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PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF TETRACYCLINE- AND BETA-LACTAM-RESISTANT *ESCHERICHIA COLI* AND *ENTEROCOCCUS* ACROSS THE POULTRY–ENVIRONMENT–HUMAN INTERFACE IN BISHOFTU, ETHIOPIA

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF PARASITOLOGY, MICROBIOLOGY, AND POULTRY HEALTH
MASTER OF SCIENCE PROGRAM IN ONE HEALTH

MSC THESIS

BY
TADELECH YILMA

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BISHOFTU, ETHIOPIA

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF TETRACYCLINE- AND BETA-LACTAM-RESISTANT *ESCHERICHIA COLI* AND *ENTEROCOCCUS* ACROSS THE POULTRY–ENVIRONMENT–HUMAN INTERFACE IN BISHOFTU, ETHIOPIA

A Thesis Submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of Science in One Health

By

Tadelech Yilma Sisay (DVM)

June, 2026

Bishoftu, Ethiopia

APPROVAL SHEET

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Parasitology, and Poultry Health

As MSc in one health research advisor, we hereby certify that we have read and evaluated this thesis prepared under our guidance by Tadelech Yilma, entitled "Phenotypic and Genotypic Characterization of Tetracycline- and Beta-Lactam-Resistant *Escherichia coli* and *Enterococcus* across the Poultry–Environment–Human Interface in Bishoftu, Ethiopia". We recommend that it be submitted as fulfilling the MSc thesis requirement for the Degree of Science in One Health.

Submitted by: **Tadelech Yilma**	_____	_____
Name of Student	Signature	Date
Hika Waktole (PhD)	_____	_____
Name of Advisors	Signature	Date

EXAMINERS' SHEET

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Parasitology and Poultry Health

As members of the Board of Examiners of the MSc open defense examination, we certify that we have read and evaluated the thesis prepared by Tadelech Yilma, entitled "Phenotypic and Genotypic Characterization of Tetracycline- and Beta-Lactam-Resistant *Escherichia coli* and *Enterococcus* across the Poultry–Environment–Human Interface in Bishoftu, Ethiopia"; we recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in One Health.

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I, Tadelech Yilma, declare that the thesis entitled "Phenotypic and Genotypic Characterization of Tetracycline- and Beta-Lactam-Resistant *Escherichia coli* and *Enterococcus* across the Poultry–Environment–Human Interface in Bishoftu, Ethiopia" is my original work and has not been previously submitted in whole or in part for the award of any degree, diploma, scholarship, or other academic title at any university or institution. All sources of information, including data, literature, and intellectual contributions used or cited in this work, have been duly acknowledged with proper citations and references. Any assistance received in the preparation of this thesis has been appropriately credited. This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in One Health program at the College of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia.

Submitted by: Tadelech Yilma Sisay Signature_____

Date_____

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LISTS OF ABBREVIATIONS AND ACRONYMS

AAU	Addis Ababa University
AMP	Ampicilin
AMR	Antimicrobial Resistance
ARGs	Antimicrobial Resistance Genes
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BEA	Bile esculin agar
blaTEM	β-lactamase TEM resistance gene
BPW	Buffered Peptone Water
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CT-SMAC	Cefixime Tellurite Sorbitol MacConkey Agar
CVMA	College of Veterinary Medicine and Agriculture
DNA	Deoxyribonucleic Acid
ddl	D-alanine: D-alanine Ligase Gene
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EMB	Eosin Methylene Blue Agar
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HLGR	High-Level Gentamicin Resistance
ISO	International Organization for Standardization
LMICs	Low- and Middle-Income Countries
MAR	Multiple Antibiotic Resistance
MDR	Multidrug Resistance
mL	Milliliter
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
spp.	Species
*stx*2	Shiga toxin 2 gene
TE	Tetracycline
*tet*M	tetracycline resistance gene M
WHO	World Health Organization

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ABSTRACT

It is evident that Antimicrobial resistance (AMR) is a growing health challenge affecting humans, animals, and the environment. Current practices involving extensive antimicrobial use and intensive poultry production systems favour the creation of important reservoirs of resistant bacteria, particularly to tetracyclines and β-lactams groups of antibiotics. However, previous studies assessing antimicrobial-resistant bacteria across poultry, environmental, and human interfaces remain limited in Ethiopia. This study aimed to investigate tetracycline- and β-lactam-resistant *Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*) across the poultry–environment–human interface in Bishoftu, Ethiopia. Accordingly, a cross-sectional study was conducted from November 2025 to June 2026 on 70 selected commercial poultry farms in Bishoftu, Ethiopia. A total of 210 cloacal, litter, and farm worker hand swab samples were collected. The isolation and identification of the bacteria were performed using standard culture, biochemical, and molecular confirmation methods. Antimicrobial susceptibility test was conducted using the Kirby–Bauer disk diffusion method following CLSI guidelines. PCR test was used to detect the *tetM*, *blaTEM*, *stx2*, and *ddl* genes in the isolated bacteria. The findings of this study disclosed that *E. coli* and *E. faecalis* were isolated from 31.0% (65/210) and 44.8% (94/210) of the samples, respectively. The occurrence of *E. coli* ranged from 30.0% to 31.4%, while *E. faecalis* ranged from 38.6% to 50.0% across the examined samples. High resistance of *E. coli* isolates was observed against tetracycline (91.5%) and ampicillin (72.3%), whereas all recovered *E. coli* strains remained responsive to gentamicin, implying 100% susceptibility. Similarly, multidrug resistance was detected in 19.1% of *E. coli* and 85.1 % of *E. faecalis* isolates. This study also revealed molecular analysis results that confirmed the presence of *tetM* and *blaTEM* resistance genes among resistant isolates of the sample domains. The *stx2* virulence gene was also detected among selected *E. coli* isolates. Findings of this study demonstrated that tetracycline and β-lactam-resistant *E.coli* and *E. faecalis* were detected in poultry, litter and human samples, indicating their distribution across the poultry production system. The presence of resistant isolates in poultry litter indicates its potential role as a reservoir for antimicrobial resistance. Those findings highlight the importance of strengthening antimicrobial stewardship, farm biosecurity, and integrated surveillance of AMR to reduce dissemination of AMR within the One Health continuum.

Keywords: *Antimicrobial resistance (AMR); E. coli; E. faecalis; Ethiopia; One Health; Poultry*

1. INTRODUCTION

1.1. Background

Penicillin discovery in the 1940s laid a cornerstone in modern medicine, revolutionizing the treatment of infectious diseases in humans and animals. At the same time, bacterial resistance to antimicrobials was largely unforeseen (Vercelli *et al*., 2022). In contrast, antimicrobial resistance (AMR) is currently recognized as a major global public health threat, where it is estimated that it causes approximately 700,000 deaths per year globally, a figure projected to increase to 10 million by 2050 if current trends continue, with cumulative economic losses exceeding $100 trillion (Tang *et al*., 2023). Antimicrobial-resistant (AMR) bacteria and antimicrobial resistance genes (ARGs) are now considered contaminants of emerging concern, posing serious risks to human, animal, and environmental health (Rizzo *et al*., 2019).

In food-producing animals, antimicrobials are extensively used for prophylactic purposes, therapy, and productivity enhancement. Livestock production accounts for nearly 70% of antimicrobial use worldwide, with poultry representing a major consumer (Van Boeckel *et al*., 2019). Among these, tetracyclines and beta-lactams are widely used because of their broad-spectrum activity, affordability, and accessibility (Azabo *et al*., 2022). The extensive use of these antimicrobials in poultry production systems contributes to the selection of resistant bacteria within the intestinal microbiota, which are subsequently shed into the farm environment through fecal and litter contamination (Pokrant *et al*., 2023; Cortés *et al*., 2025). *Escherichia coli* (*E. coli*) and *Enterococcus* species (*spp*). are commonly used as indicator organisms for monitoring AMR in animal production systems because of their ecological abundance and capacity to acquire and transfer resistance genes (Marshall and Levy, 2011; Anjum *et al*., 2021).

The frequent application and/or release of poultry litter as fertilizer into the surrounding environment facilitates the dissemination of AMR bacteria into soil, water, and farm settings (Fatoba *et al*., 2022; Lopes *et al*., 2024). Poultry production systems, especially intensive operations, have been identified as a key sources of resistant bacterial contamination in farm environments (Hubbard *et al*., 2020). This environmental dissemination increases the risk of exposure for farmworkers who have direct and frequent contact with birds, litter, and

contaminated surfaces. Occupational exposure in poultry farms has been linked with colonization by multidrug-resistant *E. coli* and *Enterococcus* spp., underscoring the public health relevance of resistance transmission across the poultry–environment–human interface (Nhung *et al*., 2018; Aworh *et al*., 2019).

The One Health framework emphasizes the interconnectedness of human, animal, and environmental health in the emergence and spread of AMR (Marshall and Levy, 2011). Although AMR has been widely reported in poultry production systems in Ethiopia, most studies have focused on single compartments such as poultry or poultry products (Gemeda *et al*., 2021; Tilahun and Efa, 2025). However, holistic investigations that simultaneously characterize resistant bacteria across poultry, environmental, and human interfaces remain limited (Zeru *et al*., 2025).

Bishoftu, as a major hub for poultry production in Ethiopia, provides an ideal setting to explore these interconnections because of intensive poultry farming, diverse litter and waste handling practices, and significant human–animal–environment interactions (Ebsa *et al*., 2019; Ismael *et al*., 2021; Berhanu *et al*., 2025). Tetracyclines and β-lactams were selected for this study because they are among the most frequently consumed antibiotic groups in poultry production, and resistant genes related to those antibiotics are widely disseminated across the animal–environment–human interface (Ajayi *et al*., 2024).

Understanding the dynamics of tetracycline- and β-lactam-resistant *E. coli* and *Enterococcus* spp. in poultry, farm environments, and farm workers is essential for informing evidence-based interventions, enhancing occupational safety, and strengthening national AMR surveillance systems. A comprehensive assessment under a One Health framework can identify critical control points where interventions can minimize the spread of AMR, protecting both public and environmental health (Cortés *et al*., 2025; Singh *et al*., 2025).

1.2. Statement of Problem

The extensive and often indiscriminate use of tetracyclines and β-lactams in poultry production systems, attributed to their affordability, accessibility, and broad-spectrum activity, has contributed to the emergence and dissemination of AMR bacteria and resistance genes within

poultry production environments, creating a serious threat to animal, human, and environmental health (Abou-Jaoudeh *et al*., 2024; Bhattarai *et al*., 2024). Resistant *E. coli* and *Enterococcus* spp. have frequently been detected in poultry litter, farm environments, poultry products, and surrounding ecosystems worldwide, with resistance genes in *E. coli* and *Enterococcus* spp. having been widely reported in poultry farms, litter, and surrounding environments, where poultry waste serves as an important reservoir for ARGs such as *tetM* and *blaTEM*, which increasingly facilitate their dissemination through environmental contamination and occupational exposure and dissemination across animal and human interfaces (Fatoba *et al*., 2022; Velhner *et al*., 2024; Agga *et al*., 2025), become a major health concern worldwide (Aminudin *et al*., 2026).

In Ethiopia, particularly in Bishoftu, where poultry production is rapidly expanding, antimicrobial use is often poorly regulated, and studies on AMR surveillance remain limited (Ephrem, 2022; Ragassa and Berhanu, 2023). Previous studies have reported multidrug-resistant *E. coli* and *Enterococcus spp.* in poultry and poultry products (Messele *et al*., 2017). However, most studies have been limited to single compartments and focused mainly on phenotypic resistance patterns without molecular characterization of resistance genes (Fujita *et al*., 2022). Consequently, information regarding the distribution of tetracycline- and beta-lactam-resistant bacteria and their resistance genes across poultry, environmental, and human interfaces remains scarce in Ethiopia, particularly in Bishoftu (Waktole *et al*., 2024).

Tetracyclines and beta-lactams were selected for this study because they remain among the most commonly used antimicrobial classes in poultry production, particularly in LMICs where veterinary antimicrobial regulation is limited (Van Boeckel *et al*., 2015; Mamphweli *et al*., 2018). Their widespread use and the increasing dissemination of transferable resistance genes among bacteria such as *E. coli* and *Enterococcus* spp. make them important indicators for studying AMR transmission across animal–environment–human interfaces (He *et al*., 2020). These bacteria are considered important indicators for AMR studies because of their ability to acquire and transfer resistance genes across multiple ecological compartments (Roberts, 2005; Thanner *et al*., 2016).

1.3. Objectives of the Study

1.3.1. General Objective

The general objective of the present study is to investigate the phenotypic and genotypic characteristics of tetracycline- and β-lactam-resistant *E. coli* and *Enterococcus* across the poultry–environment–human interface in Bishoftu, Ethiopia, within a One Health nexus.

1.3.2. Specific Objectives

- ✓ To isolate and identify *E. coli* and *E. faecalis* from poultry, farm environments, and farm workers in Bishoftu poultry farms.
- ✓ To determine the phenotypic resistance characteristics of the isolates to tetracycline and beta-lactam antibiotics
- ✓ To detect the presence of resistance and virulence genes, specifically the *bla-TEM*, *tetM*, and *stx2* through molecular analysis.
- ✓ To assess the occurrence of potential transmission of antimicrobial-resistant *E. coli* and *Enterococcus* across the poultry-farm workers and environment from a one-health perspective.

2. LITERATURE REVIEW

2.1. Global and One Health Perspective on Antimicrobial Resistance in Poultry Systems

Antimicrobial resistance (AMR) is a major global health challenge affecting humans, animals, and the environment (Djordjevic *et al*., 2024). Recent estimates indicate that resistant infections contributed considerably to worldwide mortality in 2021, and estimates indicate that the burden may increase considerably and cause an estimated ten million deaths by 2050 if effective control measures are not implemented (O'Neill, 2016; Naghavi *et al*., 2024). The widespread use and misuse of antimicrobials in human medicine, veterinary practice, and agriculture (Figure 1B) have accelerated the emergence and spread of resistant microorganisms (WHO, 2023). Poultry production systems are among the highest consumers of antimicrobials globally (Tiseo *et al*., 2020), creating conditions that favor the selection and circulation of AMR bacteria and resistant genes (ARGs) (Thongratsakul *et al*., 2023). Within the One Health framework, poultry farms serve as important interfaces linking animal, environmental, and human health directly through occupational exposure and indirectly via contaminated food, soil, and water (Gomes *et al*., 2023)

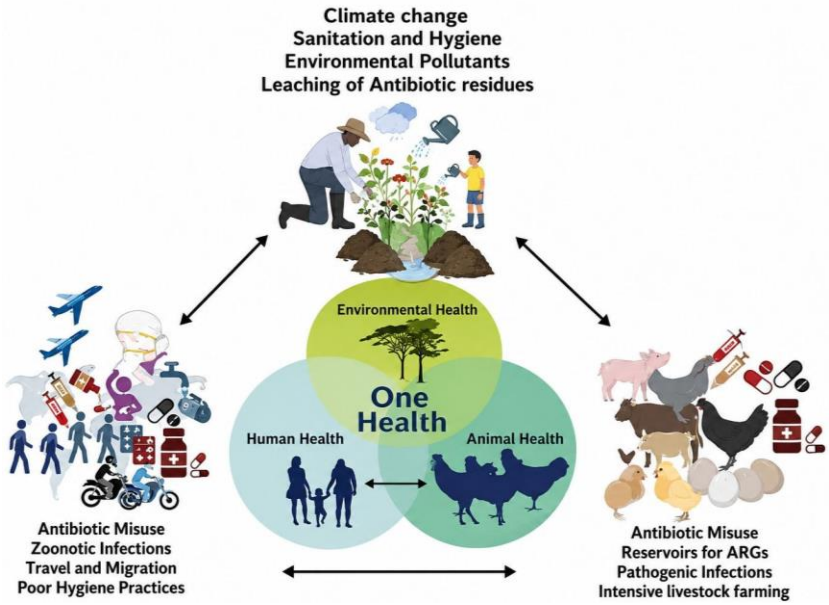


Figure 1: Mechanisms of transmission of antibiotic resistance genes in animals, the environment, and humans, which further contribute to a cycle of resistance that impacts the health of all three domains
Source: (Singh *et al*., 2025)

In Ethiopia, fragmented surveillance systems, limited residue monitoring, unregulated access to veterinary drugs, and poor farm-level biosecurity continue to hinder effective AMR control

across sectors (Fujita *et al*., 2022). Although several studies conducted across Ethiopia and other African countries have reported have reported highlevel of phenotypic AMR in *E. coli* and Enterobacteriaceae from poultry and poultry products (Table 1), most relied only on phenotypic susceptibility testing without molecular characterization or integrated assessment across poultry, environmental, and occupational interfaces (Tigabie *et al*., 2023; Zegene *et al*., 2025). Consequently, comprehensive evaluations of resistance gene distribution and cross-interface transmission remain limited, particularly in high poultry production areas such as Bishoftu (Waktole *et al*., 2024; Tilahun and Efa, 2025). These challenges may facilitate the emergence and distribution of AMR bacteria across the sectors, highlighting the need for integrated One Health surveillance and intervention strategies (Azabo *et al*., 2022).

Table 1: Status of AMR in poultry production systems in Ethiopia and selected African countries

Author (Year)	Country	Sample Source	Bacterial Species	Major Antibiotics Tested	Key AMR Findings (%)	MDR (%)
Tigabie *et al*. (2023)	Ethiopia	Poultry farms	*E. coli*	Tetracycline, Ampicillin, Ciprofloxacin	Tetracycline (83.3%), Ampicillin (75.0%)	68.8
Waktole *et al*. (2024)	Ethiopia	Poultry litter and the environment	*E. coli*, *Salmonella* spp.	Tetracycline, Ampicillin	High resistance to tetracycline (>70%) and β-lactams (>60%)	>50
Bushen *et al*. (2021)	Ethiopia	Chicken meat	*E. coli*	Tetracycline, Ampicillin, Sulfamethoxazole	Tetracycline (81.6%), Ampicillin (73.7%)	65.8
Moffo *et al*. (2021)	Cameroon	Poultry litter	*E. coli*	Tetracycline, Gentamicin, Ampicillin	Tetracycline (84.0%), Ampicillin (76.0%)	61.0
Aworh *et al*. (2019)	Nigeria	Poultry and farm workers	*E. coli*	Tetracycline, Ampicillin, Ciprofloxacin	Tetracycline (88.0%), Ampicillin (79.0%)	71.0
Noh *et al*. (2020)	South Africa	Poultry litter	*Enterococcus* spp.	Tetracycline, Erythromycin	Tetracycline (78.0%), Erythromycin (69.0%)	57.0
Mwikuma et al. (2023)	Zambia	Broiler chickens	*Enterococcus* spp.	Tetracycline, Erythromycin Ampicillin	High resistance to tetracycline and macrolides; MDR is common	>60.0
Ajibola *et al*. (2025)	Nigeria	Poultry, handlers, and environment	*E. coli*	Multiple classes, including fluoroquinolones	Fluoroquinolone resistance 66.2%	70.4
Ramatla *et al*. (2025)	South Africa	Poultry farms	*E. coli*, *Enterococcus* spp.	Tetracycline, Ampicillin, Erythromycin	Resistance exceeded 70% for tetracycline and ampicillin	>60

2.2. Tetracycline and Beta-Lactam Use in Poultry and Resistance Gene Dissemination

Antimicrobials are extensively used in poultry production, with tetracyclines and β-lactams among the most frequently administered classes worldwide (Van Boeckel *et al*., 2019; Mulchandani *et al*., 2023). Oxytetracycline and ampicillin are commonly used in broiler and layer production systems, where repeated exposure exerts selective pressure favoring resistant bacteria carrying transferable *tet* and *bla* resistance genes (Pokrant *et al*., 2023; Ramatla *et al*., 2025). Continuous antimicrobial exposure promotes the amplification and dissemination of resistant bacteria within poultry production environments and surrounding ecosystems (Aminudin *et al*., 2026).

Bacterial resistance to tetracycline arises mainly through efflux pumps of the antimicrobial agent, protection of the ribosomal target site, and enzymatic modification mechanisms (Markley and Wencewicz, 2018; Xie *et al*., 2025). Among the resistance determinants in poultry-associated bacteria, the *tetM* gene is of particular significance because it encodes ribosomal protection proteins that reduce the inhibitory effect of tetracycline on protein synthesis, and this is frequently associated with plasmids and other mobile genetic elements, enhancing its ability to spread among bacterial populations through horizontal gene transfer (Liu *et al*., 2024; Pearson *et al*., 2025). Tetracycline resistance is frequently reported in *Enterococcus faecalis* (*E. faecalis*), where mobile genetic elements contribute to the persistence and spread of resistance under antimicrobial selection pressure (Mwikuma *et al*., 2023; Tsiklauri *et al*., 2025).

Resistance to β-lactam antibiotics in Gram-negative bacteria is predominantly mediated by β-lactamase enzymes that hydrolyze the β-lactam ring and inactivate the antimicrobial agent (Bush and Bradford, 2020). In *E. coli*, *blaTEM* is among the most widely distributed β-lactamase genes and is frequently associated with transferable plasmids that facilitate dissemination among bacterial populations in poultry, litter, and surrounding environments (Ahadini *et al*., 2025; Leng *et al*., 2025). In contrast, *E. faecalis* mainly exhibits intrinsic β-lactam resistance associated with low-affinity penicillin-binding proteins rather than the acquisition of *blaTEM* (Geraldes *et al*., 2022).

The correlation of phenotypic resistance with the presence of a specific resistance gene is not always related because isolates may carry silent, unexpressed, or alternative resistance mechanisms (Channon-Wells *et al*., 2021; Rodrigues *et al*., 2025). Molecular tests such as polymerase chain reaction (PCR) give precise genotypic confirmation of resistance determinants by directly detecting specific genes, thereby improving the accuracy of AMR characterization (Banerjee and Patel, 2023; Sultana *et al*., 2024). The integration of molecular surveillance into AMR monitoring programs supports early detection of circulating resistance genes and enhances understanding of resistance distribution across the poultry–environment–human interface (Muloi *et al*., 2023; Djordjevic *et al*., 2024).

2.3. Poultry Litter as a Hotspot for Selection and Dissemination of Antimicrobial Resistance

Poultry litter consists of bird droppings mixed with bedding substrates, feathers, leftover feed, and residual antimicrobial metabolites that collectively create a favorable environment for the persistence and exchange of AMR bacteria and resistant genes (Figure 2) (Manyi-Loh *et al*., 2018; Cheng *et al*., 2019). Its high microbial density and nutrient availability facilitate horizontal transfer of resistance determinants through mobile genetic elements such as plasmids, transposons, and integrons (Wang and Chai, 2022; Zhang *et al*., 2024). Consequently, poultry litter can serve as an important reservoir for resistance genes, including *tetM*, and contribute to their maintenance and spread within poultry production systems (Zalewska *et al*., 2021; Ma *et al*., 2025).

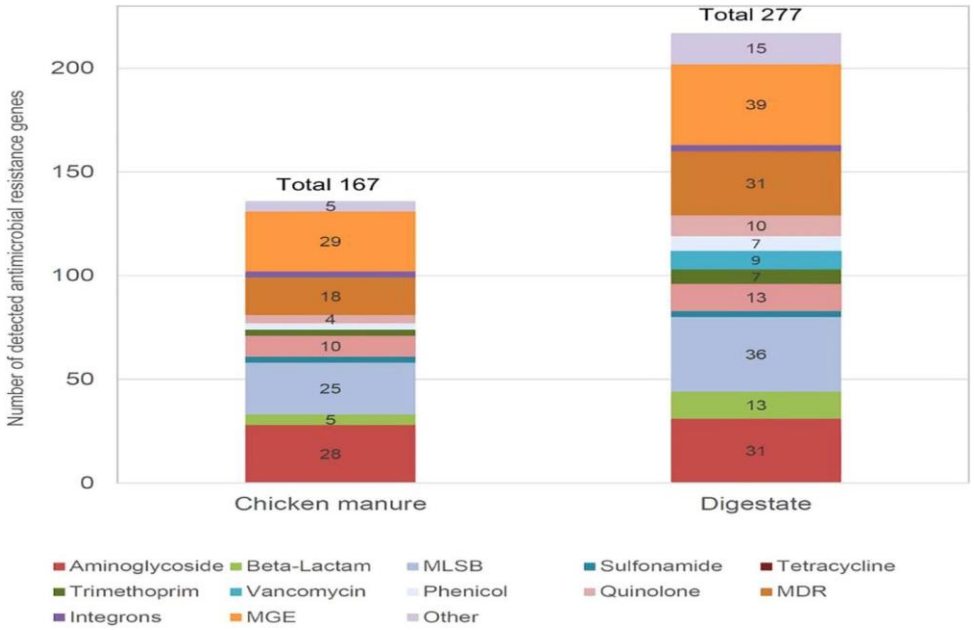


Figure 2: Number of AMR genes detected via a Resistomap HT–qPCR SmartChip in chicken manure and digestate. Multidrug resistance (MDR); macrolide-lincosamide-streptogramin B (MLSB); mobile genetic elements (MGEs)
Source: Atanasova *et al*., 2025

Many studies have reported tetracycline- and β-lactam-resistant *E. coli* and *Enterococcus* spp. in poultry litter and associated farm environments (Fatoba *et al*., 2021; Agga *et al*., 2024; Rogith *et al*., 2026). Resistant bacteria and their associated gene may survive following litter application to agricultural soils, resulting in environmental enrichment of AMR determinants (Fatoba *et al*., 2022; Agga *et al*., 2025). Metagenomic analysis has revealed that poultry litter carries diverse resistance genes capable of spreading to surrounding environments through dust, runoff, and farm equipment (Aminudin *et al*., 2026).

Inappropriate handling and disposal of poultry litter can introduce ARB and ARGs into surrounding soil and water systems, creating secondary environmental reservoirs of resistance (Berendonk *et al*., 2015). Humans may be exposed through direct contact, aerosol inhalation, contaminated water, or food chains (Huijbers *et al*., 2015). Investigations have reported similar resistance profiles among isolates recovered from poultry litter, environmental samples, and humans, supporting the role of poultry production systems in AMR spread (Aworh *et al*., 2021). In Ethiopia, antimicrobial-resistant *E. coli*, *Enterococcus*, and *Staphylococcus* spp. have been

identified in poultry-associated environments, including farms in Bishoftu (Abunna *et al*., 2022; Waktole *et al*., 2024).

2.4. *Escherichia coli* and *Enterococcus* spp. as Indicators and Hazards

E. coli and *Enterococcus* spp. are among the most important indicator organisms used to track and assess AMR in food animal production systems and environmental samples (Anjum *et al*., 2021; Mencía-Ares *et al*., 2021) due to their widespread occurrence in the intestinal tracts of animals and humans, their ability to survive in the environment, and their capacity to acquire and exchange resistance genes, making them effective sentinels for One Health AMR monitoring (Kissinga *et al*., 2018; Muloi *et al*., 2022; Gensler *et al*., 2025). In poultry production systems, resistance to tetracycline in these bacteria is mainly associated with efflux pump genes (*tetA*, *tetB*) and ribosomal protection genes such as *tetM and tetO*, which are often carried on plasmids and transposons that facilitate horizontal gene transfer (Jahantigh *et al*., 2020; Zalewska *et al*., 2024).

Poultry litter and manure often contain diverse antimicrobial resistant gene making them key reservoirs (Alam *et al*., 2023; Efriem *et al*., 2023). In poultry environments, *E. coli* functions as an important carrier of β-lactam resistance genes, notably *blaTEM*, which carries a *TEM*-family β-lactamase enzyme that can inactivate penicillins and early-generation cephalosporins (Faridah *et al*., 2023; Kendek *et al*., 2025). Studies from broiler farms have also shown that tetracycline-resistant *E. coli* frequently exhibit MDR to other antimicrobial groups, including sulfonamides, aminoglycosides, and β-lactams (Sarker *et al*., 2019; Alam *et al*., 2023). Certain strains additionally harbor virulence determinants such as the shiga toxin 2 gene (*stx2*), which increases their zoonotic importance (Oporto *et al*., 2019; Seliga-Gąsior *et al*., 2024).

Enterococci, particularly *E. faecalis* and *E. faecium*, are major opportunistic pathogens in humans and can cause bacteremia, urinary tract, and wound infections (Goh *et al*., 2017; Krawczyk *et al*., 2021). These bacteria are commonly found as commensals in poultry and are well recognized as reservoirs of resistance determinants that are transferable to human pathogens (Rajendiran *et al*., 2022; Kerek *et al*., 2024). *E. faecalis* serves as an important reservoir of tetracycline resistance genes, particularly *tetM*, which encodes a ribosome-protecting protein and

is frequently found on plasmids and the Tn916 groups of conjugative transposons, facilitating horizontal gene transfer across poultry production systems (García-Solache and Rice, 2019; Alemayehu, 2021).

One of the unique features of *E. faecalis* is its environmental persistence, allowing survival in poultry litter, soil, dust, and farm surfaces. This accelerates the circulation of resistance genes within and between flocks and increases the likelihood of occupational exposure among farm workers (Furtula *et al*., 2013; Velhner *et al*., 2024). Multiple studies have isolated tetracycline- and erythromycin-resistant enterococci from poultry litter and farm environments (Ayenit *et al*., 2016; Noh *et al*., 2020), underscoring their role as vectors of ARGs across compartments.

In Ethiopia, while antimicrobial-resistant bacteria in poultry systems have been reported, comprehensive data on the phenotypic and genotypic profiles of tetracycline and beta-lactam-resistant *E. coli* and *Enterococcus* spp. and their distributions across poultry, environmental, and human interfaces remain limited (Fujita *et al*., 2022; Zegene *et al*., 2025). A survey by Kebede and Duga (2022) and Waktole *et al*. (2024) further revealed *Salmonella* isolates from Bishoftu poultry farms with resistance profiles similar to those of *E. coli* and *Enterococcus* spp., indicating shared selective pressures in the same environment.

2.5. Isolation and Identification of *Escherichia coli* and *Enterococcus faecalis*

Isolation and accurate identification of *Escherichia coli* and *Enterococcus faecalis* are essential components of antimicrobial resistance surveillance because they provide the foundation for reliable phenotypic and molecular characterization of bacterial isolates (CLSI, 2023; WHO, 2023). These organisms are widely recognized as indicator bacteria in One Health surveillance due to their ubiquitous distribution in humans, animals, and the environment, their capacity to acquire antimicrobial resistance genes, and their importance in monitoring the dissemination of resistance across interconnected ecological compartments (Muloi *et al*., 2022; EFSA and ECDC, 2024). Consequently, reliable laboratory identification is critical for accurately determining bacterial occurrence, resistance patterns, and potential transmission pathways (Banerjee and Patel, 2023; Djordjevic *et al*., 2024).

Conventional culture-based identification remains the cornerstone of bacteriological diagnosis because it allows recovery of viable isolates for antimicrobial susceptibility testing, molecular

characterization, and epidemiological investigations (Quinn *et al*., 2011; CLSI, 2023). Phenotypic identification based on colony morphology, Gram staining, and biochemical characteristics provides a practical and cost-effective approach for routine laboratory diagnosis, particularly in resource-limited settings (Cheesbrough, 2006; Koneman *et al*., 2017). Nevertheless, closely related bacterial species may exhibit overlapping phenotypic characteristics, reducing diagnostic specificity and necessitating molecular confirmation using species-specific genetic markers (Ahmed and Gulhan, 2024; Chekole *et al*., 2025). Therefore, integrating conventional microbiological techniques with molecular approaches improves the accuracy of bacterial identification and strengthens antimicrobial resistance surveillance within the One Health framework (WHO, 2023; EFSA and ECDC, 2024).

2.6. Phenotypic Antimicrobial Susceptibility Testing

Phenotypic antimicrobial susceptibility testing is a standardized laboratory method used to determine the susceptibility of bacterial isolates to antimicrobial agents based on their observable growth response and remains the cornerstone of AMR surveillance in both clinical and veterinary microbiology (CLSI, 2023; WHO, 2023). Among the available phenotypic methods, the Kirby–Bauer disk diffusion technique is the most widely adopted because of its simplicity, reproducibility, and international standardization by the CLSI (CLSI, 2023; Jorgensen *et al*., 2015). The antimicrobial agents selected for AST should represent different antimicrobial classes of clinical and veterinary importance to provide a comprehensive assessment of resistance patterns (WHO, 2023; EFSA & ECDC, 2024). In this regard, ampicillin (β-lactam) is commonly used to assess β-lactam resistance, tetracycline to evaluate resistance associated with the widespread use of tetracyclines in animal production, ciprofloxacin (fluoroquinolone) to detect resistance affecting deoxyribonucleic acid (DNA) replication inhibitors, gentamicin (aminoglycoside) to evaluate susceptibility to an important bactericidal agent, and erythromycin (macrolide) to monitor resistance among Gram-positive bacteria, particularly *E. faecalis* (CLSI, 2023; Schwarz *et al*., 2001; WHO, 2023).

The susceptibility of bacterial isolates is interpreted by measuring the inhibition zone surrounding each antimicrobial disk and comparing the zone diameter with standardized CLSI breakpoint criteria (CLSI, 2023). Based on these criteria, isolates are classified as susceptible

(S), indicating that the organism is likely to respond to treatment with the recommended antimicrobial dosage; intermediate (I), indicating reduced susceptibility that may require increased antimicrobial exposure or may be effective at specific infection sites; or resistant (R), indicating that the antimicrobial is unlikely to inhibit bacterial growth at clinically achievable concentrations (CLSI, 2023; EUCAST, 2025). Phenotypic AST provides valuable information on resistance profiles and enables the identification of MDR bacteria; however, it cannot determine the specific genetic mechanisms responsible for resistance. Therefore, phenotypic susceptibility testing is increasingly complemented by molecular techniques for comprehensive characterization of antimicrobial-resistant bacteria in One Health surveillance programs (Magiorakos *et al*., 2012; WHO, 2023; EFSA & ECDC, 2024).

2.7. Molecular Detection of Resistance and Virulence Genes

Molecular characterization plays a crucial role in antimicrobial resistance surveillance by enabling the direct detection of resistance and virulence determinants in bacterial isolates. Unlike phenotypic antimicrobial susceptibility testing, which identifies observable resistance patterns, molecular techniques provide information on the specific genetic mechanisms responsible for resistance and pathogenicity (WHO, 2023; Banerjee and Patel, 2023). PCR is among the most widely used molecular methods for detecting antimicrobial resistance genes because of its high sensitivity, specificity, and rapid turnaround time. In poultry-associated bacteria, resistance to tetracyclines is frequently associated with genes such as *tetM*, *tetA*, and *tetB*, whereas resistance to β-lactam antibiotics is commonly mediated by β-lactamase genes including *blaTEM*, *blaSHV*, and *blaCTX-M* (Roberts and Schwarz, 2016; Liu *et al*., 2024).

Among *Enterococcus* spp., *tetM* is one of the most prevalent tetracycline resistance genes and is often carried on mobile genetic elements that facilitate horizontal gene transfer. Similarly, *blaTEM* is widely distributed among *E. coli* isolates and contributes to resistance against ampicillin and related β-lactam antimicrobials (Bush and Bradford, 2020; Leng *et al*., 2025). In addition to resistance genes, virulence genes are important indicators of the pathogenic potential of bacterial isolates. *stx2* gene is a major virulence determinant in pathogenic *E. coli* strains and is associated with severe human illnesses, including hemorrhagic colitis and hemolytic uremic syndrome (Paton and Paton, 1998; Oporto *et al*., 2019).

2.8. Occupational Exposure and Human Carriage

Individuals working in poultry production represent an important population in studies of antimicrobial resistance, as they are frequently exposed to birds, poultry litter, farm dust, contaminated surfaces, and consumption of contaminated food and water (Haas *et al*., 2022). Farm dust and aerosols can harbour resistant *E. coli*, *Enterococcus*, and other bacteria that carry resistance genes such as the *tet*, *erm*, and *bla* families (Aworh *et al*., 2021; Nguyen *et al*., 2022). Such frequent contact may facilitate the acquisition and spread of resistant bacteria and genes between poultry production environments and humans, showing that poultry workers are often considered a key interface for investigating potential transmission pathways within the one health framework (Ajibola *et al*., 2025).

Epidemiological studies have revealed comparable antimicrobial resistance patterns in bacterial isolates from poultry environments and farmworkers (Scicchitano *et al*., 2024; Ahmed *et al*., 2025). A study in the United States indicated that poultry workers were significantly more likely to carry multidrug-resistant *E. coli* and *Staphylococcus aureus* strains genetically similar to those isolated from broiler litter and farm dust (Nadimpalli *et al*., 2015). Similarly, Son *et al*. (2024) reported that poultry farmworkers in Korea carried *Enterococcus* isolates with resistance phenotypes comparable to those found in chickens, suggesting occupational transmission. More recently, Yanestria *et al*. (2024) detected identical tetracycline and macrolide resistance genes in both chicken feces and nasal swabs from poultry handlers in Indonesia.

In Africa, several studies have confirmed that livestock handlers and poultry workers may act as reservoirs and disseminators of resistant bacteria (Nyolimati *et al*., 2025). In Cameroon, high levels of resistance to tetracycline, gentamicin, and ampicillin have been reported among *E. coli* isolates from poultry litter (Moffo *et al*., 2021), reflecting resistance patterns common in farm environments. In Ethiopia, dairy farm workers carry *S. aureus* isolates with resistance phenotypes resembling those found in animals, suggesting possible bidirectional transmission (Kalayu *et al*., 2020).

2.9. Litter Management Practices and Mitigation Potential

Litter management plays a significant role in determining the maintenance of antimicrobial residues and ARB in poultry production environments (Cortés *et al*., 2025). Poultry litter is often reused for several production cycles or applied directly as fertilizer, practices that can either exacerbate or mitigate AMR risks depending on how they are managed (Esperón *et al*., 2020). The physicochemical properties of litter, such as moisture content, temperature, and pH, directly affect the survival of resistant bacteria and genes (Yu *et al*., 2019; Kim *et al*., 2023).

Proper composting is recognized as an effective strategy for reducing antimicrobial residues and resistant microorganisms in poultry litter (Okada *et al*., 2024; Esperón *et al*., 2020). Aerobic composting under thermophilic conditions (>55 °C) enhances antibiotic degradation and significantly reduces tetracycline- and β-lactam-resistance genes through thermal inactivation of bacteria and microbial community shifts during composting (Wang and Chai, 2022; Zhang *et al*., 2024). In contrast, poorly managed or low-temperature composting may incompletely degrade antimicrobial residues and fail to eliminate ARGs, thereby maintaining selective pressure for resistance (Staley *et al*., 2021; Wang *et al*., 2023).

Land application of raw or insufficiently composted poultry litter increases the abundance of tetracycline- and beta-lactam-resistant *E. coli* and *Enterococcus* in agricultural soils and surrounding environments by facilitating the maintenance and accumulation of resistance determinants such as *tetM* and *blaTEM* within soil microbial communities (Fatoba *et al*., 2021; Fatoba *et al*., 2022; Agga *et al*., 2025). Environmental factors such as rainfall and surface runoff further facilitate the spread of AMR bacteria and associated ARGs from litter-amended soils to nearby water systems, increasing their transmission potential across environmental and human interfaces (Zhang *et al*., 2023; Roddey *et al*., 2025).

At the farm level, biosecurity and sanitation practices are equally important (WHO, 2023). Using a standard biosecurity measure that targets the use of protective equipment, frequent cleaning of poultry houses, and adequate downtime between production cycles can reduce the microbial load and limit worker exposure (Moffo *et al*., 2022). In Ethiopia, the reuse of litter for multiple cycles without composting is a common practice due to resource limitations (Yévenes *et al*., 2021). The

lack of standardized litter management promotes tetracycline and beta-lactam-resistant *E. coli* and *Enterococcus* spp. in farm environments, and land application of untreated poultry litter can enrich resistance genes such as *tetM* and *blaTEM* in soils (Subirats *et al*., 2021; Fatoba *et al*., 2022; Agga *et al*., 2025).

Overall, prudent antimicrobial use, improved farm biosecurity, effective sanitation practices, and regular AMR surveillance are among the most important strategies for limiting the emergence and spread of resistant bacteria (WHO, 2023; EFSA and ECDC, 2024). In poultry production systems, antimicrobial stewardship programs encourage the responsible use of antibiotics based on veterinary prescription and disease diagnosis. Reducing unnecessary antimicrobial exposure decreases selection pressure and limits the emergence of resistant strains carrying genes such as *tetM* and *blaTEM* (Van Boeckel *et al*., 2019; Mulchandani *et al*., 2023).

2.10. Evidence Gaps and the Need for Integrated One Health Studies in Bishoftu

Despite extensive global recognition of AMR as a One Health challenge, significant knowledge gaps remain in understanding how resistant bacteria circulate across interconnected poultry, environmental, and human compartments, particularly in LMICs (Martak *et al*., 2024; Singh *et al*., 2025; Zeru *et al*., 2025). While numerous studies have documented AMR bacteria in poultry production systems, most investigations have examined single domains, either poultry, meat products, or environmental samples, without simultaneously characterizing resistance across the poultry–environment–human interface (Tilahun and Efa, 2025; Yasmin *et al*., 2025; Zeru *et al*., 2025).

In Ethiopia, research has largely focused on the phenotypic resistance characteristics of *E. coli* and other *Enterobacteriaceae* strains isolated from poultry meat, feces, or farm environments, with studies reporting elevated resistance levels in isolates recovered from broiler flocks, chicken droppings, or farm samples without integrated human or environmental sampling (Bushen *et al*., 2021; Tigabie *et al*., 2023; Tilahun and Efa, 2025). Although high levels of tetracycline and *β*-lactam resistance have been reported, many studies have relied exclusively on AST without the molecular confirmation of resistance determinants (Fujita *et al*., 2022; Tigabie *et al*., 2023; Waktole *et al*., 2024). Consequently, the distribution of key resistance genes, such as *blaTEM*

and *tetM*, and their role in mediating resistance across interconnected compartments remain insufficiently characterized (Sonola *et al*., 2022; Ugbo *et al*., 2025).

Similarly, investigations involving *E. faecalis* in Ethiopian poultry systems are limited, despite its recognized importance as a reservoir of transferable tetracycline resistance genes and its role as an opportunistic human pathogen (Donald *et al*., 2025). Molecular confirmation via species-specific markers such as the *ddl* gene and concurrent detection of resistance genes are rarely integrated into surveillance studies, limiting the resolution of transmission dynamics (Hedman *et al*., 2020; Mwikuma *et al*., 2023).

Globally, emerging evidence has demonstrated genetic overlap between resistant *E. coli* and *Enterococcus* isolates recovered from poultry, litter, farm environments, and farm workers, suggesting occupational and environmental transmission pathways, with genomics studies revealing shared resistance genes and similar resistance profiles across compartments (Aworh *et al*., 2023; Deleza *et al*., 2025; Munene *et a*l., 2025). However, comparable integrated assessment s are scarce in Ethiopian high-production hubs (Belachew *et al*., 2021). Most available studies in Bishoftu have evaluated resistance in isolated compartments or focused on pathogens such as *Salmonella*, without concurrent phenotypic and genotypic characterization of indicator organisms across poultry, soil, and human populations (Fujita *et al*., 2022; Tilahun and Efa, 2025).

Bishoftu is an important setting for AMR investigations because of its dense poultry production systems, proximity to Addis Ababa, and intensive human–animal–environment interactions that may facilitate resistance transmission (Belachew *et al*., 2021; Dugassa, 2022; Waktole, 2023). Unregulated antimicrobial access, rampant use of antimicrobials in the poultry feed system, and inconsistent biosecurity practices may further promote resistance amplification and cross-interface dissemination (Ismael *et al*., 2021). However, no comprehensive study has simultaneously assessed the occurrence, phenotypic resistance patterns, and resistance genes (*blaTEM* and *tetM*) of *E. coli* and *E. faecalis* across poultry, environmental, and human domains in the study area. Existing evidence from Ethiopia and Africa also highlights limited integrated One Health data and substantial gaps in cross-sector phenotypic and genotypic AMR surveillance (Hedman *et al*., 2020; Fujita *et al*., 2022; Nnah *et al*., 2025).

The absence of integrated molecular surveillance limits understanding of whether resistant strains observed in workers arise from independent acquisition or shared resistance pools within poultry production environments (Vallejo-Espín *et al*., 2025). Without such evidence, targeted intervention strategies and stewardship policies remain insufficiently informed (Fujita *et al*., 2022). Therefore, an integrated One Health investigation combining AST with molecular detection of resistance determinants in *E. coli* and *E. faecalis* across poultry litter, farm soil, and farm workers is essential (Singh *et al*., 2025; Zhang *et al*., 2025). This approach enables the identification of shared resistance profiles, identification of circulating resistance genes, and assessment of potential transmission pathways within the Bishoftu poultry production ecosystem. This study addresses this gap by integrating phenotypic and genotypic characterization across int erconnected compartments.

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was carried out in Bishoftu, situated in the East Shewa Zone of Oromia Regional State, approximately 47 km southeast of Addis Ababa (Figure 3). Geographically, the town lies between 8°45′N latitude and 38°56′E longitude, at an elevation ranging from 1,900 to 2,000 m above sea level (CSA, 2021), with an average annual temperature of 18–20 °C and a mean annual rainfall of approximately 1,150 mm (EMI, 2025). Bishoftu has favorable agroecological conditions for poultry production and is considered one of the major poultry production areas in central Ethiopia (Ebsa *et al*., 2019). Approximately 70 commercial poultry farms, including broiler and layer production systems, operate in and around the town (Alemayehu and Goshu, 2018; Waktole *et al*., 2023; Bishoftu Town Agriculture Office, 2025). The presence of intensive poultry production systems, hatcheries, feed processors, and veterinary services makes Bishoftu a suitable setting for investigating antimicrobial use, AMR dissemination, environmental contamination, and occupational exposure within a One Health framework (Tsegaye *et al*., 2023; Waktole *et al*., 2023).

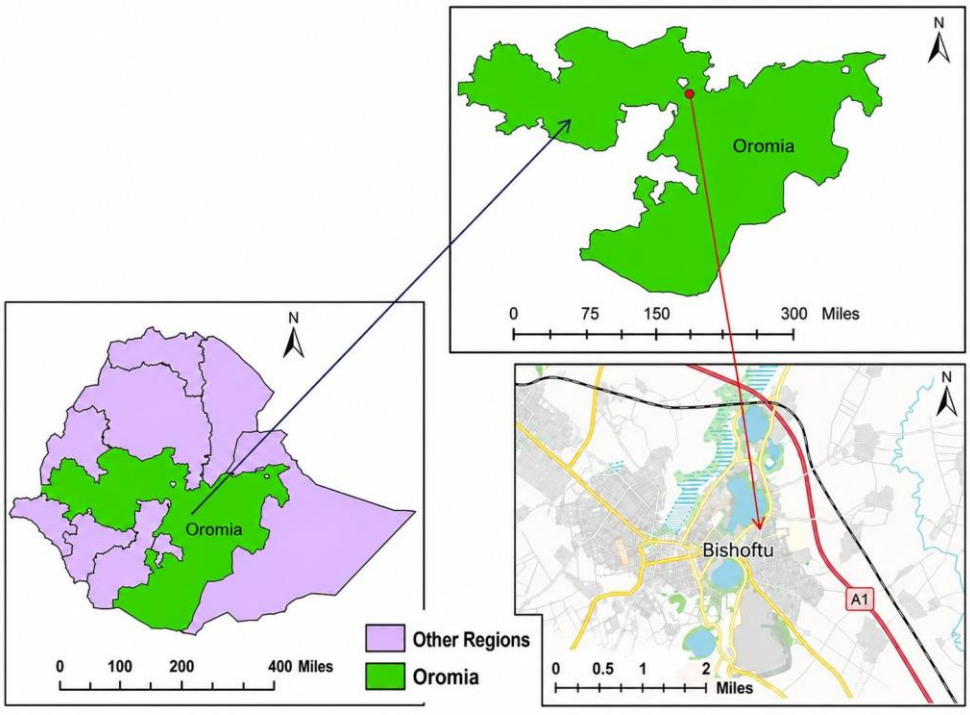


Figure 3: Map of the study area

Source: ArcGIS Pro (Esri, 2025)

3.2. Study Population

The study population consisted of three interconnected components. The poultry (animal) component included broiler and layer chickens reared on small (100-1000 birds), medium (1001-10,000 birds), and large-scale (>10,000 birds) (Wondmeneh *et al*., 2017) farms, from which pooled cloacal swab samples were obtained from 5–10 birds per poultry house to estimate flock-level prevalence of resistant *E. coli* and *E. faecalis*. Second, the environmental component consisted of poultry litter samples collected from areas with high bird density and frequent worker contact to assess the environmental persistence of resistant bacteria. The human component included farm workers involved in poultry management activities such as feeding, litter handling, and cleaning, from whom hand swab samples were collected to evaluate occupational exposure and potential cross-interface transmission of resistant *E. coli* and *E. faecalis*.

Commercial layer and broiler poultry farms operating in Bishoftu during the study period, together with their active flocks, poultry litter samples, and farm workers directly involved in poultry management activities who provided informed consent, were included in the study; whereas farms that were in accessible during the sampling period, poultry houses without active birds, and farm workers who declined participation or had no direct contact with the poultry and poultry litter were excluded from the study.

3.3. Study Design and Sampling Technique

A cross-sectional study was carried out between November 2025 and June 2026, and sampling was done from poultry, farm environments (litter) and workers in Bishoftu. To ensure representativeness and fair coverage of poultry farms, a stratified cluster sampling method was employed. Farms were stratified on the basis of size (small, medium, and large) and type of production (broiler or layer), and farms within each stratum were randomly selected for inclusion in the study.

For the selected farms, pooled poultry cloacal swab samples from five to ten (5 birds for small-scale, 7 birds for medium, and 10 birds for large-scale farms) representative birds were collected from each poultry house to account for spatial variation. Environmental litter samples were

collected from inside poultry houses, with multiple sampling points used to capture potential sources of contamination. Finally, farm workers with direct contact with poultry or litter were recruited purposively, and hand swab samples were collected from farm workers of each farm.

3.4. Sample Size Determination

The required sample size was estimated using a cluster sampling approach, in which poultry farms served as the primary sampling units. The total number of samples (n) was calculated by applying the standard formula:

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

where: Z = 1.96 for a 95% confidence level, *P* = expected prevalence (50%) used to obtain the maximum sample size, *d* = desired precision (0.1), and *DE* = design effect (1.5) to adjust for intracluster correlation.

A total of 144 samples were collected from 70 poultry farms, comprising both pooled cloacal swab samples and poultry litter samples. This corresponds to approximately 2–3 samples per farm, with pooled cloacal swabs obtained from 5–10 birds per poultry house and litter samples collected from representative sections of the poultry house to capture spatial variation. The sample size for poultry workers was estimated using the single-proportion formula of

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

which initially yielded a sample size of 96 farm workers, assuming an unknown prevalence (P = 0.5) and a precision of 10%.

Applying the finite population correction formula,$= \frac{n}{1+\frac{n-1}{N}}$, with a total worker population of approximately 350, resulted in a required sample size of 76 farm workers. However, due to logistical constraints and the clustered study design, one worker per farm across 70 farms was included, resulting in a total of 70 farm workers sampled. This corresponds to an approximate precision of 10.5% (d = 0.105) (Thrusfield, 2018; Dohoo *et al*., 2021).

3.5. Materials, Sample Collection and Management

A structured sampling format was prepared for sample collection (Annex 1). From each farm, pooled cloacal swabs, poultry litter, and hand swab samples were collected following standard aseptic procedures (Cheesbrough, 2006; Quinn *et al*., 2011). Cloacal swab samples were collected from five representative birds using Falcon tubes containing sterile BPW. Poultry litter samples were collected from 2–3 different points within poultry houses using sterile scoops and pooled into composite samples weighing approximately 10 g (Cheesbrough, 2006; Quinn *et al*., 2011). Samples were placed in sterile labeled polyethylene bags, maintained at 4 ± 2 °C in an ice box, and submitted to the Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA) Microbiology Laboratory within 24 hours following ISO sampling and transport guidelines (Cheesbrough, 2006; ISO 6887-1, 2017).

Hand swab samples were collected from one poultry worker per farm who had direct contact with birds or litter. Sterile cotton swabs moistened with sterile saline were used to swab the palms and fingers, and the swabs were transferred into sterile transport tubes according to Quinn *et al*. (2011) and ISO 6887-5 (2017). All samples were labeled with sample type, farm code, and collection date. Samples were processed within 24 hours to maintain bacterial viability (Cheesbrough, 2006). Confirmed *E. coli* and *Enterococcus* isolates were preserved in cryogenic vials at −20 °C until AST and molecular analyses were performed (Collins and Lyne, 2020; CLSI, 2023).

3.6. Sample Analysis

3.6.1. Isolation and Identification of E. coli and Enterococcus

The isolation of *E. coli* and *Enterococcus* species from environmental and human samples was conducted using standard culture-based microbiological methods (Annex 2), as recommended by the CLSI (CLSI, 2023) and ISO (ISO 6887-1:2017). All culture media were prepared as per the manufacturer's protocol, and strict aseptic precautions were used during sample handling, inoculation, and incubation to prevent cross-contamination. Representative isolates from each sample type were purified, validated using American Type Culture Collection (ATCC) control strains (ATCC 25922 for *E. coli* and ATCC 29212 for *E. faecalis*) obtained from the Ethiopian

Public Health Institute (EPHI), coded, and preserved for subsequent AST and molecular characterization.

Isolation from Pooled Cloacal Swab samples

Pooled cloacal swab samples were processed using standard culture-based microbiological procedures (Annex 2B & C). Briefly, swab suspensions in BPW were preenriched at 37 °C for 18–24 hours (ISO 16649-2, 2017). Following enrichment, aliquots were inoculated onto MacConkey agar for isolation of lactose-fermenting bacteria and then plated onto EMB agar for presumptive identification of *E. coli*, characterized by a metallic green sheen (ISO 16649-2, 2017; CLSI, 2023). For isolation of *E. faecalis*, enriched samples were streaked onto BEA and incubated at 37 °C for 24 hours, where colonies showing blackening due to esculin hydrolysis were considered presumptive isolates. Presumptive *E. coli* and *E. faecalis* isolates were further purified by subculturing onto nutrient agar and brain heart infusion agar, respectively, for subsequent analyses (Chuard and Reller, 1998).

Isolation from Poultry Litter Samples

Approximately 1g of pooled poultry litter was homogenized in 9 mL of BPW and incubated at 37 °C for 18–24 hours for preenrichment (ISO 16649-2:2017). For isolation of *E. coli*, enriched samples were streaked onto MacConkey agar and subsequently subcultured onto EMB agar, where colonies showing a metallic green sheen were considered presumptive *E. coli* (ISO 16649-2:2017; CLSI, 2023; Thongratsakul *et al*., 2023). For *Enterococcus* spp., enriched broth was inoculated onto BEA, and colonies showing blackening of the medium were considered presumptive isolates. Presumptive isolates were purified on nutrient agar and subjected to Gram staining and biochemical tests for preliminary confirmation. Confirmed isolates were preserved for AST and PCR(Annex 2B &C) (ISO 16649-2:2017; CLSI, 2023).

Isolation from Hand Swabs

Hand swab specimens were collected using sterile cotton swabs pre-moistened with BPW by swabbing the palms and fingers of farm workers (ISO 18593:2018; FSSC, 2023). Swabs were transferred into sterile tubes containing BPW and transported to the laboratory at 4 °C. Following incubation at 37 °C for 18–24 hours, samples were inoculated onto MacConkey agar for isolation of *E. coli* and BEA for *Enterococcus spp* (Annex 2B&C). Presumptive colonies

were purified on nutrient agar and subjected to preliminary identification using Gram staining and catalase testing (ISO 13307:2013; CLSI, 2023).

3.6.2. Antimicrobial Susceptibility Testing (AST)

Tetracycline and beta-lactam antimicrobials were selected for phenotypic and genotypic resistance profiling because of their widespread use in poultry production, environmental persistence, and association with transferable resistance genes, making them important indicators for evaluating AMR dissemination across the animal–environment-human interface (Roberts and Schwarz, 2016; Manyi-Loh *et al*., 2018). Antimicrobial susceptibility of *E.coli* and *Enterococcus spp* isolates was determined using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar following CLSI guidelines (CLSI, 2023). While gentamicin in *E. faecalis* isolates was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) High-level gentamicin-resistant (HLGR) screening criteria because CLSI M100 does not provide breakpoints for the 30 µg gentamicin disk (Khan *et al*., 2022). Representative isolates were validated using standard biochemical tests, and *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as quality control strains (Gajic *et al*., 2022).

Briefly, 3–5 pure colonies were suspended in sterile saline to achieve 0.5 McFarland turbidity and streaked onto Mueller–Hinton agar plates. Antibiotic discs containing tetracycline, ampicillin, ciprofloxacin, gentamicin, and erythromycin were selected based on their veterinary relevance and suitability for phenotypic resistance characterization in relation to *tetM* and *blaTEM* genes. Plates were incubated at 35–37 °C for 18–24 hours, and inhibition zones were measured and interpreted according to CLSI (2023) breakpoints (Annex 2D) (Haley *et al*., 2024). Resistance profiles of *E. coli* and *Enterococcus* isolates, particularly to tetracycline and ampicillin, were evaluated as part of the overall phenotypic and genotypic AMR assessment.

MDR was defined as resistance to at least one antimicrobial agent in three or more antimicrobial classes according to the criteria proposed by Magiorakos *et al*. (2011). The proportion of MDR isolates was determined separately for *E. coli* and *E. faecalis* and compared across sample sources. The Multiple Antibiotic Resistance (MAR) index was calculated for each isolate to assess the extent of antimicrobial exposure and resistance pressure within the study environment. The MAR index was calculated using the formula:

$$MAR = a / b$$

where:

a = number of antibiotics to which the isolate was resistant

b = total number of antibiotics tested

A MAR value greater than 0.2 was considered indicative of isolates originating from environments with frequent antimicrobial exposure and high-risk antimicrobial use practices (Krumperman, 1983). Descriptive statistics were used to summarize MDR prevalence and MAR index distributions among isolates from poultry, litter, and farm workers.

3.6.3. Molecular Detection of Resistant Bacteria and Genes

DNA extraction

Genomic DNA was extracted from confirmed *E. coli* and *E. faecalis* isolates using the QIAamp DNA extraction kit (QIAGEN) according to the manufacturer's instructions to obtain PCR-compatible DNA for molecular analysis (Queipo-Ortuño *et al*., 2007; Banar *et al*., 2024). Briefly, fresh bacterial cultures grown overnight in tryptose soy broth at 37 °C were used for extraction. A 140 µL aliquot of each culture was processed through bacterial cell lysis, DNA binding to a silica membrane, washing, and elution steps to obtain purified genomic DNA (Annex 2E). Extracted DNA was stored appropriately for subsequent PCR analysis following the manufacturer's protocol to ensure DNA quality and purity (Shehata and Hershan, 2016).

PCR Amplification

PCR was performed to detect resistance and species-specific genes, including *tetM*, *blaTEM*, *ddl*, and *stx2* (Ahmed and Gulhan, 2024; Sah *et al*., 2024; Chekole *et al*., 2025). Each reaction was prepared in a final volume of 25 µL containing PCR master mix, gene-specific primers, nuclease-free water, and 3 µL of template DNA. Thermal cycling conditions consisted of initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at gene-specific temperatures, extension at 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes (Paton and Paton, 1998; Colom *et al*., 2000; Ng *et al*., 2001; Koukos *et al*., 2016; Motohashi, 2019; Mohammed *et al*., 2024). PCR products were separated on a 1.5–2% agarose gel stained with ethidium bromide and visualized under UV illumination (Parks and Torres, 2023). Expected amplicon sizes were approximately 406 bp for *tetM*, 850 bp

for *blaTEM*, 941 bp for *ddl*, and 256 bp for *stx2* (Jones *et al*., 2006; Abdelgalel *et al*., 2025). Details of the primer pairs employed for PCR amplification, including their nucleotide sequences and expected amplicon size, are provided in Table 2.

Table 2: primer sequences and expected amplicon sizes of target genes used for PCR amplification

Target gene	Primer sequence (5'–3')	Expected amplicon size (bp)	Reference
blaTEM	F: ATGAGTATTCAACATTTCCGTG R: TTACCAATGCTTAATCAGTGAG	850	Colom *et al*., 2003
ddl (*E. faecalis*)	F: ATCAAGTACAGTTAGTCTT R: ACGATTCAAAGCTAACTG	941	Dutka-Malen *et al*., 1995
stx2	F: GGCACTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	256	Paton and Paton, 1998
tetM	F: AGTGGTTATTGATGTAGACG R: CATATGCTTTGTTGCTTG	406	Ng *et al*., 2001

3.7. Ethical Approval

Ethical permission for this study was obtained from the Institutional Review Board of AAU-CVMA for the animal component with reference number VM/ERC/08/109/18/2026 and from the AAU Aklilu Lemma Institute of Health Research (ALIHR) for the human component with reference number ALIHR IRERC-009/2018/2026 (Annex 11). Poultry farm owners and workers were provided with detailed information on the study purpose, procedures, and possible risks, and written informed consent was obtained from all participating workers. Participation was voluntary, confidentiality was maintained, and participants were allowed to withdraw at any time without any consequences. All procedures were conducted in accordance with the Declaration of Helsinki (WMA, 2013) and the national research ethics review technical guideline (Ministry of Education, 2022).

3.8. Data Management and Analysis

All laboratory and field data were recorded using structured forms (Annex 2) and entered into a secure database containing sample ID, farm code, sample type, bacterial species, AMR profile, and resistance genes. Data on farm management, antimicrobial use, litter handling, worker hygiene, and biosecurity were collected using a semi-structured questionnaire (Annex 3).

The prevalence of *E. coli* and *E. faecalis*, phenotypic resistance to tetracycline and β-lactams, and the distribution of resistance genes (*tetM* and *blaTEM*) were expressed as percentages with 95% confidence intervals. Differences among poultry, environmental, and human compartments were assessed using Chi-square, while logistic regression was used to investigate the associations between exposure factors and resistance outcomes. Statistical significance was considered at $p < 0.05$. All analyses were conducted using R Markdown within the R statistical software (Xie, 2018; R Core Team, 2023).

4. RESULTS

4.1. Descriptive Characteristics of the Study Farms and Samples

Out of the 70 poultry farms included in the study, 62.9% and 37.1% were layer and broiler farms, respectively. On the other hand, 47.1%, 38.6%, and 14.3% of the poultry farms were medium, small, and large-scale farms, respectively. Overall, a total of 210 samples comprising cloacal swabs, litter, and hand swab samples were collected equally across the poultry–environment–human interface, as shown in Table 3.

Table 3: Characteristics of the study farms and samples collected

Variable	Category	Frequency (n)	Percentage (%)
Production type	Layer	44	62.9
	Broiler	26	37.1
	Total	**70**	
Farm size	Small	27	38.6
	Medium	33	47.1
	Large	10	14.3
	Total	**70**	
Sample type	Cloacal/Fecal	70	33.3
	Litter	70	33.3
	Hand swab	70	33.3
	Sub Total	**210**	

4.2. Isolation and Identification of *E. coli* and *E. faecalis*

The two important bacteria, *E. coli* and *E. faecalis*, were successfully isolated (Annex 12 Figures 1 and 2 on pages 77 and 78) from poultry, farm environments, and farm workers. The overall occurrence of *E. coli* and *E. faecalis* was 31% (65/210) and 44.8% (94/210), respectively; across the sample types, their occurrence ranged from 30.0% to 31.4% for *E. coli*, whereas that of *Enterococcus* spp. ranged from 38.6% to 50%. The highest isolation rates were observed in the litter and hand swabs for *E. coli* and in the feces for *E. faecalis*, as shown in Figure 4 below.

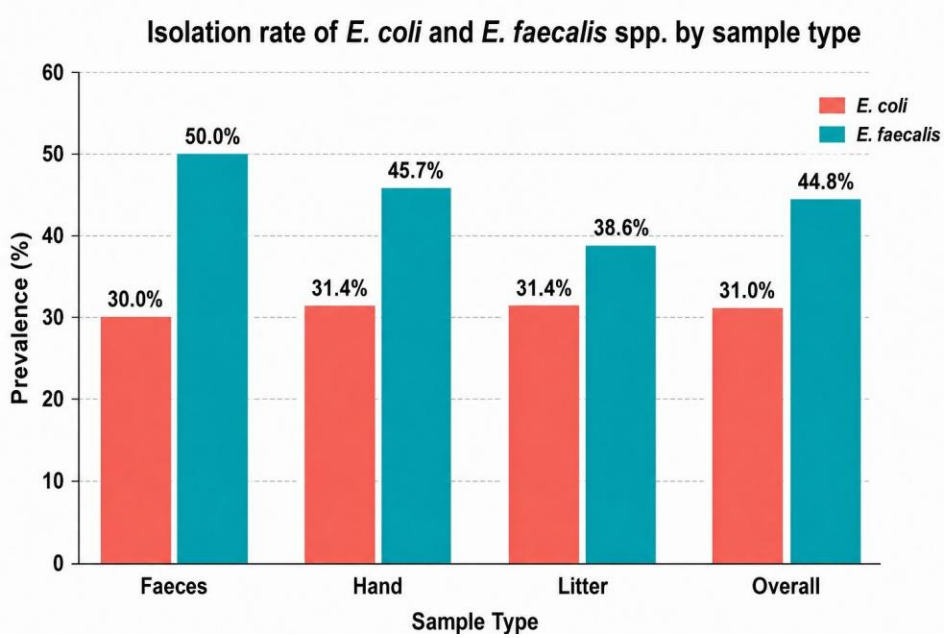


Figure 4: Overall and sample-level isolation of *E. coli* and *E. faecalis*

4.3. Antimicrobial Susceptibility Profile of Isolates

The antimicrobial susceptibility results of the *E. coli* and *E. faecalis* isolates were performed on Mueller–Hinton agar using the selected antibiotic discs, and additional information is available in Figure 3 of Annex 12, found on page 78, and is summarized in Table 4 according to the CLSI M100 (36th edition) breakpoints. *E. coli* was highly resistant (R) to ampicillin (72.3%) and tetracycline (91.5%), while all the isolates were susceptible (S) to gentamicin (100%). Ciprofloxacin demonstrated variable antimicrobial activity, as 36.2% of isolates were susceptible, 36.1% showed intermediate susceptibility (I), and the remaining 27.7% were resistant. *E. faecalis* demonstrated high resistance to tetracycline (87.2%), ampicillin (91.5%), and erythromycin (87.2%). Ciprofloxacin resulted in mixed susceptibility, with 21.3% susceptible, 40.4% intermediate, and 38.3% resistant isolates according to the CLSI criteria. Based on EUCAST HLGR screening criteria, all *E. feacalis* were categorized as HLGR negative to gentamicin (30 µg).

Table 4: Antimicrobial susceptibility profile of *E. coli* and *E. faecalis* strains

Bacteria	Antibiotic	Resistance (R %)	Intermediate (I %)	Susceptibility (S %)
	AMP	72.3	0.0	27.7
E. coli	TE	91.5	2.1	6.4
	CIP	27.7	36.1	36.2
	CN	0.0	0.0	100.0
	AMP	91.5	2.1	6.4
	TE	87.2	0.0	12.8
E. faecalis	CIP	38.3	40.4	21.3
	ERY	87.2	0.0	12.8
	CN	0.0	0.0	100.0*

Footnote: *AMP = ampicillin; CN = gentamicin; CIP = ciprofloxacin; TE = tetracycline; ERY = erythromycin.* Erythromycin was not tested for *E. coli* due to intrinsic resistance according to CLSI M100 *(36th edition) disk diffusion breakpoints criteria. * = HLGR-negative based on EUCAST screening criteria for E. faecalis using a 30 µg gentamicin disc (zone diameter ≥ 8 mm); isolates with inhibition zones < 8 mm were considered HLGR-positive.*

4.3.1. Phenotypic Antimicrobial Resistance (AMR) Patterns of E. coli and E. faecalis Isolates

The most common resistance pattern observed among *E. coli* isolates was combined resistance to ampicillin and tetracycline (AMP-TE), accounting for 48.9% of isolates, followed by MDR to ampicillin, tetracycline, and ciprofloxacin (AMP-TE-CIP), accounting for 19.1% of isolates. A smaller proportion of isolates exhibited resistance to only tetracycline (14.9%) or combined tetracycline and ciprofloxacin resistance (8.5%). Only 4.3% of the *E. coli* isolates presented no resistance to the tested antimicrobials (Table 5).

Among the *E. faecalis* isolates, the predominant resistance pattern was AMP-TE-ERY, which was observed in 48.9% of the isolates. MDR involving ampicillin, tetracycline, ciprofloxacin, and erythromycin (AMP-TE-CIP-ERY) was identified among 23.4% of the *E. faecalis* isolates. Other less frequent resistance combinations included AMP-CIP-ERY (6.4%), AMP-TE (4.3%), and AMP-TE-CIP (4.3%). Single-drug resistance patterns were uncommon (Table 5)

Table 5: Phenotypic AMR patterns of *E. coli* and *E. faecalis* Isolates

Bacteria	Resistance Pattern	Frequency (n=47)	Percentage (%)
	AMP-TE	23	48.9
	AMP-TE-CIP	9	19.1
E. coli	TE only	7	14.9
	TE-CIP	4	8.5
	AMP only	2	4.3
	No resistance	2	4.3
	AMP-TE-ERY	23	48.9
	AMP-TE-CIP-ERY	11	23.4
	AMP-CIP-ERY	3	6.4
E. faecalis	AMP-TE	2	4.3
	AMP-TE-CIP	2	4.3
	AMP only	1	2.1
	CIP-ERY	1	2.1
	TE only	1	2.1
	TE-CIP-ERY	1	2.1
	AMP-ERY	1	2.1

Note: AMP = ampicillin; TE = tetracycline; CIP = ciprofloxacin; ERY = erythromycin.

Multidrug-resistant isolates were detected across all sample domains (Table 6). Among *E. coli* isolates, 19.1% (9/47) were classified as MDR, with the highest proportion being observed in litter (25.0%), followed by hand swabs (21.4%) and cloacal swabs (14.3%). In contrast, MDR was highly prevalent among *E. faecalis* isolates, occurring in 85.1% (40/47) of isolates. The highest MDR prevalence was observed in litter samples (100.0%), followed by hand swabs (83.3%) and cloacal swabs (71.4%). The MAR index was greater in *E. faecalis* (0.61) than in *E. coli* (0.48), indicating greater antimicrobial exposure pressure.

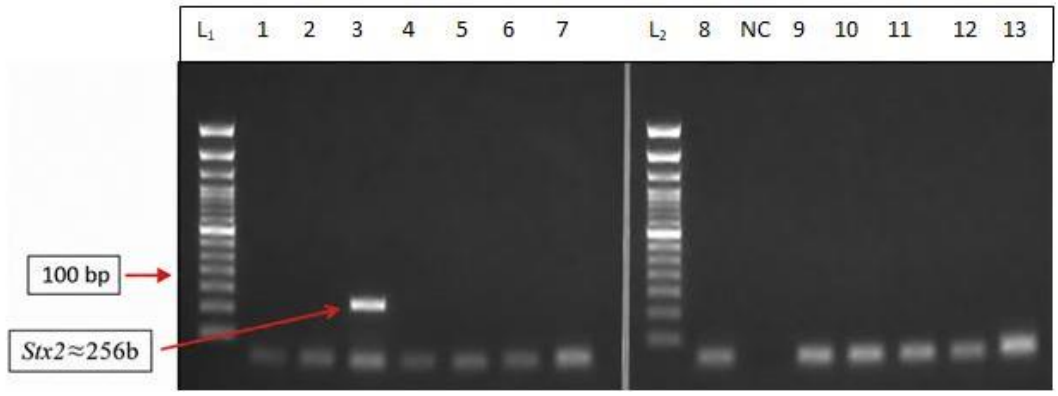
Table 6: Distribution of MDR *E. coli* and *E. faecalis* isolates across sample domains

Bacterial species	Sample domain	Total isolates (n)	MDR isolates (n)	MDR (%)	MAR Index
	Cloacal swab	21	3	14.3	
E. coli	Hand swab	14	3	21.4	
	Litter	12	3	25.0	
Total		**47**	**9**	**19.1**	0.48
	Cloacal swab	14	10	71.4	
E. faecalis	Hand swab	18	15	83.3	
	Litter	15	15	100.0	
Total		**47**	**40**	**85.1**	0.61

Note: *MDR was defined as resistance to ≥3 antibiotic classes; MAR index was calculated as the ratio of the number of antibiotics to which an isolate is resistant to the total number tested*

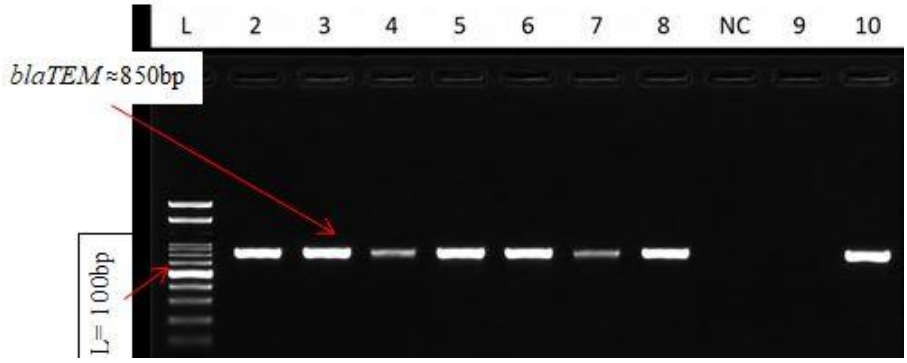
4.4. Detection and Distribution of Virulence and Antimicrobial Resistance Genes among Isolates

The PCR amplification products of the *stx2, blaTEM, ddl, and tetM* genes were visualized using agarose gel electrophoresis, as shown in Figure 5 (A, B, C, and D). Clear and specific bands at the expected molecular sizes confirmed the presence of the *stx*2, *blaTEM*, and *tet*M genes in the *E. coli* and *E. faecalis* isolates, whereas the *ddl* gene confirmed the identity of the *E. faecalis* isolates. No amplification was observed in the negative controls.



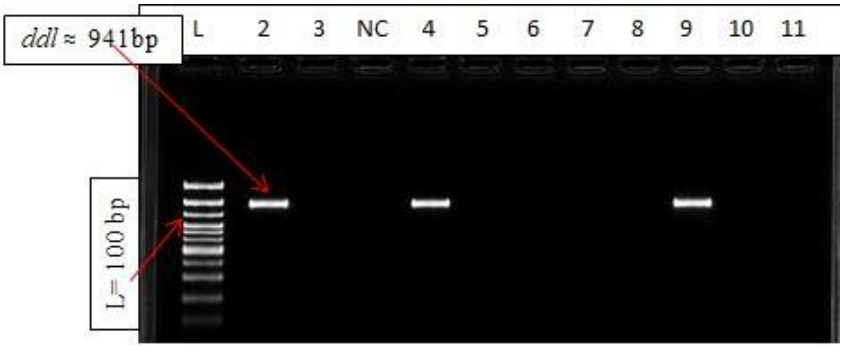
Note: ***Lane L₁****: DNA ladder 100bp;* ***Lanes 1 and 2****: no amplification detected;* ***Lane 3****:Positive amplification of stx2 at ≈ 256 bp;* ***Lanes 4, 5, 6, 7*** *and* ***8****: No amplification detected;* ***Lane L₂****: DNA ladder 100bp;* ***Lane NC****: Negative control;* ***Lanes 9, 10, 11, 12****, and* ***13****: No amplification detected.*

A: Representative gel electrophoresis image of the stx2 gene



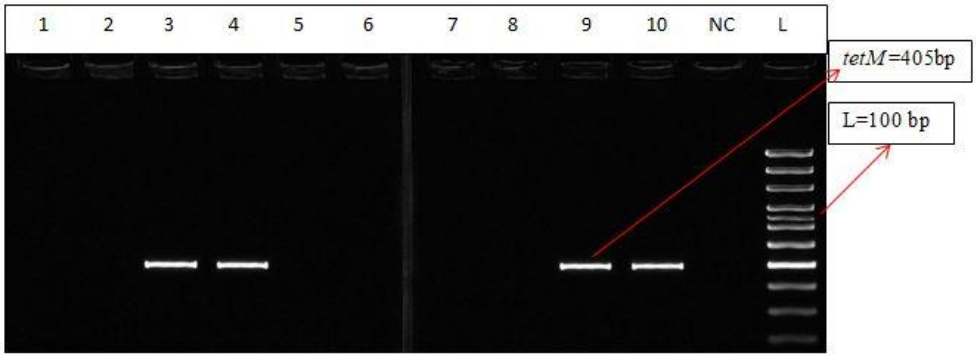
Note: ***Lane L****: DNA ladder 100bp;* ***Lanes 2,3,4,5,6,7, 8 and 10****: Positive amplification of blaTEM at ≈ 850bp;* ***Lane NC****: Negative control;* ***Lanes 9****: No amplification detected*

B: Representative image of the blaTEM gene via gel electrophoresis



*Note: **Lane L**: DNA ladder 100bp; **Lanes 2, 4, and 9**: Positive amplification of ddl at ≈ 941bp; **Lane NC**: Negative control; **Lanes 3, 5, 6, 7, 8, 10, and 11**: No amplification detected*

C: Representative image of the ddl gene via gel electrophoresis

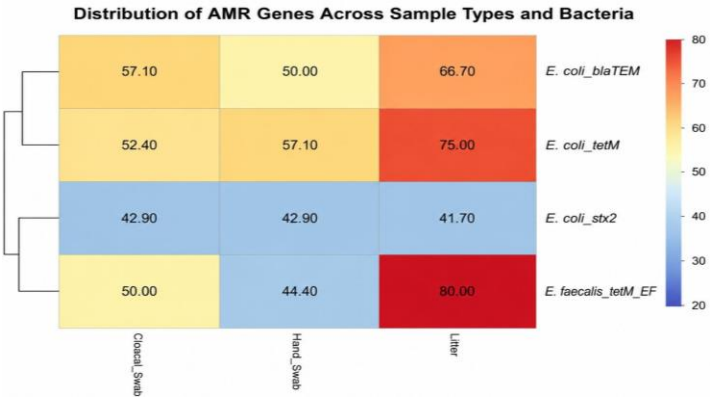


*Note: **Lane L**: DNA ladder 100bp; **Lanes 1, 2, 5, 6, 7, and 8**: No amplification detected; **Lanes 3, 4, 9, and 10**: Positive amplification of tetM at ≈ 405bp; **Lane NC**: Negative control*

D: Representative image of the tetM gene via gel electrophoresis

Figure 5: Representative gel electrophoresis images showing amplification of *stx2* (A), *blaTEM* (B), *ddl* (C), and *tetM* (D)

Detection and distribution of AMR genes among the bacterial isolates are presented in Figure 6 and Table 7.



Note: The color intensity represents the relative frequency of gene detection within each sample type

Figure 6: Heatmap illustrating the distribution of AMR genes among *E. coli* and *E. faecalis* isolates

Among the *E. coli* isolates, *blaTEM* was most frequently detected in the litter samples (66.7%), followed by the cloacal swabs (57.1%) and hand swabs (50.0%). The *tet*M gene had an overall high prevalence (59.6%), with higher detection in litter (75.0%) than in hand swabs (57.1%) and cloacal swabs (52.4%), whereas *stx*2 was moderately distributed among cloacal, litter, and hand swabs. For *E. faecalis*, *tetM* was most prevalent in the litter samples (80.0%), followed by the cloacal swabs (50.0%) and hand swabs (44.4%). Overall, the distribution of resistance genes was relatively greater in the litter and cloacal swab than in the hand swab samples (Table 7).

Table 7: PCR-based detection of *blaTEM*, *stx*2, and *tetM* resistance genes among the bacterial isolates

Bacteria	Gene	Cloacal Swab n/N(%)	Hand Swab n/N (%)	Litter n/N (%)	Overall Positivity n/N %
E. coli	*blaTEM*	12/21 (57.1)	7/14 (50.0)	8/12 (66.7)	**27/47 (57.4)**
	*tet*M	11/21 (52.4)	8/14 (57.1)	9/12 (75.0)	**28/47 (59.6)**
	*stx*2	9/21 (42.9)	6/14 (42.9)	5/12 (41.7)	**20/47 (42.6)**
E. faecalis	*tet*M	7/14 (50.0)	8/18 (44.4)	12/15 (80.0)	**27/47 (57.4)**
	ddl	9/14 (64.3)	9/18 (50)	12/15 (80.0)	**30/47 (63.8)**

4.5. Genotype–Phenotype Associations and Distribution of Multidrug-Resistant *E. coli* and *E. faecalis*

The association between phenotypic antimicrobial resistance and detection of resistance genes is presented in Table 8. Among *E. coli* isolates, a significant association was observed between phenotypic ampicillin resistance and the presence of *blaTEM* gene ($\chi^2 = 6.85$, $p = 0.009$). Likewise, tetracycline resistance was significantly associated with *tetM* detection ($\chi^2 = 6.44$, $p = 0.040$). However, no statistically significant association was seen between tetracycline resistance and *tetM* detection among *E. faecalis* isolates ($\chi^2 = 2.96$, $p = 0.085$).

Table 8: Association between phenotypic AMR and resistant gene detection in *E. coli and E. faecalis*

Bacterial species	Resistance phenotype	Resistance gene	Resistant isolates (%)	Gene-positive isolates (%)	χ^2	*p*-value
E. coli	Ampicillin resistance	*blaTEM*	72.3	57.4	6.85	0.009
	Tetracycline resistance	*tetM*	91.5	59.6	6.44	0.040
E. faecalis	Tetracycline resistance	*tetM*	87.2	57.4	2.96	0.085

***Note**: Chi-square (χ^2) analysis was used to assess the relationship between AMR phenotypes and the presence of resistance genes.*

4.6. Antimicrobial Resistance and Virulence Genes in View of One Health

The distribution of AMR and virulence genes across cloacal swabs, hand swabs, and litter samples representing the One Health continuum is presented in Table 9. For *E. coli*, the *blaTEM* gene was detected in 57.1% of the cloacal swabs, 50.0% of the hand swabs, and 66.7% of the litter samples. The *stx*2 gene was detected in 42.9% of the cloacal and hand swabs and 41.7% of the litter samples. Pairwise analysis revealed no significant differences in *blaTEM* distribution between cloacal and hand swabs (OR = 0.76, p = 0.739) or between cloacal swabs and litter samples (OR = 1.47, p = 0.719). Similarly, no significant differences were detected for stx2 between cloacal and hand swabs (OR = 1.00, p = 1.000) or cloacal swabs and litter samples (OR = 0.96, p = 0.954).

For *E. faecalis*, *tet*M gene positivity was 50.0% in cloacal swabs, 44.4% in hand swabs, and 80.0% in litter samples. Pairwise comparisons revealed no significant difference between cloacal and hand swabs (OR = 0.81, p = 0.769) or cloacal and litter samples (OR = 3.73, p = 0.129), whereas hand swabs, compared with litter, showed OR = 4.63 (p = 0.072).

Table 9: Distribution and pairwise odds ratio (OR) analysis of AMR and virulence genes among the sample types

Bacteria	Gene	Comparison (% positivity)	OR (95% CI)	*p* value
	blaTEM	Cloacal 57.1% vs Hand 50.0%	0.76 (0.19–3.05)	0.739
	blaTEM	Cloacal 57.1% vs Litter 66.7%	1.47 (0.33–7.27)	0.719
E. coli	*stx*2	Cloacal 42.9% vs Hand 42.9%	1.00 (0.24–4.06)	1.000
	*stx*2	Cloacal 42.9% vs Litter 41.7%	0.96 (0.21–4.16)	0.954
	*tet*M	Cloacal 50.0% vs Hand 44.4%	0.81 (0.19–3.40)	0.769
E. faecalis	*tet*M	Cloacal 50.0% vs Litter 80.0%	3.73 (0.74–23.48)	0.129
	*tet*M	Hand 44.4% vs Litter 80.0%	4.63 (1.02–27.46)	0.072

Note: *Cloacal swabs represent poultry samples, hand swabs represent human interface samples, and litter samples represent environmental sources. The percentages indicate gene positivity within each sample type. Odds ratios were calculated using pairwise comparisons between sample types. Fisher's exact test was applied where appropriate due to the small sample size.*

4.7. Multivariable Logistic Regression Analysis of Risk Factors Associated with Bacterial Occurrence and Resistance Determinants

Multivariate logistic regression analysis revealed several factors associated with bacterial occurrence and AMR profiles (Table 10). Production type was significantly associated with the

occurrence of *E. coli*, with layer farms having lower odds of isolation than broiler farms did (AOR = 0.54; 95% CI: 0.30–0.99; p = 0.045). Similarly, lower worker exposure was significantly associated with reduced odds of *E. faecalis* occurrence (AOR = 0.49; 95% CI: 0.25–0.97; p = 0.042). Although not statistically significant, increased odds of *stx*2 detection, *blaTEM* carriage, and *tet*M occurrence were observed in relation to layer production systems, litter reuse practices, and poor biosecurity conditions, respectively.

Table 10: Multivariable logistic regression analysis of risk factors associated with bacterial occurrence and AMR determinants

Outcome	Key Risk Factor	AOR	95% CI	*p* value	Interpretation
E. coli	Production type (Layer)	0.54	0.30–0.99	0.045*	Lower odds in layer farms
E. faecalis	Worker exposure (Low)	0.49	0.25–0.97	0.042*	Protective effect of reduced exposure
stx2	Production type (Layer)	1.59	0.89–2.87	0.115	Increased trend (not significant)
blaTEM	Litter reuse (Yes)	1.99	0.78–4.56	0.150	Possible increased gene carriage
***tet*M**	Biosecurity (Poor)	1.50	0.80–2.94	0.232	Increased resistance trend

Note: *AOR = adjusted odds ratio; CI = confidence interval. *Statistically significant at p < 0.05.*

5. DISCUSSION

The present study demonstrated widespread occurrence of antimicrobial-resistant *E. coli* and *E. faecalis* across poultry, litter, and poultry worker samples in Bishoftu, Ethiopia, confirming active circulation of resistant bacteria within the poultry–environment–human interface. The overall occurrence of *E. coli* (31%) and *E. faecalis* (44.8%) indicates considerable bacterial contamination among the investigated samples from poultry, litter, and farm workers across One Health compartments. The occurrence of *E. coli* in the current study (31%) was comparable to the 29.5% reported in Ethiopian poultry farms by Tigabie *et al*. (2023) and the 33.0% reported in Bangladesh by Sarker *et al*. (2019). However, it was lower than 50% prevalence reported in intensive poultry farms in Thailand and China (Thongratsakul *et al*., 2023). These differences may reflect and be associated with variations in antimicrobial usage, farm hygiene, stocking density, and poultry management systems. In contrast, the higher occurrence of *E. faecalis* (44.8%) observed in the present study aligns with findings from confined poultry systems by Jørgensen *et al*. (2017), where cloacal carriage of *E. faecalis* reached up to 44% in less intensive breeder system. However, this result contrasts with sixteen-year-old Ethiopian baseline findings, where *E. faecalis* has been reported at lower proportions (approximately 11.5%) among enterococcal isolates from poultry and cattle systems (Bekele and Ashenafi, 2010). The greater recovery of *E. faecalis* compared with *E. coli* may be related to the stronger environmental survival capacity of enterococci under dry and nutrient-limited conditions (García-Solache and Rice, 2019).

The detection of both bacterial species in the animals, litter and poultry workers further highlights the interconnected transmission of resistant bacteria within poultry production systems and beyond. In the current study, litter samples showed substantial bacterial recovery, supporting previous reports that poultry litter serves as a major environmental reservoir of AMR bacteria and resistance genes (Fatoba *et al*., 2021; Agga *et al*., 2025). Similarly, the recovery of isolates from poultry workers' hand swabs suggests occupational exposure and possible bi-directional transmission between poultry and humans. Comparable findings were reported by Son *et al*. (2024) in Korea and Nadimpalli *et al*. (2015) in the United States, where poultry workers carried resistant bacteria similar to poultry-associated strains. The present findings therefore reinforce

the importance of farm hygiene, litter management, and personal protective equipment (PPE) in limiting AMR dissemination.

Antimicrobial susceptibility test revealed extremely high resistance levels against tetracycline and ampicillin among both bacterial species. Resistance of *E. coli* to tetracycline reached 91.5%, which was slightly higher than the 84% reported in Ethiopia by Tigabie *et al*. (2023) and the 88% reported in Bangladesh (Sarker *et al*., 2019). Likewise, tetracycline resistance among *E. faecalis* isolates reached 87.2%, consistent with reports from poultry-associated enterococci in Zambia and Nigeria, where resistance exceeded 80% (Aworh *et al*., 2021; Mudenda *et al*., 2022). The consistently high tetracycline resistance observed across studies likely reflects the extensive use of tetracyclines in poultry production because of their affordability and broad-spectrum activity (Van Boeckel *et al*., 2019).

Ampicillin resistance was also remarkably high in the present study, reaching 72.3% in *E. coli* and 91.5% in *E. faecalis*. The *E. coli* resistance level was higher than the 61% reported in Cameroon (Moffo *et al*., 2021) but similar to findings from Ethiopia and Bangladesh, that documents complete (100%) resistance to ampicillin (Sarker *et al*., 2019; Tigabie *et al*., 2023). The exceptionally high ampicillin resistance in *E. faecalis* suggests substantial β-lactam selective pressure within the poultry environment. Similar high resistance among enterococci has been associated with long-term antimicrobial exposure and persistence of resistant strains in poultry litter and farm environments (García-Solache & Rice, 2019).

In contrast, all isolates of both *E. coli* and *E. faecalis* in the present study were found to be susceptible to gentamicin. This finding agrees with Gemeda *et al*. (2021), who similarly reported low gentamicin resistance among poultry-associated bacteria in Ethiopia. The observed susceptibility might be attributed to the lower use of aminoglycosides in poultry farms compared with tetracyclines and β-lactams classes of antimicrobials. Moderate ciprofloxacin resistance was detected in both bacterial *E.coli* (27.7%) and *E. faecalis* (38.3%) species in the current study. A similar level of ciprofloxacin resistance has been reported in poultry-associated *E.coli* isolates in Spain (32.5%) by Mencía-Ares *et al*. (2021) and in *E. faecalis* isolates from broiler breeders in South Korea (12.2%) by Noh *et al*. (2020). However, a study conducted by Das *et al*. (2023) revealed that 87.5% of *E. coli* isolates were phenotypically resistant to ciprofloxacin, indicating

emerging fluoroquinolone resistance. Available data from the literature indicate that *E. coli* and *E. faecalis* isolates from poultry were generally susceptible to ciprofloxacin, with complete susceptibility in *E coli* (Sarba *et al*., 2019) and a low level of (12.2%) resistance in *E. faecalis* (Noh *et al*., 2020). Comparable moderate resistance levels in *E. coli* and *E. feacalis* isoates obtained from human subjects have been reported in Ethiopia and South Africa (Fatoba *et al*., 2022; Fujita *et al*., 2022), raising concern because fluoroquinolones are critically important in human medicine.

The multidrug-resistant findings further demonstrate the severity of antimicrobial selection pressure within poultry production systems. In the present study, 19.1% of *E. coli* and 85.1% of *E. faecalis* isolates were multidrug resistant. The MDR prevalence observed in *E. coli* was lower than the 40–60% reported in poultry farms in Bangladesh and Cameroon (Sarker *et al*., 2019; Moffo *et al*., 2021), whereas the MDR prevalence in *E. faecalis* was considerably higher than the pooled MDR prevalence of 60.0% reported by Mesele *et al*. (2020). The dominant MDR pattern among *E. coli* isolates was AMP-TE resistance (48.9%), whereas *E. faecalis* predominantly showed AMP-TE-ERY resistance (48.9%). Similar resistance combinations have been commonly reported in poultry-associated bacteria globally and are often linked to co-selection of resistance genes located on transferable plasmids and transposons (Muloi *et a*l., 2022).

The MAR index values obtained in this study were also notably high, with values of 0.48 for *E. coli* and 0.61 for *E. faecalis*. According to Krumperman (1983), MAR values greater than 0.2 indicate high-risk environments with frequent antimicrobial exposure. The MAR values observed in the present study were considerably higher than those reported in some previous Ethiopian poultry investigations, suggesting increased antimicrobial selection pressure in the studied farms. Similar elevated MAR values have been reported in poultry farms in Nigeria and Bangladesh, where antimicrobial stewardship practices remain limited (Sarker *et al*., 2019; Aworh *et al*., 2021).

The molecular findings confirmed the circulation of important resistance genes within the poultry environment. Significant associations were observed between phenotypic resistance and the corresponding resistance genes among *E. coli* isolates, particularly tetracycline-*tetM* and ampicillin-*blaTEM*. These findings are consistent with studies from Egypt and Asia, where *tetM*

and *blaTEM* were strongly associated with phenotypic resistance among poultry-associated *E. coli* isolates (Faridah *et al*., 2023; Alam *et al*., 2023). The widespread detection of *tetM* among both *E. coli* and *E. faecalis* isolates supports previous reports indicating that tetracycline resistance genes are highly disseminated in poultry production systems worldwide (Mudenda *et al*., 2022; Liu *et al*., 2024).

The distribution of resistance genes across the different sample types further supports the existence of highly interconnected One Health transmission pathways for AMR within the poultry production system. In the current study, *blaTEM* and *stx2* genes in *E. coli* showed relatively uniform distribution across cloacal, litter, and hand swab samples, with no significant differences between compartments. Similar findings were reported in Ethiopia and Nigeria, where resistant *E. coli* strains and resistance genes circulated simultaneously among poultry, farm workers, and environmental samples (Shecho *et al*., 2017; Aworh *et al*., 2020). In contrast, *tetM* among *E. faecalis* isolates was more common in litter samples (80.0%) than in cloacal (50.0%) and hand swab samples (44.4%). Although most comparisons were not statistically significant, the higher OR for litter samples suggests environmental accumulation of tetracycline-resistant enterococci. Similar environmental enrichment of *tetM*-positive enterococci in poultry litter has been reported previously (Graham *et al*., 2009; Agga *et al*., 2020).

The multivariable logistic regression analysis identified important farm management factors associated with bacterial occurrence and resistance gene carriage. Accordingly, improved production systems, better biosecurity practices, and PPE use were associated with lower odds of bacterial occurrence and resistance detection. On the other hand, poor sanitation and increased environmental exposure increased the likelihood of detecting resistance determinants, particularly *tetM* in *E. faecalis*. Similar associations have been reported in poultry farms where inadequate hygiene, litter reuse, and weak biosecurity facilitated persistence and transmission of resistant bacteria (Noh *et al*., 2020; Fatoba *et al*., 2022). These findings suggest that management-related interventions could substantially reduce AMR dissemination within poultry systems.

The One Health approach used in this study was a major strength as poultry, environmental, and human samples were investigated simultaneously, providing stronger evidence of AMR

transmission across the prevailing interconnected compartments than previous fragemented/single-compartment Ethiopian studies. However, this study addressed and investigated only the two selected resistance genes (*tetM* and *blaTEM*), and advanced molecular typing methods such as whole-genome sequencing were not performed.

6. CONCLUSIONS AND RECOMMENDATIONS

The current phenotypic and genotypic characterization of Tetracycline- and Beta-Lactam-Resistant *Escherichia coli* and *Enterococcus* across the Poultry–Environment–Human Interface in Bishoftu, Ethiopia, revealed substantial occurrence of antimicrobial-resistant *E. coli* and *E. faecalis*. More specifically, the high levels of resistance to tetracycline and ampicillin, combined with widespread MDR patterns and detection of transferable resistance genes (*tet*M and *blaTEM*), underline the intense antimicrobial selection pressure and active circulation of resistance determinants within poultry production systems. The isolation of resistant bacteria from cloacal, litter, and worker hand swab samples highlights the interconnected nature of AMR dissemination across the poultry–environment–human interface and supports the importance of One Health approaches for AMR surveillance and control. Poultry litter was identified as an important environmental reservoir for resistant bacteria and resistance genes, whereas poultry workers represented a potential occupationally exposed population. The widespread occurrence of resistant isolates across poultry, litter, and farm workers suggests that factors include indiscriminate/prolonged antimicrobial use, inadequate biosecurity measures, improper litter management, and frequent direct contact between poultry and workers may contribute to the maintenance and transmission of AMR within the poultry production systems.

In a nutshell, the findings underscored in the current study emphasize the urgency and need for strengthened antimicrobial stewardship, improved farm biosecurity, regulated antimicrobial use, and proper litter management practices in Ethiopian poultry production systems. Based on the above concluding remarks, the following recommendations are forwarded:

- Ethiopian veterinary and public health authorities should work hand in hand to further strengthen the regulation and monitoring of antimicrobial use in poultry production systems.
- Poultry producers should implement improved litter management strategies, including proper composting and controlled disposal of poultry waste. Therefore, an adequate level of awareness and training is expected.
- Farm workers should receive regular training on biosecurity, hygiene, and personal protective equipment to reduce occupational exposure.

- Integrated One Health AMR surveillance (AST and molecular surveillance) systems linking the animal, environmental, and human health sectors should be taken as a priority and be strengthened nationally.
- Future studies should incorporate whole-genome sequencing, metagenomics, and longitudinal surveillance to better characterize transmission pathways and resistance evolution.

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8. APPENDICES

Annex 1: Participants' informed consent form

PARTICIPANT INFORMED CONSENT FORM

Name/ID of study participant: _____Age_____Sex_____

Investigator name: Tadelech Yilma Site: Commercial Poultry Farms in Bishoftu

I agree to participate in a study that plans to be conducted on **"***Phenotypic and Genotypic Characterization of Tetracycline- and Beta-Lactam-Resistant Escherichia coli and Enterococcus across the Poultry–Environment–Human Interface in Bishoftu, Ethiopia.***"** This will provide insights into the occurrence, patterns of antimicrobial-resistant bacteria (*Escherichia coli* and *Enterococcus*), and selected resistance genes in poultry farms, farm environments, and poultry workers within a One Health framework.

For this study, I have been requested to provide information related to poultry farm management practices, antimicrobial use, litter handling, hygiene practices, and other relevant factors through a questionnaire. In addition, a hand swab sample will be collected from me using a sterile cotton swab for laboratory analysis.

The investigator has clearly explained to me that there are no significant risks associated with participation in this study. The hand swab collection procedure is simple, non-invasive, and painless. I understand that there is no direct financial or medical benefit to me from participating in this research; however, the findings may contribute to a better understanding of antimicrobial resistance and support public health interventions.

I understand that my participation is completely voluntary. I have the right to refuse participation or withdraw from the study at any time without any penalty or negative consequences. All information collected from me will be kept strictly confidential and used only for research purposes. My identity will not be disclosed in any report, publication, or presentation arising from this study.

I have been given sufficient information about the study and have had the opportunity to ask questions. I understand the purpose and procedures of the study and freely agree to participate.

Name of Participant: _____Signature: _____ Date: _____

Name of Investigator: _____Signature: _____Date: _____

Annex 2: Sample Collection Format / Sampling Sheet

Farm Code	Sample Type (Litter/Soil/Hand Swab)	Sampling Date	Sample Weight/Volume	Farm Type/Size	Antibiotic (TTC) Use in Poultry (Yes/No)	Notes

Annex 3: semi-structured questionnaire for confounder control

To minimize potential confounding factors and strengthen the analytical validity of the study, the following seven questions were developed and administered at the farm and worker levels. These questions are selected on the basis of their direct relevance to tetracycline use, litter management, and antimicrobial resistance dynamics in the poultry production environment. Each question targets a key confounder likely to influence both the exposure (tetracycline residue concentration) and outcome (presence of resistant *E. coli* and *Enterococcus* isolates).

1. **Recent Tetracycline Administration**

Q1: Have any birds in the sampled house been treated with antibiotics (including tetracycline and ampicillin) within the last 30 days? ☐ No ☐ Yes (Date://20__; Route: ☐ Water ☐ Feed ☐ Injection)

2. Litter Management Practice

Q2: What best describes how litter from the sampled house is managed between production cycles?

☐ Removed and disposed of immediately

☐ Reused without treatment

☐ Composted before reuse

☐ Stored/stockpiled without composting

Antibiotic Administration Route

Q3: How are antibiotics most commonly administered to the flock in the sampled house?

☐ In drinking water

☐ Mixed in feed

☐ Individual injection

☐ Multiple routes/Other (specify): _____

4. Farm Size

Q4: What is the current number of birds housed in the sampled production house (approximate count)? _____ Birds

5. Production Type ☐ Layer ☐ Broiler

6. Worker–Litter Contact Frequency

Q5: How often does the primary sampled worker have direct contact with poultry litter during a typical working day?

☐ None

☐ 1–2 times per day

☐ 3–5 times per day

☐ More than 5 times per day

Annex 4: Sample collection and handling procedures

Purpose:

To obtain representative poultry litter, soil, and hand swab samples from poultry farms for residue, microbiological, and molecular analysis.

Materials and Equipment:

- Sterile polyethylene sampling bags (Whirl-Pak® or equivalent), Sterile 10 mL Falcon tubes, Sterile disposable gloves, Sterile plastic scoops/spatulas, Cotton swabs, BPW, Ice box with ice packs (4 ± 2 °C), Permanent markers and sample labels, Field data sheet

Reagents:

- BPW for transport and enrichment

Procedure:

1. Sterile gloves and protective clothing were worn before the poultry house was opened.
2. Using sterile scoops, litter was collected from at least three locations (near feeders, drinkers, and resting areas).
3. The subsamples were mixed thoroughly in a sterile container to form one composite sample (~100 g).
4. Pooled poultry cloacal swab samples from five representative birds were collected from each selected farm via Falcon tubes containing sterile BPW.
5. Environmental litter samples were collected using sterile scoops from 2–3 different points around poultry houses and combined to form composite samples representing each farm.

6. Approximately 10 g was transferred into sterile bags for microbiological analysis.
7. Hand swabs were collected by rotating a sterile swab moistened with BPW over both the palms and fingertips of each participant.
8. Each sample was labeled with the sample code, date, location, and type.
9. The samples were placed immediately in an ice box (4 °C) and transported to the laboratory within 6–12 hours.
10. The samples were stored for microbiological testing at 4 °C, and the residue samples were stored at −20 °C until analysis.

Annex 5: Isolation and confirmation of *E. coli*

Reference Guideline:

ISO 16649-2:2001 and CLSI M100 (2023).

Materials and Media:

- ✓ MacConkey agar, EMB agar, CT-SMAC, Nutrient agar, BPW, Sterile inoculation loops, Incubator (37 °C), Microscope, Gram stain reagents

Procedure:

1. A pooled poultry cloacal swab from at least five birds of 2-3 different points of the farm was collected using sterile cotton swap moisturize by sterilized BPW and then placed in a single Falcon tube containing sterile buffered BPW and then placed under an incubator at 37 °C for 18–24 hours for incubation.
2. A loopful of the culture was streaked onto MacConkey agar and incubated at 37 °C for 24 hours.
3. Environmental litter samples were collected using sterile scoops from 2–3 different points around poultry houses and combined to form composite samples representing each farm.
4. A 1 g litter sample was mixed with 9 mL of BPW in a sterile stomacher bag (1:10 dilution). The mixture was homogenized for 2 minutes.
5. The mixture was incubated at 37 °C for 18–24 hours for enrichment.
6. A loopful of the culture was streaked onto MacConkey agar and incubated at 37 °C for 24 hours.
7. Pink lactose-fermenting colonies were observed and subcultured onto EMB agar.

8. Colonies with metallic green were considered presumptive *E. coli*.
9. Confirm by:
   - ✓ **Gram stain:** Gram-negative rods, **Oxidase test:** Negative, **Indole test:** Positive, **Citrate test**: Negative, TSI test: yellow/A, **MR test**: Positive, and **VP test: Negative**
10. Confirmed isolates were preserved on nutrient agar slants (4 °C) and as glycerol stocks (20%) at −20 °C.

Annex 6: Isolation and confirmation of *E.faecalis*

Reference Guidelines:

ISO 7899-2:2000 and CLSI M100 (2023).

Materials and Media:

- ✓ BEA, Nutrient agar, 6.5% NaCl broth, Gram stain reagents, Incubator (37 °C)

Procedure:

1. A pooled poultry cloacal swab from at least five birds of 2-3 different points of the farm was collected using sterile cotton swap moisturize by sterilized BPW and then placed in a single Falcon tube containing sterile buffered BPW and then placed under an incubator at 37 °C for 18–24 hours for incubation.
2. A loopful of the culture was streaked onto BEA and incubated at 37 °C for 24 hours. A similar procedure applies to the hand swabs taken from each farm worker per farm.
3. Environmental litter samples were collected using sterile scoops from 2–3 different points around poultry houses and combined to form composite samples representing each farm.
4. A 1 g litter sample was mixed with 9 mL of BPW in a sterile stomacher bag (1:10 dilution). The mixture was homogenized for 2 minutes.
5. The mixture was incubated at 37 °C for 18–24 hours for enrichment and was streaked loopfuls onto BEA agar and incubated at 37 °C for 24–48 hours.
6. The blackening of the medium indicates esculin hydrolysis.
7. Perform Gram stain: Gram-positive cocci in chains.
8. Catalase test: Negative.
9. Growth in 6.5% NaCl broth confirmed the presence of *Enterococcus*.
10. The pure isolates were stored on nutrient agar slants at 4 °C and in glycerol at −20 °C.

Annex 7: Antimicrobial Susceptibility Testing

Reference Guideline:

CLSI M100, 2023 (Kirby–Bauer Disk Diffusion Method).

Materials:

MH agar plates (4 mm depth), Antibiotic disks: (tetracycline (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), and erythromycin (15 µg)), Sterile 0.85% saline, Spectrophotometer (600 nm), Sterile cotton swabs, Incubator (35 ± 2 °C)

Reference Strains:

- ✓ *E. coli* ATCC 25922, *E. faecalis* ATCC 29212

Procedure:

1. Colonies that were isolated from 18–24-hour cultures on nutrient agar were selected from 3–5 wells.
2. The mixture was suspended in 3–5 mL of sterile saline and mixed to create a homogeneous suspension.
3. The turbidity was adjusted to OD600 = 0.08–0.10 for *E. coli* and 0.10–0.13 for *Enterococcus* (equivalent to 0.5 McFarland).
4. Within 15 min of preparation, sterile swabs were dipped into the adjusted suspension.
5. The excess material was removed by pressing against the tube wall and streaking evenly over the entire MHA plate in three directions, with a 60° rotation each time for uniform growth.
6. The plates were allowed to dry for 5 minutes at room temperature.
7. The antibiotic disks were placed at least 24 mm apart via sterile forceps.
8. The plates were inverted and incubated at 35 ± 2 °C for 18–24 hours.
9. The inhibition zone diameter (mm) was measured with a ruler.
10. The results are interpreted as *susceptible, intermediate, or resistant* according to CLSI tables.
11. Verification of test performance using control strains.

Annex 8: Molecular Detection of Resistance Genes

Step 1: Preparation of Pure Isolates

- ✓ Subculture confirmed *E. coli* and *E. faecalis* isolates on nutrient agar.
- ✓ The mixture was incubated overnight at 37 °C.
- ✓ The purity was confirmed visually.
- ✓ A confirmed colony of each bacterium was then inoculated into TSB (Tryptose soy broth) and incubated for 16-18 hours at 37 °C.

Step 2: DNA Extraction (QIAamp DNA Mini Kit Method)

- ✓ Buffer AVL containing carrier RNA was prepared by adding 5.6 µL of reconstituted carrier RNA to 560 µL of Buffer AVL for each sample.
- ✓ A total of 140 µL of the bacterial suspension/sample was transferred into a 1.5/2 mL microcentrifuge tube containing 560 µL of Buffer AVL with carrier RNA.
- ✓ The mixture was mixed thoroughly by pulse vortexing and incubated at room temperature (15–25 °C) for 10 minutes.
- ✓ Briefly, the tube was centrifuged to remove drops from the inside of the lid.
- ✓ Add 560 µL of ethanol (96–100%) and mix thoroughly by vortexing.
- ✓ A total of 650 µL of the mixture was carefully added to a QIAamp Mini spin column placed in a 2 mL collection tube.
- ✓ The mixture was centrifuged at 6000 × g (≈8000 rpm) for 1 min, and the flow-through and collection tube were discarded.
- ✓ The remaining mixture was transferred into the same QIAamp Mini spin column placed in a new collection tube and centrifuged again at 6000 × g (≈8000 rpm) for 1 minute. The flow-through was discarded.
- ✓ Five hundred microliters of AW1 buffer was added to the spin column, which was subsequently centrifuged at 6000 × g (≈8000 rpm) for 1 minute. The flow-through and collection tubes were discarded.
- ✓ The spin column was placed into a new 2 mL collection tube, 500 µl of AW2 buffer was added, and the mixture was centrifuged at 20,000 × g (≈14,000 rpm) for 3 min to dry the membrane completely.
- ✓ The QIAamp Mini spin column was transferred into a clean 1.5- or 2-ml microcentrifuge tube.
- ✓ Add 80 µL of Buffer AVE directly onto the QIAamp Mini spin membrane.

- ✓ The mixture was incubated at room temperature for 1 min and centrifuged at 6000 × g (≈8000 rpm) for 1 min to elute the DNA.
- ✓ The tubes were closed properly, and the extracted DNA was stored on ice for immediate use or at −20 °C or −70 °C until PCR analysis.

Step 3: PCR reaction Preparation

✓ 25 µL of reaction mixture per sample was prepared as follows:

Component	**Volume**
Nuclease-free water	16.75 µL
PCR buffer	2.5 µL
1NTPS	0.5 µL
Forward Primer (10 pmol)	1 µL
Reverse Primer (10 pmol)	1 µL
Taq polymerase	0.25
Template DNA	3 µL

✓ Separate PCR reactions were prepared and performed for the following target genes:

Gene	Target	Purpose
tetM	*Enterococcus*	Tetracycline resistance
blaTEM	*E. coli*	β-lactam resistance
ddl	*E. faecalis*	Species confirmation
stx2	*E. coli*	Virulence detection

Step 4: Thermal Cycling Conditions

General PCR cycling program:

✓ Initial denaturation: 95 °C for 5 min

✓ 35 cycles:

- Denaturation: 94 °C for 30 sec, Annealing: 46 °C (*tet*M & *ddl*), 50 °C (*blaTEM*), and 55 °C (*stx* 2), for 30 sec (gene-specific), Extension: 72 °C for 60 sec, Final extension: 72 °C for 10 min, Hold at +4 °C

Step 5: Gel Electrophoresis

✓ A 1.5–2% agarose gel was prepared.

✓ Ethidium bromide was added.

- ✓ 5 µL of PCR product + loading dye was loaded.
- ✓ The 100 bp DNA ladder was included.
- ✓ Run at 90–120 V for ~45–60 minutes.
- ✓ The samples were visualized under an ultraviolet transilluminator.
- ✓ Document band images.

Expected Amplicon Sizes

Gene	**Expected Size**
tetM	~406 bp
blaTEM	~850 bp
ddl (*E. faecalis*)	941 bp
stx2	256 bp

Annex 9: Quality Assurance and Quality Control (QA/QC)

All media were quality-checked for sterility and performance before use.

1. Control strains (*E. coli* ATCC 25922 and *E. faecalis* ATCC 29212) were included in each batch of ASTs.
2. Duplicate samples and spiked blanks were analyzed for recovery validation.
3. Data were recorded on predesigned laboratory sheets with sample IDs, reagents, and analyst initials.

Annex 10: Biosafety and Waste Management

All work was conducted under biosafety level 2 (BSL-2) conditions.

1. PPE, such as gloves, masks, and lab coats, was applied throughout the work.
2. Workbenches were disinfected with 70% alcohol before and after each session.
3. All microbial cultures and disposable materials were autoclaved at 121 °C for 15 minutes before disposal.

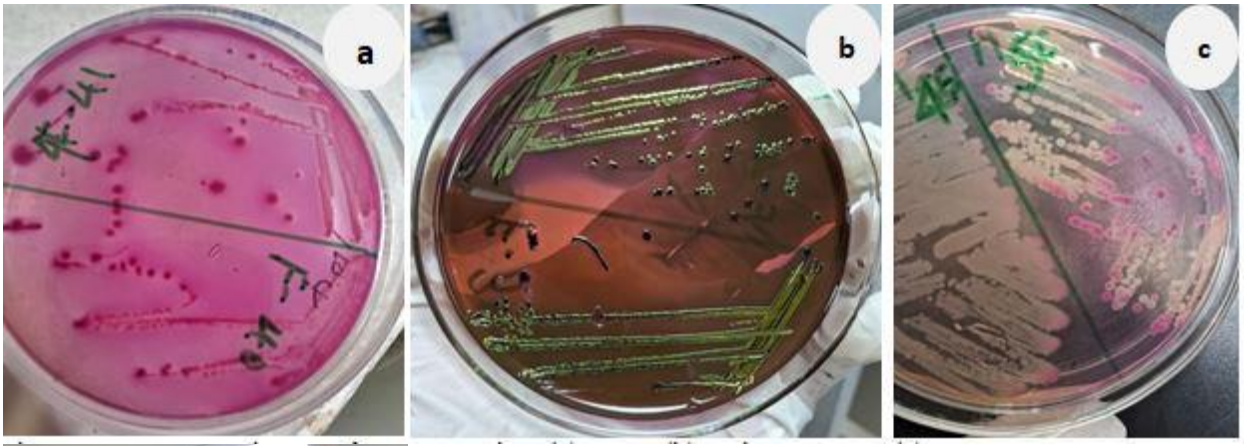
Annex 11: Documentation and Data Management

1. The laboratory data (raw results, chromatograms, AST measurements) were recorded in a bound laboratory notebook and an electronic spreadsheet.

2. Data were cross-verified by an independent analyst for accuracy and completeness, and the final data were archived digitally and backed up in an external drive and institutional cloud storage.

Annex 12: Some photos credited during research activities

A



Presumptive isolation of *E.coli* and *E.faecalis*

B

Figure 1: Presumptive E. coli (A) on MacConkey (a), EMB (b), CT-SMAC (c), and E. faecalis (B) on BEA

A: Gram staining (a) and biochemical test results (b) of *E. coli*

B: Gram staining (a) and salt tolerance (6.5% NaCl) test (b) results for *E. faecalis*

Figure 2: Gram staining (a) and biochemical test results (b) for E. coli (A) and E. faecalis (B)

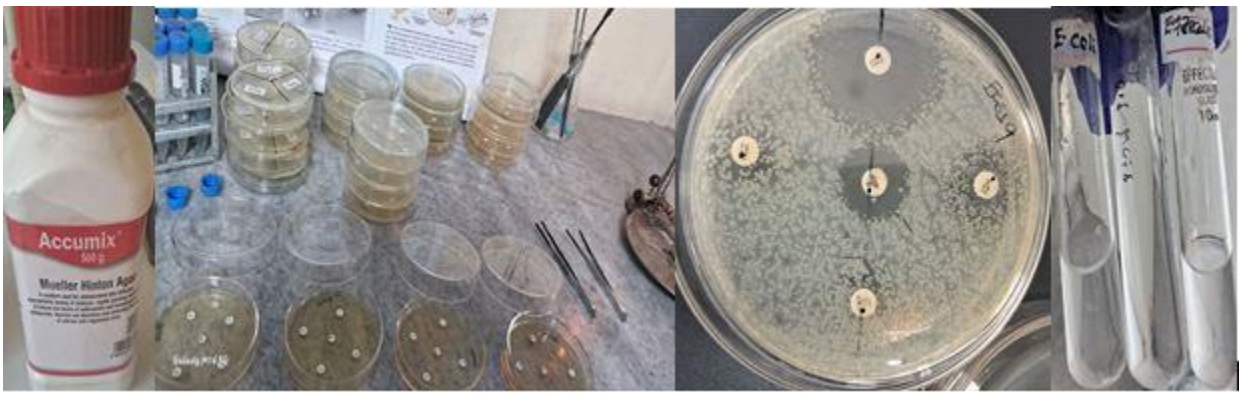


Figure 3: AST test of E. coli and Enterococcus

Annex 13: Ethical approval certificates for Animal and Human components

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/08/109/18/2026

Name of Applicant: **Tadelech Yilma Sisay (DVM, MSc Student)**

Address: Department of Microbiology, Parasitology and Poultry Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Occurrence, phenotypic and genotypic characterization of tetracycline and beta lactam resistant E. coli and Enterococcus across the poultry environment-human enterface in Bishoftu, Ethiopia*

Date of application: **November, 2025**

Nature of the project: **Field investigation**
Target animal species: **Domestic chicken**
Number of animals involved: **350**
Study area: **Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/08/18/026, 27/02/2026**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of Student Tadelech Yilma Sisay.

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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ፋክስ Fax 251-11-4339933
ስልክ Tel. +251 114338450
ፖ.ሣ.ቁ P.o.x. Box 34
ቢሾፍቱ፣ ኢትዮጵያ Bishoftu, Ethiopia

Ethical Clearance Certificate

Ref. No.: ALIHR IRERC-009/2018/2026

Date: June 9, 2026

Title: [Occurrence, Phenotypic and Genotypic Characterization of Tetracycline- and Beta-Lactam-Resistant *Escherichia Coli* and *Enterococcus* across the Poultry–Environment–Human Interface in Bishoftu, Ethiopia]

Principal Investigator: Tadelech Yilma (DVM)

Recommendation by the ALIHR -IRERC

Dear: Tadelech,

The ALIHR-IRERC has reviewed your above mentioned research proposal and noted its merit. The IRERC would like to remind you, as PI, to submit progress reports of the work every 6 months and the final report upon completion of the study. Furthermore, you are expected to notify the ALIHR-IRERC ahead of time for any amendment or modification in the protocol or premature suspension or termination of the study.

STATUS: Approved

National Ethics Review Board Clearance: not required

IRERC Chairperson: Nigatu Kebede, Prof.
Signature: _____

IRERC Secretary: Abebe Animut, PhD
Signature: _____

Approved by
Name: Prof. Wakgari Deressa, Director, ALIHR
Signature: _____
Date: _____

Director's Office
Aklilu Lemma Institute of Health Research
Addis Ababa University

CC:
- IRERC Office
- CPR Office
- ALIHR-IRERC

Annex 14: Plagiarism check report

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF TETRACYCLINE- AND BETA-LACTAM-RESISTANT ESCHERICHIA COLI AND ENTEROCOCCUS ACROSS THE POULTRY–ENVIRONMENT–HUMAN INTERFACE IN BISHOFTU, ETHIOPIA (Tadelech Y)

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