**Thesis Ref.No._____**

# EVALUATION OF SHORT- AND LONG-TERM ANTIBIOTIC TREATMENT REGIMENS ON EMERGENCE OF ANTIMICROBIAL RESISTANCE IN MICE INFECTED WITH *ESCHERICHIA COLI*

**MSc THESIS**

**BY**

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**DEPARTMENT OF BIOMEDICAL SCIENCES**

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# EVALUATION OF SHORT- AND LONG-TERM ANTIBIOTIC TREATMENT REGIMENS ON EMERGENCE OF ANTIMICROBIAL RESISTANCE IN MICE INFECTED WITH *ESCHERICHIA COLI*

Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Pharmacology

**By**

**Timotiwos Wogaso Tonga**

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**Bishoftu, Ethiopia**

**EVALUATION OF SHORT- AND LONG-TERM ANTIBIOTIC TREATMENT REGIMENS ON EMERGENCE OF ANTIMICROBIAL RESISTANCE IN MICE INFECTED WITH *ESCHERICHIA COLI***

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Timotiwos Wogaso Tonga entitled: **Evaluation of short- and long-term antibiotic treatment regimens on emergence of antimicrobial resistance in mice infected with *E. coli,*** and recommend that it be accepted as fulfilling the thesis requirement for the degree of **Masters of Science in Veterinary Pharmacology** complies with the regulations of the university, and meets the accepted standards with respect to originality and quality.

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**ABBREVIATIONS and ACRONYMS**

| | |
|---|---|
| ACT | AmpC-type cephalosporinase |
| AmpC | Ampicillin resistant Cephalosporinase |
| ARG | Antibiotic resistant gene |
| AMR | Antimicrobial resistance |
| AST | Antimicrobial susceptibility test |
| ATCC | American type colony culture |
| BLBLI | b-lactam/b-lactamase inhibitor |
| CMY ESBL | Calgary-Mirabel-York Extended spectrum beta-lactamase |
| CTX-M ESBL | Cefotaxime-Munich Extended spectrum beta-lactamase |
| DHA | De repressed hyper-production of AmpC |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxy ribonucleic acid |
| *E. coli* | *Escherichia coli* |
| EPHI | Ethiopian public health institute |
| ESBL | Extended spectrum beta-lactamase |
| ECDC | European center for disease prevention and control |
| IMP | Imipenemase |
| KPC | *Klebsiella peumoniae* carbapenemase |
| MDR | Multidrug resistance |
| MGE | Mobile gene element |
| MPs | Micro-plastic particles |
| NDH | New Delhi metallo-β-lactamase |
| OXA-48 | Oxacillinase-48 |
| PBPs | Penicillin-binding proteins |
| RT-PCR | Real time polymerase chain reaction |
| *bla*SHV | Sulfhydryl variable Extended spectrum beta-lactamase |
| *bla*TEM | Temoniera Extended spectrum beta-lactamase |
| TMP-SMX | Trimethoprim-Sulfamethoxazole |

| | |
|---|---|
| VIM | Verona integrin-encoded metallo-beta-lactamase |

**ABSTRACT**

Antimicrobial resistance (AMR) is a global issue impacting human and animal health. *E. coli* commonly found in gut serve as an indicator organism to monitor AMR and multidrug resistance (MDR) in commensal bacteria. This research investigates the impact of oxytetracycline and penstrep (a fixed combination of penicillin and dihydrostreptomycin) misuse on AMR emergence in mice infected with drug-susceptible *E. coli*, analyzing the effects of short-term and long-term ( low, optimum and high) antibiotic dosing on the AMR profiles of susceptible *E. coli* strains. The research was carried out on 40 two-month-old female mice at the AAU-CVMA mice rearing facility and microbiology laboratory from November 2023 to April 2024. The study involved infecting the mice with *E.coli*, treating them with one of those two antibiotics, sampling every seven days start from day zero, isolating *E. coli* from the samples, testing for phenotypic resistance to seven antibiotics using agar dilution test, and identifying resistance genes through PCR and lateral flow assays with anti-CTX-M and CARBA 5 monoclonal antibodies. The study revealed higher AMR in penstrep and oxytetracycline groups, with 31.4% and 24.7% resistance, respectively. Infected and naïve control groups had 1.9% and 0.5% resistance, indicating selection pressure effects. Low-dose long-term treatments showed more antibiotic resistance than optimum and higher doses for both oxytetracycline and penstrep. The low-dose penstrep group had the highest MDR at 100% on day 28, followed by the low-dose oxytetracycline group with 86%. Significant variation in AMR emergence was observed at different time points post-treatment, peak on day 28. PCR results showed *bla*TEM gene growth and detection of the *E. coli* gene, while the lateral flow assay indicated negative results for CARB-5 and CTX-M genes. The study suggests long-term low-dose antibiotic use increases AMR risk, especially fixed combination therapy (penstrep). It is recommended to use antibiotic alternatives in food animals to reduce AMR impact. If antibiotics are necessary, use an optimum dose within the nontoxic range for the shortest recommended duration.

**Key words:** *antimicrobial resistance, E. coli, oxytetracycline, penstrep, treatment regimen*

## 1. INTRODUCTION

Antimicrobial resistance (AMR) poses a major threat to human health around the world, and has become a leading cause of death globally in the coming decades (Murray *et al.,* 2022; Salam *et al.,* 2023). In the year 2019, it was estimated that around 4.95 million deaths were caused by antimicrobial resistance (AMR), and out of these, approximately 1.3 million deaths were directly linked to infections caused by resistant bacteria (Ranjbar and Alam, 2024). This number is expected to reach 10 million deaths per year worldwide by 2050 (Cong *et al.,* 2021). AMR presents a major challenge to public health worldwide, particularly in developing countries where access to effective treatment and surveillance mechanisms may be restricted (Murray *et al.,* 2022).

Antibiotics are commonly prescribed in both human and veterinary medicine to promote health, and are also utilized in agriculture and aquaculture to improve production (*Wang et al.,* 2012). However, the misuse and overuse of antimicrobials in humans, animals, and plants are the main drivers in the development of drug-resistant pathogens (WHO, 2023). This is further confirmed by evidence of higher dose of antibiotic use in bacteria correlates well with frequency of resistance (Van de Sande-Bruinsma *et al.,* 2008). Furthermore, heavy metals and other biocides could also be considered as drivers to the emergence of AMR. This is either by co-selection between biocide and antibiotic resistance genes (ARGs) provides resistance to both (Wales and Davies, 2015; Razavi *et al.,* 2017) or via cross-resistance between the biocide and antibiotic resistance mechanisms (Blair *et al.,* 2015).

Tetracycline is the class of antibiotics usually used to treat infections in animals due to their broad spectrum of activity, high rate of absorption, low toxicity, and low cost (Granados-Chinchilla *et al.,* 2017). Traditionally, tetracycline resistance in bacterial communities in food animal farms has been investigated using cultures of indicator organisms such as *E. coli* (Shin *et al.,* 2015), Salmonella (Otto *et al.,* 2018), and Campylobacter (Premarathne *et al.,* 2017). On other hand, “penstrep” is an antibacterial suspension comprised of a fixed combination of penicillin and dihydrostreptomycin. It is

also widely used in the control, prevention, and treatment of infections in animals, especially in food-producing domestic animals because of its combined efficacy against both Gram-positive and Gram-negative bacteria for empirical treatment (De Britto *et al*., 2012).

*Escherichia coli* are a bacterium that holds a unique position in the field of microbiology due to its ability to cause serious infections in both humans and animals. Additionally, it is a crucial part of the natural microbiota in various hosts and serves as a primary source of resistance genes that can lead to treatment challenges in both human and veterinary medicine (Poirel *et al*., 2018). AMR *E. coli* in developing countries were reported to be one major reason for failure of treatment of infectious diseases (Erb *et al*., 2007). Several studies conducted in Ethiopia from various human clinical settings show increments in the prevalence of AMR patterns of *E. coli* (Gebre-Sealsssiec, 2007; Yismaw *et al.*, 2010; Endalafir *et al*., 2011).

The use of antimicrobial agents, including oxytetracycline and penstrep, in veterinary medicine is widespread for prophylactic and therapeutic purposes (AbuOun *et al.*, 2020). These antibiotics are extensively used in food animals in Ethiopia non-prudently (Beyene *et al.*, 2015; Etefa *et al.*, 2021). However, the overuse and misuse of these antibiotics could contribute to the selection pressure driving the emergence and dissemination of resistant bacterial strains in humans, animal populations and the environment. Although *E. coli* infection is prevalent worldwide and exhibits numerous resistant genes towards a wide range of antibiotics, including oxytetracycline and penstrep (fixed combinations of penicillin and streptomycin), which are the most utilized drugs in Ethiopia (Tufa *et al.*, 2023), and the occurrence of AMR leads to treatment failure. Despite this, these drugs are still in use, and further research is needed to determine how improper antibiotic use contributes to the emergence of AMR. This study considered the effects of antibiotic exposure, either overuse, optimal or underuse driven by varying dosage regimens and route of administration in food animals in Ethiopia.

## Objectives

### General objective

- To investigate how antibiotic selection pressures of using different antibiotic regimens affect the emergence of AMR in mice infected with antibiotic-susceptible *E. coli*.

### Specific objectives

- To determine the effect of short-term and long-term administration of oxytetracycline and penstrep on the emergence of phenotypic resistance in mice infected with antibiotic susceptible *E. coli*.

- To detect the most common AMR genes expressed by a model bacterium due to antibiotic selection pressure by exposing to varying regimens of oxytetracycline and penstrep.

# 2. LITERATURE REVIEW

## 2.1. Antibiotics and Antimicrobial Resistance

The discovery and utilization of antibiotics may be considered as a great achievement in the realm of medicine (Gaynes, 2017). Throughout history, varieties of antibiotics have been employed for medical purposes. Initially considered as a remarkable remedy in the mid-20th century, antibiotics with a hopeful outlook that infectious diseases could be eradicated. The beginning of the modern era of antibiotics is closely linked to the work of Alexander Fleming and Paul Ehrlich. Antibiotics were seen as a powerful tool that could effectively target microorganisms that can cause diseases while protecting the host from potential harm (Aminov, 2010). The time period spanning from the 1950s to the 1970s was widely considered as the golden age for the discovery of new antibiotic classes (Davies and Davies, 2010).

Antibiotics are pharmaceutical agents that impede the proliferation of bacteria either by inhibiting their cell division or by inducing their death. While the terms antibiotic and antimicrobial are often used interchangeably, they have distinct meanings. Antibiotics are drugs derived from microbes like penicillin, whereas antimicrobial encompasses a broader range including synthetic compounds designed to eliminate various microorganisms (Guardabassi and Courvalin, 2005).

Antimicrobial resistance (AMR) refers to the ability of microorganisms, especially bacteria, to withstand the effects of antimicrobial drugs that were previously effective in treating infections caused by these organisms. The complexity of AMR arises from various factors, primarily due to the overuse and inappropriate use of antimicrobial drugs (Ajulo and Awosile, 2024). Microorganisms that develop resistance are commonly referred to as "superbugs". The emergence of AMR can occur spontaneously as a natural event, with instances of resistance to antimicrobial drugs dating back to the early days of antibiotics, such as penicillin, discovered by Alexander Fleming in 1943/44 (Economou and Gousia, 2015).

## 2.2. Emergence and Transmission of Antibiotic Resistance

During the late 1960s to the early 1980s, the pharmaceutical industry introduced numerous new antibiotics in response to the problem of resistance. However, after this period, there was a noticeable decrease in the development of new antibiotics. The reduced effectiveness of antibiotics in treating common infections has worsened in recent years. The emergence of carbapenem-resistant Enterobacteriaceae strains, which are resistant to one of the most powerful classes of antibiotics, signals the onset of a post-antibiotic era (Spellberg and Gilbert, 2014). Consequently, in late 2015, many decades after the initial use of antibiotics to treat patients, bacterial infections have again become a significant concern (Spellberg and Gilbert, 2014).

The definition of AMR as a phenomenon resulting from the excessive use of antibiotics is insufficient, as it is widely known that AMR naturally evolves over time through various mechanisms (Cepas and Soto, 2020). The excessive use of antibiotics in humans, animals, and the environment speeds up the natural process of antimicrobial resistance, leading to the increased spread of resistant bacteria (Iramiot *et al.*, 2020).

In the context of AMR gaining, resistance can be categorized into two types: natural resistance, which encompasses intrinsic and induced categories, and acquired resistance (Reygaert, 2018). The term intrinsic resistance refers to the phenomenon where bacterial species possess a natural resistance to specific groups of antibiotics without being influenced by prior exposure to these drugs (Cox and Wright, 2013). Additionally, bacteria can develop natural resistance through the activation of certain genes after being exposed to therapeutic levels of antibiotics (Sandner-miranda *et al.*, 2018). Acquired resistance can arise through two separate mechanisms: mutations that take place in the cell's DNA during replication or the transfer of DNA (Martinez, 2014). In the case of the former, the mutant strains possess the ability to transmit the mutation to subsequent generations via vertical transmission. (Ben *et al*., 2019). The second way through which bacteria acquire resistance is through transformation, transposition, and conjugation (all termed horizontal gene transfer) (Friedrich, 2019).

## 2.3. Mechanisms of Antibiotic Resistance

In order to comprehend the factors that contribute to the mechanism of resistance, it is crucial to understand the mechanism of action of the antibiotics. The mechanism of action of antimicrobial agents can be categorized based on the function that is affected by the agents, these generally included the following: inhibition of the cell wall synthesis, inhibition of ribosome function, inhibition of nucleic acid synthesis, inhibition of folate metabolism and inhibition of cell membrane function (Dowling *et al.,* 2017). Pathogens achieve resistance to antibiotics through the mode the agent has affected them. The emergence of resistance generally depends on the species, the nature of the drug and its target site. When the antibiotic interferes a particular pathway, the microorganism to avoid activity of the agent will activate a sophisticated alternative mechanism (Munita and Arias, 2016).

In addition to the complex individual mechanisms, researchers are also faced with phenomena such as cross resistance (developed resistance to antibiotics that are chemically related) and multiple drug resistance (acquired resistance to unrelated chemical compounds due to the synergistic effects of the outlined mechanisms) (Sefton, 2002). As well, individual classes of antibiotics can be resisted simultaneously through multiple mechanisms (Cox and Wright, 2013).

### *2.3.1. Enzymatic inactivation of antibiotics*

Enzymatic chemical modification of antibiotics is a key mechanism behind drug resistance, making it a particularly suitable target for chemical biology strategies. Since 1940, when the first penicillinase (b-lactamase) was identified, number of enzymes able to degrade and modify antibiotics has been identified (Jacoby, 2009). Degradation mechanisms result in either disruption of a key reactive center or structural rearrangement that is not readily reversed under normal physiological conditions. On the other hand, covalent modification of antibiotics obstructs interaction of the antibiotic with its target. This second strategy confers resistance by means of group transfer and includes O-

phosphorylation, O-ribosylation, O-glycosylation, O-nucleotidylylation as well as O- and N-acetylation (Munita *et al.,* 2016).

The phenomenon of producing enzymes that chemically alter antimicrobial compounds represents a well-established process contributing to the development of acquired antibiotic resistance in various bacterial strains, including both Gram-negative and Gram-positive bacteria. It is important to highlight that the majority of antibiotics impacted by such modifications exert their antimicrobial activity through the inhibition of protein synthesis specifically at the ribosomal level (Wilson, 2014).

*2.3.2. Efflux pump*

An alternative method through which microorganisms develop resistance to antibiotics is through the utilization of an efflux pump, a biological mechanism that expels antibiotics from the cell, preventing them from reaching their target (Willey *et al.,* 2013). The discovery of an efflux system capable of removing tetracycline from the cytoplasm of *E. coli* dates back to the early 1980s and was among the first to be documented (McMurry *et al.,* 1980). Since then, various types of efflux pumps have been identified in both Gram-negative and Gram-positive pathogens. These systems can be specific to certain substances or exhibit a broad range of substance specificity, a common trait in multidrug-resistant bacteria (Munita *et al.,* 2016).

Transport proteins located in the bacterial cell membrane facilitate the movement of nutrients and removal of harmful substances from the cell environment. The efflux mechanism is increasingly known as a primary factor in conferring resistance to multiple antibiotic classes (Soto, 2013; Varela *et al.,* 2013). Notable antibiotic classes that are known to be expelled by intrinsic bacterial efflux pumps include macrolides, β-lactams, fluoroquinolones, oxazolidinones, as well as fourth generation cephalosporins and carbapenems (Li and Nikaido, 2009; Lin *et al.,* 2015).

### *2.3.3. Reduced influx*

Many of the antibiotics used in clinical practice have intracellular bacterial targets or, in the case of Gram-negative bacteria, targets located in the cytoplasmic membrane (the inner membrane). Therefore, the compound must penetrate the outer and/or cytoplasmic membrane to exert its anti-microbial effect. Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule. This mechanism is particularly important in Gram-negative bacteria (for the reason specified above), limiting the influx of substances from the external membrane (Reygaert, 2018).

A gene mutation can result in altered pores, usually by changing the electrical charge or the physical structure, which can make it more difficult for antibiotics to enter the cell. The antibiotic is still functionally active, but it will fail to reach its target site. A microorganism can develop resistance to multiple drug classes at once in this manner. However, some gram-negative bacteria are innately resistant to large drugs like vancomycin, which is too large to pass through the pores even before a mutation occurs (Galdiero *et al.,* 2012).

### *2.3.4. Modification and protection of the target site*

An additional common approach used by bacteria to develop AMR is to evade the effects of antibiotics by disrupting their target sites. Bacterial cells contain various components that can be targeted by antimicrobial agents; these targets can be altered by bacteria to resist the drugs (Reygaert, 2009). The modifications to these targets may involve gene mutations affecting the target site, changes in enzyme activity at the binding site, or substitution/bypass of the original target. Regardless of the specific alteration, the ultimate outcome remains consistent - a reduction in the antibiotic's ability to bind effectively to the target site (Munita and Areas, 2016).

One of the main mechanisms of resistance to β-lactam antibiotics, primarily observed in gram-positive bacteria, involves changes in the structure and/or quantity of penicillin-

binding proteins (PBPs). PBPs are enzymes that play a role in synthesizing peptidoglycan in the bacterial cell wall. Variations in the number of PBPs (such as an increase in PBPs with reduced drug binding capacity or a decrease in PBPs with normal drug binding) impact the effectiveness of drug binding to the target (Beceiro *et al.*, 2013). While some genetic factors encoding proteins for target defense are located within the bacterial chromosome, the majority of clinically significant genes associated with this resistance mechanism are carried by mobile genetic elements (MGEs) (Munita and Arias, 2016).

## 2.4. Risk Factor of Antimicrobial Resistance

### *2.4.1. In-appropriate or overuse of antibiotics*

Epidemiological research has connected a clear connection between the improper or excessive use of antibiotics and the worldwide propagation or emergence of antibiotic-resistant bacterial strains (Goossens, 2009; Maltezou *et al.*, 2017; Wojkowska-Mach *et al.*, 2018). Recent research has shown that a significant portion of cases of self-medication involved obtaining antibiotics from pharmacies without a prescription, while another segment was linked to the use of leftover antibiotics at home or those obtained from acquaintances (Karakonstantis and Kalemaki, 2019).

Inadequate prescription practices and the excessive utilization of antibiotics are certainly exacerbating the obstacles presented by antibiotic-resistant bacteria (Luyt *et al.*, 2014). Studies suggest that in around 30% to 50% of cases, the diagnosis, choice of antimicrobial agents, or duration of antibiotic therapy were incorrect in critical care environments (Kollef and Fraser 2001; Roberts *et al.*, 2014). Moreover, in many developing countries where diagnostic capabilities are limited, patient care heavily depends on medication prescriptions, particularly antibiotics (Chaw *et al.*, 2018). Furthermore, numerous low-quality antibiotics are easily accessible over the counter in developing nations (Chokshi *et al.*, 2019).

### *2.4.2. Micro plastic particles and metals co- resistance*

Recent research has indicated micro plastic particles (MPs) as a novel factor that contributes to the spread of antibiotic resistance. Studies by Gao *et al.* (2015), Radisic *et al.* (2020), and Bowley *et al.* (2021) have pointed out this phenomenon. Zettler *et al.* (2013) made the groundbreaking discovery of microbial communities residing on plastic surfaces, particularly due to the hydrophobic nature of MPs that facilitates microbial biofilm formation. Furthermore, MPs play a role in the sorption of antibiotics and heavy metals, as evidenced by studies conducted by Godoy *et al.* (2019), Mammo *et al.* (2020), and Wang *et al.* (2020). They have been identified as carriers for heavy metals and as promoters of antibiotic-resistant bacteria proliferation, as shown by Rummel *et al.* (2017), Oberbeckmann *et al.* (2018), and Imran *et al.* (2019). Additionally, research by Zhang *et al.* (2020) and Song *et al.* (2020) demonstrated the presence of multidrug-resistant Vibrio species and *E. coli* on marine MPs, respectively.

While certain metals have historically been used as antimicrobial agents, their mechanisms of action differ from traditional antimicrobial agents. Heck *et al.* (2015) discussed this distinction. Bacteria have developed resistance mechanisms against metals to mitigate potential physiological damage, such as DNA damage, enzyme inactivation, disruption of membrane permeability, and oxidative damage to proteins. These mechanisms include intracellular sequestration, extracellular sequestration, bio-precipitation, biotransformation, morphology alteration, pigment production, efflux, and biofilm formation. Studies by Naik *et al.* (2012), Naik and Dubey (2013), and Sharma *et al.* (2017) have detailed these strategies. Furthermore, some bacterial isolates exhibit co-resistance or cross-resistance against critical antimicrobials, as observed in investigations by Riber *et al.* (2014), Heck *et al.* (2015), Gao *et al.* (2015), and Qian *et al.* (2016).

### *2.4.3. Agricultural chemical and antibiotic use*

Antibiotics are widely used in agriculture, livestock, poultry, fisheries, and animal husbandry. In agriculture, antibiotics are most used to prevent and cure various diseases in crops, whereas, in livestock and animal husbandry, these are most used as growth

promoting agents, and in preventing/ curing infections. There are at least 30 different antibiotics that are commonly used in agriculture and livestock, among which macrolides, penicillins and tetracyclines are the major ones (Laxminarayan *et al.,* 2015).

Annually, the average global ingestion of antibiotics per kg of livestock produced including cattle, chicken, and pigs is 45, 148, and 172 mg/kg, respectively (Van Boeckel *et al.,* 2015). It is also postulated that the worldwide ingestion of agricultural antibiotics will increase by 67% in 2030 as a consequence of the expected rise in the number of animals needed for food (Kuppusamy *et al.,* 2018).

### 2.5. Economic and Public Health Importance of Antibiotic Resistance

Antimicrobial-resistant infections have emerged as the third leading cause of mortality. Before 2015, approximately 700,000 individuals globally lose their lives due to drug-resistant infections annually (O'Neill, 2016). A recent study in January 2022 revealed that around 1.27 million deaths in 2019 were linked to antimicrobial resistance, with nearly 5 million deaths associated with drug-resistant infections in some capacity. Projections suggest that by 2050, this number could surge to 10 million per year, surpassing death due to cancer (Rabaan, 2023). The impact of AMR on morbidity and mortality in patients is significant (Founou *et al.,* 2017; Shrestha *et al.,* 2018). Resistant bacteria are twice as likely to cause severe health issues and three times more likely to result in death compared to non-resistant strains (Cecchini *et al.,* 2015). These adverse outcomes are exacerbated by the severity of resistant infections and host susceptibility (Friedman *et al.,* 2016).

The economic impact of antibiotic resistance are challenging to quantify due to various factors. Elevated resistance levels lead to increased costs related to latest costly antibiotics (as infections develop resistance to first-line antimicrobials, treatment must shift to more expensive second- or third-line drugs), specialized diagnostic equipment, prolonged hospitalization, and isolation protocols for patients. Societal costs encompass loss of life and diminished productivity (ECDC, 2009). Additionally, the gap between

developing and developed nations will widen due to AMR, resulting in heightened inequity. Those most affected by the impoverishing effects of AMR are likely to be individuals from low-income countries, underscoring the vulnerability of the world's underprivileged population to the impacts of infectious diseases (Miller-Petrie, 2017).

### 2.6. Antibiotic use in Ethiopia

In Ethiopia, a survey conducted on human subjects at hospitals revealed irrational drug use (Endale *et al.*, 2013). Similarly, in veterinary medicine, a study conducted by Beyene *et al*. (2015) on rational use of veterinary drugs at veterinary teaching hospital and the college of veterinary medicine and agriculture, and Ada district veterinary clinic showed irrational drug use. The study conducted by Kifle *et al.* (2014) demonstrated that most health workers did not adhere to the rational antimicrobial prescription guidelines. The diagnosis of a disease was generally presumptive; drug sensitivity tests were not conducted and the selection of drugs was primarily based on their availability rather than their efficacy. In addition, a few antimicrobials were commonly prescribed, and most respondents did not consider factors that could help reduce the rates of emergence of resistant pathogens.

Diverse groups of antimicrobials were used for all similar clinical signs and symptoms without reaching a final diagnosis. For instances, antibiotics such as oxytetracycline, Penstrep, and sulfa drugs were used to treat animals with clinical signs of the respiratory system (27%), the musculoskeletal system (20.8%), the digestive system (11.7%), and signs associated with systemic diseases (39.2%) (Tufa *et al.*, 2018). Review done by Alemu *et al.* (2009) and Beyene *et al*. (2016) indicates that many infectious agents have developed resistance against oxytetracycline antibiotic; Oxytetracycline (73.9%) and Penstrep (22.6%) are the most prescribed antibiotics. Similarly, these two antibiotics were commonly used as reported in previous study conducted by Beyene *et al*. (2015), 83.6% and 13.8%, respectively.

## 2.7. Historical Background and Microbiological Characteristics of *E.coli*

In 1884, Theodor Escherich identified a common commensal found in the gastrointestinal tract, which was obtained from the fecal matter of newborns and young infants, and named it *Bacterium coli* commune (Escherich, 1989). Initially labeled as *Bacillus coli* in 1895, this microorganism was later renamed *E. coli* in 1919 in honor of its original discoverer. The revised classification was officially confirmed in 1958, establishing Escherichia as a genus, with *E. coli* as its first species (Henry 2015). Moreover, as Basavaraju and Gunashree (2022) outlined, this bacterium is categorized under Domain: Bacteria, Kingdom: Bacteria, Phylum: Proteobacteria, Class: Gamma proteobacteria, Order: Enterobacterales, Family: Enterobacteriaceae, Genus: Escherichia, Species: *Escherichia coli (E. coli).*

*E. coli* are typically rod-shaped bacteria, measuring 2.0–6.0 mm in length and 1.1–1.5 mm in width, with rounded ends and a Gram-negative classification. The morphology of these bacteria can range from spherical (cocci) cells to elongated or filamentous rods. They are non-spore forming and often exhibit motility through peritrichous flagella. *E. coli* are facultative anaerobic organisms that generate gas through the fermentation of carbohydrates, as evidenced by the production of acid and gas from lactose at 37°C and 44°C (Ghernaout *et al.,* 2022).

## 2.8. Pathogenesis of *Escherichia coli*

Within the gastrointestinal tract of vertebrates, commensal *E. coli* strains are observed to inhabit the mucus layer that covers the epithelial cells, particularly in the cecum and colon (Tenaillon *et al.,* 2010). This layer of mucus is abundant in glycoproteins with diverse O-linked glycans (Marcobal *et al.,* 2013), serving as a nutrient-rich environment with adhesion sites that facilitate *E. coli* colonization. Despite *E. coli's* lack of enzymes to directly break down complex mucin-related polysaccharides, the mucolytic activity of other anaerobic commensal gastrointestinal bacteria generates essential mono- and disaccharides for *E. coli* metabolism (Conway and Cohen 2015).

"Colibacillosis" is a general term for a disease caused by the bacterium *E. coli*, which normally resides in the lower intestines of most warm-blooded mammals. Hence, *E. coli* is a versatile microorganism with a number of pathogenic isolates prone to cause intestinal and extra-intestinal infections, while most others are harmless for their host and refer to commensalism. The pathogenic *E. coli* isolates can be classified into different pathotypes, or pathovars, where each pathotype causes a different disease (Kaper *et al.*, 2004).

### 2.9. Antimicrobial Resistance Characteristics of *Escherichia coli*

The development of diverse mechanisms contributes to the resistance of *E. coli* and other Gram-negative bacteria to a variety of antibacterial drugs. In tandem with the variability of *E. coli* infections, there exists a multitude of treatment options for susceptible *E. coli* infections. These include penicillins, cephalosporins, monobactams, b-lactams, b-lactamase inhibitors (BLBLIs), fluoroquinolones, aminoglycosides, trimethoprim, and sulfamethoxazole (TMP)-SMX, among others (Paitan, 2018).

Surveillance data from the 2000s indicates the presence of antibiotic resistance in *E. coli* strains to all major classes of antibiotics. This encompasses extended-spectrum beta-lactamase (ESBL) production (such as TEM, SHV, CMY, and CTX-M), carbapenemase production (including KPC, NDM, VIM, OXA-48, and IMP), and resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole, as well as plasmid-mediated colistin resistance (Liu *et al.*, 2016; Pitout, 2012).

ESBL-producing bacteria have the capacity to interchange ESBL genes within and between species. These genes are located on transposable elements such as plasmids, integrons, or transposons, and can also be situated on bacterial chromosomes (Apata, 2009). Carbapenems may represent a final treatment option for severe infections caused by ESBL- and AmpC-producing *E. coli* strains that are frequently reported globally (Nordmann *et al.*, 2011; Poirel *et al.*, 2016; Thomson, 2010). One of the prevailing

mechanisms of carbapenem resistance involves the production of carbapenemase, a β-lactamase with broad hydrolytic potential (Queenan and Bush, 2007).

*E. coli* possesses a chromosomal gene that encodes the β-lactamase AmpC. The activity of these β-lactamases is typically limited due to a weak promoter and strong attenuator system that regulates the AmpC gene (Jacoby, 2009). Occasionally, cephamycin- and/or cephalosporin-resistant *E. coli* have been observed to utilize the chromosomally encoded AmpC cephalosporinase to produce bacterially derived, plasmid-mediated β-lactamases (Pitout, 2008).

# 3. MATERIALS AND METHODS

## 3.1. Study Area

Experimental study was conducted in Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA) laboratory animal facility, Bishoftu. Bishoftu city is in the East Shewa Zone of the Oromia Region, located in the central high lands of Ethiopia at 47 km Southeast of Addis Ababa, the capital city of Ethiopia. The town is located at $8^0$ 45′ N longitude and $38^0$ 59′ E latitude at an altitude of 1,920 meters above sea level. It has an average annual rainfall of 1150 mm of which 84% falls during the long raining season that extends from June to September, and the remaining during the short rainy season that extends from March to May. The mean annual minimum and maximum temperatures are $12.3^0$C and $28^0$C, respectively, and the mean relative humidity is 61.3% (https://en.wikipedia.org/wiki/Bishoftu accessed on March 16, 2024).

## 3.2. Study Materials, Chemical, and Drugs

Chemicals and drugs used in the study include: sterile distilled water and saline water (National veterinary institute (NVI), Bishoftu), Dimethyl sulfoxide (DMSO) and McFarland standard solution, ethanol, peptone water, Mueller-Hinton agar (Accumax, India), chromogenic orientation media, chromogenic *E.coli* selective media (CHROMagar,™ France), TSB media, antibiotics (ciprofloxacin, erythromycin, florfenicol, gentamicin, oxytetracycline, penicillin, penstrep, and trimethoprim-sulfamethoxazole) (Sigma Chemical Co, St. Louis, Mo. USA) extraction buffer, primers, PCR master mix, and ethidium bromide.

Materials used in this study include: mice cage, watering and feeding equipment's, safety cabinet, micropipette, gel electrophoresis tanks, refrigerators, incubator, Bunsen burner, thermocyclers, centrifuge, boiler, weighing balance, syringe, sterile disposable pipette tips, wire loop, sterilized cotton swab, test tubes, Petri dish, cryogenic vials, micro-

centrifuge tubes, disposable glove, beakers measuring cylinders, thermometer and Eppendorf tube.

### 3.3. Experimental Animals

The study was conducted on 40 two-month-old Swiss-Albino female mice with body weight range in between 28 - 32 gram and body temperature of $36.5^0$c to $38^0$c at different measuring time throughout the research, they acquired from Pan African Veterinary Vaccine Centre of the African Union (AU-PANVAC). The mice were housed, and cared in accordance with guidelines outlined in the eighth edition of the Guide for the Care and Use of Laboratory Animals (Edition, 2011). In this experimental study, there were two cohort study groups, each with 15 mice for oxytetracycline and penstrep. The control groups were common for both study groups; they divided into infected and naïve control groups with five mice each.

### 3.4. Study Design

The experimental study was conducted at the laboratory animal facility, veterinary microbiology, and molecular laboratory of AAU-CVMA located in Bishoftu city on mice reared from November 2023 to April 2024. During study, the mice were infected with known drug susceptible *E. coli* strain through oral exposure. Then after the confirmation of gut colonization one of these antibiotics were administered with a given dosage regimen to the randomly grouped mice. Starting from day zero of treatment, the pellet of mice was collected at seven days interval for 28 days and the samples were cultured, phenotypic antimicrobial sensitivity test (AST) was conducted by agar dilution test, and resistance genes were detected using antibody lateral flow assay and PCR. To reduce individual variation in the experimental groups, mice of the same strain, sex, and age were used. For all trials, mice were fed the same feed and housed in the same manner, and as much as possible clean environment was ensured. Contamination from the sampling process and equipment was considered, and recommended procedures were

established to mitigate bacterial contamination. Feed and water provided ad libitum. At the end of the experiment, the mice were euthanized.

### 3.5. Mice Infection, Treatment and Sample Processing

#### *3.5.1. Bacterial exposure and gut colonization detection*

Both the experimental and control groups of mice were infected orally with antimicrobial susceptible strain of known *E.coli* species (*E. coli ATCC 25922),* based on the previous study 1.5 x $10^8$ colony forming units (CFU) of bacteria recommended per mice (Ishida *et al.,* 2007), while the naïve control group was not infected. To prepare this CFU of bacteria, the refreshed culture was used and added into sterile distilled water then the cloudiness was compared to 0.5% of McFarland standard. Gut colonization of the bacteria checked after 72 hours from fecal samples of the mice. Gut colonization with the bacteria due to contamination (absence of sterility) was also checked for naïve control group. Three pellets of mice feces were collected in a sterile test tube with 10ml of peptone water, placed in an icebox, and transported to the microbiology laboratory for further analysis. The samples were then incubated at 37°C for 24 hr. The samples were cultured on CHROMagar *E.coli* selective media at 44°C for 24 hr to confirm bacterial colonization. Bacteria were identified by growing on selective media. Subsequently, the mice were divided into experimental and control groups.

#### *3.5.2. Antibiotic administration to mice*

The mice were randomly allocated into two treatment groups and two control groups. The experimental groups received one of two antibiotics, i.e., oxytetracycline and penstrep, according to the specifications outlined in Table 1. In the case of oxytetracycline, three different dosage regimens were utilized: the first group, G-O1, received a high dose in oral stat form; the second group, G-O2, received the optimal dose for three days; and the third group, G-O3, was administered a low antibiotic dose for a duration of 10 days. This dosing regimen aligns with the standard practice of dosage administration observed in veterinary clinics and farms in Ethiopia, as well as a previous study conducted on pigs (Herrero-Fresno *et al.*, 2017). Similarly, in the case of penstrep treatment in mice, group G-P1 received a high dose for one day, G-P2 received the optimal dose for three consecutive days, and G-P3 received a low dose for three consecutive days via

intramuscular (IM) injection in the thigh muscle (Table 1). This approach also aligns the common practices employed in veterinary clinics and farms in Ethiopia for administering penstrep to combat infections in animal. Table 1 describes general guidelines for the whole study including the antibiotics used in study, different dosage regimen applied, the number mice in each group and sampling interval.

**Table 1:** Experimental study design for oxytetracycline and penstrep regimens

| Drug (route of administration) | Dose and duration of therapy per day | Group (N=5) | Sample collection time (in day) |
|---|---|---|---|
| Oxytetracycline (oral) | High: 20mg/kg/day for one day | G-O1 | 0,7,14,21,28 |
| | Optimal: 10mg/kg/day for 3 days | G-O2 | 0,7,14,21,28 |
| | Low: 5mg/kg/day for 10 days | G-O3 | 0,7,14,21,28 |
| Penstrep: 400-procaine penicillin and dihydrostreptomycin (IM injection) | High 1ml/5kg/day for stat (1 day) | G-P1 | 0,7,14,21,28 |
| | Optimal: 1ml/10kg/day for 3 days | G-P2 | 0,7,14,21,28 |
| | Low: 1ml/20kg/day 3 days | G-P3 | 0,7,14,21,28 |
| Saline water (oral) | 1ml/kg for 10 days | Positive control (Fed *E. coli*, but not treated) | 0,7,14,21,28 |
| Saline water (oral) | 1ml/kg for 10 days | Naïve control | 0,7,14,21,28 |

*Key: Group 1 of oxytetracycline (G-O1), group 2 of oxytetracycline (G-O2), group 3 of oxytetracycline (G-O3), and group 1 of penstrep (G-P1), group 2 of penstrep (G-P2), group 3 of penstrep (G-P3), N= number of mice per group.*

*3.5.3. Antimicrobial susceptibility testing*

Pellets from mice in peptone water were transported to the microbiology laboratory and cultured for 24hrs at $37^0$c the bacteria on *E. coli* selective CHROM-agar media for isolation. The isolated bacterial colonies were then subjected to antimicrobial susceptibility testing (AST) using the agar dilution method. Prior to testing, stock solutions for seven antimicrobial agents were prepared in accordance with CLSI guidelines (CLSI, 2022). Mueller-Hinton II (MH-II) agar (Accumax, India) was used in AST. Antibiotics used for AST were ciprofloxacin, erythromycin, florfenicol, gentamicin, oxytetracycline, penicillin, and trimethoprim-sulfamethoxazole (TMP-SMX) (Sigma Chemical Co, St. Louis, Mo. USA). After preparing the MH-II agar in liquid form and autoclaved, then placed in water bath at 45-$50^0$C then the recommended amount of diluted antibiotic was added to media and shaken thoroughly, which dispended to petri-dish. The bacteria from fresh culture media were transferred into a sterile test tube with saline water, mixed thoroughly, compared with 0.5 McFarland solution, diluted to $10^{-1}$ in another tube with 9ml of saline water. Finally, it was dropped on media with antibiotics were incubated in an aerobic incubator at 37°C for 24 hours and any growth on media was detected on drop area.

*3.5.4. Molecular detection of resistance gene*

DNA extraction

From the resistant isolates three well grown colony was selected and dropped into Tryptic Soy Broth (TSB) media, due to the suspension used according to DNA extraction protocol. Bacterial DNA and resistance gene extraction was conducted using QIAGEN bacterial DNA extraction Mini Kit according to manufacturer's instruction, which described in detail at (Annex I). The extraction step was as follows: cultured bacterial suspension was pipetted into 1.5 mL micro-centrifuge tube and centrifuged at 7500rpm for 5 min. Animal Tissue Lysis (ATL) Buffer was added into concentrate with a total volume of 180 µL, then 20µL of proteinase K was added, mixed by vortex, and incubated at 56°C for one hr. Then 200 µL AL Buffer was added, again mixed by vortex

for 15 s, and incubated at 70°C for 10 min. To get precipitation 200µL of 90% ethanol was added in to AL and sample, and homogenized by thorough vortex for 15 s. Between each of the previous three steps, the tube centrifuged to remove drops from lid. Then the mixture of AL, alcohol and sample were added into the QIAamp Mini spin column, the cap closed, and centrifuged at 8000 rpm for 1 min. Finally, 500 µL Buffer AW1(washing buffer 1) added into QIAamp Mini spin column, and centrifuged at 8000 rpm for 1 min and 500 µL Buffer AW2 (washing buffer 2) added to the cap, then it was closed and centrifuged at 14,000 rpm for 3 min. (QIAGEN, Germany).

DNA amplification in conventional PCR machine

The PCR was conducted by conventional PCR machine; here master mix prepared for one round and reaction volume was described below. During amplification of DNA the machine were set at initial denaturation step at 95°C for 7 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45s, extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. To run on conventional PCR the total volume of amplification solution which include master mix primers, water, DMSO, extracting DNA template was prepared as described in Table 2 below.

**Table 2:** Conventional PCR master mix preparation and their volume

| No | Reagents | For one reaction | For total reaction |
|---|---|---|---|
| 1 | Go Taq Master mixes | 10 µL | 80 µL |
| 2 | Primers (forward) | 1 µL | 8 µL |
| 3 | Primers (reverse) | 1 µL | 8 µL |
| 4 | Water | 6.4 µL | 51.2 µL |
| 5 | DMSO | 0.6 µL | 4.8 µL |
| 6 | DNA template | 1 µL | 8 µL |
| | Total volume | 20 µL | 160 µL |

Agarose gel electrophoresis

The PCR product underwent analysis through electrophoresis using a 1% agarose gel. Initially, the mixture of agarose gel and TAE Buffer (Tris-Acetate-EDTA) was heated in a microwave oven and then cooled to 55°C. Subsequently, 4µl of Gel Red nucleic acid stains with loading dye was incorporated, and the resultant gel was poured onto a gel caster. Following a 45-minute solidification period, the gel was carefully positioned in the electrophoresis tank, and the comb was removed. The assessment included the utilization of a 3000bp molecular marker (ladder) in the first lane, while the subsequent lanes accommodated PCR amplified samples and non-template material. The electrophoresis process involved connecting the gel-running tank to a power supply and running it at 50 volts for 40 minutes. The examination of the gels was conducted using a UV trans illuminator gel documentation system, with the DNA bands being captured using a polaroid photographed camera. The outcome was read based on the sizes of the bands formed within the agarose gel.

*3.5.5. Lateral flow assay*

In addition to PCR, antibody coated lateral flow assay for both CTX-M and CARBA-5 (KPC, NDM, VIM, IMP, OXA-48-like) genes, were conducted which were the most known resistance genes in *E.coli* for ESBL and carbapenemase resistance, respectively.

During the test, five drops (150 µL) of extraction buffer were dispensed into one of the micro tubes provided in the kit. From the agar culture, three colonies were touched with a loop, and then suspended in the micro tube containing 150 µL of extraction buffer. The micro tube was closed, and the mixture was vortexed to homogenize it before use. Then the pouch was opened, and the device was taken out. Using the provided pipette, 100 µL of the prepared mixture was added, ensuring the sample reached the black line indicated on the pipette to accurately aspirate 100µl. The results were read at 15 minutes as described in ANEX II and III.

### 3.6. Data Analysis

The data obtained from cultured sample and susceptibility test were feed into Microsoft Excel 2010 and analysis conducted by STATA (STATA/IC version 14.0, 2015), statistical software. Descriptive statistical methods were used to describe the nature and the characteristics of the data. Association between the difference in duration of dosage regimen in different group on AMR growth and AMR growth for different antibiotics were assessed using univariate Logistic regression analysis and Chi-square ($\chi 2$) test.

### 3.7. Ethical Statements

This study received ethical clearance from Institutional Animal Research Ethical Review Committee of the Addis Ababa University College of Veterinary Medicine and Agriculture with certificate (ref. No: VM/ERC/04/22/16/2024). The certificate is attached and see details in Annex IV. All procedures were conducted according to animal research ethics.

### 3.8. Study Limitation

This study focused on the AMR development and growth characteristics of *E. coli* due to antibiotics selection pressure but not concerned on efficacy of different treatment

regimen. This make it difficult to give conclusion on which treatment regimen is best to use. Thus, future study should asses the combined characteristics of antibiotic treatment regimen. Another limitation in this study were due to lake of antibiotic disk the study conducted by agar dilution test which is insufficient to detect the strength of resistance in bacteria at different time interval.

## 4. RESULTS

This study evaluated the effect of using different dosage regimen of two widely prescribed antibiotics in food animals in Ethiopia, namely oxytetracycline and penstrep, on the development of AMR in *E. coli*. By subjecting known susceptible *E. coli* strains to varying treatment regimens of oxytetracycline and penstrep, the study elucidated the dynamics of AMR. The findings of this study showed that the adaptive responses of *E. coli* to oxytetracycline and penstrep exposure, revealing nuances in the emergence and persistence of AMR. Again, the result describes the MDR emergence and characterizes common resistance genes in bacteria due to exposure to these antibiotics.

### 4.1. Antimicrobial Susceptibility test result

From the whole sample collected and characterized to detect the resistance growth by AST, the current study revealed that higher proportion of AMR was detected in penstrep treated groups (31.4%), followed by oxytetracycline groups (24.7%). Infected and naïve control groups had 1.9% and 0.5% resistance respectively, indicating selection pressure effects. During the AMR growth detection for each antibiotic treatment, the highest proportion of resistance was detected in treatment groups, particularly in *E. coli* isolated from samples collected from penstrep treated groups, followed by oxytetracycline treatment (Figure 1).

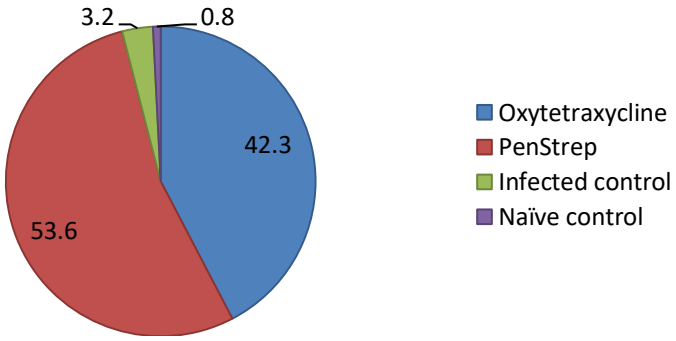


**Figure 1**: Percent of resistance for each groups from total resistance detected.

*4.1.1. Antimicrobial resistance profile for different treatment and dosage regimens*

The susceptibility profile of the bacteria (*E. coli*) to seven different classes of antibiotics was assessed. The result of the study indicated that varied percentages of resistance was detected (Table 3). Higher resistance was recorded for penicillin (70%) by isolates from G-P3 and oxytetracycline (70%) by isolates from G-O3, followed by gentamicin and TMP-SMX (60% each) by isolates from G-P3. In addition, the association of dosage regimen with AMR growth in each study model was also evaluated in relation with specific antibiotics. The result showed that gentamicin resistance growth was significantly associated with treatment regimen variation of both oxytetracycline and penstrep.

**Table 3**: Antibiotic resistance profile of *E. coli* isolated from various experimental groups against the tested seven antibiotic classes

| **Group** | **Resistance to the tested antibiotics (%)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | CIP | GEN | FL | OXT | PEN | TMP-SMX | E |
| G-O1 | 10.0 | 0.0 | 20.0 | 20.0 | 10.0 | 20.0 | 40.0 |
| G-O2 | 30.0 | 10.0 | 20.0 | 40.0 | 30.0 | 10.0 | 40.0 |
| G-O3 | 30.0 | 40.0 | 40.0 | 70.0 | 20.0 | 30.0 | 50.0 |
| *P-value* | *0.475* | *0.044* | *0.506* | *0.076* | *0.532* | *0.535* | *0.873* |
| G-P1 | 30.0 | 0.0 | 30.0 | 20.0 | 25.0 | 37.5 | 50.0 |
| G-P2 | 20.0 | 20.0 | 20.0 | 30.0 | 30.0 | 20.0 | 40.0 |
| G-P3 | 30.0 | 60.0 | 40.0 | 30.0 | 70.0 | 60.0 | 50.0 |
| *P-value* | *0.664* | *0.015* | *0.587* | *0.627* | *0.094* | *0.186* | *0.879* |
| G-7 | 0.0 | 0.0 | 0.0 | 10.0 | 30.0 | 10.0 | 30.0 |
| G-8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 |

Key: *G-7, group seven (infected control); G-8, group eight (naïve contro); G-O1, group one of oxytetracycline; G-O2, group two for oxytetracycline; G-O3, group three of oxytetracycline; G-P1, group 1 of penstrep; G-P2, group two of penstrep; G-P3, group three of penstrep; CIP, ciprofloxacin, FL, florfenicol; GEN, gentamicin; OXT, oxytetracycline; TMP-SMX, trimethoprim-sulfamethoxazole; PEN, penicillin; E- erythromycin.*

The overall resistance profile of the model bacteria isolated from treatment and control groups to seven different classes of antibiotics, including the antibiotics used in this trial were also assessed. The result showed that the oxytetracycline in orally treated groups with oxytetracycline and penicillin in penstrep injected groups have showed greatest resistance throughout the study period. In addition, gentamicin in oxytetracycline treatment model and ciprofloxacin in penstrep treatment model showed the lowest AMR growth in comparison to other antibiotics used in AST. In both control groups, there is no greater resistance detected and most of antibiotic did not show any resistance as described on Figure 2.

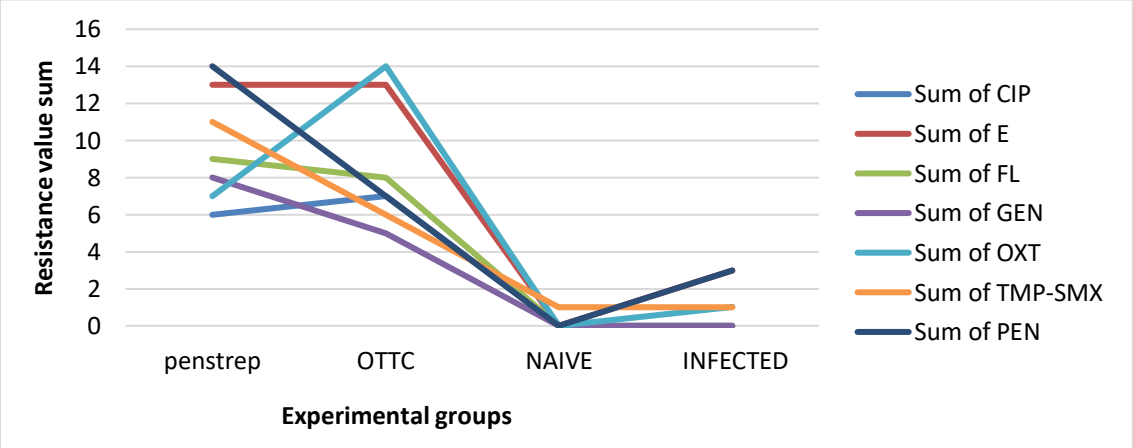


**Figure 2**: Antibiotic resistance profile in two treatment models and control groups.

*In this graph, horizontal line indicates the treatment groups, which represented as OTTC, for oxytetracycline treated mice group; Penstrep, for penstrep treated mice group; Infected, for mice group they are infected but not treated with antibiotics or they take saline for 10 days; Naïve, for non-infected and non-treated groups. The Sum of CIP- Ciprofloxacin total for each group, Sum of FL- Florfenicol total for each group, Sum of GEN- gentamycin total for each group, Sum of OXT- oxytetracycline total for each group, Sum of TMP - SMX - trimethoprim-sulfamethoxazole total of each groups, sum of PEN - penicillin total for each groups, sum of E- erythromycin total for each group.*

### *4.1.2. Antimicrobial resistance at different duration of time*

Bacteria isolated from mice under different treatment at different time duration were also evaluated for resistance. The result showed that there was no resistance detected till day

seven except for oxytetracycline and erythromycin, while the resistance status increased from time to time and reach maximum especially in erythromycin it reached 100% resistance at day 28. AMR growth against most of the tested antibiotics (GEN, FL, OXT, PEN and E) has significant association with length of time for oxytetracycline treated bacteria ($p < 0.05$) as described in Table 4. Similarly, the total resistance recorded by bacteria isolated from mice treated with different regimens of penstrep at different point of time showed that resistance was detected at day seven for penicillin and erythromycin (Table 4). The finding revealed that resistance status has increased from time to time and reach maximum (100%), particularly for erythromycin it on day 28. AMR growth has significant association with time length only for E and TMP-SMX used in susceptibility test for penstrep treated bacteria as described in (Table 4).

**Table 4**: Antibiotic resistance profile of *E. coli* isolated from oxytetracycline and penstrep based treatment model at different time intervals

| **Treatment** | **Days** | **Resistance to the tested antibiotics (%)** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **groups** | | CIP | GEN | FL | OXT | PEN | TMP-SMX | E |
| OXT | Day 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Penstrep | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| OXT | Day 7 | 0.0 | 0.0 | 0.0 | 16.7 | 0.0 | 0.0 | 16.7 |
| Penstrep | | 0.0 | 0.0 | 0.0 | 0.0 | 25.0 | 0.0 | 33.3 |
| OXT | Day 14 | 33.3 | 0.0 | 16.7 | 50.0 | 16.7 | 16.7 | 33.3 |
| Penstrep | | 33.3 | 33.3 | 33.3 | 33.3 | 33.3 | 50.0 | 50.0 |
| OXT | Day 21 | 33.3 | 33.3 | 66.6 | 66.7 | 16.7 | 33.3 | 66.7 |
| Penstrep | | 33.3 | 33.3 | 50.0 | 16.6 | 66.6 | 66.6 | 50.0 |
| OXT | Day 28 | 50.0 | 50.0 | 66.6 | 83.6 | 66.7 | 50.0 | 100.0 |
| Penstrep | | 33.3 | 50.0 | 66.6 | 50.0 | 66.6 | 66.6 | 100.0 |
| OXT | *P-value* | *0.15* | *0.048* | *0.024* | *0.020* | *0.024* | *0.132* | *0.003* |
| Penstrep | *P-value* | *0.374* | *0.194* | *0.064* | *0.349* | *0.108* | *0.032* | *0.013* |

*Key: CIP- ciprofloxacin, FL- florfenicol, GEN- gentamicin, OXT- oxytetracycline, TMP-SMX - trimethoprim- sulfamethoxazole, PEN - penicillin, E- erythromycin.*

*4.1.3. Multidrug resistance status of different dosage regimens at different time duration*

In the study, antibiotics from seven different classes (Penicillin, Tetracycline, Aminoglycoside, Fluoroquinolone, Macrolide, and Phenicol) were used to conduct AST. Isolate that showed resistance for three or more antibiotics class was considered as MDR bacteria. The MDR growth status of *E. coli* in different treatment regimens of oxytetracycline and penstrep is presented below.

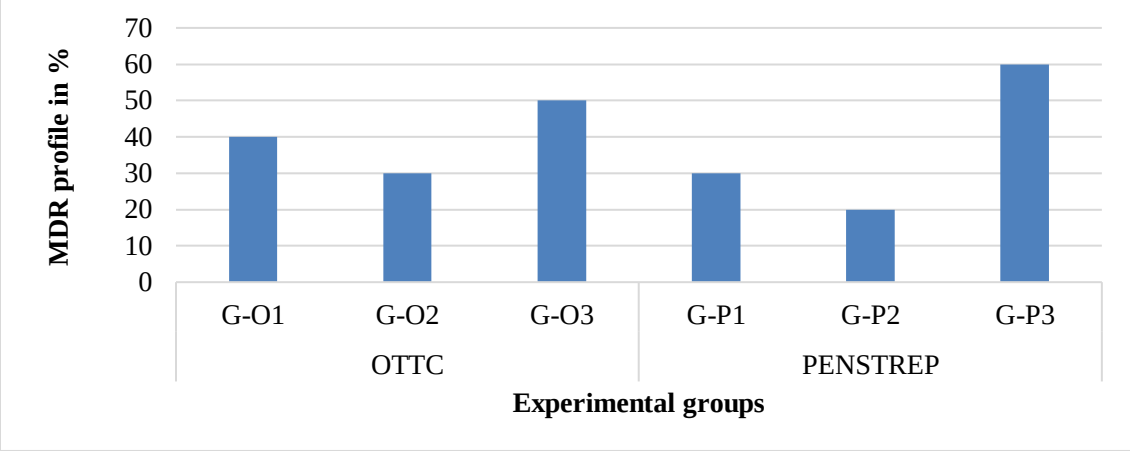


**Figure 3.** MDR profile of both oxytetracycline and penstrep treated groups with different treatment regimens.

*Key: G-O1 group one of oxytetracycline, G-O2 group two for oxytetracycline, G-O3 group three of oxytetracycline, G-P1 group 1 of penstrep, G-P2 group two of penstrep, G-P3 group three of penstrep, G-7 group seven, G-8 group eight, OTTC- oxytetracycline treated groups, PENSTREP- penstrep treated groups. The Y - axis in figure shows MDR profile of mice in different dosage regimen for oxytetracycline and penstrep in percent from total sample collected from each group (n=10).*

The result also showed that MDR was not detected at the first two rounds of sampling, while its detection was increased at the following consecutive sampling (Table 5), and it was strongly associated with duration of time in both oxytetracycline and penstrep treated groups ($p < 0.05$). Detection of MDR isolates varies in different groups and especially it increased after day 21, but there was no significant association within dosage regimen in both treatment models ($p > 0.05$). *E. coli* isolated from two control groups (infected but

not treated with antibiotics and naive) are negative for MDR detection throughout sampling period. The MDR status of *E.coli* in different treatment regimen of oxytetracycline and penstrep at different time interval is presented in Table 6.

**Table 5**: Multidrug resistance profile of *E. coli* treated with oxytetracycline and penstrep at different time interval

| **Day** | **Antibiotics showing resistance** | **Experimental group** | **Number of isolates showing resistance** |
|---|---|---|---|
| Day 1 | Not detected | Not detected | Not detected |
| Day 7 | Not detected | Not detected | Not detected |
| Day 14 | CIP E OXT | GO3 | 1 |
| | CIP E OXT PEN TMP-SMX | GP3 | 2 |
| Day 21 | E FL OXT | GO1 | 2 |
| | CIP FL OXT TMP-SMX | GO2 | 1 |
| | CIP E FL GEN OXT TMP-SMX | GO3 | 2 |
| | CIP E FL PEN | GP1 | 1 |
| | OXT PEN TMP-SMX | GP2 | 1 |
| | CIP E FL OXT PENTMP-SMX | GP3 | 2 |
| Day 28 | E FL GEN OXT | GO1 | 2 |
| | CIP E GEN OXT | GO2 | 2 |
| | CIP E FL GEN OXT TMP-SXM | GO3 | 2 |
| | E FL PEN TMP-SXM | GP1 | 2 |
| | CIP E GEN OXT PEN | GP2 | 1 |
| | CIP E FL OXT PEN TMP-SXM | GP3 | 2 |

*Key: CIP- ciprofloxacin, FL- florfenicol, GEN- gentamicin, OXT- oxytetracycline, TMP - SMX - trimethoprim-sulfamethoxazole, PEN-penicillin, E-erythromycin. G-O1 group one of oxytetracycline, G-O2 group two for oxytetracycline, G-O3 group three of oxytetracycline, G-P1 group 1 of penstrep, G-P2 group two of penstrep, G-P3 group three of penstrep, G-7 group seven, G-8 group eight.*

## 4.2. Detection of Resistance Gene

### *4.2.1. Lateral flow assay for CTX-M and CARBA-5*

In this study, 16 samples were evaluated for CTX-M while 9 randomized samples were tested for CARB-5 resistance gene (VIM, KPC, NDM, OXA, and IMP) using lateral flow assay. The result of the test showed no positive for the tested gene, as represented by letter: N, I, V, O and K, while letter C represent the control line. Therefore, the microorganisms are genotypic negative to both CTX-M and CARBA-5 gene groups (annex 5).

### *4.2.2. Molecular detection of resistance genes*

PCR was used to detect both detection of *E. coli* and resistance genes. One randomly selected *E. coli* isolate showing MDR to the tested antibiotics from each treatment groups (6 isolates randomly selected from 80 samples) was tested for both resistance genes and confirmation for being *E. coli*. The target resistance genes were *bla*SHV, *bla*CTXM, *bla*TEM or *gyr*A that could possibly expressed due to the exposure of bacteria to antibiotics. The study finding showed that all of the 6 isolates become positive for *E. coli* with of 990 bp and are positive for *bla*TEM with 500bp length (Figure 4). However, other resistance genes (*bla*SHV, *bla*CTXM, and gyrA) were not detected in this study. The current PCR analysis result was agreed with lateral flow assay test result of the current study, as all tested samples were *bla*CTXM negative.

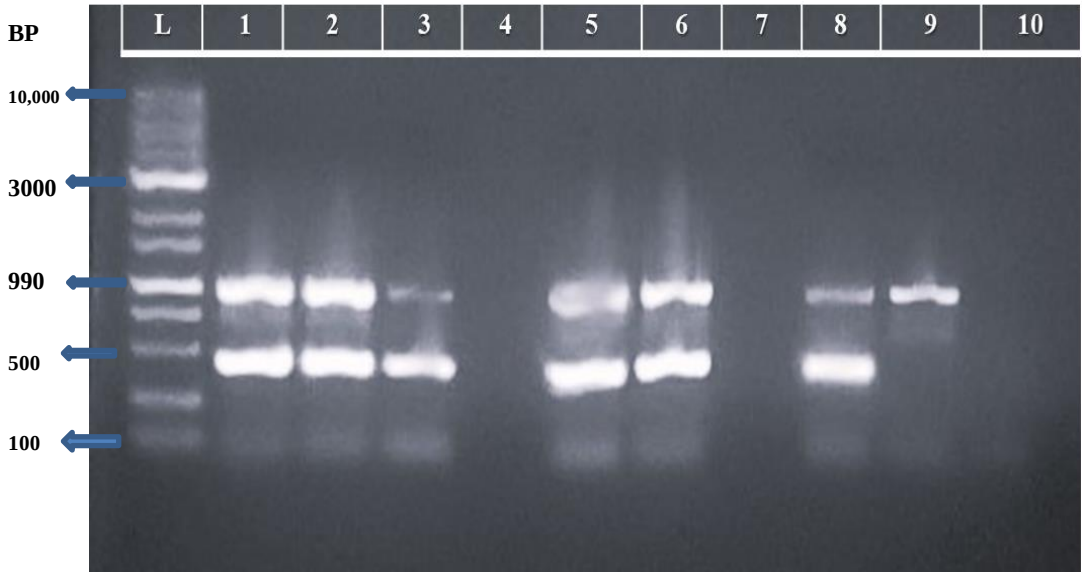


**Figure 4**: Agarose gel electrophoresis for the detection of *E. coli* and antibiotic resistance gene.

*Lane L, 10,000bp DNA ladder; Lane 1-3, 5, 6 and 8 are gene of E. coli and blaTEM gene at 990 bp and 500bp product size respectively; Lane 1, isolate from G-P3; Lane 2, isolates from G-P2; Lane 3, isolates form G-O3; Lane 5, isolates from G-O2; Lane 6, isolates from G-O1; Lane 8, isolates from G-P1; Lane 4 and Lane 7, no sample added; Lane 9, positive control for E. coli, Lane 10, negative control for E. coli*

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## 5. DISCUSSION

In this study, laboratory mice were used as model organisms to observe the emergence of AMR in a recognized susceptible strain of *E.coli* (ATCC 25922). Following the screening of mice and their cages for any prior contaminations, the presence of potential mice infections prior to the experiment was assessed. Subsequently, the resistance profiles of these bacterial isolates obtained from the collected samples were determined using the agar dilution method. Klebsiella and *E.coli* were the only detected during the testing, while the antimicrobial susceptibility testing (AST) revealed that not any isolates exhibited resistance to the various drugs prepared according to CLSI standards. Sample collection involved pooling each group into two pairs in a randomized manner, resulting in the collection of 16 samples at a single time point from six experimental and two control groups and totally 80 pooled samples were collected from the entire sampling round.

This research was consistent with a previous study conducted by Sedláková *et al.* (2014), which emphasized a clear and significant link between the exposure to antibiotics and the development of resistance in Enterobacteriaceae. Furthermore, Rovira (2023) observed that the administration of oxytetracycline led to the creation of selective pressure, resulting in an increase in the prevalence of tetracycline-resistant genes in treated animals. However, the findings of this research contrast with those of Vanbaelen *et al.* (2024), who identified no connection between the use of antimicrobials and antimicrobial resistance. Nevertheless, that study recognizes the constraints of its methodology and the brief timeframe of the investigation, which was cross-sectional format carried out in a field, unlike the controlled environment of this study.

In this study, using high concentrations of antimicrobials were shown to produce selection pressure on microbial resistance growth in both oxytetracycline and penstrep treatment model. Berge *et al*. (2005) who studied bacterial resistance patterns in *E. coli* after an aggressive treatment of a single dose observed an increase in the number of resistant *E. coli* isolates which agrees with this study. However, this study disagreed with

Singh and Tam (2011), who reported that an aggressive treatment regimen suppresses the growth of AMR bacteria even in the presence of preexisting resistance gene amplification, our study suggests otherwise. This disagreement linked to the differing methodologies employed; specifically, our study was conducted in-vivo, whereas the prior study utilized an in-vitro approach involving exposing bacteria to antibiotics for a short period.

The research demonstrates that even when oxytetracycline administered at therapeutic levels, *E. coli* develop a significant level of resistance. This finding was consistent with a study conducted by Keijser *et al.* (2019) and Pokrant *et al*. (2023) which revealed increased expression of the tetracycline resistance gene *tet*M in individuals receiving oral therapeutic doses of oxytetracycline. The resistance to therapeutic doses of oxytetracycline is due to incomplete elimination of bacteria. This resulted in sub lethal antibiotic concentrations over time that favors survival and proliferation of bacteria with resistance mechanisms. Treatment with oxytetracycline at therapeutic levels for a period of three days resulted in higher resistance (40%) to both erythromycin and oxytetracycline.

Fairchild *et al.* (2005) investigated the effects of long-term tetracycline administration on commensal bacteria from commercial poultry and found that Enterococcus species and *E. coli* were resistant to tetracycline (32.2 %). Similarly, in this study, low dose treatment for long time showed comparatively higher resistance to most antibiotic classes. In this group, oxytetracycline showed 60% resistance, which was greater than both the high dose and optimum dose treatments, and erythromycin was the second drug to record higher doses up to 50%. This result indicates that sub-inhibitory antibiotic concentrations can select antibiotic-resistant phenotypically higher than short and optimum treatments, which agrees with Tam *et al.* (2007).

In the comparison of different treatment regimens of oxytetracycline, AMR develops more in long-term treatment of antibiotics with a lower dose than the optimum dose form and treatment with a high dose in short time duration. This result aligns with those

reported by Geli *et al.* (2012) and Ahamed *et al.* (2015). This is because bacteria exposed to sub lethal concentrations have increased opportunities to adapt and develop resistance mechanisms, as antibiotics do not immediately kill them. However, there was no significant relationship between AMR growth and treatment of animals with different dosage regimens ($p > 0.05$). This was in agreement with a previous study by Herrero-Fresno *et al.* (2017) which showed that the dosage of oxytetracycline used during treatment and the mode of application did not have a significant influence on the selection of coliform bacteria. Erythromycin was particularly less effective against gram-negative bacteria due to its size and the outer membrane of these bacteria resulting in greater resistance.

Another study showed the synergistic efficacy of a penicillin-streptomycin combination in comparison with two antibiotics used alone with reducing effect of resistance (Lorian, 2005). However, similar to oxytetracycline treatment, penstrep had AMR development selection pressure in different treatment regimens in this study. This agrees with a study by Kone *et al.* 2019, which showed that treatment with penstrep increased *E. coli* species resistance to different antibiotics tested. In the aggressive treatment of penstrep for a short time or at a single dosage, the most resistant drug was erythromycin 50%, which is similar to the highest resistance status in oxytetracycline treatment and similar to the optimum dose treatment group in the penstrep. In this dosage regimen, there was no detected gentamycin resistance throughout the experimental period.

For penstrep treatment group of optimum doses for three consecutive days, the study result showed that higher resistance was emerged for erythromycin 40%. In addition, this group shows the development of 30% resistance for oxytetracycline and penicillin, which shows slight increase than an aggressive treatment regimen. At low dose of pestrep for three days most resisted antibiotics was penicillin, up to 70% of sample detected in antimicrobial susceptibility test for penicillin were become resistant. In exception from other treatment model, in this dosage regimen gentamycin shows higher resistance profile with 60% resistance.

In penstrep treatment, AMR development vary from one regimen to another in decreasing order of low treatment for three-day, aggressive treatment stat and therapeutic dose for three days. Maintaining the optimal dose over several days ensures that bacteria are continuously exposed to effective drug levels, reducing the chances of sub-therapeutic exposure that can select for resistant strains (Lees *et al.*, 2005), which showed less AMR result in this study for optimum dosage treatment than other. However, the variation in between treatment regimen and AMR growth was not significant association for all antibiotics used in antimicrobial susceptibility test except for gentamycin that have *P-value* = 0.015, but other drugs show the $P > 0.05$.

Great resistance in lower dose for long time in both oxytetracycline and penstrep treatment was due to a sub-optimal dose were not be sufficient to kill all the bacteria, leading to incomplete eradication and allowing the surviving bacteria to persist. This creates selective pressure that inhibits susceptible bacteria but is not strong enough to kill ones that are more resistant and these can multiply (Zaborskyte *et al.*, 2017). Erythromycin is particularly less effective against gram-negative bacteria due to its size in addition, the outer membrane of these bacteria resulting in greater resistance. Even if the site of action of penicillin was completely different from oxytetracycline there was resistance profile detected this was due to *E.coli* are part of species that present intrinsic resistance to penicillin G (Scott, 2009).

The resistance status between two-treatment model and control groups was compared, with oxytetracycline covering 42.3% of total resistance. Penstrep exhibited higher resistance than oxytetracycline and control groups, accounting for 53.6% of the total resistance. The two control groups did not show resistance exceeding 5% of total recorded. This result indicates the impact of antibiotic exposure on AMR development. Penstrep's broad-spectrum effect may disrupt intestinal microbiota more significantly than oxytetracycline, leading to decreased microbial diversity and domination of resistant bacteria. Penstrep targets cell wall and protein synthesis, while oxytetracycline inhibits protein synthesis by binding to the bacterial ribosome. Due to broader mechanism of

action bacteria developing resistance to these combined antibiotics may become resistant for more antibiotics.

During AMR detection of oxytetracycline, resistance growth varied over time intervals with seven-day gaps. No resistant bacteria were observed on day zero, few developments on day seven, and high resistance on days 21 and 28. The study contradicts Graesboll *et al.* (2017) by showing resistance development over time, unlike their findings of rapid non-resistance after high tetracycline doses. However, this study aligns with Rovira (2023) who found resistance at day 14 with increasing rate, suggesting bacteria may need more time for mutation or adaptation. Resistant bacteria may not dominate initially, but over time, they replicate and dominate the generation.

For penstrep treatment model the AMR status, regarding duration of time showed there was direct increase of resistance to different antibiotic class with increasing time. At day one in similar manner with oxytetracycline the bacteria do not show any resistance but increase with consecutive sampling period with variation in between groups, especially the treatment regimen of low dose for three days show up to 83% and 100% of resistance for penicillin and erythromycin respectively at day 28.

In addition to simple variations, there was a significant association between the time gaps after the exposure of bacteria to antibiotics and AMR, except for ciprofloxacin and trimethoprim-sulfamethoxazole for oxytetracycline treated groups and erythromycin, and trimethoprim-sulfamethoxazole for penstrep treated groups. Our study observed an increase in antibiotic resistance over time, which contrasts with previous findings Opatowski *et al.* (2011) and Spicknall *et al.* (2013) reported a reduction in resistance status with increased post-treatment time. This was because this study employed a longer follow-up period, allowing us to capture the long-term dynamics of bacterial populations.

Selection with one antimicrobial can have selection pressure for other antibiotic resistance because they might be co-localized on the same genetic element or may use the common AMR mechanism (Katakweba *et al.*, 2015). In this study, seven antibiotics (ciprofloxacin, florfenicol, erythromycin, penicillin, oxytetracycline, gentamycin, and

trimethoprim-sulfamethoxazole) were used to detect bacterial resistance growth different antibiotic classes. Ambrose *et al.* (2005) and Rovira (2023) showed that oxytetracycline usage was also associated with increased ARGs conferring resistance to other antibiotics. In agreement with previous studies, this study demonstrated that bacteria exhibit resistance to various antibiotics other than the administered antibiotics.

During the MDR assessment, isolates showing resistance to three or more antibiotic classes were considered multidrug resistant based on international expert proposals (Magiorakos *et al.,* 2012). In both treatment models, no MDR was detected up to day fourteen; however, at day fourteen, only the low-dose, long-term treatment showed MDR. Analyzing the association of MDR with the time elapsed after bacterial exposure to antibiotics revealed a strong significant association between time and the emergence of MDR in both antibiotic treatments, with *p-values* of *0.005* and *0.004* for oxytetracycline and penstrep, respectively. This result indicates that once bacteria are exposed to antibiotics, the incidence of MDR increases exponentially over time due to the development of adaptation mechanisms, including sub-inhibitory antibiotic concentrations, vertical and horizontal gene transfer.

In comparison between the dosage regimens, long time treatment with low dosage shows strong MDR than therapeutic and high dose treatment again they develop MDR in short time than other dosage form. In addition, the exposure of antibiotics with sub inhibitory concentration without complete withdrawal from body can act long time selection pressure on bacteria that promote more mutation in bacterial gene and adaptation to escape different antibiotic mechanism. However, there was no significant association in between dosage regimen variation and MDR growth in both penstrep and oxytetracycline treatment with *p-value 0.1599* and *0.3595*, respectively.

Regarding the MDR growth comparison between the two treatment models and the control groups, this study showed that multidrug resistance was only observed in the treatment groups, with no MDR isolates in either control group. When comparing the variation of MDR between the oxytetracycline and penstrep treatments, there was no

significant difference, with a *p-value* of 0.791. However, greater MDR was observed in the penstrep treatment than in the oxytetracycline treatment. This was likely because penstrep is a combination treatment, leading bacteria to develop mechanisms for resistance to two antibiotic classes, increasing the chance for co-selection of resistance to a broader range of antibiotics compared to oxytetracycline.

Due to the known bacterial strain administered to the mice, all samples tested became positive for the gene, confirming that the bacteria on which the AST test was conducted were *E. coli*. The study conducted to detect the common resistance genes (*bla*TEM, *bla*SHV, and *bla*CTX-M) for penstrep exposure and (gyrA) gene for oxytetracycline. The resistance gene detected in this study was the *bla*TEM gene, in both treatment models. The strong selective pressure exerted by the antibiotic ensures that bacteria acquiring the resistance gene gain a substantial survival advantage, leading to the emergence and eventual dominance of resistant strains. While oxytetracycline targets protein synthesis rather than cell wall synthesis (the target of β-lactams), spontaneous mutations can still occur, although direct mutations to *bla*TEM may be rare under oxytetracycline pressure, other resistance mechanisms or genes can still arise.

In this study, no drugs from the carbapenem class were administered or used for antimicrobial susceptibility testing of isolates due to lack antibiotics from this class. However, a lateral flow test was conducted to detect the development of carbapenem resistance genes (VIM, OXA, NDM, KPC, and IMP) in 10 randomly sampled isolates. The results showed that all samples were negative for carbapenem resistance genes. Nonetheless, this result does not necessarily indicate that all samples were susceptible to all carbapenem antibiotics, as there may still be phenotypic resistance present in the bacteria.

## 6. CONCLUSION AND RECOMMENDATIONS

This study evaluated the exposure of model bacteria to different antibiotic regimens widely practiced in the livestock with limited resources on the emergence of AMR. It was found that there was a significant likelihood of antibiotic resistance emerging in susceptible *E. coli* subjected to antibiotics pressure. The finding indicated that cross-resistance or co-resistance to antibiotics from different classes was observed in addition to resistance development for the exposed antibiotics. Additionally, this study also found that, over time, bacteria developed AMR to various antibiotic classes, leading to the detection of MDR as a result of exposure to different dosage forms with varying magnitudes. The highest selection pressure for the emergence of AMR in *E. coli* was detected when bacteria exposed to sub-inhibitory concentrations for a prolonged period. This was followed by the administration of high doses for a short duration, while using both antibiotics at their optimum (recommended) doses was linked to a decreased emergence of AMR. The study detected the *bla*TEM gene, which is responsible for resistance to beta lactam antibiotics.

Based on the conclusion of this finding, the following recommendations are forwarded:

- Use of alternatives to antibiotic treatment is recommended to reduce AMR emergence by gut microbiota that could contaminate the environment and be source of resistance genes to pathogenic bacteria. However, if antibiotic treatment becomes mandatory, reducing exposure duration with optimum dose is crucial.
- Further studies are needed to determine the optimal treatment regimen for antibiotics like penstrep and oxytetracycline, focusing on efficacy and resistance growth for the shortest duration.
- Understanding the mechanism of action of a drug is crucial before using antibiotics, as bacteria may develop resistance to previously exposed drugs.

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## 8. APPENDICES

**Annex I:** Protocol for NG-TEST CTX-M

### Culture and sampling

The samples to be assessed shall be obtained and handled according to the standardized microbiology procedures. A colony will be collected in a solid agar-based culture, then will be suspended in the extraction buffer provided into the kit. It is highly recommended to use fresh bacterial colonies for the assay performance to be optimal. Validated culture media Mueller Hinton (MH) agar, URIselect 4 (URI-4), Columbia agar + 5% horse blood, ChromID ESBL agar, Drigalski (DRIG) agar.

### Operating procedure

1. Wear protective gloves.
2. Bring the kit components at room temperature for at least 10 minutes.

Preparing the sample

1. Dispense 5 drops (150 µL) of extraction buffer in one of the micro tubes provided into the kit.
2. From a solid agar-based culture, collect a colony with a loop, and then suspend it in the micro tube containing 150 µL of extraction buffer.
3. Close the micro tube.
4. Vortex to homogenize the mixture before use.

**NOTE**: Mucous colonies can lead to migration problems, due to their high viscosity. Vortex for 3 minutes a colony in extraction buffer and incubate for 10 minutes at room temperature before performing the test.

**Conducting the test**

1. Open the pouch, and take out the device. Once opened, use the test immediately.
2. Using the provided pipette, add 100 µL of the prepared mixture (sample must reach the black line indicated on the pipette to accurately aspirate 100 µL) in the sample well-labelled "S".
3. Read the results at 15 minutes and interpret them as indicated below.

**NOTE:** Do not interpret the test results after 20 minutes, as they may vary possibly causing false positive results.

**Result interpretation**

**Negative result**

If only one red line appears on the control region (C): the sample does not contain any CTX-M enzyme or nondetectable level of this one and must be interpreted as a negative result.

**Positive result**

If two red lines appear, one on the control region (C) and one on the test region (T): the sample contains CTX-M enzyme and must be interpreted as a positive result.

**NOTE**: The intensity of the red test line (T) may vary depending on the CTX-M enzyme level in the sample. A weak line should be considered as a positive result.

**Invalid result**

If the control line (C) does not appear, the test result is invalid. Insufficient sample volume or an incorrect procedure is the most likely reasons for control line failure. Deterioration of the test kit may have occurred. Repeat the procedure using a new test. If the problem persists, do not reuse the kit, and contact your distributor.

**Annex II:** Protocol and procedure of NG-Test CARBA 5

**Culture and sampling**

The samples to be analyzed shall be obtained and handled according to Standardized microbiology procedures.

**Operating procedure**

1. Wear protective gloves and standard personal protective equipment.
2. Bring the kit components to room temperature for at least 10 minutes.

**Preparing the sample**

1. Dispense 5 drops (150 µL) of extraction buffer in one of the micro tubes provided into the kit.
2. From the agar culture, touch three colonies with a loop, and then suspend it in the micro tube containing 150 µL of extraction buffer.
3. Close the micro tube.
4. Vortex to homogenize the mixture before use.

**Carrying out the test**

1. Open the pouch, and take out the device. Once opened, use the test immediately.
2. Using the provided pipette, add 100 µL of the prepared mixture (sample must reach the black line indicated on the pipette to accurately aspirate 100 µL) in the sample well-labelled "S".
3. Read the results at 15 minutes and interpret them as indicated below.

**Note:** Do not interpret the test results after 15 minutes.

**Result interpretation**

**Negative result**

If only one red line appears in the control region (C) the sample does not contain any carbapenemase or contains carbapenemase(s) at a non-detectable level and must be interpreted as a negative result.

**Positive result**

If one red line appears in the control region (C) and one or several lines appear in the test regions K (KPC), O (OXA-48- like), V (VIM), I (IMP), N (NDM): the sample contains one or several carbapenemases and must be interpreted as a positive result. The intensity of the red test line(s) may vary. A weak line is a positive result.

**Invalid result**

If the control line (C) does not appear, the test result is invalid. Insufficient sample volume or, incorrect sample processing are the two most likely reasons for control line failure. Deterioration of the test kit may have occurred. Repeat the procedure using a new test. If the problem persists, do not use the kit, and contact your distributor.

**Annex III:** Standard Operation Procedure for DNA Extraction

Isolation of Genomic DNA from Bacterial Suspension Cultures

1. Pipet 1 mL of bacterial culture into a 1.5 mL micro centrifuge tube, and centrifuge for 5 min at 5000 x g (7500 rpm).

2. Calculate the volume of the pellet or concentrate and add Buffer ATL (supplied in the QIAamp DNA Mini Kit) to a total volume of 180 µL.

3. Follow the "Protocol: DNA Purification from Tissues

4. Add 20-µL proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

**Note**: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

5. Briefly centrifuge the 1.5 mL micro centrifuge tube to remove drops from the inside of the lid.

6. Add 200 µL Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min.

7. Briefly centrifuge the 1.5 mL micro centrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL be mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

8. Add 200-µL ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 mL micro centrifuge tube to remove drops from inside the lid.

It is essential that the sample, Buffer AL, and the ethanol be mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

9. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.* Close each spin column to avoid aerosol formation during centrifugation. It is essential to apply all the precipitate to the QIAamp Mini spin column. Centrifugation is performed at 6000-x g (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

10. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the collection tube containing the filtrate.*

11. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000-x g; 14,000 rpm) for 3 min.

12. Recommended: Place the QIAamp Mini spin column in a new 2 mL collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

13. Place the QIAamp Mini spin column in a clean 1.5 mL micro centrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µL Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000-x g (8000 rpm) for 1 min.

14. Repeat step 12. A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

**Annex IV:** Ethical Statement


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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu


Animal Research Ethical Review Committee

## *Ethical clearance certificate*

**Certificate Ref. No: VM/ERC/04/22/16/2024**

Name of Applicant: **Timotiwos Wogaso (DVM, MSc student)**

Address: Department of Biomedical Sciences, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Effect of short- and long-term selected antibiotic treatment regimens on emergence of antimicrobial resistance in mice infected with antibiotic susceptible Escherichia coli.*

| | |
|---|---|
| Date of application: | **December, 2023** |
| Nature of the project: | **Experimental study** |
| Target animal species: | **Mice** |
| Number of animals involved: | **40** |
| Study area: | CVMA-**Bishoftu, Ethiopia** |

Minutes No. and date of review: **VM/ERC/04/16/024, 16/05/2024**

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 **Timotiwos Wogaso.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature


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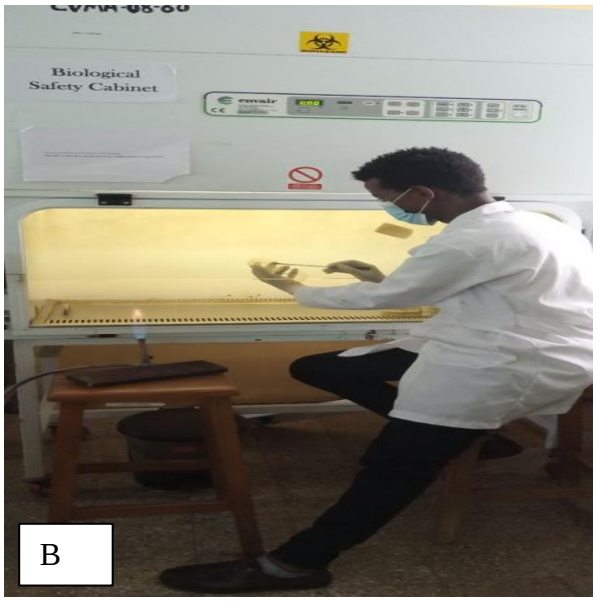
**ANNEX V:** Different Picture of the whole research activity

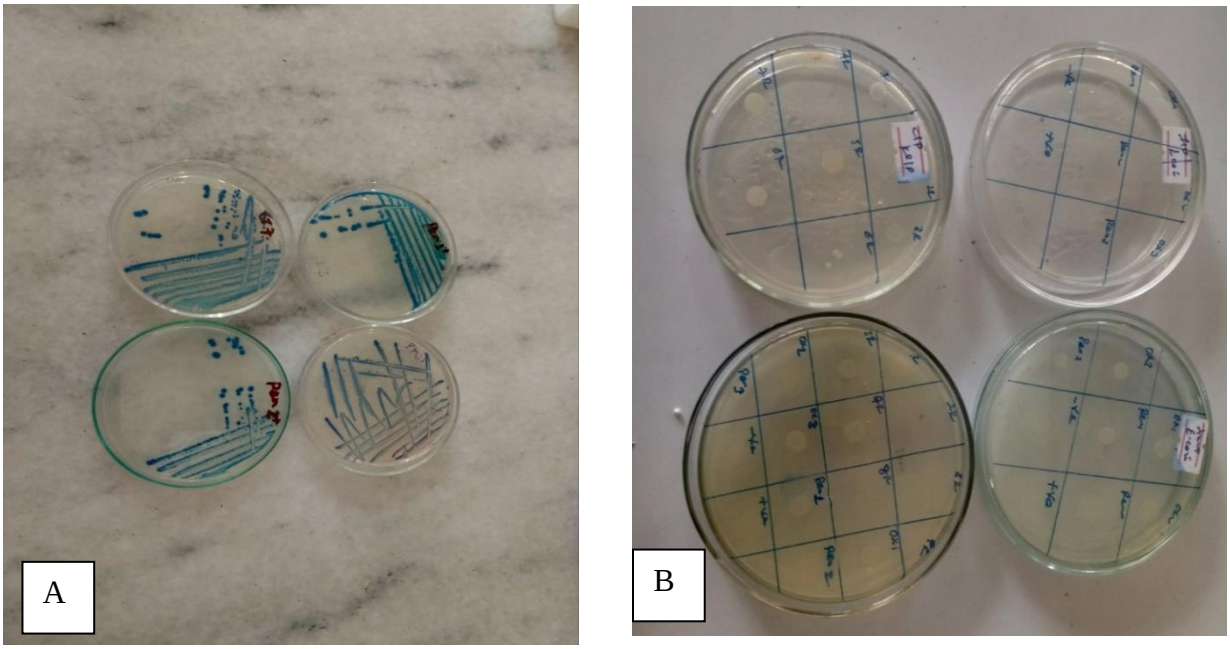




Media preparation (A) and bacteria culturing (B) in Microbiology laboratory



Mice cages (B) and handling or restraining (A) of mice at mice rearing facility.


Sample collection directly at pelleting (A) and by opening GIT (B) at last stage




*E.coli* grown on selective CHROMagar media (A) and AST on MH-II (B) result.


Antibody coated lateral flow assay for CARBA-5 resistance genes

**Annex VI**: Plagiarism Report

Evaluation of short- and long-term antibiotic treatment regimens on emergence of antimicrobial resistance in mice infected with Escherichia coli

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