PREVALENCE AND CHARACTERISATION OF CTX-M-TYPE EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING *SALMONELLA* TYPHIMURIUM IN POULTRY FARMS IN THE COPPERBELT PROVINCE, ZAMBIA

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ABSTRACT

Poultry is a major reservoir of *Salmonella* worldwide associated with increasing incidences of strains producing ESBL enzymes that are capable of inactivating a wide variety of β-lactam antibiotics. CTX-M ESBLs have been described in *S.* Typhimurium isolates with resistance genes located on transferable plasmids. The aim of this study was to determine the prevalence and antimicrobial resistance of *S*. Typhimurium, prevalence and characterise CTX-M-Type ESBL-producing *S.* Typhimurium in poultry farms in the Copperbelt Province in Zambia. A cross-section study design was used which involved five districts. One poultry farm per district was randomly selected for sampling of birds. An overall of 384 faecal samples were analysed for the presence of *S*. Typhimurium using microbiological and molecular methods. *S.* Typhimurium was detected at 17.7% prevalence in poultry farms of which 12.8% were found harboring the CTX-M-Type ESBL genes. Antibiotic use, purpose of use, withdrawal period, manure handling, hygiene and bio-security were found to be associated with this prevalence. Chingola district had a prevalence of 7.3% followed by Ndola district with a prevalence of 5.2%, Luanshya district 2.9%, Kitwe 1.6% and Mufulira 0.8%. Further findings indicated that all the isolates showed 100% resistance to tetracycline followed by erythromycin with 97.1%, ampicillin and amoxicillin with 91.2%. A study in Nigeria, reported a prevalence of 16.0% *S*. Typhimurium in poultry farms and china reported a prevalence on 17.76% CTX-M-Type producing *Salmonella* in foodborne animals which are slightly similar with findings from this study. Antibiotic resistance to third-generation cephalosporins was at 58.8% cefotaxime and 54.4% ceftazidime. This could be due to the presence of cefotaximases that have more hydrolytic activity to cefotaxime than ceftazidime.

DECLARATION

I, NAOMI KAONGA, do hereby declare to the Senate of Sokoine University of Agriculture, that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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DEDICATION

I dedicate this work to someone out there with big dreams of becoming a great scientist but is struggling with financial support and thinking how he or she would make it. Do not lose focus and determination. At the end of the tunnel there is always light.

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LIST OF ABBRIVEATIONS

AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
DNA	Deoxyribo nucleic Acid
ESBLs	extended-spectrum β-lactamases
GDP	Gross Domestic Product
MBLs	Metallo-β- Lactamases
PBPs	Penicillin-Binding Proteins
TTSS	Type Three Secretion Systems

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

In Zambia, poultry is a rapid growing sector contributing 4.8% of the Agricultural Gross Domestic Product (GDP), thus providing a significant income generating activity from selling of eggs, broiler meat and culled hens (Bronkhorst and Chongo, 2015). Despite this rapid increase, the poultry industry still faces challenges associated with emerging and re-emerging pathogens. Moreover, emergence of antimicrobial resistance bacterial strains throughout the production process is threatening the growth of the industry. Researches show that, poultry is a major reservoir of *Salmonella* worldwide and is associated with increasing incidences of enterobacterial strains producing extended-spectrum β-lactamases (ESBLs) (Gelinski *et al.,* 2014; Ziech *et al.,* 2016). ESBLs are enzymes produced by enterobacterial strains and can inactivate β-lactam compounds which include Penicillins, third-generation Cephalosporins, and Monobactams (Doi *et al.,* 2017).

Penicillins, Cephalosporins, and other β-lactams, are frequently used in treatment of *Salmonella* infections in poultry production and their misuse contributes to an emergence of a variety of multidrug resistant foodborne pathogens (Wu *et al.,* 2013). Resistant bacterial pathogens can be passed to human via direct contact with animals, spreading and exposure to animal manure, consumption of under cooked meat, and contact with meat surfaces (Shrestha *et al.,* 2017; Shamaila *et al.,* 2018). *Salmonella enterica* serovars Enteritidis and Typhimurium have been widely studied and reported the most prevalent foodborne serovars in many countries infecting both humans and animals and can be passed to humans through the food supply chain (Zhang *et al.,* 2016).

CTX-M-type (Cefotaximase-Munich) ESBLs have been described in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) isolates with resistance genes located on transferable plasmids (Tzouvelekis *et al.,* 2000). The cefotaximases can be transmitted by horizontal gene transfer mechanisms that include conjugation, transformation and transduction. These mechanisms facilitates the joining and exchange of specific genetic elements from one region to another, that is, plasmids to plasmids, chromosome to chromosome and between plasmids and chromosomes (Vaidya, 2011). In *Enterobacteriaceae*, transmission of ESBL-producing bacteria is complicated by β-lactamases that encodes on plasmids, and can be exchanged among the same and different members of this family (Stadler *et al.*, 2018).

In Zambia, studies have been conducted to assess and quantify the magnitude of bacteria associated with poultry farming, backyard chicken rearing and market ready chicken. These studies have mostly been carried out in Lusaka district which is the capital city of Zambia (Hang'ombe *et al.,* 1999; Chishimba *et al.,* 2016). However, the occurrence of CTX-M-Type ESBL-producing *S.* Typhimurium in poultry farms has not yet been established. Therefore, the aim of this study was to investigate the occurrence and antimicrobial susceptibility patterns of *S.* Typhimurium, to determine the prevalence and antimicrobial susceptibility patterns of CTX-M-Type ESBL-producing *S.* Typhimurium in poultry farms in the Copperbelt province in Zambia.

1.2 Problem Statement and Justification

The emergence and spread of antibiotic-resistance among *Salmonella* serovars originating from food-producing animals has been associated with antimicrobial usage during animal production process and has become a serious challenge in human and veterinary medicine

globally and poses a serious public health threat (Silva *et al.,* 2013). Easy access to antibiotics by Zambian farmers contributes to the abuse of these drugs in animal production, hence leading to the emergence of resistant pathogens (MNAP-AMR, 2017). In Zambia, the prevalence of CTX-M type-ESBL-producing *Salmonella* Typhimurium in poultry farms has not been well studied and there is little to no information on this subject. Previous studies by Chishimba *et al.,* (2016) reported a prevalence of 20.1% ESBL-producing *E. coli* in Market-Ready Chickens while Hang'ombe *et al.* (1999) reported a prevalence of 20.53% of *Salmonella* in processed broiler carcasses in Lusaka District.

This study was therefore carried out to establish the magnitude of CTX-M-Type ESBL-producing *S. enterica* serovar Typhimurium in poultry farms in the Copperbelt province in Zambia and to further investigate other possible contaminating factors in poultry production amongst poultry farms. Findings from this study provide baseline information on the prevalence of *S. enterica* serovar Typhimurium down the production chain in poultry farms in the Copperbelt province in Zambia and antimicrobial susceptibility and resistance patterns of this pathogen. The study also provides information on the prevalence and antimicrobial resistance and susceptibility patterns of CTX-M-type ESBL-producing *S. enterica* serovar Typhimurium down the production chain in poultry farms in the Copperbelt province in Zambia and possible sources of contamination in poultry farms.

1.3 Study Objective

1.3.1 General Objective

To investigate the occurrence and prevalence of CTX-M-Type ESBL-producing *S. enterica* serovar Typhimurium and their antibiotic susceptibility patterns in poultry farms in the Copperbelt province in Zambia.

1.3. 2 Specific objectives

i. To determine the prevalence and antimicrobial resistance of *S. enterica* serovar Typhimurium in poultry farms in the Copperbelt province in Zambia.

ii. To determine the prevalence and antimicrobial resistance of CTX-M-Type ESBL-producing *S. enterica* serovar Typhimurium.

iii. To determine possible contaminating factors affecting the prevalence of *S. enterica* serovar Typhimurium in poultry farms.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Genus *Salmonella*

Salmonella are facultative anaerobic intracellular pathogens of medical importance which can cause numerous diseases, such as typhoid fever, bacteraemia, enteric fever, salmonellosis and enterocolitis in many organisms (Wilson *et al.,* 2000). *Salmonella* infections can range from self-limiting to serious systemic diseases, based on the serotype and the infected host. Though these pathogens cause infections in different animal hosts, some serotypes like *S.* Gallinarum and *S.* Pullorum have a host range restricted to avian and causes severe fowl typhoid and pullorum disease respectively while *S.* Typhi, *S.* Paratyphi A and C cause typhoid fever only in humans and related human primates (Blondel *et al.,* 2013; Kisiela *et al.,* 2012). *Salmonella* belong to the family *Enterobacteriaceae*. These are gram negative facultative anaerobes and non-spore-forming rods that are put into serovars based on the surface molecular stuctures like lipopolysaccharide (O), flagellar protein (H), capsular and (Vi) antigens (Vanopdenbosch and Peteghem, 2013). This genus has bacterial strains that are motile with peritrichous flagella (with the exception of *S.* Gallinarum), are non-lactose fermenting, oxidase-negative, urease-negative, citrate-utilizing, acetylmethyl carbinol-negative and potassium cyanide-negative (Agbaje *et al.,* 2011). *Salmonella* has only two species, *Salmonella enterica* and *S. bongori. Salmonella enterica* has six subspecies that are identified by name or number and include: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae (*IIIa), *S. enterica* subsp. *diarizonae* (IIIb*)*, *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) while *Salmonella bongori* is designated V (CDC, 2011). *S. enterica* has over 2500 serotypes and *S. bongori* has over 23 serotypes

which are named according to the Kauffmann-White Scheme (CDC, 2011). The names of *Salmonella* serovars are presented as, *Salmonella. enterica* subsp. *enterica* ser. Pullorum or *Salmonella* ser. Pullorum or *Salmonella* Pullorum or *S*. Pullorum (Guibourdenche *et al.*, 2010).

2.2 *Salmonella* Typhimurium general virulence factors

Regardless of the genetic closeness, *Salmonella* strains have different virulence factors and disease manifestation, some are host-specific, and these mechanisms contribute to the evolution of different serovars (López *et al.,* 2012). The virulence genes of *Salmonella* are found on a specialized part of *Salmonella* chromosome called Salmonella pathogenicity islands (SPI- large segments of horizontally acquired gene sequences that are present in pathogenic species but absent from non-pathogenic species) (Blanc-potard *et al.,* 1999). DNA sequence analysis shows about 23 known SPIs and all of them contain distinct virulence genes, different GC content, presence of movable elements and association with tRNA, (Schadich, 2013). SPI-1to SPI-5 are common among all *S. enterica* serovars (Hurley *et al.,* 2014). Characteristics of *Salmonella* virulence factors, i.e, cell invasion and intracellular survival encode on these SPIs (Akyala and Alsam, 2015).

When ingested, this pathogen attaches to the mucosal cells and Peyer's patches where it activates the signalling pathways of host cells and inflammatory cytokines responses, which prevents new infections (López *et al.,* 2012). The pathogen is able to control the production and release of molecules that interrupt cellular activities of the invaded organism during infection (Yoon *et al.,* 2011). The secreted molecules will make the pathogen stay longer in the host, prolong infection duration and also facilitate its transmission to other cells of the invaded organism (Yoon *et al.,* 2011). This serovar uses

secretion stages of virulence effectors to communicate with cells of invaded organism (Yoon *et al.,* 2011). These effectors are translocated across the membrane and secreted by type three secretion systems (TTSS). The genes encoding for virulence factors as well as those encoding for TTSS are located in SPI-1 and SPI-2 (Schadich, 2013). The TTSS secretion apparatus are specialized virulence needle-like devices made up of more than 20 components that have evolved indirect translocation and releases over thirty *Salmonella* virulence effectors into host cell cytoplasm (Akyala and Alsam, 2015). SPI-1 on the *S.* Typhimurium chromosome regulates entrance of the pathogen in epithelial cells and is responsible for *Salmonella* induced macrophage apoptosis, while SPI-2 Island harbours genes required for intramacrophage survival and systemic infection (Blanc-potard *et al.*, 1999).

2.3 Beta-Lactam Antibiotics

Antibiotics are natural compounds derived from fungi, actinomycetes, and bacteria that are used to kill or restrict target microorganisms from proliferating (Rahman *et al.*, 2018). Due to their effectiveness and efficiency, β-lactams are largely used globally in treatment of infectious diseases and contributes about 60% usage to the antibiotic classes (Öztürk *et al.*, 2015). These antibiotics have wide spectrum of activity and low toxicity because they targets bacterial cell wall which has no resemblance in large organisms (Jumaa and Karaman, 2015). Effectiveness of β-lactam antibiotics includes inhibition of cell wall synthesis by attachment to penicillin-binding proteins (PBPs) or transpeptidases-(bacterial enzymes that bind covalently to penicillins and other β-lactam antibiotics during bacterial cell wall synthesis) (Jumaa and Karaman, 2015). The PBPs are responsible for elongating and crosslinking the peptidoglycan layer of the bacterial cell wall resulting to growth inhibition, damage to cell wall which can cause cell lysis and death (Nicolau,

2008). All the β-lactam antibiotics have the reactive β-lactam ring molecular structure that resembles the D-alanine-D-alanine, the substrate of PBPs and the ring act as an irreversible inhibitor of the enzyme transpeptidase (Dowling *et al.*, 2013).

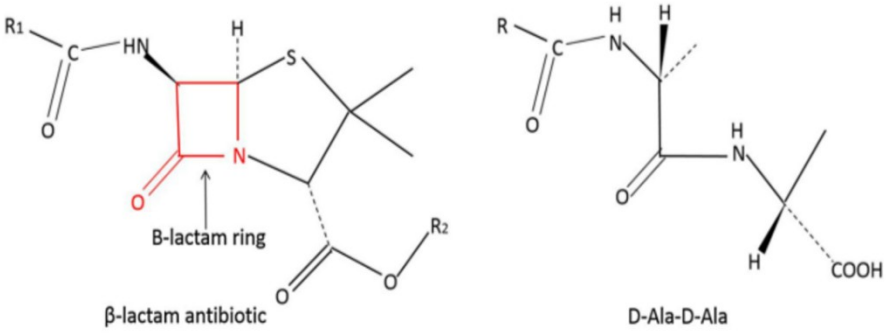

Figure 1: Molecular structure of β-lactam antibiotic (Lingzhi et al., 2018)

2.4 Classification of Beta-Lactamases

Beta-lactamases are a diversity group of enzymes which are among the most studied families of enzyme, with more than 36,076 citations in human and animal research. Research on these enzymes started as early as 1940s for their role to confer resistance in penicillin soon after its discovery. In 1940, Abraham and Chain investigated on an enzyme extracted from bacteria that was able to damage penicillins and in 1944 William Kirby was able to extract an enzyme that inactivated penicillins from penicillin resistant *Staphylococci* (Abraham and Chain, 1940; Kirby, 1944). Despite their tremendous diversity, the most common property found in these enzymes is that, they are capable of hydrolyzing compounds containing a β-lactam ring (Bush, 2018).

Presently, there are two main classification schemes that exist for categorizing β-lactamase enzymes which includes the Ambler and Bush-Jacoby-Medeiros classification. The Ambler classification put β-lactamases into four molecular classes, A, B, C, and D, based on conserved and distinguishing amino acid motifs (rests on amino acid similarity), and not

phenotypic characteristics (Ambler, 1980; Ambler, 1991). Enzymes of molecular classes A, C, and D are also known as serine B-lactamases (SBLs) of Ambler or functional groups 1 and 2 of Bush-Jacoby-Medeiros that use a catalytic serine for β-lactam ring hydrolysis, while those of molecular class B are known as metallo-β- lactamases (MBLs) or functional group 3 and employ one or two Zn^{2+} ions in the catalytic mechanism (Bonomo, 2017; Philippon *et al.,* 2016; Garau *et al.,* 2004; Widmann and Oelschlaeger, 2012). Lamotte *et al*, indicates the possibility of strong variations in catalytic properties within each class, but amino acid sequences remains clearly homologous to distribute newly discovered β-lactamases among these classes (Lamotte-brasseur *et al.,* 1994).

The Bush-Jacoby-Medeiros classification puts these enzymes into three major groups based on their functional similarities (substrate and inhibitor profile), i.e, (1) cephalosporin hydrolyzing enzymes that are not well inhibited by clavulanate; (2) penicillin, cephalosporin, and broad-spectrum enzymes that are inhibited by serine active site; and (3) metallo β-lactamases that hydrolyze penicillins, cephalosporins, and carbapenems and that are poorly inhibited by almost all β-lactam- containing molecules (Bush *et al.,* 1995; Bush, 2018). The Bush-Jacoby-Medeiros classification scheme considers β-lactamase inhibitors and β-lactam substrates of clinical significance therefore, it is of importance in a diagnostic laboratory.

2.5 Characterisation of ESBLs

ESBLs are placed in molecular class A of Ambler and functional group 2be of Bush *et al* and have a serine at the active site except OXA-type ESBLs which are placed in molecular class D and functional group 2de (Bush *et al.,* 1995). Following the Bush *et al.* (1995) classification, ESBLs can be re-defined as enzymes placed into functional group 2be that

have the capacity of hydrolyzing oxyimino-cephalosporins and are inhibited by clavulanic acid (Bradford, 2001). The class A enzymes have known amino acid sequences and they show similarities in the catalytic activities (Lamotte-brasseur *et al.,* 1994).

Hydrolysis of expanded-spectrum β-lactam compounds by ESBLs is as a result of mutations in the sequences of these enzymes (Bradford, 2001). Functional group 2b β-lactamases (TEM-1, TEM-2, and SHV-1) retain their capacity of hydrolyzing penicillin and ampicillin, and to a lesser degree carbenicillin or cephalothin (Bush and Jacoby, 2010). ESBLs are derived from group 2b, hence they are placed in group 2be and the e indicates that these enzymes have an extended spectrum to the third generation cephalosporins. When mutations occur in group 2b enzymes, resulting ESBLs differ from their progenitors by single or multiple amino acid(s) and this alters enzymatic activity of the ESBLs to start hydrolyzing the third-generation cephalosporins (Jacoby and Medeiros, 1991; Paterson and Bonomo, 2005).

Bacterial resistance to β-lactams has emerged gradually and progressing rapidly due to the production of β-lactamases (Moosavian and Ahmadkhosravy, 2016). Emergence of antibiotic resistant bacterial strains has been associated with abuse of antibiotics (Li *et al.*, 2019). Studies have showed that ESBL production is the major mechanism of influence in enterobacterial resistance to penicillins, broad-spectrum and third generation cephalosporins with an oxyimino side chain (Zhao and Hu, 2013).

The development of ESBLs is either plasmid mediated or expressed chromosomally. In gram-positive bacteria, class A genes are often chromosome-encoded while in gram-negative they are usually plasmid-encoded, with exception of some bacteria like *Klebsiella*

and *Proteus* where the genes are on the chromosome (Lamotte-brasseur *et al.*, 1994). Presently, various ESBL genotypes have been studied worldwide in *Enterobacteriaceae*; so far, over 10 families have been documented; CTX-M, SHV, TEM, PER, VEB, BES, GES, TLA, SFO and OXA (Zhao and Hu, 2013). The commonly studied families include OXA, SHV, TEM, and CTX-M types (Bradford, 2001).

2.6 Mechanism of β-Lactamase Resistance

Rossolini *et al.* (2017) outlines different mechanisms that results in β-lactamase resistance and they include: 1) the formation of β-lactamase enzymes that disrupt the ring of the β-lactam compound; 2) alterations present in Penicillin Binding Proteins which have less affinity for β-lactams such as in PBP2x of *Streptococcus pneumoniae* and Methicillin resistance in *Staphylococcus* spp (acquisition of mec element were the mecA gene, which encodes PBP2a); and 3) reduction in diffusion of the outer membrane that damage the access of β-lactam compounds to their Penicillin Binding Protein targets (Rossolini *et al.*, 2017; Bonomo, 2017). In gram-negative bacteria, production of β-lactamases is the most common and important mechanism of resistance to β-lactam antibiotics (Drawz and Bonomo, 2010). The mechanisms of β-lactam inactivation by β-lactamases are illustrated in Figure 2.

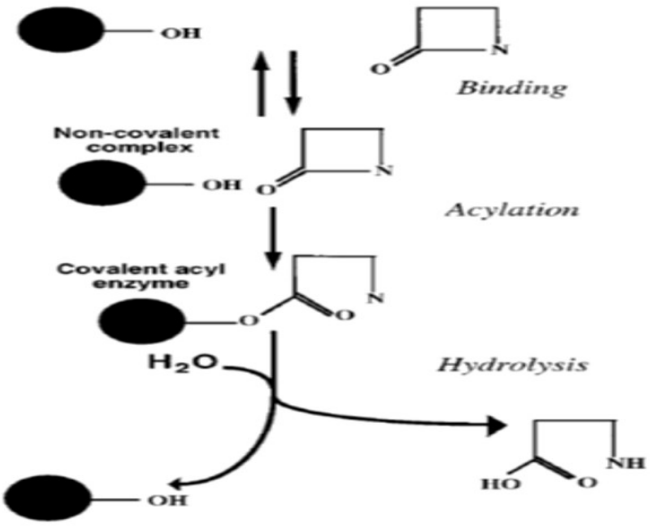

Figure 2: Mechanism of β-lactam inactivation (Livermore, 1995)

The β-lactamase starts by associating with the β-lactam to produce the non-covalent complex. An attack on the carbonyl group of the β-lactam compound is initiated by the active-site serine which results to high-energy acylation. This acylation changes to a lower-energy covalent acyl-enzyme following the addition of a proton on the β-lactam nitrogen and cleavage of the Carbon-Nitrogen bond. Activation of the water molecule takes place and attacks the covalent complex which results to a high-energy deacylation. The hydrolysed bond between the carbonyl of β-lactam compound and the oxygen of the serine regenerates the active β-lactamase and releases the hydrolysed inactive β-lactam compound (Drawz and Bonomo, 2010; Livermore, 1995).

2.7 Diversity of ESBLs

By 1970s, studies showed that SHV-type non-ESBL enzymes were produced in most *Klebsiella pneumoniae* strains, and TEM-type non-ESBL were produced in some *Escherichia coli* strains which could hydrolyze ampicillin, but not oxyimino cephalosporins (Paterson and Bonomo, 2005). In the 1980s, these non-ESBL enzymes

evolved into ESBLs through mutations at specific positions in the amino acid sequence that changed the substrate profiles to allow for inactivation of oxyimino-compounds which has led to bacteria resistance against the expanded-spectrum cephalosporins and monobactams (Doi *et al.*, 2017). These parent enzymes TEM and SHV have the capacity to undergo mutations and bestow resistance to β-lactamase inhibitors (Rossolini *et al.*, 2017). Due to resistance profile to penicillins and cephalosporins, cefotaximases (CTX-1) and ceftazidime-hydrolyzing enzyme (CAZ-1) were described (Bush and Singer, 1989). ESBLs originated from derivatives of TEM and SHV enzymes which had substitutions in their amino acid sequence resulting in an expanded substrate specificity. The most occurring mutation in both TEM-type and SHV-type ESBLs is the replacement of the amino acid Glycine 238 with Serine, Alanine or Aspartic acid (Bush and Bradford, 2019). There are two major strategies members of Enterobacteriaceae use to produce ESBLs; (1) expansion of substrate profiles of TEM- and SHV-type enzymes through mutations and amino acid substitutes at critical positions; and (ii) acquisition of new β-lactamases that encodes enzymes with active ESBL by horizontal transfer (Rossolini *et al.*, 2008). In recent years, the introduction of rapid growing CTX-M-type ESBLs worldwide has diminished the importance of TEM-type and SHV-type ESBLs (Bush and Bradford, 2019).

2.7.1 Temoniera (TEM-Type ESBLs)

The first to be discovered plasmid-mediated β-lactamase was TEM-1 in *E. coli* isolated from a patient called Temoniera in Greece in the 1960s, thus the name TEM (Bradford, 2001). Bacterial strains producing TEM-1 β-lactamases can hydrolyze ampicillin, penicillin and first-generation cephalosporins and are also cause 90% resistance of *E. coli* to ampicillin, 90% resistance of *Haemophilus influenzae* and *Neisseria gonorrhoeae* to penicillin (Livermore, 2008; Rupp and Fey, 2003). TEM-1 differs from TEM-2 by

substitution of Glutamine with Lysine at position 37 which differentiate between the ESBL enzymes derived from TEM-1 and the least occurring TEM-2 while variant TEM-13 has an additional Threonine → Methionine change at position 261 and does not affect the substrate profile (Bush and Bradford, 2019). TEM-4 and TEM-9 also have a substitution of Leucine with Phenylalanine change at position 19 and is used in processing of the mature β-lactamases (Jacoby and Medeiros, 1991).

2.7.2 Sulphydryl variable (SHV-Type ESBLs)

SHV-type ESBL is encoded on a plasmid and was initially present in *Klebsiella pneumoniae* and *E. coli*. SHV-1 gene is encoded on chromosomes in strains of *K. pneumoniae*, while in *E. coli*, the gene is encoded on plasmids (Fernandes *et al.*, 2013). Liakopoulos *et al.* (2016a) put the SHV β-lactamases into three subgroups based on their molecular characteristics or functional properties: (i) 2b enzymes; these hydrolyze penicillins and early generation cephalosporins and cannot hydrolyze clavulanic acid and tazobactam; (ii) 2br enzymes; these have a broad-spectrum and can hydrolyze clavulanic acid (iii) 2be enzymes; these are Extended Spectrum β-lactamases that confer resistance to single or multiple oxyimino β-lactams. Thus, SHV-type ESBLs can be encoded on plasmid or chromosomes and have hydrolytic activity to penicillins and first-to-third-generation cephalosporins (Rupp and Fey, 2003).

2.7.3 PER and VEB ESBLs

PER-1 and PER-2 are ESBLs that share about 25-27% amino acid sequence identity with the known TEM- and SHV-type ESBLs and where primarily found in *Pseudomonas aeruginosa* but are becoming common among members of Enterobacteriaceae (Poirel *et al.*, 2010). PER-1 was first reported in *Pseudomonas aeruginosa* and later found in other

bacterial populations like *S.* Typhimurium, *Acinetobacter* spp and various enteric bacteria (Luzzaro *et al.,* 2001). PER-2 is closely related to PER-1 by 86.4% amino acid sequence (Bauernfeind *et al.,* 1996). Bauernfeind *et al.,* (1996) detected PER-2 in *S.* Typhimurium, *Escherichia coli*, *Klebsiella pneumoniae, and Proteus mirabilis* isolated from patients in South America. PER-1 hydrolyze penicillins and cephalosporins and cannot inhibit clavulanic acid (Paterson and Bonomo, 2005). VEB-1 (Vietnamese ESBL) in *E. coli* was first isolated from a 4-months-old Vietnamese orphan child and conferred resistance to the third-generation cephalosporins and aztreonam (Poirel *et al.,* 1999).

2.7.4 OXA-Type (Oxacillinase)

This is another growing group of ESBLs but differ TEM, SHV and CTX-M-types in that they belong Ambler molecular class D and Bush-Jacoby-Medeiros functional group 2d (Table 1). These ESBLs are characterized by their strong ability to hydrolyse oxacillin and cloxacillin, confers resistance to ampicillin and cephalothin and they can be poorly inhibited by clavulanic acid and (Bush *et al.,* 1995). OXA-type ESBLs are predominately found in bacterial species such as *Pseudomonas aeruginosa* and have been reported in several species of *Acinetobacter* (Tian *et al.,* 2018). These enzymes have also showed capability to hydrolyse carbapenems in bacterial isolates of *Acinetobacter baumannii* with highest affinity for imipenem (Brown *et al.,* 2005).

2.7.5 Cefotaximase-Munich (CTX-M-Type ESBLs)

The CTX-M type ESBLs were first described in the 1980s and since 1995 they have rapidly spread worldwide (Brolund, 2015). These enzymes are plasmid-mediated acquired cefotaximases from a distinct progenitor, and are currently considered the most prevalent and rapid growing ESBLs with significant clinical impact (Zhao and Hu, 2013; Perez *et*

al., 2007). The CTX-M enzymes were originally from chromosomally encoded enzymes of environmental bacterial species of *Kluyvera* had almost no pathogenic activity towards humans (Fernandes *et al.*, 2013). Conjugation of cefotaximases in *Kluyvera* occurred on plasmids and got transferred to pathogenic species, and could move between different bacterial species (Dhillon and Clark, 2012). Genes encoding CTX-M-1 and CTX-M-2 groups have been reported in strains of *Kluyvera ascorbata*, and genes encoding CTX-M-8 and CTX-M-9 group have been detected in strains of *Kluyvera Georgiana* (Rossolini *et al.*, 2008). Some decades ago, these cefotaximases were put into two phylogroups with 20% difference in the amino acid sequences, that is, CTX-M-2, -4 and -5 and Toho-1, constituted the CTXM- 2 group; (2), CTX-M-1 group included the closely related β-lactamases MEN-1:CTXM- 1 and -3 (Tzouvelekis *et al.*, 2000).

In recent studies, CTX-M-ases have been put into 6 phylogroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC, each group having numerous minor variants and difference of less than ten percent in the amino acid sequence identity (Doi *et al.*, 2017; Zeynudin *et al.*, 2018). These genes are most prevalent in bacterial species that include, *K. pneumoniae*, *E. coli*, thyphoidal and non-thyphoidal *Salmonella*, *Shigella*, *Citrobacter freundii*, *Enterobacter*, spp. and *Serratia marcescens* (Perez *et al.*, 2007). So far, more than 90 of these enzymes have been reported (Chandramohan and Revell, 2012), and more than 10 have been detected in *Salmonella* serovars, whereas *S.* Typhimurium has been studied the most and found producing CTX-M-5 and CTX-M-15 (Jin and Ling, 2019) In terms of amino acid sequences, TEM and SHV ESBLs share an identity of about 67%, while the CTX-M-type is far distant with amino acid sequence identity of 40% to TEM and SHV (Thai *et al.*, 2009). Cefotaximases confer much hydrolytic activity against cefotaxime and ceftriaxone than ceftazidime (Doi *et al.*, 2017). Occurrence of point mutations can

increase hydrolysis against ceftazidime as in the case of CTX-M-15 and -32 which differ from CTX-M-3 and -1, by substitution of Aspartic acid-240 → Glycine (Livermore *et al.*, 2006). This family of ESBLs are mostly inhibited by clavulanic acid, tazobactam and sulbactam and cannot hydrolyze antibiotics such as carbapenems cephamycins, and temocillin (Bradford, 2001; Livermore *et al.*, 2006). Cefotaximases are morphologically related to Toho-1 and Toho-2 β-lactamases and share same hydrolytic activity against cefotaxime than ceftazidime (Paterson and Bonomo, 2005). This family of ESBLs is still growing and more variants are being discovered. A recent study done in Egypt descibes two novel CTX-M variant genes that closely matched with $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ which they named $bla_{\text{CTX-M-15.2}}$ and $bla_{\text{CTX-M-14.2}}$ (Ramadan *et al.*, 2019).

2.7.6 Clinical Relevance of CTX-M Type ESBLs

CTX-M-ases can hydrolyze to penicillins, broad-, expanded-, and extended- spectrum cephalosporins and greatly different in susceptibility profile to inhibitors from TEM and SHV ESBLs in their strong hydrolytic affinity against cefotaxime and ceftriaxone than against ceftazidime (Chen *et al.*, 2005). The first described CTX-M-type enzymes displayed a great hydrolytic activity against cefotaxime and ceftriaxone, with poor hydrolytic activity to ceftazidime. However, recent studies have showed that many newly described CTX-M variants are capable of hydrolysing cefotaxime, ceftriaxone and as well as ceftazidime (Novais *et al.*, 2010) making it difficult to treat serious infections and leaving carbapenems as the only choice of β-lactams. Occurrence of CTX-M-ases in clinical isolates is often associated with co-resistance to other antibiotic compounds that are non β-lactams in particular, to fluoroquinolones trimethoprim–sulfamethoxazol, and aminoglycosides (Zeynudin *et al.*, 2018). Inasmuch as CTX-M ESBLs have been reported to be susceptible to carbapenems, resistance to carbapenems has been detected in *K.*

pneumoniae strain producing the CTX-M-15 genes due to the loss of an outer-membrane porin (Rossolini *et al.,* 2008). Bacterial strains producing these enzymes have cefotaxime Minimum Inhibitor Concentrations (MICs) in the resistant range (>64 µg/ml), while ceftazidime MICs are mostly in the susceptible range between (2 to 8 µg/ml), though some variants of Cefotaximases can confer resistance to ceftazidime with MICs as high as 256 µg/ml (Paterson and Bonomo, 2005).

2.8 Detection of ESBLs

ESBLs have globally been reported and for this reason, various detection strategies have been developed. Kirby-Bauer Disc diffusion, microdilution and Double-Disc Synergy Test (DDST) are the most commonly used methods for enteric pathogens following the CLSI criteria (CLSI, 2018). In the Kirby-Bauer disc diffusion method 30 µg antibiotic discs of the extended-spectrum cephalosporins, are placed on the inoculated culture plate of Mueller Hinton agar, 30 mm centre to centre from the amoxicillin/clavulanic acid 20/10 µg disc. This plate is incubated at 37^{o}C for 18-24 hours and examined for an extension of the edge of zone of inhibition of antibiotic discs toward the amoxicillin/clavulanic acid disc. When this happens, the bacterium is considered an ESBL producer (Jarlier *et al.*, 1988; Carter *et al.,* 2000; Tenover *et al.,* 1999; Kumar *et al*., 2017). Similar to the above method is the cephalosporin/clavulanic acid combination disc method where single discs of cefotaxime (30 µg) and ceftazidime (30 µg) are used in combination with cefotaxime/clavulanic acid (CTX30-CA10) and ceftazidime/clavulanic acid (CAZ30-CA10). Antibiotic discs are placed on the inoculated culture plate of mueller hinton agar 2.5 cm apart from each other and incubated at 37°C for 18-24 hours. When the zone of inhibition around the ceftazidime/clavulanic acid and cefotaxime/clavulanic acid combination discs are ≥ 5 mm more than the single discs, that isolate is considered to be an

ESBL-producing bacterium (Moosavian and Ahmadkhosravy, 2016; CLSI, 2018; Kumar *et al*., 2017).

Several researchers (Cormican *et al.*, 1996; Hall *et al.*, 2002; Nijhuis *et al.*, 2012; Garrec *et al.*, 2011; Paterson and Bonomo, 2005; Bradford, 2001) have described two commercial products that have been developed for ESBL detection which include, the Vitek (bioMereiux Vitek, Hazelwood, Mo.) ESBL test card and Etest (AB Biodisk, Solna, Sweden) ESBL test strip. The Vitek and Etest are based on identification of a reduction in ceftazidime MICs in the presence of a fixed concentration (2 µg/ml) of clavulanic acid. The Etest strip is a plastic drug-impregnated strip, with one side generating a stable concentration gradient for ceftazidime and the remaining side generating a gradient of ceftazidime and clavulanic acid (MIC test range, 32 to 0.12 µg/ml). The zone of inhibition is read from two halves of the strip containing ceftazidime alone or ceftazidime plus clavulanic acid. A reduction in the MIC of ceftazidime of ≥3 dilutions in the presence of clavulanic acid is interpreted as a positive test.

PCR and DNA sequencing are the molecular tools used for the detection of ESBL producing bacteria and these are considered to be the most accurate and effective tools (Nwafia *et al.,* 2019; Nijhuis *et al.,* 2012). Thus conventional and multiplex PCR protocols are widely used in the amplification of target genes with specific primer sets and a given amplicon size following an initial denaturation, annealing and elongation step (Anbazhagan *et al.,* 2019; Moosavian and Ahmadkhosravy, 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Study Design

The study was carried out in the Copperbelt Province of Zambia, the second largest province in Zambia with the total population size of 2 480 657, covering an estimated area size of 31 328 Km^2 with 10 districts namely: Ndola, Luanshya, Kitwe, Kalulushi, Mufulira, Chingola, Chililabombwe, Masaiti, Mpongwe and Lufwanyama (CSO, 2018). The province is the mining hub of Zambia with copper being the most predominant mineral, hence the name Copperbelt. Of the 10 districts, Ndola, Kitwe, Chingola, Mufulira and Luanshya were considered for the purpose of this study. These are urban areas and the most populated, where poultry farming is widely practiced.

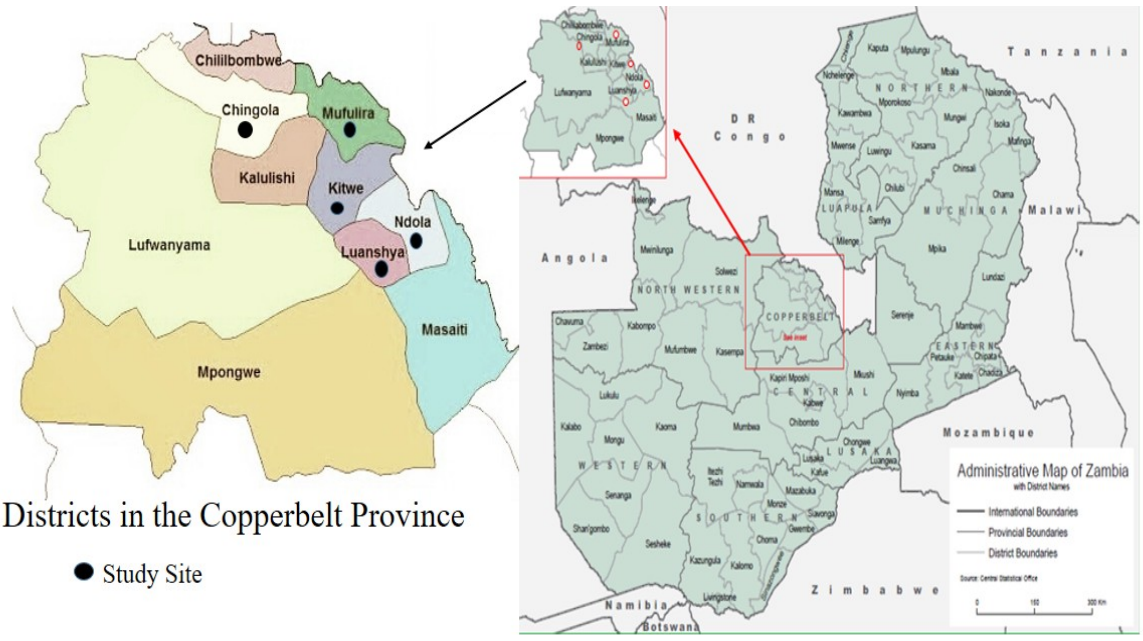

Figure 3: A map of Copperbelt province showing the study area (CSO, 2018).

A cross-sectional study design was conducted from March 2020 through May 2020, where one poultry farm was selected for sampling from each of the selected districts.

3.2 Sample Size Estimation and Sampling Techniques

Sample size was estimated using the following formula (Naing *et al.,* 2006):

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where n = expected sample size, Z = Z (1.96) statistic for a level of confidence, P = expected prevalence (0.5), and d = precision (d = 0.05).

$$n = \frac{1.96^2 \times 0.5(1-0.5)}{0.05^2} = 384$$

Fifty percent (50% = 0.5) prevalence was selected because there is no published prevalence used for CTX-M-Type ESBL-producing *S.* Typhimurium in poultry farms in the Copperbelt Province in Zambia. Previous studies by Chishimba *et al.* (2016) reported a prevalence of 20.1% ESBL-producing *E. coli* in Market-Ready Chickens while Hang'ombe *et al.* (1999) reported a prevalence of 20.53% of *Salmonella* in processed broiler carcasses in Lusaka province, Zambia.

Therefore, one poultry farm was selected for sampling from each of the five districts in the Copperbelt Province using simple random sampling techniques. The total sample size for this was 384 of which 78 cloacal swabs were collected from Ndola, 76 from Kitwe, 77 from Chingola, 76 from Luanshya and 77 from Mufulira. Cloacal swab samples were carefully collected to avoid contamination from the outside of the cloaca, and were placed in Amies with charcoal transport medium (Difco and BBL, 2009) (Fig. 4). From the farms, samples were transported on ice packs to the Microbiology laboratory at Tropical Diseases Research Centre. At farm management level, face to face questionnaire interview was used to collect data to use for risk factors assessment. Therefore, chicken population size per poultry farm, husbandry practices, antimicrobial usage and administration therapy, bio-security and hygiene practices, manure handling and feeding patterns were recorded.

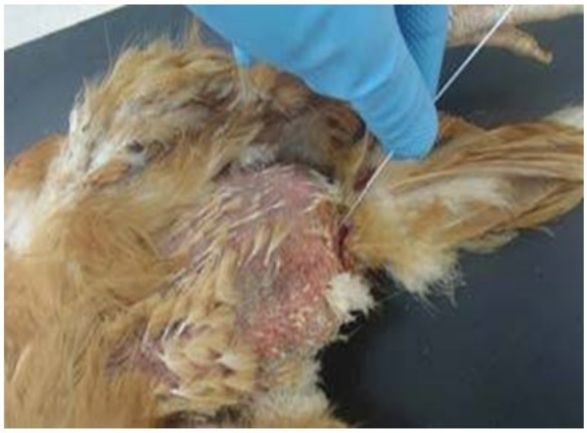

Figure 4: Cloaca swabbing of chicken (Sample collection)

3.3 Culture, Isolation and Identification of *Salmonella* Typhimurium

Isolation of *S*. Typhimurium was done using bacteriological methods described in Merck manual (Merck, 2010); Difco and BBL (2009). Cloacal swabs were first inoculated in Selenite-F broth (HiMedia Laboratories Pvt. Ltd. India) for enrichment of *Salmonella* species and incubated for 12-18 hrs at 37 ºC. From Selenite-F broth, the culture were inoculated and streaked on Salmonella-Shigella Agar (SSA) plates (HiMedia) (Merck, 2010) selective and differential media that differentiates between colonies of *Salmonella* from some *Shigella* species and incubated at 37 ºC for 18-24 hrs. *Salmonella* colonies were identified as colourless with black centres while *Shigella* colonies appear colourless.

Suspected *Salmonella* isolates were then inoculated on Brilliant Green Agar Base Modified (BGABM) plates (HiMedia), and incubated at 37 ºC for 18-24 hrs. BGABM is a highly selective medium which is used for the isolation of non-typhoidal *Salmonella* species from faeces and other materials and inhibits the growth of gram positive bacteria, *Shigella*, *S*. Typhi and Paratyphi Difco and BBL (2009). For quality control purposes, *S*. Typhimurium ATCC14028 was used.

3.4 Characterization of *Salmonella* isolates

Suspected *Salmonella* isolates were characterized through a panel of biochemical tests which included Triple Sugar Iron (TSI) and Urease. Isolates were inoculated in TSI and Urease slant tubes aseptically using a heat flamed wire loop and incubated for 18-24 hrs at 37 ^{o}C. The isolates were examined for the production of gas, hydrogen sulfide and colour change in TSI while in urease, isolates were examined for colour change.

These suspected isolates were further subjected to the Gram staining procedure. The stained slides were observed under light microscope (oil immersion) using 100x objective lens in order to categorize the isolates as gram positive or negative and identify cell morphological appearance and arrangement.

3.5 Antimicrobial Susceptibility Testing (AST) of *S.* Typhimurium

AST was done using the Kirby-Bauer disc diffusion method based on CLSI guidelines (CLSI, 2018). The antibiotic discs (HiMedia Laboratories Pvt. Ltd. India) included Cefotaxime 30µg, Ceftazidime 30µg, Penicillin 10µg, Ampicillin 10µg, Tetracycline 30µg, Gentamicin 10µg, Chloramphenicol 30µg, Norfloxacin 10µg and Amoxicillin 25µg, Nalidixic acid 30µg and Erythromycin 15µg. Direct colony suspension was employed by suspending *Salmonella* colonies in 2 mL 0.85% (w/v) normal saline and adjust the inoculum to a turbidity equivalent to a 0.5 McFarland Standard (1.5 X 10^{8} CFU/ml). These colonies were then evenly streaked on Mueller-Hinton agar (MHA) (HiMedia) plates and paper discs of the antibiotics were placed on the plate in 2.5 cm apart from each other and incubated at 37 °C for 18 -24 hrs (Moosavian and Ahmadkhosravy, 2016). Then, sensitivity and resistance patterns were examined according to the CLSI guidelines. Table 1 shows the performance standard of *Salmonella* to antibiotic discs.

Table 1: CLSI Performance standard by inhibition zone (mm) disc diffusion method for *Salmonella*

Name of Antimicrobial Agent/abbreviation	Disc content	Interpretation standards		
		Inhibition zone diameter (mm)		
		Susceptible	Intermediate	Resistant
Ampicillin (AMP)	10 µg	≥ 17	14-15	≤ 13
Amoxicillin (AMX)	25 µg	≥ 29	-	28
Ceftazidime (CAZ)	30 µg	≥ 21	18 - 20	≤ 17
Cefotaxime (CTX)	30 µg	≥ 26	23-25	≤ 22
Ceftazidime/clavulanic acid (CAZ-CA)	30-10 µg	≥ 21	18-20	≤ 17
Chloramphenicol (C)	30 µg	≥ 18	13-17	≤ 12
Erythromycin (E)	15 µg	≥ 21	16-20	≤ 15
Gentamicin (GEN)	10 µg	≥ 15	13-14	≤ 12
Nalidixic acid (NA)	30 µg	≥ 19	14-18	≤ 13
Norfloxacin (NX)	10 µg	≥ 17	13-17	≤ 12
Penicillin (P)	6 µg	≥ 29	-	≤ 28
Tetracycline (TE)	30 µg	≥ 15	13-14	≤ 12

Note: Penicillin, Erythromycin, and Amoxicillin are not included on performance standard for Salmonella but are included for other pathogens. Ceftazidime-clavulanic acid is currently not included for ESBL detection by CLSI. The breakpoint of zone of diameter where taken from (Kumar *et al.*, 2014)

Phenotypic detection of CTX-M-type ESBL- producing *S*. Typhimurium

The CTX-M-type ESBL-producing *S.* Typhimurium isolates were identified using the phenotypic combination disc method based on CLSI directions **(CLSI, 2018)**. With this method, combination discs of ceftazidime-clavulanic acid (CAZ30-CA10) were used with single discs of cefotaxime (30µg) and ceftazidime (30µg). Direct colony suspension was employed by suspending *Salmonella* colonies in 2 mL 0.85% (w/v) normal saline and adjust the inoculum to a turbidity equivalent to a 0.5 McFarland Standard (1.5 X 10^8 CFU/ml). These colonies were then evenly streaked on MHA plates and discs were placed in 2.5 cm from each other and incubated for 24 hours at 37 °C. A difference in zone of

inhibition of ≥5 mm of either of cefotaxime or ceftazidime discs and their clavulanic acid discs indicated the production of ESBLs. Confirmation of CTX-M-ESBLs was done using PCR.

3.6 DNA Extraction

DNA was extracted using the boiling method described by Reischl *et al*. (2000) where single pure bacterial colonies were suspended in a lysis buffer containing a detergent (0.1% Tween 20) of 300 µL and a buffer solution (10 mM Tris-HCl pH 8) of 300 µL in an eppendorf tube (Reischl *et al.,* 2000). This cell suspensions were boiled at 95-100°C in a boiling water bath for 10 min. The eppendorf tubes were then removed from the water bath and centrifuged for 5 min to separate the debris, from the supernatant. At this point, the samples were ready to be used for PCR. The concentration of DNA in the sample was measured using the BioDrop (BioDrop Ltd, UK) and ranged from 140 to190 µg/mL.

3.7 Detection of *Salmonella* Typhimurium and CTX-M-Type Genes by Polymerase Chain Reaction (PCR)

The detection of *Salmonella* Typhimurium and CTX-M- Type genes were achieved by serovar-specific, Typhimurium specific primers as described in (Appendix 1). The amplification was carried out in a final volume of 25 µL with the following optimized PCR contents; 12.5 µL of OneTaq Master Mix (*BioLabs*®*Inc*, England), 1.5 µL of each primer, 5 µL of template DNA, and 4.5 µL of nuclease free water. The PCR protocol was conducted under the following steps; an initial denaturation step for 4 minutes at 94 ºC, 40 cycles of 30 seconds at 94 ºC denaturation, 30 seconds at 58 ºC annealing, and 1 minute at 72 ºC extension and the final extension step for 4 minutes at 72 ºC. The positive control *Salmonella* Typhimurium ATCC 14028 was used following cycling protocol of

(Anbazhagan *et al.*, 2019). Molecular confirmation of CTX-M genes was done using two set of primers (Appendix 1). Both multiplex and conventional PCR protocols where used. *bla*$_{\text{CTX-M}}$ with 590 bp could not be amplified in the multiplex PCR due to differences in annealing temperatures.

Amplification products were detected in 1.5% agarose gel electrophoresis performed at a voltage of 100 V, current of 400 A for 60 min and visualised under UV trans-illuminator (UVP, Upland, USA).

3.8 Statistical Data Analysis

Data were entered and analysed by statistical package EPI INFO version 7.2.3.1. Frequencies and proportions in terms of percentages were computed for categorical outcomes. Fisher's exact test was performed to test the association between occurrence of *Salmonella* Typhimurium and other data such as antibiotic usage, purpose of antibiotic usage, withdrawal period, antibiotic administration and veterinarian consultation, manure handling, hygiene and bio-security practices at p-value of < 0.05 at 95% Confidence level.

3.9 Ethical Clearance

Ethical approval to conduct this study was obtained from the Research Ethics and Science Converge Committee (ERES) Institutional Review Board with reference number 2019-Dec-012. In addition, permission to visit farms was obtained from the Ministry of Livestock and Fisheries at Provincial (with reference number PFLC/CBP/101/15/1) and district levels prior to data collection.

CHAPTER FOUR

4.0. RESULTS

4.1. Culture and Isolation of *Salmonella* Typhimurium

The preliminary identification of *S.* Typhimurium gave an overall total of 130 suspected isolates from the studied farms. The identification was based on overnight cultures on Salmonella-Shigella differential (Fig. 5.1a) and Brilliant green agar base modified selective (Fig. 5.1b) media, biochemical tests and gram stain. From the 130 suspected *S.* Typhimurium isolates, 53 were from Chingola, 12 were from Mufulira, 21 were from Luanshya, 16 were from Kitwe and 30 were from Ndola.

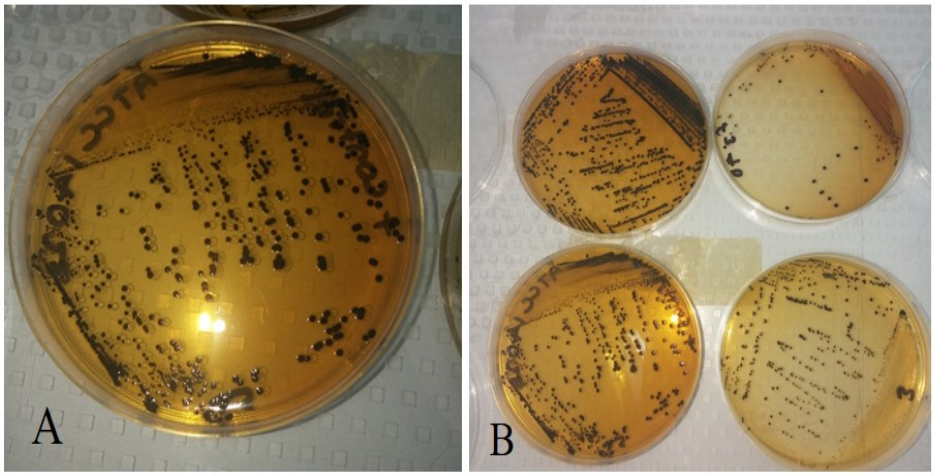

Figure 5.1(A): Bacterial growth on Salmonella-Shigella Agar

(A) Shows colonies of the positive control *S.* Typhimurium ATCC 14028 on SSA and (B) shows colonies of suspected *S.* Typhimurium isolates from poultry farms of the Copperbelt Province.

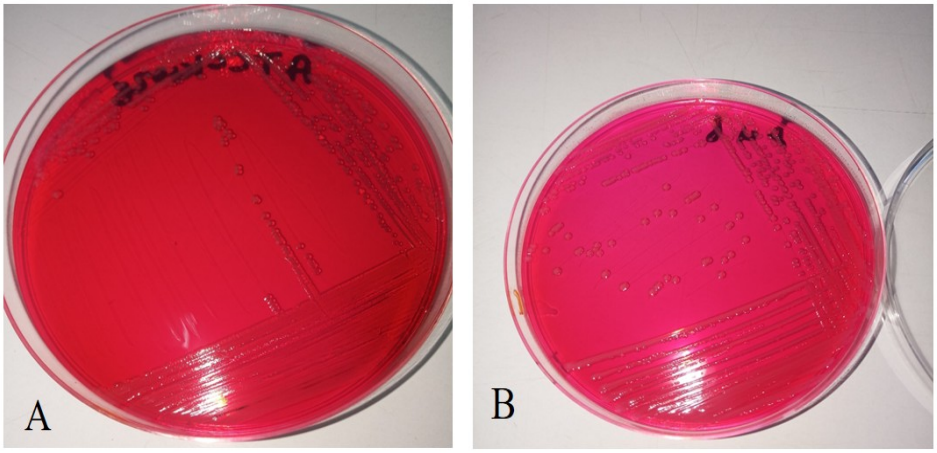

Figure 5.1(B): Bacterial growth on Brilliant Green Agar Base Modified

(A) Shows colonies of the positive control *S*. Typhimurium ATCC 14028 on BGABM and (B) shows colonies of suspected *S*. Typhimurium isolates *S*. Typhimurium isolates from poultry farms of the Copperbelt Province.

4.2 Characterization of Suspected *Salmonella* isolates

Suspected *Salmonella* isolates characterised by Triple Sugar Iron (TSI) and Urease test revealed that, out of the 384 samples tested, 146 tested positive to TSI (Fig. 5.2a) and 130 tested negative to Urease test (Fig. 5.2b). All the 130 suspected *S*. Typhimurium isolates were gram negative.

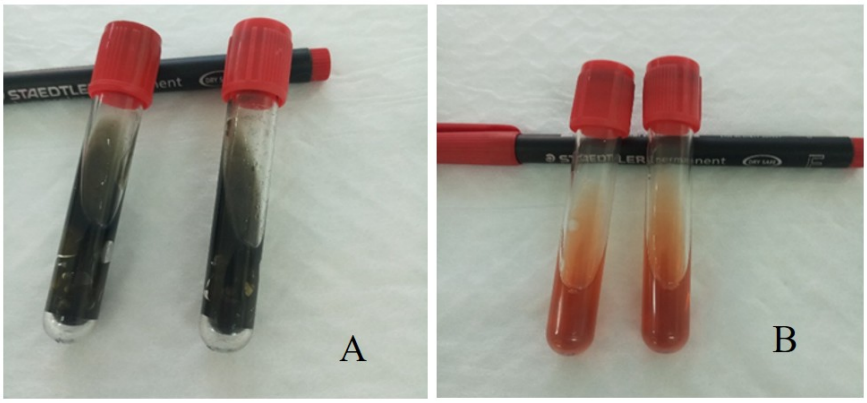

Figure 5.2 (A): Triple Sugar Iron test (a) Shows positive TSI test and (b) shows a negative TSI test.

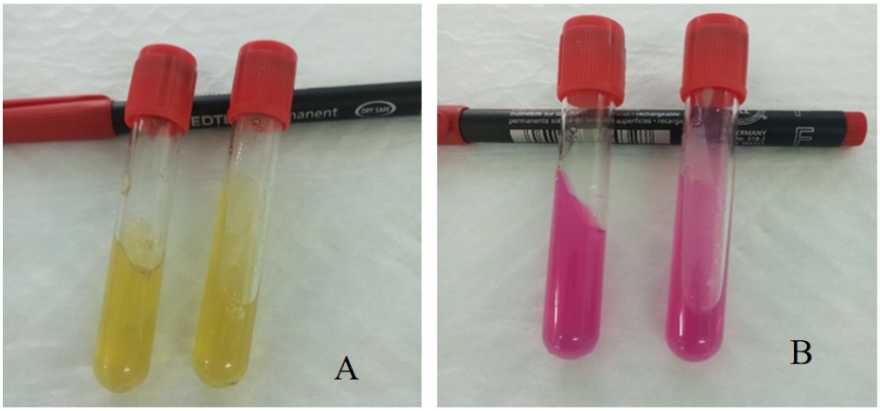

Figure 5.2(B): Urea Hydrolysis test. (a) Shows a negative urease test and (a) Shows a positive urease test.

4.3 Detection of *Salmonella* Typhimurium by PCR

Results of analysis of the 130 suspected *S.* Typhimurium isolates by PCR revealed that 68 of the isolates were *S.* Typhimurium (Fig. 6). From these findings the prevalence of *S.* Typhimurium in poultry farms in the Copperbelt Province was 17.7% (CI: 14.2%-21.8%). Amongst the districts, Chingola reported the prevalence of 7.3% *S.* Typhimurium followed by Ndola 5.2%, Luanshya 2.9%, Kitwe 1.6% and Mufulira 0.8% (Table 2).

Table 2: Distribution of *S.* Typhimurium isolated from poultry farms of the Copperbelt Province per district (n=384)

District	Total samples collected	Number of positive isolates	Prevalence	Confidence interval (95%)	
				Low limit	Upper limit
Chingola	77	28	0.0729 (7.3%)	0.0509	0.1034
Kitwe	76	6	0.0156 (1.6%)	0.0072	0.0337
Mufulira	77	3	0.0078 (0.8%)	0.0027	0.0227
Luanshya	76	11	0.0286 (2.9%)	0.0161	0.0506
Ndola	78	20	0.0521 (5.2%)	0.0340	0.0791

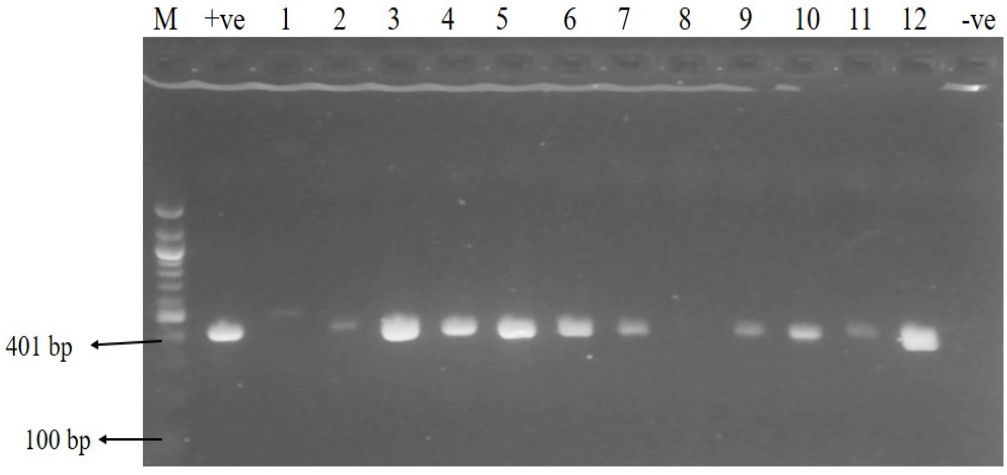

Figure 6: Detection of *S.* Typhimurium by conventional PCR at 401 bp expected band size

Key: M = 100 bp DNA ladder, +ve = positive control (*S*. Typhimurium ATCC 14028) and -ve = negative control, 1- 12 are isolates loaded for amplification. Lane 1 and 8 have no bands showing which implies that there was no amplification.

4.4 Association between Risk Factors and the Overall Prevalence

The overall prevalence of *S*. Typhimurium isolated from the Copperbelt Province was associated with eight risk factors that included, antibiotic usage, purpose of use, veterinarian consultation, antibiotic administration, withdrawal period, bio-security practice, hygiene and manure handling (Table 3). The association between the prevalence and purpose of antibiotic usage, withdrawal period, hygiene and bio-security practices was significant (p-value=0.00578499, CI: 0.0194-0.7197) with Fisher's exact test-value of 7.6164. There was also an association between antibiotic usage and manure handling with the overall prevalence (p-value = 0.00000025, CI: 0.0000- 0.1497 with Fisher's exact test of 26.592).

Table 3: Association between risk factors and the overall Prevalence (17.7%)

Risk factors	Category	Frequency	Fisher's exact test value	p-value	CI: 95%	
					Lower limit	Upper limit
Antibiotic usage	Yes	5	26.592	0.00000025	0.0000	0.1497
	No	0				
Purpose of antibiotic use	Prophylaxis	2	7.6164	0.00578499	0.0194	0.7197
	Growth promoter	3				
Veterinarian consultation	Yes	1	0.0945	0.75822758	0.0780	6.4342
	No	4				

Antibiotic administration	Veterinarian	1	0.0945	0.75822758	0.0780	6.4342
	Self	4				
Withdrawal period	Yes	2	7.6164	0.00578499	0.0194	0.7197
	No	3				
Bio-security practice	No	3	7.6164	0.00578499	0.0194	0.7197
	Yes	2				
Hygiene	Disinfectant use	2	7.6164	0.00578499	0.0194	0.7197
	Water	3				
Manure handling	Farming purpose	5	26.592	0.00000025	0.0000	0.1497
	Dispose off	0				

4.5 Antimicrobial Resistance and Susceptibility Patterns of *S*. Typhimurium Isolated from poultry farms of the Copperbelt Province

Of the 68 *S*. Typhimurium isolates tested for antimicrobial susceptibility, 88.2% (60/68) of the isolates showed resistance to one or more antimicrobial compounds. Interestingly, all the 68 *S*. Typhimurium isolates showed 100% (68/68) resistance to tetracycline followed by erythromycin with 97.1% (66/68), ampicillin and amoxicillin with 91.2% (62/68). The diversity of the antimicrobial resistance and susceptibility of the isolates are presented in Table 4. Multi-drug resistance of *S*. Typhimurium isolates per district are shown in Table 5.

Table 4: Antimicrobial susceptibility patterns of *S*. Typhimurium isolated from poultry farms of the Copperbelt Province by zone of inhibition of the isolates (n = 68 Isolates)

Antimicrobial agent	**% (n/N)**		
	Susceptible	**Intermediate**	**Resistant**
Ampicillin	2.9% (2/68)	5.9% (4/68)	91.2% (62/68)
Amoxicillin	0.0% (0/68)	8.8% (6/68)	91.2% (62/68)
Chloramphenicol	7.4% (5/68)	17.6% (12/68)	75.0% (51/68)
Erythromycin	0.0% (0/68)	2.9% (2/68)	97.1% (66/68)
Gentamicin	44.1% (30/68)	35.3% (24/68)	20.6% (14/68)
Nalidixic Acid	27.9% (19/68)	54.4% (37/68)	17.6% (12/68)
Norfloxacin	97.1% (66/68)	2.9% (2/68)	0.0% (0/68)
Penicillin	11.8% (8/68)	-	88.2% (60/68)
Tetracycline	0.0% (0/68)	0.0% (0/68)	100.0% (68/68)

KEY: - means there is no range given by CSLI guidelines for this antimicrobial agent (either susceptible or resistant).

Table 5: Multi-drug resistance of *Salmonella* Typhimurium isolates per district

District	**Number of isolates**	**Multi-Drug Resistance**
Chingola	28	Ampicillin, Tetracycline, penicillin, erythromycin and amoxicillin,
Kitwe	3	Tetracycline, erythromycin and amoxicillin
Mufulira	1	Tetracycline and penicillin
Luanshya	9	Tetracycline, erythromycin, ampicillin, chloramphenicol, amoxicillin and penicillin
Ndola	19	Tetracycline, erythromycin, ampicillin and amoxicillin

4.6 Phenotypic and molecular Detection of CTX-M-Type ESBL producing *S.* Typhimurium

Phenotypic ESBL detection showed that in combination of ceftazidime/clavulanic and cefotaxime, out of the 68 *S.* Typhimurium isolates, 18 were susceptible, 10 were intermediate, and 40 were resistant to cefotaxime. The combination of ceftazidime/ clavulanic and ceftazidime showed that 20 isolates were susceptible, 10 were intermediate and 37 were resistant (Table 6). The molecular detection of CTX-M-Type ESBL-producing *S.* Typhimurium revealed that, of the 68 *S.* Typhimurium confirmed isolates, 49 were ESBL producers carrying β-lactamase genes of $bla_{\text{CTX-M}}$ (Fig. 7) Therefore, the prevalence of CTX-M-type ESBL-producing *S.* Typhimurium in poultry farms in the Copperbelt Province was detected at 12.8% (CI: 9.8%-16.5%).

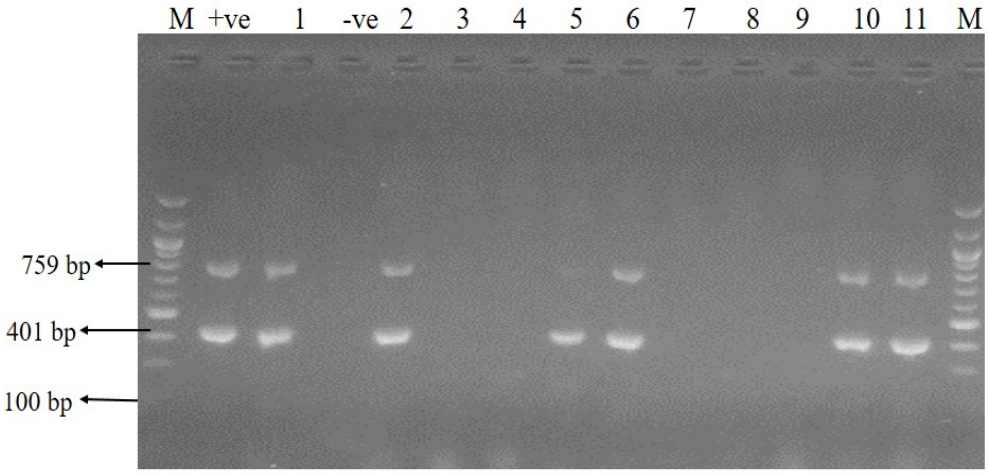

Figure 7**: Detection of CTX-M-type-ESBL producing *S.* Typhimurium by Multiplex PCR at 759 bp and 401 bp expected band sizes**

Key: M = 100 bp DNA ladder, +ve = positive control (*S.* Typhimurium ATCC 14028), 1- 11 are isolates loaded for amplification. Double bands on one lane indicates the presence of CTX-M- type ESBLs. Lanes 3, 4, and 8 show no amplification.

Table 6: Cephalosporin susceptibility patterns of *S.* Typhimurium isolated from the Copperbelt Province by zone of inhibition of the isolates (n = 68 Isolates)

Antimicrobial agent	% (n/N)		
	Susceptible	**Intermediate**	**Resistant**
Ceftazidime-clavulanic acid	100.0% (68/68)	0.0% (0/68)	0.0% (0/68)
Cefotaxime	26.5% (18/68)	14.7% (10/68)	58.8% (40/68)
Ceftazidime	29.4% (20/68)	14.7% (10/68)	54.4% (37/68)

CHAPTER FIVE

5.0 DISCUSSION

The detection of CTX-M-Type-ESBL-producing *S.* Typhimurium in poultry farms in the Copperbelt province has not been conducted before. Findings from this cross-sectional study shows that, the prevalence of *S.* Typhimurium and CTX-M-Type ESBL producing *S.* Typhimurium in poultry farms in the Copperbelt Province were 17.7% (68/383) and 12.8% (49/384). The 130 suspected *S.* Typhimurium isolates that were initially isolated based on overnight cultures and biochemical tests, could be other *S.* enterica serovars other than Typhimurium. Hang'ombe *et al.* (1999) found a prevalence of 20.52% of *Salmonella* species in chicken carcasses in Lusaka district, Zambia. In another study conducted in Lusaka, prevalences of 3.74% and 4.7% *S.* Enteritidis were reported in egg yolk and chicken carcasses respectively (Hang'ombe *et al.*, 1998).

In Tanzania a prevalence rate of 2.6% *S.* Gallinarum was reported in chickens (Mdegela *et al.*, 2000). A study on Salmonellosis in poultry farms in Nigeria, reported a prevalence of 16.0% *S.* Typhimurium (Ahmed *et al.,* 2014). In another study carried out in Egypt, *S.* Typhimurium was detected at rates of 44%, 40% and 48% in chicken meat, liver and heart, respectively (El-Aziz, 2013). A Study of *Salmonella* Typhimurium Infection in Laying Hens in Australia reported a prevalence of 7.2% *S.* Typhimurium in egg shell contamination and 5.66% in infected birds (Pande *et al.*, 2016). Another study conducted in Iran reported 43% and 36% occurrences of *S.* Enteritidis and *S.* Typhimurium respectively in poultry carcasses (Afshari *et al.*, 2018).

From the studies shown above, the occurrence of *Salmonella* in poultry, chicken carcasses and egg shell ranges from as low as 2.6% to 48%. The prevalence of *S.* Typhimurium (17.7%) detected in this study is within the range of the prevalences that have been reported in poultry farms and retail chicken meat in different parts of Africa and the world at large. This current study employed both bacteriological and molecular tools to achieve this prevalence. Overnight cultures on differential and selective media as well as biochemical tests provided a preliminary identification of *S.* Typhimurium isolates. Confirmation of these isolates was done using PCR protocols with serovar specific primers. All PCR products of *S.* Typhimurium obtained had an amplicon size of 401 bp which was similar to that obtained from studies done by Anbazhagan *et al.* (2019), Alvarez *et al.* (2004) Can *et al.* (2014), Mihaiu *et al.* (2014) and Serhat *et al.* (2016).

Salmonella enterica serovar Typhimurium is an important pathogen as it can be isolated from different animal hosts and foods. This pathogen is not host specific like *S.* Gallinarum and, *S.* Typhimurium has been identified to cause bacterial bloodstream infections in adult HIV-infections (Lê-Bury and Niedergang, 2018). In a case report by Swe *et al.*, (2008), *S.* Typhimurium meningitis was reported in an adult patient with AIDS were the pathogen was cultured from cerebrospinal fluid and blood culture specimens. *Salmonella* Typhimurium meningitis was also reported in a 5-months-old baby who was presented with acute pyogenic meningitis (Anne *et al*., 2017). In humans, this pathogen is known to cause localized gastroenteritis and systemic infections and is also mainly present in poultry products, swine and bovine meat (Anamaria *et al.,* 2018). In calves, *S.* Typhimurium causes enterocolitis and leads to dehydration (Tsolis *et al.,* 1999). The studies and findings above show the significance of *S.* Typhimurium as an important pathogen, globally, with potential of causing serious impact on animal and public health.

In this cross sectional study, an overall of 12.8% CTX-M-Type ESBL-producing *S.* Typhimurium isolates was detected in poultry farms of the Copperbelt Province. The prevalence was associated with administration of antibiotics to flocks which in turn increase the risk of higher antimicrobial resistant strains in the normal intestinal flora since these resistant genes are transferable among members of *Enterobacteriaceae*. A study conducted in China in foodborne animals, reported a prevalence on 17.76% CTX-M-Type producing *Salmonella* with $bla_{\text{CTX-M-55}}$ being the most prevalent (Zhang *et al.*, 2019), which is slightly higher than the prevalence reported in this study. In another similar study carried out in China in food producing animals, 43.4% occurrence of CTX-M-Type ESBL producing *S.* Typhimurium was reported (Zhang *et al.*, 2016).

The presence of CTX-M type ESBLs is often associated with co-resistance to other family phenotypes of antibiotic compounds in particular to fluoroquinolones trimethoprim–sulfamethoxazol, and aminoglycosides (Zeynudin *et al.*, 2018). Therefore, in this study, the isolates showed antimicrobial resistance to other classes of antibiotics (chloramphenicol, gentamicin, erythromycin, nalidixic acid and norfloxacin) other than β-lactams. In other studies, occurrence of other types of ESBLs has been reported in *S.* Typhimurium. A study carried out in Pakistan in poultry farms detected the occurrence of $bla_{\text{OXA-1}}$ (5.8%), bla_{TEM} (4.4%), bla_{SHV} (2.9%), and $bla_{\text{PSE-1}}$ (2.9%) (Wajid *et al.*, 2018). PER-1-ESBL has also been isolated in *S.* Typhimurium (Bradford, 2001). This could imply that ESBL-mediated plasmids are capable of carrying more than one type of β-lactamase genes and as such would result into high level presence of beta-lactam resistant bacteria (Wajid *et al.*, 2018).

In Zambia, poultry business is a major source of income for small and large scale farmers as well as those practicing backyard chicken rearing. In addition, there is popular demand on chicken meat and eggs by the majority of the community, small restaurant holders, big

franchises like Hungry lion, KFC and others. Therefore, this demand and the poor economic status, has resulted into many poultry farmers misusing antimicrobial agents during production process for the purpose of boosting growth and weight, and also rear the flocks in unhygienic flock houses. The manure from the poultry is used in farming of vegetables as well as other crops. The manure is a potential source of shedding ESBL-producing bacteria into the environment and bringing humans and other animals in close contact with antimicrobial resistant pathogens.

Antimicrobial testing in this study revealed interesting susceptibility patterns of the isolates to the 11 antibiotics tested. Of the 68 *S.* Typhimurium isolates analysed, 88.2% showed resistance to one or several antimicrobial compounds. Interestingly, all the 68 *S.* Typhimurium isolates showed 100% resistance to tetracycline, followed by erythromycin with 97.1%, ampicillin and amoxicillin with 91.2%. Similar results have been reported by different researchers globally. Antimicrobial resistance of *S.* Typhimurium isolated from cattle in Japan reported highest resistant patterns to ampicillin, tetracycline and chloramphenicol (Akibaa *et al.*, 2008). In Great Britain, *S.* Typhimurium in livestock was reported with resistant at 96% to ampicillin and 93% to tetracycline (Mueller-Doblies, *et al.*, 2018). A study carried out in Nigeria in poultry farms reported 100% resistance of *S.* Typhimurium to ampicillin cefotaxime, and ceftazidime (Ahmed *et al.*, 2014).

This study also reported antimicrobial resistance profiles to the third-generation cephalosporins. Though these cephalosporins are usually drugs of choice for *salmonella* infection treatment, resistance to these drugs has been found in human infections, food-producing animals, and poultry (Liakopoulos *et al.*, 2016b). This study reported the prevalence of 58.8% resistance of *S.* Typhimurium isolates to cefotaxime and 54.4%

resistance to ceftazidime. Resistance to third-generation cephalosporins was due to the production of CTX-M-type ESBLs, which were found in 68 *S*. Typhimurium isolates. These findings are similar to those of Burke *et al*. (2014) who reported the prevalence of 58% resistance of *Salmonella enterica* to cefotaxime. Therefore, the dissemination of ESBL genes of *Salmonella* isolated from poultry farms in Copperbelt Province, Zambia, is of great concern. Furthermore, this study shows the occurrence of *Salmonella enterica* serovar Typhimurium harboring CTX-M-type ESBL gene (49/68) 72.1%. These data suggest that *S.* Typhimurium may transmit antimicrobial resistance from chicken to human or to the environment or via food supply chain. Manure handling, poor hygiene and bio-security practices could be other source of ESBL dissemination and contaminating factors in these poultry farms as they were found to be associated with the overall prevalence.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This is the first study to be conducted in the Copperbelt Province in Zambia on detection of CTX-M-Type ESBL-producing *Salmonella enterica* serovar Typhimurium isolates in poultry farms. This cross sectional study has revealed the following:

An overall 17.7% prevalence of *Salmonella enterica* serovar Typhimurium isolates in poultry farms in the Copperbelt Province in Zambia which was found to be associated with the risk factors, purpose of antibiotic usage and withdrawal period and were statistically significant at p-value = 0.00578499, (CI: 0.0194-0.7197) with Fisher' exact test value = 7.6164. Chingola district had a prevalence of 7.3% *S*. Typhimurium, followed by Ndola district 5.2%, Luanshya district 2.9%, Kitwe district 1.6% and Mufulira district 0.8%. Manure handling, hygiene and bio-security practices were associated with the overall prevalence and could be possible bacterial contaminating factors in these poultry farms.

The prevalence of *S*. Typhimurium isolates harboring the CTX-M-Type ESBLs was found at 12.8% in all the districts.

S. Typhimurium isolates showed 88.2% resistance to one or several antimicrobial compounds. These isolates showed 100% resistance to Tetracycline followed by erythromycin with 97.1%, ampicillin and amoxicillin with 91.2%.

The isolates also showed resistance to the third-generation cephalosporins with prevalences of 58.8% cefotaxime and 54.4% ceftazidime resistances. These prevalences in *S*. Typhimurium isolates could be due to the presence of cefotaximases (CTX-Ms) that have more hydrolytic activity to cefotaxime than ceftazidime.

6.2 Recommendations

Now that the burden is known, based on findings from this study, the following are being recommended:

1. Further studies to quantify each of the risk factors used in this study which will assist in planning for future interventions.
2. Further studies to characterize CTX-M-type-genes and co-existence with other ESBL classes produced in *S*. Typhimurium in poultry farms.
3. Further studies to sequence and type the specific CTX-M-type genotypes that are predominant in *S*. Typhimurium in poultry farms.

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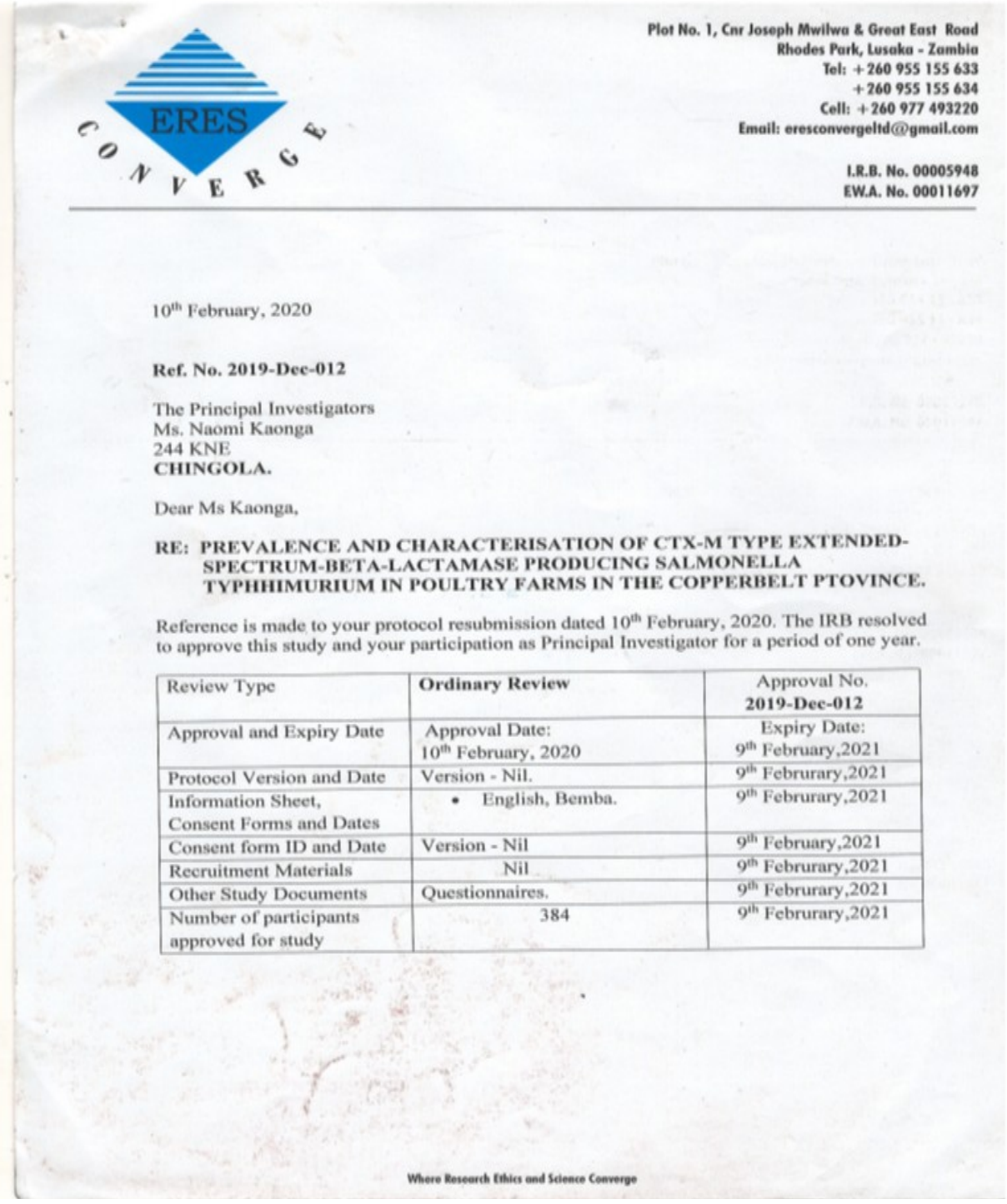
APPENDICES

Appendix 1: Primer Sequences and sizes for Typh F, Typh R bla$_{CTX-M}$ F and bla$_{CTX-M}$ R

Primer	Sequence (5'-3')	Size bp	Reference
Typh F	TTGTTCACTTTTTACCCCTGAA	401	(Anbazhagan *et al.*, 2019)
Typh R	CCCTGACAGCCGTTAGATATT		
***bla*$_{CTX-M}$ F**	ACGCTGTTGTTAGGAAGTG	759	(Mansouri and Ramazanzadeh, 2009)
***bla*$_{CTX-M}$ R**	TTGAGGCTGGGTGAAGT		
***bla*$_{CTX-M}$ F**	TTTGCGATGTGCAGTACCAGTAA	590	(Moosavian and Ahmadkhosravy, 2016)
***bla*$_{CTX-M}$ R**	CGATATCGTTGGTGGTGCCAT		

Appendix 2: Ethical Approval Letter



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10th February, 2020

Ref. No. 2019-Dec-012

The Principal Investigators
Ms. Naomi Kaonga
244 KNE
CHINGOLA.

Dear Ms Kaonga,

RE: PREVALENCE AND CHARACTERISATION OF CTX-M TYPE EXTENDED-SPECTRUM-BETA-LACTAMASE PRODUCING SALMONELLA TYPHHIMURIUM IN POULTRY FARMS IN THE COPPERBELT PTOVINCE.

Reference is made to your protocol resubmission dated 10th February, 2020. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary Review**	Approval No. **2019-Dec-012**
Approval and Expiry Date	Approval Date: 10th February, 2020	Expiry Date: 9th February,2021
Protocol Version and Date	Version - Nil.	9th Februrary,2021
Information Sheet, Consent Forms and Dates	• English, Bemba.	9th Februrary,2021
Consent form ID and Date	Version - Nil	9th February,2021
Recruitment Materials	Nil	9th Februrary,2021
Other Study Documents	Questionnaires.	9th Februrary,2021
Number of participants approved for study	384	9th Februrary,2021

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.Soc., PhD
CHAIRPERSON

Appendix 3: Participant Information Sheet

APPROVED
10 FEB 2020
ERES CONVERGE
P/BAG 128, LUSAKA

PARTICIPANT INFORMATION SHEET

Study Title
Prevalence and Characterization of CTX-M-Type Extended Spectrum Beta-Lactamase Producing *Salmonella Typhimurium* in Poultry Farms in the Copperbelt Province

Invitation paragraph
I would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Ask questions if anything you read is not clear or would like more information. Take time to decide whether or not to take part. This study is being conducted by Naomi Kaonga.

What is the purpose of the study?
The purpose of this study is to investigate the presence of genes that cause antimicrobial resistance from the common foodborne bacterium called *Salmonella Typhimurium*. Antimicrobial resistance is the ability of bacteria to stop antibiotics from working against it. As a result, normal treatments become ineffective, infections persist and may spread to others. Bacterial contamination of *Salmonella* is very common in poultry houses. This bacterium is also responsible for the production of antimicrobial resistance genes with can be transferred to humans through contamination of food.

Why have I been invited?
I have invited you to participate in this study because you are familiar with management of chicken or poultry production.

Do I have to take part?
Participation in the study is entirely voluntary. It is up to you to decide. I will describe the study and go through the information sheet, which I will give to you and will then ask you to sign a consent form to show you agreed to take part. You are free to withdraw at any time, without giving a reason and this will not affect the standard of care you receive.

What will happen to me if I take part?
Your participation will only take about three hours. During this time there will be questionnaire interviews and collection of chicken manure. Pictures will be taken during sample collection which will be used when writing up a report. No picture of you will be taken. The visit to your farm is once and for all. Participation is purely voluntary.

Expenses?
During the questionnaire interviews, snacks and drinks will be provided.

What are the possible disadvantages and risks of taking part?
There are no possible disadvantages and risks of participating in the study as it only involves sample collection of chicken manure.

What are the possible benefits of taking part?
The study will help to increase the understanding of the presence or occurrence of antimicrobial resistance genes produced by *Salmonella* Typhimurium which can be transmitted to humans through consumption of under cooked food or contamination.

Will my taking part in the study be kept confidential?
All information which is collected about you during the course of the study will be kept strictly confidential, and any information about you which leaves the university will have your name and address removed so that you cannot be recognised.

Further information and contact details:
For additional information you can contact

1. Naomi Kaonga (Principal investigator) +260 965214130
2. Professor Bernard Hang'ombe (Supervisor) +260 977326288
3. ERES Converge (Ethical Clearance Services): +260 955155634

APPROVED
10 FEB 2020
ERES CONVERGE
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Appendix 4: Questionnaire

[ ] treatment against diseases

[ ] prevention of diseases that might come in future

[ ] growth promoting factor for weight gain

3. What is the method of administration of the antibiotics?

[ ] water

[ ] feed

[ ] water and feed

[ ] others (specify) _____

4. Do you follow the withdrawal period?

[ ] Yes

[ ] No

5. Have you received training or guidance on medication safety?

[ ] Yes

[ ] No

6. Do you consult the veterinarian on antibiotic use?

[ ] Yes

[ ] No

7. Where do you usually buy antibiotics?

[ ] official pharmacy

[ ] sales representative

[ ] others (specify) _____

8. When treating diseased chickens, what is the effectiveness of medicine afterwards?

[ ] very effective

[ ] somewhat effective

[ ] not effective

9. Do you have any knowledge on antibiotics resistance?

[ ] Yes

[ ] No

10. If yes, specify what you know: _____

_____

Thank you for completing this questionnaire