ANTIBIOTIC SUSCEPTIBILITY PROFILE AND ANTIMICROBIAL RESISTANCE GENE STUDIES OF *SALMONELLA* SEROVARS FROM CHICKEN BREAST MUSCLES IN SOKOTO METROPOLIS, NIGERIA

BY

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OCTOBER, 2017

DECLARATION

I declare that the work in this Thesis entitled **"Antibiotic Susceptibility Profile and Antimicrobial Resistance Genes Studies of *Salmonella* Serovars from Chicken Breast Muscles in Sokoto Metropolis, Nigeria"** has been performed by me in the Department of Microbiology, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Muhammad Kabir GARBA

Name of student	Signature	Date

CERTIFICATION

This Thesis entitled "**ANTIBIOTIC SUSCEPTIBILITY PROFILE AND ANTIMICROBIAL RESISTANCE GENE STUDIES OF *SALMONELLA* SEROVARS FROM CHICKEN BREAST MUSCLE IN SOKOTO METROPOLIS, NIGERIA"** by Muhammad Kabir GARBA meets the regulations governing the award of Doctor of Philosophy Degree in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

Dedicated to my parents, Hajiya Aishatu Abubakar and late Alhaji Garba Jibrillah Mai-saje.

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ABSTRACT

Commercial poultry constitutes one of the largest and most important reservoirs of enteric pathogens such as *Salmonella.* Antibiotic resistance of *Salmonella* serovars to conventional and newer antibiotics have been observed in many areas of the world. Controlling enteric pathogen infections has become an important objective for the poultry industry from both public health and economic perspectives. Multidrug-resistance in *Salmonella* limits the therapeutic options available for their treatments and this has resulted in increased morbidity, mortality as well as health care costs worldwide. To determine the prevalence of *Salmonella* serovars, their antibacterial susceptibility profile as well as to determine for the presence of antibacterial resistance genes on these *Salmonella* isolates, a total of four hundred (400) Chicken breast muscle samples were randomly selected from four sampling sites in Sokoto Metropolis. The samples were screened for the presence of *Salmonella* serovars using standard techniques. Antibacterial susceptibility of the isolates recovered from the samples were determined using disk diffusion technique and the presence of antibacterial resistance genes of the isolates was determined by polymerase chain reaction (PCR) analysis. A prevalence of 6.5% *Salmonella* organisms' contamination was observed on the meat samples collected for the study. Screening for the presence of antimicrobial residues in the chicken meat samples revealed that 339 (84.75%) of the meat samples had antimicrobial residues greater than the maximum residual limit (MRL). Presumptive identification of the antimicrobial agent present in the meat sample showed that 241 (60.25%) of the meat samples had tetracycline residues and 93 (27.4%) had streptomycin residues. Antibiotic susceptibility profile of the isolates showed 96.2% susceptibility to nitrofurantoin, 92.3% to nalidixic acid and 73.1% to ofloxacin. Resistance profile of the isolates revealed 73.1% resistance to tetracycline, 65% resistance to ampicillin and a 61.5% to chloramphenicol. Multiple resistance index (MARI) of 0.25 or more was observed in

34.6% (9/26) of the bacterial isolates. Antibiotic resistance gene analysis showed the presence of ampicillin resistance gene (*bla*TEM) and tetracycline resistance gene (*tet*A) in 75% of the isolates. Presence of multiple resistance genes was also observed in 50% of the isolates. All the three resistance genes (*bla*TEM, *tet*A and *flo*R) were observed in 12.5% of the isolates. Antibiotics have been used widely in poultry to treat, prevent infections and also in feeds to promote growth. Such practice has improved poultry performance effectively and economically but has resulted in the increase in the spread of antibiotic resistant organisms. There is therefore the need for more rational use of antibtiotics in animal production and in human use.

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ABBREVIATIONS

°C Degree Celcius

AFLP Amplified Fragment Length Polymorphism

AMC Amoxicillin/clavulanic acid

AMP Ampicillin

ATM Aztreonam

BPW Buffered Peptone Water

BSA Bismuth Sulfite Agar

CAZ Ceftazidime

CDC Centers for Disease Control and Prevention

CHL Chloramphenicol

CIP Ciprofloxacin

CLSI Clinical Laboratory Standards Institute

COL Colistin

CPD Cefpodoxime

CRO Ceftriaxone

CTX Cefotaxime

CXM Cefuroxime

DNA Deoxyribonucleic Acid

DT Definitive Type

EFT Ceftiofur

EMB Eosin Methylene Blue

ESBL Extended Spectrum β-Lactamase

FFC Florfenicol

FOX Cefoxitine

GEN Gentamicin

h Hour

IMP Imipenem

Inc Incompatibility

IS Insertion Sequence

KAN Kanamycin

kb Kilobase

LB Luria Bertani

MIC Minimum Inhibitory Concentration

min Minute

MKKTTn Müller Kauffmann Thetrathionate Novobiocin Broth

MLST Multilocus Sequence Typing

NAL Nalidixic acid

NEO Neomycin

NT Non-Typable

PCR Polymerase Chain Reaction

PFGE Pulsed Field Gel Electrophoresis

PT Phage Type

QRDR Quinolone Resistance Determining Region

RAPD Randomly Amplified Polymorphic DNA

RDNC Reacting with the typing phage, but lytic pattern Did Not Correspond to any recognized phage types

RFLP Restriction Fragment Length Polymorphism

RNA Ribonucleic Acid

RVB Rappaport Vassiliadis Broth

SGI1 Salmonella Genomic Island 1

SPT Spectinomycin

STR Streptomycin

SUL Sulfonamide compounds

SXT Trimethoprim/sulfamethoksazol

TET Tetracycline

TMP Trimethoprim

Tn Transposon

WHO World Health Organization

XLD Xylose Lysine Deoxycholate

CHAPTER ONE

Introduction

Advances in poultry production practices, changes in consumer lifestyles preferences and heightened nutritional awareness have all combined to make Poultry products a leading source of protein for much of the world (Swantantra *et al*., 2014). Thus the incidence of enteric pathogens such as *Salmonella, E. coli* and *Campylobacter* infections in poultry flocks and associated incidence of antibiotic residues contamination of poultry products are of considerable public health significance (Jallailudeen *et al.*, 2015).

Enteric pathogenic infections remain among the leading sources of food-borne illness throughout the world (Laxmee and Hudaa, 2015). Pathogens such as *Salmonella E.coli* and *Campylobacter* have received considerable media attention. Infections of domestic poultry with enteric pathogens are expensive both for the poultry industry and for society as a whole. An alarming trend is the rapid emergence of antimicrobial agent-resistant enteric pathogens all over the world (David and Ban, 2001).

1.1 Classification of the Genus *Salmonella*

Salmonellae are the most complex of all the entrobacteriaceae with more than 2,200 serotypes described in the Kauffman-White scheme (Elmer *et al.,* 1992, Das, *et al.*, 2012). In this scheme, the salmonellae are grouped as A, B, C, and D on the basis of somatic (O-antigens) and sub-divided into serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, also by their flagella H-antigens (such as A1, A2, B1, B2, C1, C2, D1, E1 and G). Although most Salmonellae serotypes cannot be effectively distinguished by biochemical reactions alone, *Salmonella* Typhi possesses some unique biochemical characteristics such as its ability to produce a trace amount of hydrogen sulphide (H_2S) at the interface of the slant and butt in Kligler Iron Agar (KIA) or Triple Sugar

Iron Agar (TSI) media. *Salmonella* Typhi is specifically known to give negative reaction in the following tests; Simmon's citrate utilisation, ornithine decarboxylase, gas from glucose, fermentation of dulcitol, arabinose, rhamnose, and acetate utilization (Elmer *et al.*, 1992).

Salmonellae are Gram-negative rod-shaped bacteria, causing a wide range of human and animal diseases, such as enteric fever, gastroenteritis, endocarditis and bacteraemia (Bennasar *et al*, 2003, Liu, *et al*., 2014). The sources of infection in humans are commonly from contaminated food, water, meat or milk contaminated by human or animal faeces. Lower animals such as poultry, pigs, and pets are the principal sources of non-typhoidal Salmonellae infection in humans (Chessbrough, 1987). Several studies have shown that sources of *Salmonella* infection in the poultry include contaminated products, feeds and feed ingredients, human wastes, and rat droppings among others (Jones, 1992; Hayashi and Yamazaki, 1996). Although infections with non-typhoidal salmonellae usually cause self-limiting diarrhoeic illness, serious sequelae include meningitis, sepsis and death may occur especially among infants and elderly persons (Glaser *et al.,* 1994). Non-typhoidal *Salmonella* invasive infections are a public health concern for infants, young children and adults co-infected with HIV (Gordon, 2012). Non-typhoidal *Salmonella* serovars are a leading cause of food-borne bacterial diseases in humans throughout the world (Erol *et al.*, 2013).

In developed countries adequate sanitary measures have led to decrease in typhoidal salmonellae cases and most cases are associated with travellers returning from endemic areas of developing countries (Threifall and Ward, 2001). However, in these countries, non typhoidal salmonellosis is common and most of these cases are

associated with out-breaks from contaminated meat, dairy products and from foods contaminated with salmonellae (Glynn *et al.,* 1998; Wright *et al.,* 2005).

1.2 *Salmonella* in Nigeria

In Nigeria as well as in other developing countries of Africa and Southern Asia, *Salmonella* infection continues to pose a threat to public health with an estimated incidence of 33 million cases every year (Ivonoff, 1995; Sood *et al*., 1999). It is common in developing countries where it affects 12.5 million persons each year. Although, it is widely believed that typhoid fever is endemic in Nigeria, the burden of the disease has not been objectively ascertained (Obaro *et al*., 2015). However, in Nigeria between 10% and 50% prevalence have been reported (Uttah *et al*., 2013). Treatment of patients has been based on the use of first line antibiotics such as chloramphenicol and co-trimoxazole, and the third generation cephalosporins. The efficacy of some of these drugs has been doubtful, following the emergence of multi-drug resistance *Salmonella* strains (Akinyemi *et al.,* 2002; Smith *et al.,* 2005). The emergence and spread of *Salmonella* strains having multiple resistances to nearly all commonly used drugs is a major challenge to health care system, reducing effective treatment options, increasing treatment costs and increasing the risk of complications and death (Kariuki, 2008). Thus the high rate of hospitalisation and prolonged illness of patients with *Salmonella* associated diseases as a result of treatment failure with empirical therapy has been a great concern to the public health authorities. Poor sanitation occasioned by overcrowding, low standards of living, lack of medical access, and indiscriminate use of antibiotics and the emergence of multi-resistant strains of *Salmonella* are all factors that exacerbate the spread of the disease in developing countries (Threifall *et al*., 1999).

1.3 Antibiotics in Poultry

Antibiotics are used in the poultry industry to enhance growth, feed efficiency and reduce disease which has facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. Antibiotic usage has also enhanced the health and well-being of poultry by reducing the incidence of disease (Wageh *et al.,* 2013). The use of antimicrobials for the treatment or prevention of disease in animals closely followed their uses in humans (Gustafson and Bowen, 1997) and today antimicrobial drugs are used to control, prevent, treat infection, to enhance animal growth and feed efficiency (Tollefson and Miller, 2000). Approximately 80% of all food-producing animals receive medication for part or most of their lives (Lee *et al.,* 2001). The use of antibiotics as prophylactics and growth promoters at concentrations lower than those used for treatment is a potentially dangerous practice that can encourage the emergence of antibiotic resistant bacterial strains (Al-Mustafa and Al-Ghamdi, 2000). The selective pressure created by wide spread use of antimicrobial drugs in animals and humans may have contributed to the proliferation of resistant bacterial strains. Resistance markers may be borne on plasmids or chromosomes of resistant bacteria. Plasmids have been a major factor in the spread of antibiotic resistance between bacteria. Under antibiotic selective pressure, R-plasmids spread resistance markers between both homologous and heterogeneous bacteria (Halawani and Shohayeb, 2008). The emergence and spread of antibiotic resistance is a global concern for both human and veterinary medicine (Usui *et al.,* 2014). Potentially allergic reactions and direct toxicity of some antibiotics (such as chloramphenicol which cause fatal aplastic anemia) may have fatal consequences (Lee *et al.,* 2001). The use of antibiotic drugs in food-producing animals may result in the presence of residues in meat. Protection of public against

infection with enteric pathogens, spread of antibiotic- resistant strains and possible harmful effects of veterinary drug residues is a very important problem (Swatantra *et al.*, 2014).

1.4 Statement of the Problem

Commercial poultry constitute one of the largest and most important reservoirs of enteric pathogens such as *Salmonella, E. coli* and *Campylobacter* that can be introduced into the human food supply (Lopasovsky, *et al.*, 2015). Controlling enteric pathogenic organisms has thus become an important objective for the poultry industry for both public health and economic perspectives. In Nigeria, drug withdrawal period are not observed because, livestock and poultry owners can easily obtain drugs and dose their animals or birds at will, slaughter and offer these animals or birds for sale cheaply if sick animals or birds did not respond to therapy within a day or two. As a result, the magnitude of drug residue problem in Nigeria's food of animal origin is largely unknown (Oboegbulem and Fedelis, 1996; Kabir *et al,*. 2002; Abdulsalam *et al,*. 2010).

Drug resistance appears to be the most important hazard of drug residues (Dipeolu 2002). Sub therapeutic concentrations of drugs could perturb the gastrointestinal ecosystem causing adverse health effects. Colonization resistance sustained by indigenous anaerobes within the intestine could be compromised, increased susceptibility to invasion by pathogenic organisms and potential for over growth that could eventually lead to colitis and septicaemic conditions especially in immuno-compromised individuals (Sundlof *et al.*, 2000). Globally, antibiotic resistance among *Salmonella* isolates has increased in both developed and developing countries (Usui *et al.*, 2014). High rates of resistance of *Salmonella* serovars to conventional

and newer antibiotics have been reported with increasing frequency in many areas of the world (Su *et al.,* 2004, Chen, *et al.,* 2013). Serovars of *Salmonella* with multidrug-resistance are encountered frequently worldwide and rates of multidrug-resistance have been observed to have increased considerably over the years (Gordana *et al.,* 2012). Multidrug-resistance in *Salmonella* limits the therapeutic options available for treatments of patients infected with multi-drug resistant isolates (Glynn *et al.*, 1998; Carattoli *et al*., 2002). Antibiotic resistance in *Salmonella* has resulted in increased morbidity and mortality as well as health care costs worldwide (Sivakumar, 2012).

1.5 Justification of the Study

There is scarcity of data on *Salmonella* contamination of chicken meat particularly in Sokoto metropolis and data are also limited in Nigeria at large. Infections of domestic poultry with enteric pathogens are a source for concern both for the poultry industry and for society as a whole. This study will allow us to draw inference on the prevalence of *Salmonella* contamination of poultry meat, antibiotic residues contamination level of the meat in Sokoto and antimicrobial susceptibility pattern of the isolates.

1.6 Aim

The aim of this research is to establish the spread of *Salmonella* contamination of poultry meat in Sokoto metropolis and identify the antibiotic susceptibility and resistance gene profile of the bacterial isolates from the Chicken meat samples

1.7 Objectives

1. To isolate and characterise *Salmonella* serovars from Chicken meat samples in Sokoto metropolis
2. To determine the prevalence of *Salmonella* serovars in the Chicken meat samples
3. To evaluate the antimicrobial residues levels in the Chicken meat samples
4. To determine the antibiotic susceptibility profile of the bacterial isolates in poultry meat samples
5. To determine antibiotic resistance genes and multidrug resistance genes in bacterial isolates from the poultry meat samples in the metropolis

CHAPTER TWO

LITERATURE REVIEW

2.1 General properties of the genus *Salmonella*

The genus *Salmonella* belongs to the family Enterobacteriaceae whose members are Gram-negative, non-sporing, facultatively anaerobic, glucose fermenting, nitrate reducing, oxidase-negative and straight rods of about 0.7-1.5µm (Le Minor, 1984; ISO, 2002 and Emily *et al.,* 2011). Most *Salmonella* are motile via peritrichous flagellae except *Salmonella* Gallinarum and *Salmonella* Pullorum. Salmonellae can be isolated in the microbiology laboratory using numerous low-selective media (MacConkey agar, deoxycholate agar), intermediate-selective media (*Salmonella-Shigella* [SS] agar, Hektoen [HE] agar), and highly selective media (selenite agar with brilliant green). Salmonellae are oxidase-negative and predominantly lactose-negative. Less than 1% of nontyphoidal *Salmonella* isolates are lactose-positive (pink on MacConkey agar), but most produce hydrogen sulfide, which is detectable on HE or SS agar. As facultative anaerobes, they grow well both in bottles of standard automated systems for blood cultures and on culture media routinely used for urine, tissue, and respiratory cultures (cavallro *et al.,* 2011). Individual isolates can then be distinguished with serogrouping, pulsed-field gel electrophoresis, and bacteriophage serotyping techniques. Based on their biochemical capacities, members of the genus *Salmonella* can be differentiated from other genera within the family Enterobacteriaceae (McClelland *et al*., 2001). *Salmonella* nomenclature has changed many times and still is not stable. Traditionally, *Salmonella* species were named in accordance with the Kaufmann-White typing system, defined by different combinations of somatic O, surface Vi, and flagellar H antigens (Su and Chiu, 2017). The genus *Salmonella* was differentiated into two species: *Salmonella enterica* and

Salmonella bongori. *Salmonella enterica* (*S. enterica)* is further divided into six subspecies; *S. enterica* subsp. Enterica (1), *S. enterica* subsp. Salamae (11), *S. enterica* subsp. Arizonae (111a), *S. enterica* subsp. Diarizonae (111b), *S. enterica* subsp. Houtenae and *S. enterica* subsp. Indica (V1). Formerly, *S. bongori* was the subspecies V, but later considered as a separate species (Fluit, 2005; Tindall *et al.*, 2005). Furthermore, the genus is composed of over 2500 serotypes differentiated according to three different types of surface antigens, 99% of these serotypes belong to *S. enterica* and nearly 60% of them are in *S. enterica* subsp. Enterica. The average DNA sequence similarity between *Samonella* serotypes is 96-99% (Edwards *et al.*, 2002).

2.2 Epidemiology of *Salmonella* species

Transmission of salmonellae to a susceptible host usually occurs via consumption of contaminated foods. The most common sources of salmonellae include beef, poultry, and eggs. Improperly prepared fruits, vegetables, dairy products, and shellfish have also been implicated as sources of *Salmonella*. Almost any type food product could serve a source for infection, including peanut butter and powdered infant formula. In addition, human-to-human and animal-to-human transmissions can occur. Although the infectious dose varies among *Salmonella* strains, a large inoculum is thought to be necessary to overcome stomach acidity and to compete with normal intestinal flora. Large inocula are also associated with higher rates of illness and shorter incubation periods. In general, about 10^6 bacterial cells are needed to cause infection. Low gastric acidity, which is common in elderly persons and among individuals who use antacids, can decrease the infective dose to 10^3 cells, while prior vaccination can increase the number to 10^9 cells (World Health Organization, 2008). *Salmonella* outbreaks often result from the spread of a strain whose progeny are genetically

closely related. Thus a better understanding of epidemiology of *Salmonella* is essential for effective control of infection due to the organism. The determination of the relatedness of strains within a given *Salmonella* serotype is a prerequisite for the identification of the sources of infection and for tracing the routes of *Salmonella* dissemination in outbreaks. Since biochemical analysis is limited in further differentiation between the bacteria assigned to the same *S. enterica* subspecies, other methods have been used including serotyping and molecular analyses (Olsen, 2000; Riley, 2004). For the purpose of strain differentiation a number of typing methods have been improved. In general, they are classified into two major groups; conventional (phenotypic methods: such as serotyping and phage typing) and molecular (genotypic methods: such as plasmid probing, insertion sequence (IS) typing, ribotyping, randomly amplified polymorphic DNA analysis (RAPD), amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). An ideal typing method should fulfill the following six criteria: type ability, reproducibility, and discriminatory power, ease of interpretation, easy to use and low cost. In the choice of the most suitable methods for typing system, besides considering the limitations, the aim of the studies (e.g identification of the sources of infections, reconstruction of the chains of infection, distinguishing between an outbreak-related and unrelated strain, global epidemiological studies) can play an important role (Riley, 2004). It is obviously difficult to find a single method which is most suitable for typing *Salmonella*.

2.3 *Salmonella* Serotyping

Sero-typing is the initial step for routine diagnostics of *Salmonella* strains and performed with commercially available poly- and mono-valent antisera. Over 2500

serotypes of *Salmonella* have been identified and classified in the Kaufman-White scheme. The Kauffmann–White classification or Kauffman and White classification scheme is a system that classifies the genus *Salmonella* into serotypes based on surface antigens (Grimont, (2013). It is named after Philip Bruce White and Fritz Kauffmann First the "O" antigen type is determined based on oligosaccharides associated with lipopolysaccharide. Then the "H" antigen is determined based on flagellar proteins (H is short for the German *Hauch* meaning "breath" or "mist"; O stands for German *ohne* meaning "without"). Since *Salmonella* typically exhibit phase variation between two motile phenotypes (Chiou, 2006), different "H" antigens maybe expressed. Salmonella that can express only one "H" antigen phase consequently have motile and non-motile phenotypes and are termed monophasic, whilst isolates that lack any "H" antigen expression are termed non-motile (Grimont, 2013). Pathogenic strains of *Salmonella* Typhi, *Salmonella* Paratyphi C, and *Salmonella* Dublin carry the capsular "Vi" antigen (Vi for **vi**rulence), which is a special subtype of the capsule's K antigen (from the German word *Kapsel* meaning capsule. This scheme differentiates between O (somatic) antigens of the cell surface, H1 and H2 (flagellar) antigens of the phase 1 or phase 2, respectively (Selander *et al.*, 1996) and the Vi (capsular) antigens which however may only be present in few serotypes, such as Typhi, Paratyphi C or Dublin. Each *Salmonella* serogroup has a group specific O-antigen. Within each O-group, different serovars are distinguished by the combination of O- and H-antigens that are present. Each serotype has a specific antigenic formula where the O-antigens are indicated by Arabic numbers, the H1-antigens by lower case letters and the H2-antigens by Arabic numbers. In these formulas, underlined antigens may only be expressed once the culture is lysogenised by the corresponding converting phage whereas letters or numbers in

brackets indicate antigens which may be present or absent without relation to phage conversion (Le Minor, 1984). For most of the isolates assigned to *S. enterica* and the subspecies 1, antigenic formula corresponds to a serotype name. In contrast, serotypes identified after 1996 in the subspecies Salamae, Houtenae and Indica and in subspecies *bongori* are designated only by antigenic formula (Brenner *et al.*, 2000).

Examples

Salmonella enterica serotype Typhimurium 1,4,5,12:i:1,2

monophasic variant of Salmonella Typhimurium 1,4,5,12:i:-

"O"-group	Serovar	"O" antigens	Phase 1 "H" antigens	Phase 2 "H" antigens
A	*S.*Paratyphi A	1,2,12	A	no phase 2 antigen
	S. Paratyphi A var. Durazzo	2,12	A	no phase 2 antigen
B	*S*. Paratyphi B	1,4,5,12	B	1,2
	S. Paratyphi B var. Odense	1,4,12	B	1,2
	S. Java	1,4,5,12	B	(1,2)
	S. Limete	1,4,12,27	B	1,5
	S. Typhimurium	1,4,5,12	I	1,2
	S. Typhimurium var. Copenhagen	1,4,12	I	1,2
	S. Agama	4,12	I	1,6
	S. Abortus-equi	4,12	no phase 1 antigen	e,n,x
	S. Abortus-ovis	4,12	C	1,6
	S. Agona	4,12	f,g,s	no phase 2 antigen
	S. Brandenburg	4,12	l,v	e,n,z_{15}
	S. Bredeney	1,4,12,27	l,v	1,7
	S. Derby	1,4,5,12	f,g	no phase 2 antigen
	S. Heidelberg	1,4,5,12	R	1,2
	S. Saintpaul	1,4,5,12	e,h	1,2
	S. Salinatis	4,12	d,e,h	d,e,n,z_{15}
	S. Stanley	4,5,12	D	1,2
C_1	*S*. Paratyphi C	6,7,	C	1,5
	S. Choleraesuis	6,7	C	1,5
	S. Choleraesuis var. Kunzendorf	6,7	(c)	1,5

- Antigens in brackets are those that are rarely expressed in that serovar.

2.4 Phage Typing of *Salmonella*

Phage typing reflects the different susceptibility to a lytic pattern between strains within a serotype. A *Salmonella* strain is subjected to a specified set of typing phages and the lytic pattern obtained commonly allows the assignment to a specific phage type. The strains exhibiting a lytic pattern that does not correspond to a phage type are classified as RDNC (Reacting with the typing phage, but lytic pattern Did Not Correspond to any recognized phage types). Phage typing is mostly performed for serotypes such as *S.* Typhimurium, *S.* Enteritidis, *S.* Typhi or *S.* Paratyphi, although phage typing systems are also available for a number of additional serotypes, incluindg *S.* Virchow. In general, phage typing is only performed by the National Reference Centers, since only these institutions have access to the defined sets of typing phages. The interpretation of the results requires considerable experience (Riley, 2004). Although, phage typing in *Salmonella* epidemiology has been used since the 1950s, the stability of phage types can be limited by phage type conversion (Rabsch *et al.*, 2002), even during an outbreak (Mmolawa *et al.*, 2002). This is due to the acquisition of a temperate phage or a plasmid. Besides, host- controlled phage defense mechanisms such as restriction/modification systems and phage adsorption inhibition are also responsible for the phage typing difficulties of a *Salmonella* strain.

2.5 Molecular Methods for the Characterization of *Salmonella*

The drawbacks of phenotypic typing methods have led to the development of typing methods based on genotypic information. Molecular typing methods based on restriction endonuclease digestion, nucleic acid amplification, or nucleotide sequencing techniques have been used to characterize *Salmonella* organisms. One of the molecular typing methods effectively in use is plasmid analysis. It is particularly important, since most of the plasmids harbor virulence and antimicrobial resistance

properties in *Salmonella*. Plasmid contents of the host within the same serotype reveal the differentiation according to the profile (the number and molecular sizes of plasmid) obtained. The different plasmid profiles within a serotype points the lateral transfer by gaining or loosing the plasmid(s). The plasmids found in *Salmonella* differ in size 2 - 200kb with different functionalities (Aarts *et al.,* 2001; Porwollik and McClelland, 2003; Rychlik *et al.,* 2006).

2.5.1 Insertion sequence (IS) typing

Insertion sequence typing is based on the variation of insertion sequence from strain to strain. Insertion sequences are mobile DNA elements that are able to integrate within the bacterial genome. This diversity has been used for *Salmonella* with IS200 fingerprinting (Aarts *et al.,* 2001). Ribotyping describes the hybridization of restriction-digested DNA fragments with probes specific for rDNA. It results in profiles only consisting of a small number of bands which are easy to interpret. However, this feature also limits the ability to distinguish between closely related strains. Therefore, ribotyping is considered not suitable for local epidemiological studies or surveillance studies in restricted region (Aarts *et al.,* 2001; Riley, 2004).

DNA amplification-based typing methods which use either specific or non-specific primers yield amplicon patterns exhibiting discriminations. Randomly amplified polymorphic DNA (RAPD) is performed with short (10 bases in length) random primers at low annealing temperatures to amplify multiple fragments of bacterial DNA. Due to the amplification conditions, this method is sensitive to slight changes within amplification parameters, thus it is hard to achieve reproducibility. However, ribotyping is a supplementary tool in conjunction with other typing methods (Aarts *et al.*, 2001; Yan *et al.*, 2003).

2.5.2 Amplified Fragment Length Polymorphism (ALFP)

In another method; the amplified fragment length polymorphism (ALFP) analysis, conbines digestion of whole cell DNA with two suitable restriction endonucleases, ligation of suitable adapters and subsequent PCR amplification. The resulting amplicons are labeled by fluorescent tags and analysed using an automated sequencer. It has been considered a highly discriminative but labor and cost-intensive method (Riley, 2004).

2.5.3 Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is a method based on the partial sequencing of a set of housekeeping genes which varies due to mutation or recombination events. Nucleotide differences in the individual genes are combined and used to determine the differentiation of strains (Yan *et al.,* 2003). This method is extremely useful for long-term epidemiological studies or phylogenetic analyses. However its discriminatory power is insufficient for short-term epidemiological studies or outbreak investigations. The reason for this is based on the relatively high stability of the sequenced housekeeping genes within short periods of time. Consequently, MLST does not allow a differentiation among such stains. Besides, the costs for MLST are still high (Enright and Spratt, 1999).

2.5.4 IS200 Typing

Lam and Roth (Lam and Roth, 1983) discovered a 708 bp insertion sequence in *S.*

Typhimurium LT2, which they identified as IS200. This insertion sequence (IS) element appears to be found in a majority of the Salmonella strains examined and is absent from other enterobacteria, making it a good candidate for use in *Salmonella* spp. typing (Lam and Roth, 1983). In some cases, IS200 typing alone is

discriminatory enough to distinguish between strains. Threlfall and his colleagues (Threlfall *et al*., 1993) discovered that it was possible to differentiate between drug-sensitive and drug-resistant *Salmonella* serovar Typhi of Vi-phage types E1 and M1 based on their IS200 profiles.

2.5.5 Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) has been considered as the gold standard among other molecular typing methods. It has a considerable discriminatory power, which also fulfills the criteria of type-ability and reproducibility, since it is the best standardized method (Olive and Bean, 1999; Aarts *et al.,* 2001). By cutting the bacterial DNA with rare-cutting restriction endonucleases and running with special electrophoresis separation technique, it separates the large fragments of DNA up to 12000kb and yields strains specific patterns. It avoids the deficiency of common agarose-based DNA electrophoresis in separating large DNA fragments (> 50 kb). However, this method requires the presence of expensive specialized equipment, high quality chemicals and a considerable experience in the preparation of the DNA-containing agarose slices. Morevere, single genetic events, such as point mutations, integration, deletion or recombination events can result in differences in the fragment patterns (Tenover *et al.,* 1995; Riley, 2004; Herschleb *et al.,* 2007).

Among the molecular typing methods, PFGE is usually considered as the method of choice to determine the molecular relatedness among *Salmonella* strains. It has also been recommended as one of the methods to be used to achieve global standardization and exchange of molecular typing data via the Internet (Lindsay *et al.,* 2002)

2.6 *Salmonella* Pathogenicity and Risk of Food Contaminations

Salmonella strains are zoonotic and infect a wide range of host organisms from reptiles to human (Selander *et al.,* 1996; Tükel e*t al*., 2005; Tükel *et al.,* 2006; Tükel *et al*., 2007). Serotypes differ in their host adaptations and virulence that can be classified into different groups (Uzzau *et al.,* 2000). Isolates, which are pathogenic to humans, belong to subspecies I (Fluit, 2005). Human-adapted serotypes, such as *S.* Typhi and *S.* Paratyphi, cause severe typhoidal disease in humans, and not pathogenic to animals. Contrary to this, serotypes adapted to animals such as *S.* Gallinarum (poultry), *S.* Choleraesuis (swine), *S. Abortusovis* (sheep) or *S.* Dublin (cattle) are the ones that cause systemic illness in their primary hosts. Non-host adapted serotypes also differ in their virulence properties with high virulence, such as *S.* Enteritidis and *S.* Typhimurium, or low virulence, such as *S.* Agona, *S.* Derby and *S.* Hadar. Despite this classification, all animal origin serotypes are considered as potential pathogen in humans (Giannella, 1996). The major sources of *Salmonella* are mainly beef, poultry and eggs, pork and dairy products. Once the contaminated food is ingested and *Salmonella* survive the acidic environment of stomach, it colonizes in the gastrointestinal tract. Adherence of intestinal epithelium is followed by entry to lamina propria. At this site, the bacteria may replicate and establish a local infection, or they may be ingested by macrophages, which may disseminate the *Salmonella* to deeper tissues such as regional lymph nodes, liver, and spleen to establish a systemic infection (Selander *et al.,* 1996). Salmonellae selectively attach to specialized epithelial cells (M cells) of the Peyer patches. The bacteria are then internalized by receptor-mediated endocytosis and transported within phagosomes to the lamina propria, where they are released. Once there, salmonellae induce an influx of macrophages (typhoidal strains) or neutrophils (nontyphoidal strains). The Vi

antigen of *S typhi* is important in preventing antibody-mediated opsonization and complement-mediated lysis. Through the induction of cytokine release and via mononuclear cell migration, *S typhi* organisms spread through the reticuloendothelial system, mainly to the liver, spleen, and bone marrow. Within 14 days, the bacteria appear in the bloodstream, facilitating secondary metastatic foci. In some patients, gallbladder infection leads to long-term carriage of *S typhi* or *S paratyphi* in bile and secretion to the stool (Meltzer and Schwartz, 2017). As a rule, infection with nontyphoidal salmonellae generally precipitates a localized response, while *S typhi* and other especially virulent strains invade deeper tissues via lymphatics and capillaries and elicit a major immune response. Virulence factors of salmonellae are complex and encoded both on the organism's chromosome and on large (34-120 kd) plasmids. Several *Salmonella* pathogenicity islands have been identified that mediate uptake of the bacteria into epithelial cells (type III secretion system [TTSS]), nonphagocytic cell invasion (*Salmonella* pathogenicity-island 1 [SPI-1]), and survival and replication within macrophages (*Salmonella* pathogenicity-island 2 [SPI-2], phoP/phoQ). The severity of illness in individuals with salmonellosis is determined not only by the virulence factors of the infecting strain but also by host properties. The most common risk factors were found to be corticosteroid use, malignancy, diabetes, prior antimicrobial therapy and immunosuppressive therapy (Hohman, 2001). Infants are at a high risk of developing central nervous system (CNS) infection as a result of *Salmonella* bacteremia. Specific anatomical sites, such as an altered urinary or biliary tract, atherosclerotic aorta, or endovascular devices may facilitate persistent focal *Salmonella* infection.While typhoidal diseases occur with the invasion to blood, gastroenteritis causing *Salmonella* rarely invade blood. However, this invasion also leads to a systemic disease, which requires antimicrobial

treatment of the patient. The use of antimicrobial chemotherapy to control salmonellosis in animal husbandry has resulted in resistant microorganisms including *Salmonella* (Zhao *et al.,* 2007).

2.6.1 *Salmonella* virulence plasmids

Although, majority of the *Salmonella* species carry plasmid(s), not all of these plasmids confer a virulence phenotype to the strain. Such virulence plasmids have a low-copy number, vary in size (50 to 95 kB) (Haneda *et al*., 2001) and appear to be limited to S. enterica subspecies I serovars like Typhimurium, Enteritidis, Dublin, Choleraesius, Gallinarum, Pullorum and Abortusovis (Barrow and Lovell, 1988). Virulence plasmids have been shown to be involved in the expression of *Salmonella* virulence (Uzzau *et al.,* 2000). All virulence plasmid-containing *Salmonella* spp. share a highly conserved 8 kB operon encompassing 5 genes, designated spvRABCD (Rychlik *et al.*¸2006). It has been observed that this operon encodes the major virulence phenotype of the plasmid as the presence of this 8 kB region is sufficient to restore wild-type virulence to plasmid-cured strains. While SpvABCD show no significant homology with any known proteins, the 33 kDa SpvR protein shares sequence similarity with the MetR/LysR family of bacterial transcriptional activators. SpvR is able to activate its own expression and the expression of SpvABCD by binding to a promoter region upstream of spvA. SpvR is required for pathogenesis as spvR mutants are attenuated and unable to proliferate in macrophages. This is probably due to the fact loss of function in spvR results in the nonactivation of the downstream spvABCD. Insertional mutations in the individual genes (spvA, spvB, spvC and spvD) have resulted in reduction of virulence and attenuation (Gulig *et al.,* 1992).

2.6.2 *Salmonella* bacterial fimbriae

Bacterial fimbriae are defined as thin, filamentous appendages localised on the cell surface that mediate the attachment of bacteria to host cells (Anon, 1998). In *Salmonella* organisms, 13 fimbrial operons have been identified in the *Salmonella* genome: agf (csf), fim, bcf, lpf, pef, saf, stb, std, stc, stf, sti and stj (Clegg *et al.*, 1987) Salmonella spp. fimbrial genes have been implicated in virulence in vitro and in vivo experiments (Althouse *et al.*, 2003; De Buck et al., 2004;; Forest *et al.*, 2007) Furthermore, several studies have shown that a large number of fimbrial operons often act in concert to elicit various virulence-associated functions (Ledeboer *et al.*, 2006; Weening *et al*., 2005).

2.6.3 *Salmonella* toxins

Salmonella spp. is capable of producing both endotxins and exotoxins (van Asten and van Dijk, 2005). The lipopolysaccharide (LPS) endotoxin is a major component of the outer membrane of many enterobacteria, including *Salmonella* spp. (Raetz and Whitfield, 2002). Structural analysis of LPS has revealed that it is composed of 3 regions: the core polysaccharide, the O-specific (somatic) antigen polysaccharide side chain and the hydrophobic lipid A (Raetz and Whitfield, 2002). Although the core and Lipid A region is conserved among the *Salmonella* genus, great variation exist with the O-antigen chain, forming the basis for the Kaufmann-White serotyping scheme. LPS forms a layer around the bacterial cell and functions as a permeability barrier, selectively allowing and excluding substances (Todar, 2002). This enables the *Salmonella* bacterium to protect itself against the environment (e.g. acid and bile salts in the gastrointestinal tract) and substances (e.g. lysozyme and antimicrobial) found inside the host cell (Todar, 2002). Since it is a surface structure, LPS also

mediates host-pathogen interaction, making it important in the virulence process (Todar, 2002). Lipid A is the active portion of LPS and its interaction with the Toll-like receptors during infection activates the host innate immune system and results in the production of substances necesssary for inflammatory responses, which at high levels, leads to the development of sepsis and septic shock (Raetz and Whitfield, 2002; Todar, 2002). Biosynthesis and assembly of this molecule involves a number of genes, including lpx, pag and pmr (Ernst et al., 2001; Raetz and Whitfield, 2002). Formation of the core region involves the waa operon (Raetz and Whitfield, 2002). The rfb and wzz locus is reponsible for the synthesis and assembly of monosaccharide units into the O-antigen chain and controlling the length of the polysaccharide chain, respectively. Mutations in any of the genetic loci above have led to a loss in virulence and/or reduction in complement resistance (Murray *et al.*, 2006) Although *Salmonella* spp. are able to secrete exotoxins – cytotoxins and enterotoxins, not much is known about its exact role and function in pathogenesis (van Asten and van Dijk, 2005)

2.6.4 Variation in *Salmonella* pathogenicity

Although a majority of *Salmonella* strains are pathogenic to humans and animals their virulence appears to vary from serovar to serovar. *Salmonella* serotypes usually cause gastroenteritis, with a few (e.g. *S.* Typhi and *S.* Typhimurium) able to cause enteric fever and serovars like Dublin and Pullorum more likely to cause bacteraemia to target animals (Wallis, 2006). *Salmonella* spp. also has a wide host range: from the ubiquitous serovars like Typhimurium and Enteritidis, to host-restricted (e.g. *S.* Dublin and *S.* Choleraesuis, both capable of causing infection in cattle, pigs and humans) and host-specific ones (e.g. *S.* Typhi and *S.* Pullorum, which are fully adapted to humans and chickens, respectively) (Wallis, 2006). As pathogenicity of a

serotype is determined by various virulence genes, this diversity can be attributed to genetic variation or polymorphisms in virulence genes. Since infection is initiated by contact between the host and pathogen, it has been suggested that surface structures such as LPS and fimbriae can contribute to the pathogenic diversity of a strain (Fierer and Guiney, 2001). *Salmonella* serotypes differ structurally in the monosaccharide units that make up the O-antigen chain of the LPS and this is due to the highly polymorphic nature of the rfb region, which encodes genes responsible for the biosynthesis of the polysaccharide chain (Wang *et al.*, 2002). Genetic variation is also observed with the fimbrial operons of *Salmonella* spp. While the agf and fim fimbrial cluster is found in all *Salmonella* serotypes, some have been shown to be present in only a small number of serovars (e.g. pef and sef, whose presence correlate with serotypes frequently isolated from common domesticated animals) and others appear to be serovar-specific e.g. the *S.* Typhi fimbrial operon tcf variation seen in the SPIs is conserved within a serovar (Amavisit *et al.*, 2003). This could explain why the subspecies I strains are more able to effectively cause infections in a wide range of hosts (especially warm-blooded animals) in comparison to the other *Salmonella* subspecies. Insertion and deletion of various genes is mediated by mobile elements like transposons and IS elements (Dobrindt *et al.*, 2004). While a transposon contains both functional genes and transposase, the IS element is a small DNA fragment that encode only the transposase gene (Dobrindt *et al*., 2004). These "jumping genes" are capable of changing chromosomal location, inserting or excising genes as they "jump". By inserting itself into the sequence of virulence genes, the IS element can inactivate the gene by causing a frameshift, which disrupts gene transcription (Steinert *et al*., 2000). IS elements have also been associated with the rfb region in several *Salmonella* serovars, showing that the presence of such

mobile genes can indicate genetic polymorphisms in a specific region (Wang *et al*., 2002). Changes in the genetic sequence such as base insertion, deletion or substitution can also seriously affect expression of virulence-associated genes (Anon, 2008). Such mutations can result in the creation of a stop codon, truncating the protein (nonsense mutation) or translation of a different amino acid (misense mutation), which may alter function of the protein (Anon, 2008). The virulence plasmid of *Salmonella* spp. is sometimes referred to as a serovar-specific plasmid (Rotger and Casadesús, 1999). They are usually found in *S.* enterica subspecies I strains showing host adaptation, implying a possible correlation between the presence of the plasmid and host-specific virulence. Although virulence plasmid are particularly associated with host adapted serovars and may contribute to pathogenic diversity of *Salmonella* spp., more research needs to be done in order to gain further knowledge regarding the association between the two factors.

2.7 Antimicrobials and Antimicrobial Resistance in *Salmonella* organisms

Antibiotics have numerous applications in the veterinary and agricultural field. A large number of antibiotics are fed to animals in farms for prophylactic and therapeutic purposes. Concerns about infections due to *Salmonella* have led to the implementation of control programs for broiler flocks (Gallus gallus). In 2006, the European Union officially banned the use of antibiotics added to poultry feeds as growth promoting substances in livestock (WHO, 2011). Antimicrobials used in the therapy of infectious diseases are the drugs that either kill or suppress microorganisms such as bacteria, viruses and parasites. The actions of antibiotics are (i) inhibition of cell wall synthesis, (ii) inhibition of protein synthesis, (iii) inhibition of DNA/RNA precursor (folate) synthesis, (iv) inhibition of DNA/RNA synthesis, and (v) disruption of membrane proteins (Walsh, 2003).

There are several classes of antimicrobial drugs, however it is not the purpose of this study to classify all antimicrobial groups in detail, but the most common classes of antimicrobials that are used in the treatment of *Salmonella* infections are namely β-lactams, quinolones/fluoroquinoles, aminoglycosides, phenicols, tetracyclines, sulfonamides and trimethoprim. β-lactams are one of the critically important antibiotics in both human and veterinary medicine. For example Penicillins are used by Veterinarians as growth promoters in animal production (Li *et al.,* 2007). β-lactams are bacteriocidal by blocking the transpeptidations peptidoglycan layer of bacterial cell wall where they bind to penicillin binding-proteins. There are three major groups of β-lactams: penicillins, cephalosporins, and carbapenems. Possibly because of the widespread clinical use of penicillins, resistance to drugs such as ampicillin and methicillin has become common. In response to this problem, second class of β-lactams, the cephalosporins, was developed. Penicillins have a five-member thiazolidine ring fused to the β-lactam ring. Cephalosporins are the enzymatically converted form of penicillins by a ring expandase enzyme forming a six-member ring (a dihydrothiazine ring) fused to the β-lactam ring (Walsh 2003; Alcaine *et al.,* 2007). These changes provide cephalosporins with a broader range of activity and greater stability in the presence of β-lactamases. Both penicillins and cephalosporins are fungal secondary metabolites produced by *Penicillium chrysogenum* (Walsh 2003). There are four generations of cephalosporins, and each progressive generation is effective against a broader range of organisms. Although *Salmonella* isolates may appear susceptible to first- and second-generation of cephalosporins in vitro, the CLSI cautions that this antimicrobial drug class may not be clinically effective against Salmonella soon (Alcaine *et al.,* 2007). The latest discovered group of β-lactams is carbapenems, which differ from penicillins and

cephalosporins by lacking sulphur in five-membered ring fused to the four-member β-lactam ring. These β-lactams are sometimes paired with β-lactamase inhibitors. Carbapenems have a much broader range of activity against both Gram-negative and Gram-positive bacteria than do other β-lactams and are more stable against β-lactamases. Nevertheless, *Salmonella* isolates that possesses resistance to carbapenems such as imipenem already have been reported (Singh *et al.,* 2007). β-lactam resistance mechanism in *Salmonella* is mostly mediated by the production of the enzymes β-lactamase. These enzymes work by hydrolysing the β-lactam ring structure, yielding beta-amino acids with no antimicrobial activity. The genes encoding for β-lactamases produced by *Salmonella* are typically carried on plasmids, although most of these genes are chromosomally encoded in other bacterial species.

β-lactamases are classified by two schemes called Ambler classes A-D (Ambler 1980) and Bush classes 1-4 (Bush *et al.*, 1995). Ambler's classification scheme is based on the primary structure and amino acid sequence identity of β-lactamases. According to Ambler's classification scheme there are class A, B, C, and D beta-lactamases (Alcaine *et al.*, 2007; Li *et al.*, 2007):

In general, class A β-lactamases are the most commonly reported class of β-lactamases in *Salmonella.* They are plasmid encoded and provide a range of resistances against penicillins, early generation cephalosporins, and carbapenems. There are several different gene families encoding for enzymes in this class, and blaTEM-1 is the most prevalent among *Salmonella* isolates. Other Class A β-lactamase gene such as blaPSE-1 also has been found in a number of *Salmonella* isolates and chromosomally encoded (Li *et al.*, 2007).

The emergence of cefotaximases (CTX-M), which are class A β-lactamases conferring resistance primarily to ceftiofur, is an important trend to watch. Variants of blaCTX-M have been identified in isolates of *Salmonella* serotypes (Livermore *et al.*, 2007).

Class B β-lactamases are metallo-β-lactamases which are not commonly found in *Salmonella*.

Class C β-lactamases are typically encoded by chromosomal ampC genes and provide resistance against cephalosporins and ceftiofur. *Salmonella* has no chromosomal ampC gene; instead, these genes are harboured in plasmids. Currently researches are primarily focused on blaCMY-2, which has been associated with resistance primarily to cephoxitin. The spread of blaCMY-2 is a public health concern because the presence of this gene appears to mediate resistance or at least reduce susceptibility to ceftriaxone, another extended spectrum cephalosporin that is the drug of choice for the treatment of *Salmonella* infections in children.

Class D β-lactamases appear to be rare among *Salmonella* isolates. This class of enzymes provides resistance to β-lactams closely related to oxacillin and methicillin. The chromosomally encoded gene blaoxa-1 (=blaoxa-30) was found in *S. paratyphi, S. muenchen* and *S. typhimurium*. This group is resistant to inhibitors such as clavulanic acid (Hall and Collis, 1998; Alcaine *et al.*, 2007).

2.7.1 Quinolones and fluoroquinolones

Quinolones and fluoroquinolones are synthetic bacteriocidal drugs. In 1962, nalidixic acid became the first quinolone approved for medical use. Several generations of quinolones have been developed, with each new generation having improved action against bacterial infections. The early generation quinolones target DNA gyrase, and

the late generation quinolones both DNA gyrase and topoisomerase IV. The mode of action for quinolones is quite complex and not completely understood. Although quinolones target topoisomerases, they do not actually bind to the topoisomerase but to the double stranded DNA in the topoisomerase complex (Alcaine *et al.,* 2007).

There are documented cases of *Salmonella* isolates with resistance to nalidixic acid and low-level resistance to fluoroquinolones and emerging high-level resistance to quinolones to be emerging. Quinolone resistance of *Salmonella* isolates has been linked to two mechanisms, target gene mutations and active efflux. The first mechanism is mediated by target mutations in the quinolone resistance determining region (QRDR) of gyrA and gyrB, the two genes that encode the subunits of DNA gyrase, and in the parC subunit of topoisomerase IV. The most frequently amino acid substitutions observed in gyrA are Ser-83 (to Phe, Tyr, or Ala) or Asp-87 (to Gly, Asn, or Tyr) and in parC is Thr-57 (to Ser) (Cloeckaert and Chaslus-Dancla, 2001). The second mechanism involves changes in the expression of the AcrAB-TolC efflux system, mostly due to mutations in the genes encoding regulators of this system (e.g., marRAB) that results in over-expression and consequently decreased quinolone sensitivity. No single mutation confers high-level resistance to fluoroquinolones but resistance results from the accumulation of multiple mutations. The facts that *Salmonella* isolates must acquire multiple unlinked mutations and that some of those mutations reduce fitness, particularly those involved in the regulation of the efflux pump, may explain why this kind of resistance is so infrequent (Alcaine *et al.,* 2007). Quinolone resistance also has been linked to the expression of the plasmid-mediated qnr gene. This gene codes for a protein that appears to bind to DNA gyrase and protect it from quinolone inhibition. Research conducted on plasmids harbouring qnr revealed that this gene could be transferred from other

bacterial species to *Salmonella* via conjugation. Although documented cases of plasmid-mediated quinolone resistance in *Salmonella* isolates are rare, studies has indicated that the spread of such plasmids to *Salmonella* isolates has occurred (Kehrenberg *et al.,* 2007). The appearance of plasmid-mediated quinolone resistance in *Salmonella* isolates is a very important emerging public health concern. Plasmids harbouring qnr also can harbour other resistance genes, suggesting that the treatment of infections with *Salmonella* strains containing this plasmid may be increasingly difficult. In a study, reduced susceptibility to ciprofloxacin was observed to be conferred by a variant of the gene encoding aminoglycoside acetyltransferase AAC (6')-Ib, and it was also detected among other Enterobacteriaceae, (Kehrenberg *et al.*, 2007).

2.7.2 Aminoglycosides

Aminoglycosides were first discovered in 1944 from *Streptomyces griseus* and since then have been widely used. Examples of aminoglycosides include kanamycin, neomycin, amikacin, and gentamicin. They are hydrophilic sugars with multiple amino groups and target 16S rRNA on the 30S ribosome from the A site of aminoacyl-tRNA binding which leads to codon misreading and translation inhibition. Most aminoglycosides are bactericidal (destructive), with the exception of spectinomycin, which has a bacteriostatic (growth inhibiting) mode of action. (Walsh, 2003: Alcaine *et al.,* 2007).

Resistance to aminoglycosides in *Salmonella* is mainly associated with the modification of aminoglycoside molecules by enzymes (Sørum and L'Abée-Lund, 2002). These enzymes fall into three groups that are named according to the types of reactions they catalyse (Alcaine *et al.,* 2007):

Aminoglycoside acetyltransferases; these are enzymes that primarily acetylate aminoglycoside-amino groups. Genes encoding these enzymes are typically designated aac and these genes have been found as part of *Salmonella* genomic islands, integrons and plasmids. Aminoglycoside acetyltransferases provide resistance to gentamicin, tobramycin, and kanamycin.

Aminoglycoside phosphotransferases; these are enzymes that catalyse ATP-dependent phosphorylation of specific aminoglycoside hydroxyl groups. Most genes encoding these enzymes are designated as aph provide resistance to kanamycin and neomycin. The genes aph(3')-Ib and aph(6)-Id are commonly referred in the literature as strA and strB, respectively and provide resistance to streptomycin (Randall *et al*., 2016).

Nucleotidyltransferases also target the hydroxyl groups. Genes encoding nucleotidyltransferases are usually designated aad (for aminoglycoside adenyltransferases), although some are also designated as ant (for aminoglycoside nucleotidyltransferase). The aadA gene [or ant (3')] provides streptomycin resistance in *Salmonella* isolates. The aadB gene [or ant (2')-Ia] contributes resistance to gentamicin and tobramycin. Both aadA and aadB have been found as integron-borne gene cassettes (Randall *et al*., 2016).

2.7.3 Phenicols

Phenicols include chloramphenicol and florfenicol. Chloramphenicol was once the drug of choice for the treatment of typhoid fever. Production of chloramphenicols by *Streptomyces venezuelae* was discovered in 1947. Chloramphenicol works by binding to the peptidyltransferases centre of the 50S ribosomal unit, thus preventing formation of peptide bonds. Chloramphenicol's broad range activity against Gram-

positive and Gram-negative bacteria and its ability to cross the blood-brain barrier make it a powerful choice for the treatment of systemic infections. Its toxicity, which can lead to bone marrow damage and aplastic anemia, and widespread resistance have generally limited chloramphenicol's use to occasions where the risk of the infection, such as bacterial meningitis, is greater than the risk of adverse effects from the drug. Chloramphenicol is still widely used in developing countries because of its low cost (Walsh, 2003; Alcaine *et al.,* 2007). Chloramphenicol resistance in *Salmonella* isolates is conferred through two mechanisms: (i) the enzymatic inactivation of the antibiotic via chloramphenicol O-acetyltransferase (CAT) and (ii) the removal of the antibiotic via an efflux pump. The genes encoding for CAT are plasmid-borne and commonly found in *S. typhi* isolates. CAT genes, such as cat1 and cat2, have also been found in nontyphoidal *Salmonella* serotypes. Chloramphenicol efflux pumps in *Salmonella* isolates have been reported to be encoded by two closely related genes, *cml*A and floR. The *flo*R gene appears to be widespread in *Salmonella* isolates, whereas *cml*A is less widely distributed. The highly mobile *floR* gene has been found in *Salmonella* genomic islands and in many different plasmids. It appears to be associated with multidrug resistance.

2.7.4 Tetracyclines

Tetracyclines were discovered in the 1940s. The first tetracycline, chlro-tetracycline, was isolated from *Streptomyces aerofaciens*. Tetracyclines were popular because of their minimal adverse effects and broad-spectrum of activity. They were effective against most bacteria, including chlamydias and mycoplasmas, and even some protozoa. Tetracyclines act by preventing the binding of tRNA to the A site of the 30S ribosomal subunit, thus inhibiting protein synthesis. Unfortunately, the rise of

resistant bacteria has severely limited the use of tetracyclines (Walsh, 2003; Alcaine *et al.,* 2007).

Tetracycline resistance of *Salmonella* isolates is attributed to production of an energy-dependent efflux pump, which removes this antimicrobial drug from the bacterial cell. Other mechanism of resistance, such as modification of the ribosomal target and enzymatic inactivation of tetracycline, have been attributed to other bacterial species but have yet to be reported in *Salmonella* isolates. Deletion or inactivation of marRAB operon also has been linked to the reduced susceptibility to tetracycline in *Salmonella*. There are at least 32 different genes that confer resistance to tetracycline and oxytetracycline. Of these, *tet*(A), tet(B), *tet*(C), *tet(D), tet(G),* and *tet*(H) have been found in *Salmonella* isolates (Sivakumar *et al.,* 2012) The most commonly reported one of these genes is *tet(A).* It has been found in *Salmonella* genomic island 1, on integrons, and on transferable plasmids. The *tet(A)* gene has been detected in isolates of *Salmonella* serotypes. *tet(A), tet(B*) are located on transferable plasmids. These genes appear to be easily transferred and widespread among *Salmonella* isolates. They also tend to be found in isolates that display multidrug resistance, making them an important marker in identifying potentially dangerous *Salmonella* infections. tet(G) is linked to *Salmonella* Genomic Island 1 (SGI1).

2.7.5 Sulfonamides and trimethoprim

Sulfonamides and Trimethoprim prescribed separately and has been used in combination for the treatment of bacterial infection since the late 1960s. These compounds are bacteriostatic antimicrobial drugs that act by competitively inhibiting enzymes involved in the synthesis of tetrahydrofolic acid. Sulfonamides inhibit dihydropteroate synthetase (DHPS) and trimethoprim act by inhibiting dihydrofolate

reductase (DHFR). The combination of a sulfonamide and trimethoprim has been a popular form of treatment for decades, and although resistance among *Salmonella* isolates has emerged, this resistance does not appear to be common (Walsh, 2003; Alcaine *et al.,* 2007). Sulfonamide resistance in *Salmonella* isolates has been attributed to the presence of an extra sul gene, which expressed an insensitive form of DHPS. Three main sul genes have been identified: sul1, sul2, sul3. The sul1 gene has been found in a wide range of *Salmonella* serotypes. This gene is often associated with class I integrons that contain other resistance genes. These integron-borne gene cassettes have been found on transferable plasmids and as part of *Salmonella* genomic island variants. Although sometimes found in *Salmonella* isolates also harbouring *sul1*, *sul2* appears to be associated with plasmids, but not with class I integrons. Isolates of *Salmonella* serotypes Agona, Enteritidis, Typhimurium have been reported to carry *sul2*. The *sul3* gene has since been identified in *Salmonella*, and it has been associated with plasmids and class I integrons, suggesting that there may be further dissemination of this gene within *Salmonella* populations (Guerra *et al.,* 2004). Deletion or inactivation of the *mar*RAB also has been linked to reduced sulfonamide susceptibility. Similar to sulfonamide resistance, trimethoprim resistance is attributed to the expression of DHFR that does not bind trimethoprim. There are minivariants of the *dhfr* and *dfr* genes that encode this resistance, such as *dhfr*1, *dfrA1*, and *dhfr12*. These genes have been found as part of integron borne gene cassettes also associated with sul1 and sul3, on transferable plasmids carrying other resistance genes, and *Salmonella* genomic islands.

2.8 Mechanisms for Transmission of Antibiotic Resistance Genes

2.8.1 Horizontal transfer of antibiotic resistance

The spreading of resistance genes between members of different bacterial species and genera under natural conditions requires a horizontal transmission by conjugation, mobilization, transductions or to a lesser extent by transformation. For efficient horizontal gene transfer, two key factors are of importance: (1) a sufficiently high bacterial density, which enables the close contact of the partners between which genes are exchanged, and (2) the location of the transferred genes on mobile genetic elements. Such elements include plasmids, transposons, integrons and gene cassettes, and chromosomal genomic islands (Schwarz *et al.*, 2006). Studies have shown that one or more of these elements may be present in antimicrobial resistant *Salmonella* strains (Ogunleye *et al.*, 2013).

2.8.2 Plasmids

Plasmids are double-stranded extra chromosomal DNA elements of variable sizes. Due to their replication system, they can replicate independently from the chromosomal DNA in the bacterial cell. Plasmids may be present in single or multiple copies per bacterial cell. They can harbour resistance genes and also virulence genes or the genes for metabolic activities. Large plasmids may also carry the transferable gene complex, which enables the transfer of plasmid horizontally by conjugation. Plasmids can act as vectors for transposons and integrons/gene cassettes (Guerra *et al.*, 2000; Schwarz *et al.*, 2006). Plasmids are classified based on their replication origin into incompatibility (Inc) groups. Plasmids having same replication origin are “incompatible” whereas plasmids with different replication origin are “compatible” (Carattoli *et al.*, 2005).

2.8.3 Transposons

Transposons are also double-stranded DNA elements variable in size, which – in contrast to plasmids – cannot replicate autonomously. Since they lack an own replication system, they have to integrate into a replication-proficient molecule in the bacterial cell, such as the chromosomal DNA or plasmids. Large transposons have also been identified to be conjugative whereas small transposons can only change their host cell as part of a plasmid into which they have integrated. Transposons can change their location within the bacterial cell by transposition (Schwarz *et al.*, 2006). In this regard, transposons integrate into very specific sites whereas others just integrate into host DNA without any known preference (Waturangi *et al.*, 2003).

2.8.4 Integrons

Integrons are known as natural cloning and expression vectors that mediate the integration or excision of gene cassettes (Rowe-Magnus and Mazel, 2002; Mazel, 2006). Gene cassettes are small, double-stranded mobile genetic elements. A resistance gene cassette consists of a resistance gene and a recombination site, designated attC. This site can vary in size and contains the binding sites for the integrase which catalyses the integration and excision of the gene cassettes. After excision and before integration, gene cassettes form an intermediate circular form. The attC sites commonly have a central axis of symmetry and represent imperfectly inverted repeats. A gene cassette usually does not have its own promoter, but its cassette-borne gene is transcribed from a promoter located in the 5' conserved segment of the integron. Class 1 and 2 integrons commonly consist of two conserved segments (CS), 5'-CS and 3'-CS, which bracket a variable region that can contain one or more gene cassettes (Schwarz *et al.*, 2006). The essential components of an integron are the integrase gene (intI), the attachment site (attI) and the promoter,

which is essential for the expression of the integrated gene cassettes (Carattoli, 2001). Different classes of integrons have been defined based on the homology of the integrase proteins. Classes 1 and 2 are most commonly found in Gram-negative bacteria. The 5'-CS part contains the integrase gene, the attachment site and the promoter. The 3'-CS harbours the qacE Δ1 gene, a semi-functional derivative of the quaternary ammonium compounds resistance gene qacE, the sulfonamide gene sul1 and an open reading frame of unknown function named ORF5 in class 1 integrons whereas it contains transposition genes in class 2 integrons. The development of multiple resistance is based on the capacity of integrons to cluster the gene cassettes and to express antimicrobial resistance genes (Recchia and Hall, 1995; Carattoli, 2001; Schwarz *et al.,* 2006). Integrons are not mobile by themselves, but may be integrated into transposable elements, such as Tn1696 (Partridge et al., 2001), or located on plasmids, and then can spread with these elements. Moreover, integrons can also be located on chromosomal DNA or in chromosomal genomic islands, such as the *Salmonella* Genomic Island 1 (SGI1) (Boyd *et al*., 2002).

2.8.5 Genomic islands

Genomic islands are large elements found in the chromosomal DNA of bacteria. The best studied genomic islands associated with antimicrobial resistance are the 43-kb *Salmonella* genomic island 1 (SGI1) in various *Salmonella* serotypes (Boyd *et al.,* 2002), the 100-kb SXT element of *Vibrio cholerae* (Hochnut *et al.,* 2001), and SCCmec elements of different sizes and structures in *Staphylococcus aureus* (Katayama *et al.,* 2000). These genomic islands integrate site-specifically into the *S.* Typhimurium (Boyd *et al.,* 2002). In the meantime SGI1 was also identified in other *Salmonella enterica* serovars, including Agona, Albany, Newport, Meleagridis, and Paratyphi B (Boyd *et al.,* 2002; Ebner *et al.,* 2004). SG11 is horizontally transferable

and has been identified as an integrative mobilizable element (Doublet *et al.*, 2005). The 14 kb antibiotic resistance gene cluster within SGI1 (Briggs and Fratamico, 1999) consists of a complex integron related to the IN4 group of integrons. In most known cases, the penta-resistance phenotype associated with SGI1 includes ampicillin resistance via blapse-1, chloramphenicol/florfenicol resistance via floR, streptomycin/spectinomycin resistance via aadA2, sulfonamide resistance via sul1, and tetracycline resistance via tet(R)-tet(G). Variant clusters have been identified containing additional or other resistance genes such as dfrA1 and dfrA10 conferring resistance to trimethoprim, aadA7 conferring resistance to streptomycin, and aac(3)-Id conferring resistance to gentamicin (Boyd *et al.*, 2002; Doublet *et al.*, 2005). Resistant organisms (bacteria, fungi, viruses and some parasites) are able to withstand the attack by antimicrobial agents, such as antibiotics, antifungals, antivirals, and antimalarials. Hence, the standard treatments become ineffective, infections persist increasing risk of spread to others. The evolution of resistant strains is a natural phenomenon that happens when micro-organisms are exposed to antimicrobial agents, and resistant traits can be exchanged between bacterial species. Over use and misuse of antimicrobial medicines accelerates this natural phenomenon. A study on antimicrobial resistance in *Salmonella* isolates from animal sources have shown quinolone/fluoroquinolone resistance in *Salmonella* – with the detection of transferable genes coding for extended-spectrum β-lactamases, as well as the identification of the genetic basis of multi-resistance due to either SGI1 variants or multi-resistance integrons (Cloecckaert and Chaslus-Dancla, 2001). A vast majority of drug-resistant organisms have emerged as a result of genetic changes, acquired through mutation or transfer of genetic material during the life of the micro-organisms, and subsequent selection processes. Mutational resistance

develops as a result of spontaneous mutation in a locus on the microbial chromosome that controls susceptibility to a given antimicrobial. Resistance can also develop as a result of transfer of genetic material between bacteria. The method of resistance transfer varies for specific drug/bacteria combinations. Resistance depends on different mechanisms and more than one mechanism may operate for the same antimicrobial. Microorganisms resistant to a certain antimicrobial may also be resistant to other antimicrobials that share a mechanism of action. Such relationships, known as cross- resistance, exist mainly between agents that are closely related chemically (e.g. Neomycin- kanamycin (both Aminoglycoside), but may also exist between unrelated chemicals e.g. erythromycin (macrolide)-lincomycin (lincosamide). Micro-organisms may be resistant to several unrelated antimicrobials.

As a result of animal use of antibiotics, food borne microbes may become resistant to the antibiotics used to treat human diseases. When an animal is treated with an antimicrobial drug, a selective pressure is applied to all bacteria exposed to the drug. Bacteria that are sensitive to the antimicrobial are killed or put at a competitive disadvantage, while bacteria that have the ability to resist the antimicrobial have an advantage and are able to grow more rapidly than more susceptible bacteria. In addition, bacteria can become resistant when resistance genes are passed from a resistant bacterium to a sensitive one (Hussein and Khalil, 2013)

2.9 Use of Antimicrobial agents in Animal Production

Antimicrobial agents have not only been used for therapy of diseases but also have been used in food animal production for prophylaxis and as growth promoters. Even though all growth promoters with antimicrobial activity have been banned since 1999 in Europe especially for prophylactic applications – they still constitute a major selective pressure, which affects resistance development not only in *Salmonella*, but

also in many other bacteria. The selective pressure refers to the impact of antimicrobial use on a microbial population, in which resistant organisms gain a survival advantage over those susceptible ones (Furuya and Lowy, 2006). According to Clinical Laboratory Standards Institute (CLSI), resistance designates the isolates that are not inhibited by "usually achievable systemic concentration of the agent with normal dosage schedules and/or fall in the range where specific antimicrobial resistance mechanisms are likely (e.g. beta-lactamases) and clinical efficacy has not been reliable in treatment studies" (CLSI, 2006). A constant increase in the numbers of resistant strains has been observed since the 1950s.This is based on the distinctly higher selective pressure as imposed by the use of antimicrobial agents for various purposes in human and veterinary medicine, aquaculture and horticulture during the last 60 years (Helmuth, 2000). According to the bulletin of WHO in 2002, the mortality rate in outbreaks involving resistant strains of *Salmonella* spp. was found to be 3.4%, whereas it was only 0.2% in those sensitive strains (Smith and Coast, 2002).

2.9.1 Multi-drug resistance in *Salmonella* organisms

Another concerning aspect of antimicrobial resistance is the acquisition of multidrug resistance by pathogenic organisms, which makes the selection of antimicrobials more difficult in the clinical treatment of the disease. The most common *Salmonella* serotype having multidrug resistance is *S. typhimurium* definitive phage type DT104. It acquired multiple drug resistance with an isolate found to display a phenotype of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) so called penta-resistance. The zoonotic nature of DT104 may have provided the environment for the acquisition of the ACSSuT resistance phenotype since this resistance includes four of the five most common drug classes

used in veterinary medicine (Mulwey *et al*., 2006). Later, it was seen that multidrug resistance phenotype is emerging in other *Salmonella* serotypes too (Velge *et al.*, 2005; Pokharel *et al.*, 2006). The resistance mechanisms can be classified as (i) destruction or modification of the antimicrobial agent, (ii) pumping the antimicrobial agent out from the cell by efflux pumps, (iii) replacement or modification of the antibiotic target, and (iv) reduction in cell membrane permeability. Microorganisms are developing resistance mechanisms by accumulating mutations in the gene locations of target proteins or acquiring mobile genetic elements carrying resistance genes (Walsh, 2003).

2.9.2 Poultry products are important protein sources.

Birds are reared as broilers for meat and layers for eggs, under intensive or free range management. The administration of health-risk related substances such as growth promoting agents and veterinary drugs (antibiotics) is a recurring problem in animal production where these compounds are often used to increase the productivity and to reduce breeding costs (Toffolatti *et al.*, 2006). Uses of these drugs in broiler Chicken farms for therapeutic and performance-enhancing purposes may lead to deposit of residuals in their carcasses, particularly when the birds are slaughtered without the observance of withdrawal period of the drug (Donoghue and Hairston, 2000). Ignorance of observation of withdrawal period leads to a serious threat to human health upon exposure to these residues. Therefore, residues monitoring are required in detecting anabolic and veterinary drugs treatments for the safety of consumers. Antibiotics are used extensively in poultry industry for the treatment and prevention of several diseases, as well as to improve feed efficiency and promote growth (Di Corcia and Nazzari, 2002; McEvoy 2002). In addition assist in converting stress due to environmental changes, vaccination, debeaking and other

management practices. This wide spread use of antibiotic may cause residuals in foodstuffs, as well as the induction of allergic reactions in humans (Mubito *et al.*, 2014).

Oxytetracycline is a natural tetracycline compound that is derived from the fungus, *Streptomyces rhinosus*. It is a broad-spectrum antibiotic with bacteriostatic activity. It is poorly metabolized in target animals and excreted practically in its parent form, due to its high water solubility. Enrofloxacin is a second generation fluoroquinolone with bactericidal activity. After oral application, is well absorbed and distributed at tissue level, metabolized in the liver, generating its major active metabolite, ciprofloxacin (Swatantra et al., 2014).

2.9.3 Evaluation of antibiotic residues in poultry products

Two methods are commonly used in the determination of antibiotics residues in Food of animal origin and poultry products;

1. Qualitative evaluation: Microbiological inhibition assay using *Bacillus subtilis* as indicator organism. The level of antibiotics can be evaluated by measuring the diameter of inhibition zone observed on an agar layer seeded with a test organism with a vernier- callipers or ruler and
2. Quantitative evaluation: This is a chromatographic technique used for the determination of antimicrobial residues level in meat samples (Mohamed K., 2014)

The qualitative methods are generally microbiological methods like Agar gel diffusion test used for screening or monitoring the presence of veterinary drug residues in food of animal origin (Javadi *et al.*, 2009). However, these methods cannot be used to identify and quantify individual antibiotics. Other analytical

techniques that are used for the screening of veterinary drug residues include Radio Immunoassay (RIA), Enzyme Immunoassay (EIA), Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography and Paper Chromatography (Geidam *et al*., 2009). Confirmatory tests that are applied to screened samples that require further investigation are usually based on Mass Spectrometric (MS) techniques such as Liquid Chromatography (MS/LC) (Muhammed *et al.,* 2009; Jallailudeen *et al.,* 2015). The demand for increased testing with regard to food safety has led to the development of other technologies for screening of drug residues in food of animal origins. The advent of a Surface Plasmon Resonance (SPR) based biosensor is one of such technologies. This fully automated wizard-driven instrument is capable of analysis of wide range of analytes and it is supported by in-vitro diagnostic kits for the screening of several veterinary drug residues (Jallailudeen *et al.,* 2015). Solid Phase Micro- Extraction (SPME) and Micro dialysis Methods are also other analytical techniques used for drug residues analysis in edible animal tissues.

CHAPTER THREE

Materials and methods

3.1 Study Area

The study was conducted in Sokoto metropolis, Sokoto State, Nigeria.

Sokoto State

The state is located at the extreme North-West of Nigeria, between latitudes $4^{0}8^{1}$and $6^{0}54^{1}$ and latitude 12^{0}N and $13^{0}58^{1}$N and shares border with the Republic of Niger to the North, Kebbi State to the West and South-East, and Zamfara State to the East. The State covers a land area of 32,000 square kilometers and based on the 2006 census, it has a population of 3,696,999 (Sokoto Diary, 2008).

3.2 Sampling Sites

Three main chicken slaughter sites and a cold meat store in the metropolis were selected for the study;

I. Chicken slaughter site at the Sokoto Central Market

II. Chicken slaughter site at Digan- Agere Road

III. Chicken slaughter site at Western bypass Illela Road

IV. . Cold Meat store along Ahmadu Bello way

3.2.1 Sample size

The sample size was calculated using the following formula (Araoye, 2004)

$$N = \frac{z^2\, p\, q}{l^2}$$

Where N= sample size

Z= score for a given confidence interval usually set at 1.96 for 95% C.I

P= prevalence value of 50% (0.5)

q= (l-p) =1- 0.5 = 0.5

l= permissible error of estimation which is taken as 0.05 (5%)

$$N = \frac{(1.96)^2 \times 0.5 \times 0.5}{(0.05)^2} = 384.16$$

A prevalence of 50% observed in Etinan, Nigeria (Uttah *et al.*, 2013) was used for the calculation of the sample size.

This value was rounded up to 400 chicken's breast muscle samples, 100 samples were collected from each study location.

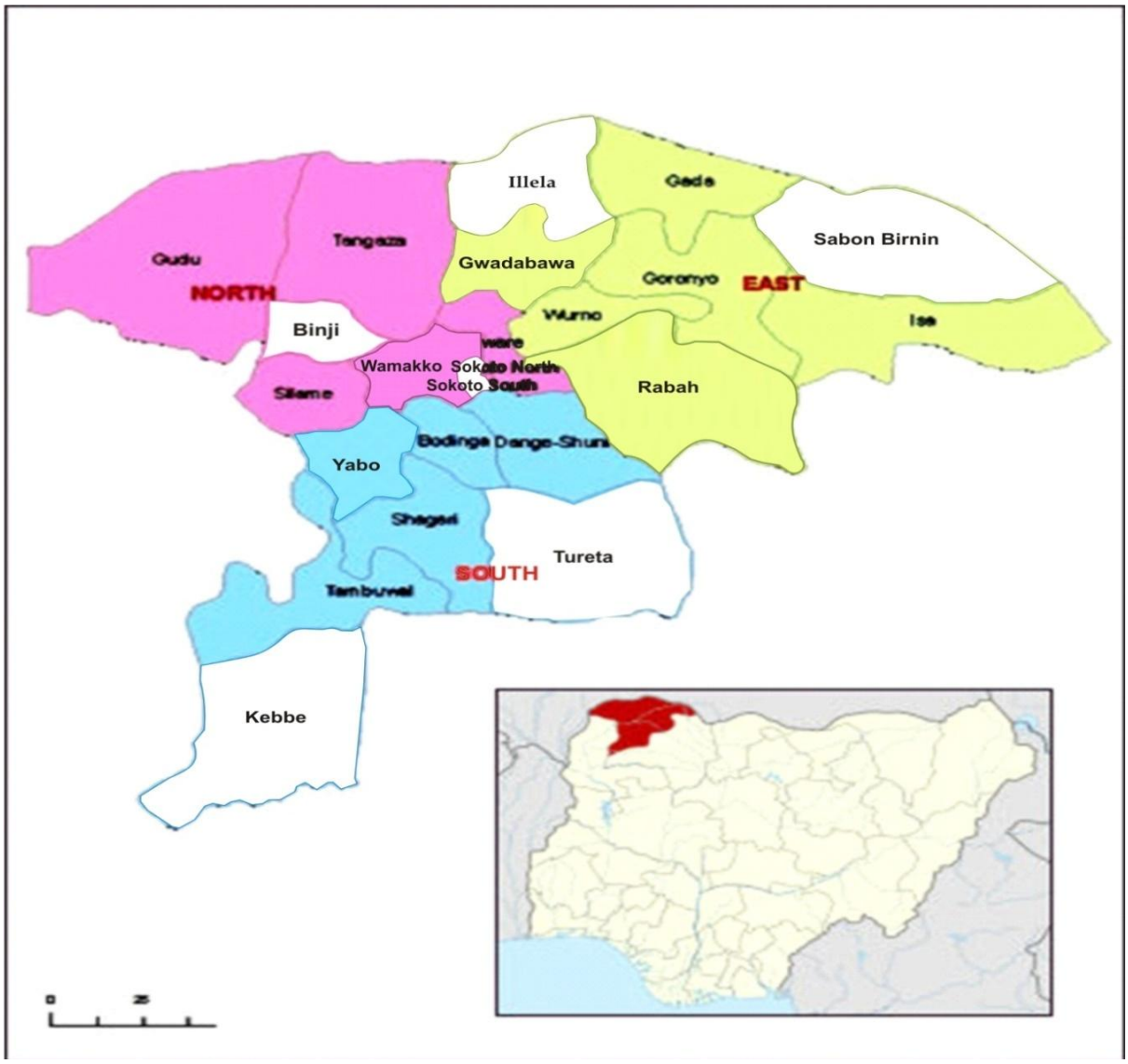


Figure I. Map showing Sokoto Metropolis and the 26 local government areas of the State

3.2.2 Chicken meat sample collection

Four hundred chicken's breast muscle samples (25g each) were randomly selected over a period of one year. Samples were collected aseptically following Standard procedures and transported in ice pack to the Laboratory of Faculty of Medical Laboratory Science, Usmanu Danfodiyo University Sokoto for preliminary storage and processing.

3.3. Isolation of *Salmonella* species from chicken meat sample

Isolation of the organism was carried out in accordance with the guide lines of the International Standard Organization (ISO 6573, 2002) manual of detection of *Salmonella* species from food and animal feeds.

3.3.1 Pre-enrichment of sample

Twenty-five gram (25g) of chicken breast muscle sample was cut into very fine pieces and pre-enriched at 37°C in 225 ml of buffered peptone water, (BPW) (Becton and Dickinson, USA) on a shaker overnight.

3.3.2 Culture

For culture, exactly 0.5 ml suspension of the pre-enriched culture was transferred to 10 ml of selenite-F broth and incubated at 37°C overnight. After overnight incubation period, one loopful of broth was sub-cultured and streaked on MacConkey's agar plate (Becton and Dickinson, U.S.A) and again incubated at 37°C for 18 hours. Non-lactose fermenting organisms that are colourless, semi-transparent or opaque colonies typical for Salmonella organisms on MacConkey agar were sub-cultured on *Salmonella-Shigella* (SS) agar (Difco) plates. The plates were then incubated at 37°C for up to 72 hours. Presumptively identified typical *Salmonella* colonies that are colourless or very light pink, opaque or semi-transparent mostly

with black centres (due to hydrogen sulphite production) on the agar plates, were sub-cultured and inoculated onto nutrient agar plates for purification prior to biochemical characterization of the isolates.

3.3.3 Biochemical characterization of the isolates

Identification of the isolated organisms was carried out as described by the International Organization of Standardization (IOS, 2002). Presumptively identified isolates were inoculated onto Triple Sugar Iron (TSI) agar, lysine iron agar (LIA), Urea agar, Simmon citrate agar slants and also inoculated into buffered glucose-peptone water for methyl-red Voges-Proskauer test, maltose, mannitol sugar utilization and motility tests were also carried out as part of biochemical tests on the isolates. Isolates that were oxidase negative, indole negative, methyl-red test positive, that shows red slant/yellow butt(alkaline/acid reaction), with or without hydrogen sulphite production on TSI slants were further characterized using Micro-gen test kit (Microbact^{TM} Gram-negative identification kit). Then, serological test to confirm the identity of the isolates as *Salmonella* organisms was carried out using polyvalent "O" and "H" serum. Microgen Salmonella latex agglutination test kit was also use as an additional support for the serological identification of the isolates.

3.3.4 Serotyping of the isolates

Biochemically identified *Salmonella* isolates were tested for somatic (O) and flagella (H) antigens with polyvalent *Salmonella* antisera (Oxoid, UK) according to Kauffmann White Scheme by slide agglutination test. For test procedure, a colony of the test organism was emulsified on clean slide with sterile saline and a drop of the appropriate serum was added and mixed properly. Presence of agglutination indicate a positive result (AppendixVI).

3.4. Evaluation for Antimicrobial Residues in the Chicken Breast Muscles Samples

For the evaluation of antimicrobial residues in the meat samples, the European Four Plate Test as described by Oboegbulem and Fedelis (1996), Karraouan *et al.,* (2009) and Farideh *et al.,* (2014) was employed for the evaluation process. This is a broad spectrum microbiological technique that involves the use of susceptible organisms for the determination of the presence of antimicrobial residues in samples. The procedure involves culturing the organism on four agar plates at different pH. Plate I at a pH of 6, plate II at a pH of 7, plates III and IV at pH of 8 respectively. The outline of the procedure is as follows;

3.4.1 Preparation of inocula and media

Pure cultures of *Bacillus cereus* and *Staphylococcus aureus* on nutrient and blood agar were used respectively. Primary cultures were sub-cultured into brain heart infusion broth for about 8 to 12 hours before inoculating onto a nutrient agar under an aseptic condition. Nutrient agar was prepared according to the manufacturer's instructions. The pH was adjusted using a pH meter (pHS-25 model) by adding drops of NaOH or HCL solutions. Exactly 16ml of the adjusted media was then introduced into universal bottles and sterilized at 121^0C for 15min. The sterilized medium was poured into Petri dishes and allowed to solidify.

3.4.2 European four plate technique for evaluation of antimicrobial residues in meat samples

For the evaluation of antimicrobial residues in the meat samples, a piece of meat sample (1x2.5mm) was applied to four plates of nutrient agar media, three of which were inoculated with *Bacillus cereus* at pH6 (Plate I), pH7 (Plate II), pH8 (Plate III) and the fourth plate (Plate IV) at pH8 was inoculated with *Staphylococcus aureus*.

The plates were incubated at 37^{o}C for 18 hours. Positive sample was indicated by a complete inhibition of growth in an annular zone of 2mm or more around the piece of meat. Less than 2mm of zone of inhibition indicate negative result. Presumptively, positive result at pH6 indicates the presence of tetracycline antibiotics, streptomycin at pH7 and penicillins at pH8. These antibiotics readily diffuse at the respective pH inhibiting bacterial growth around zone of meat. At significantly high concentration, concentrations higher than the maximum residual limit (*MRL*) the zone of inhibition is ≥ 2mm (Kabir *et al.*, 2002; Gaudi *et al.*, 2004; Ghasem *et al.*, 2015).

3.5 Antibiotic Susceptibility Testing

3.5.1 Preparation of media and bacterial inoculum

The bacterial isolates were tested for antibiotic susceptibility using Kirby-Bauer technique (Elmer, 1992). Mueller-Hinton agar medium (OXOID) was prepared in a uniform thickness (4mm) in 90mm diameter Petri dishes. An over-night broth culture of the test organism was diluted 1:100 to obtain approximately 10^{5}CFU/ml, by comparing its turbidity to a McFarland standard (0.5).

Paper disks impregnated with the following antibiotics were used; Ampicillin 10µg (Amp), Cotrimoxazole 25µg (cot), Gentamicin 10µg (gen), Nalidixic acid 30µg (nal), Chloramphenicol 30µg (chl), Tetracycline 30µg (tet), Ofloxacin 30µg (flo) and Nitrofurantoin 30µg (nit).

3.5.2 Susceptibility testing procedure

A freshly prepared Mueller-Hinton agar was inoculated with the diluted test organism using a swab and allowed to stand for 5 minutes to adsorb. The antibiotic disk was placed onto the surface of the inoculated medium and incubated at 37^{o}C for 18 hours. At the end of the incubation period, the zone of growth inhibition

surrounding the antibiotic disk was measured and interpreted using the break point of the Clinical Laboratory Standard Institute break point (CLSI, 2006).

3.6 Screening for resistance genes

Presence of known resistance genes among antibiotic resistant strains to Ampicillin, Tetracycline and Chloramphenicol was studied. For tetracycline resistant strains, *tet* (A), for ampicillin resistant strains, *bla*TEM and for chloramphenicol resistant strains *floR,* genes were sought for respectively (Table 3.1).

3.6.1 Extraction of *Salmonella* genomic DNA

Genomic DNA was extracted according to method described by Murugkar *et al*., (2003). Bacterial isolates were grown in Brain Heart Infusion (BHI; Oxoid) broth overnight at 37^{o}C and then the bacterial cells were harvested by centrifugation at 3000 rpm for one minute. The harvested cells were suspended in 3.5 ml sterile distilled water and boiled at 100^{o}C for 10 minutes. After boiling, the cell suspensions were cooled on ice and immediately tested for the presence of genes sought using PCR analysis.

3.6.2 PCR amplification of *tet* A, *bla*TEM and *flo*R genes

PCR amplification was performed using a Master-mix kit containing Taq DNA polymerase, PCR buffer, dNTPs (dATP, dTTP, dGTP) and $MgCl_2$. A volume of 2µL of the DNA template solution was added to 23 µL of the reaction mixture containing PCR buffer, 250 nmol/L primers and 1 U Taq DNA polymerase. Amplification was carried out in a thermal cycler (Biometra, Göttingen, Germany) with a temperature programme consisting of the initial denaturation (1 min at 94◦C), 40 amplification cycles (1 min 15s at 55◦C, 3min at 72^{o}C, 5min at 72◦C), and the final extension (5

min at 72◦C). A volume of 10 µL of the PCR product was analysed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. The gel electrophoresis tank was filled up to gauge mark with IX-TBE running buffer and the first well labelled M was loaded with 10µL of 100bp DNA ladder used as molecular marker. Lane 1- 8 was loaded with test samples 1-8. Voltage was set at 75v and the preparation was run for 45 minutes. The gel was observed in gel documentation unit and photographed (Protocol for agarose gel electrophoresis, Appendix VII).

Table 3.1: Primer sequence and PCR programmes for amplification of tetracycline, ampicillin and chloramphenicol resistant genes

Gene	Primer	Nucleotide sequence	amplicon size (bp)	Annealing temp.^{o}C	Reference
tetA	F	GCTACATCCTGCTTGCCTTC	210	57	Fonseca *et al.*, (2006)
	R	CATAGATCGCCGTGAAGAGG			
blaTEM	F	CATTTCCGTGTCGCCCTTAT	793	55	Randall *et al.*, (2004)
	R	TCCATAGTTGCCTGACTCCC			
floR	F	AACCCGCCCTCTGGATCAAGTCAA	548	51	Randall *et al.*, (2004)
	R	CAAATCACGGGCCACGCTGTATC			

1. 40 cycles (94^{o}C, 1m; 57^{o}C, 1m 15s; 72^{o}C, 3m; 72^{o}C, 5m)
2. 25 cycles (94^{o}C, 1m; 55^{o}C, 1m; 72^{o}C, 1m; 72^{o}C, 5m)
3. 35 cycles (94^{o}C, 1m; 51^{o}C, 1m; 72^{o}C, 2m; 72^{o}C, 7m)

CHAPTER FOUR

Results

4.1 Prevalence of *Salmonella* species Isolated in Poultry Meat in Sokoto Metropolis

Out of the four hundred (400) meat samples collected for the study, 26 samples were positive for *Salmonella* isolates, representing 6.5% prevalence (Table 4.1). Nine (2.25%) isolates were recovered from samples collected from Central Market Slaughter site, seven (1.75%) from Digan-Agere Road Slaughter site, six (1.50%) were recovered from Western-by pass road slaughter site and four (1.00%) were isolated from frozen chicken meat samples collected from a cold store (Table 4.1).

4.2 Different *Salmonella* serovars Isolated in Meat samples in the Metropolis

The frequency of *Salmonella* serovars isolated from the poultry samples collected shows that out of the 26 *Salmonella* serovars isolated, 18 (69.2%) were identified as *S.* Arizonae, 5 (19.2%) as *S.* Pullorum and 3 (11.5%) as *S.* Gallinarum (Table 4.2).

4.3 Evaluation of Antimicrobial Residues in the Meat samples

Evaluation for the presence of antimicrobial residues in the poultry meat samples revealed that 339 (84.75%) of all the meat samples collected for the study had antibiotic residues (Table 4.3).

4.4 Occurrence of Different types of Antibiotic Residues in the Meat samples

Presumptive identification of the antimicrobial agent present in the meat samples showed that 241 (60.25%) of the meat samples presumptively contain tetracycline residues, 68(20.1%) of samples from the Western by-pass sampling site had tetracycline residue similarly, 64 (18.9%) samples from Central Market sampling site were observed to have tetracycline antibiotic residues, 56(16.5 %) samples from the

Cold Store sampling site and 53(15.6%) samples collected from Digan-Agere sampling site were also observed to have tetracycline antibiotic residues respectively (Table 4.4). Streptomycin antibiotic residue was observed in 93 (27.4%) of all the samples examined in the study. A 10% (34/339) streptomycin residue was observed in samples collected from Digan-agere sampling site, which was the highest streptomycin residue that was observed in the Metropolis. This was followed by samples collected from Central market 7.7% (26/339) and samples from the Western by-pass were observed to have the least (6.5%) streptomycin residue content. Only 1.5% (5/339) of the samples examined in the study area had penicillin antibiotic residue content (Table 4.4).

4.5 Antibiotic Susceptibility Profile of the *Salmonella* serovars Isolated

Antibiotic susceptibility profile of the isolates revealed 96.2% susceptibility to nitrofurantoin, 92.3% to nalidixic acid and 73.1% susceptibility to ofloxacin and augumentin respectively. A resistance profile of the isolates showed 73.1% resistance to tetracycline, 65% to ampicillin and a 61.5% resistance to chloramphenicol (Table 4.5).

4.6 Antibiotic Susceptibility Profile of the *Salmonella* Arizonae isolates

Salmonella Arizonae isolates in the study showed 94.4% susceptibility to nitrofurantoin and nalidixic acid respectively, 77.8% susceptibility to ofloxacin and 72.2% to augumentin respectively and 66.7% susceptibility to cotrimoxazole. However, resistance profile revealed the isolates to be resistant to; tetracycline (88.9%), ampicillin (72.7%) and to chloramphenicol (66.6%) (Table 4.6).

Table 4.1: Prevalence of *Salmonella* species isolates from Chicken meat in Sokoto metropolis based on sites

Sampling site	Number Examined	Number Positive	Prevalence (%) n=400
Central Market	100	9	2.25
Digan-Agere Road	100	7	1.75
Western by-pass Illela Road	100	6	1.50
Cold Store Bello way	100	4	1.00
Total	400	26	6.50

$\chi^2 = 2.14$

df = 3

p = 0.544

Table 4.2: Distribution of *Salmonella* serovars Isolates from Chicken meat in Sokoto Metropolis based on sites

Sampling site	*Salmonella* serovars		
	S. Arizonae	*S.* Gallinarum	*S* .Pullorum
Central market	6	2	1
Digan-agere road	5	0	2
Western by-pass I	4	1	1
Cold store Bello way	3	0	1
Total (%) n=26	18 (69.2%)	3(11.5%)	5 (19.2%)

$\chi^2 = 3.05$

df = 6

p = 0.802

Table 4.3: Antibiotic residues in Chicken Meat samples in Sokoto Metropolis based on sites

Sampling site	Number Examined	Number positive	percent positive n- 400
Central market	100	93	23.25
Digan-agere road	100	89	22.25
Western by-pass I	100	90	22.50
Cold store Bello way	100	67	16.75
Total	400	339	84.75

$\chi^2 = 33.3$

df = 3

p = 0.000

Table 4.4: Occurrence Different types of Antibiotic Residues in Chicken breast Muscle in Sokoto Metropolis based on sites

Sampling sites	Plate 1	Plate II	Plate III	Plate IV
Central market	64(18.87)	26(7.67)	3(0.88)	0(0)
Digan-agere road	53(15.63)	34(10.0)	2(0.59)	0(0)
Western by-pass	68(20.06)	22(6.49)	0(0)	0(0)
Cold store	56(16.52)	11(3.24)	0(0)	0(0)
Total (%) n=339	241 (71.1)	93 (27.4)	5 (1.5)	0 (0.0)

$\chi^2 = 15.1$

df = 6

p = 0.020

4.7 Antibiotic Susceptibility Profile of the *Salmonella* Gallinarum isolates

Susceptibility profile of the *Salmonella* Gallinarum isolates in the study revealed a 100% susceptibility to nitrofurantoin and augumentin respectively. However, 50% of the isolates showed resistance to cotrimoxazole, ampicillin and tetracycline respectively (Table 4.7).

4.8 Antibiotic Susceptibility Profile of the *Salmonella* Pullorum isolates

Antibiotic susceptibility profile of the *Salmonella* Pullorum isolated in the study showed a 100% susceptibility to nitrofurantoin, nalidixic acid respectively and a 66.7% susceptibility to ofloxacin. A 50% resistance profile was shown to ampicillin and chloramphenicol respectively (Table 4.8).

4.9 Multi-drug Resistance Profile of the *Salmonella* Isolates in the study area

Antibiotic resistance profile of the isolates, showed 61.5% of the isolates showed resistance to only one type of antibiotic agent, a multi-drug resistance profile revealed that 19.2% of the isolates showed resistance to a combination of two different antibiotic agents, 7.7% showed resistance pattern to a combination of three different antibiotic agents, only 1 (3.9%) isolate showed resistance pattern to a combination of four different antibiotics and none of the isolates shows resistance to a combination of more than four different antibiotics (Table 4.9).

4.9.1 Multiple-antibiotic-resistance index (MARI)

Multiple-antibiotic-resistance index (MARI) was calculated for the tested isolates by dividing the number of antibiotics the isolate shows resistance against the number of antibiotics tested. In our study, a multiple-antibiotic- resistance index of 0.25 was observed in 19.2% (5/8) of the isolates, 0.38 MARI in 7.7% (2/8) of the isolates and 0.5 MARI in 3.9% (1/8) of the isolates in the study area (Table 4.9).

4.10 Antibiotic Resistance Gene Studies

Ampicillin resistance gene (*bla*TEM), tetracycline resistance gene (*Tet*A) and Chloramphenicol resistance gene (*flo*R) were all detected in the tested *Salmonella* isolates (Plate V).

Ampicillin resistance gene (*bla*TEM) and tetracycline resistance gene (*Tet*A) were detected in 6 (75%) of the isolates respectively. Presence of multiple resistance genes was also observed in this study. In 4 (50%) isolates, both Ampicillin *(bla*TEM) and tetracycline *(Tet*A) resistance genes were detected. Ampicillin *(bla*TEM) and Chloramphenicol *(flo*R) resistance genes were observed in 3 (37.5%) of the isolates. Two (25%) isolates were observed to harbour both Chloramphenicol (*flo*R) and tetracycline (*Tet*A) resistance genes. Presence of all the three resistance genes (*blaTEM, Tet*A *and flo*R) was observed in 12% of the tested isolates (Table 4.10).

Table 4.5: Antibiotic susceptibility profiles of the *Salmonella* serovars isolated from the Chicken meat samples collected for the study

Antimicrobial agent (disc potency)	Susceptibility profile (n=26)		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Cotrimoxazole (25µg)	16 (61.5)	3 (11.5)	7 (27.0)
Ampicillin (10µg)	8 (30.8)	1 (3.8)	17 (65.4)
Nitrofurantoin (30µg)	25 (96.2)	0 (0)	1 (3.8)
Nalidixic acid (30µg)	24 (92.3)	2 (7.7)	0 (0.0)
Oflxacin (30µg)	19 (73.1)	6 (23.1)	1 (3.8)
Augmentin (10µg)	19 (73.1)	7 (26.9)	0 (0.0)
Chloramphenicol (30µg)	9 (34.6)	1 (3.8)	16 (61.5)
Tetracycline (30µg)	6 (23.1)	1(3.8)	19 (73.1)

Key: Susceptible: Diameter of zone of growth inhibition ≥ 21 mm

Intermediate susceptibility: Diameter of zone of growth inhibition 17- 20mm

Resistant: Diameter of zone of growth inhibition ≤ 16mm

Table 4.6: Antibiotic susceptibility profile of *Salmonella* Arizonae isolated from Chicken meat samples in Sokoto metropolis

Antimicrobial agents (disc potency)	Susceptibility profile (n=18)		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Cotrimoxazole (25µg)	12 (66.7)	2 (11.1)	4 (22.2)
Ampicillin (10µg)	4 (22.2)	1 (5.6)	13 (72.2)
Nitrofurantoin (30µg)	17 (94.4)	0 (0.0)	1 (5.6)
Nalidixic acid (30µg)	17 (94.4)	1 (5.6)	0 (0.0)
Oflxacin (30µg)	14 (77.8)	3 (16.7)	1 (5.6)
Augmentin (10µg)	13 (72.2.)	5 (27.8)	0 (0.0)
Chloramphenicol (30µg)	6 (33.3)	0 (0.0)	12 (66.6)
Tetracycline (30µg)	2 (11.1)	0 (0.0)	16 (88.9)

Table 4.7: Antibiotic susceptibility profile of *Salmonella* Gallinarum isolated from the Chicken meat samples in Sokoto Metropolis

Antimicrobial agent (disc potency)	Susceptibility profile n=2		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Cotrimoxazole (25µg)	1 (50.0)	0 (0)	1 (50.0)
Ampicillin (10µg)	1 (50.0)	0 (0)	1 (50.0)
Nitrofurantoin (30µg)	2 (100)	0 (0)	0 (0)
Nalidixic acid (30µg)	1 (50.0)	1 (50.0)	0 (0)
Oflxacin (30µg)	1 (50.0)	1 (50.0)	0 (0)
Augmentin (10µg)	2 (100)	0 (0)	0 (0)
Chloramphenicol (30µg)	0 (0)	1 (50.0)	1 (50.0)
Tetracycline (30µg)	1 (50.0)	0 (0)	1 (50.0)

Table 4.8: Antibiotic susceptibility profile of *Salmonella* Pullorum isolated from Chicken meat samples collected in Sokoto metropolis

Antimicrobial agent (disc potency)	Susceptibility profile n=6		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Cotrimoxazole (25µg)	3 (50.0)	1 (16.7)	2 (33.3)
Ampicillin (10µg)	3 (50.0)	0 (0)	3 (50.0)
Nitrofurantoin (30µg)	6 (100)	0 (0)	0 (0)
Nalidixic acid (30µg)	6 (100)	0 (0)	0 (0)
Ofolxacin (30µg)	4 (66.7)	2 (33.3)	0 (0)
Augmentin (10µg)	3 (50.0)	3 (50.0)	0 (0)
Chloramphenicol (30µg)	3 (50.0)	0 (0)	3(50.0)
Tetracycline (30µg)	3 (50.0)	1(16.7)	2 (33.3)

Table 4.9: Multi-drug Resistance Profile of the *Salmonella* Organisms Isolated from Chicken Meat in Sokoto Metropolis

Number of antibiotic the Isolates were resistant to	MARI	Number of resistant isolates (%) n=26	Resistance patterns
1	0.125	16 (61.5)	amp., tet., chl., nit.,
2	0.25	5 (19.2)	amp-tet, chl-tet, amp-chl, amp-aug, tet-aug
3	0.375	2 (7.7)	amp-tet-chl, tet-amp-aug
4	0.50	1 (3.9)	amp- chl- aug- tet
≥5	0	0	

Key: MARI- Multiple –Antibiotic Resistance Index

Amp- Ampicillin

Tet- Tetracycline

Chl- Chloramphenicol

Aug- Augumentin

Nit- Nitrofurantoin

Ofl- Ofloxacin

Nal- Nalidixic acid

Cot- Cotrimoxazole

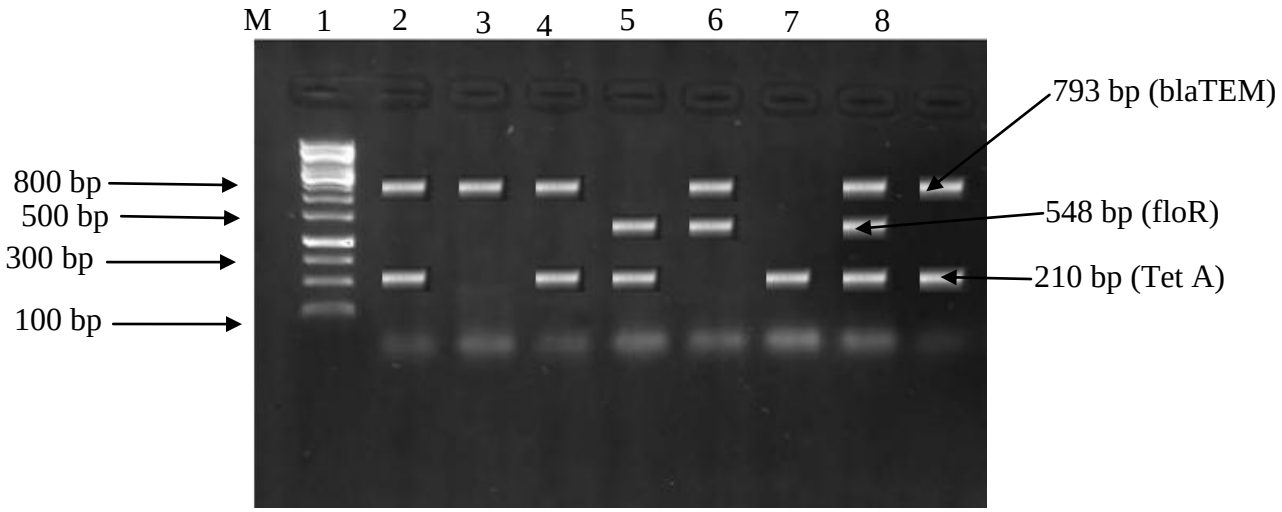


Plate I: Agarose gel electrophoresis of *bla*TEM, *flo*R and *tet*A genes after multiple-flex PCR test

M= Molecular maker/ ladder

Lanes 1- 8 are the samples loaded with lines 1, 2, 3, 5, 7 and 8 showing amlicons for the blaTEM gene

Lanes 4, 5 and 7 showing amplicons for the *flo*R gene

Lanes 1, 3, 4, 6, 7 and 8 showing amplicons for *tet* A gene

65

Table 4.10: Antibiotic resistance gene profile of *Salmonella* species isolated from Chicken meat samples in Sokoto metropolis

Antimicrobial agent	Gene tested	Number of resistant Isolates n=8 (%)
Amp	*bla*TEM	6 (75.0)
Tet	*tet*A	6 (75.0)
Chl	*flo*R	3 (37.5)
Amp, Tet	*bla*TEM, *tet*A	4 (50.0)
Amp, Chl	*bla*TEM, *flo*R	2 (25.0)
Tet, Chl	*tet*A, *flo*R	2 (25.0)
Amp, Tet, Chl	*bla*TEM, *tet*A, *flo*R	1 (12.5)

Key

Amp- ampicillin

Tet- tetracycline

Chl- chloramphenicol

Table 4.11: Antimicrobial resistance gene profile among the multi-drug resistant *Salmonella species* from Chicken meat in Sokoto metropolis

No. of resistant genes detected	Number of resistant isolates (%)	Gene combination
1	1(12.5)	*blaT*EM
	1(12.5)	*tet*A
2	3(37.5)	*bla*TEM-*tet*A,
	1(12.5)	*flo*R-*tet*A,
	1(12.5)	*bla*TEM-f*lo*R
3	1(12.5)	*bla*TEM-*flo*R-*tet*A

Table 4.12: Occurrence of Multi- resistance genes among the antibiotic-resistant isolates

Antimicrobial agents isolates	Terget gene	No, of resistant n=8 (%)
Ampicillin- Tetracycline	*bla*$_{TEM}$ - *tet*A	4 (50.0)
Ampicillin-Chloramphenicol	*bla*$_{TEM}$ – *flo*R	2(25.0)
Tetracycline-Chloranphenicol	*tet*A-*flo*R	2(25.0)
All the three antibiotics	*bla*$_{TEM}$–*tet*A- *flo*R	1(12.5)

CHAPTER FIVE

Discussion

A prevalence of 6.5% of *Salmonella* was observed in this study area, this is higher than the 4.0% prevalence reported by Al-Nakhi *et al.,* (1999) in Riyadh and by Medeiros *et al.,* (2011) who recorded a prevalence rate of 2.7% in Brazil. However the prevalence is slightly lower than 9% prevalence reported by Muhammed *et al.,* (2010) and 10.9% by Agada *et al.,* (2014) both in Jos Plateau state, Nigeria. Differing *Salmonella* prevalence rates are not uncommon with differing regions, areas and sanitary conditions. The slightly higher prevalence rate observed in this study compared to observations made in Brazil and Riyadh might be a reflection of the differences in the sanitary conditions associated with the different countries in which the researches were conducted. It might also be attributed to lack of knowledge on the transmission of *Salmonella* infection and improper orientation on bio-security measures among the meat handlers in the different study areas. The slightly lower prevalence rate observed in this study compared to the work of Agada *et al.* (2014) in Jos might be as result of the differences in the sources of samples. While Agada *et al*. worked on chicken droppings as the source of sample, in this study raw meat from freshly dressed chicken was the source of our sample. The scalding process during dressing of the chicken might reduce or kill most of the bacterial organisms and thus lowering the rate of isolation.

Salmonella Gallinarum and *Salmonella* Pullorum have been reported in poultry, animals and food products. These serovars are known to pose zoonotic risk to man and they are known to cause fowl typhoid and pullorum disease in birds thus incurring heavy economic losses to the poultry industries. In this study low prevalence rate of *S.* Gallinarum (11.5%) and *S.* Pullorum (23.1%) was observed

compared to the report of Mbuko *et al* (18.4%) and Agada *et al., (2014)* who reported a prevalence rate of 23.3% for *S.* Gallinarum from poultry droppings. The slightly lower prevalence rate observed for *S.*Gallinarum in this study might be as a result of the differences in the source of samples from which the isolation was made.

Chicken meat has high quality proteins and low fat, along with important vitamins and minerals (Givens, 2005). An increased demand for chicken meat has force poultry industry to increase the use of drugs such as antibiotics for growth and promote weight gain in chickens (Payad and Mahmoudi, 2008). It is known that drugs residues on animal product especially meat is an important problem in most countries in world (Swatantra *et al.,* 2014). Potentially allergic reactions and direct toxicity of some antibiotics may have fatal consequences (Lee *et al.,* 2001). Differing ranges of antibiotic residues in Chicken meat from 2.5% - 90% have been observed by many workers (Ghasemi, *et al.,* 2014).

In our study antimicrobial residue contamination was observe in 84.75% of all the Chicken meat samples examined. This is closely in line with the findings of Al-Ghamdi *et al.* (2000) who observed 87% raw poultry meat contamination with antibiotic residues in a research conducted in 33 poultry farms in Saudi Arabia. However, our result was slightly lower than what was observed by David *et al*. (2015) who detected 66% antibiotic residue contamination level of poultry meat in their research. It is known that drugs residues on animal product especially meat is an important problem in most countries in the world. Existence of antibiotic residues in food stuff can pose hazards to human health. Among them are sensitivity to antibiotics, allergic reactions, imbalances of microflora, bacterial resistance to antibiotics in microorganisms and losses to the food industry (Swantantra, *et al.,* 2014). Antimicrobial resistance in *Salmonella* organisms is a cause of serious

concern in both human and veterinary practice. The drugs of choice for the treatment of salmonellosis are usually ampicillin, amoxicillin/clavunate cephalosporins or fluoroquinolones, tetracyclines, but the increasing emergence of resistance to these drugs limits their therapeutic use (Toro *et al.*, 2011). In our study antibiotic susceptibility profile of the isolates showed 96.2% susceptibility to nitrofurantoin acid, 92.3% susceptibility to nalidixic acid, 73.1% susceptibility to augumentin and ofloxacin respectively. However, 73.1% of the isolates showed resistance to tetracycline. Varying tetracycline resistance profile among *Salmonella* species has been observed by many workers (Gordana, *et. al.*, 2012). Tetracycline resistance among *Salmonella* organisms seemed to vary according to the animal source and the geographical origin of the isolates (Frech and Schwarz, 2000). The result observed in this study is slightly lower to what was obtained by order researchers. Frech and Schwarz reported 92% tetracycline resistance in poultry. Tetracycline drugs are by far the most frequently used antimicrobial agents in veterinary medicine, β-lactam antibiotics and aminoglycosides rank in third and fourth place. Resistance to aminoglycosides and β-lactams was frequently encountered among the tetracycline-resistant isolates (Frech and Schwarz, 2000, Toro *et al.*, 2011). Sixty-five point four percent (65.4%) of the isolates in this study were resistant to ampicillin the result is slightly lower than what was reported by Agada *et al*., (2014). They reported a resistance of 96.0% to ampicillin. However, a slightly higher (61.5%) chloramphenicol resistance was observed among the *Salmonella* isolates in this study compared to 42.9% reported by Agada *et al*. (Agada, *et al.*, 2014). The observe increase in resistance to chloramphenicol in our study might be as a result of the continued miss-use and over-use of the drug by the public. Furthermore, wide spread use of antibiotics in medical, veterinary and agricultural practices have resulted in

resistance to large spectrum of antibiotics leading to the proliferation of antibiotic resistance genes in the horizontal gene pool (Meervenne, *et al.,* 2012, Mubito *et al*., 2014). The World wide increase in the use of antibiotics as an integral part of the poultry and livestock production industry has lead to the problem of the development of antibiotic resistant bacterial strains. Scientific evidence has shown that resistance to antibiotics is not only due to the natural ability of a tiny fraction of the bacteria with un-usual trait to survive antibiotic attack, enabling resistant strains to multiply but also stems from the transmissibility of acquired resistance to their progeny and across to other unrelated bacterial species through extra-chromosomal DNA. The circumstances of occurrence and spread of antibacterial drug resistance is complex, however, genetic analysis has indicated that the source of resistance is frequently a transferable plasmid (Lin, *et al.,* 2004; Abdullahi *et al.,* 2015).In the study, genotypic identification of *bla*TEM, floR and *tet*A genes were made among the 8 multi-drug resistant isolates. It was observed that among the multi-drug resistant isolates 3 (37.5%) of the isolates were found to harbor both *bla*TEM and *tet*A genes together, 2 (25%) of the isolates were found to harbour either *bla*TEM or *tet*A genes responsible for ampicillin and tetracycline respectively. One (12.5%) isolate was found to harbour all the three resistance genes tested (*bla*TEM, *tet*A and *flo*R genes). This finding is in line with the findings of Huque *et al*., (2001); Asma *et al.,* (2005) and Abdullahi *et al*., (2015), whose findings reported the presence of multiple resistance genes in *Salmonella* isolates. Application of antibiotics in poultry production brings about an increase in resistance to antibiotics not only in pathogenic bacterial strains but also in commensal bacteria. In this respect, gastrointestinal commensal bacteria constitute a reservoir of resistance genes for pathogenic bacteria. Poultry and meat are common reservoirs of emerging antibiotic resistance available to bacteria

inhabiting humans. It can be supposed that the transmission of antibiotic resistant bacteria to people who got in contact with these sources through direct ingestion or handling results in an increase in the human reservoir of these strains which can rapidly spread to the community. The emergence and spread of resistant bacterial strains like *Salmonella* from poultry products to consumers put humans at risk to new strains of bacteria that resist antibiotic treatment. Some of the significant implications of introducing antibiotic resistant bacteria to humans include increasing cost of treatment and suffering to individuals, families and the entire community. The emerging resistant bacterial strains will adversely affect the efficacy of antibiotic chemotherapy and will furthermore encourage the need for more expensive and toxic medication. The strategy to slow down the development of antibiotic resistant pathogens is to regulate antibiotic usage and also put in place resistance surveillance programs in the country.

CHAPTER SIX

Conclusion and Recommendations

6.1 Conclusion

Antibiotics have been used widely in poultry to treat, prevent infections and also in low concentration in feeds to promote growth. Such practice has improved poultry performance effectively and economically but has resulted in the increase in the spread of resistant bacterial strains. In our study we observed a prevalence of 6.5% and 84.8% of the meat samples collected for the study were contaminated with antimicrobial residues. Antibiotic susceptibility profile of the isolates revealed 96.2% susceptibility to nitrofurantoin, 92.3% to nalidic acid and 73.1% susceptibility to ofloxaci and augmentin respectively. However resistance profile of the isolates showed 73.1% resistance to tetracycline, 65% resistance to ampicillin and 61.5% resistance to chloramphenicol. Resistance gene analysis of the resistant isolates shows that 75% of the isolates harbored ampicillin resitance gene (*bla*TEM) or tetracycline resistance gene (*tet*A) respectively. Presence of multiple resistance genes was also observed in this study. In 50% f the isolates, a combination of both ampicillin (*bla*TEM) and tetracycline (*tet*A) or chloramphenicol (*flo*R) resistance genes were detected. One (12.5%) isolate was observed to harbor all the three (*bla*TEM, *tet*A and *flo*R) resistance genes tested in the study. There is therefore the need for more rational use of antibtiotics in animal production and more prudent use in humans. It is important to put in place antibiotic resistance surveillance programs in the country and improve on its capacity world-wide with a view to monitoring the emerging resistance genes and their transfer in both animal and human strains. In addition alternative to antibiotic growth promoters in poultry practice should be explored.

6.2 Recommendations

1. There should be an improvement in the hygienic practice of handling raw animal products and adequate heat treatment should be use in cooking meat to eliminate the possibility of antibiotic resistant bacteria being consume or spread in the community
2. There should be an increase interest by both the public and Government in phasing out inappropriate antibiotic usage in animal husbandry
3. Action should be focused on putting in place antibiotic resistance surveillance programs in the country
4. It is also important to monitor prevalence of *Salmonella* and bacterial resistance to antibiotics not only in humans but also in animals in order to detect transfer of resistant bacteria or resistance genes from animal to human and vice-versa
5. Alternative approach to sub-therapeutic antibiotics in poultry, especially the use of probiotic organisms that can positively influence poultry health and produce safe edible products should be encouraged in poultry practice.

6.3 Contribution to knowledge

1. A 6.5% *Salmonella* prevalence on chicken meat was observed
2. 84.8% of the meat samples collected in the study area were found to contain antibiotic residues.
3. In our study, we observed that 73.1% the isolates were resistant to tetracycline, 65.4% were resistant to ampicillin and 61.5% were resistant to chloramphenicol
4. Ampicillin resistance gene (*bla*TEM) and tetracycline resistance gene (*tet*A) were detected in 75% of the isolates. While in 37.5% of the isolates chloramphenicol resistance gene (*flo*R) was detected in the *Salmonella* strains isolated in the study area.
5. One of the isolates was found to harbor all the three resistance genes (*bla*TEM, *tet*A and *flo*R).

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APPENDICES

Appendix 1: A sample of the Microgen test kit that was used for the identification of the *Salmonella* organisms



Un-inoculated Micogen test kit

Appendix II

Microgen test kits inoculated and incubated at 37^{o}C for 18 hours



1



2

Inoculated Microgen kits (1 and 2) after incubation at 37^{o}C for 18 hours

Appendix III

Biochemical characterizations of Salmonellae isolated from chicken meat samples collected in Sokoto metropolis based on the sampling sites

Sample number	Biochemical identification															serovar
	Oxi	mot	urea	lys	orn	H_2s	glu	man	xlo	ONPG	Ind	Vp	Cit	TDA		
CM8	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CM15	-	-	-	+	+	+	+	+	-	+	-	-	-	-		*S.* Gallinarum
CM52	-	-	-	+	+	+	+	+	+	-	-	-	-	-		*S.* Pullorum
CM53	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CM63	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CM76	-	-	-	+	-	+	+	+	-	+	-	-	-	-		*S.* Gallinarum
CM82	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CM87	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CM95	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
DA40	-	-	-	+	+	+	+	+	+	-	-	-	-	-		*S.* Pullorum
DA61	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
DA73	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
DA79	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
DA81	-	-	-	+	+	+	+	+	+	-	-	-	-	-		*S.* Pullorum
DA86	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
DA91	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
WP3	-	-	-	+	+	+	+	+	+	-	-	-	-	-		*S.* Pullorum
WP5	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
WP18	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
WP39	-	-	-	+	-	+	+	+	-	+	-	-	-	-		*S.* Gallinarum
WP75	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
WP88	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CS20	-	-	-	+	+	+	+	+	+	-	-	-	-	-		*S.* Pullorum
CS50	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae
CS79	-	+	-	+	+	+	+	+	+	+	-	-	+	-		*S.* Arizonae

Appendix IV



Plate II: *Bacillus cereus* and *Staphylococcus aureus* culture plates for the Evaluation of Antibiotic residues in the Meat samples

Appendix V

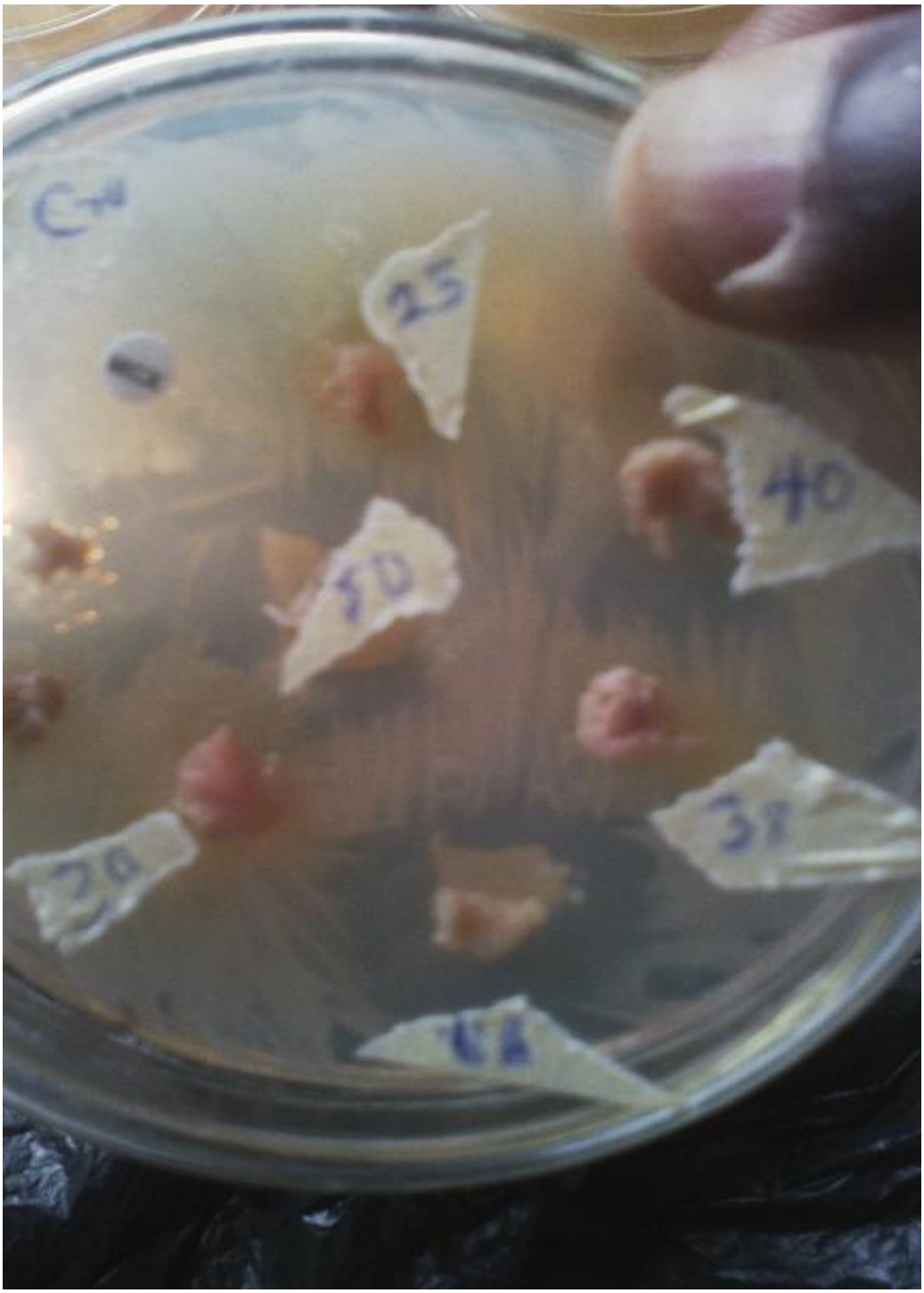


Plate III: A *Bacillus cereus* culture plate showing zone of growth inhibition surrounding Meat sample number 68 (indicating presence of antibiotic residue in the Meat sample)

Appendix VI

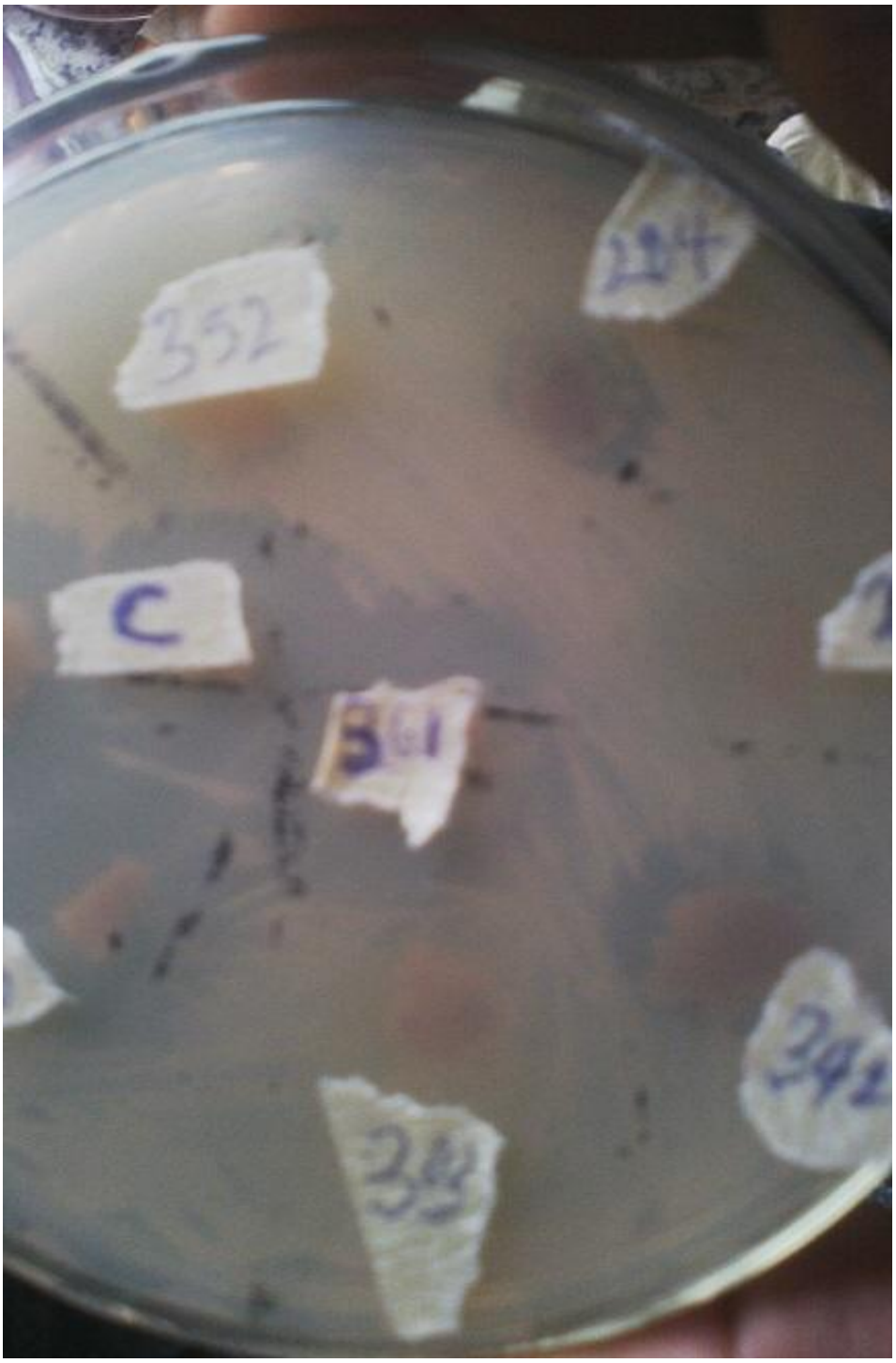


Plate IV: A *Staphylococcus aureus* culture plate showing no zone of growth inhibition surrounding Meat sample number 38 (indicating absence of antibiotic residues on this Meat sample)

Appendix VII



Plate V: A *Bacillus cereus* culture plate showing zone of growth inhibition surrounding most the Meat samples (indicating the presence of antibiotic residues in most of these samples)

APPENDIX VIII

Antibiotic susceptibility test plates

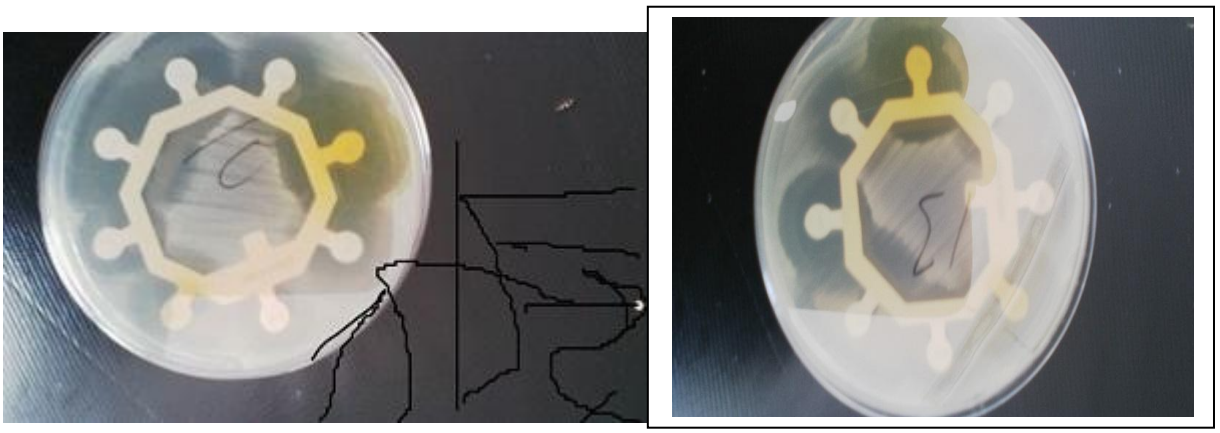




Plate: VI Susceptibility test plates showing zone of growth inhibition

Appendix IX

PREPARATION OF A 0.5 MCFARLAND STANDARD

Reagents

1% (wt/vol) anhydrous Barium Chloride ($BaCl_2$)

1% (vol/vol) cold pure sulfuric acid (H_2SO_4)

To prepare a 0.5 McFarland Standard:

0.5 mL of 1.0% $BaCl_2$ was added to 99.5 mL of 1% H_2SO_4 solution and Stirred to maintain a suspension. The mixture was thoroughly mixed and 0.5 ml was distributed into screw-top tubes. The diameters of these tubes were the same as those used for adjusting the density of culture suspensions prior to inoculation. When these standards are thoroughly shaken the turbidity equals that of a culture containing about 1.5×10^8 cells / ml. The tubes containing the 0.5 McFarland Standard were stored in a dark at room temperature.

Appendix x

Microgen Salmonella latex slide agglutination test procedure

Test kit presentation

Reagent- *Salmonella* latex reagent, 2.5ml (blue cap) M40a, latex particles coated with rabbit antiserum against *Salmonella* antigens. Preserved with 0.099% sodium azide

Control (pos.) - positive control 0.5ml (black cap) M40b, inactivated preparation of *Salmonella* antigens preserved with 0.099% sodium-azide

Isotonic saline- 0.88% sodium chloride 5ml (white cap), M40

Test procedure

1. A drop of saline (M40) was added onto a clean slide
2. Using a sterile wireloop a colony of the test organism was transferred and emulsified in the drop of saline to produce a heavy smooth suspension
3. Then a drop of Microgen *Salmonella* latex reagent (M42a), was added and mixed gently
4. The slide was rocked for up to 2 minutes and checked for agglutination

Interpretation of the test

Agglutination within 2 minutes is a positive result and indicates the presence of *Salmonella* in the sample.

Appendix XI

Protocol for agarose gel electrophoresis

1. 2 grams of agarose powder was weighted and put into a 100ml conical flask
2. 100ml of IX-TBE was added mixed and dissolved in a microwave oven
3. The mixture was cooled to 45^{o}C
4. Gel casting was assembled and the combs were positioned
5. 5µL of ethidium bromide was added to the agarose and swirled gently
6. The mixture was carefully poured into the casting try and allowed to solidify
7. The combs were carefully removed from the casting try
8. The casting try was transferred into the gel electrophoresis tank
9. The tank was filled up to the gauge mark with IX-TBE running buffer
10. The first well labeled M was loaded with 5 µL of 100bp DNA ladder
11. Lane 1- 8 were loaded with samples 1- 8 respectively
12. Voltage was set 75v
13. The preparation was run for 45 minutes
14. The gel was then observed in gel documentation unit and photographed