COMPARATIVE ANALYSES OF BACTERIAL BIOBURDEN AND THE ANTIBIOTIC SUSCEPTIBILITIES OF SELECTED BACTERIA FROM FAECAL DROPPINGS OF COMMERCIAL AND FREE-RANGE CHICKENS, SAMARU, ZARIA

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JULY, 2014

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BY

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DEPARTMENT OF MICROBIOLOGY,
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JULY, 2014

DECLARATION

I hereby declare that the work in this thesis entitled "Comparative analysis of bacterial bioburden and the antibiotic susceptibilities of selected bacteria from faecal droppings of commercial and free-range chickens, Samaru, Zaria." was carried out by me in the Department of Microbiology, Faculty of Sciences, Ahmadu Bello University, Zaria, Nigeria under the supervision of Prof. O.S. Olonitola and Prof. S.E. Yakubu. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has previously been submitted for the award of a degree or diploma at this institution or any other.

_____ _____

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CERTIFICATION

This thesis entitled "Comparative analysis of the bioburden and antibiotic susceptibilities of selected bacteria isolated from faecal droppings of commercial and free-range chickens in samaru, zaria" by Hilda, INABO(Msc/Scie/1180/2010 – 2011) meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to God the Father, the Son and the Holy Spirit.

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ABSTRACT

The widespread use of antibiotics against bacterial diseases in the poultry industry has been associated with the development of antibiotic resistance in pathogenic bacteria. This poses a threat to the public worldwide since they are in contact directly when handling animals and indirectly through food. The purpose of this study was to determine the bacterial load, prevalence and antibiotic susceptibilities of *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* and *Enterococcus* sp. Faecal droppings were randomly taken from one hundred (100) broilers and one hundred (100) free-range (local) chickens. The inocula were cultured on various media and incubated for 24-48 hours at 37^0C for identification. The pure isolates were subjected to antibiotic susceptibility tests using the disc diffusion technique. It was established that there was no significant difference between the total plate counts of faecal droppings from commercial and free-range chickens. The comparison of the antibiotic susceptibilities of the isolates from commercial and free-range chickens showed no significant difference statistically except in streptomycin where *S. aureus* from free-range chickens were more susceptible than those from commercial chickens. High susceptibility to ciprofloxacin, gentamicin, chloramphenicol and high resistance to cloxacillin, cotrimoxazole and tetracycline by the isolates were observed. The source of antibiotic resistance may not necessarily be originating from the feeds, but from the immediate environment of the chickens. This may be as a result of misuse of antibiotics during treatment and control of diseases affecting chicken and other domesticated animals.

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CHAPTER ONE

1.0 INTRODUCTION

Poultry rearing contributes to the livelihood of most households in developing countries by providing income, source of protein and employment. They are easily and readily fed more than any other animals with antibiotic supplemented feeds to promote growth and for prophylaxis. Poultry rearing is seen to be appealing to individuals with low income because it requires minimal capital and a small area for raising the birds (MAAIF/UBOS, 2009). Presently, poultry rearing has taken a new dimension in terms of increased number of poultry farms in the urban centres and this is as a result of increase in human population, migration from rural to urban centres and improved livelihood in the urban centres (NOAH, 2009).

Chickens are known to constitute a vast reservoir of enteric bacteria with the general problem of environmental contamination. Adeleke and Omafuvbe, (2011) isolated the following bacteria from the faecal droppings of commercial and free-range chickens: *Alcaligenes faecalis*, *Acinetobacter mallei, Bacillus alvei, Corynebacterium kutseri, Corynebacterium ulcerans, Escherichia coli, Staphylococcus aureus, Proteus morganii, Salmonella* Arizonae, and *Pseudomonas fluorescens.*

Escherichia coli is an aerobic Gram-negative bacterium, usually motile rod. It is commonly isolated from human and animal waste, thereby contaminating the environment and thus constituting a public health hazard (Smith *et al*., 2003). *Escherichia coli* can be commonly found in the intestinal tract of humans and animals. It is used as an indicator bacterium because it acquires antibiotic resistance faster than other bacteria. Thus, changes

in the resistance of this organism serve as a good indicator of resistance in potentially pathogenic bacteria (Kijima-Tanaka *et al*., 2003). Furthermore, as an essential part of the gastrointestinal flora, it is known to be capable of accepting and transferring plasmids to other enteric bacteria; it is considered as an important reservoir of transferable antibiotic resistance.

Salmonella species are facultative aerobic and gram-negative rods. With the exception of *S.* Pullorum-Gallinarum, all salmonellae are actively motile. They are non-sporing and with the exception of *S.* Typhi, are non-capsulated. *Salmonella* species are particularly important because of their prevalence as agents of foodborne enteritis (Gast and Stephens, 1988). *Salmonella* sp. and their r-plasmids have been studied in poultry processing wastes and were found to be capable of interspecies r-plasmid transmission including transfer of antibiotic-resistance to *E. coli.*

Enterococcus species are Gram positive cocci, occurring in pairs or short chains. They are non-capsulated and the majority are non-motile. They are widely distributed in the environment, inhabiting mainly the gastrointestinal tract of humans and animals (Moreno *et al*., 2006). They are intrinsically resistant to many antibiotic agents such as tetracycline or macrolides through the acquisition of resistance genes or transposons or plasmids.

Staphylococcus species are Gram positive cocci of uniform size, occurring characteristically in groups but also singly and in pairs. They are non-motile and non-capsulated. They have the ability to become resistant to several antibiotics. The spread of resistance in *S. aureus* is largely due to the acquisition of plasmids and/or transposons (Lyon and Skurray, 1987).

Farmers administer antibiotic drugs in water or chicken feeds to treat or prevent infections (Food and Agriculture Organization, 2008). Antibiotic drugs are also used for the

promotion of growth in animals (Poppe *et al*., 2001). Studies have shown that this practice promotes the emergence of resistant clones and resistance plasmids in strains of *E. coli* in chickens which eventually infect people in close contact (Van den Bogaard *et al.,* 2001).

1.1 Statement of the problem

Presently, antibiotic resistance is a serious clinical and public health problem on a global basis. In the poultry industry, the widespread use of antibiotics against bacterial diseases has been associated with the development of antibiotic resistance in pathogenic bacteria. These microorganisms in the faeces are released into the environment via manure, wastewater or sewage sludge which contaminates agricultural soils and growing vegetables (Acar and Rostel, 2001). Furthermore run-off water from farms where such contaminated manure is used can flow into streams used by the public for drinking and recreational activities, thus serving as a public health threat. The ingestion of antibiotic contaminated food (vegetables, meat) has the potential of altering the human flora, thus favoring bacterial infections such as *Salmonella* (Cabello, 2003; 2004; 2006). Excessive use of antibiotics in poultry farms has had negative consequences on human and animal health, and has also caused damage to the environment (Grave *et al*., 1999, Wolf, 2004).

1.2 Justification

Livestock do function as a reservoir of resistant bacteria for environmental contamination. Bearing in mind the consequences on human health, concerns have been raised about the contamination of surface water with resistant bacteria from livestock activities. Infection of humans and animals with antibiotic resistant bacteria and contamination of the environment with resistant bacteria has become of great concern. The use of anti-microbial drugs in food animals have been shown to lead to the emergence of resistant strains of pathogens which

may be transmitted to humans through food in the developed countries, the contribution of anti-microbial use in food animals to resistance in bacteria infecting humans or vice versa in developing countries remain unclear. This study was necessitated by the fact that resistance of bacteria to available antibiotics are increasing which is adversely affecting the health of birds, their production and thus constitute a public health importance to man.

1.3 Aim

The aim of this study was to comparatively analyze the bioburden and determine the antibiotic susceptibilities of selected bacteria in the faecal droppings of commercial and free-range birds from poultry houses in Samaru, Zaria, Nigeria.

1.4 Objectives

1. To determine the microbial load of the faecal droppings (bioburden) from the commercial and free range chickens.
2. To isolate and biochemically characterize the selected bacteria from the faecal droppings of the commercial and free range chickens.
3. To determine the antibiotic susceptibility patterns of the selected bacteria isolated from the faecal droppings of the commercial and free-range chickens.

1.5 Research questions

1. Are the selected bacteria present in the faecal droppings of the commercial and free range birds?
2. Are the selected bacteria susceptible or resistant to the antibiotics to be used in the study?

1.6 Research hypothesis

$H_{o:}$ Continuous exposure to antibiotics has no effect on the antibiotic susceptibilities of the selected bacteria from the faecal droppings of commercial and free-range chickens.

H_A: Continuous exposure to antibiotics has an effect on the antibiotic susceptibilities of the selected bacteria from the faecal droppings of commercial and free-range chickens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The poultry industry

The poultry industry is one of the largest and fastest growing agro-based industries in the world. There is increasing demand for poultry meat mainly due to its acceptance by most societies and its relatively low cholesterol content. One of the major problems of the poultry industry is the accumulation of large amount of wastes, especially manure and litter generated by intensive production (Bolan *et al.,* 2010). Large-scale accumulation of these wastes may pose disposal and pollution problems, microbial hazards and public health nuisance unless strict measures, adequate environmental and economically sustainable management technologies are evolved (Power and Dick, 2000; Kelleher *et al.,* 2002; Sharpley *et al.,* 2007). Bio-aerosols (i.e. microbes suspended in air) generated at production has become a major environmental and health concern of the poultry industry, in addition, manure storage facilities and during land spreading of poultry litter constitute the most frequent source of complaints against animal-based industries (Millner, 2009).

2.2 Poultry litter production

Poultry population is increasing annually giving rise to a large amount of poultry litter (Amanullah *et al.,* 2010), and the utilization of poultry waste is a common practice especially in developing countries. Poultry faeces are a waste product excreted by the poultry chicken. It is a by-product that results from the digestion of food intake by poultry birds (Adegunloye, 2006). The chemical composition supports the growth of microorganisms which contaminate eggs and meat. The presence of these microorganisms

causes various diseases in chickens and by extension humans. Poultry waste (litter and excreta) are generally added to the soil as fertilizer. The final step in the poultry farming management strategy entails a big risk for the environment due to the nutrients and microorganisms contained in high concentrations in these waste materials.

2.2.1 Quality of Poultry Litter

The quality of poultry litter produced in a poultry unit depends on the litter (i.e. bedding material) management, and feed intake and its digestibility. A range of materials including wood shavings, cereal straw, husk and paper clipping are used as bedding materials (Swain and Sundaram, 2000). Bernhart *et al*. (2010) mentioned three common practices adopted for litter management in poultry units. They include:

2.2.1.1 *Single use litter*

The single use litter involves the total clean-out of the house after each flock and replacement of the bedding material (Bolan *et al.,* 2010).

2.2.1.2 *Partial re-use litter*

The partial re-use litter involves the removal of litter from the brooding section for spreading on the grower section of the house. New bedding material is then spread on the brooding section. The partially spent litter is often composted for a few days to elevate its temperature to kill pathogens. Some of the spent litter may be removed after each batch, and after two to five batches, the house is totally cleaned out (Bolan *et al.,* 2010).

2.2.1.3 *Multi-use litter*

With the multi-use litter, only caked material is removed (Sistani *et al.,* 2003) and the house is disinfected. The litter in the brooding section is either left untouched or covered with 25 to 50 mm of fresh bedding material. The multi-flock litter may increase the incidence of pathogenic microbes and parasites.

2.3 Poultry wastes

Poultry faeces are a waste product excreted by the poultry fowl. It is a by-product resulting from the digestion of food intake by poultry birds. The colour of droppings vary among species of birds, some are whitish, ashes and dark brown in colour, while the droppings can be in form of semi-solid or water (Adegunloye, 2006). The chemical composition of poultry litter supports the growth of numerous microorganisms which contaminate various products of poultry such as eggs and meat.

2.3.1 Types of Poultry Waste

There are varying types of poultry waste. These include deep litter waste, broiler waste, cage waste and high risk waste (Amanullah *et al.,* 2010)

2.3.1.1 *Deep litter poultry waste*

This refers to the dropping produced by layers during egg production period. This form of poultry waste usually consists of wood shavings in a layer of 10-15 cm deep. Deep litter is the accumulation of the material used for litter with poultry droppings. In addition, the droppings of the birds gradually combine with the wood shavings to build up the litter (Amanullah *et al.,* 2010).

2.3.1.2 *Broiler house waste*

This is similar to deep litter poultry waste, but the litter is changed more frequently, which gives rise to less ammonia due to restricted ammonia but enhance nitrogen production than that observed in the deep litter waste (Overcash *et al.,* 1983).

2.3.1.3 *Cage poultry waste*

This waste contains reasonable amount of moisture. However, enormous amount of ammonia is lost if the droppings are not used immediately (Overcash *et al.,* 1983).

2.3.1.4 *Deep pit or high-rise poultry waste*

The deep pit poultry waste are kept dry by drains and external grading which direct water from the droppings, so as to ensure natural composition. In this case, droppings can be stored for several months before removal (Amunallah *et al.,* 2010).

2.4 Components of poultry litter

Poultry waste is a mixture of excreted manure mixed with bedding material. Analysis has shown that poultry waste contains potentially valuable plant nutrients making it suitable for use as organic manure. The major components of poultry litter include the bedding material, feather, manure and the spilt feed. The litter contains plant nutrients, pesticide residues, pharmaceuticals, endocrine disruptors and microorganisms (Bolan *et al.,* 2010).

2.4.1 Plant nutrient

The major plant nutrients in poultry manure include nitrogen, phosphorus, potassium and calcium, magnesium and sulphur (Bolan *et al.,* 2010). There are several reports on the

chemical composition of poultry manure and litter (Sato *et al.,* 2005, Diaz *et al.,* 2008). The huge variability in nutrient concentration may be attributed to a number of factors including feed use efficiency, bedding material used, and litter management practices.

2.4.2 Pesticides and pharmaceutical residues

There are several antibiotics and chemicals used in the poultry production. Antibiotics used in poultry production include amongst others bacitracin, chlortetracycline, erythromycin, neomycin, oxytetratcycline, penicillin, streptomycin, tetracycline and tylosin (Bhattacharya and Taylor, 1975). Similarly, several chemicals are used to control internal protozoan parasites that cause coccidiosis (Hobson-Frohock and Johnson, 2006). Chemicals are often included in diets in poultry operations to control insects in the litter. Such chemicals include zoalene, unistat, furazolidone, nitrofurazone and cyromazine (Sims and Wolf, 1994). Some of these antibiotic and chemical residues have been measured in poultry manure wastes (Nahm, 2005; Karci and Balcioglu, 2009). Although the use of antibiotics as growth promoters in poultry in feed in some developed countries has been reduced, however, the potential environmental risks of proliferation and transport of antibiotic-resistant bacteria via land application of poultry manure is less documented than human impacts to antibiotic exposures (Singer and Hofacre, 2006; Powers and Angel, 2008; Brooks *et al.,* 2007).

2.4.3 Endocrine disrupting compounds

Endocrine disrupting compounds (EDCs) are a group of compounds either synthesized or naturally present in the environment suspected to have adverse effects in animals and humans (Bolan *et al.,* 2010). These EDCs include several natural hormones excreted by the

animal, and include pesticides, herbicides, plant oestrogens, and other compounds that disrupt endocrine systems (Powers and Angel, 2008). The EDCs affects organisms by interfering with the binding of hormones to their receptors or disrupting the endocrine system by mimicking natural hormones (Colborn *et al.,* 1993).

2.4.4 Microbial load

Microorganisms have existed on planet earth for more than 3.5 billion years, they exhibit the greatest genetic and metabolic diversity (Byarugaba, 2010). It is believed that microorganisms make up about 50 % of the living biomass. They have evolved mechanisms that enable them to respond accordingly to survive the selective pressure exerted by various environments and competitive challenges. Humans' struggle for survival has sought to deprive disease pathogens from their habitat using antibiotic agents, hence, these pathogens have responded by developing resistance to survive and fight off this offensive phenomenon.

2.5 Bacteria in poultry litter

Poultry waste contains a large diverse population of microorganisms (bacteria, fungi, protozoans and viruses). Microbial load in poultry litter can exceed 10^{10} cells (Cook *et al.,* 2008) and Gram-positive bacteria account for nearly 90 % of the microbial diversity. Pathogenic bacteria present in poultry litter are of great importance to the poultry industry (Bolan *et al.,* 2010). Culture and molecular-based studies reveals that poultry litter is a reservoir for several zoonotic pathogens, including *Escherichia coli*, *Salmonella* species, *Campylobacter jejunum*, *Listeria monocytogenes*, and *Clostridium perfringens* (Bull *et al.,* 2006; Line and Bailey, 2006). Contact between the birds and/or litter or between humans

and the litter represents a major route of contamination, with carcass contamination representing a dominant source of pathogen introduction into meat processing plant. Pathogen destruction may be required in some situations prior to land spreading a potential major source of pathogen introduction into the environment (Millner, 2009).

2.6 Uses of poultry litter

2.6.1 Nutrient source

Poultry litter is often used as an organic nutrient source in forage, cereal and fibre crop production (Bolan *et al.,* 2010). The addition of poultry litter to tall fescue, orchard grass, bermuda grass has been shown to increase dry matter production (McGrath *et al.,* 2009). Excessive application of poultry litter in crop production can cause undesirable effects on forage crops and animals consuming the forage (Mcginley *et al.,* 2003) such as problems with grass tetany and fat necrosis in beef cattle.

2.6.2 Soil amendment

Continuous cultivation of arable soils results in the deterioration of soil structure leading to reduced crop yield (Bolan *et al.,* 1992). The addition of poultry manure to soil will increase crop yield, improving the chemical fertility status of cultivated soil. Using poultry manure alone may be enough to achieve the potential yield of crop in arable soils by improving the physical fertility of the cultivated soil. In addition, poultry manure is used to commonly as mulching materials for agricultural and horticultural crops to conserve soil moisture and to protect the surface feeding roots from dying during summer periods (Agbede and Ojeniyi, 2009). Furthermore, organic manures such as poultry litter are increasingly being used in the rehabilitation of disturbed land resulting from mining and other industrial activities.

2.6.3 Animal feed

Poultry manure, either on its own or when mixed with feed grains, has been found to be valuable feed for cattle and fish (Bolan *et al.,* 2010). Ruminants are able to utilize the urea nitrogen (uric acid) in poultry manure. However, from a hygienic perspective, unprocessed poultry waste contains potential pathogenic microorganisms such as *Escherichia coli, Clostridium* sp. and *Salmonella* sp. (Adegunloye, 2006). Hence, proper processing to reduce the number of these microorganisms or render the waste free of pathogens is required (Kawata *et al.,* 2006). Although disease problems have not been reported when poultry manure are fed to farm animals under acceptable conditions, however, copper toxicity has been found to be a major challenge especially in sheep (Bolan *et al.,* 2010). Sheep are less tolerant than other livestock species to high dietary levels of copper when fed poultry litter (Sharma *et al.,* 2005).

2.6.4 Fuel source

Poultry litter can be burnt directly as a fuel source to produce heat energy (Adegunloye, 2006; Oyewole, 2010). The anaerobic digestion of poultry waste yields biogas, a combustible gas composed of approximately 60 % methane, 38 % carbon dioxide, and a mixture of water vapor, ammonia and hydrogen sulphide (Hansen, 2001; Charles *et al.,* 2003). Biogas (also called 'producer' gas) may be used as an energy source for burning as heat or as fuel for internal combustion engines to generate electricity (Blake *et al.,* 2007). Poultry litter combustion has been used as a method for producing heat and electricity at large centralized facilities (Turnell *et al.,* 2007; Fibrowatt, 2008)

2.7 Management and beneficial prospects for poultry litter

There is need to formulate strict regulations governing the safe disposal and handling of poultry waste in order to minimize its health impact. Guidelines on specific land application, heavy metals, antibiotics and coccidiostats in poultry waste are required. Recycling of poultry waste in fish and cattle feed and in electricity generation should be given consideration for efficient and profitable management of poultry waste (RIRDC, 2009). Management programmes for poultry litter should reflect both the potential value of the litter as a resource and realistic appraisal of the negative effects litter constituents may have on human health and the environment (Bolan *et al.,* 2010).

2.8 Common bacterial pathogens found in poultry litter

2.8.1 *Escherichia coli*

Escherichia coli historically was first isolated from the feces of a child in 1885 by Theodor Eschrich and have since remained the best studied bacterium. Presently, it is a common inhabitant of the gastrointestinal tract of animals and humans (Kaper *et al.,* 2004). There are two broad types of *E. coli*, the *E. coli* strains that are harmless commensals of the intestinal tract and others are that are major pathogens of human and animals, while the pathogenic *E. coli* strain is divided into those causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites (Kaper *et al.,* 2004; Sousa, 2006). *Escherichia coli* is found secondarily in soil and water due to fecal contamination. The bacterium can be cultured easily in the laboratory, but the different pathogenic genotypes can be identified through virulence gene detection methods.

2.8.2 Classification of *Escherichia coli*

Domain: Eubacteria

Class: Gammaproteobacteria

Order: Enterobactriles

Family: *Enterobacteriaceae*

Genus: *Escherichia*

Species: *coli*

Bionomial: *Escherichia coli*

2.8.3 Evolution and habitat of *Escherichia coli*

Genome sequencing of three different *E. coli* strains such as the laboratory K-12 strain MG1655, enterohaemorrhagic 0157:07 strain EDL933, and an uropathogenic isolate, CFT073 reveals unambiguous conservation of nearly 40 % of the core genes sequences among the three isolates. The synteny of the genes around the circular chromosomes is nearly intact and representative of the classic *E. coli* K-12 gene map (Berlyn, 1998). The organisms are reported to be common inhabitants of the small intestine and large intestine in human and animals. Also, they are the most abundant facultative anaerobes in the environment (Bermudez and Hazen, 1988; Hooper and Gordon, 2001).

2.8.4 Taxonomy, phylogeny and characteristics

Comparative analysis of ribosomal RNA sequences suggest that *E. coli* originated from a common ancestor with *Salmonella* between 120 and 160 million years ago, and this coincide with the origin of mammals (Ochman and Wilson, 1987). *Escherichia coli* is placed within the family *Enterobacteriaceae*. Besides *E. coli,* there are other species within the genus. However, little is known about their distribution, biology or interrelatedness of these species (Selander, 1987).

Escherichia coli is a Gram negative anaerobic or facultative anaerobic bacterium. It is a non-spore forming short rod. However, Eosin methylene blue agar is the selective media for the organism. The bacteria is cultivated with an optimum temperature of 37^{o}C (Buxton and Fraser, 1977), however, other strains of *E. coli* can multiply at a temperature of 49^{o}C. *Escherichia coli* is a facultative gram negative bacilli, it ferments lactose. Each strain has a typical gram-negative cell wall containing lipopolysaccharide. These organisms possess antigen H (flagella), K (capsular) and O (somatic). Some possess fimbriae which serve as appendages for adhesion. *Escherichia coli* inhabit the gastrointestinal tract of animals (warm birds and mammals) and humans. The bacterium is present wherever there is fecal contamination (Feng *et al.,* 2002).

2.8.5 Cell Structure and Physiology of *Escherichia coli*

Escherichia coli is approximately 0.5 µm and 1.0-3.0 µm in diameter an length respectively with a peptidoglycan single layer within the periplasm. *Escherichia coli* is commonly motile by means of peritrichous flagella and are fimbriated (Eisenstein, 1987). Furthermore,

they are capable of growing over a wide range of temperature (Whifield and Roberts, 1999; Braun and Braun, 2002).

2.8.6 Pathogenic Strains of *Escherichia coli*

2.8.6.1 *Enteroaggregative Escherichia coli*

The enteroaggregrative *E. coli* (EAEC) are a heterogenous group of bacteria that display a wide array of virulence factors. The EAEC strains are pathogens associated with persistent diarrhea in the developing world. They adhere to the tissue cells often in stacks or aggregates. They have been implicated as causes of both outbreaks and sporadic diarrhea among AIDS patients in developed countries (Sousa, 2006). The infection with the strain of EAEC depicts varying clinical features which include watery and mucoid diarrhea, low grade pyrexia with or without vomiting (Bhan *et al.,* 1989).

2.8.6.2 *Enterohaemorrhagic Escherichia coli*

The common type of enterohaemorrhagic *E. coli* (EHEC) is *E. coli* O157:H7. The EHEC was first observed as a cause of illness in 1982, and since then the infections have been reported with increasing frequency (Fitzpatrick, 1999). Prescott *et al.* (2000) reported the presence of attaching effacing region on this strain which causes haemorrhagic colitis with associated abdominal cramps and pains accompanied by bloody diarrhea. *Escherichia coli* O157:H7 is reported to have been responsible for several outbreaks of haemorrhagic colitis in developed countries especially the United States of America, with a minimum occurrence of about 200, 000 and 250 cases of morbidity and mortality respectively (Prescott *et al.,* 2000). Enterohaemorrhagic *E. coli* is due to a cytotoxin that damages

vascular endothelial cells and is associated with serogroup O157:H7. It is seldom called verocytotoxin producing *E. coli* because of its toxicity to vero monkey cells in cultures.

The EHEC strains are associated with food-borne diseases principally due to ingestion of uncooked minced meat, contaminated meat products and raw milk. Serotype O157:H7 is the prototype of increasing importance and is associated with hemorrhagic colitis, watery diarrhea, bloody diarrhea and a potentially fatal condition called hemolytic uremic syndrome (HUS) characterized by acute renal failure (Wilson *et al.,* 1997). The recognition and perception of *E. coli* O157 associated diarrhea as declining has changed (Griffin and Tauxe, 1991). A variety of *E. coli* serotypes have been linked with human illness, of which *E. coli* O157:H7 is reported to be the most important (Aibinu *et al.,* 2007). This serotype is readily isolated from human and animal wastes that pollute the environment, hence, it is of great public health significance (Smith *et al.,* 2003; 2005).

2.8.6.3 *Enteroinvasive Escherichia coli*

The pathogenesis of disease caused by enteroinvasive *E. coli* (EIEC) involves cellular invasion and spread that requires specific chromosomal and plasmid-borne virulence genes. The EIEC have the ability to invade the epithelial cells. This strain is associated with the presence of a large plasmid and has the ability to also produce a cytotoxin and enterotoxin. The EIEC causes a broad spectrum of human diseases. They cause an invasive inflammatory colitis, and may also elicit a watery diarrhea syndrome distinguishable from that caused by other pathotypes via the penetration and multiplication of the bacterium within the epithelial cells of the intestine (Casalino *et al.,* 2003; Todar, 2007).

2.8.6.4 *Enteropathogenic Escherichia coli*

The enteropathogenic *E. coli* (EPEC) strains attach to the brush border of intestinal cells. The affinity for this site results in cell damage which is specific and causing effacing lesions or attaching effacing lesions. These lesions results in the destruction of the brush border of microvilli adjacent to attaching bacteria. The destruction of the brush border of the microvilli by this pathogen leads to diarrhea. Furthermore, it has been reported that this strain of *E. coli* is an important aetiology of the causes of diarrhea in paediatrics especially in developing nations (Nataro and Kaper, 1998). The terminology attaching effacing lesions is solely associated and used to describe the effect of this strain.

2.8.6.5 *Enterotoxigenic Escherichia coli*

The enterotoxigenic *E. coli* (ETEC) strain produces one or both of two distinct (the heat stable and heat labile) plasma-mediated enterotoxins responsible for diarrhea. The heat stable enterotoxin binds to a glycoprotein receptor that is coupled to guanylatecyclase which stimulates the production of cyclic guanosine monophosphate (cGMP), this leads to the release of electrolytes and water into the lumen of the small intestine resulting in watery diarrhea. This is a distinct characteristic of ETEC (Sears and Kaper, 1996) while the heat labile enterotoxin have affinity and binds to specific gangliosides on the epithelial cells and activates membrane-bound adenylase monophosphate by the choleratoxin resulting in hypersecretion of electrolytes and water into the lumen of the intestine.

2.8.7 Laboratory diagnosis of Escherichia coli

Microscopic examination of stool samples at X100 magnification shows clusters of Gram negative rods. Then Eosin methylene blue and MacConkey agar are inoculated with the

stool. On EMB medium, there are black colonies with greenish-black metallic sheen while on MacConkey agar, pink or deep red colonies are produced to confirm the lactose fermenter organism resulting to drop in pH, leading to darkening of the medium. Biochemical tests used for confirmation of *E. coli* include Triple Sugar Iron Agar (A/A/g+/H2S), IMViC; indole (red ring for positive) and Methyl red (bright red for the positive), but Voges-Proskaeur negative (no colour changes) and Citrate negative (colour remains greenish).

For *E. coli* 0157:H7 in stool, ELISA test, direct immunoflorescent microscopy of filters and immunocapture techniques using magnetic beads are screening tools that precede rapid testing for the presence of *E. coli* without prior culturing of the stool (Boer and Heuvelink, 2000).

2.8.8 Future prospects for Escherichia coli comprehension

Physiologically, *E. coli* is versatile well adapted to its characteristic habitats. The organism can respond to environmental signals such as chemicals, pH, temperature, osmolarity, and stimulants in a number of very remarkable ways considering its nature as a single-celled organism. Knowledge of the pathogenic mechanisms of *E. coli* pathotypes has led to the development of rational interventions for the treatment and prevention of *E. coli*-induced diseases (Sousa, 2006). Furthermore, due to its natural habitat and its ability to subvert, circumvent and/or evade the immune defenses, the survival of this bacterium is enhanced and safeguarded in nature. However, continuous research and investigations into *E. coli* epidemiology, virulence, and biology may provide useful insights into the origins, evolution and epidemiology of this versatile bacterial pathogen in our environments.

Moreover, with the emergence and spread of antibiotic resistance in bacteria, *E. coli* is considered an important reservoir of transferable antibiotic resistance (Cohen, 2001). Therefore, active surveillance of the antibiotic resistant pattern of *E. coli* serotypes found in poultry wastes/feces should be of great public health interest because colonization of the intestinal tract with resistant *E. coli* from poultry has been shown in humans, giving empirical and historical evidence that animals are reservoir for *E. coli* found in humans (Sayah *et al.,* 2005). Furthermore, the spread of antibiotic resistance plasmids in *E. coli* from poultry to human or of antibiotic resistant microorganisms from animals to humans has been reported in various countries including Nigeria (Fang *et al.,* 2008; Nsofor *et al.,* 2012). The ability of *E. coli* to transfer antibiotic resistance is well documented with several studies describing antibiotic agent resistance profiles of *E. coli* strains isolated from foods of animal origin, various species of animals, and humans (Schroeder *et al.,* 2002).

2.9 *Salmonella* species

The genus *Salmonella* contains two species; *Salmonella* Enterica *and Salmonella* Bongori. The former *S.* Enterica consists of six subspecies; *S.* Enterica subsp. Enterica*, S.* Enterica subsp Salamae*, S.* Enterica subsp Arizonae*, S.* Enterica subsp Diarizonae*, S.* Enterica subsp Houtenae*, S.* Enterica subsp Indica (Brenner and Mcwhorter-Murlin, 1998; Zhang-Barber *et al.,* 1999).

2.9.1 History of *Salmonella*

Salmonella was named after Daniel Elmer Salmon an American Veterinary Pathologist while Theobald Smith also an American discovered the type bacterium (*Salmonella* Enterica var Cholera-suis) from pigs. The generic name was given to this bacterium in

1900 by Lignieresi in honour of Dr. Daniel Elmer Salmon, who at the time was the administrator of the United States Department of Agriculture (USDA) Research Program (FDA, 2008).

2.9.2 Taxonomy of *Salmonella*

Salmonella belongs to the Kingdom: Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Enterobacteriales, Family: *Enterobacteriaceae* and Genus: *Salmonella.* The Family *Enterobacteriaceae* to which *Salmonella* belongs consists of other groups namely: *Escherichia, Klebsiella, Proteus, Edwardsiella, Citrobacter, Enterobacter, Ewingella, Hafnia, Kluyvera, Providencia, Morganella, Shigella, Serratia, Tatumella* and *Yersinia* (Fox, 2011).

2.9.3 Nomeclature of *Salmonella*

After the discovery of the first *Salmonella* strain, scientists have used different systems for *Salmonella* nomenclature. However, uniformity in *Salmonella* nomenclature is necessary for communication between scientists, health officials and the public (Yan *et al.,* 2003). Present usage often combines several nomenclatural systems that inconsistently divide the genus into species, sub-species, sub-genera, groups, sub-groups and serotypes (serovars) which causes confusion (Brenner *et al.,* 2000). The nomenclature of *Salmonella* evolved from the initial one serotype–one species concept proposed by Kauffmann. Serotyping is based on the identification of somatic (O) and flagellar (H) antigens using specific antisera. Each serotype is considered as a separate species (for example *S.* Paratyphi A*, S.* Newport*, S.* Enteritidis)*;* if this concept were

used, it would result in 2,501 species of *Salmonella* (Popoff, 2001). The number of *Salmonella* species, subspecies serotypes and their habitats is as shown in Table 2.1.

Table 2.1: Actual number of Salmonella species, sub-species, serotypes and their usual habitats (Popoff, 2001).

***Salmonella* species and subspecies**	**Usual habitat**	**Number of serotypes within subspecies**	
		1998	**2001**
***Salmonella enterica* subsp. Enterica**	Warmblooded animals	1,454	1,478
***Salmonella enterica* subsp. Salamae**	Cold blooded animals and the environment	489	498
***Salmonella enterica* subsp. Arizonae**	Cold blooded animals and the environment	94	94
***Salmonella enterica* subsp. Diarizonae**	Cold blooded animals and the environment	324	327

***Salmonella enterica* subsp. Houtenae**	Cold blooded animals and the environment	70	71
***Salmonella enterica* subsp. Indica**	Cold blooded animals and the environment	72	12
***Salmonella* Bongori**	Cold blooded animals and the environment	20	21
Total		2,463	2,501

Other taxonomic proposals have been based on the clinical role of a strain, on the biochemical characteristics that divide the serotypes into subgenera and ultimately, on genomic relatedness (Ewing, 1986; Farmer III, 1999). In 1986, the subcommittee of *Enterobacteriaceae* of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology unanimously recommended that the name of the species for *Salmonella* be changed to *S.* Enterica, a name coined by Kauffmann and Edwards in 1952, because no serotype shares this name.

The number of *Salmonella* species and *Salmonella* nomenclature currently seen in the literature is as summarized in by Jones *et al.* (2000) in Table 2.1. The antigenic formulae of *Salmonella* serotypes are defined and maintained by the World Health Organisation (WHO) Collaborating Centre for Reference and Research on *Salmonella*

at the Pasteur Institute, Paris, France (WHO Collaborating Centre). The new serotypes are listed in annual updates of the Kauffman-White Scheme (Popoff *et al.,* 2000). Currently, there are over 2,500 identified serotypes of *Salmonella* (Foley and Lynne, 2008). A relatively small number of these serotypes have been documented to be host adapted, characteristically producing systematic disease in a restricted number of animal species. These serotypes include *S.* Gallinarum and *S.* Pullorum in chicken; *S.* Dublin in cattle; *S.* Cholera-suis in pigs (Zhang-Barber *et al,* 1999).

2.9.4 Salmonella infections

Salmonella infections are the most common widespread cause of salmonellosis among animals and humans globally (Mead *et al.,* 1999). Animal used for food production especially poultry often carry *Salmonella* (Santos *et al.,* 2003).

2.9.5 Risk Factors associated with *Salmonella* Infections in Poultry

Numerous environmental factors can influence the likelihood and outcome of infections of poultry with *Salmonella*. Lengthy environmental persistence of pathogens can generate extended opportunities for horizontal transmission within and between flocks (Gast, 2007). Contaminated feed and litter are high risk factors in *Salmonella* infections in poultry (Davies and Breslin, 2003). *Salmonella* was able to survive in litter and feed for more than two years after the removal of an infected flock (Davies and Breslin, 2003). *Salmonella* infections have been found to be higher in stressful housing or flock management.

Large hatcheries, feed mills, a higher number of poultry houses on a farm and rearing during the wet and cold seasons are risk factors for *Salmonella* infections. Age of the flock

can serve as a risk factor as older birds are more likely to be contaminated by *Salmonella* (Namata *et al.,* 2008). Lack of general hygiene is a risk factor for *Salmonella* infections in poultry farms. Inefficient cleaning and disinfection after depopulation for flock renewal is another risk factor of *Salmonella* infections (Davies and Breslin, 2003).

2.9.6 Pathogenicity of *Salmonella* Infection

Infective *Salmonella* must have the ability to survive peristalsis and pH to invade the gastro-intestinal mucosa, attach and invade the intestinal epithelia and payers patches, cause diarrhoea/ disseminate from the intestine, multiply and survive within the reticulo-endothelial system (D'Aoust, 1997). Both host and microbial factors are known to affect the ability of *Salmonella* to cause disease. Host factors include resistance in adult animals (Rotger and Casadesus, 1999), innate immunity in the young, activity of macrophages (Taub *et al.,* 2012), cytokines/proteins (Lalmanach and Lantier, 1999), temperature differences, pH and organic/inorganic environments (Slauch *et al.,* 1997).

2.9.7 Possible sources of *Salmonella* Infection in Animals

The possible sources of *Salmonella* infection in animals and human include the following:

2.9.7.1 *Animal feed*

Animal feed is a known source of *Salmonella* infection in farm animals (Häggblom and Aspán, 2002; Boqvist *et al.,* 2003). Manufacturers of compound feed for livestock use different types of feed materials. The origin, transport, processing and storage of feed materials may result in contamination of the feed with several food-borne pathogens (Whyte *et al.,* 2003). In countries with a low *Salmonella* prevalence in animals, feed is

considered to be the major source of this pathogen. However, it is difficult to quantify the public health risk associated with the presence of *Salmonella* in feeds in countries with high prevalence rates (EFSA, 2008). *Salmonella* present in feed, represent a group of zoonotic bacteria that may cause human disease and, hence, animal feed may act as an indirect source of infection for humans consuming food of animal origin (Salomonsson *et al.,* 2005).

2.9.7.2 *Water*

Salmonella has been isolated from water released into the environment from human wastewater treatment plants (Berge *et al.,* 2006). *Salmonella* can be frequently found in sewage, sea, and river water and can contaminate a variety of foods (Mastroeni *et al.,* 2000). Many serovars of *Salmonella* have been isolated in natural environments contaminated by human and animal feces (Threlfall, 2002).

2.9.7.3 *Poultry litter*

Litter may introduce pathogens into the primary production environment although treatment and testing prior to use can greatly reduce or eliminate pathogen carriage (Ivanov, 2001). Increase in litter pH can bring about a decrease in the survival of *Salmonellae*. Prolonged use of litter, with multiple batches of birds, is considered a far greater problem, as it can harbor pathogens (Vicente *et al.,* 2007). Effective composting of spent litter has been shown to eliminate *Salmonella*, thus enabling recycling as fertilizer or animal feed (Mohee *et al.,* 2008).

Salmonella is known to survive in litter and feed for more than two years after the removal of an infected flock (Davies and Breslin, 2003). Levels of moisture in poultry houses have

been shown to highly influence the persistence of *Salmonella*. It is also known that *Salmonella* numbers in poultry litter are usually highest in areas of poultry houses with reduced airflow (Myint *et al.,* 2005).

2.9.7.4 *Poultry faecal droppings*

Faecal shedding of *Salmonella* is often greatest during the first few weeks of life, when the susceptibility of chicks to infection is highest (Waltman *et al.,* 1998). Faecal shedding of *Salmonella* by infected birds can be detected by testing environmental samples from poultry houses and it is a strategy useful for monitoring the introduction of *Salmonella* into poultry houses by vectors, personnel, equipment and other sources (Gast, 2007). Faecal samples have sometimes not been reliable for testing as intermittent shedding of the organism by infected birds occurs.

2.9.8 Laboratory diagnosis of Salmonella infection

Laboratory diagnosis of *Salmonella* can be obtained by the use of culture media and molecular tools.

2.9.8.1 *Culture media for isolation of Salmonella*

Salmonella requires selective enrichment broths which are formulated to selectively inhibit other bacteria while allowing it to multiply to levels that may be detected after plating. There are currently three major types of selective enrichment broth media namely: tetrathionate, selenite and Rappaport-Vassiliadis (RV). There are also different formulations within each type of enrichment (The Oxoid, 2006). Selective plating media such as Salmonella-Shigella agar, MacConkey agar, Bismuth sulphite agar,

Brilliant green agar (BGA) and Brilliant green sulphapyridine agar, Xylose deoxycholate agar (XLD) and Deoxycholate Citrate Lactose Sucrose agar are used (OIE, 2008). Such media are used to assist the selective enrichment broth to inhibit other bacteria (Waltman *et al.,* 1998). They are aerobic to facultative anaerobes. The optimum temperature for growth is 37^0C, but some can grow at a temperature range of 5– 45^0C. The pH for growth is 4 – 9 (Gast and Beard, 1990).

2.9.8.2 *Colonial morphology*

Typical *Salmonella* colonies on agar media are about 2-4 mm in diameter with round smooth edges, slightly raised and glistering. On blood agar, they form moist colonies at 2-3 mm in diameter. They appear red to pink on Brilliant green agar, Brilliant sulphapyridine and Brilliant green novobiocin, black metallic sheen on Bismuth sulphite agar, red with black centre on Xylose lysine tergitol 4 and Xylose lysine deoxycholate agar, colourless with black centre on Deoxycholate agar and Salmonella-Shigella agar, Blue-green with black centre on Hecktoen enteric agar, colourless on MacConkey, crimson with pale borders on Rambach agar (Waltman *et al.,* 1998). The viability of *Salmonella* can be maintained for years in simple media such as peptone or nutrient agar (Gast and Beard, 1990).

2.9.8.3 *Staining and microscopic appearance*

Salmonella are straight, non-spore forming rods measuring 0.7 – 1.5 x 2.0 – 5.0µm**.** They are Gram-negative but the cells are readily stained with common dyes such as methylene blue or carbolfuchsin (Gast and Beard, 1990). They are motile by petrichous flagella (except *S.* Pullorum and *S.* Gallinarum) and are non-capsulated.

2.9.8.4 *Biochemical properties of Salmonella*

Typical *Salmonella* colonies found on the selective agar should be confirmed by biochemical tests. The principal biochemical tests by which *Salmonella* can be identified are as follows: nitrate reduction, glucose, oxidase, mannitol, oxidation/fermentation, maltose, urea hydrolysis, lactose, sucrose, salicin, dulcitol, adonitol, indole, H_2S production, citrate utilization, sodium malonate, growth on KCN, lysine decarboxylase, methyl red, Voges-Proskauer, arginine dihydrolase, ornithine decarboxylase, gelatin liquefaction. Testing all these reactions requires considerable resources. Therefore, it makes sense to use composite media such as triple sugar iron (TSI) agar which is prepared in tubes for further use. It contains glucose, lactose and sucrose, a H_2S detection system and an indicator (Jones *et al.,* 2000). When glucose not lactose or sucrose is fermented by the microorganisms, a change of colour from red to yellow can be observed.

The colour of fermentation reaction 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 H_2S production is characterized by blackening of the medium (Jones *et al.,* 2000). Some *Proteus* sp. 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 TSI. The typical reactions of six different strains of *Enterobacteriaceae* in TSI are as shown in Table 2.2.

Table 2.2: Biochemical reactions of some Enterobacteriaceae on Triple Sugar Iron (TSI) agar. (Jones *et al*., 2000)

Organism	Butt	Slope/slant	H_2S	Gas
Enterobacter	AG	A	-	+
Escherichia coli	AG	A	-	+
Proteus	AG	A	+	+/d
Morganella	A or G	NC or ALK	-	d
Shigella	A	NC or ALK	-	-
Salmonella	AG	NC or ALK	+	+

KEYS:

AG - acid (yellow) and gas formation;

A - acid (yellow);

NC - no change;

ALK - alkaline (red);

\+ - hydrogen sulphide (black) or gas;

\- - no hydrogen sulphide (no blackening) or gas;

d - variation in different strains.

NB: *S. typhi* does not produce gas (Jones *et al.,* 2000).

The common biochemical characters of *Salmonella* are as follows although most strains conform to this pattern and many of the characters are subject to variation therefore, no organism should be excluded from the group on the result of a single test (Wilson and Miles, 1966). The usual reactions include: fermentation of glucose, maltose, mannitol and sorbitol with the production of acid and gas, absence of fermentation of lactose, sucrose, salicin and adonitol, failure to produce indole, to hydrolyse urea, to deaminate phenyl alanine, to use malonate, or to grow in potassium cyanide medium.

2.9.8.5 *Culture and identification*

Salmonella infections are detected based on culture and identification of the organism. Organs/tissues as well as environment samples are collected aseptically and enriched using appropriate pre-enrichment, enrichment and selective media to grow the bacteria at 37^0C or at 42^0C depending on the sample(s) taken. This procedure takes several days (Waltman *et al.,* 1998). The isolation of *Salmonella* involves the following stages:

Pre-enrichment:

Pre-enrichment is done to revive sub-lethally injured *Salmonella* organisms and to increase numbers of organisms to levels that will readily multiply in selective media. Some of the agents used include buffered peptone water (BPW), a non-selective pre-enrichment broth that promotes the recovery of those bacteria that may be injured (Waltman *et al.,* 1998). Peptone in the media supplies nitrogenous compounds needed for the growth of bacteria. The phosphate salts provide buffering capacity to maintain the pH. Maintenance of the pH is important when attempting to recover injured bacteria, as low pH can be detrimental to the repair of the damaged cells (Vanderzant and Splittstoesser, 1992).

Selective enrichment:

Selective enrichment is done to selectively enhance the growth of *Salmonella* in relation to other organisms in the sample. Some of the media used for selective enrichment include Rappaport Vassiliadis medium, Selenite broth, and Muller Kauffman Tetrathionate medium (OIE, 2010).

Isolation:

Isolation is carried out to obtain typical *Salmonella* colonies on solid isolation medium. There are different media used for isolating *Salmonella*. These include Salmonella-Shigella agar (SSA), Brilliant Green Agar (BGA), Xylose Lysine Tergitol 4 (XLT4) and Xylose Lysine Deoxycholate (XLD) (OIE, 2010).

Confirmation:

Various biochemical, serological and molecular tests can be applied to the pure culture to provide a definitive confirmation of an isolated strain. *Salmonella* possess antigens designated somatic (O), flagellar(H) and virulence (Vi), which may be identified by specific typing sera and the serovar may be determined by reference to the antigenic formulae in the Kauffman–White scheme (Popoff *et al.,* 2001)

2.9.8.6 *Serological tools in Salmonella diagnosis*

Serological tools used in *Salmonella* diagnosis include whole blood plate (most commonly used test for pullorum-typhoid), rapid serum plate test, standard macroscopic tube agglutination test, micro agglutination test and microantiglobulin test (OIE, 2010). Enzyme linked immunosorbent assays (ELISA) have been developed for detecting antibodies to

various salmonellae (OIE, 2010). ELISA has been said to be an appropriate method of indicating that infection (or exposure through vaccination) has occurred in the past and it can also detect infected chickens that are excreting *Salmonella* organism intermittently (Hassan *et al.,* 1990). ELISA is recognized as a rapid and highly sensitive test for detecting a number of avian pathogens including *Salmonella* infections (Seo *et al.,* 2003; Holt *et al.,* 2006). However, there are concerns about their specificity (Waltman *et al.,* 1998).

2.9.8.7 *Molecular tools for diagnosis of Salmonella*

Polymerase Chain Reaction (PCR) assay has proven to be an effective method for *Salmonella* detection in meat and poultry products with high specificity and sensitivity and more importantly a less time-consuming procedure (Malkawi, 2003). Using PCR, *Salmonella* detection could be achieved within 24-36 hours compared to 3-8 days for the conventional microbiological methods. Several target genes are used to produce specific primers for *Salmonella* sp. such as *inv*A, *agf*A, *IS*200, *hin*, *H-li*, *iag*AB, *spv*R, *via*B, *mkf*A,*omp*C and *ori*C (Bäumler *et al.,* 1997). The *inv*A gene is usually used because of its presence in almost all *Salmonella* serotypes and its association with the pathogen invasion ability (Santos *et al.,* 2005).

For efficient identification of *Salmonella*, there is a need for fast and reliable detection and typing methods with the ability to discriminate between strains (Lofstrom *et al.,* 2004). A number of DNA-based typing methods for *Salmonella* sp. have been developed including amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) and ribotyping (Soto *et al.,* 1999; De Cesare *et al.,* 2001).

2.9.8.8 *Serotyping in Salmonella diagnosis*

Serotypes of *Salmonella* are defined based on the antigenic structure of both somatic or cell wall (O) antigens and flagellar (H) antigens. These antigens are detected using slide and tube agglutination tests with commercially produced antisera, the O antigens using a suspension of growth from an agar plate while the H antigens using a suspension of broth culture. The serotype is deduced from the specific pattern of agglutination reactions using the Kauffmann-White classification scheme (Popoff *et al.,* 2001). Most strains acidify dulcitol promptly but exceptions include *S. typhi* and some of the d-tartarate-positive Paratyphoid B Bacilli.

Gas is not formed by *S.* Typhi and *S.* Gallinarum or by occasional strains of a number of other serotypes. Maltose is not usually fermented by *S.* Pullorum strains and *S.* Typhisuis does not attack mannitol. Occasionally, strains which ferment lactose, sucrose or salicin (e.g. *S.* Arizona (subgroup III) and *S.* Houtenae) or produce indole have been recorded. The urease, phenyl alanine, potassium cyanide, methyl-red and Voges-Proskauer reactions appear however to be constant.

Most *Salmonella* grow with citrate as the sole carbon source (Jones *et al.,* 2000), though important exceptions include the Typhoid and Paratyphoid A bacilli, *S.* Typhisuis, *S.* Sendai, and the pullorum variety of *S.* Gallinarum (Wilson and Miles, 1964). *Salmonella* reduce trimethylamine oxide to trimethylamine and nitrate to nitrite. They are catalase positive, oxidase negative and normally do not liquefy gelatin, pectate and alginate. Inositol is utilized by numerous strains. Sodium malonate is not utilized and acid is produced in Jordan's tartarate medium. They form arginine dihydrolase, esculin

is not hydrolysed, and growth occurs in sodium acetate medium (Al-hindawi and Rished, 1979).

The statement is often made that the production of H_2S is characteristic of the *Salmonella* group. Practically all *Enterobacteriaeae* form some H_2S under optimal conditions of growth. The ability to cause blackening of certain media in which heavy metallic salts are incorporated in an agar base is however, confined to the *Salmonella*, *Citrobacter* and *Proteus* groups. Thus, most *Salmonella* give a positive reaction for H_2S in Kligler's medium and in ferrous chloride gelatin, but exceptions include *S.* Paratyphi A, the diphasic variety of *S.* Cholerae-suis, *S.* Typhi-suis, *S,* Sendai, *S.* Beria and some strains of the typhoid bacillus. Nearly all *Salmonella* produce lysine, arginine and ornithine decarboxylase, but no glutamic acid decarboxylase. The only noticeable exceptions are the typhoid bacillus and some strains of *S.* Paratyphi B var Odense which form no decarboxylase for ornithine. Most *Salmonella* rapidly attack d-tartarate, citrate and mucate, though there are many exceptions. Several types, notably *S.* Paratyphi A, *S.* Sendai and the human type of *S.* Paratyphi B are d-tartarate negative. Numerous others attack this substance late irregularly (Kauffman and Petersens, 1956).

Strains of subsp IIIa generally differ from *Salmonella* in that they attack lactose, have no action on dulcitol, liquefy gelatin, and fail to ferment d-tartarate, citrate or mucate in 24 hours. With a few exceptions they are malonate positive (Shaw, 1956). Many gelatin-liquefying *Salmonella* of African origin are, however, also able to use malonate and are slow or irregular in their attack on the organic acids. Some other biochemical reactions are variable within *Salmonella*, and are at times useful for the identification of individual serotypes, or for subdividing them further. They include the fermentation of

rhamnose, xylose, arabinose, trehalose and inositol. Most strains attack these sugars, and it is the occasional absence of fermentation which is of significance (Wilson and Miles, 1966).

2.9.9 Salmonella infections in human and animals

Salmonella causes serious diseases such as gastritis and typhoid in humans (Lamont *et al.,* 2002). This disease affects all domestic animals including mammals, fowls, and reptiles with symptoms such as gastritis, diarrhoea, and septicaemia. However, the host susceptibility and development of carrier states vary widely among species (Wigley *et al.,* 2002). Common serovars causing disease in humans and food animals belong to the subspecies Enterica, while other subspecies are found in cold blooded animals and in the environment but are occasionally associated with human disease (OIE, 2010).

2.9.9.1 *Salmonella infection in poultry*

Domestic poultry are known to be the largest single reservoir of *Salmonella* organisms existing in nature (Jemmi *et al.,* 2000). Poultry are highly susceptible to infection by *Salmonella* even at very low exposure doses, for several days after hatching, but they rapidly become much more resistant during the first few weeks of life (Waltman *et al.,* 1998). This transition is mostly attributable to the gradual acquisition by chicks and poults of a complete intestinal micro-flora from their environment. Of the more than 2,500 serotypes of *Salmonella*, only about 10 % have been isolated from poultry and even smaller proportion of serotypes are commonly found in poultry (Gast, 2007). Many of the serotypes that are prevalent in humans (such as *S.* Typhimurium and *S.* Enteritidis) are also common

in poultry, this suggests a possible epidemiologic link between poultry and human reservoirs of the salmonellae (Ferris *et al.,* 2003; Van Duijkeren *et al.,* 2004).

2.9.9.2 *Salmonella Infections in other Animals*

Salmonellae have been isolated from many animal species including, cows, chickens, turkeys, sheep, pigs, dogs, cats, horses, donkeys, seals, lizards and snakes (Mastroeni *et al.,* 2000). Host-specific *Salmonella* infections occur in other animals as host specific infections such as *S.* Choleraesuis in pigs, *S.* Abortus-ovis in sheep, *S.* Abortusequi in horses and S. Dublin and *S.* Newport in cattle (Velge *et al.,* 2005; OIE, 2010).

2.9.9.3 *Salmonella Infection in Humans*

Salmonellosis is a major cause of bacterial enteric illness in both humans and animals. Each year an estimated 1.4 million cases of salmonellosis occur among humans in the United States (CDC, 1999). In approximately 35,000 of these cases, *Salmonella* isolates are serotyped by public health laboratories and the results are electronically transmitted to the Centers for Disease Control and Prevention (CDC). This information is used by local and state health departments and CDC to monitor local, regional, and national trends in human salmonellosis and to identify possible outbreaks of salmonellosis. Over the past 25 years, the National *Salmonella* Surveillance System has provided valuable information on the incidence of human salmonellosis in the United States and trends in specific serotypes. The recently implemented *Salmonella* Outbreak Detection Algorithm, another valuable tool for the recognition of outbreaks (Hutwagner, 1997), allows users to detect increases in human infections due to specific *Salmonella* serotypes. *Salmonella* surveillance activities depend upon the accuracy of serotype identification and are facilitated by standardized

nomenclature. The National *Salmonella* Reference Laboratory at CDC assists public health laboratories in the United States in serotype identification by providing procedure manuals, training workshops, updates, and assistance with the identification of problem isolates.

It primarily causes two types of diseases: gastroenteritis characterized by the development of bacteria in the intestinal tract and typhoid fever that result from the invasion of the systemic compartment (Parry *et al.,* 2002). Typhoid and paratyphoid fevers are caused by *Salmonella* Typhi and *Salmonella* Paratyphi, respectively (WHO, 2008) though *Salmonella* Typhimurium is also one of the major causes of food-borne human salmonellosis (Thorns, 2000). Typhoid fever (enteric fever) caused by *Salmonella* Typhi is an endemic disease and is one of the most widespread zoonoses in the tropic and sub-tropic. It has become a major public health problem in developing countries of the world with an estimated annual incidence of 540 per 100,000. The annual incidence of typhoid is estimated to be about 17 million cases worldwide (WHO, 2008). It is often encountered in tropical countries including Nigeria where they constitute serious sources of morbidities and mortalities (Ibekwe *et al.,* 2008).

2.9.9.4 *Sources of Salmonella infections in humans*

Salmonella infections in humans often results from the ingestion of contaminated foods, such as poultry, beef, pork, eggs, milk, seafood, and fresh food produce (Braden, 2006; Meldrum *et al.,* 2007). Direct contact with animals also results in transmission of *Salmonella* to humans (Bacigil *et al.,* 2007) because food animals harbor a wide range of *Salmonella* serotypes and so act as a source of contamination, which is of paramount epidemiological importance in non-typhoid human salmonellosis (Acha and Szyfres,

2001). Contamination of meat by *Salmonella* may occur at abattoirs from the excretion of symptomless animals, contaminated abattoir equipment, floor and personnel, and the pathogen can gain access to meat at any stage during butchering (Adesiyun and Oni, 1989). Cross-contamination of carcasses and meat products could continue during subsequent handling, processing, preparation and distribution.

2.9.9.5 *Clinical signs of Salmonella infection in humans*

The most common manifestations of salmonellosis in humans are diarrhoea, abdominal cramps and fever within eight to seventy-two hours. Additional symptoms may be chills, headache, nausea and vomiting that can last up to seven days. In humans, S*almonella* infections can be life threatening especially for infants, the frail or elderly and persons with chronic disease or undergoing chemotherapy (for example HIV/AIDS patients) resulting in immunosuppression which favours the growth of S*almonella*.

2.9.9.6 *Mode of transmission of Salmonella in humans and animals*

Salmonella sp. are carried asymptomatically in the intestines or gall bladder of many animals, and are continuously or intermittently shed in the faeces (Kim *et al.,* 2009). Poultry can become infected by horizontal transmission through infected litter, faeces, feed, water, dust, fluff, insects, equipment, fomites, diseased chicks and rodents contaminated with *Salmonella*. They can also be transmitted by other animals, wild birds and personnel (Muhammad *et al.,* 2010).

2.9.10 Salmonella Infection and HIV/AIDS in Nigeria

Despite efforts to clamp down the pandemic of Human Immunodeficiency Virus (HIV) infections and Acquired Immunodeficiency Syndrome (AIDS) in most developing countries, its impact is still alarming. Many infections including typhoid are known to complicate HIV/AIDS in sub-Saharan Africa. Non-typhoidal *Salmonella* (NTS) invasive infections are indeed a public health concern for infants, young children and young adults suffering from malnutrition, malaria and HIV infection (Gordon, 2008; Vandenberg *et al.,* 2010).

The clinical manifestation of HIV secondary infections in developing countries, including Nigeria, shows a high prevalence of infections of the skin, gut, respiratory tract, tuberculosis, malnutrition (Akolo and Ukoli, 2005) and gastrointestinal diseases caused by enteric bacterial pathogens characterized by high rates of bacteraemia and frequent occurrence of diarrhoea. Disseminated infections with *Salmonella* Typhimurium*, S.* Enteritidis*, S.* Arizona*, S.* Dublin and other non-typhoidal *Salmonella* serotypes were recognized early in the HIV epidemic (Adeleye *et al.,* 2008).

Salmonella Typhimurium and *S.* Enteritidis are the two most common serotypes isolated from blood of patients with AIDS in the United States (Levine *et al.,* 1991) and Africa (Kariuki, 2006; Adeleye *et al.,* 2008). Mortality for non-typhoid *Salmonella* is reported to be as high as 60% in African patients with HIV (Boyle *et al*., 2007). Agwu *et al*. (2009) confirmed that typhoid is highly prevalent among HIV/AIDS patients from his study in Ekpoma with a prevalence of 73 (13.0%) from 560 consenting known HIV/AIDS patients examined for the presence of *S.* Typhi in their peripheral blood.

2.9.11 Prevention and control of Salmonella infection

The goals of *Salmonella* prevention are to minimize exposure and maximize resistance by adhering to the following rules: observation of strict biosecurity measures to prevent introduction of *Salmonella* into the herd, quarantine of new arrivals for at least two weeks, isolation of sick animals, restriction of animate (birds, rodents, insects) and inanimate objects into and out of the farm, provision of hygienic environment for animals especially calves, disinfection of equipment used between animals, maintenance of good personal hygiene by personnel, prevention of feed and water contamination with faeces, purchase of feed from reputable sources. In addition, prevention of herd stresses such as excessive heat and overcrowding as well as implementation of a sound general herd vaccination program will be of importance (NYSCHAP, 2006).

2.9.11.1 *Treatment of Salmonella Infections*

It is common practice to use antibiotics for both therapeutic and prophylactic purposes (Aarestup, 2004; EFSA, 2008). *Salmonella*, being an intracellular organism, require drugs that have intracellular activity to be effective (Sirinavin and Garner, 1999). Non absorbable drugs such as neomycin and colistin were used initially followed by the broad spectrum antibiotics such as chloramphenicol, ampicillin, amoxycillin, tetracycline and cotrimoxazole which do not have substantial intracellular activity. Newer drugs that are absorbable with potent intracellular activity like the 5 fluoroquinolone compounds such as norfloxacin, ofloxacin and ciprofloxacin are the drugs of choice in *Salmonella* infections (Sirinavin and Garner, 1999).

2.9.11.2 *Vaccination of poultry*

Vaccination has been shown to be an important tool in countries with high prevalence of *Salmonella* in poultry (Berge, 2011). The immune response of poultry reduces the duration and severity of *Salmonella* infections and helps to prevent re-infection and this response provides the basis for efforts to protect birds through vaccination (Gast, 2007). Development of safe and effective vaccines is a priority in developing countries, as the only strategy for *Salmonella* infections is prophylactic treatment with antibiotics, whereas many western countries have succeeded with elimination of the disease by test and slaughter (Olsen *et al*., 2007). The development of an effective vaccine is dependent on an understanding of how *Salmonella* sp. infect their hosts and the host response to infection.

2.9.11.3 *Strict biosecurity measures in poultry farms*

The control of *Salmonella* infections should primarily focus on the farm since most of the risk factors are on the farm. Some ways of control include preventing contact between poultry and contaminated feed and visitors, wearing house-specific clothing by poultry staff, thorough cleaning and disinfection of the houses, vaccination and rodent control programmes (Namata *et al.,* 2008). Others are personnel and equipment control, routine screening of flocks for *Salmonella*. Chicks should be sourced from farms that are *Salmonella*-free. This can be achieved through constant fumigation and observance of good hygiene and use of disinfectant measures in poultry premises and routine screening of flocks (EFSA, 2008). The source of water needs to be checked so as to ensure safe drinking water for the birds since water contamination by *Salmonella* is known to occur (Threlfall, 2002).

2.9.11.4 *Eradication of Salmonella infections in poultry*

Western countries have succeeded with elimination of *Salmonella* infection by test and slaughter of positive flocks from the production chain (Olsen, 2007). Several specific recommendations are common features of nearly all *Salmonella* control programmes which include:

i. Eggs and chicks should be obtained from breeding flocks proven to be *Salmonella* free.

ii. Hatching eggs should be properly disinfected and hatched under strict hygienic conditions.

iii. Biosecurity practices should be enforced on farms to restrict movement of equipment and staff.

iv. Insects and rodents should be controlled in and around poultry farms.

v. Thorough cleaning and disinfection of poultry farms between flocks should be done

vi. Water used in poultry farms should come from treated sources

vii. Feed should be pelleted or it should not contain animal proteins.

viii. The pest control system for rodents, wild birds and insects from poultry farms should be used.

ix. The all in all out policy for raising birds and appropriate times between flocks should be used.

x. Nutrition and immune status in the birds should be optimized.

xi. Protective mechanisms of the individual birds should be maximized by optimizing the intestinal flora using probiotics and vaccinations where possible (Nurmi and Rantala, 1973).

2.9.11.5 *Treatment of Salmonella infection in human*

Treatment includes the use of fluids and electrolytes, antibiotic and non-steroidal anti-inflammatory drugs. However, the use of antibiotics for *Salmonella* Enteritis without septicaemia (bacteraemia) is controversial because the population of normal intestinal bacterial microflora may be altered as well as the possible development of antibiotic resistance by *Salmonella* organisms (Randall, 2001).

2.10 *Staphylococcus aureus*

Staphylococcus aureus was first described in 1871 by von Recklinghausen but not isolated. Alexander Ogston, a surgeon in 1881documented two kinds of micrococci: one was arranged in chains (Streptococci) and the other cocci arranged in clusters. The cocci cluster was named *Staphylococci* because “Staphyle” in Greek means "bunches of grapes" and “kokakos” meaning a "berry". The description of the genus was not provided by Ogston, therefore it was not recognized. In 1884, Rosenbach successfully isolated and grew *Staphylococcus aureus* from pus. Rosenbach is credited with proposing the genus *Staphylococcus* and the species *Staphylococcus aureus* (Paniker, 2006). He kept the genus name *Staphylococcus* because the bacterium was similar to that studied by

Ogston. Rosenbach proposed the nomenclature for *Staphylococcus aureus* based on the yellow pigmentation of the colony.

2.10.1 Taxonomy of Staphylococci

Staphylococci belong to the Phylum: Firmicutes, Class: Bacilli, Order: Bacillales, Family *Staphylococcaceae*, Genus: *Staphylococcus* and Species: *aureus*, with the binomial *Staphylococcus aureus.* The family to which *Staphylococci* belong consists of lesser known genera namely: *Gamella, Macrococcus* (Kwok and Chow, 2003) and *Salinicoccus.* The best-known of its nearby phylogenetic relatives are the members of the genus *Bacillus* in the family *Bacillaceae,* which is on the same level as the family *Staphylococcaceae.* The *Listeriaceae* are also a nearby family.

2.10.2 Morphology and biochemical characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, catalase-positive, oxidase-negative, non-motile microorganism that does not form spores. It is a facultative anaerobe capable of generating energy by aerobic respiration and by fermentation which yields mainly lactic acid. It has the ability to produce from lactose, maltose, mannitol, fructose, trehalose and sucrose but cannot produce from xylose, cellobiose, raffinose and mannose. It is nitrate-positive and susceptible to novobiocin. (Barrow and Felthan, 1993) It forms smooth, convex, lustrous, circular colonies reaching a size of 0.5-1.5 μm in diameter and growing in an irregular three-dimensional bunch of grapes-like clusters of cells.

On an ordinary medium, the organism can grow within a temperature range of 10-42°C. The optimum pH ranges in between pH 7.4-7.6. It thrives best in an oxygen rich

environment and can grow on all common laboratory media such as milk, nutrient gelatin or agar. When grown on an agar-based culture medium and incubated for 24 hours, the colonies appear to be 2-4mm in diameter (Paniker, 2006).The colony pigmentation varies from grey, grey-white with yellowish to orange shades with typical β-haemolysis on the blood agar depending on the growth conditions provided (Asperger and Zangerl, 2003; Bremer *et al.,* 2004). *Staphylococcus aureus* is very tolerant of high concentrations of sodium chloride, up to 1.7 molar.

2.10.3 Habitat and requirement of *Staphylococcus aureus*

Staphylococcus aureus is a commensal associated with the skin, skin glands, and mucous membranes, particularly in the noses of healthy individuals (Crossley and Archer, 1997). About 20–30% of the human population are said to be carriers of *S. aureus* (Heyman, 2004). On a rich medium, *S. aureus* forms medium size "golden" colonies. On sheep blood agar plates, colonies of *S. aureus* often cause β-hemolysis (Ryan and Ray, 2004). The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (Liu *et al.*, 2005). It requires B vitamins (thiamine and niacin), inorganic salts and amino acids as a nitrogen source, especially arginine, cysteine, proline and valine for growth. Glutamic acid, leucine and tyrosine are not required for growth, but they are essential for enterotoxin production. Arginine seems to be essential for enterotoxin B production (Baird-Parker, 2000; Jay, 2000).

The organism produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin. Blood coagulation is used to

distinguish *S. aureus* from others members of the genus, which are collectively designated as coagulase-negative staphylococci (Ryan and Ray, 2004). This organism is one of the most intensively studied bacterial species since it is a frequent etiological agent of human diseases and exhibits resistance to a growing number of therapeutic agents,

Most strains have the capacity to hydrolyse native animal proteins (casein, gelatine, fibrin), lipids, phospholipoproteins and tween. They can also coagulate animal plasma with the assistance of a coagulase and the clumping factor. The typical enzymatic activity of *S. aureus* includes production of coagulase, alkaline phosphatase, proteases, lipases, and esterases and some strains also produce lecithinase (Baird-Parker, 2000, Asperger and Zangerl, 2003).

2.10.4 Genome organization of *Staphylococcus aureus*

The first genome sequences of *S. aureus* strains Mu50 and N315 were published in 2001 (Kuroda *et al.*, 2001). At present, complete genomic sequences of *S. aureus* strains are available, and the genomes of several others have been partially determined (Gill *et al.*, 2005; Diep *et al.*, 2006). The genome of *S. aureus* is a circular chromosome that is 2.8–2.9 million base pairs in size, with a G+C content of about 33% (Crossley and Archer, 1997). The chromosome encodes approximately 2700 CDSs (protein coding sequences) as well as structural and regulatory RNAs.

2.10.5 Cell wall structure

The staphylococcal cell wall is a semi-rigid structure composed of three components: peptidoglycan, teichoic acids and surface proteins. Peptidoglycan is the major component which builds the murein sacculus and it structurally forms a macromolecular net in which

glycan strands are cross-linked by short peptides. The glycan strands are composed of repeating disaccharide units of β-1-4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid (Labischinski, 1992). The majority of glycan chains have a length of 3–10 disaccharide units. Extending from the carboxyl residue of the β-1-4 linked acetylglucosamine moiety is the stem peptide with the sequence: l-alanyl-d-isoglutaminyl-l-lysyl-d-alanyl-d-alanine (Scheffers and Pinho, 2005). A series of five l-glycine residues are attached to the l-lysine of the stem peptide which is a characteristic feature of the *S. aureus* cell wall. Teichoic acid is an antigenic component that aids adhesion of the cocci to the host cell surface.

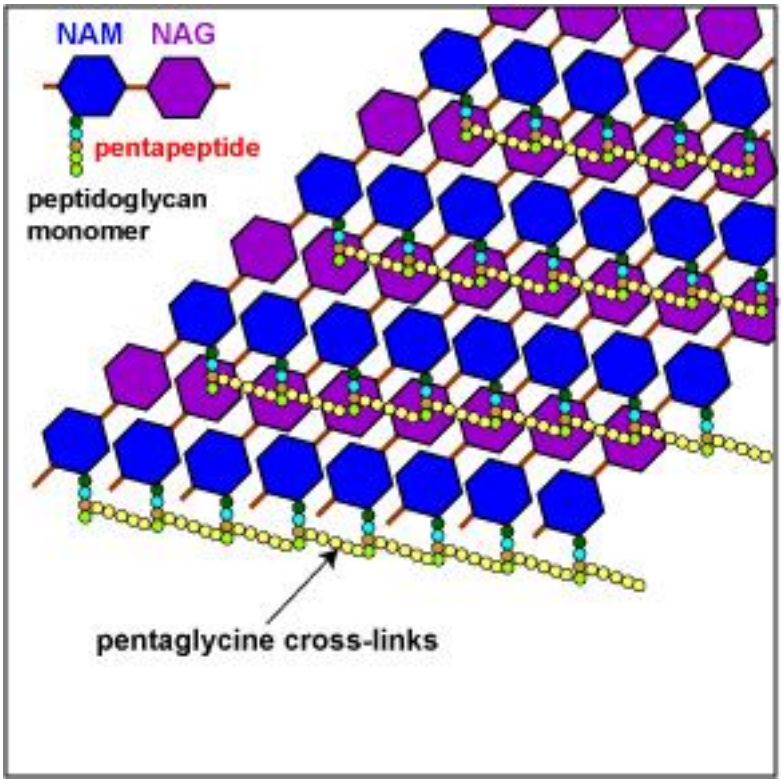


Figure 1.1: Biochemical structure of the cell wall of *Staphylococcus aureus*

2.10.6 Human infection with Staphylococcus aureus

Staphylococcus aureus is a member of the normal flora of the human skin, respiratory tract and gastrointestinal tract. The anterior nares are the major site of colonization in humans. About 20–30% of individuals are persistent carriers of *S. aureus* and 30% are intermittent carriers (colonized transiently) (Wertheim *et al.*, 2005). Colonization significantly increases the risk of infections since it provides a reservoir of the pathogen from which bacteria are introduced when host defense is compromised (Kluytmans *et al.*, 1997). Patients with *S. aureus* infections are usually infected with the same strain that they carry as commensal.

This organism is reported as one of the main causes of hospital-and community-acquired infections with serious consequences (Diekema *et al.*, 2001). Nosocomial *S. aureus* infections affect the bloodstream, skin, soft tissues and lower respiratory tracts. The organism cause central venous catheter-associated bacteremia and ventilator-assisted pneumonia. It also causes serious deep-seated infections, such as endocarditis and osteomyelitis (Schito, 2006). In addition to the infections listed above, *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal food borne diseases (SFD). Hospitalized patients are particularly exposed to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections (Lindsay and Holden, 2004).

Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portal of entry may be a hair follicle or most times a break in the skin (minute needle-stick or a surgical wound). Foreign bodies, including sutures, are readily colonized by staphylococci making infections difficult to control. Another portal of entry is the respiratory tract. The localized host response to

staphylococcal infection is inflammation, characterized by an elevated temperature at the site, swelling, the accumulation of pus, and necrosis of tissue (Brooks *et al.*, 2007). Around the inflamed area, a fibrin clot may form, walling off the bacteria and leukocytes as a characteristic pus-filled boil or abscess. More serious infections of the skin may occur, such as furuncles or impetigo. Localized infection of the bone is called osteomyelitis. Serious consequences of staphylococcal infections occur when the bacteria invade the blood stream which results to septicemia that may be rapidly fatal; a bacteremia may result in seeding other internal abscesses, other skin lesions, or infections in the lung, kidney, heart, skeletal muscle or meninges (Todar, 2005).

2.10.7 Virulence factors associated with pathogenesis of Staphylococcus aureus infections

Staphylococcus aureus is equipped with a great variety of virulence factors, which include both structural and secreted products such as attachment improving agents participating in pathogenesis of infection.

2.10.7.1 *Microbial surface components recognizing adhesive matrix molecules*

Staphylococcus aureus carries numerous surface proteins named "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) that mediate attachment to host tissues and initiate colonization leading to an infection (Gordon and Lowy, 2008). Fibronectin binding proteins A and B (Fnbp A and Fnbp B) participate in attachment of bacterial cells to an extra-cellular matrix component, fibronectin, and to plasma clot. Collagen binding protein, Cna, is necessary for adherence of *S. aureus* to collagenous tissues and cartilage (Switalski *et al.*, 1993) and it has been shown that antibodies against Cna block the bacteria attachment to those tissues (Patti *et al.*, 1994).

2.10.7.2 *Clumping factors*

Clumping factor A and B mediate clumping and adherence of bacterial cells to fibrinogen in the presence of fibronectin. They play a significant role in wound and foreign body infections and it has been shown that *clfA* mutant is less virulent than the wild type isogenic strain (Foster and Hook, 1998).

2.10.7.3 *Protein A*

Protein A is a cell wall-associated protein that binds to the Fc domain of immunoglobin G (IgG) in a "wrong orientation" on the surface of *S. aureus* cells which disrupts opsonization and phagocytosis (Switalski *et al.*, 1993). It exhibits an ability to bind to von Willebrand factor, a protein present at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases by *S. aureus* (Hartleib *et al.*, 2000).

2.10.7.4 *Cytolytic toxins*

Cytolytic toxins form β-barrel pores in the cytoplasmic membranes causing leakage of the cell's content and lysis of the cell (Foster, 2005). *Staphylococcus aureus* secretes several cytolytic toxins which includes alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL). Alpha-hemolysin inserts into eukaryotic membranes and oligomerizes into a β-barrel that forms a pore which causes osmotic cytolysis. Alpha-hemolysin is particularly cytolytic toward human platelets and monocytes (Menestrina *et al.*, 2001). The PVL is classified as a bicomponent cytolysin because it is dependent on two secreted proteins (LukF-PV and LukS-PV) that insert into the host's cytoplasmic membrane and hetero-oligomerize to form a pore. Furthermore, PVL exhibits a high affinity toward leukocytes and is mostly associated with community-acquired methicillin resistant *S. aureus* (CA-MRSA) which causes severe necrotizing pneumonia and contagious skin infections (Foster, 2005). Other bicomponent toxins,

gammahemolysin (Hlg) and leukocidin (Luk), are cytotoxic toward erythrocytes and leukocytes respectively.

2.10.7.5 *Superantigen toxin*

Staphylococcus aureus generates a group of powerful immuno-stimulatory proteins implicated in gastroenteritis and toxic shock syndrome. They are resistant to heat denaturation and proteases. These toxins have the ability to cross-link major histocompatibility complex (MHC) class II molecules located on antigen-presenting cells with T-cell receptors, forming a tri-molecular complex. Formation of the complex induces intense T-cell proliferation in an antigen-independent manner resulting in massive cytokine production and release which causes capillary leak, epithelial damage and hypotension (Baker and Acharya, 2004).

The primary function of super antigens is to weaken the host's immune system sufficiently to allow the pathogen to propagate and the disease to progress (Kotzin *et al.*, 1993). The staphylococcal enterotoxins A, B, C, D, E, G, and Q are responsible for staphylococcal foodborne diseases and toxic shock syndrome, while TSST-1 is the cause of toxic shock syndrome.

2.10.8 Pathogenesis of *Staphylococcus aureus* in chickens

The routes of infection can be through a break in the skin (a wound caused by injury, beaking trimming or needle injections etc), the respiratory tract (as a result of poor air quality or hot respiratory vaccines) and possibly the gut. Once in circulation, staphylococci have a high affinity for collagen-rich surfaces, such as the articular surface of joints, and synovial sheaths located around joints and tendons and also tend to localize in the growth

plate of actively growing bones. This explains the higher incidence of femoral head necrosis and osteomyelitis in young chickens versus more mature chickens (Skeeles, 1997). Staphylococcal disease is evidenced by depressed chicks, high early mortality (first two weeks), wet navels and omphalitis. Infection related to toe trimming is seen as swollen footpads that may be associated with a reddish discoloration extending from the trimmed toe to the hock. Infection of the bone (osteomyelitis) may also occur, often causing the development of femoral head necrosis (Jensen, 1990). Chickens with arthritis and tenosynovitis will experience lameness (Glisson and Smith, 1990). Typically, the hock joint is involved and will be swollen and warm to the touch. The stifle joint is the second most commonly affected joint. When opened, the joint and surrounding tissue will contain a white- to yellow-colored purulent exudate. Inflammation may also be evident on the articular surface and nearby tendons (Butterworth, 1999).

Studies have shown that early infection with *Staphylococcus aureus* can cause inflammation of the hock joint and nearby tendons (Hill *et al.,* 1989). Chronic infection of the tendons with either organism leads to progressive fibrosis and calcification. Weight gained by the chickens may lead to the rupturing of the tendons which results to the permanent loss of use of the affected leg. Initially, the area surrounding the ruptured tendon (typically seen in the gastrocnemius tendon located above the hock) will discolor, turning reddish-purple from hemorrhage, then green, before returning to a normal color. A large knot (fibrosis) will form at the site of the rupture.

Staphylococcal infections of the footpad and toes ("bumblefoot") are common in adult chickens. The severe swelling of the footpad and toes results in subsequent lameness.

Osteomyelitis (infection of the bones) may cause either lameness or paralysis depending on whether a long bone or the spine is affected. Bone sites most frequently involved are the proximal head of the tibiotarsus, proximal femur and the thoracic vertebrae (T3-T5). Birds with infections in the leg bones are reluctant to walk. (McNamee and Smyth, 2000) When the femur is affected, the proximal head of the femur will separate from the shaft when disarticulated from the hip joint (femoral head necrosis). Infections of the joint spaces between vertebrae may cause paralysis of the legs. Gross lesions may include a yellow-brown exudate or necrosis in the head of the femur (metaphysis).

2.10.9 Prevention and control of Staphylococcus aureus infection

2.10.9.1 *Prevention and control in humans*

Regular hand washing is the most important means of preventing transmission of *S. aureus*. Persons should routinely wash hands with soap and running water before eating, after using the bathroom, when hands are visibly dirty, before and after physical contact with clients, and when there has been contact with blood or other body fluids, mucous membranes, or broken skin. Persons should wash hands with soap and running water for at least 20 seconds. Individuals in facilities where there may be close physical contact (e.g., children at play**,** martial arts classes, many athletic activities) must have access to needed supplies and sufficient opportunities for good personal hygiene (Department of State Health Services and Correctional Facilities Workgroup, 2009).

Effective laundering procedures are of necessity since *S. aureus* can survive on clothing. The dilution and agitation of laundered items for 20 minutes at any water temperature removes some bacteria. When laundry is be to done at cool water temperatures (less than 72^0F or 22.2^0C), a detergent formulated for cold water should be used. The disinfectant capability of chlorine bleach is well established. Its use is the most effective means of

reducing the bacterial count in laundered items at any temperature. Thorough drying in a dryer reduces the number of bacteria.

Methicillin Resistant *Staphylococcus aureus* (MRSA) can exist on environmental surfaces, including in bathrooms, therefore, all washable (non-porous) surfaces of bathrooms and living areas should be cleaned routinely (Berger-Bachi and Tschierske, 1998). Cleaning should be done with a 1:100 bleach solution (1 tablespoon bleach in 1 quart water slightly exceeds this concentration) Shared items (telephones, computer keyboards, remote controls, combs, brushes, scissors, clippers, toys, exercise equipment, furniture) may provide opportunities for transmission. Persons using exercise equipment should use barriers to protect bare skin, such as a clean towel or shirt while using exercise equipment.

2.10.9.2 *Prevention and control of Staphylococcus aureus in poultry*

Staphylococcal infections in young birds are best controlled by strict sanitation, including proper egg handling practices at the breeder farm and in the hatchery. Hatchery processing procedures, such as beak and toe trimming and dubbing, must be carried out using proper technique. Breeders on feed restriction do experience a precarious nutritional balance. If not managed carefully, the additional stress of restricted amounts of feed will play an important role in the appearance of staphylococcal infections. Insufficient feeder space will lead to increased aggression by dominant birds, which may cause more injuries and staphylococcal problems. The environment in the chicken house should be managed to prevent scratches that may allow staphylococcal bacteria to enter through the skin. Management of the amount of light, feed and water are necessary to prevent scratches (Valle, 1998).

Actions must be taken to reduce the chance of injury because wounds provide a significant portal of entry for staphylococcal bacteria. Sharp objects, such as rocks, wire, metal edges on equipment, nails and splinters, must be removed. Slats and equipment should be well maintained. Setting the slat height correctly (22 inches) and using ramps will reduce leg and foot injuries. Maintaining good litter quality will reduce footpad damage. Proper ventilation, good quality shavings, removal of caked litter, and using a water restriction program or nipple drinkers help maintain litter quality.

2.10.10 Treatment of Staphylococcal infections

2.10.10.1 *Treatment of humans infected with S. aureus*

Hospital acquired infection is often caused by antibiotic resistant strains (MRSA) and can only be treated with vancomycin or an alternative (Berger-Bachi and Tschierske, 1998). These organisms are uniformly resistant to penicillins and cephalosporins. The infections have been treated with combination therapy using sulfa drugs and minocycline or rifampin (Todar, 2005).

2.10.10.2 *Treatment of chickens infected with S. aureus*

Treatment varies in efficacy, but may be cost-effective, as well as advantageous for bird welfare. The majority of *S. aureus* isolated from poultry are sensitive to penicillin but sensitivity tests should always be performed, as antibiotic resistance is common. Penicillin resistance may develop quickly in birds undergoing treatment, so it may be beneficial to switch to another antibiotic after five to seven days of penicillin therapy. Other water-soluble antibiotics that may be effective include erythromycin, lincomycin and tetracyclines. There may be need to consult a poultry veterinarian for advice on antibiotic selection, as some antibiotics can only be used when prescribed by a licensed veterinarian

(Jensen and Miller, 2001). Preventive treatment programs with feed-grade antibiotics have been used with varying degrees of success. Antibiotics are most effective if given early in the disease. Established infections, especially with arthritis and osteomyelitis, will not respond to medication due to the amount of damage that has already been done to the joint and the bone.

2.11 *Enterococcus* species

Enterococcus (formerly called group D streptococci) has been recognized since 1899, when Thiercelin identified it as an intestinal organism (Stiles and Holzapfel, 1997). The name *Enterococcus* was derived from the French word "enterocoque" meaning they are cocci of intestinal origin. Characteristic features of *Enterococcus* sp. include their presence in faeces and sewage, their ability to cause endocarditis and urinary tract infections and survive unfavorable environmental conditions such as drying, high temperatures, and exposures to some antiseptics (Murray, 1990; Wade, 1997).

2.11.1 Taxonomy of *Enterococcus* species

Many attempts have been made to distinguish *Enterococcus* species from *Streptococcus* species. Classification of *Streptococcus* species into four subgroups: faecal streptococci (enterococci), dairy streptococci, viridans group and pyogenous streptococci was done by Sherman in 1937. Sherman (1937) noted that the enterococci subgroup included the Lancefield group D streptococci; he also suggested that the latter could be differentiated by haemolytic and proteolytic reactions, however, Stiles and Holzapel (1997) reported such action as inappropriate as haemolysis may only be determined by a plasmid.

Other methods such as biotyping, serotyping and phage typing brought about questions as to which of the *Streptococcus* species actually belonged to the genus *Enterococcus* (Saeedi *et al*., 2002). In 1984, the species *Streptococcus faecium* and *Streptococcus faecalis* were established to be distinct from the other streptococci which were designated to another genus called *Enterococcus* through the use of DNA hybridization and 16S rRNA sequencing (Foulquie Moreno *et al*., 2006). This meant that the D group antigen is found in both streptococci and enterococci. Therefore, nine species were transferred from the *Streptococcus* groups into the genus *Enterococcus* which has 28 species (Foulquie Moreno *et al*., 2006). The molecular data that were collected using 16S rRNA sequencing of *Streptococcus* enabled the construction of a16S rRNA-dendrogram showing the relationship between *Streptococcus*, *Enterococcus* and *Lactococcus* species.

The method also allowed the grouping of *Enterococcus* species. The *Enterococcus faecalis* species group includes *E. faecalis, E. haemoperoxidus and E.moraviensis* while the *Enterococcus faecium* species group includes *E. faecium, E. durans, E. hirae, E. mundtii, E. porcinus and E. villorum* (Klein, 2003). *Streptococcus bovis, Streptococcus alactolyticus and Streptococus equinus* are serogroup D using the the Lancefield group D antigen to differentiate between enterococci and streptococci. These groups can be distinguished from *Enterococcus* species by the lack of growth in 6.5% (w/v) sodium chloride at 10^{o}C. It is harder to distinguish *Enterococcus* species from other cocci that do not express the D group antigen such as *Pediococcus, Lactococcus or Tetragenococcus* species because no other phenotypic differences have been reported that allow distinction. Thus the use of fermentation patterns enzyme activities such as pyroglutamyl aminopeptidase (PYRase) (Domig *et al*., 2003), growth at defined temperatures and physiological characteristics were essential in the identification of *Enterococcus* species (Shanks *et al*., 2006).

2.11.2 Biochemical characteristics of *Enterococcus* species

The genus *Enterococcus* consists of Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria that can occur both as single cocci and in chains. *Enterococcus* species grow at a range of temperatures from 5 to 50^{o}C on brain heart infusion (BHI) agar in aerobic conditions (Van den Berghe *et al*., 2006), and can also grow under anaerobic conditions (Martinez *et al.,* 2003). The ability to survive heating at 60^{o}C for 30 minutes makes these species distinguishable from other closely related genera such as *Streptococcus* (Foulquie Moreno *et al*., 2006). Trypticase soy agar or Columbia agar with 5% (v/v) defibrinated sheep blood may be used to assess the haemolysis produced by enterococci. If human or horse blood is used, haemolysis is based on cytolysin activity and causes a β-haemolytic reaction. They grow in a wide range of pH (4.6–9.9), with the optimum being 7.5 (Van den Berghe *et al*., 2006). They can tolerate and grow in the presence of 40% (w/v) bile salts (Gardin *et al.,* 2001; Nakajo *et al.,* 2005). They are able to grow in 6.5% NaCl solution and have a cation homeostasis which contributes to its resistance to pH, salt, metals and desiccation.

2.11.3 Epidemiology and transmission of *Enterococcus* species

Enterococci have several intrinsic characteristics that allow them to grow and survive under harsh environmental conditions. They are wide spread in nature i.e. they can be found in soil, plants, food, and water and in animals (Devriese *et al.,* 1992, Aarestrup *et al*., 2002). Furthermore, they are essential part of the gastrointestinal tract of humans and animals, and their distribution is very similar in these sources. *Enterococcus faecium* and *E. faecalis* are the most common in the human gastrointestinal tract, *E. faecium* in farm animals while *E.*

mundtii and *E. casseliflavus* in plant. The isolation of *E. faecium* and *E. faecalis* is less prevalent from livestock than from human faeces (Franz *et al*., 1999). Transmission is via the oral and/or aerosol routes as well as from skin wounds. Infection may result in septicemia. Endocarditis can occur when the infection progresses to a subacute or chronic stage.

2.11.4 Resistance to antibiotics in *Enterococcus* sp.

Resistance of *Enterococcus* species to several antibiotics is their fundamental characteristic as observed in *E. faecalis* and *E. faecium* commonly associated with human infections (Huycke *et al.,* 1998, Kwow and Chow, 2003). Their antibiotic intrinsic resistance is related to inherent or naturally chromosomally encoded characteristics present in all or most of the enterococci (Billstrom *et al.,* 2008). The various intrinsic (inherent) traits expressed by enterococci include resistance to semisynthetic penicillinase-resistant penicillins, cephalosporins, low levels of aminoglycosides, and low levels of clindamycin (Murray, 1998). In acquired resistance, its occurrence is more variable resulting from either a mutation in existing DNA or acquisition of new genetic determinants found in plasmids or transposons (Strausbaugh and Gimlore, 2000). Examples of acquired resistance include resistance to chloramphenicol, erythromycin, high levels of clindamycin, tetracycline, high levels of aminoglycosides, penicillin by means of penicillinase, fluoroquinolones, and vancomycin (Murray, 1990).

Enterococci are commonly resistant to macrolides, cephalosporins and tetracycline and often exhibit high-level resistance to gentamicin, precluding synergy with β-lactams or glycopeptides which are important in human medicine (Lukasova and Sustackova, 2003;

Brown *et al*., 2008). The evidence for dissemination of resistant enterococci from animals to man have been provided by some studies and more importantly, the exchange of resistance genes between poultry and human enterococci (van den Bogaard *et al*., 2002). Multidrug resistance is common among enterococci and presents a formidable treatment problem (Donabedian *et al*., 2003). Food animals are implicated as reservoirs for antibiotic resistant enterococci, following the use of antibiotic growth promoters and prophylactics (Bates *et al*., 1993; Waites *et al*., 2006).

Resistant strains of enterococci could also originate from humans and some studies have shown that isolates in poultry originated from humans with a history of poultry exposure (Borgen *et al*., 2002). Carriage of resistant enterococci in healthy poultry slaughterers and farmers has been described (Borgen *et al*., 2000), and transmission of resistant strains is likely to occur between poultry and humans in these settings (Borgen *et al*., 2002). The antibiotic resistance of *Enterococcus* varies depending on the geographical site, national and local antibiotic usage politics, and usage intensity.

2.11.5 *Enterococcus* sp. Infection in humans and animals

2.11.5.1 *Enterococcus sp. infection in humans*

Enterococcal infections include urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia and neonatal sepsis (Poh *et al.*, 2006). In Europe, infection with *Enterococcus* species was considered harmless to humans for a long time but recently, enterococci have been reported as the second most common cause of wound and urinary tract infection and the third most common cause of bacteraemia (De Fátima Silva Lopes *et al.*, 2005). In 2005, in the UK there were 7066 reported cases of Enterococcal bacteraemia, sixty-three percent of these cases being due to *E. faecalis* and twenty-eight

percent to *E. faecium,* both of which have increasing antibiotic resistance (HPA, 2007). In the USA approximately twelve percent of the hospital-acquired infections are *Enterococcus* species with *E. faecalis* as the most common species associated with clinical infection while *E. faecium* poses the higher antibiotic resistance threat (Giraffa, 2002). *Enterococcus* species with the highest virulence are medical isolates, followed by food isolates and then starter strains (Busani *et al*., 2004; Ben Omar *et al*., 2004).

2.11.5.2 *Enterococcus sp. infection in poultry*

Enterococcal infections in poultry are usually secondary to another disease and can result in either an acute or subacute/chronic form. In the acute form of enterococcosis, clinical signs are related to septicemia and include depression, lethargy, ruffled feathers, diarrhea, and a decrease in egg production. In the subacute/chronic form, depression, lameness, and head tremors may be noted. If untreated, most affected birds die. Egg transmission or fecal contamination of hatching eggs can often lead to late embryo mortality, and an increased number of hatchlings are unable to "pip" through the shell at hatch. Acute enterococcosis lesions include splenomegaly, hepatomegaly, enlarged kidneys, and congestion of subcutaneous tissue. Multifocal, whitish tan areas of necrosis may be observed on the liver and spleen. Omphalitis or enlarged yolk sacs may be seen in infected chicks or poults. In the subacute/chronic form, lesions include pericarditis, perihepatitis, airsacculitis, arthritis and/or tenosynovitis, osteomyelitis, myocarditis, and valvular endocarditis. An enlarged, flaccid heart with pale to hemorrhagic areas in the myocardium has also been reported along with infarcts throughout the internal organs. Focal granulomas can be found in many tissues as a result of septic emboli. Gram-positive bacterial colonies are readily observed in thrombosed vessels and within areas of necrosis (MVM, 2012).

2.11.6 Factors determining virulence of *Enterococcus* sp.

Many factors are responsible for the virulence of *Enterococcus* species these factors include the following (Franz *et al*., 1999).

i. ability to colonize the gastrointestinal tract, which is the normal habitat;
ii. ability to adhere to a range of extracellular matrix proteins, including thrombospondin, lactoferrin and vitronectin; and
iii. ability to adhere to urinary tract epithelia, oral cavity epithelia and human embryo kidney cells.

2.11.7 Pathogenesis of *Enterococcus* species

The chemical substances that are involved in the pathogenesis and facilitate disease in Enterococci infection are as stated below:

2.11.7.1 *Aggregation substance*

The aggregation substance (Agg) on the surface of *E. faecalis*, has been shown in vivo to form large aggregates thereby contributing to pathogenesis. The presence of Agg increases the hydrophobicity of the enterococcal cell surface which induces localization of cholesterol to the phagosomes, delaying fusion with lysosomal vesicles (Eaton and Gasson, 2002). Agg is a pheromone-inducible surface glycoprotein and mediates aggregate formation during conjugation, thus aiding in plasmid transfer as well as adhesion to an array of eukaryotic surfaces (Koch *et al*., 2004).

2.11.7.2 *Adhesion of collagen*

Ace (adhesion of collagen) is another cell-surface protein present in *E. faecalis*. It is a collagen-binding protein that belongs to the Microbial Surface Components Recognizing

Adhesive Matrix Molecules (MSCRAMM) family. This protein (Ace) according to Koch *et al.* (2004) may play a role in the pathogenesis of endocarditis.

2.11.7.3 *Extracellular surface protein*

Extracellular surface protein (Esp) is a cell-wall-associated protein first described in *Enterococcus* species by Shankar *et al*. (1999). It promotes adhesion, colonization and evasion of the immune system, and also play some role in antibiotic resistance (Foulquie Moreno *et al*., 2006). It contributes to the formation of enterococcal biofilm which could lead to resistance to environmental stresses, and adhesion to eukaryotic cells such as those of the urinary tract (Borgmann *et al*., 2004). The ability of enterococci to produce biofilms is very paramount in causing endodontic and urinary tract infections, as well as endocarditis. The formation of pili by enterococci is necessary for biofilm formation, the gene cluster associated with this being ebp (endocarditis- and biofilm-associated pili).

2.11.7.4 *Cytolysin*

Cytolysin (haemolysin) is a bacterial toxin whose the genes are located on pheromone-responsive plasmids (Koch *et al*., 2004). Cytolysin has β-haemolytic properties in humans and is bactericidal against other Gram-positive bacteria. Cytolysin is regulated by a quorum-sensing mechanism involving a two-component system.

2.11.7.5 *Enzymes*

A group of hydrolytic enzymes including hyaluronidases, gelatinase and serine protease are involved in the virulence of *Enterococcus* species (Semedo *et al*., 2003). Hyaluronidase acts on hyaluronic acid and is a degradative enzyme which is associated with tissue damage. Hyaluronidase depolymerizes the muco-polysaccharide moiety of connective tissue, thus facilitating spread of enterococci as well as their toxins through host tissue.

Hyaluronidase is encoded by the chromosomal hyl gene. The main role of both gelatinase and serine protease in enterococcal pathogenesis is in the provision of nutrients to the bacteria by degradation of host tissue, although they also have some function in biofilm formation. Gelatinase (GelE) is an extracellular zinc metallo-endopeptidase secreted by *E. faecalis* (Koch *et al*., 2004). It is able to hydrolyse gelatin, casein, haemoglobin and other bioactive peptides. The gene (gelE) encoding GelE is located on the chromosome and is regulated in a cell-density-dependent manner.

2.11.8 Prevention and control of *Enterococcus* species

Prevention and control requires reducing stress and preventing immunosuppressive diseases and conditions. Proper cleaning and disinfection can reduce environmental enterococcal resident flora to minimize external exposure (MVM, 2012).

2.11.9 Treatment of Enterococcal infections

2.11.9.1 *Treatment in human*

Cell wall-active agents with limited or no activity against enterococci include nafcillin, oxacillin, tircarcillin, ertapenem, most cephalosporins and aztreonam. Enterococci are relatively resistant to aminoglycosides, and their serum concentrations required for bactericidal activity are much higher and cannot be achieved safely in humans. The simultaneous use of a cell-wall active gent is needed to raise the permeability of the cell so that the aminoglycoside concentration can be achieved (Baddour *et al.,* 2005). Prior to the use of penicillin or ampicillin for endocarditis or life threatening enterococcal infections such as meningitis, the isolate should be screened for beta-lactamase production with nitrocefin, a chromogenic cephalosporin even if the isolate is susceptible to ampillicin. Also

the isolates from such life-threatening infections should be tested for high resistance to gentamicin and streptomycin (Caballero-Granado *et al.,* 1998).

2.11.9.2 *Treatment in poultry*

In the poultry industry, the treatment of *Enterococcus* infection includes the use of penicillin, erythromycin, novobiocin, oxytetracycline, chlortetracycline, or tetracycline in acute and subacute infections. Clinically affected birds respond well early in the course of the disease. As the disease progresses within a flock, treatment efficacy decreases. Antibacterial sensitivity should be performed on bacterial isolates in any clinical cases of enterococcosis before treatment begins. There is no treatment for poultry with bacterial endocarditis (MVM, 2012).

2.12 Antibiotics

This is a term used to describe antibiotic agents (usually antibacterial) that can be used to treat infections.

2.12.1 Historical discovery of antibiotics

Antibiotic discovery started in the 1928 by Alexander Fleming who observed the inhibition of growth on agar plate containing *Staphylococcus* species. The bacterium, Penicillium neonatum was found to be responsible for the inhibition of the *Staphylococcus* species. This marked the genesis of the discovery of penicillin and other antibiotic drugs. This discovery has saved millions of humans and animals from infectious disease-causing organisms (Byarugaba, 2010). The discovery of antibiotics in the 19^{th} century reduced the threat of infectious diseases against human race.

2.12.2 Use of Antibiotics in Animal Production

Antibiotics are routinely used in livestock production to treat and prevent diseases. In addition, subtherapeutic concentration are commonly added to animal feed and/or drinking water as growth promoters, and have been a regular part of poultry production and other food animals (Cromwell, 2001) when used in this manner, antibiotics can selected for resistant bacteria in the gastrointestinal tract of production animals, providing a potential reservoir for dissemination of drug-resistant bacteria into other animals, humans and the environment (Andremont, 2003). The potential for long-term, cummulative inputs of antibiotics and correspondingly, their potential effects on acquisition and maintenance of antibiotic resistance mechanisms in bacteria suggest a huge impact on the occurrence, persistence and mobility of resistance genes in natural environments (Andremont, 2003).

A number of reviews, reports and opinion papers have emerged to address the possible link between antibiotic use and the impact on antibiotic resistance development (Seveno *et al.,* 2002; Kummerer, 2004). These reviews have highlighted various issues related to antibiotic use in agriculture, often focusing on the link to emerging antibiotic-resistant bacteria, gene transfer mechanisms and consequent risks to human and animal health.

2.12.3 Indications for antibiotic use in food animals

Antibiotics are used in food animals to treat or prevent disease and also promote growth. Various sources provide data on such uses in animals (Arello, 1998). Therapeutic treatments are intended for animals that are diseased. In food animal production, individual animals may be treated, but it is more efficient treating entire groups by medicating feed or

water such as in poultry (AVMA, 2001). Producers administer antibiotics to food animals to promote growth and to enhance feed efficiency (NAS, 1999).

2.12.4 Quantity of antibiotic used in livestock production

Reliable data for antibiotic use in livestock are not publicly available, this make it difficult to determine which drugs are used in what quantity and for what purpose (Mellon *et al.,* 2001). Accurate data are needed for each drug used in feed animals, purpose (e.g., therapy, growth promotion), and route of administration and duration of treatment (Bager, 2000). Volume of estimates and other sample comparisons between antibiotics used for animals and humans can give only a rough idea of the potential effect of those uses on the development of antibiotic resistance and human health.

2.12.5 Antibiotic use practices in poultry production

Many antibiotics are approved for treatment or growth promotion in poultry production (USDA, 2001). Many problematic infectious diseases are controlled with antibiotics. Antibiotics approved for growth promotion and feed efficiency in poultry include bacitracin, chlortetracycline, penicillin, virginomycin, fluoroquinolones (Tanner, 2006). Treatment entails administration of the antibiotic in water to an entire flock, because single bird treatment is not practical.

2.12.6 Entry of antibiotics into the environment

Antibiotics used in poultry production can enter the environment via a number of routes including the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drugs (Andremont,

2003,Beausse, 2004; Boxall *et al.,* 2002; 2006). The excretion of waste products by animals, atmospheric dispersal of feed and manure dust containing antibiotics and the incident release of products from spills or discharges are also potential pathways of antibiotic residue entry into the environment (Nielsen *et al.,* 2000).

Many antibiotics are not completely absorbed in the gut, resulting in the excretion of the parent compound and its breakdown metabolites (Boxall *et al.,* 2004). In a report, Elmund *et al.* (1971) observed that about 75 % of the antibiotics administered to animals could be excreted into the environment. Also, Feinman and Matheson (1978) suggested that about 25 % of oral dose of tetracycline is excreted in faeces and another 50-60 % is excreted unchanged or as an active metabolite in urine. Furthermore, oral administration of the macrolide tylosin resulted in a maximum of 67 % of the antibiotics excreted, mainly in faeces (Feinman and Matheson, 1978).

The practice of land application of livestock manure provides large-scale areas for introduction of antibiotics into the environment. Once released into the environment, antibiotics can be transported either in a dissolved phase or adsorbed to colloids or soil particles into surface water and groundwater (Yang and Carlson, 2003; Krapac*et al.,* 2004). However, manure potentially contains significant amounts of antibiotics and their presence can persist in soil after land application.

2.12.7 Mode of action of antibiotic agents

Most antibiotic agents are classified or categorized according to their principal mode of action (Neu, 1992).

2.12.7.1 *Interference with cell wall synthesis*

Some antibacterial drugs work by inhibiting bacterial cell wall synthesis such as penicillin, cephalosporins, vancomycin. They inhibit synthesis of the bacteria cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer of the cell wall while others such as vancomycin interfere with cell wall synthesis by binding to the terminal D-alanine residues of the nascent peptidoglycan chain, thereby preventing the cross-linking steps required for stable cell wall synthesis (McManus, 1997).

2.12.7.2 *Inhibition of protein synthesis*

Macrolides, aminoglycosides, tetracyclines, chloramphenicol and streptomycin produce antibacterial effects by inhibiting protein synthesis. These drugs bind to the 30s and 50s subunit of the ribosome (Neu, 1992).

2.12.7.3 *Interference with nucleic acid*

Antibiotic drugs such as fluoroquinolones exert their antibacterial effects by disruption of DNA synthesis causing lethal double stranded DNA breaks during DNA replication (Drlica and Zhao, 1997).

2.12.7.4 *Inhibition of metabolic pathway*

Sulphonamides and trimethoprim block the pathway for folic acid synthesis which ultimately inhibits DNA synthesis (Yao and Moellering, 2003; Petri, 2006). The common drug combination of trimethoprim and sulphonamide inhibits two steps in the enzymatic pathway for bacterial folate synthesis (Tenover, 2006).

2.12.7.5 *Disruption of bacterial membrane structure*

Polymyxins are postulated to exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial contents (Petri, 2006).

2.13 Antibiotic resistance

Antibiotic drug resistance has been recognized as an emerging global problem in both human and veterinary medicine (Sayah *et al.,* 2005). Antibiotic drugs are considered the most important factor for the emergence, selection, and dissemination of antibiotic agent-resistant bacteria (Witte, 1998). Bacteria in the guts of humans and animals are subjected to different types, concentrations, and frequencies of antibiotic agents which results in the development of resistance (Prescott *et al.,* 2000). Over time, selective pressure picks out resistant bacteria that have specific fingerprints for resistance to the antibiotic agents that has been previously used (Prescott *et al.,* 2000; Troy *et al.,* 2002).

Bacteria gain antibiotic resistance genes through mobile elements, such as plasmids, transposons, and integrons (Prescott *et al.,* 2000) which results in mutations in genes responsible for antibiotic drug uptake or binding sites or activation of proteins of bacteria chromosomes (Alekshun and Levy, 1999). Antibiotic usage is possibly the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant microorganisms in human and animals. This acquired resistance occurs not only in pathogenic bacteria but also in endogenous flora of exposed individuals (Daniels *et al.,* 2009; Nsofor and Iroegbu 2012).

In extensively reared poultry, antibiotics may be administered to whole flocks rather than individual animals, and antibiotic agents may be fed continuously to birds as growth promoters giving rise to antibiotic selection pressure for such drug resistance in the animals and subsequently their fecal flora will contain a relatively high proportion of resistant bacteria (Whitworth *et al.,* 2008). Due to intricate balance of microflora of different habitats within the ecosystem, the transfer of resistance genes among bacteria occupying different habitats has the potential to occur frequently (Davies *et al.,* 2009).

Widespread dependence on antibiotics in livestock production has led to considerable rise in antibiotic resistant strains of bacteria, thereby complicating treatment of infectious diseases in animals and human. This has led to serious changes in the perceptions and priorities of regulatory agencies with regard to antibiotic usage as growth promoters and prophylactic agents (Call *et al.,* 2008).

2.13.1 Genesis of antibiotic resistance

After the discovery of penicillin, a number of treatment failures ensued and some bacteria were no longer sensitive to penicillin (Byarugaba, 2010). This was the genesis of resistance to antibiotics. Increasing prevalence of resistance has been reported in many pathogens globally including developing countries. The resistance has been attributed to changing microbial characteristics, selective pressures of antibiotic use, and societal and technological changes that enhance the development and transmission of drug-resistant organisms (Walsh, 2000). Antibiotic resistance as a natural biological phenomenon is enhanced as a consequence of adaptation by infectious agents to exposure to antibacterial agents used in humans or agriculture. It is a general opinion that the use of antibiotics is a

single most significant factor responsible for the increase and spread of antibiotic-resistance (Aarestrup *et al.,* 2004; Byarugaba, 2004).

2.13.2 Mechanisms of antibiotic resistance in bacteria

There has been a continual battle between the multitude of organisms and human from time immemorial (Tenover, 2006). Infectious diseases have affected substantial portion of human population, causing significant morbidity and mortality. However, major advances in antibacterial drug development to control infection helped turn the tide in favor of the human population. The excitement over the potential conquest of infectious diseases by these antibacterial drugs was short lived because these bacteria responded by the manifestation of various forms of resistance. Furthermore, as the use of antibacterial drugs increased, so did the level and complexity of the resistance mechanisms by bacterial pathogens. Unfortunately, as bacteria evolve ever more clever mechanisms of resistance, the number of scientists involved in research and development of new antibacterial drugs are dwindling (Tenover, 2006).

2.13.3 Antibiotic selective pressure

Selective pressure imposed by the use of antibiotics in both human and veterinary medicine promotes the spread of multiple antibiotic resistances resulting in the growing problems of infections that are difficult to treat (Carattoli, 2003). Microbial resistance to some β-lactams, tetracyclines, chloramphenicol, or trimethoprim is reported with increasing proportion (Velonakis *et al.,* 2001). Selective pressure on antibiotics results in adverse effect of antibiotic resistance, and this has been recognized as treatment failure. The disease

caused by the pathogen may worsen significantly due to the incurred resistance (Clarence and Nosakhare, 2008).

2.13.4 Bacterial resistance to antibiotic drugs

Microbes manifest resistance to antibacterial drugs through different mechanism. Some bacteria are innately resistant to more than one class of antibiotic drugs (Tenover, 2006). In such cases, all strains of that bacteria species are likely to develop resistance to all members of those antibacterial classes. Cases of acquired resistance are of greater concern, where bacteria populations initially susceptible become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that drug (WHO, 1998; 2000).

Varied mechanisms of antibiotic resistance are readily spread to several bacteria genera. First, the organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect (Bester and Essack, 2010). Secondly, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect (Poole and Srikumar, 2001; Schweizer, 2003). Thirdly, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacteria cell walls that no longer contain the binding site for the antibiotic agent, or bacteria may acquire mutations that limit access of antibiotic agents to the intarcellular targets site via down-regulation of porin genes (Singh *et al.,* 2005; Maurelli, 2006; Bester and Essack, 2010). This may occur either through one of several genetic mechanisms such as transformation, conjugation or transduction (Bester and Essack, 2010).

Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacterial with resistance to more than one or three antibacterial drug classes (multiple drug resistance) have become a serious global concern (Tenover, 2006). Strains of bacteria carrying resistance conferring mutations are selected by antibiotic use, which kills the susceptible starins but allows the newly-resistant starins to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical evolution (Bester and Essack, 2010; Roh *et al.,* 2010). Bacteria also acquire resistance through the acquisition of new genetic materials from other resistant organisms. This is termed horizontal evolution, and may occur between strains of the same species or between different species or genera.

The mechanism of genetic exchange includes conjugation, transduction and transformation (McManus, 1997; Bester and Essack, 2010). For each of these processes, transposons may facilitate the transfer and incorporation of the acquired resistance genes into the host's genome or into plasmids.

2.13.5 Pathways for antibiotic resistance transfer

Direct (primary) and indirect (secondary) pathways are linked to the problem of acquired antibiotic resistance. The primary pathways are mutations in the gene encoding resistance against the mechanisms of action of particular antibiotics (Dessen *et al.,* 2001; Ghosh and LaPara, 2007) while secondary pathways are gaining of small fragments of DNA coding for resistance. These secondary pathways are further categorized into three: transformation, conjugation and transduction (Catry *et al.,* 2003).

2.13.5.1 *Transformation pathway*

Transformation is the process by which bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis (Bester and Essack, 2010). Mutation and selection, together with the mechanisms of genetic exchange, enable many bacterial species to adapt quickly to the introduction of antibiotic agents into their environment (McGowan, 2001). The DNA in a natural transformation comes from a donor bacterium. Consideraable amounts of DNA are released into surrounding environment when bacteria lyse. These fragments may be relatively large and contain several genes. If a fragment contacts a "competent cell"ie a cell that is able to take up DNA and be transformed, the DNA can be bound to the cell and taken inside. The transformation frequency of very competent cells is around 10^{-3} fro most genera when an excess of DNA is used i.e. about one cell in every thousand will take it up and intergrate the gene. Competency is a complex phenomenon and is dependent on several conditions. Bacteria need to be in a certain stage of growth eg Streptococcus pneumoniae becomes competent during the expotential phase when the population reaches about 10^{7}-10^{8} cells per ml. When a population becomes competent, bacteria such as S.pneumoniae secrete a small portion called competence factor that stimulates the production of 8-10 new proteins required for transformation.

2.13.5.2 *Conjugation pathway*

During conjugation, a Gram negative bacterium transfers plasmid containing resistance genes to an adjacent bacterium, often via an elongated proteinaceous structure termed pilus, which joins the two organisms while conjugation in Gram positive bacteria is usually

initiated by production of sex pheromones by the mating pair, which facilitate the clumping of donor and recipient organisms allowing the exchange of DNA (Roh *et al.,* 2010). The conjugation pathway allows transmissible plasmids and chromosomal DNA of very large sizes to be transferred from cell to cell, either within or between species, mediating the transmitted genetic material through various specified enzyme activities (Catry *et al.,* 2003).

This pathway does not select DNA material simply from the environment, but has a direct cell-to-cell exchange of genetic material, where one cell is the donor of the genetic material and the other the new host or the recipient (Bester and Essack, 2010). This pathway uses a very specific, hair-like attachment on the surface of the bacterial cell, constructed mainly of oligomericpilin protein, which acts as a bridge pulling the two cells together. Enzyme activities further initiates the transportation of a single DNA strand to the new host, where both cells synthesis duplicates, and both cells can act as donor cells (Catry *et al.,* 2001; Bester and Essack, 2010). Conjugation plasmids that have the ability to replicate independently in the recipient or newly acquired host have greater opportunity to spread through a bacterial flora community than those without conjugative abilities (Maurelli, 2006). It is unclear whether the transfer of genetic elements through conjugation occurs voluntarily.

2.13.5.3 *Transduction pathway*

Transduction is the frequent mode of horizontal gene transfer in nature and is mediated by viruses. It is the transfer of bacterial genes by viruses. Bacterial genes are incorporated into a phage capsid because of errors made during the virus life cycle. The virus containing

these genes then injects them into another bacterium completing the transfer (Willey *et al*., 2008). There are two different kinds of transduction

i. Generalized transduction: This occurs duringbthe ltic cycle of virulent and some temperate phages and can transfer any part of the bacterial genome. Durig the assembly stage, when the viral chromosomes are packaged into protein capsids, random fragments of the partially degraded bacterial chromosome also may be packaged by mistake. The DNA is left behind because the capsid can contain only a limited quantity of DNA. The quantity of bacterial DNA depends primarily on the size of the capsid.

ii. Specialized transduction: In this type of transduction, the transducing particle carries only specific portions of the bacterial genes. It is made possible by an error in the lysogenic life cycle of phages that insert their genomes in the specific site in thhist chromosome. Excision is sometimes crried ot improperly. The resulting phage genome contains portions of the bacterial chromosome (about 5-10% 0f the bacterial DNA) next to the intergration site. A transducing phage genome is usually defective and lacks some part of its attachment site. The transducing particle will inject bacterial genes into anthor bacterium, even though the defective phage can not reproduce withot assistance i.e. bacterial genes may become stably incorporated under the proper circumstances.

Current knowledge postulates that transduction is a feature mainly of bacteriophage (Bester and Essack, 2010).

2.14 Emergence and spread of resistant bacteria due to antibiotic use in animals

In animals, antibiotic resistance in zoonotic enteropathogens (e.g., *Salmonella*, *Staphylococcus* and some strain of *Escherichia coli* such as *E. coli* O157:H7) and commensals (enterococci and most generic *E. coli*) are of great concern to public health, because, these bacteria are most likely to be transferred to the zoonotic enteropathogens (WHO, 1997; 1998). There is considerable evidence that antibiotic use in animals selects for resistance in commensals and in zoonotic enteropathogens (Jacob-Rietsma *et al.,* 1994; Low *et al.,* 1997).

Antibiotic use in animals apparently contributes to the selection and spread of resistance among populations of bacteria in animals. Some antibiotic animal-treatment practices may exert selective pressures for resistance than others. Feeding birds growth promoters, which entails exposing bacteria to sub-lethal concentrations of drugs over long periods, would appear conducive to selecting and maintaining resistance organisms (Barragry, 1994). Gentamicin resistance in some serotypes of salmonellae in poultry appears to have been amplified, at least in part, by this practice (NASMP, 1998). Faecal waste from poultry reared under intensive production often is spread as fertilizer or spread on pasture lands, sometimes after composting are implicated in the contamination of the environment with resistant bacteria (Chee-Sanford *et al.,* 2001).

2.15 Animal reservoirs of antibiotic resistance

Livestock production is mainly incriminated as the hotspot for reservoirs of antibiotic resistance. Production animals are often subjected to preventive, therapeutic or prophylactic treatment with antibiotic agents (Ghosh and LaPara, 2007). Nevertheless, sub-therapeutic

use of antibiotics in livestock production is reported to be a major contributor to proliferation of antibiotic resistant amongst environmental bacteria (Bester and Essack, 2010). Ghosh and LaPara (2007) reported in a study that the clearance of animal pens of manure, followed by its subsequent disposal, had a greater impact on the lateral (horizontal) transfer for genes coding for antibiotic resistance in soil bacteria. Generally, animals also have a high level of exposure to their environment, especially the soil environment, which makes it easier for them to be infected with bacteria that carry antibiotic resistance gene (D'Costal *et al.,* 2006).

2.16 Concern on antibiotic resistance in animal and human health

Several reasons exist why antibiotic resistance is a concern not only to physicians and veterinarians alone but also the public. Foremost, resistant bacteria such as *E. coli,* enterococci and staphylococci are commonly associated with health care institutions and farms (Karlowsky *et al.,* 2003; NNIS, 2004). Bacterial resistance often results in treatment failure which may result in serious consequences (Tenover, 2006). Inadequate empirical antibacterial therapy (the initial use of an antibacterial drug to which the causative agent may not be susceptible), has been associated with increased mortality rates in patients with bloodstream infections due to resistant bacteria. Resistant bacteria may also spread and become broader infection-control problem (Woodford *et al.,* 2004). The spread of resistant microorganisms poses additional problems for infection control globally and places added burden on healthcare costs (McGowan *et al.,* 2001).

Antbiotic resistance is also a concern for animal health. Resistance among animal pathogens reduces the effectiveness of some drugs (Wray and Gnanou, 2000). This effect

could potentially affect public health if drug use in food animals increases to compensate for this drop in effectiveness or if alternative drugs that are crucial to human health are used to treat animals (NAS, 1999). Antibiotic resistance has been reported in a wide variety of animal pathogens e.g., *E. coli* of calves, pigs and poultry (Martel and Coudent, 1993). Generally, there is high variability among animal pathogens in different geographical areas (Dargatz *et al.,* 2000; Wells *et al.,* 2001). Furthermore, some isolates of pathogens such as *E. coli* are resistant to multiple antibiotics.

2.17 Emerging zoonoses due to antibiotic resistance

Zoonotic bacteria with pathogenic capabilities are a threat to human health especially when they become resistant to antibiotics (Geonaras *et al.,* 2001). Bacteria normally associated with animals, have qualities that allow them to replicate in humans with grave disease consequences. These bacteria have a greater chance of being carriers of antibiotic resistant genes (Van Vuuren *et al.,* 2007). In addition to zoonotic bacteria acquiring antibiotic resistance, pathogenicity can also be increased by the addition of virulent genetic elements (WHO, 1997; Aquino *et al.,* 2002).

In rural areas in developing countries, there is a high risk of exposure to food animals, and thus exposure to zoonotic bacteria (Gummow, 2003), even though the use of antibiotic growth promoters may not be a normal farming practice.

2.18 Prospects for the control of antibiotic resistance

In the last decades, the frequency and spectrum of antibiotic resistant infections have increased in communities and hospitals (Cohen, 1992), this has resulted in increased morbidity and mortality, and health-care costs (Hooper, 2001; Aibinu *et al.,* 2003b).

Poultry remains an important vehicle of bacteria pathogens transmission to humans, occurring mainly via contaminated meat, and environment (Marcus *et al.,* 2007; Alali *et al.,* 2010). Furthermore, many scientists/researchers points to antibiotic use in agriculture (e.g., poultry) as the driving force behind development and dissemination of antibiotic-resistant pathogens (Rajashekara *et al.,* 2000; Alali *et al.,* 2010). Therefore, it is assumed that most conventional poultry enterprise may be beset with the problem of antibiotic resistance because, poultry production focuses on reducing cost and maximizing production through weight gain and feed efficiency (Sundrum, 2006), and this is mainly achieved by the sub-therapeutic (growth promotion) or therapeutic antibiotic use .

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area consisted of some poultry houses in Samaru, Zaria, Nigeria.

3.2 Study Population

Commercial and free-range birds belonging to owners who are willing to participate in this study forms the included population for the study and the exclusion criteria comprises of commercial and free-range birds belonging to owners who are not willing to participate in this study. The quasi experimental design was used for the collection of samples.

3.3 Sample size

A total of two hundred faecal samples were randomly collected from intensive poultry houses (100 samples) and extensive free range chickens (100 samples).

3.4 Sample collection and processing

The faecal samples were collected early hours of the morning from the poultry houses and the free-range chickens on a weekly basis. The faecal samples were placed in clean glass containers. They were taken to the laboratory in the Department of Microbiology under ambient conditions and processed immediately. This study was carried out between April, 2012 and June, 2013.

3.5 Preparation of media

The media used in this study include: Eosin Methylene Blue Agar, Salmonella-Shigella Agar, Bile Esculin Agar, Plate Count Agar, Mannitol Salt Agar, Citrate Agar,

Methyl Red-Voges Proskauer medium, Triple Sugar Iron Agar, Blood Agar Base, and Mueller-Hinton Agar. They were all prepared according to manufacturers' instructions and the prepared media were then stored at 4°C for use during cultivation.

3.6 Enumeration of total bacterial load in the faecal droppings

Enumeration of the total bacterial load was done using the Plate Count Agar (PCA) by the conventional spread plate technique. Serial dilutions of the faecal samples were carried out by transferring 1ml of the suspension made by mixing one gram of the faecal droppings with nine mls of distilled water in a test tube. After mixing thoroughly, one ml of the suspension was transferred from dilution 10^{-1} to 10^{-2} and this was done up to dilution 10^{-8}. The suspensions from dilution 10^{-8} was inoculated on the Plate Count Agar plates and spread out aseptically on the surface of the agar medium using a bent glass rod after being dipped into methylated spirit and flamed. The plates were inverted and incubated at 37°C for 24 hours for enumeration of total bacterial load (F.A.O. Manual, 1979).

3.7 Inoculation of samples on the differential media

The samples collected were aseptically streaked onto Eosin Methylene Blue Agar, Mannitol salt agar and Bile Esculin Agar for *E. coli*, *S. aureus* and *Enterococcus* sp. The plates were inverted and incubated for 24 hours at 37°C under aerobic conditions. The faecal samples were inoculated in buffered peptone water for enrichment at 37^0C for 18hours and the broth was inoculated on Salmonella-Shigella agar for *Salmonella* sp. The plates were incubated at 37^0C for 24 hours (Chessebrough, 2006).

3.8 Isolation of target bacteria

The plates were examined after 24 hours and colonies with greenish metallic sheen, grayish colonies with black centres, yellow colonies, and brownish colonies were

considered tentatively as *E. coli*, *Salmonella* sp., *Staphylococcus aureus* and *Enterococcus* sp. respectively pending the biochemical characterization. The presumptive isolates were gram-stained and were purified by sub-culturing on the appropriate media. Discreet colonies were picked and streaked on nutrient agar slants, incubated for 24hours and stored at 4^{o}C until required for characterization (Ruzaukas *et al*., 2010, Pan *et al*., 2010, Oluduro, 2012).

The PCA plates were also examined after 24 hours. Plates containing 30 - 300 colonies were counted and the average counts were calculated. They were expressed as Colony Forming Units/ml of the sample (CFU/ml) (F.A.O. Manual, 1979).

3.9 Biochemical Characterization of the presumptive isolates

The presumptive isolates stored on nutrient agar slants were subjected to biochemical tests which were conventionally carried out on the isolates.

3.9.1 Biochemical tests for presumptive *E. coli* isolates

3.9.1.1 *Indole test*

Two to three colonies of the *E. coli* isolates were inoculated in test tubes containing Sulphur Indole Motility medium using a wire loop and incubated for 48 hours. Two drops of Kovac's reagent were added to the medium and shaken. Formation of a pink layer was recorded as a positive result (Chessebrough, 2000).

3.9.1.2 *Methyl-Red (MR) test*

Two to three colonies of the isolates were inoculated into Methyl red-Voges Proskauer broth (MR-VP) and incubated for 24 hours. Few drops of Methyl red were added to the

broth and shaken. It was recorded positive since there was formation of a pink-red product within 5-15minutes (Chessebrough, 2000).

3.9.1.3 *Voges-Proskauer (VP) test*

Two to three colonies of the isolates were inoculated into Methyl red-Voges Proskauer broth (MR-VP) and incubated for 24hours. After incubation, 0.6ml of α-naphthol solution and 0.2ml of 40% potassium hydroxide were added to 2.5ml of the culture broth and were shaken well. The tube was placed in a sloping position to allow air-liquid interface. It was recorded negative since there was no formation of a pink-red product within an hour (Chessebrough, 2000).

3.9.1.4 *Citrate utilization test*

Few colonies of the isolates were streaked on the slope of the Simmon's Citrate agar medium and stabbed to the butt using a sterile straight wire. They were incubated at 37^0C for 24 hours. The medium remained green in colour indicating a negative test (Chessebrough, 2000).

3.9.2 Biochemical characterization of presumptive *Salmonella* sp. isolates

3.9.2.1 *Indole test*

Two to three colonies of the *Salmonella* sp. isolates were inoculated in test tubes containing Sulphur Indole Motility medium using a wire loop and incubated for 24 hours. Two drops of Kovac's reagent were added to the medium and shaken (Chessebrough, 2000). Formation of a yellow layer was recorded as a negative result.

3.9.2.2 *Methyl-Red (MR) test*

Two to three colonies of the isolates were inoculated into Methyl red-Voges Proskauer broth (MR-VP) and incubated for 24 hours. Few drops of Methyl red were added to the broth and shaken. The tube was placed in a sloping position to allow air-liquid interface (Chessebrough, 2000). It was recorded positive since there was formation of a pink-red product within 5-15minutes.

3.9.2.3 *Voges-Proskauer (VP) test*

Two to three colonies of the isolates were inoculated into Methyl red Voges-Proskauer broth (MR-VP) and incubated for 24hours. After incubation, 0.6ml of α-naphthol solution and 0.2ml of 40% potassium hydroxide were added to 2.5ml of the culture broth and were shaken well. The tube was placed in a sloping position to allow air-liquid interface (Chessebrough, 2000). It was recorded negative since there was no formation of a pink-red product within 5-15minutes.

3.9.2.5 *Triple Sugar Iron Utilization Test*

Few colonies of the isolates were inoculated into the Triple Sugar Iron medium using a straight wire and incubated at 37^0C for 24 hours (Chessebrough, 2000). The formation of a red colour (K) at the slant and yellow colour (A) at the butt with or without production of gas (G) and Hydrogen sulphide (H_2S) was recorded as a positive test.

3.9.3 Biochemical Characterization of presumptive *Staphylococcus aureus* isolates

3.9.3.1 *Catalase test*

Two loopfuls of the hydrogen peroxide solution were placed on a sterile clean glass slide. A few colonies of the isolates were taken from the culture plate using a sterile wire loop and

immersed in the hydrogen peroxide solution (Chessebrough, 2000). Active bubbling indicates a positive test.

3.9.3.2 *Coagulase test*

A drop of distilled water was placed on each end of a slide. A colony of the test organism (previously checked by Gram staining) was placed in each of the drops to make two thick suspensions. A loopful of the sheep plasma was added to one of the suspensions, and mixed gently. The slide was held and tilted back and forth for one minute. The apparent clumping of cells in the bacterial suspension mixed with plasma showed a positive test (Chessebrough, 2000).

3.9.4 Biochemical Characterisation of Presumptive *Enterococcus* sp.

3.9.4.1 *Bile Esculin (BE) test*

Few colonies of the presumed *Enterococcus* sp isolates were inoculated on Bile Esculin Medium and incubated at 37^0C for 24 hours (Chessebrough, 2000). The light-dark brown colonies with a brown–black surrounding (hydrolysis of esculin) were recorded as positive.

3.9.4.2 *Growth in 6.5% Sodium Chloride broth*

Few colonies of the isolates were inoculated into the 6.5% Sodium Chloride broth and incubated at 42^0C for seven days. (Murray *et al*., 2003). Increase in turbidity in the broth was recorded as positive.

3.9.4.3 *Voges-Proskauer (VP) test*

Two to three colonies of the isolates were inoculated into Methyl red-Voges-Proskauer broth (MR-VP) and incubated for 24hours. After incubation, 0.6ml of α-naphthol solution and 0.2ml of 40% potassium hydroxide were added to 2.5ml of the culture broth and

shaken. The tube was placed in a sloping position to allow air-liquid interface (Murray *et al*., 2003). Formation of a pink-red product within 5-15min was recorded as positive.

3.9.4.4 *Haemolysis test*

A colony was picked from a 24hr culture and streaked on Blood agar. The plates were incubated at 37^0C for 24 hours. Colonies with α-haemolysis, β-haemolysis (colonies surrounded by a clear zone), δ-haemolysis (colonies that have no clear zone) were recorded (Murray *et al*., 2003). Colonies with α-haemolysis, β-haemolysis (colonies surrounded by a clear zone), δ-haemolysis (colonies that have no clear zone) were recorded.

3.9.5 Biochemical Characterisation using Microgen^{TM} Kits

The isolates were further confirmed using Microgen^{TM} GnA- ID, STAPH-ID and STREP-ID Kits. The data obtained by the Microgen GN-ID A microwell strip was designed to generate a 4 digit octal code which was used to interpret the result by the Microgen Identification System Software (Appendix IV).

3.9.5.1 *Confirmation of E. coli and Salmonella sp. using Microgen^{TM} GnA-ID Kit.*

A colony was emulsified from an 18-24 hour culture in 3ml sterile 0.85% saline for the GN A microwell test strips. The adhesive tape sealing the microwell test strips were carefully peeled. Three to four drops of the bacterial suspension were added to each well of the microwell test strips. After inoculation, wells 1, 2, 3 and 9 were overlaid with 3-4 drops of mineral oil. The top of the microwell test strips were sealed with adhesive tape and were incubated at 35-37^0C. The microwell test strips were read after 18-24 hours incubation. The adhesive tape was removed and all positive reactions were recorded with the aid of the colourful chart. Two drops of Kovac's reagent were added to well 8 and read after 60

seconds. One drop each of VP I and VP II reagent were added to well 10 and read after 15-30 minutes. One drop of TDA reagent was added to well 12 and read after 60 seconds.

3.9.5.2 *Confirmation of Staphylococcus aureus using Microgen^{TM} STAPH-ID Kit*

A colony was emulsified from an 18-24 hour culture in 3ml sterile 0.85% saline for the GN STAPH-ID microwell test strips. The adhesive tape sealing the microwell test strips were carefully peeled. Three to four drops of the bacterial suspension were added to each well of the microwell test strips. After inoculation, wells 10 and 11 were overlaid with 3-4 drops of mineral oil. The top of the microwell test strips were sealed with adhesive tape and were incubated at 35-37^{0}C. The microwell test strips were read after 18-24 hours incubation. The adhesive tape was removed and all positive reactions were recorded with the aid of the colourful chart. One drop of PYR was added to well 12 and read after 10 minutes. The nitrate reduction was performed on well 9 after reading the β-Glucuronidase reaction (one drop of Nitrate A reagent was added and one drop of Nitrate B reagent to the well and read after 60 seconds.

3.9.5.2 *Confirmation of Enterococcus sp. using Microgen^{TM} STREP-ID Kit.*

Gram-staining of the isolates and catalase test were carried out. Haemolysis produced on blood agar by the isolates were noted and recorded. The isolated colonies from the primary isolation medium were emulsified in the suspending medium supplied in the kit to produce suspension equivalent to 2.0 MacFarland and mixed thoroughly. The adhesive tape sealing the microwell test strips were carefully peeled. Three to four drops of the bacterial suspension were added to each well of the microwell test strips. After inoculation, wells 10 and 11 were overlaid with 3-4 drops of mineral oil. Three drops of Hippurate solution were added to an empty tube and one or two colonies were emulsified in the Hippurate solution

in the capped tubes and were incubated at 18-24 hours at 35-37^{0}C. The top of the microwell test strips were sealed with adhesive tape and were incubated at 35-37^{0}C. The microwell test strips were read after 18-24 hours incubation. The adhesive tape was removed and all positive reactions were recorded with the aid of the colourful chart. One drop each of VP I and VP II reagent were added to well 8 and read after 15-30 minutes. One drop of PYR reagent was added to well 11 and read after 5-10 minutes. Three drops of the ninhydrin reagent was carefully to the Hippurate test and incubate at room temperature for 10-15 minutes.

3.10 Antibiotic susceptibility testing

The antibiotic susceptibility testing of the isolates were carried out using the disc diffusion technique (Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966) according to the methods recommended by the Clinical Laboratory and Standards Institute, (2011) to determine the susceptibility or resistance profiles of the isolates. About 3-5 discrete colonies of the isolates were inoculated into 5ml of nutrient broth and incubated overnight at 37^{o}C. The overnight culture was then standardized with 0.5 MacFarland standard suspensions. Sterile cotton swab was used in inoculate the bacterial suspension to freshly prepared pre-dried Mueller-Hinton agar plates prepared according to manufacturer's instructions. The antibiotic sensitivity discs were aseptically and spaciously placed on the inoculated Mueller-Hinton agar plates. The antibiotics used in this study include: Ciprofloxacin (5µg), Chloramphenicol (10µg), Cloxacilin (5µg), Cotrimoxazole (25µg), Gentamicin (10µg), Erythromycin (5µg), Nalixidic acid (5µg), Streptomycin (10µg), Tetracycline (10µg) and Vancomycin (30µg) (Abtek Biologicals Ltd, UK, Lot no: LC01/P,

Expiry date: July, 2013; Liofilchem Diagnostici, Italy, Lot no: 030212013, Expiry date: March, 2014).

The reference standard strains used as control in this study include *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. They were obtained from the National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria.

3.10.1 Measurement of diameter of the inhibition zone

After incubation, the test plates were examined for confluent growth and zone of inhibition. The diameter of each zone of inhibition was measured in millimetre (mm) using a ruler on the underside of the plate. The interpretation of the measurement as sensitivity, intermediate and resistant was made according to National Committee for Laboratory Standards (1997, 1998) and Clinical Laboratory Standards Institute (CLSI, 2011)manual.

3.10.2 Multi-antibiotic resistance index

The multiple-antibiotic resistance (MAR) index was determined for each isolate by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotics tested (Olayinka *et al*., 2004).

$$\text{MDR index} = \frac{\text{Number of antibiotics the isolate is resistant to}}{\text{Total number of antibiotics tested.}}$$

3.11 Statistical analysis

The ststistical tool employed for the comparsion of the total plate counts of the commercial and free chickens and the comparsion of the antibiotic activities on the isolates from the commercial and free-range chickens was the t-test (SPSS, version 19).

CHAPTER FOUR

4.0 RESULTS

A total of thirty nine (39) isolates identified as *E. coli* (8), *Salmonella* sp. (5), *S. aureus* (10) and *Enterococcus* sp. (16) were obtained from two hundred (200) samples of chicken droppings - consisting of 100 samples from the commercial farms and 100 samples from homes that rear free-range chickens). Table 4.1 shows the overall distribution of selected bacteria in the faecal droppings of chickens. Table 4.2 shows the comparative analysis of the bioburden (microbial load) present in faecal droppings of commercial and free-range chickens. There is no significant difference in comparison of the microbial load.

4.1.1 Comparison of the antibiotic activity on *E. coli* isolated from faecal droppings of commercial and free-range chickens

Fig 4.1 shows the comparison of the antibiotic activity on *E. coli* isolated from faecal droppings of commercial and free-range chickens. There was no statistical significant difference observed in the susceptibility of *E. coli* isolated from the commercial and free-range chickens' faecal droppings ($p > 0.05$). It also shows the isolates from the free-range chickens exhibited larger zones of inhibition compared to those of commercial chickens except in the case of cloxacillin where they have the same zone of inhibition and in the case of gentamicin where the zones of inhibition for the isolates from the commercial chickens was larger than those of free-range chickens.

4.1.2Antibiotic susceptibility profile of *E. coli* isolated from faecal droppings of commercial and free-range chickens

Fig 4.2 shows the antibiotic susceptibility profile of *E. coli* isolated from the faecal droppings of commercial and free-range chickens. The antibiotic with the highest

antibacterial activity for commercial chickens was gentamicin with 100% activity against all the isolates. This was followed by a moderate antibacterial activity to ciprofloxacin, chloramphenicol, nalixidic acid and streptomycin against 50% of the isolates. Resistance was seen in the activity of tetracycline and cloxacillin. In the case of the free-range chickens, the antibiotic with the highest antibacterial activity was chloramphenicol with 100% activity against all the isolates which was followed by moderate susceptibility to gentamicin and ciprofloxacin (83%) and nalixidic acid (67%). Minimal resistance was observed in resistance to the activity of cotrimoxazole (23%). All isolates were found to be resistant to tetracycline and cloxacillin.

4.1.3 Resistance patterns of *E. coli* isolated from faecal droppings of commercial and free-range chickens

The resistance pattern of *E. coli* isolated from faecal droppings of commercial and free-range chickens is shown in Table 4.3. No resistance pattern was obtained with the single antibiotic resistance in the isolates of the commercial chickens as they were found to be resistant to more than one antibiotic. Two multiple resistance patterns were obtained with varying combinations of 3 and 7 antibiotic combinations. In the case of free-range chickens, six multiple resistance patterns with varying combinations of 2,3,5,6 antibiotic combinations were obtained. The highest frequency (2) was found in the combinations of 2 antibiotics.

4.1.4 Multiple antibiotic resistance and indices of *E. coli* isolated from faecal droppings of commercial and free-range chickens

Multi-antibiotic resistance (MAR) can be regarded as resistance to two or more antibiotics. All the *E. coli* isolates from the commercial and free-range chickens exhibited MAR. Multi

antibiotic resistance index (MARI) of the isolates from the faecal droppings of commercial and free-range chickens can be seen in Fig 4.3.

None of the isolates had a MAR index ≤ 0.2. Fifty percent of the isolates exhibited a MAR index of 0.4 and fifty percent of the isolates exhibited a MAR index of 0.9. Thirty-three percent of the isolates from free-range chickens exhibited a MAR indices of 0.3 and 0.6 while seventeen percent of the isolates from commercial chickens exhibited a MAR indices of 0.4 and 0.8(Fig. 4.3).

Table 4.1 Distribution of organisms isolated from faecal droppings of commercial chickens and free-range chickens

Management Systems	*E. coli*	*Salmonella* sp.	*S.aureus*	*Enterococcus* sp.	Total (%)
CC (n=100)	2(2)	1(1)	7(7)	9(9)	19(19)
FRC (n=100)	6(6)	4(4)	3(3)	7(7)	20(20)
Total (200)	8(4)	5(2.5)	10(5)	16(8)	39(19.5)

KEY: CC=Commercial chickens, FRC= Free-range chickens, n=number of chicken,

A (B)= number (percentage) of isolates

Table 4.2 Comparative analysis of the bacterial bioburden present in faecal droppings of commercial and free-range chickens

Management system	No. of faecal samples	Mean±S.E.M (log cfu g^{-1})
Commercial Chickens	100	9.86±0.22
Free-range Chickens	100	9.51±0.12

t= 1.4198, p=0.1572, S.E.M. = Standard Error Mean, cfu g^{-1}= coliform unit per gram

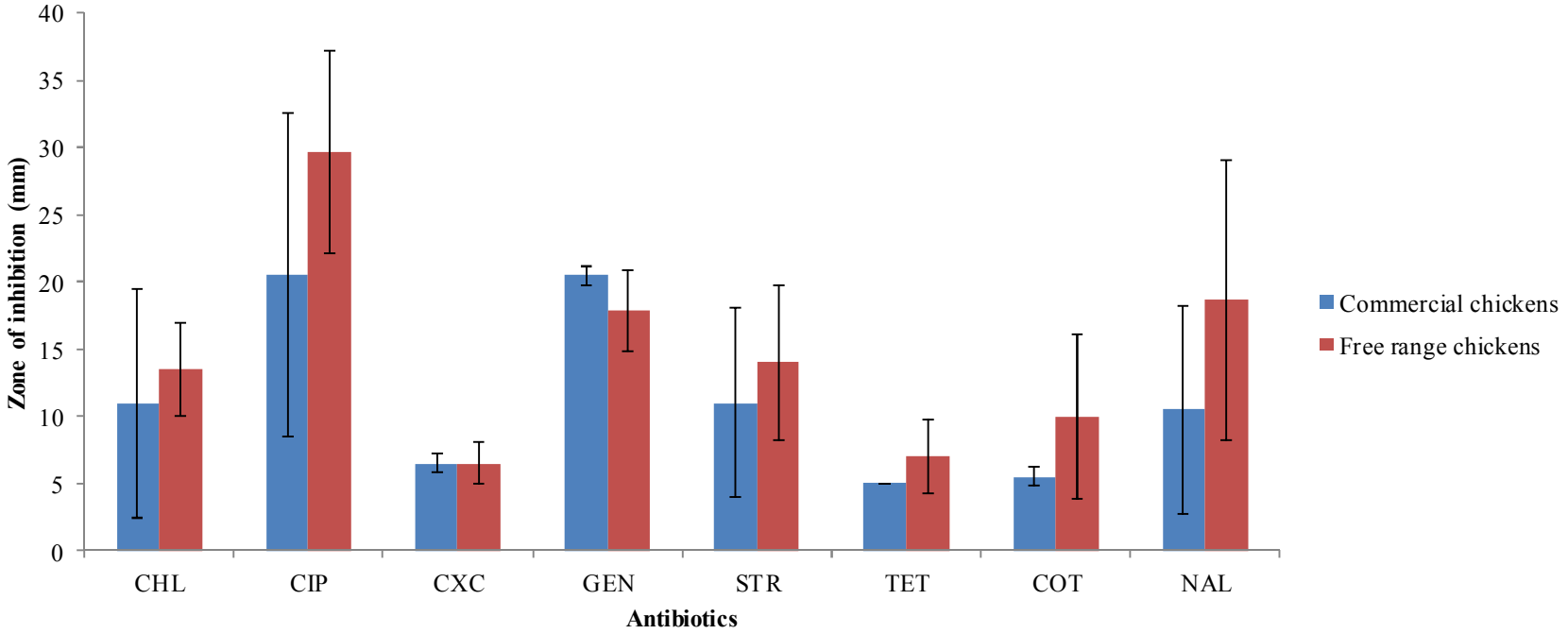


Figure 4.1: A comparison of the antibiotic activity on *E. coli* isolated from the faecal droppings of commercial and free-range chickens. Key:CHL- Chloramphenicol, CIP-Ciprofloxacin, CXC- Cloxacillin, GEN- Gentamicin, TET- Tetracycline, COT- Cotrimaxazole, NAL- Nalixidic acid

Values are means ± SD (standard deviation). No significant difference exists ($p>0.05$) between the susceptibility patterns of the isolates.

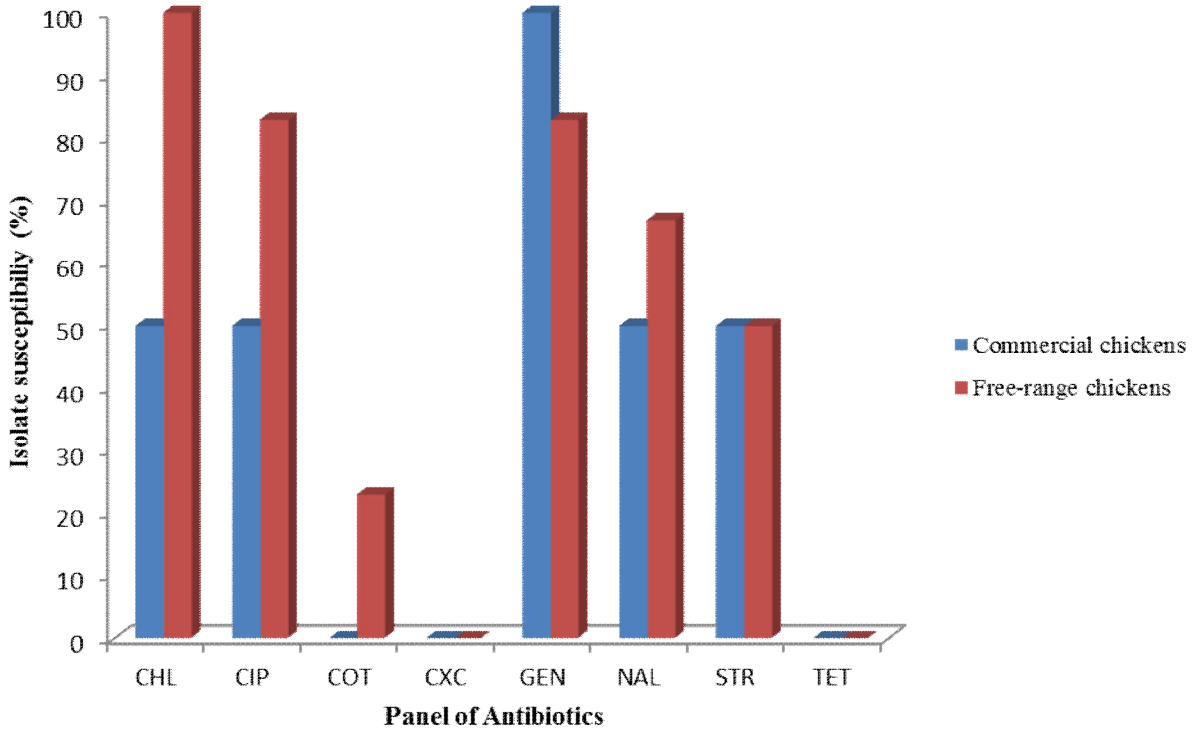


Fig 4.2 Antibiotic susceptibility profile of *E. coli* isolated from faecal droppings of commercial and free-range chickens

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimoxazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline

Table 4.3 Resistance patterns of *E. coli* isolated from faecal droppings of commercial and Free-range chickens

Single antibiotic Resistance		Multiple Antibiotic Resistance		
No. of isolates	Resistance Pattern	No. of Antibiotic Combinations	No. of isolates (%) with pattern	Resistance pattern
Commercial Chickens				
0(0)	Nil	2	Nil	Nil
		3	1(50)	COT, CXC, TET
		4	Nil	Nil
		5	Nil	Nil
		6	Nil	Nil
		7	1(50)	COT,NAL,CXC,CIP, CHL, TET, STR
Free-range chickens				
0(0)	Nil	2	2(33)	CXC, TET
		3	1(17)	COT, TET, CXC
		4	Nil	Nil
		5	1(17)	COT, CXC, GEN, TET, STR
			1(17)	COT, NAL, CXC, TET, STR
		6	1(17)	COT, NAL, CXC, CHL, TET, STR

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimoxazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline CC=Commercial chickens, FR=Free-range chickens

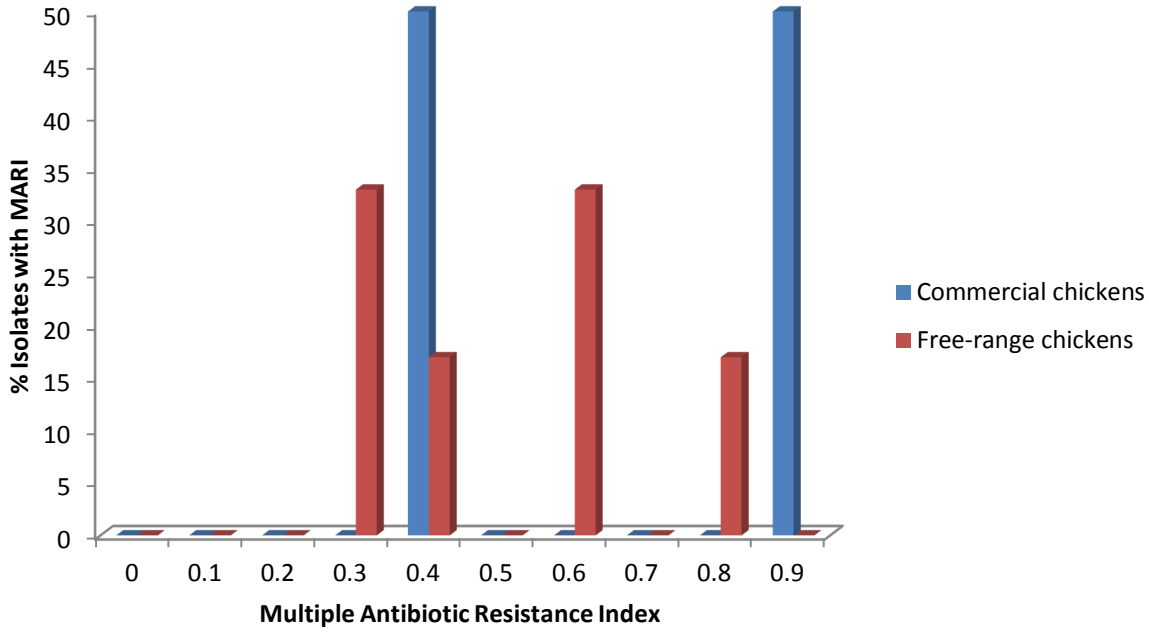


Fig. 4.3: Multiple antibiotic resistance indices of *E. coli* isolated from faecal droppings of commercial and free-range chickens.

4.2.1 Comparison of the antibiotic activity on *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens

Fig 4.4 shows the comparison of the antibiotic activity on *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens. There was no statistical significant difference observed in the susceptibility of *Salmonella* sp. isolated from the commercial and free-range chickens' faecal droppings ($p > 0.05$). All isolates from the commercial chickens exhibited larger zones of inhibition with chloramphenicol and cotrimoxazole compared to those from the commercial chickens. The isolates from the free-range chickens exhibited larger zones of inhibition with cloxacillin, gentamicin, streptomycin and tetracycline.

4.2.2 Antibiotic susceptibility profile of *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens

Antibiotic susceptibility profile of *Salmonella* sp. isolated from faecal droppings of commercial and free range chickens is shown in Fig 4.5. The antibiotics with the highest antibiotic activity for commercial chickens were ciprofloxacin, chloramphenicol, gentamicin, nalixidic acid, and streptomycin against 100% of the isolates. Resistance was observed in the activity of tetracycline and cloxacillin. Antibiotics with the highest antibacterial activity from the free-range chickens were ciprofloxacin, cotrimoxazole, gentamicin, nalixidic acid, streptomycin against 100% of the isolates. This is followed by a moderate activity by ciprofloxacin (75%). All isolates were resistant to tetracycline and cloxacillin.

4.2.3Resistance patterns of *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens

The pattern of resistance of *Salmonella* sp. isolated from fecal droppings of commercial and free-range chickens is shown in Table 4.4. No resistance pattern was obtained with the single antibiotic resistance type in the isolates from the commercial and free-range chickens. One multiple resistance pattern was obtained with varying combinations of 2 antibiotics while in the case of free-range chickens, two multiple resistance patterns were obtained with varying combinations. The highest frequency (3) was found in the combinations of 2 antibiotics.

4.2.4Multiple antibiotic resistance and indices of *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens

All the isolates from the commercial and free-range chickens exhibited multi-antibiotic resistance. The multiple antibiotic resistance index (MARI) of the isolates from the faecal droppings of commercial and free-range chickens can be seen in fig. 4.6. None of the isolates from the commercial and free-range chickens exhibited a MAR ≤ 0.2. The isolate from the commercial chickens exhibited a high MAR index of 0.3. Twenty-five percent of the isolates exhibited a MAR of 0.4 while seventy-five percent exhibited a MAR of 0.3.

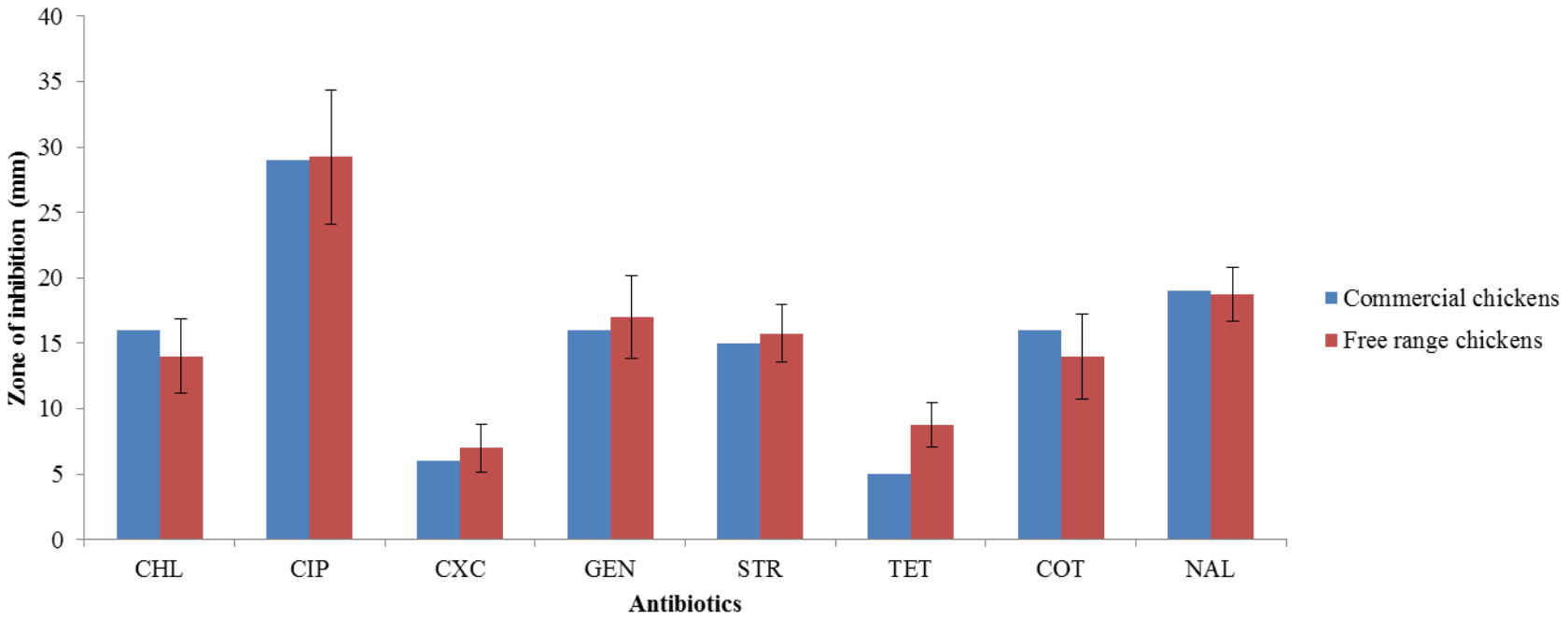


Fig. 4.4: A comparison of the antibiotic activity on *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens. KEY: CHL=Chloramphenicol, CIP=Ciprofloxacin, CXC=Cloxacillin, GEN=Gentamicin, STR=Streptomycin, TET=Tetracycline, COT=Cotrimoxazole, NAL=Nalixidic acid

Values are means ± SD (standard deviation). No significant difference exists ($p>0.05$) between the susceptibility patterns of the isolates.

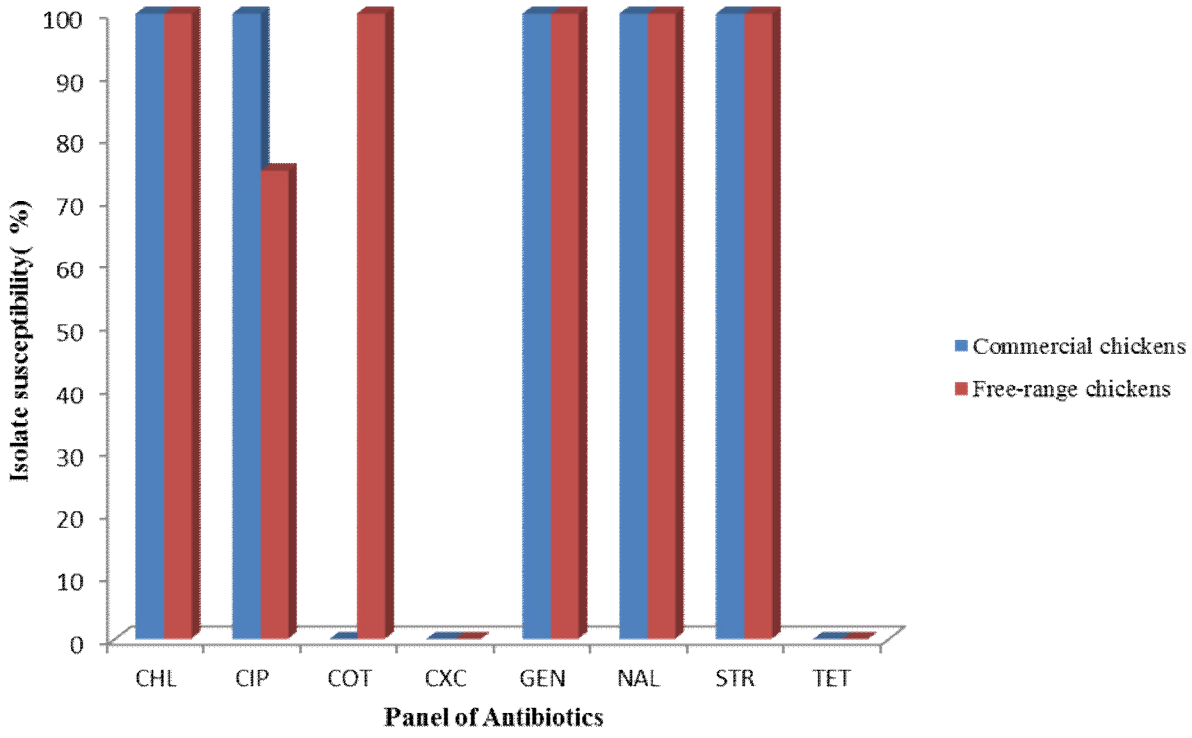


Fig. 4.5 Antibiotic susceptibility profile of *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimoxazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline

Table 4.4 Resistance pattern of *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens

Single antibiotic resistance		Multiple antibiotic resistance		
No. of isolates	Resistance Pattern	No. of antibiotic Combination	No. of isolates(%) with pattern	Resistance pattern
Commercial chickens				
0(0)	Nil	2	1(100)	CXC, TET
Free-range chickens				
0(0)	Nil	2	3(100)	CXC, TET
		3	1(33)	CXC,CHL,TET

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, CXC=Cloxacillin, TET=Tetracycline

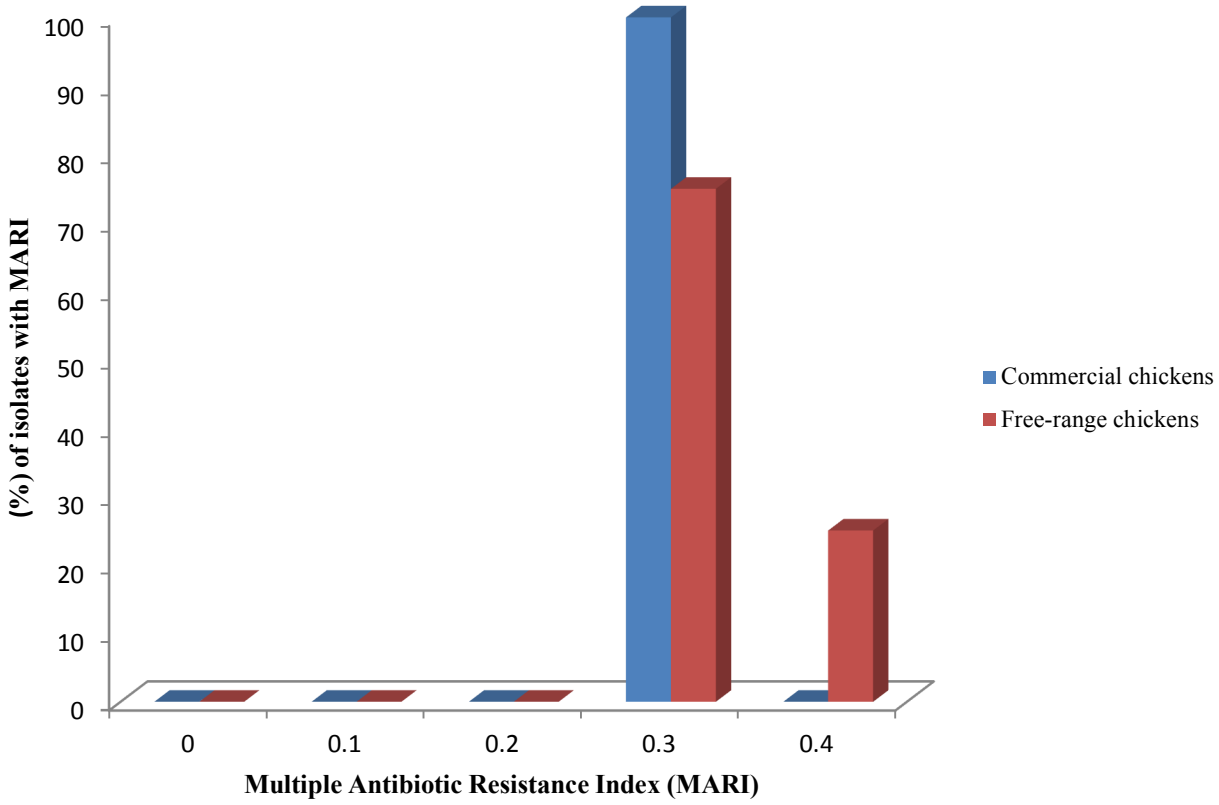


Fig. 4.6: Multiple antibiotic resistance indices of *Salmonella* sp. isolated from faecal droppings of commercial and free-range chickens.

4.3.1 Comparison of the antibiotic activity on *S. aureus* isolated from faecal droppings of commercial and free-range chickens

Fig 4.7 shows the comparison of the antibiotic activity on *S. aureus* isolated from faecal droppings of commercial and free-range chickens. There was a statistical significant difference observed in the susceptibility of *S. aureus* isolated from the commercial and free-range chickens' faecal droppings from the activity of streptomycin ($p < 0.05$). The isolates from the free-range chickens exhibited larger zones of inhibition from the activities of ciprofloxacin, gentamicin, erythromycin and streptomycin compared to the isolates from the commercial chickens. The isolates from the commercial chickens exhibited larger zones of inhibition from cloxacillin and tetracycline. The isolates from the commercial and free-range chickens had similar zones of inhibition in case of vancomycin.

4.3.2 Antibiotic susceptibility profile of *S. aureus* isolated from faecal droppings of commercial and free-range chickens

Antibiotic susceptibility profile of *S. aureus* isolated from faecal droppings of commercial and free range chickens is shown in Fig 4.8. The antibiotics with the highest antibiotic activity for commercial chickens were ciprofloxacin and gentamicin against all the isolates (100%). This is followed by vancomycin and streptomycin (88%). Antibiotics with moderate activity were chloramphenicol (57%) and erythromycin (43%). The antibiotic with lowest activity was tetracycline (14%). Resistance was seen in the activity of cloxacillin. In the case of free-range chickens, antibiotics with the highest antibacterial activity were chloramphemicol, gentamicin, streptomycin and vancomycin against all the isolates (100%). This is followed by a moderate activity by ciprofloxacin and erythromycin (67%). The isolates were resistant to tetracycline and cloxacillin.

4.3.3 Resistance patterns of *S.aureus* isolated from faecal droppings of commercial and free-range chickens

The pattern of resistance of *S. aureus* isolated from faecal droppings of commercial and free-range chickens is shown in Table 4.5. No resistance pattern was observed with the single antibiotic resistance type in the isolates from the commercial and free-range chickens. Three (3) multiple resistance patterns were observed from commercial chickens with varying combinations of two (TET,CXC), three (TET,ERY,CXC and CHL,TET,CXC), four(CHL,TET,ERY,CXC and VAN,ERY,CXC,STR) antibiotics while in the case of free-range chickens, two (2) multiple resistance patterns were obtained with varying combinations of two (TET, CXC) and four (CHL,TET,ERY,CXC) antibiotics. The highest frequency (3) was found in the combinations of 4 antibiotics in commercial chickens while that of free-range chickens, the combinations were of the same frequency (1).

4.3.4 Multiple antibiotic resistance indices of *S. aureus isolated* from faecal droppings of commercial and free-range chickens

All the isolates from the commercial and free-range chicken exhibited multi antibiotic resistance. The multiple antibiotic resistance index (MARI) of the isolates from the faecal droppings of commercial and free-range chickens can be seen in fig.4.9. None of the isolates from both commercial and free-range chickens had a MAR $\leq$ 0.2. Forty-two percent of the isolates from the commercial chickens had MAR index of 0.5 while twenty-nine percent had MAR of 0.3 and 0.4. Sixty-seven percent of the isolates from the free-range chickens had MAR index of 0.5 while thirty-three percent had MAR of 0.3.

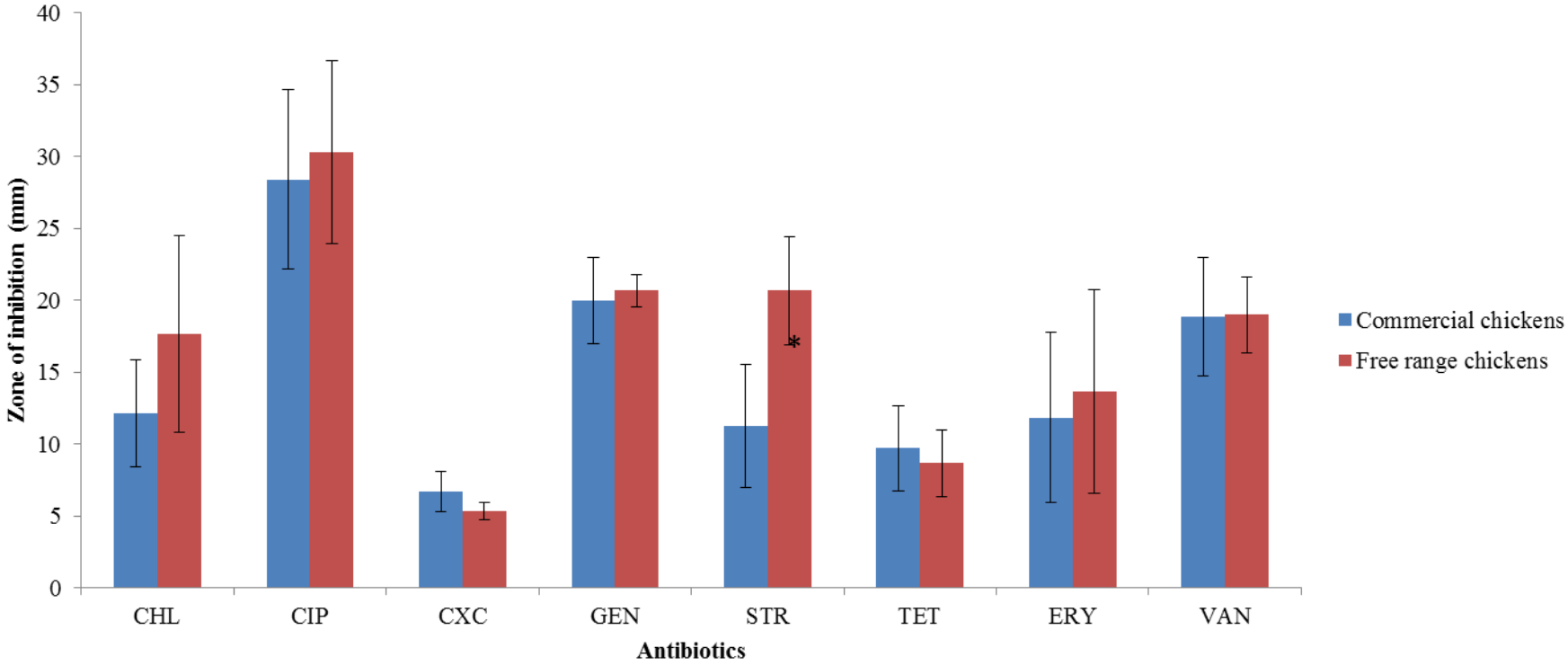


Fig 4.7: A comparison of antibiotic activity on *S. aureus* isolated from the faecal droppings of commercial and free-range chickens.

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, CXC=Cloxacillin, ERY=Erythromycin, GEN=Gentamicin, STR=Streptomycin, TET=Tetracycline, VAN=Vancomycin

Values are means ± SD (standard deviation). *= significant difference exists ($p<0.05$) between the susceptibility patterns of the isolates.

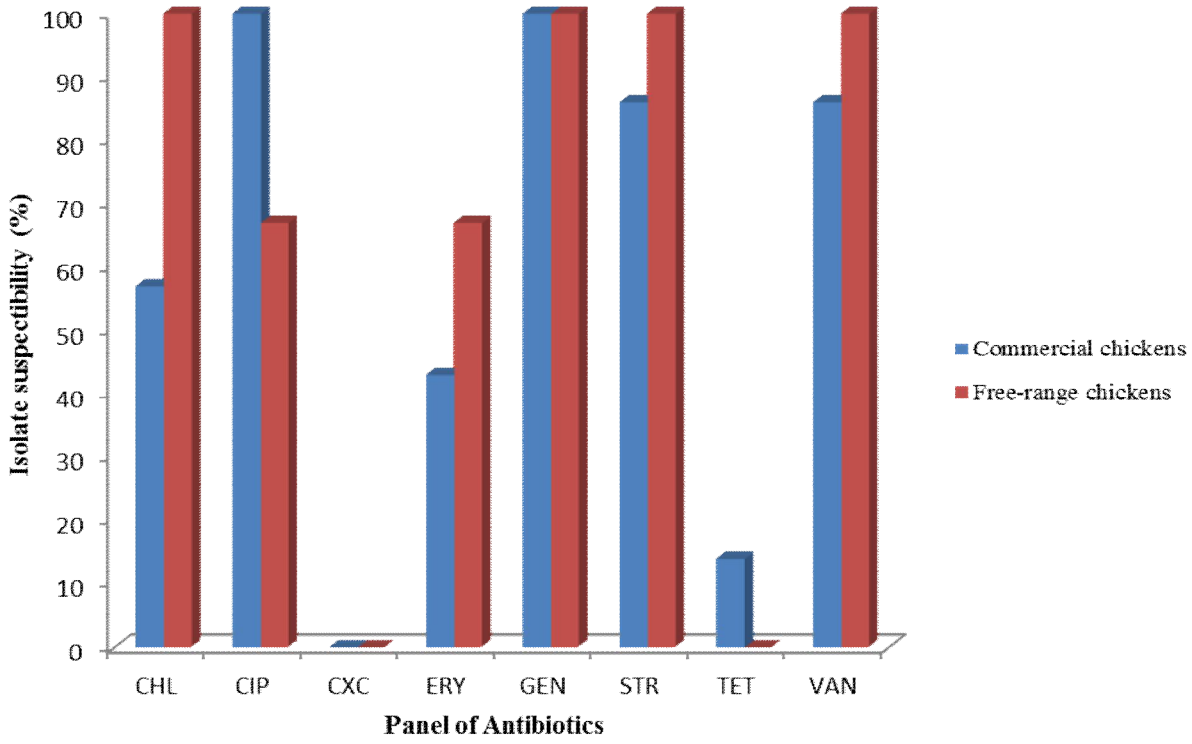


Fig. 4.8 Antibiotic susceptibility profile of *S.aureus* isolated from faecal droppings of commercial and Free-range chickens

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, CXC=Cloxacillin, ERY=Erythromycin GEN=Gentamicin, STR=Streptomycin, TET=Tetracycline, VAN=Vancomyin

Table 4.5 Resistance patterns of *S. aureus* isolated from faecal droppings of commercial and free-range chickens

Single antibiotic resistance		Multiple antibiotic Resistance		
No. of isolates (%)	Resistance Pattern	No. of antibiotic Combination	No. of isolate (%) with pattern	Resistance Pattern
Commercial chickens				
0(0)	Nil	2	2 (29)	TET, CXC
		3	1(14)	TET, ERY, CXC
			1(14)	CHL, TET, CXC
		4	2(29)	CHL, TET, ERY,CXC
			1(14)	VAN, ERY, CXC, STR
Free-range chickens				
0(0)	Nil	2	2(67)	TET, CXC
		3	Nil	Nil
		4	1(33)	CHL, TET, ERY, CXC

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, CXC=Cloxacillin, Van=Vancomycin, STR=Streptomycin, TET=Tetracycline

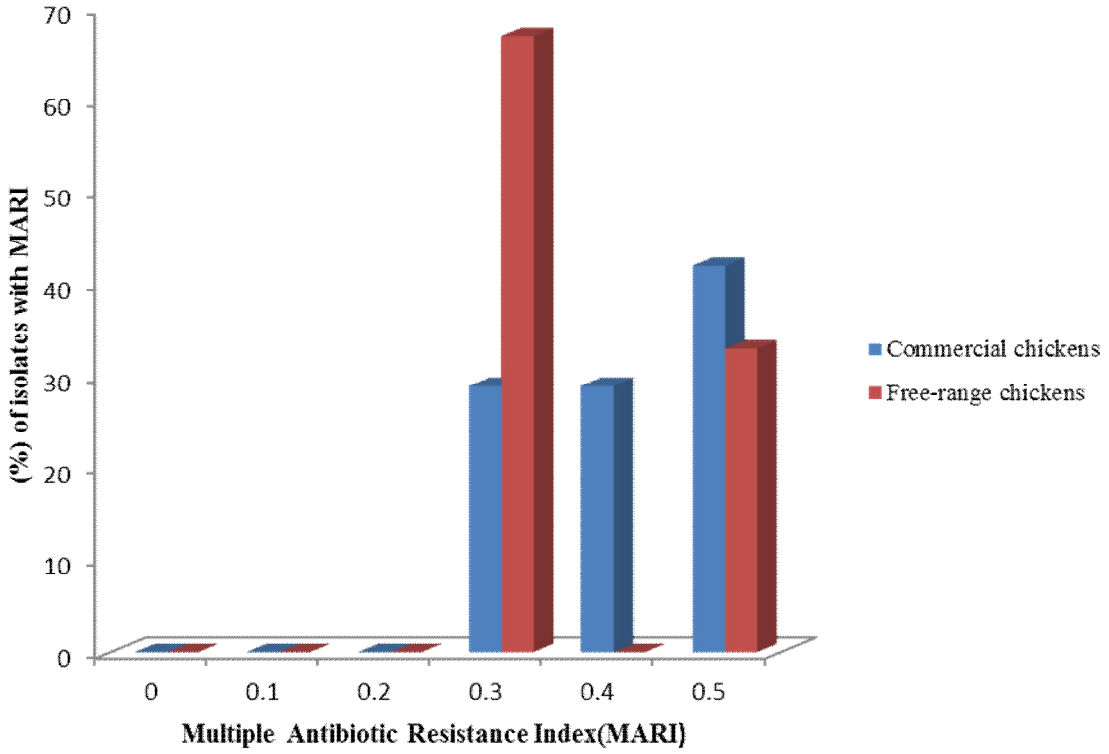


Fig. 4.9: Multiple antibiotic resistance indices of *S. aureus* isolated from faecal droppings of commercial and free-range chickens.

4.4.1 Comparison of the antibiotic activity on *Enterococccus* sp. isolated from faecal droppings of commercial and free-range chickens

Fig 4.10 shows the comparison of the antibiotic activity on *Enterococcus* sp. isolated from faecal droppings of commercial and free-range chickens. There was no statistical significant difference observed in the susceptibility of *Enterococcus sp.* isolated from the commercial and free-range chickens' faecal droppings ($p > 0.05$). The isolates from the commercial chickens had larger zones of inhibition from the activities of chloramphenicol, ciprofloxacin, erythromycin and gentamicin while the isolates from the free-range chickens had larger zones of inhibition from the activities of cloxacillin, streptomycin and tetracycline. All the isolates from both commercial and free-range chickens had similar zones of inhibition.

4.4.2 Antibiotic susceptibility profile of *Enterococcus* sp. isolated from faecal droppings of commercial and free-range chickens

Antibiotic susceptibility profile of *Enterococcus* sp. isolated from faecal droppings of commercial and free range chickens is shown in Fig 4.11. Antibiotic with the highest antibacterial activity was gentamicin against all the isolates from the commercial chickens. This was followed by streptomycin (67%). Antibiotic with moderate activity was vancomycin (50%). Antibiotics with low activity were ciprofloxacin and chloramphenicol (33%) and erythromycin (17%). Resistance was seen in the activity of tetracycline and cloxacillin. In the case of free-range chickens; there was no antibiotic with 100% activity against the isolates. Therefore, the highest antibacterial activity was seen in gentamicin and ciprofloxacin (89%). This is followed by a moderate activity by chloramphenicol and vancomycin (44%). Lowest activity can be seen in erythromycin and streptomycin (22%). The isolates were resistant to tetracycline and cloxacillin.

4.4.3 Resistance patterns of *Enterococcus* sp. isolated from faecal droppings of commercial and free-range chickens

The pattern of resistance of *Enterococcus sp.* isolated from the fecal droppings of commercial and free-range chickens is shown in table 4.6. No resistance pattern was observed with the single antibiotic resistance type in the isolates from the commercial and free-range chickens. Six (6) multiple resistance patterns were obtained from commercial chickens with varying combinations of 3, 4, 5, 6 antibiotics while in the case of free-range chickens, six (6) multiple resistance patterns were observeded with varying combinations of 3, 4, 5, 6 antibiotics. The highest frequency (3) was found in the combinations of 4 antibiotics in commercial chickens while that of free-range chickens, the highest frequency (2) was in the combinations of 3 and 5 antibiotics.

4.4.4Multiple antibiotic resistance indices of *Enterococcus* sp. *isolated* from faecal droppings of commercial and free-range chickens

All the isolates from the commercial and free-range chicken exhibited multidrug resistance. The multiple antibiotic resistance indices (MARI) of the isolates from the faecal droppings of commercial and free-range chickens can be seen in fig.4.12. None of the isolates from both commercial and free-range chickens exhibited MAR $\leq$0.2. Thirty-three percent of the isolates from the commercial chickens exhibited MAR indices of 0.4 and0.6 while seventeen percent exhibited indices of 0.5 and 0.8. Thirty-three percent of the isolates from the free-range chickens exhibited indices of 0.6 and 0.8, twenty-two percent exhibited MAR index of 0.4 and eleven percent exhibited MAR index of 0.5.

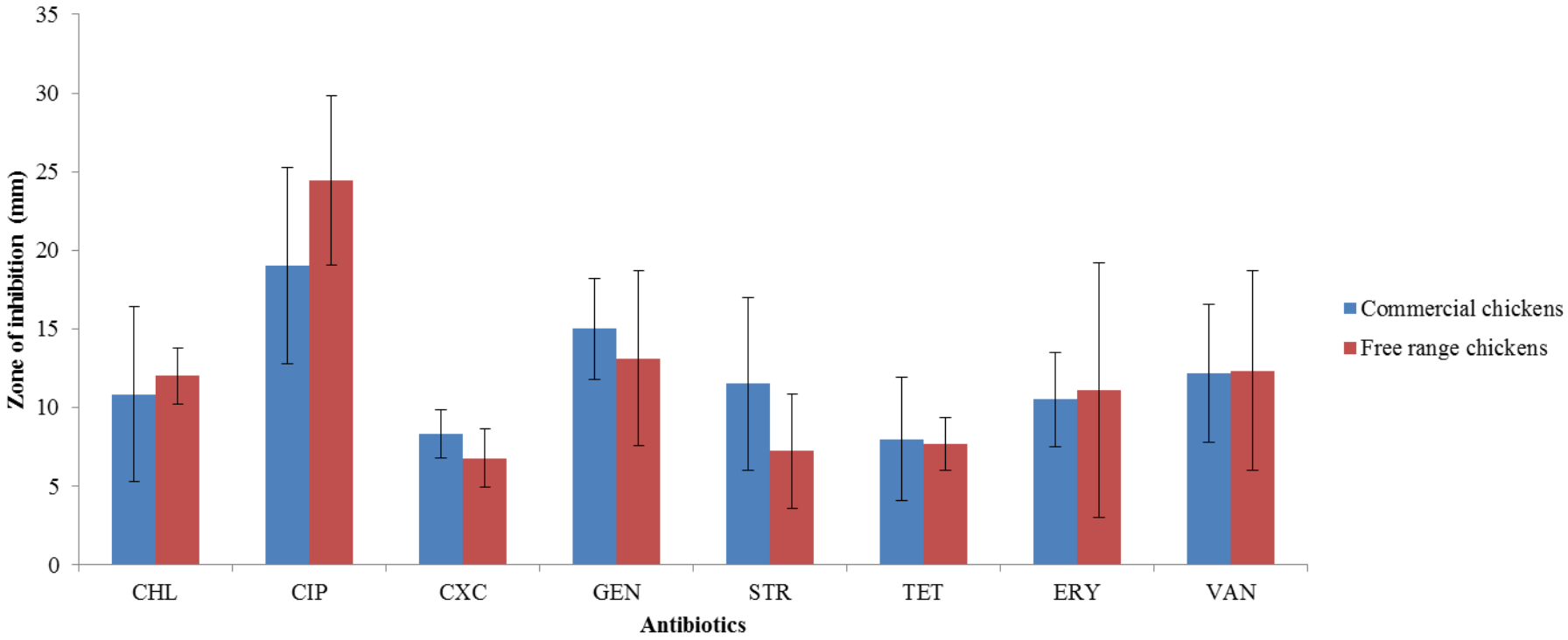


Fig. 4.10: A comparison of the antibiotic activity on *Enterococcus* sp .isolated from faecal droppings of commercial and free-range chickens.

Key: CHL=Chloramphenicol, CIP=Ciprofloxacin, CXC=Cloxacillin, GEN=Gentamicin, STR=Streptomycin, TET=Tetracycline, ERY=Erythromycin, VAN=Vancomycin

Values are means ± SD (standard deviation). No significant difference exists ($p>0.05$) between the susceptibility patterns of the isolate

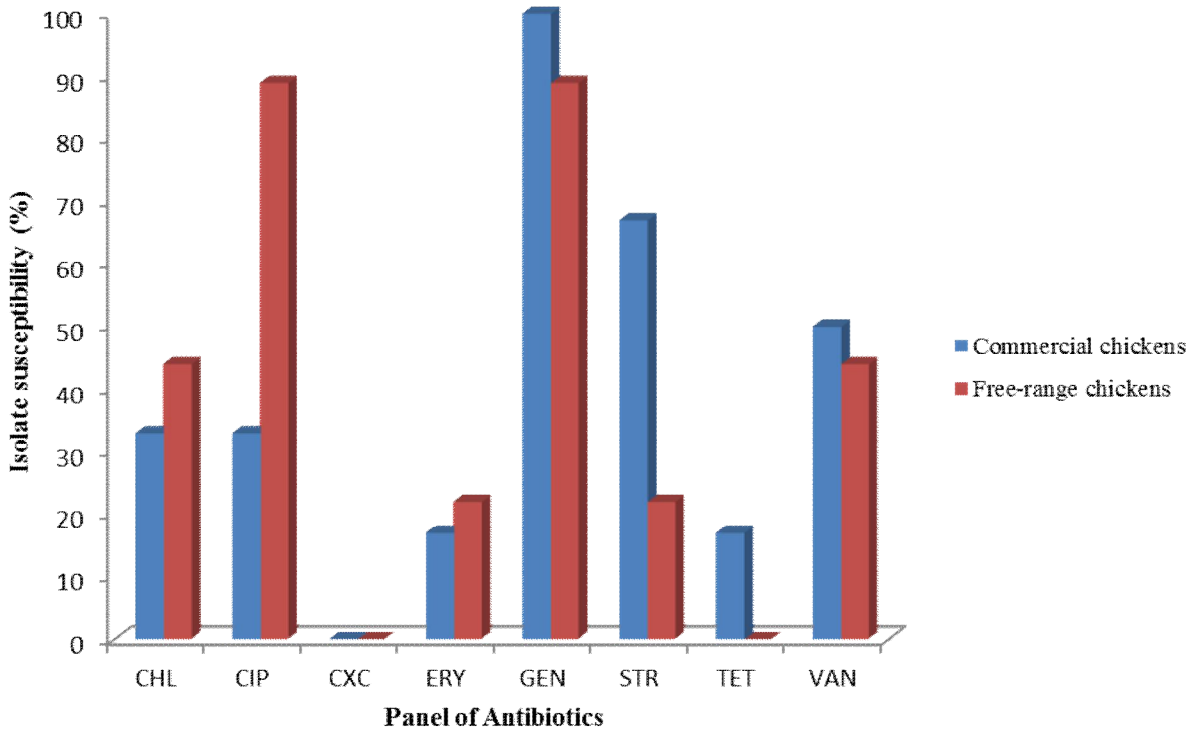


Fig 4.11 Antibiotic susceptibility profile of *Enterococcus* sp. isolated from faecal droppings of commercial and free-range chickens

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, CXC=Cloxacillin, ERY=Erythromycin GEN=Gentamicin, STR=Streptomycin, TET=Tetracycline, VAN=Vancomycin

Table 4.6 Resistance pattern of *Enterococccus* sp. isolated from faecal droppings of commercial and free-range chickens

Single antibiotic Resistance		Multiple antibiotic Resistance		
No. of isolates (%)	Resistance Pattern	No. of antibiotics Combination	No. of isolates (%) with pattern	Resistance Pattern
Commercial chickens				
0(0)	Nil	2	Nil	Nil
		3	1(17)	CHL, CXC, STR
		4	1(17)	CHL, TET, ERY,CXC
			1(17)	TET, ERY, CXC, STR
			1(17)	CIP,TET,ERY,CXC
		5	1(17)	VAN,CHL,CIP,TET,ERY
		6	1(17)	VAN,CIP,CHL,TET,CXC,ERY
Free-range chickens				
0(0)	Nil	2	Nil	Nil
		3	1(11)	VAN, TET, CXC
			1(11)	TET, CXC, STR
		4	1(11)	CHL, TET, ERY, CXC
		5	2(22)	CHL,TET,ERY,CXC,STR
			1(11)	VAN,TET,ERY,GEN,STR
		6	3(33)	VAN,CHL,TET,ERY,CXC,STR

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, CXC=Cloxacillin, GEN=Gentamicin, ERY=Erythromycin, STR=Streptomycin, TET=Tetracycline, VAN=Vancomycin

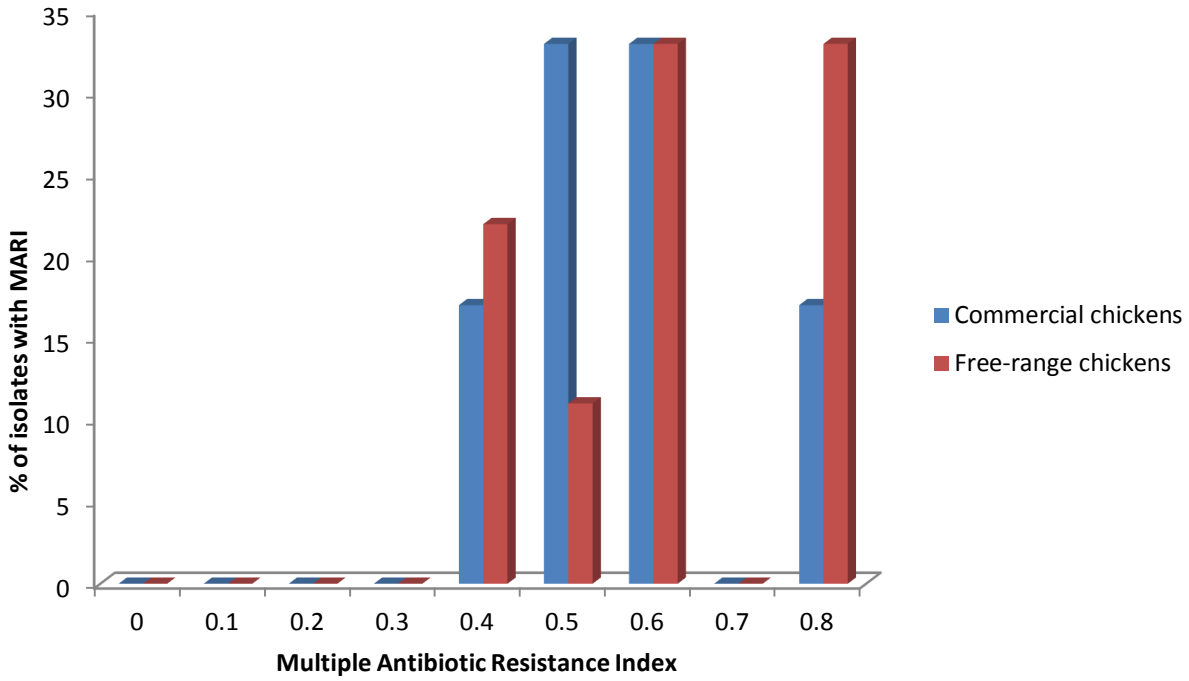


Fig. 4.12: Multiple antibiotic resistance indices of *Enterococcus* sp. isolated from faecal droppings of commercial and free-range chickens.

CHAPTER FIVE

DISCUSSION

The comparative analysis of the bioburden and antibiotic susceptibilities of selected bacteria from faecal droppings of commercial and free-range birds in Samaru, Zaria was studied. Total plate counts for faecal microbial load in commercial and free-range chickens in this study are 9.86±0.22 log $cfu^{-1}$and 9.51±0.12 log cfu^{-1} respectively. There was no significant difference ($p > 0.05$) when compared to each other. These observations are slightly higher than 6.15±2.49 log cfu^{-1} and 7.18±0.49 log cfu^{-1} reported by Adeleke and Omafuvbe (2011) in cockerel and layers's faecal droppings respectively; however, it was lower than 103.5±3.62 log cfu^{-1} reported by Nasrin *et al*. (2007). The variation in microbial load observed in faecal droppings of commercial and free-range chickens in this study may presumably be due to the differences in the level of contamination of the environment, feed and water these chickens are exposed to.

In this study, the prevalences of *Escherichia coli* from faecal droppings of commercial and free-range chickens were 5% and 11%. This observation is lower than the prevalence of 61.3%, 62.3%, 83% and 100% reported by Kolar *et al*. (2002), Nazir (2004), Samuel *et al*. (2010) and Bashar *et al*. (2011) in commercial chickens in Czech republic, Bangladesh, Kampala respectively and 81.4%, 56.5% and 20% reported by Nwiyi and Erumaka, (2012), Ojo *et al*. (2012), and Oluduro *et al*. (2012) in free-range chicken in Abia State, Ogun State and Osun State, Nigeria. The antibiotic susceptibilities of *E. coli* isolates in this study were observed to be similar to the report of Mutsami (2011) but do not agree with that of Siwela *et al.* (2007).

The antibiotic susceptibility of *E. coli* in this study reveals that all the isolates obtained from commercial chickens were susceptible to gentamicin. This agrees with the observation of Masuder *et al*. (2008), and Samuel *et al*. (2010). This observation perhaps may be due to the limited use of gentamicin in the poultry industry (Bywater *et al*., 2004).

Fifty percent of *E. coli* isolates were moderately susceptible to ciprofloxacin and chloramphenicol. This concurs with the reports of Masuder *et al*. (2008), Mamza *et al*. (2010), and Oluduro *et al*. (2012) where they observed moderate susceptiblility of *E. coli* to both ciprofloxacin and chloramphenicol in Bangladesh, Borno State and Osun State while 50% of the isolates were susceptible to streptomycin and nalixidic acid. This result is higher than the 49.7%; 63.2% and 30% reported by Kijima-Tanaka *et al*. (2003) and Akond *et al*. (2009) in Japan and Bangladesh respectively. The variation in susceptibilities of *E. coli* in these antibiotics may not be unconnected with their occasional usage in poultry enterprise, as chloramphenicol in particular has been banned in poultry production due to its causes of aplastic anaemia in humans. The *E. coli* isolates from the commercial chickens were resistant to cotrimoxazole, cloxacillin and tetracycline. This observation agrees with the reports of Chah *et al*. (2000) and Nsofor and Iroegbu, (2012). This indicates that these compounds have become seriously compromised and are of little value in the treatment of infections due to the organism. Cotrimoxazole is used for treatment of secondary infection in HIV/AIDS patients; therefore the transmission of resistant strains from poultry to immuno-compromised individuals could lead to complicated diseases and eventually increase morbidity and mortality (Samuel *et al*. 2010).

The *E. coli* isolates from free-range chickens in this study were high susceptibility to chloramphenicol, ciprofloxacin and gentamicin. This observation concurs with the reports of Okoli *et al.* (2005) and Nwiyi and Erumaka, (2012) in Imo State and Abia State where they observed high susceptibility of the organism to the above antibiotics. Sixty percent of *E. coli* isolates in free-range chickens were moderately susceptible to nalixidic acid and streptomycin. This is similar to the report of Okoli *et al*. (2005). Thirty percent of the isolates were susceptible to cotrimoxazole, which agrees with the report of Okoli *et al*. (2005), but lower than the 48.3% and 56% obtained by Mutsami, (2011) and Oluduro *et al*. (2012).

The *E. coli* isolates in faecal droppings in free-range chicken were highly resistant to cloxacillin and tetracycline. This agrees with the reports of Tabaatabaei *et al*. (2010), Nwiyi and Erumaka, (2012), and Ojo *et al*. (2012). Even though, free-range chickens hardly receive the attention of veterinarians; they maintain close contact with these antibiotics through various sources in the environment and also from humans that may have been exposed to these antibiotics.

None of the *E. coli* isolates in this study were resistant to a single antibiotic. This is in agreement with the reports of Akond *et al*.(2009), Mamza *et al*. (2010), Nwiyi and Erumaka, (2012), Ojo *et al*. (2012) and Oluduro *et al*. (2012) where high multi-antibiotic resistance were observed in commercial and free-range chickens. The most predominant pattern was COT-CXC-TET and it corresponds to a similar study in Kenya (Mitema and Kikuri, 2004) and Bangladesh (Akond *et al*., 2009) where the *E. coli* isolates showed multi-antibiotic resistance to ampillicin, cotrimoxazole and tetracycline. The high incidence of

multi-antibiotic resistance in *E. coli* may be due to the continuous exposure to antibiotics and close contact with their environment. The isolates from both commerical and free-range birds had multiple-antibiotic index (MARI) above 0.2 which infers that the isolates had arisen from environment were these antibiotics are frequently used, thereby indicative of a high antibiotic selective pressure in the environment (Adeleke and Omafuvbe, 2011; Hemen *et al*. (2012).

Several antibiotics are used to treat clinical infections and are currently in use sub-therapeutically in animals, particularly in livestock and poultry. This has promoted the emergence of resistant *E. coli* strains that can infect humans in close contacts with faecal droppings of chickens (Van der Bogaard *et al*., 2001) and render treatment less effective (Hart and Kariuki, 1998).

In this study, the prevalence of *Salmonella* sp. from commercial chicken farms was found to be 1%. This observation is similar with the report of Mutsami (2011) where *Salmonella* sp. was isolated from faecal droppings of commercial broilers in Kenya, and lower than the prevalences reported by Suh and Odeh, (2008), Begum *et al*. (2010), Enabulele *et al*. (2010), Nagwa *et al*. (2012) and Wafaa *et al*. (2012) while in free-range chickens, a prevalence of 4(4%) was observed. This prevalence is similar to the reports of Liu *et al*. (2005) and Pan *et al*. (2010), higher than that of Mutsami, (2011), lower than that of Siemon *et al*. (2007), Alali *et al*. (2010) and Ojo *et al*. (2012). This difference in prevalence could be due to the sampling procedure, sampling locations and the technique used for isolating the organism (Bryan and Doyle, 1995; Kishama *et al*., 2008).

Previous studies have shown an increase in cases of human salmonellosis as a result of infections caused by antibiotic resistant *Salmonella* (Foley and Lynne, 2008). The antibiotic susceptibility profile of *Salmonella* sp. shows that all the isolates obtained from commercial and free-range chickens were highly susceptible to chloramphenicol, ciprofloxacin, and gentamicin. This result is in line with the reports of Pan *et al*. (2010), Alali *et al*. (2010), Begum *et al*. (2010) and Enabulele *et al*. (2010) but do not agree with the findings of Suh and Odeh (2008) and Ojo *et al.* (2012). The high antibiotic susceptibility observed in this study may presumably be due to the fact that these antibiotics are not commonly used in the treatment of poultry diseases in this environment, and more so ciprofloxacin, an expensive antibiotic may not be within the reach of most poultry farmers. *Salmonella* isolates in this study were highly susceptible to nalidixic acid and streptomycin. These results concur with that of Alali *et al.* (2010) and Enabulele *et al*. (2010), and contradict the reports of Siemon *et al*. (2007), Pan *et al*. (2010), and Payne *et al*. (2010). *Salmonella* isolates in this study were resistant to both tetracycline and cloxacillin. This is similar to the reports of Payne *et al*. (2006) and Begum *et al*. (2010). The resistance of this organism to these antibiotics may be due to their widespread use especially as additives in feed for growth promotion and prevention of avian diseases (Muirhead, 1999; Rajashekara *et al*., 2000). Previous studies have shown that tetracycline persists in the environment longer than any other antibiotic (Frost, 1991).

In this study, *Salmonella* isolates from faecal droppings of free-range chickens were susceptible to cotrimoxazole, while that of commercial chickens was resistant to the drug. This result agrees with the reports of Alali *et al*. (2010) and Pan *et al*. (2010), and contradicts those of Begum *et al*. (2010) and Enabulele *et al*. (2010). None of the

Salmonella isolates was resistant to a single antibiotic. This result agrees with the report of Ojo *et al*. (2012), but disagrees with those of Siemon *et al*. (2007) and Alali *et al*. (2010). The isolates in the present study were found to exhibit one pattern of resistance pattern and two patterns of resistance patterns from commercial and free-range chickens respectively. This finding does not agree with the reports of Payne *et al*. (2006), Pan *et al*. (2010) and Ojo *et al*. (2012). The variations observed in multi antibiotic resistance of *Salmonella* in this study may be due to differences in the choice and concentrations of the antibiotics administered to the types of chicken studied. Multi-antibiotic resistance in *Salmonella* renders treatment with antibiotics ineffective (Rossiter *et al*., 2002). However, all *Salmonella* isolates in this study exhibited high multiple antibiotic resistance index, this is indicative of the probable source of the isolates, and reveals the likelihood of their origin, which may be an environment where these antibiotics are frequently used (Hemen *et al*., 2012).

Prevalence of *S. aureus* from faecal droppings of commercial and free-range chickens in this study was found to be 7% and 3% respectively. These prevalences are similar to those of Shareef *et al*. (2009), but lower than those of Kolar *et al*. (2002), Otalu *et al*. (2011), Omojowo and Omojasola, (2013). The antibiotic susceptibility tests of *S. aureus* to selected antibiotics in this study reveals that the *S. aureus* isolates (100%) from faecal droppings of free-range chickens were susceptible to ciprofloxacin, streptomycin and vancomycin while the isolates from commercial chickens were susceptible to gentamicin. These results agree with the reports of Kolar *et al.* (2002) and Otalu *et al.* (2011), but disagree with the report of Adeleke and Omafuvbe (2011). The results obtained in this study concur with previous observations that these antibiotics are less abused and are recommended for treatment of *S.*

aureus infections in chickens (Kolar *et al.,* 2002; Shryock and Richwine, 2010; Otalu *et al.,* 2011). In this study, the isolates from commercial and free-range chickens were moderately susceptible to chloramphenicol and erythromycin. This is in line with the observations of Omojowo and Omojasola (2013), but disagrees with those of Nasrin *et al.* (2007), Omololu-Aso *et al*. (2011) and Adeleke and Omafuvbe, (2011). *Staphylococcus aureus* isolates in this study were highly resistant to tetracycline. This is because antibiotics such as chlortetracycline and oxytetracycline alone or in combination with other compounds are found in most animal feeds in the market (Quinsey *et al.,* 1990). This is in agreement with the findings of Mamza *et al*. (2010), Otalu *et al*. (2011) and Omojowo and Omojasola, (2013).

All the *S. aureus* isolates in this study were highly resistant to cloxacillin. The high resistance of *S. aureus* to cloxacillin (a closely related *β*-lactam antibiotic to methicillin) may be attributed to the production of *β*-lactamase, an enzyme that inactivates penicillin and closely related antibiotics. This may probably explained the results obtained in this study (Schwarz *et al*., 1998; Alekshun and Levy, 2000). Multiple-antibiotic resistant Staphylococci that are resistant to beta-lactam antibiotics have been suspected of being methicillin-resistant strains (Van Duijkeren *et al*., 2004). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a critically important human pathogen and an emerging concern in veterinary medicine and animal agriculture. All the isolates in this study were found exhibit multi-resistance (2-4 antibiotics). This is similar to the report of Otalu *et al*. (2011), and may be due to the transfer of genes coding for resistance from the environment. All the isolates from faecal droppings of commercial and free-range chickens in this study had a multi-antibiotic resistance greater than 0.2, which concur with the report of Adeleke

and Omafuvbe, (2011). This shows that the isolates may have originated from an environment where these antibiotics are commonly being used.

The prevalence of *Enterococcus* sp. in this study is 6% and 9% from commercial and free-range chickens respectively. These findings are similar to the reports of Kolar *et al*. (2002) and lower than those of Jung *et al*. (2007), Ruzauskas *et al*. (2010) and Sapkota *et al*. (2011). The *Enterococcus* sp. observed in this study were *E. gallinarium* (86.7%) and *E. faecium* (13.3%).

The antibiotic susceptibility tests of *Enterococcus* sp. from faecal droppings of commercial farm chickens and free-range chickens were susceptible to gentamicin. This may due to the fact that gentamicin is expensive and therefore may not be readily abused. Gentamicin is not registered for use in poultry and may also explain the isolation of non-resistant *Enterococcus* sp. This antibiotic is less abused and is recommended for treatment of *Enterococcus* infections in chickens. These findings are in agreement with the reports of Tejedor-Junco *et al*. (2005). The most frequent resistance properties of *Enterococcus* sp. observed from commercial farms and free range chickens were resistances to cloxacillin and tetracycline (100%), erythromycin (83% and 78%), ciprofloxacin (67% and 11%), chloramphenicol (67% and 56%) and vancomycin (50% and 44%). Enterococci are intrinsically resistant to macrolides (erythromycin), penicillinase-resistant penicillins (cloxacillin) and tetracycline and often exhibit high-level resistance to glycopeptides (vancomycin). These findings are in consonance with the reports of Van den Bogaard *et al*. (2002), Lukasova and Sustackova (2003), Brown *et al*. (2008) and Ruzauskas *et al*. (2010).

The higher fluoroquinolone (ciprofloxacin) resistance (67%) of *Enterococcus* spp observed in broilers reared in commercial farms compared with that of the free-range birds (11%) is most likely due to use of quinolones during the rearing period. This has also been reported earlier by Van den Bogaard *et al*. (2001). In contrast, the report of Sapkota *et al*. (2011) showed lower prevalence of antibiotic-resistant enterococci among broilers reared on farms adopting certified organic practices (which exclude the use of antibiotics as growth promoters).

The resistance profile of *Enterococcus* sp. to Vancomycin in broiler flocks raised in commercial farms was observed to be 50%. Similar reports of Vancomycin Resistant Enterococci (VRE) identified in birds raised in commercial farms include those of Bender *et al*. (2009) and Yurdakui *et al*. (2013). Avoparcin and Vancomycin are glycopeptide antibiotics. Previous studies have shown that avoparcin confers cross-resistance to vancomycin (i.e the (mis)use of avoparcin selects VRE where it is used in chicken feeds (Bager *et al*., 2000). Poultry are increasingly been associated with the carriage of multi-resistant bacteria that may be responsible for infections in humans. The use of antibiotics in poultry has been related with dissemination of resistant *Enterococcus* strains.

The *Enterococcus* sp. isolates in this study were observed to be multi-antibiotic resistant (3-6 antibiotics). This is in agreement with Sapkota *et al*. (2011). The isolates from commercial and free-range chickens had multiple drug resistance indices greater than 0.2 which suggests the probability that these isolates may have originated from environments where antibiotics are often being used. There are great concerns over microbial antibiotic resistance in poultry and this has led to the banning of antibiotic use in feeds for poultry

and livestock in various countries of Europe (Hayes *et al.*, 2004) as well as in the U.S.A. However not much has been done in Nigeria. Antibiotics have been widely used for the purpose of antibiotic therapy, prophylaxis and growth promotion in farm animals (Mathew *et al*., 2007; Waters *et al*., 2011). This increasing handling of antibiotics has led to a worldwide problem in the development of antibiotic resistance among bacterial populations during recent decades (Shryock and Richwine, 2010).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has shown high bacterial burden in the faecal droppings of both commercial and free-range chickens which is not affected by the type of poultry farming system. Isolates from faecal droppings of free-range chickens are more susceptible to antibiotics than those from commercial chickens. This perhaps is because free-range chickens do not really receive veterinary services as compared to their commercial counterparts; they scavenge for food in the environment thereby exposing themselves to a myraid of microogranisms in the environment. The study has established the presence of antibiotic-resistant bacteria in free-range chickens which could be as a result of acquisition of these antibiotic resistant bacteria by contact with carriers or ingestion of food and water contaminated by faecal droppings of other animals with previous exposure to antibiotics. This could constitute a hazard to the public because of possible transmission to humans via contact and consumption of contaminated food or water. The study has also indicated the presence of bacteria with multiple drug resistance patterns. The multiple antibiotic resistance indices of the isolates indirectly suggests that they may have arisen from sources of high level of antibiotic selective pressure which results from the misuse of antibiotics.

6.2 Recommendations

Based on the conclusions above, the following recommendations are made:

i. The public should be enlightened about the importance of maintaining hygienic standards in their environment and the importance of proper cooking of poultry meat and products which may be contaminated with fecal matter.

ii. Poultry farmers should be educated on how to get maximum profit without excessive use of drugs for growth promotion, prevention and treatment of diseases.

iii. Poultry farmers should be enlightened on how antibiotics are good when used as required but can become a serious enemy if they are misused and also on the possibility of acquiring genes from the poultry they keep and vice-versa.

iv. Findings from this study has necessitated the need for effective surveillance systems for monitoring agents used as feed additive and keeping track of the emergence and spread of multi-antibiotic resistance in both commensal and pathogenic bacteria likewise possible transmission of zoonotic resistant bacteria to humans. This will keep the public abreast on the efficacy of chemotherapy and disease prevention both in human and livestock.

v. Further research should be carried out to determine the type of isolates (as in the study) found in the gut of poultry handlers in order to ascertain the correlation between the isolates from the poultry handlers and the poultry birds.

vi. Further studies should be carried out on isolates that showed intermediate susceptibility to the selected antibiotics to ascertain the trend in relation to their effectiveness in terms of disease.

vii. Alternatives to antibiotic use for maintenance of animal health and productivity such as new vaccination techniques, improved animal nutrition and genetic strategies, helping to reduce the potentially negative consequences of limiting the subtherapeutic use of antibiotics.

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APPENDICES

APPENDIX I: Microgen GN A substrate reference table

Well	Reaction	Description	Positive	Negative
1.	Lysine	Lysine decarboxylase-Bromothymol blue changes to green/blue indicating the production of the amine cadaverine.	Green/Blue	Yellow
2.	Ornithine	Ornithine decarboxylase- Bromothymol blue changes to blue indicating the production of the amine putrescin.	Blue	Yellow/Green
3.	H_2S	H_2S production-Thiosulphate is reduced to H_2S that reacts with ferric salts producing a black precipitate.	Brown/black	Straw
4. 5. 6.	Glucose Mannitol Xylose	Fermentation- Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation.	Yellow	Blue/green
7.	ONPG	Hydrolysis-ONPGHydrolysis by B-galactosidase results in the production of yellow ortho-nitrophenol	Yellow	Colourless
8.	Indole	Indole is produced from tryptophan and gives a pink/red complex when Kovac's reagent is added.	Pink/red	Colourless
9.	Urease	Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which turns phenol red from yellow to pink/red.	V.Deep Pink	Straw to pale salmon pink colour
10.	VP	Acetoin production from glucose is detected by the formation of a pink /red complex after the addition of alpha naphthol and creatine in the presence of KOH.	Deep Pink/Red	Colourless to Pale Pink
11.	Citrate	Utilization of citrate (only carbon source)	Blue	Yellow/Pale Green

		leading to a colour change in bromothymol blue from green to blue.		
12.	TDA	Indolepyruvic acid is produced from tryptophan by tryptophan deaminase giving a cherry red colour when ferric ions are added. Indole positive isolates may give a brown colour- this is a negative result.	Cherry red	Straw colour

Key: H_2S-Hydrogen sulphide; ONPG-Ortho-nitrophenol- galactosidase; TDA-Tryptophan deaminase acid

APPENDIX II: Microgen STAPH-ID substrate reference table

Well	Reaction	Description	Positive	Negative
1.	Sucrose			
2.	Trehalose			
3.	Mannitol	Fermentation-Phenol r4red changes from red to yellow as a result of acid produced from the carbohydrate fermentation.	Yellow-Yellow/Orange	Red
4.	N-Acetyl Glucosamine			
5.	Mannose			
6.	Turanose			
7.	Alkaline Phophatase	Hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase results in the production of yellow p-nitrophenol	Yellow	Colourless
8.	Glucosidase	Hydrolysis of p-nitrophenyl β Dglucuronide by β glucosidase results in the production of yellow o-nitrophenol	Yellow	Colourless
9.	Glucuronidase	Hydrolysis of o-nitrophenyl β Dglucuronide by β glucuronidase results in the production of yellow p-nitrophenol	Red	Yellow
10.	Nitrate	Nitrate is reduced to nitrite which forms a deep red complex after the addition of α-Naphthylamine and sulphanilic acid	Red	Yellow
11.	Urease	Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which bturns phenol red from yellow to pink/red	Very deep pink	Straw to pale salmon pink colour
12.	Arginine	Arginine is converted to ornithine, ammonia and CO_2 by arginine dihydrolase resulting in an increase in pH and a change in colour of the bromothymol blue from green to blue. At 48 hours, green reactions are negative	Green/blue Blue	Yellow Yellow/Green
13.	PYR	Hydrolysis of L-pyrrolidonyl-α-naphthylamine by the enzyme pyyrolidonyl arylamidase	Red/Deep Pink	Colourless/very pale pink

14.	LAT	Latex aggluntination test for clumping factor and protein A, or a slide or tube coagulase test
15	CPG	The development of visible colony pigmentation (cream to golden in colour)

KEY: PYR: L-pyrrolidonyl-α-naphthylamine, LAT: Latex aggluntination CPG: colony pigmentation

APPENDIX III: Microgen STREP-ID substrate reference table

Well	Reaction	Description	Positive	Negative
1.	Melibiose			
2.	Sorbitol			
3.	Inulin	Sugar Fermentation-Phenol red changes from red to yellow as a result of acid production produced from carbohydrate fermentation.	Yellow-Yellow/Orange	Red/Deep Orange
4.	Lactose			
5.	Arabitol			
6.	Ribose			
7.	Esculin	Hydrolysis of esculin to glucose and esculetin which produces a black compound in the presence of ferric ions	Black	Brown
8.	Voges Proskauer	Acetoin production from glucose is detected by the formation of a pink/red complex after the addition of alpha naphthol and creatine in the presence of potassium hydroxide.	Pale Pink/Red	Clear
9.	Alkaline phosphatase	Hydrolysis of p-nitrophenylnphosphate by alkaline phosphatase results in the productionof yellow p-nitrophenol.	Yellow	Colourless
10.	β-Galactosidase	Hydrolysis-p-nitrophenyl-β-D-galactopyranoside by B-galactosidase results in thye production of yellow ortho-nitrophenol.	Yellow	Colourless
11.	PYR	Hydrolysis of L-pyrrolidonyl-α-naphthylamide by the enzyme pyrrolidonyl arylamidase.	Pale Pink/ Red	Colourless
12.	Arginine	Arginine is converted to ornithine, ammonia and CO_2 by arginine dihydrolyase resulting in an incrase in pH and a change in colour of the bromothymol blue from green to blue. At 48 hours, green reaction are negative	Green/Blue	Yellow
13.	Hippurate	Hippurate is hydrolysed to the amino acid Glycine which is detected using the Ninhydrin reagent	Blue Purple	Clear
14.	α-Haemolysis	Visual reading of haemolysis on blood agar	(1)	
15.	β-Haemolysis	Visual reading of haemolysis on blood	(2)	

agar

KEY: PYR: L-pyrrolidonyl-α-naphthylamine

(1). α-Haemolysis – Presents as a zone of partial haemolysis of red blood cells on blood agar medium.

(2) β-Haemolysis- Presents as a zone of complete haemolysis of red blood cells on blood agar medium.

APPENDIX IV: MICROGEN IDENTIFICATION RESULTS

APPENDIX IVa: Microgen GN A Identification result (*E. coli*)

CODE	Lys	Orn	H_2S	Glu	Man	Xyl	ONPG	Ind	Ure	VP	Cit	TDA	Octal code	ID
CC28	+	+	-	+	-	+	+	+	-	-	-	+	6561	*E. coli*
CC87	+	+	-	+	-	-	+	+	-	-	-	-	6060	*E .coli*
FR69	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E. coli*
FR71	+	+	-	+	-	-	+	+	-	-	-	-	6460	*E. coli*
FR73	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E. coli*
FR74	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E. coli*
FR81	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E. coli*
FR84	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E. coli*

KEY:

Lys- Lysine, Orn- Ornithine, H2S- Hydrogen sulphide , Glu-Glucose, Man-Mannitol, Xyl- Xylose, ONPG- Ortho-nitrophenol- galactosidase, Ind - Indole, Ure- Urease, VP- Voges Proskauer, Cit- Citrate, TDA- Tryptophan deaminase acid , ID-Identification

APPENDIX IVb: Microgen GN A Identification result (*Salmonella* sp.)

CODE	Lys	Orn	H_2S	Glu	Man	Xyl	ONPG	Ind	Ure	VP	Cit	TDA	Octal code	ID
CC 43	+	+	+	+	+	+	-	-	-	-	+	-	5702	*Salmonella* sp.
FR 23	+	+	+	+	+	+	-	-	-	-	+	-	5702	*Salmonella* sp.
FR 32	+	+	+	+	+	+	-	-	-	-	+	-	5702	*Salmonella* sp.
FR 34	+	+	+	+	+	+	-	-	-	-	+	-	5702	*Salmonella* sp.
FR 67	+	+	+	+	+	+	-	-	-	-	+	-	5702	*Salmonella* sp.

KEY:

Lys- Lysine, Orn- Ornithine, H2S- Hydrogen sulphide , Glu-Glucose, Man-Mannitol, Xyl- Xylose, ONPG- Ortho-nitrophenol- galactosidase, Ind - Indole, Ure- Urease, VP- Voges Proskauer, Cit- Citrate, TDA- Tryptophan deaminase acid , ID-Identification

APPENDIX IVc: Microgen STAPH-ID Identification result (*Staphylococcus aureus*)

Code	lat	Cpg	nit	suc	tre	man	nag	Mns	tur	pho	βgl	βgn	Ure	Arg	pyr	Octal Code	ID
CC5	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	77667	*S. aureus*
CC20	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	77667	*S. aureus*
CC53	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	73267	*S. aureus*
CC58	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	77667	*S. aureus*
CC92	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	77667	*S. aureus*
CC96	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	77667	*S. aureus*
CC100	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	77667	*S. aureus*
FR16	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	77667	*S. aureus*
FR24	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	77663	*S. aureus*
FR29	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	77666	*S. aureus*

KEY

lat- Latex agglutination, cpg- Colony pigmentation, nit-Nitrate, suc- Sucrose, tre- Trehalose, man-Mannitol, nag- N-acetylglucosamine, mns- Mannose, tur-Turanose, pho-Alkaline phosphatase, βgl- Glucosidase, βgn- Glucuronidase, ure-Urease, arg-Arginine, pyr-L-pyrrolidonyl-α-napthylamidase, ID-identification

APPENDIX IVd: Microgen STREP-ID Identification result (*Enterococcus* sp.)

Code	Hip	ahe	bhe	mel	Sor	Inu	Lac	Ara	Rib	esc	vp	phs	Bga	Pyr	Arg	Octal code	ID
CC8	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	47773	*E. gallinarium*
CC22	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	47772	*E. gallinarium*
CC31	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	57773	*E. gallinarium*
CC60	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	50377	*E. durans*
CC88	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	57773	*E. gallinarium*
CC91	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	57773	*E. gallinarium*
FR4	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	47773	*E. gallinarium*
FR32	+	+	-	+	+	+	+	+	+	+	+	+		+	+	67773	*E. gallinarium*
FR35	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	46777	*E. faecium*
FR46	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	47777	*E. gallinarium*
FR47	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	47673	*E. gallinarium*
FR74	+	-	-	+	+	-	+	+	+	+	+	+	-	+	+	47773	*E. gallinarium*
FR75	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	47773	*E. gallinarium*
FR78	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	57773	*E. gallinarium*
FR85	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	56773	*E. faecium*

KEY: hip- hippurate, ahe-α-haemolysis, bhe-β-haemolysis, mel- Melibose, sor- Sorbitol, inu- Inulin, lac- Lactose, ara- Arabitol, rib-Ribose, esc- Esculin, vp- Voges Proskauer, phs-Alkaline phosphatase, βga- β-galactosidase,pyr- L-pyrrolidonyl-α-napthylamidase, arg-Arginine, ID-Identification

APPENDIX Va: Zone Diameter Interpretative Standard Breakpoints (for *Enterobacteriaceae*) of antibiotics used in this study.

Antibiotic Agents	Disk potency (µg)	Zone diameter (mm)			Testing conditions
		R≤	I	S ≥	
Ampicillin	5	13	14-16	17	**Medium**
Chloramphenicol	30	18	13-17	12	Mueller-Hinton agar
Ciprofloxacin	5	13	14-20	21	
Cotrimoxazole	25	10	11-15	16	**Inoculum**
Gentamicin	10	12	13-14	15	Growth method or direct colony suspension equivalent to a 0.5 McFarland Standard
Nalidixic acid	5	13	14 -18	19	
Sterptomycin	10	11	12-14	15	
Tetracycline	30	14	15-18	19	**Incubation**
					35 ± 2^0C,
					ambient air;
					16-18hr

Key: R= Resistant I=Intermediate S=Sensitive

Source: NCCLS, 1997, 1998; CLSI, 2011

APPENDIX Vb: Zone Diameter Interpretative Standard Breakpoints (for *Staphylococcus aureus)* for the antibiotics used in this study

Antibiotic Agents	Disk potency (µg)	Zone diameter (mm)			Testing conditions
		R≤	I	S ≥	
Ampicillin	10	13	14-16	17	**Medium**
Chloramphenicol	10	12	13-17	18	Mueller-Hinton agar
Gentamicin	10	12	13-14	15	
Streptomycin	10	6	7-9	10	**Inoculum**
Ciprofloxacin	5	15	16-20	21	Growth method or direct colony suspension equivalent to a 0.5 McFarland Standard
Erythromycin	5	13	14-22	23	
Tetracycline	10	14	15-18	19	
Vancomycin	30	14	15-16	17	**Incubation** 35 ± 2^0C, ambient air; 16-18hr

Key: R= Resistant I=Intermediate S=Sensitive

Source: NCCLS, 1997, 1998; CLSI, 2011

APPENDIX Vb: Zone Diameter Interpretative Standard Breakpoints (for *Enterococcus* sp.) for the antibiotics used in this study

Antibiotic Agents	Disk potency (µg)	Zone diameter (mm)			Testing conditions
		R≤	I	S ≥	
Ampicillin	10	16	-	17	**Medium**
Chloramphenicol	10	12	13-17	18	Mueller-Hinton agar
Gentamicin	10	12	13-14	15	
Streptomycin	10	6	7-9	10	**Inoculum**
Ciprofloxacin	5	15	16-20	21	Growth method or direct colony suspension equivalent to a 0.5 McFarland Standard
Erythromycin	15	13	14-22	23	
Tetracycline	10	14	15-18	19	
Vancomycin	30	14	15-16	30	**Incubation** 35 ± 2^0C, ambient air; 16-18hr

Key: R= Resistant I=Intermediate S=Sensitive

Source: NCCLS, 1997, 1998; CLSI, 2011

APPENDIX VI: Media Formulation

1. Eosin methylene blue (EMB) agar (Oxoid, England)

g/L	
Peptone	10.0
Lactose	5.0
Dipotassium hydrogen phosphate	2.0
Eosin-Y	0.4
Metyhlene blue	0.065
Agar	15.0

pH 6.8 ±0.2 @25^{o}C

2. Salmonella-Shigella Agar (Oxoid, England)

g/L	
Lab-Lemco powder	5.0
Peptone	5.0
Lactose	10.0
Bile Salts	8.5
Sodium citrate	10.0
Sodium thiosulphate	8.5
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	15.0

pH 7.0±0.2 @25^{o}C

3. Mannitol Salt Agar (Oxoid, England)

Lab-Lemco powder	1.0
Peptone	10.0
Mannitol	10.0

Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
pH 7.5±0.2 @25^{o}C	

4. Bile Aesulin Agar (Oxoid, England)

g/L	
Peptone	14.0
Bile salts	15.0
Ferric citrate	0.5
Aesculin	1.0
Agar	14.0
pH 7.1±0.2 @25^{o}C	

5. Blood agar (Oxoid, England)

Proteose peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0
pH 7.4±0.2 @25^{o}C	

6. Triple Sugar Iron Agar (Oxoid, England)

g/L	
Lab-Lemco powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024

Agar	12.0
pH 7.4±0.2 @25°C	

7. Methyl Red Voges Proskauer Broth (Scharlau, Spain)

g/L	
Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0
pH 7.0 ± 0.2 @25°C	

8. Mueller-Hinton-Agar (Merck, Germany)

g/L	
Infusion from meat	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar-Agar	13.0
pH 7.4 ± 0.2 @25°C	

9. Nutrient broth (agar) (Oxoid, England)

g/L	
Lab- Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
(Bacteriological agar)	15.0
pH 7.4 ± 0.2 @25°C	

10. Plate count agar (Biotec, U.K.)

	g/L
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar No2	12.0

pH 7.0 ± 0.2 @25°C

11. SIM medium (Oxoid, England)

	g/L
Tryptone	20.0
Peptone	6.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Agar	3.5

pH 7.3 ± 0.2 @25°C

12. Simmon's citrate agar (Biomark, India)

	g/L
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

pH 6.8 ± 0.2 @25°C

13. Buffered Peptone Water **(Oxoid, England)**

g/L	
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5

pH 7.2 ± 0.2 @25^{o}C

APPENDIX VII: Composition of reagents

1. Kovàc's Indole Reagent

Para-dimethylaminobenzaldehyde	2g
Isoamyl alcohol (3-methyl-1-butanol)	30ml
Hydrochloric acid, concentrated	10ml

2. Methyl red solution

Methyl red (pH indicator)	0.05g
Absolute ethanol	28ml
Distilled water	22ml

3. 5%-alpha-napthol

Alpha-napthol	5g
Absolute ethanol	100ml

4. 40% Potassium hydroxide

Potassium hydroxide	40g
Distilled water	100ml

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APPENDIX VIII: STATISTICAL ANALYSIS

APPENDIX VIIIa: Comparison of the antibiotic activities against *E. coli* isolates from faecal droppings of commercial and free-range chickens in Samaru, Zaria.

	MGT SYS	N	MEAN	Std deviation	Std. error mean
CHL	Commercial	2	11.00	8.485	6.000
	Free-range	6	13.50	3.391	1.384
CIP	Commercial	2	20.50	12.021	8.500
	Free-range	6	29.67	7.501	3.062
CXC	Commercial	2	6.50	0.707	0.500
	Free-range	6	6.50	1.517	0.619
GEN	Commercial	2	20.50	0.707	0.500
	Free-range	6	17.50	3.061	0.619
STR	Commercial	2	11.00	7.071	5.000
	Free-range	6	14.00	5.797	2.366
TET	Commercial	2	5.00	0.000	0.000
	Free-range	6	7.00	2.757	1.125
COT	Commercial	2	5.50	0.707	0.500
	Free-range	6	10.00	6.099	2.490
NAL	Commercial	2	10.50	7.778	5.500

	Free-range	**6**	**18.67**	**10.424**	**4.256**
ERY	**Commercial**	**0[a]**			
	Free-range	**0[a]**			
VAN	**Commercial**	**0[a]**			
	Free-range	**0[a]**			

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	Levene's Test for Equality of Variences		t-test for Equality of Means	
	F	Sig.	t	df
CHL Equal variences assumed	4.509	0.078	0.659	6
Equal variences not assumed			0.406	1.109
CIP Equal variences assumed	0.861	0.389	-1.333	6
Equal variences not assumed			-1.015	1.272
CXC Equal variences assumed	1.200	0.315	0.000	6
Equal variences not assumed			0.000	4.365
GEN Equal variences assumed	0.778	0.412	1.163	6
Equal variences not assumed			1.982	5.965
STR Equal variences assumed	0.027	0.875	-0.610	6
Equal variences not assumed			-0.542	1.483
TET Equal variences assumed	9.187	0.023	-0.973	6
Equal variences not assumed			-1.777	5.000
COT Equal variences assumed	5.063	0.065	-0.989	6
Equal variences not assumed			-1.772	5.368
NAL Equal variences assumed	0.097	0.766	-0.997	6
Equal variences not assumed			-1.174	2.385

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality of Means			
				95% confidence
	Sig. (2-tailed)	Mean difference	Std error difference	Lower
CHL Equal variences assumed	0.534	-2.500	3.793	-11.782
Equal variences not assumed	0.749	-2.500	6.158	-64.752
CIP Equal variences assumed	0.231	-9.167	6.879	-25.998
Equal variences not assumed	0.465	-9.167	9.035	-79.493
CXC Equal variences assumed	1.000	0.000	1.155	-2.825
Equal variences not assumed	1.000	0.000	0.796	-2.139
GEN Equal variences assumed	0.289	2.667	2.293	-2.945
Equal variences not assumed	0.095	2.667	1.346	-0.631
STR Equal variences assumed	0.565	-3.000	4.922	-15.043
Equal variences not assumed	0.658	-3.000	5.532	-36.813
TET Equal variences assumed	0.368	-2.000	2.055	-7.028
Equal variences not assumed	0.136	-2.000	1.125	-4.893
COT Equal variences assumed	0.361	-4.500	4.552	-15.639
Equal variences not assumed	0.133	-4.500	2.540	-10.896
NAL Equal variences assumed	0.357	-8.167	8.191	-28.209
Equal variences not assumed	0.344	-8.167	6.954	-33.905

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality
	95% Confidence
	Upper
CHL Equal variences assumed	6.782
Equal variences not assumed	59.752
CIP Equal variences assumed	7.665
Equal variences not assumed	61.160
CXC Equal variences assumed	2.825
Equal variences not assumed	2.139
GEN Equal variences assumed	8.278
Equal variences not assumed	5.964
STR Equal variences assumed	9.043
Equal variences not assumed	30.813
TET Equal variences assumed	3.028
Equal variences not assumed	0.893
COT Equal variences assumed	6.639
Equal variences not assumed	1.896
NAL Equal variences assumed	11.876

Equal variences not assumed		**17.572**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

APPENDIX VIIIb: Comparison of the antibiotic activities against *Salmonella* spp. isolates from faecal droppings of commercial and free-range chickens in Samaru, Zaria.

	MGT SYS	N	MEAN	Std deviation	Std. error mean
CHL	Commercial	1	16.00	2.828	1.414
	Free-range	4	14.00		
CIP	Commercial	1	29.00	5.123	2.562
	Free-range	4	29.25		
CXC	Commercial	1	6.00	1.826	0.913
	Free-range	4	7.00		
GEN	Commercial	1	16.00	3.162	1.581
	Free-range	4	17.00		
STR	Commercial	1	15.00	2.217	1.109
	Free-range	4	15.75		
TET	Commercial	1	5.00	1.708	0.854
	Free-range	4	8.75		
COT	Commercial	1	16.00	3.266	1.633
	Free-range	4	14.00		
NAL	Commercial	1	19.00	2.062	1.031
	Free-range	4	18.75		

ERY	**Commercial**	**0[a]**
	Free-range	**0[a]**
VAN	**Commercial**	**0[a]**
	Free-range	**0[a]**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	Levene's Test for Equality of Variences		t-test for Equality of Means	
	F	Sig.	t	df
CHL Equal variences assumed			**0.632**	**3**
Equal variences not assumed				
CIP Equal variences assumed			**-0.044**	**3**
Equal variences not assumed				
CXC Equal variences assumed			**-0.490**	**3**
Equal variences not assumed				
GEN Equal variences assumed			**-0.283**	**3**
Equal variences not assumed				
STR Equal variences assumed			**-0.303**	**3**
Equal variences not assumed				
TET Equal variences assumed			**-1.964**	**3**
Equal variences not assumed				
COT Equal variences assumed			**0.548**	**3**
Equal variences not assumed				
NAL Equal variences assumed			**0.108**	**3**
Equal variences not assumed				

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality of Means			
				95% confidence
	Sig. (2-tailed)	Mean difference	Std error difference	Lower
CHL Equal variences assumed	**0.572**	**2.000**	**3.162**	**-8.064**
Equal variences not assumed		**2.000**		
CIP Equal variences assumed	**0.968**	**-0.250**	**5.728**	**-18.480**
Equal variences not assumed		**-0.250**		
CXC Equal variences assumed	**0.658**	**-1.000**	**2.041**	**-7.498**
Equal variences not assumed		**-1.000**		
GEN Equal variences assumed	**0.796**	**-1.000**	**3.536**	**-12.252**
Equal variences not assumed		**-1.000**		
STR Equal variences assumed	**0.782**	**-0.750**	**2.479**	**-8.640**
Equal variences not assumed		**-0.750**		
TET Equal variences assumed	**0.144**	**-3.750**	**1.909**	**-9.827**
Equal variences not assumed		**-3.750**		
COT Equal variences assumed	**0.622**	**2.000**	**3.651**	**-9.621**
Equal variences not assumed		**2.000**		
NAL Equal variences assumed	**0.920**	**0.250**	**2.305**	**-7.085**
Equal variences not assumed		**0.250**		

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

APPENDIX VIIIc: Comparison of the antibiotic activities against *Staphylococcus aureus* isolates from faecal droppings of commercial and free-range chickens in Samaru, Zaria.

	MGT SYS	N	MEAN	Std deviation	Std. error mean
CHL	Commercial	7	12.14	3.716	1.405
	Free-range	3	17.67	6.807	3.930
CIP	Commercial	7	28.43	6.214	2.349
	Free-range	3	30.33	6.351	3.667
CXC	Commercial	7	6.71	1.380	0.522
	Free-range	3	5.33	0.577	0.333
GEN	Commercial	7	20.00	3.000	1.134
	Free-range	3	20.67	1.155	0.667
STR	Commercial	7	11.29	4.271	1.614
	Free-range	3	20.67	3.786	2.186
TET	Commercial	7	9.71	2.984	1.128
	Free-range	3	8.67	2.309	1.333
COT	Commercial	0[a]			
	Free-range	0[a]			
NAL	Commercial	0[a]			
	Free-range	0[a]			

ERY	**Commercial**	**7**	**11.86**	**5.928**	**2.241**
	Free-range	**3**	**13.67**	**7.095**	**4.096**
VAN	**Commercial**	**7**	**18.86**	**4.140**	**1.565**
	Free-range	**3**	**19.00**	**2.646**	**1.528**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	Levene's Test for Equality of Variences		t-test for Equality of Means	
	F	Sig.	T	df
CHL Equal variences assumed	2.118	0.184	-1.709	8
Equal variences not assumed			-1.324	2.530
CIP Equal variences assumed	0.043	0.842	-0.442	8
Equal variences not assumed			-0.437	3.767
CXC Equal variences assumed	2.421	0.158	1.628	8
Equal variences not assumed			2.231	7.933
GEN Equal variences assumed	1.849	0.211	-0.363	8
Equal variences not assumed			-0.507	7.998
STR Equal variences assumed	0.556	0.477	-3.272	8
Equal variences not assumed			-3.452	4.345
TET Equal variences assumed	0.002	0.966	0.536	8
Equal variences not assumed			0.600	5.028
ERY Equal variences assumed	0.010	0.922	-0.420	8
Equal variences not assumed			-0.388	3.278
VAN Equal variences assumed	0.890	0.373	0.054	8
Equal variences not assumed			0.065	6.145

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality of Means			
	Sig. (2-tailed)	Mean difference	Std error difference	95% confidence Lower
CHL Equal variences assumed	**0.126**	**-5.524**	**3.232**	**-12.978**
Equal variences not assumed	**0.293**	**-5.524**	**4.173**	**-20.317**
CIP Equal variences assumed	**0.670**	**-1.905**	**4.312**	**-11.848**
Equal variences not assumed	**0.686**	**-1.905**	**4.354**	**-14.296**
CXC Equal variences assumed	**0.142**	**1.381**	**0.849**	**-0.576**
Equal variences not assumed	**0.057**	**1.381**	**0.619**	**-0.049**
GEN Equal variences assumed	**0.726**	**-0.667**	**1.837**	**-4.902**
Equal variences not assumed	**0.626**	**-0.667**	**1.315**	**-3.700**
STR Equal variences assumed	**0.011**	**-9.381**	**2.867**	**-15.992**
Equal variences not assumed	**0.023**	**-9.381**	**2.717**	**-16.694**
TET Equal variences assumed	**0.606**	**1.048**	**1.953**	**-3.457**
Equal variences not assumed	**0.575**	**1.048**	**1.746**	**-3.434**
ERY Equal variences assumed	**0.685**	**-1.810**	**4.306**	**-11.740**
Equal variences not assumed	**0.722**	**-1.810**	**4.669**	**-15.980**
VAN Equal variences assumed	**0.958**	**-0.143**	**2.637**	**-6.225**
Equal variences not assumed	**0.950**	**-0.143**	**2.187**	**-5.463**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality
	95% Confidence
	Upper
CHL Equal variances assumed	**1.930**
Equal variences not assumed	**9.270**
CIP Equal variences assumed	**8.039**
Equal variences not assumed	**10.486**
CXC Equal variences assumed	**3.338**
Equal variences not assumed	**2.811**
GEN Equal variences assumed	**3.568**
Equal variences not assumed	**2.367**
STR Equal variences assumed	**-2.770**
Equal variences not assumed	**-2.067**
TET Equal variences assumed	**5.552**
Equal variences not assumed	**5.529**
ERY Equal variences assumed	**8.121**
Equal variences not assumed	**12.360**
VAN Equal variences assumed	**5.939**
Equal variences not assumed	**5.178**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

APPENDIX VIIId: Comparison of the antibiotic activities against *Enterococcus* sp. isolates from faecal droppings of commercial and free-range chickens in Samaru, Zaria.

	MGT SYS	N	MEAN	Std deviation	Std. error mean
CHL	Commercial	6	10.23	5.565	2.272
	Free-range	9	12.00	1.803	0.601
CIP	Commercial	6	19.00	6.229	2.543
	Free-range	9	24.44	5.364	1.788
CXC	Commercial	6	8.33	1.506	0.615
	Free-range	9	6.78	1.856	0.619
GEN	Commercial	6	15.00	3.225	1.317
	Free-range	9	13.11	5.555	1.852
STR	Commercial	6	11.50	5.468	2.232
	Free-range	9	7.22	3.667	1.222
TET	Commercial	6	8.00	3.899	1.592
	Free-range	9	7.67	1.658	0.553
COT	Commercial	0[a]			
	Free-range	0[a]			
NAL	Commercial	0[a]			
	Free-range	0[a]			

ERY	**Commercial**	**6**	**10.50**	**3.017**	**1.232**
	Free-range	**9**	**11.11**	**8.085**	**2.695**
VAN	**Commercial**	**6**	**12.17**	**4.401**	**1.797**
	Free-range	**9**	**12.33**	**6.344**	**2.115**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	Levene's Test for Equality of Variences		t-test for Equality of Means	
	F	Sig.	T	df
CHL Equal variences assumed	0.780	0.008	-0.594	13
Equal variences not assumed			-0.496	5.707
CIP Equal variences assumed	0.638	0.439	-1.808	13
Equal variences not assumed			-1.751	9.687
CXC Equal variences assumed	0.505	0.490	1.706	13
Equal variences not assumed			1.784	12.344
GEN Equal variences assumed	2.059	0.175	0.747	13
Equal variences not assumed			0.831	12.870
STR Equal variences assumed	2.089	0.172	1.825	13
Equal variences not assumed			1.681	7.998
TET Equal variences assumed	4.770	0.048	0.230	13
Equal variences not assumed			0.198	6.222
ERY Equal variences assumed	1.639	0.223	-0.175	13
Equal variences not assumed			-0.206	10.928
VAN Equal variences assumed	2.072	0.174	-0.056	13
Equal variences not assumed			-0.060	

12.935

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality of Means			
	Sig. (2-tailed)	Mean difference	Std error difference	95% confidence Lower
CHL Equal variences assumed	**0.563**	**-1.167**	**1.966**	**-5.413**
Equal variences not assumed	**0.638**	**-1.167**	**2.350**	**-6.989**
CIP Equal variences assumed	**0.094**	**-5.444**	**3.011**	**-11.949**
Equal variences not assumed	**0.111**	**-5.444**	**3.109**	**-12.402**
CXC Equal variences assumed	**0.112**	**1.556**	**0.912**	**-0.414**
Equal variences not assumed	**0.099**	**1.566**	**0.872**	**-0.339**
GEN Equal variences assumed	**0.468**	**1.889**	**2.527**	**-3.571**
Equal variences not assumed	**0.421**	**1.889**	**2.272**	**-3.025**
STR Equal variences assumed	**0.091**	**4.278**	**2.344**	**-0.785**
Equal variences not assumed	**0.131**	**4.278**	**2.545**	**-1.591**
TET Equal variences assumed	**0.821**	**0.333**	**1.447**	**-2.793**
Equal variences not assumed	**0.849**	**0.333**	**1.685**	**-3.754**
ERY Equal variences assumed	**0.864**	**-0.611**	**3.485**	**-8.140**
Equal variences not assumed	**0.840**	**-0.611**	**2.963**	**-7.138**
VAN Equal variences assumed	**0.956**	**-0.167**	**2.992**	**-6.630**
Equal variences not assumed	**0.953**	**-0.167**	**2.775**	**-6.165**

KEY: CHL-chloramphenicol, CIP-ciprofloxacin, CXC-cloxacillin, GEN-gentamicin, STR-streptomycin, TET-tetracyline, COT-cotrimoxazole, NAL-nalixidic acid, ERY-erythromycin, VAN-vancomycin

	t-test for Equality
	95% Confidence
	Upper
CHL Equal variences assumed	3.080
Equal variences not assumed	4.656
CIP Equal variences assumed	1.060
Equal variences not assumed	1.513
CXC Equal variences assumed	3.525
Equal variences not assumed	3.450
GEN Equal variences assumed	7.348
Equal variences not assumed	6.802
STR Equal variences assumed	9.341
Equal variences not assumed	10.147
TET Equal variences assumed	3.460
Equal variences not assumed	4.421
ERY Equal variences assumed	6.918
Equal variences not assumed	5.916
VAN Equal variences assumed	6.296
Equal variences not assumed	5.831

ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF *ESCHERICHIA COLI* FROM FAECAL DROPPINGS OF COMMERCIAL AND FREE-RANGE CHICKENS IN SAMARU, ZARIA.

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Antibiotic susceptibility patterns of *E. coli* from faecal droppings of commercial and free-range chickens were determined. Seven *E. coli* isolates were recovered from 200 faecal droppings of live commercial and free- range chickens on Eosin Methylene Blue. They were confirmed using standard biochemical tests. Susceptibility of the isolates was carried out by Disc Diffusion test using the following antibiotics: chloramphenicol, ciprofloxacin, cloxacillin, cotrimazole, gentamicin, nalixidic acid, streptomycin, tetracycline. There was no significant difference ($p < 0.05$) in antibiotic susceptibility patterns of *E. coli* isolates from both faecal droppings. This study revealed that 100% of isolates from commercial chickens were resistant and susceptible to cloxacillin, cotrimazole, tetracycline and gentamicin respectively, while 50% were susceptible to chloramphenicol, ciprofloxacin, nalixidic acid, and streptomycin. However, one isolate exhibited multi-antibiotic resistance. A 100% resistance and susceptibility to cloxacillin and chloramphenicol respectively was observed from free-range chickens while 83% were susceptible to both gentamicin and ciprofloxacin, 67% and 23% were susceptible to nalixidic acid and streptomycin respectively, and three isolates were observed to exhibit multi-antibiotic resistance. The study has established the presence of antibiotic susceptibility, resistance and multi-antibiotic resistance of *E. coli* isolates from both commercial and free-range chickens to antibiotics in Samaru, Zaria. Multi-antibiotic resistance was observed to be higher in free-range chickens than in commercial chickens. This underscores the importance of the rational use of antibiotics in chicken to forestall zoonotic transmission of antibiotic resistance to humans.

Key words: Antibiotics, antibiotic susceptibility, *Escherichia coli*, commercial chickens, free-range chickens, multi-antibiotic resistance, fecal droppings.

INTRODUCTION

Escherichia coli is an aerobic Gram-negative bacterium, usually motile rod. It is commonly isolated from human and animal waste, thereby contaminating the environment and thus constituting a public health hazard (Smith *et al*., 2003). *Escherichia coli* can be commonly found in the intestinal tract of humans and animals. It is used as an indicator bacterium because it acquires antibiotic resistance faster than other bacteria. Thus, changes in the resistance of this organism serve as a good indicator of resistance in potentially pathogenic bacteria (Kijima-Tanaka *et al*., 2003). Furthermore, as an essential part of the gastrointestinal flora, it is known to be capable of accepting and transferring plasmids to other enteric bacteria; it is considered as an important reservoir of transferable antibiotic resistance.

MATERIALS AND METHODS

Sample collection

Two hundred (200) faecal samples were randomly collected from commercial poultry houses (100 samples) and homes that rear free range chickens (100 samples). The faecal samples were placed in clean glass containers. They were taken to the microbiology laboratory of the Department of Microbiology, Ahmadu Bello University, Nigeria under ambient conditions and processed immediately.

Isolation and identification of *E. coli*

The samples collected were aseptically streaked onto Eosin Methylene Blue Agar (EMB) for *E. coli*. The plates were inverted and incubated for 24 hours at 37°C under aerobic conditions. (Chessebrough, 2006). The plates were examined after 24 hours and colonies with greenish metallic sheen were considered tentatively as *E. coli* pending the biochemical characterization. The presumptive isolates were gram-stained and were purified by sub-culturing on the appropriate media. Discreet colonies were picked and streaked on nutrient agar slants, incubated for 24hours and stored at 4^{o}C until required for characterization (Oluduro *et al*., 2012). The suspected isolates were biochemically identified using triple sugar iron agar (TSI), Simmon citrate agar, sulphur indole motility medium (SIM), and Methyl red-Voges proskauer broth (MR-VP). Further confirmation was done using biochemical tests kits (Microgen^{TM} GnA- ID System) and accompanying computer software package (Microgen ID Version 1.2.5.26).

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of the isolates were carried out using the disc diffusion technique (Kirby-Bauer disk diffusion method) according to the methods recommended by the Clinical Laboratory and Standards Institute (6). About 3-5 discrete colonies of the isolates were inoculated into 5ml of nutrient broth and incubated overnight at 37^{o}C. The overnight culture was then standardized with 0.5 MacFarland standard. A sterile cotton swab was used in inoculating the culture to freshly prepared pre-dried Mueller-Hinton agar

plates prepared according to manufacturer's instructions. The antibiotic discs were aseptically and spaciously placed on the inoculated Mueller-Hinton agar plates. The plates were inverted and incubated aerobically at 37^{o}C for 18-24hr. The antibiotics discs used include: Ciprofloxacin (5µg), Chloramphenicol (10µg), Cloxacilin (5µg), Cotrimoxazole (25µg), Gentamicin (10µg), Nalixidic acid (5µg), Streptomycin (10µg), Tetracycline (10µg) (Abtek Biologicals Ltd, UK). For each isolate, the zone of inhibition around each disk was measured, after incubation at 37^{0}C for 24h.

RESULTS

Prevalence rates of *E. coli* are as shown in Table 1. Antibiotic susceptibility profile of *E. coli* isolated from faecal droppings of commercial and free range chickens is shown as in Figure 1. The antibiotic with the highest antibacterial activity for commercial chickens was gentamicin with 100% activity against all the isolates. This was followed by a moderate antibacterial activity to ciprofloxacin, chloramphenicol, nalixidic acid and streptomycin against 50% of the isolates. Resistance was seen in the activity of tetracycline and cloxacillin. In the case of the free-range chickens, the antibiotic with the highest antibacterial activity was chloramphenicol with 100% activity against all the isolates which was followed by moderate susceptibility to gentamicin and ciprofloxacin (83%) and nalixidic acid (67%). Minimal resistance was observed in resistance to the activity of cotrimoxazole (23%). All isolates were found to be resistant to tetracycline and cloxacillin. Multi-antibiotic resistance pattern of *E. coli* isolated from fecal droppings of commercial and free-range chickens is shown in Table 2. Two multiple resistance patterns were obtained with varying combinations of 3 and 7 antibiotic combinations. In the case of free-range chickens, six multiple resistance patterns with varying combinations of 2,3,5,6 antibiotic combinations were obtained. The highest frequency (2) was found in the combinations of 2 antibiotics. The multiple antibiotic resistance index (MARI) of the isolates can be seen in Figure 2. None of the isolates had a MAR index ≤ 0.2. Fifty percent of the isolates exhibited a MAR index of 0.4 and fifty percent of the isolates exhibited a MAR index of 0.9. Thirty-three percent of the isolates from free-range chickens exhibited a MAR indices of 0.3 and 0.6 while seventeen percent of the isolates from commercial chickens exhibited a MAR indices of 0.4 and 0.8.

Table 1. Prevalence of *E. coli* from the faecal droppings of commercial and free-range chickens

Management system	No. of samples	No. of isolates (%)
CC	100	2(2%)
FRC	100	6(6%)

CC= Commercial chickens, FRC= Free-range chickens

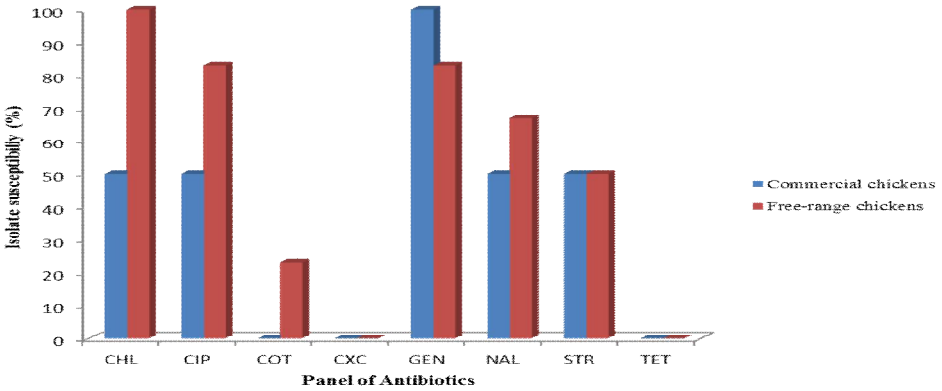


Fig. 1. Antibiotic susceptibility profile of *E. coli* isolated from faecal droppings of commercial and free-range chickens

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimoxazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline

Table 2. Resistance patterns of *E. coli* isolated from faecal droppings of commercial and Free-range chickens

Single antibiotic Resistance		Multiple Antibiotic Resistance		
No. of isolates	Resistance Pattern	No. of Antibiotic Combinations	No. of isolates (%) with pattern	Resistance pattern
Commercial Chickens				
0(0)	Nil	2	Nil	Nil
		3	1(50)	COT, CXC, TET
		4	Nil	Nil
		5	Nil	Nil
		6	Nil	Nil
		7	1(50)	COT,NAL,CXC,CIP, CHL, TET, STR
Free-range chickens				
0(0)	Nil	2	2(33)	CXC, TET
		3	1(17)	COT, TET, CXC

4	Nil	Nil
5	1(17)	COT, CXC, GEN, TET, STR
	1(17)	COT, NAL, CXC, TET, STR
6	1(17)	COT, NAL, CXC, CHL, TET, STR

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimoxazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline CC=Commercial chickens, FR=Free-range chickens

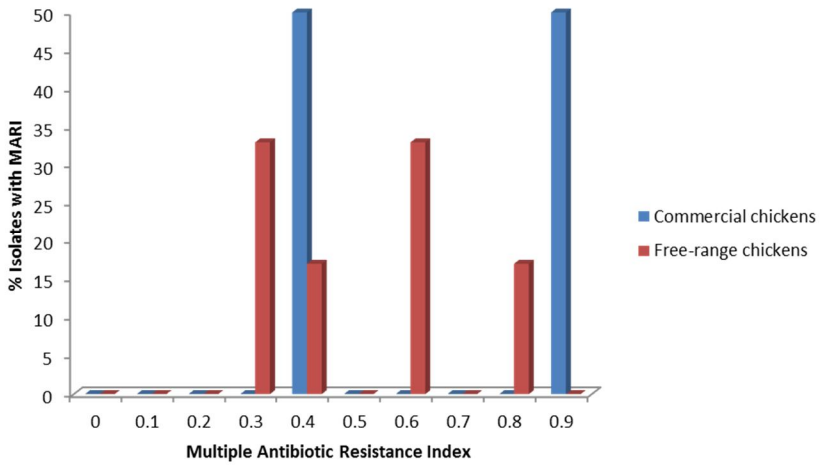


Fig. 2: Multiple antibiotic resistance indices of *E. coli* isolated from faecal droppings of commercial and free-range chickens.

Discussion

In this study, the prevalences of *Escherichia coli* from faecal droppings of commercial and free-range chickens were 5% and 11%. This observation is lower than the prevalence of 61.3% reported by Kolar *et al*. (2002) in commercial chickens in Czech republic and 81.4% reported by Nwiyi and Erumaka, (2012) in free-range chicken in Abia State, Nigeria. The antibiotic susceptibilities of *E. coli* isolates in this study were observed to be similar to the report of Mutsami (2011) but do not agree with that of Siwela *et al.* (2007).

The antibiotic susceptibility of *E. coli* in this study reveals that all the isolates obtained from commercial chickens were susceptible to gentamicin. This agrees with the observation of Masuder *et al*. (2008), and Samuel *et al*. (2010). This observation perhaps may be due to the limited use of gentamicin in the poultry industry (Bywater *et al*., 2004).

Fifty percent of *E. coli* isolates were moderately susceptible to ciprofloxacin and chloramphenicol. This concurs with the report of Mamza *et al*. (2010) where they observed moderate susceptiblility of *E. coli* to both ciprofloxacin and chloramphenicol in Borno State, Nigeria while 50% of the isolates were susceptible to streptomycin and nalixidic acid. This result is higher than the 49.7%; 63.2% and 30% reported by Kijima-Tanaka *et al*. (2003) and Akond *et al*. (2009) in Japan and Bangladesh respectively. The variation in susceptibilities of *E. coli* in these antibiotics may not be unconnected with their occasional usage in poultry enterprise, as chloramphenicol in particular has been banned in poultry production due to its causes of aplastic anaemia in humans. The *E. coli* isolates from the commercial chickens were resistant to cotrimoxazole, cloxacillin and tetracycline. This observation agrees with the reports of Chah *et al*. (2000) and Nsofor and Iroegbu, (2012). This indicates that these compounds have become seriously compromised and are of little value in the treatment of infections due to the organism. Cotrimoxazole is used for treatment of secondary infection in HIV/AIDS patients; therefore the transmission of resistant strains from poultry to immuno-compromised individuals could lead to complicated diseases and eventually increase morbidity and mortality (Samuel *et al*. 2010).
The *E. coli* isolates from free-range chickens in this study were high susceptibility to chloramphenicol, ciprofloxacin and gentamicin. This observation concurs with the reports of Okoli *et al.* (2005) in Imo State where high susceptibility of the organism to the above antibiotics was observed. Sixty percent of *E. coli* isolates in free-range chickens were moderately susceptible to nalixidic acid and streptomycin. This is similar to the report of Okoli *et al*. (2005). Thirty percent of the isolates were susceptible to cotrimoxazole, which agrees with the report of Okoli *et al*. (2005), but lower than the 48.3% and 56% obtained by Mutsami, (2011) and Oluduro *et al*. (2012).
The *E. coli* isolates in faecal droppings in free-range chicken were highly resistant to cloxacillin and tetracycline. This agrees with the reports of Tabaatabaei *et al*. (2010). Even though, free-range chickens hardly receive the attention of veterinarians; they maintain close contact with these antibiotics through various sources in the environment and also from humans that may have been exposed to these antibiotics. None of the *E. coli* isolates in this study were resistant to a single antibiotic. This is in agreement with the reports of Ojo *et al*. (2012) and Oluduro *et al*. (2012) where high multi-antibiotic resistance were observed in commercial and free-range chickens. The most predominant pattern was COT-CXC-TET and it corresponds to a similar study in Kenya (Mitema and Kikuri, 2004) and Bangladesh (Akond *et al*., 2009) where the *E. coli* isolates showed multi-antibiotic resistance to ampillicin, cotrimoxazole and tetracycline. The high incidence of multi-antibiotic resistance in *E. coli* may be due to the continuous exposure to antibiotics and close contact with their environment. The isolates from both commerical and free-range birds had multiple-antibiotic index (MARI) above 0.2 which infers that the isolates had arisen from environment were these antibiotics are frequently used, thereby indicative of a high antibiotic selective pressure in the environment (Hemen *et al*. (2012).

Conclusion

This study has established the presence of resistant strains of *E. coli* isolated from both commercial and free-range chickens to some antibiotics used both in humans and animals. The presence of high MARI in *E. coli* isolates from faecal droppings of commercial and free-range chickens connotes the frequent use of these antibiotics in the environment.

Resistance was common to antibiotics frequently and commonly used by both humans and in the poultry industry in Nigeria.

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ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF *SALMONELLA SPP.* ISOLATED FROM FAECAL DROPPINGS OF COMMERCIAL AND FREE-RANGE CHICKENS IN SAMARU, NIGERIA.

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ABSTRACT

Antibiotic susceptibility patterns of *Salmonella* spp. from faecal droppings of commercial and free-range chickens were determined. Five *Salmonella* spp. isolates were recovered from 200 samples of faecal droppings from live commercial and free- range chickens on *Salmonella-Shigella* Agar. They were confirmed using standard biochemical tests. Susceptibility of the isolates was carried out by Disc Diffusion test using the following antibiotics: chloramphenicol, ciprofloxacin, cloxacillin, cotrimazole, gentamicin, nalixidic acid, streptomycin, and tetracycline. This study revealed that 100% of isolates from commercial chickens were susceptible to chloramphenicol, ciprofloxacin, gentamicin, nalixidic acid, streptomycin and resistant to cloxcaillin, cotrimazole and tetracycline. However, the isolate obtained exhibited multi-antibiotic resistance to cloxacillin and tetracycline. Hundred percent of the isolates from free-range chickens were susceptible to ciprofloxacin, cotrizamole, gentamicin, nalixidic acid and streptomycin while 75% were susceptible to chloramphenicol. However, 100% were resistant to cloxacillin and tetracycline and all the isolates were observed to exhibit multi-antibiotic resistance. The study has established the presence of antibiotic susceptible, resistant and multi-antibiotic resistant *Salmonella* spp. isolates in commercial and free-range chickens to antibiotics in Samaru, Zaria. Multi-antibiotic resistance was higher in free-range chickens than in commercial chickens.

Key words: Antibiotics, antibiotic susceptibility, *Salmonella* spp., commercial chickens, free-range chickens, multi-antibiotic resistance, fecal droppings.

INTRODUCTION

Salmonella is a genus of bacteria that can cause foodborne infections in humans and animals. They are well distributed in nature and can survive in a variety of foods. Domestic animals particularly poultry are considered to be reservoirs of the organism which play an important role in the spread of infection. Transmission of the organism from animals to human can be through the food chain. The use of antibiotics in sub-therapeutic and therapeutic doses for poultry has helped in promoting growth, protection and cure from diseases (NOAH, 2009). Consequently, this has led to the emergence of antimicrobial-resistant strains which are of great global concern. The aim of this study was to determine the antibiotic susceptibility profile of *Salmonella* spp. from the faeces of commercial and free-range chickens.

MATERIALS AND METHODS

Sample collection

Two hundred (200) faecal samples were randomly collected from commercial poultry houses (100 samples) and homes that rear free range chickens (100 samples). The faecal samples were placed in clean glass containers. They were taken to the microbiology laboratory of the Department of Microbiology, Ahmadu Bello University, Nigeria under ambient conditions and processed immediately.

Isolation and identification of *Salmonella*

Fecal samples were inoculated into peptone water for enrichment at a ratio of 1gram to 9ml of broth and incubated at 37^0Cfor 18-24hrs. The broth was inoculated on *Salmonella-Shigella* agar (SSA) and incubated at 37^oC for 18-24hrs. After incubation, the SSA plates were examined for bacterial growth. The suspected isolates were subjected to gram-staining and biochemically identified using triple sugar iron agar (TSI), Simmon citrate agar, sulphur indole motility medium (SIM), and Methyl red-Voges proskauer broth (MR-VP). Further confirmation was done using biochemical tests kits (Microgen^{TM} GnA- ID System) and accompanying computer software package (Microgen ID Version 1.2.5.26).

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of the isolates were carried out using the disc diffusion technique (Kirby-Bauer disk diffusion method) according to the methods recommended by the Clinical Laboratory and Standards Institute (6). About 3-5 discrete colonies of the isolates were inoculated into 5ml of nutrient broth and incubated overnight at 37^oC. The overnight culture was then standardized with 0.5 MacFarland standard. A sterile cotton swab was used in inoculating the culture to freshly prepared pre-dried Mueller-Hinton agar plates prepared according to manufacturer's instructions. The antibiotic discs were aseptically and spaciously placed on the inoculated Mueller-Hinton agar plates. The plates were inverted and incubated aerobically at 37^oC for 18-24hr. The antibiotics discs used include: Ciprofloxacin (5µg), Chloramphenicol (10µg), Cloxacilin (5µg), Cotrimoxazole (25µg), Gentamicin (10µg), Nalixidic acid (5µg), Streptomycin (10µg), Tetracycline (10µg). For each isolate, the zone of inhibition around each disk was measured, after incubation at 37^0C for 24h.

RESULTS

Prevalence rates of *Salmonella* spp. is as shown in Table 1. Antibiotic susceptibility profile of *Salmonella* spp. isolated from faecal droppings of commercial and free range chickens is shown as in Figure 1. Ciprofloxacin, chloramphenicol, gentamicin, nalixidic acid, and streptomycin had the highest antibiotic activity against the isolate (100%) and was resistant to tetracycline and cloxacillin. In free-range chickens, ciprofloxacin, cotrimazole, gentamicin, nalixidic acid, streptomycin had the highest antibiotic activity against all the isolates (100%) while the isolates were susceptible (75%) to chloramphenicol. However all the isolates were resistant to tetracycline and cloxacillin.

Multi-antibiotic resistance pattern of *Salmonella* spp. isolated from fecal droppings of commercial and free-range chickens is shown in Table 2. No antibiotic resistance phenotype was observed with the single antibiotic resistance type in the isolates from the commercial and free-range chickens. One multiple resistance phenotype was obtained with varying combinations of 2 antibiotics while in free-range chickens, two multiple resistance phenotypes were obtained with varying combinations. The highest frequency of 3 was observed in the combinations of 2 antibiotics.

All isolates from both commercial and free-range chickens exhibited multidrug resistance. The multiple drug resistance index (MDRI) of the isolates can be seen in Figure 2. All isolates from commercial chickens and free-range chickens had a MDR index greater than 0.2.

Table 1. Prevalence of *Salmonella* sp. from the faecal droppings of commercial and free-range chickens

Management system	No. of samples	No. of isolates (%)
CC	100	1(1%)
FRC	100	4(4%)

CC= Commercial chickens, FRC= Free-range chickens

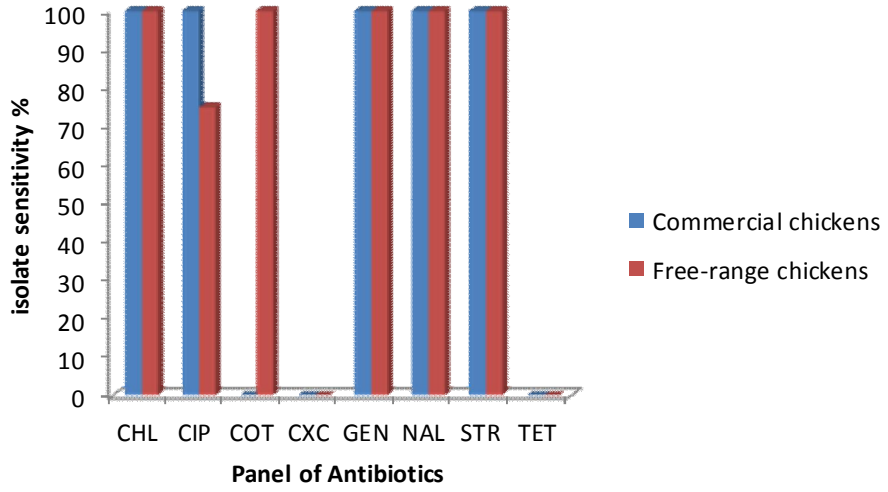


Figure 1. Antibiotic susceptibility profile of *Salmonella spp.* isolated from faecal droppings of commercial and free-range chickens

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline

Table 2.Multidrug-resistance pattern of *Salmonella* spp. isolated from the faecal droppings of commercial and free-range chickens

Single antibiotic resistance		Multiple antibiotic resistance		
No. of isolates	Resistance Phenotype	No. of antibiotic Combination	No. of isolates(%) with pattern	Resistance phenotype
CC 0(0)	Nil	2	1(100)	CXC, TET
FR 0(0)	Nil	2	3(100)	CXC, TET
	Nil	3	1(100)	CXC,CHL,TET

Key: CIP=Ciprofloxacin, CHL=Chloramphenicol, COT=Cotrimazole, CXC=Cloxacillin, GEN=Gentamicin, NAL=Nalixidic acid, STR=Streptomycin, TET=Tetracycline CC= Commercial chickens, FRC= Free-range chickens

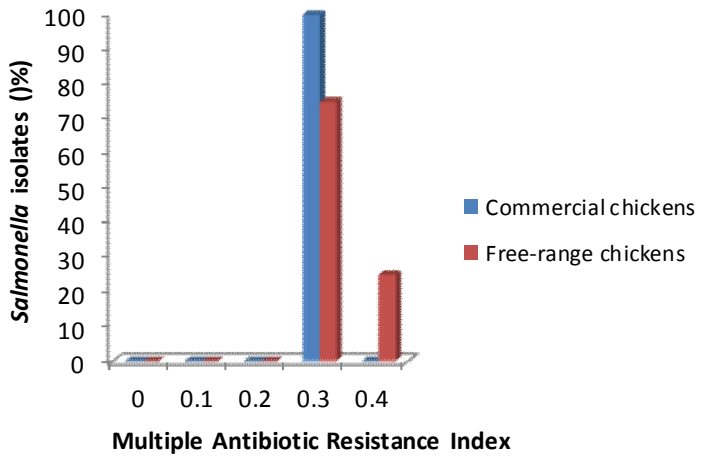


Figure 2: Multiple antibiotics resistance indices of *Salmonella spp.* isolated from faecal droppings of commercial and free-range chickens

4. DISCUSSION

The antibiotic susceptibility pattern of *Salmonella* from faecal droppings of commercial and free-range chickens in Samaru, Nigeria was studied. Prevalence of *Salmonella* spp. from faecal droppings of commercial chickens was found to be 1%. This finding is similar to a report by Mutsami, 2011 where *Salmonella* spp. (0.1%) was isolated from the faeces of broilers in Kenya and below the number reported by Wafaa *et al* 2012. The prevalence of *Salmonella* spp. from free-range chickens in this study was (4%), this agrees with the report of Pan *et al*. (9) but not in consonance with the prevalence reported by Mutsami, 2011 and Ojo *et al*., 2012. Mutsami,2011 reported a prevalence of 0.1% and Ojo *et al*., 2012, 11% .The variation in the prevalence rates could be due to sampling procedures, location of samples and isolation methods used (Kishima *et al*., 2008).

Poultry producers depend on antibiotics for treatment and in low doses for growth promotion. Studies have shown that increase in cases of human salmonellosis is due to antibiotic resistant *Salmonella* (Foley and Lynne, 2008). Antibiotic susceptibility of *Salmonella* spp. to selected antibiotics in this study shows that all the isolates obtained from commercial and free-range chickens were highly susceptible to chloramphenicol, ciprofloxacin, and gentamicin. This observation concur with report of Begum *et al*. 2010 but disagrees with the results obtained by Ojo *et al*., 2012 and Suh and Odeh, 2008 where *Salmonella* spp. isolated from commercial and free-range chickens were susceptible to chloramphenicol, ciprofloxacin and gentamicin. This may be due to the difficulty encountered during injection and the cost of the drugs. The high susceptibility of *Salmonella* isolates to nalixidic acid and streptomycin in this study agrees with the result obtained by Enabulele *et al*., 2010. However, it contradicts the reports of Payne *et al*., 2006 and Siemon *et al*.,2007. The resistance of the isolates to both tetracycline and cloxacillin agrees with previous reports of Begum *et al*., 2010 and Payne *et al*., 2006 but disagrees with the observation of Ojo *et al*., 2012 and Alali *et al*., 2010 in commercial and free-range chickens. Resistance to tetracycline is very common. Tetracycline is one of the most widely used antibiotic especially in feed as feed additives for growth promotion and for the

prevention of other diseases of bacterial origin (Muirhead, 1999; Rajashekara, 2000). Besides, studies have shown that tetracycline persists in the environment longer than any other antibiotic (Frost, 1991). This contradicts the reports of studies done by All the isolates from the free-range chickens were susceptible to cotrimazole while that of the commercial chickens was resistant. This is agreeable with Alali *et al*., 2010.This contradicts with Begum *et al*., 2010 and Enabulele *et al*., 2010.

The multidrug resistance of *Salmonella* spp. to several antibiotics observed in this study agrees with the results of Ojo *et al*., (10), but disagrees with reports of Alali *et al*., 2010 and Siemon *et al*., 2007. Furthermore, the isolates from the study exhibited one and two patterns of resistance phenotypes from commercial and free-range chickens respectively. Both patterns contradict the observations of Payne *et al*., 2006, Ojo *et al*., 2012 and Pan *et al*., 2010. This variation in the pattern of resistant phenotypes may be due to differences in the choice and concentrations of the antibiotics administered. Multidrug resistance in *Salmonella* renders treatment with antibiotics ineffective, thereby resulting in grave zoonotic implication. In addition, the high MARI observed in this study is suggestive of the fact that the probable source of the multidrug antibiotic. *Salmonella* isolates may likely to have originated from environment when these drugs frequently used (Hemen *et al*., 2012).

CONCLUSION

This study has established the presence of resistant strains of *Salmonella* spp. isolated from both commercial and free-range chickens to some antibiotics used both in humans and animals. The presence of high MARI in *Salmonella* spp. isolates from faecal droppings of commercial and free-range chickens connotes the frequent use of these antibiotics in the environment. Resistance was common to antibiotics frequently and commonly used by both humans and in the poultry industry in Nigeria.

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