OCCURRENCE OF *SALMONELLA SPP* IN CHICKENS AND *ESCHERICHIA COLI* IN TABLE EGGS IN KADUNA AND ZARIA, NIGERIA

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES AHMADU BELLO UNIVERSITY INPARTIAL FULFILLMENTS OF THE REQUIRMENTS FOR THE AWARD OF MASTER OF SCIENCE

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DECEMBER, 2010

DECLARATION

I declare that the work in the thesis entitled **Occurrence of *Salmonella spp* in Chickens and *Escherichia coli* in Table eggs in Zaria and Kaduna** has been performed by me in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Prof. J. K. P. Kwaga, Dr. J. Kabir and Prof. V. J. Umoh.

The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

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Name of student Signature Date

CERTIFICATION

This thesis entitled '**OCCURRENCE OF *SALMONELLA SPP* IN CHICKENS AND *ESCHERICHIA COLI* IN TABLE EGGS IN ZARIA AND KADUNA'** by **Akpabio Uduak** meets the regulations governing the award of the degree of Master of Science in Veterinary Public Health and Preventive Medicine of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents, David and Eno Akpabio, who have always encouraged me.

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ABSTRACT

This study assessed the biosecurity and other preventive measures carried out on poultry farms. Deep litter system of management was commonly practiced and there was report of multiple antibiotics usage in feed. There was low level of biosecurity in most of the farms surveyed. A total of 3820 pooled egg, 114 cloacal and 140 ovarian follicle samples were tested for *Salmonella*. *Salmonella* was not isolated in any of the samples. Other organisms were isolated such as *Serratia plymuthica, Enterobacter agglomerans, and Serratia liquefaciens*. Also 100 pooled egg samples were analyzed for total aerobic counts, total coliform counts and *Escherichia coli.* Of the 100 pools, 3 were positive for *E. coli* for which antimicrobial susceptibility patterns were tested using a panel of 12 antimicrobial agents. There was no significant difference ($P > 0.05$) in the mean total aerobic counts and coliform counts. There was a negative correlation of the total aerobic and coliform counts ($r = -0.028$), total aerobic counts and antibiotics usage ($r = -0.05472$) as well as correlation of coliform counts and antibiotics use ($r = -0.187$). The bacterial isolates were resistant to multiple antibiotics.

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INTRODUCTION

Chapter 1

.1 Background Information

Salmonella is one of the major bacterial organism that cause foodborne infections in humans worldwide (Acheson and Homann, 2001; Humphrey, 2000). Poutry and poultry products that are contaminated with these organisms have been implicated as a major source of human salmonellosis (Humphrey, 2000; Bailey *et al*., 2001). Poultry that are infected with *Salmonella* but show no clinical illness maybe important in the spread of infection between flocks and as a source of food poisoning in humans (Cogan and Humphrey, 2003; Carli *et al*., 2001). *Salmonella* Typhumurium and *Salmonella* Enteritidis are the commonest cause of non- typhoidal salmonellosis in humans (Acheson and Homann, 2001; Guard- Petter, 2001). The global spread of *Salmonella enterica* serovar Enteritidis in chickens (Ward *et al*., 2000; Rabsch *et al*., 2001) has resulted in an international food poisoning pandemic (Rodrigue *et al*., 1990; ACMSF, 2001; Almonacid *et al*., 2002), contaminated eggs and egg products remain the main source of infection (Molbak and Neimann, 2002).

Although *Salmonella* is ubiquitous, the primary reservoir is the intestinal tract of animals and the colonisation is favoured by intensive animal production. Poultry products are frequent vehicles in the transmission of *Salmonella*, dominating other foods of animal origin as potential source of infection (Bryan and Doyle, 1995; D'Aoust, 1997).

Salmonella is transmitted both vertically and horizontally, thereby causing problems at all levels of poultry breeding and production. Infected hens can shed live bacteria into eggs,

contaminating both table eggs and chicks. Horizontal transmission of *Salmonella* can take place from even a very small number of shedders (Lister, 1988; Cason *et al*., 1994).

The special ability of *S*. Enteritidis to cause prolonged infection of the avian reproductive tract has been a major factor in vertical transmission of the organism from breeding flocks and internal contamination of eggs is thought to have been the major factor in its spread (Guard-Petter, 2001). *S*. Enteritidis localizes in glandular parts of the reproductive tract, such as the magnum and isthmus and ovarian granulosa cells (Thiagarajan *et al*., 1994; Keller *et al*., 1995), in a way that other food poisoning serovars such as *Salmonella* Typhimurium do not (Baker *et al*., 1980; Okamura *et al*., 2001). Birds infected at day old can remain as life-long carriers, with infection increasing during stress, especially during the onset of lay (Gast and Holt, 1998; Berchieri *et al*., 2001).

The widespread use of antimicrobial agents in food animal production has contributed to the occurrence of resistant bacteria in animals, including zoonotic pathogens, which can be transmitted to humans via the food chain (WHO, 1997; Aarestrup, 1999; Van den Bogaard and Stobberingh, 1999; Tollefson and Miller, 2000). Since most *Salmonella* infections are acquired from ingestion of contaminated foods of animal origin, a likely cause for the increasing prevalence of antimicrobial-resistant *Salmonella* is the use of antimicrobial agents in food animals (Angulo *et al*., 2000). In recent years, the occurrence of this disease in humans has increased (NIAID, 2005). Tauxe (1996) and Thong *et al*. (2002) reported that most infections have been attributed to consumption of poultry meat and eggs. In addition to *Salmonella* Enteritidis and *Salmonella* Typhimurium, many other serovars have been associated with food borne infections from contaminated poultry meat (Davies *et al*., 2001). Numerous outbreaks of salmonellosis have been reported usually involving the

consumption of raw or undercooked eggs (Anonymous, 1990; Anonymous, 1996a; 1996b). In eggs, a strain of major concern is *S. enterica subsp enterica* serotype Enteritidis (Henzler *et al*., 1994).

When associated with food borne outbreaks, *Salmonella* infection usually leads to gastroenteritis. Ingestion of as few as one to ten cells of a disease causing strain of *Salmonella* can lead to penetration of the epithelial lining of the small intestine. Growth in the underlying tissue causes destruction of the epithelial lining of the small intestine after an incubation period of 8-72 hours. Affected individuals usually show signs of diarrhoea and abdominal pain with or without fever, and the disease is life threatening in infants, immune compromised individuals and the elderly (Zwadyk, 1992; D'Aoust, 1997). Diarrhoea, a common symptom of human salmonellosis, kills about 3million children each year in developing countries (White *et al*., 1997).

The commercial poultry industry has a common turnover rate of forty two days in a broiler setting which can lead to a number of possible contamination sites such as litter, air, or feed (Humphrey, 2000). Birds are also raised in close proximity with other birds, which increases the likelihood of horizontal transfer. Since *Salmonella* has several opportunities to be introduced to the host, there is an increased risk of *Salmonella* reaching the consumer. It is believed that chickens have a chronic carrier state similar to that of Typhoid Mary. Typhoid Mary was a cook in New York City back in the 1900's, who killed her employers by contaminating their food with *Salmonella* Typhi (Salyers and Whitt, 2002). The actual mechanism of this chronic carrier state is still unknown; however, if there is a large incidence of these carriers in the poultry industry, this could also be a large source of contamination. Due to the number of food borne illness cases evolving from poultry and

poultry products, it is important to understand how the bacteria are invading the product and ultimately causing illness in humans.

Coliforms were historically used as indicator microorganisms to serve as a measure of faecal contamination, and thus potentially of the presence of enteric pathogens in foods. Although coliform bacteria themselves are not pathogenic, their presence indicates possible faecal contamination and the corresponding presence of intestinal pathogens responsible for a variety of diseases. Within the coliforms *Escherichia coli* is of interest since when present in foods it indicates direct or indirect faecal contamination. It is also an indicator of the possible presence of enteric pathogens in water, shellfish, dairy products and other foods. High counts of *E. coli* and total coliforms in foods usually indicate lack of hygiene in handling and production operations, inadequate storage and post-process contamination (Blood and Curtis, 1995; DeSousa *et al.*, 2002).

E. coli is a bacterium whose natural habitat is the enteric tract of humans and warm-blooded animals (Kaspar *et al.*, 1987). *E. coli* and total coliform enumeration are used as food-quality parameters. *E. coli* typically colonizes the infant gastrointestinal tract within hours of life, and, thereafter, *E. coli* and the host derive mutual benefit (Draser and Hills, 1974). *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrhoeal disease (Nataro and Kaper, 1998).

E. coli is the most important agent causing secondary bacterial infection in poultry and may also be a primary pathogen (Gross, 1994). Colibacillosis is the most frequently reported disease in surveys of poultry diseases or condemnations at processing (Saif, 2003). It refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (APEC). The most common form of colibacillosis is colisepticaemia and is responsible for significant economic losses in the poultry industry in most parts of the world (Barnes and Gross, 1997). In the past few years, both the incidence and severity of colibacillosis have increased rapidly, and current trends indicate that it is likely to continue and become an even greater problem in the poultry industry (Altekruse *et al*., 2002; Blanco, 1997).

In some parts of Africa, *Salmonella* Hadar, *Salmonella* Brancaster and *Salmonella* Enteridids have been implicated to be the most prevalent serovars in poultry (Cardinale *et al*., 2003; 2004). In Nigeria Adene, (1997) and Saidu *et al*. (1994) have reported that salmonellosis causes financial losses to farmers. A serological survey for *S. pullorum* antibody in chickens around Zaria showed the overall prevalence rate to be 37.1% (Adesiyun *et al*., 1984). Salmonellae have been isolated by various workers in Nigeria from the following sources, cattle (Collard and Sen, 1956; Plowright, 1957; Johnson, 1958, Corke *et al*., 1975; Olayemi, 1978, Kwaga *et al*., 1985), poultry (Falade and Ehizokale, 1981), pigs (Falade *et al*., 1983), sheep and goats (Johnson, 1958; Ojo, 1972; Corke *et al*.,1975; Falade, 1978; Tabukun, 1990), dogs (Kwaga *et al*.,1989). Antibiotics resistant *Salmonella* and *E. coli* in day old chicks Anyanwu *et al*. (2010).

1.2 Statement of Research Problem

Salmonellosis is an economically important disease of poultry and other birds, with high mortality in young birds. Hens may become chronic carriers and pass the disease to their embryos by egg transmission (OIE, 2004). Poultry meat and its derivatives are among the food products that are of great concern to public health authorities, owing to the associated risks of bacterial food poisoning (Luiz *et al.*, 2004). It is a serious septicaemic disease of domestic poultry, and causes economic losses with mortality of about 30%, morbidity of 100% and drop in egg production (Berchieri *et al.*, 1997; Shivaprasad, 2000)

Salmonella which can cause severe disease in humans have emerged as food borne zoonotic pathogens in which egg and poultry are the most common sources of infection (Linam and Gerber, 2007; Braden, 2006). *Salmonella enterica* serovar Enteritidis is transmitted to the human food supply through eggs of hens that appear healthy (Porwollik *et al.*, 2005).

Food safety is of particular concern in developing countries not only because of the high prevalence of the foodborne illness and other hazards associated with food, but also because of the considerable economic and social cost that, in turn, reflects prevailing levels of economic development. The majority of trade-limiting factors in developing countries relate to economics, poor infrastructure and lagging skills; food safety is still mainly the responsibility of the consumers. Improving food safety along western standards, however, may be expensive and may not be affordable by the poor (Veen, 2005).

Apart from the impact on the health of the individuals, economic losses on international and national trade due to *Salmonella* in the poultry products and product recalls have direct

economic and public perception effects on the processing industries. The recalls also have a negative effect on the demand of the product and affect a move towards non-meat products. In the United States, although the incidence has decreased, *Salmonella* accounts for 5% of USDA recalls (Kramer *et al.*, 2005).

Colibacillosis is an economically important disease and is prevalent throughout the world (Margie and Lawrence, 1999). *E. coli* causes a lot of disease conditions in poultry such as colisepticaemia, coligranuloma, air sacculitis, peritonitis, pericarditis, omphalitis and oophoritis and these account for about 5-50% mortality in poultry flocks (Roy *et al.*, 2006).

Salmonella and *E. coli* are enteric pathogens of economic importance in poultry industry and the findings from this study will be used in proferring solutions.

1.3 Justification

Poultry represents an important source of protein throughout the world and eggs are important sources of cheap protein, which has led farmers to rear birds such as broilers for meat and layers for eggs in Nigeria (Kabir *et al*., 2004).

Eggs and egg products are nutritious foods and they form an important part of the human diet. Consuming eggs, however, has been associated with negative health impacts. Eggs and egg products that are improperly handled can be a source of food borne diseases, such as salmonellosis (Anon, 2004).

Salmonellosis is a leading food borne disease worldwide, and a wide range of foods have been implicated in such disease. Estimates from the US Centers for Disease Control and Prevention (CDC) indicate that there are approximately 1.4 million cases of salmonellosis

resulting in 17,000 hospitalizations and 585 deaths each year in the United States (Mead *et al*., 1999; Voetsch *et al*., 2004).

However, foods of animal origin, especially poultry and poultry products, including eggs, have been consistently implicated in sporadic cases and outbreaks of human salmonellosis (FAO/WHO, 2002). In recent years, public health problems associated with *Salmonella* infection have increased, in particular, the risk to public health posed by *Salmonella* infection of poultry and outbreaks attributed to poultry products (Zhang-Barber *et al*., 1999).

In Nigeria poultry production is characterized by low biosecurity levels, with many small backyard flocks producing commercial eggs. Veterinary drugs are widely used often without veterinary supervision resulting in residues and high concentration of drugs in table eggs. There is also paucity of information on salmonellae and *E. coli* in eggs in the study area.

It is therefore necessary to undertake a research that will investigate the occurrence of *Salmonella* and *E. coli* in eggs that cause food borne diseases in humans.

1.4 Aim of the Study

The aim of the study is to determine the public health risks posed by table eggs, ovarian follicles and cloacal swabs due to contamination with *Salmonella* and also the aerobic and coliform bacteria in table eggs.

1.5 Objectives

The objectives were to

1. Assess management practices carried out on the farms that could influence the occurrence of *Salmonella* in eggs
2. Determine the level of contamination of table eggs with *Salmonella*, aerobic and coliform bacteria.
3. Determine the prevalence of *Salmonella* infection in laying and slaughtered hens.
4. Determine antimicrobial susceptibility of *Salmonella* and *E. coli* isolated from table eggs.

Chapter 2 LITERATURE REVIEW

2.1 General Characteristics of Shell Eggs

The chicken (*Gallus domesticus*) is the most important bird for the production of eggs for human consumption around the world; and eggs are a unique well-balanced source of nutrients in the human diet. Egg proteins have a high biological value, and are often used as the standard to compare the quality of other proteins in foods. In addition, eggs contain unsaturated fatty acids, iron, phosphorus, trace minerals, and vitamins (Stadelman, 1995a; Watkins, 1995). Shell eggs consist of 9.5% shell, 63% albumen, and 27.5% yolk (Li-Chan *et al.*, 1995). Egg formation is a process that occurs in the ovary and the oviduct of the chicken's female reproductive system. Formation of the unfertilized egg starts with generation of the yolk (*ovum*) in the ovary, followed by its release to the upper part of the oviduct. Subsequently, yolk membrane, albumen, and shell are produced during the passage of the yolk through the different sections of the long tubular oviduct. Laying chickens produce a complete shell egg approximately every 24 hours, which is the time required for the egg to reach its full size and shape (Romanoff and Romanoff, 1949; Stadelman, 1995b).

The yolk structure consists of the latebra, the germinal disc or blastoderm, and a series of layers of light and dark yolk, which are enclosed by the vitelline membrane. The albumen is made of four layers, from the inside to the outside of the egg, that includes the chalaziferous that extends as a rope-like structure and keeps the yolk in the center of the egg, the adjacent inner thin layer, the dense albuminous sac, and the surrounding outer thin layer. The outer covering consists of two keratin-like inner and outer membranes, with 0.01-1.02 mm total thickness, encircled by the shell. The eggshell is composed of 94% calcium carbonate, 1% magnesium carbonate, 1% calcium phosphate, and 4% protein. The

shell is a porous structure (~ 10,000 pores/shell), has an average thickness of 0.31 mm, and is covered by the cuticle, which is a protein-rich coating that constitutes the most external layer of the egg (Romanoff and Romanoff, 1949; Stadelman, 1995b; Okubo *et al.*, 1997).

2.1.0 Background of *Salmonella*

2.1.1 History

Before the nineteenth century, human enteric or typhus fever was often confused with typhus which is a rickettsial disease. The two diseases were pathologically distinguished by P.Ch.A. Louis in France (1829) and William Jenner in the United States (Scherer and Miller, 2001). Further investigation by European workers lead to the isolation and characterization of the typhus bacillus responsible for typhoid fever and to the development of a serodiagnostic test for the detection of the disease agent (D'Aoust, 1989; Le Minor, 1981). During the first quarter of the 20^{th} century, great advances occurred in the serological detection of somatic and flagella antigens within the *Salmonella* group. *Salmonella* is a generic term coined by Lignieres in 1900 (Le Minor, 1981). An antigenic scheme for the classification of *Salmonella* was first proposed by White (1926) and subsequently expanded by Kauffman (1941) into the Kauffman White scheme.

2.1.2 Taxonomy

There are many different references on the taxonomy of *Salmonella*. According to World Health Organization (WHO) collaborating centre for Reference and Research on *Salmonella* (Institute Pasteur Paris) (D'Aoust *et al*., 2001), the genus *Salmonella* contains two species: *S. enterica* and *S. bongori* (formerly subspecies V). In the ninth edition of Bergey's manual, all of the *Salmonella* serovars belong to two subspecies: *S. bongori* and

S. Cholerasuis. More than 2500 serovars are all part of the *S.* Cholerasuis which is divisible both phenotypically and genetically, into 6 subspecies (Holt *et al*., 2002).

Table 2.1: Species within the *Salmonella* genus

***Salmonella* spp and subspecies**	**No of serovars**
S. enterica subsp *enterica* (I)	1478
S. enterica subsp *salamae* (II)	498
S. enterica subsp *arizonae* (IIIa)	94
S. enterica subsp *diarizonae* (IIIb)	327
S. enterica subsp *houtenae* (IV)	71
S. enterica subsp *indica* (VI)	12
S. bongori (V)	21
Total	2501

(Popoff, 2001)

S. enterica is further divided into six subspecies which are referred to by a Roman numerals and a name (I, *S. enterica* subsp *enterica*; II, *S. enterica* subsp *salamae*; IIIa, *S. enterica* subsp *arizonae*; IIIb, *S. enterica* subsp diarizonae ; IV, *S. enterica* subsp *houtenae*; VI, *S. enterica* subsp *indica*). *S. enterica* subsp are differentiated biochemically and by genomic relatedness (Brenner *et al*., 2000).

Depending on this classification system, the correct names for the formerly called *S. enteritidis* and *S. typhimurium* are *S. enterica subsp. enterica*. *S. bongori* which was

initially classified as subsp V is generally considered a separate species due to its divergence from other *Salmonella* (Reeve *et al*., 1989).

According to Popoff and Le Minor (1997), the name of the *Salmonella* serotype is related to the geographical area where it was first isolated. The serotype name is written in roman letters not italicized e.g. *Salmonella* serotype Typhimurium or *Salmonella* Typhimurium.

Classification and detection of this bacteria is based on serology and phage susceptibility assays. DNA-based typing methods that have contributed to the re-classification of the serovars of *Salmonella* to a new subspecie group scheme include random amplified polymorphic DNA (RAPD) technique, ribotyping, pulsed field gel electrophoresis (PFGE), and multi locus enzyme electrophoresis (MLEE) (Scherer and Miller, 2001).

Epidemiologically, salmonellae can be classified into three major groups (WHO Expert Committee, 1988):

Those that infect humans only such as *S. typhi, S. paratyphi* A and S, *paratyphi* C. This group includes the agent of typhoid and paratyphoid fever, which are the most severe of all the diseases caused by *Salmonella*.

The host adapted serovars some of which are human pathogens and may be contracted from foods including *S. gallinarium* (Poultry), *S. dublin* (Cattle), *S. abortus equi* (Horses), *S. abortus ovis* (Sheep) and *S. cholera suis* (Pigs).

The host-unadapted serovars, have no host preference, which are pathogenic agents for humans. This group mainly includes food borne serovars.

2.1.3 Structure, Morphology and Biochemical Reactions

Salmonella are small, gram negative, facultative anaerobes, straight, rod- shaped, bacteria belonging to the family Enterobacteriaceae, measuring 0.7-1.5 um in width and 2-5 um in length. The *Salmonella* cell has three common compartments. The first compartment is the cytosol where genetic replication and protein expression occurs. The second compartment is a cell envelope, containing a cell wall and cytoplasmic membranes which is critical to the structure and function of the organism. The third compartment anchors surface structures that lie external to the cell envelope (e.g. capsule; O or K antigen, flagella; H antigen) (McClane *et al*., 1999; Murray *et al*., 2002).

They have the capacity to grow under either aerobic or anaerobic conditions and are non-capsulated and non- sporular. The optimal growth temperature on ordinary culture media is 37°C, with small colonies 2-4 mm in diameter which are smooth shiny and homologous (Krieg and Holt, 1984).

It grows within a pH range of 4.5 to 9.5 depending on the surrounding conditions. *S*. Typhimurium has been said to be growing at a temperature range of 2-54°C. Regarding available moisture, growth inhibition has been reported for water activity (aw) values below 0.93 (Doyle *et al*., 2001). A salt content of 3-4% inhibits the growth of *Salmonella* but increasing temperature increases salt tolerance. A salt content above 8% is bactericidal for *Salmonella* (Jay *et al*., 2005).

Salmonella are catalase positive, oxidase negative, indole and voges proskauer negative, methyl red and simmons citrate positive (Holts *et al*., 2002). In a triple sugar iron medium, typical *Salmonella* isolate will produce acid and gas from glucose and would not utilize

lactose and sucrose in differential plating media such as Xylose lysine desoxycholate and Brilliant green agar. Salmonellae readily produce an alkaline reaction from the decarboxylation of lysine to cadaverine in lysine iron agar; generate hydrogen sulphide gas in triple sugar iron and lysine iron media and does not hydrolyse urea (D'Aoust and Purvis, 1998).

Though most salmonellae are motile, *S. gallinarium* and *S. pullorum* are non-motile. The motile ones may lose their ability to develop flagella under the effect of sub lethal stress, which is caused by external physicochemical influences such as refrigeration or temperature (Krieg and Holt., 1984; Doyle *et al*., 2001).

Most salmonellae are aerogenic; however *S. typhi* is an important exception, it never produces gas. Anaerobic variants of gas producing *Salmonella* serovar may occur e.g *S. dublin*. Majority of salmonellae produce hydrogen sulphide, but a few do not produce this gas e.g. some strains of *S. cholerasuis* and most strains of *S. paratyphi* A (Krieg and Holt, 1984).

2.1.4 Serotyping of *Salmonella*

Salmonella express flagellar, polysaccharide and capsular antigens which determine strain pathogenicity and therefore variation of these antigens has formed the basis for *Salmonella* serotyping. The Kauffmann-White scheme, which was first published in 1929, divides *Salmonella* into more than 2500 serotypes according to their antigenic formulae (Mortimer *et al.*, 2004). Currently, 57 O antigens and 117 H antigens have been identified and more than 2500 serotypes have been described. Some of the H antigens share common antigenic factors. These antigens are clustered in five complexes, the E, G, L, Z4 and 1 complex.

Salmonella H antigens are expressed in different phases. Most serotypes are diphasic, and minor parts are monophasic. *Salmonella* Gallinarum is the only serotype in the Kauffmann-White scheme that does not express any flagella antigen and is therefore non-motile (Sonne-Hansen *et al.,* 2005). *Salmonella* serotyping methods recognize 63 distinct phase 1 flagellar antigenic factor and 37 phase 2 flagellar antigenic factors, although the latter are not always present. Some antigenic factors, denoted by square brackets in formulae, may be present or absent without affecting serotype designation. Serotyping methods are stable, reproducible and have high typability, yet there are several drawbacks, particularly the dependence on availability of antisera, considering the ethics, cost and quality control measures necessary to maintain such a supply (Mortimer *et al.,* 2004).

Salmonella isolates can be differentiated from one another in a wide variety of ways, and the number of *Salmonella* continues to increase. Epidemiologically, it is important to be able to distinguish *Salmonella* isolates, because definitive typing of *Salmonella* isolates may assist in tracing the source of an outbreak and monitoring trends in antimicrobial resistance associated with a particular type (Yan *et al.,* 2003). In addition to the conventional antigen-based serotyping, there are advanced techniques for serotyping currently being used to enhance the tracing of the individual isolates. The following techniques are currently available for serotyping.

(1) Conventional method

(2) Phage typing

(3) Molecular typing

a. Restriction digestion based techniques
b. Amplification based techniques
c. Nucleotide sequencing based techniques

2.1.5 Conventional serotyping

Conventional serotyping of *Salmonella* is the most commonly used method to differentiate strains, which are epidemiologically the smallest bacterial unit from which isolates share the same phenotypic and genotypic traits (Yan *et al.,* 2003). In most clinical studies, initial serotyping is done using polyvalent O antisera to allow *Salmonella* isolates to be grouped into different O groups designated in capitalized letters. Many *Salmonella* show diphasic production of flagellar antigens and each strain can spontaneously and reversibly vary between these two phases with different sets of H antigens. In phase 1 or the specific phase, the different antigens are designated by small letters, and in phase 2 or the group phase, the antigens first discovered are numbered. In a single cell, usually only one antigen is expressed at a time (Yan *et al*., 2003). Conventional serotyping using the autoagglutination method has some limitations, such as limitations in detection of Vi antigens (Wain *et al*., 2005), strains which are not typeable (Rasschaert *et al.*, 2005); it only allows detection of a single antigen-antibody reaction at a time, requires well-experienced technologists to perform, consumes relatively high amount of reagents, and takes a longer time (Cai *et al.*, 2005).

The antigenic formulae for some salmonellae serovars are shown in Table 2.

Table 2.2: Antigen of some *Salmonella* serotypes

Serotype	Serogroup	Somatic (O) antigens	Flagella H antigens Phase1	Phase 2
S. Paratyphi	A	1, 2, 12	A	(1,5)
S. Typhimurium	B	1, 4 (5), 12	i	1, 2
S. Agona	B	4, 12	f, g, s	-
S. Derby	B	1, 4, (5), 12	f, g	(1,2)
S. Typhi	D	9, 12, (Vi)	c	1,2
S. Enteritidis	D	1, 9, 12	g, m	(1,7)

(Krieg and Holt, 1984; Jay, 1992)

In some cases, O and H factors are variably present. This is indicated in the generic serotype formula for underlining when the factor is encoded on a bacteriophage (e.g., 1) weakly recognized antigens are indicated by paratheses eg., (k). The absence of an H antigen is indicated by a minus sign (-).

2. 2 Salmonellosis

2. 2. 1 Epidemiology

The primary reservoir of salmonellae is the intestinal tract of humans and animals, particularly in poultry and swine. As intestinal forms, the organisms are excreted in faeces from which they may be transmitted by insects and other creatures to water, soil and kitchen surfaces. Eggs, poultry and raw meat products are the most important food vehicles

of *Salmonella* infection in humans, with *S*. Typhimurium and *S*. Enteritidis being the most commonly isolated food borne serovars (Jay, 1992).

Information about the incidence and serotype distribution of salmonellae in domestic animal populations is essential for understanding the relationship within and among the reservoirs of salmonellae in animals and humans that are ultimately responsible for zoonotic disease transmission (Gast, 1997).

Salmonella infection is mainly acquired by ingestion of contaminated food and drink. Any food product is a potential source of human infection. *Salmonella* can be transmitted directly from human to human or from animal to human without the presence of contaminated food or water, but this is not a common mode of transmission. The true incidence of *Salmonella* infection is difficult to determine. Reported cases represent only a small proportion of the actual number because only large outbreaks are investigated and documented. Hence, sporadic cases are underreported because it is only patients with protracted diarrhoea that report cases for microbiological evaluation (Hanes, 2003).

2.2.2 **Incidence**

Human gastroenteritis is caused by many serotypes of *Salmonella*, the most common of which in the United States are *S*. Enteritidis and *S*. Typhimurium (Alkatruse *et al*., 1997). Cases of gastroenteritis are usually due to contamination of food with animal waste. Undercooked eggs, meat and seafoods are common causes of salmonellosis, although the contamination of fresh produce with animal waste is also a significant problem (Tauxe, 1991).

In the United States, approximately 2-4 million cases of *Salmonella* related gastroenteritidis occur per year. Children are more likely infected with salmonellosis. The most likely individuals to have severe infections with salmonellosis include young children, the elderly and the immune-compromised. It is estimated that approximately 600 people die each year with acute salmonellosis, and it's more common in the summer than winter (CDC, 2004).

Salmonellosis may occur in small, contained outbreaks in the general population or in large outbreaks in hospitals, restaurants, or institutions for children or the elderly. While the disease is found worldwide, health experts most often report cases in North America and Europe. Each year CDC receives reports of 40,000 cases of salmonellosis in the United States. The agency estimates that 1.4 million people in that country are infected and 1,000 people die each year due to salmonellosis. *S*. Typhimurium and *S*. Enteritidis are the most commonly found serovars in the United States (NIAID, 2005).

2.2.3 **Transmission**

Salmonella can grow on any food such as poultry eggs and meat, seafoods, dairy products and vegetables. Foods prepared on surface previously in contact with raw meat or meat products can become contaminated with the organism. This is known as cross contamination. In recent years the Centres for Disease Control and Prevention has received reports of several cases of salmonellosis from eating raw alfalfa sprout grown on contaminated soil. *Salmonella* infection usually occurs after handling pets, particularly reptiles like snakes, turtles and lizards (NIAID, 2005).

Infection occurs by ingestion of the organism in food derived from infected animals or contaminated by faeces of animals or man. This includes raw and undercooked egg and egg

products, raw milk and raw milk products, contaminated water, meat and meat products, poultry and poultry products. Epidemics may also be traced to foods such as meat and poultry products that have been processed or prepared with contaminated utensils and work surfaces. *S*. Enteritidis infection of chicken and eggs has caused outbreaks and single cases especially in the Northeastern US and Europe, and is responsible for the majority of cases of this serotype in the US. Temperature abuse of food during its preparation and cross contamination during food handling are the most important risk factors (Washington State Department of Health, 2002).

Salmonellosis can become a chronic infection in people who may not have symptoms. Though they may have no symptoms, they can spread the disease by not washing their hands before preparing food for others (NIAID, 2005).

2. 2. 4 **Salmonellosis in humans**

Human salmonellosis continues to be a major international problem both in terms of morbidity and economic losses (Barnass *et al*., 1989). Salmonellosis is primarily associated with consumption of food products contaminated with *Salmonella*. The Robert Koch Institute in Germany reported 21,596 cases of salmonellosis during the first 8 months of 2008 (Anon, 2008). In Germany a batch of contaminated dessert resulted in a salmonellosis outbreak causing at least 239 illnesses and one death (Anon, 2007). *Salmonella*-contaminated vegetables and fruits were also identified as a widespread source of *Salmonella* infections in humans (Brandl, 2006). The incubation period is usually within the range of 12-72 hours, but occasionally may extend up to a week. In some outbreaks, where large numbers of organisms are believed to have been consumed, incubation periods as short as 2.5 hours have been reported (Stevens *et al*., 1989). *Salmonella* infection most

commonly results in a gastroenteritic illness with diarrhoea and, from time to time, a number of other symptoms can occur. In the majority of cases the acute gastrointestinal symptoms will clear with supportive therapy only within 4-5 days. Malaise, lassitude, and weakness may continue for substantially longer time. A possible complication of acute salmonellosis is a reactive arthritis.

2.2.5 ***Salmonella*** **in chicken**

2.2.5.1 *Distribution and importance in foods*

Infection with bacteria of the genus *Salmonella* are responsible for a variety of acute and chronic diseases in poultry. Infected poultry, moreover, comprise one of the most important reservoirs of salmonellae that can be transmitted through food chains to humans. Isolations of *Salmonella* are reported more often from poultry and poultry products than from any other animal species (Gast, 1997). Poultry can become colonized by pathogens via drinking water, feed or pecking in contaminated soil and water (ICMSF, 1998).

Although the proportion of food poisoning outbreak and cases in which the sources of infections can be positively identified is small, poultry and poultry products are repeatedly implicated in human outbreaks. *Salmonella* organisms from poultry sources currently enter the human food chain mainly as a result of carcass contamination from infected faecal material or eggs. Several *Salmonella* serovars have been isolated from poultry (Gast, 1997).

Occurrence of food poisoning related to *Salmonella* contaminated eggs and chicken meat has been frequent in humans (Mead *et al*., 1999). Eating raw or undercooked eggs has also been considered a major risk factor for food poisoning with salmonellae in some situations (Molback and Neiman, 2002). *S*. Enteritidis (SE) and *S*. Typhimurium (ST) are amongst the

most important paratyphoid salmonellae associated with chicken meat and eggs (Tavechio *et al*., 2002; Taunay *et al*., 1996). *S*. Enteritidis is the most frequently isolated *Salmonella* from poultry products in Brazil (Fiorentin, 2004).

2.2.6 **Public health and economic impacts**

Typhoid and non-typhoid salmonellosis are major public health problems and are the most economically important food-borne disease. Incidence of typhoid salmonellosis is stable, with very low numbers of cases in developed countries, but cases of non-typhoid salmonellosis are increasing worldwide. Non-typhoid cases account for 1.3 billion cases of acute gastroenteritis/diarrhoea with 3 million deaths and for 16 million cases of typhoid fever with nearly 600,000 deaths (Pang *et al*., 1995).

In many countries, the incidence of salmonellosis has increased greatly, however a paucity of good surveillance data exist. An estimated 12-33 million cases of typhoid fever occur globally each year and the disease is endemic in developing countries of the Indian subcontinent, South and Central America, and Africa (Zapor, 2005).

2.2.7 **Prevention and control of Salmonella in poultry**

Prevention and control measures should be designed based on the two distinct categories, *Salmonella* infections that have a direct negative impact on the poultry population, and *Salmonella* infections of importance to public health (Breytenbach, 2004). Prevention of salmonellosis by the implementation of hygienic measures is difficult and use of antibiotics may give rise to the emergence of resistance problems (Mastroeni and Menager, 2003). Reducing *Salmonella* prevalence requires a multi-hurdle approach at all stages of breeding, hatching, grow-out, transportation and processing. Nothing can be added at a single point in

production or processing that will completely eliminate *Salmonella* on chickens. As such, some researchers describe "competitive exclusion" as the most effective and harmless method to control *Salmonella* in poultry (Ragione and Woodward, 2003; Schneitz, 2005). Competitive exclusion (CE) is a term that has been used to describe the protective effect of the natural or native bacterial flora of the intestine in limiting the colonization of some bacterial pathogens. Attenuated DNA recombinant live *Salmonella* vaccines (Kang *et al.*, 2002), combined with comprehensive control strategy in animals, feed and animal feed stuffs with restrictions on the infected flocks until they have been cleaned up from infections (Boqvist and Vagsholm, 2005), will help reduce salmonellosis. The prevention of paratyphoid *Salmonella* infection which has greater public health consequences requires a comprehensive control strategy including regular monitoring, strict biosecurity, sourcing feed containing no animal protein, and vaccination (Breytenbach, 2004).

2.2.8 **Antibiotic Resistance**

Resistance of *Salmonella* spp to antibiotics used in human medicine thereby reducing therapeutic options and threatening the lives of infected individuals is a major public health concern (Tacket *et al.*, 1985). Multidrug resistant strains have emerged in India and Southeast Asia. In India 50- 70% of strains are resistant to chloramphenicol and other antibiotics (Pang *et al.*, 1995; Rowe *et al*., 1997).

In some parts of the world, antibiotics are increasingly being used in the agricultural and aquaculture industries as therapeutic, prophylactic and growth promoters to promote animal vigour. New evidence indicates that growth promoters such as apramycin, avoparcin and tylosin can cause bacterial resistance to gentamicin, vancomycin and erythromycin respectively. The approved veterinary use of enrofloxacin in several European countries

(1987-1994) and sarafloxacin in the United States (1995) was considered unfortunate, because the agents used led to the emergence and spread of fluoroquinolone-resistant *Salmonella, Campylobacter* and other bacterial pathogens in foods and in consumers (D'Aoust, 2001).

Recently, the emergence of antibiotic resistant *S.* Typhimurium strains, particularly the penta-resistant strain DT104, which is a more virulent than sensitive strain, is troublesome in the United States (Glynn *et al*., 1998). This strain has been isolated from numerous species of animals, both wild and domesticated, and it is resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline. There have been reports of resistance to two other antibiotics namely trimethoprim and fluoroquinolones, in Great Britain (Scherer and Miller, 2001).

2. 2. 8.1 *Resistance to beta lactams*

Resistance to beta-lactam antimicrobials is mainly due to inactivation by beta lactamases (Livermore, 1995) and decreased ability to bind to penicillin-binding proteins (Georgeopapadakou, 1993). However, beta-lactam resistance may also be a result of decreased uptake of the drug due to permeability barriers or increased efflux via multidrug transporters (Paulson *et al*., 1996; Quintiliani *et al*., 1999). The inactivation of beta-lactams is primarily due to the cleavage of the amino bond in the beta-lactam ring by a beta lactamase enzyme (Bush *et al*., 1995; Livermore, 1995; Bush, 2001; Wiegand, 2003). Genes encoding beta-lactamases are located on either plasmids or the bacterial chromosome. Examples of specific gene variants for the beta-lactamase family in gram negative bacteria include *amp*C, *tem*, *shv*, *oxa* and *ctx*-M (Aarts *et al*., 2006).

2. 2. 8.2 *Resistance to tetracyclines*

Tetracycline resistance is almost always a result of the uptake of new genes (Chopra and Roberts, 2001). There are 23 efflux genes (which code for energy dependent efflux of tetracyclines), 11 ribosomal protection genes (which code for protein that protects bacterial ribosomes), 3 genes that code for enzymes that modify and inactivate tetracycline, and 1 gene that have an unknown mechanism (Schwarz *et al*., 2006).

Currently only the first two mechanisms are important in bacteria of veterinary importance. The efflux resistance genes *tet*A, *tet*B, *tet*C, *tet*D and *tet*H are most wide spread in gram negative bacteria and are located on transposons (Allmeier *et al*., 1992; Chalmers *et al*., 2000; Lawley *et al*., 2000) and plasmids (Schwarz *et al*., 2006). The *tet*B gene confers resistance to both tetracycline and minocylcine, but not to the new glycyclines, while the other efflux proteins confer resistance only to tetracycline (Chalmers *et al*., 2000; Chopra and Roberts, 2001). Resistance to minocycline and glycyclines are relevant as they are newer drugs that play a role in human medicine (Fech and Scjwarz, 2000; Kehrenberg *et al*., 2001; Aminov *et al*., 2001; Ng *et al*., 2001; Guerra *et al*., 2004; van Hoek *et al*., 2005).

Ribosomal protection genes are a second important way for tetracycline resistance development. They are of gram positive origin but can also be found in gram negative genera (Schwarz *et al*., 2006). An example of a ribosomal protection gene is the *tet*M gene which has a wide range of hosts and is located on a conjugative transposon (Flannagan *et al*., 1994; Chopra and Roberts, 2001; Salyers *et al*., 1995). Other less well described mechanisms of tetracycline resistance include enzymatic inactivation, 16S rRNA mutation, other mutations, and multidrug transporters (Schwarz *et al*, 2006).

2. 2. 8.3 *Resistance to quinolones and fluoroquinolones*

Quinolones and fluoroquinolones are potent inhibitors of bacterial DNA replication (Schwarz *et al*., 2006). The two major mechanisms of resistance development to fluoroquinolone antimicrobials are point mutations and decreased intracellular accumulation (Schwarz *et al*., 2006). Several recent reviews deal with the molecular basis and epidemiology of quinolone resistance in *E. coli* and *Salmonella* spp. of animal origin (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1999; Bager and Helmuth, 2001; Cloeckaert and Chaslus-Dancla, 2001; Webber and Piddock, 2001; Ruiz, 2003). Briefly, point mutations in the target genes *gyr*A and *gyr*B coding for DNA gyrase and or for *par*C and *par*E coding for DNA topoisomerase IV are frequent in quinolone and fluoroquinolone resistance (Schwarz et al., 2006). Detection of these point mutations in the region of the *gyr*A, *gyr*B, or *par*C and *par*E genes can be accomplished through PCR (Aarts *et al*., 2006) while microarrays have been used to assess multidrug efflux systems. Resistance genes associated with multidrug efflux pumps vary depending on the organism involved (Schwarz *et al*., 2006) and they may lead to high levels of resistance to quinolones and other antimicrobials where multidrug efflux pumps and decreased membrane permeability are involved (Lee *et al*., 2000). Quinolone and fluoroquinolone resistance can also result from interaction between different resistance mechanisms, decreased drug uptake and DNA gyrase protection (Schwarz *et al*., 2006).

2. 2. 8.4 *Resistance to aminoglycosides*

The main mechanism for aminoglycoside resistance is enzymatic inactivation (Shaw *et al*., 1993; Mingeot-Leclercq *et al*., 1999), but reduced uptake and chromosomal mutations conferring high levels of resistance to streptomycin have also been described (Quintiliani *et*

al., 1999). Aminoglycoside resistance is mediated by more than 50 aminoglycoside modifying enzymes that are classified as either aminoglycoside acetyltransferases (*aac*), aminoglycoside adenyltransferases (*aad* or *ant*), and aminoglycoside phosphotransferases (*aph*) (Shaw *et al*., 1993; Mingeot-Leclercq et al., 1999; Aarts *et al*., 2006). Most *aac*, ant and *aph* genes are located on mobile genetic elements such as plasmids, transposons, or gene cassettes (Shaw *et al*., 1993; Recchia and Hall, 1995; Davies and Wright, 1997; Mingeot-Leclercq *et al*., 1999; Wright, 1999; Sandvang and Aarestrup, 2000).

2. 2. 8. 5 *Resistance to chloramphenicol*

Both enzymatic and non-enzymatic chloramphenicol resistance genes have been described (Aarts *et al*., 2006), but enzymatic inactivation is the predominant method (Shaw, 1983; Murray and Shaw, 1997; Schwarz *et al*., 2004) of resistance development. Enzymatic resistance genes are primarily encoding acetyltranfereases and are the *cat* genes (Aarts *et al*., 2006). Non-enzymatic gene coding of chloramphenicol includes the *cml* genes on transposon TN1696 and the *flo*R gene (Aarts *et al*., 2006). Efflux systems conferring resistance to chloramphenicol alone or in combination with florfenicol (Schwarz *et al*., 2004), permeability barriers, and multidrug transporters (Paulsen *et al*., 1996; Schwarz *et al*., 2004) as well as other minor mechanisms of resistance have also been identified for this class of antimicrobials (Schwarz *et al*., 2006).

2. 2. 8. 6 *Resistance to sulphonamides and trimethoprim*

Sulphonamides and trimethoprim are competitive inhibitors of different enzymatic steps in folate metabolism (Schwarz *et al*., 2006). Sulphonamide resistance can result from chromosomal mutations in the dihydropteroate synthase (*fol*P) gene or by acquisition of resistant dihydropteroate synthase genes (*sul* genes) (Aarts *et al*., 2006; Schwarz *et al*.,

2006). Three *sul* genes have been described in gram negative bacteria (Swedberg and Skold, 1980; Radstrom and Swedberg, 1988; Aarts *et al*., 2006). The *sul*I gene is associated with class 1 integrons and, therefore, is often linked to other genes. It is spread in gram negative species as part of transposons or as conjugative plasmids (Sundstrom *et al*., 1988). The *sul*II gene often occurs with streptomycin resistance genes *str*A and *str*B on conjugative or nonconjugative plasmids (Radstrom and Swedberg, 1988; Kehrenberg and Schwarz, 2004), while the *sul*III gene can be found on conjugative plasmids (Perreten and Boerlin, 2003). Trimethoprim resistance is primarily mediated by acquisition of *dfr* gene encoding resistant dihydrofolate reductase (Aarts *et al*., 2006; Schwarz *et al*., 2006). Transferable trimethoprim resistance has been identified in a variety of gram negative bacteria and several of these genes are part of plasmids, transposons, or gene cassettes (Recchia and Hall, 1995; Skold, 2001; Ito *et al*., 2004). Other potential mechanisms of trimethoprim resistance for some bacteria include permeability barriers and efflux pumps (Kohler *et al*., 1996; Huovinen, 2001) and *dhfr* and folate auxotrophy (Quintiliani *et al*., 1999).

2. 3 **Microbiological detection**

The cultural isolation of *Salmonella* is most common in animal-production and food processing industries. Most of the methods were developed for the diagnosis of clinical salmonellosis in humans and animals (Fedorka-Cray *et al*., 2000). The microbiological isolation and detection of *Salmonella* involves the use of the following culture media.

2.3.1 **Pre-enrichment media**

Several pre-enrichment media have been proposed (D'Aoust, 1981). Lactose broth was perhaps the first to receive widespread use. But there has been concern that lactose broth,

because of the fermentation of lactose and resulting acidity, would allow the pH to fall to a level that is inhibitory or lethal to *Salmonella* (Hiker, 1975). Buffered peptone water (BPW) has been used without a fermentable sugar and with greater buffering capacities. BPW was found to be more appropriate than lactose broth for isolating *Salmonella* (Fricker, 1987). The pH of the lactose broth cultures after incubation ranged from 4.8-5.5, whereas the range for BPW was 5.8-6.4, respectively. BPW has been the pre-enrichment broth of choice for use in conjunction with Rappaport-Vassiliadis (RV) enrichment media (Waltman, 2000).

2.3.2 **Selective-enrichment broth**

Selective-enrichment broths are formulated to selectively inhibit other bacteria while allowing *Salmonella* to multiply to levels that may be detected after plating. There are currently three major types of selective-enrichment media: tetrathionate, selenite and Rappaport-Vassiliadis (RV). There are also different formulations within each type of enrichment (Waltman, 2000).

2.3.3 **Selenite enrichment media**

Leifson (1936) formulated the first selenite enrichment media, commonly known as selenite F (SF). Bacteria reduce selenite, which increases the pH and reduces the toxicity of selenite. North and Bartram (1953) modified SF by adding cystine (selenite-cystine (SC)). Stokes and Osborne (1955) modified SF by changing the carbohydrate source from lactose to mannitol and adding sodium taurocholate and brilliant green (selenite brilliant green (SBG)). In a comparative study, Waltman *et al.* (1995) compared SF, SC, and SBG with tetrathionate and RV enrichment broths. The tetrathionate and RV enrichment results were

better than the results from the use of selenite enrichment. In addition selenite is reported to be toxic and a mutagen (Andrews, 1996).

2.3.4 **Tetrathionate enrichment media**

Muller (1923) described a selective enrichment broth that contained iodine and sodium thiosulphate, which he combined to form tetrathionate. Kauffmann (1935) modified the tetrathionate brilliant green (TBG) enrichment broth of Muller by adding ox bile and brilliant green (MKTT). Several studies have shown that tetrathionate enrichment is better than selenite enrichment (Waltman *et al*., 1995; D'Aoust *et al*., 1992).

2.3.5 **Rappaport-Vassiliadis (RV) enrichment media**

Rappaport *et al*. (1956) described an enrichment medium based on the ability of *Salmonella* to: survive relatively high osmotic pressures (using magnesium chloride), multiply at relatively low pH (pH 5.2), and selectively grow in presence of malachite green (106 mg L-1), and grow with minimal nutritional requirements (5 g peptone L-1). Vassiliadis *et al*. (1970; 1976) modified the medium by reducing the concentration of malachite green, which made the medium suitable for incubation at 43°C. Several studies have shown that RV enrichment was better than either tetrathionate or selenite enrichment broths (Waltman *et al*., 1995; Oboegbulem, 1993). RV enrichment medium has been approved for isolating *Salmonella* from raw, highly contaminated food and animal feeds, replacing the use of selenite enrichment media (June *et al*., 1996).

Goossens *et al*. (1984) developed a semi-solid medium based on RV enrichment broth (modified semi-solid Rappaport-Vassiliadis (MSRV)). The MSRV plate is incubated at 41.5°C and the motility of *Salmonella* further selects and differentiates *Salmonella* from

other microorganisms. Several studies have shown the advantages of the MSRV culture method (Davies and Wray, 1994; Read *et al*., 1994; Aspinall *et al*., 1992).

2.3.6 **Plating media**

Plating media should be judged not only on their ability to selectively grow *Salmonella,* but also on their ability to differentiate colonies of *Salmonella* from those of other bacteria. Many selective plating media are available for the isolation of *Salmonella*. The plates are incubated at 35-37°C for 20-24 h and colonies suspected to represent *Salmonella* are taken for further investigations (Waltman, 2000). Brilliant green (BG) agar was developed by Kristensen *et al*. (1925) and later modified by Kauffmann (1935). The selectivity of the agar derives from the presence of the brilliant green dye and the presence of lactose and sucrose (BPLS), which is the basis for the differential capabilities of the media. Several investigators have proposed the use of BPLS (Waltman *et al*., 1995). Rambach agar (RAM) was developed based on the finding that *Salmonella* produce acid from propylene glycol (Rambach, 1990). The selective ability results from the presence of bile salts. The differential characteristics involve the fermentation of propylene glycol by *Salmonella* and resulting in a change in colour of the colonies to pink red. This is due to the precipitation of the neutral red dye present in the media and also change in pH to acidic conditions. A chromogenic indicator system for β-galactosidase activity is used to differentiate *Salmonella* from other lactose fermenting members of the family Enterobacteriaceae. In presence of lactose fermenters the chromogenic substance, X-Gal (5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside), results in the formation of blue-green colonies. But some serovars of *Salmonella* do not produce the characteristic red colonies, e.g. *S.* Pullorum, *S.* Gallinarum, *S.* Abortus, and *S.* Dublin (Pignato *et al*., 1995; Kühn *et al*., 1994). *Salmonella*

give colourless colonies with black centres on *Salmonella-Shigella* (SS) agar; blue-green with black-centred colonies are seen on Hektoen agar. Typically *Salmonella* species will appear as black or red to pink colonies with a black center on Xylose lysine tergitol 4 (XLT4) media due to the ability to reduce thiosulfate to hydrogen sulfide. *Salmonella* grow as red colonies with black centres on Xylose lysine desoxycholate (XLD) agar; giving yellow colonies on Gassner agar and red colonies on Brilliant green agar (BGA).

2.3.7 **Biochemical identification**

Typical *Salmonella* colonies isolated on any of the selective plating media should be confirmed by biochemical tests. Testing for all these reactions requires considerable resources. Therefore it is more convenient to use composite media such as triple sugar iron agar (TSI), which is prepared in tubes. It contains glucose, lactose and sucrose, a H2S detection system and an indicator (Jones *et al*., 2000).

TSI is a differential medium that contains lactose, sucrose, a small amount of glucose (dextrose), ferrous sulfate, and the pH indicator phenol red. It is used to differentiate enteric bacteria based on the ability to reduce sulfur and ferment carbohydrates. As with the phenol red fermentation broths, if an organism can ferment any of the three sugars present in the medium, the medium will turn yellow. The medium is inoculated by stabbing the loop into the centre of the agar down to the base of the reaction tube. The loop is pulled back and the rest of the inocular is distributed on the slope of the agar surface. The tube is incubated at 37°C for 24 h. When glucose (not lactose or sucrose) is fermented by the microorganisms a change of colour from red to yellow can be observed. The intensity of the colour depends on the amount of glucose, which is converted. However, the reaction under the aerobic

conditions on the slant reverts and becomes alkaline (red) because of protein breakdown in the medium. Under anaerobic conditions inside the agar at the bottom of the tube, the medium remains acid (yellow) and H2S production is characterized by blackening of the medium (Jones *et al*., 2000). This happens with *Salmonella* and *Shigella*. Some *Proteus* spp. and other species may give a similar reaction, but can be distinguished by their ability to hydrolyse urea. Urea agar in another reaction tube should always be used in parallel. With recent, developments in the area of biochemical detection of *Salmonella* it is now possible to rapidly carry out these biochemical tests and obtain results within hours using test cards, like the VITEK GNI+ card used for the biochemical identification of Gram-negative bacteria (de la Torre *et al*., 2005).

2.3.8 **Immunology-based methods**

Immunological detection with antibodies is perhaps the only technology that has been successfully employed for the detection of bacterial cells, spores, viruses and toxins alike (Iqbal *et al*., 2000). Methods based on antigen–antibody bindings are widely used for determining food-borne pathogens. Immunology-based methods have been used for the detection of *Escherichia coli* (Aldus *et al*., 2003; Sunwoo *et al*., 2006), *Salmonella* (Abdel-Hamid *et al*., 1999; Schneid *et al*., 2006; Valdivieso-Garcia *et al*., 2003). In order to ensure the reliable detection of food-borne pathogens using antibody-based methods, the influence of stress on antibody reactions should be thoroughly examined and understood first as the physiological activities in cells are often altered in response to a stress (Hahm and Bhunia, 2006). Polyclonal antibodies are able to detect *L. monocytogenes* (Jung *et al*., 2003), Campylobacter species (Hochel *et al*., 2007). Monoclonal antibodies are often more useful for specific detection of a molecule than polyclonal antibodies because they provide an

indefinite supply of single antibody. Monoclonal antibodies are used to detect wide range of pathogens in food. Examples are *E. coli* O157 (Zhao and Liu, 2005), *Salmonella* Enteritidis (Schneid *et al*., 2005), *Salmonella* Typhi (Kumar *et al*., 2003) etc. In spite of their number of advantages over polyclonal antibodies, the monoclonal antibodies have few disadvantages as well.

The problems with monoclonal antibodies are, there is a need for a skilled technician and specialized growth apparatus for tissue culturing, and also expensive to produce. But recombinant antibodies fill this void since they can be produced in reasonable quantities in short periods of time from bacterial expression systems. While the immunological-based detection is not much specific and sensitive than nucleic acid-based detection, it is faster, and has the ability to detect not only contaminating organisms but also their biotoxins that may not be expressed in the organism's genome (Iqbal *et al*., 2000). Examples of immunological techniques include the enzyme immunoassay (EIA) (Borck *et al*., 2002), enzymelinked immunosorbent assay (ELISA) (Beumer and Brinkman, 1989; Mattingly *et al*., 1988; Palumbo *et al*., 2003), flow injection immuno-assay (Abdel-Hamid *et al*., 1999), enzyme-linked fluorescent assay (ELFA), bioluminescent enzyme immunoassay (BEIA) (Valdivieso-Garcia *et al*., 2003), enzyme-linked immunomagnetic chemiluminescence (ELIMCL) (Gehring *et al*., 2006), immunomagnetic separation (Hudson *et al*., 2001), agglutination test (Matar *et al*., 1997), radio-immunoassays (RIA), western blot test (Rasooly and Rasooly, 1998) and technically modified western blot include the line immunoassay (LIA) and the recombinant immunoblot assay (RIBA).

2.3.9 **Polymerase chain reaction (PCR)**

This assay is based on the ability of *Salmonella* specific primers, through complementary DNA base pairing, to anneal only to the target sequence. Thermostable DNA polymerase (*Taq* polymerase) recognizes the template primer complex as a substrate, which results in the simultaneous copying of both strands of the segment of DNA between the two annealed primers. The denaturation, annealing and elongation steps take place in a cyclical fashion, relying on the thermostability of the *Taq* polymerase, until the target sequence is amplified to detectable amounts (van der Zee and Huis, 2000). Before starting the first cycle in the thermocycler the DNA, primers, the polymerase, deoxynucleotide triphosphates (dNTPs), and a buffer are mixed in a reaction tube. The targeted region of the *Salmonella* genome is amplified by repetition of a three-step process:(a) Denaturation of the double-stranded DNA into single strands by heating (b) annealing specific, complementary, oligonucleotide primers to the single stranded DNA by cooling and (c) enzymatically extending the primers to produce an exact copy of the original double-stranded target sequence. This process from (a) to (c) is usually repeated in 30 to 40 cycles. In the final step the detection of the amplified target DNA is done using agarose gel electrophoresis. Before PCR it is often necessary to first grow the bacteria on an enrichment medium and then extract and purify the DNA (Hernandez *et al.*, 1995). Zhang and Weiner (2000) reported a method of bacterial DNA extraction from liquid media by using the cetyltrimethylammonium bromide (CTAB). PCR-based assays are the latest technique for the detection of pathogens including *Salmonella* in food samples (Nakano *et al.*, 2003).

2. 4 ***Escherichia coli***

Escherichia coli were first described in 1885 by Theodor Escherich. Escherich, a Bavarian paediatrician, had performed studies on the intestinal flora of infants and had discovered a normal microbial inhabitant in healthy individuals, which he named *Bacterium coli commune*. In 1919, the bacterium was renamed in his honour to *Escherichia coli* (Kaper, 2005).

The species *E. coli* comprises gram-negative, oxidase-negative straight cylindrical rods measuring 1.1-1.5 x 2.0-6.0 μm. They are aerobic and facultative anaerobic, motile by peritrichous flagella, or non-motile (Scheutz and Strockbine, 2005). *E. coli* can be characterized by serotyping, a method based on differences in antigenic structure on the bacterial surface. The serotype is defined by the bacterium's O-antigen (Ohne), a polysaccharide domain in the bacterium's lipopolysaccharide (LPS) in the outer membrane, and the H-antigen (Hauch) consisting of flagella protein. Serotyping may also include the K-antigen (Capsular) and the F-antigen (Fimbriae). There are many known O, H, K and F antigens and the existing number of different serotypes is known to be very high. Serotyping is an important tool which can be used in combination with other methods to distinguish pathogenic *E. coli* strains as specific pathogenicity attributes are often linked to certain serotypes. (Gyles, 2007; Kaper, 2005; Scheutz and Strockbine, 2005). *E. coli* can be recovered easily from clinical specimens on general or selective media at 37°C under aerobic conditions. *E. coli* in stool are most often recovered on MacConkey or Eosin methylene-blue agar, which selectively grow members of the *Enterobacteriaceae* and permit differentiation of enteric organisms on the basis of morphology(Balows *et al*., 1991).

Enterobacteriaceae are usually identified using biochemical reactions. These tests can be performed in individual culture tubes or by using test "strips" which are commercially available. Either method produces satisfactory results (Nataro and Kaper, 1998).

For epidemiologic or clinical purposes, *E. coli* strains are often selected from agar plates after presumptive visual identification. However, this method should be used only with caution, because only about 90% of *E. coli* strains are lactose positive; some diarrheagenic *E. coli* strains, including many of the EIEC strains, are typically lactose negative. The indole test, positive in 99% of *E. coli* strains, is the single best test for differentiation from other members of the *Enterobacteriaceae* (Nataro and Kaper, 1998).

2. 4.1 **Antibiotics Resistance of *E. coli***

Antimicrobial therapy is an important tool in reducing the incidence and mortality associated with avian colibacillosis (Freed *et al*., 1993; Watts *et al*., 1993). *E. coli* may be sensitive to many antibiotics. However, isolates of *E. coli* from poultry are frequently resistant to one or more antibiotics, especially if they have been widely used in poultry industry over a long time (e.g., tetracyclines) (Allan *et al*., 1993; Blanco *et al*., 1997; Watts *et al*., 1993). Antibiotics that were effective at controlling *E. coli* infections are no longer effective as the bacterium has acquired resistance to these compounds. Resistance to two or more classes of antibiotics is common in both veterinary (Gonzalez and Blanco, 1989; Harnett and Gyles, 1984; Irwin *et al*., 1989) and human medicine (Dennesen *et al*., 1998). Resistance usually occurs following response to prior contact with the antimicrobial but can occur naturally in the absence of previous exposure. Resistance to florfenicol an antibiotics related to chloramphenicol that has never been used in poultry in United States was found in *E. coli* isolates in chickens (Keyes *et al*., 2000). Antibiotic usage is possibly the most

important factor that promotes the emergence, selection and dissemination of antibiotics resistance micro-organism in both veterinary and human medicine (Neu, 1992; Witte, 1998). Possible harmful effects on humans through the use of drugs in agriculture are increased microbial drug resistance, drug residues in foods, allergic reactions and drug toxicity (Bazile-Pham-Khac *et al*., 1996). Antibiotic resistance and its transmission to human pathogens are important because these resistant bacteria may colonize the human intestinal tract and may also contribute resistance genes to human endogenous flora.

Colonization of the intestinal tract with resistant *E. coli* from chicken has been shown in human volunteers (Linton *et al*., 1977). The result of a study carried out by Van den Bogaard *et al.* (2001) indicates that transmission of resistant clones and resistant plasmids of *E. coli* from poultry to humans occurs. Furthermore, in some previous studies, spread of an antibiotic resistance plasmid, pSL222-6, in *E. coli* from chickens to human handlers (Levy, 1976), finding the same O serotype in chickens from a commercial rearing centre, in oven ready birds and in humans (Linton *et al*., 1977), direct transmission of *E. coli* resistant to streptomycin, sulphonamides and tetracycline from poultry to poultry attendants in Nigeria (Ojeniyi, 1985, Ojeniyi, 1989), evidence that animals are a reservoir for *E. coli* found in humans (Cooke, 1971), chickens as a source of antibiotic resistance in humans in Saudi Arabia, Morocco and Northern India (Al Ghamdi *et al.,* 1999; Amara *et al*., 1995; Singh *et al.,* 1992) was described. In contrast, others have concluded that human and poultry isolates belong to two distinct pools of resistant *E. coli* (Bass *et al*., 1999; Caya *et al.,* 1999; Kariuki *et al*., 1997; Nijsten *et al.*, 1995; Parsonnet and Kass, 1987). Fluoroquinolones are highly efficacious antimicrobial agents and because of their low toxicity and relatively broad-spectrum coverage, are extremely valuable for treating human

infections (Angulo, *et al.,* 2000; Livermore *et al.,* 2002). In the past years they have been used in treatment of certain bacterial diseases in animals, including acute bovine respiratory disease and avian colibacillosis (Hooper, 2001; White *et al.,* 2000; Yang *et al.,* 2004). Some studies have reported an association between the emergence of fluoroquinolone-resistant zoonotic pathogens, such as *Salmonella*, *E. coli*, and *Campylobacter*, and the subsequent approval and use of these agents in veterinary medicine (Blanco *et al.,* 1997; Guerra *et al.,* 2003; Saenz *et al.,* 2003; Yang *et al.,* 2004).

2.4.2 **Serotyping of *E. coli***

A specific combination of O and H antigens defines the "serotype" of an isolate. *E. coli*. According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Edward and Ewing 1986; Lior 1996). A total of 170 different O antigens, each defining a serogroup, are recognized currently. The presence of K antigens was determined originally by means of bacterial agglutination tests: an *E. coli* strain that was inagglutinable by O antiserum but became agglutinable when the culture was heated and was considered to have a K antigen. The discovery that several different molecular structures, including fimbriae, conferred the K phenotype led experts to suggest restructuring the K antigen designation to include only acidic polysaccharides (Lior, 1996). Proteinaceous fimbrial antigens have therefore been removed from the K series and have been given F designations (Orskov *et al*., 1982).

Specific serogroups can be associated reproducibly with certain clinical syndromes, but it is not in general the serologic antigens themselves that confer virulence. Rather, the serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Whittam *et al*., 1993).

Chapter 3 MATERIALS AND METHODS

3.0 **Materials**

The following materials were used in this study;

Tetrathionate broth Base CM0029 (OXOID), xylose lysine deoxycholate Agar VM603087618 (MERCK), Nutrient Agar, Triple Sugar Iron Agar (OXOID), Urea Agar Base Cm0053 (OXOID), M.R.V.P CM0043 (OXOID), MacConkey agar, Sterile normal saline, Stomacher Blender 400 (Model No. BA 6021), Distilled Water, Sterile swab sticks, cotton wool, bijou bottles, universal bottles, Petri dishes, Pasteur pipettes, polythene bags, peptone water Microbact Reagent Set D MB1082A (OXOID), Microbact Reagent Nitrate A MB0186A (OXOID), Microbact Reagent Nitrate B MB0187A, Microbact GNB 24E, Salmonella Test kit (OXOID DR1108A), Refrigerator, Test Tubes, adonitol, mannitol, arabinose, rhamnose, dulcitol, meso-inositol, sucrose, lactose, glucose, d-sorbitol, salicin, raffinose, arginine, lysine, ornithine, Oxoid antibiotics disks and Oxoid antibiotics dispense.

3. 1 **Methods**

3.1.1 **Study area**

Sampling for this study covered four Local Government Areas (LGAs) in Kaduna State which includes Kaduna North, Kaduna South, Sabon Gari and Zaria. The LGA were selected by convenience.

3.1.2 **Geographical location of Kaduna state**

Kaduna State is located within the semi-arid and sub-humid regions of the north western zone of Nigeria. It lies between longitude E 006.5^0-E 008.6^0 (East of Greenwich meridian) and latitude N 09.2^0-N 11.3^0 (North of equator).

3.1.3 **Assessment of management practices**

Questionnaires were designed and administered to farms within the LGAs sampled. This was carried out in order to determine the biosecurity and other management practices carried out on the farms that could promote or hinder the occurrence of *Salmonella* in eggs. Information sought from the farms include system of management, source of birds, number of birds, age of birds, week in lay, percentage production, source of feed, inclusion of additives, source of drinking water, availability of foot bath, disposal of dead birds, presence of pests, other animals on the farm, record of disease outbreak and control measures to prevent outbreak (Appendix I).

3.1.4 **Sample size**

The sample size was determined by using the formula outlined by Mahajan (1997).

$$N = \frac{Z^2 Pq}{L^2}$$

Where Z = 1.96

P = prevalence (37.1%)

q = 1- P

L^2 = allowable error (5%)

N = 358

3.1.5 **Sampling**

The number of farms in each LGA to be sampled were not equal, so proportionate probability sampling technique was used i.e. 25% proportion of registered farms in each of the LGAs were selected for sampling. There are 90 registered farms in these LGAs and this gave a total of 23 farms to be sampled. Two major commercial egg depots were also sampled from each LGA making a total of eight collection points where eggs were obtained from major egg marketers. Two major slaughter slabs were visited in Kaduna and Zaria. Sampling was carried out every two weeks for three months.

Cloacal swabs were collected from ten birds using a sterile swab in each of the farms visited and these were placed in sterile universal bottles containing 20 ml peptone water. Ten eggs were also bought from each farm; all the egg samples from each of the farms and markets were pooled in tens to form a sample. Ovarian follicles were collected from slaughtered hens using a sterile polythene bag.

All the samples were transported to the Bacterial Zoonoses Laboratory of Department of Veterinary Public Health and Preventive Medicine in sterile polythene bags.

3.1.6 **Preparation of Media**

Each medium used was prepared from commercially available powder according to the Manufacturer's instructions. Sterilization was by autoclaving at 121℃ for 15 minutes. Pippetes and petri dishes were packed in canisters and sterilized in hot air oven at 160℃ for 60 minutes. Aseptic conditions were maintained throughout sample analysis in order to prevent contamination of the samples and media.

3.1.7 **Sample processing**

3.1.8 **Isolation of Salmonellae from samples**

The egg shells were disinfected with 70% ethanol, the taper end of each egg was broken aseptically using a thumb forceps. The contents of the pool of ten eggs i.e. egg yolk and egg white were emptied into a sterile polythene bag and homogenized using a stomacher. 10ml of the homogenate was inoculated into 90 ml of tetrathionate broth enrichment medium and incubated at 37 ℃ for 24 hours.

Cloacal swab samples in peptone water which served as pre-enrichment medium were incubated at 37 °C for 24 hours. 1ml of the pre-enrichment broth was inoculated into 9ml of tetrathionate broth and incubated at 37 °C for 24 hours.

Ovarian follicles were weighed and 1g of each was decontaminated by dipping into boiling water for 5-10 seconds. They were then chopped into tiny pieces using sterile scissors and forceps and each added to 9 ml tetrathionate broth and incubated at 37 ℃ for 24 hours.

3.1.9 **Selective plating and identification of *Salmonella* isolates**

A loopful of the inoculum from each enrichment broth above was transferred onto xylose lysine deoxycholate agar. The inoculum was streaked on the agar and incubated at 37 ℃ for 24 hours. On this medium, *Salmonella* colonies appeared as pinkish colonies with or without dark centre. All non-lactose fermenting organisms were picked from the plate and inoculated into nutrient agar slants, incubated at 37 ℃ for 24 hours, and stored in a refrigerator at 4°C pending further studies. The stored slants were considered to be presumptive isolates of *Salmonella*.

3.1.10 **Preliminary and complete biochemical testing**

All agar-based media and substrates were prepared according to the Manufacturer's instructions. Sugars and amino acids were also prepared using standard procedures. The purity of the isolates were ascertained by plating on xylose lysine deoxycholate agar before stabbing and streaking on triple sugar iron agar and urease agar slants.

Colonies were picked and streaked on triple sugar iron agar and urease agar slopes while the butts of TSI agar were stab-inoculated. These were incubated at 37°C for 24 hours. *Salmonella* suspected organisms gave alkaline over acid reaction in TSI agar slants, with or without H_2S and gas respectively. A positive urease test was indicated by pinkish discolouration of the slant.

Complete biochemical testing of the isolates were carried out using the following sugars; salicin, mannitol, rhamnose, meso-inositol, adonitol, arabinose, raffinose, glucose, sucrose, lactose, sorbitol and dulcitol. The following amino acids were also tested for decarboxylation, arginine, lysine and ornithine.

3.1.11 **Sugar fermentation and amino acid utilization test**

The sugars were tested by using standard procedures with bromothymol blue as the indicator. The amino acids were prepared at 1% concentration and 1ml of sterile paraffin added to each tube. Bromocresol-purple was used as indicator for amino acid utilization. These were inoculated with the test organisms and incubated at 37°C for four days and checked for colour change (Cowan and Steel, 1993).

Changes in colour from green to yellow for the sugars indicated a positive reaction, while the green colour indicates a negative reaction. Amino acid utilization tests were performed

using the modified Falkows methods. Using sterile wire loop, tubes containing 1% concentration of lysine, ornithine and arginine broth overlaid with 1ml sterile paraffin were inoculated with the test organisms and incubated at 37°C for four days and examined daily for colour changes from yellow to purple for positive test results.

3.1.12 **Identification of *Salmonella* using Microbact GNB 24E**

Prior to testing, all isolates were streaked on xylose lysine deoxycholate agar plates and incubated at 37°C for 24 hours. Sterile normal saline was prepared and 5ml dispensed into each test-tube. Using a sterile loop, 1-3 colonies of the culture were picked and emulsified in the 5ml sterile saline, and mixed thoroughly to prepare a homogenous suspension to yield a turbidity equivalent to 0.5 McFarland's standard. The wells of the individual substrate sets was exposed by cutting the end tag of the sealing strip and slowly peeled backward. The plate was placed in a holding tray and using a sterile Pasteur pipette, four drops of the bacterial suspension was added to each well set.

Using a sterile pipette the substrates underlined on the holding tray were overlayed with mineral oil i.e. wells 1, 2, 3, 20 and 24. The inoculated rows were resealed with the adhesive seal. The specimen number was written on the end tag with a marker pen. The plate was incubated at 37°C for 24 hours. The tray was removed from the incubator after 24hours, the adhesive seal peeled; Nitrate, Kovacs, VP and TDA reagents were added to wells 7, 8, 10 and 12 respectively. The results were interpreted as stipulated by the Manufacturerers, using the supplied software version Microbact^{TM} 2000 Identification package V2.03 (Windows^{TM}).

3.1.13 **Identification of *Salmonella* strains**

Isolates suspected to be *Salmonella* were serologically tested using *Salmonella* polyvalent 'O' group A-Z antiserum latex kit according to the instruction of the Manufacturer (OXOID). All isolates were streaked on xylose lysine deoxycholate agar plates and incubated at 37℃ for 24 hours. Latex reagents were brought to room temperature, one drop of the test latex reagent was dispensed onto a circle on the reaction card, and a drop of saline was placed on the circle distant from the latex. Using a loop, a portion of the colony of presumptive *Salmonella* spp on xylose lysine deoxycholate agar plates was emulsified in the saline drop on a portion of the circle on the card. The test latex and the resulting smooth suspension were mixed together and spread to cover the reaction card using the loop. The card was then rocked in a circular motion observing for agglutination within two minutes.

3. 1.14 **Isolation of *E. coli* from eggs**

One hundred pooled egg samples were collected from the farms and tested for *E. coli*. The egg shells were disinfected with 70% ethanol, the taper end of each egg was broken aseptically using a thumb forceps. The contents of the pool of ten eggs i.e. egg yolk and egg white were emptied into a sterile polythene bag and homogenized using a stomacher. The homogenate was serially diluted by ten (10) fold into universal bottles containing sterile normal saline i.e.1 ml of the homogenate was inoculated into 9 ml sterile normal saline.

3.1.15 **Total aerobic counts**

The diluted sample (0.1ml) from 10^{-1} dilution was aseptically transferred into nutrient agar plates. The inoculum was spread using an aseptic glass rod spreader. The plates were incubated at 37℃ for 24 hours. Total aerobic counts were determined and recorded from nutrient agar plates.

3.1.16 **Total coliform counts**

The diluted sample (0.1ml) from 10^{-1} was aseptically transferred into MacConkey agar plates. The inoculum was spread using an aseptic glass rod spreader. The plates were incubated at 37℃ for 24 hours. Total coliform counts were made from the MacConkey agar plates. Lactose fermenting organisms from the MacConkey agar plates were identified and picked from the plates and inoculated into nutrient agar slants incubated at 37℃ for 24 hours, and thereafter stored in a refrigerator at 4℃ pending further studies.

3.1.17 **Preliminary and Complete Biochemical Testing**

All agar based media and substrates used were prepared according to Manufacturer's instructions. The sugars and amino acids were also prepared using standard procedures. The purity of the isolates was ascertained by plating on MacConkey agar before stabbing and streaking. Colonies were picked and inoculated on triple sugar iron agar, urea agar, Simmons citrate agar, S.I.M medium and M.R.V.P broth as reported above.

Complete biochemical testing of the isolates were carried out using the following sugars, salicin, mannitol, rhamnose, meso-inositol, adonitol, arabinose, raffinose, glucose, sucrose, lactose, sorbitol. Amino acids tested for decarboxylation included arginine, lysine and ornithine. Nitrate reduction was also tested.

3.1.18 **Sugar fermentation and amino acid utilization test**

The sugars were tested using standard procedures and bromothymol blue was used as indicator. Similarly the amino acids were prepared at 1% concentration and overlaid with 1ml of sterile paraffin added to each tube. These were inoculated with the test organism and incubated at 37℃ for four days and observed for colour changes (Cowan and Steel, 1993).

Changes in colour from green to yellow for the sugars indicated a positive reaction, while green colouration indicated a negative reaction. Colour changes from yellow to purple for positive test results for amino acids.

3.1.19 **Antimicrobial susceptibility testing of bacterial isolates**

This was performed using a panel of 12 antimicrobial agents by disk diffusion method following CLSI guidelines (CLSI 2002) and cultured on Mueller Hinton agar.

Sterile nutrient broth was prepared according to the Manufacturer's instruction; the test isolates were inoculated into the broth and incubated at 37°C for 24 hours. The broth culture was adjusted with sterile saline to obtain turbidity optically comparable to 0.5 McFarland standards. Mueller Hinton agar was inoculated with 0.1 ml of the nutrient broth culture and spread over the entire sterile agar surface. The drug impregnated disks used (OXOID) contained Sulphamethoxazole/trimethoprim (25 μg), Lincomycin (10 μg), Nitrofurantoin (50 μg), Gentamicin (10 μg), Amoxicillin/clavulanic acid (50 μg), Ciprofloxacin (5 μg), Chloramphenicol (30 μg), Kanamycin (30 μg), Ampicillin (10 μg), Streptomycin (10 μg), Tetracycline (30 μg) and Penicillin G(10 units) were placed individually on the surface of inoculated agar plates using a dispenser (OXOID) and incubated at 37°C for 18 hours. The zones of inhibition were measured to the nearest millimeter using a ruler.

3.2 **Statistical Analysis**

Data was organized in Microsoft excel and the general descriptive analaysis and coefficient correlation was used to analyse occurrence and extent of factors and the statistical

relationship using Microsoft excel and statistical package for social science (SPSS) windows 16.0 (Standard Version SPSS Inc., Chicago, IL, USA).

Chapter 4 RESULTS

4.1 Result of evaluation of farm management practices

The distribution of management systems practiced by the poultry farmers, sources of birds and number of birds are provided in Table 4.1 A total of 41 (82%) of the farms visited practiced deep litter system, while 9 (18%) of the farms practiced battery cage system. 49 (98%) of the farms obtained their birds from commercial hatcheries, while only 1(2%) got their birds from a different farm at point of lay. About 39 (78%) of the farms visited had less than 2500 birds, while 7 (14%) farms had more than 2500 - 5000 birds on their farms, 2 (4%) farms had greater than 5000 -7500 birds on their farms.

Table 4.2 shows the age of birds, number of weeks in lay and the percentage production. It was found that 5 (10%) of farms had birds within the age range of 20-40 weeks old, while 33 (66%) of the farms had birds within the age range of 40-60 weeks old and 12 (24%) farms had birds within the age range of 60-80 weeks old. Also 6(12%) of the farms were within 10-20 weeks in lay, 18(36%) were above 20-30 weeks in lay, 15(30%) of the farms were above 30-40weeks in lay, 7(14%) of the farms were greater than 40-50weeks in lay and 4(8%) of the farms were above 50-60weeks in lay. About 29(58%) of the layers were producing below 70%, 14(28%) of the farms were producing about 70-80% and 7(14%) of the farms were producing more than 80%.

Table 4.3 shows the sources of feeds, inclusion of feed additives and sources of drinking water for the farms. About 30 (60%) of the farms placed their birds on commercial feeds, while 20 (40%) of the farms compounded their feeds. 12 (24%) of the farms included additives to the feeds while there was no additives used in 38 (76%) of the farms; 29 (58%)

of the farms used bore-hole as their source of drinking water, 12 (24%) used pipe-borne water while the remaining 9 (18%) used well water. Table 4.4 shows the use of foot bath, various methods of disposal of dead birds, presence of pests and other animals on the farms. The data showed that 33 (66%) of the farms used foot bath on their farms while 17 (34%) did not use foot bath on their farms; 18 (36%) of the farms burnt their dead birds, 26 (52%) buried dead birds around the farm and 6 (12%) of the farms prepared dead birds for guard dogs. The results showed that 33(66%) of the farms had pests and 17 (34%) did not have pests on their farms; 22 (44%) of the farms had other animals, while 28 (56%) did not have other animals on the farms.

Table 4.1: Management system practiced, source of and no of birds in the farms surveyed

Description	Frequency	Percent
System of management		
Deep litter	41	82.0
Battery cage	9	18.0
Total	50	100.0
Source of birds		
Bought at point of lay	1	2.0
Commercially hatched	49	98.0
Total	50	100.0
Number of birds		
< 2500	39	78.0
>2500-5000	7	14.0
>5000-7500	2	4.0
>7500-10,000	2	4.0
Total	50	100.0

Table 4.2: Distribution of birds by age, week in lay and percentage production

Description	Frequency	Percent
Age of birds (Weeks)		
>20-40	5	10.0
>40-60	33	66.0
>60-80	12	24.0
Total	50	100.0
Week in lay		
<20-30	6	12.0
>20-30	18	36.0
>30-40	15	30.0
>40-50	7	14.0
>50-60	4	8.0
Total	50	100.0
Percentage production		
<60-70	29	58.0
>70-80	14	28.0
>80-90	7	14.0
Total	50	100.0

Table 4.3: Distribution of sources of feed, drinking water and inclusion of feed additives

Description	Frequency	Percent
Source of feed		
Locally compounded	20	40.0
Commercial	30	60.0
Total	50	100.0
Inclusion of additives		
Yes	12	24.0
No	38	76.0

Total	50	100.0
Source of drinking water		
Well	9	18.0
Bore-hole	29	58.0
Pipe-borne	12	24.0
Total	50	100.0

Table 4.4 Use of foot-bath, disease outbreak, and disposal of dead birds, presence of pest and other animals on the farm

Description	Frequency	Percent
Present of Footbath		
Yes	33	66.0
No	17	34.0
Total	50	100.0
Disease outbreak		
Yes	13	26.0
No	37	74.0
Total	50	100.0
Disposal of dead birds		
Burning	18	36.0
Burrying	26	52.0
Prepared for dogs	6	12.0
Total	50	100.0
Presence of pest		
Yes	33	66.0

No	17	34.0
Total	50	100.0
Other animals		
Yes	22	44.0
No	28	56.0
Total	50	100.0

Figure 4.1 shows the distribution of antibiotics used in the farms surveyed. Tetracycline was the most commonly used drug; 45 (90%) of the farms used tetracycline. Other drugs that were used included chloramphenicol in 15 (30%) of the farms, neomycin in 18 (36%) of the farms, erythromycin in 18 (36%) of the farms, ciprofloxacin in 6 (12%) of the farms, enrofloxacin in 5 (10%) of the farms and gentamicin 3(6%) of the farms. There was a negative correlation between disease outbreak and antibiotics use ($r = -0.35509$).

Figure 4.2 shows the distribution of farms and number of antibiotics used. Seventeen farms (34%) used one antibiotic, 7(14%) of the farms used 2 antibiotics, 21 (42%) of the farms used 3 antibiotics and 4 (8%) of the farms used 4 antibiotics.

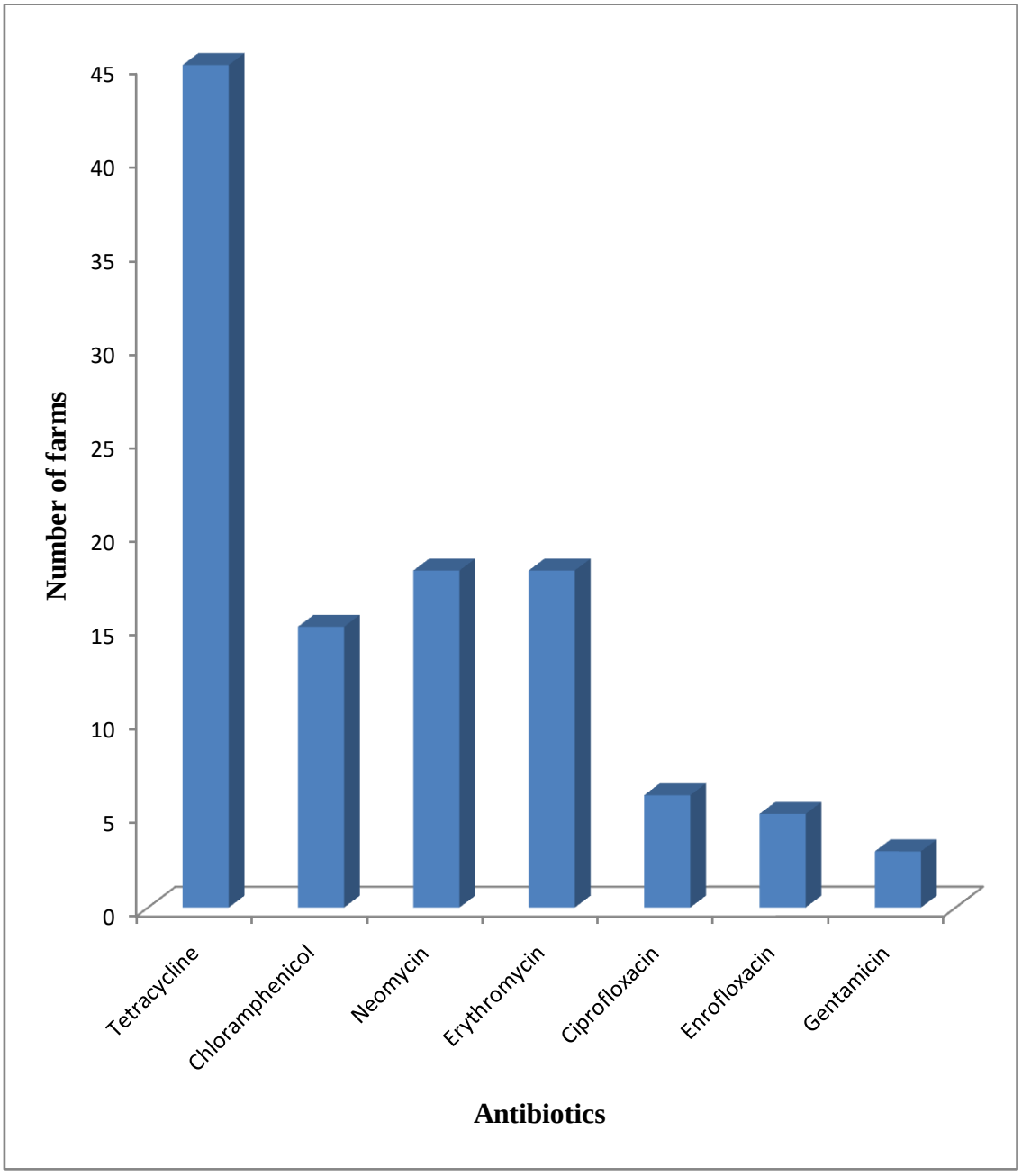

Figure 4.1: Antibiotics usage in the farms surveyed

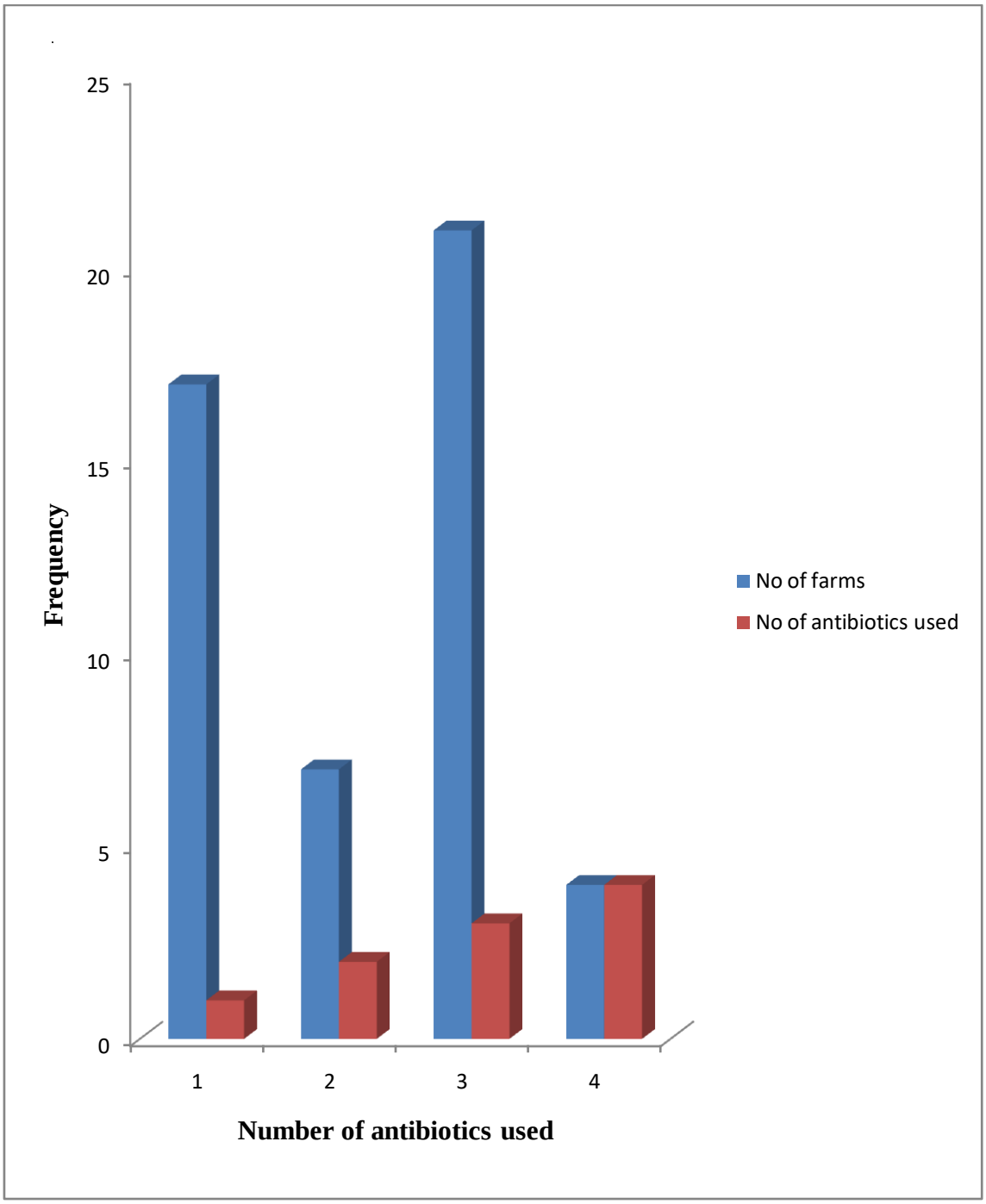
25
20
15
10
5
0
Frequency
1
2
3
4
Number of antibiotics used
No of farms
No of antibiotics used

Figure 4.2: Number of antibiotics used in the farms surveyed

4. 2 **Frequency of isolation of bacteria from eggs**

A total of 3820 pooled egg samples were analyzed for the presence of *Salmonella*. There were 12 suspects, consisting of 3 egg samples from Kaduna north LGA, 1 sample from Kaduna south LGA and 8 from Sabon-gari LGA. None of the isolates was identified as *Salmonella*. The following organisms were found *Serratia plymuthica, Serratia liquifaciens* and *Enterobacter agglomerans.*

4. 3 **Frequency of isolation of bacteria from cloacal swabs and ovarian follicles**

A total of 114 cloacal swab and 140 ovarian follicle samples were analyzed for the presence of *Salmonella*. There were 21 suspects, 1 cloacal swab sample from Kaduna north LGA, 10 from Sabon-gari LGA, 2 from Zaria LGA and 8 ovarian follicle samples from Sabon-gari LGA. No *Salmonella* was isolated from these samples.

4. 4 **Total aerobic counts, total coliform counts and isolation of *E. coli.***

A total of 100 pooled eggs were tested from farms in the four LGAs. Twenty six pooled samples had aerobic growth and 21 had coliforms. *E. coli* was identified from the coliforms. There were 3 isolates of *E. coli*. There was no significant difference ($P > 0.05$) in the mean total aerobic counts and coliform counts (Table 4.5). There was a negative correlation of the total aerobic and coliform counts ($r = -0.028$) (Table 4.6). There was a negative correlation of the total aerobic counts and antibiotics usage ($r = -0.05472$), and correlation of coliform counts and antibiotics use was also negative ($r = -0.187$).

4. 5 **Antimicrobial susceptibility of bacterial isolates**

Table 4.5 shows the sample identification, location of farm, antibiotics used on the farm and resistance profile of *Serratia plymuthica*, *Serratia liquefaciens* and *Enterobacter agglomerans* and the three *E. coli* isolates.

4.6 **Diameter of growth inhibition (mm) of antimicrobials**

Table 4.6 shows the diameter of growth inhibition of the antimicrobial agents tested in milimeter.

4.7 **Antimicrobial break points**

Table 4.7 shows the antimicrobial agents, disc content, and zone diameter to the nearest whole milimeter.

Table 4.5 Antimicrobial drug susceptibilities

Sample ID	Isolate	Location of Farm	Antibiotics Used on the farms	Resistance profile of isolates
EX7	*Serratia plymuthica*	Samaru	ENR, TE	AMC, AMP, MY P
EX8	*Serratia liquefaciens*	Samaru	C, CIP, TE	AMP, MY, P, S, TE
EX10	*Enterobacter agglomerans*	Samaru	C, ENR, TE	AMC, AMP, MY,P, TE
KS3E2	*Escherichia coli*	Kaduna South	TE	C, MY, P, TE
KN3E2	*Escherichia coli*	Kaduna North	TE	C, MY, TE
OKE2	*Escherichia coli*	Samaru	C, TE	C, MY, P, TE

Table 4. 6 Diameter of growth inhibition (mm) of antimicrobials

Antibiotics/Isolates	KS3E2	KN3E2	OKE2	EX7	EX8	EX10
Amoxicillin and Clavulanic acid	20	30	24	11	19	10
Nitrofurantoin	15	22	16	17	17	10
Sulphamethoxazole/Trimethoprim	19	21	20	29	28	20
Gentamicin	22	20	23	20	17	25
Ciprofloxacin	26	30	26	31	29	22
Ampicillin	21	34	22	0	0	0
Chloramphenicol	11	8	12	27	21	21
Tetracycline	0	9	0	20	10	12
Kanamycin	21	17	24	19	20	22
Lincomycin	0	9	0	0	0	0
Streptomycin	13	20	15	16	10	18
Penicillin G	9	36	11	0	0	0

KS = Kaduna south

KN = Kaduna north

EX = Samaru market

OKE = Okey farms, Samaru

Table 4.7 Antimicrobial break points

Antimicrobial agent	Disk content	Zone Diameter, Nearest whole mm		
Amoxicillin /Clavulanic acid	30 µg	R ≤13	I 14 – 17	S ≥ 18
Nitrofurantoin	50 µg	≤14	15 – 16	≥17
Sulphamethoxazole/Trimethoprim	25 µg	≤10	11 – 15	≥16
Gentamicin	10 µg	≤12	13 -14	≥15
Ciprofloxacin	5 µg	≤15	16 -20	≥21
Ampicillin	10 µg	≤13	14 – 16	≥17
Chloramphenicol	30 µg	≤12	13 – 17	≥18
Tetracycline	30µg	≤14	15 – 18	≥19
Kanamycin	30 µg	≤13	14 – 17	≥18
Streptomycin	10 µg	≤10	12 -14	≥15
Penicillin G	10 I.U	≤20	21-25	≥26

R= Resistant, I= Intermediate and S= Sensitive.

Chapter 5 DISUCUSSION

This study investigated the occurrence of *Salmonella* and *E. coli* in commercial eggs and assessed the management practices carried out on 23 farms in Kaduna State that could influence the occurrence of *Salmonella*. Majority of the farms surveyed were small to medium scale and the management system commonly practiced was deep litter. This is a reflection of the predominance of small to medium sized flocks within the Nigerian poultry production system (FAO, 2006). Generally large scale farmers were found to practice strict biosecurity measures and reluctant to allow access to their farms. Similar findings were reported by Akidarju *et al.* (2010) in Maiduguri where 82.7% of the farmers practiced deep litter and Ovwigho *et al*. (2009) in Delta state where very few farms practiced battery cage system of management. Small scale farms are characterized by low levels of biosecurity and are more proned to the introduction of *Salmonella* infections (Akidarju *et al*., 2010).

There was low level of biosecurity in most of the farms surveyed in this study. Drinkers and feeders were not cleaned regularly; most farms indicated that the drinkers and feeders were washed once in a week, litter was not changed frequently, some farms buried dead carcasses around the farm, and most of the personnel in the farms did not use protective clothing. Some of the farms compounded their feed locally; which can also be a possible source of *Salmonella* infection in poultry. Some farms (66%) reported pests such as rodents and lizards. Rodents have been reported to be mechanical transmitters of *Salmonella* in poultry farms (Valiente *et al.*, 2009). This low level of biosecurity is characterized by poor hygiene and unclean environment and utensils that would allow rapid spread of infection once introduced. Rapid spread could occur through drinkers contaminated with faecal material from infected birds (Jafari *et al*., 2006; Babiker *et al.*, 2009). Sometimes the contamination of water could occur indirectly from contaminated litter (Sasipreeyajan *et*

al., 1996). Eventually the whole flock and the environment becomes infected and contaminated respectively with *Salmonella*. It is difficult to establish how *Salmonella* enters the flock particularly in the farming environments of developing countries such as Nigeria. It is safer to assume that new birds, farm inputs such as feed, water, personnel and pests are all potential sources of infection. Several farms compounded their own feeds partly in response to increasing cost of poultry feed in Nigeria. Some of the feed ingredients are derived from abattoir by-products produced in suspicious environments and conditions that could allow the transmission of *Salmonella* infection in the feed. Various studies around the world have identified feed as an important source of *Salmonella* (Alvarez *et al*, 2003; Charlotta *et al*., 2004; Maciorowski *et al*., 2000; Grey *et al.,* 1996). Although raw materials used for the preparation of feed may harbour the pathogen, pelleting, heating and other specific treatments such as adding volatile fatty acids to feeds are generally successful in eliminating *Salmonella* (Al-Natour and Alshawabkeh, 2005). Such technologies are not common within the Nigerian poultry industry. The final feed may be re-contaminated and hence allowing *Salmonella* multiplication during transportation or during storage at the farm (Gast, 2003). Feed may serve as a reservoir that may contaminate incoming flocks. Between 1 and 5 % of all animal feed produced and 31% of all animal by-products may be contaminated with *Salmonella* spp (Maciorowski *et al*., 2000; Khalil *et al*., 2006).

One alternative that is rapidly gaining popularity amongst feed millers in Nigeria is the use of fish protein sources. Many farmers are integrating poultry and fish production to optimize production. Previous studies such as that of Abdel- Hamid *et al* (1985) recovered 6 isolates of *Salmonella* from 37 (16.2%) imported broiler fish meal. Similarly Veldman *et al* (1995) reported that mash feed used for layer breeders contained fish and bone meal and

were more frequently contaminated with *Salmonella*. Interest in the contamination of feed components with *Salmonella* tends to be focused on raw materials of animal origin. These findings confirm that salmonellae are ubiquitous and that the control of *Salmonella* in feedstuffs has to be extended to raw materials of plant origin as those of animal origin (Harris *et al*., 1997). The type of feed ingredients used in compounding feed within the farms in this study were not investigated, but there is sufficient reason to be concerned about these practices being a source of *Salmonella* to poultry in Nigeria. A key strategy employed by farmers and feed millers in minimizing inherent risks of infection due to feed is by using feed additives.

Feed additives are included in diets for poultry in order to increase production by improving nutrient availability. Antibiotics as feed additives act as growth promoters by preventing disease occurrence thus improving the efficiency of animal production. Antibiotics have been used in livestock farming for several decades in combating bacterial infections. The use of antibiotics as growth promoter in diets for layers has been discouraged. In the European Union as in many developed countries, the inclusion of antibiotics in animal feed is forbidden because residues of these drugs may appear in eggs and thus constituting potential health hazards to the consumers in addition to risks of promoting antibiotics resistance (Schothrost et al., 1978; DuPont and Steel 1987; Lu *et al*., 2004).

In this study, 24% of the farms were found to use feed additives; farms that compounded their feed locally reported the addition of antibiotics as feed additives. Also related to the use of antibiotic feed additives, the farms studied in this work were also found to be widely and frequently using antibiotics. In particular tetracycline, erythromycin, neomycin and

chloramphenicol were misused in most of the farms surveyed. It was a common practice by farmers in the study area to administer drugs without consulting a veterinarian and the drug withdrawal period was not observed.

The level of *Salmonella* contamination in table eggs was determined in this study. *Salmonella* was not isolated from eggs in this study; this may not be unconnected with the gross misuse of antibiotics in the study area. A similar finding was also reported by Ansah *et al*., (2009) in Ghana. In that study *Salmonella* was not isolated from eggs due to multiple antibiotics use; however other bacterial genera isolated in that study included *Streptococcus*, *Staphylococcus*, *Escherichia coli*, *Corynebacteria*, *Micrococcus*, *Diplococci*, and Gram negative bacilli.

The use of antibiotics could have interfered with the isolation of *Salmonella* from cloacal swabs due to intermittent bacterial excretion or antibiotics use in feed and water or as a prophylactic treatment. Betancor *et al*. (2010) reported a similar finding; in which they also failed to isolate *Salmonella* from cloacal swabs that were also attributed to antibiotic suppression. Raufu *et al.* (2009) reported a 23% prevalence of *Salmonella* from cloacal swabs which are at variance with our study.

Despite the fact that *Salmonella* may occur due to well known factors especially the low level of biosecurity that was observed in the study area, no *Salmonella* was isolated in this study from all the samples investigated.

In this study, 21 of the samples were positive for coliforms and 26 were positive for aerobic bacterial growth. *E. coli*, *Serratia liquefaciens*, *Serratia plymuthica*, and *Enterobacter agglomerans* were isolated even though in very few numbers perhaps may be due to the

pressure of antibiotic use. The bacterial isolates were resistant to tetracycline, chloramphenicol, lincomycin, ampicillin, amoxicillin and clavulanic acid, streptomycin and peniciliin G. These multiple resistance was an indication of the need for the control of excessive antibiotics use. When the intensity of antibiotics use was compared with the aerobic and coliform counts in the eggs tested in this study, a negative correlation was seen suggesting that the more the antibiotic use, the less the bacterial counts in the eggs. Though not categorically determined, there is greater tendency for the aerobic and coliform bacteria from the eggs to be highly resistant to antibiotics.

Salmonella was reported to have been isolated from yolk and albumin of eggs (Cox *et al.*, 2005) but their numbers must be large enough to survive the antibiotic pressure. Davies and Breslin (2000) reported an overall minimum contamination of combined shell and content of eggs to be 0.24%. Miyamato *et al*. (1997; 1998) also reported that *Salmonella* can ascend from the cloaca through the vagina into the lower part of the oviduct and subsequently contaminate the egg.

Table 4.5 shows that all three *E. coli* isolates were all resistant to tetracycline, chloramphenicol and lincomycin. This was in contrast to the study of Shtylla *et al* (2009) in Albania who reported that the highest level of resistance was observed for erythromycin (100%), amoxicillin (99.1%) and tetracycline (96.07%). It was also in contrast with the report of Mohammed *et al* (2009) in Bangladesh who reported a resistance rate of 30% for chloramphenicol and 52% for tetracycline from poultry and the report of Anyanwu *et al* (2010) in Jos who observed that *E. coli* were sensitive to chloramphenicol for samples collected from day old chicks. The result of the present study is similar to the report of Okoli *et al.* (2005) and Chah *et al.* (2000) in Owerri who also recorded one hundred percent

resistance to tetracycline, chloramphenicol, nitrofurantoin, cotrimoxazole and ampicillin and 93.3%, 90.0%, 70.0% and 60% resistance to ampicillin, tetracycline, chloramphenicol, and nitrofurantoin respectively for isolates from commercial layers. These organisms may constitute enormous reservoirs of genes encoding resistance against these antibiotics and foci for continual spread of these mechanisms (Stern, 1992; On *et al*., 1998). Though the isolates were from different areas, multi drug resistance (MDR) was a common feature in these isolates, highlighting the fact that the resistance genes for these drugs may be linked on plasmids (Schroeder *et al*., 2002).

In poultry farms, wild birds and rodents consume antibiotic containing feed or poultry droppings contaminated with multi-antibiotic-resistant *E. coli*. Down the line of the food chain, predatory animals such as stray dogs and snakes also feed on rodents, escaped chicken and chicken carcasses (Krumperman, 1983). These feeding behaviours can also cause the spread and maintenance of multi-antibiotic-resistant *E. coli* in the environment. Raw chicken manure also used as fertilizer for crops further contaminate the environment with multi- antibiotic-resistant organisms which can also be picked up by other animals (Saleha *et al*., 2009). In this study all the farms that were surveyed sold their litter to crop farmers for use as manure which may serve as a source of muti- antibiotic- resistance in the environment as mentioned above.

Chapter 6 SUMMARY, CONCLUSIONS, RECOMMENDATIONS

6. 1 Summary

This study assessed the biosecurity and other preventive measures carried out on poultry farms. Deep litter system of management was commonly practiced and there was documentation of multiple antibiotics usage in feed. There was low level of biosecurity in most of the farms surveyed. A total of 3820 pooled egg, 114 cloacal and 140 ovarian follicle samples were tested for *Salmonella*. *Salmonella* was not isolated in any of the samples. Other organisms were isolated such as *Serratia plymuthica, Enterobacter agglomerans, and Serratia liquefaciens*. Also, 100 pooled egg samples were analyzed for total aerobic counts, total coliform counts and *Escherichia coli.* Of the 100 pools, 3 were positive for *E. coli* for which antimicrobial susceptibilities were tested using a panel of 12 antimicrobial agents. There was no significant difference ($P > 0.05$) in the mean total aerobic counts and coliform counts. There was a negative correlation of the total aerobic and coliform counts ($r = -0.028$), total aerobic counts and antibiotics usage ($r = -0.05472$) as well as correlation of coliform counts and antibiotics use ($r = -0.187$). The bacterial isolates were resistant to multiple antibiotics.

6. 2 Conclusions

The findings in this study show the risk facors that could lead to the occurrence of *Salmonella* in poultry production in Nigeria due to the low level of biosecurity that was observed in the farms. This low level of biosecurity is attributed to high level of antimicrobial usage in poultry production.

This study did show that most poultry farmers were not observing strict biosecurity measures on their farms. *Salmonella* was not isolated from eggs, ovarian follicles and cloacal swabs in this study. The eggs were contaminated with other organisms including *Serratia liquefaciens, Serratia plymuthica, Enterobacter agglomerans, Klebsiella ozoanae, Enterobacter hafnei* and *Citrobacter freundii.*

Consumers of eggs from these farms are at risks of exposure to drug residues in view of the wide spread use of antimicrobials.

6. 3 **Recommendations**

The farmers should be educated about the public health significance of good farming practices. Animal health personnel should promote adoption of biosecurity measures. Poultry houses should be thoroughly cleaned and disinfected. Rodent and insect control measures should be incorporated into the design of the poultry house and management. The use of protective clothing as a means of biosecurity to reduce the risks of pathogen being passed from workers to birds should be enforced. The use of antibiotics in laying birds for the purpose of growth promotion should be discouraged.

Consumption of raw eggs and improperly/under-cooked eggs should be discouraged especially by young children, elderly persons, and persons with weakened immune systems or debilitating illness because the content of eggs can be contaminated with various micro-organisms. The hands of handlers, cooking utensils, and food preparation surfaces should be washed with soap and water after contact with raw eggs. Eggs should be cooked until both the white and the yolk are firm and eaten promptly after cooking. Assessment of poultry products in Nigeria should not rely entirely on bacteriological methods alone. A

more systematic/detailed study using more sensitive techniques for detection of *Salmonella* in poultry and poultry products should be carried out. Efforts should be made in preventing poultry farmers from the use of drugs without prior consultation of a veterinarian, through enforcement of existing regulations and advocacy.

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APPENDIX I

SAMPLE QUESTIONNAIRE FOR POULTRY FARMS

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

Dear Respondent,

This questionnaire is designed as part of a scientific study of occurrence of *Salmonella* and *E.coli* in Nigerian commercial poultry eggs. Please, all information obtained will be treated strictly and confidential and information provided would be solely for this purpose!

Thank you for your cooperation.

Dr. Akpabio Uduak

Date……..

Name of farm…….

Town/Village……..

LGA……

1. System of management practiced on the farm
   (i)Deep Litter
   (ii)Battery cage
   (iii)Others please specify

2. Source of birds
   (i)Locally hatched
   (ii)Bought at point of lay
   (iii)Commercially hatched
   (iv)Others please specify

3. No of birds……..

4. Age of birds……..
5. Week in lay……
6. % production……..
7. Source of feed
   (i)Locally compounded
   (ii)Commercial
   (iii)Others please specify
8. Inclusion of feed additives Yes….. No……

If yes please specify the type of additive added……..

9. Who adds the feed additive (a)manufacturer (b)farmer
10. Source of drinking water (a) Well (b) borehole (c)pipe borne (d) others please specify
11. How often do you clean your drinkers and feeders?……
12. How often do you change your litter?.....
13. Where do you dispose your litter?.....
14. Where do you dispose dead birds?.....
15. Are there pest on the farm? Yes No Pls specify type
16. Are there other animals on the farm Yes No Please specify
17. Nature of personnel on the farm (a)permanent (b) casual
18. What kind of clothing do the personnel use on the farm (a) farm clothing (b) personal clothing?
19. Do you practice all in all out system? Yes No
20. Do you disinfect before bringing in new birds Yes No
21. Presence of footbath Yes No
    Content of the footbath if present……
22. Are visitors allowed into the farm? Yes ……. No……..
23. Do you have any record of disease outbreak Yes ……. No…….
    If yes please specify……
    ……
    ……
    ……
    ……
    ……
24. What preventive and control measures do you take to reduce disease outbreak?.....
    .....
    .....
    .....
    .....
    .....
    .....
    .....
    .....
25. Where the birds vaccinated and at what age?.....
    .....
    .....
    .....
    .....
    .....
    .....
26. Who administered the vaccine?.....

APPENDIX II: Total aerobic counts in the four LGAs

Sample code	Colony forming unit/ml
KNIE1	4×10^2
KN3E2	11×10^2
KN6E2	60×10^2
KSIE2	5×10^2
KS3E2	75×10^2
FRE2	6×10^2
OKE2	15×10^2
KSIE3	4×10^2
KS2E3	35×10^2
KS3E3	3×10^2
HSE3	10×10^2
KS4E3	6×10^2
ABE4	7×10^2
SUE3	2×10^2
KNIE4	2×10^2
ABE3	30×10^2
KN3E5	2×10^2
FRE6	7×10^2
OKE6	10×10^2
SUE6	25×10^2
SUE6	25×10^2
OCE6	15×10^2
HSE6	18×10^2
ACE6	9×10^2
KN2E6	7×10^2
KN3E6	6×10^2

APPENDIX III: Total coliform counts

Sample code	Colony forming unit/ml
KNIE1	52×10^2
FRE1	2×10^2
OCE1	1×10^2
KN3E2	3×10^2
KN6E2	42×10^2
KSIE2	4×10^2
KS3E2	72×10^2
SFE2	62×10^2
FRE2	4×10^2
OKE2	24×10^2
KS2E3	20×10^2
KS3E3	10×10^2
ABE3	22×10^2
KS2E4	50×10^2
KS3E4	1×10^2
KN3E5	9×10^2
KSIE5	2×10^2

KN3E5	9×10^2
KSIE5	2×10^2
FRE6	1×10^2
ABE6	3×10^2
ACE6	1×10^2
KN3E6	2×10^2

APPENDIX IV:

Lists of antibiotics

AMC = Amoxicillin/Clauvulanic acid

F = Nitrofurantoin

SXT= Sulphamethaxazole/trimethoprim

CN = Gentamicin

CIP = Ciprofloxacin

AMP = Ampicillin

C= Chloramphenicol

TE = Tetracycline

K = Kanamycin

MY = Lincomycin

S = Streptomycin

P = Penicillin G
