EXTENDED SPECTRUM BETA-LACTAMASE (ESBL) PRODUCING *Escherichia coli* IN POULTRY AND WATER IN LUSAKA DISTRICT.

BY

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DECLARATION

I, **KENNEDY CHISHIMBA**, do hereby declare that this dissertation represents my own original work. It has been presented in accordance with the guidelines for MSc dissertation of the University of Zambia. It has not been submitted before for the award of any degree or examination in any other University.

Signed: _____Date: _____

CERTIFICATE OF APPROVAL

The University of Zambia approves the dissertation submitted by **KENNEDY CHISHIMBA**, as fulfilling the partial requirements for the award of the Master of Science Degree in One Health Analytical Epidemiology by the University of Zambia.

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ABSTRACT

Extended Spectrum Beta-Lactamase are enzymes that hydrolyse the beta-lactam ring on most of the beta-lactam antibiotics which comprise of penicillins, cephalosporins, and the monobactam aztreonam thereby rendering the antibiotics ineffective to treatment. These antimicrobial resistant strains have become a global public health challenge affecting both humans and domestic livestock such as cattle, pork and poultry. In Lusaka, poultry is considered as a universal protein source consumed by a larger population of people. Coupled with this is a relatively increased number of shallow well water points located in various peri-urban communities within Lusaka district where part of the people access drinking water from. Antimicrobial resistant ESBL-producing *Escherichia coli* bacteria have been detected in some domestic poultry and shallow well water sources world over. In addition, the frequent usage and administering of antibiotics in the treatment of various diseases in poultry could contribute to the emergence of antimicrobial resistant *E .coli* strains in the normal intestinal flora. The objective of the research was to detect the presence of extended-spectrum β-lactamase (ESBL) producing *E. coli* in poultry and water in Lusaka district. This study was conducted in Lusaka district, the capital city of Zambia with a total population of 1,747,152. A total of 384 poultry faecal samples and 145 shallow well water samples were collected between September 2014 and March 2015 and later submitted for laboratory analysis by the Laboratory Technicians at the University of Zambia, School of Veterinary Medicine, Department of Paraclinical Studies, Microbiology Laboratory. Mapping of unprotected shallow wells was conducted using GPS and spatial distribution of ESBL producing *E. coli* isolates was determined. Seventy seven potential ESBL producing *E. coli* isolates were later characterized by polymerase chain reaction (PCR) for detection of ESBL genes and also tested for antimicrobial sensitivity. Overall 54.5% (95% CI; 43.2 – 65.5%) of total samples analyzed possessed ESBL genes, 42.9% for poultry and 11.6% for water. 85.7% (95% CI; 75.7 – 92%) of the total samples analyzed for antibmicrobial disc sensitivity suggested that ESBL producing *E. coli* isolates had conferred resistance to beta-lactum antibiotics and other common antimicrobial agents. The results obtained require strengthening and reinforcement of the antibiotic administering policy for poultry and surveillance on the emergence of antimicrobial resistance in other food animal products.

DEDICATION

This study is dedicated to my father, Mr. Luke Chishimba, my mother Mrs. Esther Navile Chishimba, my wife Justina, and children Daniel, Joshua, Samuel and Elijah. Their encouragement and continuous prayers have necessitated the successful completion of this study. To them I say, thank you very much and may God richly bless you. Above all I acknowledge the grace and the eternal love of the Almighty God for the gift of life.

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LIST OF ABBREVIATIONS

Amp C	Alpha-methoxy-cephalosporins (Cephamycins)
AMP	Ampicillin
bla	Beta Lactamase
β	Beta
bp	Base Pair
CI	Confidence interval
CTX-M	Cefotaxime – Munich
CAZ	Ceftazidime
CHL	Chloramphenicol
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EARSS	European Antimicrobial Resistance Surveillance System
E. coli	*Escherichia coli*
ECOFF	Epidemiological Cut-Off
ESBL-E	Extended Spectrum Beta Lactamase *Enterobacteriaceae*
ESBL	Extended Spectrum Beta Lactamase
ESC-R	Extended-Spectrum-Cephalosporin-Resistant
EUCAST	European Committee on Antimicrobial Sensitivity Testing
GEN	Gentamicin
GPS	Geographical Position System
≥	Greater or equal to
IMViC	Indole Methyl red, Voges-Proskauer and Citrate
LCC	Lusaka City Council
MIC	Minimum Inhibitory Concentration
n	Number of isolates or samples
NOR	Norfloxacin
PEN	Penicillin
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis

R	Resistance
ROC	Receiver Operating Characteristic
SHV	Sulphydryl variable
ST	Strain
TE Buffer	Tris –Ethylenediaminetetraacetic acic buffer
TEM	Temoniera
TET	Tetracycline
TSI	Triple Sugar Iron
UV	Ultraviolet
UTI	Urinary tract infection
WHO	World Health Organization
ZEMA	Zambia Environmental Management Agency

CHAPTER ONE

INTRODUCTION

1.1 Background

Extended-Spectrum Beta-Lactamase (ESBL) producers are Gram-negative bacteria that produce enzymes that bestow resistance to most beta-lactam antibiotics which include penicillins, cephalosporins, and the monobactam aztreonam (Silvia and Jacoby, 2014). These ESBL producers have been noticed mainly in the *Enterobacteriaceae* family of bacteria and the commonly encountered ones are *Escherichia coli* and *Klebsiella pneumoniae* (Barthelemy *et al*., 1985).

Poultry is one of the major protein sources consumed by a larger population of people in the city of Lusaka with no religious restrictions. As a result of this, the poultry industry has recorded an unprecedented demand for chicken meat and other products (Zulu, 2015). Coupled with this are a relatively increased number of shallow well water points located in various peri-urban communities within Lusaka District which are a major source of water (LCC, 2008). Waste effluents from backyard poultry slaughters and abattoirs are regularly discharged into these water points resulting in serious environmental and public health hazards (Nafarnda *et al*., 2012). This contributes to frequent outbreaks of water-borne diseases, particularly cholera and typhoid fever, which result in severe morbidity and mortality. The presence of faecal matter in the water bodies necessitates the dissemination of resistant antibiotic ESBL producers (Meerambika *et al.*, 2013).

E. coli is ubiquitous and the antimicrobial resistant *E. coli* asymptomatically colonizes the intestinal flora of food animals and has a likelihood of becoming infectious agents to humans if consumed through the food chain (von Baum and Marre, 2005). There has been an increased worldwide distribution of *E. coli* ESBL producers in food producing animals conferring resistance to β-lactam antimicrobials (Reich *et al*., 2013). These antimicrobial agents used in the treatment of food animals in veterinary medicine range from penicillins, first to fourth generation cephalosporins and β-lactamase inhibitors (Li

et al., 2007). The ESBL producers are also resistant to most antibiotics such as aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulphonamides and quinolones (Hunter *et al*., 2010). Studies have shown that chickens are associated with the presence of quinolone resistant *E. coli* with β-lactamase CTX-M genes (Warren *et al*., 2007).

Infections associated with ESBL can affect the respiratory tract (pneumonia), urinary tract and bladder, skin and soft tissue, blood, gastrointestinal tract, reproductive organs, and central nervous system which could contribute to a higher mortality rate and lengthy hospitalization (Kayange *et al*., 2010 and Bloomberg *et al*., 2005).

1.2 Statement of the Problem

The emergence and spread of ESBLs among members of the *Enterobacteriaceae* family originating from food-producing animals has become a major public health issue worldwide and a serious threat, particularly as a cause of nosocomial (acquired from prolonged stay in hospital) infections (Johann *et al.*, 2007). Food animals colonized with ESBL-producing bacteria are capable of enhancing the spread of ESBL producing *E. coli* at the community level (Valeria *et al*., 2010). It is imperative to note that these notorious organisms have been identified to confer resistance to many other antimicrobial agents recommended for the treatment of infections caused by *E. coli*, such as fluoroquinolones and trimethoprim-sulphamethoxazole (Rodriguez-Baño *et al.*, 2008). There is also an increased risk factor for acquisition of ESBL producing organisms due to heavy usage of antibiotics world over and Zambia has not been spared (Mshana *et al*., 2009b). Furthermore the uncontrolled monitoring of high usage of off-label broad-spectrum antibiotics for prophylactic treatment of poultry and other food producing animals has facilitated the spread of drug resistant ESBL producers (Grave, 2010). In addition, an increased number of backyard food animal waste products gain entry into the water body such as shallow wells influencing the dissemination of these antimicrobial resistant ESBL producers into the environment (Blaak, 2011; Nafarnda *et al*., 2012). Studies have further shown an increase in isolation of ESBL producing isolates and that community and

hospital-acquired ESBL-producing *Enterobacteriaceae* have continued to pose a serious public health threat (Silvia and Jacoby, 2014).

1.3 Justification of Study

Zambia is one of the sub-Saharan African countries mostly affected by neglected bacterial resistant infectious diseases which have contributed to increased morbidity and mortality cases and the majority of studies have however placed attention on hospitalized patients although there is strong evidence that the community can be affected as well (Woerther *et al*., 2013). In Zambia data on ESBL is not available particularly on environment and food products of animal origin. A few studies on antimicrobial resistance are limited with only a few focussing on bacteria inhabiting commercial livestock (Mubita *et al.*, 2008). A baseline study has therefore been initiated to determine the carriage of ESBL in food and environmental foci's. This study will also be important in stimulating further research on this important public health problem.

1.4 Research Question

What is the rate of ESBL producing *E. coli* bacteria in poultry and shallow well water found in Lusaka District, Zambia?

1.5 General Objectives

To detect the presence of Extended Spectrum Beta-lactamase producing *E. coli* in poultry and shallow well water in Lusaka District.

1.6 Specific Objectives

1.6.1 To determine the presence of ESBL producing bacteria in poultry and shallow well water.

1.6.2 To determine the susceptibility pattern among the ESBL-producing *E. coli* isolates to beta lactam antibiotics and other common antimicrobial agents.

1.6.3 To map the distribution of ESBL producing *E. coli* in shallow wells in Lusaka District.

CHAPTER TWO

LITERATURE REVIEW

2.1 Definition of Extended Spectrum Beta – Lactamases (ESBL)

There is no agreement on the exact definition of ESBLs, however, a universal working definition is that ESBLs are chromosomal or plasmid-mediated β-lactamases which are enzymes that cleave the β-lactam ring which have mutated from pre-existing broad-spectrum β-lactamases, hence resulting into the extensive use of third generation cephalosporins as well as aztreonam (Shukla *et al*., 2004; Giriyapur *et al*., 2012).

The term "Extended broad-spectrum Beta-lactamases" describes enzymes conferring (transferable) resistance to newer beta-lactam agents. In this case newer Beta-lactam agents are extended spectrum cephalosporins; third generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime in opposition to the broad spectrum enzymes mainly TEM-1 which could hydrolyze penicillins and broad spectrum penicillins, e.g. ampicillin (Giske *et al*., 2009).

The abbreviation "ESBL" has now become common and describes resistance to β-lactams conferred through production of beta-lactamase enzymes which break down β -lactam antibiotic molecules (Sibhghatulla *et al*., 2015). These ESBL producing bacteria can cause severe infections that encompass the urinary tract, septic conditions, wound, meningitis and respiratory tract (Paterson and Bonomo, 2005).

2.2 Worldwide Distribution of ESBL

Worldwide, about 150 million people are diagnosed with urinary tract infections each year, costing the global economy in excess of 6 billion US dollars (Gonzalez and Schaettes, 1999). *Enterobacteriaceae* producing β-lactamases have been established to be a worldwide problem and considerably noticed mainly in *Escherichia coli* and *Klebsiella pneumoniae* strains (Figure 1.1) in most of the countries (Ben-Ami, 2006). Currently *E. coli* strains have emerged and confirmed as a frequent cause of ESBL community-

acquired infections and continues to escalate worldwide in different varying rates (Rodriguez-Baño, 2008).

A study conducted in Asia-Pacific region established that ESBL-producing *Enterobacteriaceae* was more prevalent compared to other continents (Hawser *et al*., 2009). Generally, the infection rate due to ESBL producers is higher in nations with low income and poor economic conditions (Casellas, 1999; Sader, 2006). An increase in self prescription of antibiotics sold over the counter and unhygienic hospital environments results into high colonization rates and infections with *Klebsiella* species (Villegas *et al*., 2008).

ESBLs have a complex epidemiology and the most prominent bacteria that are involved include *E. coli* and *K. pneumoniae* whose reservoirs include the environment (soil and water), wild animals, farm animals, food and pets (Carattoli, 2008). Currently there have been a large number of reported cases of nosocomial outbreaks related to clonally ESBL strains (Alsterlund *et al.*, 2009). For instance in Sweden, a report compiled by the European Antimicrobial Resistance Surveillance System (EARSS), revealed that 2.6% of *E. coli* and 1.7% of *K. pneumoniae* strains were established to be resistant to third-generation cephalosporins (EARSS, 2011). A widespread outbreak of *Enterobacteriaceae* producing the CTX-M-14 enzyme was reported in Calgary, Canada (Pitout *et al.*, 2005). An extensive study that was conducted in the United States of America also established that 5.3% of *E. coli* had ESBLs (Winokur *et al*., 2001).

In the African context, a study carried out at a tertiary hospital in Nigeria revealed that 35% of the ESBL isolates were of community origin while 65% was from hospitals and these isolates were established to be highly resistant to tetracycline, gentamicin, pefloxacin, ceftriaxone, cefuroxime, ciprofloxacin and Augmentin (Ruth *et al*., 2011). Another research conducted at a tertiary hospital in Mwanza, Tanzania, established an overall prevalence of ESBLs in all Gram-negative bacteria at 29%. Overall the ESBL prevalence for *K. pneumoniae* was 64% while that for *E. coli* was reported to be 24% (Mshana *et al*., 2009). In Madagascar, the majority of the ESBL isolates detected from

non-hospitalized patients were the CTX-M-15 type and this was mainly associated with poverty (Fatemeh *et al*., 2012).

Most of the countries in the Middle East have reported alarming numbers of ESBL-producing strains as high as 61% of *E. coli* produced ESBLs comprising of the CTX-M-14, CTX-M 15, and CTX-M 27 types with an additional TEM type enzyme (Al-Agamy *et al*., 2006). Generally, the prevalence of ESBL-producers differs between countries. However, the highest prevalence of ESBL-producing *K. pneumoniae* in the world has been noticed in Latin America with 36.7% of *K. pneumoniae* isolates and 20.8% of 932 *E. coli* isolates (Rossi *et al*., 2008). Figure 1.1 shows the distribution of ESBL producing *E. coli* and *K. pneumoniae* isolates detected from each country in the world.

2.3 Different Classification Schemes for ESBLs

2.3.1 Temoniera (TEM-type)

The first plasmid-mediated β-lactamase in Gram-negative bacteria was discovered in Greece in the 1960s, called TEM, and was named Temoniera a patient from whom the bacteria were isolated (Data and Kontomichalou, 2005). There are about 140 TEM-type enzymes that have been described. However, the most common ones include TEM-10, TEM-12, and TEM-26 (Villegas *et al*., 2008). In *E. coli*, the most commonly encountered β-lactamase is TEM-1 with about 90% resistance to ampicillin antibiotics (Paterson, 2003). TEM-53 and TEM-63 enzymes have been isolated in *K. pneumoniae* in South Africa while TEM-3 enzymes have been isolated in *S. typhimurium* in Morocco (Ndugulile *et al*., 2005). TEM-type β-lactamases have also been detected in other Gram-negative bacteria species increasing in occurrence with its amino acid substitutions responsible for the ESBL phenotype clustering around the active site of the enzyme (Bradford, 2001).

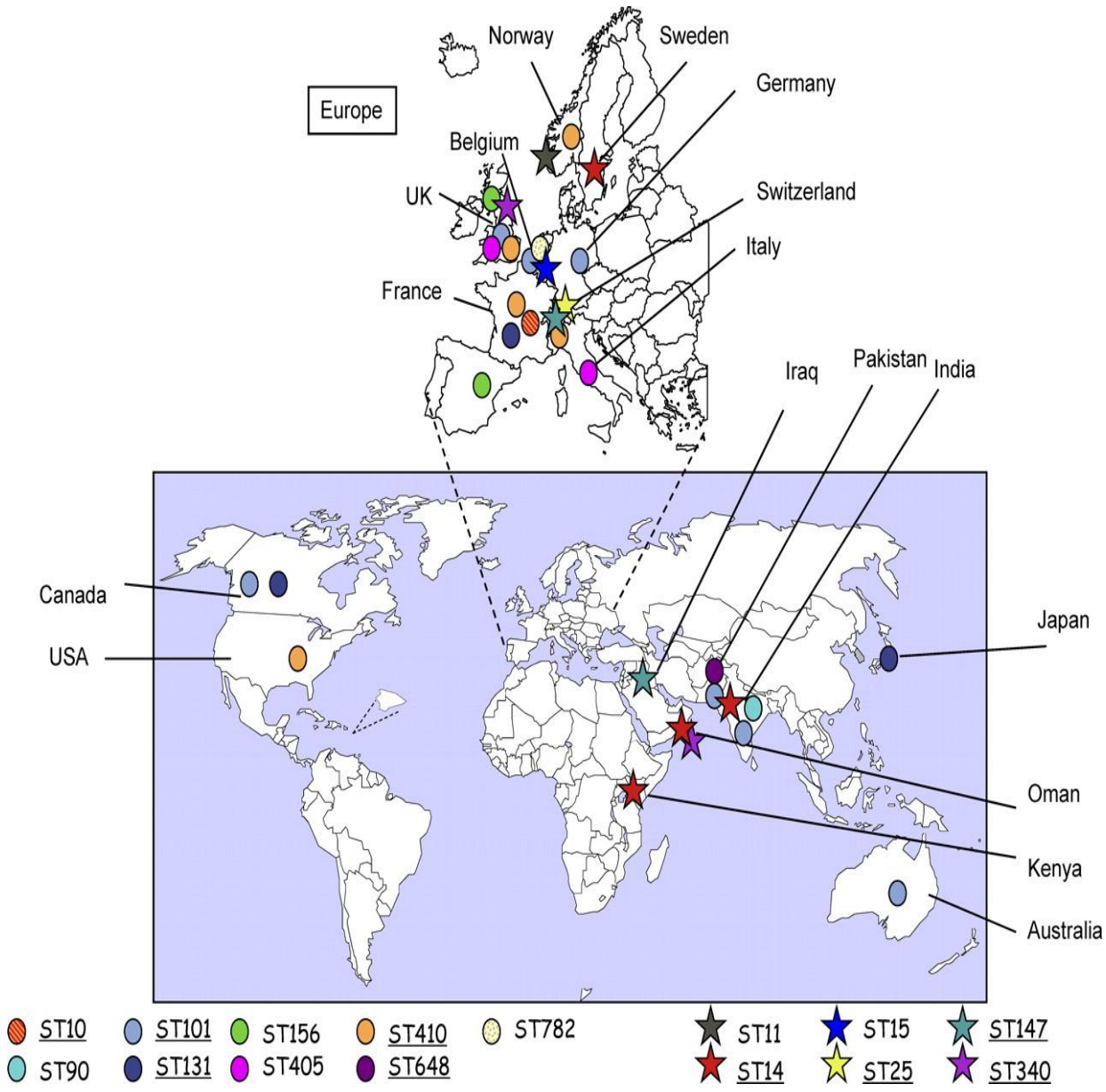


Figure 1.1: Worldwide distribution of ESBL producing *E. coli* (circles) and *K. pneumoniae* (stars) isolates with colour codes for country detected (Laurent *et al*., 2011).

2.3.2 Sulphydryl variable (SHV-type)

SHV refers to Sulphydryl variable (SHV)-type and are all derived from SHV-1 by point mutations, with more than 90 SHV ESBL variants so far described (Paterson *et al*., 2003). SHV-1 confers resistance to a wide range of penicillins such as ampicillin, tigecycline and piperacillin but not necessarily to oxyimino substituted cephalosporins (Livermore, 1995).

The most frequently detected enzymes include SHV-5 and SHV-12 which have been detected in a wide range of *Enterobacteriaceae* and outbreaks due to SHV–producing *Pseudomonas* and *Acinetobacter* species, (Mshana, 2011). SHV-2, SHV-5, SHV-19, SHV-21 and SHV-12 enzymes have been isolated in *K. pneumoniae* in African countries such as South Africa and Tanzania while in Morocco, SHV-12 enzymes have been isolated in *S. enteric* serotype (Ndugulile *et al*., 2005).

2.3.3 Cefotaximase–Munchen (CTX-M)-type

CTX-M-type enzymes are a group of class A ESBL and are rapidly spreading among *Enterobacteriaceae* worldwide and these enzymes exhibit powerful activity against cefotaxime and ceftriaxone but generally not against ceftazidime and considered the most prevalent ESBLs in *Enterobacteriaceae* (Rossolini *et al*., 2008). CTX-M ESBLs have turned out more prevalent with a larger infiltration into *E. coli* (David *et al*., 2006). In African countries such as Tanzania, a high prevalence of CTX-M-15 gene was isolated among the ESBL producing *K. pneumoniae* (Mshana *et al*., 2013)

2.4 Reservoirs of ESBL Infection

In the community setup, the reservoir for ESBL can be detected in the faecal flora of children and these are present on highly transmissible plasmids (Birgy *et al*., 2012). Community-acquired infections with ESBL-producing organisms are associated with foodstuffs, animal consumption of antibiotics, and frequent patient contact with health care facilities (Paterson and Bonomo, 2005). Food animals are reservoirs for resistant faecal flora, particularly *E. coli*, an important indicator for environmental contamination likely to spread through the food chain (Carattoli, 2008). A study conducted in Germany, established that CTX-M genes were dominant in healthy broiler chickens which in the long run spread ESBLs from the environment into the food chain (Reich *et al.*, 2013). Samples of stool collected from different environmental locations in Spain, ranging from animal farms, livestock meat products and sewage have also revealed the widespread presence of *Enterobacteriaceae* ESBL-producing bacteria carriages (Mesa *et al*., 2006). Apart from poultry, CTX-M-producing isolates have also been detected in cattle, pigs and

dogs which indicate that food-producing animals and domestic pets might act as a reservoir for the acquisition of resistant organisms (Horton, *et al.*, 2011).

A number of researchers have identified widespread dissemination of ESBL-producing *E. coli* in healthy food producing animals and in particular food products such as meat, fish and raw milk (Nadine *et al*., 2012). An exploratory study revealed a high presence of CTX-M-1 colonization by ESBL-producing bacteria largely linked to the frequent consumption of pork meat (Rasmus *et al*., 2013). Contamination of meat products during slaughtering, dressing and evisceration of internal contents is another risk factor that can result into further spread of resistant ESBLs genes within the human population (Lavilla *et al*., 2008; Reich *et al.*, 2013).

A number of studies have documented the presence of ESBL in food and water. These pathogens gain entry into human by the faecal-oral route, with the common source being water contaminated with animal excreta and food contaminated with faecal pathogens (Walsh *et al*., 2011; Kluytmans *et al*., 2013). These studies have shown a genetic relationship between ESBL-genes found in food, animals and in humans (Kluytmans *et al*., 2013). The spread of extended-spectrum-cephalosporin-resistant (ESC-R) *E. coli* in poultry meat may also be associated with veterinary usage of different classes of antibiotics for poultry treatment (Kola *et al*., 2012). The dissemination of faecal matter into the water bodies by dumping of sewage mostly from hospitals or public defaecation, also adds to the propagation of drug resistant ESBLs (Meerambika *et al.*, 2013).

A study conducted in Spain identified the presence of ESBL-producing bacteria in poultry and *E. coli* strains comprising of ESBL CTX-M-14, CTX-M-9, and SHV-12 were isolated from faecal samples of healthy and sick poultry (Brinas *et al*., 2003). High prevalence's of ESBL genes detected in chicken meat could confer a drastic effect on future treatment options with a probable threat to humans (Overdevest *et al*., 2011). A study conducted in Britain established that 54.5% of CTX-M-carrying *E. coli* were isolated from twenty two broiler abattoirs sampled and of the 388 ceacal samples from individual broilers 3.6% had CTX-M carrying *E. coli* (Randall *et al*., 2010).

There is need to evaluate the role of poultry in the transmission of highly resistant ESBL-producing bacteria in humans (Kola *et al*., 2012). The distribution of ESBL/*AmpC* enzymes among *E. coli*, with respect to geographical and host origin are shown in figure 1.2. AmpC beta-lactamases (AmpC) are enzymes which convey resistance to penicillins, second and third generation cephalosporins and cephamycins. They also result in resistance to combinations of these antibiotics and substances which are actually intended to inhibit the effect of beta-lactamases. They do not however, convey resistance to fourth generation cephalosporins. Most of these studies are from European countries, with a prevalence of various ESBL/*AmpC* types ranging between 0.6% and 44.7%. There are also quite a number of reports from Asia, with rates from 1.7% to 11.8% of ESBL/*AmpC*-producing *E. coli* and *Salmonella* species in poultry. However data from North, South America and African countries is very limited (Ewers *et al*., 2012).

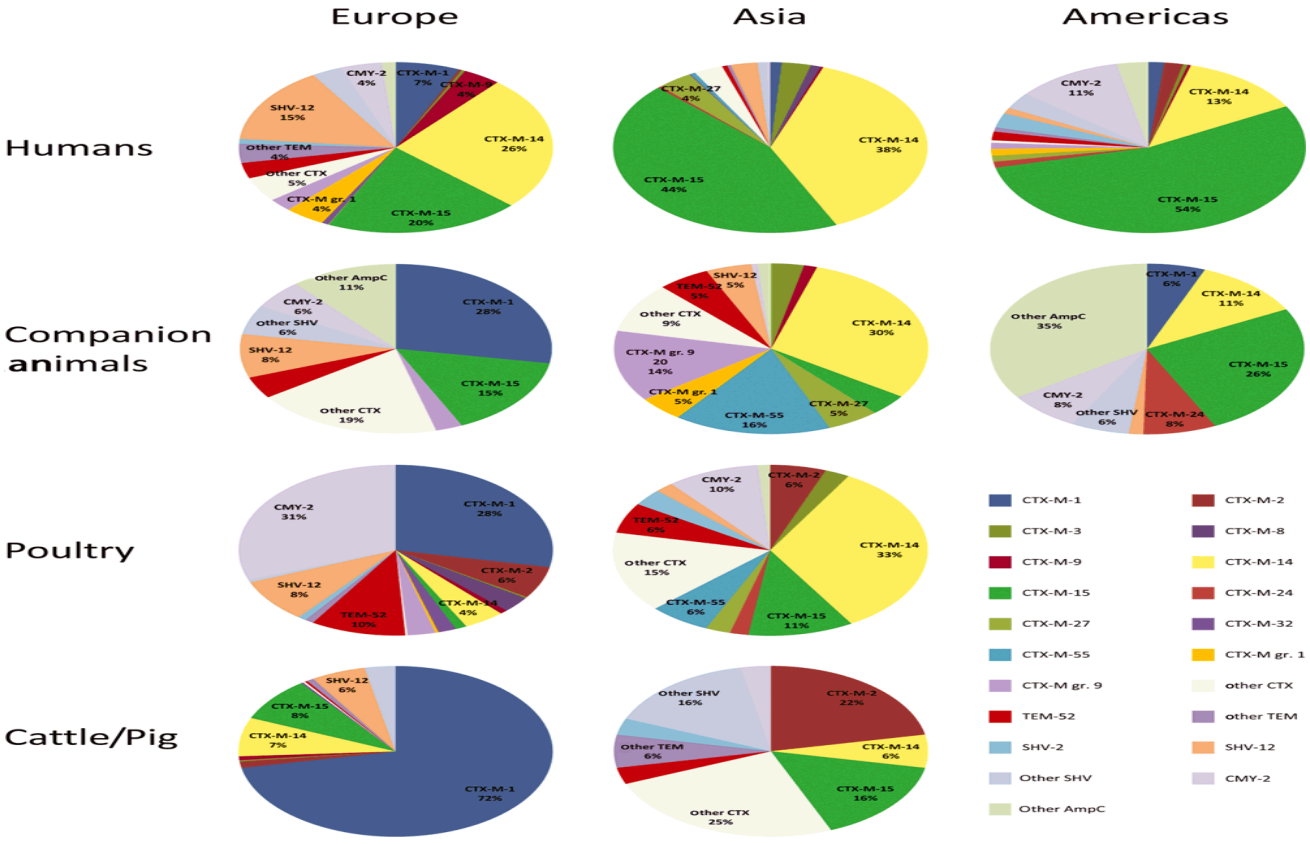


Figure 1.2: Spatial and host distribution of *Escherichia coli* extended-spectrum β-lactamase (ESBL)/*AmpC* types (Ewers *et al*., 2012)

2.5 ESBL-Producing Bacterial Carriage in Urban Water Supply

An increased influx of people migrating from the rural areas into the urban areas puts pressure on existing land sites resulting into creation of unplanned settlements exacerbating the rapid spread of infectious diseases (Abraham, 2010). Urban water supply is particularly prone to environmental hazards ranging from physical, chemical and biological contaminants (Shehani and Sui, 2013). ESBL-producing *Enterobacteriaceae* are present in environmental water bodies, though the diversity of bacterial species and ESBL genes they harbour are infrequently documented (Lu *et al*., 2010).

Generally faecal bacteria emanating from humans carry with them antibiotic resistant genes that are discharged into the environment (Allen *et al*., 2010). The occurrence of ESBL pathogenic bacteria in drinking water harbouring drug resistant genes creates a greater risk of community acquired infections which could lead to disease outbreaks and later transferring of drug resistant bacteria to humans resulting in a public health hazard (Odumosu and Akintimehin, 2015).

In terms of safe distances between wells and sanitation units, the "traditional" guideline is 15m and on the basis of statistical associations between latrines and nitrate concentrations in water sources, recommended distances of 20m, 36m and 48m for pit latrines that are in use for less than 1 decade, 1–2 decades, and greater than two decades, respectively (Tandia *et al*., 1999). Banks *et al*., (2002) further suggested that pit latrines should be located no less than 15–30 m from groundwater abstraction points and should terminate no less than 1.5–2.0m above the water table. Wells are likely to be contaminated if pit latrines are less than 12m away (Vinger *et al*., 2012). A number of community-acquired pathogens that commonly cause diarrhoea have been found to be ESBL producers especially the ESBL-producing *Salmonella* infections (AitMhand *et al*., 2002). Due to the cosmopolitan nature of cities, there is a likelihood of exposure to TEM, CTX-M and SHV carrying pathogenic species in food products through the use of contaminated water supply (EFSA, 2011).

2.6 Transmission Risk of ESBL/*AmpC* Carrying Organisms

There is no clear evidence to support the spread of ESBL/*AmpC*-carrying organisms through direct contact with animals or indirectly through the environment. However, ESBL/*AmpC* carrying organisms isolated either in animals or humans could have an indirect association in as far as antimicrobial resistance is concerned (Wright, 2007). Resistant bacteria detected in faeces and soil in the farm environment could possibly be transferred from these sources to animals and humans through the food chain (Silbergeld *et al*., 2008). A study conducted by Moodley and Guardabassi (2009) established strong evidence in the transmission of CTX-M1 between pigs and pig farmers.

Another study confirmed that people working with poultry had a higher risk of harbouring intestinal ESBL-producing bacteria suggesting that direct transmission from poultry to humans may also be a possible route of transmission (Dierikx *et al*., 2010). Unhygienic preparation of cooked foods in the kitchen is another established source of ESBL-producing *Enterobacteriaceae.* Placement of raw and already cooked foods in a contaminated environment exacerbates the transmission of ESBL-producers and also on hands through the use of kitchen utensils such as cutting boards (Tschudin-Sutter *et al.*, 2014).

Contamination of fresh food of plant origin has been increasingly recognized in many parts of the world as a source of pathogens (Lynch *et al*., 2009). Moreover, these food products may serve as a vehicle of ESBLs, transmission as demonstrated by the recent description of *Enterobacteriaceae* carrying CTX-M genes in spinach, parsnip, bean sprouts and radish (Raphael *et al*., 2011; Reuland *et al*., 2011) and SHV-2 in ready-to-eat salads (Campos *et al.*, 2011). The possibility of human acquisition of bacteria producing ESBL or *AmpC*, may also be influenced by the increasing consumption of fresh produce (Lynch *et al*., 2009).

2.7 Antimicrobial Resistance in ESBL Producing organisms

Heavy use of antibiotics has been determined to be a risk factor for acquisition of ESBL producing organisms (Mshana *et al*., 2009). ESBL-producing strains of *Enterobacteriaceae* are becoming a key problem in hospitalized as well as community based patients (Rodriguez-Baño *et al.*, 2004). The *Enterobacteriaceae* organisms producing ESBLs such as *K. pneumoniae* and *E. coli* cause failure of treatment with cephalosporin antibiotics (Bradford, 2001). A review of published and unpublished literature from Democratic Republic of Congo, Mozambique, Tanzania and Zambia revealed an increased trend of resistance to commonly used antibiotics namely; ampicillin, co-trimoxazole, gentamicin, erythromycin, tetracycline and third generation cephalosporins (Mshana *et al.*, 2013).

There has been an increase in multidrug antimicrobial resistance especially in developing countries due to *E. coli* (Wang and Chen, 2005; Kennedy *et al*., 2008). Most of the ESBL-producing isolates have a high resistant rate to ampicillin, chloramphenicol, gentamicin, streptomycin, tetracycline and cefotaxime and this makes available therapeutic choices limited (Black *et al*., 2005). Animals, food and the environment are potential reservoirs for multidrug resistance for Gram negative bacteria (Kuenzli *et al*., 2014). Food animals largely contribute a considerable proportion of the *E. coli* in the human gastrointestinal tract, including drug-resistant strains and are moderately host specific with a demonstrated ability of animal origin drug-resistant strain such as fluoroquinolone-resistant *E. coli* from chickens with a probability of either colonizing or causing infection in humans (Collignon *et al*., 2009).

The uncontrolled use of antibiotics as growth promoters in food animals has contributed greatly to the development of antibiotic resistance status (Wegener *et al.*, 1999). Furthermore, bacteria harbouring ESBLs demonstrate additional resistances to other antimicrobial classes such as the quinolones, cotrimoxazole, trimethoprim, and aminoglycosides, which create therapeutic treatment problems (Chopra *et al*., 2008). In addition, horizontal gene transfer plays a significant role in disseminating genes that are related to antimicrobial resistance between bacteria and bacteria species and these are

situated on plasmids (Lester *et al*., 2006). For instance, horizontal gene transfer of ESBLs can occur in sewage treatment plants as they handle big volumes of sewage containing high densities of bacteria facilitating dissemination of resistance genes (Shakibaie *et al*., 2009).

2.8 Laboratory Detection of ESBL

2.8.1 β-Lactamase Genes Detection

Faecal or food sample isolates are first inoculated on MacConkey agar which is a selective growth media containing 2mg/l concentrations of either cefotaxime or ceftriaxone and incubated at 37°C for 24 hours. Lactose fermenting positive colonies are picked and confirmed by the triple sugar iron and Indole Methyl red, Voges-Proskauer and Citrate (IMViC) test. Isolates are cultured in Lauria broth and incubated at 37°C for 24 hours. Thereafter DNA is prepared by the boiling method. Suspected isolates are identified and confirmed as ESBL by dedicated susceptibility tests and for molecular identification as ESBL-producing *E. coli*, targeted genes such as bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{OXA} are detected using PCR amplification and then characterized using specific group primers and sequence analysis (Batchelor *et al*., 2005; Rayamajhi *et al*., 2008). Some of the specific group primer target genes are shown in Table 2.1.

Table 2.1: Primers used for amplification of targeted genes and significance of the gene in ESBL producers

Gene	Primer	Gene Significance
bla$_{SHV}$	5-ˈCGC CGG GTT ATT CTT ATT TGT-3ˈ	Shares 68% of its amino acids with TEM-1. Commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance (Paterson *et al*., 2003).
bla$_{TEM}$	5-ˈATA AAA TTC TTG AAG ACG AAA-3ˈ	Up to 90% of ampicillin resistance in *E. coli* due to the production of TEM-1. The amino acid substitutions responsible for the ESBL phenotype cluster around the active site of the enzyme and change its configuration, allowing access to oxyimino-beta-lactam substrates. (Boyd *et al*., 2004).
bla$_{CTX-M}$	5-ˈCGTTAACGGCACGATGAC-3ˈ	Ability to hydrolyse cefotaxime and are located on highly transmissible plasmids, facilitating fast and efficient spread of resistance (Boyd *et al*., 2004; Bonnet, 2004)

2.8.2 Molecular Typing of ESBL-Producing E. coli strains

This is done using the Pulsed-Field Gel Electrophoresis (PFGE) a laboratory technique used for separation of large deoxyribonucleic acid (DNA) molecules if electric current that periodically changes direction is applied to it. The PFGE is the "gold standard technique" used in this discipline of molecular epidemiological studies and it is basically the comparison of large genomic DNA fragments after digestion with a restriction

enzyme that cuts infrequently. Since the bacterial chromosome is typically a circular molecule, the digestion by the enzyme yields several linear large DNA molecules (Schwartz and Cantor, 1984).

For molecular typing, chromosomal DNAs of ESBL-producing *E. coli* strains are subjected to PFGE analysis using a contour-clamped homogeneous electric field (CHEF) apparatus. An agarose plug containing bacterial DNA is prepared and processed for PFGE. Restriction analysis of chromosomal DNA with *Xba*1 is later performed and separation of DNA is carried out by using 1.5% low melting agarose. The DNA banding patterns are visually compared and interpreted accordingly (Ranjbar *et al.*, 2008). After viewing the banding patterns, the results are compared and analyzed by manual visualization from a computer monitor following previously established criteria and these guidelines are used for the interpretation of PFGE (Tenover *et al*., 1995). The banding pattern difference of up to three fragments could occur due to a single genetic event and thus these isolates are classified as highly related. Isolates that differ by three fragments in PFGE analysis may represent epidemiologically related subtypes of the same strain. Conversely, isolates differing in the positions of more than three restriction fragments may represent a more tenuous epidemiological relation (Sader *et al.,* 1993; Tenover *et al.*, 1995).

2.8.3 Double-Disc Diffusion Test

The disc diffusion test has extensively been used for ESBLs resistance determination to antibiotics. Detection of synergy between cefotaxime and clavulanate is by placing a disc of amoxicillin/clavulanate (20 μg/10 μg, respectively) and a disc of cefotaxime (30 μg), 30 mm apart (centre to centre) on an inoculated agar plate and a clear extension observed at the edge of the cefotaxime inhibition zone towards the disk containing clavulanate is interpreted as synergy, indicating the presence of an ESBL (Paterson and Bonomo, 2005). The double-disc diffusion has also been utilized for genotypic confirmation of ESBL producers or non-producers with higher sensitivities and specificities (Brown *et al.*, 2000). Though the double-disc diffusion test is technically uncomplicated, the interpretation is quite biased but however the sensitivity can be condensed when action of

ESBL is exceedingly low resulting into large zones of inhibition around the cephalosporin and aztreonam disks (Vercauteren *et al*., 1997).

2.8.4 Isolation and Identification of ESBL and *AmpC* producing *E. coli*

ESBL producing organisms can be cultured and identified on MacConkey agar supplemented with 1 mg/L cefotaxime (CTX) or ceftriaxone. The use of low concentrations will result in optimum sensitivity to detect all relevant β-lactamase families. Pre-enrichment may be performed in a general broth like Mueller-Hinton, Brain Heart Infusion or Luria-Bertani broth with 1 mg/L cefotaxime or ceftriaxone. Identification is performed by determination of susceptibility to cefotaxime, ceftazidime and cefoxitin. ESBL producers are resistant to cefotaxime variable to ceftazidime and susceptible to cefoxitin. Confirmation of ESBL is performed by testing for synergy with clavulanic acid by combination disks, ESBL E-test or broth microdilution including cefotaxime or ceftazidime as single drugs and in combination with clavulanic acid. This is performed by determination of susceptibility to cefepime; *AmpC* producers are susceptible to cefepime and resistant to cefotaxime, ceftriaxone and cefoxitin (Henrik *et al*., 2014).

2.8.5 Interpretive criteria and Minimum Inhibitory Concentration (MIC)

The interpretive criteria for *Enterobacteriaceae* ESBL-producers is shown in table 2.2 where the Minimum Inhibitory Concentration (MIC) breakpoints or epidemiological cut-off (ECOFF) values of the European Committee on Antimicrobial Sensitivity Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) are presented.

Table 2.2: Antibiotic MIC-breakpoints (S/R) as prescribed by EUCAST (European Committee on Antimicrobial Sensitivity Testing) and CLSI (Clinical and Laboratory Standards Institute) for *Enterobacteriaceae*

Reference organization	Cefotaxime (mg/L)	Ceftazidime (mg/L)	Ceftriaxone (mg/L)	Cefoxitin (mg/L)	Cefepime (mg/L)	Ceftiofur (mg/L)
EUCAST*	≤1/>2	≤1/>4	≤1/>2	NA**	≤1/>4	NA**
EUCAST	≤0.5 – 0.064#	≤ 0.125 – 2#	≤0.125– 0.5#	≤ 8	≤ 0.12	≤ 1 – 2#
ECOFF§ CLSI*	≤1/≥ 4	≤4/≥ 16	≤1/≥ 4	≤8/≥32	≤8/≥32	NA**

*Clinical breakpoints are not primarily intended to detect resistance mechanisms. Cut-offs is adequate for this objective. Values are not provided for all species associated with ESBL or *AmpC*s.

#Range comprises values corresponding to different *Enterobacteriaceae* species.

§ECOFF: This value separates microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to the agent in question. The ECOFF is the lowest possible value for the clinical breakpoint.

**NA – not available Although CLSI has recently redefined MIC breakpoints for third and fourth generation cephalosporins, the R-breakpoints for ceftazidime, cefoxitin and cefepime are still one to two dilution steps higher than those defined by EUCAST. To harmonize the interpretation of susceptibility data and for optimum phenotypic detection of ESBL and/or *AmpC* producers, it is advised to use EUCAST clinical breakpoints for interpretation of susceptibility or resistance and EUCAST ECOFF's to determine if an isolate belongs to the wild-type population or not (EFSA, 2011).

2.9 Treatment and Control of ESBL Associated Infections

Most of the infections attributed to ESBL-*Enterobacteriaceae* are important in clinical practice as they are largely associated with increased morbidity and mortality (de Kraker *et al*., 2011). Treatment challenges in ESBL-*Enterobacteriaceae* infections relate to co-resistance which may be as a result of the ESBL plasmid harbouring several antibiotic resistance determinants making treatment more difficult leading to co-selection of several resistant determinants (Rottier *et al*., 2012). High mortality has been noticed in patients diagnosed with ESBL- *Enterobacteriaceae* producing organisms who did not receive proper antibiotic therapy compared to patients with non-ESBL-*Enterobacteriaceae* producing organisms of the same species (Paterson and Bonomo, 2005). Thus, most of the infections caused by the presence of resistant ESBL producers are linked to high morbidity and mortality rates (Tumbarello *et al.*, 2010). A number of outbreaks of infections as a result of ESBL-*Enterobacteriaceae* producing organisms have been reported in South Africa and also in North African countries (Bell *et al*., 2002). Other outbreaks in Nigeria and Kenya due to *Klebsiella* infections with strains that are resistant to third-generation cephalosporins have also been reported (Paterson and Bonomo, 2005).

There are limited therapeutic options for ESBL-producing organisms as most ESBLs confer resistance to most β-lactam antibiotics with an exception of cephamycins and carbapenems. For the treatment of uncomplicated urinary tract infections (UTIs) fluoroquinolones may be used when established to be susceptible. Studies that have been conducted have proved carbapenems to be superior to quinolones for treatment of serious infections caused by ESBL-producing organisms (Endimiani *et al*., 2004). Some infections that are resistant to ceftazidime may be susceptible to cefotaxime or ceftriaxone but respond to treatment with alternative cephalosporins. Nevertheless, the MICs of these agents may rise considerably as the inoculum is increased (Chaudhary and Aggarwal, 2004).

Even though ESBL activity is inhibited by clavulanic acid, β-lactam/ β-lactamase inhibitor combinations are not considered optimal therapy for serious infections caused by ESBL producers as their clinical effectiveness is controversial. The majority of ESBL-

producing organisms produce more than one β-lactamase, often in different amounts. Additionally, it is well known that ESBL-producing organisms may continue to harbour parent enzymes for example, SHV-1 or TEM-1 (Akhan *et al.*, 2001; Paterson and Bonomo, 2005). The β-lactam/ β-lactamase inhibitor combinations are subject to rising MICs as inoculum rises. As a result, infections with high organism burden (intra-abdominal collections, sepsis) may be associated with sufficient β-lactamase production to overcome the effects of the β-lactamase inhibitor. However, they may be useful for less serious infections, such as uncomplicated non-bacteremic lower urinary tract infection, because the infection is localized and the antibiotic is excreted in large amounts through the urine. The advantage of using β-lactamase inhibitors is that by inhibiting ESBLs they appear to impair the emergence and spread of *Klebsiella*-carrying resistance plasmids. Furthermore, administration of inhibitors may exert *in vitro* pressure on ESBLs, thereby facilitating their reverse mutation to less harmful enzymes (Chaudhary and Aggarwal, 2004).

Uncomplicated infections like non-bacteremic urinary tract infections can be managed with a variety of antibiotics, depending on their susceptibility. These include oral antibiotics like trimethoprim, nitrofurantoin, fosfomycin, co-amoxiclav, mecillinam; or intravenous agents like aminoglycoside (gentamicin, amikacin) and inhibitor combinations (Bhattacharya, 2006). Among these carbapenems are the drugs of choice for serious infections with ESBL producers. Imipenem and meropenem are preferred in nosocomial infections, while etrapenam is preferred in community-acquired infections (Shah and Isaacs, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Study Area

The study was conducted in Lusaka District the Capital City of Zambia as shown in Figure 3.1 below. Lusaka district has a total population of 1,747,152 with about 358,871 households and has seven constituencies namely; Chawama, Kabwata, Kanyama, Lusaka Central, Mandevu, Matero and Munali (CSO, 2011). Lusaka city covers an estimated area of 360 km^2 and is located at 15º30' latitude south and 28º17' longitude east. The city lies on a plateau 1280m above sea level (MFNP, 2005). Three constituencies, namely Kanyama, Mandevu and Matero were considered for the purpose of shallow well water sampling. The selected communities were picked on the basis of their dense populations and high annual number of diarrhoeal cases annually. The inadequate provision of wholesome water supply was also an important fact in considering the safety of water used in these areas.

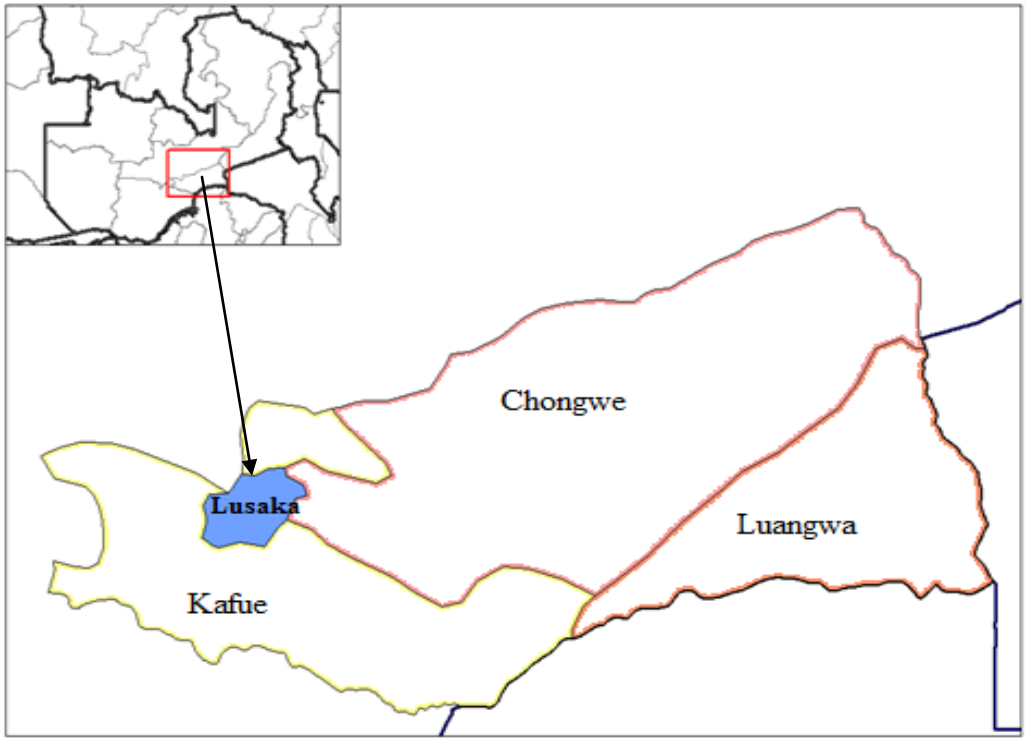


Figure 3.1: Location of Lusaka District where the study was conducted.

3.1 Study Design and Sampling

A cross-sectional study design was used and the duration of the sampling was from September 2014 to March 2015. A total of 384 poultry faecal swabs and 145 unprotected shallow wells were sampled. In this study, a shallow well was defined as a hole which is less than 15 meters deep which had been dug, bored, driven or drilled into the ground for the purpose of extracting water (Gronwall *et al*., 2010). A typical shallow well from the sampling site is shown in Figure 3.2

Figure 3.2: Example of the study shallow wells from Kanyama and George compound sampling sites.

3.2 Sample Size Calculation

Sample size was calculated using the following formula as described by Cochran (1963).

$$S(n) = \frac{X^2 NP(1-P)}{d^2 (N-1) + X^2 P(1-P)}$$

Where S (n) = Desired sample size

P = expected frequency of factor under study at 0.5

d = degree of accuracy 5%

X = Confidence level of 1.96 for a 2-sided test at the 0.05 level

$$S(n) = \frac{1.96^2 \ (1, 747, 152)\ 0.5\ (1-0.5)}{0.05^2\ (1, 747, 152-1) + 1.96^2(0.5)\ (1-0.5)}$$

$$= 384 \text{ (Poultry samples)}$$

The estimate poultry sample size was adjusted for fine population according to the formula below: $n' = \frac{1}{\frac{1}{n}+\frac{1}{N}}$

Thus, a sample size of eight (8) sampling units comprising of seven (7) peri-urban communities and one (1) poultry abattoir was considered.

3.3 Poultry Sample Collection

3.3.1 Poultry Abattoir

At the slaughterhouse, cecal samples were collected from different batches before the carcasses were chilled. From each batch consisting of 270 poultry carcasses 10% of faecal swabs were sampled.

3.3.2 Peri-Urban Community

From each peri-urban community, 10% of live poultry chickens available for sale were sampled. Faecal swabs were collected from each live poultry chicken offered for sale to the general public.

3.4 Water Sample Collection

A sampling frame for each peri-urban community with an estimated number of shallow well water points existing was developed. A random sampling method was employed in which 10% of shallow well water samples from each peri-urban community were collected. These peri-urban communities included Chawama, Chipata, Chunga, Garden, George, Kanyama and Matero. The distance between the shallow well water point and the nearest pitlatrine existing was measured and the majority of the distances ranged approximately from 5 – 7m. The sampling was done using a 200ml sterile bottle

supported by a string tied around its neck which was first disinfected with 70% alcohol. The sterile bottle was carefully lowered into the well ensuring that it did not have contact with the walls of the well. When full, the bottle was pulled out and immediately capped. Samples were labelled appropriately and placed in a box with ice and transported to the School of Veterinary Medicine, Department of Paraclinical Studies microbiology laboratory, University of Zambia. Samples were processed within 24 hours of collection.

3.5 Isolation of ESBL-producing *E. coli* bacterial strain

The 384 poultry faecal swabs and 145 shallow well water samples were inoculated on MacConkey agar containing 2 mg/L of cefotaxime for preliminary screening of ESBL producing bacteria. The plates were incubated at 37°C for 24 hours. The colonies that grew on MacConkey agar were identified as lactose fermenters or non-lactose fermenters. The lactose fermenters were identified and selected for further analysis. Identification of *E. coli* lactose fermenting positive colonies was done using phenotypic characteristics and confirmed by the Triple Sugar Iron (TSI) and IMViC tests as previously described (Batchelor e*t al*., 2005; Rayamajhi *et al*., 2008). For genetic detection and characterization, *E. coli* isolates were cultured on brain heart broth at 37°C for 24 hours. After incubation, DNA was prepared by boiling methods. A 1.5 ml of bacterial suspension was later centrifuged at 5, 800 x g for 5 minutes. After the centrifugation, the supernatant was discarded. The cell pellet was washed with 500 µl normal saline and centrifuged at 13, 000 x g for 15 minutes and the supernatant was later discarded. Immediately after washing, the cell pellet was suspended in 500 µl of TE buffer (pH 8.0) and was boiled at for 10 minutes and then immediately transferred to ice for 10 minutes. Cell debris was removed by centrifugation at 13,000 x g for 15 minutes. The supernatant was transferred into a new microfuge tube and kept at - 20°C until use.

3.6 PCR Analysis

The *E. coli* isolates were subjected to PCR for confirmation of resistance genes TEM (Temoniera), SHV (Sulphydryl Variable) and CTX-M (Cefotaxime –Munich). The PCR (Finnzymes Piko) was performed using a total reaction volume of 10 µl consisting of 5 µl Phusion, 2µl sterile distilled water, 2 µl primers (forward and reverse) and 1 µl bacterial

DNA template. PCR reaction was performed using the rapid cycle DNA amplification method comprising of an initial denaturation step at 98ºC for 30 seconds, followed by 35 cycles of template denaturation at 98ºC, primer annealing at 60ºC for 5 seconds at 72ºC for 1 second and a final extension at 72ºC for 10 seconds. The PCR products were viewed with ethidium bromide after electrophoresis through 1.5% agarose gel as described by Ranjbar *et al*., (2008).

3.7 Antimicrobial Susceptibility Test and ESBL Producing Strain Detection

The antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method based on the Clinical Laboratory Standard Institute (CLSI) guideline (CLSI, 2009). The antibiotic discs (Becton, Dickinson and Company, MD, USA) used included ampicillin (10 µg), sulfamethoxazole/trimethoprim (1.25/23.75 µg), streptomycin (300µg), ciprofloxacin (5µg), tetracycline (30 µg), gentamicin (10µg), nalidixic acid ((30 µg), chloramphenicol (30 µg), ceftazidime (30 µg), norfloxacin (10µg) and cefotaxime (30 µg). The phenotypic confirmation of ESBL isolates was done by the combination of disc approximation method using either ceftazidime (30 µg) or cefotaxime (30 µg) alone followed by over- night incubation at 37°C for 18 – 24 hrs. Interpretation of susceptibility patterns on other anti-microbial disks was done using guidelines laid down in the Clinical Laboratory Standards Institute (CLSI), which provides break points corresponding to zone of inhibition diameter. An increase in zone diameter of $\geq$ 5mm for either ceftazidime or cefotaxime indicated ESBL production (Mshana, 2009; CLSI, 2009). Quality control standard laboratory procedures were strictly adhered to so as to avoid contamination. *E. coli* ATCC 25922 was used as quality control organisms.

3.8 Spatial Distribution of ESBL in Shallow Wells

Mapping the distribution of ESBLs in shallow wells was done using GPS reader GPS, Garmin e30 equipment. The parameters recorded include the location of shallow well water, number of shallow wells and the associated coordinates and altitude.

3.9 Data Management and Analysis

Laboratory data was entered into Microsoft excel and then exported to the STATA version 13.0 software for analysis according to the objectives of the study. To explore the data, descriptive statistical analysis of quantitative bacterial counts, measurements of location were used to describe the outcome. Results were presented in percentages/proportions and the difference in distribution of predictor variables was considered significant if p-value was less than 0.05. A logistic regression was also used to determine multiple effects of predictor variables on the presence of resistant genes. .

3.9.1 Ethics Considerations

Authority to conduct the study was approved by the University of Zambia and permission to collect water and poultry faecal swabs was obtained from Ministry of Community Development Mother and Child Health.

CHAPTER FOUR

RESULTS

4.0 Detection of ESBL Producing *E. coli*

A total of 77 suspected ESBL-producing *E. coli* isolates were detected out of which 42 (54.5%, 42/77; 95% CI; 43.2 – 65.5%) were confirmed to be ESBL-producing *E. coli* isolates (Figure 4.1). The breakdown showed that 33 (42.9%) ESBL-producing *E. coli* isolates were found in poultry while 9 (11.6%) ESBL-producing *E. coli* isolates were found in shallow wells (Figure 4.2).

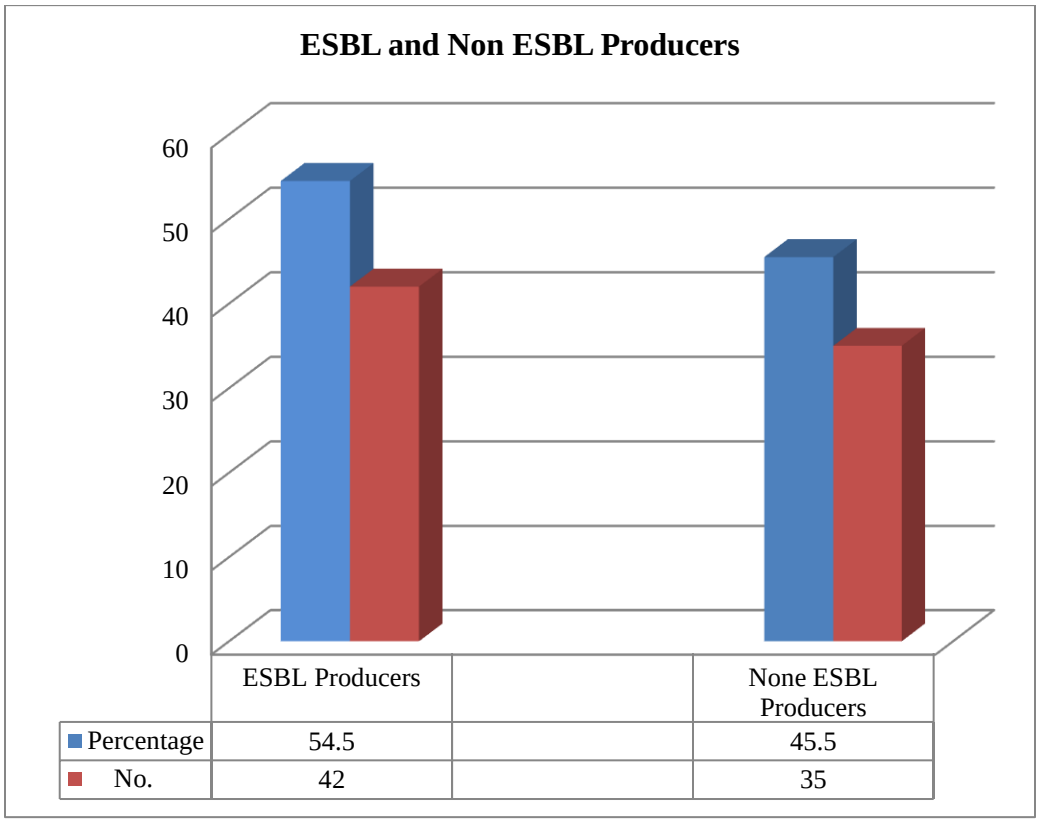


Figure 4.1: ESBL producers and non-ESBL producers in chickens and sampled water.

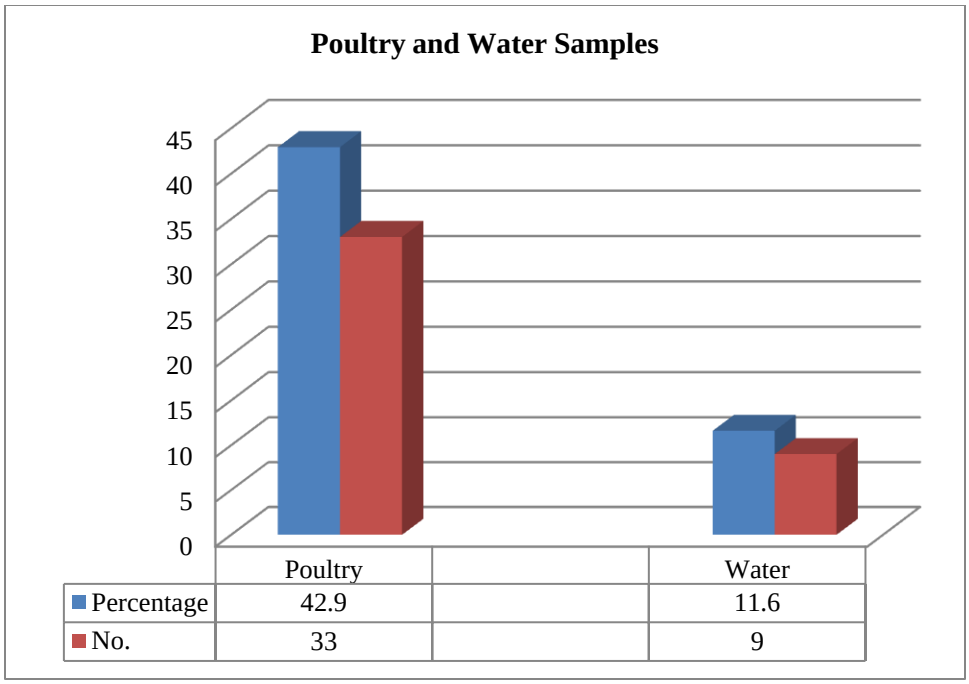


Figure 4.2: Proportion of *E. coli* ESBL-producers for poultry and water

4.1.2 Proportion of ESBL *E. coli* Producers in Poultry and Water According to Location

In general the slaughter house (abattoir) had a significantly higher proportion of ESBL-producing *E. coli* isolates with 31.2% (95% CI = 21.7 – 42.6). This was followed by ESBL-producing *E. coli* isolates from Chipata and Matero compounds, which all recorded 13% (95% CI = 7 – 22.6). The third ESBL-producing *E. coli* isolates from Chawama and George compounds showed 11.7% (95% CI = 6.1 – 21.2). The fourth ESBL-producing *E. coli* isolates were from Garden Compound which showed 9% (95% CI = 4.3 – 18.1), while Kanyama compound showed 7.8% (95% CI = 3.5 – 16.5). The lowest was Chunga Compound at 2.6% (95% CI = 0.6 – 10.1) (Table 4.1).

Table 4.1 Proportion of ESBL Producers According to Location

Location	**Sample Proportion %**	**Classification of *E. coli* isolates**		**(95% CI)**
		Positive	Negative	
Abattoir	31.2	9		(0.217 - 0.426)
			15	
Chawama	11.7	6		(0.061 - 0.212)
			3	
Garden	9	3		(0.043 - 0.181)
			4	
Chipata	13	6		(0.070 - 0.227)
			4	
Kanyama	7.8	2		(0.035 - 0.165)
			4	
George	11.7	7		(0.061 - 0.212)
			2	
Matero	13	7		(0.070 - 0.227)
			3	
Chunga	2.6	2		(0.006 - 0.101)
			0	
Total	100	42	35	

4.1.3 PCR Detection of ESBL Genes

The seventy seven (77) *E. coli* isolates analyzed by PCR revealed that 54.5% (42 isolates) were ESBL-producing *E. coli* isolates carrying the β-lactamase genes of either $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} or a combination (Table 4.2). The major genes detected on PCR was $bla_{\text{CTX-M}}$-cluster (Figure 4.3a), followed by bla_{SHV}-cluster (Figure 4.3b) and bla_{TEM}-cluster (Figure 4.3c). A combination of $bla_{\text{CTX-M}}$ + bla_{TEM}-cluster showed 13% (10 isolates). This was followed by a combination of $bla_{\text{CTX-M}}$ + bla_{SHV}-cluster, $bla_{\text{CTX-M}}$ + bla_{SHV} + bla_{TEM}-

cluster with 2.6% (2 isolates), respectively and the lowest was a combination of *bla*$_{TEM}$ + *bla*$_{SHV}$-cluster with 1.3% (1 isolate) (Table 4.2).

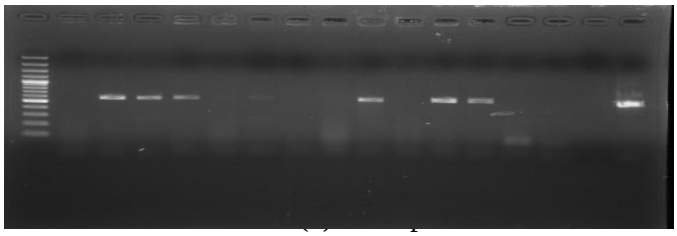
Table 4.2: Confirmed ESBL producers using PCR Gene Detection from Poultry and Shallow Water samples

Detected gene (s)	**Number of *E. coli* isolates**	**% *E. coli* isolates (n = 77)** [a]
SHV	4	5.2
CTX-M	19	24.7
TEM	4	5.2
CTX-M and TEM	10	13
CTX-M and SHV	2	2.6
TEM and SHV	1	1.3
CTX-M, SHV, and TEM	2	2.6
None[b]	35	45.5
Proven ESBL producers	42	54.5

[a]Seventy seven *E. coli* isolates suspected of being ESBL producers were examined.

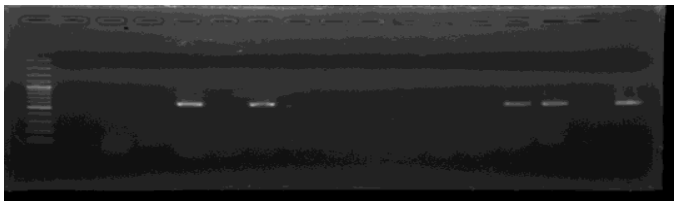
[b]Negative in all PCRs.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



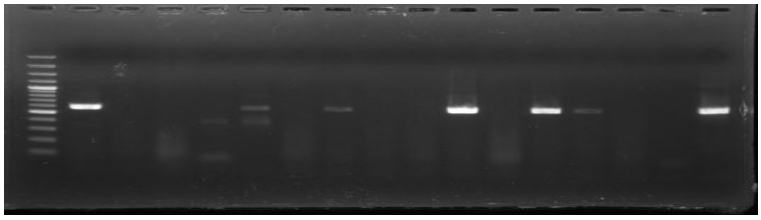
(a) 500bp

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



(b) 500bp

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



(c) 600bp

Figure 4.3: Agarose gels showing results of PCR products after amplification from the DNA of ESBL producing *E. coli*. (a) CTX-M gene, (b) SHV gene and (c) TEM gene detected with 15 and 16 as negative and positive controls respectively. Lane M is a molecular marker, where as lane 1 to 14 are samples being analysed.

4.1.4 Detected ESBL genes from Water *E. coli* isolates

The results showed that 9/77 (11.6%) ESBL-producing *E. coli* isolates analyzed on PCR confirmed the presence of ESBL-producing *E.coli* isolates and the most frequently encountered gene was a combination of $bla_{\text{CTX-M}}$ + bla_{TEM}-cluster (5.2%) (4 isolates), followed by $bla_{\text{CTX-M}}$-cluster, bla_{SHV}-cluster with 2.6% (2 isolates) and bla_{TEM}-cluster with 1.3% (1 isolate) (Table 4.3).

Table 4.3: Confirmed ESBL producers (using PCR and ESBL genes) from Water isolates

Detected gene (s)	**Number of *E. coli* isolates**	**% E. coli isolates (n = 9) [a]**
SHV	2	2.6
CTX-M	2	2.6
TEM	1	1.3
CTX-M and TEM	4	5.2
Proven ESBL producers	9	9

[a]Nine *E. coli* isolates suspected of being ESBL producers were examined.

Out of the 145 unprotected shallow well water samples from the peri-urban communities in Lusaka District, nine harboured ESBL-producing *E. coli* as indicated in Figure 4.4. Map showing the locations of shallow well water samples collected from peri-urban communities in seven study sites (Chawama, Chipata, Garden, Mazyopa, Kanyama, George and Chunga) in Lusaka district.

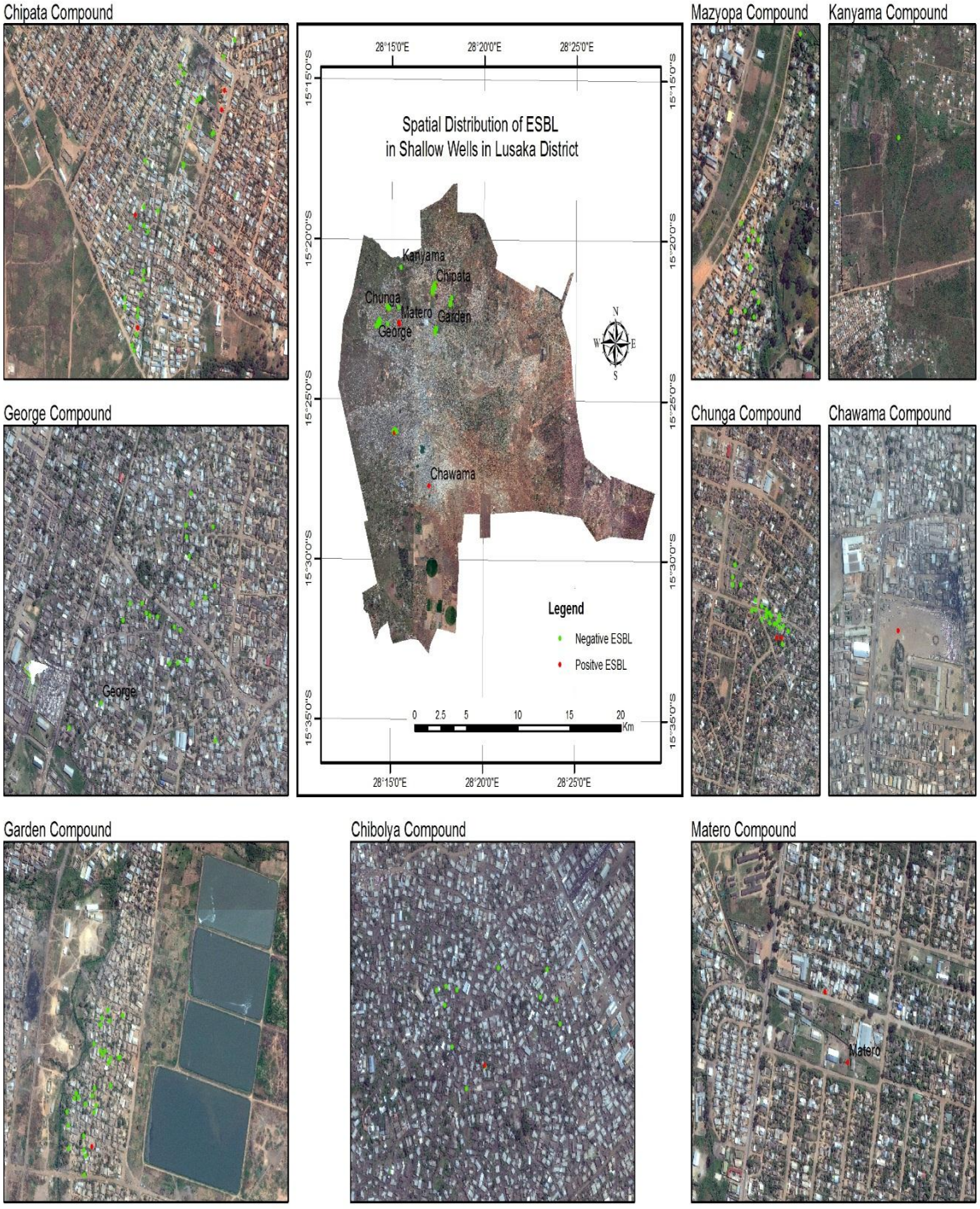


Figure 4.4: Spatial distribution of ESBL- producing *E. coli* in unprotected shallow wells in the peri-urban communities of Lusaka District.

4.1.5 Antimicrobial Resistance Pattern

Of the 77 *E. coli* isolates analyzed, 85.7% (66/77; CI: 75.7 – 92) were resistant to one or several antimicrobial compounds, and the rest 14.3% (11/77; CI: 8 – 24.3) *E. coli* isolates were found to be completely susceptible to the tested antibiotics (Table 4.4). Of the 66 *E. coli* isolates exhibiting resistance, 86.4%, (57/66) were from poultry faecal samples while 13.6%, (9/66) were from shallow well water samples (Figure 4.5).

The diversity of the antibiotic resistance and susceptible *E. coli* isolates are presented in Table 4.6. Interestingly, high resistance rates were noticed in AMP (79.2%), CTX/CAZ (67.5%), TET (59.7%), CHL (57.1%) and NOR (54.5%) (Table 4.7). It was further observed that in poultry swabs multidrug-resistance (MDR) *E. coli* isolates to six or more drugs was most frequent (45.5%, 35/77) followed by resistance to five drugs (11.7%, 9/77) and four drug resistance (11.7%, 9/77) (Table 4.8).

In water multidrug-resistance (MDR) *E. coli* isolates to six or more drugs was most frequent (3.9%, 3/77) together with resistance to five drugs (3.9%, 3/77) followed by four drug resistance (2.6%, 2/77) (Table 10).

Table: 4.4 Antibiotic Sensitivity Test Results

Results	**N**	**Sample Proportion%**	**95% C.I**
Resistance	66	85.7	(75.7 - 92)
Susceptible	11	14.3	(8–24.3)

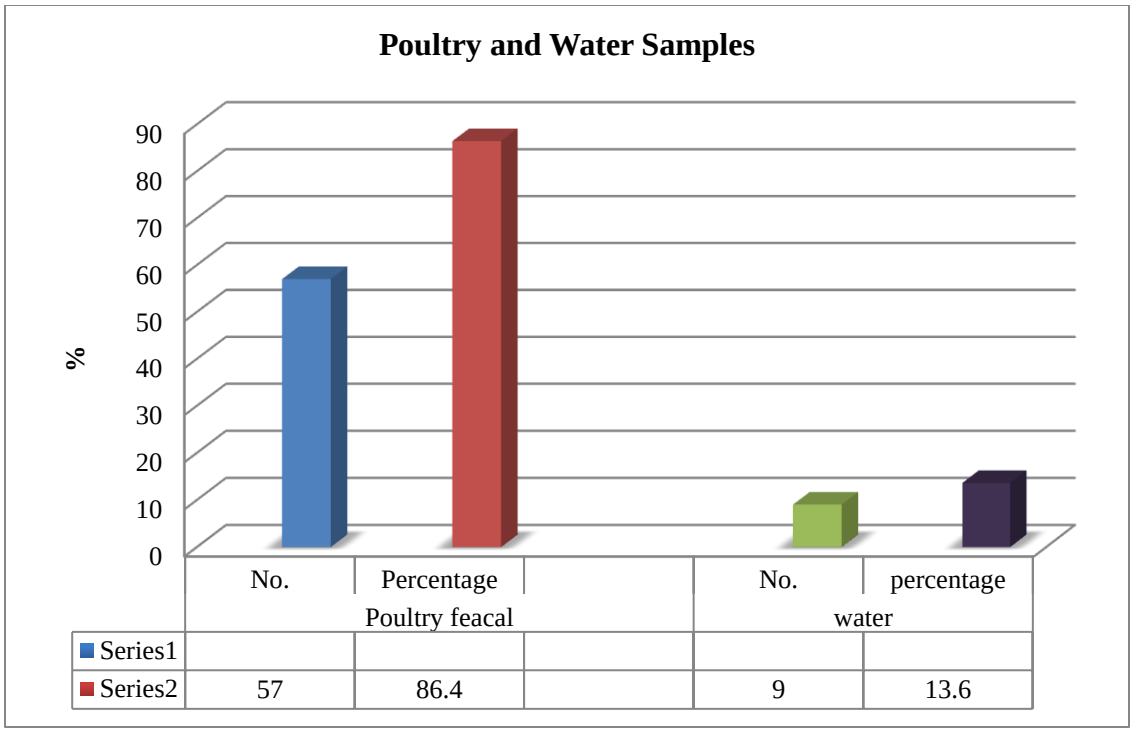


Figure 4.5: Frequency and percentage of poultry and water samples positive for ESBL producing *E. coli* subjected to antibiotic sensitivity.

Table 4.5: Antibiotic Susceptibilities of the isolates n = 77

Antibiotic	**Resistant Number (%)**	**Sensitive Number (%)**
Cefotaxime/Ceftazidime	52 (67.5%)	25 (32.5%)
Ampicillin	61(79.2%)	16(20.8%)
Chloramphenical	44 (57.1%)	33 (42.9%)
Ciprofloxacin	37 (48.1%)	40 (51.9%)
Gentamicin	29 (37.7%)	58 (62.3%)
Nalidixic Acid	37 (48.1%)	40 (51.9%)
Norfloxacin	42 (54.5%)	58(45.5%)
Streptomycin	15 (20.8%)	62 (80.5%)
Sulfamethoxazole/Trimethoprime	32 (41.6%)	45 (58.4%)
Teteracycline	46 (59.7%)	31 (40.3%)

Table 4.6: Antibiotic resistance patterns of *E. coli* isolates from Poultry

Antibiotic combination	**Number of resistant isolates**	**%**	**Observation**
AMP+TET	1	1.3	Resistant to two antibiotics
AMP+TET+CHL+CAZ	3	3.9	Resistant to four antibiotics
AMP+CAZ+CHL+NOR	3	3.9	
AMP+CHL+CXT+SXT	3	3.9	
AMP+STR+GEN+TET+CXT	9	11.7	Resistant to five antibiotics
AMP+STR+TET+CXT+NAL+CAZ+NOR+CIP	35	45.5	Resistant to six antibiotics

Abbreviations: AMP, ampicillin; TET, tetracycline; CXT, cefotaxime; CHL, chloramphenicol; CAZ, ceftazidime; NOR, norfloxacin; SXT, trimethoprim-sulphamethoxazole; NAL, nalidixic acid; STR, streptomycin; GEN, gentamicin; CIP, ciprofloxacin.

Table 4.7: Antibiotic resistance patterns of *E. coli* isolates from Water

Antibiotic combination	**Number of resistant isolates**	**%**	**Observation**
AMP+CXT+GEN	1	1.3	Resistant to three antibiotics
AMP+CAZ+CHL+SXT	2	2.6	Resistant to four antibiotics
AMP+STR+GEN+TET+CXT	3	3.9	Resistant to five antibiotics
AMP+STR+TET+CXT+NAL+CAZ+NOR+CIP	3	3.9	Resistant to six or more antibiotics

Abbreviations: AMP, ampicillin; TET, tetracycline; CXT, cefotaxoime; CHL, chloramphenicol; CAZ, ceftazidime; NOR, norfloxacin; SXT, trimethoprim-sulphamethoxazole; NAL, nalidixic acid; STR, streptomycin; GEN, gentamicin; CIP, ciprofloxacin.

4.2 Logistic Regression Analysis of ESBL Detection Variables on PCR Results

A binary logistic regression model was used to determine variables that could be useful predictors for the outcome of PCR results. Variables with p-value less than 0.05 were considered significant.

The results of the logistic regression analysis after adjusting for the effect of Chipata location showed that George, Matero and Chawama locations were the only significant predictors on PCR results. The odds of PCR results for George compound was established to be 22.4 (95% CI = 2.42 – 207.53) times more likely to positively influence the PCR results (P< 0.006). Matero was determined to be 14.9 (95% CI = 1.87 – 119.23) times more likely to positively influence the PCR results (P< 0.011) and Garden was also statistically significant with (P<0.017) and was found to be 12.8 (95% CI = 1.57 – 104.46) times more likely to positively influence the PCR results (Table 4.8)

A non-significant Hosmer-Lemeshow goodness of fit test (chi2 = 0.73; Prob > X2 = 0.97) indicated that the model fitted the data (Figure 4.6).

Table 4.8: Logistic regression model

Location	**Odds Ratio**	**Z**	**P>z**	**[95% Conf. Interval]**
Chipata	5.0	1.75	0.081	0.82 - 31.26
George	22.4	2.74	0.006	2.42 - 207.53
Matero	14.9	2.55	0.011	1.87 - 119.23
Chawama	12.8	2.38	0.017	1.57 - 104.46
Constant	1.0	0.06	0.955	0.5 - 2.08

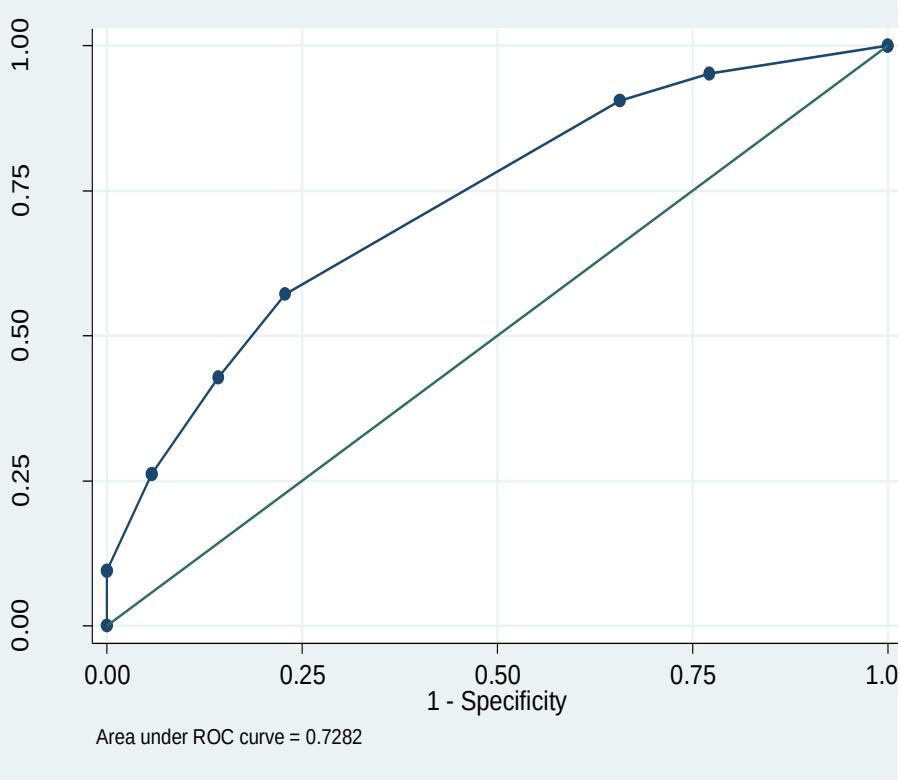


Figure 4.6: ROC curve analysis for presence PCR results

CHAPTER FIVE

DISCUSSION

Detection of ESBL-producing *E. coli* isolates in poultry and shallow well water have not been conducted in Lusaka District and generally in Zambia. In this cross sectional study an overall of 54.5% ESBL-producing *E. coli* isolates were detected in poultry and shallow well water. The presence of ESBL-producing *E. coli* isolates in this study may have been due to faecal contamination discharges to poultry and water (Walsh *et al*., 2011, Kluytmans *et al.*, 2013). The majority of the ESBL-producing *E. coli* isolates were found in poultry. The high prevalence observed in poultry may be due to frequent administration of antimicrobial drugs to poultry food animals which in turn increases the risk of higher antimicrobial resistant *E. coli* strains in the normal intestinal flora. This is in agreement with a study conducted by Carattoli, (2007) which established that food animals were possible reservoirs for resistant faecal flora, particularly *E. coli.* Conversely, the presence of ESBL-producing *E. coli* detected in shallow well water frequently utilized by some families residing in the peri-urban communities of Lusaka District could be to a lesser extent due to domestic animal excreta gaining access into ground water thereby contributing to the contamination of these water sources with faecal pathogens. This is in line with studies conducted by Walsh *et al*., (2011) and Kluytmans *et al*., (2013).

The study further revealed that the poultry abattoir had recorded a relatively high number of ESBL-producing *E. coli* isolates compared to the other market places in the peri-urban communities namely Chawama, Garden, Chipata, Kanyama, George, Matero and Chunga where live poultry are offered for sale to the general public. This high colonization rate could be attributed to cross contamination of poultry and other meat products particularly during slaughtering and dressing which is a potential risk factor that could exacerbate the transmission rate of ESBL-producing *E. coli* resistant genes (Lavilla *et al*., 2008 and Reich *et al*., (2013). In this study the factors responsible for the high levels of ESBL-

producing *E. coli* isolates was not fully known as the study did not focus on the potential risk factors.

From the 42 ESBL-producing *E. coli* isolates for poultry and water, the study revealed the presence of the following genes *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ or a combination responsible for β -lactam resistant phenotypes. The predominant ESBL-producing *E. coli* gene identified in this study was *bla*$_{\text{CTX-M}}$-cluster with 24.7% which was relatively low in relation to 54.5% of *bla*$_{\text{CTX-M}}$ carrying *E. coli* isolated from poultry chickens in Britain (Randall *et al*., 2010). In Zambia, the economic situation has resulted in many residents in the peri-urban communities in Lusaka District resorting to rearing of broiler chickens as an income generating venture with unhygienic backyard poultry houses. This brings humans into close contacts with poultry livestock which could be another possible route of shedding ESBL-producers into the environment. However, no detailed studies have yet been conducted in Zambia to specifically describe the types of ESBL-producing *Enterobacteriaceae* present in health poultry chickens and other food animals.

In addition, the study also showed that ESBL-producing *E. coli* isolates carried multiple types of β-lactamase genes and that the combination of *bla*$_{\text{CTX-M}}$ + *bla*$_{\text{TEM}}$-cluster was predominant followed by *bla*$_{\text{SHV}}$-cluster and *bla*$_{\text{TEM}}$-cluster. This, therefore, confirms that ESBL-mediated plasmids are capable of carrying more than one beta-lactamase genes and as such would result into high level presence of beta-lactam resistant phenotypes as described by Rottier *et al*., (2012). Previous studies in Spain identified *E. coli* strains comprising of *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-9}}$, and *bla*$_{\text{SHV-12}}$as the predominant ESBL-producing subtypes isolated among poultry (Brinas *et al*., 2003).

The frequent use of shallow well water sources in the peri-urban communities in Lusaka District increases the risk of acquiring ESBL-producing *E. coli* isolates. Water from these shallow wells is drawn using a bucket and rope method and this practice is capable of introducing contaminants into the water. The uses of shallow well water in almost all the peri-urban communities includes laundry and washing of kitchen utensils, cooking and washing of fruits and vegetables, and thus the transfer of ESBL resistant strains may

either be directly through the consumption of contaminated fruits and vegetables or indirectly through contact with contaminated shallow well water (Raphael *et al*., 2011; Reuland *et al*., 2011). However, little attention has been given to the transfer of resistance genes through water and vegetables. The majority of these shallow wells are poorly constructed and sited near pit latrines making them vulnerable to contamination. The average distance between the shallow wells and sanitary structures ranged from about 1 to 7 m in contrast to recommendations stated by Banks *et al*., (2002) which suggested a distance of not less than 15–30. In addition, the study sites have overcrowded inhabitants leaving them with limited distances between wells and sanitary structures. The presence of *Enterobacteriaceae* detected in the various unprotected shallow well water sources particularly in Chunga, Garden and Kanyama Compounds could possibly be due to heavy contamination of faecal origin bacteria emanating from heavy rainfall patterns resulting into floods and indiscriminate disposal of garbage which poses a potential public health hazard (Ramphal and Ambrose, 2006). The incidence of these ESBL-producing bacteria in water supplies is of greater risk without effective treatments and this calls for attention because these bacterial pathogens have been reported to be associated with serious human infections globally (Odumosu and Akintimehin, 2015). This entails that shallow well water could play a significant role in the dissemination of these ESBL producing *E. coli* organisms.

Antimicrobial susceptibility testing revealed interesting patterns with resistance rates observed in the majority of the eleven antibiotics tested. From the antimicrobial susceptibility results, 85.7% (66/77; CI: 75.7 – 92) of the isolates were resistant to ampicillin (10 μg), chloramphenicol (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (300 μg), sulfamethazole/trimethoprime and tetracycline. The majority (49.4%, 38/77) were found to be resistant to six (6) or more classes of antibiotics mainly ampicillin, streptomycin, tetracycline, cefotaxime, nalidixic acid, ceftazidime, norfloxacin and ciprofloxacin. The findings are similar to studies conducted by Kuenzli *et al*., (2014) on food animals and water enteric bacteria where high antibiotic resistant profiles were established. For instance, a review of published and unpublished literature

for Democratic Republic of Congo, Mozambique, Tanzania and Zambia revealed an increased trend of resistance to ampicillin, co-trimoxazole, gentamicin, erythromycin, tetracycline and third generation cephalosporins (Mshana *et al.,* 2013). In this study, high resistance rates to ampicillin (79.2%), cefotaxime and ceftazidime (67.5%), tetracycline (59.7%), chloramphenical (57.1%) and norfloxacin (54.5%) were observed among the isolates investigated. The development of high antibiotic resistance status could be attributed to frequent misuse of β-lactum antibiotics in treatment and management of poultry diseases and also the uncontrolled use of antibiotics as growth promoters in poultry farms (Johnson *et al.,* 2006) and also due to presence of extended- spectrum β-lactamases which are either transferable through mobile genetic elements or could be chromosomally mediated (Lester *et al*., 2006). Unfortunately, in this study it was not possible to identify the type of antimicrobial drug frequently used for poultry feed and growth promotion as it was beyond the scope of this study.

In this study a binary logistic regression model was used to test the significance of some of the independent variables to the presence of ESBL genes/producers. The most significant findings from the logistic regression model were that George, Matero and Chawama locations played an important role in the presence of ESBL-producers. This agrees with studies conducted by Mesa *et al*., (2006) in which samples of stool collected from different environmental locations revealed the widespread presence of *Enterobacteriaceae* ESBL-producing bacteria carriages.

This study has detected the presence of ESBL-producing *E. coli* isolates in poultry and water. It is, therefore, clear that poultry and contaminated shallow well water would be a major possible element in the transmission of ESBL- resistant genes to the general public.

CHAPTER SIX

CONCLUSION

This is the first study to be conducted in Lusaka District on detection of ESBL-producing *E. coli* isolates in poultry and shallow wells in the peri-urban communities. The cross sectional study has revealed the following:

1) The presence of ESBL-producing *E. coli* isolates in poultry (42.9%) and shallow wells (11.6%).

2) Poultry is a major and potential reservoir for the antimicrobial ESBL producing *E. coli* resistant genes which could be spread by the food chain.

3) The study has also shown widespread occurrence of multi drug resistance *E. coli* strains.

4) Location is significantly associated with the presence of ESBL producers in Lusaka District.

CHAPTER SEVEN

RECOMMENDATIONS

The following are the recommendations based on the study findings observed:

1. There is need to strengthen the enforcement of existing antibiotic usage policy in poultry production.

2. There is also need to strengthen surveillance on the emergence of antimicrobial resistance in food animal products.

3. There is need to ensure that food producing animals and food supply chains are monitored regularly to reduce food-borne antibiotic resistance and contamination of food by antimicrobial resistant bacteria.

4. There is need urgent need for concerned stakeholders and government wings to immediately bury all the shallow wells in the peri-urban communities and provide them with wholesome piped water supply.

5. There is also need to provide proper sewage system to avoid contamination of food and water by human excreta.

6. Further investigations should be conducted by sequencing the ESBL – producing *E. coli* isolates to identify the phylogenetic relationship in nature.

7. A comprehensive study involving the associated risk factors of ESBL–producers from food animals to humans should be conducted extensively.

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