CHARACTERIZATION OF *SALMONELLA* ISOLATES FROM SELECTED POULTRY FARMS IN KWARA STATE, NIGERIA

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BY

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JULY, 2017

Declaration

I declare that the work in this thesis, entitled "**Characterization of *Salmonella* Isolates from Selected Poultry Farms in Kwara State, Nigeria**" has been performed by me in the Department of Veterinary Microbiology, Ahmadu Bello University, Zaria under the supervision of Prof. C. N. Kwanashie, Prof. M. A. Raji and Dr P. H. Mamman. The information derived from the literature has been duly acknowledged in the list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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Sign Date

Certification

This thesis, **entitled "CHARACTERIZATION OF *SALMONELLA* ISOLATES FROM SELECTED POULTRY FARMS IN KWARA STATE, NIGERIA"** by AKEEM OLAYIWOLAAHMED meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria and is approved for its' contribution to knowledge and literary presentation.

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Dedication

I passionately dedicate this work to Allah, the owner of all that exist, who has made me what I am and to my family whose prayers will always remain with me.

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Abstract

Salmonellosis is a major threat facing the poultry industry in Nigeria. This study was conducted to characterize *Salmonella* from selected poultry farms in Kwara state and determine their resistance patterns to routinely used antimicrobials. A total of 900 samples were collected between December, 2015 and May, 2016 from poultry environment, live chickens, and dead chickens. The samples were subjected to standard bacteriological methods. Presumptive *Salmonella* isolates were tested for specific serovars, antimicrobial resistance profile and PCR testing of 24 randomly selected isolates for the genes *fim*A, *fli*C, *rfbsg* and *Sef*C. Of the 900 samples, 58 (6.4 %) were positive for *Salmonella*and the isolation rate was only statistically significant ($p < 0.05$) in live birds. The isolates are comprised of 13 different serovars. The three predominant serovars; *Salmonella entrica* ser. 6, 7:d:- (29.0 %), *S.* Agama (28.0 %) and *S.* Typhimurium (16.0 %), were isolated from all the three sources.Rare serovars such as *S.* Albany, *S*. Colindale, *S.* Istanbul, *S.* Larochelle, *S.* Nigeria and *S.* Orion were also isolated from chickens in this study.A high frequency of antimicrobial resistance was generally observed with all the isolates exhibiting a total (100 %) resistance to ampicillin, cefotaxime and ceftazidime. Varying distribution patterns of the studied genes were observed among the isolates.While, *fim*Awas found in all the 24 strains, *sef*Cwas found only among the *Salmonella enterica* ser. 45: d: 1,7.This study documents for the first time, isolation of *Salmonella entrica* ser. 6, 7:d:- from chicken. The studyalso documents a high frequency of fluoroquinolone resistance among the isolates which may be consequence of selective pressure in the environment.The results of this study has shown that the *fim*Agene was widely distributedamong *Salmonella* irrespective of the serovars. However, the *sefC*gene appears to be serovar specific.*Salmonella* serovars 6, 7: d: -, Agama and Typhimurium were the major serovars associated with poultry and environmental

contamination in the Kwara State, north-central region of Nigeria.These serovars constitute a health risk to poultry, environment and human population in the region, therefore,there is need to institute measures to control their spread.

Table of Contents

Tittle Page ……………………..……………………………………………………….....iii

Declaration iii

Certification iv

Dedication v

Acknowledgements vi

Abstract viii

Table of Contents x

List of figures xv

List of Tables xvi

List of Abbreviations xviii

CHAPTER ONE 1

1.0 INTRODUCTION 1

1.1 Back Ground Information 1

1.2 Statement of Research Problem 4

1.3 Justification of the Study 6

1.4 Aim and Objectives 7

1.4.1 Aim of the Study 7

1.4.2 Objectives of the Study 7

1.5 Research Questions 7

CHAPTER TWO 8

2.0 LITERATURE REVIEW 8

2.1 Nomenclature of Salmonella 8

2.2 Morphology of Salmonella 11

2.3 Growth Requirement and Survival characteristics of Salmonella 11

2.4 Mode of Transmission.. 13

2.5 Carrier and Sources of Infection 15

2.6 Epidemiology of Salmonella 16

2.7 Predisposing Factors 18

2.8 Pathogenesis of Salmonellosis 19

2.9 Chromosomal and Plasmid Mediated Salmonella Virulence.. 20

2.10 Economic Implication of Salmonellosis.. 24

2.11 Public Health Significance 25

2.12 Clinical Manifestation.. 27

2.13 Immunity 28

2.14 Diagnosis 30

2.15 Treatment and Control 31

2.16 The Disease in Man 32

2.17 Antimicrobial Resistance ... 34

2.18 Current Situation of Avian Salmonellosis in Nigeria ... 36

CHAPTER THREE ... 37

3.0 MATERIALS AND METHODS ... 37

3.1 Materials ... 37

3.1 Study Area ... 37

3.2 Study Design ... 40

3. 2 Sampling and Sample Collection ... 42

3.3 Isolation and Identification of Salmonella Species ... 44

3.4 Biochemical Tests ... 45

3.4.1 Indole test ... 45

3.4.2 Methyl red test ... 45

3.4.3 Voges Proskauer test ... 45

3.4.4 Citrate utilization test using Simmon's citrate agar ... 46

3.4.5 Urease test ... 46

3.4.6 Triple sugar iron agar (TSI) test ... 46

3.4.7 Motility test ... 47

3.5 Serotyping of Salmonella Isolates ... 47

3.6 Antibiotic Susceptibility Testing ... 49

3.7 Polymerase Chain Reaction Testing of the Isolates 50
3.7.1 DNA extraction..... 50
3.7.2 Primers..... 51
3.7.3 PCR assay 53
3.8 Statistical Analysis..... 56

CHAPTER FOUR..... 57
4.0 RESULTS 57
4.1 Bacterial Isolates from the Different Sources..... 57
4.2 Serovars Distribution..... 59
4.3 Detection of Salmonella Genes in isolates from selected poultry farms in Kwara State Using PCR 62
4.4 Antimicrobial Susceptibility Testing..... 65
4.5 Frequency of Resistance Patterns of Salmonella Isolates to Antimicrobial Agents67

CHAPTER FIVE 69
5.0 DISCUSSION..... 69

CHAPTER SIX 75
6.0 CONCLUSION AND RECOMMENDATIONS..... 75

6.1 CONCLUSION 75

6.2 RECOMMENDATIONS 76

REFERENCES 77

APPENDIX 99

List of figures

Title **Page**

Figure 2.1: Virulence factors involved in the intracellular survival of *Salmonella*..................... 23

Figure 3.1: Map of Kwara State showing location of the sample sites within selected Local Government Areas……………………………………………………..…………….…39

List of Tables

Title **Page**

Table 3.1: Farms selected from different local Government areas of Kwara State 41

Table 3.2: Types and number of samples collected from poultry farms in Kwara State. 43

Table 3.3: Primers sequence for PCR amplification of selected genes of *Salmonella* isolates from poultry farms Kwara State. 52

Table 3.4: PCR programs used for genes sefC and fimA 54

Table 3.5: PCR programs used for genes fliC and rfbsg 55

Table 4.1: Number of samples collected and rate of *Salmonella* isolation rate per farm per farm 58

Table 4.2: Occurrence of *Salmonella* species from different sources in poultry farms in Kwara State.. 59

Table 4.3: Distribution of *Salmonella* serovars in selected poultry farms in Kwara state............ 60

Table 4.4: Distribution of Salmonella serovars by different sources from selected poutry farms in Kwara State 61

Table 4.5: Antimicrobial resistance profile of *Salmonella* serovars from selected poultry farms in Kwara State 66

Table 4.6: Multidrug resistance patterns detected among *Salmonella* isolates from poultry farms in Kwara State 68

List of Plates

Title Page

Plate 4.1: Detection of *fim*A gene by polymerase chain reaction 634

Plate 4.2: Detection of *sefC* gene polymerase chain reaction. 65

Plate I: Typical colonies of *Salmonella* on Xylose lysine Deoxycholate (XLD). 100

Plate II: Reaction of *Salmonella* species on TSI slants.. 101

Plate III: Reaction of *Salmonella* species to Methyl Red test.. 102

Plate IV: Reaction of *Salmonella* species on Simmon Citrate slants... 103

Plate VI: Reaction of *Salmonella* species on Urea agar slants 104

Plate V: Incubation of of PCR cocktails in a thermocycler and loading of PCR products on agarose gel well.. 105

List of Abbreviations

CDC: Centres for Disease Control and Prevention

CLSI: Clinical and Laboratory Standard ainstitute

DNA: Deoxyribonucleic Acid

DT: Definitive Phage Type

ERK: Extracellular Signal-regulated Kinases

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

ICMSF: International Commission on Microbiological Specifications for Foods

ISO: International Standard Organization

JNK: Jun N-terminal Kinases

MTOC: Microtubule Organising Centre

NAFDAC: National Agency for Food and Drug Administration an Control

NBS: National Bureau of Statistics

NIAID: National Institute of Allergy and Infectious Diseases

OIE: Office Internationale Epizootic

PCR: Polymerase Chain reaction

SCV: *Salmonella* Containing Vacuole

SPI: *Salmonella* Pathogenicity Island

T3SS: Type III Secretion System

TSI: Triple Sugar Iron

WHO: World Health Organization

XLD: Xylose Lysine Deoxycholate

CHAPTER ONE

1.0 INTRODUCTION

1.1 Back Ground Information

Members of the genus *Salmonella* are gram-negative, non-lactose fermenting and non-spore formingand facultatively anaerobic, rod-shaped bacteria (Cheesbrough 2000). Phenotypically and phylogenetically (Wyatt *et al.,* 1993), the genus *Salmonella* represents a homogeneous taxon within the family *Enterobacteriaceae* (Aslanzadeh 1992) within the delta subclass of proteobacteria. All salmonellae are actively motile except *Salmonella* Pullorumand *Salmonella* Gallinarum (Cheesbrough 2000). They are also, non-capsulated with the exception of *Salmonella* Typhi (Cheesbrough, 2000; Perilla, 2003).The genus comprises two species, *Salmonella enterica* and*Salmonella bongori*. *Salmonella enterica*is further divided into 6subspecies: *enterica*, *salmae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (Brenner *et al*., 2000). So far, more than 2610 serovars of *Salmonellaenterica* have been recognized from all over the world, and almost all are able to cause illness in humans and animals (Guibourdenche *et al*., 2010). The genus was named after Daniel Elmer Salmon, an American veterinarian(FDA/CFSAN, 2009).

Salmonella can be divided into two major groups of clinical importance: Group one, includes members of the genus that are involved as aetiologic agents of enteric fever (typhoidal salmonellosis): *Salmonella* Typhi and *Salmonella* Paratyphi,while the group two are the aetiologic agents of food poisoning (non typhoidal salmonellosis): *Salmonella* Typhimurium and more recently phagetype DT 104. Other members of the non typhoidal *Salmonella*are *Salmonella* Enteritidis,*Salmonella* Hiduddify,*Salmonella* Heidelberg, *Salmonella* Agona, *Salmonella*

Newport, *Salmonella* Hadar, and *Salmonella* Dublin (Arora, 2001; Adkins and Sandiago, 2006, Raufu *et al.,* 2009).

Salmonellosis in poultry is endemic worldwide, causing morbidity and mortality and economic losses (Akter *et al.,* 2007; Kwon *et al.,* 2010; Abiodun *et al.,* 2014 Mamman*et al.,* 2014). The disease is very significant by virtue of the fact that *Salmonella* can betransmitted vertically from parent to offspring. The control of salmonellosis in the poultry industry is complicated because, in addition to vertical transmission from parent stock to offspring, horizontal transmission on farms is also common; this makes its control problematic (Dawoud *et al.,* 2011; Hannah *et al.,* 2011; Abiodun *et al.,* 2014).Poultry can contract*Salmonella* infection by horizontal route through contaminated litter, faeces, feed, water, dust, equipment, fomites contaminated with *Salmonella* or via diseased chicks and rodents infected with*Salmonella*(Poppe, 2000; Mohammed*et al.,* 2009). They can also be transmitted by other animals, wild birds and personnel. *Salmonella* may contaminate young chicks directly through ovarian transmission or penetrate the egg shell after the egg has been laid (Cox *et al.,* 2000; Mohammed*et al.,* 2009). A number of studies in Nigeria have shown that *Salmonella* infection is endemic in many parts of the country (Onunkwo *et al.,* 2001; Fashae *et al*., 2010; Raufu *et al*., 2013; Mamman*et al.,* 2014). The wide host range, the large number of convalescents, chronic health carriers and environmental sources in the community increase the reservoir status of *Salmonella* infection and enhance its endemicity especially, in areas with low environmental hygiene (Onunkwo *et al.,* 2001). Poultry farms and poultry products are the major sources for *Salmonella* contamination (Todd, 1980; Hamphrey *et al*., 1988; Robberts, 1991; Hussein *et al.,* 2009). Reports on various poultry diseases occurring in some parts of this country showed that salmonellosis is one of the major threats facing poultry production in Nigeria (Abdu *et al*., 1985; Garba, 2005; Saidu *et al*., 2006 and Mamman*et al.,*

2014). Animal droppings have been shown to be a potential reservoir for many enteric organisms (Mara and Caincross 1989; Raufu *et al.,* 2014). Salmonellosis is an infection caused by ingesting *Salmonella*in food that is contaminated by faeces of animals or humans directly or indirectly. Salmonellosis is one of the most important food-borne bacterial zoonotic diseases worldwide. Some of the symptoms of human salmonellosis are diarrhoea, vomiting, fever, and abdominal pain. These occur 12-36 hours after eating infected food, in acute infection, blood and mucous are present in faecal specimens (Al – jurayyan *et al.,* 2004). *Salmonella* is known to be a major public health problem all over the world. For instance, in the year 2000, it was reported to have caused 41,800 cases of human food borne diseases in England and Wales (Adak *et al.,*2002). In the USA, about one million human salmonellosis cases are recorded per year, with 94% associated with a food borne origin (Scallan *et al.,* 2011).

The virulence of *Salmonella* species is associated with a combination of chromosomal and plasmid factors (Oliveira *et al*., 2003), and many studies have identified genes that encode these factors. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Some genes are known to be involved in adhesion and invasion viz., *pef* (plasmid encoded fimbriae), *spv* (*Salmonella* plasmid virulence) or *inv* (invasion gene)(Clouthier *et al*.,1993). Others are associated with the survival in the host system- *mgt*C (Magnesium transport C) (Blanc-Potard and Groisman 1997) or in the actual manifestation of pathogenic processes viz., *stn* (*Salmonella* toxin), *pip* A, B, D (Marcus *et al*., 2000). Most *Salmonella* strains have structural gene *fli*C (Flagella) that encodes flagellins. Non-motile strains generally harboured these structural genes, but are unable to build up a functional flagellum (Paiva, 2009). The primers for allele-specific Polymerase Chain Reaction (PCR) amplification of *rfb*Sg (Rough *Salmonella* gene) and *rfb*Sp (Rough *Salmonella* gene) genes were based on *rfb*S, these primers

are used in differentiation of *Salmonella* Gallinarum from *Salmonella* Pullorum (Shah *et al.,* 2005).

1.2 Statement of Research Problem

Salmonellosis is considered to be one of the major bacterial disease problems in the poultry industry world-wide. *Salmonella* species are responsible for a variety of acute and chronic diseases in both poultry and humans (Majowicz *et al*., 2010; Okwori *et al*., 2013). To date, more than 2610 serovars of *Salmonellaenterica* have been recognized from all over the world, and almost all are able to cause illness in humans and animals (Guibourdenche *et al*., 2010). Salmonellosis is a very important disease in the poultry industry because of the fact that *Salmonella* can betransmitted vertically from parent to offspring. The control of salmonellosis in the poultry industry is complicated because, in addition to vertical transmission, horizontal transmission on farms is also common; this makes its control a problematic (Dawoud *et al.,* 2011; Hannah *et al.,* 2011; Abiodun *et al.,* 2014).

Apart from the known poultry-adapted non-motile *Salmonella* serotypes namely, *Salmonella* Gallinarum and *Salmonella* Pullorum(Montville and Mattews, 2008), some other serotypes have been known to be commonly associated with poultry and poultry products. According to the USDA *Salmonella* serotyping quarterly reports from January to June 2011, the following ten serotypes: Kentucky, Enteritidis, 4, 5, 12: i: -, Heidelberg, Brandenburg, Typhimurium, Berta, Montevideo, Thompson and Worthington predominated the list of identified serotypes from broilers (USDA, 2011). *Salmonella* Ituri, which is naturally found in reptiles, was one of the 41 *S. enterica* isolated from poultry disease outbreaks between April, 2005 and August, 2007 in Oyo state, Nigeria (Ogunleye *et al*., 2010). A study conducted in 2009 to determine the prevalence of *Salmonella* serovars in chickens and poultry meat products in rural areas in Nigeria

yielded 39 *Salmonella* Hiduddify (Raufu *et al.,* 2009). This study documented for the first time the isolation of *Salmonella* Hiduddify in chickens and shows that this serovar is widespread in rural areas in Nigeria.

In the last 20 years, there has been a worldwide emergence of multidrug-resistant phenotypes among *Salmonella* serotypes, in particular *Salmonella* Typhimurium (Threlfall *et al.,* 2000) and, more recently, *Salmonella* serotype Newport (CDC, 2002). *Salmonella*phage type Typhimurium DT104, which is resistant to at least five antimicrobials, namely, ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline, has caused severe infections and deaths in animals and humans worldwide (Glynn *et al*., 1998; Grob *et al*., 1999). Emergence of multiple drug resistant *Salmonella* serotypes from poultry and asymptomatic poultry workers has been reported (Fasure *et al.,* 2013). Improperly cooked poultry products and unpasteurized milk are known to contain food borne pathogens and many of them are resistant to different antibiotics (Walsh *et al*., 2005; Okpalugo *et al*., 2008; Novakova *et al*., 2010). Raufu *et al.* (2014a) reported that beyond quinolone resistance, additional resistance to streptomycin, spectinomycin, gentamicin, sulfamethoxazole, and tetracycline were observed in some CIP^R*S.* Kentucky isolates from poultry and poultry sources over time from two regions (Maiduguri and Ibadan) in Nigeria.

1.3 Justification of the Study

Salmonellosis is one of the major threat facing poultry production in Nigeria causing huge economic losses in terms of morbidity, reduced production and mortality (Abdu *et al*., 1985; Garba, 2005; Saidu *et al*., 2006; Mamman*et al.,* 2014). Other costs associated with avian salmonellosis include various direct expenses producers incur as a consequence of treating *Salmonella* infection in avian flocks (Agada *et al.,* 2014a). Moreover, *Salmonella* contamination of food products can significantly reduce consumer demand and affect producer profits (Namata *et al*., 2008). In Nigeria, a more accurate cost of salmonellosis is difficult to determine because normally only large outbreaks are investigated whereas sporadic cases are under-reported (Nwabor *et al*., 2015).

According to Centres for Disease Control and Prevention, *Salmonella* alone affects about 1.4 million people each year in the United States with about 16,000 hospitalizations and more than 500 deaths annually. In 1996, the United States Department of Agriculture (USDA), Economic Research Services estimated that the total costs for medical care and lost productivity, resulting from foodborne *Salmonella* infections of humans was between 0.6-3.5 billion dollars annually (CDC, 2009; Majowicz *et al*., 2010). In Nigeria, World Health Organization (WHO) estimates 200,000 deaths from diarrhoea each year, as many as 70 % of which may be attributable to food and water contaminated by *Salmonella* (WHO, 2008).

Salmonellosis can be controlled by controlling source of contamination and transmission. To achieve this, it is necessary to isolate and serotype *Salmonella* species. Speciation can be used to identify possible transmission pathways and apply intervention measures at critical points to reduce or eliminate the disease in poultry facilities (Mohammed*et al*., 2009). Knowing the

prevalence and local patterns of antimicrobial susceptibility of *Salmonella* species is important for reducing the burden of the diseases.

There is paucity of information in Kwara state regarding the status of salmonellosis in poultry farm, hence the need for this study.

1.4 Aim and Objectives

1.4.1 Aim of the Study

The aim of this study was to isolate and characterize *Salmonella* from selected poultry farms in Kwara State, Nigeria.

1.4.2 Objectives of the Study

1. To isolate and identify *Salmonella*serovars from selected poultry farms in Kwara State.
2. To determine the antimicrobial susceptibility of the isolates to routinely used antimicrobial agents.
3. To detect the presence of *fliC, fim*A *Sef*C and *rfbsg* genes in the isolates using PCR.

1.5 Research Questions

1. What serovars of *Salmonella* are present in poultry farms in Kwara state?
2. Are the *Salmonella* isolates from poultry farms in Kwara state resistant to routinely used antimicrobial agents?
3. Do *Salmonella* from poultry farms in Kwara state possess one or more of *fli*C*, fim*A *Sef*C and *rfbsg* genes?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Nomenclature of Salmonella*

Salmonella is a genus of the family *Enterobacteriaceae* and comprises a large and closely related population of veterinary and medically important pathogens. It has long been associated with a wide spectrum of infectious diseases, including typhoid fever and nontyphoid salmonellosis, which cause public health problems worldwide. Despite recognition of *Salmonella* as an important pathogen, the sophisticated nomenclature system of *Salmonella* remains unfamiliar to clinicians (Lin-Hui and Cheng-Hsun, 2007). *Salmonella* was named after an American veterinarian, D. E. Salmon, who first isolated "*Salmonellacholeraesuis*"from porcine intestine in 1884 (Smith, 1894). The organism was originally called "*Bacillus choleraesuis*," which was subsequently changed to "*Salmonellacholeraesuis*" by Lignieres in 1900. Salmonellae are a group of bacterial organisms with a high genetic similarity and are differentiated by their serotyping results. The antigenic classification system of various *Salmonella* serotypes used today is a result accumulated from 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 (Popoff and Le Minor, 2001).Based on the serologic classification determined using arrays of specific antisera, many salmonellae were differentiated by their serotyping results. Although "serotype" and "serovar" were both frequently used, according to the Rules of the *Bacteriological Code* (1990 Revision) established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes, the term "serovar" is preferred to the term "serotype". Kauffmann proposed that each serovar be considered a separate species

(Kauffmann, 1966). Thus, *Salmonella* serovars identified after 1966 were designated mainly by their antigenic formula and multiple species within the genus *Salmonella* were generally accepted. However, some clinically important salmonellae identified before 1966 had been given specific names either according to the disease and/or the animal from which the organism was isolated, such as *S.*Typhiand *S.* Typhimurium, or by the geographical area where the strain was first isolated, e.g., *S.* London and *S.* Panama. These names had been used for a number of years and therefore were adopted without being amended into the new antigenic formula system.

Because of the complexity of multiple *Salmonella* species, it was proposed that the genus *Salmonella* be subdivided into three species, *S.choleraesuis* (the type species), "*S. typhosa*" (*S.*Typhi), and "*S. kauffmannii*," with the last containing all the other serovars (Borman *et al.,* 1944). Later, "*Salmonella enterica*" was proposed by Kauffman and Edwards to encompass all salmonellae (Kauffmann, and Edwards, 1952). In 1966 a similar three-species model was proposed, with "*Salmonella enteritidis*" representing all serovars other than *S. typhi* and *S.choleraesuis* (Ewing, 1972). Another proposal in 1970 recommended that Kauffmann's "subgenera" be considered a species, i.e., "*S. kauffmannii*" for "subgenus" I, *S. salamae* for "subgenus" II, *S. arizonae* for "subgenus" III, and *S. houtenae* for "subgenus" IV (Le Minor *et al* 1970). Serovars of "*S. kauffmannii*" would be designated by their species names followed by that of their serovar (e.g. "*S. kauffmannii*" serovar Typhi), and serovars of the other three species would be designated by their species names followed by their antigenic formulae.

In 1973, on the basis of DNA-DNA hybridization experiments, Crosa *et al*. demonstrated that all *Salmonella* strains should belong to a single species (Crosa *et al*., 1973). In 1982, on the basis of numerical taxonomy and DNA relatedness studies, Le Minor *et al.* proposed the name "*Salmonella choleraesuis*" for thesingle *Salmonella* species and six subspecies were defined (Le

Minor *et al*., 1982). Because of confusion caused by using "choleraesuis" as a name for both a species and a serovar, in 1986 "*Salmonella enterica*" was proposed again as the type species of *Salmonella* by the Subcommittee of *Enterobacteriaceae* of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology (Penner, 1988). The proposal was formally made to the Judicial Commission of the International Committee of Systematic Bacteriology in 1987 by Le Minor and Popoff of the World Health Organization (WHO) Collaborating Centre (Le Minor and Popoff, 1987). The epithet "enterica" was recommended because it has not been used previously for a serovar (Le Minor and Popoff, 1987). They also proposed that the seven subgenera of *Salmonella* be referred to as subspecies (subspecies I, II, IIIa, IIIb, IV, V, and VI). Subgenus III was divided into IIIa and IIIb by DNA similarity and phenotypic characteristics. The suggestion was accepted by the Centres for Diseases Control and Prevention (CDC) and other experts and laboratories (Ewing *et al.,* 1986; Old, 1992) but denied by the Judicial Commission due to concerns that the status of *Salmonella* serovar Typhi might be overlooked.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*. A third species "*Salmonella subterranea*" was recognized in 2005, and the CDC may incorporate it in their system in the near future. *S.enterica* consists of six subspecies (Brennerand McWhorter-Murlin, 1998; Brenner *et al*., 2000; Popoff, and Le Minor, 2001). I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*.

2.2 Morphology of *Salmonella*

Salmonella is a facultative intracellular pathogen that causes a variety of infectious diseases. It is a facultative anaerobe, Gram-negative straight rod, 0.7-1.5 x 2.0-5.0 µm in shape and size,most of which are motile by means of peritrichous flagella with the exception of the non-motile serotypes: *S.* Gallinarum and *S.* Pullorum (Montville and Mattews, 2008). The colonies are usually 2-4 mm in diameter. They reduce nirate to nitrites, produce gas from glucose and Hydrogen Sulphide from tripple sugar iron agar.

Originally, inclusion into the genus *Salmonella* was on the basis that the organisms were related to one another antigenically. However, since these organisms have a large number of biochemical characteristics in common, more emphasis was put on their biochemical characteristics than their respective antigenic structure (Parker, 1983). They are indole and urease negative and usually utilize citrate as only carbon source (Cheesbrough, 2000). The non-flagellated variants, such as *Salmonella* Pullorum and *Salmonella* Gallinarum are non-motile strains resulting from dysfunctional flagella (D'Aoust, 1997). With the development of DNA based methods, the genetic relationships can now be studied in order to decide which organism belongs to which genus.

2.3 Growth Requirement and Survival characteristicsof *Salmonella*

Salmonella has relatively simple nutritional requirements and can survive for long periods of time in foods and other substrates. The growth and survival of *Salmonella* is influenced by a number of factors such as temperature, pH, water activity and the presence of preservatives. The temperature range for growth of *Salmonella* is 5.2 ^{0}C–46.2 ^{0}C, with the optimal temperature being 35 ^{0}C–43 ^{0}C (ICMSF, 1996). Although freezing can be detrimental to *Salmonella* survival, it does not ascertain destruction of the organism. There is an initial rapid decrease in the number

of viable organisms at temperatures close to the freezing point as a result of the freezing damage. However, at lower temperatures *Salmonella* has the ability to survive long term frozen storage (Jay *et al.,* 2003). Strawn and Dayluk (2010) showed that *Salmonella* was able to survive on frozen mangoes and pawpaw stored at -20 ^{0}C for at least 180 days.

Heat resistance of *Salmonella* in food is dependent on the composition, pH and water activity of the food. The heat resistance of *Salmonella* increases as the water activity of the food decreases. Foods which are high in fat and low in moisture, such as chocolate and peanut butter, may have a protective effect against heat. In low pH conditions, the heat resistance of *Salmonella* is reduced (Jay *et al.,*2003; Shachar and Yaron, 2006; Podolak *et al.,*2010). *Salmonella* will grow in a broad pH range of 3.8–9.5, with an optimum pH range for growth of 7–7.5 (ICMSF, 1996). The minimum pH at which *Salmonella* can grow is dependent on temperature, presence of salt and nitrite and the type of acid present. Volatile fatty acids are more bactericidal than organic acids such as lactic, citric and acetic acid. Outside of the pH range for growth, cells may become inactivated, although this is not immediate and cells have been shown to survive for long periods in acidic products (Bell and Kyriakides, 2002; Jay *et al.,* 2003). Water activity has a significant effect on the growth of *Salmonella* with the optimum water activity being 0.99 and the lower limit for growth being 0.93. *Salmonella* can survive for months or even years in foods with a low water activity (such as black pepper, chocolate, peanut butter and gelatine) (ICMSF, 1996; Podolak *et al.,* 2010).*Salmonella*, like other Gram-negative bacteria in regard to susceptibility to preservatives commonly used in foods,growth of *Salmonella* can be inhibited by benzoic acid, sorbic acid or propionic acid. The inhibition of *Salmonella* is enhanced by the use of a combination of several factors, such as the use of a preservative in conjunction with reduction in pH and temperature (ICMSF, 1996; Ha *et al*., 2004; Banerjee and Sarkar, 2004). *Salmonella* is

classified as facultative anaerobic organisms and can grow in the presence or absence of air. Growth under nitrogen is only slightly less than that under air. Grows at 8-11 ^{0}C in the presence of 20-50% CO_2(Jay *et al*., 2003).

2.4 Mode of Transmission

There has been variation in the occurrence of *Salmonella*serotypes in different geographical locations at different times, with some serotypes becoming widespread in a geographical area for a given period of time and then declining in incidence (Mohammed*et al*., 2009). This behaviour has been attributed to microbial competition (Nogrady *et al*., 2003). Epidemiological studies have demonstrated a variety of routes through which *Salmonella* can be disseminated within a poultry enterprise (Nayak *et al*., 2004). Poultry can contract *Salmonella* infection by horizontal route through contaminated litter, faeces, feed, water, dust, equipment, fomites contaminated with *Salmonella* or via diseased chicks and rodents infected with *Salmonella*(Poppe, 2000;Bastiaan and Aize, 2007; Raufu *etal*., 2009). The most important vectors for *Salmonella* transmission are rodents, especially mice (Henzler and Opitz, 1992; Davies and Wray, 1995; Kinde *et al*., 1996; Guard-Petter *et al.,* 1997; Liebana *et al.,*2003; Davies and Breslin, 2004), but also flies (Olsen and Hammack, 2000) have been shown to contribute to the transmission of *Salmonella*.They can also be transmitted by other animals, wild birds and personnel. *Salmonella* may contaminate young chicks directly through ovarian transmission or penetrate the egg shell after the egg has been laid (Cox *et al*., 2000). Studies have also shown that when *Salmonella*-contaminated and *Salmonella*-free eggs were incubated together the horizontal transmission of *Salmonella* occurred during hatching (Cason *et al*., 1994, 2000). Once *Salmonella* traverses the membrane, it becomes very difficult to destroy or prevent further invasion of the egg and developing embryo (Cason *et al*., 1994).*Salmonella* is very persistent. In a study on the survival

of *S.* Enteritidisin poultry units and poultry feed it was found that the organisms could persist for at least one year in a trial house stocked with broilers (Highlights of Layers '99 Study results, 2005; Bastiaan and Aize, 2007).Moreover, it was shown that *S.* Enteritidiscould survive at least 26 months in artificially contaminated poultry feed (Davies and Wray, 1996). Inhalation of the bacterium during close confinement in high humidity environments such as hatching and brooder operations, direct contact with infected birds and animals, and insects are other demonstrated transmission routes for salmonellosis (Gast, 1997).

People generally acquire salmonellosis through food-borne exposure, although direct contact with infected animals is another possible route (Mead *et al*., 1999; LPlym and Wierup, 2006). Human salmonellosis is an infection caused by ingesting salmonellae in food that is contaminated by faeces of animals or humans directly or indirectly. Common sources of infection include poultry meat and meat products, eggs and egg products (Al – jurayyan *et al*., 2004). Transmission of *Salmonella* to human is usually between humans, from animal via food supply and from water and the environment. Foods contaminated with *Salmonella* usually look and taste normal (Bell and Kyriakides, 2002). On occasions, multiple strains of *Salmonella* have been found associated with food raw material implicated in outbreak (Leh macher *et al.,* 1995). Vegetables have also been incriminated in the transmission of*Salmonella*. Raufu *et al.* (2014) in their work found the prevalence of *Salmonella* from vegetables to be 6.3% and the serovars obtained were *Salmonella* Hadar, *Salmonella*serovar 47: mt: -, 5 and *Salmonella* Vinohrady.

2.5 Carrier and Sourcesof Infection

Salmonellae are distributed all over the world. Multiplication outside the body of the host is facilitated by high temperatures and presence of protein (for example, in residual waters). Therefore, the most important points of transmission of *Salmonella* are tropical and subtropical regions, as well as places where there is a large concentration of animals and people. *Salmonella* may also be found in products refrigerated at 2 °C; the microorganism is able to remain viable in frozen products for long periods (Eliana *et al*., 2015). Wild birds and mammals are generally regarded as the main reservoir for *Salmonella*in the environment. These warm-blooded animals can carry the bacteria in their intestinal tracts, mostly without showing any clinical signs of disease (Blaser *et al*., 1983). Infected animals can then cause transmission of pathogens from the farm environment to food animals, as is often mentioned in studies on *Salmonella* epidemiology (Davies and Wray, 1995; Davies and Wray, 1996). Laboratory studies have proved that rodents can in principle be infected with *Salmonella*. The primary sources of *Salmonella* are gastrointestinal tracts of humans, domestic and wild animals, rodents and birds. Consequently, they are widespread in the natural environment including soil and water in which they do not multiply significantly but may survive for long periods, that is, many months in the soil and dried animal faeces. Transmission to humans is usually between humans, from animals via food supply and from water and the environment (Bell and Kyriakides, 2002).

Transportation of animals, overcrowding, and administration of corticosteroids, parturition and concurrent viral and protozoan infections have all been shown to increase susceptibility of animals to disease (Clarke and Gyles, 1993). The carriersof *Salmonella* are domestic (including poultry) and wild animals, tortoise and other pets. Human patients both sick and convalescence, and subclinical carriers may also shed organisms. Other sources include whole eggs, especially

duck eggs, egg products, meat and meat products, poultry and fertilizers and animal feeds prepared from bones, fish meals and meat (Carter and Chengappa, 1986).

2.6 Epidemiology of *Salmonella*

The primary sources of *Salmonella* are gastrointestinal tracts of humans and animals. As a result, they are diffused in the natural environment including soil and water in which they do not reproduce significantly but may survive for months in the soil and dried animal faeces (Bell and Kyriakides, 2002).As intestinal forms, the organisms are excreted in faeces from which they may be transmitted by insects and other creatures to a large number of places such as to water, soil and kitchen surfaces. Eggs, poultry and raw meat products are the most important food vehicles of *Salmonella* infection in humans, with *Salmonella* Typhimurium and *Salmonella* Enteritidis being the most frequently isolated food borne serovars (Jay, 1992). The true incidence of *Salmonella* infection is difficult to determine. Reported cases represent only a small proportion of actual number because it is only large outbreaks that are investigated and documented. Hence, sporadic cases are under reported because it is only patients with protracted diarrhoea that take cases for microbiological evaluation (Hanes, 2003). Infectious diseases spread through food or beverages are common, distressing, and sometimes life-threatening problem for millions of people around the world. The Centres for Disease Control and Prevention (CDC) estimates 76 million people suffer food borne illnesses each year in the United States, accounting for 325,000 hospitalizations and more than 5000 deaths. Food borne disease is extremely costly. Health experts estimate that the yearly cost of all food borne diseases in the United States is five to six billion dollars in direct medical expenses and lost productivity. Infections with *Salmonella* alone account for one billion dollars yearly, in direct and indirect medical costs (NIAID Fact Sheet, 2005).

Salmonella is one of the microorganism most frequently associated with food borne outbreaks of illness. Meat products in general and poultry in particular are the most common sources of food poisoning by *Salmonella*(D'Aoust, 1997; Antunes *et al.,* 2003). A study conducted in 2009 to determine the prevalence of *Salmonella* serovars in chickens and poultry meat products in rural areas in Nigeria yielded 39 *Salmonella* Hiduddify (Raufu *et al.,* 2009). That study documented for the first time the isolation of *Salmonella* Hiduddify in chickens and shows that this serovar is widespread in rural areas in Nigeria. The Centres for Disease Control and Prevention (CDC) in 1999 estimated that there were about 1.5 million cases with 5000 deaths associated with the consumption of food contaminated with *Salmonell*a (Schneider *et al.,* 2003). In many countries, the incidence of salmonellosis had markedly increased; however, there is paucity of good surveillance data. An estimated 12-33 million cases of typhoid fever occur globally each year, and the disease is endemic in many developing countries of the Indian subcontinent, South and Central America, and Africa (Zapor, 2005). In the Netherlands, which has a population of 15.8 million, 50,000 cases of salmonellosis are reported each year (Van Pelt and Valkenburgh, 2001). In Nigeria, the Federal Ministry of Health reported 90,000 cases of food poisoning in 2007. The World Health Organization (WHO) estimates 200,000 deaths from diarrhoea each year in Nigeria (WHO 2008), as many as 70% of which may be attributable to*Salmonella*contaminated food and water. Other costs associated with Salmonellae include various direct expenses producers incurred as a consequence of treating *Salmonella* infection in aviary (Agada *et al.,* 2014a). An incidence in the frequency and severity of non-typhoidal strains has been reported in Patients with AIDS (Sperber and Schleupner, 1987). The most common species isolated are *S*. Typhimurium and *S*. Enteritidis (Levine *et al.,* 1991) and infection often presents as recurrent diarrhoea with bacteraemia which relapses frequently despite therapy.

2.7 Predisposing Factors

Salmonellaeare zoonotic agents and a wide variety of animals have been identified as reservoirs (Mead *et al.,* 1999; Adak *et al.,* 2002; Hald *et al.,* 2007). *Salmonella* species cause asymptomatic intestinal infections in birds but acute outbreaks exhibiting clinical disease along with high levels of mortality occur in chicks younger than 2 weeks old (Duchet-Suchaux *et al*., 1995). In laying hens *Salmonella* can be highly invasive leading to systemic infections that can potentially be deposited in the internal contents of eggs by trans-ovarian transmission following colonization of the intestinal tract (Woodward *et al*., 2005).

Salmonellosis in humans is often associated with gastroenteritis, which is usually self-limiting. In some cases, particularly in children, pregnant women, infants, the elderly, and immunocompromised patients, *Salmonella* infection can lead to invasive and focal infections that can be severe (Hald *et al.,* 2007). The risk to salmonellosis is increased due to the following factors; absence of effective vaccines, modifying hand washing behaviour after defecating to control prolonged community outbreaks and identifying high risk groups and targeting prevention measures (Perilla, 2003).The ability of *Salmonella* to cause invasive infection varies with the serovar, the age of patient, and species of animal. For example, while *S.* Typhimurium colonization in humans commonly produces gastroenteritis, this same organism causes lethal enteric fever in mice (Carter and Collins,1974;Miller *et al*., 1995). The colonization of *S.* Typhimuriumin chickens may elicit gastroenteritis in young birds; however, adult birds can serve as lifetime hosts for this organism without showing signs of infection (Barrow *et al*., 1989). In contrast, *Salmonella* Pullorumis a host-specific avian pathogen whose colonization in chicken results in a septic disease that kills young birds (Snoeyenbos, 1991). *S.* Pullorumis not often associated with disease in any other species (Taylor and McCoy, 1969). *S.* Enteritidis, in

particular, has shown a greater ability to colonize the vaginal epithelium of laying hens compared to other serotypes (Woodward *et al*., 2005).

2.8 Pathogenesis of Salmonellosis

Salmonella is an intracellular, facultative pathogen that infects a variety of hosts, leading to several manifestations of disease, including enteric fever, bacteremia, and gastroenteritis (Goldberg and Rubin, 2008). Following oral ingestion, *Salmonella* enters the mucosal epithelium of the small intestine, interacting with columnar epithelial cells and micro fold cells overlaying the Peyer's patches (Carter and Collins, 1974). Interaction between this organismand the epithelium triggers the chemotaxis of phagocytic cells to the infected site (Ruitenberg *et al.,* 2001). This cellular response involves both neutrophils and macrophages migrating to the lumen surface where they begin eradication of the bacterial pathogen (Ozawa *et* al., 1993). Penetration of microfold cells lead to the presentation of *Salmonella* to macrophages residing in the lymphoid follicles (Kraehenbuhl and Neutra, 1992). *Salmonella* has been shown to survive and replicate within macrophages from many hosts, including mice and chickens (Carrol *et al.,* 1979; Buchmeier and Heffron, 1989; Abshire and Neidhardt, 1993a;Abshire, and Neidhardt, 1993b; Alpuche-Aranda *et al*., 2005). Previous studies have shown that macrophages participate in the dissemination of *Salmonella* to organs of the reticuloendothelial system, such as mesenteric lymph nodes, liver, and spleen (Goldberg and Rubin, 2008). Survival within macrophages is essential for the full expression of *Salmonella* virulence in mice (Ozawa *et al.*, 1993).

Although *Salmonella* pathogenesis has been well characterized in the mammalian model, limited information is available on specific mechanisms of the disease course in the avian species. Examinations of intestines taken from chickens experimentally infected with various *Salmonella* species under light and electron microscopes showed similar cellular responses to these

organisms, including the influx of heterophils and macrophages to the luminal surface of the intestine (Turnbull and Snoeyenbos, 1973; Barrow *et al.,* 1987). Heterophils are considered to be the avian counterpart of mammalian neutrophils in their action as tissue phagocytes, and their importance to host defense against bacterial infections is well known (Brune *et al*., 1972). The capabilities of the heterophils and avian macrophages to kill *Salmonella* have been demonstrated through bactericidal assays performed in vitro (Stabler *et al.,* 1994). If *Salmonella*are not cleared by the immune system, colonization of the intestine occurs, and they are able to move through and colonize other cells by inducing them to take up the bacteria (Lucas and Lee, 2000). Studies have shown that at least *Salmonella* used to experimentally infect birds will migrate from the intestine to the liver, spleen, and ovaries (Thiagarajan *et* al., 1994; Holt, 1995; Ricke, 2003). This indicates that the pathogenesis of *Salmonella* in experimental avian model infections involves a sequential dissemination in the internal organs that is similar to what has been established in the mammalian model.

2.9 Chromosomal and Plasmid Mediated *Salmonella* Virulence

Salmonellaeare facultative intracellular bacteria that are found within a variety of phagocytic and non-phagocytic cells *in vivo*. The virulence of *Salmonella* species is associated with a combination of chromosomal and plasmid factors (Oliveira *et al*., 2003), and many studies have identified genes that encode these factors. Following intestinal colonization,*Salmonella* penetrates enterocytes, M cells and dendritic cells (DCs) in the intestinal epithelium. Subsequently *Salmonella* that reach the submucosa can be internalized by resident macrophages and rapidly disseminate through the blood stream accumulating in mesenteric lymph nodes and, ultimately, the spleen (Salcedo *et al*., 2001). Altogether the ability of *Salmonella* to survive in a variety of host cells is vital to its success as a pathogen.

Internalization of *Salmonella* into host cells can occur via at least two distinct processes.Phagocytes such as macrophages utilize phagocytic uptake to efficiently recognize and internalize bacterial pathogens. *Salmonella* can also actively invade both phagocytic and non-phagocytic cells using a type III secretion system (T3SS), T3SS1 (Kumar *et al*., 2009). Whereas phagocytosis is an essential innate immune function that has developed to sample a potentially vast array of different pathogens, T3SS1-mediated invasion by *Salmonella* is a highly specific process that depends on the tightly regulated expression of a number of bacterial factors (Takaya *et al*., 2005; Kage *et al*., 2008). In a remarkably co-ordinated process, a small group of effector proteins (SipA, SipC, SopB/SigD, SopD, SopE2 and SptP) induce dramatic rearrangement of the actin cytoskeleton resulting in massive localized membrane ruffles and rapidinternalization of the bacteria (McGhie *et al*., 2009). In addition to phagocytosis and T3SS1-mediated invasion, fimbriae and/or non-fimbrial adhesins on the surface of *Salmonella* may also mediate attachment and internalization via a T3SS1-independent process (Guo *et al*., 2007).Following internalization,*Salmonella* survive and replicate within a modified phagosome known as the *Salmonella*containing vacuole (SCV), which initially is marked by the accumulation of early endosome markers. These 'early'markers are then rapidly removed and within 60–90 min post invasion, SCVs become highly enriched in markers of late endosomes and lysosomes particularly lysosomal glycoproteins (Steele-Mortimer *et al*., 1999). Concomitantly, the SCV moves from the cell periphery to a juxtanuclear position at the microtubule-organizing centre (MTOC) (Salcedo and Holden, 2003; Deiwick *et al*., 2006). The onset of intracellular replication is accompanied in some cell types by the appearance of *Sif*s (*Salmonella*-induced filaments), a network of dynamic membrane tubules that radiate from the SCV (Drecktrah *et al*., 2008).

In addition to two T3SSs.Salmonellaehave a type I secretion system and other factors such as fimbriae, flagella and ion transporters that have important roles in establishing and maintaining the intracellular niche. Many virulence factors are encoded on *Salmonella* Pathogenicity Islands (SPI) on the chromosome. Most notably, T3SS1 and T3SS2 are encoded on SPI1 and SPI2 respectively. Invasion and early post-invasion processes are modulated by T3SS1, flagella, fimbriae and non-fimbrial adhesins; subsequently the T3SS2 and factors involved in nutrient acquisition and avoidance of antibacterial mechanisms are induced. In reality, the system is rather more complex and there is considerable temporal overlap (Antonio and Olivia, 2009). At least 15 effectors can be translocated by T3SS1 into the host cell (McGhie *et al*., 2009). Four of these, SopE/SopE2, SopB and SipA, cooperatively induce the actin rearrangements required for invasion but almost all of the others have been implicated in a variety of post-invasion processes, including host cell survival,SCV biogenesis and modulation of the inflammatory response. Accumulating evidence suggests that many effector proteins have multiple activities within host cells.

Two genes, *spvB* and *spvC*, encode the principal factors for plasmid-mediated virulence of serovar Typhimurium (Matsui *et al*., 2001). Both are translocated via the T3SS2 into host cells (Browne *et al*., 2008; Mazurkiewicz *et al*., 2008). *Spv*B code for factors which activate ADP-ribosylates actin that destabilizes the cytoskeleton and is associated with host cell cytotoxicity (Browne *et al*., 2008; Antonio and Olivia, 2009). *Spv*C has phosphothreonine lyase activity and can inactivate the mitogen activated protein kinases Erk1/2, JNK and p38 in mammalian cells (Li *et al*., 2007; Mazurkiewicz *et al*., 2008).

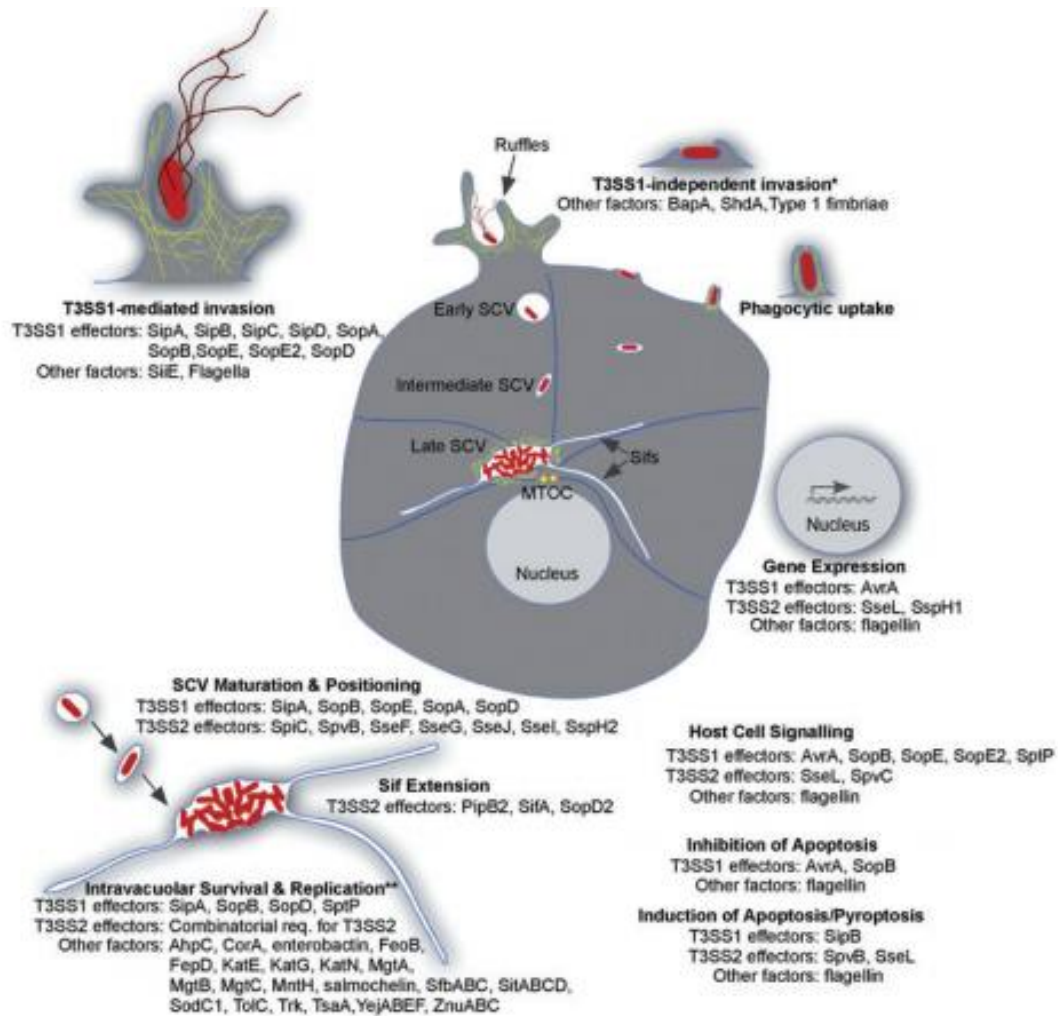

Figure2.1: Virulence factors involved in the intracellular survival of *Salmonella* (Antonio and Olivia, 2009).

2.10 Economic Implication of Salmonellosis

Salmonella is an important pathogen in poultry industry and receives a considerable attention for its economic importance. Poultry farms and poultry products are the major sources for *Salmonella* contamination (Robberts, 1991; Hamphrey *et al*., 1988; Todd, 1980). *Salmonella* causes diseases in chickens which results in huge economic losses as infected chickens have a high mortality rate, succumbing to septicaemia, enteritis, and haemolytic anaemia (Shivaprasad, 1997). In poultry, salmonellae give rise to pullorum diseases and fowl typhoid, resulting in increased mortality and poor-quality chicks hatched from infected eggs, anaemia, depression, laboured breathing and diarrhoea (Todar, 2005). 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 plague the poultry industry in Nigeria, salmonellosis constitutes the most important ones (Onunkwo and Onoviran, 1978; Mbuko *et al.,* 2009).*Salmonella* infection is one of the most serious problems that affect poultry industry in Nigeria causing high economic losses not only due to high mortality in young birds but also for the debilitating effect which predisposes to many other diseases (Banasser *et al*., 2000).Other costs associated with salmonellae include various direct expenses producers incurred as a consequence of *Salmonella* infection in chicken flocks. Control measures such as biosecurity practices, cleaning and disinfecting of facilities, rodent control programs, vaccination and testing, all can significantly increase production costs. Moreover, *Salmonella* contamination of food products can significantly reduce consumer demand and affect producer profits (Namata *et al*., 2008). In a retrospective study of postmortem records of the Zonal Investigation Laboratory of the National Veterinary Research Institute, Yola by Garba*et al*., (2013),40.8% of all post-mortem poultry cases diagnosedbetween 2007 and 2009 were salmonellosis. Mamman (2000), found that fowl

typhoid and pullorum disease accounted for 32.7 % of the total mortality recorded from all the cases of poultry diseases reported in Zaria and environs.

Salmonella species are responsible for a variety of acute and chronic diseases in both poultry and humans (Majowicz *et al.,* 2010; Okwori *et al.,* 2013). In humans, salmonellosis results in enteric fever (typhoid) and acute gastroenteritis that can range from mild to severe infection (Todar, 2005).

2.11 Public Health Significance

Salmonellosis is an important socioeconomic problem in several countries, mainly in developing countries, where this *Salmonella* is reported as the main agent responsible for foodborne disease outbreaks (Alves *et al.,* 2001). There are reports of foodborne salmonellosis in humans since the 19th century, caused by the ingestion of contaminated bovine meat (Barrow, 1991). It is one of the most problematic zoonosis in terms of public health all over the world because of the high endemicity, difficulty in controlling it (Antunes *et al*., 2003; Santos *et al*., 2002), and the significant morbidity and mortality rates (Cardoso *et al*., 2002). According to the World Health Organization (WHO), *Salmonella* is the bacterial agent most frequently involved in cases of foodborne disease all over the world. The agent is normally transmitted to humans by means of foods of animal origin, such as meat, eggs and milk (Nascimento *et al*., 2003). In the past, the main motivations for controlling *Salmonella* species infections in poultry were the losses caused by clinical (pullorum disease and fowl typhoid) and subclinical diseases (paratyphoid infections) (Calnek, 1997). Nowadays, due to the public health implications, prevention of foodborne transmission of *Salmonella* species is a priority for the poultry sector (Oliveira and Silva, 2000).*Salmonella* species is an intestinal bacterium responsible for severe foodborne

intoxications. It is one of the most important agents involved in outbreaks reported in several countries (Tessari *et al.,* 2003).

According to the WHO, *Salmonella* is one of the pathogens that causes the greatest impact on population health, and is associated with outbreaks and with sporadic cases of foodborne disease. According to data of the Brazilian Ministry of Health, 6,602 foodborne disease outbreaks were recorded between 1999 and 2008, and *Salmonella* species was associated with 43% of the cases in which the aetiological agent was identified (Medeiros, 2011). Historically, *Salmonella* Typhimurium was the most common agent of the foodborne disease in humans, although in the past decades *Salmonella* Enteritidis has been most frequently involved in salmonellosis outbreaks (Berchieri Jr and Freitas, 2009; Kottwitz, *et al.,* 2010). There is a growing concern about human infections caused by other serovars, such as Infantis, Agona, Hadar, Heidelberg and Virchow (Freitas Neto *et al*., 2010). In the European Union, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Infantis, *Salmonella* Hadar and *Salmonella* Virchow are considered by the European Food Safety Authority, as the most important serovars in terms of public health (EFSA, 2007). In Japan, between 1999 and 2002, 32% of the cases of foodborne infection were due to *Salmonella*, with Enteritidis, Typhimurium and Infantis as the predominant serovars. In 2005, in the US, the serovars that were most frequently isolated from human sources were *Salmonella*Typhimurium, *Salmonella* Enteritidis, *Salmonella* Newport, *Salmonella* Heidelberg and *Salmonella* Javiana(CDC, 2007). In Denmark, *Salmonella* Infantis was isolated from samples of pork, which was implicated as the source of human infection (Wegener and Baggesen, 1996). In several industrialized countries, cases of human infection caused by this serovar have been described (Pelkonen *et al*., 1994). In Finland, *Salmonella* Infantis was described as the third most important serovar; it infects humans, and it is the most frequently

isolated serovar in poultry (Pelkonen *et al*., 1994). In Hungary, the rate of occurrence of *Salmonella* Infantis has increased in the past years both in the poultry industry and in humans (Nógrády *et al.,* 2008).

2.12 Clinical Manifestation

Chicks hatched from *Salmonella* infected eggs are dead in the shell or die shortly after hatching. *Salmonella* infected chicks and poults develop nonspecific signs such as weakness, depression, dehydration, innappetite, drooping wings, huddling, somnolence and ruffled feathers. Laboured breathing or gasping, as well as diarrhoea and matting of the vent feathers, may be seen. The droppings can be white and viscous in pullorum disease. In somewhat older birds, pullorum disease can be subacute, and lameness and joint swelling may be apparent. Blindness has also been described. Birds that survive may be underweight and poorly feathered, and may not mature into productive adults (Nógrády *et al.,* 2008).

In growing birds and adults, *Salmonella* Pullorum infections are likely to be inapparent. Fowl typhoid can occur in older as well as young birds. The clinical signs may include decreased appetite, depression, dehydration, weight loss, ruffled feathers, and watery to mucoid diarrhoea. A progressive loss of condition can lead to anaemia with pale, shrunken combs. Occasionally, *Salmonella* Pullorum may cause a disease similar to fowl typhoid in older birds; the most common signs are anorexia, depression, diarrhea and dehydration. *Salmonella*Gallinarum and *Salmonella* Pullorum can cause decreased egg production, fertility or hatchability in inapparent carriers as well as in birds with systemic signs (Nagarajan *et al*., 2009).

Some of the symptoms of human salmonellosis are diarrhoea, vomiting, fever, and abdominal pain, these occur 12-36 hours after eating contaminated food, in acute infection, blood and

mucous are present in faecal specimens (Al – jurayyan *et al*., 2004). Salmonellae can be isolated from blood, stool, urine, bone marrow, duodenal aspirates and rose spots. From blood, the organisms can usually be detected in 75-90% of patients during the first ten days of infection, and in about 30% of patients during the third week (Cheesbrough, 2002).

2.13 Immunity

Host-specific *Salmonella* infections in poultry cause systemic disease and are primarily caused by *Salmonella enterica* serovar Gallinarum and serovar Pullorum, which cause fowl typhoid and pullorum disease, respectively (Chappell *et al.,* 2009), and are often avirulent in mammals. The immune response to *Salmonella* infections is very complicated and involves the interaction of many components of the immune system, including the innate and the adaptive immune systems (Nagarajan *et al*., 2009). Although progress has been made in understanding immune responses against *Salmonella* infections, further research is needed to understand the complete role of humoral and cell-mediated immunity because until now, no consistent pattern has been observed. Pathogenic bacteria have evolved mechanisms to invade the epithelial cell barrier and survive within host tissues. *Salmonella* maintains genes organized within pathogenicity islands that encode virulence factors that allow adherence, invasion, and dissemination in the host (Aziz *et al*., 2007). Toll-like receptors (TLR) are cell receptors that recognize structural motifs on pathogens and initiate signaling cascades controlling the development of innate immune response (Chaussé *et al*., 2011). These receptors contribute to host resistance to microbial pathogens and can drive the evolution of virulence mechanisms (Arpaia *et al*., 2011) and can promote adaptive immunity through control of dendritic cell maturation (Iwasaki and Metzhitov, 2004). The consequences of *Salmonella* infection on the expression of the different TLR, and particularly TLR4, have been widely studied (Crhanova *et al*., 2011).*Salmonella* Gallinarum

does not induce an inflammatory response and may not be limited by the immune system, leading to severe systemic disease (Kaiser *et al*., 2000). Invasion of *Salmonella* Gallinarum results in little or no production of IL-6, suggesting that the pathogenesis and host specificity of *Salmonella* Gallinarum infection in the chicken may be related, to some extent, to the lack of an inflammatory response in the early stages of the infection in the gut (Kaiser *et al*., 2000).

Chickens infected with enteric *Salmonella* serovars shows high levels of specific antibodies, a T-cell response, cytokines, and chemokines. Within cell populations, their function can be further discriminated by the presence of cellular determinants, such as Cd4+ (T helper cells) and Cd8+ (T cytotoxic cells), which are associated with helper and cytotoxic functions, respectively (Jeurissen *et al*., 2002). The local immune response in the gut has been shown to be more effectively involved in the clearance of *Salmonella* Enteritidis from the gastrointestinal tract than in thesystemic response (Desmidt *et al*., 1998). An important role of local cell-mediated immunity in the defense ofchickens against *Salmonella* exposure has beensuggested (Berndt and Methner, 2001),describing that modifications of T-cell populations, especially Cd8+TcR1+(γδ) cells (T-cell receptor-bearing cells) in caeca, occur a few days after the inoculation of 1-day-old chickens with the serovar Typhimurium.It has been suggested that intestinal secretory IgA (SIgA) responses partially contributes to the elimination of *Salmonella* Enteritidis later from the gut, and the humoral systemic and local immune responses seem to be related to the cecal colonization (Berthelot-Hérault *et al*., 2003). Cell-mediated immunity is responsible for tissue clearance, but how this mechanism could be responsible for intestinal clearance remains unclear (Zhang-Barber *et al*., 1999). The role of T-cell responses in the clearance of enteric salmonellae has not been proven.

2.14 Diagnosis

Diagnosis of salmonellosis is based on isolation of the organism from either tissues or fluids collected aseptically from necropsy, faeces, milk, blood, rectal swabs or environmental samples (Davies and Wray, 2000). However, a tentative diagnosis can be made, based on the flock history, clinical signs, mortality and lesions (Shivaprasad, 2000). Positive serological findings can also be of great value in detecting infection; however, negative results should not be considered adequate for a definitive diagnosis, because of the delay of three to ten or more days in appearance of agglutinating antibodies following infection(Shivaprasad *et al*., 1990; Gast and Beard, 1990). When infection of reproductive organ or conception occurs, it is necessary to culture foetal intestinal content, vagina swabs and placenta and in case of poultry, egg contents. However, salmonellosis is particularly difficult to determine in clinically normal carrier animals. Because of multiplicity of *Salmonella* serovars, serotyping is important. It represents an important prerequisite for the detection of the source of infection and route of transmission (Mohr and Pollex, 1998).

Salmonellae may be isolated by various techniques which may include pre-enrichment in non-selective medium, enrichment media that contain inhibitory substances, and selective plating agars to differentiate salmonellae from other *Enterobacteriaceae*(OIE, 2000). Various biochemical and serological tests can be applied to the pure culture to provide definitive confirmation of isolated strains. Salmonellae possess antigens designated somatic (O), flagellar (H) and virulent (Vi), which may be identified by specific typing sera and serovar may be determined by reference to antigenic formulae in the Kauffman-White scheme. Serology can be used to detect infected flocks and estimate the prevalence of infection within a flock. The rapid

whole blood plate agglutination test can identify reactors in the field. Agglutinating antibodies appear from three to ten or more days after infection. This test is not reliable in turkeys and ducks, due to false positives. Other serological tests include the rapid serum agglutination test, tube agglutination, microagglutination, microantiglobulin, immunodiffusion, hemagglutination, and enzyme–linked immunosorbent (ELISA) assays. Serological tests are useful for the identification of infected herds but are inadequate for identification of persistently infected animals (OIE, 2000).

Over the last decades, several molecular methods have increasingly become the preferred tools for diagnosis of foodborne pathogens, including *Salmonella.* The molecular methods used for diagnosis of *Salmonella* include Polymerase Chain Reaction (PCR), Southern blotting (DNA-DNA hybridization) and genotyping (finger printing) (Gebreyes, 2003). PCR has been one of the most promising new methods proved to be well suited for both rapid and sensitive detection of *Salmonella* contamination in foods (Ferreti *et al*., 2001; Olivera *et al*., 2002).Methods based on genotyping tend to have high discriminating power and offer rapid and sensitive subtyping, complementing conventional approaches that are based on phenotypes (Gebreyes, 2003).

2.15 Treatment and Control

Every effort should be made to eradicate salmonellosis, and treatment should be the last option. Various sulphonamides, followed by nitrofurans and several other antibiotics have been found to be effective in reducing mortality from salmonellosis. However, no drug or combinations of drugs have been found to be capable of eliminating infection from treated flocks (Shivaprasad, 2000). Sulphonamides that have been used in treatment of salmonellosis include sulphadiazine, sulphamerazine, sulphathiazole, sulphamethazine and sulphaquinoxaline (Mullen, 1946; Bottorff and Kiser, 1947). However, most studies have shown that despite treatment, a significant number

of infected birds can survive and become carriers (Pomeroy *et al*., 1948).Various other antibiotics that can be used for controlling and treating salmonellosis include furaltodone, furazolidone, chloramphenicol, biomycin, apramycin, gentamicin andchlorotetracycline (Aziz *et al*., 1997). Resistance to some of these antibiotics has been reported (Stuart, *et al*., 1967; Raufu *et al*., 2014).

Prevention and control programs for infections caused by paratyphoid salmonellae aimed at protecting the health of the birds, safety of the consumers, and strengthening the reliability of the poultry production chain. In the case of *Salmonella*, measures recommended for prevention and controls are not specific due to the large number of species and their complex epidemiological behavior. Similarly, variability in the implementation of these measures depends on the requisites determined by the international market, or the adaptation of the industry to the chronogram of production (Eliana *et al*., 2015).The epidemiological complexity of the disease, which involves vertical transmission, faecal excretion, horizontal transmission, environmental contamination and presence of carriers in different species, make salmonellosis control difficult to be achieved (Soncini and Back, 2001).

2.16 The Disease in Man

Clinical syndromes in human caused by salmonellae can be divided into two major groups: Group one, includes members of the genus that are involved as aetiologic agents of enteric fever (typhoidal salmonellosis): *Salmonella*Typhi and *Salmonella*Paratyphi. Group two, includes members of the genus that are involved as aetiologic agents of food poisoning (non typhoidal salmonellosis): *Salmonella*Typhimurium and more recently phagetype DT 104. Other members of non typhoidal *Salmonella* are *Salmonella* Enteritidis, *Salmonella* Heidelberg, *Salmonella* Agona, *Salmonella*Newport, *Salmonella* Hadar, and *Salmonella* Dublin (Arora, 2001; Adkins

and Sandiago, 2006).After entering the digestive system together with contaminated food and water, salmonellae reach the intestines, where they attach to intestinal cells and multiply. Depending on the host species and age, and on the pathogenicity of the microorganism and its adaptation to the host, salmonellae may cause severe disease, or go unnoticed and remain in the host for months or years. In this case, the host will be a reservoir of the bacteria for susceptible animals (Eliana *et al*., 2015). The most common symptoms include diarrhoea, abdominal pain, vomiting and nausea, and may occur together with prostration, muscle pain, drowsiness and fever. Although symptoms generally disappear after 5 days, the microorganisms may be excreted in the faeces for many weeks (Jay, 1992). Children, mainly those younger than 1 year of age, elderly and immunocompromised patients are much more susceptible to the disease, and may present more severe infections, such as sepsis, which may lead to death (Gomez and Cleary, 1998; Pinto *et al.,* 2004; Ahmed *et al*., 2016). Salmonellosis is not limited to intestinal infection and gastroenterocolitis. The microorganism may infect other organs; as salmonellae are able to reach the circulation, they may cause diffuse extraintestinal infections, such as meningitis, osteomyelitis, arthritis, pneumonia, cholecystitis, peritonitis, pyelonephritis, cystitis, endocarditis, pericarditis, vasculitis and other disorders (Gelli, 1995). Salmonellae have been shown to cross the intestinal epithelium, and reach the lamina propria where they multiply (Franco and Landgraf, 2004). They are phagocyted by macrophages and monocytes, causing an inflammatory response as a consequence of the hyperactivity of the reticuloendothelial system. Different from what happens in typhoid fever, penetration of *Salmonella* species is limited to the lamina propria in cases of enterocolitis. In these cases, sepsis or systemic infections are rarely observed, and infection is restricted to the intestinal mucous membrane. Inflammatory response

is also related to the release of prostraglandins, which stimulate adenylate cyclase, leading to increased secretion of water and electrolytes and watery diarrhea (Franco and Landgraf, 2004).

2.17 Antimicrobial Resistance

Microbial resistance is related to strains of microorganisms that are able to multiply in the presence of concentrations of antimicrobial compounds even higher than those given as therapeutic doses to humans or animals. Development of resistance is a natural phenomenon that followed the introduction of antimicrobial agents in clinical practice. The irrational and widespread use of these agents has added to the problem, and resistance rates vary from one region to another, depending on the local use of antibiotics (Eliana *et al*., 2015).One of the major concerns of the poultry industry is maintaining the sanitary status of the flock. In the incubators where birds are hatched, there is an attempt to reduce contamination to minimum levels in all phases of the process. Lack of contact with natural biota soon after birth interferes with the normal developmentintestines of bird (Silva, 2005). Acquired resistance in *Salmonella*can originate from chromosomal mutation or from acquisition of transferable genetic materials (Akbarmehr, 2012).Many scientists have reported that the main cause of acquired resistance is the use of antibiotics in poultry for different purposes for growth promotion, prophylaxes or therapeutics (Cardoso *et al*., 2006; Al-Ferdous *et al.,* 2013).

Until 1960's, almost all salmonellae were sensitive to a wide range of antimicrobial agents, but since 1962 emergence of resistance, frequently plasmid mediated, have appeared in *Salmonella*worldwide (Demissie *et al*., 2014; Ahmed *et al*., 2016).*Salmonella*Typhimurium definitive phage type 104 (DT104), resistant to streptomycin, cloramphenicol, amoxicillin, sulfonamides, and tetracyclines, is one of the strains that most frequently displays multiple resistance characteristics (Kristiansen *et al*., 2003).Additional resistance to quinolones, such as

nalidixic and ciprofloxacin acid, has been described in recent years, thus increasing the importance of antimicrobial resistance (Piddock, 2002; Raufu *et al.,* 2014a). Since antimicrobials started to be widely used by humans at the end of the 1940s, the emergence of resistant strains was observed in most bacterial species, and against all drugs available (Flemming, 2005).

Strains of *Salmonella* Enteritidis may become resistant because of the indiscriminate use of the drugs in their country of origin, imports of foodstuffs contaminated with bacteria carrying resistance genes, or infected people returning from international trips. Finnish researchers (Hakanen *et al*., 2001) observed increased antimicrobial resistance in strains of *Salmonella*Enteritidis isolated from travelers after they came back from Asian countries where quinolones were used indiscriminately. There was an increase from 3.9% to 23.5% in the resistance to fluoroquinolones in samples analyzed between 1995 and 1999 in Finland. These facts, suggest that drug resistance genes may be associated with virulence, or that human strains have an improved resistance profile compared with *Salmonella* of animal origin, making the whole situation even more worrisome from a public health viewpoint. The frequency and extent of *Salmonella* resistance to antimicrobials vary based on the use of antibiotics in humans and animals, and on ecological differences in the epidemiology of*Salmonella* infections (McDermott, 2006). Globally, *Salmonella* exhibits extensive resistance profiles which have been associated both with higher rates of morbidity and mortality and the use of antimicrobials in food-producing animals (Angulo *et al*., 2004). Antibiotics are known to suppress normal intestinal microbiota, breaking its protective effect, increasing the competitive advantage of antibiotic-resistant *Salmonella*, and favoring the occurrence of salmonellosis (Eley, 1994).

2.18 Current Situation of Avian Salmonellosis in Nigeria

There has been rapid expansion in poultry industry in Nigeria in the past years despite facing many problems such as disease outbreak, the global financial crisis and inadequate credit facilities and poor infrastructures (FAO, 2008). The Nigerian poultry industry increased by 28 % within the period of 5 years, that is, it grew from 150,700 million chickens in 2005 to 192,313 million in 2010(FAO, 2015).Poultry meat and eggs are the major sources of animal protein in Nigeria, as in many developing countries, because of their affordability and acceptability (Bettridge *et al*., 2014). This source is, however, being jeopardised by diseases such as salmonellosis and avian influenza (FAO, 2008).*Salmonella* species are responsible for a variety of acute and chronic diseases in poultry (Majowicz *et al.,* 2010; Okwori *et al.,* 2013). Salmonellosis is one of the major bacteria threat facing poultry production in Nigeria causing huge economic losses in terms of morbidity, reduced production and mortality (Abdu *et al*., 1985; Garba, 2005; Saidu *et al*., 2006; Mamman*et al.,* 2014). Okwori *et al.* (2007) obtained 37.9 % of positive results from a serological survey of *Salmonella* Gallinarum antibodies in chickens in Jos, Plateau State, Nigeria.Mbuko *et al.* (2009) conducted a five-year retrospective study (2003 - 2007) on the prevalence of fowl typhoid and other poultry diseases diagnosed at the Avian Unit of the Veterinary Teaching Hospital (VTH), Ahmadu Bello University, Zaria, Kaduna State, Nigeria. In a total of 700 cases of poultry diseases documented, 129 (18.4%) cases were diagnosed positive for fowl typhoid. Mamman (2000), found that fowl typhoid and pullorum disease accounted for 32.7 % of the total mortality recorded from all the cases of poultry diseases reported in Zaria and environs.In a retrospective study of postmortem records of the Zonal Investigation Laboratory of

the National Veterinary Research Institute, Yola by Garba *et al*., (2013), 40.8% of all post-mortem poultry cases diagnosed between 2007 and 2009 were due salmonellosis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

These are some of the materials used for the study; Xylose Lysine Deoxycholate (XLD) agar, Rappaport Vasiliadis (RV) broth, Nutrient agar, TSI agar, Urease agar, Simmon's citrate agar, MRVP broth, tryptophan agar, Motility agar, Mueller Hinton agar, sample bottles, sample collection box, Marker, antibiotic discs among others.

3.1 Study Area

The study was conducted in Kwara state. Kwara state is located between latitudes (8° 30′N) and longitudes (5° 00′E). The state has an elongated shape running from west to east and covering an area of about 32,500 sq. km and has River Niger as its natural boundary along its northern and eastern margins and shares a common internal boundary with Niger State in the North, Kogi State in the East, Oyo, Ekiti and Osun States in the South and an international boundary with the Republic of Benin in the west. Kwara state lies within a region described as tropical climate and characterized by double rainfall maxima and has tropical wet and dry climate (Olanrewaju, 2009). Kwara State is a summer rainfall area, with an annual rainfall range of 1000 mm to 1500 mm. The rainy season begins at about the end of February and lasts until early October, while the dry season begins in late October and ends in late February. Temperature is uniformly high and ranges between 25 ^{0}C and 30 ^{0}C throughout wet the season except in July – August when the clouding of the sky prevents direct insulation (heatstroke) while in the dry season it ranges between 33 ^{0}C to 34 ^{0}C (NBS, 2010). The elevation on the western side varies from 273m to

333m above sea level while on the Eastern side it varies from 273m to 364m (Ajadi *et al.*, 2011). The climate of Kwara state supports tall grass interspersed with short scattered trees. This attribute predisposes the people of Kwara State to take up farming as their major occupation. Food crops produced in the state are mostly root crops namely yam, cassava, water yam and sweet potato and they constitute the main staple food aside cereals (Ajadi*et al*., 2011).

Figure3.1: Map of Kwara State showing locations of the sampling sites within selectedLocal Government Areas.

3.2 Study Design

Six local Governments, namely, Moro, Asa, Irepodun, Offa, Ilorin West and Ilorin South were purposively selected for the study. The local Government areas were selected because most of the large poutry farms are located within these local Government areas. Two farms were selected randomly by balloting from each of the local Government areas from the list of farms registered with University of Ilorin Veterinary Teaching Hospital and Kwara state Veterinary clinic. The farms selected are as shown in table 3.1.

Table 3.1: Farms selected from different local Government areas of Kwara State

S/N	Local Government Areas	Selected Farms
1	Ilorin West	Gerewu farm, Al-Abrar farm
2	Ilorin South	Remsec farm, Unilorin farm
3.	Offa	Fasal farm, Yanfi farm
4.	Asa	Fabis farm, Angel farm
5	Irepodun	Landmark farm, Mudlad farm
6	Moro	Albarka farm, Khalilat Anike farm

3.2 Sampling and Sample Collection

A total of 900 samples were collected (360 samples from 72 dead birds, 315 samples of cloacal swabs from live birds and 225 samples from poultry environment) from 9 of the 12 farms during the period December, 2015 to October, 2016. The other 3 farms were consented and agree to participate in the study initially but later declined at the time of sampling (Table 3.2).

Table 3.2: Types and number of samples collected from poultry farms in Kwara State.

	Type of sample	No. of samples/farm
Dead birds	Liver	8
	Spleen	8
	Heart	8
	Ovarian follicle	8
	Caecum	8
Live birds	Cloacal swab	35
Poultry Environment	Feed from feeder	5
	Feed from feed store	5
	Water from drinker	5
	Water from water tank	5
	Litter swabs	5
Total		100

Samples of organs from dead birds were collected by opening the birds, and placed in sterile polythene bag, swabbing was done using sterile swab stick and approximately 5 g of feed was collected in sterile polythene bag while 5 ml of water was collected in sterile sample bottle. All samples were labeled properly and kept in a cool box containing ice packs and were transported, within 2-5 hours, to Veterinary Microbiology laboratory, University of Ilorin for analysis.

3.3 Isolation and Identification of *Salmonella* Species

1 g of each solid sample was added to 9 ml of buffered peptone water, whereas, swabs were inoculated into 10 ml buffered peptone water and these were incubated at 37 ^{0}C for 18-24 hours (Mitchell and Shane 2000; Al-Abadi *et al*., 2011). After pre- enrichment, 1 ml of enriched cultures of all samples was transferred to 9 ml of Rappaport Vasiliadis (RV) broth and incubated at 37^{0}C for 18-24 hours (Al-Abadi *et al*., 2011).

Samples that became turbid on Rappaport vasiliadis were sub-cultured on Xylose lysine Deoxycholate medium (XLD) plates, the plates were incubated at 37 ^{0}C for 24-48 hours and were checked for growth of typical colonies of *Salmonella* species (Menghistu *et al*., 2011). The samples that were positive on XLD showed discrete pinkish colonies with/without dark centres (Plate I). All suspected non-lactose fermenting *Salmonella* colonies were inoculated on nutrient agar and the following biochemical tests were performed for presumptive identification of *Salmonella* species; Triple sugar Iron (TSI) agar, urease, citrate, methyl red, voges proskauer, motility and indole tests (Cheesbrough, 2002; Perilla, 2003).

3.4 Biochemical Tests

3.4.1 Indole test

Peptone water was prepared according to manufacturer's directive; the medium was then inoculated with the test isolates and incubated at 37 ^{0}C for 18 to 24 hours. After which 0.5 ml of the Kovacs reagent (4-dimethyl-aminobenz-aldehyde) was added and mixed gently before examining for possible red coluor formation on the surface layer of the mixture (MacFaddin**,** 2000). A positive indole test showed the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium withinfew minutes of adding the reagent. *Salmonella*is indole negative (MacFaddin,2000).

3.4.2 Methyl red test

Methyl Red Voges Proskauer's (MRVP) broth was inoculated with the test isolates and incubated at 37 ^{0}C for 24 hours. Following incubation, 4-5 drops of Methyl Red Reagent was added to the culture, and the tube shaken in order to homogenize the mixture. The colour development was then observed in the medium. The test is considered positive if it turns to red and negative if it remains yellow(Plate III). Salmonellae are methyl red positive (ISO, 2002).

3.4.3 Voges Proskauer test

Methyl Red Voges Proskauer's broth was prepared according to manufacturer's instruction. The medium was inoculated with the isolates and incubated at 37 ^{0}C for 24 hours. 0.5 ml of α-naphtol reagent was added to the tube. This was then followed with addition of 3-5 drops of 40 % of KOH.The tube was allowed to stand for up to 30 minutes for maximum oxygen exposure to the reaction mixture. The test is positive if the medium acquires a pink-violet colour, forming at the

top of the tube. If the test is negative, there is no colour formation. *Salmonella* is Voges Proskauer negative (ISO, 2002).

3.4.4 Citrate utilization test using Simmon's citrate agar

Using sterile wire loop, a colony of test isolates was streaked on Simmon's citrate agar slants andthese were inoculated and incubated at 37 ^{0}C 24 hours. Positive samples turned the initial green colour of the sample to bright blue colour(Plate IV) (MacFaddin, 1985).

3.4.5 Urease test

Test for urease enzyme activity was carried out using Christensen's urea agar slants in bijou bottles prepared according to manufacturer's recommendations. The test isolate was inoculated on the surface of the agar and the butt was stabbed and incubated at 37 ^{0}C for 24 hours. There was no colour change from intial yellow to pink colour indicating negative testwhich denoted that *salmonella* is urease negative (Plate V) (Cowan and Steel, 2002).

3.4.6 Triple sugar iron agar (TSI) test

Triple sugar iron test is used to determine the ability of an organism to attack specific carbohydrates present in a basal medium with or without production of gas along with determination of possible Hydrogen Sulphide (H_2S) production. Typical *Salmonella* colonies from XLD plates were selected using a sterile wire loop. TSI medium was prepared according to manufacturer's recommendation and inoculated by stabbing the butt and streaking the slants. The tubes were then incubated at 37 ^{0}C for 24 hours (Cowan and Steel, 2002). Blackening along the stab line or throughout the slant indicates hydrogen Sulphide (H_2S) production. *Salmonella* forms a red/pink slant (alkaline) and yellow (acidic) butt with H_2S production and with/without gas (Plate II) (Cheesbrough, 2002).

3.4.7 Motility test

Motility agar was prepared and inoculated with a presumptive isolate from XLD using sterile Pasteur loop making a single stab about 1-2 cm down into the medium. After incubation at 37 ^{0}C for 24 hours,the motility medium was examined. Isolates positive for motility showed diffuse growth (appearing as colouring of the medium) away from the line of inoculation. All *Salmonella* species are motile except *Salmonella* Pullorum and *Salmonella* Gallinarum. (Cheesbrough, 2002; Perilla, 2003).

3.5 Serotyping of *Salmonella* Isolates

All the isolates were serotyped at the WHO National *Salmonella* and *Shigella*Centre, Bangkok, Thailand, by slide agglutination. O and H antigens were characterized by agglutination with hyperimmune sera (S & A Reagents Laboratory, Ltd., Bangkok, Thailand) and serotypes were assigned according to the Kauffmann–White scheme (Popoff and Minor, 2007).

A loopful of saline was placed on a clean glass slide with the aid of a 5 µl disposable wire loop; a second loopful of saline was placed next to the first drop on the same glass slide. From the inoculated nutrient agar plate, a loopful of colony growth was picked and mixed with the first saline drop on the slide and this was repeated on the second saline drop (Negative control test) ensuring that a smooth opaque session was obtained in both drops. A drop of polyvalent O antiserum was added to the first drop. The antiserum and the antigen (the culture) were mixed with a 5 µl disposable wire loop for up to 1 minute without tipping the slide. The appearances of the two drops were then compared. Agglutination was observed in the first drop as particulate matter forming within the drop. If there is lump formation on the second slide, it means autoagglutination and no further typing can be done.

Loopful of culture from nutrient agar and a drop of O-serum on a slide were mixed together and the slide was gently rocked for a period of 2 minutes. Appearance of lump was recorded as a positive reaction while the appearance of homogenous suspension was a negative reaction. The strains were first tested in the O-sera pool, afterwards, the strains were tested in the individual O-serum represented in the positive O-pool, and O-antigens detected were noted. Both positive and negative reactions were also noted.

For Flagella (H)-antigen, on day 1, the isolate was sub-cultured from nutrient agar to swarm agar and this was inoculated in one spot at the centre of the swarm agar and incubated over night at 37 ^{0}C. On day 2, drops of saline was placed with a plastic loop next to each other on a glass slide and loopful of growth was removed from the edge of the motility zone on swarm agar and mixed into the first drop of saline. This was repeated for the second drop (negative control) ensuring an opaque suspension in both drops. A drop of polyvalent H-antiserum was added to the first suspension and mixed with a loop for approximately 1 minute without tipping the slide. A loopful of growth from the edge of the motility zone and a drop of H-serum was carefully mixed on a slide, the slide was gently rocked for a maximum of two minutes and homogenous suspension was recorded as negative result while formation of lump was recorded as positive reaction.

The strain was first tested in the H-antisera pools. Afterwards, the strains were tested in the individual H-serum represented in the positive H-pool, and H-antigens detected (Phase 1) were noted. Both positive and negative reactions were also noted.10 µl antisera against detected H-antigen (Phase 1) was added to petri dish together with 5 ml swarm agar (56-60 ^{0}C), this was observed until the agar has solidified, and inoculated in one spot at the centre of the agar followed by incubation at 37 ^{0}C overnight.

On the day 3, phase H-antigen were detected by the same method as described for phase 1, H-antigen (phase 2) detected were noted. Both positive and negative reactions were equally noted. Serotype identifications were carried out combining both the O- and H-reactions and the specific type (the serovar) in the "Kauffmann-White scheme were identified.

3.6 Antibiotic Susceptibility Testing

The isolates that were identified as *Salmonella* species by serotyping were tested for antibiotic susceptibility using Kirby-Bauer disc diffusion assay (Bauer *et al.,* 1966) following guidelines of Clinical and Laboratory Standards Institute (CLSI, 2016) and culturing on Mueller Hinton agar. The drug impregnated disks (OXOID) included; Ampicillin (10µg), Compound sulfonamide (300 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Ceftazidime (30 µg) Cefotaxime (30 µg) Neomycin (30 µg), Nalidixic acid (30 µg), Streptomycin (10 µg) and Tetracycline (30 µg). Using a sterile wire loop, 1-2 well isolated colonies were picked and emulsified in 5 ml of normal saline in a test tube and mixed gently. The turbidity of the saline was then matched with a turbidity standard (0.5 McFarland) to have an equivalent turbidity. The suspension wasdispensed on prepared and dried Mueller Hinton agar plate, spread evenly and allowed to dry. Sterile forcep was then used to place the antimicrobial discs on the inoculated plates. Within 30 minutes after applying the disc, the plate was incubated at 37 ^{0}C for 16-18 hours. By using a ruler on the underside of plate, the diameter of each zone of inhibition was measured in millimeter and recorded. Zone diameter for each drug was compared with Clinical and Laboratory Standards Institute standard; Interpretative chart was then used to interpret the zone sizes of Inhibition. Results were recorded as susceptible, Intermediate or resistant based on the zone sizes of each antimicrobial disc used (WHO, 2004; Andrews *et al*., 2005; CLSI, 2016).

3.7 Polymerase Chain Reaction Testing of the Isolates

3.7.1 DNA extraction

After confirming the isolates as *Salmonella* by serological tests, 24 of the 58 isolates (these were selected randomly by balloting) were sub-cultured on nutrient agar and incubated at 37 ^{0}C for 24 hours. DNA extraction was carried out in accordance with manufacturer's instruction using ZR fungal/bacterial DNA miniPrep^{TM} (ZYMO RESARCH CORP. USA) at bacteria zoonosis laboratory, Department of Veterinary Public Health and Preventive Medicine, University of Ilorin.

Briefly; 6-8 discrete colonies from 24-hour-old nutrient agar plate was suspended in 200 µl of sterile distilled water in a BashingBead^{TM} lysis tube after which 750 µl of lysis solution was added. The bead was then secured in the canister of vortex mixer and vortexed at 5000 revolutions per minute (rpm) for 5 minutes. The tube was then centrifuged at 1000 rpm for 1 minute. 400 µl of the supernatant was transferred to a Zymo-Spin^{TM} IV Spin Filter fixed with a collection tube and centrifuged at 7000 rpm for 1 minute. 1200 µl of Bacteria DNA binding buffer was added to the filtrate in the collection tube. 800 µl of this mixture was transferred into Zymo-Spin^{TM} IIC Column fitted with a collection tube and centrifuged at 10,000 rpm for 1 minute. The flow through from the collection tube was discarded and the previous process was repeated. A new collection tube was fitted to Zymo-Spin^{TM} IIC Column and 200 µl of DNA pre-wash buffer was added to the Zymo-Spin^{TM} IIC Column and then centrifuged at 10000 rpm for 1 minute. 500 µl of bacterial DNA wash buffer was then added to Zymo-Spin^{TM} IIC Column and centrifuged at 10000 rpm for 1 minute. The Zymo-Spin^{TM} IIC Column was transferred in to a 1.5 microcentrifuge tube and 100 µl of DNA elution buffer was added and centrifuged at 10000 rpm for 30 Seconds to elute the DNA. The DNA eluted were stored at -18 ^{0}C until used.

3.7.2 Primers

The sets of primer pairs that were used in the PCR assay are as shown in Table 3.3. The primers were synthesized at Inqaba biotech (QN 201502628) South Africa.

Table 3.3: **Primers sequence for PCR amplification of selected genes of*Salmonella* isolates from poultry farms Kwara State.**

Primer		Sequence: F-(5'-3') R-(3'-5')	Product size (bp)	Reference
***fli*C**	**F**	CTGGTGATGACGGTAATGGT	197	(Paiva *et*
	R	CAGAAAGTTTCGCACTCTCG		*al*.,2009)
rfbsg	**F**	GTA TGG TTA TTA GAC GTT GTT	187	(Shah *et al*.,
	R	TAT TCA CGA ATT GAT ATA CTC		2005)
***fim*A**	**F**	CCT TTC TCC ATC GTC CTG AA	670	Huguette *et al*.,
	R	CA CGA TCC GTC TAT TGT TGG		1996
***sef*C**	**F**	GCGAAAACC AAT GCG ACT GTA	1103	Murugkar *et al*.,
	R	CCCACC AGA AAC ATT CAT CCC		2003

3.7.3 PCR assay

Polymerase Chain Reaction (PCR) analysis was carried out in Bioscience Laboratory unit of International Institute for Tropical Agriculture (IITA), Ibadan. Polymerase chain reaction amplifications were performed according to method previously described by Guo*et al.* (2000). The PCR reaction contained a final volume of 25 µL included 12.5 μl master mix (Inqaba Biotech, S. Africa) containing (5 U/ul) Taq DNA polymerase, 2.5 mMeach of dATP, dCTP, dTTP and dGTP, 50mM $MgCl_2$ and PCR buffer, 1.25μl (1μM) each of foward and reverse primers and 2.5 μl of template DNA (100ng/µl DNA) and 7.5 μl of deionized water. The cocktail was used for each sample. PCR incubation was performed in a thermocycler (Perkin-Elmer, USA) (Plate VI). The PCR programs used for the primers are shown in tables 3.4 and 3.5.

Table 3.4: PCR programs used for genes *sef*C and *fim*A

		40 Cycles			
Process	Initial den.	Denaturation	Annealing	Extension	Final Extension
Temp (^{0}C)	94	94	55	72	72
Time	5 mins	30 secs	45 secs	45 secs	7 mins

Table 3.5: PCR programs used for genes *fli*C and *rfbsg*

	9 Cycle (Torch down)				35 Cycles			
Process	Initial den.	Denat.	Annealing	Extension	Denat.	Annealing	Extension	Final Exte.
Temp (^0C)	94	94	55	72	94	45	72	72
Time	5min	30sec	45sec	45sec	30sec	45sec	45sec	7min

15 μl of each PCR products were loaded on 2.0% agarose gelcontaining 0.5 μl/ml ethidium bromide (Pharmacia, Sweden) (Plate VI). The ladders used were50bp (Thermo Scientific) for *fim*A, *fli*C &*rfbsg* and 100 bp(Invitrogen, NY) for *sef*C. The gel picture was captured using Gel doc 2000 documentation system.

3.8 Statistical Analysis

The rate of isolation was calculated by dividing the number of samples positive for *Salmonella* by total number of samples processed. The significance ($p<0.05$) of differences between isolation rates of *Salmonella* in various sources were calculated using Chi-square test for independent proportion (OPENEPI version 2009).

CHAPTER FOUR

4.0 RESULTS

4.1 *Bacterial Isolates from the Different Sources*

This study showed that out of the 900 samples collected from three different sources comprising of poultry environment, dead birds and live birds, 58 were positive for *Salmonella* giving an overall rate of isolation of 6.4 %. The isolation rate of *Salmonella*species varied among the farms ranging from 4.0 % in farms7 and 8,to 16.0 % in farm 9(Table 4.1). The higest frequency of isolation of *Salmonella* was obtained from live birds (8.0 %) while the least was from dead birds (4.7 %). Among different sampling units in the poultry environment, feed from feeding trough recorded the highest rate of isolation of 11.1 % while the lowest was obtained from water from both drinker and reservoir (4.4 % each). Considering different sampling units in dead birds, highest frequency of isolation was obtained from the liver (8.3 %) (Tables 4.2).The isolation rate was only statistically significant ($p< 0.05$) in live birds.

Table 4.1: Number of samples collected and rate of*Salmonella*isolation rate per farm per farm

Farm No	No of samples collected	No. of positive samples(%)
F1	100	5(5.0)
F2	100	8(8.0)
F3	100	6(7.0)
F4	100	5(5.0)
F5	100	5(5.0)
F6	100	5(5.0)
F7	100	4(4.0)
F8	100	4(4.0)
F9	100	16(16.0)
Total	900	58(6.4)

Table 4.2: Occurrence of *Salmonella* species fromdifferent sources in poultry farms in Kwara State

Farm No	Number of positive samples/farm (%)													
	Poultry environment					*P*	Dead birds					*p*	Live birds	*p*
	Litter	Feed		Water			Liver	Spleen	Ovarian	Caecum	Heart		Cloacal Swabs	
		F	S	D	R									
F1	0(0)	1(20)	0(0)	0(0)	0(0)		0(0)	0(0)	1(13)	0(0)	0(0)		3(9)	
F2	1(20)	0(0)	1(20)	1(20)	1(20)		1(13)	1(13)	0(0)	0(0)	0(0)		2(6)	
F3	1(20)	1(20)	0(0)	0(0)	0(0)		1(13)	1(13)	1(13)	0(0)	1(13)		0(0)	
F4	0(0)	0(0)	0(0)	0(0)	0(0)		2(25)	2(25)	0(0)	1(13)	0(0)		0(0)	
F5	1(20)	0(0)	0(0)	1(20)	0(0)		1(13)	0(0)	0(0)	0(0)	2(25)		0(0)	
F6	0(0)	1(20)	0(0)	0(0)	0(0)		1(13)	0(0)	0(0)	0(0)	0(0)		3(9)	
F7	0(0)	0(0)	2(40)	0(0)	0(0)		0(0)	0(0)	0(0)	1(13)	0(0)		1(3)	
F8	0(0)	0(0)	0(0)	0(0)	0(0)		0(0)	0(0)	0(0)	0(0)	0(0)		4(11)	
F9	0(0)	2(40)	1(20)	0(0)	1(20)		0(0)	0(0)	0(0)	0(0)	0(0)		12(34)	
Subtotal	3(6.7)	5(11.1)	4(8.8)	2(4.4)	2(4.4)	0.224	6(8.3)	4(5.6)	2(2.8)	2(2.8)	3(4.2)	0.086	25(8.0)	0.05**
Total/source			16(7.1)						17(4.7)				25(8.0)	

KEY: F= Feeding trough, S= Feed store, D= Drinking trough, R= Water reservoir, f= farm

**$p<0.05$

4.2 Serovars Distribution

The 58 *Salmonella* isolates revealed thirteen different serovars which included*Salmonella* Agama (*S.* Agama), *S.* Albany, *S.* Colindale, *S.* enterica ser. 4,5,12:i: -, *S.*enterica ser. 4,12, 27: z:-, *S.* enterica ser. 6, 7: d:-, *S. enterica* ser. 45: d: 1,7, *S.* Istanbul, *S.* Larochelle, *S.* Muenster, *S.* Nigeria, *S.* Orion and *S.* Typhimurium. *Salmonella entrica* ser. 6, 7: d: -was the most frequently isolated, accounting for (17/58) 29 % of all the serovars. *S.* Agama accounted for (16/28) 28 % while *S.* Typhimurium accounted for (9/58) 16 % of the *Salmonella* serovars. *Salmonella*Agama was isolated from all the farms while *S. entrica* ser. 6, 7: d: - was isolated from six (6) of the nine (9) farms sampled (Table 4.3).

Eight (8) different serovars were isolated from dead birds representing the highest number of serovars from a single source. Majority of the most prevalent serovars were obtained from multiple sources. *Salmonella enterica* ser. 6, 7:d:- was isolated from all the samples except from liver and caecum. *Salmonella* Agama was obtained from poultry environment (feed, water), dead birds (liver, spleen and ovarian follicle), and from apparently healthy birds (cloaca swabs), while *S.* Typhimurium was isolated from feeds, dead birds (liver, spleen and caecum), and live birds (cloaca swabs) (Table 4.4).

Table 4.3: **Distribution of *Salmonella* serovars in selected poultry farms in Kwara state**

Serovars	No(%) of serovar/farm									Total
	F1	F2	F3	F4	F5	F6	F7	F8	F9	
***S.* Agama**	1(6)	1(6)	1(6)	1(6)	2(13)	5(31)	1(6)	1(6)	3(19)	16(28)
***S.* Albany**	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	1(2)
***S.* Colindale**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	1(2)
***S.* enterica ser. 4,5,12: i: -**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	1(2)
***S.* enterica ser. 4,12,27: z: -**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	1(2)
***S.* enterica ser. 6,7: d: -**	4(22)	5(28)	1(6)	0(0)	1(6)	0(0)	0(0)	2(11)	4(22)	17(29)
***S. enterica* ser. 45: d :1,7**	0(0)	0(0)	2(40)	0(0)	0(0)	0(0)	0(0)	0(0)	3(60)	5(9)
***S.* Istanbul**	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(2)
***S.* Larochelle**	0(0)	0(0)	0(0)	1(50)	0(0)	0(0)	0(0)	0(0)	1(50)	2(3)
***S.* Muenster**	0(0)	2(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(3)
***S.* Nigeria**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	1(2)
***S.* Orion**	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(2)
***S.* Typhimurium**	0(0)	0(0)	0(0)	3(33)	1(11)	0(0)	0(0)	1(11)	4(44)	9(16)
Total	5(9)	8(14)	6(10)	5(9)	5(9)	5(9)	4(7)	4(7)	16(28)	58(100)

Table 4.4: Distribution of *Salmonella* serovars by different sources from selected poutry farms in Kwara State

Serovars	Source and number of serovars											
	Poultry Environment					Dead birds					Live birds	Total
	Litters	Feed		Water		Liver	Spleen	Ovarian	Caecum	Heart	C/ swabs	
		F	S	D	R							
***S.* Agama**	1	1	-	-	-	2	1	1	-	-	10	16
***S.* Albany**	-	-	-	-	-	-	-	-	-	1	-	1
***S*. Colindale**	-	-	-	-	-	-	-	-	-	-	1	1
***S.enterica* ser. 4,5,12: i: -**	-	-	1	-	-	-	-	-	-	-	-	1
***S*. *enterica* ser. 4,12,27: z: -**	-	-	-	-	-	-	1	-	-	-	-	1
***S.enterica* ser. 6,7:d:-**	1	1	2	2	1	2	-	1	-	2	5	17
***S. enterica* ser. 45: d :1,7**	1	1	-	-	-	1	-	-	-	-	2	5
***S.* Istanbul**	-	-	-	-	-	-	1	-	-	-	-	1
***S.* Larochelle**	-	-	-	-	-	-	-	-	-	-	2	2
***S.* Muenster**	-	-	-	-	1	-	-	-	1	-	-	2
***S.* Nigeria**	-	-	-	-	-	-	-	-	-	-	1	1
***S.* Orion**	-	1	-	-	-	-	-	-	-	-	-	1
***S.* Typhimurium**	-	1	1	-	-	1	1	-	1	-	4	9
Total	3	5	4	2	2	6	4	2	2	3	25	58

KEY: F= Feeding trough, S= Feed store, D= Drinking trough, R= Water reservoir.

4.3 Detection of *Salmonella* Genes in isolates from selected poultry farms in Kwara State Using PCR

In this study, PCR assay was performed for the detection of four differentgenes fortwenty-four (24) randomly selected *Salmonella* serovars from three different sources. These genes included*fim*A, *sef*C, *fli*Cand *rfbsg*.

*fim*A gene which encodes the major fimbrial subunit in *Salmonella* was found to be present in all the isolates demonstrated by the presence of a 670 bp PCR product (Plate 4.1). The *sef*C gene wasdetected by the presence of 1103 bp PCR product (Plate4.2), whichwas found in only five(5) of the twenty-four (24) isolates which were confirmed to be *Salmonella enterica* subspecies *enterica* ser. 45: d: 1,7. The other two genes (*fliC* and *rfbsg*) were not amplified.

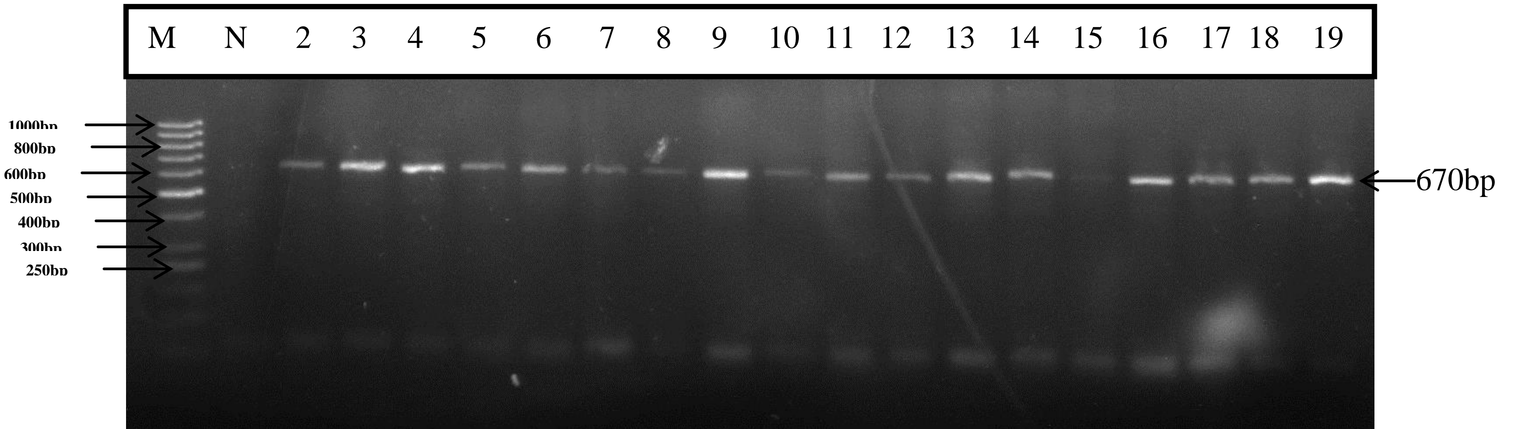

Plate 4.1: Detection of *fim*A gene by polymerase chain reaction (PCR). Lane M-(50 bp) DNA ladder molecular weight marker, lane N-negative control (deionized water), 2 to 24- Test isolates

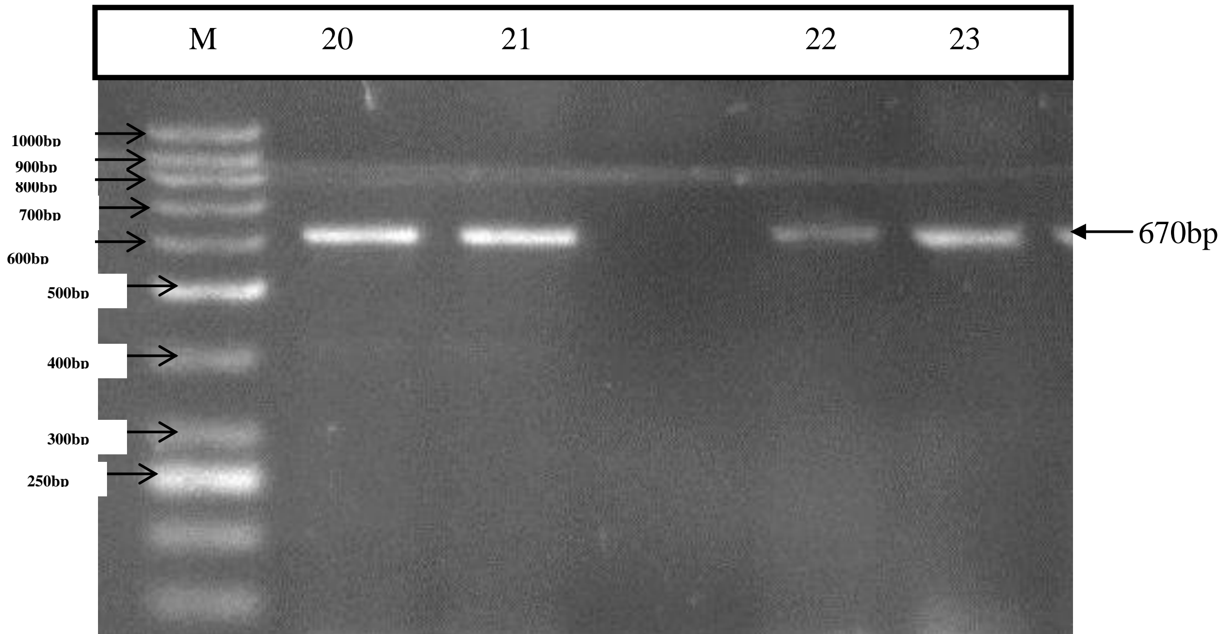

Plate 4.1: Continued

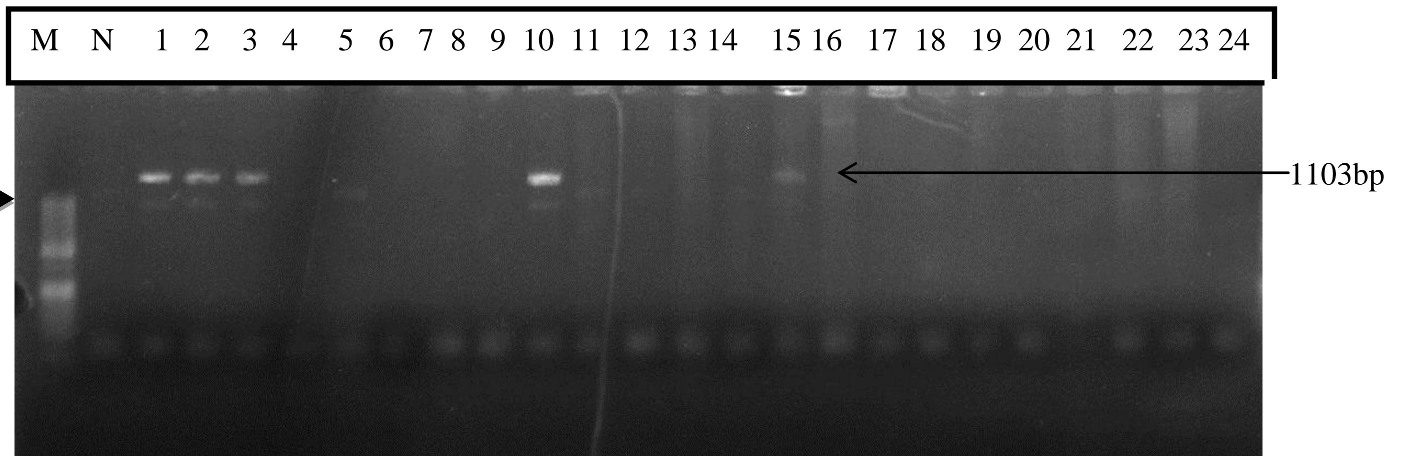

Plate4.2:Detection of *sefC* gene by PCR. Lane M-(100 bp) DNA ladder molecular weight marker, lane N-negative control (deionized water), 1 to 24-Test isolates.

4.4 Antimicrobial Susceptibility Testing

In general, a high percentage of resistance to the tested antimicrobials was observed across all the serovars. All the serovars(100 %) were resistant to ampicillin, ceftazidime and cefotaxime. The *Salmonella*Albany isolatedwas resistant to all antimicrobials used except chloramphenicol, neomycin, compound sulfonamides and tetracycline. *Salmonella*Nigeria and *S. enterica* ser. 4, 5, 12: i: - were resistant to all the antimicrobials except neomycin. All the isolates showed high resistance to ciprofloxacin and nalidixic acid (≥ 50 %) except *Salmonella*Muenster which was susceptible to ciprofloxacin, and*Salmonella*Colindale which was susceptible to the two.*S. enterica* subspecies *enterica* ser. 4, 12, 27: z: - showed resistance to all antimicrobial agents used (Table 4.5).

Table 4.5: Antimicrobial resistance profile of *Salmonella* serovars from selected poultry farms in Kwara State

Serovar	**No of positive**	Number (%) of isolates showing resistance to the following antimicrobials										
		AMP10	C30	CAZ30	CIP5	CN30	CTX30	N30	NA30	S3	S10	TE30
S. Agama	16	16(100)	8(50)	16(100)	8(50)	8(50)	16(100)	2(13)	11(69)	7(44)	11(69)	11(69)
S. Albany	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)
S. Colindale	1	1(100)	0(0)	1(100)	1(100)	0(0)	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)
S.enterica ser. 4,5,12: i:-	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)
S.enterica ser. 4,12,27: z:-	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
S.enterica ser. 6,7: d:-	17	17(100)	8(47)	17(100)	11(65)	9(53)	17(100)	5(35)	11(65)	8(47)	11(65)	9(53)
S. enterica ser. 45: d: 1,7	5	5(100)	1(20)	5(100)	3(60)	2(40)	5(100)	1(20)	1(20)	1(20)	1(20)	2(40)
S. Istanbul	1	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)
S. Larochelle	2	2(100)	1(50)	2(100)	2(100)	1(50)	2(100)	1(50)	2(100)	2(100)	1(50)	2(100)
S. Muenster	2	2(100)	2(100)	2(100)	0(0)	2(100)	2(100)	2(100)	1(50)	1(50)	2(100)	1(50)
S. Nigeria	1	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)
S. Orion	1	1(100)	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)
S. Typhimurium	9	9(100)	5(56)	9(100)	6(67)	5(56)	9(100)	6(67)	3(33)	6(67)	7(78)	7(78)

The codes of the antimicrobials were AMP10, ampicillin (10 µg); C30, chloramphenicol (30 µg); CAZ30, ceftazidime (30 µg); CIP5, ciprofloxacin (5 µg); CN30, gentamicin (30 µg); CTX30, cefotaxime (30 µg); N30, neomycin (30 µg); NA30, nalidixic acid (30 µg); S3, compound sulfurnamides (300 µg); S10, streptomycin (10 µg); and TE30, tetracycline (30 µg).

4.5 Frequency of Resistance Patterns of *Salmonella* Isolates to Antimicrobial Agents

Generally, the *Salmonella* isolates tested showed 18 different resistance patterns.All the 58 isolates (100 %)exhibited multidrug resistance. The predominant patterns detected were AMP-CAZ-CTX (100 %), AMP-CAZ-CTX-CIP and AMP-CIP (63.8 % each).1 (1.7 %) isolate eachexhibited AMP-C-CAZ-CIP-CN-CTX-N-NA-S_3-S_{10}-TE, AMP-C-CAZ-CIP-CN-CTX-NA-S_3-S_{10}-TE and AMP-C-CAZ-CN-CTX-NA-N-S_3-S_{10}-TE (Table 4.6).

Table 4.6: **Multidrug resistance patterns detected among *Salmonella* isolates from poultry farmsin Kwara State**

S/N	Resistance Pattern detected[a]	No. (%) of *Salmonella* isolates[b]
1	AMP-C	28(48.3)
2	AMP-CIP	37(63.8)
3	CAZ-CN	32(55.2)
4	CTX-NA	36(62.1)
5	AMP-CAZ-CTX	58(100)
6	TE-C-GN	18(31.0)
7	TE-C-CIP-CN	19(32.8)
8	CIP-CN-N-NA	19(32.8)
9	AMP-CAZ-CTX-TE	34(58.6)
10	AMP-CAZ-CTX-S_3	28(48.3)
11	AMP-CAZ-CTX-NA	36(62.1)
12	AMP-CAZ-CTX-N	19(32.8)
13	AMP-CAZ-CTX-CN	32(55.2)
14	AMP-CAZ-CTX-CIP	37(63.8)
15	AMP- C-CAZ-CTX	28(48.3)
16	AMP-C-CAZ-CIP-CN-CTX-NA-S_3-S_{10}-TE	1(1.7)
17	AMP-C-CAZ-CN-CTX-NA-N-S_3-S_{10}-TE	1(1.7)
18	AMP-C-CAZ-CIP-CN-CTX-N-NA-S_3-S_{10}-TE	1(1.7)

[a] AMP, ampicillin; C, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; CTX, cefotaxime; N, neomycin; NA, nalidixic acid; S_3, compound sulfurnamides; S_{10}, streptomycin; and TE, tetracycline.

[b] Of a total of 58 isolates tested for susceptibility.

CHAPTER FIVE

5.0 DISCUSSION

Salmonella serovars were isolated from all the three sources sampled, suggesting the endemicity of *Salmonella* in the farms and/or farm environments. *Salmonella* is an important zoonotic pathogen and it's occurence in animals poses a continuous threat to man (Muragkar *et al.*, 2005). The isolation rate (6.4 %) of *Salmonella* from this study was high whichis similar to the rates that have been reported from Maiduguri, northeastern Nigeria with a rate of 7.0 % (Raufu *et al*., 2013) and Ibadan south western Nigeria with a rate of 10 % (Fashae *et al*., 2010). Higher rate (37 %) of *Salmonella* contamination of broiler farms had equally been reported from Algeria (Elgroud *et al*., 2009) thus, suggesting chickens and poultry environmentsare important reservoirs of *Salmonella* in Nigeria.

The overall frequency of isolation of *Salmonella* from the cloacae of birds was relatively high (8.0 %) in this study which is in agreement with the range (4 to 12%) reported in Algeria by Ammar *et al*. (2010). However, it was higher than 0.9 % reported in Trinidad and Tobago (Adesiyun *et al*., 2014). Swabs of litter in poultry farms had high frequency (6.7 %) of *Salmonella* contamination which indicates that freshly laid eggs in the studiedfarms have a higher risk of being contaminated by *Salmonella.* The overall isolation rate of *Salmonella* from poultry environment in the present study (7.1 %) was within the range of 0.95 % to 33.3 % reported by other researchers (Shirota *et al.,* 2013; Adesiyun *et al*., 2014). Feeds, particularly in a deep litter management system, can be a source of contamination of eggs laid in the litter (Shirota *et al.,* 2013; Adeiyun *et al*., 2014). The

isolation rate of *Salmonella* from feed samples in this studywas high (10.0 %) and might be due to the fact that feed being considered the costliest component of poultry production in Nigeria. The feed is therefore, usually formulated using blood meal, fish meal, bone meal, egg shells (animal), groundnut cake and soya bean cake (plant) as sources of protein and calcium. In most cases, these ingredients are not stored properly or un-hygienically processed, thus serving as a source of contamination in feed. The climatic weather in Kwara is warm and humid and *Salmonella* organisms can, under these circumstances, multiply in the feed especially during storage in the farms (Jones and Richardson, 2004).

In this study, the predominant serovars were *Salmonella enterica* ser. 6, 7:d:- (29.0 %),*S.* Agama (28.0 %) and *S*. Typhimurium (16.0 %). *Salmonella enterica* ser. 6, 7:d:- has similar antigenic formula with*Salmonella* Kivu(6,7:d:1,6). *Salmonella* Kivu was first characterized in 1961 in Congo (Van*et al*., 1961) and was reported to cause human sporadic diarrhoea in Durban, South Africa (Govinden *et al.,* 2008).*Salmonella* Kivu had also been reported albeit at lower level (0.62 %) in poultry processing environments in Malaysia (Nidaullah *et al.,* 2017).To the best of our knowledge, no study has been published to date on whether there is genotypic relationship between the serovar Kivu and the one isolated in this study (6, 7:d:-).*S*. Typhimurium is also a common serovar in chickens and has been reported by other workers in Nigeria (Mike *et al*., 2005; Fasure *et al*., 2013). It has also been documented from poultry in Trinidad and Tobago and Algeria (Adesiyun *et al.,* 2014; Jakirul *etal*., 2016). There has been report ofan epidemic increase in prevalence of *S*. Typhimurium which has been linked to circulation of a particular MLST clone, ST313, in sub-Saharan African countries (Kingsley *et al*., 2009); however, it has not been determined if the *S*. Typhimurium ST313 clone has spread to Kwara state,

Nigeria. Further study(ies) will reveal if these isolates belong to the previously described clone of phage type U282 in Nigeria (Ojeniyi and Montefiore 1986). *S.* Agama was characterized in 1956 as a new serotype of *Salmonellaenterica* from faeces of the agama lizard (*Agama agama*) in Nigeria (Collard and Montefiore, 1957). Subsequently, *S.* Agama was isolated from geckos and mammals in Africa (Collard and Sen, 1960; Oboegbulem and Okoronkwo, 1990; Orji *et al.*, 2005) and the United Kindgom (Wilson *et al.,* 2003; Davies and Breslin, 2004). It was also reported as a contaminant in poultry feed mills in UK (Davies and Wales, 2010). Human infections with *S.* Agama was reported in Nigeria and related to the lizards as a possible reservoir (Collard and Sen, 1960).It was also reported to have caused traveler's diarrhoea in Gabon (Swinton *et al*., 2007) and France, in a 9-month-old child with fever and diarrhoea (Appas *et al*., 1966). It was also recently incriminated in neonatal meningitis in UK (Paul *et al.*, 2015) and human sporadic diarrhoea in Okinawa, Japan (Jun *et al*., 2006).Although,*S*. Agama was reported recently in Nigeria from faecal droppings and poultry feeds,it was not among the major serovars (3.7 %) isolated in the study ofIdowu *et al*. (2017).*S.* Agama was isolated from all the sampling units except water in this study. This is of public health importance as the infected birds and poultry environment can be sources of infection to man. The occurrence of *Salmonella* Agama in chickens and the poultry environment in the study area might be due to the abundance of Agama lizards around all habitations, including the poultry pen and the poultry environment. *Salmonella* Enteritidis was not isolated from chickens in this study, which is similar to the report by Fashae *et al.* (2010) in which they did not isolate *S.* Enteritidis from chickens in Ibadan. In contrast, it was one of the serovars isolated from chickens in Awka, Southern Nigeria (Orji *et al.*, 2005) and Maiduguri, North eastern

Nigeria (Raufu *et al*., 2013). This variation may be due to differences in climate, feed and feeding patterns, or production systems. In another report by Raufu *et al.* (2009), *S.* Hiduddify, a rare serovar, was found to be the only serovar in chickens in Maiduguri, North eastern Nigeria, but was not isolated in this study. However, the chickens studied by Raufu *et al.*(2009) were largely free range in contrast to the intensively reared chickens in this study.

In food products or clinical samples, potentially pathogenic bacteria like *Salmonella* may be present in low numbers, which requires their isolation by cultural techniques and is time-consuming and labuor intensive. Control of infection depends on the availability of rapid methods and precise diagnostic tests for monitoring animal production, food-producing factories, and final products (Huguette *et al.,* 1996). PCR has become a potentially powerful alternative in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy (Pickup *et al.,* 2003). In this study, *fimA* gene fragment was amplified in all the 24 *Salmonella* isolates selected for further testing by PCR. These finding is in agreement with earlier reports by Huguette *et al.* (1996), and Alaa and Adnan (2011) which both found that the *fim*Agene contains sequences unique to *Salmonella* isolates and demonstrate that this gene is a suitable PCR target for the detection of *Salmonella* strains. Bacterial adherence (Kurkkonen *et al*., 1993) is considered to be a baseline requirement for infection, and there is proof that many bacteria have surface appendages, such as fimbriae (Clegg*et al.,* 1985; Aslanzadeh and Paulissen, 1992) or pili, that mediate binding to specific receptors on the epithelial cell surface, or intestinal mucus. Although certain types of *Escherichia coli* fimbriae are known to be important for virulence, only type 1 fimbriae (*fim*A) have been implicated in *Salmonella* pathogenicity.

Salmonella Enteritidis fimbriae 14 (SEF14) is encoded by the *sef* operon, which contains *sef*C gene. It plays important role in the pathogenicity of the bacterium because it promotes attachment to intestinal epithelium and caecal colonization which is important in egg contamination (Allen-Vercoe and Woodward, 1999). It is made up of 4 major protein subunits SefA (14 kd), SefB (28 kd), SefC (90kd), and SefD (10 kd). In the present study, the *sef*C gene was detected in 20.8 % (5/24) of the isolates selected for PCR and these isolates were confirmed to be *Salmonellaenterica*subsp. *enterica* ser. 45:d:1,7. The present finding is in contrast with the findings of Rahman (1999) and Murugkar *et al*. (2003) which showed that except for strains of *S.* Enteritidis and *S.* Gallinarum, none of the other serotypes (*S*. Typhimurium, *S*. Newport, *S.* Kentucky, *S.* Weltevreden and *S.* Indiana) tested were found to harbor these genes. These differences might be due to the fact that the serotype shown to contain this gene in our present study was not includedin the serovars studied by Rahman (1999) and Murugkar *et al*. (2003).

The high level of resistance tomost of antimicrobials tested in this study especially nalidixic acid and ciprofloxacin is worrisome because fluoroquinolones are used strategically, in the treatment of salmonellosis. This resistance may be due to indiscriminate use of antimicrobials at recommended doses or at subtherapeutic doses in feed as growth promoters, and as chemotherapeutic agents to control epizootics on the farms.The lack of policy to control the use of antimicrobials in poultry especially fluoroquinolones, including ciprofloxacin, enrofloxacin and ofloxacin in Nigeria, may have contributed to rapid spread of resistance in the poultry industries(Parry and Threlfall, 2008). These findings agreed with the report of Fashae *et al*. (2010) which equally reported high level of resistance to nalidixic acid and reduced susceptibility to ciprofloxacin. The

resistance to cephalosporins (ceftazidime and cefotaxime) is in agreement withVincent *et al*.(2008), Agada *et al*. (2014b) and Ahmed *et al*. (2016). This was not surprising, in view of the high level of resistance observed for all the *Salmonella* serovars isolated in this study.Cephalosporins are major antimicrobials used to treat serious *Salmonella* infections. However, their effectiveness is being compromised by the emergence of extended-spectrum beta-lactamases (ESBLs) and plasmid mediated cephalosporinases (Vincent *et al*., 2008). The low level of resistance by most of the isolates to Neomycin might be due to the fact that the farmers in the study area have neglected this drug and opted for some alternate effective antimicrobials like ciprofloxacin. *Salmonella* Agama, which is of zoonotic significance, was one of the most prevalent serovars in this study and showed high level of resistance to most of the commonly used antimicrobials. These observations call for regulation of antibiotic usage in Nigeria to ameliorate the spread of resistance to antimicrobials.

The occurrence of a high frequency of multidrug resistance reported in this study, in addition to the occurence of a total of 18 multiresistance patterns, was an evidence of a potential for therapeutic failure that may be associated with the use of antimicrobial agents in the area. These findings corroborate the report on *Salmonella* isolates from poultry houses in Pakistan and Trinidad and Tobago(Mir *et al*., 2010; Adesiyun *et al*., 2014). From one region to another, multiresistance patterns are known to vary and to reflect the use of antimicrobial agents in the respective poultry industries (Mir *et al*., 2010; Makaya *et al*., 2012).

CHAPTERSIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The following conclusions can be drawn from this study

1. The overall isolation rate of *Salmonella* serovars from poultry farms in Kwara state was found to be 6.4 %.
2. Thirteen(13) different serovars of *Salmonella* were identified from poultry farms in Kwara State with *Salmonella* 6,7:d: - (29.0 %) being the predominant serovar followed by*Salmonella* Agama (28.0 %).
3. The study established isolation of *Salmonella* 6,7:d:- for the first time from chickens in Nigeria.
4. The study also established that *Salmonella* serovars from poultry farms in Kwara State exhibited high level of resistance to critically important antimicrobials like fluoroquinolones and cephalosporins.
5. Multidrug resistance patterns were evident and very high

6. The study reaffirmed the applicability of PCR amplification of*fim*A for identification of *Salmonella* species.

6.2 RECOMMENDATIONS

From the results of the present study, the following recommendations are made:

- Poultry farmers should be educated on the appropriate useand effect of abuse of antibiotics especially fluoroquinolones and cephalosporins**.**
- There is need for concerned authorities like National Agency for Food and Drug Administration an Control (NAFDAC) in Nigeria to formulate a policy that regulates the use of antimicrobials in agricultural settings to reduce the spread of multidrug-resistant *Salmonella* in animals and humans.The use of certain drugs that are reserved for important usage in human, such as fluoroquinolones should be prohibited in agricultural settings.
- There is need for collaboration between researchers and government/funding agencies in order to fund research and use the outcomes of the research to promulgate policies that enhance effective and efficient management and control of diseases.

- Further research should be carried out to determine the genotypic relationship between *Salmonella* Kivu and *Salmonella* 6,7:d:- and also determine the pathogenicity of this serovar (*Salmonella* 6,7:d:-).

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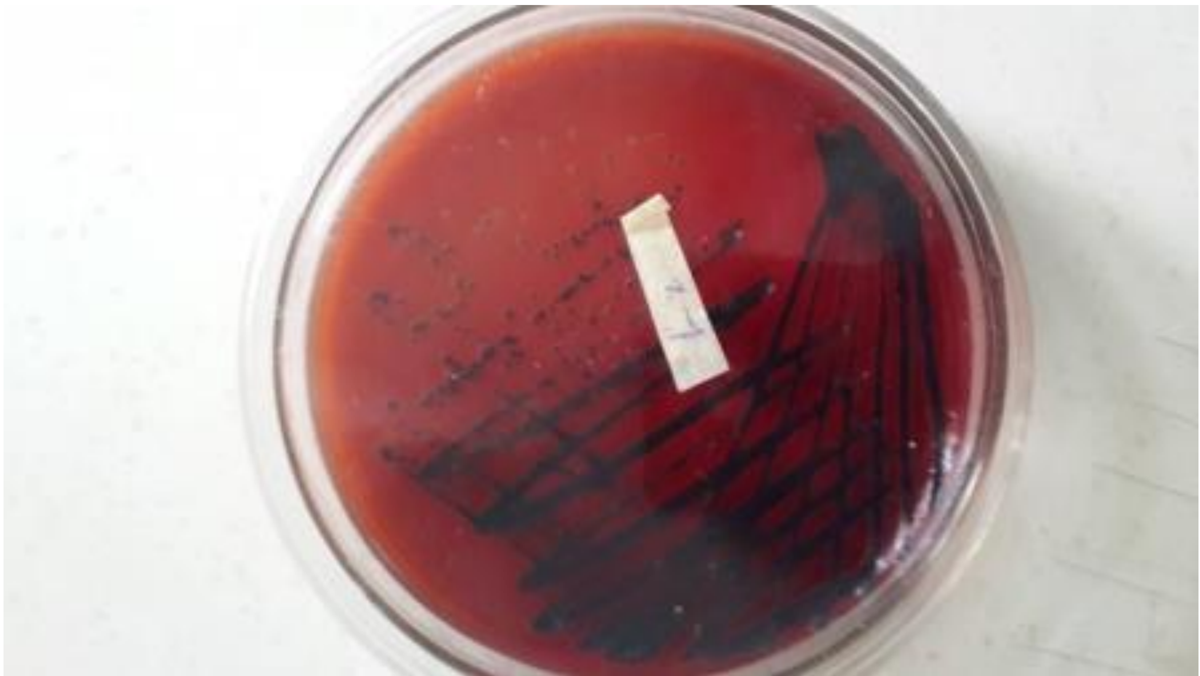
APPENDIX



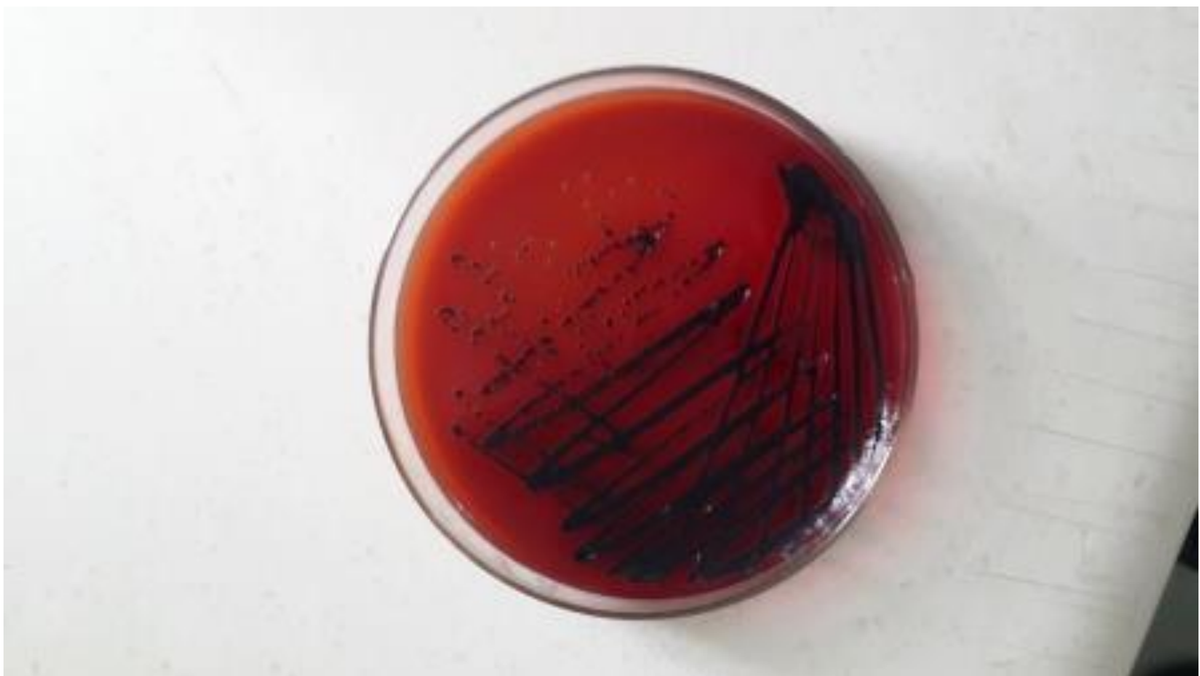
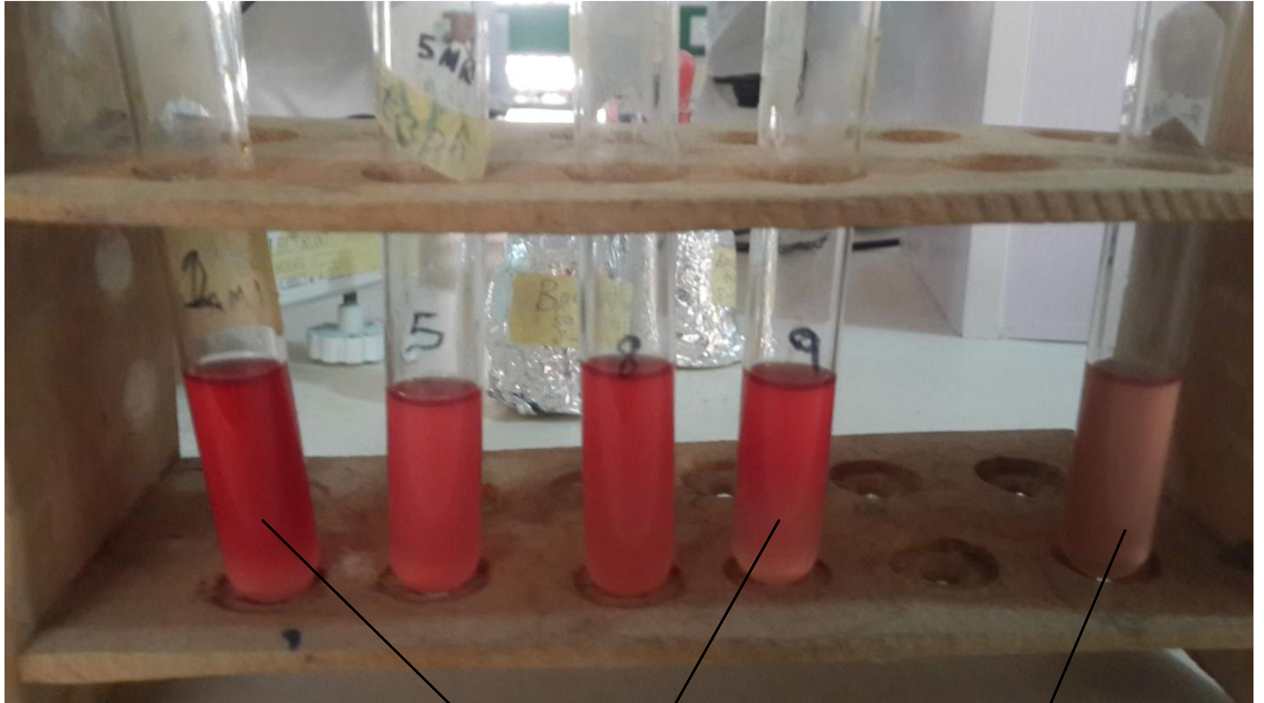


Plate I: Typical colonies of *Salmonella* species (pinkish with dark centre) on Xylose Lysine Deoxycholate Agar (XLD)

Picture A

Picture B

Plate II: Typical reaction of *Salmonella* species on Tripple Sugar Iron (TSI)
Picture A (*Salmonella* species); Picture B (Non *Salmonella* organism)

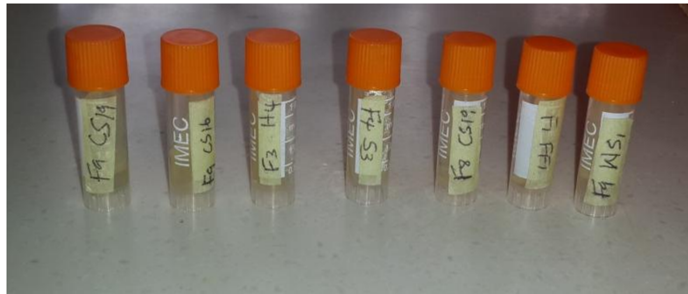


Methyl Red Positive (*Salmonella* species)

Methyl Red Negative (Non *Salmonella* species)

Plate III: Reaction of *Salmonella* species to Methyl Red Test

Plate IV: Reaction of *Salmonella* species on Simmon citrate Agar

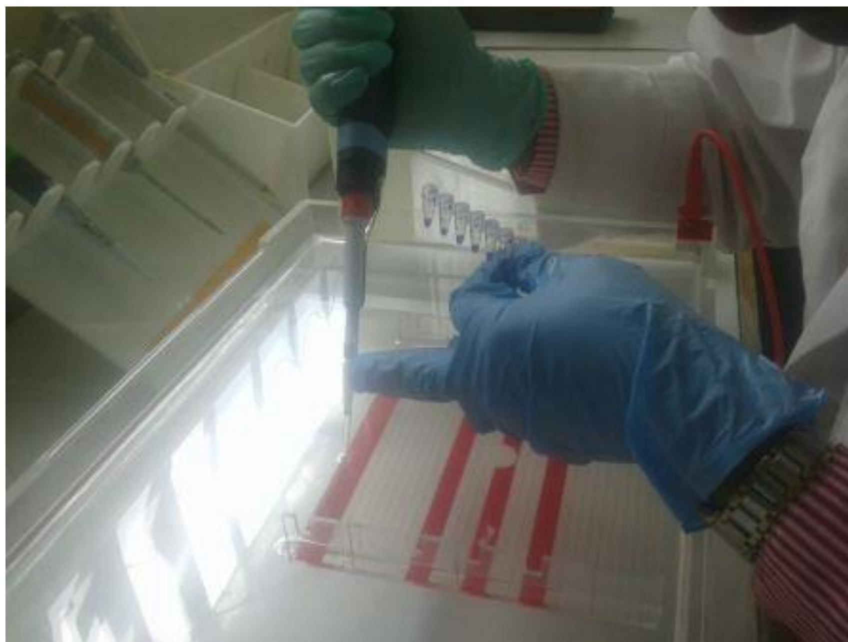


(Urease negative: *Salmonella* positive)

(Urease positive: *Salmonella* negative)

Plate V: Reaction of *Salmonella* species on Urease agar

(Picture C)



(Picture D)

Plate VI: Incubation of PCR coctails in a thermocycler (Picture C) and loading of PCR products on agarrose gel well (Picture D)