EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* IN MILK AND SOIL FROM ENVIRONMENT ON DAIRY FARMS IN LUSAKA, ZAMBIA

By

CILUVYA KAVIMBA KALUBA

A dissertation submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in One Health Laboratory Diagnostic Sciences

THE UNIVERSITY OF ZAMBIA

Lusaka

DECLARATION

I, **Ciluvya Kavimba Kaluba**, declare that this Dissertation is my work and that all sources I have cited herein have been indicated and acknowledged using complete references. I further declare that this Dissertation has not been previously submitted for a diploma, degree or any other qualifications at this or another university.

Signature…….

Date……

CERTIFICATE OF APPROVAL

This dissertation of **Ciluvya Kavimba Kaluba** has been approved as partial fulfillment of the requirements for the award of the Degree of Master of Science in One Health Laboratory Diagnostic Sciences by the University of Zambia.

Prof. John B. Muma……

Supervisor Signature …….

Date…….

Examiner I

Signature……...

Date……

Examiner II

Signature……...

Date……

Examiner III

Signature……..

Date……

ABSTRACT

Escherichia coli and *Klebsiella* spp. are the most common coliforms that cause clinical mastitis and are responsible for the disease in about 40% of cases in dairy animals. The presence of *E. coli* and *K. pneumoniae* in raw milk poses a threat to human health as consumption of contaminated raw milk may cause diseases such as diarrhoea, haemolytic colitis, haemolytic uremic syndrome, urinary tract infections (UTIs), wound infections, nosocomial infections, meningitis in infants, pyogenic liver abscess, necrotizing fasciitis, endophthalmitis and severe pneumonia. This cross-sectional study aimed to generate knowledge on the presence of *E. coli* and *K. pneumoniae* in dairy cattle milk and soil. A total of 180 (90 milk and 90 soil environmental) samples were collected from 30 farms around Lusaka province. Samples were collected and processed using standard microbiological and molecular laboratory procedures. A total of 69 isolates were identified, of which (62) were *E. coli* (51.6%: milk and 48.4%: soil) and (7) *K. pneumoniae* (85.7%: milk and 14.3%: soil) and confirmed using *uid*A and KP-27 genes, respectively. The isolates were subjected to eight antimicrobials for susceptibility testing, with the highest resistance recorded for *E. coli* and *K. pneumoniae* to ampicillin (84%) and ceftazidime (86%), respectively. Isolates that were resistant to tetracycline, cephalosporins and trimethoprim-sulphamethoxazole were subjected to PCR to detect resistance genes and these showed that 33%of them had *tet* (A), 27% *bla*CTX-M, 37.8% *bla*TEM-1, 21.6% *bla*TEM-2 and 80% for *dfrA7*, encoding tetracycline, cephalosporin, and trimethoprim-sulphamethoxazole resistance, respectively. In order to presumptively screen for Extended Spectrum Beta Lactamases (ESBLs), isolates resistant to cephalosporins were sub-cultured onto MacConkey agar supplemented with cefotaxime. Isolates that grew on MacConkey-cefotaxime agar were further tested for the detection of ESBL genes using PCR. Three out of five isolates (60%) showed the presence of ESBL resistance genes *bla*CTX-M (1/3) and *bla*TEM-1 gene (2/3). The presence of antimicrobial-resistant *E. coli* and *K. pneumoniae* isolated from milk and the environment indicates poor hygienic conditions and the importance of the One Health approach. In addition, the findings demonstrate the need to control the usage of antibiotics in veterinary medicine better and implement effective surveillance programs.

DEDICATION

I especially dedicate this piece of scientific literature to every researcher out there who is invested in the fight against antimicrobial resistance, which continues to be a significant problem in our societies today. I further dedicate this work to my family and friends for their tremendous support during the program.

ACKNOWLEDGEMENTS

I wish to show profound gratitude to people, particularly my research supervisor, Prof. John. B. Muma and co-supervisors Dr. Kaunda Yamba and Dr. Mulemba Samutela, whose ingenious and uncompromised input helped bring this research process to success.

Special and heartfelt thanks also go to:

- The Africa Centre of Excellence for Infectious Diseases in Humans and Animals (ACEIDHA) for sponsoring my studies at the University of Zambia.
- The Dean, School of Veterinary Medicine; Assistant Dean, Postgraduate; Department of Disease Control for the scholarly support rendered throughout my study period.
- Dr. Kaunda Yamba and Mr. Henry Chimana, thank you for the gifts of polymerase chain reaction amplification primers.
- Mr. Andrew Mukubesa and Mr. Patrick Katemangwe were responsible for the technical support.
- My family, for all the sound moral, emotional and material support/encouragement they incessantly offered during my study period.
- The 2021 Postgraduate cohort (School of Veterinary Medicine) for being such an academic resource.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMU	Antimicrobial use
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ARG	Antimicrobial resistant genes
ATCC	American Type Culture Collection
CLSI	Clinical & Laboratory Standards Institute
CPS	Capsule polysaccharide
CTX-Mac	MacConkey agar with cefotaxime
DAEC	Diffusely adherent *E. coli*
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ESBL	Extended spectrum beta lactamase
ExPEC	Extra-intestinal *E. coli*
GIT	Gastroinstestinal tract
GUD	Glucuronidase
HGT	Horizontal gene transfer
NMEC	Neonatal meningitis *E. coli*
UPEC	Uropathogenic *E. coli*
FBD	Foodborne diseases
K. pneumoniae	*Klebsiella pneumoniae*
MDR	Multi-drug resistant

RNA	Ribonucleic acid
SIM	Sulphur, Indole, Motility
TSI	Triple Sugar Iron
UTI	Urinary tract infections
WHO	World Health Organisation

OPERATIONAL DEFINITIONS

- **Antimicrobial resistance**: Occurs when bacteria, viruses, parasites and fungi change over time and no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death.
- **Antibiotic resistance**: loss of susceptibility of bacteria to the killing or growth-inhibiting properties of an antibiotic agent
- **Extended Spectrum Beta Lactamases**: Enzymes that confer resistance to most beta-lactam antibiotics.
- **Multi-drug resistant**: Resistance to at least one antibiotic in three or more drug classes.
- **Foodborne diseases**: Illnesses caused by the contamination of food and beverages with bacteria.
- **One Health**: An integrated approach of solving problems to improve health by balancing the health of humans, animals and the ecosystem.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Foodborne diseases (FBD) cause a substantial public health, economic and social burden worldwide (Havelaar et al., 2015). One study showed that about one out of 10 people get ill from food contaminated with microbial or chemical agents, resulting in 600 million illnesses, 420,000 deaths and the loss of 33 million healthy years of life (Pires et al., 2021). FBDs cause an estimated 48 million illnesses yearly in the United States, including 9.4 million caused by known pathogens such as *Escherichia coli* (*E. coli*) and *Salmonella* (Gould et al., 2013). Surveillance carried out by the World Health Organization (WHO) showed that Africa had the highest FBD burden, with an estimated 1300 per 100,000 population with disability-adjusted life years (Havelaar et al., 2015). In Zambia, diarrheal FBD ranks in the top five causes of morbidity and mortality in all ages (Kapaya et al., 2018). In order to combat these FBD, food strategies must not merely be directed at ensuring food security for all; they must also include the consumption of adequate quantities of safe and good quality foods (Vasileska and Rechkoska, 2012).

Humans in all age groups consume milk and its products as a source of protein and calcium, and it is a valuable food for bone health (Turck, 2013). There is a long-standing tradition of cattle keeping and milk consumption in Eastern and Southern Africa, with milk consumption being an intervention to combat malnutrition in children and promote bone health (Hetherington et al., 2017). However, several factors have been found to negatively impact milk production, such as the presence of high moisture and an almost neutral pH in milk products that favours the growth and multiplication of multiple bacteria that may lead to infection (Uddin et al., 2011). In developing countries, industrialization brought a series of problems along with the much-appreciated progress, with the mass collection and distribution of milk from various sources playing the role of a potential vehicle for disease transmission (Dhanashekar et al., 2012).

Escherichia coli is among the 31 major pathogens that cause foodborne diseases with adverse effects on human health, while *Klebsiella pneumoniae* (*K. Pneumoniae*) is one of the clinically significant organisms that have acquired public health concern as it is considered one of the opportunistic pathogens showing an

increasingly frequent acquisition of resistance to antibiotics (Effah et al., 2020; Madani et al., 2022). Antimicrobial-resistant pathogens have been found to be transferred from animals to humans through the food chain and, therefore, pose a threat to the increase in antimicrobial resistance (AMR) (Saini et al., 2012). *E. coli* and *K. pneumoniae* are reportedly resistant to cephalosporins, fluoroquinolones, monobactams and aminoglycosides (Gebremeskel et al., 2023). The severity of clinical episodes such as fever, shock, and inflammation of mammary glands in animals leads to decreased milk production in cattle, poor response to vaccination such as the J5 vaccine for mastitis and lack of effective treatment, thus making *K. pneumoniae* more troublesome than *E. coli*. Therefore, identifying potential sources of these organisms is important for implementing preventive measures to decrease exposure and limit the risk of infection (Hisaeda et al., 2011; Munoz et al., 2008; Wilson et al., 2007).

In order to ensure the safety of milk, some developed countries have put in place several measures through the use of food safety, quality assurance and control measures based on scientific analysis and good management practices (Dhanashekar et al., 2012). These include hygiene in milk handling, storage and transportation (Girma et al., 2014). However, Zambia still has potential risk factors along the dairy value chain, such as hygiene deficiencies at all stages (udder, personnel, environment and equipment hygiene) and lack of refrigeration during storage and transportation for long hours (Phiri et al., 2021). Therefore, this study aims to determine the public health significance of *E. coli* and *K. pneumoniae* in dairy cattle milk and the milking environment. The study will highlight gaps needed to control the spread of pathogens and improve food safety.

1.2 Statement of the Problem

Raw or unpasteurized milk is a vehicle for transmitting pathogens from animals to humans (Pant et al., 2013). The consumption of raw milk is relatively common in Zambia, especially among milk producers (Moll et al., 2007). Therefore, determining the presence of *E. coli* and *K. pneumoniae* in milk and the milking environment is important in assessing the risk posed to human health due to consuming contaminated raw milk with either organism (Badri et al., 2017). *E. coli*

and *K. pneumoniae* are among the major causes of clinical mastitis in cattle (Schukken et al., 2012) and predominant causes of blood stream infections and urinary tract infections in humans (Badri et al.). Antibiotic resistance in microbes isolated from milk samples shows AMR to be a growing problem, posing a threat to the health of humanity (Pant et al., 2013). Zambia continues to face high rates of AMR in microorganisms isolated from hospital settings, animal health and the environment against commonly available antibiotics (Kasanga et al., 2023; Nowbuth et al., 2023). A study by Saini and colleagues revealed an emergence in the transfer of antibiotic-resistant bacteria from animals to humans (Saini et al., 2012). Additionally, a study examining the prevalence of antibiotic-resistant *E. coli* isolated from Zambian dairy cattle showed a high prevalence of resistance to antibiotics used in human health across commercial farms (Mainda et al., 2015). Moreover, evidence suggests that certain resistance in the human population may be attributed to an exchange of genotypes between organisms of human origin and those from cattle (Mainda, 2016). However, there is still lack of data from the Zambian diary milk chain on the presence and public health significance of *E. coli* and *K. pneumoniae* and the transfer of AMR genes.

1.3. Significance of the study

The milk contamination and the milking environment with *E. coli* and/or *K. pneumoniae* threaten animal and human health. These microorganisms can cause disease directly through consumption or cross-contamination. In order to reduce microbial cross-contamination and ensure the safety of milk and milk products, there is a need to evaluate the presence of both *E. coli* and *K. pneumoniae* in dairy milk and the milking environment. The increased prevalence of antimicrobial-resistant *E. coli,* isolated from dairy cattle and milk in Zambia, justifies the need to further study these organisms' antimicrobial resistance patterns and the resistance genes in their genome. The information generated from this study will add new knowledge on the antimicrobial resistance patterns in Zambia and guide infection prevention measures.

1.4 Research Question

Is antimicrobial-resistant *E. coli* and *K. pneumoniae* in milk and the milking environment of public health significance?

1.5 Study Objectives

1.5.1 General Objective

To evaluate the public health significance of *E. coli* and *K. pneumoniae* from dairy cattle milk and the milking environment.

1.5.2 Specific Objectives

1.5.2.1 To evaluate the prevalence of *E. coli* and *K. pneumoniae* from dairy milk and the milking environment.

1.5.2.2 To determine the antibiotic susceptibility patterns of *E. coli* and *K. pneumoniae* in dairy milk and the milking environment.

1.5.2.3 To detect the presence of extended-spectrum β-lactamases (ESBLs) and resistance genes in both *E. coli* and *K. pneumoniae*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Milkborne Diseases

Foodborne diseases have been reported to be a major public health concern worldwide. Many foodborne pathogens can be transmitted through various routes, such as food, water, soil, or air, by direct contact between people or between people and animals (Havelaar et al., 2015). Dairy products, which include milk, contribute significantly to FBD and are the most important, accounting for about 20 disability adjustable life years per 100,000 population (Grace et al., 2020). According to a study carried out in Korea on adults and adolescents, it revealed that the most common reasons for consumption of milk were for bone health (59.6%) and as a meal substitute (34.8%) (Park et al., 2019). Similarly, milk is highly valuable for a balanced diet and contributes to food security in Zambia, with the traditional dairy value chain accounting for a large share of the total milk supply (Phiri et al., 2021). Most FBD outbreaks are caused by *Campylobacter* spp, *Salmonella* spp, *Listeria* spp, *E. coli*, and *Staphylococcus aureus* (Gourama, 2020). Milkborne diseases that affect both cattle and humans include Brucellosis caused by *Brucella* species (spp*)* (Piao et al., 2020), tuberculosis, which is caused by *Mycobacterium bovis*, known to survive in yoghurt and cheese made from raw milk for upto 100 days (Gourama, 2020), campylobacteriosis caused by *Campylobacter jejuni* which usually makes its way into milk through faecal contamination (Christidis et al., 2016) and salmonellosis caused by nontyphoidal Salmonella serovars responsible for zoonotic transmission (Singh et al., 2018). Besides these, other milk-borne diseases include colibacillosis, which is caused by *E. coli* as a result of unhygienic processing of milk (Rahman et al., 2017), *Staphylococcus aureus*, the most common aetiological agent of mastitis that also leads to deterioration of the quality of the milk (Neelam et al., 2022) and finally streptococcus-induced mastitis which may be caused by *Streptococcus spp* (*S. agalactiae, S. uberis* and *S. dysgalactiae*) which may contaminate milk from cow-to-cow and through the environment (Schmelcher et al., 2015). Possible sources of milk contamination include the udder and its exterior, milk handling, milk equipment, storage equipment, faeces and the soil (Uddin et al., 2011). In order to ensure the safety of milk and milk products, potential

microbiological contaminations need to be minimized to the greatest extent achievable over the entire food chain (Phiri et al., 2021).

2.2 General Characteristics of *E. coli* and *K. pneumoniae*

E. coli and *K. pneumoniae* are both members of the *Enterobacteriaceae* family. *E. coli* is a Gram-negative, non-sporulating facultative anaerobe. It usually inhabits the intestines and faeces of warm-blooded animals and reptiles as a commensal bacteria (Tenaillon et al., 2010). Although most *E. coli* live harmlessly in the intestines, many pathogenic strains can cause intestinal or extraintestinal diseases in healthy and immunocompromised individuals (Gomes et al., 2016). Eight pathogenic strains have been studied, and these have been classified into intestinal or diarrhoeagenic *E. coli* and extraintestinal *E. coli* (ExPEC). Diarrhoeagenic *E. coli* are seven and include enterohaemorrhagic *E. coli* (EHEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) which are common bacteria of the gut but can lead to extensive abdominal discomfort and bloody or non-bloody diarrhoea. ExPEC includes neonatal meningitis (NMEC) and uropathogenic *E. coli* (UPEC), the most common cause of UTIs and neonatal meningitis. (Croxen and Finlay, 2010; Leung et al., 2019).

K. pneumoniae is a rod-shaped, Gram-negative, lactose-fermenting bacillus with a prominent capsule. It is an opportunistic pathogen found in the mouth, skin and intestines, and environment such as surface water, sewage and soil (Guerra et al., 2022). Based on its capsule antigens, *K. pneumoniae* can be distinguished and classified by serotyping. There are about 82 serotypes as of 2018, and among these, K1 and K2 are the most virulent serotypes due to the polysaccharide capsule they pose that helps them evade phagocytosis (Kaur et al., 2018; Li et al., 2014).

2.3 Transmission

The transmission of enteric pathogens normally occurs through the fecal-oral route, and host-to-host transmission in a hospital setting is a major source of infection (Young et al., 2020). The faecal-oral route includes consuming contaminated foods, such as raw or undercooked ground meat products and raw milk (Bezirtzoglou, 2000). Furthermore, most outbreaks are associated with the consumption of fruits and vegetables that may have been contaminated from with faeces from domestic or wild

animals (García and Heredia, 2017). Waterborne transmission has been reported, from contaminated drinking-water as well as recreational waters through faecal contamination (Ashbolt, 2015). In addition, cross-contamination during food preparation (with beef and other meat products, contaminated surfaces and kitchen utensils), also leads to infection (Kennedy et al., 2011). *E. coli* strains have been isolated from the environment such as bodies of water, wells and water troughs, and has been found to survive for months (Cho et al., 2020).

2.4 Pathogenesis and Clinical Presentation

E. coli and *Klebsiella* spp. are the most common coliforms that cause clinical mastitis and are responsible for the disease in about 40% of cases (Schukken et al., 2012). The presence of *E. coli* and *K. pneumoniae* in raw milk poses a threat to human health as consumption of contaminated raw milk may produce pathogenic conditions such as diarrhoea, haemolytic colitis, haemolytic uremic syndrome, urinary tract infections (UTIs), wound infections, nosocomial infections, meningitis in infants pyogenic liver abscess, necrotizing fasciitis, endophthalmitis and severe pneumonia (Badri et al., 2017; Virpari et al., 2013; Li et al., 2014). The production of cytotoxins and the ability of *E. coli* to attach to the intestinal epithelium are related to the pathogenicity of various strains of this organism (Assumpção et al., 2015). In humans, EHEC colonises the large intestines and releases Shiga toxin, which binds to endothelial cells, allowing absorption into the blood stream and dissemination of the toxin to other organs(Lee et al., 2021). Cattle are a major reservoir of EHEC and can, therefore, transport this pathogenic *E. coli* to humans (Nguyen and Sperandio, 2012). In cattle, pathogenic *E. coli* also releases Shiga toxin, but unlike in humans, the toxin binds to the epithelial cells, leading to inflammatory responses and cytokine production responsible for the clinical symptoms (Sheldon et al., 2010).

In humans and cattle, *K. pneumoniae* produces the capsule polysaccharide (CPS), which suppresses inflammatory responses and plays an important role in resisting phagocytosis (Li et al., 2014). After that, attachment and colonization of the mucosal surfaces of the gastrointestinal tract occur through the aid of fimbriae and the production of enterobactin, which competitively binds to iron sequestered by the host as a mechanism to combat the bacterial infection (Kaur et al., 2018).

2.5 Diagnosis of *E. coli* and *K. pneumoniae*

The conventional method to identify bacterial pathogens includes bacterial isolation through culture combined with biochemical characteristics detection and analysis (Li et al., 2020). The diagnosis of both *E. coli* and *K. pneumoniae* is usually confirmed through culture of specimens on selective and differentiating media such as MacConkey agar and incubating at 35-37 °C under aerobic conditions for 18-24 hours (Newell and La Ragione, 2018; Yu and Chuang, 2015). Biochemical characteristics are detected by carrying out biochemical tests, which include Triple Sugar Iron (TSI), Lysine Iron Agar (LIA), Sulphur Indole Motility (SIM), Simmon's citrate agar, urease and oxidase (Mladenović et al., 2018). Furthermore, identification and speciation of bacterial pathogens can be achieved by using molecular techniques such as PCR combined with gel electrophoresis, which improves the detection efficiency (Li et al., 2020). Identification using PCR involves exponential amplification of specific DNA sequences, allowing for detecting low concentrations of the target organisms (Molina et al., 2015). In addition to all these methods of diagnosis, universal DNA sequencing is another method that can be used in the diagnosis of bacteria, especially for nonculturable bacteria and novel bacteria (Cai et al., 2014).

2.6 Treatment

In animals, clinical mastitis can be treated with a variety of antibiotics. The use of low-dose antibiotics such as tetracycline leads to selective pressure, which enables the multiplication of resistant bacteria. It is estimated that nearly an equal tonnage of antimicrobial agents is used in humans and agriculture worldwide (Pant et al., 2013). Only a few antimicrobials have adequate pharmacokinetics and pharmacodynamics for treating mastitis caused by Gram-negative bacteria. Treatment of *E. coli* mastitis in cattle is commonly achieved using broad-spectrum antibiotics. Some broad-spectrum antibiotics include trimethoprim–sulfonamides, oxytetracycline, fluoroquinolones, cefquinome, and ceftiofur (Suojala et al., 2013). In human medicine, the increased resistance in *E. coli* to amoxicillin or ampicillin has resulted in using aminoglycosides, fluoroquinolones and third-generation cephalosporins as empiric therapy (Rottier et al., 2015). However, growing

resistance to these classes of antibiotics further complicates the treatment of infections (Seiffert et al., 2013).

Treatment of *K. pneumoniae* infections is quite challenging as few trials have been done to determine the efficacy of antimicrobials against this pathogen, especially ESBL-producing *K. pneumoniae*. However, cephalosporins, piperacillin/tazobactam, and carbapenems such as imipenem and meropenem have been seen to be effective against Multi-drug resistant (MDR) *K. pneumoniae* infections, including UTIs and pneumonia (Gupta et al., 2003). Like human infection, several studies have suggested that *E. coli* responds better to treatment than *K. pneumoniae* in cattle. However, ceftiofur and cefquinome have improved the treatment outcomes of Klebsiella mastitis compared to first-generation cephalosporins (Klaas and Zadoks, 2018). Other than treatment, past studies show that it was a common practice to use antimicrobial therapy to prevent and control mastitis. However, bacterial cures still proved difficult due to antimicrobial resistance (Barkema et al., 2006).

2.7 Prevention and Control of *E. coli* and *K. pneumoniae*

In general, strategies for the prevention and control of the spread of *E. coli* and *K. pneumoniae* should include access to clean and safe water, good handling practices to reduce the risk of food contamination, sanitation measures, public education and vaccinations (Hartantyo et al., 2020; Mielke, 2010). Access to safe water is the primary target for the prevention of infections whilst appropriate storage and cooling temperatures are measures that could be used to prevent infections from food products (Allocati et al., 2013). Prevention of *E. coli* and/or *K. pneumoniae infections* involves coordinated infection-prevention and control interventions such as contact precautions, hand hygiene and active surveillance for patients at risk of infection and carriage (Li et al., 2019). Additionally, due to the presence of both bacteria in soil and farms, it is important that fresh vegetables are thoroughly washed and raw meat thoroughly cooked to prevent infection (Hartantyo et al., 2020). In humans, hospital measures that have been put in place to limit the spread of pathogens include the prevention of cross-contamination through the implementation of strict hygiene standard protocols as well as the control of antimicrobial use (Mielke, 2010). The spread of infections in hospitals is usually due to contaminated medical devices and the hands of health workers; therefore, it

is important to promote hand hygiene to prevent cross-contamination (Allocati et al., 2013).

Furthermore, vaccination may be an important primary prevention strategy for humans against the most harmful strains of *E. coli*, such as ETEC, UPEC and NMEC. However, no effective vaccine is available yet to prevent these infections (Seib et al., 2012). Although this maybe so in humans, the J5 vaccine has proven to be effective in the prevention of coliform mastitis caused by *E. coli* in animals, though not effective against *K. pneumoniae* mastitis (Dosogne et al., 2002; Gorden et al., 2018). Various vaccination strategies are underway to prevent infection with the different *E. coli* pathotypes as well as *K. pneumoniae* infection (Pokharel et al., 2023; Rafi et al., 2023).

2.8 Antimicrobial Resistance

2.8.1 Mechanisms of AMR Development

The three major mechanisms of AMR include the inactivation of the antibiotic by bacterial enzymes, modification of the antibiotic target site and lastly, active efflux of the antibiotic via the transmembrane efflux pumps, which keep the concentration of the antibiotic within the bacterial cell below the toxic threshold (Walsh and Wright, 2005). Bacteria can exhibit resistance using any of these mechanisms, either through intrinsic resistance or acquired resistance. Intrinsic resistance refers to the ability to resist antibiotics due to an inherent structural of functional characteristic (Blair et al., 2015). This type of resistance usually utilizes enzymes to destroy the drug or prevent intracellular drug binding with the target organism (Ali et al., 2018). On the other hand, acquired resistance is the ability of a bacteria to confer resistance through mutations in chromosomal genes and horizontal gene transfer (Blair et al., 2015). The most common site for antibiotics to inhibit transcription and translation is within the 23s rRNA, making mutations of particular interest (Ali et al., 2018). Figure 2.1 shows how mutations lead to resistance by altering the target site.

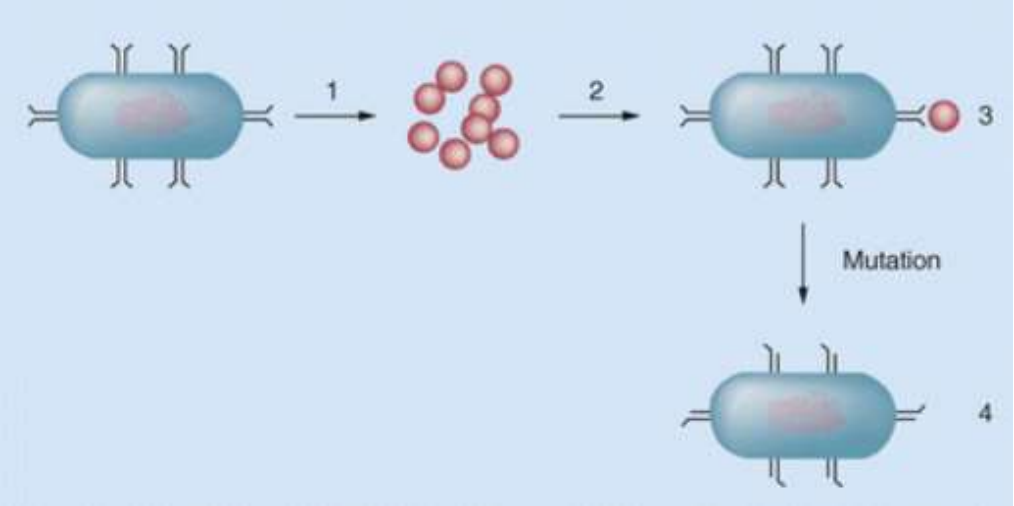


Figure 2.1. Showing how mutations lead to AMR. (1) A wild-type bacteria. (2) Antibiotics bind and destroy target bacteria. (3) Antibiotic binds and destroys target bacteria. (4) After mutation occurs, the binding site is altered, and the antibiotic cannot bind the mutant bacteria. Mutant bacteria proliferate, creating a new resistant colony (Von Wintersdorff et al., 2016).

Horizontal gene transfer (HGT) allows bacteria to exchange their antibiotic resistance genes (ARG) among diverse bacterial species, and this can occur through conjugation, transduction and transformation (Sun et al., 2019). HGT via conjugation involves the transfer of genetic material through cell-to-cell contact using surface pili or adhesins. The donor cell identifies a recipient and, through its pilus, brings both cells into close contact by forming a mating bridge or pore between the cells. This triggers the transfer of DNA to the recipient cell, where it is recircularized, replicated and established (Arutyunov and Frost, 2013). Transduction is another HGT mechanism which uses bacteriophages to transfer resistant genes between two cells (Von Wintersdorff et al., 2016). Generalized transduction occurs during the phage lytic cycle when the bacterial genome is packaged accidentally into the phage particle. When released upon the donor bacterial cell lysis, this transducing phage transfers this genetic material by injecting a recipient bacterial cell (Leclerc et al., 2022). Lastly, the transformation mechanism involves genetic recombination from a dead and degraded bacterium, which acts as the donor cell. DNA is taken by the recipient cell from the extracellular environment and incorporated into their genome of inheritance (Mao and Lu, 2016). Figure 2.2 shows the mechanisms of HGT.

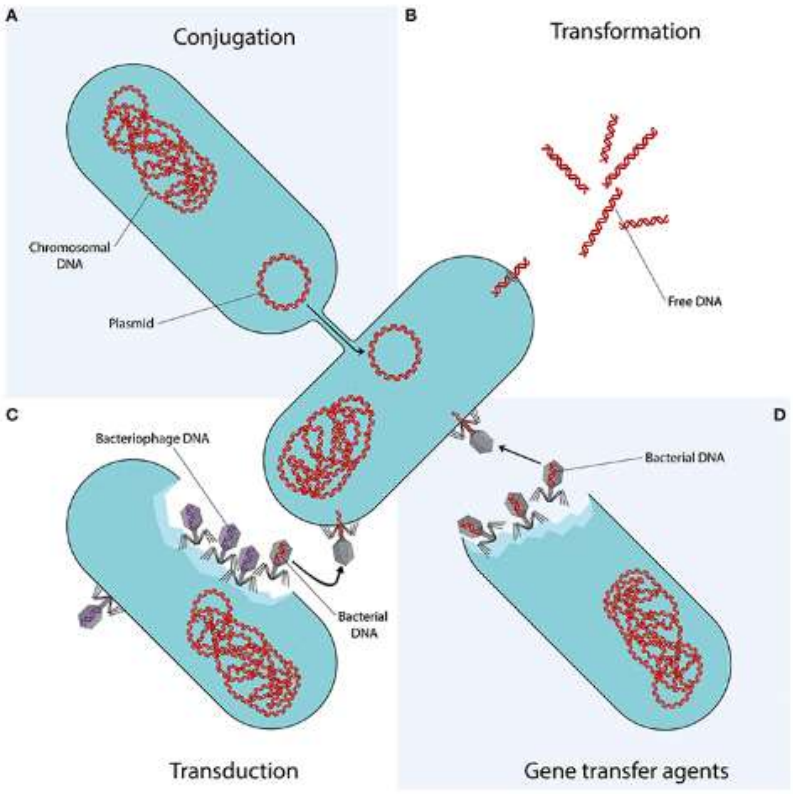


Figure 2.2 shows the mechanisms of HGT (A), which is a conjugation mechanism requiring cell-to-cell contact via pili. (B) Transformation mechanism involving uptake and integration of naked fragments of extracellular DNA. (C) The transduction mechanism involves transferring bacterial DNA from donor to recipient through a bacteriophage. (D) Gene transfer agents are bacteriophage-like particles that carry random pieces of the producing cell's genome (Von Wintersdorff et al., 2016).

2.8.2 Development of Antimicrobial Resistance in Animals

Antimicrobials are used in humans and agriculture to treat and prevent infections. In agriculture, antimicrobials are also used as growth promoters in animal feed (Parkhill, 2022). Various antimicrobials used in human treatment, such as fluoroquinolones, are also used in livestock, poultry and aquaculture, which may jeopardize the efficacy of these antibiotics due to the development of resistance in pathogens of zoonotic potential (Lekshmi et al., 2017). In addition, overuse and misuse of antimicrobials in livestock feed has rapidly increased antimicrobial resistance. MDR has created major economic and health concerns, affecting livestock industries and human healthcare, thereby failing conventional clinical treatments (Mellata, 2013).

Furthermore, the impact of antibiotic overuse continues to spread further as antibiotic residues, resistant bacteria and genetic resistance elements subsequently spread to adjacent environments through waste streams (Von Wintersdorff et al., 2016). It has been reported that the regular use of antimicrobials in food animals results in the development of antimicrobial resistance in commensal bacteria. A study that was carried out in Africa to assess the use of antimicrobials in food animals found a total of 14 antimicrobials being used, some of which included tetracycline, aminoglycosides, penicillin and macrolides (Kimera et al., 2020). The percentage of antimicrobial use in farms ranged from 77.6% in Nigeria to 100% in Tanzania, Cameroon, Zambia, Ghana and Egypt (Kimera et al., 2020).

2.8.3 Mechanism of Spread of AMR Between Animals, Humans and the Environment

Livestock can be reservoirs for resistant genes, including those associated with producing extended-spectrum β-lactamases (ESBLs) in *Enterobacterales* that could be transferred to humans. The emergence and transfer of antimicrobial-resistant bacteria or genetic determinants from animals to human populations via the food chain is a growing concern (Saini et al., 2012). Transmission of AMR determinants between food animals and humans has been stated to occur very rapidly. This was seen in a study that was carried out in China, where a mobile piece of DNA encoding resistance to colistin (*mcr-1*) was first discovered in *E. coli* isolated in pigs in 2013 (Liu et al., 2016). Less than five years after these findings, genomic data showed that the *mcr-1* gene had been found in at least six different genera of bacteria isolated from humans and animals worldwide (Wang et al., 2018). Evidence of the transfer of AMR bacteria has also been presented in a study that was carried out by Davis *et al*. (2015), where genomics was used to analyse *K. pneumoniae* in retail meat and urinary tract infections within the same city in the United States. This study found that closely related strains recovered from both animals and humans shared a sequence type (ST), as defined by multi-locus sequence typing (MLST). The authors suggested that these findings indicated the recent transfer of these strains (Davis et al., 2015).

Some studies have shown that environmental mastitis is also a common and costly form of mastitis that is of significance to public health. Bovine faeces, the environment and used pasture are major sources of mastitis pathogens (Klaas and

Zadoks, 2018). The role of food-producing environments in the spread of antimicrobial-resistant bacteria in the European Union plant-based food production, terrestrial animals and aquaculture was assessed (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021). It was found that the potential sources for spread to animals were humans and feed. Several antimicrobial-resistant bacteria of highest priority for public health, such as carbapenem or extended-spectrum cephalosporin and/or fluoroquinolone-resistant Enterobacterales (including *Salmonella enterica*), fluoroquinolone-resistant *Campylobacter* spp., methicillin-resistant *Staphylococcus aureus* and glycopeptide-resistant *Enterococcus faecium* and *E. faecalis* were identified in different sources, at primary and post-harvest level, particularly faeces/manure, soil and water (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021).

2.9 *E. coli* and *K. pneumoniae* in Livestock and Susceptibility Patterns to Antimicrobials

The basis for all antimicrobial therapies should be that the pathogen is susceptible to the antimicrobial drug used during treatment (Suojala et al., 2013). In recent years, multidrug-resistant *E. coli* strains have become a significant public health problem worldwide. In Bangladesh, 70 *E. coli* isolates from faecal samples from cattle were subjected to 10 antimicrobials, and results showed that all the isolates were resistant to tetracycline and sulphamethoxazole. The majority of the isolates showed resistance to amoxicillin (90%), ampicillin (87%), nalidixic acid (86%) and erythromycin (83%). Among the tested isolates, 83%, 73%, 68% and 64% were susceptible to chloramphenicol, gentamicin, ciprofloxacin and ampicillin, respectively (Gupta et al., 2017). A study that carried out antimicrobial susceptibility testing (AST) on *E. coli* from cow's raw milk found that out of 42 isolates, 50% were resistant to amoxicillin, whilst most were susceptible to chloramphenicol (95.2%), azithromycin (92.9%), gentamycin (83.3%), imipenem (73.8%), sulphamethoxazole/trimethoprim (71.4%), tetracycline (61.9%), ceftriaxone (59.5%) and ciprofloxacin (54.8%) (Adzitey et al., 2022).

A study in various towns in South Africa isolated 196 *E. coli* and *K. pneumoniae* from faeces and raw meat (Montso et al., 2019). In Montso and others' (2019) study, all isolates were subjected to AST, and the number of isolates resistant to the

different antimicrobial agents was translated into percentages. About 85-100% of the isolates from all the sampling sites except from Mafikeng (54.5%) and Boshoek (66.7%) were resistant to ampicillin, while 66.7–100% of the isolates from Stella and Boshoek were resistant to cefotaxime, piperacillin, ceftazidime, and aztreonam (Montso et al., 2019). Ninety per cent of the isolates from Potchefstroom were resistant to amoxicillin, cephalothin, and piperacillin (Montso et al., 2019). A study that was carried out in Germany to determine antimicrobial susceptibility profiles of 24 *K. pneumoniae* isolates from milk found that 100% were susceptible to ciprofloxacin, amikacin, tigecycline, trimethoprim/Sulfamethoxazole, cefepime, cefotaxime, ceftazidime, ceftazidime/avibactam, imipenem, and ertapenem. Of the 24, 33% showed resistance to piperacillin and 4.2% to chloramphenicol (Wareth et al., 2022). These findings were different from a study in Iraq that subjected 20 *K. pneumoniae* isolates from UTIs to different antimicrobials and recorded high levels of resistance, with 87.5% of the isolates being MDR and 65% resistant to amoxicillin-clavulanic, 65% to cefixime and 60% to cefotaxime, and no resistance to imipenem (Pishtiwan and Khadija, 2019). Lastly, a study in South Africa that isolated *K. pneumoniae* from cow and buffalo mastitic milk found that out of the nine isolates, 82.6% were chloramphenicol and 87.5% were erythromycin. Those susceptible included 60.9% isolates to trimethoprim-sulfamethoxazole, 91.3% to nitrofurantoin and 100% gentamicin (Osman et al., 2014).

3.10 *E. coli* and *K. pneumoniae* Resistance Gene Determinants

ESBLs are enzymes Gram-negative bacteria produce and can hydrolyse extended-spectrum cephalosporins, penicillins and monobactams (Gundogan and Avci, 2013). ESBLs are mediated by plasmids and are the products of point mutations at the active site of TEM, SHV, CTX-M and OXA enzymes. However, CTX-M and TEM are the most common ESBLs (Biswas et al., 2013). Studies done in different countries to determine the ESBL-resistant genes in *E. coli* have found that the most common ESBL gene was CTX-M, with $bla_{CTX\text{-}M\text{-}15}$ being prevalent in Riyadh (Alqasim et al., 2018), $bla_{\text{CTXM-14}}$ in East Asia, and $bla_{\text{CTX-55}}$ mainly in Japan and China (Berglund et al., 2018; Matsumura et al., 2015). Similarly, a study conducted in Saudi Arabia found the $bla_{\text{CTX-M-15}}$ gene was the most predominant ESBL-resistant gene among their isolates (Yasir et al., 2020).

The ability of *K. pneumoniae* to synthesize large amounts of capsular polysaccharide (CPS) is an important correlate of virulence (dos Santos Goncalves et al., 2014). One study conducted in Italy reported that seven K. pneumoniae isolates were isolated from bovine milk samples. Three of these isolates were found to be MDR ESBL-producing isolates, two of which had the CTX-M-15 gene, two with SHV-187 and one with the TEM-1B gene (Bonardi et al., 2023). A study in Iraq that isolated 28 *K. pneumoniae* from beef found that all the isolates had at least one resistant gene. Of the 28 isolates, 85.7% contained bla_{TEM}, 89.2% bla_{SHV}, and 100% $_{bla}$CTX-M genes (Aldabbagh, 2022). Another study that sequenced nine *K. pneumoniae* isolates taken from hospitalized patients in South Africa reported that 100% of the isolates had the bla_{TEM} and $bla_{\text{CTX-M-15}}$ resistance gene,89% had the $bla_{\text{SHV-1}}$ and 44.5% had the $bla_{\text{OXA-1}}$ gene (Founou et al., 2019).

CHAPTER THREE

3.0METHODOLOGY

3.1 Study Design

This cross-sectional study was carried out from February 2023 to November 2023.

3.2 Study Sites

The study was conducted on traditional dairy farms around Lusaka province from selected districts, including Lusaka, Chilanga, and Chongwe. These sites were selected because of the many diary farmers in these areas.

3.3 Study Frame

The study included dairy cattle from Lusaka Province.

3.4 Inclusion Criteria

Lactating dairy cows from Lusaka Province.

3.5 Exclusion Criteria

Dairy cows that were sick were not included in the study.

3.6 Sample size

It was estimated that there are approximately 100 dairy farms in the Province of Lusaka (Mainda et al., 2015). The study aimed to obtain a 10% sampling fraction, which resulted in sampling 30 farms, ten from each district (Table 3.6.1). From each farm, milk samples were collected from the randomly selected animals. Similarly, environment samples (soil) from the kraals from each farm were collected.

Table 3.1: Sample collection plan: number of farms and samples by district

District	Number of farms	Three milk samples per farm	Three soil samples per farm
Chilanga	10	30	30
Lusaka	10	30	30
Chongwe	10	30	30
Total	**30**	**90**	**90**

3.7 Experimental Approach

3.7.1 Sample Collection

Milk samples were collected randomly from cows from different independent owners during milking. About 20 ml of raw milk samples were aseptically collected into sterile screw cap containers from the teats of each selected animal. The samples were transported to the laboratory for processing in a cooler box with ice packs (Gebeyehu et al., 2022). For environmental samples, approximately 10g of soil was collected from at least three different points of the kraals of the cattle. The soil samples were collected with a sterile spatula and put into sterile screw-cap containers. The soil samples were transported on ice packs to the laboratory and processed (Sadiqi et al., 2022).

3.7.2 Evaluation of the Presence of *E. coli* and *K. pneumoniae*

3.7.2.1 Phenotypic Identification of *E. coli* and *K. pneumoniae*

Isolation and detection of both *E. coli* and *K. pneumoniae* involved standard microbiological procedures. The milk and soil samples from the environment were inoculated in peptone water for 24 hours at 37°C. After that, samples were sub-cultured using a ten µl sterile loop on MacConkey agar and incubated at 37°C for 24 hours. After 24 hours of incubation, plates were examined for colony morphology. A single isolated dark pink colony suspected to be *E. coli* and a pinkish mucoid colony suspected to be *K. pneumoniae* were further purified on MacConkey agar and incubated for 18-24hrs at 35-37°C. Simultaneously, a smear was prepared for Gram-stain using the same colony. Those that were Gram-negative and rod-shaped were suspected to be *E. coli* and *K. pneumoniae*. The suspected isolates were further confirmed by subculturing pure colonies on Eosin Methylene Blue (EMB) agar. Those that gave a metallic green sheen were suspected to be *E. coli*, and those with large mucoid pink-to-purple colonies were suspected to be *K. pneumoniae*. These colonies were later subcultured from EMB agar onto Nutrient agar in preparation for biochemical and antimicrobial susceptibility testing (AST) tests.

3.7.2.2 Biochemical Identification of *E. coli* and *K. pneumoniae*

Biochemical tests were performed on both *E. coli* and *K. pneumoniae* using Simmon's Citrate Agar, Triple Sugar Iron Agar (TSI), Sulphide Indole Motility

(SIM) Test and Urease (Soomro et al., 2002). *E. coli* isolates were negative on Simmon's citrate agar and for the Urease test. The isolates on SIM were positive for indole and motile with no hydrogen sulphide production. On TSI, the isolates produced an acid butt and acid slant with gas production (Hsu et al., 2010). *K. pneumoniae* isolates were positive on Simmon's citrate agar and for the urease test. The isolates on SIM were non-motile, negative for Indole production, and negative for hydrogen sulphide production and on TSI, they showed an acid butt and slant with gas production (Hansen et al., 2004).

3.7.2.3 Molecular Identification of *E. coli* and *K. pneumoniae*

The presumptive *E. coli* and *K. pneumoniae* isolates were then subjected to PCR for confirmation, using the *uid*A gene for *E. coli* and the *rcsA* gene for *K. pneumoniae,* respectively (Dong et al., 2015; Jang et al., 2017). The primer sequences used for the species identification are shown in Table 3.7.2. Identification of *E. coli* was based on the presence of the uidA gene that encodes the β-D-glucuronidase (GUD) enzyme (Feng et al., 1991). The *rcsA,* a gene specific to *K. pneumoniae,* regulates the capsule polysaccharide (CPS) synthesis and is the target gene of choice for detecting *K. pneumoniae.* The KP27 primer was used to detect the *rcsA* gene (Dong et al., 2015).

Table 3.2. Primer sequences were used to confirm *E. coli* and *K. pneumoniae.*

Organism	**Gene Oligo Name**	**Primer sequence 5'-3'**	**Annealing Temp (°C)**
E. coli	*uidA* F	CGGAAGCAACGGCTAAACTC	50
	uidA R	TGAGCGTCGCAGAACATTACA	
K. pneumoniae	KP-27 F3	GGATATCTGACCAGTCGG	59
	KP-27 R3	GGGTTTTGCGTAATGATCTG	

*F: Forward primer; R: Reverse primer

3.7.3 Determination of the Antimicrobial Susceptibility Patterns

After isolating and identifying the organisms, they were subjected to AST using the Kirby-Bauer disk diffusion method. Bacterial suspensions equivalent to the 0.5 McFarland standard were prepared by suspending bacterial colonies into 0.85% normal saline. Using a sterile cotton swab, the bacterial suspension was streaked on two Mueller-Hinton agar plates (Liu et al., 2014). Both *E. coli* and *K. pneumoniae* were subjected to 8 antimicrobial disks listed in Table 3.3. This was done using sterile forceps to place the disks onto the Mueller Hinton agar plates. They were then incubated at 35-37°C for 18-24 hours, after which the zone of inhibition was measured using a ruler and interpreted according to the 2020 Clinical Laboratory Standards Institute (CLSI) guidelines for *Enterobacterale* breakpoints. The zones of inhibition were defined as resistant (R), intermediate (I) and susceptible (S). The control strains used were ATCC 25922 for *E. coli* and ATCC 700603 for *K. pneumoniae*.

Table 3.3 Antimicrobial agent name and disk content used

Antibiotic name	**Concentration**
Ampicillin	10 μg
Ciprofloxacin	5 μg
Ceftriaxone	30 μg
Ceftadizime	30 μg
Cefepime	30 μg
Imipenem	10 μg
Tetracycline	30 μg
Trimethoprim/sulphamethoxazole	23/75 μg

3.7.4 Determination of Resistance Genes

In this study, the isolates found to be resistant to trimethoprim-sulfamethoxazole, tetracycline and third-generation cephalosporins were considered MDR.

Furthermore, the isolates resistant to third-generation cephalosporins were screened for the presence of ESBLs. All the isolates resistant to the third-generation cephalosporins were subjected to PCR assays to determine the presence of ESBL genes. Furthermore, the isolates resistant to the third-generation cephalosporins were subjected to ESBL testing by inoculating the suspected ESBL-producing isolates on MacConkey agar with cefotaxime (CTX-Mac). Isolates that grew on CTX-Mac agar were then subjected to PCR to detect the ESBL resistance genes (Gundogan and Avci, 2013). Resistance genes detected in the isolates were detected by PCR using primers shown in Table 3.4 (Badri et al., 2017).

The crude method was briefly used to extract the deoxyribonucleic acid (DNA) from the isolates. This involved the addition of bacterial colonies that were cultured on Nutrient Agar to 200μl of nuclease-free water. The mixture was then heated in a water bath at 89.1°C for 10 minutes. This was later centrifuged at 2500rev/min for 4 minutes, and the supernatant containing the DNA was stored at -80°C. In order to perform PCR, a final volume of 20μl master mix was made, which constituted

10µl OneTaq®Quick-Load® 2X Master Mix with Standard Buffer, 2.5mM MgCl, 0.16mM dNTPs premixed, 0.5µl of each primer, 6µl of nuclease-free water and 2µl of bacterial DNA. Nuclease-free water was used as a negative control, and ATCC 25922 and ATCC 700603 were used as positive controls for *E. coli* and *K. pneumoniae,* respectively. After that, the PCR amplification was carried out to detect the presence of the resistance genes in the MDR and ESBL-producing isolates (Shams et al., 2015). An initial denaturation step at 95°C for 30 seconds was followed by 35 cycles of 95°C denaturation for 1 minute, specific annealing temperature for the specific primers used for 30 seconds, 72°C extension for 30 seconds, and a final extension step at 72°C for 5 minutes after 35 cycles. The amplification products were then run in an electrophoresis system in parallel with a 100bp ladder molecular weight marker by adding 5µl of the PCR products in each well on 1% agarose gel in 0.5 x Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer stained with 0.5µl ethidium bromide for 30minutes at 100V. A picture of the gel was taken under ultraviolet light in a UV illuminator, and all negative reactions were repeated.

Table 3.4: Primer sequences that will be used in the detection of ESBL and antimicrobial resistance genes

Antimicrobial name	**Gene Oligo Name**	**Primer Sequence 5'-3'**	**Annealing temp (°C)**
β-Lactams & ESBLs	*bla*TEM-2 F	AAGTAAAAGATGCTGAAGATAAGTTGG	57
	*bla*TEM-2 R	GATCTGTCTATTTCGTTCATCCATAG	
	*bla*CTX-M F	GTGAAACGCAAAAGCAGCTG	55
	*bla*CTX-M R	CCGGTCGTATTGCCTTTGAG	
	*bla*SHV-1 F	GCGTTATATTCGCCTGTGTATTAT	55
	*bla*SHV-1 R	GCCTGTTATCGCTCATGGTAATG	
	*bla*OXA-48 F	ATGGCAAGAAAACAAAAGTTGG	53
	*bla*OXA-48R	TTGAGCACTTCTTTTGTGATGG	
Trimethoprim-sulfamethoxazole	dfrA2d F	CGGTTCGCATTCCCATCAA	55
	dfrA2d R	GGACTGAGCCTGGGTGAGA	
	dfrA7 F	AAATGGCGTAATCGGTAATG	52
	dfrA7 R	GTGAACAGTAGACAAATGAAT	
	sul1 F	CGGCGTGGGCTACCTGAACG	56
	sul1 R	GCCGATCGCGTGAAGTTCCG	
	sul2 F	GCGCTCAAGGCAGATGGCATT	53
	sul2 R	GCGTTTGATACCGCCACCCGT	
Tetracyclines	Tet (A) F	GGTTCACTCGAACGACGTCA	53
	Tet (A) R	CTGTCCGACAAGTTGCATGA	
	Tet (B) F	CCTCAGCTTCTCAACGCGTG	55
	Tet (B) R	GCACCTTGCTCATGACTCTT	

3.8 Data Analysis

The AST results were entered into WHONET; the susceptibility patterns were presented in graphs and tables. The proportion of resistant *E. coli* and *K. pneumoniae* was determined. All descriptive data was presented as percentages in graphs and tables.

3.9 Ethical Considerations

The study was carried out with ethical approval obtained from the Excellence in Research Ethics and Science (ERES) Converge Institutional Review Board (IRB) committee in Lusaka under reference 2023-Jun-018 and approved by the National Health Research Authority (NHRA) under registration number NHRA-956/21/02/2024. Permission to collect the milk and soil samples was sought from the Ministry of Livestock. Furthermore, consent was sought from farm owners, and for confidentiality purposes, the names of farms and animals were replaced with codes.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of *E. coli* and *K. pneumoniae* from Dairy Milk and Soil

A total of 62 isolates of *E. coli* were isolated, of which 32 (51.6%) were isolated from milk and 30 (48.4%) from soil. Seven *K. pneumoniae* were isolated, of which six (85.7%) were from milk and one (14.3%) from soil.

4.2 Molecular Detection of *E. coli* and *K. pneumoniae* in Milk and Soil

4.2.1 Molecular Detection and Identification of *E. coli* in Milk and Soil

All 62 *E. coli* isolates subjected to PCR were positive for the *uidA* gene with an amplicon size of 147 base pairs (Figure 4.1).

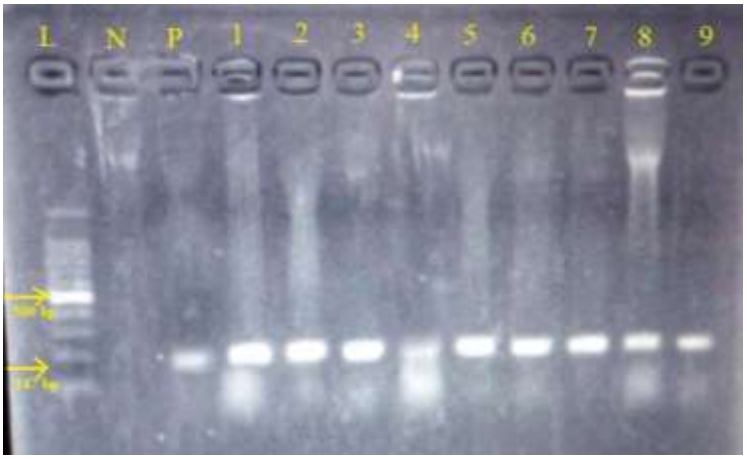


Figure 4.1. Gel showing species confirmation of representative *E. coli* isolates using *uidA* primers. KEY: L: 100bp ladder; N: Negative control; P: Positive control ATCC 25922. Lanes 1, 2, 3, 4, 5, 6, 7, 8 and 9: *uidA* gene positive.

4.2.2. Molecular Detection and Identification of *K. pneumoniae* in Milk and Soil.

The seven *K. pneumoniae* isolates subjected to PCR were positive for the *rcsA* gene with an amplicon size of 176 base pairs (Figure 4.2).

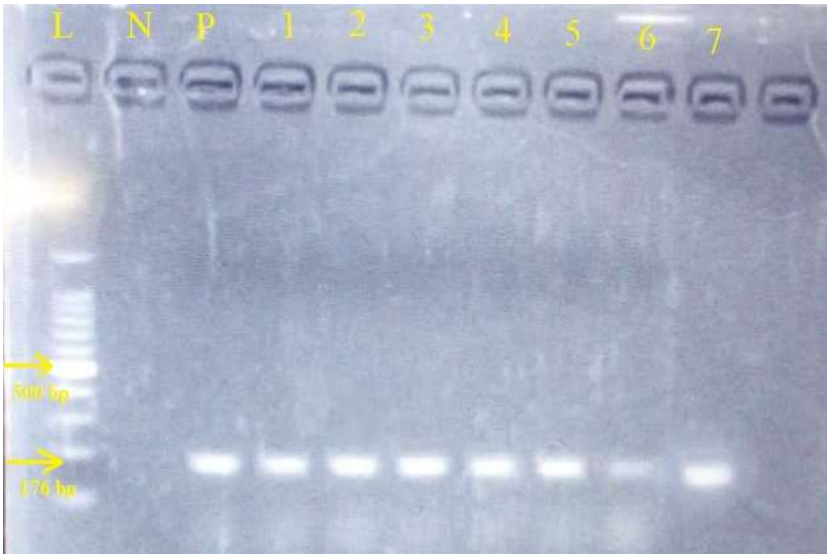


Figure 4.2. Confirmation of *K. pneumoniae* isolates using KP-27 primers. KEY: L: 100bp Ladder; N: Negative control; P: Positive control ATCC 700603; Lanes 1, 2, 3, 4, 5, 6 and 7: *rcsA* gene positive

4.3 Antimicrobial Susceptibility Patterns of *E. coli* and *K. pneumoniae*

4.3.1 Antimicrobial Susceptibility Patterns of *E. coli* in Milk and Soil

All 62 *E. coli* isolates were subjected to antimicrobial susceptibility testing with eight (8) antimicrobials (Figure 4.1). We found that out of the 62 isolates, 84% were resistant to ampicillin, 38% to ceftazidime and 35% to ciprofloxacin. Imipenem was seen to be the most effective antimicrobial, with 98% of the isolates being susceptible. This was followed by 90% susceptibility to trimethoprim-sulfamethoxazole, 81% to tetracycline, and 62% to ceftriaxone.

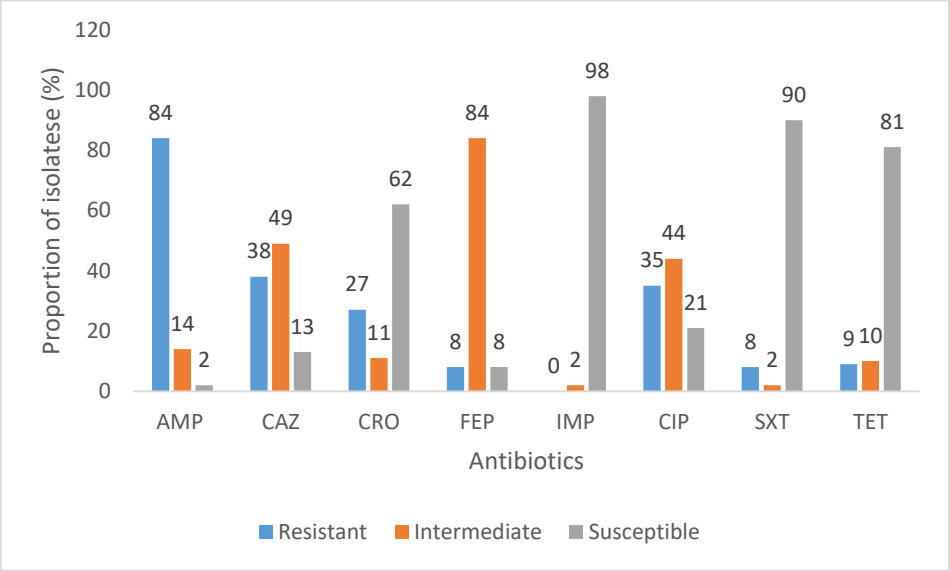


Figure 4.1. Overall antimicrobial susceptibility patterns of *E. coli* in milk and soil. AMP = Ampicillin, CAZ = Ceftazidime, CRO = Ceftriaxone, FEP = Cefepime, IMP = Imipenem, CIP = Ciprofloxacin, SXT = Trimethoprim-sulfamethoxazole, TET = Tetracycline.

4.3.2 Resistance Pattern of *E. coli* in Milk and Soil

Of the 62 *E. coli* isolates obtained from milk and soil, the highest resistance was seen to AMP from milk and soil samples at 91% and 78%, respectively (Figure 4.2). This was followed by 34% and 35% resistance to ciprofloxacin from isolates from milk and soil (Figure 4.2). The least resistance for isolates from milk and soil was to tetracycline, with 9% and 10%, respectively (see Figure 4.2).

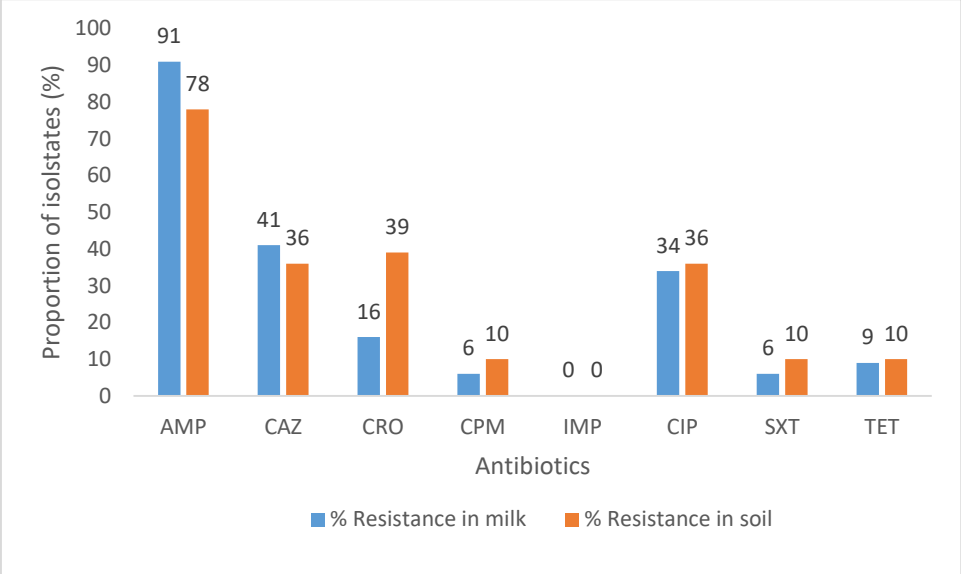


Figure 4.2. Comparison of the resistance patterns of *E. coli* in milk and soil. AMP = Ampicillin, CAZ = Ceftazidime, CRO = Ceftriaxone, FEP = Cefepime, IMP = Imipenem, CIP = Ciprofloxacin, SXT = Trimethoprime-sulfamethoxazole, TET = Tetracycline.

4.3.4 Antimicrobial Susceptibility of *K. pneumoniae* in Milk and Soil

Of the seven *K. pneumoniae* isolates, 100% were susceptible to imipenem and 86% to ciprofloxacin and trimethoprim-sulfamethoxazole (Figure 4.3). The highest resistance was to ceftazidime at 86% (Figure 4.3).

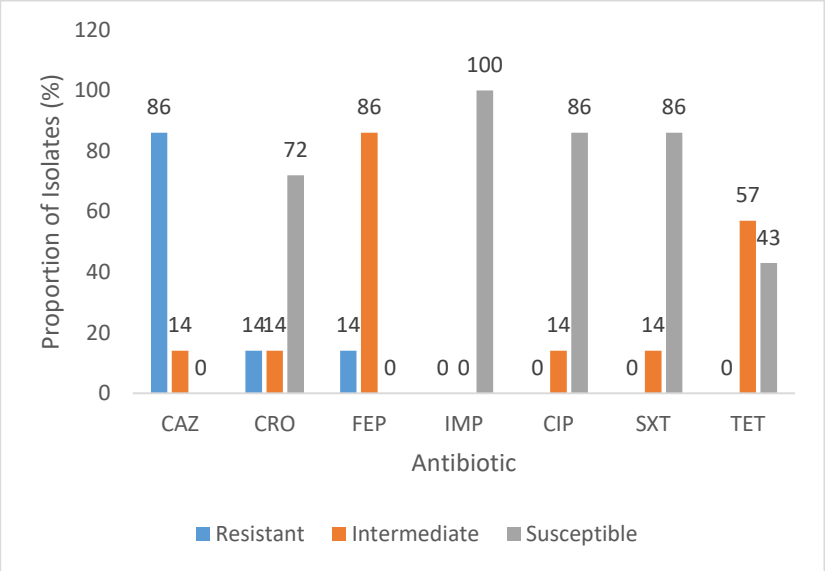


Figure 4.3. Overall antimicrobial susceptibility patterns of *K. pneumoniae* in milk and soil. CAZ = Ceftazidime, CRO = Ceftriaxone, FEP = Cefepime, IMP = Imipenem, CIP = Ciprofloxacin, SXT = Trimethoprime-sulfamethoxazole, TET = Tetracycline.

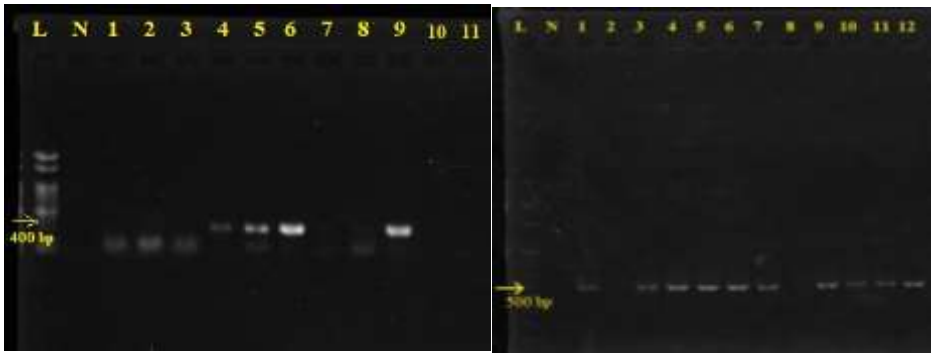
4.4 Determination of ESBL and ESBL Resistance Genes

4.4.1 Determination of ESBL Resistance

Five *E. coli* isolates (three from milk and two from soil) grew on CTX-Mac.

4.4.2 Determination of ESBL Resistance Genes

Out of the five, 60% (3/5) of the isolates were positive for two of the genes that were tested, that is, *bla*CTX-M (1/3) isolates (Figure 4.4), *bla*TEM-1 gene (2/3) isolates (Figure 4.4) and none for *bla*SHV.



(A) (B)

Figure 4.4. (A) Gel showing isolates with ESBL resistance genes and *bla*CTX-M gene (400 bp). Lanes 4, 5, 6 and 9: *bla*CTX-M gene positive. (B) Gel showing ESBL resistance genes and presence of *bla*TEM-1gene (500 bp). Lanes 1, 3, 4, 5, 6, 7, 9, 10, 11 and 12: *bla*TEM-1 gene positive. KEY: L; 1000 bp molecular weight ladder; N: Negative control.

4.5 Determination of Antimicrobial Resistance Genes

A total of 37 *E. coli* isolates and one *K. pneumoniae* had genes conferring resistance to either one or more of the following antibiotics: ceftazidime, ceftriaxone, cefepime, tetracycline and trimethoprim-sulfamethoxazole.

The five PCR assays to determine resistance genes in the 38 isolates are shown in the figures below (Figure 4.5). A total of 27% (10/37) *E. coli* isolates were positive for the *bla*CTX-M gene (Figure 4.5.A), 37.8% (14/37) for *bla*TEM-1 gene (Figure 4.5.B), and 21.6% (8/37) for *bla*TEM-2 gene (Figure 4.5 C). A total of 80% (4/5) isolates were positive for the *dfrA7* gene (Figure 4.5 D) and 33% (2/6) for the *tet*(A) gene (figure 4.5 E). No tet(B), dfrA2d, blaOXA, blaSHV and sul-1 were detected. The single isolate of *K. pneumoniae* showed no resistance genes present.

Table 4.1. Showing the distribution of antimicrobial resistance genes detected.

Genes screened	**Positive isolates**		
	E. coli N (%)	*K. pneumoniae* N (%)	Total
***bla*CTX-M**	10 (27)	1 (100)	11
***bla*TEM-1**	14 (37.8)	-	14
***bla*TEM-2**	8 (21.6)	-	8
dfrA7	4 (80)	-	4
***tet*(A)**	2 (33)	-	2

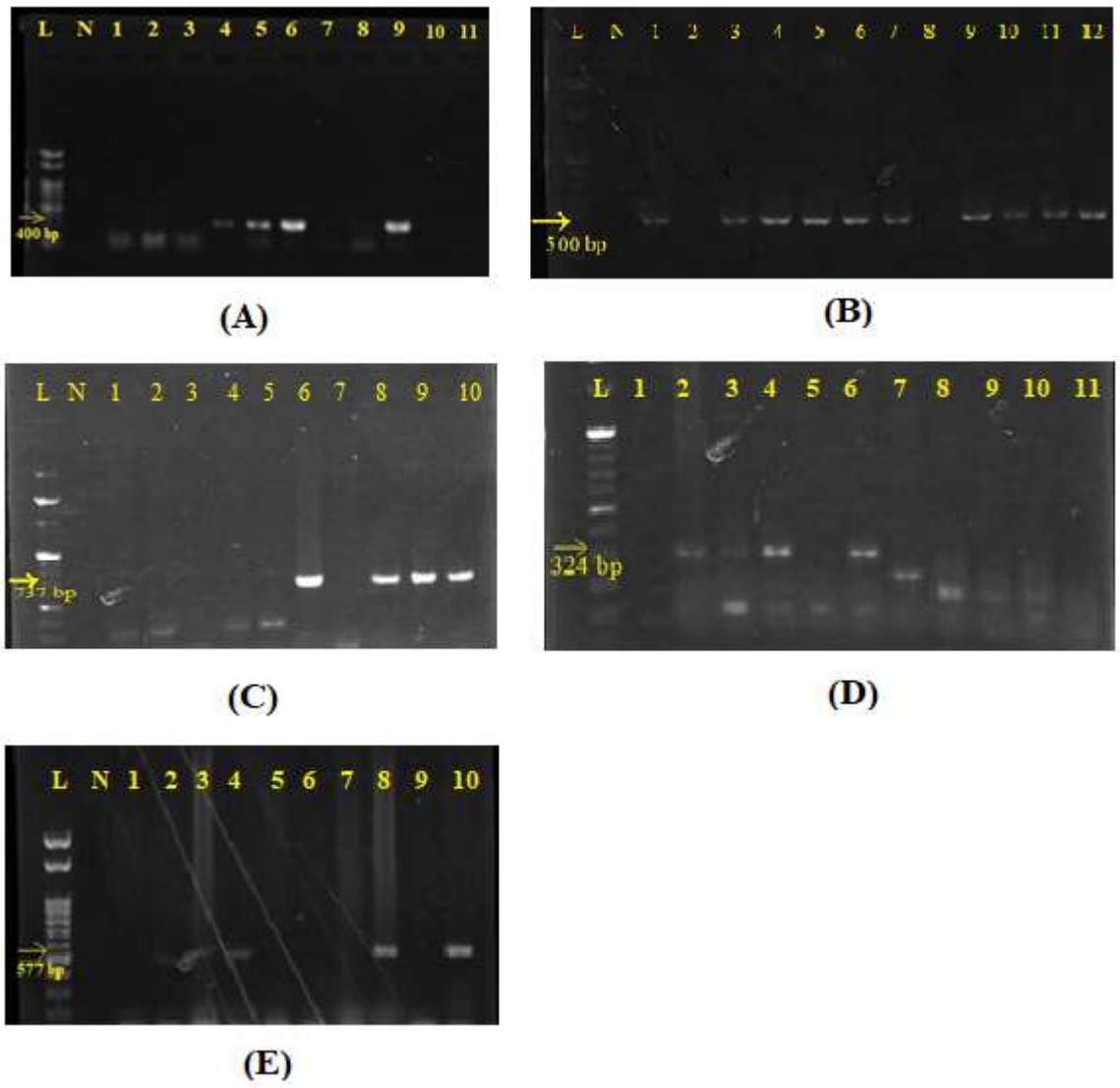


Figure 4.5. (A) Gel showing some of the isolates with ESBL resistance genes and presence of *bla*CTX-M gene, expected amplicon size 400 bp; Lanes 4, 5, 6 and 9: *bla*CTX-M gene positive. (B) Gel showing some of the isolates with ESBL resistance genes and presence of *bla*TEM-1gene, expected amplicon size 500 bp. Lanes 1, 3, 4, 5, 6, 7, 9, 10, 11 and 12: *bla*TEM-1 gene positive. (C) Gel showing some of the isolates with ESBL resistance and presence of the *bla*TEM-2 gene, expected amplicon size 737 bp; Lanes 6, 8, 9 and 10: *bla*TEM-2 gene positive. (D) Gel showing the presence of the *dfrA7* gene, expected amplicon size 323 bp; Lanes 1, 2, 3 and 5: *dfrA7* gene positive. (E) Gel showing the presence of *tet*(A) gene, expected amplicon size 577 bp. Lanes 8 and 10: *tet*(A) gene positive. KEY: L: 1000 bp molecular weight ladder; N: Negative control.

CHAPTER FIVE

5.0 DISCUSSION

This study was carried out to evaluate the public health significance of *E. coli* and *K. pneumoniae,* determine antimicrobial susceptibility profiles and detect ESBL resistance genes and other antimicrobial resistance genes.

The prevalence of *E. coli* was 48% higher than *K. pneumoniae* at 8% in milk and soil samples. The findings of the prevalence of these organisms in our study could suggest contamination from the milking process. These findings are similar to findings from studies done in Sudan, Ghana and South Africa by Badri et al. (2017), Adzitey et al. (2022) and Caine et al. (2014) that isolated 38% *E. coli* from 70 samples, 40% *E. coli* from 250 samples and 47% *E. coli* from 400 samples, respectively. However, the findings from this study were in contrast with a study carried out in Egypt by Ombarak et al. (2016) that isolated 76.4% of *E. coli* from 72 raw milk samples and also a study by Lubote et al. (2014) in Tanzania that isolated 90.7% out of 75 raw milk samples. The differences in the findings may be attributed to high faecal contamination and unhygienic practices in both Egypt and Tanzania, such as the use of unboiled water to clean the udder and equipment (Ombarak et al., 2016), as well as the absence of control measures before distribution of raw milk (Lubote et al., 2014).

Our study recorded a low prevalence of *K. pneumoniae* from both milk and soil, similar to studies from China, the Middle East and Egypt that isolated 9.8%, 7.2%, and 11.9% of *K. pneumoniae* isolates from raw milk, respectively (Yang et al., 2021; Tartor et al., 2021; Osman et al., 2014). Similarly, Zimbabwe recorded 11% prevalence of *K. pneumoniae* isolates from milk (Gran et al., 2003). The findings in this study were, however, contrary to the findings in studies done in New York, North-east India and Sudan that isolated *K. pneumoniae* from raw milk at a prevalence of 61.9%, 38% and 62%, respectively by Munoz et al., (2007) , Koovapra et al., (2016) and Badri et al., (2017). The higher prevalence of *K. pneumoniae* reported in other studies could indicate contamination at the hands of personnel and a lack of strict preventive measures for hygiene practices. Few studies have reported to have isolated *E. coli* from soil (Nautiyal et al., 2010; Navab-Daneshmand et al., 2018; Oliveira et al., 2012; Xing et al., 2019) as well as

K. pneumoniae (Kumar et al., 2021; Samanta et al., 2018; Swetha et al., 2022). The presence of both *E. coli* and *K. pneumoniae* in soil proves that the environment can be a suitable habitat for the survival of these organisms. In addition, the isolation of *E. coli* and *K. pneumoniae* from the soil in our study shows that the soil does pose a risk of transmission of these organisms to both animals and humans through their interaction with the environment (Bolton et al., 2011).

Antimicrobial resistance in *Enterobacterales* has increased worldwide, mainly due to an increase in the use of antimicrobials (Badri et al., 2017). Most *E. coli* isolates (84%) resisted ampicillin in the present study. The high resistance to ampicillin could result from misuse and overuse of ampicillin/amoxicillin in human and animal health and an indication of transmission of AMR organisms between humans and animals (Rahman et al., 2022). These results are similar to previous studies from China (43.8%), the Czech Republic (30.4%) and Nigeria (89.3) that have reported ampicillin to be the least effective antimicrobial against *E. coli* (Liu et al., 2021; Skočková et al., 2015; Ghali-Mohammed et al., 2023). Contrary to our findings, other studies that determined ampicillin resistance in *E. coli* isolated from milk recorded low resistance, 7.6% in Thailand, 9.1% in Saudi Arabia, and 18.9% in Egypt (Hinthong et al., 2017; Alharbi et al., 2019; Ombarak et al., 2016). In our study, the highest susceptibility of both *E. coli* and *K. pneumoniae* was observed to imipenem, trimethoprim-sulphamethoxazole and tetracycline. This was in agreement with studies done in Thailand, China, the Czech Republic, Spain and Zambia (Hinthong et al., 2017; Liu et al., 2021; Skočková et al., 2015; Navajas-Benito et al., 2017; Mwasinga et al., 2023). Additionally, low resistance to trimethoprim-sulfamethoxazole in *K. pneumoniae* isolates was reported in Japan (2.6%) and Brazil (9.5%) (Tsuka et al., 2021; Nobrega et al., 2021). Overall, the isolation of bacteria conferred low resistance to antimicrobials in our study, showing that we still have treatment options for infections caused by *E. coli* and *K. pneumoniae* that may affect animals.

Studies carried out in several countries such as Nigeria, China, and the Middle East found *E. coli* isolates from milk to be highly resistant to trimethoprim-sulphamethoxazole and tetracycline, ranging between 20-100% (Ghali-Mohammed et al., 2023; Vicar et al., 2019; Yang et al., 2021; Tartor et al., 2021; Amosun et al.,

2012). The low resistance to trimethoprim-sulphamethoxazole and tetracycline found in our study indicates that treating *E. coli* and *K. pneumoniae* with these antimicrobials may be effective and contribute to preventing outbreaks. Furthermore, the low resistance may reduce the need to use broad-spectrum antimicrobials in livestock farming, positively impacting food safety and public health. Additionally, the disparities in the results from our study compared to the other studies may be due to the genetic variation among the isolated bacterial populations and the environmental conditions in different geographical locations. These variations may actually affect the prevalence of these bacteria, thus leading to different antimicrobial options in different regions.

This study recorded an emergence of resistance to third-generation cephalosporins, which was largely attributed to ESBL production, which mediate resistance to antibiotics such as ceftazidime, ceftriaxone, cefotaxime and aztreonam, which have an oxyamino group (Badri et al., 2017). This study observed less than 40% resistance in *E. coli* to third-generation cephalosporins, with 8% cefepime, 27% ceftriaxone, and 38% ceftazidime. However, a higher resistance rate (86%) was observed in *K. pneumoniae* isolates to ceftazidime. These results may indicate both raw milk and the environment of the cattle being potential sources for the isolation of *E. coli* and *K. pneumoniae* isolates with increasing resistance to β-lactam antibiotics. Consumption of milk contaminated with resistant bacteria may lead to infections that may be difficult to treat, and hence, the presence of resistance to β-lactam antibiotics such as third-generation cephalosporins is a significant public health concern. The risk of infection can result from ingesting contaminated milk and cross-contamination from the cattle's environment during milking. The findings in this study are similar to findings from other studies that found an overall *E. coli* resistance of 8% to third-generation cephalosporins in Saudi Arabia, 4.5% in Egypt and 0.7% in the Czech Republic (Alharbi et al., 2019; Ombarak et al., 2016; Skočková et al., 2015). The findings of our study are in contrast with those reported in a study done in Kenya that found an overall *E. coli* resistance of 81% to β-lactams (Ngaywa et al., 2019). For *K. pneumoniae* isolated from milk, China and Brazil recorded 1.52% and 7% resistance to third-generation cephalosporins, respectively (Yang et al., 2021; Nobrega et al., 2021). The contrast in the AMR findings between

our study and other studies may be attributed to the choice of antibiotics used in these countries and varying restrictions and regulations in accessing certain antibiotic classes that prevent misuse and overuse, thereby preventing selective pressure (Mittal et al., 2020). The high resistance of *K. pneumoniae* observed to ceftazidime in comparison to that observed by the other third-generation cephalosporins may be due to a specific resistance mechanism or certain mutations that the isolates may have acquired (Abdelraouf et al., 2020).

In this study, resistance to third-generation cephalosporins was mostly due to the *bla*TEM-1 gene (27%), followed by the *bla*CTX-M gene (24.3%), with the least being the *bla*TEM-2 gene (16.2%). Our findings are similar to a study in Turkey that found that 28.5% of the *E. coli* isolates harboured the *bla*TEM gene. However, the same study had contrary findings regarding the *bla*CTX-M gene found in 62.3% of the isolates (Kürekci et al., 2019). Another study in Indonesia on *E.coli* from milk found the most detected gene to be *bla*TEM genes. However, the prevalence of 77.8% of *bla*TEM genes was higher than recorded in this study (Widodo et al., 2023). Similarly, a study done in Kenya detected 98% *bla*TEM and 16% *bla*CTX-M (Ngaywa et al., 2019).

In the present study, the identified resistance gene determinant to tetracycline was the *tet*(A) gene (33%), while that to trimethoprim-sulfamethoxazole was the *dfrA*7 gene (80%). There were no *tet*(B), dfrA2d, *bla*OXA, *bla*SHV and sul-1 detected. Our findings in this study are similar to a study that was done by Skocková et al. (2012) that found *tet*(A) to be the most dominant gene responsible for tetracycline resistance in comparison to *tet*(B). However, Skocková et al. (2012) found a higher incidence of *tet*(A) (81.3%) among their *E. coli* isolates, which was about the same in South Africa (70%) (Iweriebor et al., 2015) in comparison to our study. Conversely, only 1.7% of the isolates were found to harbour the *tet*(A) resistance genes in a study from Japan (Suzuki et al., 2022). The presence of these resistance gene determinants in the *E. coli* isolates retrieved from our study's milk and environmental samples indicate that they can be reservoirs for antibiotic resistance and possibly transfer these genes to other microorganisms. The incidence of antimicrobial resistance genes ARG that were detected in our study was very low compared to other studies. This may indicate a low exposure of the cattle to

antibiotics. However, despite the low incidence, the presence of these organisms with resistance genes in milk can actually contribute to the spread of ARGs to humans and to their gastrointestinal tract (GIT) microbiota. The resistance genes in isolates from the environment may be indicative of cross-infection or spreading of resistant pathogens to the environment.

In this study, ESBL-producing *E. coli* and *K. pneumoniae* from raw milk and samples from the kraals environment highlighted five (8%) *E. coli* ESBL-producing isolates and none for *K. pneumoniae*. The prevalence of ESBL-producing *E. coli* is similar to findings from Indonesia (1.7%) in milk, Malaysia 8.5% in milk and 1.3% in the environment (Tyasningsih et al., 2022; Kamaruzzaman et al., 2020). ESBL-producing *E. coli* in raw milk may indicate exposure to antibiotics and is of particular public health concern. ESBL-producing *E. coli* retrieval from milk may also indicate environmental contamination, which correlates with our study's findings, which isolated ESBL-producing isolates from the environment. In our study, one of the three isolates possessed the blaCTX-M gene (33.3%), whilst two had the *bla*TEM-1 gene (66.7%). Neither the *bla*SHV nor the *bla*OXA genes were detected in any of our isolates. The presence of ESBL-producing *E. coli* strains in the milk of healthy animals demonstrates the need to control the usage of antibiotics in veterinary medicine better and implement effective surveillance programs. Our study's findings are similar to those of a study carried out in Malaysia that detected the *bla*CTX-M gene in 22.2% of the isolates, and no isolates showed the presence of *bla*SHV and *bla*OXA. However, detecting the *bla*TEM gene (11.1%) findings was contrary to our study (Kamaruzzaman et al., 2020). In contrast to our findings, a study on food from healthy animals by Alonso et al. (2017) found that all isolated ESBL-producing *E. coli* strains were associated with the *bla*CTX-M gene. There is a need for research and surveillance of AMR and ARGs to understand how they are transmitted within the food chain and prevent cross-contamination and transmission between animals and the environment, which would ultimately pose as a risk to humans due to their interaction with animals and the environment.

Study Limitations

Most farmers were skeptical about participating due to fear of finding their milk

contaminated with strains of *E. coli* and *K. pneumoniae,* as milk production was their source of income. During sample processing, the major limitation was the lack of primers that would have enabled the detection of the resistance genes from all resistant isolates.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study isolated 69 *E. coli* (62) and *K. pneumoniae* (7) isolates from milk and the environment. This shows that there may be a transfer of bacteria to raw milk from the exterior of the udder during the milking milk collection process or from environmental contamination. Furthermore, the presence of these organisms in soil highlights that there is a potential for cross-contamination from the soil to animals through interaction.

A total of 38 isolates showed the presence of ARG, and these findings suggest that AMR can be spread through ARG transfers between bacteria. This, therefore, may lead to the spread of infections that may be difficult to treat due to limited treatment options.

6.2 Recommendations

The findings of our study highlight the presence of antimicrobial-resistant strains of *E.coli* and *K.pneumoniae* in milk and environment, including MDR strains such as ESBLs that harbour ARGs. In order to combat AMR in dairy farming in our settings, we recommend enhanced monitoring and surveillance of AMR bacteria in milk and milk products and to raise awareness among various populations on the risks associated with AMR. Furthermore, to reduce and prevent cross-contamination, we recommend improving hygienic practices by encouraging good sanitation and emphasizing the importance of disinfecting milking equipment. We also recommend increasing/incorporating surveillance on zoonotic pathogens that are normal flora but can also cause infections in animals and humans.

REFERENCES

Abdelraouf, K., Chavda, K.D., Satlin, M.J., Jenkins, S.G., Kreiswirth, B.N., Nicolau, D.P., 2020. Piperacillin-tazobactam-resistant/third-generation cephalosporin-susceptible Escherichia coli and Klebsiella pneumoniae isolates: resistance mechanisms and in vitro-in vivo discordance. Int. J. Antimicrob. Agents 55, 105885.

Adzitey, F., Yussif, S., Ayamga, R., Zuberu, S., Addy, F., Adu-Bonsu, G., Huda, N., Kobun, R., 2022. Antimicrobial susceptibility and molecular characterization of Escherichia coli recovered from milk and related samples. Microorganisms 10, 1335.

Aldabbagh, S.Y., 2022. Molecular characterization of extended spectrum beta-lactamase producing Klebsiella pneumoniae isolated from cows in Mosul city, Iraq. Iraqi J. Vet. Sci. 36, 375–380.

Alharbi, N.S., Khaled, J.M., Kadaikunnan, S., Alobaidi, A.S., Sharafaddin, A.H., Alyahya, S.A., Almanaa, T.N., Alsughayier, M.A., Shehu, M.R., 2019. Prevalence of Escherichia coli strains resistance to antibiotics in wound infections and raw milk. Saudi J. Biol. Sci. 26, 1557–1562. https://doi.org/10.1016/j.sjbs.2018.11.016

Ali, J., Rafiq, Q.A., Ratcliffe, E., 2018. Antimicrobial resistance mechanisms and potential synthetic treatments. Future Sci. OA 4, FSO290.

Allocati, N., Masulli, M., Alexeyev, M.F., Di Ilio, C., 2013. Escherichia coli in Europe: an overview. Int. J. Environ. Res. Public. Health 10, 6235–6254.

Alonso, C., Zarazaga, M., Ben Sallem, R., Jouini, A., Ben Slama, K., Torres, C., 2017. Antibiotic resistance in Escherichia coli in husbandry animals: the African perspective. Lett. Appl. Microbiol. 64, 318–334.

Alqasim, A., Abu Jaffal, A., Alyousef, A.A., 2018. Prevalence of multidrug resistance and extended-spectrum β-lactamase carriage of clinical uropathogenic Escherichia coli isolates in Riyadh, Saudi Arabia. Int. J. Microbiol. 2018.

Amosun, E., Olatoye, I., Adetosoye, A., 2012. Antimicrobial resistance in Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa isolated from milk of dairy cows in three Nigerian cities. Niger. Vet. J. 33.

Arutyunov, D., Frost, L.S., 2013. F conjugation: Back to the beginning. Spec. Issue Based Int. Soc. Plasmid Biol. Meet. Santander 2012 70, 18–32. https://doi.org/10.1016/j.plasmid.2013.03.010

Ashbolt, N.J., 2015. Microbial contamination of drinking water and human health from community water systems. Curr. Environ. Health Rep. 2, 95–106.

AssumpÇÃo, G.L.H., Cardozo, M.V., Beraldo, L.G., Maluta, R.P., Silva, J.T., Avila, F.A.D., McINTOSH, D., Rigobelo, E.C., 2015. Antimicrobials resistance patterns and the presence of stx1, stx2 and eae in Escherichia coli. Rev. Bras. Saúde E Produção Anim. 16, 308–316.

Badri, A., Ibrahim, I., Mohamed, S., Garbi, M., Kabbashi, A., Arbab, M., 2017. Prevalence of extended spectrum beta lactamase (ESBL) producing Escherichia coli, and Klebsiella pneumoniae isolated from raw milk samples in Al Jazirah state, Sudan. Mol Biol 7, 201.

Barkema, H., Schukken, Y., Zadoks, R., 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. J. Dairy Sci. 89, 1877–1895.

Bezirtzoglou, V.M., Chrissoula Voidarou, Arsenis Tsiotsias, Maria Papapetropoulou, Eugenia, 2000. Food-borne intestinal bacterial pathogens. Microb. Ecol. Health Dis. 12, 96–104.

Biswas, S., Mia, M., Ara, N., Ibrahim, M., Nasir, T., Yunus, S., 2013. Comparison of three dimensional test and double disc synergy test for detection of extended spectrum?-lactamase (ESBL) producing Gram negative bacteria. Pulse 6, 12–19.

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J., 2015. Molecular mechanisms of antibiotic resistance. Nat. Rev. Microbiol. 13, 42–51.

Bolton, D.J., Monaghan, A., Byrne, B., Fanning, S., Sweeney, T., McDowell, D.A., 2011. Incidence and survival of non-O157 verocytotoxigenic Escherichia coli in soil. J. Appl. Microbiol. 111, 484–490. https://doi.org/10.1111/j.1365-2672.2011.05057.x

Bonardi, S., Cabassi, C., Fiaccadori, E., Cavirani, S., Parisi, A., Bacci, C., Lamperti, L., Rega, M., Conter, M., Marra, F., 2023. Detection of carbapenemase-and ESBL-producing Klebsiella pneumoniae from bovine bulk milk and comparison with clinical human isolates in Italy. Int. J. Food Microbiol. 387, 110049.

Cai, H.Y., Caswell, J.L., Prescott, J.F., 2014. Nonculture Molecular Techniques for Diagnosis of Bacterial Disease in Animals: A Diagnostic Laboratory Perspective. Vet. Pathol. 51, 341–350. https://doi.org/10.1177/0300985813511132

Caine, L.-A., Nwodo, U.U., Okoh, A.I., Ndip, R.N., Green, E., 2014. Occurrence of Virulence Genes Associated with Diarrheagenic Escherichia coli Isolated from Raw Cow's Milk from Two Commercial Dairy Farms in the Eastern Cape Province, South Africa. Int. J. Environ. Res. Public. Health 11, 11950–11963.

Cho, S., Jackson, C., Frye, J., 2020. The prevalence and antimicrobial resistance phenotypes of Salmonella, Escherichia coli and Enterococcus sp. in surface water. Lett. Appl. Microbiol. 71, 3–25.

Christidis, T., Pintar, K., Butler, A., Nesbitt, A., Thomas, M., Marshall, B., Pollari, F., 2016. Campylobacter spp. prevalence and levels in raw milk: a systematic review and meta-analysis. J. Food Prot. 79, 1775–1783.

Croxen, M.A., Finlay, B.B., 2010. Molecular mechanisms of Escherichia coli pathogenicity. Nat. Rev. Microbiol. 8, 26–38. https://doi.org/10.1038/nrmicro2265

Davis, G.S., Waits, K., Nordstrom, L., Weaver, B., Aziz, M., Gauld, L., Grande, H., Bigler, R., Horwinski, J., Porter, S., 2015. Intermingled Klebsiella pneumoniae populations

between retail meats and human urinary tract infections. Clin. Infect. Dis. 61, 892–899.

Dhanashekar, R., Akkinepalli, S., Nellutla, A., 2012. Milk-borne infections. An analysis of their potential effect on the milk industry. Germs 2, 101.

Dong, D., Liu, W., Li, H., Wang, Y., Li, X., Zou, D., Yang, Z., Huang, S., Zhou, D., Huang, L., 2015. Survey and rapid detection of Klebsiella pneumoniae in clinical samples targeting the rcsA gene in Beijing, China. Front. Microbiol. 6, 519.

dos Santos Goncalves, M., Delattre, C., Balestrino, D., Charbonnel, N., Elboutachfaiti, R., Wadouachi, A., Badel, S., Bernardi, T., Michaud, P., Forestier, C., 2014. Anti-biofilm activity: a function of Klebsiella pneumoniae capsular polysaccharide. PLoS One 9, e99995.

Dosogne, H., Vangroenweghe, F., Burvenich, C., 2002. Potential mechanism of action of J5 vaccine in protection against severe bovine coliform mastitis. Vet. Res. 33, 1–12.

Effah, C.Y., Sun, T., Liu, S., Wu, Y., 2020. Klebsiella pneumoniae: an increasing threat to public health. Ann. Clin. Microbiol. Antimicrob. 19, 1. https://doi.org/10.1186/s12941-019-0343-8

EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis, K., Allende, A., Álvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Herman, L., 2021. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. EFSA J. 19, e06651.

Feng, P., Lum, R., Chang, G.W., 1991. Identification of uidA gene sequences in beta-D-glucuronidase-negative Escherichia coli. Appl. Environ. Microbiol. 57, 320–323.

Founou, R.C., Founou, L.L., Allam, M., Ismail, A., Essack, S.Y., 2019. Whole genome sequencing of extended spectrum β-lactamase (ESBL)-producing Klebsiella pneumoniae isolated from hospitalized patients in KwaZulu-Natal, South Africa. Sci. Rep. 9, 1–11.

García, S., Heredia, N., 2017. Microbiological safety of fruit and vegetables in the field, during harvest, and packaging: A global issue. Glob. Food Secur. Wellness 27–48.

Gebeyehu, A., Taye, M., Abebe, R., 2022. Isolation, molecular detection and antimicrobial susceptibility profile of Salmonella from raw cow milk collected from dairy farms and households in southern Ethiopia. BMC Microbiol. 22, 84. https://doi.org/10.1186/s12866-022-02504-2

Gebremeskel, L., Teklu, T., Kasahun, G.G., Tuem, K.B., 2023. Antimicrobial resistance pattern of Klebsiella isolated from various clinical samples in Ethiopia: a systematic review and meta-analysis. BMC Infect. Dis. 23, 643. https://doi.org/10.1186/s12879-023-08633-x

Ghali-Mohammed, I., Odetokun, I.A., Raufu, I.A., Alhaji, N.B., Adetunji, V.O., 2023. Prevalence of Escherichia coli O157 isolated from marketed raw cow milk in Kwara State, Nigeria. Sci. Afr. 19, e01469. https://doi.org/10.1016/j.sciaf.2022.e01469

Girma, K., Tilahun, Z., Haimanot, D., 2014. Review on milk safety with emphasis on its public health. World J Dairy Food Sci 9, 166–83.

Gomes, T.A., Elias, W.P., Scaletsky, I.C., Guth, B.E., Rodrigues, J.F., Piazza, R.M., Ferreira, L., Martinez, M.B., 2016. Diarrheagenic escherichia coli. Braz. J. Microbiol. 47, 3–30.

Gorden, P., Kleinhenz, M., Ydstie, J., Brick, T., Slinden, L., Peterson, M., Straub, D., Burkhardt, D., 2018. Efficacy of vaccination with a Klebsiella pneumoniae siderophore receptor protein vaccine for reduction of Klebsiella mastitis in lactating cattle. J. Dairy Sci. 101, 10398–10408.

Gould, L.H., Walsh, K.A., Vieira, A.R., Herman, K., Williams, I.T., Hall, A.J., Cole, D., 2013. Surveillance for foodborne disease outbreaks—United States, 1998–2008. Morb. Mortal. Wkly. Rep. Surveill. Summ. 62, 1–34.

Gourama, H., 2020. Foodborne pathogens. Food Saf. Eng. 25–49.

Grace, D., Wu, F., Havelaar, A.H., 2020. MILK Symposium review: Foodborne diseases from milk and milk products in developing countries—Review of causes and health and economic implications*. J. Dairy Sci. 103, 9715–9729. https://doi.org/10.3168/jds.2020-18323

Gran, H., Wetlesen, A., Mutukumira, A., Rukure, G., Narvhus, J., 2003. Occurrence of pathogenic bacteria in raw milk, cultured pasteurised milk and naturally soured milk produced at small-scale dairies in Zimbabwe. Food Control 14, 539–544.

Guerra, M.E.S., Destro, G., Vieira, B., Lima, A.S., Ferraz, L.F.C., Hakansson, A.P., Darrieux, M., Converso, T.R., 2022. Klebsiella pneumoniae Biofilms and Their Role in Disease Pathogenesis. Front. Cell. Infect. Microbiol. 12, 877995. https://doi.org/10.3389/fcimb.2022.877995

Gundogan, N., Avci, E., 2013. Prevalence and antibiotic resistance of extended-spectrum beta-lactamase (ESBL) producing Escherichia coli and Klebsiella species isolated from foods of animal origin in Turkey. Afr. J. Microbiol. Res. 7, 4059–4064.

Gupta, A., Ampofo, K., Rubenstein, D., Saiman, L., 2003. Extended Spectrum β Lactamase-producing Klebsiella pneumoniae Infections: a Review of the Literature. J. Perinatol. 23, 439–443. https://doi.org/10.1038/sj.jp.7210973

Gupta, M.D., Islam, M., Sen, A., Sarker, M.S., Das, A., 2017. Prevalence and antibiotic susceptibility pattern of Escherichia coli in cattle on Bathan and intensive rearing system. Microbes Health 6, 1–4.

Hansen, D.S., Aucken, H.M., Abiola, T., Podschun, R., 2004. Recommended test panel for differentiation of Klebsiella species on the basis of a trilateral interlaboratory evaluation of 18 biochemical tests. J. Clin. Microbiol. 42, 3665–3669.

Hartantyo, S.H.P., Chau, M.L., Koh, T.H., Yap, M., Yi, T., Cao, D.Y.H., Gutierrez, R.A., Ng, L.C., 2020. Foodborne Klebsiella pneumoniae: virulence potential, antibiotic resistance, and risks to food safety. J. Food Prot. 83, 1096–1103.

Havelaar, A.H., Kirk, M.D., Torgerson, P.R., Gibb, H.J., Hald, T., Lake, R.J., Praet, N., Bellinger, D.C., De Silva, N.R., Gargouri, N., 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med. 12, e1001923.

Hetherington, J.B., Wiethoelter, A.K., Negin, J., Mor, S.M., 2017. Livestock ownership, animal source foods and child nutritional outcomes in seven rural village clusters in Sub-Saharan Africa. Agric. Food Secur. 6, 9. https://doi.org/10.1186/s40066-016-0079-z

Hinthong, W., Pumipuntu, N., Santajit, S., Kulpeanprasit, S., Buranasinsup, S., Sookrung, N., Chaicumpa, W., Aiumurai, P., Indrawattana, N., 2017. Detection and drug resistance profile of Escherichia coli from subclinical mastitis cows and water supply in dairy farms in Saraburi Province, Thailand. PeerJ 5, e3431.

Hisaeda, K., Arima, H., Sonobe, T., Nasu, M., Hagiwara, K., Kirisawa, R., Takahashi, T., Kikuchi, N., Nagahata, H., 2011. Changes in Acute-Phase Proteins and Cytokines in Serum and Milk Whey from Dairy Cows with Naturally Occurring Peracute Mastitis Caused by Klebsiella pneumoniae and the Relationship to Clinical Outcome. J. Vet. Med. Sci. 73, 1399–1404. https://doi.org/10.1292/jvms.10-0403

Hsu, B.-M., Wu, S.-F., Huang, S.-W., Tseng, Y.-J., Ji, D.-D., Chen, J.-S., Shih, F.-C., 2010. Differentiation and identification of Shigella spp. and enteroinvasive Escherichia coli in environmental waters by a molecular method and biochemical test. Water Res. 44, 949–955.

Iweriebor, B.C., Iwu, C.J., Obi, L.C., Nwodo, U.U., Okoh, A.I., 2015. Multiple antibiotic resistances among Shiga toxin producing Escherichia coli O157 in feces of dairy cattle farms in Eastern Cape of South Africa. BMC Microbiol. 15, 1–9.

Jang, J., Hur, H., Sadowsky, M.J., Byappanahalli, M., Yan, T., Ishii, S., 2017. Environmental Escherichia coli: ecology and public health implications—a review. J. Appl. Microbiol. 123, 570–581.

Kamaruzzaman, E.A., Abdul Aziz, S., Bitrus, A.A., Zakaria, Z., Hassan, L., 2020. Occurrence and characteristics of extended-spectrum β-lactamase-producing Escherichia coli from dairy cattle, milk, and farm environments in Peninsular Malaysia. Pathogens 9, 1007.

Kapaya, F., Mwansa, F.D., Sakubita, P., Gama, A., Langa, N., Chewe, O., Mutale, L., Nanzaluka, F., Gershom, C., Chola, M., 2018. A foodborne disease outbreak investigation experience in a College in Lusaka, Zambia, 2017. Pan Afr. Med. J. 29, 1–9.

Kasanga, Maisa, Kwenda, G., Wu, J., Kasanga, Maika, Mwikisa, M.J., Chanda, R., Mupila, Z., Yankonde, B., Sikazwe, M., Mwila, E., 2023. Antimicrobial resistance patterns

and risk factors associated with ESBL-producing and MDR Escherichia coli in hospital and environmental settings in Lusaka, Zambia: implications for One Health, antimicrobial stewardship and surveillance systems. Microorganisms 11, 1951.

Kaur, C.P., Vadivelu, J., Chandramathi, S., 2018. Impact of Klebsiella pneumoniae in lower gastrointestinal tract diseases. J. Dig. Dis. 19, 262–271.

Kennedy, J., Nolan, A., Gibney, S., O'brien, S., McMahon, M., McKenzie, K., Healy, B., McDowell, D., Fanning, S., Wall, P., 2011. Deteminants of cross-contamination during home food preparation. Br. Food J. 113, 280–297.

Kimera, Z.I., Mshana, S.E., Rweyemamu, M.M., Mboera, L.E., Matee, M.I., 2020. Antimicrobial use and resistance in food-producing animals and the environment: an African perspective. Antimicrob. Resist. Infect. Control 9, 1–12.

Klaas, I.C., Zadoks, R.N., 2018. An update on environmental mastitis: Challenging perceptions. Transbound. Emerg. Dis. 65, 166–185. https://doi.org/10.1111/tbed.12704

Koovapra, S., Bandyopadhyay, S., Das, G., Bhattacharyya, D., Banerjee, J., Mahanti, A., Samanta, I., Nanda, P., Kumar, A., Mukherjee, R., 2016. Molecular signature of extended spectrum β-lactamase producing Klebsiella pneumoniae isolated from bovine milk in eastern and north-eastern India. Infect. Genet. Evol. 44, 395–402.

Kumar, P., Dash, B., Suyal, D.C., Gupta, S.B., Singh, A.K., Chowdhury, T., Soni, R., 2021. Characterization of Arsenic-Resistant Klebsiella pneumoniae RnASA11 from Contaminated Soil and Water Samples and Its Bioremediation Potential. Curr. Microbiol. 78, 3258–3267. https://doi.org/10.1007/s00284-021-02602-w

Kürekci, C., Osek, J., Aydın, M., Tekeli, İ.O., Kurpas, M., Wieczorek, K., Sakin, F., 2019. Evaluation of bulk tank raw milk and raw chicken meat samples as source of ESBL producing Escherichia coli in Turkey: Recent insights. J. Food Saf. 39, e12605.

Leclerc, Q.J., Wildfire, J., Gupta, A., Lindsay, J.A., Knight, G.M., 2022. Growth-Dependent Predation and Generalized Transduction of Antimicrobial Resistance by Bacteriophage. Msystems 7, e00135-22.

Lee, K.-S., Jeong, Y.-J., Lee, M.-S., 2021. Escherichia coli Shiga toxins and gut microbiota interactions. Toxins 13, 416.

Lekshmi, M., Ammini, P., Kumar, S., Varela, M.F., 2017. The Food Production Environment and the Development of Antimicrobial Resistance in Human Pathogens of Animal Origin. Microorganisms 5. https://doi.org/10.3390/microorganisms5010011

Leung, A.K., Leung, A.A., Wong, A.H., Hon, K.L., 2019. Travelers' Diarrhea: A Clinical Review. Recent Pat. Inflamm. Allergy Drug Discov. 13, 38. https://doi.org/10.2174/1872213X13666190514105054

Li, B., Zhao, Y., Liu, C., Chen, Z., Zhou, D., 2014. Molecular pathogenesis of Klebsiella pneumoniae. Future Microbiol. 9, 1071–1081.

Li, M., Wang, X., Wang, J., Tan, R., Sun, J., Li, L., Huang, J., Wu, J., Gu, Q., Zhao, Y., 2019. Infection-prevention and control interventions to reduce colonisation and infection of intensive care unit-acquired carbapenem-resistant Klebsiella pneumoniae: a 4-year quasi-experimental before-and-after study. Antimicrob. Resist. Infect. Control 8, 1–10.

Li, P., Zhang, D., Li, H., Pang, J., Guo, H., Qiu, J., 2020. Establishment and application of multiplex PCR for simultaneously detecting Escherichia coli, Salmonella, Klebsiella pneumoniae, and staphylococcus aureus in minks. Front. Vet. Sci. 7, 588173.

Liu, H., Meng, L., Dong, L., Zhang, Y., Wang, J., Zheng, N., 2021. Prevalence, antimicrobial susceptibility, and molecular characterization of Escherichia coli isolated from raw milk in dairy herds in Northern China. Front. Microbiol. 12, 730656.

Liu, P.-Y., Shi, Z.-Y., Tung, K.-C., Shyu, C.-L., Chan, K.-W., Liu, J.-W., Wu, Z.-Y., Kao, C.-C., Huang, Y.-C., Lin, C.-F., 2014. Antimicrobial resistance to cefotaxime and ertapenem in Enterobacteriaceae: the effects of altering clinical breakpoints. J. Infect. Dev. Ctries. 8, 289–296.

Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect. Dis. 16, 161–168.

Lubote, R., Shahada, F., Matemu, A., 2014. Prevalence of Salmonella spp. and Escherichia coli in raw milk value chain in Arusha, Tanzania.

Madani, A., Esfandiari, Z., Shoaei, P., Ataei, B., 2022. Evaluation of Virulence Factors, Antibiotic Resistance, and Biofilm Formation of Escherichia coli Isolated from Milk and Dairy Products in Isfahan, Iran. Foods 11. https://doi.org/10.3390/foods11070960

Mainda, G., 2016. Molecular epidemiology of antimicrobial resistance (AMR) and Shiga toxin producing E. coli (STEC) in dairy herds of central Zambia.

Mainda, G., Bessell, P.R., Muma, J.B., McAteer, S.P., Chase-Topping, M.E., Gibbons, J., Stevens, M.P., Gally, D.L., deC. Bronsvoort, B.M., 2015. Prevalence and patterns of antimicrobial resistance among Escherichia coli isolated from Zambian dairy cattle across different production systems. Sci. Rep. 5, 12439. https://doi.org/10.1038/srep12439

Mao, J., Lu, T., 2016. Population-Dynamic Modeling of Bacterial Horizontal Gene Transfer by Natural Transformation. Biophys. J. 110, 258–268. https://doi.org/10.1016/j.bpj.2015.11.033

Mellata, M., 2013. Human and avian extraintestinal pathogenic Escherichia coli: infections, zoonotic risks, and antibiotic resistance trends. Foodborne Pathog. Dis. 10, 916–932.

Mielke, M., 2010. Prevention and control of nosocomial infections and resistance to antibiotics in Europe–Primum non-nocere: elements of successful prevention and control of healthcare-associated infections. Int. J. Med. Microbiol. 300, 346–350.

Mittal, A.K., Bhardwaj, R., Mishra, P., Rajput, S.K., 2020. Antimicrobials misuse/overuse: adverse effect, mechanism, challenges and strategies to combat resistance. Open Biotechnol. J. 14.

Mladenović, K.G., Muruzović, M.Ž., Žugić Petrović, T., Stefanović, O.D., Čomić, L.R., 2018. Isolation and identification of Enterobacteriaceae from traditional Serbian cheese and their physiological characteristics. J. Food Saf. 38, e12387.

Molina, F., López-Acedo, E., Tabla, R., Roa, I., Gómez, A., Rebollo, J.E., 2015. Improved detection of Escherichia coli and coliform bacteria by multiplex PCR. BMC Biotechnol. 15, 1–9.

Moll, H.A.J., Staal, S.J., Ibrahim, M.N.M., 2007. Smallholder dairy production and markets: A comparison of production systems in Zambia, Kenya and Sri Lanka. Agric. Syst. 94, 593–603. https://doi.org/10.1016/j.agsy.2007.02.005

Montso, K.P., Dlamini, S.B., Kumar, A., Ateba, C.N., 2019. Antimicrobial Resistance Factors of Extended-Spectrum Beta-Lactamases Producing Escherichia coli and Klebsiella pneumoniae Isolated from Cattle Farms and Raw Beef in North-West Province, South Africa. BioMed Res. Int. 2019, 4318306. https://doi.org/10.1155/2019/4318306

Munoz, M.A., Bennett, G.J., Ahlström, C., Griffiths, H.M., Schukken, Y.H., Zadoks, R.N., 2008. Cleanliness Scores as Indicator of Klebsiella Exposure in Dairy Cows. J. Dairy Sci. 91, 3908–3916. https://doi.org/10.3168/jds.2008-1090

Munoz, M.A., Welcome, F.L., Schukken, Y.H., Zadoks, R.N., 2007. Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New York State. J. Clin. Microbiol. 45, 3964–3971.

Mwasinga, W., Shawa, M., Katemangwe, P., Chambaro, H., Mpundu, P., M'kandawire, E., Mumba, C., Munyeme, M., 2023. Multidrug-Resistant Escherichia coli from Raw Cow Milk in Namwala District, Zambia: Public Health Implications. Antibiotics 12, 1421.

Nautiyal, C.S., Rehman, A., Chauhan, P.S., 2010. Environmental Escherichia coli occur as natural plant growth-promoting soil bacterium. Arch. Microbiol. 192, 185–193. https://doi.org/10.1007/s00203-010-0544-1

Navab-Daneshmand, T., Friedrich, M.N.D., Gächter, M., Montealegre, M.C., Mlambo, L.S., Nhiwatiwa, T., Mosler, H.-J., Julian, T.R., 2018. Escherichia coli Contamination across Multiple Environmental Compartments (Soil, Hands,

Drinking Water, and Handwashing Water) in Urban Harare: Correlations and Risk Factors. Am. J. Trop. Med. Hyg. 98, 803. https://doi.org/10.4269/ajtmh.17-0521

Navajas-Benito, E.V., Alonso, C.A., Sanz, S., Olarte, C., Martínez-Olarte, R., Hidalgo-Sanz, S., Somalo, S., Torres, C., 2017. Molecular characterization of antibiotic resistance in Escherichia coli strains from a dairy cattle farm and its surroundings. J. Sci. Food Agric. 97, 362–365.

Neelam, Jain, V., Singh, M., Joshi, V.G., Chhabra, R., Singh, K., Rana, Y., 2022. Virulence and antimicrobial resistance gene profiles of Staphylococcus aureus associated with clinical mastitis in cattle. PLoS One 17, e0264762.

Newell, D., La Ragione, R., 2018. Enterohaemorrhagic and other Shiga toxin-producing Escherichia coli (STEC): Where are we now regarding diagnostics and control strategies? Transbound. Emerg. Dis. 65, 49–71.

Ngaywa, C., Aboge, G.O., Obiero, G., Omwenga, I., Ngwili, N., Wamwere, G., Wainaina, M., Bett, B., 2019. Antimicrobial resistant Escherichia coli isolates detected in raw milk of livestock in pastoral areas of northern Kenya. Food Control 102, 173–178.

Nguyen, Y., Sperandio, V., 2012. Enterohemorrhagic E. coli (EHEC) pathogenesis. Front. Cell. Infect. Microbiol. 2, 90.

Nobrega, D.B., Calarga, A.P., Nascimento, L.C., Vasconcelos, C.G.C., de Lima, E.M., Langoni, H., Brocchi, M., 2021. Molecular characterization of antimicrobial resistance in Klebsiella pneumoniae isolated from Brazilian dairy herds. J. Dairy Sci. 104, 7210–7224.

Nowbuth, A.A., Asombang, A.W., Tazinkeng, N.N., Makinde, O.Y., Sheets, L.R., 2023. Antimicrobial resistance from a One Health perspective in Zambia: a systematic review. Antimicrob. Resist. Infect. Control 12, 15. https://doi.org/10.1186/s13756-023-01224-0

Oliveira, M., Viñas, I., Usall, J., Anguera, M., Abadias, M., 2012. Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. Int. J. Food Microbiol. 156, 133–140. https://doi.org/10.1016/j.ijfoodmicro.2012.03.014

Ombarak, R.A., Hinenoya, A., Awasthi, S.P., Iguchi, A., Shima, A., Elbagory, A.-R.M., Yamasaki, S., 2016. Prevalence and pathogenic potential of Escherichia coli isolates from raw milk and raw milk cheese in Egypt. Int. J. Food Microbiol. 221, 69–76.

Osman, K.M., Hassan, H.M., Orabi, A., Abdelhafez, A.S., 2014. Phenotypic, antimicrobial susceptibility profile and virulence factors of Klebsiella pneumoniae isolated from buffalo and cow mastitic milk. Pathog. Glob. Health 108, 191–199.

Pant, R., Nirwal, S., Rai, N., 2013. Prevalence of antibiotic resistant bacteria and analysis of microbial quality of raw milk samples collected from different regions of Dehradun. Int. J. PharmTech Res. 5, 804–810.

Park, J., Lee, H.S., Lee, C., Lee, H.-J., 2019. Milk consumption patterns and perceptions in Korean adolescents, adults, and the elderly. Int. Dairy J. 95, 78–85. https://doi.org/10.1016/j.idairyj.2019.03.011

Parkhill, J., 2022. Antimicrobial Resistance Exchange Between Humans and Animals: Why We Need to Know More. Eng. Beijing China.

Phiri, B.S.J., Sakumona, M., Hang'ombe, B.M., Fetsch, A., Schaarschmidt, S., 2021. The traditional dairy value chain in Zambia and potential risk factors to microbiological food safety. Food Control 124, 107885. https://doi.org/10.1016/j.foodcont.2021.107885

Piao, D., Li, Z., Yang, X., Tian, G., Zhao, H., Jiang, H., 2020. Emerging Brucellosis Outbreaks Associated with Unpasteurized Milk in China. China CDC Wkly. 2, 888.

Pires, S.M., Desta, B.N., Mughini-Gras, L., Mmbaga, B.T., Fayemi, O.E., Salvador, E.M., Gobena, T., Majowicz, S.E., Hald, T., Hoejskov, P.S., 2021. Burden of foodborne diseases: Think global, act local. Curr. Opin. Food Sci. 39, 152–159.

Pishtiwan, A.H., Khadija, K.M., 2019. Prevalence of blaTEM, blaSHV, and blaCTX-M genes among ESBL-producing Klebsiella pneumoniae and Escherichia coli isolated from thalassemia patients in Erbil, Iraq. Mediterr. J. Hematol. Infect. Dis. 11.

Pokharel, P., Dhakal, S., Dozois, C.M., 2023. The diversity of escherichia coli pathotypes and vaccination strategies against this versatile bacterial pathogen. Microorganisms 11, 344.

Rafi, M.O., Al-Khafaji, K., Mandal, S.M., Meghla, N.S., Biswas, P.K., Rahman, M.S., 2023. A subunit vaccine against pneumonia: targeting S treptococcus pneumoniae and Klebsiella pneumoniae. Netw. Model. Anal. Health Inform. Bioinforma. 12, 21.

Rahman, M., Rahman, A., Islam, M., Alam, M., 2017. Antimicrobial resistance of Escherichia coli isolated from milk, beef and chicken meat in Bangladesh. Bangladesh J. Vet. Med. 15, 141–146.

Rahman, M.M., Alam Tumpa, M.A., Zehravi, M., Sarker, M.T., Yamin, M., Islam, M.R., Harun-Or-Rashid, M., Ahmed, M., Ramproshad, S., Mondal, B., 2022. An overview of antimicrobial stewardship optimization: the use of antibiotics in humans and animals to prevent resistance. Antibiotics 11, 667.

Rottier, W.C., Bamberg, Y.R., Dorigo-Zetsma, J.W., van der Linden, P.D., Ammerlaan, H.S., Bonten, M.J., 2015. Predictive value of prior colonization and antibiotic use for third-generation cephalosporin-resistant enterobacteriaceae bacteremia in patients with sepsis. Clin. Infect. Dis. 60, 1622–1630.

Sadiqi, S., Hamza, M., Ali, F., Alam, S., Shakeela, Q., Ahmed, S., Ayaz, A., Ali, S., Saqib, S., Ullah, F., Zaman, W., 2022. Molecular Characterization of Bacterial Isolates from Soil Samples and Evaluation of their Antibacterial Potential against MDRS. Molecules 27. https://doi.org/10.3390/molecules27196281

Saini, V., McClure, J.T., Léger, D., Keefe, G.P., Scholl, D.T., Morck, D.W., Barkema, H.W., 2012. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. J. Dairy Sci. 95, 4319–4332. https://doi.org/10.3168/jds.2012-5373

Samanta, A., Mahanti, A., Chatterjee, S., Joardar, S.N., Bandyopadhyay, S., Sar, T.K., Mandal, G.P., Dutta, T.K., Samanta, I., 2018. Pig farm environment as a source of beta-lactamase or AmpC-producing Klebsiella pneumoniae and Escherichia coli. Ann. Microbiol. 68, 781–791.

Schmelcher, M., Powell, A.M., Camp, M.J., Pohl, C.S., Donovan, D.M., 2015. Synergistic streptococcal phage λSA2 and B30 endolysins kill streptococci in cow milk and in a mouse model of mastitis. Appl. Microbiol. Biotechnol. 99, 8475–8486.

Schukken, Y., Chuff, M., Moroni, P., Gurjar, A., Santisteban, C., Welcome, F., Zadoks, R., 2012. The "other" gram-negative bacteria in mastitis: Klebsiella, Serratia, and more. Vet. Clin. Food Anim. Pract. 28, 239–256.

Seib, K.L., Zhao, X., Rappuoli, R., 2012. Developing vaccines in the era of genomics: a decade of reverse vaccinology. Clin. Microbiol. Infect. 18, 109–116.

Seiffert, S.N., Hilty, M., Perreten, V., Endimiani, A., 2013. Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: an emerging problem for human health? Drug Resist. Updat. 16, 22–45.

Shams, E., Firoozeh, F., Moniri, R., Zibaei, M., 2015. Prevalence of plasmid-mediated quinolone resistance genes among extended-spectrum β-Lactamase-producing Klebsiella pneumoniae human isolates in Iran. J. Pathog. 2015.

Sheldon, I.M., Rycroft, A.N., Dogan, B., Craven, M., Bromfield, J.J., Chandler, A., Roberts, M.H., Price, S.B., Gilbert, R.O., Simpson, K.W., 2010. Specific strains of Escherichia coli are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. PloS One 5, e9192.

Singh, P., Singh, R., Gupta, B., Tripathi, S.S., Tomar, K.S., Jain, S., Sahni, Y., 2018. Prevalence study of Salmonella spp. in milk and milk products. Asian J. Dairy Food Res. 37, 7–12.

Skočková, A., Bogdanovičová, K., Koláčková, I., Karpíšková, R., 2015. Antimicrobial-resistant and extended-spectrum β-Lactamase–producing Escherichia coli in raw cow's milk. J. Food Prot. 78, 72–77.

Skocková, A., Cupáková, S., Karpísková, R., Janstová, B., 2012. Detection of tetracycline resistance genes in Escherichia coli from raw cow's milk. J. Microbiol. Biotechnol. Food Sci. 1, 777.

Soomro, A., Arain, M., Khaskheli, M., Bhutto, B., 2002. Isolation of Escherichia coli from raw milk and milk products in relation to public health sold under market conditions at Tandojam. Pak. J. Nutr. 1, 151–152.

Sun, D., Jeannot, K., Xiao, Y., Knapp, C.W., 2019. Horizontal gene transfer mediated bacterial antibiotic resistance. Front. Microbiol. 10, 1933.

Suojala, L., Kaartinen, L., Pyörälä, S., 2013. Treatment for bovine E scherichia coli mastitis–an evidence-based approach. J. Vet. Pharmacol. Ther. 36, 521–531.

Suzuki, Y., Hiroki, H., Xie, H., Nishiyama, M., Sakamoto, S.H., Uemura, R., Nukazawa, K., Ogura, Y., Watanabe, T., Kobayashi, I., 2022. Antibiotic-resistant Escherichia coli isolated from dairy cows and their surrounding environment on a livestock farm practicing prudent antimicrobial use. Int. J. Hyg. Environ. Health 240, 113930.

Swetha, P., Mathew, B., Latha, C., Sethulekshmi, C., 2022. Occurrence of Klebsiella pneumoniae and Salmonella spp. in environmental samples.

Tartor, Y.H., El-Aziz, A., Norhan, K., Gharieb, R., El Damaty, H.M., Enany, S., Soliman, E.A., Abdellatif, S.S., Attia, A.S., Bahnass, M.M., 2021. Whole-genome sequencing of gram-negative bacteria isolated from bovine mastitis and raw milk: the first emergence of colistin mcr-10 and fosfomycin fosA5 resistance genes in Klebsiella pneumoniae in middle east. Front. Microbiol. 3596.

Tenaillon, O., Skurnik, D., Picard, B., Denamur, E., 2010. The population genetics of commensal Escherichia coli. Nat. Rev. Microbiol. 8, 207–217.

Tsuka, T., Ozaki, H., Saito, D., Murase, T., Okamoto, Y., Azuma, K., Osaki, T., Ito, N., Murahata, Y., Imagawa, T., 2021. Genetic characterization of CTX-M-2-producing Klebsiella pneumoniae and Klebsiella oxytoca associated with bovine mastitis in Japan. Front. Vet. Sci. 8, 659222.

Turck, D., 2013. Cow's milk and goat's milk. Evid.-Based Res. Pediatr. Nutr. 108, 56–62.

Tyasningsih, W., Ramandinianto, S.C., Ansharieta, R., Witaningrum, A.M., Permatasari, D.A., Wardhana, D.K., Effendi, M.H., Ugbo, E.N., 2022. Prevalence and antibiotic resistance of Staphylococcus aureus and Escherichia coli isolated from raw milk in East Java, Indonesia. Vet. World 15, 2021.

Uddin, M.A., Motazzim-ul-Haque, H.M., Noor, R., 2011. Isolation and identification of pathogenic Escherichia coli, Klebsiella spp. and Staphylococcus spp. in raw milk samples collected from different areas of Dhaka City, Bangladesh. Stamford J. Microbiol. 1, 19–23.

Vasileska, A., Rechkoska, G., 2012. Global and Regional Food Consumption Patterns and Trends. XI Int. Conf. Serv. Sect. Terms Chang. Environ. 27-29 Oct. 2011 Ohrid 44, 363–369. https://doi.org/10.1016/j.sbspro.2012.05.040

Vicar, E.K., Feglo, P.K., Acquah, S.E., Williams, W., Saba, C.K., Mensah, G.I., 2019. Antibiotic resistant bacteria in raw cow milk and milk products retailed in the northern region of Ghana; a food safety challenge. J. Food Saf. Hyg. 5, 206–213.

Virpari, P.K., Nayak, J., Thaker, H., Brahmbhatt, M., 2013. Isolation of pathogenic Escherichia coli from stool samples of diarrhoeal patients with history of raw milk consumption. Vet. World 6, 659–663.

Von Wintersdorff, C.J., Penders, J., Van Niekerk, J.M., Mills, N.D., Majumder, S., Van Alphen, L.B., Savelkoul, P.H., Wolffs, P.F., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 173.

Walsh, C., Wright, G., 2005. Introduction: antibiotic resistance. Chem. Rev. 105, 391–394.

Wang, R., Van Dorp, L., Shaw, L.P., Bradley, P., Wang, Q., Wang, X., Jin, L., Zhang, Q., Liu, Y., Rieux, A., 2018. The global distribution and spread of the mobilized colistin resistance gene mcr-1. Nat. Commun. 9, 1179.

Wareth, G., Linde, J., Hammer, P., Pletz, M.W., Neubauer, H., Sprague, L.D., 2022. WGS-based phenotyping and molecular characterization of the resistome, virulome and plasmid replicons in Klebsiella pneumoniae Isolates from powdered milk produced in Germany. Microorganisms 10, 564.

Widodo, A., Lamid, M., Effendi, M.H., Tyasningsih, W., Raharjo, D., Khairullah, A.R., Kurniawan, S.C., Yustinasari, L.R., Riwu, K.H.P., Silaen, O.S.M., 2023. Molecular identification of blaTEM and blaCTX-M genes in multidrug-resistant Escherichia coli found in milk samples from dairy cattle farms in Tulungagung, Indonesia. J. Vet. Res. 67, 381.

Wilson, D.J., Grohn, Y.T., Bennett, G.J., González, R.N., Schukken, Y.H., Spatz, J., 2007. Comparison of J5 Vaccinates and Controls for Incidence, Etiologic Agent, Clinical Severity, and Survival in the Herd Following Naturally Occurring Cases of Clinical Mastitis. J. Dairy Sci. 90, 4282–4288. https://doi.org/10.3168/jds.2007-0160

Xing, J., Wang, H., Brookes, P.C., Salles, J.F., Xu, J., 2019. Soil pH and microbial diversity constrain the survival of *E. coli* in soil. Soil Biol. Biochem. 128, 139–149. https://doi.org/10.1016/j.soilbio.2018.10.013

Yang, Y., Peng, Y., Jiang, J., Gong, Z., Zhu, H., Wang, K., Zhou, Q., Tian, Y., Qin, A., Yang, Z., 2021. Isolation and characterization of multidrug-resistant Klebsiella pneumoniae from raw cow milk in Jiangsu and Shandong provinces, China. Transbound. Emerg. Dis. 68, 1033–1039.

Yasir, M., Farman, M., Shah, M.W., Jiman-Fatani, A.A., Othman, N.A., Almasaudi, S.B., Alawi, M., Shakil, S., Al-Abdullah, N., Ismaeel, N.A., Azhar, E.I., 2020. Genomic and antimicrobial resistance genes diversity in multidrug-resistant CTX-M-positive isolates of Escherichia coli at a health care facility in Jeddah. J. Infect. Public Health 13, 94–100. https://doi.org/10.1016/j.jiph.2019.06.011

Young, T.M., Bray, A.S., Nagpal, R.K., Caudell, D.L., Yadav, H., Zafar, M.A., 2020. Animal model to study Klebsiella pneumoniae gastrointestinal colonization and host-to-host transmission. Infect. Immun. 88, 10–1128.

Yu, W.L., Chuang, Y.C., 2015. Clinical features, diagnosis, and treatment of Klebsiella pneumoniae infection. Calderwood Date.

APPENDICES

Appendix A. Informed Consent Form

Project title: EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE IN MILK AND THE MILKING ENVIRONMENT ON DAIRY FARMS IN LUSAKA.

This informed consent form is for farmers who keep dairy cattle in Lusaka district and whom we invite to participate in research on the evaluation of the public health significance of *E. coli* and *K. pneumoniae* in milk and the milking environment on dairy farms in Lusaka.

Name of Principal Investigator: Ciluvya Kavimba Kaluba

Name of Organization: University of Zambia

Name of Proposal: EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE IN ILK AND THE MILKING ENVIRONMENT IN DAIRY FARMS IN LUSAKA.

Introduction

I am Ciluvya Kavimba Kaluba, doing a Master of Science in One Health Laboratory Diagnostic Sciences at the University of Zambia, School of Veterinary Medicine. The research focuses on the presence of *E. coli* and *K. pneumoniae* in milk and the environment in cattle in the Lusaka district, which seems to be a public health concern. I will give you information and invite you to be part of this research. You do not have to decide today whether you will participate in the research. Before you decide, talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the collection process, and I will take time to explain. If you have questions later, you can ask them of me or any other staff.

Purpose of the research

The tradition of consumption of raw milk in Zambia is fairly common especially among the producers (Moll et al., 2007). Therefore, determining the presence of *E. coli* and *K. pneumoniae* in milk and milking environments is important to human

health. This information will be cardinal in coming up with appropriate interventions meant to improve our hygiene practices regarding cattle if need be.

Participant selection

We are inviting farmers who keep dairy cattle to participate in this study

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You may change your mind later and stop participating even if you agreed earlier.

Procedures and Protocol

We will collect milk from the cattle udder and environmental samples from the kraal of the same cattle. And all samples will be analysed in the public health laboratory at the University of Zambia.

Risks

This study will not pose more than minimal risk.

Benefits

If you participate in this research and are interested in the findings of *E. coli* and/or *K. pneumoniae* in your cattle at your farm, that information will be shared with you regarding your cattle alone.

Reimbursements

You will not be given any money or gifts to participate in this research.

Confidentiality

We will not be sharing the identity of those participating in the research. This includes the farm owners, cattle identities and name of the farm.

The information that we collect from this research will be kept confidential. Information about you that will be collected during the research will be put away, and no one but the researchers will be able to see it. Any information about you will

have a number instead of your name. Only the researchers will know your number, and we will lock that information up with a lock and key.

Sharing the Results

The knowledge we get from this research will be shared with ERES, Veterinary services and the University before it is made widely available to the public. Confidential information will not be shared. We will also publish the results so that other interested people may learn from our research.

Right to Refuse or Withdraw

You do not have to participate in this research if you do not wish to, and refusing to participate will not affect you. You may stop participating in the research at any time that you wish.

Whom to Contact

If you have any questions, you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact the following:

1. Ciluvya Kavimba Kaluba
   Master of Science student in One Health Laboratory Diagnostic Sciences
   The University of Zambia
   Lusaka, Zambia
   Phone number: 0977417833
   kalubakc@gmail.com
2. ERES Converge IRB
   Ethics Research Board
   0955155633/ 0955155633
   eresconverge@yahoo.co.uk
3. Prof John Muma(Supervisor)
   Public health lecturer and research
   University of Zambia
   0977912099
   jmuma@unza.zm

Appendix B. Participant Consent form (English)

Dear participant,

My name is CILUVYA KAVIMBA KALUBA; I am enrolled in the Master of Science in One Health Laboratory Diagnostic Sciences programme at the School of Veterinary Sciences, University of Zambia. In partial fulfilment of my studies, I am required to undertake a research project. My research topic is "EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ESCHERICHIA COLI AND PNEUMONIAAE FROM MILK AND THE MILKING ENVIRONMENT OF DAIRY CATTLE IN LUSAKA PROVINCE, ZAMBIA".

Consent

The information concerning this study has been fully explained to me, and I have been able to ask questions, all of which have been answered to my satisfaction. I understand that my participation in this study is voluntary and that I am free to withdraw at any time, without giving reason and without cost and that I am free to skip any questions I may deem personal or otherwise. I have been assured that my information will be kept private and strictly confidential. I voluntarily agree to take part in this study.

…….. or ……

…../…../……

Participant Signature Thumb print Date

I, the undersigned, have taken the time to explain to the above participant the nature and purpose of this study in a way they could understand. I have explained the possible benefits of this study and I invited the participant to ask questions and make any clarifications concerning this study.

…… ……. ……./……/…….

Name Signature Date

Appendix C: Participant Consent Form (Nyanja)

Wokondewa wanga ofuna kuthengako mbali,

Dzina langa ndine Ciluvya Kavimba Kaluba, mwana wa sukulu wapa University of Zambia (UNZA) Ndichita masters mu One Health Laboratory Diagnostic Sciences mu sukulu ya veterinary sciences. Iyi khani yofufudza ifunika kuti nisilize sukulu yanga. Muntu wa iyi khani yofufudza ndi "EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ESCHERICHIA COLI AND PNEUMONIAE FROM MILK AND THE MILKING ENVIRONMENT OF DAIRY CATTLE IN LUSAKA PROVINCE, ZAMBIA"

Kuvomekeza kuthengako mbali

Khani ya kufufudza iyi yalondoledwa kuli ine ndipo mafuso yanga yonse yayankidwa bwino bwino. Kutegako mbali muli iyi kani ya kufufudza ndikufana kwanga sono ndinga leke nthawi ili yonse kopanda kulondolola kuli konse.

_____ ____/__/____ _____

Otengako mbali signature/kufwatika Tsiku

Ine mwine wa khani iyi yo fufudza na fotokodza bwino bwino kuli ulionse ofuna kuthengako mbali kuli iyi khani ya kufufudza zawibwino otengako mbali kuli khani yofufudza.

Appendix D: Institution Approval, Ethical Approval, NHRA Approval and Permission from the Ministry of Livestock and Fisheries

THE UNIVERSITY OF ZAMBIA
SCHOOL OF VETERINARY MEDICINE
OFFICE OF THE ASSISTANT DEAN (POSTGRADUATE)

Telephone: 293727
Telegrams: UNZA LUSAKA
Telex: UNZALU ZA 44370
Fax: 293727/253952
School Fax: 293727
Vet. Clinic Telephone: 291515

P.O. Box 32379
Lusaka, Zambia

Your Ref:

Our Ref:

24th March, 2023

Kaluba Kavimba Ciluvya
University of Zambia
Department of Paraclinical Studies
LUSAKA

Dear Ciluvya

SUBJECT: APPROVAL OF RESEARCH CONCEPT NOTE

I am writing on behalf of the Board of Graduate Studies to inform you that your research concept note entitled– ***'Evaluation of the public health significance of escherichia coli and Klebsiella pneumoniae in milk and the milking environment in dairy cattle from dairy farms in Lusaka, Zambia"*** was tabled and considered on 23rd March, 2023 by the Board of Graduate Studies of the School of Veterinary Medicine. I am therefore, pleased to inform you that your research concept was subsequently approved by the Board.

On behalf of the Board, I wish you success as you proceed with your research proposal development and all relevant related research activities.

Yours sincerely

Dr. Andrew Kataba (PhD)
ASSISTANT DEAN (PG), SCHOOL OF VETERINARY MEDICINE
Cc Director, DRGS
Dean, School of Veterinary Medicine
Assistant Registrar, School of Veterinary Medicine
Head, Paraclinical Studies
File

Plot No. 272, Cnr Olive Tree Meanwood Road,
Meanwood Ibex
Lusaka - Zambia
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 977 493 220
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

28 September 2023

Ref. No. 2023 -Jun-018
The Prinicpal Investigator
Ms. Kaluba, Ciluvya Kavimba

Dear Ms. Kaluba, Ciluvya Kavimba,
RE: KAVIMBA
Reference is made to your protocol. The IRB has resolved to approve "KAVIMBA" and your participation as Principal Investigator for a period of one year.

Review Type	**Approval No. 2023 -Jun-018**
Approval Date: 2023-09-28	Expiry Date:
Protocol Version	Version- Nil
Information Sheet, Conscent Forn and Dates	-English
Other Study Documents	Questionnaire

Number of Participants Approved for the Study-
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

NATIONAL HEALTH RESEARCH AUTHORITY

Lot No. 18961/M, off Kasama Road, Chalala, P.O. Box 30075, LUSAKA

Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

Ref No:NHRA-956/21/02/2024 **Date:23rd February, 2024**

The Principal Investigator,
Ciluvya Kavimba Kaluba,
University of Zambia,
School of Veterinary Medicine,
Lusaka, Zambia.

Dear Ms Kaluba,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Evaluation of the Public Health Significance of *Escherichia coli* and *Klebsiella pneumoniae* in Milk and the Milking Environment on Dairy Farms in Lusaka, Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA bi-annually from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof Victor Chalwe
Acting Director/Chief Executive Officer
National Health Research Authority

All correspondence should be addressed to
The Director Veterinary Services
Tel: 0211-252608

In reply please quote
No.....
DVS/101/1/15

REPUBLIC OF ZAMBIA

MINISTRY OF FISHERIES AND LIVESTOCK

DEPARTMENT OF VETERINARY SERVICES
MULUNGUSHI HOUSE
P.O. Box 50060
RIDGEWAY 15100
LUSAKA - ZAMBIA

11th December, 2023

Ciluvya Kavimba Kaluba
Scholl of Veterinary Medicine
University of Zambia
Lusaka

REF: REQUEST TO CONDUCT A RESEARCH IN LUSAKA DISTRICT

The Department of Veterinary Services is in receipt of your letter dated 10th November, 2023 in which you were requesting for clearance to conduct a study entitled "***Evaluation of the Public Health Significance of Escherichia Coli and Klebsiella Pneumoniae from Dairy Cattle in Lusaka Province, Zambia".***

After reviewing your request, the Department hereby conditionally grants authorisation of the research to go ahead as long as the following are met;

1) Submission of a final report to the Department of Veterinary Services on the findings and recommendations of the study.
2) You collaborate closely with our local District Veterinary Officials in the study areas to ensure smooth implementation.

Yours Sincerely,

Dr. Paul Fandamu
Acting Director
DEPARTMENT OF VETERINARY SERVICE

Cc: Prof. John Bwalya Muma , University of Zambia, School of Vet. Medicine