13	1.08	0.30	0.68-6.67
Drinkers maintenance						
Once a week	27	43	0.81	0.36	0.55	0.46-1.42
Twice a week	45	53	1.09	0.06	0.81	0.67-1.77
Others	83	107	1.00 ref	0.01	0.92	0.67-1.50
Feeders maintenance						
Once a week	48	66	0.97	0.00	1.00	0.60-1.56
Twice a week	32	37	1.15	0.13	0.72	0.66-2.02
Others	75	100	1.00 ref	0.01	0.91	0.65-1.53
Feeding frequency						
Once a day	11	20	0.78	0.20	0.65	0.36-1.69
Twice a day	107	151	1.00 ref	0.008	0.93	0.70-1.42
Three times daily	32	28	1.61	2.32	0.13	0.92-2.84
Others	5	4	1.74	0.25	0.62	0.46-6.72

infection were farm fencing (OR =0.49; 95% CI: 0.31-0.77), daily gathering of eggs (OR =0.50; 95% CI: 0.31-0.79) and staff having protective clothing on the farm (OR=0.54; 95% CI: 0.33-0.88).

Using a multivariable logistic regression model (Table 4.5) to calculate the adjusted odds ratio (AOR), factors found to remain statistically significant are type of housing (AOR = 2.84; 95% CI: 1.00 - 8.03), age of farmer (AOR = 2.79; 95% CI 1.33 - 5.86), crate type (AOR = 1.74; 95% CI 1.05 - 2.88) and PAN membership (AOR= 1.67; 95% CI 1.07 - 2.62). Others are farm fencing (AOR= 0.46; 95% CI: 0.29 - 0.73) and staff having protective clothing on the farm (AOR = 0.54; 95% CI: 0.33 - 0.89). All other factors which were found to be associated with *Salmonella* infection by bivariate analysis were not statistically significant by logistic regression.

4.4 Results of Antimicrobial Susceptibility Testing of *Salmonella* Strains Isolated in the Commercial Layer Farms in the Six Selected States in Nigeria

MIC was performed on 200 isolates out of which 97 (48.5%) from 63 different farms revealed various resistance profiles. *S.* Kentucky had the highest number of resistant isolates 44 (45.4%). Five isolates of *S.* Isangi, three of *S.* Kentucky, two of *S.* Poona and one of *S.* Saintpaul were resistant to seven (7) of the fourteen (14) antibiotics tested. Resistance to ciprofloxacin was common in all the multi-drug resistant isolates (Table 4.6) while all the isolates were susceptible to Cefotaxime, Ceftazidime and Meropenem (Table 4.7). The MAR index was used to calculate the multiple antibiotic resistances of the isolates with the resistance index ranging from 0.07-0.5 (Table 4.6).

Table 4.5: Multivariate analysis (logistic regression) of the factors associated with *Salmonella* occurrence in poultry farms in six states of Nigeria.

Variables	Adjusted odds ratio	95% Confidence Interval	P-Value
Farm fencing			
Yes	**0.46**	**0.29-0.73**	**0.001**
No			
Work clothing			
Never	0.62	0.35 - 1.09	0.09
Sometimes	**0.54**	**0.33 - 0.89**	**0.02**
Always	1.00 ref	1.00ref	1.00ref
PAN membership			
Yes	**1.67**	**1.07-2.62**	**0.02**
No			
Age (years)			
20-30	**2.79**	**1.33 - 5.86**	**0.01**
31-40	1.73	0.98-3.07	0.06
41-50	0.92	0.52-1.61	0.76
Above 50	1.00ref	1.00ref	1.00ref
Crate type			
Plastic	**1.74**	**1.05 - 2.88**	**0.03**
Carton and Plastic	2.15	0.88-5.30	0.10
Carton (paper)	1.00ref	1.00ref	1.00ref
Type of housing			
Deep litters and battery cage	**2.84**	**1.00 - 8.03**	**0.05**
Battery cage	1.39	0.82-2.36	0.22
Deep litters	1.00ref	1.00ref	1.00ref

Table 4.6: Resistance profiles (antibiograms) of *Salmonella* isolates from commercial layer farms in six selected States in Nigeria.

S/N	Serotype	Antibiotic resistance pattern (s)	No. (%) of isolates	MAR index
1	*S.* Kentucky	CHL, CIP, NA	1	0.21
		CIP, GEN, NA, SMX, TET	32	0.36
		AMP, CIP, GEN, NA, SMX, TET,	8	0.43
		AMP, CHL, CIP, GEN, NA, SMX, TET	3	0.5
2	*S.* Poona	CIP, NA	1	0.14
		GEN, TMP	1	0.14
		AMP, CHL, CIP, SMX	1	0.29
		AMP, CHL, SMX, TET, TGC	1	0.36
		AMP, CHL, CIP, COL, SMX, TET, TGC	1	0.5
		AMP, CHL, CIP, NA, SMX, TET, TGC	1	0.5
3	*S.* Nigeria	CIP, NA, TET, TGC	2	0.29
4	*S.* Isangi	CIP, NA	5	0.14
		AZI, CHL, CIP, NA, SMX, TET, TMP	5	0.5
5	*S.* Saintpaul	CHL, CIP, TET,	1	0.21
		AMP, AZI, CHL, CIP, SMX, TET, TMP	1	0.5
6	*S.* Chomedey	CIP, SMX, TET	2	0.21
		CIP, NA, SMX, TET	3	0.29
7	*S.* Virchow	CIP, NA, SMX, TET, TMP	7	0.36
8	*S.* Larochelle	CIP	2	0.07
		CIP, NA	4	0.14
		CIP, GEN, NA	1	0.21
		AMP, CHL, CIP, GEN, SMX	1	0.36
9	*S.* Telelkebir	CHL, CIP, SMX, TET	2	0.29
10	*S.* Kingston	CIP, SMX, TET	1	0.21
11	*S.* Schwarzengrund	SMX, TET	3	0.14
12	*S.* Bonariensis	CHL, SMX	1	0.14
13	*S.* Lomita	CIP, NA	1	0.14
14	*S.* Muenster	CIP, NA	1	0.14
15	*S.* Chichester	SMX	1	0.07
16	*S.* Give	AZI	1	0.07
17	*S.* Liverpool	COL	1	0.07
18	*S.* Enteritidis	COL	1	0.07
Total			97	

Table 4.7: Number and percentage resistance of the most common *Salmonella* serovars to 14 antibiotics used in *Salmonella* treatment.

SEROTYPES	Percentage of the most common serotype that are resistant to each antibiotic (%)														Total
	AMP	AZI	CHL	CIP	COL	GEN	NAL	SMX	TET	TGC	TMP	MERO[b]	FOT[c]	TAZ[d]	
S. Kentucky [a,]	25 (11/44)	0 (0/44)	9.1 (4/44)	100 (44/44)	0 (0/44)	97.7 (43/44)	100 (44/44)	97.7 (43/44)	97.7 (43/44)	0 (0/44)	0 (0/44)	0 (0/44)	0 (0/44)	0 (0/44)	37.7 (232/616)
S. Poona	67 (4/6)	0 (0/6)	67 (4/6)	67 (4/6)	17 (1/6)	17 (1/6)	33 (2/6)	67 (4/6)	50 (3/6)	50 (3/6)	17 (1/6)	0 (0/6)	0 (0/6)	0 (0/6)	32.1 (27/84)
S. Nigeria	0 (0/2)	0 (0/2)	0 (0/2)	100 (2/2)	0 (0/2)	0 (0/2)	100 (2/2)	0 (0/2)	100 (2/2)	100 (2/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	28.6 (8/28)
S. Isangi	20 (2/10)	50 (5/10)	50 (5/10)	100 (10/10)	0 (0/10)	0 (0/10)	100 (10/10)	50 (5/10)	50 (5/10)	0 (0/10)	50 (5/10)	0 (0/10)	0 (0/10)	0 (0/10)	33.6 (47/140)
S. Saintpaul	50 (1/2)	50 (1/2)	100 (2/2)	100 (2/2)	0 (0/2)	0 (0/2)	0 (0/2)	50 (1/2)	100 (2/2)	0 (0/2)	50 (1/2)	0 (0/2)	0 (0/2)	0 (0/2)	35.7 (10/28)
S. Chomedey	0 (0/5)	0 (0/5)	0 (0/5)	100 (5/5)	0 (0/5)	0 (0/5)	60 (3/5)	100 (5/5)	100 (5/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)	25.7 (18/70)
S. Virchow	0(0/7)	0(0/7)	0(0/7)	100 (7/7)	0(0/7)	0(0/7)	100 (7/7)	100 (7/7)	100 (7/7)	0(0/7)	100 (7/7)	0(0/7)	0(0/7)	0(0/7)	35.7 (35/98)
S. Larochelle	12.5 (1/8)	0 (0/8)	12.5 (1/8)	100 (8/8)	0 (0/8)	25 (2/8)	62.5 (5/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	0 (0/8)	15.2 (17/112)
S. Schwarzengrund	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	100 (3/3)	100 (3/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	14.3 (6/42)
S. Telelkebir	0 (0/2)	0 (0/2)	100 (2/2)	100 (2/2)	0 (0/2)	0 (0/2)	0 (0/2)	100 (2/2)	100 (2/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	28.6 (8/28)
S. Kingston	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	21.4 (3/14)
S. Lomita	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	14.3 (2/14)
S. Muenster	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	14.3 (2/14)
S. Chichester	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	7.1 (1/14)
S. Give	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	7.1 (1/14
S. Liverpool	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	7.1 (1/14
S. Enteritidis	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	7.1 (1/14
S. Bonariensis	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	14.3 (2/14)
Total	19.6 (19/97)	7.2 (7/97)	19.6 (19/97)	89.7 (87/97)	3.1 (3/97)	47.4 (46/97)	77.3 (75/97)	75.3 (73/97)	75.3 (73/97)	5.2 (5/97)	14.4 (14/97)	0 (0/97)	0 (0/97)	0 (0/97)	14.2 (189/1330)

AMP = Ampicillin: AZI = Azithromycin: CHL = Chloramphenicol: CIP = Ciprofloxacin: COL = Colistin: GEN = Gentamicin: NAL = Nalidixic acid: SXM = Sulphamethoxazole: TET = Tetracycline: TGC = Tigecycline: TMP = Trimethoprim: MERO = Meropenem: FOT = Cefotaxime: TAZ = Ceftazidime.

[a] = All the S. Kentucky isolates are susceptible to Ciprofloxacin and Nalidixic acid.

[b] = All the isolates are resistant to Meropenem.

[c] = All the isolates are resistant to Cefotaxime.

[d] = All the isolates are resistant to Ceftazidime.

4.5 Results of Antimicrobial Resistance Target Genes of Salmonellae Isolated from Commercial Layer Farms in the Six Selected States in Nigeria

The PCR and sequencing results of resistance genes shows the presence of targeted genes (Table 4.8). The detection of *pmrA* gene (Plate III), *gyrA* gene (Plate IV), *parC* genes (Plate V) *qnr* genes (Plate VI), *tem* (Plate VII), *catA1, cmlA1* and *floR* genes (Plate VIII), *dfrA5-14* gene (Plate IX), *sul2* (Plate X), *aac(3)-le* gene (Plate XI), *tetA* gene (Plate XII) as well as mutations in the nucleotide of *parC* (Figure 4.3), *tem* (Figure 4.6) and *pmrA* (Figure 4.8) with subsequent amino acid substitution of *parC* (Figure 4.4), *gyrA* (Figure 4.5), *tem* (Figure 4.7) and *pmrA* (Figure 4.9) provides an insight to the resistance nature of these isolates to antimicrobials.

4.6 Results of Sequence Analysis to Determine Point Mutation(s) in Target Resistance Genes for Isolates that were Resistant to Quinolones, Polymixins and *Beta*-lactams

All *S.* Kentucky isolates were resistant to at least 3 classes of antibiotics. In particular, the predominant resistance pattern, GenSulTetCipNal, was observed in 60% (12/20) of the isolates. All *S.* Kentucky isolates showed resistance to fluoroquinolone and quinolone (ciprofloxacin and nalidixic acid) and this resistance was due to a double mutation of *gyrA* (Ser83 and Asp87) genes in 95% of the isolates (19/20). One isolate showed only one mutation in the *gyrA* (Ser83) gene and simultaneously having the *qnrS* and *qnrA* plasmidic genes. Only 2 isolates of *S.* Kentucky showed the presence of *qnrB* genes in addition to the chromosomal mutations occurring on *gyrA* gene. The *tetA* and *aac(3)-le* genes were present in all 20 isolates. Ninety five percent (95%) of *S.* Kentucky isolates showed *sul1* gene (19/20) and only one isolate was positive for *sul2* gene. Eight isolates showed phenotypic resistance to ampicillin with subsequent detection of bla_{TEM} gene. The following TEM variants were observed TEM1, TEM57 and TEM30 (Figure 4.7).

Table 4.8: Polymerase chain reaction (PCR) and sequence results showing the detected target genes and the amino acid substitutions (mutation) at different positions for the most common *Salmonella* serovars isolated in the six selected states in Nigeria.

Serotypes	Polymerase Chain Reaction (PCR) analysis							Sequencing		
	AMP *(tem)*	**SMX *(sul)***	**TET *(tet)***	**GEN (*aac(3)-le*)**	**TMP (dfrA)**	**CHL/FFN**	**(PMQR) *qnr***	**(QRDR) *gyrA***	**(QRDR) *parC***	**COL (*pmrA*)**
Kentucky	*tem1; tem30; tem57*	*sul1; sul2*	*tetA*	*aac(3)-le*		*cmlA1*	*qnrA; qnrB; qnrS*	Ser83→Phe; Asp87→Gly; Ser83→Tyr	-	-
Poona	*tem57*	*sul3*	*tetA*	-	-	*cmlA1*	*qnrB*	-	Thr57→Ser	T89→S; V93→G
Isangi	-	*sul2*	*tetA*	-	*dfrA5-14*	*catA1*	*qnrB; qnrS*	Leu55→Phe	-	-
Saintpaul	*tem1*	*sul2*	*tetA*	-	*dfrA5-14*	*catA1; floR*	*qnrS*	-	Thr57→Ser	-
Virchow	-	*sul1*	*tetA*	-	*dfrA5-14*	-	-	Ser83→Tyr	-	-
Chomedey	-	*sul2*	*tetA*	-	-	-	*qnrB*	-	Thr57→Ser	-
Nigeria	-	-	*tetA*	-	-	-	*qnrB*	-	Thr57→Ser	-
Larochelle	-	-	=	-	-	-	*qnrB*	-	Thr57→Ser	-
Lomita	-	-	-	-	-	-	*qnrB*	-	Thr57→Ser	-
Bonariensis	-	*sul3*	-	-	-	*cmlA1*	-	-	-	-
Muenster	-	-	-	-	-	-	*qnrA*	Ser83→Tyr	-	-

mPCR= Multiplex Polymerase Chain Reaction
QRDR= Quinolone Resistance Determining Region
PMQR= Plasmid Mediated Quinolone Resistance
PCR SUL= Polymerase chain reaction for *sul* genes (Sulphamethoxazole)
PCR GEN= Polymerase chain reaction for aac(3)-le gene (Gentamycin)
PCR TMP= Polymerase chain reaction for *dfrA5-14* gene (Trimethoprim)
PCR COL= Polymerase chain reaction for *pmrA* genes (Colistin)

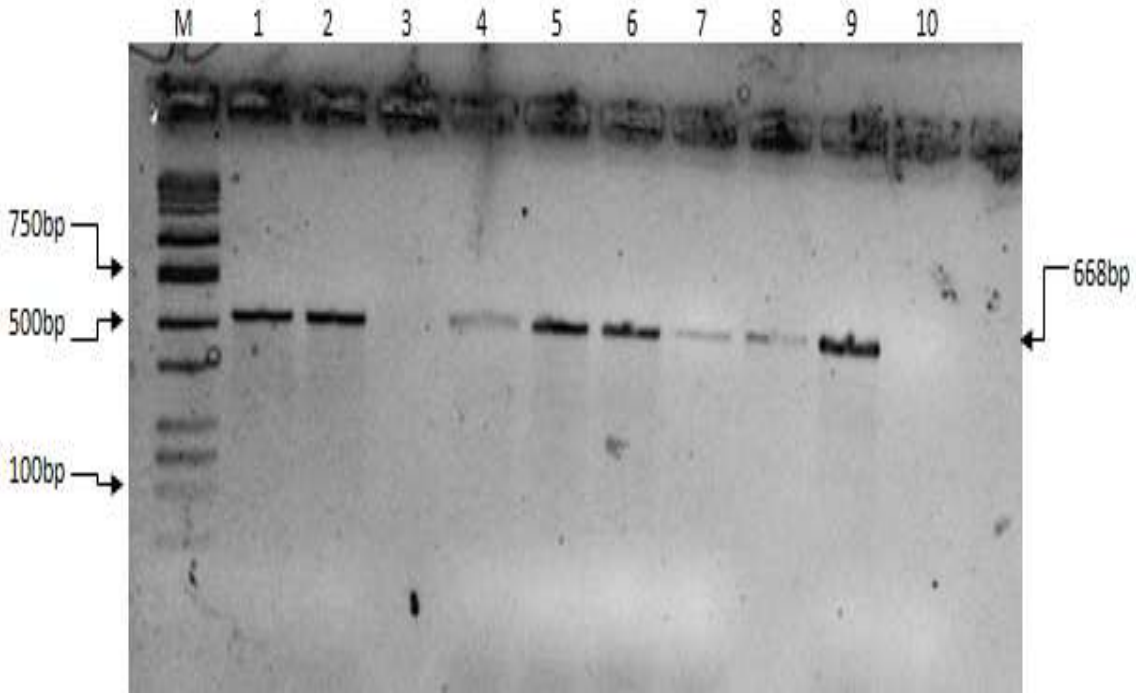


Plate III: PCR gel picture for detection of Colistin resistance gene (*pmrA*= 668bp). Lane M is the molecular weight marker, Lanes 1-8 (isolates 1804, 1852, 1845, 1846, 2522, 2528, 2530 and 2536 respectively) are test isolates. Lane 9 is a positive control and lane 10 is the negative control (water).

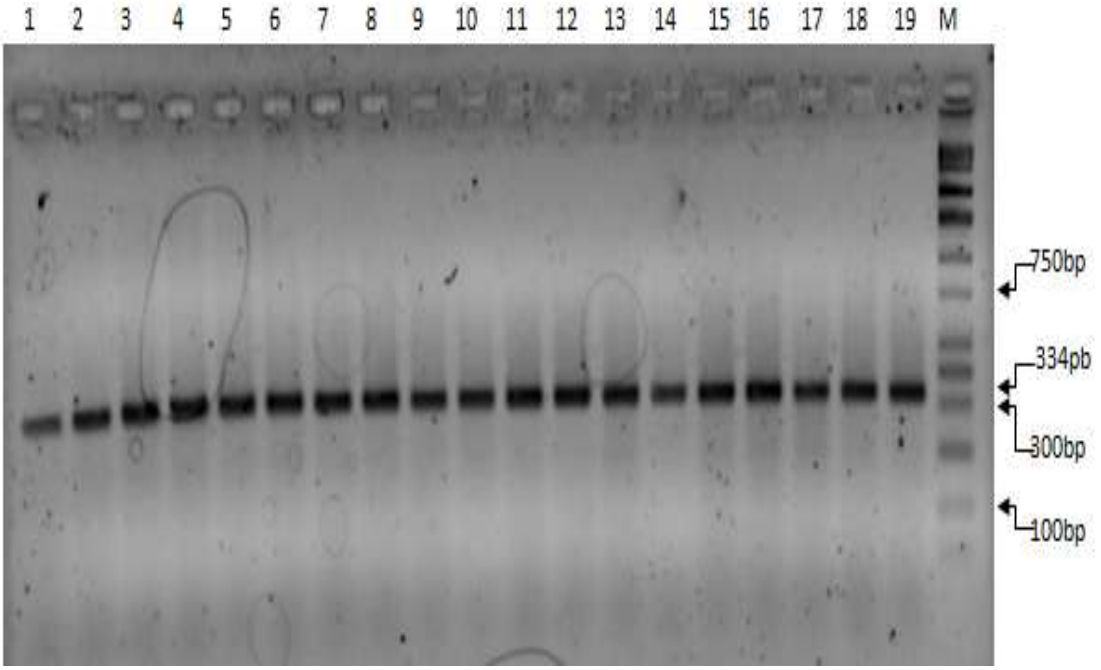


Plate IV: PCR gel picture for amplification of Quinolone resistance gene (*gyrA*= 334bp). Lane M is the molecular weight marker and lanes 1-18 (isolates 2255, 2258, 2259, 2231, 2238, 2308, 2310, 2311, 1800, 1802, 1807, 1814, 1818, 1819, 1822, 1824, 1835 and 1843 respectively) are test isolates. Lane 19 is the positive control.

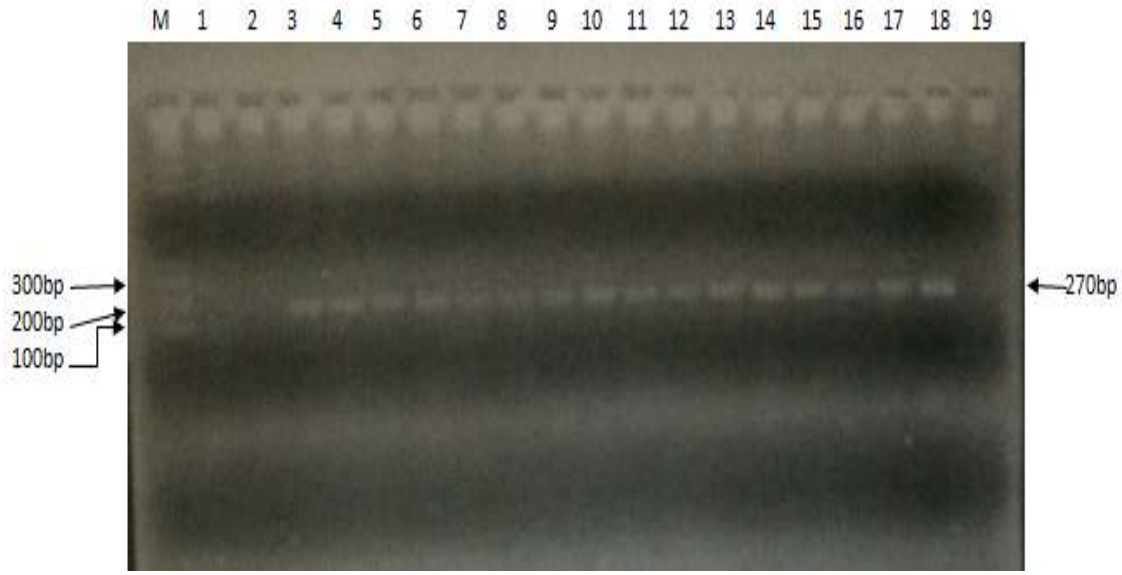


Plate V: PCR gel picture for detection of Quinolone resistance gene (*parC*= 270bp). Lane M is the molecular weight marker and lanes 1-17 (isolates 2255, 2258, 2259, 2231, 2238, 2308, 2310, 2311, 1800, 1802, 1807, 1814, 1818, 1819, 1822, 1824 and 1835 respectively) are test isolates. Lane 18 is a positive control and lane 19 is the negative control (water).

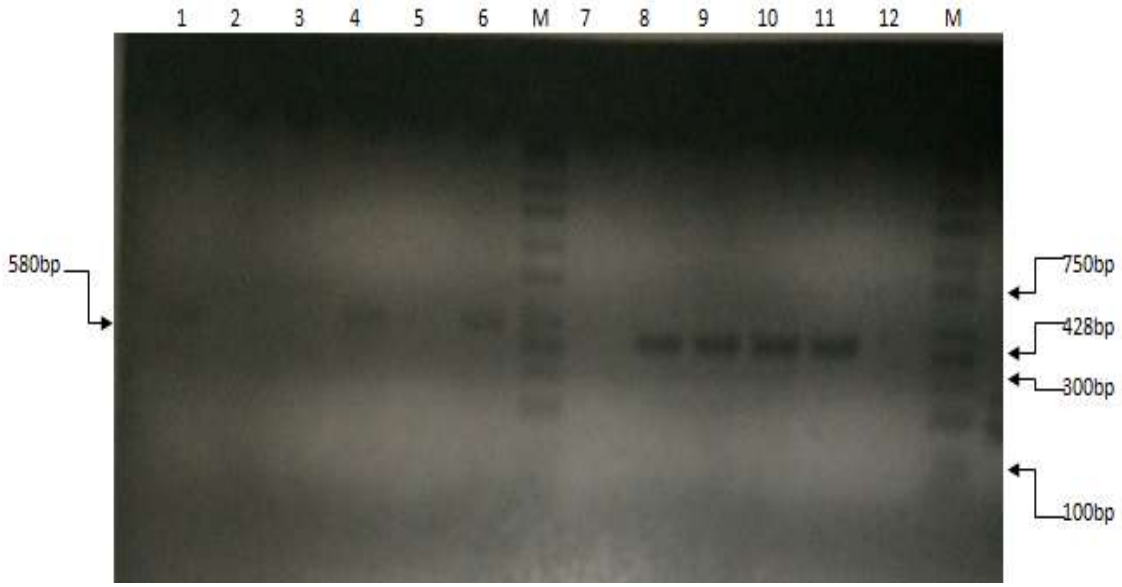


Plate VI: PCR gel picture for the detection of PMQR genes (*qnrA*=580bp and *qnrS*=428bp). Lane M is the molecular weight marker, lane 1 is *qnrA* positive control, lane 11 is *qnrS* positive control, lanes 2 and 12 are negative controls and lanes 3-10 (isolates 1800, 1802, 1807, 1814, 1818, 1819, 2225 and 1128 respectively) are test isolates.

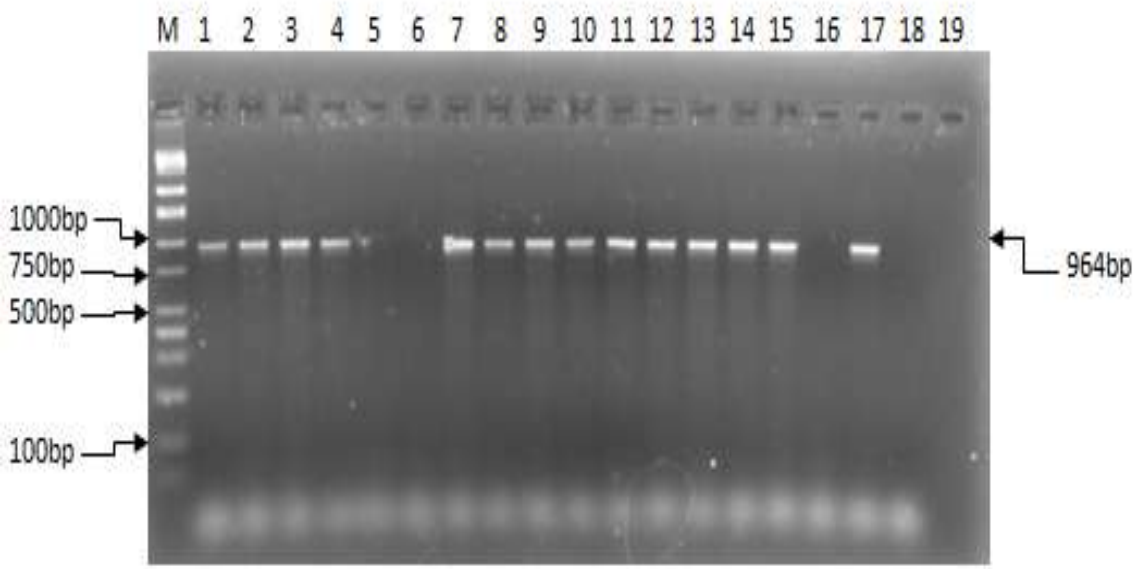


Plate VII: PCR gel picture for detection of Ampicillin resistance gene (*tem* =964bp). Lane M is the molecular weight marker and lanes 1-16 (isolates 2231, 2238, 2239, 2310, 1800, 1802, 1848, 1851, 2511, 2522, 2524, 2529, 2530, 2739, 2744 and 2528 respectively) are test isolates. Lane 17 is a positive control and lane 18 is the negative control (water).

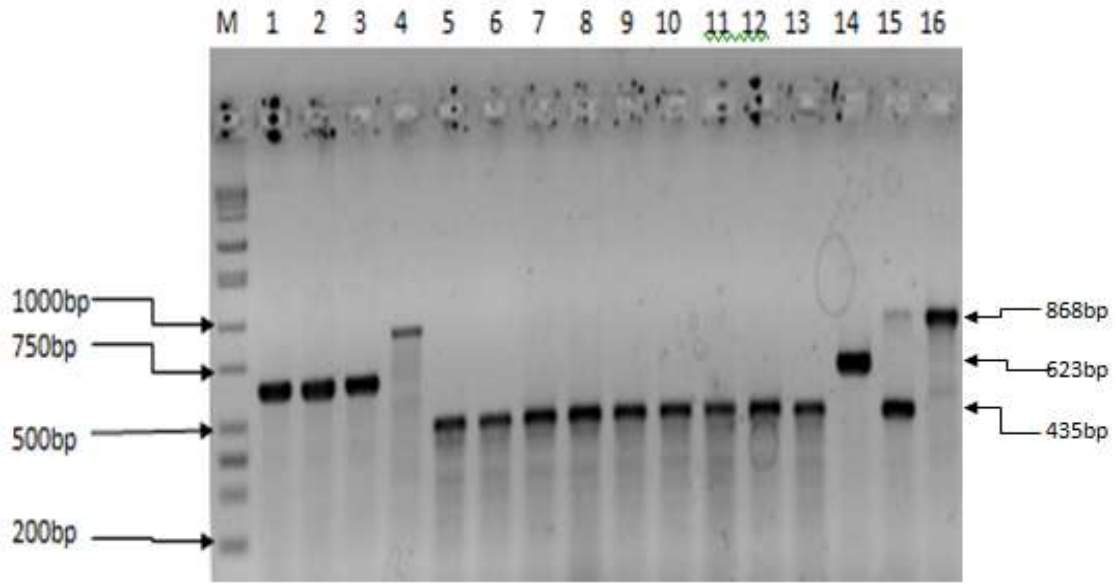


Plate VIII: Multiplex PCR gel picture for amplification of Chloramphenicol resistance genes (*catA1*=623bp, *cmlA1*= 435bp and *floR*= 868bp). Lane M is the molecular weight marker and lanes 1-13 (isolates 1800, 1802, 1851, 2498, 2511, 2522, 2524, 2526, 2529, 2530, 2739, 2744 and 2528 respectively) are test isolates. Lane 14 is *catA1* positive sample, lane 15 is *cmlA1* positive control and lane 16 is *floR* positive control.

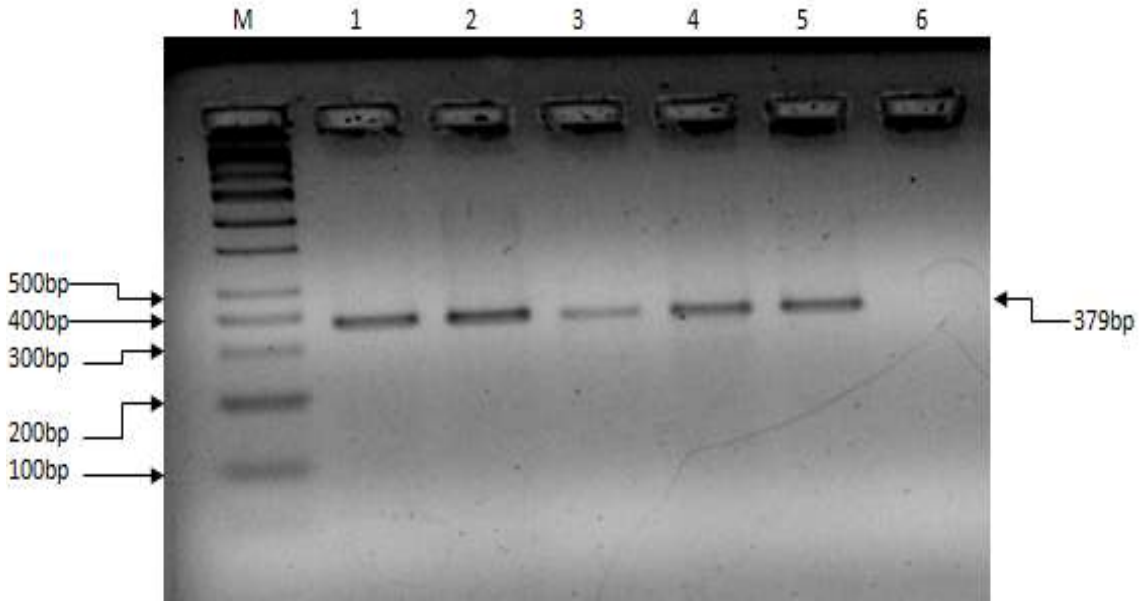


Plate IX: PCR gel picture for detection of Trimethoprim resistance (*dfrA5-14*=379bp). Lane M is the molecular weight marker and lanes 1-4 (isolates 1800, 1802, 1824 and 1835 respectively) are test isolates. Lane 5 is *dfrA5-14* positive sample and lane 6 is a negative control (water).

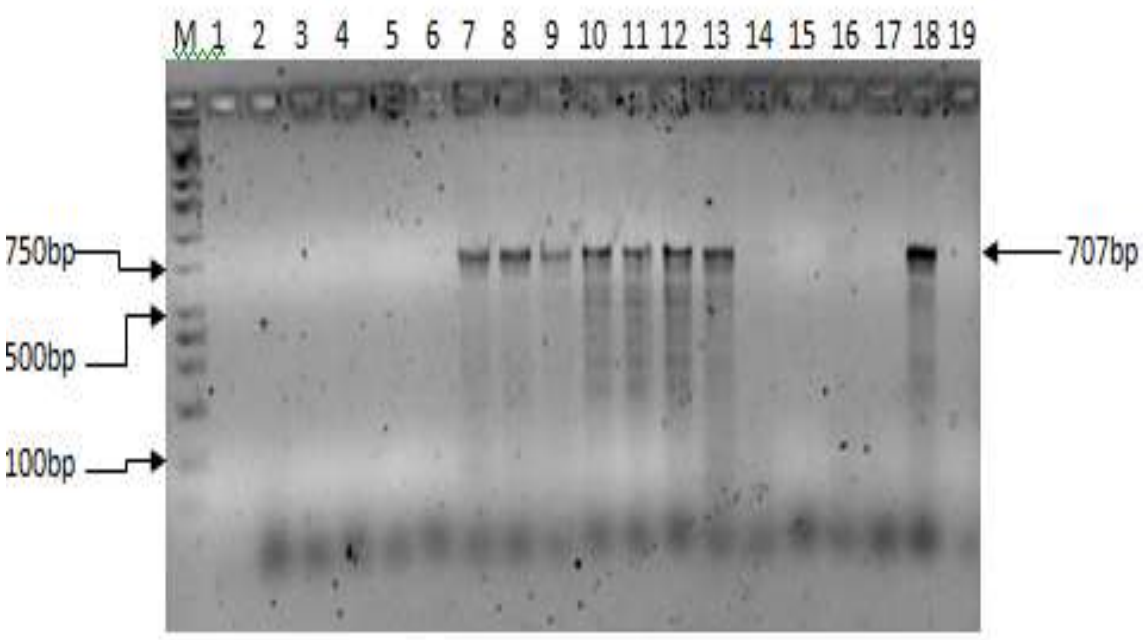


Plate X: PCR gel picture for detection of Sulphamethoxazole resistance (*sul 2*= 707bp). Lane M is the molecular weight marker and lanes 1-17 (isolates 2229, 2231, 2238, 2239, 2210, 2311, 1800, 1802, 1807, 1814, 1818, 1822, 1824, 1835, 1843, 1845 and 1847 respectively) are test isolates. Lane 18 is *sul2* positive sample and lane 19 is a negative control (water).

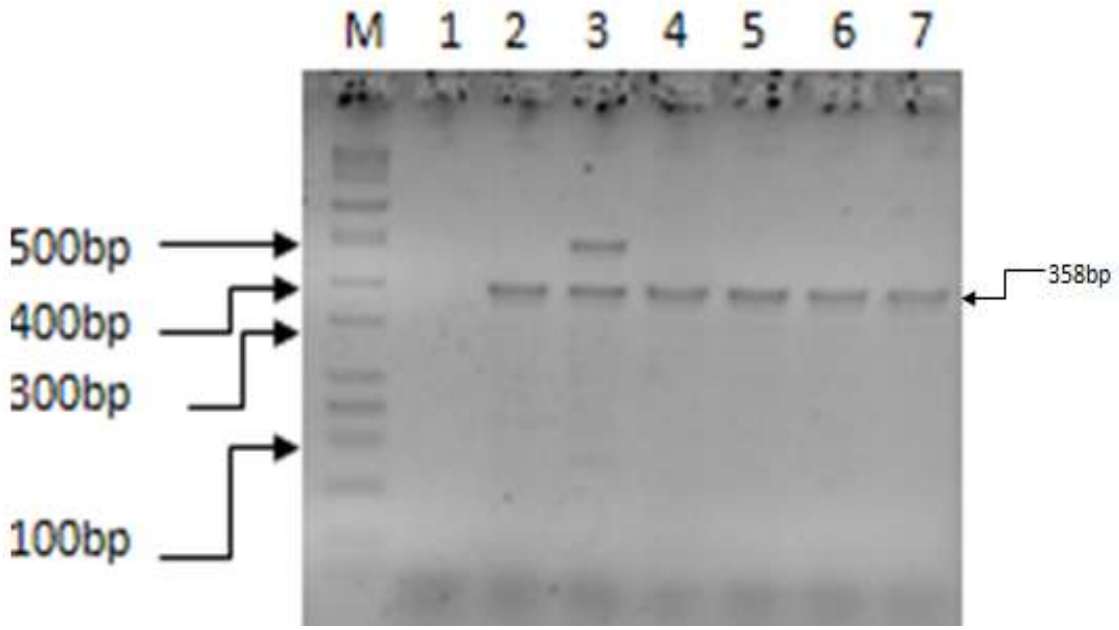


Plate XI: PCR gel picture for detection of Gentamicin resistance gene (*aac (3)-Ie* gene = 358bp). Lane M is the molecular weight marker and lane 1 is negative control. Lane 2 is the positive control and lanes 3-7 (isolates 2229, 2231, 2238, 2239 and 2310 respectively) are the test isolates.

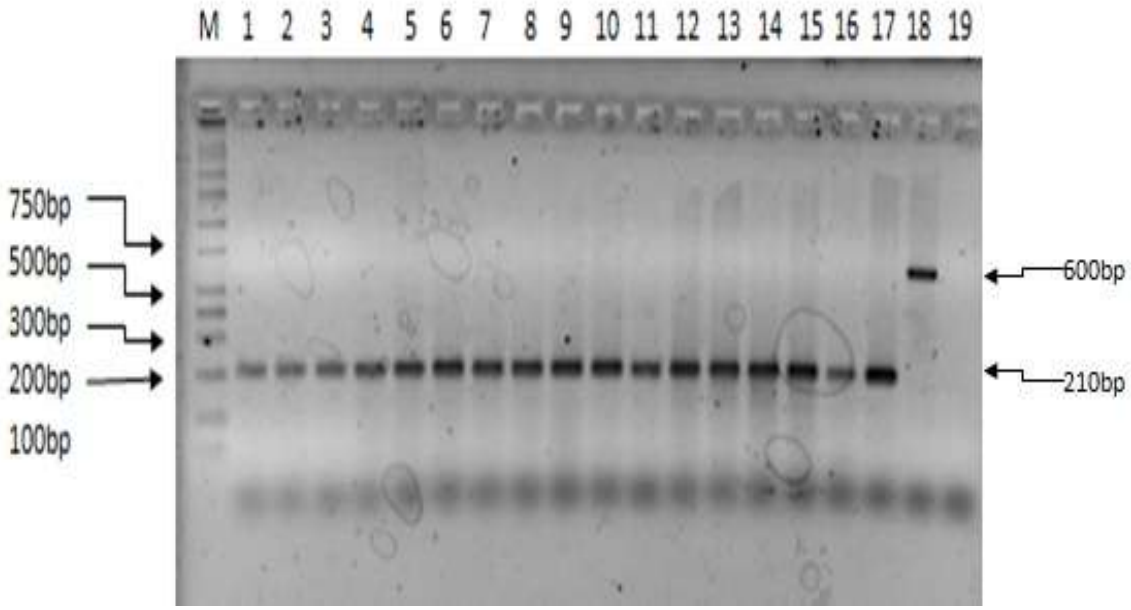


Plate XII: Multiplex PCR gel picture for the detection of Tetracycline resistance genes (*tetA*=210bp and *tetB*=600bp). Lane M is the molecular weight marker and lanes 1-16 (isolates 2229, 2231, 2238, 2239, 2210, 2311, 1800, 1802, 1807, 1814, 1818, 1822, 1824, 1835, 1843 and 1845) are test isolates. Lane 17 is *tetA* gene positive control, lane 18 is *tetB* gene positive control and lane 19 is a negative control (water).

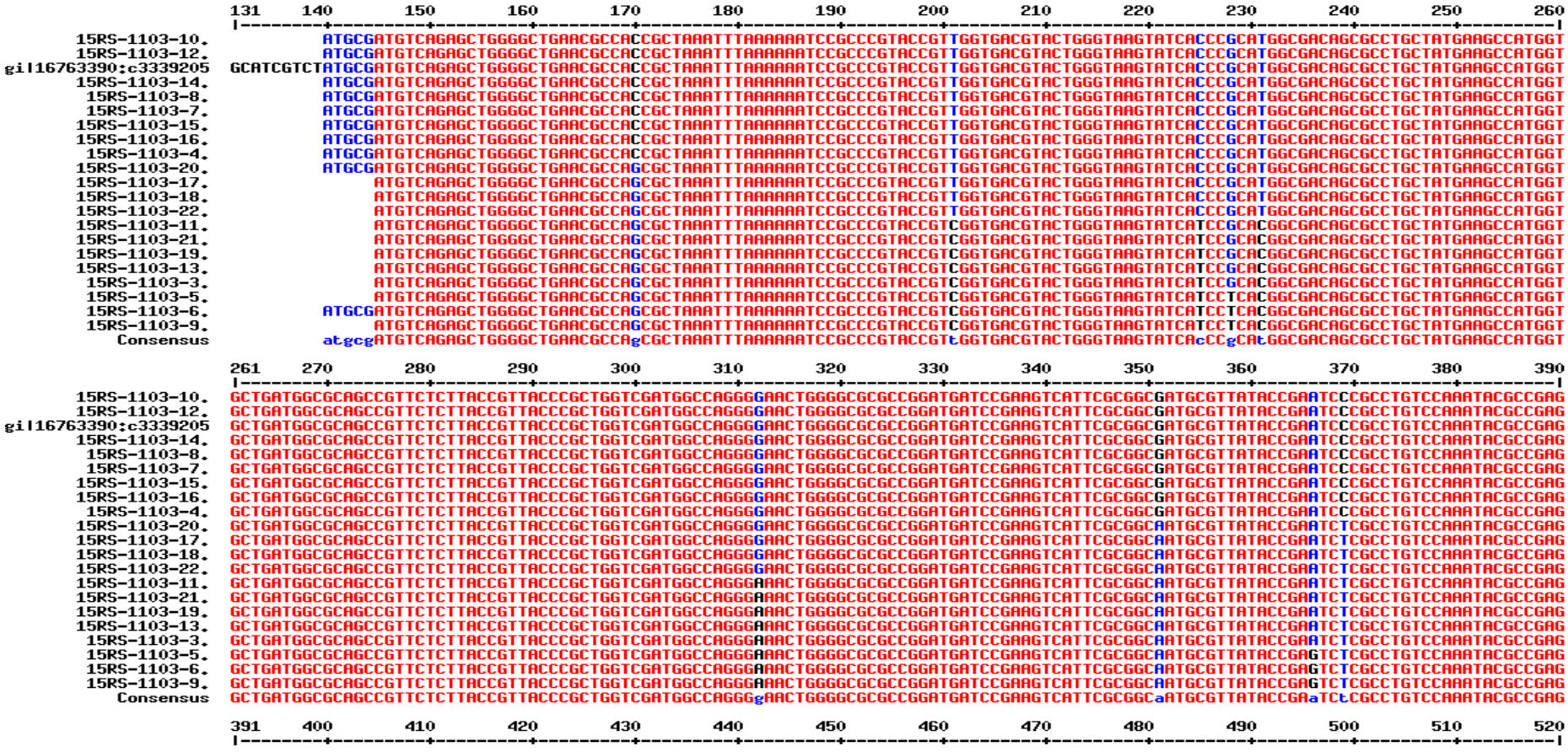


Figure 4.3: Nucleotide sequence of a portion of *parC* gene showing substitution of (C) with (G), (T) with (C), (C) with (T), (G)with (T), (T) with (C), (G) with (A), (G) with (A), (A) with (G) and (C) with (T) at positions 170, 201, 225, 228, 231, 312, 351, 366, 369. C = Cytosine, G = Guanine, T = Thymine, A = Adenine.

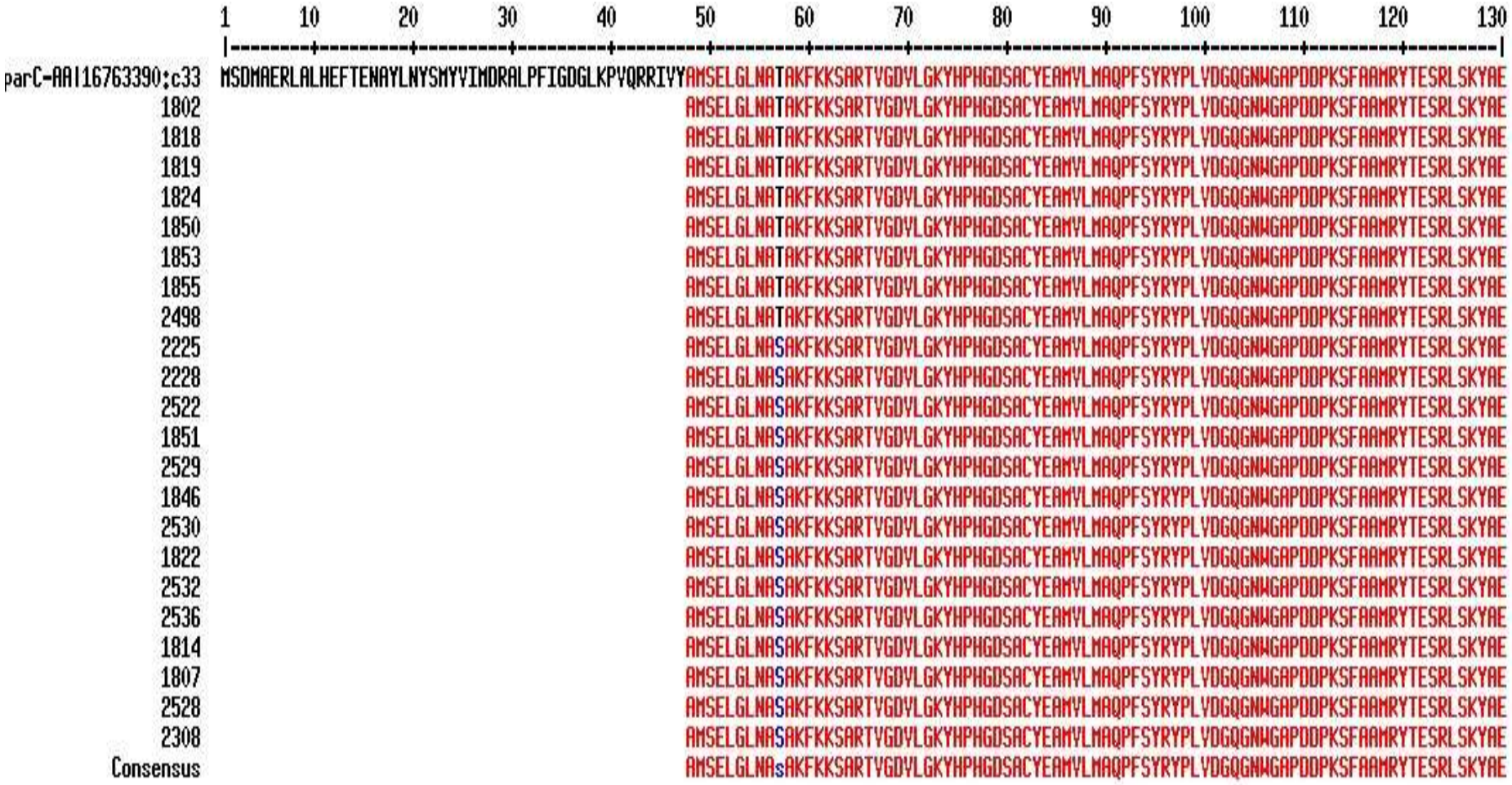


Figure 4.4: Amino acid sequence of a portion of *parC* gene showing substitution of threonine (T) with serine (S) at position 57.

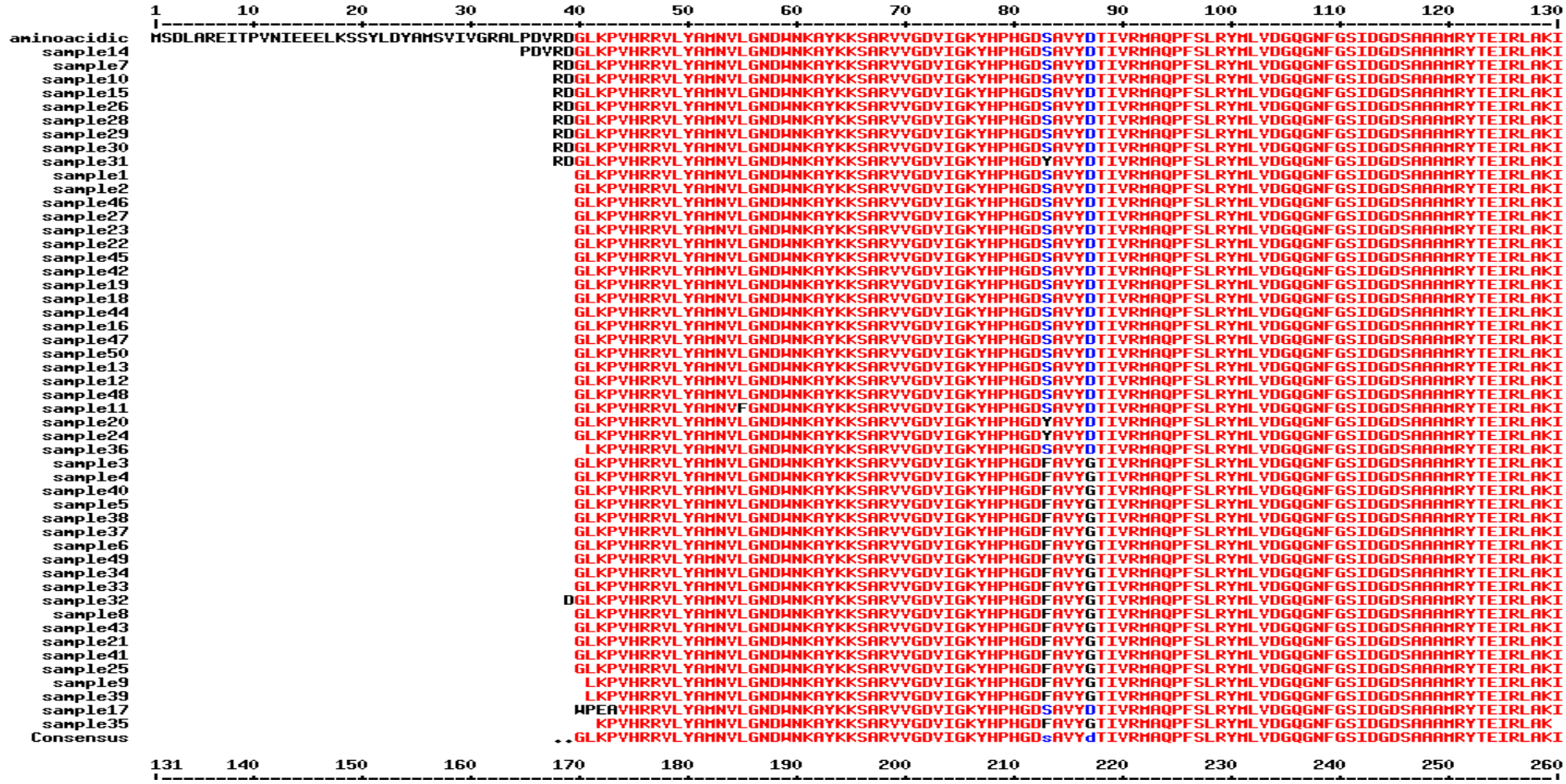


Figure 4.5*:* Amino acid sequence of a portion of *gyrA* gene showing substitution of L for F, S for Y and F, and D for G at positions 55, 83 and 87 respectively. L = Leucine, F = Phenylalanine, S = Serine, Y = Tyrosine, D = Aapartic acid, G = G.

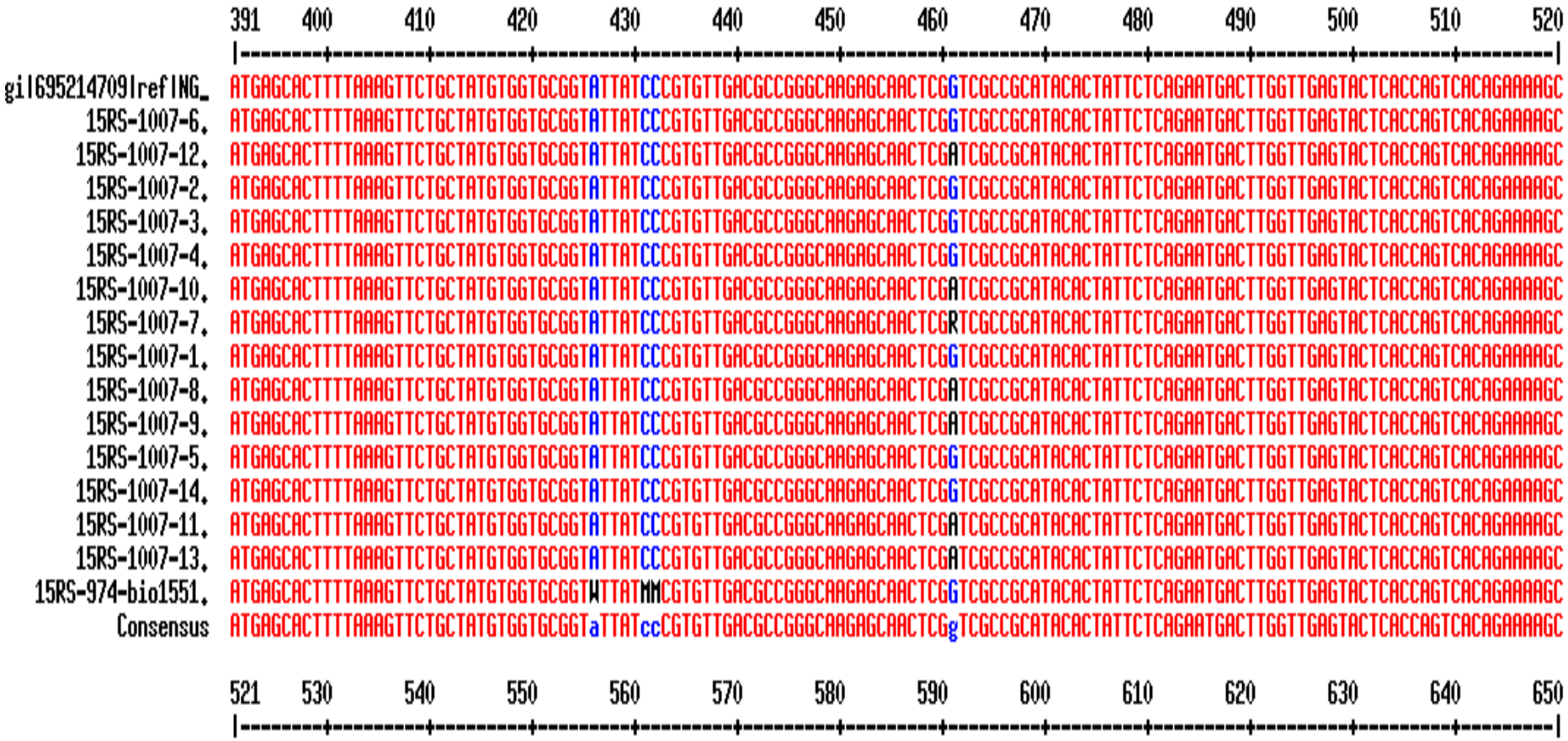


Figure 4.6: Nucleotide sequence of a portion of *tem* gene showing substitution of (A) with (W) at position 426, (C) with (M) at position 431,432, and G with A and R at position 461. A = Adenine, C = Cytosine, G = Guanine, R = Purine (A or G), M = Amino (A or C), W = Weak A or T.

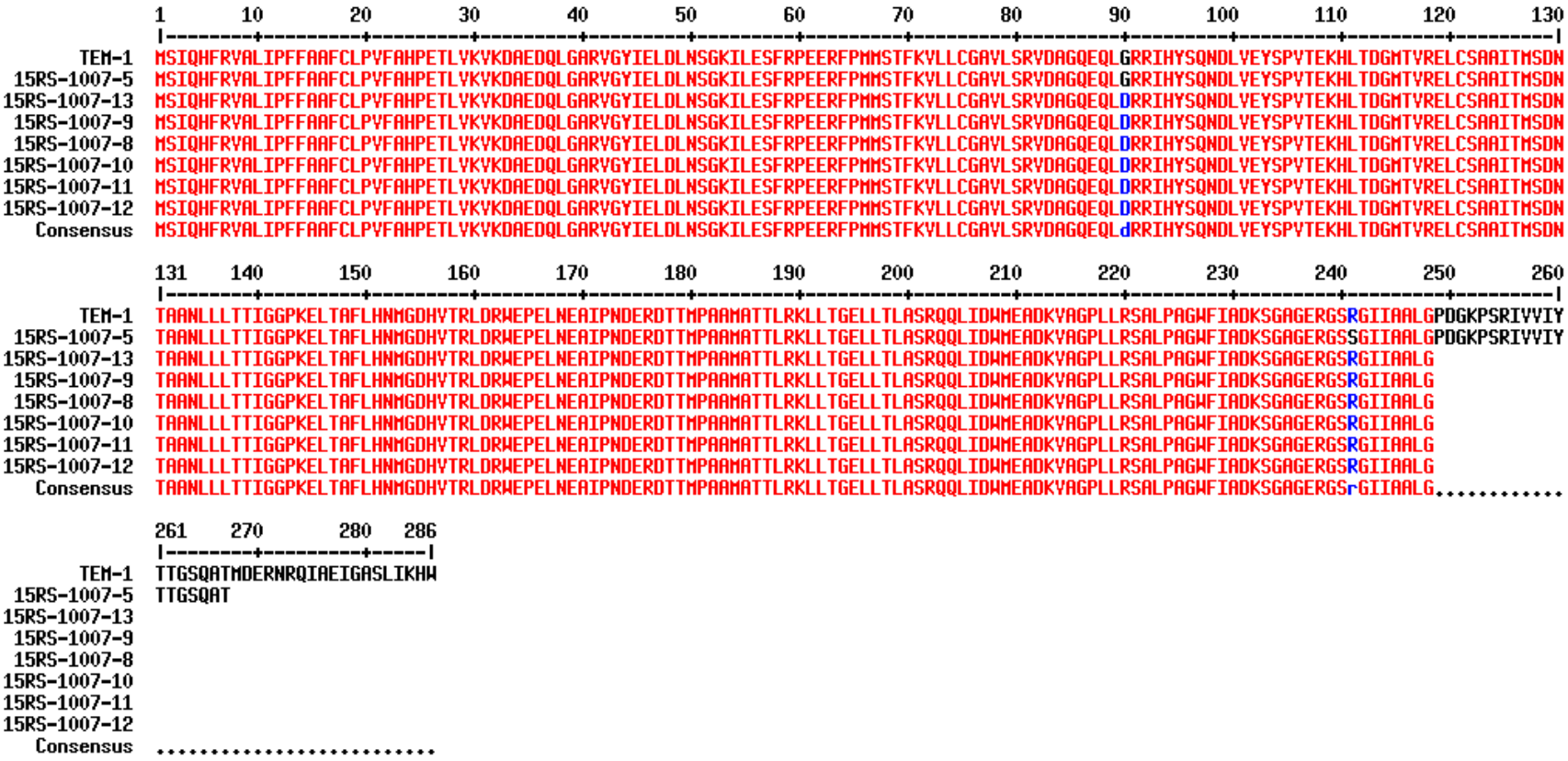


Figure 4.7: Amino acid sequence of a portion of *tem* gene showing substitution of Glycine (G) with Aspartic acid (D) [tem57] and Arginine (R) with Serine (S) [tem30] at position 90 and 241 respectively.

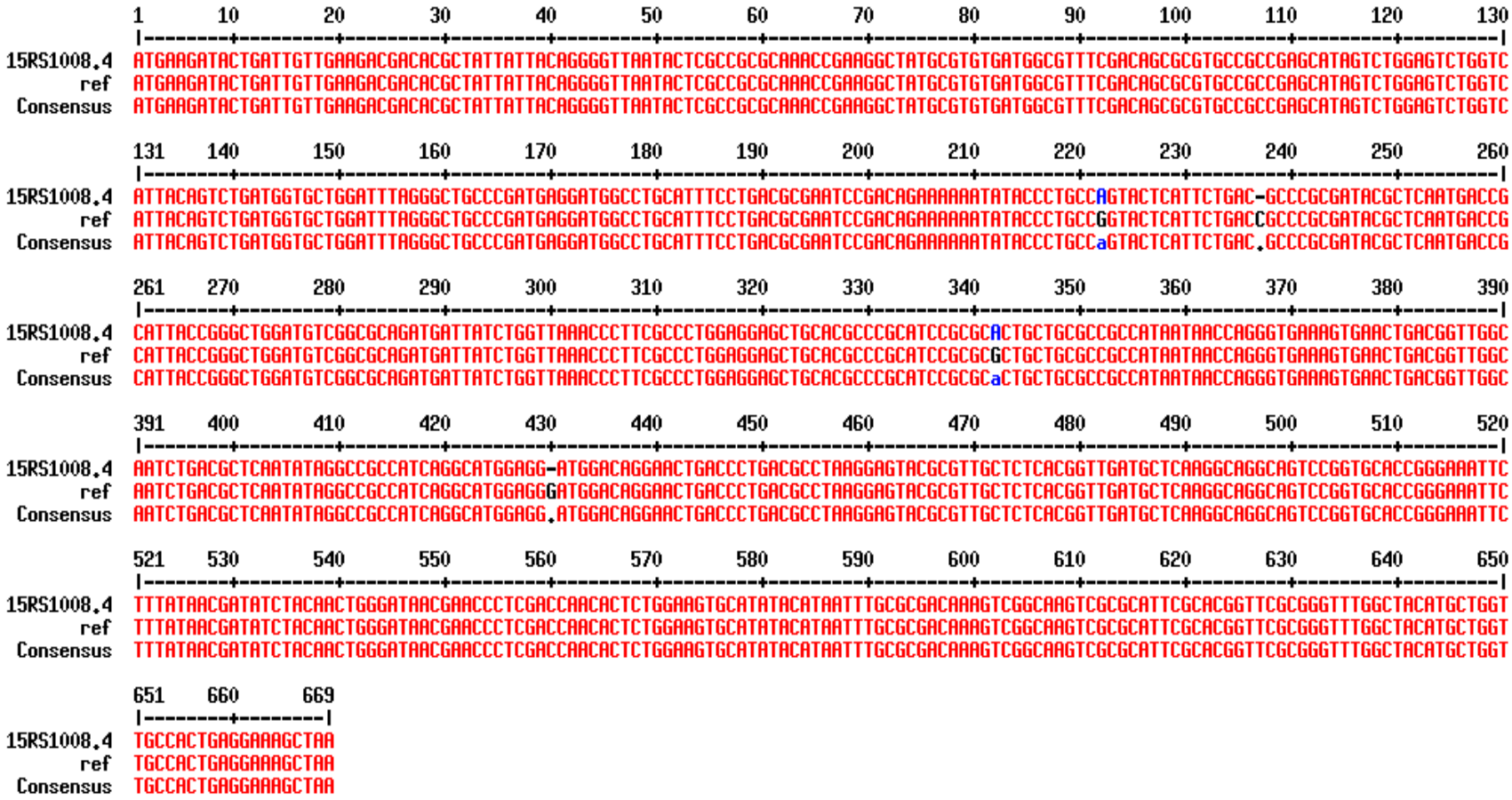


Figure 4.8: Nucleotide sequence of a portion of *pmrA* gene showing substitution of Guanine (G) with Adenine (A) at position 222, 242 and a deletion of Cytosine (C) at position 237.

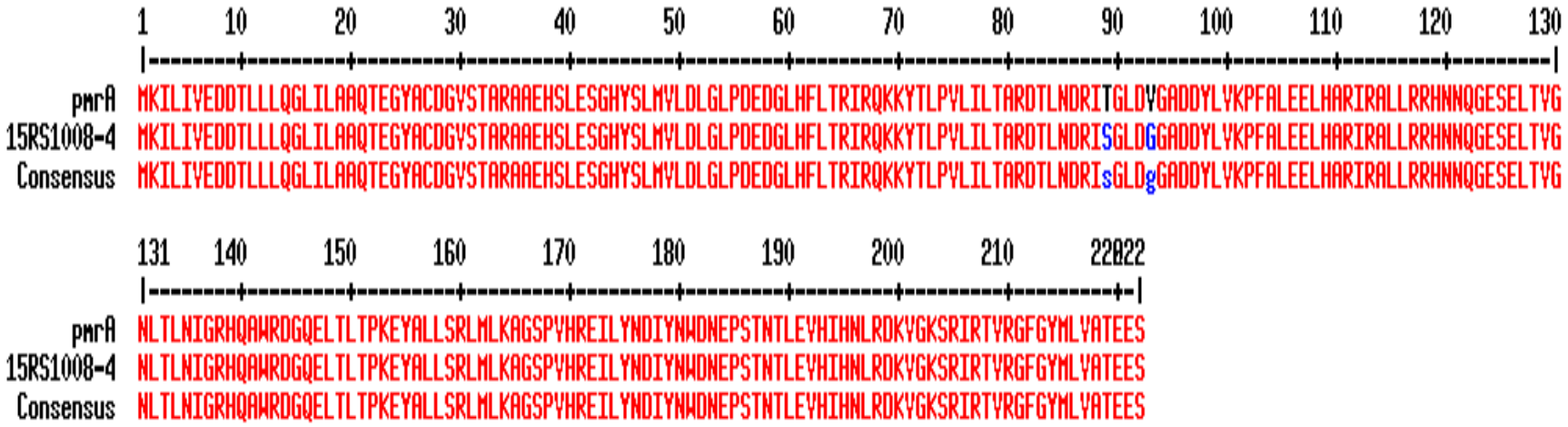


Figure 4.9: Amino acid sequence of a portion of *pmrA* gene showing substitution of threonine (T) with serine (S) and Valine (V) for Glycine (G) at position 89 and 93.

Most of the *S.* Poona isolates were resistant to ampicillin, chloramphenicol, tetracycline, ciprofloxacin, tigecycline and sulphomethoxazole. Resistance to ciprofloxacin was seen in almost all (5/6) the *S.* Poona isolates and the resistance were as a result of mutation in the *parC* (Thr53) gene in addition to the presence of plasmid mediated resistance gene *qnrB.* Five isolates showed phenotypic resistances to chloramphenicol, ampicillin and sulphamethoxazole with the subsequent detection of *cmlA1, tem57 and sul3* genes respectively. One isolate showed double mutation in *pmrA* (T89 and V93) for colistin (Figure 4.9).

All the (5/5) *S.* Isangi isolates were resistant to the quinolones (Ciprofloxacin and Nalidixic acid) and in addition, two isolates were resistant to ampicillin, chloramphenicol, sulphamethoxazole, tetracycline and trimethoprim. All but one of the isolates had both *qnrB* and *qnrS* genes. One of the isolate had mutation in the *gyrA* (Leu55) in addition to having the plasmidic genes *qnrB* and *qnrS*. Resistance genes *sul2* (Sulphomethoxazole), *tetA* (Tetracycline), *dfrA5-14* (Trimethoprim) and *catA1* (Chloramphenicol) were seen in two of the isolates (Table 4.8).

The two *S.* Saintpaul isolates showed resistances to chloramphenicol, ciprofloxacin and tetracycline. In addition, one of the isolate had resistance to ampicillin, azithromycin, sulphamethoxazole and trimethoprim. Mutation was seen in *parC* (Thr57) gene in one of the isolate. Both isolates had resistance genes *qnrS* and *tetA*. Resistance genes *tem1, sul2, dfrA5-14, catA1* and *floR* were seen in one isolate. Similarly, resistance genes *sul3* and *cmlA1* to sulphamethoxazole and chloramphenicol respectively were detected in the *S.* Bonariensis isolate.

The two *S.* Virchow isolates had a resistance pattern TrimSulTetCipNal with mutation seen at the *gyrA* (Ser83) in one of the isolate. Resistance genes *sul1*, *tetA, dfrA5-14* were

detected in both isolates. All the *S*. Chomedey isolates were resistant to ciprofloxacin, sulphamethoxazole and tetracycline with additional resistance to Nalidixic acid in three of the isolates. Mutation was observed in *parC* (Thr57) gene in the three isolates that were resistant to Nalidixic acid. All the isolates had resistance genes *sul2*, *tetA* and *qnrB.*

Both *S.* Larochelle and *S.* Lomita had resistances only to fluoroquinolones. Resistance was as a result of mutation seen in *parC* (Thr57) in addition to the plasmid mediated quinolone resistance gene *qnrB. S.* Mueuster also had resistance only to fluoroquinolones; however, resistance was as a result of mutation seen in *gyrA* (Ser83) in addition to the plasmid mediated quinolone resistance gene *qnrA.*

4.7 Result showing genetic diversity/relatedness amongst *Salmonella* Serovars from the Six Selected States in Nigeria using pulsed field gel electrophoresis (PFGE) and multilocus variable tandem repeat analysis (MLVA)

The PFGE gel documentation results of *S*. Poona (Plate XIII), *S*. Kentucky (Plate XIV), *S.* Isangi (Plate XV), *S*. Larochelle (Plate XVI), *S*. Typhimurium and Nigeria (Plate XVII), *S*. Virchow (Plate XVIII) and *S.* Saintpaul (Plate XIX) showed varying degrees of similarity/difference between the molecular weights (bands) of the restricted segments. The PFGE dendrogram was constructed using the Dice coefficient (F) and UPGMA clustering parameters. The comparison of PFGE profiles obtained from 110 isolates tested in this study identified PFGE cluster patterns for each of the eight serotypes investigated with significant ranges of genetic similarities.

Salmonella Kentucky strains were classified into three clusters with different genetic similarity (Figure 4.10). In particular, 4 out of 44 strains (3 of them were

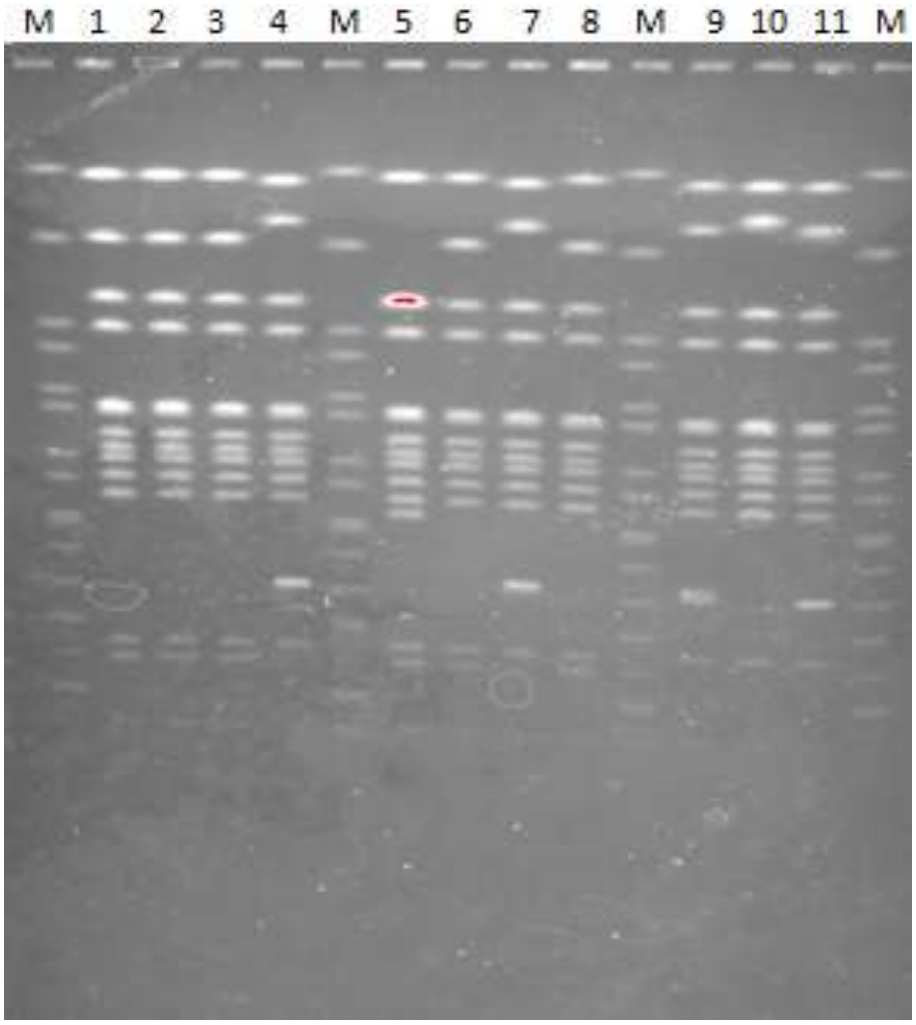


Plate XIII: PFGE gel documentation of *Salmonella* Poona (1,2,3,4,5,6,7,8,9,10,11). *S*. Braenderup (M) was control

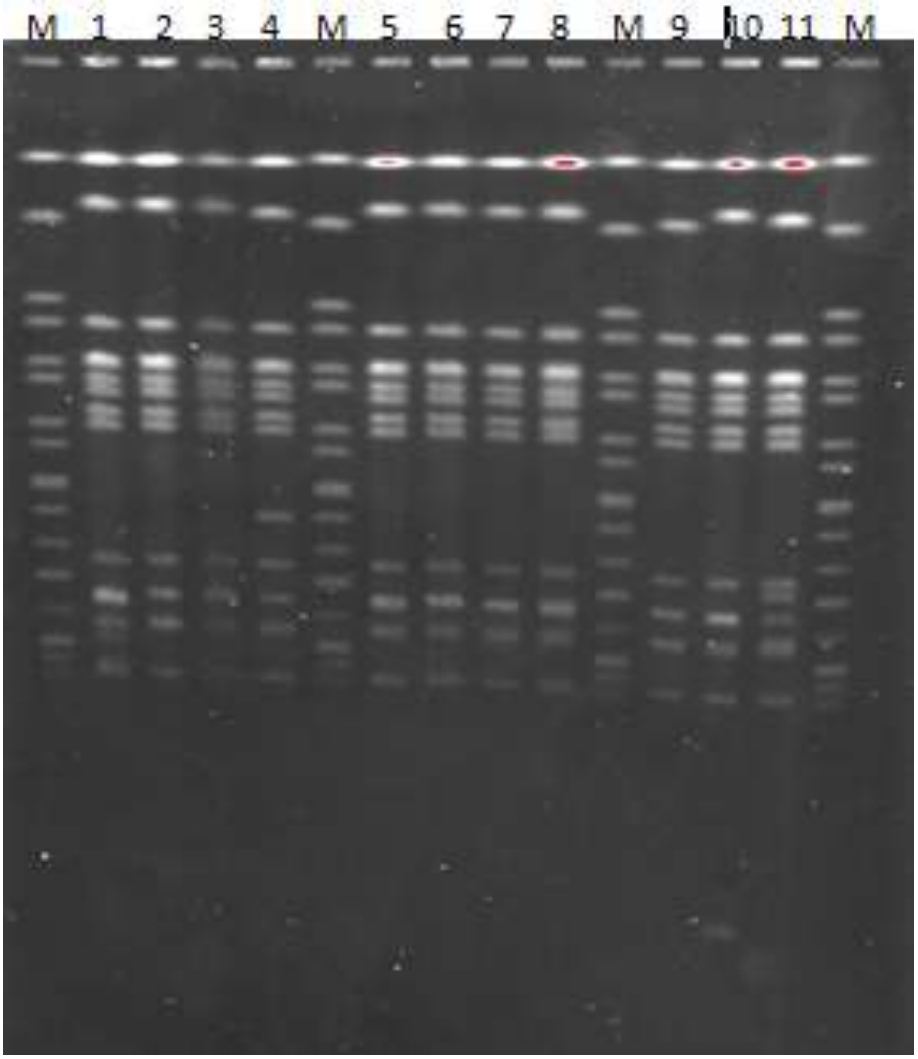


Plate XIV: PFGE gel documentation of *Salmonella* Kentucky (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11). *S*. Braenderup (M) was the control.

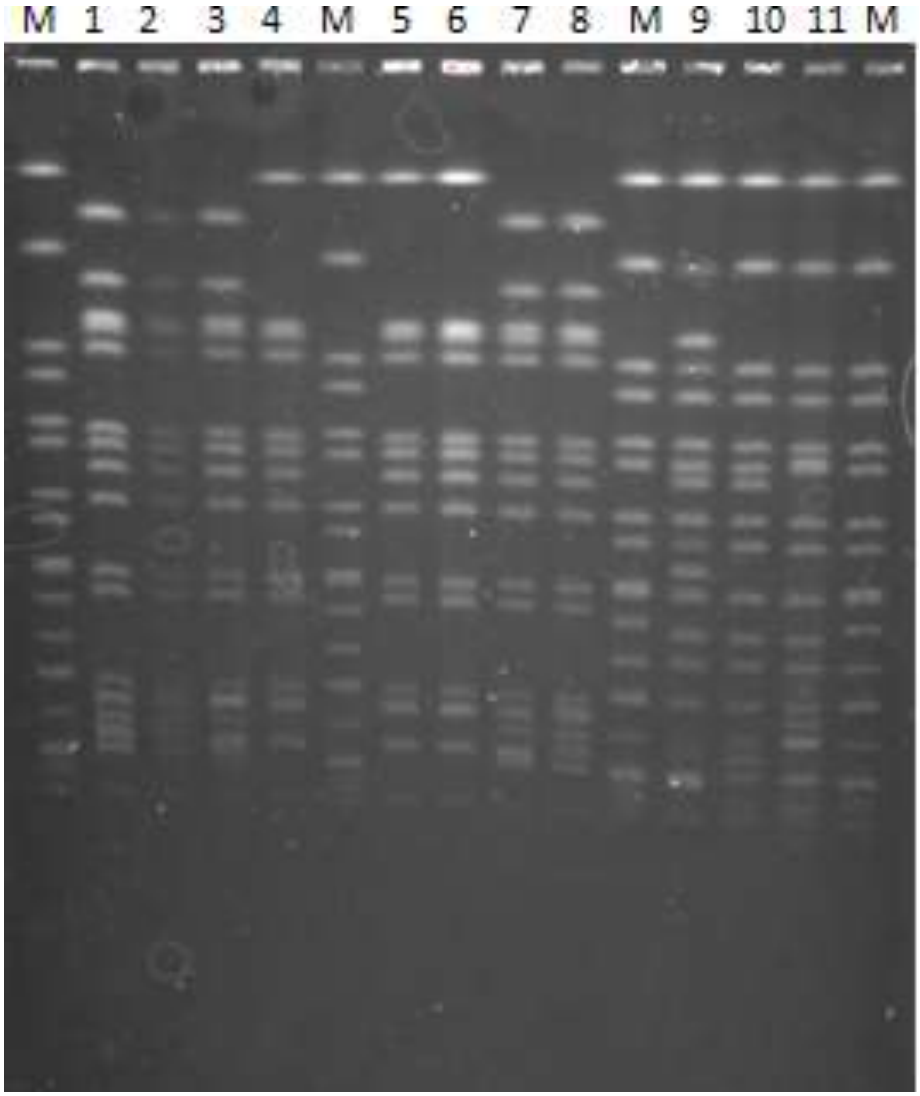


Plate XV: PFGE gel documentation of *S.* Isangi (1, 2, 3, 4, 5, 6, 7, 8), Larochelle (9, 10 and 11). *S*. Braenderup (M) was the control

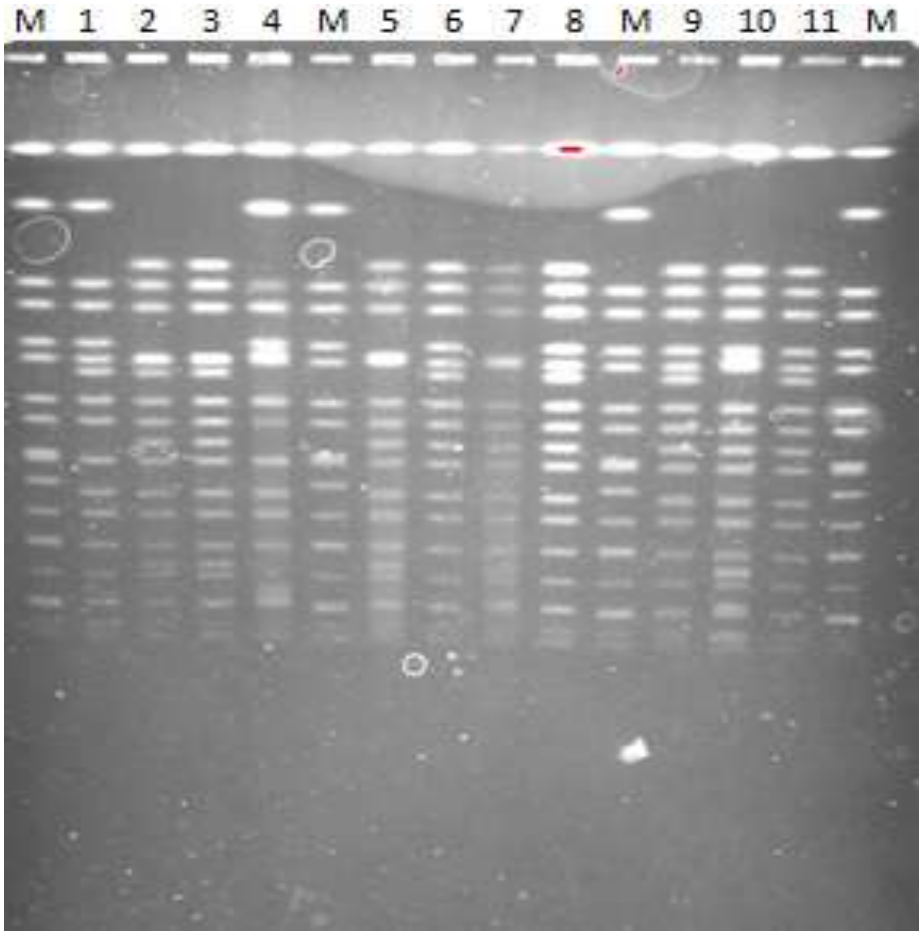


Plate XVI: PFGE gel documentation of *S*. Larochelle (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11), *S*. Braenderup (M) was the control.

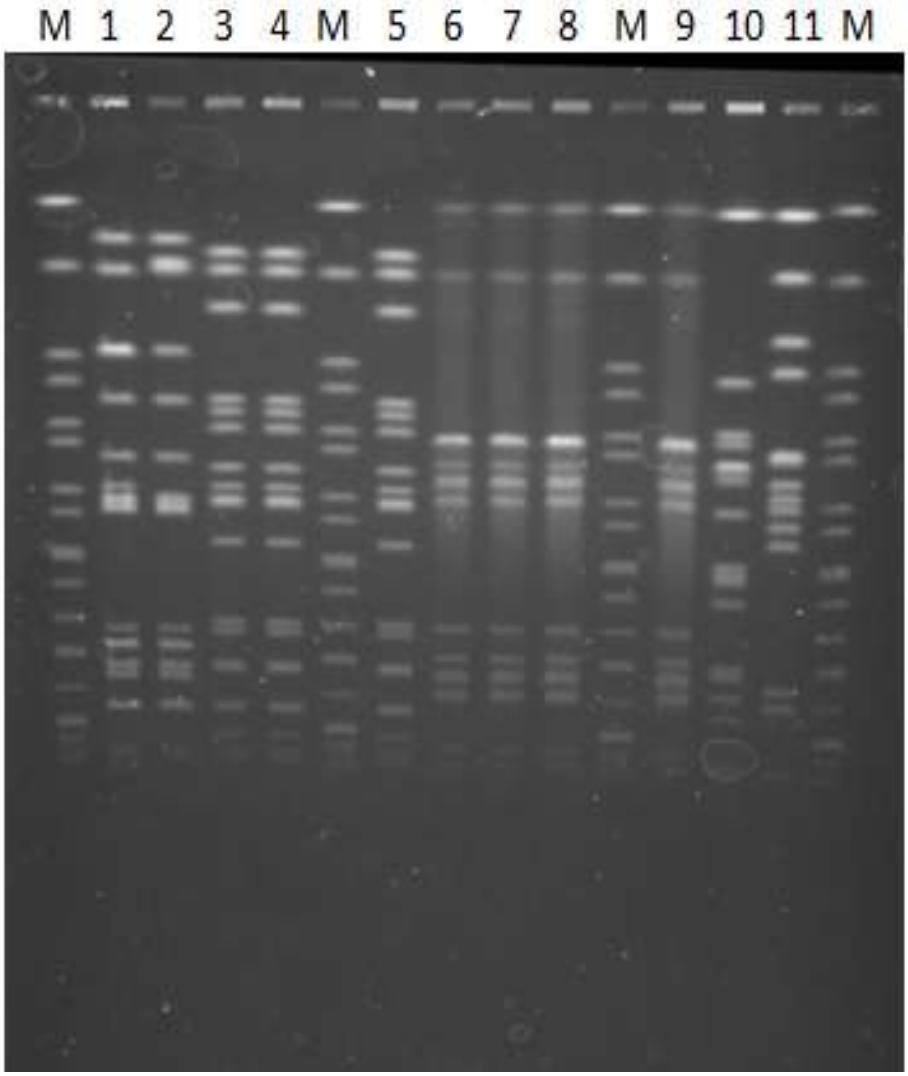


Plate XVII: PFGE gel documentation of *S*. Typhimurium (1, 2, 3, 4, 5), *S*. Nigeria (6, 7, 8, 9, 10) and *S*. Poona (11). *S*. Braenderup (M) was the control

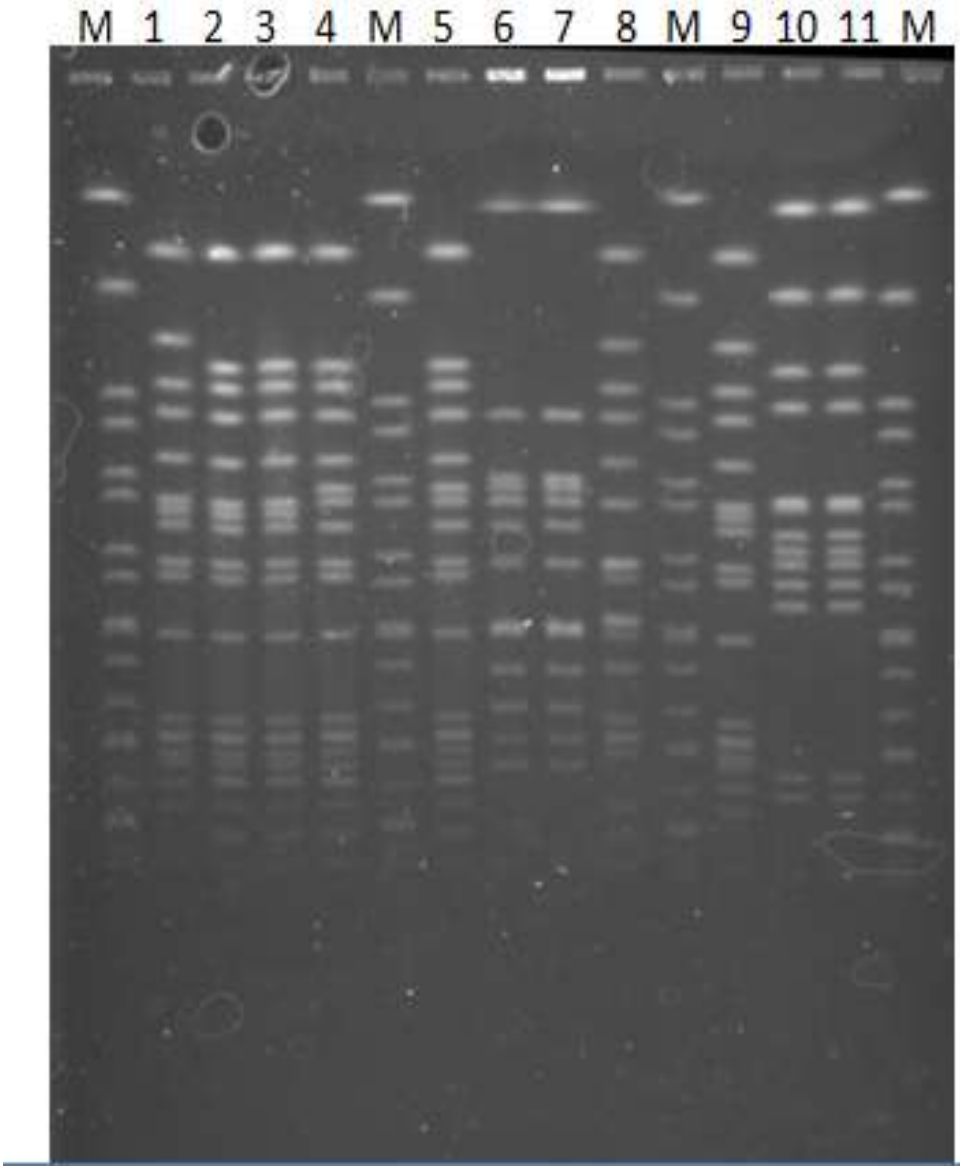


Plate XVIII: PFGE gel documentation of *S*. Virchow (1, 2, 3, 4, 5, 6, 7, 8, 9). *S*. Poona (10, 11).*S*. Braenderup (M) was the control

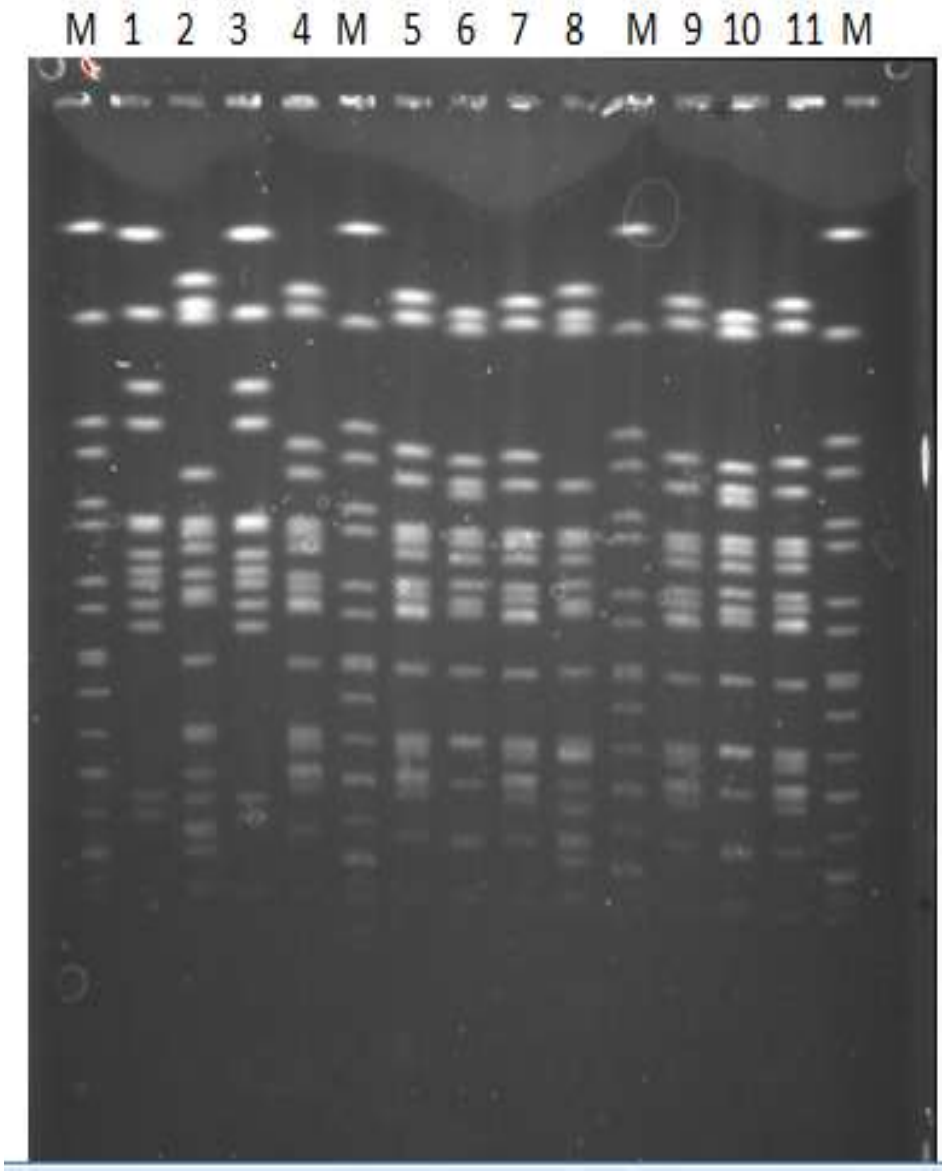


Plate XIX: PFGE gel documentation of *S*. Saintpaul (1, 2, 3, 4, 5, 6, 7, 8, 9). *S*. Poona (10, 11).*S*. Braenderup (M) was the control.

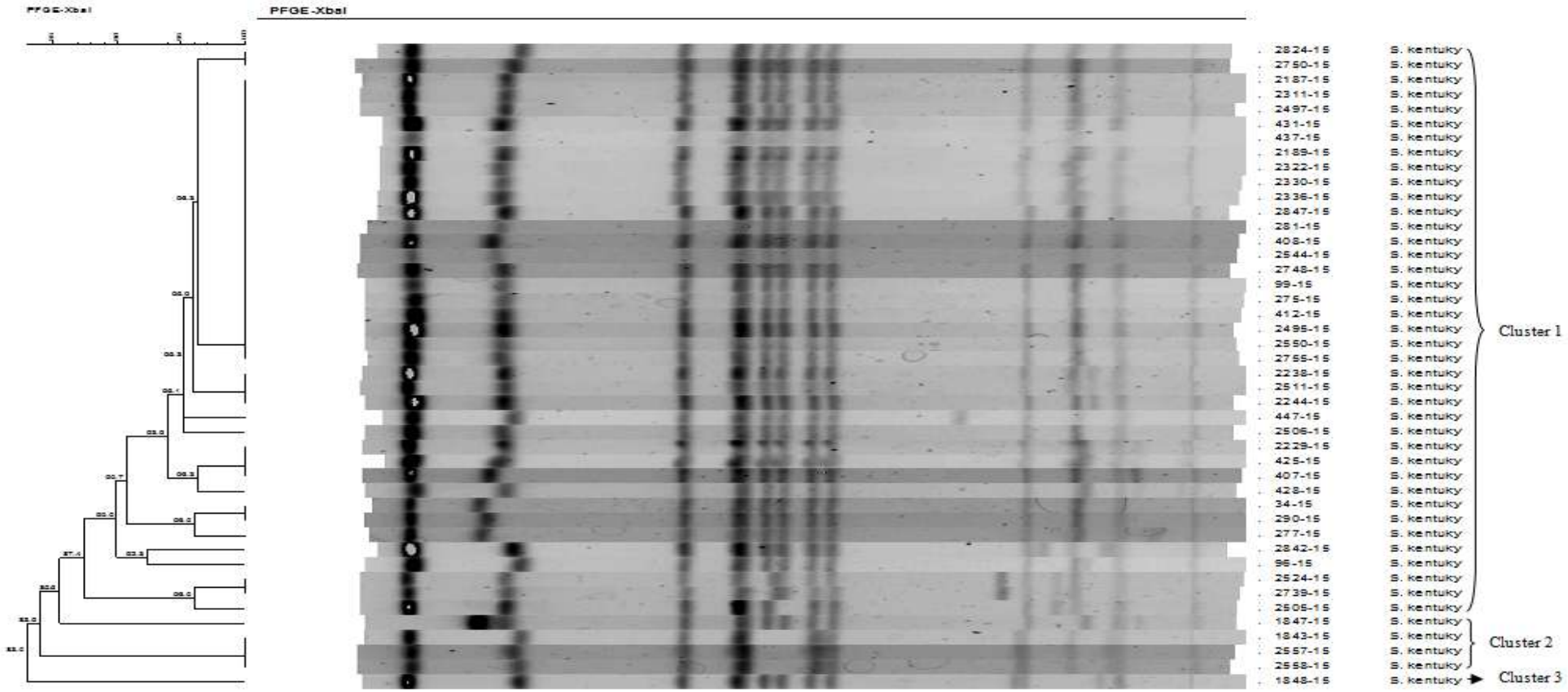


Fig.4.10: Dendrogram showing a cluster analysis of 44 *Salmonella* Kentucky strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

undistinguishable, Dice coefficient = 100%) were grouped into two different clusters (Dice coefficient = 83.0%). The remaining 40 strains were grouped into one cluster with 13 different pulsotypes that are characterized by high degree of genetic similarity (Dice coefficient ≥ 85.5 %). Among these, 45% of the total number of strains analyzed were undistinguishable (Dice coefficient = 100%).

Salmonella Poona strains were grouped into two main clusters that are probably related (Dice coefficient = 83.9 %). Each cluster contains pulsotypes (groups) with genetic similarity of 92% (isolates 1820-15, 2522-15, 2529-15, 2746-15, and 2532-15) and 92.3% (isolates 38-15, 119-15, 2326-16, 2528-15, 447-15, 1829-15, 40-15, 2441-15, 278-15, 307-15 and 1817-15), meaning that they are strictly related genetically. Moreover, each group contains sub-groups characterized by undistinguishable strains. Among the strains of one of these sub-groups (in cluster 2), undistinguishable strains were common in 10 out of 16 strains (38-15, 119-15, 2326-16, 2528-15, 447-15, 1829-15, 40-15, 2441-15, 278-15, 307-15) analyzed (Figure 4.11).

Salmonella Isangi strains were classified into two different clusters, probably related (Dice coefficient = 82.8 %). Each of the clusters has pulsotypes that are indistinguishable (Figure 4.12). *Salmonella* Larochelle were classified into two main clusters that are probably related (Dice coefficient = 83.2 %). In one of the clusters, the groupings contain different pulsotypes with variable genetic similarity, but > 85%, meaning that they are strictly related genetically (Figure 4.13). One of the pulsotype in cluster 1 has subgroup with undistinguishable strains (isolates 2536-15, 2513-15, 2751-15, 2504-15).

Salmonella Virchow strains were classified into three unrelated clusters (Dice coefficient = 60.3%). Cluster 1 has two pulsotypes with three subgroups that are strictly

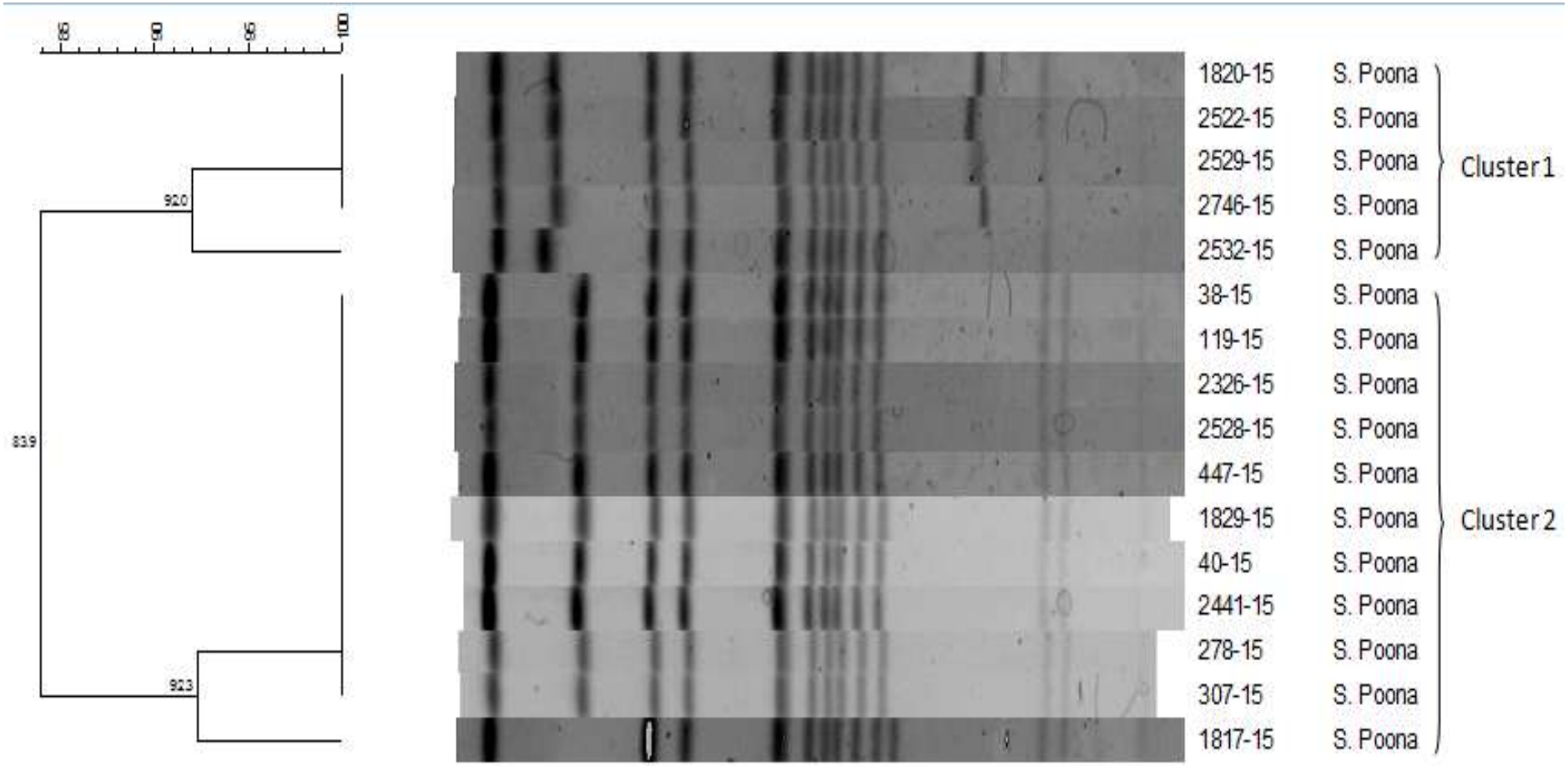


Fig.4.11: Dendrogram showing a cluster analysis of 16 *Salmonella* Poona strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

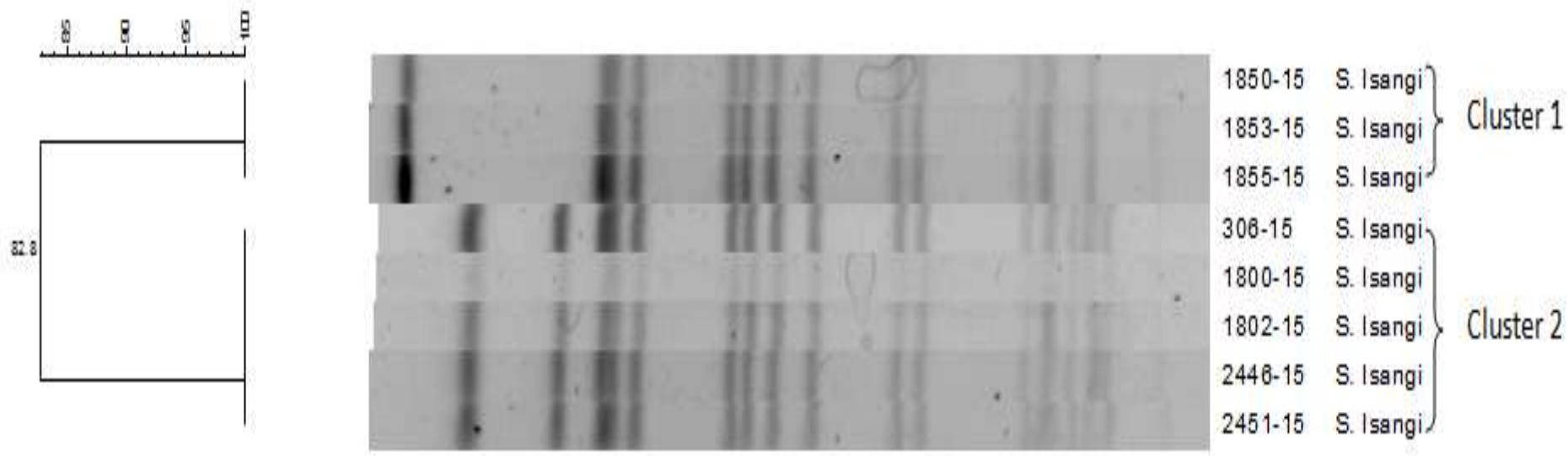


Figure 4.12: Dendrogram showing a cluster analysis of 8 *Salmonella* Isangi strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

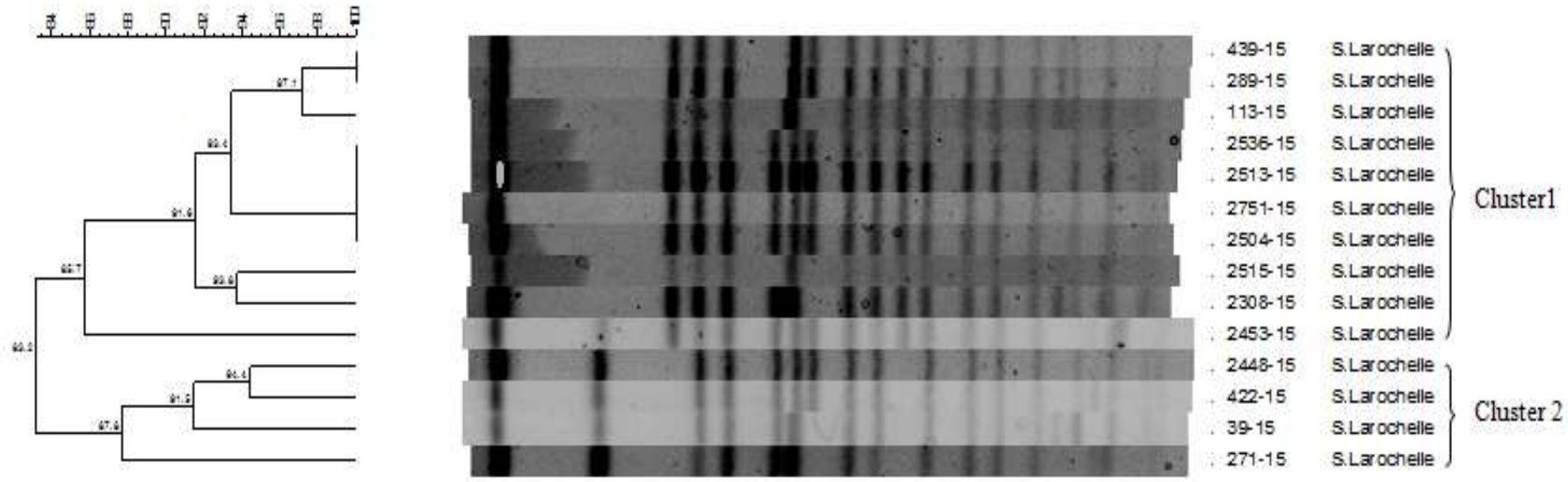


Figure 4.13: Dendrogram showing a cluster analysis of 14 *Salmonella* Larochelle strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

related (89.2% and 93.4%) and each of the subgroups is characterized by undistinguishable strains (Dice coefficient =100%). Clusters 2 and 3 are characterized by strains that are probably related (Dice coefficient ≥ 77 %). Cluster 3 has two isolates that are undistinguishable (Figure 4.14).

Salmonella Typhimurium strains analyzed were classified into two clusters (Figure 4.15) that are unrelated (Dice coefficient = 56.6%). Cluster 2 is characterized by 3 undistinguishable strains while cluster 1 is characterized by 2 strains that are strictly related (Dice coefficient = 92.9%). *Salmonella* Nigeria strains revealed two different clusters (Dice coefficient = 59.3 %). Cluster 1 is characterized by 4 strains that are undistinguishable (Dice coefficient = 100 %). Cluster 2 has just one strain (Figure 4.16).

Salmonella Saintpaul strains were classified into two main clusters probably related genetically (Dice coefficient = 79 %). In the first cluster, each pulsotypes is characterized by subgroups with strains that were genetically similar (86.1%), meaning that they are strictly related. Moreover, within each group there were undistinguishable strains (dice coefficient =100%). The most prevalent pulsotype was shared by 5 out of 9 *S.* Saintpaul strains investigated (Figure 4.17).

The MLVA result showed that isolates 1428/1 and 1430/1 had the same allelic genes STTR9 and STTR3 but differed in the rest of the allelic genes (Table 4.9). Isolates 1222/1, 1387/1 and 1388/1 were 100% genetically related. They had identical allelic genes STTR9, STTR5 and STTR3 and lacked allelic genes STTR6 and STTR10. They are also completely unrelated to the other two isolates (Figure 4.18).

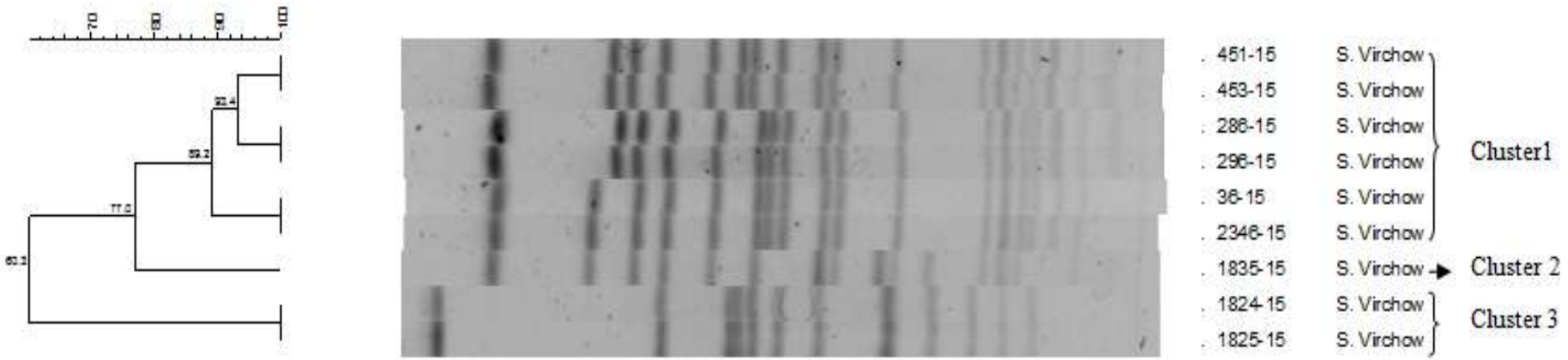


Figure 4.14: Dendrogram showing a cluster analysis of 9 *Salmonella* Virchow strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

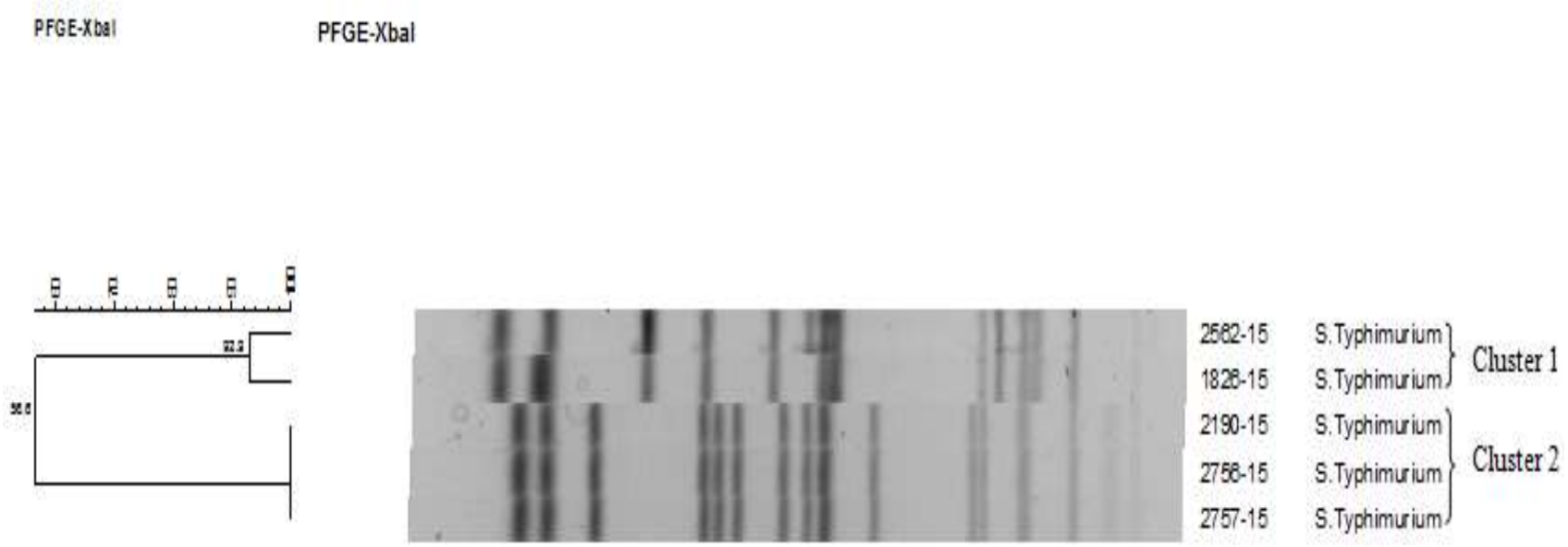


Figure 4.15: Dendrogram showing a cluster analysis of 5 *Salmonella* Typhimurium strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

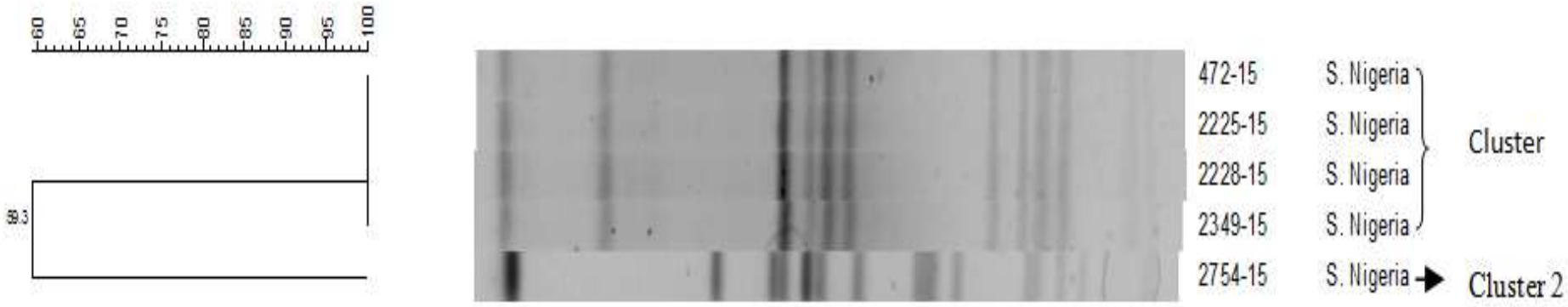


Figure 4.16: Dendrogram showing a cluster analysis of 5 *Salmonella* Nigeria strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

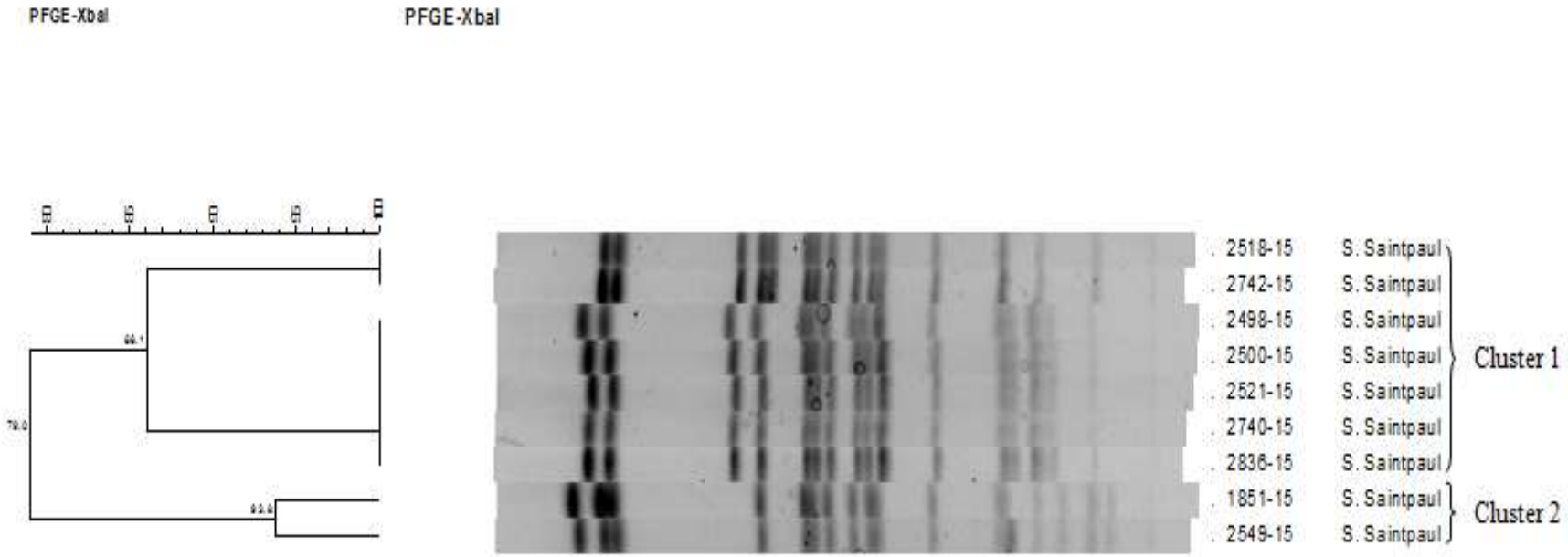


Figure 4.17: Dendrogram showing a cluster analysis of 9 *Salmonella* Saintpaul strains based on the PFGE profiles of the XbaI-digested chromosomal DNA of the bacterial strains isolated from six selected States in Nigeria. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters.

Table 4.9: The MLVA tandem repeat (STTR) analysis at five different loci for five *S.* Typhimurium isolates from some of the selected states in Nigeria.

Isolate	Sample data					Corrected sizes					New nomenclature of alleles				
Number	STTR9 - bp	STTR5 - bp	STTR6 - Bp	STTR10 - bp	STTR3 - bp	STTR 9	STTR 5	STTR 6	STTR1 0	STTR 3	STTR 9	STTR 5	STTR 6	STTR1 0	STTR 3
STMSSI30	162	209	288	395	370	160.75	210.33	286.44	389	368.06	2	5	4	13	0009
1428/1	171	259	331	353	490	169.63	259.16	329.78	347.75	488.33	3	13	11	6	0211
1430/1	171	259	300	432	517	169.63	259.16	298.62	424.61	515	3	13	6	19	0311
1222/1	153	277			531	151.75	277.4			528.92	1	16			0113
1387/1	153	277			531	151.75	277.4			528.92	1	16			0113
1388/1	153	277			531	151.75	277.4			528.92	1	16			0113

STTR = *Salmonella* Typhimurium Tandem Repeat and the numbers 3, 5, 6, 9 and 10 are the loci that were amplified.

STMSS130= Positive control

1428/1, 1430/1, 1222/1, 1387/1, 1388/1 = Isolates

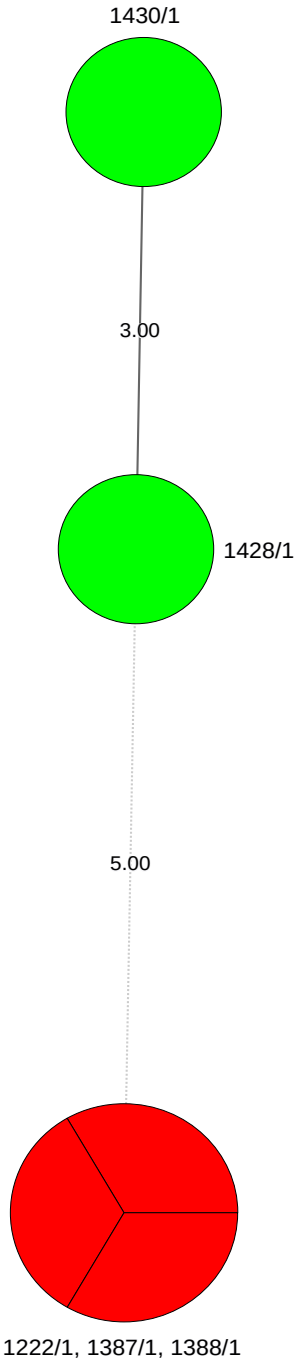


Figure 4.18: Minimum spanning tree of MLVA types identified from *S.* Typhimurium isolated from commercial layer farms in the selected states in Nigeria. Each circle in the tree represents a different MLVA type. Short lines connecting two MLVA profiles denote types differing by MLVA loci. The colours in the circles indicate *Salmonella* Typhimurium isolates with a particular MLVA profile.

CHAPTER FIVE

DISCUSSION

This is one of few comprehensive studies on the occurrence and distribution of *Salmonella* in commercial layer farms from all the six regions of Nigeria with an overall farm prevalence of 43.6% and sample prevalence of 14.1%. The study also reported a large number of circulating *Salmonella* serotypes which has not been isolated in Nigeria hitherto. A relatively high farm prevalence (43.6%) of *Salmonella* was detected from commercial poultry farms in Nigeria when compared to the 11.7% reported in the United Kingdom (Snow *et al.*, 2007). The frequency of *Salmonella* in poultry can vary depending on the location, the nature of the production system and the specific control measures that have been put in place. Flocks from some EU countries are virtually free (2.7%) from *Salmonella* (EFSA, 2014b) whereas in countries like the US, contamination rate of up to 60% has been reported (NARMS, 2011; Mølbak *et al*., 2006). In Japan, *Salmonella* was isolated from 36% of the broiler faecal samples (Ishihara *et al*., 2009). In the Gambia, Dione *et al*. (2011) reported the detection rate of *Salmonella* in chicken faeces that was higher (67%) than in the present study (16.8%). This study recorded a slightly higher (13.4%) sample prevalence of *Salmonella* infection from chicken reared under intensive system in comparison to the 11% reported by Fashae *et al*. (2010). Differences in isolation rates can be due to differences in region, sample types, collection seasons, culture methods, isolation methodologies, culture media and local environmental conditions (European Centre for Disease Prevention and Control, 2013).

The prevalence of *Salmonella* was insignificantly higher in faecal samples (16.8%) than in other sample types. This result does not agree with the work of Tabo *et al*. (2013) who reported the highest isolation rate of 15.63% in litter than in faecal samples from Chad Republic. The high prevalence in this study may be attributed to the generally

poor infrastructure and low biosecurity measures in controlling stray animals, rodents, reptiles, amphibians and even humans. By observational analysis, the majority of the infections seem to be attributed to persistent contamination of the farms. A significant improvement in the sanitary condition at flock level will reduce *Salmonella* contamination in poultry farms. Thus, ensuring *Salmonella* free feed and water, effective cleaning and disinfection of the farms, applying appropriate measures against animated and unanimated vectors are some of the several measures suggested that can limit vertical and horizontal transmissions of *Salmonella* on farms (Humphrey, 2006; Wales *et al*, 2007).

The size of flocks did not appear to be associated with the risk of *Salmonella* contamination. This does not support the report of other studies (Mollenhurst *et al.*, 2005; Namata *et al*., 2008; Snow *et al*., 2010) in which the size of the flock was shown to be a risk factor. However, majority (89.7%) of farms in this study were small flocks with between 200 and 10,000 birds. Large scale farms with over 10,000 birds are generally few in Nigeria. The isolation rate relative to both the small farm and the large farms were the same (43.3% and 43.2% respectively).

Type of housing and presence of wire mesh to prevent wild bird access were found to be associated with an increased risk of *Salmonella* although wire mesh against wild bird access was not significant by multivariate analysis. This supports the work of others where the type of housing was reported to be a risk factor (Henken *et al.,* 1992; Carrique-Mas *et al*, 2009b; Snow *et al*., 2010). Battery cage housing system which was found to be a risk factor may be due to the inability of the farmers to regularly maintain good sanitary conditions in poultry houses when compared to the deep litter system. Farmers using the battery cage system admitted that poultry waste are disposed after 3-6

months. Other authors (Akinyemi *et al*., 2011; Dekker *et al*., 2015) have implicated water as a risk factor, but the source of water and staff serving in more than one farm which were significant by bivariate analysis in this study were found not to have any impact in the spread of *Salmonella* infection. This may be as a result of the use of chlorine water sanitizer (water guard$^{(R)}$) by most farmers that is relatively cheap and effective. In fact, similar use of water sanitizer (acetic acid) has been reported to decrease the risk of *Salmonella* infection (Le Bouquina *et al*., 2010).

Feed additives which were found to increase the risk of *Salmonella* had no significant impact by multivariate analysis. Feed additives which include digestive enzymes, mycotoxin binders, amino acids, vitamins and antioxidants may be contaminated with *Salmonella*. Contaminated feed is recognized as a major source of infection with *Salmonella* for poultry flocks (Berchieri and Barrow, 1996; Davies and Hinton, 2000; Jones and Richardson, 2004). *Salmonella* can be introduced into the feed with contaminated ingredients, but contamination can also be acquired during processing, transport, storage and distribution (Davies and Hinton, 2000). Moreover, *Salmonella* organisms can, under some circumstances, multiply in the feed especially when it is damp and the temperature is high, which may occur during farm storage and administration (Jones and Richardson, 2004). The inclusion of coccidiostats which is a common practice among commercial farmers did not appear also to have any significant effect on the risk of *Salmonella* contamination.

Frequent access to the pen houses to gather eggs and the crate types (plastic crates as opposed to paper) were shown to increase the risk of *Salmonella* infection, although the regular access to poultry houses for egg collection was not significant. The likelihood of

introduction of an infection is higher when un-disinfected egg crates and personnel access the pen house regularly.

Membership of the Poultry Association of Nigeria (PAN), a non-governmental support organisation for commercial poultry farmers, was found to be associated with the risk of *Salmonella*. This increased risk may be due to the fact that these members, though, aware of the need to control *Salmonella* on farms and having a better understanding of the need for biosecurity and the need to adopt new technologies for disease control may be unwilling to do so because of the cost implications and/or their unwillingness to adhere to expert advice from professionals. Also, members of this association share equipment and facilities which make it easier to spread infection between member's farms. Young farmers (ages 20-30 years) were found to be associated with the spread to *Salmonella* in this study. Inexperience of the young farmers as oppose to older ones may be a reason for the infection in this group of farmers.

General vaccination of poultry was carried out by 96.7% of farmers, while routine vaccination for fowl typhoid (*Salmonella* Gallinarum) was practised by only 36.3% of farmers. Fowl typhoid is endemic in Nigeria and is often assumed to be the sole cause of *Salmonella* infections in poultry (Mamman *et al.,* 2014). Almost, 64% of farmers did not vaccinate against *Salmonella* Gallinarum, for which there is a locally produced *Salmonella* Gallinarum 9R strain vaccine which could induce cross-protective immunity against *Salmonella* of the same serogroup. Even though the vaccine is effective, *Salmonella* Gallinarum 9R vaccine still possesses some residual virulence for chickens (Kwon and Cho, 2011). Farmers indicated that this residual virulence accounted for their reluctance to vaccinate their birds as this would translate into a loss of egg production. On the other hand, there is no cross-protection between serotypes belonging to different

serogroups (Lee *et al.,* 2001). Vaccination against other serotypes of *Salmonella* is generally not practiced in Nigeria even though it is known that the immune response of poultry to vaccination could reduce the duration and severity of *Salmonella* infections and help prevent reinfection (Gast, 2007).

Studies by Rose *et al*. (1999) and Gotter *et al*. (2012) have shown that good cleaning and disinfection are effective in reducing *Salmonella* contamination especially those transmitted horizontally. Interestingly in the present study, cleaning and disinfection did not appear to have any significant impact on the risk of *Salmonella*. It is plausible that there was reporter bias which is a particular issue in studies that rely on questionnaires for information gathering. It is unclear how accurately cleaning and disinfection practices were recollected. It may also be possible that there was a failure by the disinfectant used to penetrate organic matter in the poultry environment owing to the quality of disinfectant as the more potent disinfectants are expensive and not economical for small scale farmers to use. Many farmers (69%) in the study areas reported that they were not able to afford the more potent disinfectants due to costs. There was also a growing problem with a lack of consistency (19%) in applying disinfection from this study. Also, vertical transmission of *Salmonella* have been reported in chicks in Nigeria (Muhammad *et al.,* 2010) where these birds shed the organism at day-old and therefore control of *Salmonella* contamination using only disinfectant may not be too effective.

Losses suffered from previous episodes of *Salmonella* were found to be associated with an increased risk of *Salmonella*. This is not surprising as other studies have demonstrated that in spite of comprehensive management programmes, *Salmonella* could still persist. In a study by Carrique-Mas *et al.* (2009b), they reported that despite cleaning and disinfection, they found that *Salmonella* was present in previously studied

flocks when the next batch of birds was introduced. While fresh faeces provide an indication of current infection, positive dust samples may be indicative of previous infection. *Salmonella* has been reported to survive in dust for up to 53 weeks (Davies and Wray, 1996) and up to 26 months in litter, dried faeces and feed (Davies and Breslin, 2003). *Salmonella* found in faeces and or dust is usually an indication of concurrent infection in a flock (Carrique-Mas *et al.*, 2008).

Personnel sanitation such as the presence of foot dips, lavatories and changing rooms did not appear to have any significant impact on the risk of *Salmonella* contamination. However, wearing of work clothing significantly reduced the risk of *Salmonella* infection in this study. This was because the chances of introduction of the *Salmonella* infection from outside through this medium are reduced. Some studies (Funk and Gebreyes, 2004; Lo *et al*., 2004) reported that access to toilets, specific working materials (e.g. clothing) and hand-washing facilities were associated with a decrease in *Salmonella* prevalence on pig farms. However, Amass *et al.* (2000a) have shown that the use of disinfectants in foot dips without using a boot brush to remove organic matter from the boots may not effectively remove the bacteria.

The prevalence of *Salmonella* serotypes differed slightly among sample types and states investigated. However, most of the serovars were isolated in all the sample types. Serogroups B, C1 and C2-C3 belonging to subspecies I were dominant. This may be due to the ubiquitous nature of serovars in this sub specie and the result was consistent with what was obtainable in the USA (Antunes *et al*., 2011). Therefore, control of salmonellosis in Nigeria should therefore involve a multivalent vaccine targeting members of these serogroups. *S.* Enteritidis and *S.* Typhimurium which are the two most frequently isolated serovars worldwide (EFSA, 2011), were not commonly isolated in

this study. Only one *S.* Enteritidis belonging to phage type PT1 and five *S.* Typhimurium belonging to phage types DT1 (1/5), DT161 (1/5) and NT (3/5) were isolated in this study.

A total of 63 different serotypes were identified using both serological (microtitre and slide agglutination) and molecular methods (Beads microarray). Three different isolation media were used per sample in this study. So, in some cases, more than one serovar were isolated because the performance of any plating medium is also dependent on the serovar distribution in that livestock population (Carrique-Mas *et al.*, 2009a). Therefore, the distribution of serovars based on sample type had a slightly higher number than the distribution of isolates based on sample type.

The most predominant serotypes were *S.* Kentucky (16.73 %), *S.* Poona (7.43%) and *S.* Larochelle (4.46%). While *S.* Kentucky had a widespread distribution in five of the six states sampled, others were not as widespread as this serovar. Previous studies carried out in Nigeria also reported *S.* Kentucky as one of the most prevalent *Salmonella* serovars in poultry (Muhammad *et al*., 2009; Ogunleye and Carlson, 2012; Raufu *et al*, 2013). This serovar which was initially thought to be endemic in poultry industries in Africa is rapidly spreading across different continents of the world and increasingly becoming a public health concern (Le Hello *et al*., 2011; Le Hello *et al*., 2013). The lack of regulations and strict enforcement of laws on the importation of uncertified poultry and poultry product may be a problem in Nigeria. The high prevalence and presence of these multiple *Salmonella* serovars especially in Southern states may be due to poor sanitary conditions of poultry farms, high human movement, and lack of enforcement of laws on the ban of importation of poultry and poultry products as well as the poorly managed (porous) borders with neighbouring countries. An improvement on risk factors

together with improved cleaning and disinfection and vaccination could have a significant impact on reducing *Salmonella* levels on farms in Nigeria. Though, vaccination is still regarded as an important part of the overall control strategy for *Salmonella*, it is, however, advocated that routine vaccination for *Salmonella* control should not stop at fowl typhoid control alone but should include other serotypes which could be easily transmitted in eggs meant for human consumption.

All (100%) the isolates tested were susceptible to three antibiotics namely Meropenem, Cefotaxime and Ceftazidime which belong to the third generation cephalosporins. This shows that there is presently minimal abuse of this class of antibiotic in Nigeria and implication of this is that, even though they may be the drug of choice for the treatment of salmonellosis in Nigeria, care should be taken not to misuse/abuse these drugs so as not to allow the development of resistance strains. This study showed 27 different resistance patterns with the maximum MAR index of 0.5. High rates of resistance to Ciprofloxacin (89.7%), Nalidixic acid (77.3%), Sulphamethoxazole (75.3%), Tetracycline (75.3%) and Gentamicin (47.4%) were found with an overall percentage resistance of 14.2% to a panel of 14 antibiotics. Resistance to Chloramphenicol, Ampicillin, Trimethoprim, and, Azithromycin, Tigecycline and Colistin ranged from 19.6% to 3.1%. A comparative study in Ibadan, Nigeria, reported a high frequency of resistance of 87%, 93%, 81% and 87% to ciprofloxacin, tetracycline, Nalidixic acid and sulphamethoxazole respectively among chicken isolates (Fashae *et al*., 2010). In another study, Akyala *et al*. (2013) reported a 100% resistance to fluoroquinolones for clinical isolates from northern part of Nigeria while Adesiji *et al*. (2014) reported only one isolate from chicken that was resistant to ciprofloxacin out of thirty isolates. Four isolates in this study showed reduced susceptibility to ciprofloxacin and high susceptibility to Nalidixic acid as was earlier reported by Fashae *et al*. (2010).

All the *S.* Kentucky isolates (100%) were resistant to Ciprofloxacin and Nalidixic acid, 97.7% resistance to Tetracycline and Sulphamethoxazole. The multiple-resistance displayed by *Salmonella* isolates in this study may suggest the emergence of co-resistance and/or cross-resistance. For example, colistin showed low levels of resistance (3.1%); this may be due to cross-resistance from ciprofloxacin, Tetracycline, and Sulphonamides (Livermore, 2002; Cantón and Ruiz-Garbajosa, 2011). Co-resistance to fluoroquinolones and third-and fourth-generation cephalosporins has also been identified in *Salmonella* isolated from humans (Zhang *et al.*, 2014).

Raufu *et al.* (2014) reported a significant contamination of poultry with multidrug-resistant *S.* Kentucky in Nigeria. The association of *S.* Kentucky with poultry dates back to 1937 in United States where it was isolated from chickens. Though, Multilocus sequence typing (MLST) was not carried out in this study, the patterns of resistance to antimicrobials including ciprofloxacin and Nalidixic acid, reaffirmed the initial speculation of the existence of a prevailing circulating poultry-associated STI98-X1 CIP *S.* Kentucky clone in Africa, including Nigeria (Le Hello *et al*., 2012).

The lack of policy to control the use of antimicrobials in poultry especially fluoroquinolones, including ciprofloxacin, enrofloxacin and ofloxacin in Nigeria (Adetosoye, 1980), may have contributed to its rapid spread in poultry farms. In *Salmonella,* acquisition of resistance genes was likely to have occurred by conjugation, usually with other *Enterobacteriaceae* and through the transfer of plasmids (EFSA, 2014b). Bacteria that develop resistance via ESBLs could become a reservoir of resistance genes, which may enter the food chain (EFSA, 2012). Screening of resistance genes have proved to be a useful tool in epidemiologic tracing of genes between

bacterial species, prediction of future outbreaks in different regions, treatment and control strategies of bacterial diseases (Samadi *et al.*, 2015).

Resistance to gentamicin involves enzymatic modification of amino glycosides (Yu *et al.,* 2011). This includes phosphorylation, adenylation and acetylation of amine and hydroxyl groups. Although aminoglycoside modifying enzymes are the most common and major methods of resistance, plasmid mediated 16S rRNA-methylases (16S-RMTases) have been increasingly reported in recent years (Yamane *et al.*, 2007). It was, however, not studied in this work. There are scarce reports on aminoglycoside resistance genes in *Salmonella* species (Folster *et al.,* 2009; Samadi *et al.*, 2015), however, we detected the aminoglycoside modifying enzymes, *aac(3)-le* gene in 47.4% of the isolates screened for gentamicin resistance. This aminoglycoside gene is similar to the one detected in *E. coli* in China (Xiao *et al.*, 2012) but different (*aac(6')-Ib*) from that of Yu *et al.* (2011). Aminoglycoside-resistant isolates from foods of animal origin is of public health importance in developing countries because they are used to treat a wide variety of infections (Davis *et al*., 2002).

Tetracycline-resistance genes occurred most frequently in this study and all of the tetracycline-resistant isolates yielded targeted amplicons with *tetA* primers, but there was no amplification products detected by *tetB* and *tetG* primers. Tetracycline-resistance genes were also detected in most tetracycline-susceptible and resistant isolates. Thus the present results and those of Deekshit *et al*. (2012) and Maynard *et al*. (2003) agree that some antimicrobial-resistance genes are "silent" in bacteria *in-vitro*; it further provides an indication that these silent genes can spread to other bacteria or be turned on *in-vivo*, especially under selection pressure of antibiotic use (Yaqoob *et al*., 2007). All *Salmonella* isolates that were resistant to Sulphamethoxazole carried either the *sul 1*,

sul2 and sul3 genes with *sul1* being the most predominant. Most of the isolates that were resistant to Sulphamethoxazole were also resistant to tetracycline, Ciprofloxacin and Nalidixic acid. The present study detected only *dfrA5-14* gene in isolates that were resistant to Trimethoprim. The *catA1* (3/13), *cmlA1* (9/13) and *floR* (1/13) genes were detected amongst the Chloramphenicol resistant isolates tested. Chloramphenicol used to be the drug of choice in the treatment of *Salmonella*-related infections in Nigeria after which a survey revealed 72.4–89.2% increase of resistance from 1997 to 2007, thus limiting its therapeutic value (Akinyemi *et al.*, 2006; Adesiji *et al.*, 2014).

Sequencing of the bla_{TEM} genes revealed three variants. These included TEM 1 (the reference sequence), TEM 30 (substitution of amino acid R with S at position 241) and TEM 57 (substitution of amino acid G with D at position 90). Bae *et al.* (2015) reported that ampicillin and/or cephalosporin resistant *Salmonella* strains can carry the bla_{TEM} gene, with or without the bla_{CTX-M} or bla_{OXA} gene. However, only the bla_{TEM} gene was detected in this study. This further confirms the low rates of resistance by these *Salmonella* isolates to the extended beta-lactam antibiotics especially the cephalosporins. Mutation was observed in *pmrA* gene (T89→S and V93→G) for Colistin resistance. This amino acid substitution for *pmrA* gene has also been reported by Sun *et al*. (2009).

Resistance to fluoroquinolones typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV), changes in drug entry/efflux and plasmid mediated quinolone (*qnr*) resistance which protects the quinolone target proteins from inhibition (Jacoby, 2005). However, the genes regulating the influx and efflux of quinolones into the cells were not studied in this work. The genetic mutation in fluoroquinolone resistance is a step-wise phenomenon, affecting both *gyrA* and *parC*

genes (Le Hello *et al*., 2011). Most investigators have reported various substitutions of amino acid at different positions in the *gyrA* gene (Le Hello *et al.*, 2013; Nüesch-Inderbinen *et al*., 2015; Wasyl *et al*., 2015), but the substitution in this study which has a combination of Leu55→ Phe in *gyrA* subunit is extremely rare. Contrary to the report of Wang *et al*. (2015), mutation at position 87 has no comparative advantage of ciprofloxacin resistance over mutation at position 83. Although *gyrB* or *parE* gene mutations were not analysed in this study, the data presented here indicate these particular substitutions in g*yrA* and *parC* may be considered a major quinolone resistance mechanism as most work have not been able to detect mutations in the *gyrB* or *parE* genes (Le Hello *et al*., 2011; Le Hello *et al.*, 2013; Wasyl *et al*., 2015).

Different plasmid mediated quinolone resistance (PMQR) genes (*qnrA, qnrB, qnrC, qnrD, qnrS, qepA, aac(60)-Ib-cr*) have been described (Wasyl *et al*., 2014), but only *qnrA, qnrB* and *qnrS* genes were detected in this study. While *S.* Kentucky, *S.* Saintpaul and *S.* Isangi harboured the *qnrS* gene, only *S.* Kentucky and *S.* Muenchen had the *qnrA* gene. The PCR and sequencing results showed that plasmid mediated quinolone resistance (*qnr*) genes were responsible for the resistances seen in four of the isolates that were resistant to ciprofloxaxin but susceptible to nalidixic acid even though two of the isolates had single mutation on the *parC* gene.

Fluoroquinolones and third-generation cephalosporins are now commonly used for treatment due to widespread resistance to chloramphenicol, ampicillin, and cotrimoxazole. Fluoroquinolones are often the last resort for treatment and are listed by the WHO as critically important antimicrobials (Collignon *et al*., 2009). Prescription pattern, availability, and cost-effectiveness of quinolones as drugs that are usually prescribed in the management of most resistant bacterial infections were suggested as

factors that could be responsible for continued rapid evolution of fluoroquinolone resistant bacteria (Lamikanra *et al*., 2011; Wannaprasat *et al*., 2011).

In previous studies, researchers have demonstrated the relationship between the increased prevalence of antimicrobial-resistant bacteria and (a) the increased use of antimicrobials in human and veterinary medicine, (b) greater movement of people and animals, and (c) increased industrialization (Cheng *et al*., 2012). The southern part of Nigeria, especially Ogun is central to poultry production in Nigeria. This area not only meets the protein needs of its own people through the production of day-old chicks and poultry products, but also supplies other areas and imports animals feeds and ingredients from other regions (Killebrew *et al.,* 2010). This movement is a potential contributor to the spread of antibiotic-resistant bacteria (Kuang *et al.*, 2015). The environment itself also contains a variety of bacteria that potentially represent an immense pool of antibiotic-resistance genes, which if transferred between bacteria could cause human and animal disease (D'Costa *et al*., 2006). High level of resistance observed in this study reaffirms the importance of the need for strengthening collaboration between veterinary and public health sectors on appropriate detection and reporting of zoonotic foodborne pathogens (Adesiji and Fagbami, 2006). Despite bans and legislation, some antibiotics are still routinely fed to livestock prophylactically to increase profits and to limit potential bacterial infections in stressed and crowded livestock (Ndi and Barton, 2011). Antibiotic-resistant bacteria will inevitably follow wherever antimicrobials are used; therefore, a coordinated multi-disciplinary approach will be required to address this issue (Smith *et al*., 2009). Monitoring antimicrobial resistance trends among bacteria isolated from food, animals, and humans is necessary to inform public policy regarding the appropriate use of antimicrobial agents in veterinary and human medicine (Cummings *et al*., 2013).

Many studies have reported the successful use of PFGE for assessing genetic diversity of *Salmonella* isolates (Liebana *et al.,* 2002) and the clonal transmission of isolates in a country, a food industry, or a herd (Mammina *et al.,* 2003; Wonderling *et al.,* 2003; Liljebjelke *et al.,* 2005; Langvad *et al.,* 2006). The PFGE-based database remains useful for veterinarians, food producers, and risk managers for tracking sources of contamination on farms and in food processing plants. PFGE is also a powerful tool for foodborne disease investigation. The results of PFGE on 110 isolates (*S.* Kentucky = 44; *S.* Virchow = 8; *S.* Isangi = 9; *S.* Larochelle = 14; *S.* Poona = 16; *S.* Saintpaul = 9; *S.* Nigeria = 5; *S.* Typhimurium = 5) showed genetic similarities. Twenty isolates (45%) of *S.* Kentucky were indistinquishable (epidemiologically related) and were isolated from four different states (Ogun, n = 9; Kaduna, n = 6; Plateau, n = 3 and Bauchi, n = 2). The 110 isolates investigated by PFGE in this work were divided into 41 pulsotype patterns (Kentucky, n = 15; Larochelle, n = 9; Poona, n = 4; Virchow, n = 4; Saintpaul, n = 3; Nigeria, n = 2; and Typhimurium, n = 2). PFGE has been successfully used for the sub-typing of several *Salmonella* serotypes (Punia *et al.,* 1998; Garaizar *et al.,* 2000; Cardinale *et al.,* 2005). It has also been used in determining *Salmonella* serovars when there is a database with a large number of PFGE patterns covering a wide range of different serovars and which uses a highly stringent tolerance for serovar grouping (Gaul *et al.,* 2007; Chiou *et al.,* 2015). This result of study therefore provided a baseline molecular PFGE database of the most prevalent *Salmonella* serotypes in poultry in Nigeria for which other research work can be built upon..

Some serotypes of *Salmonella* are known to be associated with specific sources (Valdezate *et al.,* 2005). *Salmonella* Derby, *Salmonella* Montevideo and *Salmonella* Kottbus have often been linked to pig, cattle, and poultry production systems, respectively. Conversely, other serotypes are associated with isolates of various origins.

However, most of the PFGE patterns of these serotypes were unrelated to a specific sample type. For example all the 20 indistinguishable *S.* Kentucky isolates were from the five sample types analysed.

The MLVA results showed that three of the five *S.* Typhimurium isolates were 100% identical (same clone) and completely unrelated to the other two isolates which were related to each other (40% identical) at two loci (STTR9 and STTR5). Epidemiologically, two of these identical isolates were from the same state but from different farms while the other three isolates were from a different state. MLVA is useful in genotyping and is increasingly used in the detection and investigation of food-borne outbreaks caused by *S.* Typhimurium and other bacterial pathogens. MLVA data analysis usually relies on simple clustering approaches involving an appreciable isolates (Dimovski *et al*., 2014). Unfortunately, very few *S.* Typhimurium were isolated, thereby making interpretation of results difficult. However, this result was in agreement with that of serotyping, phage typing and the PFGE.

This study provided an epidemiological picture of the prevalence of *Salmonella* in Nigerian poultry. However, an analytical cross sectional study such as described here although well-suited to descriptive analysis may not be able to differentiate between the potential risk of contamination and the risk of the persistence of the organism. This is because samples were only collected once in this study. In order to be able to differentiate between risk factors for the persistence of *Salmonella* and those for the introduction of the organism, detailed longitudinal studies are required. Environmental sampling of the poultry houses is regarded as a cost effective method of the isolation of *Salmonella* from a large number of other competing bacteria (Aho, 1992; Davies and Wray, 1996). The most common samples collected from poultry houses include faeces,

litter and dust (Carrique-Mas *et al.*, 2009b). Naturally pooled faeces and dust is superior to sampling individual faeces for detection of *Salmonella* in laying flocks (Carrique-Mas *et al.*, 2008). Several investigators have demonstrated that rodents may be infected with *Salmonella* and often with the same serotypes as the livestock being investigated (Davies and Wray, 1995; Davies and Breslin, 2001; Barber *et al*., 2002). This study did not collect rodent samples because of the difficulties in trapping and preserving such samples.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The study recorded a farm *Salmonella* prevalence of 43.6% and a sample prevalence of 13.4% for commercial farms in the six selected states in Nigeria.
2. Sixty three different *Salmonella* serotypes were isolated in this study with *S.* Kentucky being the most prevalent, widespread and commonly resistant to antibiotics of all the serovars.
3. Factors found to be responsible for *Salmonella* infection and contamination of farm were type of housing ($p < 0.05$), crate type ($p < 0.03$), age of farmer ($p < 0.01$) and being a member of PAN ($p < 0.02$) while fencing farm ($p < 0.001$) and use of protective clothing ($p < 0.02$) was found to reduce the risk of *Salmonella* infection.
4. All (100%) the *Salmonella* isolates were susceptible to Meropenem, Cefotaxime and Ceftazidime.
5. Resistance of *Salmonella* to traditional antibiotics (AMP = 19.6%, TET = 75.3%, GEN = 47.4% and SUL = 75.3%) were high.
6. Resistance to ciprofloxacin and Nalidixic acid were high (89.7% and 77.3% respectively) in most of the isolates screened for antibiotic resistance and all (100%) *S.* Kentucky isolates were resistant to both antibiotics.
7. Resistance genes detected in this study included *tetA* (tetracycline), *sul1, sul2, sul3* (sulphamethoxazole), *catA1, cmlA1, floR* (chloramphenicol), *dfrA5-14* (Trimethoprim), *aac(3)-le* (gentamicin), *tem1, tem30, tem57* (ampicillin) and *qnrA, qnrB, qnrS* (quinolones).

8. Mutations (substitutions) were detected on the *gyrA* gene (Ser83→Phe, Asp87→Gly, Ser83→Tyr, Leu55→Phe), *parC* gene (Thr57→Ser) and *pmrA* gene (Thr89→Ser, Val93→Gly).
9. Comparison of PFGE profiles obtained from 110 *Salmonella* isolates tested identified PFGE cluster patterns for each of the eight serotypes investigated with significant ranges (56.6% - 100%) of genetic similarities.
10. The MLVA result showed that two *S.* Typhimurium isolates have the same allelic genes STTR9 and STTR3 but differ in the rest of the allelic genes while the other three isolates which were 100% genetically related have identical allelic genes STTR9, STTR5 and STTR3 but lacks allelic genes STTR6 and STTR10.

6.2 Recommendations

From the findings of this study, the following recommendations were made:

1. Antibiotic sensitivity testing should be done routinely by clinicians on *Salmonella* isolates in order to determine their susceptibilities before priscribing or administrating antibiotics.
2. Antibiotics such as Meropenem, Cefotaxime and Ceftazidime were effective against all the *Salmonella* isolates and calls for their preservation (by regulatory authorities) for use in the treatment of salmonellosis.
3. Further studies especially on the pathogenicity of the three most commonly isolated *Salmonella* serovars should be carried out by researcher.
4. There is need for the introduction of two or three of the most common serovars isolated in this study to the fowl typhoid (*Salmonella* Gallinarum) vaccine by the

National Veterinary Research Institute with the aim of developing a multivalent *Salmonella* vaccine that will be more cross protective.

5. There is need for regulatory authorities to monitor antimicrobial resistance trends among bacteria isolated from food, animals, and humans in order to inform public policy regarding the appropriate use of antimicrobial agents in veterinary and human medicine.
6. High rate of antibiotic resistance by *Salmonella* observed in this study reaffirms the importance of the need for strengthening collaboration between veterinary and public health sectors on appropriate detection and reporting of zoonotic food borne pathogens.
7. Farmers should maintain good farm biosecurity practises such as movement control (e.g. farm fencing), rational antibiotic usage and vaccination.
8. Government should regulate the importation of poultry and poultry products into the country and maintain a good border control to reduce the introduction of new *Salmonella* serovars which were hitherto not present in Nigeria such as *S.* Elisabethville, *S.* Larochelle.

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Appendix I: Survey of Risk Factors of *Salmonella* Infections in Commercial Poultry Flocks in Nigeria

INTERVIEWER-ADMINISTERED QUESTIONNAIRE

1. General Farm Data

1.1	Date of Survey (dd/mm/yy)	
1.2	Name of Investigator	
1.3	Name of farm	
1.4	Age of farm	
1.5	State & LGA	
1.6	Phone number	
1.7	Location (GIS Coordinates)	

2. Farmer/Farm Manager Characteristics

2.1	Age	☐ 1. 20-30 ☐ 2.31- 40 ☐ 3.41-50 ☐ Above 50
2.2	Gender	☐ Male ☐ Female
2.3	Marital status	☐ Married ☐ Single
2.4	Education	☐ Informal/Adult education ☐ Qur'anic ☐ Primary ☐ Secondary ☐ Tertiary
2.5	Primary profession	☐ 1. Poultry farmer ☐ 2. Civil servant ☐ 3. Private sector ☐ 4. Other specify
2.6	Membership of a poultry association or cooperative society?	☐ 1. Yes ☐ 2. No
2.7	Contact with animal health or extension staff	☐ 1. Yes ☐ 2. No
2.8	If yes	☐ 1. Once ☐ 2. Twice ☐ 3. More (specify)
2.9	How long have you been a poultry farmer	☐ 1. Less than 1 year ☐ 2. Up to 5 years ☐ 3. Up to 10 years ☐ 4. More than 10 yrs

3. Awareness of the Salmonellosis

3.1	Are you aware of *Salmonella* (Typhoid/Pullorum) infection in poultry farms?	☐ 1. Yes ☐ 2. No
3.2	Have you ever had *Salmonella* infection on your farm?	☐ 1. Yes ☐ 2. No
3.3	Signs/symptoms	☐ 1. Diarrhoea ☐ 2. Egg production ☐ 3. Mortality ☐ 4. Chick mortality
3.4	Losses suffered	☐ 1. Egg production ☐ 2. Mortality ☐ 3. Culling ☐ 4. Treatment ☐ 5. Revaccination
3.5	How many episodes of *Salmonella* in the last two years	☐ 1. Once ☐ 2. Twice ☐ 3. More (specify)

4. Farm Stock Details

4.1	House No.	Age of birds	Breed	Type of production	Type of Housing

5. Sample Stock Detail

5.1	Flock size	
5.2	Breed of flock	
5.3	Age of flock (weeks)	
5.4	Source of day-old chick	
5.5	Egg gathering system	☐ 1. Manual ☐ 2. Automatic
5.6	Number of egg gatherings per day	☐ 1. Once ☐ 2. Twice ☐ 3. More than twice
5.7	Egg packing crates	☐ 1. Carton ☐ 2. Plastic
5.8	System of Production	☐ 1. Deep litter ☐ 2. Battery cage Litter type ☐ i Wood shaving ☐ ii Sawdust ☐ iii Other specify

6. Disease Management

6.1	Vaccinations	☐ 1. Yes Names of vaccines ☐ 2. No
6.2	Any disease outbreak during rearing period?	☐ 1. Yes ☐ 2. No
6.3	Any treatment for outbreak	☐ 1. Yes Name of medication ☐ 2. No
6.4	Antibiotic treatment during the last two weeks	☐ 1. Yes Name of antibiotic ☐ 2. No
6.5	Treatment of sick birds	☐ 1. Vet ☐ 2. Self ☐ 3. Other (specify)
6.6	Management of dead birds	☐ 1. On-farm ☐ 2. Off-farm

7. Farm Biosecurity

7.1	Farm Fenced	☐ 1. Yes ☐ 2. No
7.2	Floor Type	☐ 1. Concrete ☐ 2. Other
7.3	Poultry waste management	☐ 1. On-farm ☐ 2. Off-farm
7.4	Poultry by-product disposal	☐ 1. On-farm ☐ 2. Off-farm
7.5	Rodent Control (Observation/bait)	☐ 1. Yes ☐ 2. No
7.6	Presence of other livestock on farm	☐ 1. Yes ☐ 2. No
7.7	Presence of other poultry farms within 1km radius	☐ 1. Yes ☐ 2. No
7.8	Do wild birds have access to the farm	☐ 1. Yes ☐ 2. No
7.9	Presence of other livestock farms within 1km radius	☐ 1. Yes ☐ 2. No
7.10	Are there foot dips provided at the entrance of houses?	☐ 1. Yes ☐ 2. No
7.11	How frequently are the disinfectants used in the foot dips changed?	☐ 1. Every week ☐ 2. Every month ☐ 3. Other (specify)
7.12	Do the doors, windows and other openings of the houses have wire mesh?	☐ 1. Doors only ☐ 2. Windows only ☐ 3. Drain pipes only ☐ 4. All openings ☐ 5. No opening is wire-meshed
7.13	Is there a changing room on the farm?	☐ 1. Yes ☐ 2. No
7.14	Is there a lavatory on the farm	☐ 1. Yes ☐ 2. No
7.15	Do staff of the farm work on other farms	☐ 1. Yes ☐ 2. No

8. Personnel & Sanitation

8.1	Are workers specifically and permanently assigned to work in each unit of the farm?	☐ 1. Always ☐ 2. Sometimes ☐ 3. Never
8.2	Do workers wear specific clothing during work on the farm?	☐ 1. Always ☐ 2. Sometimes ☐ 3. Never
8.3	Do workers wear specific footwear during work on the farm?	☐ 1. Always ☐ 2. Sometimes ☐ 3. Never
8.4	Do personnel leave the clothing/footwear on the farm?	☐ 1. Always ☐ 2. Sometimes ☐ 3. Never
8.5	Do workers wear boots when working inside the houses all the time	☐ 1. Always ☐ 2. Sometimes ☐ 3. Never
8.6	Are workers who have direct contact with the birds aware of the importance of personal hygiene in poultry farms?	☐ 1. Yes ☐ 2. No

9. Farm Sanitation

9.1	How are poultry houses cleaned after depopulation?	☐ 1. Washing only ☐ 2. Washing & disinfection ☐ 3. Disinfection only ☐ 4. Fumigation
9.2	Cleaning of poultry farm surroundings	☐ 1. Once a week ☐ 2. Twice a week ☐ 3. Other (specify)
9.3	Number of weeks poultry house is left empty after previous flock	☐ 1. Less than a month ☐ 2. More than a month ☐ 3. One month ☐ 4. Other (specify)

10. Feed and Water Management

10.1	Source of feed	☐ 1. Compounds own feed ☐ 2. Commercial feed
10.2	Additives in feed	☐ 1. Yes Name of additive _____ ☐ 2. No
10.3	Coccidiostat in feed	☐ 1. Yes ☐ 2. No
10.4	Source of Water	☐ 1. Public ☐ 2. Bore hole ☐ 3. Well ☐ 4. Others (Specify)
10.5	Type of drinker	☐ 1. Metal ☐ 2. Plastic ☐ 3. Other
10.6	Drinker maintenance	☐ 1. Once a week ☐ 2. Twice a week ☐ 3. Other (specify)
10.7	Feeder Maintenance	☐ 1. Once a week ☐ 2. Twice a week ☐ 3. Other (specify)
10.8	Number of feedings per day	☐ 1. Once a day ☐ 2. Twice a day ☐ 3. Three times a day ☐ 4. Other (specify)

Appendix II: Polymerase chain reaction (PCR) Procedures.

A. Multiplex PCR - Identification of tem, pse, oxa Genes Responsible for Ampicillin Resistance

PCR reaction (MIX) was prepared, using a 2 ml Dnase free tube. To the tubes, 45 µl of PCR mix was added, which was briefly vortexed before adding 5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	200 µM	1 µl
Primer *tem* fw (503bp)	10 µM	1 µM	5 µl
Primer *tem* rv	10 µM	1 µM	5 µl
Primer *pse* fw (419bp)	10 µM	1 µM	5 µl
Primer *pse* rv	10 µM	1 µM	5 µl
Primer *oxa* fw (708bp)	10 µM	1 µM	5 µl
Primer *oxa* rv	10 µM	1 µM	5 µl
Taq Gold	5U/µl	2.5 U	0.5 µl
H2O		4.5 µl	
DNA		5 µl	
Total volume		50	

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 55°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primers sequence

Name	Sequence	Accession number	Reference
tem fw	5'-TTGGGTGCACGAGTGGGT-3'	AF126482	Guerra *et al*., 2001
tem rv	5'-TAATTGTTGCCGGGAAGC-3'		
pse fw	5'-CGCTTCCCGTTAACAAGTAC-3'	M69058	Sandvang *et al*., 1997
pse rv	5'-CTGGTTCATTTCAGATAGCG-3'		
oxa fw	5'-AGCAGCGCCAGTGCATCA-3'	AJ009819	Guerra *et al*., 2000
oxa rv	5'-ATTCGACCCCAAGTTTCC-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons are:

tem gene: 503 bp

pse gene: 419 bp

oxa gene: 708 bp

B. Multiplex PCR - Identification of tetA, tetB, tetG Genes Responsible for Tetracycline Resistance

PCR reaction (MIX) was prepared, using a 2 ml Dnase free tube. To the tubes, 45 µl of PCR mix was added, which was briefly vortexed before adding 5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	200 µM	1 µl
Primer *tetA* fw (210bp)	10 µM	1 µM	5 µl
Primer *tetA* rv	10 µM	1 µM	5 µl
Primer *tetG* fw (500bp)	10 µM	1 µM	5 µl
Primer *tetG* rv	10 µM	1 µM	5 µl
Primer *tetB* fw (600bp)	10 µM	1 µM	5 µl
Primer *tetB* fv	10 µM	1 µM	5 µl
Taq Gold	5U/µl	2.5 U	0.5 µl
H2O		4.5 µl	
DNA		5 µl	
Total volume		50 µl	

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 55°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primers sequence

Name	Sequence	Accession number	Reference
tetA fw	5'-GCTACATCCTGCTTGCCT-3'	X01367	Ng *et al*., 1999
tetA rv	5'-CATAGATCGCCGTGAAGA-3'		
tetB fw	5'-TTGGTTAGGGGCAAGTTTTG-3'	J01830	Ng *et al*., 1999
tetB rv	5'-GTAATGGGCCAATAACACCG-3'		
tetG fw	5'-GCTCGGTGGTATCTCTGC-3'	852437	Ng *et al*., 1999
tetG rv	5'-AGCAACAGAATCGGGAAC-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons are:

- *tetA* gene: 210 bp
- *tetB* gene: 600 bp
- *tetG* gene: 500 bp

C. Multiplex PCR - Identification of qnrA, qnrB and qnrS Genes Responsible for plasmidic quinolone Resistance

PCR reaction (MIX) was prepared, using a 2 ml Dnase free tube. To the tubes, 45 µl of PCR mix was added, which was briefly vortexed before adding 5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	3 µl
dNTPs	10 mM	200 µM	1 µl
Primer *qnrA* fw (580bp)	10 µM	0,2 µM	5 µl
Primer *qnrA* rv	10 µM	0,2 µM	5 µl
Primer *qnrB* fw (264bp)	10 µM	0,2 µM	5 µl
Primer *qnrB* rv	10 µM	0,2 µM	5 µl
Primer *qnrS* fw (428bp)	10 µM	0,2 µM	5 µl
Primer *qnrS* fv	10 µM	0,2 µM	5 µl
Taq Gold	5U/µl	2.5 U	0.5 µl
H2O		29,5 µl	
DNA		5 µl	
Total volume		50 µl	

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 10';

35 cycles: (95°C 1', 54°C 1', 72°C 1');

Post-extension: 72°C 10'.

Primers sequence

Name	Sequence	Accession number	Reference
qnrA fw	5'-TCAGCAAGAGGATTTCTCA-3'	AY070235	Wang *et al.,* 2003;
qnrA rv	5'-GGCAGCACTATTACTCCCA-3'		Cattoir *et al*., 2007
qnrB fw	5'-ATGACGCCATTACTGTATTA-3'		Jacoby *et al.,* 2006;
qnrB rv	5'-GATCGCAATGTGTGAAGTTT-3'		Cattoir *et al*., 2007
qnrS fw	5'-ACGACATTCGTCAACTGCAA-3'		Gay *et al*., 2006;
qnrS rv	5'-TAAATTGGCACCCTGTAGGC-3'		Cattoir *et al*., 2007

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons are:

qnrA gene: 580 bp

qnrB gene: 264 bp

qnrS gene: 428 bp

D. Multiplex PCR - Identification of catA1, cmlA1 and floR Genes Responsible for chloramphenicol Resistance

PCR reaction (MIX) was prepared, using a 2 ml Dnase free tube. To the tubes, 45 µl of PCR mix was added, which was briefly vortexed before adding 5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	400 µM	2 µl
Primer *catA1* fw (623bp)	10 µM	1 µM	5 µl
Primer *catA1* rv	10 µM	1 µM	5 µl
Primer *cmlA1* fw (435bp)	10 µM	1 µM	5 µl
Primer *cmlA1* rv	10 µM	1 µM	5 µl
Primer *floR* fw (868bp)	10 µM	1 µM	5 µl
Primer *floR* fv	10 µM	1 µM	5 µl
Taq Gold	5U/µl	2.5 U	0.5 µl
H2O		3.5 µl	
DNA		5 µl	
Total volume		50 µl	

PCR conditions (Thermal profile):

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 55°C 30'', 72°C 40'')

Post-extension: 72°C 5'.

Primers sequene

Name	Sequence	Accession number	Reference
catA1 fw	5'-CCACCGTTGATATATCCC-3'	U40780	Guerra *et al.*, 2001
catA1 rv	5'-CCTGCCACTGATCGCAGT-3'		
cmlA1 fw	5'-TGTCATTTACGGCATACTCG-3'	M64550	Guerra *et al.*, 2001
cmlA rv	5'-ATCAGGCATCCCATTCCCAT-3'		
floR fw	5'-CACGTTGAGCCTCTATAT-3'	AF071555	Ng *et al*., 1999
floR rv	5'-ATGCAGAAGTAGAACGCG-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons are:

- *catA1* gene: 623 bp
- *cmlA1* gene: 435 bp
- *floR* gene: 868 bp

E. One-Target PCR - Identification of sul1 Gene Responsible for Sulfamethoxazole Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	2,5 µl
MgCl2	25 mM	2 mM	2µl
dNTPs	10 mM	200 µM	0,5 µl
Primer *sul1* fw (436bp)	10 µM	1 µM	2,5 µl
Primer *sul1* rv	10 µM	1 µM	2,5 µl
Taq Gold	5U/µl	1.5 U	0,3 µl
H2O		12.2 µl	
DNA		2,5 µl	
Total volume		25 µl	

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 65°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primer sequence

Name	Sequence	Accession number	Reference
sul1 fw	5'-CTTCGATGAGAGCCGGCGGC-3'	X12869	Sandvang *et al.*, 1997
sul1 rv	5'-GCAAGGCGGAAACCCGCGCC-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 436

F. One-Target PCR - Identification of sul2 Gene Responsible for Sulfamethoxazole Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	2,5 µl
MgCl2	25 mM	2 mM	2µl
dNTPs	10 mM	200 µM	0,5 µl
Primer *sul2* fw (707bp)	10 µM	1 µM	2,5 µl
Primer *sul2* rv	10 µM	1 µM	2,5 µl
Taq Gold	5U/µl	1.5 U	0,3 µl
H2O		12.2 µl	
DNA		2,5 µl	
Total volume		25 µl	

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 58°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primer sequence

Name	Sequence	Accession number	Reference
sul2 fw	5'-TCAACATAACCTCGGACAGT-3'	M36657	Chu *et al.*, 2001
sul2 rv	5'-GATGAAGTCAGCTCCACCT-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 707bp

G. One-Target PCR - Identification of sul3 Gene Responsible for Sulfamethoxazole Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	2,5 µl
MgCl2	25 mM	2 mM	2 µl
dNTPs	10 mM	200 µM	0,5 µl
Primer *sul3* fw (789bp)	10 µM	1 µM	2,5 µl
Primer *sul3* rv	10 µM	1 µM	2,5 µl
Taq Gold	5U/µl	1.5 U	0,3 µl
H2O		12.2 µl	
DNA		2,5 µl	
Total volume		25 µl	

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 51°C 30'', 72°C 40'')

Post-extension: 72°C 5'.

Primer sequence

Name	Sequence	Accession number	Reference
sul3 fw	5'-GAGCAAGATTTTTGGAATCG-3'	M36657	Chu *et al.*, 2001
sul3 rv	5'-CATCTGCAGCTAACCTAGGGCTTTGGA-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 789

H One-Target PCR - Identification of gyrA Gene

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	240 µM	1,2 µl
Primer *gyrA* fw (334bp)	20 µM	1 µM	2,5 µl
Primer *gyrA* rv	20 µM	1 µM	2,5 µl
Taq Gold	5U/µl	1,5 U	0,3 µl
H2O		29,5 µl	
DNA		5 µl	
Total volume		50 µl	

PCR conditions (Thermal profile):

Pre-denaturation: 95°C 5';

35 cycles: (95°C 30'', 52°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primer Sequence

Name	Sequence	Accession number	Reference
gyrA fw	5'-CCAGATGTHCGHGATGG-3'	U28377	Guerra *et al.*, 2003
gyrA rv	5'-ACGAAATCAACSGTYTCTTTTTC-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 334bp

J. One-Target PCR - Identification of dfrA5-14 Gene Responsible for Trimethoprim Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	200 µM	1 µl
Primer *dfrA5-14* fw (379bp)	10 µM	1 µM	5 µl
Primer *dfrA5-14* rv	10 µM	1 µM	5 µl
Taq Gold	5U/µl	2.5 U	0,5 µl
H2O		24,5 µl	
DNA		5 µl	
Total volume			

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 55°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primer sequence

Name	Sequence	Accession number	Reference
dfrrA5-14 fw	5'-GATTGGTTGCGGTCCA-3'		Frech *et al.*, 2003
dfrrA5-14 rv	5'-CTCAAAAACAACTTCGAAGG-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 379bp

K. One-Target PCR - Identification of aac(3)-le Gene Responsible for Gentamycin Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	400 µM	2 µl
Primer *aac(3)-le* fw (358bp)	10 µM	1 µM	5 µl
Primer *aac(3)-le* rv	10 µM	1 µM	5 µl
Taq Gold	5U/µl	2.5 U	0,5 µl
H2O		23,5 µl	
DNA		5 µl	
Total volume			

PCR conditions (Thermal profile):

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 55°C 30'', 72°C 30'');

Post-extension: 72°C 5'.

Primer sequence

Name	Sequence	Accession number	Reference
aac(3)-le fw	5'-GCAACAAGCCGTCATCAA-3'	AY463797	B. Aranda unpublished
aac(3)-le rv	5'-CTCTGCTCAACCGCAATGTC-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 358bp

L One-Target PCR - Identification of pmrA Gene Responsible for colistin Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	200 µM	1 µl
Primer *pmrA* fw (668bp)	5 µM	0,8 µM	8 µl
Primer *pmrA* rv	5 µM	0,8 µM	8 µl
Taq Gold	5U/µl	2,5 U	0,5 µl
H2O		18,5 µl	
DNA		5 µl	
Total volume			

PCR conditions (Thermal profile):

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 55°C 30'', 72°C 40'');

Post-extension: 72°C 7'.

Primer sequence

Name	Sequence	Accession number	Reference
pmrA fw	5'-CGCGAATTTCGTGCATGATATG-3'		Sun *et al.*, 2009
pmrA rv	5'-ATGTCCCGATGCTCATTTGGC-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 668bp

M One-Target PCR - Identification of pmrB Gene Responsible for colistin Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	2 mM	4 µl
dNTPs	10 mM	400 µM	2 µl
Primer *pmrB* fw (1070bp)	5 µM	0,8 µM	8 µl
Primer *pmrB* rv	5 µM	0,8 µM	8 µl
Taq Gold	5U/µl	2,5 U	0,5 µl
H2O		17,5 µl	
DNA		5 µl	
Total volume			

PCR conditions (Thermal profile):

Pre-denaturation: 95°C 5';

30 cycles: (95°C 30'', 52°C 30'', 72°C 1');

Post-extension: 72°C 10'.

Primer sequence

Name	Sequence	Accession number	Reference
pmrB fw	5'-AGGAAATTCTGGGCGAGCA-3'		Sun *et al.*, 2009
pmrB rv	5'-CGTTTTCAGCGAAGAGCGA-3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 668bp

N. One-Target PCR - Identification of parC Gene Responsible for quinolone Resistance

PCR reaction (MIX) was prepared, using a 1.5 ml Dnase free tube. To the tubes, 22.5 µl of PCR mix was added, which was briefly vortexed before adding 2.5 µl of DNA preparation. The tubes were then inserted firmly into the thermal cycler and covered with the lid for DNA amplification. The amplicons were run in a 2% agarose gel, stained with ethidium bromide before viewing and reading the results

Reagents	Initial concentration	Final concentration	Quantity per sample
Buffer Taq Gold	10X	1X	5 µl
MgCl2	25 mM	1,5 mM	3 µl
dNTPs	10 mM	200 µM	1 µl
Primer *parC* fw (270bp)	5 µM	0,5 µM	5 µl
Primer *parC* rv	5 µM	0,5 µM	5 µl
Taq Gold	5U/µl	1 U	0,2 µl
H2O		25,8 µl	
*DNA		5 µl	
Total volume			

PCR conditions (Thermal profile)

Pre-denaturation: 95°C 5';

35 cycles: (95°C 30'', 53°C 30'', 72°C 30'') ;

Post-extension: 72°C 5'.

Primers sequence

Name	Sequence	Accession number	Reference
parC fw	5' -CTATGCGATGTCAGAGCTGG -3'		Eaves *et al*., 2002
parC rv	5'- TAACAGCAGCTCGGCGTATT -3'		

The PCR products are visualised by gel electrophoresis in 2% agarose gel and stained with ethidium bromide solution. Observation and images are carried out using a UV light source. The expected amplicons is 270bp