

**Detection of Extended Spectrum Beta Lactamase producing *Enterobacteriales* from diseased chickens and estimation of antimicrobial usage from antibiotics importation and sales data in Lusaka district, Zambia**

**By**

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**Thesis submitted to the University of Zambia in fulfilment of the requirements for the award of a Degree of Master of Science in Veterinary Public Health**

**SCHOOL OF VETERINARY MEDICINE**

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## DECLARATION

I, **Chikwanda Chileshe**, declare that the contents of the thesis being submitted herein are my original work and have not been previously submitted to any University for the award of a degree or any other qualification.

Signature

Date

## CERTIFICATE OF APPROVAL

This thesis submitted by **Chikwanda Chileshe** is approved as fulfilling the requirements for the award of the degree of Master of Science in Veterinary Public Health at the University of Zambia.

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## ABSTRACT

Poultry products in Zambia form an integral part of the human diet in many households, as they are more affordable and easier to produce as compared to other sources of animal protein. However, the burden of poultry diseases has remained a major challenge, hindering the growing consumer demand for poultry products in Zambia. This has resulted in non-prudent antimicrobial use on farms, with the intent to prevent and treat poultry diseases for growth optimization and maximising profits. This study aimed to identify the different types of extended-spectrum  $\beta$ -lactamase (ESBL) producing Gram negative bacteria (GNB) causing diseases in chickens in Lusaka and to assess possible association between antibiotic resistance and antibiotic consumption. A cross-sectional study was conducted in Lusaka, which involved the collection of samples from diseased chickens at three different post-mortem facilities. Importation data was gathered from antimicrobial usage (AMU) data collection report from the Ministry of Fisheries and Livestock (MOFL) whilst sales data was collected from the largest outlet of veterinary pharmaceutical products in Zambia. A total of 215 samples were collected and screened for GNB. Of these samples, 103 tested positive for various clinically relevant Enterobacterales, including *Enterobacter* (43/103, 41.7%), *Escherichia coli* (20/103, 19.4%), *Salmonella* (10/103, 9.7%), and *Shigella* (8/103, 7.8%). Other isolated bacteria included *Yersinia*, *Morganella*, *Proteus*, and *Klebsiella*, which accounted for 21.4%. Only *E. coli*, *Enterobacter*, *Salmonella*, and *Shigella* were subjected to antimicrobial susceptibility testing (AST) using the Kirby-Bauer disc diffusion due to their public health significance. The AST results revealed that *E. coli*, *Enterobacter* and *shigella* were highly resistant to tetracycline, ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole, while *Salmonella* showed complete susceptibility to all tested antibiotics. The observed resistance patterns correlated with antimicrobial usage estimated from the importation and sales data. The broth microdilution test was used to quantify for cefotaxime resistance. Results from this test showed that only fourteen (14/103, 13.5%) were resistant to cefotaxime. Eight of the fourteen isolates were *Enterobacter* whilst the remaining six were *E. coli*. The polymerase chain reaction (PCR) technique was then used to screen for the genes *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>OXA</sub> and *bla*<sub>SHV</sub>. All Six (6/14, 42.9%) of the *E. coli* isolates carried the gene *bla*<sub>CTX-M</sub> whilst only four carried the gene *bla*<sub>TEM</sub>. On the other hand, all eight (8/14, 57.1%) of the *Enterobacter* isolates carried the gene *bla*<sub>TEM</sub> whilst only four of the isolates harboured the gene *bla*<sub>CTX-M</sub>. Sanger sequencing of the PCR products revealed that five (5/6, 83.3%) of *E. coli* isolates carried the *bla*<sub>CTX-M-15</sub> allele.

These results suggest the presence of pathogenic ESBL-producing *Enterobacteriaceae* in poultry, threatening public health.

## **DEDICATION**

This work is especially dedicated to my husband, Emmanuel Manda, for his incredible support during my studies at the University of Zambia. I thank him immeasurably for his moral support when I could not avail myself as much during the period of my studies.

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## LIST OF ABBREVIATIONS

<b>AMR</b>	Antimicrobial resistance
<b>AST</b>	Antibiotic susceptibility testing
<b>CSLI</b>	Clinical and Laboratory Standards Institute
<b>CTX-M</b>	Cefotaxime Munich
<i>E. coli</i>	<i>Escherichia coli</i>
<b>ESBL</b>	Extended spectrum beta-lactamase
<b>HGT</b>	Horizontal gene transfer
<b>HRS</b>	Hours
<b>MIC</b>	Minimum inhibitory concentration
<b>MDR</b>	Multidrug resistance
<b>PCR</b>	Polymerase chain reaction
<b>SHV</b>	Sulfhydryl variable
<b>TEM</b>	Temoneira
<i>bla</i> <sub>CTX-M</sub>	Cefotaxime munich gene
<i>bla</i> <sub>TEM</sub>	Temoneira gene
<i>bla</i> <sub>OXA</sub>	Oxacillinases gene
<i>bla</i> <sub>SHV</sub>	Sulphhydryl Variable gene

## UNIT OF MEASUREMENTS

<b>%</b>	Percentage
<b>g</b>	Grammes
<b>ml</b>	Millilitres
<b>µl</b>	Microlitres
<b>°C</b>	Degrees Celsius

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background

Poultry production in Zambia is one of the most important activities in the livestock sector, with the chicken population estimated at 94 million broilers, 15 million village chickens, and 5.8 million layers (Goma et al., 2019). Moreover, poultry products in Zambia, like in other developing African countries, form an integral part of the human diet in many households, as it is a cheaper source of animal protein and is easier to produce compared to other foods of animal origin (Muonga et al., 2019; Caudell et al., 2020;).

As the Zambian population progressively expands, the demand for meat and other foods increases, leading to food security problems. As a result, the Government relies on agricultural industries to heighten animal production and address food insecurity (Goma et al., 2019). These industries usually raise large numbers of animals by boosting production through extensive farming methods that involve antimicrobial growth promoters (Gonzalez et al., 2017; Miles et al., 2006). Although such livestock intensification approaches are essential for alleviating food shortages, they are also associated with the emergence and spread of antimicrobial resistance (AMR) (Palma et al., 2020). This is further exacerbated by poorly monitored animal husbandry practices that result in frequent infections requiring antimicrobial use, leading to AMR (Agyare et al., 2019; Mehdi et al., 2018). Other contributing factors to AMR involve practices by farmers who sometimes deliberately underdose their livestock because of the high cost associated with antibiotics, thereby worsening the problem (Bumbangi, et al., 2023).

The most common poultry infections in Zambia are caused by *E. coli* and *Salmonella* (Chishimba et al., 2016; Muonga et al., 2019; Phiri et al., 2019; Mwansa et al., 2023). These infections arise mainly from the lack of proper biosecurity measures associated with backyard poultry farms. *E. coli* infections usually occur when the immune system of the host is suppressed, or when gastro-intestinal barriers are violated (Roth et al., 2019). However, there are certain *E. coli* strains coined as avian pathogenic *E. coli*, these spread into various internal organs and cause colibacillosis which is characterized by reduced appetite and poor growth

rates (Lutful et al., 2010; Roth et al., 2019). *Salmonella* spp. are equally one of the commonest microbial contaminants in the poultry industry (Kaonga et al., 2019). Transmission of the disease in birds can be vertical (transovarian) but also occurs through direct or indirect contact with infected birds via respiratory route or fecal matter or contaminated feed, water, or litter (Mubita et al., 2020; Sackey et al., 2001). The above bacterial diseases call for the use of antibiotics such as tetracycline and penicillins as treatment options (Lutful et al., 2010). The indiscriminate use of antimicrobials in poultry farming is likely to accelerate the development of AMR in pathogens, as well as in commensal organisms. Additionally, AMR in poultry pathogens is likely to lead to economic losses, derived from the expenditure on ineffective antimicrobials, as well as the burden of untreated poultry disease (Nhung et al., 2017).

AMR may be encoded chromosomally; this is a process where the resistance comes from genes and the resistance present in the bacteria does not depend on previous exposure to antibiotic (Peterson et al., 2018). This mechanism mainly depends on the decreased permeability of the external wall of the bacteria, however, the production of plasmid-mediated extended-spectrum  $\beta$ -lactamases (ESBLs) is more common (Ayinla et al., 2023). There are nine ESBL classes, but common ones include variants of the CTX-M-type and derivatives of SHV-1, TEM-1, and TEM-2 (Rodríguez-Baño et al., 2008; Dirar et al., 2020; Ayinla et al., 2023). ESBL-producing *Enterobacteriaceae*, resistant to third generation cephalosporins like cefotaxime (CTX), are dreaded profoundly because of their extensive geographic distribution and adverse health impacts. While ESBLs are more prevalent among hospital isolates, poultry has emerged as an important reservoir for possible zoonotic transmission (Falgenhauer et al., 2019). This reservoir includes ESBL-encoding genes harboured by commensal and pathogenic strains and may disseminate to humans via two main mechanisms. Firstly, ESBL genes may be transmitted by horizontal gene transfer, and the treatment implications depend on the pathogenicity of the recipient bacterial strain (Smillie et al., 2010). Of greater concern is the direct transmission of disease-causing pathogens by clonal expansion, potentially leading to clinical disease and treatment failure. Therefore, understanding the zoonotic transmission of poultry-associated ESBLs requires a multipronged approach that considers both healthy and diseased chickens. However, in Zambia, most studies have focused on non-pathogenic bacteria isolated from asymptomatic chickens, leaving a gap in the presence of ESBL-producing GNB status in sick chickens. Some public and private veterinary institutions conduct routine post-mortems on chickens suspected to have died of infection. Taking advantage of this development, we

characterized ESBL-producing GNB isolated from post-mortems from three different laboratories in Lusaka.

All antibiotics for veterinary usage are imported into the country as Zambia does not manufacture any antibiotics (Ministry of Fisheries and Livestock, 2022). Data on the importation of antibiotics in Zambia can be sought from the Zambia Medicines Regulatory Authority (ZAMRA) and the MOFL. Estimation of antibiotic consumption is a challenge as the country lacks comprehensive surveillance systems resulting from shortage of veterinarians and the reluctance of livestock industry (food animal producers and animal feed producers) to give the comprehensive reports on antimicrobial consumption (Caudell et al., 2020).

## **1.2 Statement of the problem**

In Zambia poultry forms an integral part of the human diet. In order to meet demand of the ever-growing population there has been a shift to a more intensive kind of poultry farming which has seen the mushrooming of backyard poultry farms with limited biosecurity. The limitations in biosecurity leave room for infectious diseases to sprout thereby increasing the need to use antibiotics (Bumbangi, et al., 2023). In Zambia, several poultry farmers acquire antimicrobials from agro-vet shops without prescriptions, an indication of easy access to antimicrobials as a result of weak enforcement of regulations (Caudell et al., 2020; Malama, et al., 2022). Further, there have been reports of farmers medicating their animals without advice from a veterinarian (Chilawa et al., 2023). Farmers also have limited access to veterinary services leading them to get advice and assistance from fellow farmers (Caudell et al., 2020; Chilawa et al., 2023). This has contributed to the imprudent use of antimicrobials, consequently emanating in AMR. The general policy direction is not to use antimicrobials as feed additives to promote growth. However, in areas with a high disease burden, it is permitted to use feed additives containing antimicrobials prophylactically to lessen the disease burden.

In addition, the animal health sector in Zambia lacks adequate prescription and treatment guidelines to help professionals make informed decisions and directed therapy. This has been due to limited resources to improve diagnostics and provide technical support to professionals, non-trained handlers and users of antimicrobials. As a result of these various drivers of antimicrobial overuse and AMR, there is a growing concern about the safety of chicken and chicken products.

### **1.3 Rationale of the study**

Currently, the data on antimicrobial resistance in various bacterial pathogens in poultry is limited in Zambia. The lack of updated data on AMR in bacterial pathogens in poultry, impedes the understanding of the extent and complexity of the problem, which cripples the prospect of putting up appropriate preventive and control intervention measures. Furthermore, data from this study will make veterinary practitioners more aware of the current situation pertaining to antibiotic resistance and give them the proper insight into which antibiotics to use to treat bacterial diseases. Knowing which antibiotics are effective will further reduce the cost of treatment which is usually exacerbated by the trial-and-error usage of drugs.

### **1.4 Research question**

What is the prevalence of ESBL producing *Enterobacterales* in diseased chickens presented on post-mortem in Lusaka?

### **1.5 Objectives**

#### 1.5.1 General objectives

To determine the presence of ESBL producing *Enterobacterales* among diseased chickens presented for post-mortem and assess if there is possible association between antibiotic consumption and the resistant patterns observed.

#### 1.5.2 Specific objectives

1. To estimate poultry antimicrobial usage through antibiotic importation and sales data.
2. To isolate and identify the GNB species found in diseased chickens in Lusaka district.
3. To detect ESBL-producing *Enterobacteriaceae* phenotypically and confirm the presence of ESBL-encoding genes by PCR and sequencing.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 General overview of *Enterobacterales*

The order *Enterobacterales* is a diverse group of GNB, facultatively anaerobic, non-spore-forming, rod-shaped bacteria within the class gammaproteobacteria (Brenner et al., 2015). Members of this group inhabit a number of different ecological niches including the environment. Under the order *Enterobacterales*, *Enterobacteriaceae* are the largest group of clinically relevant GNB rods belonging to the class gammaproteobacteria and the phylum pseudomonadota (Brenner et al., 1983). They reside in the large intestines as commensals and symbionts. However, they have the ability to become opportunistic pathogens and cause disease. These bacteria are also widespread in the environment and are a common finding in the soil. Typically, *Enterobacteriaceae* are 1-5µm, with fimbria that allows them to adhere to their host. All are non-capsulated apart from *Klebsiella* (Rai et al, 2020). They are motile except for *Shigella* and *Klebsiella* and are non-spore forming. Physiologically they reduce nitrate to nitrates, are oxidase negative, catalase positive and non-fastidious. Their cultural characteristics include the ability to grow on MacConkey media and some species ferment glucose whilst others do not (Adeolu et al., 2016). The glucose fermenters drop the pH changing the colour of the agar to pink. Some *Enterobacteriaceae* that colonise the gastrointestinal tract have been found to carrying resistance genes with a possibility of colonize the gastrointestinal system transferring the resistant gene to the endogenous flora, thereby jeopardizing future treatment protocols for infections (Adeolu et al., 2016).

##### 2.1.1 Structure of *Enterobacteriaceae*

*Enterobacteriaceae* are made up of an impermeable outer membrane that enables them to withstand injury from environmental factors such as antibiotics (Lerouge et al, 2002). This outer membrane contains a lipopolysaccharide layer made up of three components. The outer most layer that interacts intimately with the environment is a glycan polymer known as the O antigen (Adeolu et al., 2016). The O antigen is heat stable and inhibits phagocytosis. The next layer is made up of short sugar chains and is known as the core oligosaccharide. The core oligosaccharide connects the O antigen to the inner most layer called Lipid A which is responsible for eliciting inflammatory reactions and pathophysiology's associated with *Enterobacteriaceae* (Rai et al, 2020).

### **2.1.2 Antigenic structures**

To colonise their host, the outer membrane of *Enterobacteriaceae* is coated with antigens that elicit immune activation. The three main antigens found on the outer membrane are the O antigen, H antigen and K antigen (Farmer et al., 2015). The H antigens are heat labile and are found on the flagellar. However, they are absent in non-motile *Enterobacteriaceae* like *Shigella* and *Klebsiella* whilst the K antigens are capsule antigens found on the outer layer of the outer membrane and are responsible for phagocytosis. The F antigens are found on the fimbriae and adhere to the mucosa of its host (Lerouge et al, 2002).

### **2.2 AMR transmission dynamics**

In any large population of bacteria, a few cells will be present which possess traits that enable them to survive in the presence of noxious substances, in this case, the ability to fend off the action of antimicrobials (Apata, 2009). Susceptible organisms, i.e., those lacking this trait, will be eliminated, leaving the resistant populations behind. This increased prevalence of resistance may be viewed as part of the Darwinian principle of survival of the fittest (Apata, 2009). Bacteria can evolve and acquire resistance genes that provide them with protection against the effects of antibiotics used against them. There are two principal biological pathways involved in the evolution and development of AMR (Sun, 2018). Firstly, bacterial cells accumulate resistance genes during the evolution process and transfer the resistance gene to progeny via vertical gene transfer leading to innate resistance. The second scenario is known as acquired resistance or horizontal gene transfer (HGT) involves the acquisition of new resistance genes harboured on mobile genetic elements such as plasmids, integrons, and transposons within bacterial species (Smillie et al., 2010). The genetic materials are transferred through transduction, conjugation, and transformation

Transduction refers to the transfer of DNA fragments from one bacterium to another by a bacteriophage (Ozeki et al., 1967). During transformation, genes are transferred from one bacterium to another as naked DNA, which occurs when the cells die, and DNA is released into the surrounding environment. Other bacteria in proximity can then scavenge this free-floating DNA and incorporate it into their own DNA (Motro et al., 2009). Transformation involves homologous recombination, which is a recombination between DNA having the same nucleotide sequences or amongst closely related strains (Frost et al., 2010). Another mechanism

that confers emergence of AMR is known as point mutation. This involves the changing of a single nucleotide base by insertion, deletion, or substitution. Information on the occurrence of resistance to drugs, changes over time because bacteria can be considered to have access to a comprehensive tool kit that provides them with the potential to remodel and mix and match their genes (Morto et al., 2009). It is, therefore, of vital importance that the magnitude of resistance at each point in time is understood to establish a baseline of action (Rowe-Magnus et al., 2002).

### **2.3 Production systems in poultry**

Antimicrobial use in intensive poultry farms has become a common occurrence within low-income countries like Zambia (Caudell et al., 2020). This is done to increase the throughput of both meat and eggs. Intensive poultry farming can be divided into large scale intensive farming and family poultry husbandry. Large scale intensive can be defined by various characteristics which include routine AMU and high densities of more than 1000 birds. For broilers, birds under the large-scale intensive system are kept on litter with automated drinkers and feeders (Hedman et al., 2020). Furthermore, farmers under this system require a high capital input to start this venture and are fully market oriented as compared family poultry husbandry who raise birds mainly for home consumption. On the other hand, family poultry husbandry is divided into four categories small extensive, extensive, semi-intensive and intensive (Hedman et al., 2020). These subdivisions vary based on inputs, chicken breeds, gender and biosecurity measures. Small-extensive and extensive scavenging poultry farming typically involve local breeds of poultry and are generally raised without routine antimicrobial therapy. Small-extensive and extensive farming systems are generally managed by female heads of households and children (Hedman et al., 2020). On the other hand, semi-intensive and intensive operations raise broiler or layer chickens with antimicrobials administered in commercial feed. Intensive family operations typically lack the financial resources to support minimal biosecurity measures thus increasing the risk of infection and consequently the need to use antibiotics. Unfortunately, intensive poultry farming serves as a double-edged sword as it alleviates food security whilst exacerbating the need to use antimicrobials (Jeni et al., 2021). The need to use antimicrobials springs from the shortcomings in biosecurity. Most of the intensive farming establishments are placed near households, this potentially leads to the spread of AMR due to poor water sanitation and regular human to animal interactions (Castellini et al., 2012).

#### **2.4 Common bacterial infections caused by *Enterobacteriaceae* in Zambia.**

Poultry infections pose a major constraint on small scale poultry farmers in Zambia. Bacterial diseases, like colibacillosis and salmonellosis, are responsible for most the chicken losses in small scale settings (Hedman et al., 2020). Coliform infections are often caused by strains of *E. coli*. *E. coli* infections range from severe acute infections with sudden and high mortality to mild infections of a chronic nature with low morbidity and mortality in Zambia (Chishimba et al., 2016). The disease may result from a coliform infection alone as in primary infection or as a secondary infection arising from other complications. The primary routes of invasion by the organism are the respiratory system and the gastrointestinal tract. Infections in young birds usually result from entry through the unhealed navel or penetration of the egg shell prior to incubation (Nhung et al., 2017). *E. coli* infections affect birds of all ages. Another common infection in Zambia is salmonellosis is mainly caused by *Salmonella typhimurium* and *enteritidis*. These particular serovars are commonly found in the gastro-intestinal tract of chickens. The contamination of chicken meat and eggs by from salmonella-infected chickens is the main source of infection in humans. In some cases, eggs may become infected transovarially. Humans become infected by ingesting contaminated meat or eggs. These infections are zoonotic and of major public health concern (Van Boeckel et al., 2015). Small scale poultry farmers in Zambia are susceptible to high levels of these disease because smallholder poultry farms are often lack adequate sanitation and appropriate biosecurity measures, thereby, facilitating the transmission and spread of diseases (Jeni et al., 2021). This widespread presence of diseases imposes an economic burden on small scale farmers. In order to counter the effects of these infections farmers resort to the use antimicrobials (Belmahdi et al., 2016). The use of antimicrobials in poultry fosters AMR, which can be transmitted to humans.

#### **2.5 Use of antimicrobials in the management of *Enterobacteriaceae* infections in poultry**

Majority of the antimicrobials used in poultry are administered orally as poultry is usually kept in large batches making it impractical to treat individual birds (Simoneit et al., 2015). The mass medication of poultry via feed or water, targets both the healthy and sick chickens (Gyles, 2008). However, the uptake of water and feed in sick chickens is reduced and this exposes the healthy chickens to unwarranted uptake of antimicrobials. In poultry, antimicrobials are used to treat infections. However, at sub therapeutic levels, antimicrobials act as growth promoters by modifying and increasing the growth of microbiota in the gut, leading to increased

absorption of nutrients which in turn causes an increase in weight gain (Mehdi et al., 2018). A practise that further worsens AMR.

With the notable increase in intensive farming, backyard poultry barns have significantly contributed to the increased risk of AMR development, which may be attributed to the close human-animal interface (Hedman et al., 2020). In areas with poor water sanitation, overflow of human excreta tends to interact with the underground water systems being used in the poultry houses allowing for anthro-p-zoonotic transmission of AMR. Such interactions often lead to the development of uncommon resistance to antibiotics like fluoroquinolones that are seldomly used in poultry (Moyaert et al., 2014).

## 2.6 Current antimicrobial usage situation in Zambia

In Zambia the most imported veterinary antibiotics are tetracyclines, sulphonamides, penicillins, and aminoglycosides, for use in food animals (Goma et al., 2019). Of the 5721 kg of antibiotics imported for animal use in Zambia in 2020, the major importers were the agro-veterinary shops (Figure 1). The bulk of these antibiotics were imported from Europe and South Africa, with a few from Egypt and China.

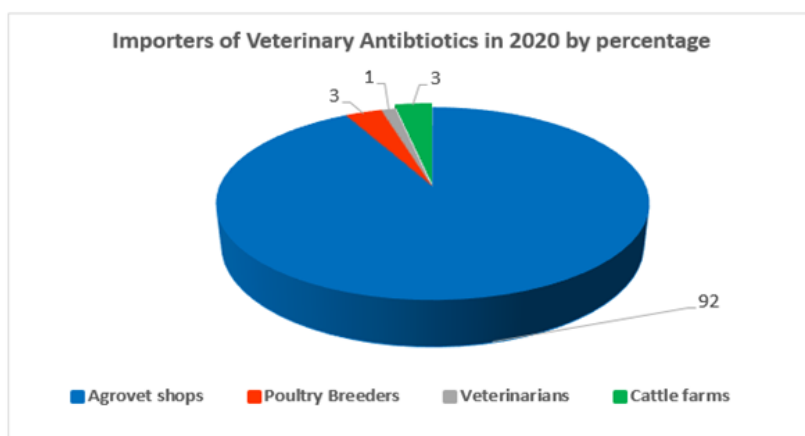


Figure 2.1. Importers of antibiotics in Zambia in 2020 (Ministry of Fisheries and Livestock, 2022).

Under current legislation in Zambia, only veterinarians can prescribe antimicrobials for animals/livestock. However, the shortage of veterinary personnel makes it challenging to have veterinary practitioners' presence everywhere, resulting in farmers accessing antimicrobials without prescriptions (Caudell et al., 2020). In Zambia, it is understood that the majority of farmers access antimicrobials from pharmacies and agro-veterinary shops (Mukosha, et al., 2022). Agro-veterinary shops fall into three registered categories based on the ZAMRA

classification namely: Class 1) The shop has a veterinary doctor at all times, Class 2) a shop has a veterinary paraprofessional (VPP) available at all times but is overseen by a veterinarian, and Class 3) the shop is run by a suitably qualified person (agro-vet shop seller) based on the Veterinary Council of Zambia requirements, and a VPP may be available for advice. The Caudell et al. (2020) study indicated other sources of antibiotics besides agro-vet shops by farmers, such as private veterinarians' friends and feed distributors, this was besides agro-vet shops by farmers (Caudell et al., 2020). However, the majority is still from agro-vet shops. Their study, which focussed on antimicrobial usage (AMU) in the livestock sector across five African countries, also found that farmers in Zambia (as well as Tanzania) reported the highest AMU (Caudell et al., 2020).

### **2.7 AMR in Humans, Animals and the Environmental sectors.**

AMR in humans as with poultry is highly exacerbated by the heavy prescription of antibiotics by general practitioners, coupled with the easy access of antibiotics in pharmacies (Pokharel et al., 2020). The raise in AMR cases in hospitals has led to an increase treatment failure of simple infections hence the need to urgently reduce the spread of AMR (Malama, et al., 2023). AMR in humans is linked to AMR in animals and environmental populations (Queenan et al., 2016). The One Health philosophy recognizes the connection of environmental, animal and human health sectors (McEwen et al., 2018). as well as the possibility that the abuse of antibiotics in one area may have an impact on the others. AMR can spread between people, animals, and the environment through a variety of channels, including direct contact, food chain transmission, and environmental pollution (McEwen et al., 2018). The issue is worsened by the shared use of antibiotics in human and veterinary sectors, as resistant bacteria can develop and spread between species. Additionally, environmental contamination aids in the emergence and spread of AMR via wastewater and agricultural runoff. The possible transfer of antibiotic-resistant microorganisms from animals to humans highlights the need of a well-coordinated One Health strategy in order to control and slow down antibiotic consumption across sectors (Velazquez-Meza et al., 2022).

### **2.8 AMR resistance in poultry**

The constant exposure of poultry to antibiotics through clinical treatment or preventative therapy has contributed to the development of AMR (Nhung et al., 2017).  $\beta$  lactams have been identified as the most commonly used antibiotics used in the treatment of poultry infections

(Belmahdi et al., 2016). Reports in the livestock sector have indicated high levels (38-100%) of resistance to broad-spectrum cephalosporins mediated by extended-spectrum beta-lactamase (ESBL)-producing organisms such as *Escherichia coli* and *Salmonella* in the poultry value chain (Chishimba et al., 2016). Resistance in poultry strains has also been reported in some of the crucial antimicrobials used as second-line treatment of human infections, such as 3rd generation cephalosporins and fluoroquinolones (Chishimba et al., 2016; Phiri et al., 2019; Ziba et al., 2020). Most of the available data on AMR in poultry is mainly on *Salmonella* spp and *E. coli*. A study on broiler chickens by (Ziba et al., 2020) reported *Salmonella* species resistant to various antimicrobial classes, including penicillins, fluoroquinolones, aminoglycosides, tetracyclines, polymyxins and dihydrofolate reductase inhibitors (Ziba et al., 2020). Another study on poultry reported *Salmonella* spp resistance to penicillins and cephalosporins and *E. coli* resistance to tetracyclines, penicillins, and dihydrofolate reductase inhibitors (Phiri et al., 2019). Other studies in Zambia have reported isolated bacterial pathogens resistant to tetracyclines, penicillins, dihydrofolate reductase inhibitors, and cephalosporins (Muonga et al., 2019; Phiri et al., 2020). Based on the abovementioned reports, the antimicrobial classes with the highest resistance include tetracyclines and penicillins (Muonga et al., 2019; Phiri et al., 2020). However, there is limited data on bacterial pathogens resistant to other antimicrobial classes, such as carbapenems and monobactams. Therefore, the presence of AMR in Zambia remains a public health concern.

## **2.9 $\beta$ -lactam antibiotics and production of $\beta$ -lactamases**

$\beta$  lactam antimicrobials are the most commonly used treatment choice for infections caused by GNB in poultry worldwide (Lontie et al., 1992).  $\beta$  lactam antibiotics include all penicillins, monobactams, carbapenems, and cephalosporins that contain a chemical structure called a  $\beta$ -lactam ring (Paterson et al 2005). Nitrogen is attached to the  $\beta$  carbon relative to the carbonyl ring, hence the name. This structure is capable of binding to the enzyme that cross-links peptidoglycans, interfering with the cross-linking and thus preventing bacterial cell wall synthesis (Lontie et al., 1992). The persistent exposure of bacteria to a multitude of  $\beta$  lactam has led to the development of resistance. One way in which bacteria become resistant to  $\beta$ -lactams is by the production of enzymes capable of inactivating or modifying the drug before it exerts its effects on the bacteria (Sun, 2018). Depending on the bacterial species, the genes encoding resistance may exist on the chromosome or plasmids. The bacteria can pass these AMR genes to other bacteria by HGT (e.g., conjugation) (McMillan et al., 2019). This could

occur when two bacteria come into contact with each other and a small channel is created between them, allowing for the exchange of a copy of the resistance plasmid. If the plasmid is transcribed and translated, the bacteria will begin to produce drug-inactivating enzymes. These enzymes can destroy  $\beta$ -lactam antibiotics and are known as  $\beta$ -lactamases (Lontie et al., 1992).

### 2.9.1 Classification of $\beta$ -lactamases

$\beta$  lactamases were first classified in 1940 in *E. coli*; however, over the past years, more than 890  $\beta$ -lactamases have been identified in naturally occurring bacteria (Bush et al., 2010)..  $\beta$ -lactamases are primarily classified according to either the Ambler molecular classification or the Bush -Jacoby -Medeiros functional classification (Paterson et al, 2005). The Ambler classification is the most widely used classification, which divides  $\beta$ -lactamases into four classes (A, B, C, D) based on amino acid sequences. Classes A, C, and D, are serine  $\beta$  lactamases that hydrolyse their substrates by forming an acyl enzyme through an active site serine, whilst class B are metallo beta-lactamases that require a bivalent metal ion, usually  $Zn^{2+}$  for activation (Saliu et al., 2017).

The Bush-Jacoby-Mederiros classification scheme groups  $\beta$ -lactamases into three groups according to their functional similarities. Group 1, also known as cephalosporinases, encompasses the Ambler class C (Bush et al., 2010). This group is not inhibited by clavulanic acid and is more active on cephalosporins than benzylpenicillin. Group 2 includes the Ambler classes A and D and is the largest group due to the increasing identification of ESBLs. This group is referred to as the serine  $\beta$ -lactamases. Finally, group 3 comprises Ambler class B and is not inhibited by clavulanic acid (Bush et al., 2010).

### 2.9.2 ESBL classification

Generally, ESBL genes are located on plasmids and are known for their ability to hydrolyse oxyimino-cephalosporins and monobactams but not cephamycins such as cefoxitin and carbapenems like imipenem (Ayinla et al, 2023). ESBLs of class A include TEM, SHV, and CTX-M, while OXA belongs to class D (Rodríguez-Baño et al., 2008). Initially, TEM and SHV were the most predominant ESBLs and were frequently associated with *Klebsiella pneumoniae*. However, it was not until the early 2000s that CTX-M took precedence and started what is now coined as the CTX-M pandemic (Cantón et al., 2012). This group derived its name from the observation that the original CTX-M ESBLs were more effective against CTX rather than ceftazidime. Phylogenetic studies now show that CTX-M did not originate by

mutations from previous plasmid-mediated enzymes but through the mobilization of chromosomal genes from the *Kluyvera* species, particularly the *Kluyvera ascorbata* and *Kluyvera georgiana* (Cantón & Coque, 2006). CTX-M 15 and CTX-M 14 have been reported worldwide and carry the most importance as they affect animals, humans, and the environment (Ferreira et al., 2016).

## **2.10 History of ESBL detection in poultry in Zambia**

CTX-M has recently taken over as the main ESBL in both the hospital and poultry sector (Shawa et al., 2021). However, documentation of ESBL-producing bacteria in poultry is still a novel field in Zambia. A few studies regarding ESBLs in poultry were done in healthy chickens (Chishimba et al., 2016; Kaonga et al., 2019). However, the current study focuses on diseased chickens. One study revealed *E. coli* carrying the ESBL genes *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, or a combination (Chishimba et al., 2016). Another study identified *Salmonella Typhimurium* as a carrier of the gene *bla*<sub>CTX-M</sub> on commercial farms in the Copperbelt province (Kaonga et al., 2019). The dissemination of genes encoding for ESBL from bacteria of poultry origin to a human strain is possible via the transfer of plasmids by conjugation. A study by Shawa et al. (2021) compared multidrug-resistant (MDR) *E. coli* collected from poultry in Lusaka against *E. coli* found in patients at the University Teaching Hospital. Their results suggested clonal dissemination of MDR *E. coli* between poultry and humans, with the independent acquisition of the *bla*<sub>CTX-M</sub> gene (Shawa et al., 2021).

Chickens are the target populations for AMR surveillance and animal research because they are the most widely consumed proteins (Van Boeckel et al., 2015). The first round of AMR surveillance by the Fleming Fund and most studies in Zambia have been conducted on zoonotic pathogenic commensal bacteria found in the gastrointestinal tracts of healthy chickens (Nowbuth et al., 2023). The Fleming Fund sits under the Antimicrobial Resistance Coordinating Committee (AMRCC) at the Zambia National Health Institute. The AMRCC is mainly responsible for implementing the National Action Plan on antimicrobial resistance. Further, the constant application of antimicrobials in poultry production tends to result in an increase in AMR in both pathogenic and commensal bacteria, with commensal bacteria acting as a resistant gene pool reservoir for pathogenic bacteria (Tang et al., 2017). In this regard, commensal gastrointestinal bacteria can be considered as an indicator for the selection pressure for AMU (Nhung et al., 2017). However, very little is known about the status of AMR in disease-causing bacteria in poultry in Zambia, thus the current study aimed at identifying the

different kinds of bacteria found in diseased chickens in Lusaka, determine the ESBL genes found in various bacteria and estimate AMU and correlate it with the observed AMR pattern.

## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.0 Study site and design

This cross-sectional study was conducted in Lusaka district, the capital city of Zambia, with a total human population of 20,569,737, making it the most dense and diverse city (Central Statistical Office, 2022). Lusaka has an area of 360 km<sup>2</sup> and sits 1280m above sea level. It is located 15°25'South of the equator. Lusaka district is the leading region of intensive poultry activity in Zambia, having five hatcheries that produce day-old chicks. Of the total chick production, 65 % is sold in the Lusaka district (Ministry of Fisheries and Livestock, 2022).

Samples were collected from various veterinary post-mortem facilities which included LSCS, Agrivet Africa and the University of Zambia School of Veterinary Medicine pathology department (UNZA). The clients submitting the samples came from Lusaka Central, Kanyama, Munali, Matero, Chawama, and Mandeveu areas. Sampling was carried out between April 2021 to December 2021.

#### 3.1 Sample size calculation

The desired sample size was calculated using the Yamane formula.

$$n = N / (1 + Ne^2)$$

- **n** is the sample size
- **N** is the average number of chickens brought in a period of three months for postmortems from the three facilities
- **e** is the margin of error

95% confidence level was used and 0.05 margin of error.

$$n = 135 / (1 + 135 * 0.05^2)$$

$$n = 100.9$$

A total of 215 different internal organs (heart, liver, lung, and spleen) were collected from 91 diseased broiler chickens aged between three to five weeks. On post-mortem, different criteria

such as pneumonia, pericarditis, congestion of the heart, congestion of the lungs, perihepatitis, necrosis and bronzy liver were used to collect the samples.

### **3.2 Retrieval of antibiotic importation and sales data**

Antibiotic importation data was retrieved from the AMU data collection report (Goma et al., 2019). Importation data was obtained because as there no pharmaceutical industries for veterinary antibiotic manufacturing in the country. This was done in order to establish importation trends of veterinary antibiotics. Reliable antimicrobial use data for animals are currently not available in the country, making it difficult to determine which drugs are used in what quantities per species. Therefore, a focus was placed on powdered antibiotics and not injectables as injectables are seldomly used in poultry. In order to have oversight on what antibiotics are used in poultry, retrospective sales data was collected from (LSCS), the largest retail and wholesale outlet of veterinary pharmaceuticals in the country that dispenses antimicrobials on prescription basis. This was done to establish the trend in antimicrobial consumption. The product's brand name, active pharmaceutical ingredients and the pack size of powdered antibiotics was obtained and put into an excel sheet Microsoft Excel, 2010). The data was then transferred to R studio (Version 3). were the antibiotics were grouped according to the various antibiotic classes which included tetracyclines, penicillins, aminoglycosides and sulfonamides.

### **3.2 Bacterial isolation and identification of *Enterobacteriaceae***

#### **3.2.1 Media Preparation**

To make the agar, appropriate amounts of media (Table 1) which included MacConkey (Oxoid LTD, Hampshire UK), Slantez (Oxoid LTD, Hampshire UK), Xylose lysine deoxycholate (Oxoid LTD, Hampshire UK) and Nutrient Agar (Oxoid LTD, Hampshire UK) were weighed and dissolved into 500 ml of distilled water; this solution was brought to boil by heating in the microwave for 8 minutes and then autoclaved at 121 °C for 15 minutes. The media was then cooled, poured onto Petri-plates, and left to solidify overnight at 42°C.

Table 1: Quantities of media used to make agar.

<b>MEDIA</b>	<b>QUANTITY</b>
MacConkey (Oxoid LTD, Hampshire UK)	25g
Slantez (Oxoid LTD, Hampshire UK)	21g
Xylose lysine deoxycholate (Oxoid LTD, Hampshire UK)	28.5g
Nutrient Agar (Oxoid LTD, Hampshire UK)	14g

### 3.2.2 Sample inoculation

A sterile blade was used to incise into the tissue samples, after which a sterile loop was used to swab the incised tissue and streaked back and forth onto various agar. The agar plates were turned upside down and left to incubate at 37°C for 24hrs.

### 3.2.3 Biochemical tests

After culture, single colonies of the various bacteria were selected and subjected to different biochemical tests namely the sulfur indole motility test, triple sulfur iron test, the lysine iron agar, Simmons citrate agar test and the urease test for phenotypic characterisation. This was done by inoculating a single colony of bacteria into the different biochemical sugars that were made as described below:

Sulfur Indole Motility (SIM) media (Oxoid LTD, Hampshire UK); 18.115 g of SIM agar was weighed and dissolved in 500mls of distilled water and was brought to the boil by heating in the microwave for 8 minutes, then dispensed in 5 ml test tubes. The media was then autoclaved at 121 °C for 15 minutes, the test tubes were left in an upright position for 24 hours for the media to solidify.

Triple Sulfur Iron (TSI) media (Oxoid LTD, Hampshire UK); 30.3g of TSI agar was weighed and dissolved in 500mls of distilled water brought to the boil by heating in the microwave for 8 minutes, then dispensed 5mls into test tubes. This media was then autoclaved at 121 °C for 15 minutes, and the test tubes were then slanted and left to solidify for 24 hours.

Lysine Iron Agar (LIA) media (Oxoid LTD, Hampshire UK); 17g of LIA agar was weighed and dissolved in 500mls of distilled water and brought to the boil by heating in the microwave

for 8 minutes, then dispensed 5mls into test tubes. This media was then autoclaved at 121 °C for 15 minutes; the test tubes were slanted and left to solidify for 24 hours.

Simmon's citrate agar (Oxoid LTD, Hampshire UK); 11.5g of citrate agar was weighed and dissolved in 500mls of distilled water and brought to the boil by heating in the microwave for 8 minutes, then dispensed 5mls into test tubes. This media was then autoclaved at 121 °C for 15 minutes. Finally, the test tubes were slanted and left to solidify for 24 hours.

Urease media (Oxoid LTD, Hampshire UK); 12.6g of urease agar was weighed and dissolved in 500mls of distilled water and brought to the boil by heating in the microwave for 8 minutes, then autoclaved at 121 °C for 15 minutes. After the media was autoclaved, it was left to cool off up to 45-50 °C; 4 g of urea crystals were added to 10 ml of distilled water to make a 40% urea solution. Next, 25 ml was drawn and added to the urease media together with three drops of hydrochloric acid.

After media preparation, a single pure colony of bacteria was picked using a sterile inoculating needle and stabbed into the medium and then streaked on the surface the of TSI agar slants. The tubes were then incubated at 37°C for 18–24h with loosened caps and results recorded. Change in color of the butt and slant from red to yellow was noted, as well as presence or absence of gas and hydrogen sulfide. Materials from the same pure colonies of bacteria used for TSI test were later used for all the other biochemical tests. Sterile inoculating needle was used for each subsequent test. Bacteria was lightly stabbed into the Citrate agar slant ~1 cm from the bottom of the tube and made sure not to twist the needle. The tubes were then incubated at 37°C for 18–24 h with loosened caps and results recorded. Change in colour of the media from green to blue was noted as a positive result while no change in colour of the media was noted as a negative result. A single stab of bacteria was made into the tube of SIM agar ~1 cm from the bottom of the tube and made sure not to twist the needle. The tubes were then incubated at 37°C for 18–24 h with loosened caps and results recorded. Motility and presence or absence of hydrogen sulfide were noted.

### **3.3 Antimicrobial susceptibility testing (AST) of *Enterobacteriaceae***

AST was done using the Kirby Bauer disc diffusion method. Briefly, a suspension was made by suspending the various bacteria colonies in 2 ml of normal saline and streaking on Mueller-Hinton agar plates made by dissolving 19 g of Mueller-Hinton powder (Becton, Dickinson and Company, Franklin lakes, USA) in 500ml of distilled water and sterilised by autoclaving it for 15 minutes at 121°C. The mixture was poured into Petri-dishes and left to solidify for 24 hrs.

Later, paper discs impregnated with antibiotics were placed 2.5 cm apart and incubated aerobically at 37°C for 24hrs. The antibiotics disks (Becton, Dickinson and Company, Franklin Lakes, USA) used included tetracycline (30µg), ampicillin (10µg), erythromycin (10µg), cotrimoxazole (10µg), amoxicillin (10µg), gentamicin (10µg), and penicillin (30µg). Zones of inhibition were measured with a digital vernier callipers, and endpoints were determined based on the areas showing no bacterial growth visible to the naked eye. Interpretation of Resistant or Sensitivity was based on the 2021 Clinical and Laboratory Standards Institute (CLSI) guidelines (Patel et al., 2021.).

### **3.4 Cefotaxime MIC**

To determine cefotaxime resistance, the isolates were grown on cefotaxime infused Luria Bertani (LB) agar (Oxoid LTD, Hampshire, UK). This was done by adding 12.5g of LB to 500 ml distilled water and stirred. The mixture was then sterilized by autoclaving it for 20 minutes. Once cooled, CTX was added to the LB to a final concentration of 1 µg/ml, poured onto Petri dishes, and left to solidify. Once solidified, the bacterial samples were streaked onto the plates and incubated for 18 hrs at 37°C.

After the incubation process, 3 ml of the CTX-supplemented LB (1µg/ml) was transferred into test tubes. Single colonies of the bacteria that grew on the solid cefotaxime infused LB agar plates were picked using sterile loops, transferred to the test tubes, and vortexed. The test tubes were loosely sealed, placed in a shaker at 175 rpm at 37°C, and left to incubate for 18 hrs. The overnight cultures that showed growth were then diluted 10<sup>4</sup>-fold and added in triplicates of a serial dilution of cefotaxime in a 96 well plate. The 96 well plate was then incubated at 37°C for 18hrs.

### **3.5 DNA extraction**

#### **3.5.1 Preparation of cell pellets for gDNA extraction**

An overnight bacterial culture was centrifuged at 4500xg for 10 minutes at 4°C. The supernatant was pipetted out, and the pellet was subjected to gDNA extraction using the Zymo® DNA extraction kit, according to the manufacturer's instructions.

### 3.5.2 DNA extraction using the Zymo® DNA extraction kit.

The pellet was added to bashing lysis tube were 750µl of lysis solution was added and capped tightly. The lysis tube was secured in a bead beater at maximum speed for 5 minutes. After which the lysis tubes were centrifuged at 10000xg for one minute. A total volume of 400µl of the supernatant was transferred to a collection tube through the spin filter and centrifuged at 7000xg for one minute. 1200µl of DNA binding buffer was then added to the filtrate in the collection tube. 800µl of the mixture was the transferred to a lIC-Z column in a collection tube .and centrifuged at 10000xg for one minute. The flow through was discarded from the collection tube and the step was repeated. 400µl of DNA wash buffer 1 was added to the lIC-Z column in a collection tube and centrifuged at 10000xg for one minute and the flow was discarded. 200µl of DNA wash buffer 2 was then added to the lIC-Z column and centrifuged at 10000xg for one minute. The lIC-Z column was transferred to a 1.5µl microcentrifuge tube and 100µl of DNase free water was added directly to the column matrix and incubated for one minute. This was then centrifuged at 10000 x g to elute the DNA. The lll-HRC filter was placed in a new collection tube. 600µl of HRC prep solution was added and centrifuged at 8000xg for three minutes. The eluted DNA was transferred to a prepared lll-HRC filter in a clean 1.5ml microcentrifuge tube and centrifuged at 16000xg for three minutes before being stored at -30°C

### 3.6 Detection of *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub>, and *bla*<sub>SHV</sub> by PCR

In order to detect ESBL encoding genes, PCR was performed with a total reaction volume of 50µl consisting of 5µl 10x ExTaq buffer, 4µl of DNTP mixture, 2µl of DNA template, 5µl of both forward and reverse primers (10µM each) (Table 2), 28.75µl of nuclease free water and 0.25µl of Takara Ex Taq HS. PCR reactions conditions for *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA</sub> were: denaturation at 98°C for 2 minutes, followed by 25 cycles of template denaturation at 98°C for 10 seconds, annealing at 60.5°C for 5 seconds and extension at 72°C for 1 minute with a final extension at 72°C for 8 minutes. The PCR conditions for *bla*<sub>TEM</sub> were 94°C for 7 minutes, followed by 30 cycles of template denaturation at 94°C for 30 seconds, primer annealing at 57°C for 30 seconds and 72°C for 5 minutes, whilst the final extension of 72°C was for 5 minutes. The PCR products were visualized on 1.5% agarose gel coated with ethidium bromide.

Table 2 Primer sequences for the genes *bla<sub>TEM</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>SHV</sub>*, and *bla<sub>OXA</sub>*

PRIMERS	TARGET GENE	SEQUENCE 5'-3'	EXPECTED AMPLICON SIZE
TEM1F TEM1R	<i>bla<sub>TEM</sub></i>	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	864
SHVF SHVR	<i>bla<sub>SHV</sub></i>	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	865
CTX-MA1 CTX-MA2	<i>bla<sub>CTX-M</sub></i>	*SCSATGTGCAG <sup>‡</sup> YACCAGTAA CCGC <sup>‡</sup> RATATGRTTGGTGGTG	544

Note: \*S = G or C, <sup>‡</sup>Y = C or T, <sup>‡</sup>R = A or T.

### 3.6 Sequencing of PCR Products

#### 3.6.1 Purification of DNA

The wizard SV gel and PCR clean-up recovery kit was used to purify *bla<sub>CTX-M</sub>* and *bla<sub>TEM</sub>* PCR products. Briefly, 53µl of membrane binding solution was added to the PCR product, incubated at room temperature for 1 minute, and then transferred to a spin column for centrifugation at 16000xg for 1 minute. Next, the flow through was discarded, and 500µl DNA wash buffer was added and centrifuged at 16000xg for 5 minutes. As in the previous step, the flow through was discarded, and this step was repeated one more time. Finally, 30µl of DNA elution buffer was added directly to the column, inserted in a new tube, and centrifuged for 1 minute to elute and collect the purified PCR product.

#### 3.6.2 Cycle sequencing reaction

The Big Dye™ Terminator V3.1 sequencing kit (Thermo Fisher Scientific) was used to sequence the purified PCR product. The reaction volumes were as follows; 0.5µl big dye, 3.8µl sequencing buffer, 0.5µM primer, 13.5µl nuclease-free water, and 4µl (template) purified DNA. The thermal cycler conditions were set under the following conditions: initial denaturation at 96°C for 45 seconds, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes.

### **3.6.3 Ethanol precipitation and capillary electrophoresis**

Excess buffers and dNTPs were removed from the cycle sequencing products using the ethanol precipitation method. For 20ul of each sample, 2µl of EDTA, 2µl of acetate, and 90 µl of absolute alcohol were added and then incubated for 10 minutes in the dark. The mixture was then centrifuged at 15000xg for 20 minutes and the supernatant discarded. 200µl of 70% alcohol was added and then centrifuged for 5 minutes, and the supernatant was discarded. After this, the samples were wrapped in aluminum foil, vacuum dried for 10 minutes, dissolved in 20µl of highly ionized formaldehyde, and denatured at 95°C for 5 minutes. The purified and denatured samples were then subjected to capillary electrophoresis using the seq studio analyzer.

### **3.7 Data and sequence analysis**

All graphs and tables were generated using R version 4.2.0. Nucleotide sequences were assembled using GENETYX software version 4.0 (GENETYX cooperation Tokyo). The sequences were subjected to the BLAST tool on the National Centre for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST>), which was used for comparing and analysing the nucleotide sequences. The *bla*<sub>CTX-M</sub> nucleotide sequences were then aligned with other nucleotide sequences obtained from GenBank using Clustal X2 incorporated in Genetyx version 4.0 (GENETYX cooperation Tokyo).

### **3.8 Ethical clearance**

Ethical clearance was sought from the Excellence in Research Ethics and Science (ERES), reference number 2023-Mar-012. In addition, permission to collect samples was obtained from the head of departments from the different institutions.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.0 Isolation of Enterobacteriaceae

A total of 215 samples were collected from diseased broiler chickens aged between three to five weeks across Lusaka. A total of 103 (103/215, 43%) tested positive for pathogenic GNB. The major pathogenic *Enterobacteriaceae* of clinical relevance identified were *Enterobacter* (43/103, 41.7%), *E. coli* (20/103, 19.4%), *Salmonella* spp (10/103, 9.7%), *Proteus* spp (10/103, 9.7%) and *Shigella* spp (8/103, 7.8%) (Table 3). Other isolated bacteria included *Yersinia* spp, *Morganella* spp and *Klebsiella* spp, which accounted for 19.5%.

Table 3 Bacterial species identified from diseased chickens collected from UNZA, LSCS and Agrivet.

Species	Facility			Total
	UNZA	LSCS	AGRIVET	
<i>Enterobacter</i>	2	26	15	43
<i>E. coli</i>	5	11	4	20
<i>Salmonella</i>	2	8	0	10
<i>Klebsiella</i>	0	1	0	1
<i>Shigella</i>	0	5	3	8
<i>Yersinia</i>	0	2	0	2
<i>Citrobacter</i>	0	4	0	4
<i>Vibrio</i>	1	0	0	1
<i>Proteus</i>	4	5	1	10
<i>Morganella</i>	0	4	0	4
Total	14	66	23	103

#### 4.1 Antibiotic sensitivity testing

From the above-identified *Enterobacteriaceae*, five randomly picked *Enterobacter* isolates, five *E. coli* samples, four *Salmonella* samples, and two samples of *Shigella* were subjected to antibiotic sensitivity testing (AST) against tetracycline, gentamycin, amoxicillin, ampicillin and cotrimoxazole. The discs were selected based on the highest selling antibiotics from LSCS.

Findings from this study showed the highest resistance was to tetracycline (11/16, 68.8%), amoxicillin (10/16, 62.5%), ampicillin (9/16 56.2%), cotrimoxazole (7/16,43.8 %) and gentamycin (1/16, 6.2%) (Figure 2). In addition, MDR was observed in *E. coli* (3/5, 60%), *Enterobacter* (2/5, 40%), and *Shigella* (1/2, 50%), while *Salmonella* showed complete susceptibility to all antibiotics (Figure 3). MDR was defined as antimicrobial resistance shown when a bacterial isolate was resistant to three or more classes of antibiotics. Based on the location of the sampling facility (Magiorakos et al., 2012), most of the samples showing resistance were from LSCS (Table 4), and this can be attributed to its central location compared to the other locations.

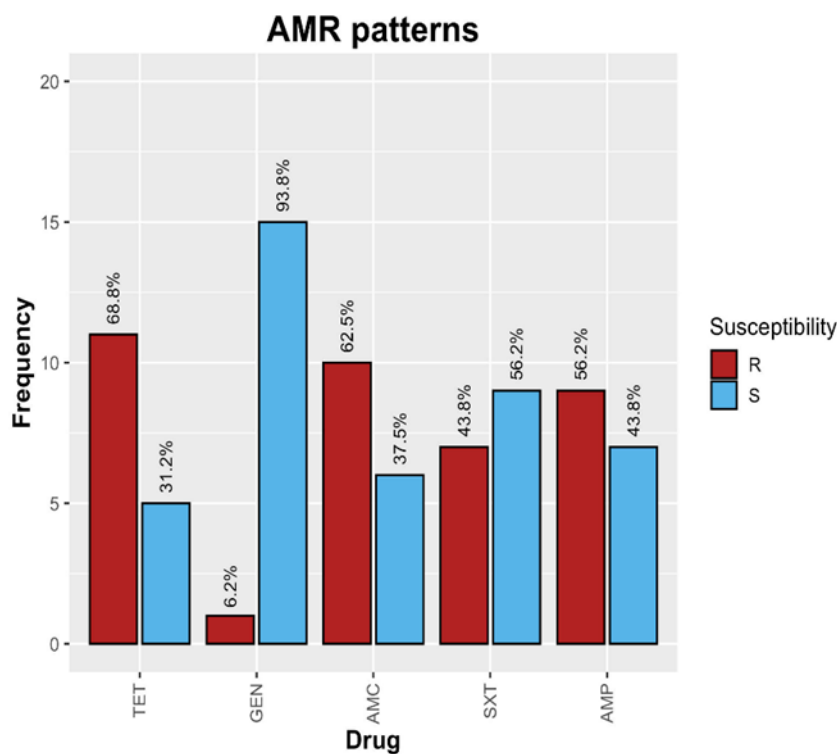


Figure 2. A graph illustrating the antibiotics to which the bacteria were resistant

Table 4 Sampling points for isolates for AST

FACILITY	PATHOGENS			
	<i>Enterobacter</i>	<i>E. coli</i>	<i>Salmonella</i>	<i>Shigella</i>
LSCS	3	3	3	1
Agrivet	1	1	0	1
UNZA	1	1	1	0

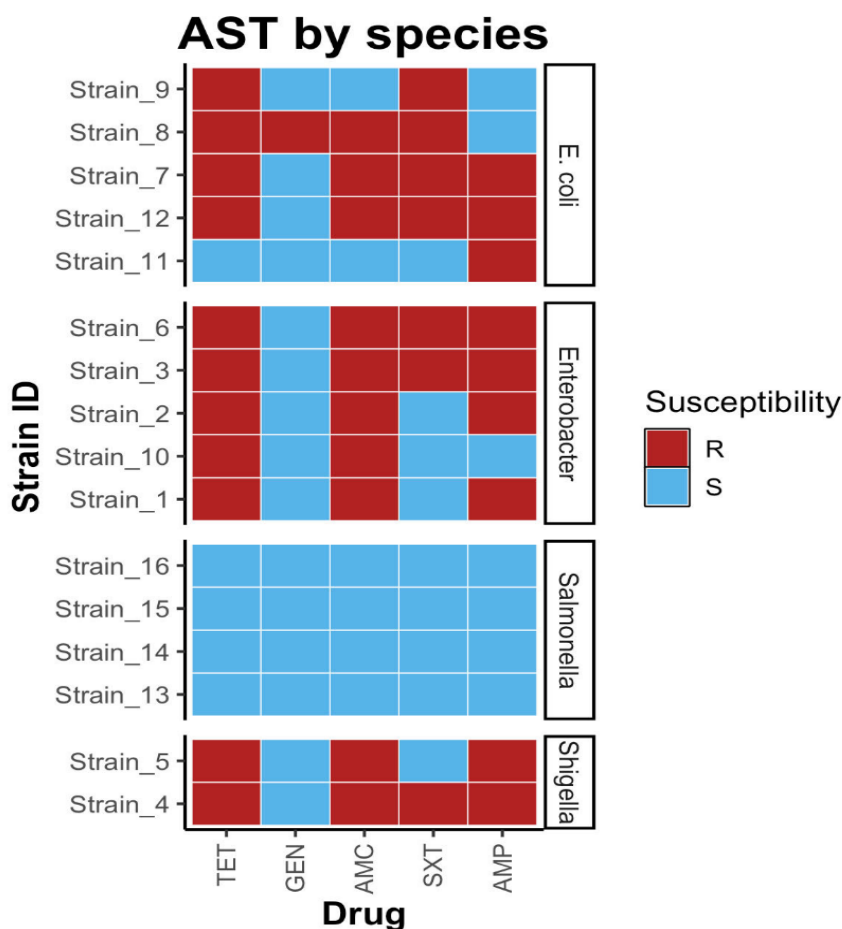


Figure 3 A graph illustrating the AST results by species

#### 4.2 High-level CTX resistance among *E. coli* and *Enterobacter*

To determine cefotaxime resistance and screen for ESBLs, the 103 positive isolates were inoculated on CTX-supplemented LB agar. Single colonies of the samples that showed growth were picked and transferred to CTX-supplemented LB broth. Of the 103 isolates, fourteen isolates were resistant to cefotaxime. Of the 14 isolates, eight (8/14, 57.1%) were *Enterobacter* and six (6/14, 42.9%) *E. coli* showed growth after the overnight culture in the CTX-supplemented LB liquid media. To quantify CTX resistance, the 14 isolates were subjected to broth microdilution. All samples showed resistance to CTX, with CTX MICs > 1µg/ml. Notably, one *Enterobacter* and three *E. coli* strains exhibited high-level resistance with CTX MICs of at least 512µg/ml.

PCR tests revealed that all six of the *E. coli* isolates were positive for *bla*<sub>CTX-M</sub>. However, only four (4/6, 66.7%) of these *E. coli* positives isolates carried both the *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> genes.

On the other hand, all eight of the *Enterobacter* were positive only for *bla*<sub>TEM</sub> whilst only four harboured both *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>. None of the isolates harboured the *bla*<sub>OXA</sub> and *bla*<sub>SHV</sub> genes (Figure 4).

Table 5. CTX MICs for *E. coli* and *Enterobacter*

SAMPLE ID	ORGANISM	CTX MIC	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>CTX-M</sub>
LS 1	<i>Enterobacter</i>	2	+	-
LS 2	<i>E. coli</i>	4	-	-
LS 3	<i>Enterobacter</i>	2	+	-
UZ 1	<i>E. coli</i>	≥ 512	+	+
LS 4	<i>Enterobacter</i>	2	+	-
AV 1	<i>E. coli</i>	2	+	-
LS 5	<i>E. coli</i>	≥ 512	+	+
LS 6	<i>Enterobacter</i>	128	+	+
AV 2	<i>Enterobacter</i>	≥ 512	+	-
AV 3	<i>Enterobacter</i>	2	+	-
UZ 2	<i>Enterobacter</i>	2	+	+
LS 7	<i>E. coli</i>	≥ 512	-	+
LS 8	<i>E. coli</i>	16	+	+
LS 9	<i>Enterobacter</i>	128	+	-

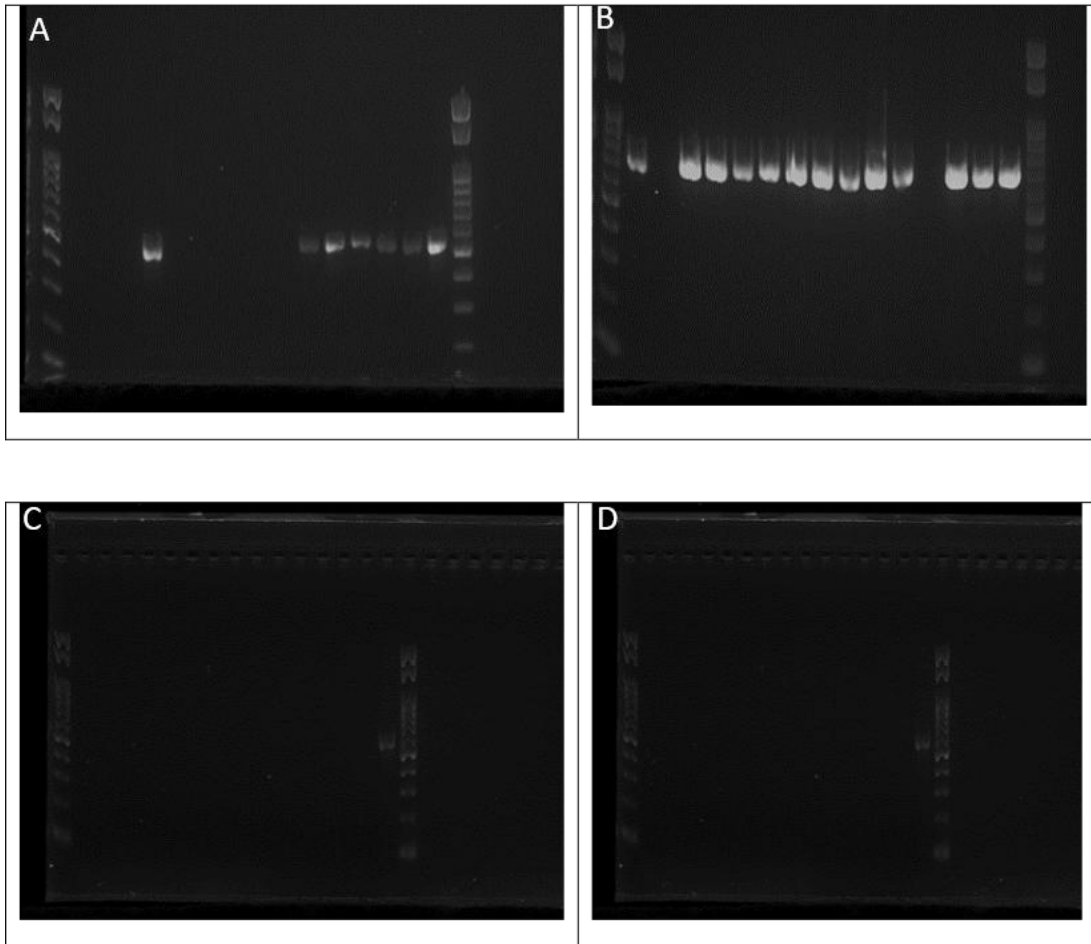


Figure 4: Gel images showing PCR results: A shows positive *bla*<sub>CTX-M</sub>, B shows positive *bla*<sub>TEM</sub>, C shows no positive *bla*<sub>SHV</sub>, D shows no positive *bla*<sub>OXA</sub>.

#### 4.3 Sequencing of the *bla*<sub>CTX-M</sub> gene

Amplicon sequencing of the six *bla*<sub>CTX-M</sub> positive *E. coli* strains showed that 5/6 (83.3%) of the sequences were highly similar with a nucleotide homology of 86.6% to 97.95% to *bla*<sub>CTX-M-15</sub> allele identified in *E. coli* from Egypt (MN096663.1).

Table 6: Sequencing results of the gene *bla<sub>CTX-M</sub>* from the *E. coli* isolates.

SAMPLE ID	LOCATION	ORGANISM	<i>bla<sub>CTX-M</sub></i> allele
UZ 1	Lusaka	<i>E. coli</i>	<i>bla<sub>CTX-M-15</sub></i>
LS 5	Lusaka	<i>E. coli</i>	<i>bla<sub>CTX-M-15</sub></i>
LS 6	Lusaka	<i>E. coli</i>	<i>bla<sub>CTX-M-15</sub></i>
LS 7	Lusaka	<i>E. coli</i>	<i>bla<sub>CTX-M-15</sub></i>
LS8	Lusaka	<i>E. coli</i>	<i>bla<sub>CTX-M-15</sub></i>

#### 4.4 Antibiotic importation and sales

Antibiotic importation data from the livestock and aquaculture report showed that tetracyclines, sulphonamides and penicillins were the most imported antibiotics (Figure 5). The group of tetracyclines imported included doxycyclines, oxytetracyclines and chlortetracyclines. The high influx of tetracyclines could be attributed to their inexpensive nature, broad spectrum activity and minimal side effects. The second most imported group were the sulphonamides (Figure 5) which included sulfamethoxazole, trimethoprim and the sulfadiazine and trimethoprim combination.

The sales data obtained from a major wholesale and retail pharmaceutical company (Figure 6) for the period between March 2021 to February 2022 showed that all poultry antimicrobials sold were for oral administration and were backed by a prescription from a veterinarian. Throughout the year the trend in the sales remained the same with tetracyclines being the most sold antibiotics followed by sulphonamides, penicillins and aminoglycosides (Figure 6).

Overall, the data showed high importation levels of tetracyclines, sulphonamides and penicillins, and this was in agreement with the sales data (Figure 6) which equally showed high sales volumes of the named antimicrobials. This information was used to select antibiotics for use in the follow up susceptibility tests.

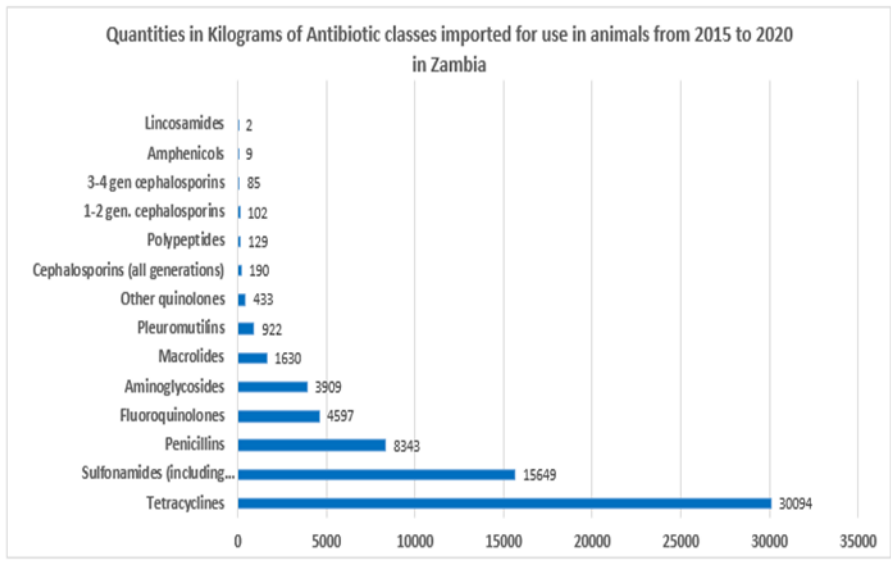


Figure 5 Importation of antibiotics from 2015 to 2020 retrieved from the AMU data collection report.

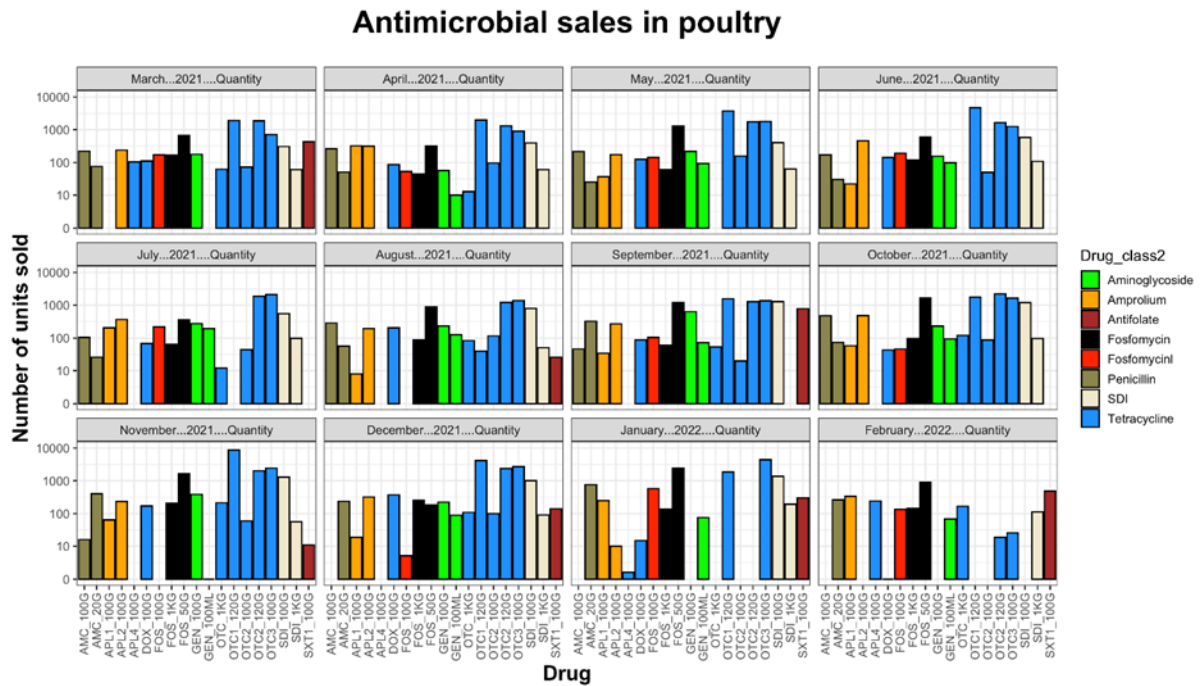


Figure 6 Sales data from March 2021 to February 2022 retrieved from LSCS.

Note: SDI= sulphonamides

## CHAPTER FIVE

### 5.0 DISCUSSION

Over the past few years AMR among the *Enterobacteriaceae* family has skyrocketed in Zambia. Studies pertaining to AMR have frequently been reported in *E. coli* and *Salmonella* in healthy chickens (Muonga et al., 2019; Phiri et al., 2020). In this study, the most predominant species isolated was *Enterobacter* (43/103,47%) which showed multidrug resistance to tetracyclines, sulphonamides, ampicillins and amoxicillin. This was consistent with findings in Bangladesh that revealed the presence of *Enterobacter* showing resistance to ampicillin, sulphonamides and penicillin (Nandi et al., 2013). A similar AMR profile was observed in Europe where all samples were resistant to ampicillin, sulphonamides and erythromycin (Cornaglia et al., 2004).

In our study 20/103,19.4% of *E. coli* was detected. These results are similar to the findings of Ibrahim et al and Ameen-Ur-Rashid et al who found about 34% and 35% *E. coli* isolates in diseased chickens in Jordan and Pakistan respectively (Ameen-Ur-Rashid, 2016; Ibrahim et al., 2019). Higher percentages of *E. coli* (75.5%) isolates observed in a study by Engy Ahmed Hamed et al (Hamed et al., 2021) which could be attributed to the fact that his study was done over a four-year period. Our *E. coli* isolates showed multidrug drug resistance to tetracycline's, penicillin's and sulphomonamides, a similar observation was made in Arusha however, most of the *E. coli* isolates in Arusha were more resistant to sulphonamides which can be explained by them being cheaper in price in the Arusha region (Kiiti et al., 2021).

Ten (10/103,9.7%) of *Salmonella* was detected in this study, these finds were lower compared to a study on the Copperbelt, which reported 17.7% occurrence of *Salmonella* in commercial poultry farms Interestingly, the four *Salmonella* strains sampled in this study showed susceptibility to all antibiotics. Future studies must target larger numbers to account for rare phenotypes and genotypes.

Over the recent years, *Enterobacter* has emerged as the third *Enterobacteriaceae* showing resistance to third generation cephalosporins after *E. coli* and *klebsiella* (Nandi et al., 2013). This study revealed the presence of CTX resistance not only in *E. coli* but *Enterobacter* as well. One (1/8, 12.5%) of *Enterobacter* isolates and three (3/6, 50%) of *E. coli* showed significantly high levels of CTX MICs of at least 512µg/ml. These results were consistent with findings in Korea which detected high MICs of 512 in *E. coli* (Seo et al 2021), Despite bacteria having

different mechanism of acquiring AMR, the cumulative effect of using third generation cephalosporins in poultry contributes to increased levels of CTX resistance (Apatha, 2009). The oral treatment of chickens has contributed to increased levels of resistance as this is the main treatment route which allows for the uptake of antimicrobials by both infected and healthy chickens. Oral treatment regimens in chickens are prone to contamination with antimicrobials either by application or exposure to excreted feces from treated chickens (Simoneit et al., 2015).

This is the first study to describe the presence of ESBL encoding genes in diseased chickens in Zambia. This study revealed the presence of CTX-M- and TEM in *E. coli* (6/14,42.8%) and TEM in *Enterobacter* (8/14,57.1%) isolates. Similarly, 21% of *E. coli* isolated in rural Nepal harbored the gene *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> (Subramanya et al., 2020). Studies done in Zambia reported 13% prevalence of *bla*<sub>CTX-M</sub> in *E. coli* isolated from market ready chickens (Muonga et al., 2019). Another study done on commercial poultry farms on the Copperbelt (Kaonga et al., 2019), revealed a 12.8% occurrence of *bla*<sub>CTX-M</sub> in *Salmonella*. Both findings were slightly lower compared to this study which showed 42% presence of CTX-M in *E. coli*. The increase in the presence of CTX-M could be attributed to the general increase in antimicrobial usage over recent years. However, both these studies did not determine what allele of CTX-M was present amplicon sequencing of *bla*<sub>CTX-M</sub> revealed the presence of *bla*<sub>CTX-M-15</sub> in 5/6, 83% of the *E. coli* isolates. These findings are in line with the fact that CTX-M 15 is the most widely spread ESBL genotypes globally (Shams et al., 2019). Studies done in Korea also detected the presence of *bla*<sub>CTX-M-15</sub> in 33% of the *E. coli* isolates (Seo et al, 2021). The selective pressure caused by the indiscriminate use of antimicrobials promotes its global dominance. The emergence of CTX-M-15 could also be attributed to the plasmid dissemination and clonal spread of *E. coli*. The CTX-M-15 *E. coli* positive isolates found in this study also portrayed a MDR phenotype as well as a co-resistance to tetracyclines and sulfonamides. The prevalence of MDR observed in this study could be attributed to the fact that in Zambia, farmers are still using antibiotics to optimize on their production (Mudenda et al, 2022). This is supported by antibiotic sales data that shows that large amounts of antibiotics belonging to various classes are sold (Figure 6). Moreover, MDR can also be selected by only one antibiotic since AMR genes usually reside together on mobile genetic elements, allowing for simultaneous selection by a single drug (Magiorakos et al., 2012). MDR bacteria pose significant danger to the public

as common infections which were once easily treatable become fatal owing to the expensive nature of stronger antimicrobials.

*bla*<sub>CTX-M-14</sub> genes have been detected in human *E. coli* isolates that cause urinary tract infections (Irrgang et al., 2017). The transfer of ESBL encoding genes from poultry *E. coli* to *E. coli* from a human recipient through conjugation has been recorded throughout the world (Ferreira et al., 2016; Zeynudin et al., 2018). In Zambia the gene *bla*<sub>CTX-M-15</sub> was also detected in human *E. coli* by Shawa et al, who further went on to suggest that there could be clonal dissemination of MDR *E. coli* between poultry and humans with possible independent acquisition of the *bla*<sub>CTM-M</sub> gene (Shawa et al., 2021). This could be attributed to the poor handling of poultry in abattoirs or the increase in backyard poultry barns. These findings have led to the assumption poultry acts as a reservoir for ESBL producing bacteria. In Ghana the ESBL gene *bla*<sub>CTX-M-15</sub> was found in both poultry (96%) and human (92%) *E. coli* isolates., which suggested clonal transmission between poultry and human reservoirs (Falgenhauer et al., 2019). Similarly, studies from Sweden and Romania found evidence for the transmission of *bla*<sub>CTX-M</sub> via plasmids of *E. coli* isolates between poultry and humans.

In this study all six isolates of *E. coli* carried the *bla*<sub>CTX-M</sub> and 4/6 carried *bla*<sub>TEM</sub> gene. This suggested that ESBL plasmids are capable of carrying more than one  $\beta$  lactamase gene (Rottier et al., 2012). The co-occurrence of the genes *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> have mostly been reported in chickens with disease associated symptoms as can be seen in this study (Maciucă et al., 2015).

The co-occurrence of *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> in *E. coli* poses a major challenge in the treatment of infections as resistance is now conferred to both penicillins and cephalosporins (Maciucă et al., 2015).

Unlike *E. coli*, the clinical relevance of *Enterobacter* in poultry has not been well documented in Zambia which can be attributed to the fact that *Enterobacter* rarely causes disease in immunocompetent chickens (Nandi et al., 2013). Data from this study has shown that *Enterobacter* does indeed carry the ESBL encoding gene *bla*<sub>TEM</sub> which may in turn be transmitted to humans. The observed *bla*<sub>TEM</sub> confers resistance to penicillin and first generation cephalosporins like cephaloridine. The MDR phenotype profile associated with TEM in *Enterobacter* is alarming and calls for the urgent need for diagnostics before dispensing antimicrobials. This will allow for the more prudent use of antimicrobials and in turn limit the

spread of AMR. Since *Enterobacter* rarely causes disease in immunocompetent chickens, appropriate biosecurity measures in poultry houses play a vital role in preventing its spread (Subramanya et al., 2020).

The documentation of antimicrobial importation and sales data is vital as it serves as a basis for prevention programs and policy decisions. In this study, antibiotic disc selection was based on sales data from March 2021 to February 2022 from one of the country's largest wholesale and retail outlets of animal pharmaceuticals. The highest group of antibiotics sold belonged to the tetracyclines, followed by the penicillins, which corresponds to the AST results that showed that 68.8% of the isolates were resistant to tetracyclines, 62.5% to amoxicillin, and 56.2% to ampicillin. The high influx of sulphonamides could be attributed to their use in the treatment of coccidiosis and colibacillosis which are amongst the most common poultry infections. The penicillin group included amoxicillins and ampicillins (Azabo et al., 2022). The high levels of tetracycline and penicillin resistance observed in this study are similar to what has been reported previously (Chishimba et al., 2016). This is attributed to huge quantities of tetracyclines, and penicillins being imported and sold in the country, as reflected in the sales data presented in this study. This concurs with a study done in Tanzania and Cameroon where tetracyclines, penicillins and sulfonamides were the most used antimicrobials in poultry production (Azabo et al., 2022; Kamini et al., 2016).

The cumulative rise in AMR could be attributed to the use of antibiotics for infection control rather than treatment of disease. This is done by introducing antibiotics in the first week of the chick's life to counter infections that may occur due to breaches in biosecurity (Jeni et al., 2021). Despite large agro shops dispensing antimicrobials by prescription, farmers still have access to antimicrobials in smaller outlets in the central business district. The lack of knowledge by farmers is a major attribute to the development of MDR as some farmers are of the belief that the use of different antibiotics lowers the chances of AMR development (Malama, et al., 2022). To avoid profit losses from infections some farmers continue to medicate chickens despite being aware of the withdraw periods (Chilawa et al., 2023).

## CHAPTER SIX

### 6.0 CONCLUSION AND RECOMMENDATIONS

#### 6.1 Conclusions

This is the first study to be conducted in diseased chickens in Lusaka. In addition, *Enterobacter*, *E. coli*, *Salmonella*, *Shigella*, *Yersinia*, *Morganella*, *Proteus*, and *Klebsiella* were also isolated from diseased chickens in Lusaka. MDR as well as ESBL genes (*bla<sub>CTX-M</sub>* and *bla<sub>TEM</sub>*) were observed in both *Enterobacter* and *E. coli*. Overall, a possible relationship between AMU and AST patterns was observed with high levels of tetracycline, sulfonamide and penicillin resistance observed, coinciding with the high sales of these antimicrobials.

#### 6.1 Recommendations

- This study revealed the presence of multi drug resistant ESBL producing *Enterobacteriaceae*. Therefore, interventions should aim to reduce exposure of broilers to ESBL producing bacteria thereby reducing the need to use antibiotics. This can be done by improving biosecurity.
- Heightening national AMR awareness campaigns targeted at both farmers and veterinary professionals. Farmers should be made aware of the consequences of using antibiotics indiscriminately as this leads to selection of resistant bacteria in the intestinal flora of birds thereby contributing to AMR development. However, human behaviour will need to be monitored as acquisition of knowledge does not necessarily translate to new practices.
- Strengthening surveillance and research by the government is key as it plays an important role in early detection of resistant strains of pathogens and informs treatments decisions. This is a key step to combating AMR as it determines the resistance burden from different ecological niches. The information attained from surveillance aids in the formation of evidence-based policy.

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## **APPENDICES**

Appendix A-ethical approval letters



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Tel: +260 955 155 633  
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24<sup>th</sup> April, 2023.

**Ref. No. 2023-Mar-012**

The Principal Investigator  
Chikwanda Chileshe  
The University of Zambia  
School of Veterinary Medicine  
P.O. Box 32379  
Lusaka, Zambia.

Dear Chikwanda Chileshe

**RE: THE DETECTION OF EXTENDED SPECTRUM BETA LACTAMASES IN DISEASED CHICKENS IN LUSAKA.**

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	Ordinary	Approval No. <b>2023-Mar-012</b>
Approval and Expiry Date	Approval Date: 24 <sup>th</sup> April, 2023	Expiry Date: 23 <sup>rd</sup> April, 2024
Protocol Version and Date	Version - Nil.	23 <sup>rd</sup> April, 2024
Information Sheet, Consent Forms and Dates	• English	23 <sup>rd</sup> April, 2024
Consent form ID and Date	Version - Nil	23 <sup>rd</sup> April, 2024
Recruitment Materials	Nil	23 <sup>rd</sup> April, 2024
Other Study Documents	-	23 <sup>rd</sup> April, 2024
Number of participants approved for study		23 <sup>rd</sup> April, 2024

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered

to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

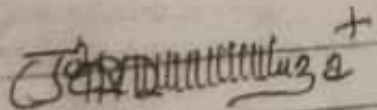
### **Conditions of Approval**

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,  
**ERES CONVERGE IRB**



Dr. Jason Mwanza  
Dip. Clin. Med. Sc., BA., M.Sc., PhD  
**CHAIRPERSON**