**Characterization of Antimicrobial Resistant Salmonella and *Escherichia coli* in Retail Broiler Chickens in Lusaka Province of Zambia**

**By**

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A dissertation submitted to the University of Zambia, in Full Fulfilment of the Requirements for the Degree of Master of Science in Veterinary Public Health (Food Microbiology)

**The University Of Zambia**

**Lusaka**

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# **DECLARATION**

I, **Elizabeth Muligisa-Muonga,** hereby declare that the project report titled **“Characterization of Antimicrobial Resistant *Salmonella* and *Escherichia coli* in Retail Broiler Chickens in Lusaka Province of Zambia”** submitted to the University of Zambia is entirely original work done by me under the guidance of my supervisors. I further declare that, to the best of my knowledge, the information given in the dissertation is authentic, and that the work of others has been duly acknowledged. Also that, the dissertation has not been submitted for any other degree or professional qualification. No part of this dissertation may be reproduced, stored in a retrieval system, or transmitted in any form, electronic, mechanical, photocopying, recording or otherwise, without prior written consent of the author, except for research purposes.

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# **ABSTRACT**

The steady increase in the demand for poultry in Zambia and the world over has put a strain on poultry producers leading to the introduction of new practices such as the use of antibiotics in order to boost production. However, in many resource poor countries, the use of these antibiotics is often not monitored. When antibiotics are misused it can result in the emergence of antibiotic resistance in organisms that could find their way into poultry meat such as *Salmonella* and *Escherichia coli* (*E. coli*). These organisms, as well as others, that could be found in poultry meat, form a reservoir of Antimicrobial Resistant Genes that can be spread from one bacterium to another through horizontal gene transfer, whether pathogenic, commensal or environmental in nature. This spread is facilitated by cross-contamination of the poultry meat when in contact with other surfaces or products. A cross-sectional study was undertaken at the retail level in four districts of Lusaka province (Lusaka, Chilanga, Chongwe and Kafue) in order to determine the proportion of antimicrobial resistant *Salmonella* and *E. coli* from retail broiler chickens and to determine the genes that confer the resistance. Sampling was done between January and May 2018. A total of 250 whole broiler carcasses were purchased from 26 open markets (n=134) and 37 supermarkets (n=116). An open market was defined as an unrestricted competitive market in which any buyer and seller is free to participate, while a supermarket was defined as a self-service shop offering a wide variety of food and household products organized into aisles. Upon purchase, all samples were transported in a cooler box containing ice packs to the UNZA Veterinary Public Health Laboratory. Laboratory isolation included a whole carcass rinse in buffered peptone water (Oxoid), pre-enrichment and subsequent incubation at 37OC overnight. Ten microliter (10µL) of the incubated broth was then transferred to MacConkey agar (Oxoid UK) for *E. coli* isolation while 1ml was also transferred to Rappaport Vassiliadis (Oxoid UK) and later subcultured on Xylose-Lysine Deoxycholate agar (Oxoid UK) for isolation of *Salmonella*. Biochemical tests were performed on all suspected isolates using Analytical Profile Index (API 20E) (Biomerieux®). Further identification of the isolates was done using 16S rRNA sequencing for bacteria and Antimicrobial susceptibility tests were performed using the Kirby Bauer disk diffusion technique using a panel of 10 different antibiotics. The panel of antibiotics comprised of Ampicillin (10µg), Amoxicillin/Clavulanic acid (30µg), Imipenem (10µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Trimethoprim/Sulfamethoxazole (25µg), Colistin (10µg), Chloramphenicol (30µg), Tetracycline (30µg) and Cefotaxime (30µg). Analysis of the disk diffusion results was done using WHONET 2018 software. A total of 148 *E. coli* isolates were identified and subjected to antimicrobial susceptibility testing. Fifty three percent (53%) (n=79) of the isolates were from Open markets while 47% (n=69) were from Supermarkets. Tetracycline recorded the highest resistance of 76.4%, followed by Ampicillin 51.4%, Trimethoprim Sulphamethoxazole 44.6%, Nalidixic Acid 23.6%, Chloramphenicol 14.2%, Cefotaxime 12.8%, Ciprofloxacin 8.1%, Amoxicillin/Clavulanic acid 5.4%, Colistin 5.4% and Imipenem 0.7%. Five *Salmonella* isolates was recovered and two were resistant to Tetracycline and Ampicillin, but were susceptible to the other eight antimicrobials. The three target genes of resistance were Tet1, Sul1 and CtxM. The resistant isolates had at least one of these genes encoded in their DNA, with the beta-lactam gene being the highest. This study has demonstrated the presence of resistant *E. coli* isolates on broiler chicken from both open and supermarkets. Such resistance patterns pose a threat to public health, hence the need to find the predisposing causes.

**Keywords**: *Antimicrobial resistance, E. coli, Salmonella, Poultry, Zambia*

# **DEDICATION**

I dedicate this research to my ever-loving friend and husband Chikumbi Alphonse Muonga, my parents: Bernard and Merab Muligisa, my three brothers: Bernard Francis Muligisa Junior, Victor Anthony Muligisa and Ronald Kenneth Muligisa for their love and support. I would also like to dedicate this research to my son Theodore Kalumbu Muonga.

# **CERTIFICATE OF COMPLETION**

The undersigned have read, and hereby certify, that this dissertation titled “**Characterization of Antimicrobial Resistant *Salmonella* and *Escherichia coli* in Retail Broiler Chickens in Lusaka Province of Zambia”** that is being submitted by **Elizabeth Muligisa-Muonga** for the degree of Master of Veterinary Public Health (Food Microbiology), meets the prescribed guidelines for post-graduate studies’ dissertations for the University of Zambia and recommend it for examination.

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# **APPROVAL**

The University of Zambia, School of Veterinary Medicine approves this Dissertation submitted by **Elizabeth Muligisa-Muonga** in full fulfilment of the requirements for the award of degree of Master of Veterinary Public Health (Food Microbiology).

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**Chairperson Signature Date**

**(Board of Examiners)**

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**Supervisor Signature Date**

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May God richly bless you All.

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# **ABBREVIATIONS AND ACRONYMS**

**Abbreviations/Acronyms Meaning**

ACEIDHA African Centre of Excellence in Infectious Diseases of Humans and

Animals

AGISAR Advisory Group on Integrated Surveillance of Antimicrobial

Resistance

AMR Antimicrobial Resistance

FBDs Foodborne Diseases

HGT Horizontal Gene Transfer

ETEC Enterotoxigenic *Escherichia coli*

XLD Xylose Lysine Deoxycholate Agar

UTI Urinary Tract Infection

EMB Eosin Methylene Blue Agar

ExPEC Extraintestinal Pathogenic *E. coli*

NMEC Neonatal Meningitis *E. coli*

EPEC Enteropathogenic *E. coli*

EHEC Enterohemorrhagic *E. coli*

EIEC Enteroinvasive *E. coli*

EAEC Enteroaggregative *E. coli*

DAEC Diffusely Adherent *E. coli*

DNA Deoxyribonucleic Acid

FDA Food and Drug Administration

API Analytical Profile Index

rRNA Ribosomal Ribonucleic Acid

PCR Polymerase Chain Reaction

HACCP Hazard Analysis Critical Control Points

# **DEFINITION OF TERMS**

Antibiotic A substance or chemical used produced by a microorganism that

kills or retards the growth of another microorganism

Antimicrobial A substance or chemical that inhibits or stops the growth of a

microorganism

Antimicrobial resistance The ability of a microorganism (like bacteria, viruses, and some

parasites) to stop an antimicrobial (such as antibiotics, antivirals and

antimalarials) from working against it.

Commensal An organism that is part of the normal flora of the host

Extra-intestinal diseases Diseases that occur at locations other than the gastro-intestinal tract

Facultative anaerobes Organisms that grow under strict anaerobic conditions (in the

absence of oxygen)

Food-borne diseases Disease acquired by the intake of food contaminated with

Microorganisms.

Gene A sequence of nucleotides that code for a particular trait

Genotype Genetic characteristic of an organism that determine their physical

characteristics

Open market An unrestricted competitive market not housed in a building, where

foodstuffs are sold often exposed and in which any buyer and seller

was free to participate.

Pathogenic organism An organism that has the potential to cause disease

Pathotype A group of organisms that have the same pathogenic characteristics

Phenotype Characteristics of an organism that can be seen and are as a result of

genetic characteristics

Supermarket A market housed in a closed building with modernized facilities that

offered a wide variety of food and household products, organized

into aisles

Zoonotic diseases Diseases that can spread from other animal species to humans

# **CHAPTER ONE**

# **INTRODUCTION**

## **Background**

Poultry meat has long been considered a delicacy in many parts of the world. It forms an integral part of the diet, especially in developing countries, where families live on less than a dollar a day. The popularity of poultry meat is because it is a cheaper source of protein compared to other meat such as beef and pork and also because it is easier to produce (Musaba and Mseteka, 2014). However, this high demand for poultry meat puts a strain on producers, who have to meet the ever-growing demand and at the same time realize profits in a competitive market environment (Ahuja and Sen, 2007). Therefore, in order to be competitive, farmers have to produce broiler chickens that visibly look attractive to the customers by weight and other aesthetic characteristics, but also make profit. One way in which this is achieved is by the use of antibiotics. Antibiotics are used to prevent and treat diseases of poultry in order to optimize growth (Apata, 2009). When the birds are disease-free they can grow at optimum rates thereby increasing the market-potential and value of the birds, which gives the producer advantage in a competitive market.

In Zambia, Lusaka Province is one such competitive market for poultry products. It has a population of about 2.777 million people and has a number of poultry producers and processers. Being the industrial capital of Zambia, the province is both a major source of poultry distributed to other provinces and a retail destination for poultry from other provinces of the country. It hosts both small-scale and commercial poultry producers (AgriProFocus Zambia, 2015). However, as is the case with any competitive trade environment, this comes with challenges. In case of poultry trade, the challenges are related to assurance of safety, and in this case, to the monitoring of the use of antibiotics in both small-scale and commercial production systems. In the absence of a coordinated national surveillance system on the use of antimicrobials, it is impossible to know whether the antimicrobials are being handled properly (WHO Global Report, 2014). Responsible use of antimicrobials entails that the farmer administers the right doses to sick birds and withdrawal periods are adhered to. It also precludes the use of antimicrobials for growth promotion. Where necessary, antimicrobials should be administered up to a certain period of time (usually at least five days and at most fourteen days before slaughter depending on the type of antimicrobial) in order to allow the antimicrobials to be completely metabolized before consumption by human beings (Manyi-Loh *et al.*, 2018). Sometimes these withdraw periods may not be observed, especially if the birds get sick when they are close to market weight.

Poultry may suffer from a number of diseases, broadly classified according to the infectious agent. They are classified as viral, bacterial or parasitic diseases. Of these three categories, bacterial diseases are of utmost importance to public health because they are the commonest cause of foodborne infections (Pattison *et al.*, 2008). Some of the important diseases include: colibacilosis, fowl cholera, infectious synovitis, necrotic enteritis, and salmonellosis (fowl typhoid). These diseases are often spread in the poultry house by ingestion of contaminated feed and water. The contaminated feed and water may also contain antibiotic resistant bacteria that may also be ingested by the birds. When ingested, these resistant organisms may transfer their resistance to other organisms within the gut of the birds via horizontal gene transfer. This becomes a problem for public health because consumption of poultry that has been contaminated with antibiotic resistant organisms can also facilitate the transfer and spread of resistance between bacteria that are in close proximity in the human consumer. When this happens, it could lead to difficulties in the treatment of foodborne diseases, especially where treatment options are limited (Bennett, 2008).

In humans, bacterial food-borne diseases (FBDs) account for a high number of deaths in developing countries such as Zambia (Torgerson *et al.*, 2015). FBDs are acquired by the ingestion of contaminated raw and processed foods. Meat, such as poultry, is a favorable environment for bacterial growth and multiplication due to its high protein and moisture content, among other factors. Statistics from hospitals and other medical centers suggest that there is an increase in the number of individuals hospitalized for food-borne diseases (Kelly *et al.*, 1996). These diseases affect both healthy and immune-compromised individuals such as those suffering from chronic diseases, children under the age of five and the elderly. FBDs are a major concern in immune-compromised individuals because they are likely to advance to life-threatening stages, which can eventually lead to death.

A number of organisms causing FBDs have been isolated from patients in hospitals and among the most incriminated are *Campylobacter*, *Listeria*, *Salmonella* and *Escherichia coli* (*E. coli*) (Scallan *et al.*, 2015). These bacteria also happen to be zoonotic in nature and cause diseases such as typhoid fever, listeriosis and colibacilosis in humans and are, therefore, of public health importance. It should also be noted that resistance to antibiotics has been observed in these organisms (Mshana *et al*, 2013).

In recent decades pathogenic organisms have been shown to acquire and spread antibiotic resistance among themselves. Resistance can occur naturally, by mutation or by the transfer of resistant genes from one species to another. Resistance which can be passed on from one species to another is very critical to public health (Von Wintersdorff *et al.*, 2016). It has also come to scientists’ attention that not only are resistant genes being passed on or acquired from other pathogenic bacteria, but also that these genes can be acquired and spread to any other bacteria, whether pathogenic, commensal or environmental in nature. This forms a reservoir of Antimicrobial Resistant Genes (ARGs) from which pathogenic bacteria can acquire resistance via horizontal gene transfer (HGT) (Chadha, 2012).

Food often comes into contact with bacteria that can be pathogenic or non-pathogenic in nature. Some of the organisms that are commonly found contaminating food are *Salmonella spp.* and *E. coli*. This contamination of food can occur anywhere along the food continuum from the farm during production to the consumption of the food (farm-to-folk). Therefore, it is important for the control and prevention strategies of contamination to be applied throughout the food chain (Voidarou *et al.*, 2011; Wabeto et al, 2017).

*Salmonella* causes a disease broadly classified as salmonellosis. Clinical signs usually include diarrhea, fever and abdominal cramps 12 to 72 hours after infection. These symptoms usually last 4-7 days with most people recovering without treatment. However, some cases may be severe and the patient may have to be hospitalized. In such cases, the infection may spread from the intestines to other parts of the body such as the blood where they can cause death if not treated effectively. These severe forms of illness are usually experienced by people with compromised or impaired immunity such as infants, people suffering from chronic disease and the elderly (Reller, 2017).

Despite *E. coli* being an innocuous resident of the digestive system, it can also be pathogenic and cause severe intestinal and extra-intestinal diseases (Diarrassouba *et al.*, 2007). These pathogenic forms of *E. coli* (pathotypes) have been known to cause a lot of morbidity and mortality worldwide. While there are many strategies that these pathotypes employ to colonize the intestinal lining and cause disease, the course, onset, and implications vary greatly. Outbreaks are common in both developed and developing countries and can sometimes be life-threatening (Kabwe *et al.*, 2016). This is another major public health concern because the pathotypes usually have a low infectious dose and are easily transmitted through ingestion of contaminated food and water (Croxen and Finlay, 2010).

This study focuses on two food-borne pathogens associated with market-ready broiler chickens; *E. coli* and *Salmonella*, in an effort to provide information on the positivity rate of antimicrobial resistance in these pathogens and to determine the genes that confer this resistance.

## **Problem Statement**

It is estimated that about 48 million people get sick from foodborne illness every year, the majority of these cases are hospitalized and some of these die (Torgerson *et al.*, 2015). *Salmonella spp.* are among the most incriminated when it comes to bacterial food poisoning, especially those from poultry meat. Human infections caused by *Salmonella* are usually acquired through the handling of raw poultry and poultry products, as well as through eating undercooked poultry meat (Panisello *et al.*, 2000).

Pathogenic forms of *E. coli* such as *Enterotoxigenic E. coli* (ETEC) also cause significant diarrheal illness. It is the leading cause of travelers' diarrhea and other diarrheal illnesses in developing countries, especially among children (Nataro and Kaper, 1998).

*Salmonella* and *E. coli* infections can be treated using relevant antibiotics, but there is also accumulating evidence of the consequences of drug resistance with regards to public health in thesepathogens and other foodborne bacteria. These consequences have a lot to do with a the reduction in the efficiency of treatment with first-line drugs and limited choices after microbiological diagnosis (Clarke *et al.*, 2012; Mshana *et al*, 2013).

In Zambia, recurrent typhoid outbreaks as well as recent findings which showed that *Salmonella* species and *E. coli* were among the most detected pathogens causing bacterial diarrheal disease in children between the ages of 0-59 months at the University Teaching Hospital (Chiyangi *et al.*, 2017). This suggests that foodborne pathogens, poor hygiene and sanitation and other food safety risks such as the emergence of antimicrobial resistance in foodborne pathogens are having a negative impact on public health (Mainda *et al.*, 2015). Illness as a result of these bacteria also leads to a reduction in the nation’s productive hours, ultimately affecting a nation’s economy.

Developing countries have recorded high antibiotic resistance rates in bacteria originating from meat, probably due to uncontrolled use of antibiotics in livestock farming. Therefore, the study of antibiotic resistance in developing countries is important because the information from such studies could enhance correct and controlled use of antibiotics in food production (Mshana *et al*, 2013; Chishimba *et al.*, 2016).

## **Justification**

Information on the phenotypes and genotypes of antimicrobial resistant foodborne microorganisms is largely restricted to developed countries and there is little or no information on what is happening in developing countries (Hart and Kariuki, 1998; Ayukekbong *et al*, 2017). For Zambia, the extent of the burden of antimicrobial resistance in foodborne pathogens isolated from poultry products remains largely unknown. This is partly because there is no national surveillance system currently in place for AMR, a situation that makes it difficult to implement any control measures. Despite reports indicating that there is misuse and abuse of antimicrobials, the extent of the resulting AMR is not yet defined (Mainda *et al.*, 2015).

This study will therefore provide information on the burden of antimicrobial resistance of two foodborne pathogens; *Salmonella* and *E. coli,* in Lusaka province. The study will also provide base-line information, which could inform future AMR surveillance efforts and implementation of control strategies for emergence of antimicrobial resistance of foodborne pathogens.

## **Research Question**

What are the antimicrobial resistant profiles of Salmonella and *E. coli* in market-ready poultry products in Lusaka Province of Zambia?

## **General Objective**

To characterize the phenotypes and genotypes of antimicrobial resistant *E. coli* and Salmonella in retail broiler chickens in Lusaka Province of Zambia.

## **Specific Objectives**

1. To determine the proportion of antimicrobial resistant *E. coli* and *Salmonella* species from retail broiler chickens in Lusaka province.
2. To determine the genes that confer resistance to antimicrobials in retail broiler chickens in Lusaka province.

# **CHAPTER TWO**

# **LITERATURE REVIEW**

## **2.1. Bacterial Food borne diseases**

The term foodborne diseases is used to refer to diseases of the digestive system that are as a result of consuming contaminated food or drink. They are responsible for the suffering of millions of people worldwide every year and the social and economic strain of many developing countries (Ayukekbong *et al*, 2017).

Zoonotic diseases are diseases that can be transmitted to human from other animal species. Foodborne zoonotic diseases are as a result of consuming food or drinking water contaminated by disease causing microorganisms such as bacteria, the toxins that they produce, viruses and parasites. They are usually ingested and find their way into the digestive system where they cause disease. These diseases can manifest themselves as just mild symptoms or can progress to life-threatening forms in humans. Many of these microorganisms can cause disease in humans even though they are commensals in the intestines of other species of animals (Voidarou *et al.*, 2011).

The foodborne bacteria include *Salmonella*, *Campylobacter*, *Escherichia coli* (*E. coli*), *Listeria monocytogens*, *Yersinia enterocolitica*, *Staphylococcus*, *Arcobacter*, *Clostridium perfringens*, *Clostridium botulinum* and *Bacillus cereus* (Dhama *et al.*, 2013). Among the most virulent are *Campylobacter*, *E. coli*, *Salmonella* and *Shigella* species (Scallan *et al.*, 2015).

Diarrhea is a major and common symptom of FBDs. It is usually a symptom of infection in the digestive system and can be caused by bacteria, viruses or parasites. Diarrhea is the second leading cause of death in children under five years old, killing around 760 000 children every year (Tian *et al.*, 2016). It can last several days, leading to dehydration. Most deaths are as a result of excessive fluid loss leading to dehydration. Immune-compromised individuals are at risk of getting severe forms of diarrhea that can lead to death (Irena *et al*, 2011).

Infection is spread through ingestion of contaminated food or drinking water, or from person-to-person due to poor hygiene conditions. Most of these infections are spread when water used for drinking, cooking and cleaning is contaminated with feces. *Rotavirus* and *E. coli* are the most incriminated microorganism causing diarrhea in developing countries. When food is prepared or stored in unhygienic conditions it can also cause diarrheal disease and this is a major cause of diarrhea worldwide, especially in developing countries (Kelly *et al.*, 1996; Bosomprah *et al.*, 2016).

### 2.1.1. *Salmonella* foodborne infections

### 2.1.1.1. Etiology and Structure

*Salmonella* is a rod-shaped gram-negative bacterium belonging to the family Enterobacteriaceae and is the causative agent of salmonellosis (Reller, 2017). They are straight rods which are approximately 0.7-1.5µm in width × 2-5µm in length. They are motile facultative anaerobes and have peritrichous flagella. They are mesophilic in nature and have an optimum growth temperature of 37°C (Gruenberg, 2015).

### 2.1.1.2. Ecology

Members of the genus *Salmonella* can colonize both vertebrate and invertebrate hosts. Salmonellosis is usually associated with *Salmonella enterica* in warm-blooded vertebrates. These warm-blooded animals are able to carry the pathogen for a long period of time without showing any signs of infection. Diseases caused by *Salmonella enterica* can be classified as either systemic septicemia, as known as typhoid, or enteritis. However, there can also be other clinical presentations such as abortion, arthritis, respiratory disease, necrosis of extremities, and meningitis (Gruenberg, 2015; Kalonda *et al.*, 2015).

### 2.1.1.3. Clinical signs

Non-typhoidal *Salmonella* species are responsible for causing gastroenteritis and bacteremia, eventually leading to secondary infection. These bacteria are a problem in immune-compromised individuals such as patients with malignancy, human immunodeficiency virus, diabetes, and those receiving medication for anti-inflammatory diseases. Infections of the bloodstream and deep bone or visceral abscesses are usually difficult to treat. The infection site and immune status guide treatment choices (Román *et al*, 2013).

### 2.1.1.4. *Salmonella* diagnosis

### Culture

Laboratory diagnosis of *Salmonella* usually involves bacterial culture in a selective pre-enrichment broth such as Rappaport Vassiliadis media, selenite F broth or tetrathionate broth, selective growth on solid media such as Xylose Lysine Deoxycholate (XLD) Agar or MacConkey Agar and further biochemical testing for identification (U.S. Food and Drug Administration (FDA), 2001). *Salmonella* colonies on XLD agar appear either pink with a black center, completely black or just pink without a black center. Colonies that exhibit these characteristics are then subjected to biochemical tests for confirmation.

### Biochemical and Serological tests

*Salmonella* species are generally oxidase negative, catalase positive, indole negative, Voges-Proskauer negative and produce acids and hydrogen sulfide. They are also citrate positive and are non-lactose fermenters (Löfström *et al.*, 2015). The *Salmonella* bacterium has three major antigens on its surface; the O antigen that is found on the somatic cell, the H antigen that is found on the flagella and the Vi antigen that is found on the capsules of a few serovars that form capsules. Antigen classification of *Salmonella* is done based on the O and H antigens (Reller, 2012).

### 2.1.2. *Escherichia* foodborne infections

### 2.1.2.1. Etiology and Structure

*Escherichia* organisms are also gram-negative rod-shaped bacteria that exist singly or in pairs. It is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), and traveler's diarrhea, and other clinical infections such as neonatal meningitis and pneumonia. *E coli* is facultatively anaerobic with a type of metabolism that is both fermentative and respiratory. Each bacterium measures approximately 0.5µm in width × 2µm in length. They are either non-motile or motile by peritrichous flagella. Some pathogenic forms of *E. coli* are able to form capsules around the cell. They are mesophilic in nature, growing at an optimum temperature of 37°C (Dupont *et al*, 2009).

### 2.1.2.2. Ecology

*E. coli* is a common inhabitant of the gastro-intestinal tract of many mammals, some birds and can also be found in the environment such as in soil. Despite it being a commensal, it is also responsible for a number of enteric infections (Frankel *et al.*, 1998). *E coli* is a major facultative inhabitant of the large intestine (Johnson, 1991). *E. coli* infections are widely distributed among poultry of all ages. They are primarily related to poor hygienic conditions, neglected technological requirements or to respiratory and immunosuppressive diseases (Stromberg *et al.*, 2017).

### 2.1.2.3. *E. coli* diagnosis

### Culture

Laboratory diagnosis usually involves culture on solid media such as MacConkey agar for identification of lactose fermenters and use of other selective media such as Eosin Methylene Blue agar on which *E. coli* gives a characteristic metallic green sheen. Confirmation is usually done by biochemical tests (Gomes *et al.*, 2016).

### Biochemical and Serological tests

*E. coli* bacteria are lactose fermenters, catalase positive, oxidase negative, indole positive and citrate negative. Like *Salmonella*, *E. coli* are also grouped based on three antigens; the O and H antigens are found on the somatic and flagella, respectively, while the K antigens are either envelope or capsular antigens (Jann *et al*, 2011).

### 2.1.2.4. Disease manifestation

*E. coli* strains can cause diarrhea, extraintestinal (ExPEC) infections such as urinary tract infections caused by neonatal meningitis *E. coli* (NMEC). Enteric *E. coli* infections can be divided into six groups based on their pathogenicity: Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC, including *Shigella spp*.), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Diffusely Adherent *E. coli* (DAEC) (Clements *et al.*, 2012).

### 2.1.3. Other important bacterial foodborne infections

*Campylobacter* infections are among the top five leading foodborne infections worldwide (Acheson, 2015). They are mostly acquired from eating food of avian origin such as poultry. The species that affects humans most is *Campylobacter jejuni*, though other species, such as *Campylobacter coli* are also known to cause infections of the gastro-intestinal system. Contamination of poultry meat with *Campylobacter* can occur right from production, during processing or at retail level. Resistance to antimicrobials has also been observed in this genus of foodborne pathogens (Silva *et al.*, 2011).

*Listeria* disease outbreaks have been reported worldwide. They are usually associated with the ingestion of raw and processed foods of animal origin and vegetables that are contaminated with the bacteria. The most incriminated is Listeria monocytogens (Mehdizadeh *et al.*, 2010). A recent outbreak in South Africa claimed a number of lives of which the highest percentage were children under the age of five (WHO, 2018).

## **2.2. Antibiotic usage in management of *E. coli* and *Salmonella* pathogens**

## 2.2.1. Antibiotic Treatment

Enteric bacteria such as *E. coli*, in general, can cause a variety of infections in different locations of the body. Some of these are urinary tract infections that can be caused by *E. coli* and *Klebsiella* species. These infections are treated using antimicrobials such as nalidixic acid, amoxicillin-clavulanic acid, ampicillin, gentamycin, ciprofloxacin and co-trimoxazole (Trimethoprim-Sulphamethoxazole) (Atlas of the universe, 2005). Other infections caused by *E. coli*, *Salmonella* and other enteric bacteria include lower respiratory tract infections, sinus infections, gastro-intestinal tract infections, meningitis, bone infections, upper respiratory tract infections, bacterial conjunctivitis, complicated intra-abdominal infections, infectious diarrhea, severe infections of the kidney and bacterial septicemia. Some of the other antibiotics used to treat such infections are imipenem, cefotaxime, metronidazole, tetracycline and colistin. Colistin is used as a last resort antibiotic for infections such as those caused by *Klebsiella pneumonia*. It is a reserve antibiotic and, therefore, its resistance is critical to human health and must be closely monitored (Li *et al.*, 2006; Ah *et al*, 2014).

## 2.2.2. Mechanism of action of antimicrobials

There are three main ways in which antimicrobials function. They can target bacterial cell wall biosynthesis, bacterial protein synthesis or bacterial DNA replication and repair. Basically they interfere with some structure or process essential to bacterial growth or survival without harming host cells (Blair *et al.*, 2015).

### 2.2.2.1. Cell wall biosynthesis

The cell walls of bacteria are made up of peptides and glycan (peptidoglycan). The higher the fraction of peptides, the stronger the cell wall. The strength of the cell wall has an effect on the osmotic lysis of the cell. The stronger the cell wall, the harder it is for osmotic lysis to take place as a result of changes in osmotic pressure. Therefore, the enzymes that are responsible for adding peptidoglycan subunits during cell wall biosynthesis are the target sites for betalactam antibiotics such as penicillins and cephalosporins. There are five generations of cephalosporins depending on when the drugs were developed and on the target bacterial group. The molecules in these antimicrobials are similar in structure to the substrates that are supposed to bind to the enzymes responsible for adding peptidoglycan subunits to the cell wall. When they bind to the enzymes they are acylated slowly resulting in a weak cell wall that is vulnerable to changes in osmotic pressure, which ultimately leads to the lysis of the cell. Examples of antimicrobials that employ this mechanism are penicillin, cefotaxime, augmentin (amoxicillin-clavulanic acid), ampicillin and imipenem (Blair *et al.*, 2015; Ruppé *et al*, 2015).

### 2.2.2.2. Disruption of Protein Synthesis

This mechanism is employed at different stages of ribosome action during protein synthesis. Each target site has a different inhibitor. Examples of classes of antimicrobials that employ this strategy are macrolides, tetracyclines and aminoglycosides. Examples of antimicrobials that fall in these groups are oxytetracycline, gentamycin and erythromycin (Blair *et al.*, 2015).

### 2.2.2.3. Disruption of DNA replication and repair

This group of antimicrobials kill bacteria by targeting DNA gyrase which is responsible for un-coiling twining circles of double-stranded bacterial DNA. Inhibiting DNA gyrase causes accumulation of double-stranded breaks, which sets off the SOS repair mechanism that results in cell death. Fluoroquinolone antibiotics fall into this category. Examples of fluoroquinolone antibiotics are ciprofloxacin and nalidixic acid (Redgrave *et al.*, 2014).

## **2.3. Antimicrobial Resistance**

### 2.3.1. Origins and Spread of Antibiotic Resistance

Since their invention, antibiotics have been thought to be a wonder discovery (Davies, 2011). However, the use of these antibiotics has resulted in the emergence of drug resistance, especially in zoonotic bacteria originating from food-producing animals. These bacteria are passed on to humans through the food chain (Walsh and Fanning, 2008).

Although microbial resistance is primarily as a result of therapeutic agents, a variety of other factors contribute to the emergence and spread of resistance. Among these factors are; over-prescription of antimicrobials by physicians, use of various antimicrobial agents in animals raised commercially for food such as poultry, pigs and cows, patient self-medication and noncompliance with recommended treatments and various practices common in hospitals which include the failure by health workers to practice simple control measures such as hand washing and changing of gloves after examining a patient (Levy, 2002). Food may represent a dynamic environment for the continuing transfer of antibiotic resistance determinants between bacteria (Walsh and Fanning, 2008).

Several genes, many of which can be transferred between bacteria, encode antibiotic resistance. New resistance mechanisms are constantly being described, and new genes and vectors of transmission are identified on a regular basis (Wallace *et al.*, 2017). Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer (Blair *et al.*, 2015). The intrinsic resistance of a bacterial species to a particular antibiotic is the ability to resist the action of that antibiotic as a result of inherent structural or functional characteristics.

Pathogenic, commensal as well as non-pathogenic bacteria form a reservoir of Antimicrobial Resistant Genes from which pathogenic bacteria can acquire resistance via horizontal gene transfer (Von Wintersdorff *et al.*, 2016). Horizontal gene transfer is the process of swapping genes between bacteria that are in close proximity. Many of the antibiotic resistance genes are carried on mobile elements such as plasmids, transposons and integrons that can act as vectors that transfer these genes to other bacteria whether the recipient bacteria are members of the same bacterial species or not.

Horizontal gene transfer may occur via three main mechanisms; transformation, transduction or conjugation. Transformation involves uptake of short fragments of naked DNA by naturally transformable bacteria. Transduction involves transfer of DNA from one bacterium to another via bacteriophages. Conjugation involves transfer of DNA via sexual pilus and requires cell-to-cell contact. DNA fragments that contain resistance genes from resistant donors can then make previously susceptible bacteria express resistance as coded by these newly acquired resistance genes (Von Wintersdorff *et al.*, 2016).

### 2.3.2. Antimicrobial Resistance of *Salmonella* and *E. coli* in Poultry

One study was carried out in Oyo State, Nigeria to establish the levels of *Salmonella* and *E. coli* contamination of poultry meat from a processing plant and retail markets. *Salmonella* and *E. coli* levels were calculated to be 33% and 43.4% prevalent, respectively. Antibiotic sensitivity of the isolates using eight different antibiotics showed different resistance patterns. *Salmonella enterica* showed 93% resistance to tetracycline and 100% resistance to augmentin and amoxicillin, while *E. coli* showed 100% resistance to augmentin and amoxicillin (Adeyanju and Ishola, 2014). Another study was undertaken to describe the bacterial contamination of poultry meat packaged at a Zambian poultry abattoir. *E. coli* and *Salmonella spp.* were predominant. The mean percentage of isolations of *E. coli* and *Salmonella* were 56.5% and 27.5%, respectively (Hang’ombe *et al.*, 1999). Another study was carried out to isolate bacteria from broiler and layer chicks in Zambia. The major bacteria species isolated were *E. coli* (84.58%) and *Salmonella spp.* (46.15%). Detection of *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella dublin* indicates that poultry has the potential of transmitting zoonotic pathogenic bacteria to humans (Munang’andu *et al.*, 2012). These studies have all shown that resistance to frequently used antibiotics is rising right from production through to processing and consumption.

### **2.4. Poultry Sub-Sector in Zambia**

### 2.4.1 Structural organisation

An annual report by the Poultry Association of Zambia in 2014 showed that the poultry sector is continuously growing at annual rates of 8% for broilers and 10% for layers. It also reported that poultry production in 2014 reached over 1 billion eggs and 73 million broilers. These growth rates are as a result of increase in demand and supply. Due to the rapidly growing middle-income population, the demand for poultry has increased resulting in tremendous advances in poultry breeding, production and processing due to the introduction of modern technology (Voidarou *et al.*, 2011). However, 60% of the poultry production farms are small and medium scale enterprises that do not have enough investment to modernize production. Such is the case with backyard poultry production. Village chicken production is also another growing enterprise in rural and urban areas of Zambia. However, village chickens are resistant to most poultry diseases (Syakalima *et al*, 2017).

### 2.4.2. Use of antimicrobials

Antimicrobials have long been used in livestock production for both therapeutic and preventive purposes (Van Boeckel *et al.*, 2015). Poultry production is no exception. The use of these antimicrobials has been observed at all levels of poultry production. The most commonly used antimicrobials include Oxytetracycline, Sulfadimidine, Enrofloxacin, Trimethoprim-sulphamethoxazole, Amoxicillin/clavulanic acid, Amprolium and Gentamycin. Mostly these antimicrobials are used for respiratory and gastro-intestinal tract infections. Diagnosis is usually done by clinical signs and post-mortem inspections. In rare circumstances, laboratory diagnosis is also used (Porter, 1998). The procurement of these antimicrobials is often done over-the-counter and without prescription. Laws that guide the acquisition and use of antimicrobials are present but are not followed, especially in the veterinary sector. Regulation of veterinary drugs is not monitored in the livestock sector. This means drugs are easily accessible because farmers are able to diagnose and buy veterinary drugs with little or no involvement of a veterinarian (Ayukekbong *et al*, 2017). Antimicrobial resistance has been observed to some of these commonly used drugs in both the veterinary and human sectors (Mainda *et al.*, 2015; Macvane, 2017).

## **2.5. Risk factors for AMR**

Among the risk factors that predispose the organisms to antimicrobial resistance are excessive use of antimicrobials for preventive purposes, poor hygiene that facilitates the transfer of resistant organisms from one surface to another and poor infection-control strategies, such as hand-washing and disinfection. The slow pace of developing newer antimicrobials is also a pre-disposing factor because when new antimicrobials are not developed regularly, existing antimicrobials are over-used, thereby facilitating the development of resistance by organisms that are exposed to the antimicrobials (Ayukekbong *et al*, 2017).

## **2.6. Resistance Genes**

Changes in DNA can lead to the acquiring of antibiotic resistance. When changes to DNA lead to changes in protein shape at a location where an antibiotic is supposed to act, the protein may lose its functional abilities because it is no longer able to recognize where it should perform its functions (Lowe, 1982; Blair *et al.*, 2015). This change prevents the entry of an antibiotic into the cell or, if already within the cell, the antibiotic in unable to act on the cell. These mutant genes that code for resistance may be passed on to other bacteria by horizontal gene transfer. Though studies are still being conducted to identify more genes, some of those that have been identified are genes that code for resistance to sulfonamides such as the sul1 gene, genes that code for resistance to tetracyclines such as TetA, genes that code for resistance to beta-lactam antibiotics such as CtxM (Scaria *et al*, 2005; Diarrassouba *et al.*, 2007).

## **2.7. Knowledge gaps**

The topic of antibiotic resistance is of international public health concern. However, developing countries such as Zambia have little to no data on certain aspects of AMR. One of the knowledge gaps is that despite it being known that certain bacteria (*E. coli* and *Salmonella*) causing foodborne diseases are resistant to antibiotics, little is known about the molecular patterns of resistance in Zambia. There is lack of knowledge on the genes that are conferring the resistance. Similar studies that have been done in other developing countries have established some antibiotic resistant genes of interest (Adesiji and Deekshit, 2014). However, it is not known whether these are similar to the genes that are responsible for spreading resistance in Zambia. Furthermore, it is not known whether bacteria are developing resistance to antibiotics being used in the livestock sector that are crucial for the treatment of human diseases. Another area that has not been explored adequately is the relationship between the antibiotics used during livestock production and those used during human chemotherapy. It is not known whether the high resistance being observed in human affections is as a result of ingestion of food contaminated with antibiotic resistant organisms. Even though there are speculations, there is no scientific evidence to back up the theory (Okeke *et al.*, 2005; Mainda *et al.*, 2015).

# **CHAPTER 3**

# **MATERIALS AND METHODS**

## **3.1. Study Area and Design**

A cross-sectional study involving four districts of Lusaka Province in Zambia (Lusaka, Chilanga, Chongwe and Kafue) was undertaken to investigate antimicrobial resistant *E. coli* and Salmonella in market-ready broiler meat. The study was conducted between August 2017 and May 2018. Lusaka province was purposely selected because it is the industrial capital and is a retail destination for many poultry products from other provinces of the country. The primary sampling units were the markets (broadly classified as Open markets and Supermarkets) and the secondary sampling units were individual dressed broiler chickens. An open market was defined as an unrestricted competitive market not housed in a building, where foodstuffs are sold often exposed and in which any buyer and seller was free to participate, while a supermarket was defined as a market housed in a closed building with modernized facilities that offered a wide variety of food and household products, organized into aisles. Proportion stratified random sampling was employed where Open markets and Supermarkets were the strata. From the information collected from Lusaka city council, there were 47 supermarkets and 33 open markets in Lusaka Province at the time the study was conducted. This formed the sampling frame from which a study population was drawn.

## 3.2. Sample Size Calculation

The sample size for estimation of a single proportion was calculated using Epi tools software ([www.epitools.ausvet.com](http://www.epitools.ausvet.com)).

The formula: n=

Where: Z = confidence level; p = estimated prevalence; e2 = error

The sample size was estimated using the following assumptions:

Z = 1.96

p = 0.25

1-p = 0.75

e2= 0.05

Using the above formula and the assumptions, the total number of supermarkets and open markets to be included in the study was calculated to be 63 markets, proportionally distributed as follows:

58.75% = Supermarkets

41.25% = Open markets

The estimated study population was adjusted according to the finite population (n=80), resulting in a sample size of 63 (37 supermarkets and 26 open markets).

### 3.2.1. Sample size from the open markets

Twenty-six open markets from Lusaka, Chongwe, Chilanga and Kafue districts that had shops and/or tables trading in dressed broiler chickens were selected. From each open market, three shops trading in dressed broiler chicken carcasses were identified for inclusion into the study and from each shop two dressed broiler carcasses were sampled (26×2×2=104). Also at the markets, three stands that traded in dressed broiler chickens were included in the study (one dressed broiler carcass from each stand/table).

Number of samples from open markets (stands/tables) = 26×3×2 = 156

The total number of samples from open markets was therefore 104+156 = 260

### 3.2.2. Sample size from the Supermarket

Thirty-seven supermarkets that traded in dressed broiler chickens from Lusaka, Kafue, Chilanga and Chongwe districts were selected. From each supermarket, three different brands of dressed broiler chickens were sampled (one of each brand). Therefore, the number of broiler carcasses from supermarkets = 37×3 = 111.

The total number of dressed broiler carcass samples for both open markets and supermarkets = 260+111 = 371.

### **3.2.3. Sample Collection and Laboratory Analysis**

### 3.2.3.1. Sample Collection

Upon collection, all samples were immediately put on ice in a cooler box and transported to the School of Veterinary Medicine, Public Health Laboratory for analysis within 8 hours of collection. A summary distribution of the samples that were collected from the four districts and the distribution of the samples by strata are presented in Table 5.

### 3.2.3.2. Media Preparation and Quality Control

All media was prepared using the manufacturer’s instructions and sterilized at 121°C and 15 lbs pressure. Autoclave tape was used with every batch of media that was prepared to ensure that sterilization conditions were being met by the autoclave. From every batch of prepared solid media, one plate was inoculated with the *E. coli* 25922 (ATCC) reference strain used as a control and incubated overnight while another plate was incubated un-inoculated to check for contamination. The thickness of the Mueller Hinton (Oxoid) plates that were prepared for drug susceptibility testing was measured using digital Vernier calipers to ensure a thickness of 4mm. All media was prepared on the ‘clean bench’ that did not come into contact with any samples or cultures during laboratory analysis.

### 3.2.3.3. Carcass Rinse Technique

The Carcass Rinse technique outlined by the USDA Food Safety and Inspection Service was used with a few modifications (D’Aoust et al, 1982). Whole chicken carcasses were aseptically placed in sterile stomacher/ziplock bags. 450mL of sterile buffered peptone water (Oxoid UK) was poured into each of the bags containing the carcasses. The open ends of the bags were then twisted to hold the bags closed and the entire carcasses was rinsed using a repeated rocking motion 30 times. The bags were then opened and the carcasses removed. The rinsate was then collected in sterile containers by puncturing the “v”-end of the bags and used for analysis (Figure 1).

Figure 1: Stages of the carcass rinse technique

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#### 3.2.3.4. Isolation and Identification of Salmonella from market-ready poultry

The methods proposed by the Food and Drug Administration’s Bacteriological Analytical Manual (U.S. Food and Drug Administration (FDA), 2001) were used; whereby 1mL of the rinsate was transferred into 9mL of Rappaport Vassiliadis Media (Oxoid UK) and incubated for 48 hrs at 42oC. All broths that showed a change in color from green to yellow were then inoculated onto Xylose-Lysine-Deoxycholate (XLD) agar plates (Oxoid UK) using the streaking method and left to incubate at 37oC for another 24hours. After incubation, colonies that appeared black in the center and pink on the outline were stained using the Gram’s staining technique and examined under a light microscope (Figure 2). The black colonies were then sub-cultured onto Blood Agar plates (Oxoid UK) to rule out *Proteus* species (*Proteus spp*. swarm on blood agar). Those isolates that did not swarm on blood agar and which showed Gram-negative short rods were then subjected to biochemical tests using Analytical Profile Index (API 20E) kits (Biomerieux). All batches were run against a *Salmonella typhimurium* (ATCC 14028) reference strain used as a control. Further confirmation of the isolates was done using 16S rRNA sequencing. Confirmed *Salmonella* isolates were then assessed for drug susceptibility (Refer to Section 3.2.3.6.). The isolates were then suspended in 10% glycerol-peptone solution for storage at -80°C.

### 

Figure 2: Salmonella colonies on XLD agar plate

### 3.2.3.5. Isolation and identification of *E. coli* from market-ready poultry

The methods proposed by The Food and Drug Administration’s Bacteriological Analytical Manual (U.S. Food and Drug Administration (FDA), 2001) were used with a few modifications. 1mL of the rinsate was put in 9ml of buffered peptone water (Oxoid UK) and incubated overnight at 37oC for initial enrichment. A loopful of the suspension was then inoculated onto MacConkey agar plates (Oxoid UK) using the streaking method and incubated at 37oC for another 24 hours. After incubation small pink colonies were stained using the Gram’s staining technique and examined under a light microscope. Colonies that showed Gram-negative short rods were then sub-cultured onto Eosin Methylene Blue (EMB) agar (Oxoid UK) plates. Colonies that showed a metallic green sheen (Figure 3) were subjected to biochemical tests using API 20E kits (Biomerieux) and later sequenced using 16S rRNA sequencing for confirmation. Confirmed *E. coli* isolates were then assessed for drug susceptibility (Refer to section 3.2.3.6.). The isolates were then suspended in 10% glycerol-peptone solution for storage purposes.



Figure 3: E. coli metallic green sheen on EMB agar plate

### 3.2.3.6. Drug Susceptibility Testing

The Kirby-Bauer disk diffusion technique described by Bauer et al (Kirby-bauer, 1961) for Drug Sensitivity Testing was used on all confirmed *Salmonella* and *E. coli* isolates. The isolates were prepared by sub-culturing the isolates onto blood agar (Oxoid UK) overnight at 37oC. A Gram’s stain was then done to identify the organisms and to check for purity. One or two colonies were then suspended in 4mL of 0.9% sodium chloride solution (normal saline) and their turbidity compared to that of a 0.5 McFarland’s turbidity standard. An inoculum of the suspension was then spread on two Muellar Hinton agar (4 ml thickness) plates (Oxoid UK) until the entire surfaces of the plates were covered and 5 antibiotic wafers placed on the surface of each of 2 plates using the applicator (Oxoid). Two plates were used for each isolate to accommodate the 10 antibiotics that were used. The list of antibiotics and their concentrations is as outlined in Table 1 below. The list of antibiotics was chosen from a list of antibiotics that was obtained from a major poultry antibiotic distributor in Lusaka Province as being the most frequently purchased antibiotics. Those antibiotics that are also used in the treatment of human infections were then chosen for antibiotic susceptibility testing. The plates were then incubated at 37oC for 24hrs and the diameters of the zones of inhibition recorded using a digital Vernier calipers. The diameters of the zones of inhibition were then entered and analyzed in WHONET 2018 software. All isolates that showed resistance were then forwarded for molecular determination of the genes that conferred the resistance (Refer to Section 3.2.3.8. and 3.2.3.9).

Table 1: Antibiotics used and their concentrations

|  |  |
| --- | --- |
| **Antibiotic** | **Concentration (µg)** |
| Tetracycline | 30 |
| Ampicillin | 10 |
| Chloramphenicol | 30 |
| Trimethoprim-Sulfamethoxazole | 25 |
| Colistin Sulphate | 10 |
| Amoxicillin-Clavulanic Acid | 30 |
| Ciprofloxacin | 5 |
| Cefotaxime | 30 |
| Nalidixic Acid | 30 |
| Imipenem | 10 |

### 3.2.3.7. DNA Extraction

Isolates were prepared for molecular work by first extracting the DNA. DNA extraction was done by heat extraction. A few pure colonies were suspended in 100µL of nuclease free water and heated at 80°C for 10minutes. The suspension was then centrifuged at 60000*g* for 3 minutes at 4°C. The supernatant was then subjected to 16S rRNA sequencing for identification.

### 3.2.3.8. Molecular Identification of bacteria using the 16S RNA sequencing

16S rRNA sequencing is a molecular tool that can be used to identify bacteria based on the genetic sequence of the 16S ribosomal subunit (Weisburg *et al.*, 1991). During transcription the 30S ribosomal subunit is cleaved by the enzyme ribonuclease into 3 subunits. These are 16S, 5S and 23S. These 3 tend to be stable within a bacterial species. The most stable is the 16S subunit and can, therefore, be used to identify bacterial species. The 16S rRNA sequencing procedure generally involves a nested polymerase chain reaction in which the DNA sequence is first amplified using primers that are general for all bacteria and then a portion of the DNA product containing the 16S gene is then sequenced and compared with sequences stored in a gene bank. The comparison is done using PubMed Blast.

### 3.2.3.9. Determination of the genes that confer resistance

Determination of genes conferring resistance was performed according to the method described (Adesiji *et al*, 2014). The mastermix volumes and PCR reaction were as outlined in Table 2 and Table 3. Multiplex PCR was used to determine the genes that conferred resistance to selected antibiotics. The target genes were selected based on the antimicrobial susceptibility results and the genes that were outlined as genes of importance for antimicrobial susceptibility. The 3 target genes were TetA, Sul1 and CtxM. The primer specifications are as outlined in Table 4.

Table 2: PCR mastermix volumes

|  |  |
| --- | --- |
| **Item** | **Volume (µL)** |
| 10X ExTaq Buffer | 2 |
| DNTPs | 1.6 |
| Forward Primer | 0.8 |
| Reverse Primer | 0.8 |
| ExTaq HS Enzyme | 0.1 |
| Nuclease-Free Water | 13.7 |
| Template | 1 |
| Total Volume | 20 |

Table 3: PCR conditions

|  |  |  |  |
| --- | --- | --- | --- |
| **Stage** | **Temperature (°C)** | **Time (Mins)** | **Number of cycles** |
| Initial Denaturation | 95 | 5 |  |
| Denaturation | 95 | 1 | 35 |
| Annealing | 58 | 1 |  |
| Extension | 72 | 1 |  |
| Final Extension | 72 | 1 |  |
| Hold | 4 |  |  |

Table 4: Primer sequences for the 3 target genes encoding resistance

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers** | **Sequence (5’-3’)** | **Annealing Temp** | **Expected Band Size** |
| TetA (Forward) | GTAATTCTGAGCACTGTCGC | 58 | 494 |
| TetA (Reverse) | CTGCCTGGACAACATTGCTT | 58 |
| Sul1 (Forward) | TGAGATCAGACGTATTGCGC | 58 | 793 |
| Sul2 (Reverse) | TTGAAGGTTCGACAGCACGT | 58 |
| CtxM (Forward) | CGATGTGCAGTACCAGTAA | 55 | 585 |
| CtxM (Reverse) | TAAGTGACCAGAATCAGCGG | 58 |

### 3.2.4. Process Quality Control

The laboratory processing benches were disinfected before use with 70% ethanol and after use with 3.5% Sodium hypochlorite solution (JIK). Every batch of samples was processed along with a positive and negative control. The ATCC reference strains *E. coli* 25922 (ATCC) and *Salmonella typhimurium* 14028 (ATCC) were used as control strains. During the carcass rinse technique, 450mL of sterile buffered peptone water was also poured into an empty bag that did not contain a carcass. The rinsate was incubated overnight and later streaked onto MacConkey agar plate (Oxoid, UK) to ensure that the batch of bags was sterile and that the organisms isolated were indeed from the chicken carcasses and not the bags.

# **CHAPTER 4**

# **RESULTS**

## **4.1. Descriptive statistics**

A total of 250 dressed broiler carcasses were collected from both supermarkets and open markets. Table 5 shows the distribution of samples by district and market types. Majority of the samples were obtained from Lusaka district because it had the highest number of markets.

Table 5: Summary of broiler samples collected by strata and district

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| District | No. of open markets | No. of Super market | Total No. of markets | Samples from Open Markets | Samples from Supermarkets | Total No. of samples |
| Chilanga | 2 | 1 | 3 | 8 | 4 | 12 |
| Chongwe | 1 | 0 | 1 | 11 | 0 | 11 |
| Kafue | 1 | 2 | 3 | 2 | 6 | 8 |
| Lusaka | 22 | 34 | 56 | 113 | 106 | 219 |
| Total | 26 | 37 | 63 | 134 | 116 | 250 |

## **4.2 Antimicrobial sensitivity**

A total of 148 *E. coli* and five *Salmonella spp.* were isolated and identified. All the isolates were subjected to drug susceptibility testing using the Kirby-Bauer disc diffusion technique. The zones of inhibition were entered and analyzed using WHONET 2018 software. Tetracycline, Ampicillin and Trimethoprim-Sulfamethoxazole recorded the highest resistance of 76.4% (n=113 isolates), 51.4% (n=76 isolates) and 44.6% (n=66 isolates) respectively with regards to *E. coli* isolates while 40% (n=2) of the *Salmonella* isolates were resistant to Ampicillin and Tetracycline (Table 6 and Figure 4).

Table 6: Antibiotic resistance profiles for E. coli isolates as summarized by WHONET 2018 software

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Antibiotic name** | **Breakpoints** | **Number** | **%R** | **%I** | **%S** | **%R 95%C.I.** |
| Amoxicillin/Clavulanic acid | 14 - 17 | 148 | 5.4 | 3.4 | 91.2 | 2.5-10.7 |
| Ampicillin | 14 - 16 | 148 | 51.4 | 2 | 46.6 | 43.1-59.6 |
| Cefotaxime | 23 - 25 | 148 | 12.8 | 4.7 | 82.4 | 8.1-19.5 |
| Chloramphenicol | 13 - 17 | 148 | 14.2 | 4.7 | 81.1 | 9.2-21.1 |
| Ciprofloxacin | 16 - 20 | 148 | 8.1 | 4.1 | 87.8 | 4.4-14.0 |
| Colistin | S >= 11 | 148 | 5.4 | 0 | 94.6 | 2.5-10.7 |
| Imipenem | 20 - 22 | 148 | 0.7 | 2 | 97.3 | 0-4.3 |
| Nalidixic acid | 14 - 18 | 148 | 23.6 | 7.4 | 68.9 | 17.2-31.4 |
| Tetracycline | 12 - 14 | 148 | 76.4 | 1.4 | 22.3 | 68.6-82.8 |
| Trimethoprim/Sulfamethoxazole | 11 - 15 | 148 | 44.6 | 0.7 | 54.7 | 36.5-53.0 |

A graphical presentation of the results is outlined in Figure 4 below.

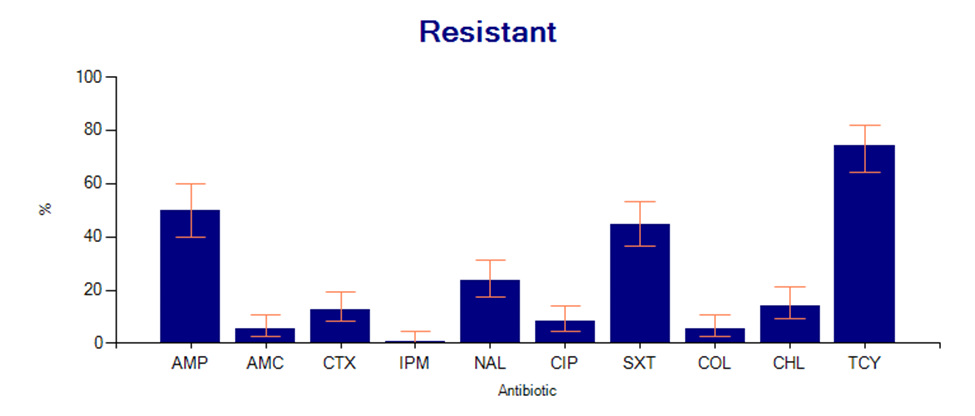


Figure 4: Graphical Presentation of Antibiotic Profiles for E. coli isolates (Note:

AMP=ampicillin, AMC=amoxicillin-clavulanic acid, CTX=cefotaxime, IPM=imipenem, NAL=nalidixic acid, CIP=ciprofloxacin, SXT=trimethoprim/sulfamethoxazole, COL=colistin, CHL=chloramphenicol, TCY=tetracycline.

Table 7: Antimicrobial Resistance Profiles for Salmonella spp.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Antibiotic name** | **Breakpoints** | **Number** | **%R** | **%I** | **%S** | **%R 95%C.I.** |
| Ampicillin | 14 - 16 | 5 | 60 | 0 | 40 | 17.0-92.7 |
| Amoxicillin/Clavulanic acid | 14 - 17 | 5 | 0 | 20 | 80 | 0.0-53.7 |
| Cefotaxime | 23 - 25 | 5 | 0 | 20 | 80 | 0.0-53.7 |
| Imipenem | 20 - 22 | 5 | 0 | 0 | 100 | 0.0-53.7 |
| Nalidixic acid | 14 - 18 | 5 | 0 | 20 | 80 | 0.0-53.7 |
| Ciprofloxacin | 21 - 30 | 5 | 0 | 20 | 80 | 0.0-53.7 |
| Trimethoprim/Sulfamethoxazole | 11 - 15 | 5 | 0 | 0 | 100 | 0.0-53.7 |
| Colistin | S >= 11 | 5 | 0 | 0 | 100 | 0.0-53.7 |
| Chloramphenicol | 13 - 17 | 5 | 0 | 0 | 100 | 0.0-53.7 |
| Tetracycline | 12 - 14 | 5 | 40 | 20 | 40 | 7.3-83.0 |

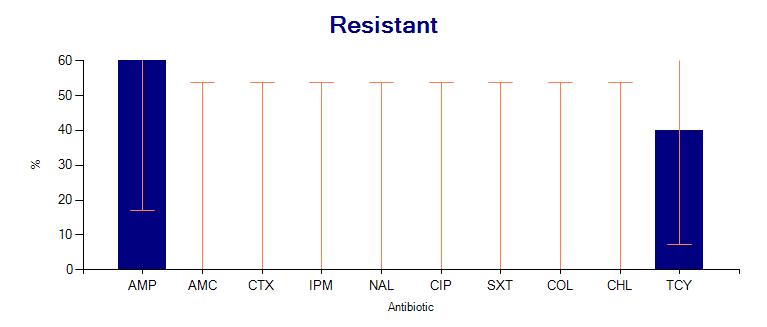


Figure 5: Graphical Presentation of Antibiotic Profiles for Salmonella isolates (Note: AMC=Amoxicillin-clavulanic acid, CTX=Cefotaxime, IPM=Imipenem, NAL=Nalidixic acid, CIP=Ciprofloxacin, SXT=Trimethoprim/sulphamethoxazole, COL=colistin, CHL=chloramphenicol, TCY=Tetracycline

Isolates from broiler carcasses obtained from open markets had a higher resistance of 83.8% (n=67) while supermarkets recorded 65.8% (n=48) percentage resistance. The overall resistance for both the open markets and supermarkets was calculated to be 75.2% (n=115). The summary of the percentage resistance according to the two strata is summarized in **Table 8**.

Table 8: Resistance patterns of Salmonella and E. coli by strata

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Open Markets** | **Supermarkets** | **Total** |
| ***E. coli*** | 79 | 69 | 148 |
| ***Salmonella*** | 1 | 4 | 5 |
| **Number resistant** | 67 | 48 | 115 |
| **% Resistance** | **83.8** | **65.8** | **75.2** |

Figure 6 below shows the resistance profile for isolates from the Open Markets. Tetracycline, Trimethoprim/Sulfamethoxazole and Ampicillin had the highest resistance, while Imipenem, Amoxicillin-Clavulanate and Colistin had the lowest resistance.

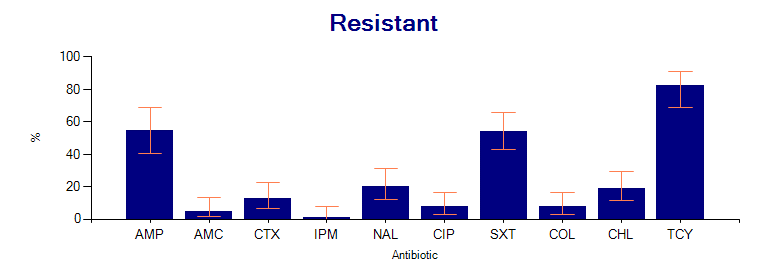


Figure 6: Graphical presentation of resistance profiles of samples collected from open markets

Figure 6 below shows resistance profiles for the isolates from supermarkets. Tetracycline had the highest resistance, followed by Ampicillin, Trimethoprim/Sulfamethoxazole and Nalidixic Acid.

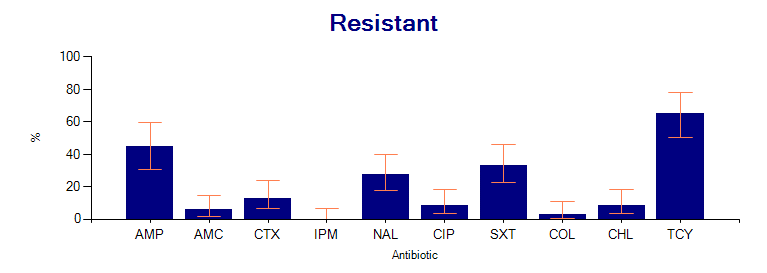


Figure 7: Graphical presentation of resistance profiles of samples collected from supermarkets

The resistance profiles according to the surrounding human settlements was as outlined in Table 9 below.

Table 9: Resistance profiles according to surrounding human settlements

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **District** | **Area** | **Human Settlements Around** | **# of Isolates** | **% Resistance** | **Highest Resistance** | **Top Resistant Antibiotics** |
| Lusaka | A | Chelstone, Kaunda Square, Munali, Jezmondine | 4 | 100 | Open Markets | Tet, Sxt, Amp |
|  | B | Arcades, Northmead, Manda Hill, East Park, Long Acres | 16 | 81 | Supermarkets | Tet, Amp, Nal |
|  | C | Kabulonga, Woodlands, Chilenje | 13 | 69 | Open Markets | Tet, Sxt, Amp |
|  | D | Kabwata, Libala | 6 | 33 | Supermarkets | Tet, Amp, Nal |
|  | E | Matero, George, Lilanda | 24 | 83 | Open Markets | Tet, Sxt, Amp |
|  | F | Kanyama, Downtown, Makeni, Kafue Road | 21 | 71 | Open Markets | Sxt, Nal, Cip |
|  | G | Mumbwa road, Chawama, Kamwala | 6 | 83 | Open Markets | Tet, Sxt, Ctx |
|  | H | Town Centre | 11 | 64 | Open Markets | Tet, Sxt, Amp |
|  | J | Foxdale, Kalundu | 4 | 75 | Open Markets | Sxt, Ctx |
|  | K | Chaisa, Mandevu, Emmasdale, Kabanana | 16 | 75 | Open Markets | Tet, Sxt, Amp |
|  | Z | Mtendere, Kalingalinga | 10 | 90 | Open Markets | Tet, Sxt, Amp |
| Kafue | A | C5 and C7 | 7 | 43 | Supermarkets | Tet, Sxt, Amp |
| Chongwe | A | Dam Area | 5 | 60 | Open Markets | Tet, Sxt, Amp |
| Chilanga | A | Chilanga | 5 | 100 | Supermarkets | Tet, Sxt, Amp |

The highest resistance AMR profiles were recorded in open markets that were located in high-density areas. Tetracycline, Trimethoprim/Sulphamethoxazole and Ampicillin were the most resistant in most open markets while areas with a larger number of supermarkets had a higher resistance to Nalidixic acid than that of Trimethoprim/sulphamethoxazole, in addition to Tetracycline and Ampicillin. Areas that were either further away or located near low-density areas recorded the least resistance. Most isolates were resistant to at least one antibiotic with some isolates being resistant to up to 5 antibiotics.

## **4.3. Determination of Antimicrobial resistance genes**

The isolates that recorded resistance on phenotyping were further subjected to multiplex PCR. The Beta-lactam gene (CtxM) was the most detected in the isolates (Table 10).

Table 10: Summary of resistant genes according to strata

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Target Gene | Open Markets | | Supermarkets | |
| *E. coli* | Salmonella | *E. coli* | Salmonella |
| TetA, Sul1, CTXM | 4 | 0 | 1 | 0 |
| TetA, Sul1 | 2 | 0 | 1 | 0 |
| Sul1, CTXM | 0 | 0 | 0 | 0 |
| TetA, CTXM | 3 | 0 | 2 | 1 |
| TetA | 7 | 0 | 4 | 0 |
| Sul1 | 0 | 0 | 0 | 0 |
| CTXM | 9 | 0 | 13 | 0 |

# **CHAPTER 5**

# **DISCUSSION**

The aim of the study was to characterize the phenotypes and genotypes of antimicrobial resistant *E. coli* and *Salmonella* in retail broiler chickens in Lusaka Province of Zambia. The study observed that AMR is a problem in bacteria contaminating broiler chickens in Lusaka, a situation that requires interventions. Studies within Zambia have shown that the resistance levels to both *E. coli* and *Salmonella* in humans, especially children under the age of five and adults at referral hospitals, is also quite high (Clarke *et al.*, 2012; Kabwe *et al.*, 2016; Mainda, 2016; Chiyangi *et al.*, 2017).

## **5.1. Isolation and Identification of *E. coli* and *Salmonella***

A total of 148 *E. coli* and five *Salmonella spp.* were isolated and identified using both biochemical tests and 16S rRNA sequencing. Though a higher number of *Salmonella* suspected organisms were initially identified and subjected to confirmatory tests, only five isolates were confirmed to be *Salmonella*. The low recovery rate of *Salmonella* could be attributed to intermittent shedding patterns of *Salmonella* in broiler chickens (Van Immerseel *et al.*, 2004). However, most of the *Salmonella* isolates that were recovered were isolated from supermarkets (n=4) while only one isolate was from open markets. Most of the broiler chickens that are sold in the supermarkets come from commercial abattoirs while those sold in open markets come from backyard poultry houses. The recovery of Salmonella could have been as a result of cross-contamination of the carcasses during processing at one of the commercial abattoirs or as a result of infection of the live birds before slaughter. Studies done at commercial poultry abattoirs have shown the presence of *Salmonella spp.* on abattoir bench surfaces and because large batches of chickens are processed within a day, the transfer of bacteria from the abattoir bench surfaces to the carcasses is facilitated (Rusul *et al.*, 1996). The sampling strategy that was applied at the supermarkets could also have reduced the recovery rates of *Salmonella*. Other studies that were conducted at retail level in a similar setup showed a higher positivity rate for *Salmonella* in poultry than what was observed during this study probably due to the reduced sampling time-frame (Tuchili *et al.*, 1996; Hang’ombe *et al.*, 1999; Mshana *et al*, 2013; Chotinun *et al.*, 2015; Shrestha *et al.*, 2017). The recovery of *E. coli* was higher than that of *Salmonella* and this is in line with the recovery rates of *E. coli* that have been documented from other similar studies (Mainda *et al.*, 2015; Chishimba *et al.*, 2016; Stromberg *et al.*, 2017). As opposed to *Salmonella*, chickens continuously shed *E. coli*. Furthermore, *E. coli* is found in the environment and can easily contaminate the broiler carcasses (Dierikx *et al.*, 2013). This means contamination could have occurred during processing or handling of the carcasses. *E. coli* has been isolated both in commercial abattoirs and on surfaces at retail level (Geornaras *et al.*, 1995; Adeyanju and Ishola, 2014). The recovery of *E. coli* from Open Markets was higher than that from supermarkets probably due to differences in contamination from the production source and unhygienic handling by the retailers. Commercial abattoirs, which are the main source of poultry supplied to supermarkets, are largely mechanized with a good understanding of hygiene and food safety management such as HACCP (Hazard Analysis Critical Control Points) principles (Giovannucci *et al.*, 2000). Chickens traded at the Open markets, on the other hand, are mostly supplied by small and medium sized enterprises whose processing methods are less hygienic. Most of the poultry processing among the small-scale processors happens either in the backyard or at small abattoirs that often do not adhere to hygienic principles. A study that supports this theory was conducted in a modern administrative region similar in organization to Lusaka Province in northwestern Greece (Voidarou *et al.*, 2011). Bacterial contamination of carcasses purchased from supermarkets was lower than that of chickens that were processed in households. The study pointed out that the slaughtering conditions in the rural households were poor.

## **5.2. Phenotypic Resistance profiles of Antibiotics**

The resistance to tetracyclines, sulfonamides and beta-lactam antibiotics was generally high, with the highest resistance being recorded in open markets. This could be attributed to the fact that, at the present, use of antibiotics in both livestock and human is not well regulated and is subject to misuse and abuse, especially among small scale producers. In Zambia, there is poor regulation of veterinary drugs and antibiotics, whereby farmers are able to purchase antibiotics over the counter without a prescription (Mainda, 2016; Manyi-Loh *et al.*, 2018). Furthermore, the poor hygienic processing methods that are employed at small and medium scale may facilitate the contamination of the carcasses with antimicrobial resistant organisms. The handling of the carcasses during slaughter, rinsing, transportation and sale may all introduce resistant organisms from humans and the environment. Broiler carcasses that originate from commercial abattoirs, however, may get contaminated mostly from the abattoir bench surfaces and intestines of the broilers during processing (Voidarou *et al.*, 2011). Most carcasses from supermarkets were highly resistant to Nalidixic acid, in addition to Tetracycline and Trimethoprim/sulfamethoxazole. This could be as a result of the use of fluoroquinolone antibiotics such as Enrofloxacin and Ciprofloxacin during poultry production at commercial level. Similar trends have been noticed in other parts of the world where similar studies have been undertaken. (Donado-Godoy *et al.*, 2012). Open markets that were surrounded by shanty over-populated areas recorded the highest number of resistant isolates from dressed chickens. These were also the areas where some of the broiler carcasses were sold mostly on tables and thus subject to contamination and proliferation of intrinsic bacteria in the absence of a cold chain. Moreover, most traders in the open markets sourced their birds from small producers who probably abused or misused antibiotics (Apata, 2009). It has also been documented that Tetracycline and Sulfadimidine are among the most commonly used antibiotics for therapy, especially at small-scale production. Over-time, farmers have learnt about these drugs and tend to self-prescribe whenever they have a disease situation when raising the birds. This overuse and misuse of antibiotics in livestock production has been reported to cause antimicrobial resistance (Lowe, 1982; Ngoma *et al.*, 1993; Koluman and Dikici, 2013; Kalonda *et al.*, 2015; Ayukekbong, Ntemgwa and Atabe, 2017).

## **5.3. Resistance According to Location**

In general, retail outlets that were located in highly populated areas recorded a higher resistance than those located in low-density areas. These also happen to be the areas that had more open markets than supermarkets to cater for the large population. Sanitation and hygiene in these areas could be a major contributing factor to the acquisition and spread of antibiotic resistance through cross-contamination, especially at retail points that are in the open such as tables. Most of these retail points received carcasses that were not pre-packed individually but came as batches in one containment vessel such as a large polyethylene bag. The processing methods and transportation of the carcasses to the market could have also facilitated the spread of resistant organisms from one carcass to another. These carcasses rarely go through abattoirs; they are usually directly transported after being processed in the backyard straight to the market where they are either sold whole or cut into pieces that the nearby customers can afford. The sanitation at such locations could also be a major source of contamination because waste is not disposed of in a proper manner, leaving piles of waste within the market environment. Unfortunately such dump sites within the markets may be in close proximity with the shops or stands where the broiler carcasses were sold and flies and other insects may act as vectors carrying resistant organisms directly from the waste to the carcasses and other fresh foods of animal origin sold in the open nearby (Songe *et al.*, 2017). In Lusaka, cholera has frequently been reported in high-density areas. This is an indication of poor sanitary conditions (Sasaki *et al.*, 2009).

## **5.4. Comparison of Phenotypic and Genotypic Resistance Profiles**

Of the three resistant genes that were targeted, the most detected was that of the beta-lactams (CtxM gene). The phenotypic and genotypic results of resistance profiles were similar, confirming the efficiency of the Kirby-bauer disk diffusion method. This implies that the beta-lactam gene of interest that was targeted is similar to the one that was found in other countries where similar studies were undertaken (Adesiji, Deekshit and Karunasagar, 2014; Chishimba *et al.*, 2016; Ramachandran, Bhanumathi and Singh, 2017). Though the other two genes for resistance to sulfonamides and tetracyclines (Sul1 and TetA) were also detected, the detection rates were not as high as that of the beta-lactam gene of interest. These discrepancies could be attributed to differences in target sequences of the resistant genes that were being targeted.

# **CHAPTER 6**

# **CONCLUSION AND RECOMMENDATIONS**

# **6.1. Conclusion**

Tetracyclines, beta-lactams, sulfonamide and fluoroquinolone antibiotics recorded the highest resistance in both open markets and supermarkets. This could be attributed to both the overuse of these drugs for therapeutic reasons at both commercial and small-scale levels of production. The presence of these resistant organisms in both open markets and supermarkets is a major public health concern because this could lead to the spread of resistance to humans in households where these carcasses end up. The spread of this resistance to pathogenic bacteria is a major concern.

# **6.2. Recommendations**

There is need to regulate the use of these antibiotics during production. The resistance genes to tetracyclines, sulfonamides and beta-lactam antibiotics were present is some of the isolates. There is need to do more molecular work that can give a complete understanding of the actual genes that are conferring resistance in Zambia. An understanding of the genes will be beneficial in the event that there is need for new drug formulation or combination of drugs.

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# **APPENDICES**

## **Appendix 1: Selection of Primary and Secondary Sampling Units**

**SELECTION OF PRIMARY SAMPLING UNITS**

A list of the supermarkets and council markets in Lusaka Province was obtained from Lusaka City Council and was the Sampling Frame. Supermarkets and Open markets were the two strata. 37 supermarkets and 26 open markets were randomly selected for inclusion into the study by writing them on a piece of paper and placing them in a hat. The sampling sites were then be picked at random out of the hat. This was done separately for the supermarkets and open markets and formed the primary sampling units.

**SELECTION OF SECONDARY SAMPLING UNITS**

From each open market, three shops trading in frozen broiler meat were identified for inclusion into the study and from each shop two broiler carcasses were sampled (26×3×2=156). Also at the markets, 3 stands that trade in poultry products were included in the study (1 frozen broiler carcass sampled from each stand/table). From each supermarket, three different poultry meat brands were sampled (one of each brand). Therefore, the number of samples from Supermarkets = 37×3 = 111.. The birds form the secondary sampling units. Upon arrival at the retail outlets, consent was sought and whole broiler carcasses were purchased as outlined above and immediately put in a cool box with icepacks at 4oC. The samples were then transported to the laboratory within 24 hours. This ensured uniformity for all samples collected whether from supermarkets or open markets. The carcass rinse technique was then done on all carcasses as outlined in **Appendix 2**. The summary of the sampling techniques and laboratory procedures is as outlined in the flow diagram below.

## **Appendix 2: Laboratory Protocols**

* 1. **Materials:**

• Antibiotic discs

**.** Falcon tubes

• Scissors

• Plain swabs

• Blood agar base

• XLD agar Base

• EMB base

• MacConkey agar base

• Peptone water base

• Rappaport Vasilliadis Media

• Gram staining reagents

• Frosted glass slides

• Immersion oil

• Gloves

• Petri dishes

• Methylated spirit

• Permanent markers

• Large stomacher bags

• Cryovials

• PCR and sequencing Reagents

• Biochemical test kits

• Micropipette Tips

• Glycerol

• Citrate agar media

1. ***CARCASS RINSE TECHNIQUE.***

The Carcass Rinse technique outlined by the USDA Food Safety and Inspection Service were used with a few modifications.

**Materials:**

* Sterile examination gloves
* 50mL sterile falcon tubes
* 400mL sterile Buffered Peptone Water
* Large Stomacher bags
* Sterile scissors
* Cotton wool
* 70% ethanol

**Procedure:**

* Wear sterile gloves and disinfect the working bench.
* Carefully open the stomacher bag and lay it on its side.
* Using one gloved hand, pick up the selected chicken carcass by the legs and place the carcass inside the bag without touching the inside of the bag with either hand.
* Add 400mL of sterile buffered Peptone Water.
* Expel most of the air from the bag and firmly hold the bag closed.
* Rinse the entire carcass using a repeated rocking motion to invert the bird 30 times (approximately 1 minute).
* Carefully open the bag and remove the bird.
* Using the “V” formed by the bag at the lower corner, carefully pour the rinsate into an open sterile container.
* Collect 50mL of this rinsate for subsequent analysis.
* Incubate the rinsate for 24hrs at 37oC.
* Store broth at 4oC.

1. ***LABORATORY PROTOCOL FOR THE ISOLATION OF SALMONELLA FROM MARKET-READY FROZEN POULTRY MEAT.***

The methods proposed by Andrews W. H, Wang H, Jacobson A, and Hammack T. S and the Food and Drug Administration’s Bacteriological Analytical Manual were used.

**Materials:**

* Examination gloves
* Distilled Water
* 70% Ethanol
* Cotton wool
* Buffered Peptone Water
* Petri-dishes
* Rappaport Vassiliadis Enrichment Broth
* Xylose Lysine Deoxycholate (XLD) Agar
* Blood Agar
* Glass slides
* Gram Stain reagents
* Kovac’s Reagent
* Sulfur Indole Mortility (SIM) Media
* Triple Sugar Ion (TSI) Media
* Simmons Citrate Agar
* Urea broth
* 10% glycerol-peptone broth.

**Procedure:**

* Transfer 1mL of the rinsate broth into 9mL of Rappaport Vassiliadis (RV) enrichment broth and votex. Also include a positive control (Typical *Salmonella*) and a Negative control (*Proteus spp.*).
* Incubate the broths at 42oC for 48hrs.
* Vortex tube and transfer 3mm loopful (10µL) of RV medium to XLD agar and streak.
* Incubate plates at 37oC for 24hrs.
* Examine plates for pink colonies with or without black centers.
* Do gram stains for these colonies. *Salmonella* *spp.* are small gram negative rods.
* Sub-culture colonies with black centers onto Blood Agar plates.
* Plates with swarming should be discarded and only those that are not swarming should be forwarded for biochemical tests.
* Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI slant by streaking slant and stabbing butt.
* Store the selected XLD agar plates at 5-8°C
* Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H2S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H2S (blackening of agar) in TSI.
* With sterile needle, inoculate growth from each presumed-positive TSI slant culture into tubes of urea broth. Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control. Incubate for 24hrs at 37°C.
* Inoculate Simmons Citrate agar using needle containing growth from TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate for 96hrs at 37°C. Read results as follows:

1. Positive — presence of growth, usually accompanied by color change from green to blue. Most cultures of *Salmonella* are citrate-positive.
2. Negative — no growth or very little growth and no color change.

* Inoculate SIM media with suspected *Salmonella* colony. Incubate at 37oC for 24hrs. Add 0.2-0.3 ml Kovacs' reagent to the top of the broth. Aerobic areas turn red due to alkaline conditions while anaerobic areas remain yellow.
* Store isolates in 10% glycerol-peptone solution.

1. ***LABORATORY PROTOCOL FOR THE ISOLATION OF E. COLI FROM MARKET-READY BROILER CARCASSES.***

The methods proposed by Feng P., Weagant S. D, Grant M. A and Burkhardt W and The Food and Drug Administration’s Bacteriological Analytical Manual were used with a few modifications.

**Materials:**

* Examination gloves
* Distilled Water
* 70% Ethanol
* Cotton wool
* Buffered Peptone Water
* Petri-dishes
* Glass slides
* Levine's Eosin-Methylene Blue (EMB) agar
* MacConkey agar
* Simmons Citrate Agar
* Nutrient Agar
* Gram Stain reagents
* 10% glycerol-peptone broth.

**Procedure:**

* Transfer 1mL of the rinsate broth into 9mL of Buffered Peptone Water and votex. Also include a positive control (Typical *Escherichia coli*) and a Negative control (*Citrobacter spp.*).
* Incubate the broths at 37oC for 24hrs.
* Vortex tube and transfer 3mm loopful (10µL) of Peptone Water broth to MacConkey agar plates and streak.
* Incubate plates at 37oC for 24hrs.
* Examine plates for pink/brick red colonies which are lactose fermenters.
* Do gram stains for these colonies. *E. coli* are small gram negative rods.
* Store plates with suspected colonies at 4oC.
* Inoculate pink colonies onto EMB agar and incubate at 37oC for 24hrs.
* Typical lactose-fermenting colonies on EMB agar appear dark centered and flat, with or without metallic sheen. Both *E. coli* and *Citrobacter spp.* may present a metallic green sheen.
* Transfer suspicious colonies to Simmons Citrate Agar. A negative reaction (media remains green) differentiates *E. coli* from *Citrobacter spp.* which give a positive result (Media turns blue).
* Sub-culture colonies on Nutrient Agar incubate at 37oC for 24hrs.
* Store isolates in 10% glycerol-peptone broth.

1. ***DRUG SENSITIVITY TESTING OF ISOLATES.***

The Kirby-Bauer disk diffusion technique for Drug Sensitivity Testing will be employed.

**Materials:**

* Examination gloves
* Antibiotic disk wafers (8 different antibiotics)
* Petri-dishes
* Glass slides
* Gram Stain reagents
* Blood Agar
* Nutrient Agar
* Sterile Forceps

**Procedure:**

* Sub-culture confirmed *Salmonella* and *E. coli* isolates onto Blood Agar plates for 24hrs at 37oC to isolate a pure culture of the bacteria.
* Perform a Gram Stain on the pure colonies before proceeding with the disk diffusion technique.
* Using a sterile cotton swab, sweep the blood agar plate with the colonies and spread the bacteria on the nutrient agar plates until the plate is completely covered and there are no visible spaces.
* Using sterile forceps carefully place the antibiotic wafers on the media and tap lightly to secure it onto the agar.
* Incubate the plates at 37oC for 24hrs.
* Inspect the plates and measure the diameter of the zones of inhibition for each antibiotic.
* Compare the diameter of the zones of inhibition for each antibiotic to the standard diameter of the zones of inhibition. Standard zones of inhibition have been established for each antibiotic. If the diameter is smaller than the standardized then resistance is evident.

1. ***MOLECULAR IDENTIFICATION OF SALMONELLA ISOLATES AND DETERMINATION OF THE GENES THAT CONFER RESISTANCE.***
2. **DNA Extraction**

DNA extraction was done by heat. The methods previously described by were used with a few modifications.

**Materials:**

* Sterile toothpicks
* Sterile 20-200µL tips
* 20-200µL Micropipette
* Water bath
* Micro centrifuge
* Eppendorf rack
* Laboratory gloves
* 2.5mL Eppendorf tubes
* Fine tip permanent marker

**Reagents:**

* TE buffer or deionized water

**Procedure:**

* Label the Eppendorf tubes with laboratory accession numbers and place them in a rack
* Switch on the water bath and set the temperature at 97oC.
* Dispense 100µL of sterile TE buffer or sterile deionized water into each labeled Eppendorf tube and place it back on the rack
* Using a sterile toothpick, pick 1-5 individual pure colonies of the isolates and transfer them into the 100µL deionized water.
* Gently break the colonies with the toothpick so that they are visually broken into smaller fragments.
* Set a timer at 5-10 minutes before you do the next step
* Place the tubes containing the cell suspension into the water bath once you have checked that it has reached 97oC
* Start the timer
* When the timer goes off, remove the tubes from the water bath and switch it off if it is not going to be used again.
* Switch on the micro centrifuge and set it at 15,000 ×g for 5-15 minutes
* Label another set of eppendorf tubes with the laboratory IDs on the tubes containing the cell suspension
* Place the tubes in the centrifuge and press the start button
* Remove the supernatant containing the DNA and transfer it into a clean labeled eppendorf tube.

Note: An aliquot of the DNA suspension (1-5µL) can immediately be added to a PCR reagent mix or stored at -78oC if not being used immediately. It will be best to use TE buffer if DNA will be stored for long periods of time under refrigeration.

1. **DNA Amplification**

Multiplex PCR was employed.

**Materials:**

* Sterile laboratory gloves
* Sterile eppendorf tubes
* Micro pipettes
* Sterile pipette tips
* Eppendorf tube racks
* Waste disposal container

**Reagents:**

* 3µL of 10× buffer
* 50µmol/L of each of the four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, dTTP)
* 10pmol of each primer pair
* 1.0U of Taq DNA polymerase
* 2µL template DNA
* Double distilled water

**Procedure:**

* Make a primer mix by adding 10pmol of each primer pair to an eppendorf tube.
* Make a master mix by adding the PCR reagents to an eppendorf tube starting with the largest volume and ending with the smallest.
* Add primer mix, master mix to a 96 well plate.
* Add the template DNA to the plate.
* Amplify in a thermocycler using desired pcr parameters outlined below:
* Initial denaturation at 95oC for 5 minutes
* 35 cycles of denaturation at 95oC for 1 minute
* Annealing at 55oC for 1 minute
* Extension at 72oC for 1 minute
* Final extension at 72oC for 10 minutes

1. **Resolution of Amplified PCR Products by Agarose Gel Electrophoresis**

**Materials:**

* Two 1 litre orange-cap bottles
* 250mL flask
* Volumetric cylinders
* Spatula
* Gel casting tray
* Gel combs
* Tape
* Electrophoresis tank
* Power supply and cables
* Microwave

**Reagents:**

* 1× TAE Buffer
* Ladder
* 6× loading dye
* Agarose powder

**Procedure:**

*(a)Making the gel*

* Weigh 2.5g of the agarose powder and transfer it into a 250mL flask.
* Measure 100mL of TAE buffer and add it to the flask. Swirl to mix.
* Place the flask in a microwave and time for 2 minutes.
* Remove the flask and check to see whether the gel has dissolved. Typically when it has dissolved completely it appears more transparent or clear. If it has not dissolved completely, place it back into the microwave for a few seconds longer until you are satisfied that it is ready.
* Remove the ethidium bromide from the refrigerator and carefully add 3 drops into the gel and mix by gently swirling. Be careful as you handle ethidium bromide because it is a carcinogenic substance and should not come in contact with bare skin.
* Place the gel combs in a gel casting tray and pour in the agarose gel making sure the combs are well submerged in the gel.
* Cover the gel and allow it to polymerise
* When the gel has polymerized, carefully remove the comb to expose the sample wells.

*(b) Running the gel*

* Place the gel into the tank
* Add EtBr to the ~900mL 1× TAE to make it 0.05µg/mL. Pour the buffer into the tank high enough to cover the gel. Be careful since the buffer now has ethidium bromide in it.
* Add 1µL loading dye per 5µL sample.
* Add the ladder to the first well
* Add samples to the dye and transfer into the wells starting with the ladder
* Connect the tank to the power supply and run for about an hour or until the faster dye migrates most of the way through the gel

*(c) Imaging the gel*

* Carry the gel in a casting tray
* Open the plastic cover
* Transfer the gel from the casting tray by sliding it into the transilluminator
* Close the plastic cover
* Turn on UV to 100%. You should see bands at this point.

## **Appendix 3: Laboratory Results**

Table 11: Some of the genes Detected After Multiplex PCR

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Isolate Number** | **Area** | **Type of Market** | **Resistant Antibiotics** | **Genes Detected** |
| 1 | Chilanga A | Supermarket | Tet, Sxt, Amp | CtxM |
| 2 | Lusaka E | Supermarket | Tet, Amp | CtxM |
| 3 | Lusaka E | Open Market | Tet, Sxt, Amp | CtxM |
| 4 | Lusaka D | Open Market | Cip, Tet, Sxt, Amp, Nal | CtxM, TetA |
| 5 | Lusaka B | Supermarket | Tet, Sxt, Amp | TetA |
| 6 | Lusaka C | Open Market | Tet, Sxt, Chl, Amp | CtxM |
| 7 | Lusaka C | Supermarket | Tet, Sxt, Chl, Amp, Nal | TetA, CtxM, Sul1 |
| 8 | Kafue A | Supermarket | Tet, Sxt, Amp | CtxM |
| 9 | Chilanga A | Supermarket | Tet, Sxt, Amp | CtxM |
| 10 | Lusaka E | Supermarket | Tet, Nal | CtxM |
| 11 | Lusaka C | Supermarket | None | CtxM |
| 12 | Lusaka E | Open Market | Tet, Chl, Amp | CtxM, TetA |
| 13 | Chilanga A | Supermarket | Cip, Tet, Amp, Nal | CtxM |
| 14 | Lusaka E | Open Market | Tet, Sxt | CtxM |
| 15 | Lusaka E | Open Market | Tet, Sxt, Amp | TetA |
| 16 | Lusaka E | Supermarket | Chl, Ctx, Amp | TetA, CtxM |
| 17 | Chilanga A | Supermarket | None | None |
| 18 | Lusaka A | Open Market | Tet, Sxt, Amp | CtxM |
| 19 | Lusaka A | Open Market | None | None |
| 20 | Lusaka H | Supermarket | Tet, Amp | None |
| 21 | Lusaka A | Open Market | Ctx, Amp | Sul1, TetA |
| 22 | Lusaka K | Open Market | Tet, Sxt, Amp | Sul1, TetA |
| 23 | Kafue A | Supermarket | None | None |
| 24 | Lusaka K | Open Market | None | CtxM |
| 25 | Lusaka B | Supermarket | Tet, Ctx, Amp | CtxM |
| 26 | Lusaka K | Open Market | Tet, Sxt | CtxM |
| 27 | Lusaka K | Open Market | Tet, Sxt, Amp | CtxM |
| 28 | Lusaka E | Supermarket | Tet, Amp, Nal | CtxM, TetA |
| 29 | Lusaka K | Open Market | Tet, Sxt, Chl, Amp | None |
| 30 | Lusaka K | Open Market | Tet, Sxt, Chl, Amp | CtxM, TetA, Sul1 |
| 31 | Lusaka K | Supermarket | Cip, Tet, Sxt, Amp, Nal | None |
| 32 | Lusaka B | Supermarket | Tet, Ctx, Amc, Amp, Nal | TetA, Sul1 |
| 33 | Lusaka C | Supermarket | None | None |
| 34 | Lusaka B | Supermarket | Tet | None |
| 35 | Lusaka K | Open Market | Tet, Sxt, Amp | Sul1, TetA, CtxM |
| 36 | Lusaka E | Open Market | Tet, Amp | Sul1, TetA, CtxM |
| 37 | Lusaka C | Supermarket | Tet, Sxt, Amp | None |
| 38 | Lusaka K | Open Market | None | None |
| 39 | Lusaka K | Open Market | Tet, Amp | CtxM, TetA |
| 40 | Lusaka E | Open Market | Tet, Sxt, Nal | Sul1, TetA, CtxM |
| 41 | Lusaka C | Open Market | Tet, Sxt, Amp | CtxM |
| 42 | Lusaka C | Supermarket | None | CtxM |
| 43 | Kafue A | Supermarket | None | TetA |
| 44 | Lusaka E | Open Market | Tet, Sxt, Amp | TetA |
| 45 | Lusaka C | Open Market | Tet | TetA |
| 46 | Lusaka K | Supermarket | Cip, Tet, Sxt, Chl, Amp, Nal | None |
| 47 | Lusaka B | Supermarket | None | None |
| 48 | Lusaka C | Open Market | Tet | TetA |
| 49 | Kafue A | Open Market | Tet, Chl, Nal | CtxM |
| 50 | Lusaka C | Supermarket | None | None |
| 51 | Lusaka E | Open Market | Tet, Sxt, Amp | None |
| 52 | Lusaka E | Open Market | Tet | None |
| 53 | Lusaka B | Supermarket | Tet | None |
| 54 | Lusaka E | Open Market | Tet | TetA |
| 55 | Lusaka E | Open Market | Tet, Chl, Amp | None |
| 56 | Lusaka E | Open Market | Tet, Amc, Amp | CtxM |
| 57 | Kafue A | Supermarket | Tet, Sxt, Amp | CtxM |
| 58 | Kafue A | Supermarket | None | None |
| 59 | Lusaka E | Supermarket | Tet, Amp | CtxM |
| 60 | Lusaka E | Supermarket | Tet, Nal | None |
| 61 | Chilanga A | Supermarket | None | None |
| 62 | Lusaka B | Supermarket | None | None |
| 63 | Chilanga A | Supermarket | Tet, Sxt, Amp | CtxM |
| 64 | Lusaka B | Supermarket | Tet | TetA |
| 65 | Kafue A | Open Market | Tet, Chl, Nal | TetA |
| 66 | Lusaka E | Open Market | Tet, Sxt, Amp | None |
| 67 | Lusaka D | Supermarket | Tet | TetA |
| 68 | Lusaka E | Open Market | Tet | None |
| 69 | Lusaka E | Supermarket | Amp | CtxM |
| 70 | Chongwe A | Open Market | Amp | CtxM |