TIGECYCLINE RESISTANCE AMONG *ESCHERICHIA COLI* ISOLATED FROM POULTRY AND RED MEATS

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TIGECYCLINE RESISTANCE AMONG *ESCHERICHIA COLI* ISOLATED FROM POULTRY AND RED MEATS

submitted by **FİLİZ YENİKÖYLÜ** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Food Engineering, Middle East Technical University** by,

Prof. Dr. Naci Emre Altun
Dean, **Graduate School of Natural and Applied Sciences** _____

Prof. Dr. Hami Alpas
Head of the Department, **Food Engineering** _____

Prof. Dr. Yeşim Soyer Küçükşenel
Supervisor, **Food Engineering, METU** _____

Prof. Dr. Cemil Kürekci
Co-Supervisor, **Food Hygiene and Technology, Hatay Mustafa Kemal University** _____

Examining Committee Members:

Prof. Dr. Behiç Mert
Food Engineering, METU _____

Prof. Dr. Yeşim Soyer Küçükşenel
Food Engineering, METU _____

Prof. Dr. Cemil Kürekci
Food Hygiene and Technology, Hatay Mustafa Kemal University _____

Prof. Dr. İlkay Şensoy
Food Engineering, METU _____

Assoc. Prof. Dr. Eda Demirok Soncu
Food Engineering, Ankara University _____

Date: 18.12.2024

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name : Filiz Yeniköylü

Signature :

ABSTRACT

TIGECYCLINE RESISTANCE AMONG *ESCHERICHIA COLI* ISOLATED FROM POULTRY AND RED MEATS

Yeniköylü, Filiz
Master of Science, Food Engineering
Supervisor: Prof. Dr. Yeşim Soyer Küçükşenel
Co-Supervisor: Prof. Dr. Cemil Kürekci

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Antimicrobial resistance is one of the major concerns globally and poses a great threat to public health. Although resistance gain occurs naturally through genetic changes over time, misuse and overuse of antimicrobials in clinical, livestock and aquaculture settings accelerate antimicrobial resistance gain (WHO, 2019). Tigecycline is the first approved glycylcycline class of antimicrobial agents which are derivatives of the tetracycline antibiotics, modified structurally to have enhanced activity and used as last resort antibiotic in the treatment of complicated infections caused by multidrug resistant Gram-positive and Gram-negative bacteria. Novel plasmid-borne *tet*(X) genes responsible for resistance to tigecycline, pose a serious risk to food safety and human health. In this study, the plasmid mediated tigecycline resistance gene *tet*(X4) was detected in 14 *Escherichia coli* isolates and identified. 13 of them were collected from poultry meat and 1 from red meat samples. The *tet*(X4) positive *E. coli* were characterized by using antimicrobial resistance test (MIC), Pulsed Field Gel Electrophoresis (PFGE) method and whole genome sequencing with Illumina HiSeq 2500 platform. PFGE subtyping method with 80% similarity margin differentiated isolates into 4 different pulsotypes. Similarly, 4

different MSLT types were identified after typing, ST206 being the most prevalent one (n=11), ST744(n=1), ST609(n=1) and ST189(n=1). *E. coli* ST206 found to have similar genotypic antimicrobial resistance profiles, few being common with the *E. coli* ST744, ST609 and ST189 isolates. All of the isolates have shown similar antimicrobial resistance profiles phenotypically. These results were confirmed with whole genome sequencing data. In addition, 12 of the isolates harbored antimicrobial resistant gene carrying plasmids with IncFIA, IncFIB, IncFIC(FII) and IncX1 replicons and diversely other two isolates were found to harbor IncFIA(HI1) and IncFIB(K). Our study provided insight information that expands the understanding on distribution of tigecycline resistant bacteria, as well as on their diversity, resistance mechanisms and epidemiology, found on poultry and red meat in Türkiye. The increased tigecycline resistance in commensal bacteria may also increase the frequency of tigecycline resistance transmission to pathogenic bacteria. Therefore, the prevention methods and no antimicrobial usage regulations on the farm level should be strictly followed and the surveillance systems should screen antimicrobial resistance in bacteria under One Health umbrella.

Keywords: Food Safety, Antibiotic Resistance, Tigecycline Resistance, Genomic Characterization, Foods of Animal Origin

ÖZ

KÜMES HAYVANLARI VE KIRMIZI ETLERDEN İZOLE EDİLEN *ESCHERICHIA COLI*'LERDE TİGESİKLİN DİRENCİ

Yeniköylü, Filiz
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Prof. Dr. Yeşim Soyer Küçükşenel
Ortak Tez Yöneticisi: Prof. Dr. Cemil Kürekci

Aralık 2024, 76 sayfa

Antimikrobiyal direnç, küresel olarak endişe yaratan en önemli konulardan biridir ve halk sağlığı için büyük bir tehdit oluşturmaktadır. Direnç kazanımı zamanla genetik değişiklikler yoluyla doğal olarak gerçekleşse de antimikrobiyallerin klinik ortamlarda, çiftlik ve akuakültür ortamlarında yanlış kullanımı ve aşırı kullanımı, antimikrobiyal direnç kazanımını hızlandırır. Tigesiklin, tetrasiklin antibiyotiklerinin türevi olan, arttırılmış aktiviteye sahip olacak şekilde yapısı modifiye edilmiş ve çoklu ilaca dirençli Gram pozitif ve Gram negatif bakterilerin neden olduğu komplike enfeksiyonların tedavisinde kullanılan glisilsiklin sınıfı antimikrobiyal ajanların ilk onaylı üyesidir. Yeni plazmid kaynaklı *tet*(X) genleri, gıda güvenliği ve insan sağlığı için ciddi bir risk oluşturmaktadır. Bu çalışmada 14 *Escherichia coli* izolatında plazmid aracılı tigesiklin direnç geni *tet*(X4) tespit edildi. Bu izolatlardan 13'ü kanatlı etlerinden, 1'i ise kırmızı et örneklerinden toplandı. *tet*(X4) pozitif *E. coli* izolatları antimikrobiyal direnç (Minimum İnhibitör Konstantrasyon – MİK) testi, Pulsed Field Gel Electrophoresis (PFGE) yöntemi kullanılarak karşılaştırıldı, Illumina HiSeq 2500 platformu ile tüm genom dizilimi kullanılarak kararterize edildi. %80 benzerlik marjına sahip PFGE alt tipleme yöntemi izolatları 4 farklı pulsotipe ayırdı. Benzer şekilde, tiplendirme sonrasında

ST206 en yaygın olmak üzere (n=11), ST744(n=1), ST609(n=1) ve ST189(n=1) olarak 4 farklı MSLT tipi tanımlandı. *E. coli* 'nın benzer genotiple antimikrobiyal direnç profillerine sahip olduğu, birkaçının *E. coli* ST744, ST609 ve ST189 izolatlarıyla ortak olduğu bulundu. Tüm izolatlar fenotipik olarak benzer antimikrobiyal direnç profilleri gösterdi. Bu sonuçlar tüm genom dizileme verileriyle doğrulandı. Ek olarak, izolatların 12'sinde IncFIA, IncFIB, IncFIC(FII) ve IncX1 replikonları bulunan antimikrobiyal dirençli gen taşıyan plazmidler bulunurken, diğer iki izolatın IncFIA(HI1) ve IncFIB(K) içerdiği bulundu. Çalışmamız, Türkiye'de kümes hayvanları ve kırmızı ette bulunan tigesiklin dirençli bakterilerin dağılımı, çeşitliliği, direnç mekanizmaları ve epidemiyolojisi hakkında anlayışı genişleten içgörü bilgileri sağladı. Komensal bakterilerdeki artan tigesiklin direnci, patojenik bakterilere tigesiklin direnci bulaşma sıklığını da artırabilir. Bu nedenle, çiftlik düzeyinde önleme yöntemleri ve antimikrobiyal kullanım düzenlemeleri kesinlikle takip edilmeli ve gözetim sistemleri Tek Sağlık çatısı altında bakterilerdeki antimikrobiyal direnci taramalıdır.

Anahtar Kelimeler: Gıda Güvenliği, Antibiyotik Direnci, Tigesiklin Direnci, Genotipik Karakterizasyon, Hayvansal Gıdalar

To my beloved family and supportive friends

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ABBREVIATIONS

AMR: Antimicrobial Resistance

BAP: Bilimsel Araştırma Projeleri

BHI: Brain Heart Infusion

CDC: Centers for Disease Control and Prevention

CFU: Colony Forming Units

CLC: Cell Lysis Buffer

CLSI: Clinical & Laboratory Standards Institute

CSB: Cell Suspension Buffer

DNA: Deoxyribonucleic Acid

EDTA: Ethylene Diamine Tetra Acetic Acid

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FAD: Flavin Adenine Dinucleotide

FDA: Food and Drug Administration

HSP: High-Scoring Segment Pair

LB: Luria Bertani

MALDI-TOF MS: Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

MDR: Multi Drug Resistant

METU ID: Middle East Technical University Identification Code

MIC: Minimum Inhibitory Concentration

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

ORFs: Open Reading Frames

PCR: Polymerase Chain Reaction

PFGE: Pulsed Field Gel Electrophoresis

ProK: Proteinase K Enzyme

RNA: Ribonucleic Acid

RPM: Revolutions per Minute

SKG: SeaKem Gold (Agarose)

SDS: Sodium Dodecyl Sulfate

TBE: Tris Borate EDTA Buffer

TE: Tris EDTA Buffer

TÜBİTAK: Türkiye Bilimsel ve Teknolojik Araştırma Kurumu (The Scientific and Technological Research Council of Türkiye)

UPGMA: Unweighted Pair Group Method with Arithmetic Means

UV: Ultraviolet

WGS: Whole Genome Sequencing

XbaI: Restriction enzyme with recognition sequence 5'TCTAGA 3'AGATCT

CHAPTER 1

INTRODUCTION

Antibiotic resistance is a serious concern worldwide. In fact, antibiotic usage can result in residues in animal foods, as well as lead to antibiotic resistance in pathogenic bacteria, and cause dangerous health problems. Based on the estimation antimicrobial resistance was found to be associated with nearly 5 million deaths in 2019 worldwide, and according to the Centers for Disease Control and Prevention's (CDC) 2019 Antibiotic Resistance report, more than 2.8 million people in the United States are infected with antibiotic resistant bacteria each year, and more than 35.000 people die as a result of it. For this reason, it is necessary to monitor antibiotic residues and resistance in the food chain.

Antibiotics are used to treat and prevent a variety of bacterial infections in animals and humans. They are also used as animal growth promoting agent in livestocks (Landers et al., 2012), even though antibiotic utilization as feed additive has been banned in many countries, including Türkiye since 2006 (European Commission, 2005; T.C. Resmî Gazete, 2006). In terms of their effects on the cell, antibiotics can affect the cell wall or cell membrane, affect the mechanisms that make nucleic acids, DNA and RNA, and can affect protein synthesis by binding to ribosome (Kohanski et al., 2010). Tigecycline, being a tetracycline derivative, acts by disrupting protein synthesis.

Tigecycline can be used as an alternative to colistin in infections with multiple drug resistance and this makes it an important last resort antibiotic. Although there was no significant difference found in clinical trials between colistin and tigecycline in terms of better response or reducing the rate of recurrent infection, nephrotoxicity

(renal toxicity) was found to be lower in the use of tigecycline (Abushanab et al., 2022; Kim et al., 2016).

The acquisition of antimicrobial resistance occurs naturally with genetic changes over time. Misuse and overuse of antimicrobials in clinical, livestock and aquaculture accelerate this process. He et al. and Sun et al. both showed the emergence of plasmid-mediated tigecycline resistance genes *tet*(X3) and *tet*(X4) in bacteria belonging to the Enterobacteriaceae family, especially *Escherichia coli*, isolated from animals, meat and humans as well as *Acinetobacter* spp. and *Myroides* spp., *Raoultella ornithinolytica*, *Empedobacter brevis*, *Sphingobacterium multivorum* and *Providencia rustigianii* (Fang et al.,2020; He et al., 2019; Sun et al., 2019).

While the prevalence of the tigecycline resistance gene in bacteria of animal origin has been reported to reach 43.3% in some regions in China, its prevalence in bacteria of human origin was reported to be 0.07% and most of them being *Escherichia coli* (He, T. et al., 2019). In a clinical study conducted by Çalışkan et al., antimicrobial resistance patterns in 1844 *Escherichia coli* strains isolated from urine cultures from Pamukkale University Health Research and Application Hospital were evaluated and the resistance rate to tigecycline was found as 1.12% (Çalışkan et al., 2024). Even though the tigecycline resistance in *Escherichia coli* is still comparatively low, the development of transferable resistance genes such as *tet*(X4), which also threatens the clinical efficacy of the entire tetracycline family of antibiotics, calls for constant monitoring to maintain the effectiveness of this vital antibiotic and thus surveillance of *tet*(X) variants requires urgent attention.

The aim of this thesis study is to screen and isolate tigecycline resistant *Escherichia coli* isolates among poultry and red meat samples and then further characterize the tigecycline resistant *Escherichia coli* isolates, especially isolates with *tet*(X) gene, by using phenotypic and genotypic methods.

CHAPTER 2

LITERATURE REVIEW

2.1 Enterobacteriaceae

Enterobacteriaceae is a large family of facultatively anaerobic, bacilli (rod-shaped), non-spore forming, Gram-negative bacteria which are typically 1-5 μm in length. Most are motile except for a few genera. They are generally catalase positive, oxidase negative and capable of reducing nitrate. The family is within the order Enterobacterales under the class of Gammaproteobacteria under *Proteobacteria* phylum (Adeolu et al., 2016; Octavia & Lan, 2014).

Enterobacteriaceae can be found everywhere in nature. Numerous species within this family can live freely in a variety of terrestrial and aquatic environments, while other species are living inside the intestines of animals and linked to plants or insects exclusively. Enterobacteriaceae family includes many important pathogens that cause a variety of illnesses in humans, other animals, and/or plants. Medically important genera of *Enterobacteriaceae* family includes *Citrobacter* species, *Enterobacter* species, *Escherichia* species, *Hafnia* species, *Klebsiella* species, *Morganella* species, *Plesiomonas shigelloides*, *Proteus* species, *Providencia* species, *Salmonella* species, *Serratia* species, *Shigella* species and *Yersinia* species (Octavia & Lan, 2014; Tankeshwar, 2022). Many of these pathogenic species have been known to reappear repeatedly as a result of acquiring virulence components, which are mobile and encoded by plasmids, prophages, and pathogenicity islands. Enterobacteriaceae have been found to possess a wide variety of virulence factors (Chen et al. 2011; Croxen & Finlay 2009; Schmidt and Hensel 2004). Similar virulence mechanisms are employed across species and genera, as

well as across plant, animal and human pathogens in Enterobacteriaceae family (Hacker & Kaper 2000; Schmidt & Hensel 2004).

Among the pathogenic species, *Escherichia coli* is the most well-known and extensively researched genus with several pathogenic forms, including enteropathogenic, enterohemorrhagic, enteroinvasive, enteroaggregative, and extraintestinal *E. coli* (Croxen & Finlay 2009). Every pathogenic type has been demonstrated to have several distinct lineages (Wirth et al. 2006).

2.1.1 *Escherichia coli*

Theodor Escherich, who first isolated a common bacterium from the fecal material of newborns, it was named as *Bacterium coli commune*. To honor the name Escherich, *Escherichia coli* was proposed as a name for the common colon bacillus *Bacterium coli commune* by Castellani and Chalmers in 1919, but it was not officially recognized until 1958 (Castellani & Chalmers 1919; "Etymologia: *Escherichia coli*," 2015; Yu et al., 2021).

E. coli is the most well-known of the species of *Escherichia* and is frequently found in the digestive tracts of both humans and other animals. Additionally, *E. coli* is a significant pathogen that causes intestinal and extraintestinal infections in humans and other animals (Croxen & Finlay 2010).

Like the other members of the Enterobacteriaceae family, *E. coli* is a rod-shaped, facultatively anaerobic Gram-negative coliform bacterium. *E. coli* commonly inhabits the lower intestines of warm-blooded animals, where the bacteria and host have a mutualistic relationship. While most *E. coli* strains are commensal, some can cause severe infections as several strains have acquired virulence factors that make them pathogenic in both humans and animals (Yu et al., 2021). Normal gut microbiota of many animals including humans contains facultative anaerobes like *E. coli* and the main way that disease-causing strains of the bacteria spread is through fecal contamination. Pathogenic *E. coli* can be distinguished into several pathotypes

based on pathogenicity, the intestinal infection causing ones which are enteropathogenic (EPEC), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC), diffuse-adherent (DAEC), and the extraintestinal pathogenic (ExPEC) *E. coli*. Uropathogenic *E. coli* (UPEC) is a subset of ExPEC and causes urinary tract infections (UTIs). Additionally, bloodborne *E. coli* (BBEC), which causes septicemia in both people and animals, and meningitic *E. coli*, including neonatal meningitic *E. coli* (NMEC), which causes meningitis in neonates are subsets of ExPEC (Yu et al., 2021). EHEC is a subset of Shiga toxin producing *E. coli* (STEC). Within an existing population, genetic material can spread horizontally due to bacteria's capacity to transfer DNA through conjugation or transduction. A new pathogenic form emerged in 2011 with the outbreak in German of O104:H4 strain that is a Shiga toxin producing enteroaggregative *E. coli* (Chen et al., 2011; Mellmann et al. 2011; Yu et al., 2021).

2.2 Antibiotic Resistance

Antimicrobial resistance (AMR) poses a threat to the effective prevention and treatment of an increasing number of diseases caused by bacteria, parasites, viruses, and fungi. AMR occurs naturally when bacteria, viruses, fungi, and parasites change over time and become less responsive to drugs, making diseases harder to treat and increasing the risk of severe illness, death, and disease spread. AMR is primarily caused by the overuse or misuse of antibiotics in healthcare, veterinary practices and animal husbandry, but it is also influenced by several interrelated factors that increase its prevalence and spread such as globalization, travel and environmental contamination.

World Health Organization (WHO) reports the antimicrobial resistance (AMR) in the top 10 threats to global health. Antibiotic resistance is a problem that seriously affects public health, as well as creating socioeconomic burden. Modern medicine relies heavily on the effectiveness of antibiotics; however, difficulties in treating infections associated with multi drug resistant (MDR) bacteria have been widely

reported worldwide (WHO, 2022). According to the CDC's 2019 Antibiotic Resistance (AR) Threats Report, over 2.8 million Americans contract infections caused by antibiotic-resistant bacteria each year, and over 35,000 of them die from these infections (CDC, 2019). *E. coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa* were detected to be the six most common pathogens responsible for resistance related deaths and bacterial AMR led to 4.95 millions of fatalities globally in 2019 and was estimated to be directly responsible for around 1.27 million deaths (Murray et al., 2022). Additionally, *M. tuberculosis, Enterococcus faecium, Enterobacter* spp., *Streptococcus agalactiae* (group B *Streptococcus*), *Salmonella* Typhi, and *Enterococcus faecalis*, each were reported to be accountable for 100 thousand to 250 thousand deaths due to antibiotic resistance (Murray et al., 2022). In 2021, an estimation of 4.71 millions deaths were associated with bacterial AMR and by 2050, approximately 8.22 millions AMR related fatalities are predicted worldwide and an estimated 1.91 million deaths owing to AMR (Naghavi et al., 2024). From the point of financial burden, according to the World Bank estimations, in case of high impact resistance scenario, AMR could cost the world's economy up to 3.8% of its (global gross domestic product) GDP by 2050 (The World Bank, 2017).

Antibiotics work by going after important bacterial cell functions, such as breaking down the cell wall or membrane, preventing the synthesis of nucleic acids (DNA and RNA), or interfering with the ribosome and its related parts required to produce proteins (Kohanski et al., 2010). Antibiotics are widely used in the production of food animals in addition to human medicine. They were being used to prevent and control common disease outbreaks, treat clinical diseases, and to promote animal growth (Landers et al., 2012). However, various countries like European countries including Turkey, United States of America, Canada and China are combating antibiotic resistance through bans and restrictions on the use of antibiotics as growth promoters, as a part of global efforts to address this issue (European Commission, 2005; T.C. Resmî Gazete, 2006; Center for Veterinary Medicine, 2017; Bean-

Hodgins & Kiarie, 2021; Wen et al., 2022). Nonetheless, the extensive use of antibiotics in animals leads to the emergence of antimicrobial resistance, underscoring the necessity of their prudent use in both medical and agricultural contexts.

2.3 Tigecycline

Tigecycline is the first approved member of the glycylcycline class of antimicrobial agents, which are derivatives of tetracycline antibiotics. Tetracycline has been structurally modified to have enhanced activity and obtained tigecycline is used in the treatment of complicated infections caused by Gram-positive and Gram-negative bacteria with multiple antibiotic resistance (Yaghoubi et al., 2021). Glycylcyclines share the same core four-ring carbocyclic structure as tetracyclines, which is necessary for their antibacterial activity. When it comes to binding strength to the ribosome, glycylcyclines outperform tetracyclines five-fold (Moffa & Brook, 2015). The substitution of the N-alkyl-glycylamido group at position 9 in the D ring of the basic tetracycline structure broadens the range of action of tigecycline and confers the ability to overcome most tetracycline resistance mechanisms, such as tetracycline-specific cellular firing pumps such as the *tet*(A) gene and ribosomal protection mechanisms such as the *tet*(M) gene (Pankey, 2005; Yaghoubi et al., 2021).

The parenteral form is approved as monotherapy for complicated skin and skin structure infections (excluding diabetic foot infection), complicated intra-abdominal infections, and acquired bacterial pneumonia in adults, and in addition, recent evidence suggests that it is effective in treating serious *Clostridioides difficile* infections, with in vitro susceptibility observed in *Coxiella* spp., *Rickettsia* spp., and MDR *Neisseria gonnorrhoeae* strains (Yaghoubi et al., 2021).

Tigecycline shares a similar mechanism of antimicrobial action to other tetracycline group of antibiotics. Tigecycline halts protein synthesis by binding to the helical

region (H34) on the bacterial 30S ribosomal subunits, blocking the entry of aminoacyl tRNA molecules into the A site of the ribosome, thereby preventing the addition of amino acids into elongating peptide chains (Bennett et al., 2020; Moffa & Brook, 2015; Yaghoubi et al., 2021). Bacteriostatic effects result from this inhibition, which stops bacteria from making vital proteins required for growth and replication.

2.3.1 Resistance Mechanisms of Tigecycline in Bacteria

Tigecycline is a last-resort antibiotic used to treat infections caused by Gram-positive and MDR Gram-negative bacteria (Cheng et al., 2021). The use of last-resort antibiotics like tigecycline in the treatment of medical illnesses is significantly impacted by the rising rate of bacterial resistance to the drug, even though tigecycline overcomes the tetracycline-specific efflux pumps and ribosome protection due to steric hindrance caused by the 9-glycyl substitution (Moffa & Brook, 2015). Resistance to tigecycline in human clinical isolates has been on the rise since its initial report in 2007 (Korczak et al., 2024).

As stated by Zhang et al. antibacterial mechanism and drug resistance mechanisms of tigecycline can be examined under four main mechanisms (Zhang et al., 2022). The first one being the efflux pump overexpression, second one is the cell membrane porin mutation, third one being the ribosomal protection and fourth one is the degrading enzyme mechanism. According to Wang et al., earlier studies has linked tigecycline resistance in Enterobacterales strains to mutations in the plasmid-mediated Tet proteins Tet(A), Tet(K), and Tet(M), overexpression of efflux pumps (*AcrAB-TolC* and *OqxAB*), and a ribosomal S10 protein mutation (*rpsJ*) (Wang et al., 2021). According to Hammerstrom et al. (2015) the genes *ade*S, *gna*, *rrf* and *msb*A may also play a role in the adaptation to tigecycline (Hammerstrom et al., 2015).

Tet(X) is a flavin adenine dinucleotide (FAD) dependent monooxygenase enzyme which requires O_2, Mg^{2+}, and NADPH for function. Tet(X) protein modifies tigecycline by forming 11a-hydroxytigecycline to hinder translation, as the important area for binding of antibiotic crucial for coordinating magnesium undergoes hydroxylation (Cheng et al., 2021; Moore et al., 2005).

Occasional reports exist globally that the chromosomal *tet*(X) and variant *tet*(X2), which originates from *Bacteroides* spp., give tigecycline resistance (Wang et al., 2021). He et al. reported new plasmid-borne *tet*(X) genes, *tet*(X3) and *tet*(X4), which have been found to confer a significant level of tigecycline resistance in several Enterobacterales which are *E. coli*, *Enterobacter asburiae, K. pneumoniae, Proteus mirabilis, Proteus vulgaris, Acinetobacter* species which are *A. baumannii, A johnsonii, A. indicus, A. towneri, A. lwoffi*; and six other species, which are *Miroides* spp., *Raoultella ornithiolytica*, *E. brevis, Sphingobacterium multivarum, Providenca rustigianii, Aeromonas caviae*, isolated from meat animal in China (Fang et al.,2020; He et al., 2019).

The recently discovered plasmid mediated variants of the Tet(X), inactivating enzymes, tetracycline destructases, have the ability to give high-level tigecycline resistance besides inactivating most of the first generation and the second generation tetracyclines. Moreover, these Tet(X) enzymes also decrease the sensitivity to aminomethylcycline, and fluorocycline antibacterial agents, omadacycline, and eravacycline, which are the fourth-generation tetracycline derivatives that are made to circumvent efflux pumps and ribosomal protection mechanisms of resistance to tetracyclines and received FDA approval in 2018 (Chen et al., 2019; Fang et al., 2020; Sun et al., 2019).

The *tet*(X3) and *tet*(X4) genes also found to be able to coexist alongside other resistance genes, such as $bla_{\text{NDM-1}}$, $bla_{\text{OXA-58}}$, *mcr*-1, *cfr*, *flo*R, *mef*B, *sul*3 and *tet*(A), some of which encodes resistance for last resort antibiotics like carbapenems, colistin and chloramphenicol–florfenicol (Fang et al., 2020; Lu et al.,2022; Li et al., 2018;

Wang et al., 2022; Korczak et al., 2024; Zhang et al., 2020; Zhang et al., 2021; Zhang et al., 2022).

Mechanisms for tigecycline resistance may be found on genetic vehicles like plasmids or encoded on chromosomes. Resistance genes' coexistence on a single plasmid may have a positive impact on persistence and broad diffusion under selective pressure in the environment (Korczak et al., 2024). Some metals and heavy metals which may also provide antimicrobial properties, damage cellular structures, and contribute to resistance (Korczak et al., 2024). He et al. point out that heavy metals, which can be present in food animal feces and wastes from plants, have notable effect on the dissemination of mobile *tet*(X) variants and might enhance the conjugation capacity of plasmids containing the *tet*(X3) and *tet*(X4) genes by increasing the permeability of the bacterial outer membrane, upregulating the transcription of genes encoding the type IV secretion system (T4SS) on plasmids containing *tet*(X) variants and putting environment under selective stress (He et al., 2023). The danger of gene transfer in the environment associated with animal manure is pointed out since the conjugation promoting heavy metal concentrations are below the maximum limits specified in the national standard for fertilizers (He et al., 2023).

The *tet*(X2), and its orthologs mostly *tet*(X3) and *tet*(X4) were predominantly found in humans and food animals (Zhang et al., 2021). Het al. points out that while tigecycline has never been utilized in animal husbandry, oxytetracycline, tetracycline, chlortetracycline, and doxycycline have been widely used for years in production of animals including chickens, cows, pigs, lambs, and rabbits as growth-promoter or preventative action against spread of infections (metaphylactic use) (He et al., 2019). Prior to China banning the use of antibiotics in animal feed in 2020, the use of veterinary tetracyclines as growth promoters was allowed which led to the spread of *tet*(X) genes (Korczak et al., 2024).

In a study by Li et al., 864 *tet*(X) positive *E. coli* isolates from humans, animals, and the environment worldwide were subjected to a thorough genomic analysis. These

isolates, which were obtained from 13 distinct hosts, were reported in 25 different countries (Li et al., 2023). The majority of *tet*(X) positive isolates were reported from China (71.76%), followed by Thailand (8.45%) and Pakistan (5.19%) *tet*(X4) being the most common *tet*(X) variation. It was shown that the most significant reservoirs are pigs with 53.93%, humans with 17.41% and chickens with 17.41% for the isolates. Additionally, Lv et al. also discovered another new plasmid-mediated efflux pump gene cluster from *K. pneumoniae* strains, named *tmexCD1-toprJ1*, that provides resistance to several medications, including tigecycline (Lv et al., 2020).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Materials

The analytical quality of all chemicals and materials utilized in the experiments was meticulously chosen. Table A.1 and Table A.2 present a list of materials and chemicals with their respective commercial manufacturers.

3.2 Isolation of Tigecycline Resistant Bacteria

Meat samples were weighed 25 gr into stomacher bags and homogenized for 120 seconds after 1:10 dilution in 225 ml LB broth + tigecycline (1 mg/L) according to the standard microbiological aseptic process. The homogenate then incubated for 24 hours at 37°C for enrichment. After incubation, 10 µl was taken from the filtered part of the stomacher bag and inoculated on MacConkey agar prepared with 1 mg/L tigecycline and then incubated at 37°C for 24 hours. Red/dark pink colored lactose positive colonies with different morphologies that have shown growth were placed on blood agar for purification and identification procedure. After purification, a colony of bacterial samples were taken into BHI broth and incubated overnight at 37°C for the freezing step. Bacteria grown in BHI broths mixed with glycerol to obtain 20% glycerol concentration in cryotubes and stored at -80°C. The source, type of meat and production facilities of the resistant isolates were recorded for further analysis.

Sample collection was conducted in three provinces Ankara, Hatay, and Sivas in Türkiye between August and December 2022. The poultry meat samples were sourced from 14 major producer companies, coded from A to O.

3.3 Determination of Tigecycline Minimum Inhibitory Concentration (MIC) Values and Phenotypic Antibiotic Resistance Profiles of Isolates

Colonies that have shown growth on tigecycline containing media and isolated in Ankara are tested for minimum inhibitory concentration (MIC). The isolates were inoculated onto Müller-Hinton agar plates approximately 16-18 hours before the experiment. 0.9% NaCl solution for preparing bacterial suspension and Müller-Hinton Broth for dilution and as a growth medium were prepared in glass tubes no more than 12 hours before the experiment (Kowalska-Krochmal, B. & Dudek-Wicher, R., 2021). Colonies were suspended in NaCl solution, and the concentration was adjusted to optical density of 0.5 McFarland, which corresponds to Enterobacterales 2021). Tigecycline solution was prepared and diluted from the stock solution to 0.25, 0.50, 1, 2, 4, 8, 16 and 32 mg/L concentrations with Müller-Hinton Broth and final bacterial concentrations were adjusted to be $5x10^5$ CFU/mL in the 96-well plate. Broth control, NaCl control, bacterial control wells for each isolate were also prepared and the 96-well plate was incubated at 37°C for 18 hours. According to EUCAST clinical breakpoint tables (2024), the resistance cutoff concentration for Enterobacterales is >0.5 mg/L. The isolates showing growth over 0.5 mg/L tigecycline concentration deemed resistant.

Along with the other isolates from Sivas and Hatay that have shown growth under tigecycline resistance, isolates were sent to Veterinary Faculty at Hatay Mustafa Kemal University to be identified and confirmed for their tigecycline resistance in scope of the TUBITAK Project No: 121N855. The MIC values for all isolates were confirmed again using broth microdilution method with concentration range 0.125 mg/L to 128 mg/L at Hatay Mustafa Kemal University. The antibiotic concentration in the last well where no bacterial growth was observed was determined as the MIC value. All the strains were tested against a panel of antibiotics, including ampicillin, meropenem, ciprofloxacin, azithromycin, amikacin, gentamicin, tigecycline, ceftazidime, cefotaxime, chloramphenicol, colistin, nalidixic acid, tetracycline, tigecycline, trimethoprim, and sulfamethoxazole in Hatay Mustafa Kemal

University. Antibiotic susceptibility testing was conducted using Sensititre™ plates (EUVSEC3; Thermo Fisher Scientific) and interpreted according to threshold values determined by Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2020) and EUCAST (EUCAST, 2024). In all tests, ATCC 25922 and the tigecycline resistant *E. coli* strain isolated in a previous study by Kürekci et al. (2022) and characterized by whole genome sequencing were used as control strains. Isolates with the confirmed MIC values and being tigecycline resistant were used in further studies.

3.4 Confirmation of *E. coli* from tigecycline resistant isolates collected from Ankara Province with *rpoB* gene

From samples collected from Ankara, suspicious *E. coli* colonies on BHI agar were tested with polymerase chain reaction (PCR) for molecular confirmations. One colony from the suspicious isolates on BHI solid medium was taken to check marker gene for *E. coli, rpoB,* is present or not with a sterile loop and transferred to PCR tubes containing 95 µL of sterile distilled water. Then, the cells were lysed at 95°C for 10 minutes. The prepared PCR reaction solution is given in Table 3.1 for each isolate.

Table 3.1 PCR Master Mix and *rpoB* Primer Sequences

Component			Amount
dH_2O			16.25 µL
5X Go Taq Flexi buff			2.5 µL
$MgCl_2$ [25mM]			1.5 µL
dNTPs [10mM]			0.5 µL
10 µM forward primer	*rpoB*-F	GTATGTCCAATCGAAACCCCT	1.0 µL
10 µM reverse primer	*rpoB*-R	GGTAGTGAATTTCGTCAGTTACA	1.0 µL

Table 3.1 (Cont'd)

Taq DNA Polymerase	0.25 µL
Target DNA	2 µL
Total	25 µL

A total of 2 µL of the lysed cell solutions were transferred to PCR tubes containing 23 µL of PCR master mix. A positive control and a negative control with pure water instead of target DNA were also prepared in the tubes using the same method and after the PCR tubes were placed, they were run in the thermal cycler with the appropriate amplification program (Table 3.2).

Table 3.2 Amplification conditions for PCR

95°C for 10 minutes (1 cycle)	x 1 cycle
95°C for 30 seconds 58°C for 30 seconds 72°C for 30 seconds	x 25 cycles
72°C for 5 minutes	x 1 cycle
4°C ∞	∞

After the amplification was completed, the marker, positive control, negative control and isolates were placed in the wells of the agarose gel prepared with 1.8 g agarose and 0.5X TBE buffer, as 10 µL each. The agarose gel was placed in the electrophoresis tank and ran at 120 volts for 45 minutes. After the time was up, the gel was stained in 10 mg/mL Ethidium Bromide (EtBr) solution for 5 min and then kept in distilled water for 30 min and visualized under UV light.

3.5 Identification of All Isolated Tigecycline Resistant Bacteria with MALDI-TOF

Identification of all the bacteria isolated under the TUBITAK project (Project No: 121N855) in species level was done by peptide mass fingerprint analysis where Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF MS; Bruker Daltonics GmbH, Germany) was used in Hatay Mustafa Kemal University.

3.6 Polymerase Chain Reaction (PCR) for *tet*(X) variants and *tmexCD1-toprJ1* Genes

Plasmid mediated tigecycline resistance genes *tet*(X), *tet*(X2), *tet*(X3), *tet*(X4) and *tmexCD-toprJ1* were searched in all isolates using the PCR (Bartha et al., 2011; He et al., 2019; Li et al., 2021) in Hatay Mustafa Kemal University. The forward and reverse primers used for polymerase chain reaction (PCR) of tigecycline resistant isolates given in Table 3.3 specific for *tet*(X), *tet*(X2), *tet*(X3), *tet*(X4) and *tmexCD-toprJ1* genes that mediate tigecycline resistance.

Table 3.3 Primers used in PCR

Gene	Primer	Sequence (5' to 3')	Reference
tmexCD1-toprJ1	*tmex*-F	CTGCTGGTCATTCCGTTCCT	Li et al., 2021
	tmex-R	ATGATCCGCTCGACGTTCT	
tet(X)*	*tet*(X)-F	TTAGCCTTACCAATGGGTGT	Bartha et al., 2011
	tet(X)-R	CAAATCTGCTGTTTCACTCG	
tet(X3)	*tet*(X3)-F	TGCCATAGTCAGTCCAACG	He et al., 2019
	tet(X3)-R	ATTTCAATGCTTGCCCAC	
tet(X4)	*tet*(X4)-F	AGTCCAACGGGTCCACCAC	He et al., 2019
	tet(X4)-R	TGCTCATTTGATGCCTCCTT	

Table 3.3 (Cont'd)

tet(X4)**	*tet*(X4)-F	CAACGACCGAGAGGCAAGAA	Li et al. #
	tet(X4)-R	CATCAACCCGCTGTTTACGC	

*Specific primers for *tet*(X) and *tet*(X2) genes, **Sequencing primer, # Primer is designed by Project partner Prof. Dr. Ruichao Li and his team.

3.7 Subtyping of Isolated Tigecycline Resistant *tet*(X4) Positive *E. coli* with Pulsed Field Gel Electrophoresis (PFGE)

All the isolates obtained from poultry meat and red meat samples in the project that exhibited tigecycline resistance and positive for *tet*(X4) were collected in Middle East Technical University, Ankara for further subtyping.

The subtyping of tigecycline resistant, *tet*(X4) positive isolates was done using Pulsed Field Electrophoresis (PFGE). Bacteria were seeded on BHI agar and incubated for 14-18 hours at 37°C. Each isolate was suspended into a 10 mL cell suspension buffer (CSB) and cell concentrations were adjusted with the help of a spectrophotometer at a wavelength of 610 nm so that the optical density was between 1.3-1.4. Then, 400 µL of cell suspensions were taken into microcentrifuge tubes and incubated at 37°C for 10 minutes. 20 µL of 20 mg/mL Pro K solution and 400 µL of the prepared 1% SeaKem Gold Agarose:1% SDS mixture were added to each microcentrifuge tube by pipetting, mixing, and poured into the plug mold to set. Falcon tubes were labeled and 5 mL of cell lysis buffer (CLB) + 25 µL of ProK solution were added to each tube and frozen plugs were incubated in these tubes for 1.5-2 hours at 54°C/170 RPM. After pouring the CLB/Pro K solution with perforated screw caps, 10 ml of sterile deionized water was used two times to wash the plugs in the tubes and the falcon tubes were incubated for 10 minutes at 50°C/70 RPM. After deionized water is drained with perforated screw caps, 10 mL of Tris EDTA buffer (TE buffer) were added to the falcon tubes and incubated for 15 minutes at 50°C/70

RPM. This step was repeated three more times. Fresh sterile 5 mL of TE buffer was added to the falcon tubes to store the plugs at 4°C.

The prepared plugs were taken from the falcon tubes and cut with a 2 mm wide scalpel, taken into labeled microcentrifuge tubes, 200 µL of H buffer solution was added and incubated at 37°C for 10 minutes. Then, the H buffer solution was removed from the tubes and 200 µL of XbaI solution was added to each tube and the tubes were incubated at 37°C for 4 hours.

The electrophoresis chamber was cleaned with 2.2 L deionized water for 5-10 minutes, then the water was drained. 1.5 g of SKG agarose + 7.5 mL of 10X TBE + 150 mL of deionized water was mixed and melted in the microwave, after waiting at 55°C for 10 minutes and poured into a gel mold. The liquid part of the tubes, whose incubation is completed for 4 hours, were drawn with a pipette and the plugs were placed in the wells of the prepared gel. After the running buffer was filled into the electrophoresis, the system was allowed to reach 14°C and the prepared gel was placed into the electrophoresis. After pouring 2*836 µL of thiourea solution onto gel, electrophoresis was run at the appropriate settings as in Table 3.1.

Table 3.4 PFGE Running Conditions

Parameter	Value
DNA size interval	50 kb – 400 kb
% agarose	% 1
Voltage	6.0 V/cm
Run time	19 h
Temperature set	14°C
Included angle	120°
Initial switch time	6.76 s
Final switch time	35.38 s
Pump speed	70 (1L/min)

After 19 hours, the gel was stained in 10 mg/mL Ethidium Bromide (EtBr) solution for 45 minutes, then kept in distilled water for 60 minutes and photographed under UV light. The resulting images were uploaded to the BioNumerics program, bands on the images are marked carefully and similarity of banding patterns analyzed using Dice's similarity coefficient with 2.5 % band position tolerance and 1.5 % optimization. By using the unweighted pair group method with arithmetic means (UPGMA) dendrogram is generated.

3.8 Whole Genome Sequencing

Whole genome sequencing (WGS) was done commercially for *tet*(X4) positive *E. coli* isolates from chicken meats and a red meat sample. High-purity bacterial genome was obtained with the help of a commercial extraction kit. After quantification by Qubit 4 (Invitrogen), Illumina HiSeq 2500 platform used to generate 2x150 bp paried-end reads.

3.9 Bioinformatic Analyses of Sequenced Isolates

Data obtained from WGS was quality checked with fastQC tool and was filtered by using trimmomatic tool. The short-read raw sequences were de novo assembled using SPAdes in order to obtain draft genomes (Bankevich et al., 2012). Using prokka v1.14.6, the sequence assembly was annotated/curated by an external service provider.

Bioinformatic analyses for *tet*(X) positive *E. coli* isolates were performed on the sequences to determine key characteristics of the isolates (Figure 3.1). Multi-Locus Sequence Typing (MLST) were applied and ST types identified using MLST-2.0 Server of Center for Genomic Epidemiology (Larsen et al., 2012) and the MLST tool on Galaxy.eu also had been used to confirm. Plasmid replicons were detected using both the Plasmid Finder-2.0 server provided by the Center for Genomic Epidemiology (Carattoli and Hasman, 2020) and PlasmidFinder tool available on

Galaxy.eu. Resistance genes were identified and confirmed using both the Staramr tool on Galaxy.eu (Bharat et al., 2022), which integrates searches across the ResFinder and PlasmidFinder databases and, ResFinder (version 4.6.0) platform provided by Center for Genomic Epidemiology (Bortolaia et al., 2020).

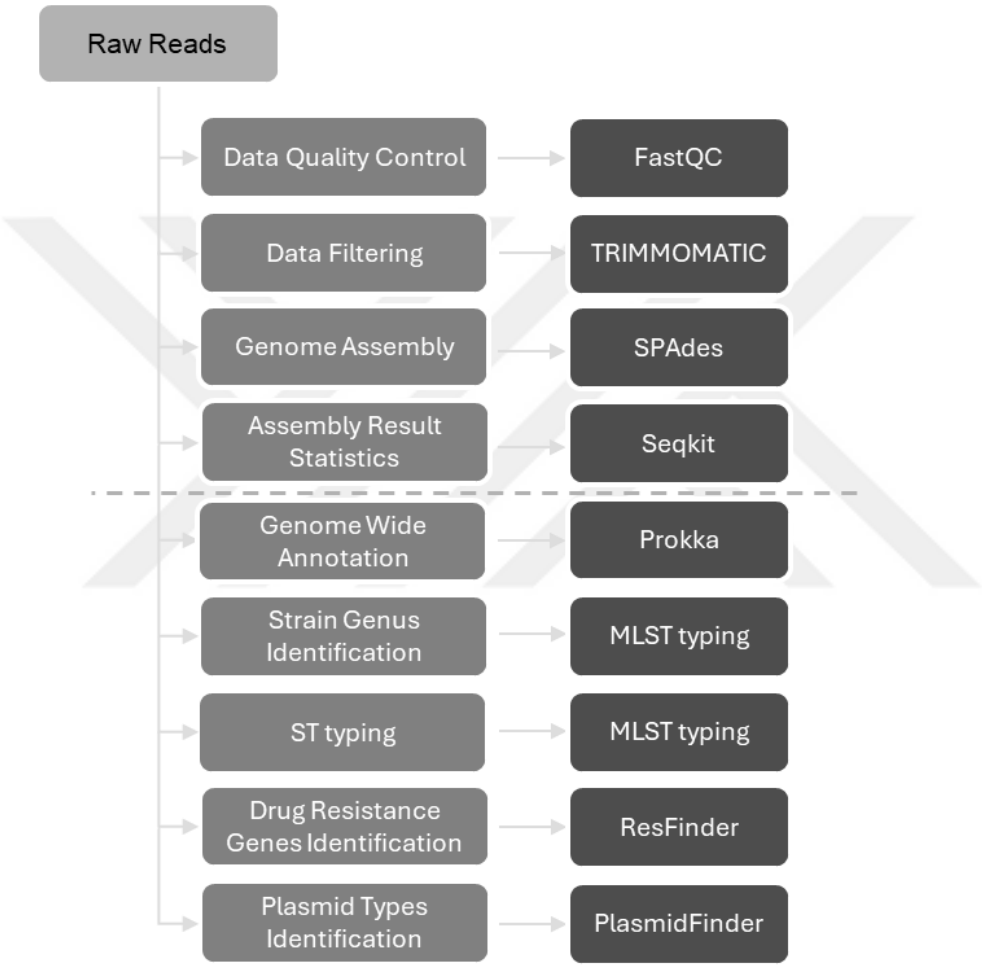

Figure 3.1. Pipeline for Bioinformatic Analysis

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Bacteria in Enterobacteriaceae Family

Between October 11, 2022, and December 25, 2022, a total of 50 bacterial colonies were obtained from 23 different meat samples collected from various butcher shops, markets, and farms in Ankara province. Colonies showing growth on MacConkey agar medium were isolated and purified. Of these isolates, 28 were isolated from poultry meat samples while 22 from red meat samples. The meat samples from Ankara province and production locations from which these 50 isolates were obtained (Table 4.1). The brands and manufacturers of the meat samples gathered in Ankara province were initially named differently than the project. For identifying, packaged products from markets were labeled with M-, while butchers were labeled with K-.

Table 4.1 Bacteria Isolated from Ankara Province

Isolate ID	Source	Production Location	Brand
DE1	Veal	Kaman/Kırşehir	K-A
DE2	Veal	Kaman/Kırşehir	K-A
DK1	Ground Beef	Kaman/Kırşehir	K-A
DK2	Ground Beef	Kaman/Kırşehir	K-A
TE1	Chicken Breast	Sakarya	M-B
TE2	Chicken Breast	Sakarya	M-B
TE3	Chicken Breast	Sakarya	M-B
TE4	Chicken Breast	Sakarya	M-B
TE5	Chicken Breast	Sakarya	M-B

Table 4.1 (Cont'd)

TD1	Chicken Wing	Kemalpaşa/İzmir	M-C
TD2	Chicken Wing	Kemalpaşa/İzmir	M-C
KK1	Meatball	Bursa	K-D
KK2	Meatball	Bursa	K-D
TE6	Chicken Meat	Kemalpaşa/İzmir	M-E
TE7	Chicken Meat	Kemalpaşa/İzmir	M-E
TE8	Chicken Meat	Eşme/Uşak	M-F
TE9	Chicken Meat	Eşme/Uşak	M-F
KE1	Lamb Meat	Afyon & Kayseri	K-L
DE3	Beef Chunks	Afyon & Kayseri	K-L
KE2	Lamb Meat	Balıkesir	K-A
DE4	Beef Chunks	Amasya	K-A
TE10	Chicken Breast	Bolu	K-G
TE11	Chicken Breast	Bolu	K-G
KE3	Lamb Meat	Polatlı/Ankara	K-G
KE4	Lamb Meat	Polatlı/Ankara	K-G
KE5	Lamb Meat	Polatlı/Ankara	K-G
DE5	Veal	Amasya	K-G
DE6	Veal	Amasya	K-G
DE7	Veal	Amasya	K-G
TE12	Chicken Meat	Kemalpaşa/İzmir	M-C
TE13	Chicken Meat	Kemalpaşa/İzmir	M-C
DK3	Ground Beef	Ankara	K-M
DK4	Ground Beef	Ankara	K-M
DK5	Ground Beef	Ankara	K-M
TE14	Chicken Breast	Göynük/Bolu	M-I
TE15	Chicken Breast	Göynük/Bolu	M-I
TE16	Chicken Breast	Göynük/Bolu	M-I
HE1	Turkey Breast	Bolu	M-I

Table 4.1 (Cont'd)

HE2	Turkey Breast	Bolu	M-I
HE3	Turkey Breast	Bolu	M-I
TE17	Chicken Meat	Bandırma/Balıkesir	M-J
TE18	Chicken Meat	Bandırma/Balıkesir	M-J
TE19	Chicken Meat	Bandırma/Balıkesir	M-K
TE20	Chicken Meat	Bandırma/Balıkesir	M-K
DK6	Ground Beef	Tekeli/Menderes/İzmir	K-H
HE4	Turkey Meat	Ankara	K-N
HE5	Turkey Meat	Ankara	K-N
HE6	Turkey Meat	Ankara	K-N
DE8	Veal Chunks	Ankara	K-O
DE9	Veal Chunks	Ankara	K-O

In parallel, samples from poultry and red meats were collected in the scope of the TUBITAK project in Sivas and Hatay. From the 159 broiler chicken meat samples, 31 of them showed growth in presence of tigecycline and samples belonged to 8 companies and no growth is observed from the 4 turkey meat samples. A total of 33 Enterobacterales strains were obtained from the chicken meat samples in which 20 of them being *E. coli*. Of the 172 red meat samples 5 *E. coli* were isolated.

4.2 Determination of MIC Values in Isolates

The minimum inhibitory concentration (MIC) test against tigecycline antibiotic was performed for the 50 isolates collected from Ankara province. According to the EUCAST clinical breakpoint tables dated 2024, the resistance concentration for Enterobacterales was accepted as >0.5 mg/L. The broth microdilution test revealed that only 16 of the 50 isolates that developed on solid medium with 1 mg/L

tigecycline were resistant (MIC > 0.5 mg/L). 6 of the resistant isolates were obtained from poultry meat samples and 10 from various cattle and sheep red meat samples.

The fact that only 16 of the 50 isolates growing on solid media containing 1 mg/L tigecycline showed resistance to tigecycline (>0.5 mg/L) in the MIC test conducted in Ankara indicating that the effectiveness of the antibiotic in isolation media may have decreased depending on the preparation or storage conditions. As stated by Amann et al. (2020), the sensitivity of tigecycline to light and oxygen may affect the reliability of the results. Temperature fluctuations and exposure to light, especially during storage of the media, may have adversely affected the stability of tigecycline. This creates a limitation on the accuracy of MIC test results. Therefore, it is highly recommended to prepare tigecycline fresh on the day of test for preparation of medium and MIC test.

PCR was performed on the isolates that have been observed to be resistant in the MIC tests to determine whether they are *E. coli*. 7 of the 16 resistant isolates were identified as *E. coli* as the result of confirmation with the *rpoB* gene via PCR. Isolates that gave positive results were labelled with METU ID codes. All the isolated bacteria with measured MIC values >0.5 gm/L and *E. coli* isolates having METU IDs are given in Table 4.2.

Table 4.2 Isolated Bacteria from Ankara Province Having MIC Values >0.5 gm/L

METU ID	Isolate ID	MIC TGC (mg/L)
-	DE1	0,5
MET A1-439	DE2	1
MET A1-400	DK1	2
-	DK2	1
-	TE1	0,25
-	TE2	0,25

Table 4.2 (Cont'd)

-	TE3	1
-	TE4	0,25
-	TE5	1
MET A1-442	TD1	1
-	TD2	2
MET A1-441	KK1	2
-	KK2	2
-	TE6	0,25
-	TE7	0,25
MET A1-443	TE8	1
-	TE9	1
-	KE1	1
MET A1-440	DE3	1
-	KE2	1
-	DE4	1
-	TE10	0,5
-	TE11	0,25
-	KE3	0,25
-	KE4	0,5
-	KE5	0,5
-	DE5	0,5
-	DE6	0,5
-	DE7	0,5
-	TE12	0,5
-	TE13	0,5
-	DK3	0,25
-	DK4	0,25
MET A1-401	DK5	1
-	TE14	0,25
-	TE15	0,5

Table 4.2 (Cont'd)

-	TE16	0,25
-	HE1	0,25
-	HE2	0,25
-	HE3	0
-	TE17	0,5
-	TE18	0,25
-	TE19	0,25
-	TE20	0,5
-	DK6	0,25
-	HE4	0
-	HE5	0,25
-	HE6	0,5
-	DE8	0,25
-	DE9	0,25

Isolates showing tigecycline resistance were sent for confirmation MIC values to Hatay Mustafa Kemal University. Of the 16 isolates from Ankara province, only two isolates from red meat samples were further found to have MIC >0.5 mg/L for tigecycline with using EUVSEC3 test plates. This was also attributed to the freshness of tigecycline before the use in MIC test.

4.3 Tigecycline Resistant Isolates of the Project with Confirmed MIC Values

In the project, among all tigecycline resistant isolates from Ankara, Sivas and Hatay, a total of 27 *E. coli*, 13 *K. pneumoniae*, 1 *S. marcescens*, 1 *E. cloacae* belonging to Enterobacteriaceae family and 1 *P. aeruginosa* which belongs to Pseudomonadaceae family identified from chicken meat and red meat samples showed tigecycline resistance, with confirmed MIC values exceeding 0.5 mg/L (EUCAST, 2024). A total of 22 *E. coli* isolates were obtained from chicken meat and 5 from red meat.

Commensal bacteria might gain some virulence traits via different molecular mechanisms and might lead to serious infections in immunocompromised people, as well as causing extended hospital stays, complications in organ and bone marrow transplants, and primary immunodeficiencies (Riccardi et al., 2019). Some *E. coli*, *Klebsiella* spp., *P. aeruginosa*, *Serratia* spp. and *E. cloacae* are linked to opportunistic infections (Davin-Regli & PagÃ¨S, 2015; Riccardi et al., 2019). The MDR in these species of bacteria, isolated in the scope of the project, is a significant threat to patients with weakened immune systems.

Resistant isolates in this study having MIC values >0.5 mg/L according to EUCAST (2024) epidemiological cutoff values, were screened for the presence of plasmid mediated *tet*(X4) and *tmexCD1-toprJ* genes. The *tet*(X4) gene was detected exclusively in *E. coli* isolates. Table 4.3 shows the ID codes, MALDI-TOF results, MIC values for tigecycline, *tet*(X4) and *tmexCD1-toprJ1* positivity of tigecycline resistant *E. coli* isolated from poultry and red meat samples in this project.

The *E. coli* isolates, positive for *tet*(X4), from meat samples (n=22) with the confirmed MIC values above 0.5 mg/L for tigecycline that were sent to METU for further studies. These isolates were stored in METU isolate database under METU ID codes with the phenotypic antibiotic resistance profile information.

Table 4.3 MIC Values of Tigecycline Resistant *E. coli* Isolates Obtained from Meat Samples in the Study and Presence of Plasmid Mediated Resistance Genes *tet*(X4) and *tmexCD1-toprJ1*

METU ID	Project ID	MALDI-TOF	MIC (mg/L)	*tet*(X4)	*tmexCD1-toprJ1*
MET A1-375	Tig294	*Escherichia coli*	2	-	-
MET A1-377	Tig438	*Escherichia coli*	2	-	-
MET A1-378	Tig452	*Escherichia coli*	>8	+	-
MET A1-379	Tig454	*Escherichia coli*	8	+	-

Table 4.3 (Cont'd)

MET A1-380	Tig455	*Escherichia coli*	>8	+	-
MET A1-390	Tig488	*Escherichia coli*	8	+	-
MET A1-381	Tig457	*Escherichia coli*	>8	+	-
MET A1-382	Tig458	*Escherichia coli*	>8	+	-
MET A1-383	Tig459	*Escherichia coli*	>8	+	-
MET A1-384	Tig460	*Escherichia coli*	8	+	-
MET A1-385	Tig463	*Escherichia coli*	>8	+	-
MET A1-386	Tig465	*Escherichia coli*	>8	+	-
MET A1-387	Tig470	*Escherichia coli*	8	+	-
MET A1-388	Tig473	*Escherichia coli*	>8	+	-
MET A1-389	Tig487	*Escherichia coli*	>8	+	-
MET A1-392	Tig516	*Escherichia coli*	1	-	-
MET A1-393	Tig518	*Escherichia coli*	1	-	-
MET A1-394	Tig519	*Escherichia coli*	1	-	-
MET A1-395	Tig520	*Escherichia coli*	1	-	-
MET A1-396	Tig521	*Escherichia coli*	1	-	-
MET A1-397	Tig522	*Escherichia coli*	1	-	-
MET A1-398	Tig523	*Escherichia coli*	2	-	-
MET A1-376	Tig437	*Escherichia coli*	4	+	-
MET A1-399	Tig524	*Escherichia coli*	1	-	-
MET A1-391	Tig514	*Escherichia coli*	2	-	-
MET A1-400	Tig525	*Escherichia coli*	2	-	-
MET A1-401	Tig526	*Escherichia coli*	1	-	-

All *tet*(X4) positive *E. coli* isolates had MIC values ≥8 mg/L, with the exception of one isolate MET A1-376 having MIC of 4 mg/L, highlighting the association between the presence of *tet*(X4) gene and elevated tigecycline resistance. Additionally, none of the screened tigecycline resistant isolates were positive for the *tmexCD1-toprJ1* gene PCR, which is also found to be located on mobile genetic elements in some bacterial strains (Lv et al., 2020).

Alongside tigecycline resistance, all the isolates that were tested for susceptibility to tetracycline, ampicillin, meropenem, ciprofloxacin, azithromycin, amikacin, gentamicin, ceftazidime, cefotaxime, chloramphenicol, colistin, nalidixic acid, trimethoprim, and sulfamethoxazole antibiotics demonstrated high levels of resistance to tetracycline and ampicillin. Analysis revealed that *E. coli* exhibited resistance rates of 88.9% to chloramphenicol, 70.4% to ciprofloxacin, 81.5% to nalidixic acid, 88.9% to trimethoprim, 85.2% to sulfamethoxazole and resistance to gentamicin and colistin were 14.8%. All *E. coli* were susceptible to azithromycin, amikacin, meropenem, ceftazidime and cefotaxime.

4.4 Pulsed Field Gel Electrophoresis Subtyping of *tet*(X4) Carrying *E. coli* Isolates

Subtyping of the 14 tigecycline resistant *tet*(X4) carrying *E. coli* isolates from meat samples collected in the scope of the project was performed at METU using Pulsed Field Gel Electrophoresis (PFGE) technique and the resulting cluster analysis dendrogram is given in Figure 4.1. The PFGE analysis of 14 *E. coli* isolates with the *tet*(X4) gene revealed that, based on the 80% similarity rate, they were separated into four distinct pulsotypes. It was discovered that one isolate (MET A1-376) from a red meat sample and two isolates (MET A1-387 and MET A1-384) from chicken meat samples from two different companies (A and E respectively) were clonally distinct from others due to their distinct band patterns.

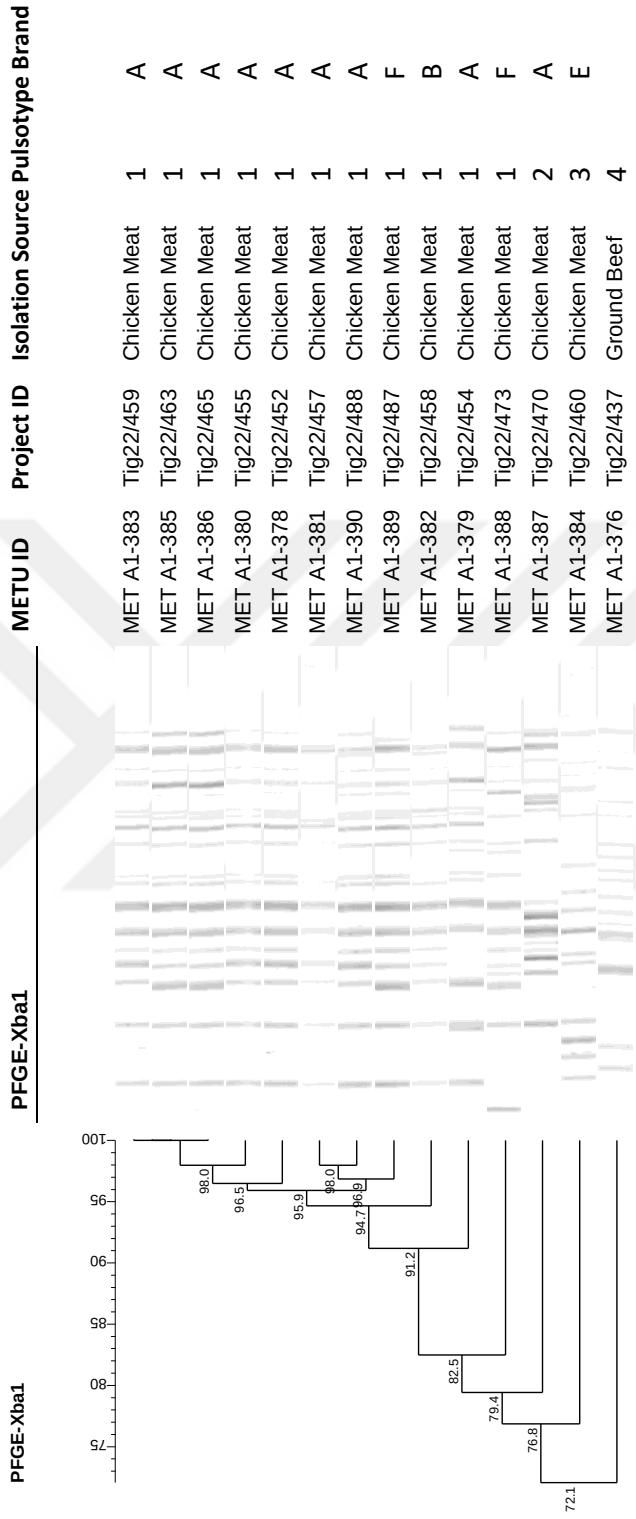

Figure 4.1. Dendrogram of *tet*(X4) Positive *E. coli* Isolates

4.5 Bioinformatic Analyses of Sequenced Isolates

The genomic data derived from the sequenced isolates underwent bioinformatic analyses to describe their genetic characteristics, such as sequence types, antibiotic resistance genes and plasmid replicons. *E. coli* isolates that were positive for *tet*(X4) gene subjected to these investigations in order to determine the genetic background of tigecycline resistance and its correlation with plasmids. Multilocus sequence typing (MLST) was utilized to evaluate the genetic diversity of the isolates by determining sequence types, and tools like ResFinder, Staramr and PlasmidFinder were utilized to identify resistance genes and plasmid replicons, respectively. The results of these studies are described in detail in the sections that follow.

4.5.1 MLST Typing of Isolates

Bioinformatic analyses on whole genome sequences of 14 *tet*(X4) positive isolates identified four Multi-Locus Sequence Typing (MLST) types, shown in Table 4.4, with ST206 being the most prevalent sequence type observed in 11 of the isolates. The other three isolates identified as ST189, ST609 and ST744. The PFGE analysis was supported by the WGS analysis of *tet*(X4) positive isolates, which showed that the majority of isolates had an MLST type of ST206 (n=11), ST189 (n=1) for MET A1-376, ST744 (n=1) for MET A1-387 and ST609 (n=1) for MET A1-384.

Table 4.4 MLST Typing Results of Sequenced *E. coli* Isolates

METU ID	**Project ID**	**Brand**	**MLST Type**	**Housekeeping Genes**						
				adk	***fumC***	***gyrB***	***icd***	***mdh***	***purA***	***recA***
MET A1-376	Tig437	NA	ST189	10	27	5	10	12	8	49
MET A1-378	Tig452	A	ST206	6	7	5	1	8	18	2
MET A1-379	Tig454	A	ST206	6	7	5	1	8	18	2
MET A1-380	Tig455	A	ST206	6	7	5	1	8	18	2

Table 4.4 (Cont'd)

MET A1-381	Tig457	A	ST206	6	7	5	1	8	18	2
MET A1-382	Tig458	B	ST206	6	7	5	1	8	18	2
MET A1-383	Tig459	A	ST206	6	7	5	1	8	18	2
MET A1-384	Tig460	E	ST609	8	7	1	8	8	7	6
MET A1-385	Tig463	A	ST206	6	7	5	1	8	18	2
MET A1-386	Tig465	A	ST206	6	7	5	1	8	18	2
MET A1-387	Tig470	A	ST744	10	11	135	8	8	8	2
MET A1-388	Tig473	F	ST206	6	7	5	1	8	18	2
MET A1-389	Tig487	F	ST206	6	7	5	1	8	18	2
MET A1-390	Tig488	A	ST206	6	7	5	1	8	18	2

NA: Not Available

Multi locus sequence typing (MLST) has been the common tool in surveillance on evolution of bacterial populations and identification of bacterial clones (Feil & Enright, 2004) as the specific housekeeping genes on the core genome selected for MLST provides a population framework (Turner & Feil, 2007). Isolates having the same or similar MLST types means that they are closely related strains from a common ancestor, which may help in tracing the source of bacteria. On the other hand, traits like antibiotic resistance which are often encoded on accessory genes can transfer between unrelated strains by means of horizontal gene transfer. MSLT data can be used together with PFGE data, plasmid type and resistance gene information to identify correlations and patterns of dissemination of antimicrobial resistance.

As expected, the clonal relationship of the isolates determined by the PFGE analysis were also confirmed by the MLST types. Nine poultry meat isolates were obtained from Brand A, one isolate (MET A1-381) was obtained from Brand B, one isolate (MET A1-384) was obtained from Brand E and two isolates (MET A1-388 and MET A1-389) were obtained from Brand F. Among the isolates from Brand A, eight of them were identified as ST206 and one of them as ST744. The three other isolates from Brand B and Brand F were all identified as ST206. The isolate from Brand E

poultry meat was identified as ST609 and the isolate obtained from red meat was identified as ST189.

In a study by Zhang et al. 1286 avian origin samples from migratory birds, ducks and geese were taken in seven different regions of Sichuan, China and 21 *tet*(X4) positive *E. coli* isolates with serious resistance to multiple antibiotics were obtained (Zhang et al., 2023). According to MLST, ST206 was reported to be the most prevalent type with eleven isolates followed by ST761 with five, ST767 with two, ST155, ST1638 and ST542 are all reported to be one isolate each (Zhang et al., 2023). Although ST10 being the most reported MLST type in various studies and worldwide, amongst the isolates from animals, environment and humans (Li et al., 2023; Zeng et al., 2022) the ST types identified in *tet*(X4) positive *E. coli* are highly diverse. In a study by Fang et al. (2022) ST189 was reported to be one of the most common ST types identified in Guangxi, China and ST206 was reported to be the most common in Ningxia, China. Kürekci et al. (2022) identified four samples from wastewater in Türkiye where the ST type is reported as ST609.

4.5.2 Screening of Resistance Genes Using ResFinder

In addition to *tet*(X4), other antimicrobial resistance encoding genes were also detected from whole genome sequencing data by using ResFinder. 20 other resistance genes were detected in the fourteen sequenced *E. coli* isolates, providing resistance to various antibiotics, including aminoglycosides, aminocyclitols, β-lactams, amphenicols, lincosamides, fluoroquinolones, sulfonamides, and tetracyclines (Table 4.5). All the sequenced isolates were bearing tetracycline resistance gene *tet*(A), the aminoglycoside resistance gene *aad*A2, the β-lactam resistance gene $bla_{\text{TEM-1B}}$, the phenicol resistance gene *flo*R and sulfonamide resistance gene *sul*2. Except for isolates MET A1-376, MET A1-387 and MET A1-387, the other eleven isolates, all of them representing ST206, carried the same antibiotic resistance genes. Only one isolate MET A1-387 (ST744) also carried the *dfr*A36 gene, which provides resistance to trimethoprim and the $bla_{\text{EC-8}}$ gene,

whereas one other isolate MET A1-376 (ST189) had *bla*$_{EC-5}$ gene which both provide resistance to β-lactam group of antibiotics. Additionally, MET A1-384, representing ST609, had two more resistance genes *oqx*A and *oqx*B, which are associated with resistance against fluoroquinolones.

Table 4.5 ResFinder Results of Resistance Genes on Sequenced Isolates.

METU ID / Project ID		Antibiotic Classes and Resistance Genes																				
		aminoglycosides					β-lactams				phenicols		antifolates		lincosamides	fluoroquinolones		quinolones	sulfonamides		tetracyclines	
		*aad*A1	*aad*A2	*aph*(3'')-Ib	*aph*(3')-Ia	*aph*(6)-Id	*bla*$_{EC-15}$	*bla*$_{EC-5}$	*bla*$_{EC-8}$	*bla*$_{TEM-1B}$	*cml*A1	*flo*R	*dfr*A12	*dfr*A36	*lnu*(F)	*oqx*A2	*oqx*B	*qnr*S1	*sul*2	*sul*3	*tet*(A)	*tet*(X4)
MET A1-376	Tig437		■					■		■		■	■	■				■	■	■	■	■
MET A1-378	Tig452	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-379	Tig454	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-380	Tig455	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-381	Tig457	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-382	Tig458	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-383	Tig459	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-384	Tig460		■				■			■		■	■			■	■	■	■	■	■	■
MET A1-385	Tig463	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-386	Tig465	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-387	Tig470		■	■		■	■		■	■		■		■	■				■		■	■
MET A1-388	Tig473	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-389	Tig487	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■
MET A1-390	Tig488	■	■	■	■	■	■			■	■	■	■		■			■	■	■	■	■

The gene's presence is shown by dark-shaded boxes, while its absence is indicated by unshaded boxes.

To better understand the resistance profiles of the *tet*(X4) positive isolates, both genotypic and phenotypic antibiotic resistances were evaluated in the study. By examining whole genome sequencing data and identifying particular resistance genes, genotypic resistance was identified (Table 4.6). On the other hand, phenotypic resistance was evaluated using antimicrobial susceptibility testing, method of broth

microdilution to determine MIC values. 14 *tet*(X4) positive isolates exhibited resistance phenotypically to tetracycline, ampicillin, ciprofloxacin, chloramphenicol, nalidixic acid, trimethoprim and sulfamethoxazole. Genotypic AMR profiles of those isolates have *aad*A1, *aad*A2, *aph*(3')-Ia, *aph*(3")-Ib, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *Inu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A) and *tet*(X4) genes conferring resistance to aminoglycoside, β-lactam, amphenicol, antifolate, phenicol, sulfonamide, fluoroquinolone and tetracycline classes, respectively. As a result, a strong correlation was observed between the presence of known resistance genes and resistance to corresponding antibiotics.

Table 4.6 Genotypic and Phenotypic Resistance Profiles of *tet*(X4) Positive *E. coli*

METU ID / Project ID	**Brand**	**Phenotypic AMR profile**	**Genotypic AMR profile**
MET A1-376 Tig437	NA	AMP, CHL, TET, TGC, TMP, SMX	*aad*A2, bla_{EC-5}, bla_{TEM-1B}, *dfr*A12, *flo*R, *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-378 Tig452	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-379 Tig454	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-380 Tig455	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)

Table 4.6 (Cont'd)

MET A1-381 Tig457	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-382 Tig458	B	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-383 Tig459	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-384 Tig460	E	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A2, bla_{EC-15}, bla_{TEM-1B}, *dfr*A12, *floR*, *oqx*A2, *oqxB*, *qnr*S1, *sul2*, *sul3*, *tet*(A), *tet*(X4)
MET A1-385 Tig463	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, $bla_{EC-15,}$ bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-386 Tig465	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-387 Tig470	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A2, *aph*(3")-Ib, *aph*(6)-Id, bla_{EC-15}, bla_{EC-8}, bla_{TEM-1B}, *dfr*A36, *flo*R, *lnu*(F), *sul2*, *tet*(A), *tet*(X4)
MET A1-388 Tig473	F	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul2*, *sul3*, *tet*(A), *tet*(X4)

Table 4.6 (Cont'd)

MET A1-389 Tig487	F	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)
MET A1-390 Tig488	A	AMP, CHL, CIP, NAL, TET, TGC, TMP, SMX	*aad*A1, *aad*A2, *aph*(3")-Ib, *aph*(3')-Ia, *aph*(6)-Id, bla_{EC-15}, bla_{TEM-1B}, *cml*A1, *dfr*A12, *flo*R, *lnu*(F), *qnr*S1, *sul*2, *sul*3, *tet*(A), *tet*(X4)

AMP: Ampicillin, CHL: Chloramphenicol, CIP: Ciprofloxacin, NAL: Nalidixic Acid, TET: Tetracycline, TGC: Tigecycline, TMP: Trimethoprim, SMX: Sulfamethoxazole

4.5.3 Plasmid Distribution of *tet*(X4) Positive Isolates

Sequenced isolates harbored multiple plasmid types. Replicons of the IncF family were consistently detected across all isolates (Table 4.7). IncFIA, IncFIB (accession: AP001918), and IncFIC(FII) with high sequence identities ranging from 93.56%, 100% and 99.60% respectively were present in 12 of the isolates. MET A1-376 and MET A1-384 were found to have IncFIA(HI1) and IncFIB(K) replicons unlike the other 12 isolates. Resistance genes including *tet*(X4), *tet*(A), bla_{TEM-1B}, *aad*A2, *dfr*A12, *flo*R, *sul*2, *sul*3, and *qnr*S1 were found on IncFIA(HI1) and IncFIB(K) plasmids in a study by Kürekci et al. (2022). IncF plasmids are often large (>100 kb), low copy number plasmids, and carry many replicons (FII, FIA, and FIB) that promote replication initiation (Villa et al., 2010) and are commonly found in Enterobacteriaceae family. The IncF family is highly adaptable and can carry a diverse array of resistance genes alongside virulence factors (Villa et al., 2010). Tend to accumulate resistance genes due to their large size and modular structure, making them major players in MDR.

On the other hand, IncX1 plasmid was also prevalent in the 12 isolates, except MET A1-376 and MET A1-384, exhibiting 98.93% to 100% sequence identity over a length of 374 bp (accession: EU370913). IncX plasmids are medium sized plasmids

with a narrower host range compared to IncF and are known for efficient conjugation and dissemination of resistance genes especially among Enterobacteriaceae (Johnson et al., 2012). During the acquisition of a resistance determinants, which are typically genes, some other genetic mutations or associated regulatory elements that influence the expression of resistance traits can also be included in plasmids which increase the probability that one of the successful plasmids will proliferate and persist in different hosts and settings. IncX plasmids are also known to encode conjugal transfer genes which enables conjugative transfer of their own and accessory functions like biofilm formation to their host bacteria alongside the resistance genes (Johnson et al., 2012; Norman et al., 2008; Zalewska et al., 2024). The occurrence of plasmids that belong to not so largely prevalent plasmid families in naturally occurring bacteria can increase locally because the acquired resistance gene offers selective advantages when antimicrobials are used (Carattoli, 2013).

Certain isolates were found to have distinct plasmid replicons, MET A1-379, MET A1-385 and MET A1-386 additionally contained ColE10, IncB/O/K/Z and IncFII(p96A) plasmids other than the prevalent ones. IncI1-I(Alpha) with 100% sequence identity found in MET A1-381, IncQ1 with 100% sequence identity found in MET A1-387, Col156 with 100% identity in MET A1-376, IncFII(pECLA) with 81.93% identity in MET A1-381, IncFII(pCoo) with 99.62% identity in MET A1-384. In the isolates MET A1-381 and MET A1-388, p0111 plasmids with 99.10% and 98.87% sequence identity respectively (accession: AP010962) were found. ColpVC replicon with 99.48% identity (accession: JX133088) was also found in MET A1-388. Col(MG828) plasmid was found in MET A1-381 and MET A1-382 with 77.10% and 100% identity respectively.

Numerous plasmid varieties, including IncX1, IncHI1 B/A, IncFIA/B, IncQ1, IncFII, IncX4, IncX3, IncN, F-:A18:B-, Col, p0111 and multi-replicon fusion plasmids or hybrid plasmids like IncX1-IncFIA/B-IncY, IncX1-IncFIA(HI1)-IncFIB(K), IncFIA18-IncFIB(K)-IncX1 and IncX1-IncN have been shown to contain *tet*(X4) in various studies and some of which were also detected in the 14 *tet*(X4) positive *E. coli* isolates in this study (Fang et al., 2020; Shafiq et al., 2022; Wang et al., 2022;

Zhai et al., 2022). In a study conducted by Zhang et al. (2022) using the public NCBI database, it was discovered that 399 isolates of *E. coli* that produce Tet(X4) protein have plasmid replicon genes and there were found to be 21 different plasmid replicons. IncHI1A and IncHI1B were the most prevalent kinds, making up 41.4% and 41.9% of all that identified, respectively, followed by IncFIA, IncFII, IncN, IncR, IncI1, IncQ1, Col, IncHI2, IncX4, IncY, IncL/M, IncA/C2, IncX3, IncB/O/K/Z, IncFIB, IncI2, IncP1, IncHI2A, and IncU which were the other plasmid types identified. Furthermore, it was shown that 191 isolates (27.3%) were found to carry at least three replicon genes, 63 isolates (15.8%) each harbored at least four plasmid replicon genes, and 5 of the isolates (1.3%) harbored six or more replicon genes. The *tet*(X4) is often associated with insertion sequences such as IS26, IS*Vsa3*, *rdmc*, IS*CR2* and the *catD* gene, facilitating its regional and interspecies spread (Du et al., 2020; Fang et al., 2020; Zeng et al., 2022, Zhang et al., 2023).

Table 4.7 PlasmidFinder Results of Plasmid Replicons on Sequenced Isolates

METU ID	Project ID	Plasmid	%Identity
MET A1-376	Tig437	Col156	100.00
		IncFIA(HI1)	100.00
		IncFIB(K)	100.00
		IncQ1	100.00
MET A1-378	Tig452	ColRNAI	97.69
		IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncX1	98.93
MET A1-379	Tig454	ColE10	100.00
		ColRNAI	100.00; 97.69
		IncB/O/K/Z	94.38
		IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncFII(p96A)	73.60
		IncX1	98.93

Table 4.7 (Cont'd)

		ColRNAI	97.69
		IncFIA	99.72
MET A1-380	Tig455	IncFIB	98.39
		IncFIC(FII)	95.79
		IncX1	98.93
		Col(MG828)	77.10
		ColRNAI	97.69; 100.00
		IncFIA	99.72
		IncFIB	98.39
MET A1-381	Tig457	IncFIC(FII)	95.79
		IncFII(pECLA)	81.93
		IncI1-I(Alpha)	100.00
		IncX1	98.93
		p0111	99.10
		Col(MG828)	100.00
		ColRNAI	97.69; 100.00
MET A1-382	Tig458	IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncX1	98.93
		ColRNAI	97.69
		IncFIA	99.72
MET A1-383	Tig459	IncFIB	98.39
		IncFIC(FII)	95.79
		IncX1	98.93
		IncFIA(HI1)	100.00
MET A1-384	Tig460	IncFIB(K)	100.00
		IncFII(p96A)	70.60
		IncFII(pCoo)	99.62
		ColE10	100.00
		ColRNAI	90.77;97.69;100.00
		IncB/O/K/Z	94.38
MET A1-385	Tig463	IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncFII(p96A)	73.60
		IncX1	98.93
		ColE10	100
		ColRNAI	97.69
		IncB/O/K/Z	94.38
MET A1-386	Tig465	IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncFII(p96A)	73.60
		IncX1	98.93
		IncFIA	99.72
		IncFIB	98.39
MET A1-387	Tig470	IncFIC(FII)	95.79
		IncQ1	100.00
		IncX1	98.93

Table 4.7 (Cont'd)

MET A1-388	Tig473	ColRNAI	97.69
		ColpVC	99.48
		IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncX1	98.93
		p0111	98.87
MET A1-389	Tig487	ColRNAI	97.69; 66.92
		IncFIA	99.72
		IncFIB	98.39
		IncFIC(FII)	95.79
		IncX1	98.93
MET A1-390	Tig488	ColRNAI	97.69
		IncFIA	93.56
		IncFIB	100.00
		IncFIC(FII)	99.60
		IncX1	100

The eleven *tet*(X4) positive *E. coli* having ST206 type have similar antimicrobial resistance gene profiles and harbor similar plasmid replicons which may also further indicate clonal transmission. Additionally, IncX1 plasmid was found in both ST189 and ST206 *E. coli* isolates and $bla_{\text{TEM-1B}}$, *qnr*S1, *oqx*AB, *flo*R, *aph*(3′)-IIa and *tet*(A) were detected as the most common antibiotic resistance genes along with tigecycline resistance (Feng et al., 2022).

Li et al. analyzed the public databases geographically and found that tigecycline resistance due to *tet*(X) variants were most prevalent in Asian countries, majority being from China, followed by Thailand and Pakistan (Li et al., 2023). The *tet*(X) positive isolates also reported from Europe, America, Africa and Oceania. The pigs were reported to be the primary source followed by humans and chickens. It can be speculated from these findings that the *tet*(X4) gene may be disseminating to different parts of the world by migratory birds, travel and transport of goods. Horizontal gene transfer poses a high risk of dissemination of resistance genes across species, including serious pathogens. Zhai et al. (2022) isolated MDR eight *tet*(X4) positive *E. coli* from stool samples of humans in a tertiary class A hospital in Beijing, China in 2019. The study further supports that *tet*(X4) positive *E. coli* showed clonal spread as bioinformatic analyses show that four of the isolates collected in different

times from various departments of the hospital were close phylogenetically. Furthermore, three isolates found close to previously identified animal origin and environment origin isolates which further emphasizes the high risk on passage of resistance to humans. All the isolates in the study were reported to be bearing IncX1 plasmid replicon and the team also demonstrated successful transfer of tigecycline resistance to recipient strain in three isolates indicating the horizontal gene transfer being another significant mechanism for transfer of *tet*(X4) (Zhai et al., 2022). Analyses of public databases on February 8, 2023, by Li et al. also pointed the horizontal gene transfer and *tet*(X4) positive *E. coli* were significantly diverse with 166 ST types reported (Li et al., 2023).

Plasmid identification within the bacterial genome assemblies could not be done in this study as it can be challenging due to identifying which contigs are plasmid derived being trivial as plasmids and chromosomal DNA are frequently indistinguishable with short Illumina sequences. PlasmidFinder is a tool created to detect and categorize plasmid replicons according to how closely they resemble sequences in curated plasmid databases (Carattoli et al., 2014), however, it cannot relate plasmids with antibiotic resistance genes, like *tet*(X4). Using PlasmidSPAdes to identify individual plasmids in the WGS failed, it is most likely because of difficulties with fragmented assemblies, or inadequate read coverage for plasmid contigs as explained by Antipov et al. (2016). The lack of accurate identification of resistance genes and plasmids in this study can be attributed to the method employed, and the use of long-read whole genome sequencing with hybrid assembly is suggested.

Conjugation experiments and long reads with Oxford nanopore on three isolates which are Tig437 (MET A1-376), Tig460 (MET A1-384) and Tig488 (MET A1-390) were performed for plasmid characterization within the scope of the TUBITAK project and the results were evaluated in project report.

CHAPTER 5

CONCLUSION

In this study, it was aimed to investigate the existence, prevalence and distribution of plasmid-mediated mobile *tet*(X) gene variants, which show high-level tigecycline resistance among the bacteria obtained from meats in the food chain in Türkiye with focus on the Enterobacteriaceae family, particularly *E. coli*.

A total of 42 bacteria from Enterobacteriaceae family and 1 *P. aeruginosa* tigecycline resistant isolate were obtained from collected poultry and red meat samples in the study. However, the plasmid mediated tigecycline resistance gene *tet*(X4) was only detected in 14 *E. coli* isolates in this study.

Bioinformatic investigations have indicated that the dissemination of *tet*(X4) gene can be related to both clonal spread and horizontal gene transfer as same ST types observed in different brands as well as different ST types, ST206 and ST744, isolated from the same brand. Comparison with literature also points out that the resistance genes spreading clonally can also be spreading through being carried via humans, animals, food products, environment and even it can be carried via different bacteria from Enterobacteriaceae.

The clustering of resistant isolates in a specific brand highlights the possibility of localized resistance gene transfer, likely facilitated by shared environmental or operational factors. Host bacterial cells might not suffer a major fitness penalty from keeping the genes or mobile genetic elements that confer resistance to antibiotics, such as *tet*(X4). Under selective pressure like antibiotics or antimicrobials, expression of certain resistance genes can be induced in the presence of antibiotics, and this may also help other resistance genes remain and proliferate in bacterial populations. This situation points out the need for strict regulations and supervision on antibiotic and antimicrobial use on farms and production facilities.

The discovery of many plasmid replicons, some of them being common, on isolates that were *tet*(X4) positive, in addition to a large number of resistance genes, emphasizes how important mobile genetic factors are in the transmission of resistance. Plasmid-mediated horizontal gene transfer, which permits genes to move among many bacterial strains and species, may thus be a major cause of resistance.

This study contributes valuable insights to tracing and combatting antimicrobial resistance especially against last resort antibiotics like tigecycline in the food chain in Türkiye. Antibiotic use on farms should be subjected to strict regulations, as it has been observed to be widespread on farm basis. Future studies should focus on elucidating the fitness cost of plasmids carrying *tet*(X4) genes, the nearby structures and their role in dissemination across different bacterial species and environments.

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APPENDICES

A. CHEMICALS AND MATERIALS

Table A.1 List of materials and the manufacturers

Materials	**Manufacturer**
Petri Dishes	ISOLAB
Glass Tubes	ISOLAB
Sterile Pipette Tips	METTLER TOLEDO-RAININ
Cotton Swabs	LP ITALIANA
Eppendorf Tubes	ISOLAB
Falcon Tubes	ISOLAB
96-Well Microplate	SARSTEDT
Glass Bottles	KAVALIER
Erlenmeyer Flask	ISOLAB
Stomacher Bags	interscience
Scalpel	AESCULAP
Spatula	Thermo Fisher Scientific
Sterile Loops	ISOLAB
Cuvettes	ISOLAB

Table A.2 List of chemicals and the manufacturers

Chemicals, enzymes, and mediums	**Manufacturer**
Trizma Base (Tris)	Sigma-Aldrich (St. Lois, MO, USA)
Trizma Hydrochloride (Tris-HCL)	Sigma-Aldrich (St. Lois, MO, USA)
EDTA	Merck (Darmstadt, Germany)
Boric Acid	Sigma-Aldrich (St. Lois, MO, USA)
Luria-Bertani (LB) Broth	Becton, Dickinson and Company (Le Pont de Claix, France)
Müller-Hinton Media	Oxoid (Hampshire, UK)
Glycerol	Merck (Darmstadt, Germany)
Sodium Chloride (NaCl)	Merck (Darmstadt, Germany)
Brain Hearth Infusion (BHI) Broth	Condalab (Madrid, Spain) Oxoid (Hampshire, UK)
MacConkey Agar Medium	Merck (Darmstadt, Germany)
Blood Agar Base	Becton, Dickinson and Company (Le Pont de Claix, France)
Tigecycline	Sigma-Aldrich (St. Lois, MO, USA)
Forward and Reverse Primers	Oligomer Biyoteknoloji (Ankara, Türkiye)
Taq Polymerase Enzyme	Biomatik (Wilmington, DE, USA)
XbaI Enzyme	Roche (Mannheim, Germany) Thermo Fisher Scientific (Vilnius, Lithuania)
Proteinase K	Roche (Mannheim, Germany) Thermo Fisher Scientific (Vilnius, Lithuania)

Table A.2 (Cont'd)

H Buffer	Roche (Mannheim, Germany) Thermo Fisher Scientific (Vilnius, Lithuania)
Bacteriological Agar	Condalab (Madrid, Spain)
Ethidium Bromide (EtBr)	Merck (Darmstadt, Germany)
Thiourea	Merck (Hohenbrunn, Germany)
SeaKem Gold Agarose	Lonza (Rockland, ME, USA)
N-Lauryl sarcosine Sodium Salt	Merck (Darmstadt, Germany)
Sodium Dodecyl Sulfate (SDS)	Merck (Darmstadt, Germany)

B. PREPARATION OF MEDIA

Table B.1 Brain Hearth Infusion (BHI) Agar

BHI Medium	18.5 g
Bacteriological Agar	7.5 g
Distilled H_2O (dH_2O)	500 mL

Table B.2 Luria Bertani (LB) Agar

LB Medium	20 g
Distilled H_2O (dH_2O)	1 L

Table B.3 0.5M EDTA Stock Solution, pH: 8

EDTA	93.05 g
Double Distilled H_2O (ddH_2O)	450 mL
pH of the solution adjusted to 8 using 10M NaOH solution and ddH_2O added to bring total volume to 500 mL	

Table B.4 10X Tris Borate EDTA Stock (TBE) Solution

Tris Base	54 g
Boric Acid	27.5 g
Ethylene Diamine Tetra Acetic Acid (EDTA)	4.65 g
Double Distilled H_2O (ddH_2O)	500 mL

Table B.5 1M Tris Stock Solution, pH:8

Tris-HCL	78.8 g
Double Distilled H_2O (ddH_2O)	450 mL
pH of the solution adjusted to 8 using 10M NaOH solution and ddH_2O added to bring total volume to 500 mL	

Table B.6 Cell Suspension Buffer (CSB)

1M Tris Stock Solution, pH:8	20 mL
0.5M EDTA Stock Solution, pH: 8	40 mL
Double Distilled H_2O (ddH_2O)	140 mL

Table B.7 Cell Lysis Buffer (CLB)

1M Tris Stock Solution, pH:8	25 mL
0.5M EDTA Stock Solution, pH: 8	50 mL
N-Lauryl Sarcosine Sodium Salt	5 g
Sterile Double Distilled H_2O (ddH_2O)	400 mL
The mixture is heated to 60°C and mixed for 30 min and 25 mL more sterile ddH_2O added	

Table B.8 Tris EDTA (TE) Buffer, pH:8

1M Tris Stock Solution, pH:8	5 mL
0.5M EDTA Stock Solution, pH: 8	1 mL
Double Distilled H_2O (ddH_2O)	450 mL
pH of the solution adjusted to 8 using 10M NaOH solution and ddH_2O added to bring total volume to 500 mL	

Table B.9 10 mg/mL Ethidium Bromide Solution

Et-Br	40 µL
Distilled H_2O (dH_2O)	400 mL

Table B.10 20 mg/mL Proteinase K (ProK) Stock Solution

ProK	0.01 g
Sterile Double Distilled H_2O (ddH_2O)	500 µL
The mixture is stored at -20°C	

Table B.11 20% Sodium Dodecyl Sulfate (SDS) Solution

SDS	10 g
Sterile Double Distilled H_2O (ddH_2O)	500 mL
The mixture is heated to 45°C and mixed	

Table B.12 10 mg/mL Triourea Solution

Thiourea	0.5 g
Sterile Double Distilled H_2O (ddH_2O)	50 µL

Table B.13 Running Buffer

10X TBE	110 mL
Double Distilled H_2O (ddH_2O)	2090 mL

Table B.14 Mueller Hinton Broth

Müller-Hinton Media	21 g
Distilled H_2O (dH_2O)	1 L

Table B.15 Mueller Hinton Agar

Müller-Hinton Media	21 g
Bacteriological Agar	15 g
Distilled H_2O (dH_2O)	1 L

Table B.16 MacConkey Agar

MacConkey Agar Medium	50 g
Distilled H_2O (dH_2O)	1 L

Table B.17 Blood Agar Base Infusion Agar

Blood Agar Base Infusion Agar	40 g
Distilled H_2O (dH_2O)	1 L

C. PCR GEL IMAGE

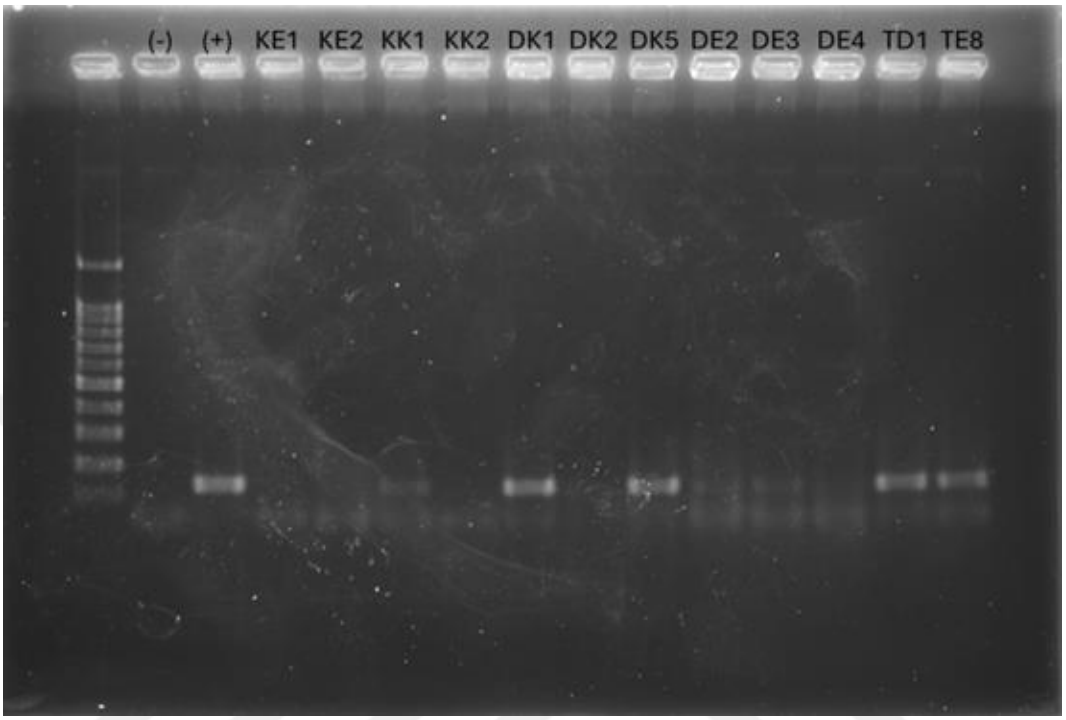

Figure C.1 PCR gel image for *E. coli* confirmation with *rpoB* gene.

PCR gel image. Lane 1: 100bp DNA Ladder; 2: Negative control; 3: Positive control; Lanes 4 to 20: Tested isolates

D. PFGE GEL IMAGES

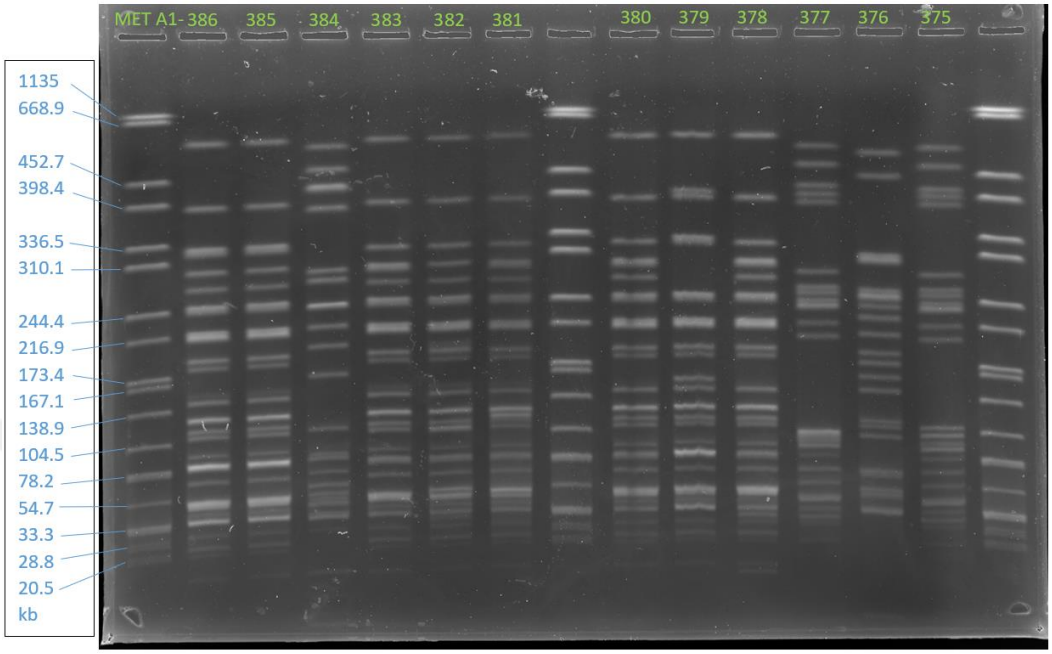

Figure D.1 PFGE gel image of *Escherichia coli* isolates from MET A1-386 to MET A1-375.

PFGE gel image of *Escherichia coli* isolates. Lanes 1,8,15: MET S1-713 (*Salmonella* Braenderup) as standard PFGE reference; 2: MET A1-386, 3: MET A1-385, 4: MET A1-384, 5: MET A1-383, 6: MET A1-382, 7: MET A1-381, 9: MET A1-380, 10: MET A1-379, 11: MET A1-378, 12: MET A1-377, 13: MET A1-376, 14: MET A1-375

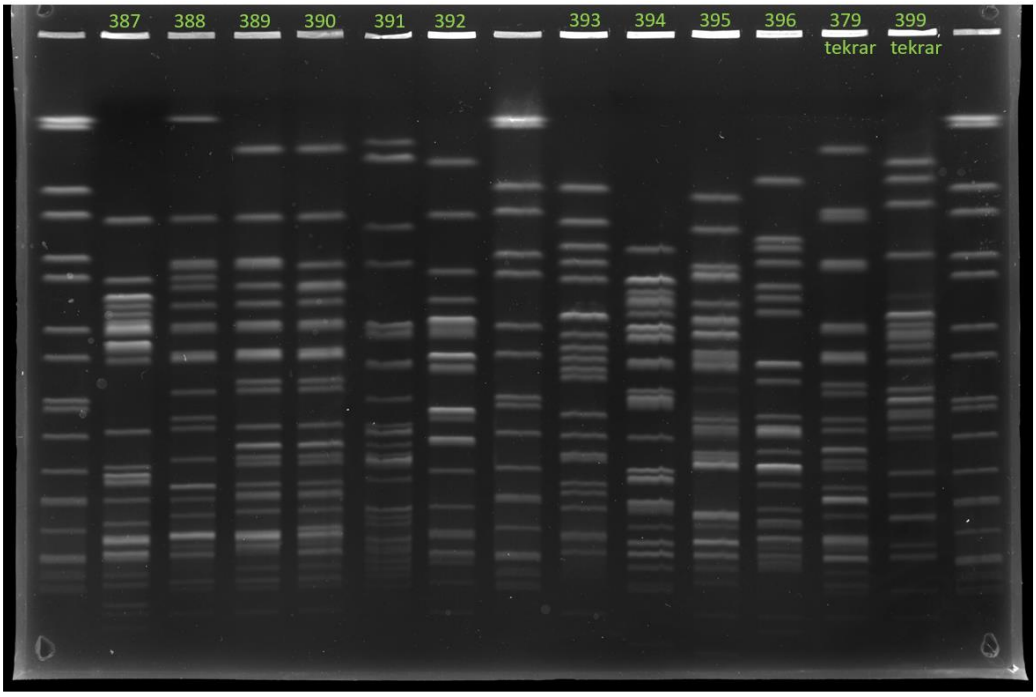

Figure D.2 PFGE gel image of *Escherichia coli* isolates from MET A1-387 to MET A1-396, MET A1-379 (repeated) and MET A1-399 (repeated).

PFGE gel image of *Escherichia coli* isolates. Lanes 1,8,15: MET S1-713 (*Salmonella* Braenderup) as standard PFGE reference; 2: MET A1-387, 3: MET A1-388, 4: MET A1-389, 5: MET A1-390, 6: MET A1-391, 7: MET A1-392, 9: MET A1-393, 10: MET A1-394, 11: MET A1-395, 12: MET A1-396, 13: MET A1-379 (repeated), 14: MET A1-399(repeated)

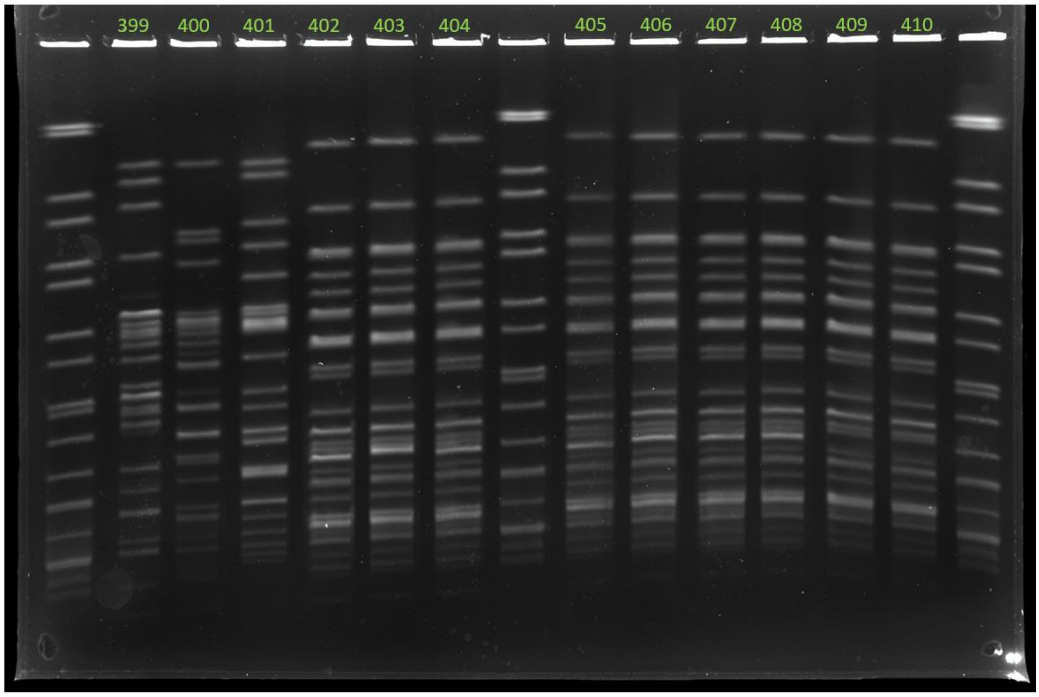

Figure D.3 PFGE gel image of *Escherichia coli* isolates from MET A1-399 to MET A1-410.

PFGE gel image of *Escherichia coli* isolates. Lanes 1,8,15: MET S1-713 (*Salmonella* Braenderup) as standard PFGE reference; 2: MET A1-399, 3: MET A1-400, 4: MET A1-401, 5: MET A1-402, 6: MET A1-403, 7: MET A1-404, 9: MET A1-405, 10: MET A1-406, 11: MET A1-407, 12: MET A1-408, 13: MET A1-409, 14: MET A1-410

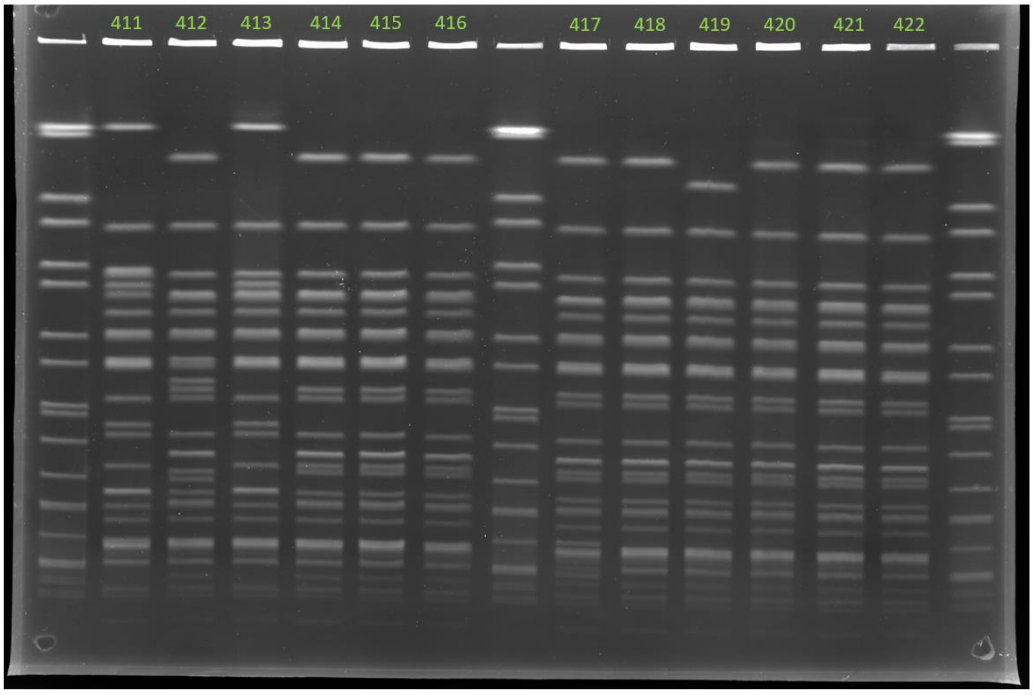

Figure D.4 PFGE gel image of *Escherichia coli* isolates from MET A1-411 to MET A1-422.

PFGE gel image of *Escherichia coli* isolates. Lanes 1,8,15: MET S1-713 (*Salmonella* Braenderup) as standard PFGE reference; 2: MET A1-411, 3: MET A1-412, 4: MET A1-413, 5: MET A1-414, 6: MET A1-415, 7: MET A1-416, 9: MET A1-417, 10: MET A1-418, 11: MET A1-419, 12: MET A1-420, 13: MET A1-421, 14: MET A1-422

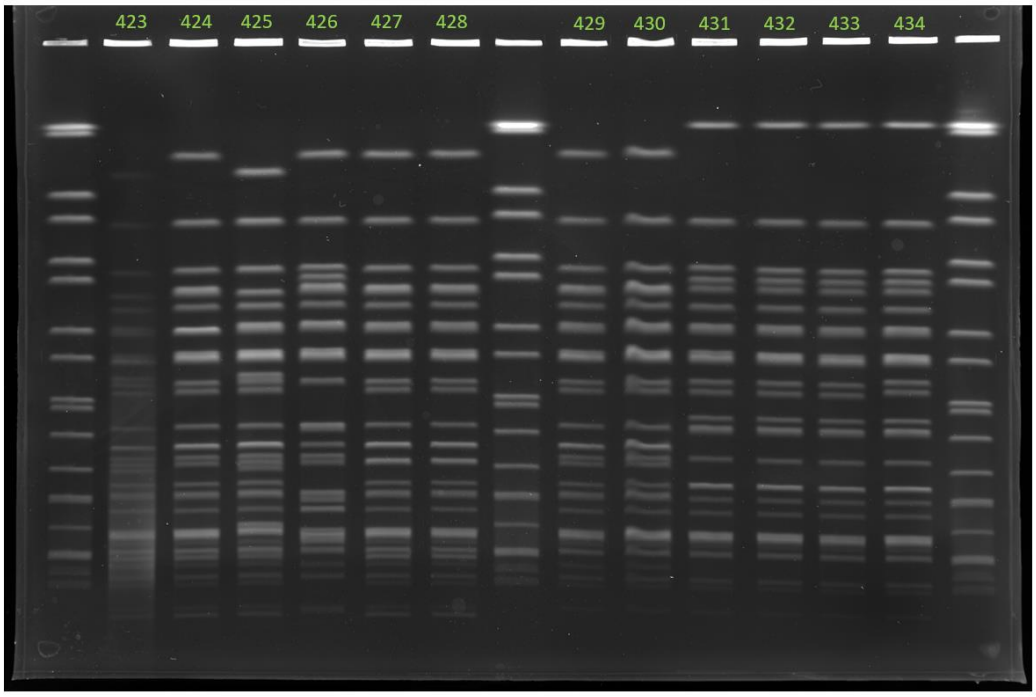

Figure D.5 PFGE gel image of *Escherichia coli* isolates from MET A1-423 to MET A1-434.

PFGE gel image of *Escherichia coli* isolates. Lanes 1,8,15: MET S1-713 (*Salmonella* Braenderup) as standard PFGE reference; 2: MET A1-423, 3: MET A1-424, 4: MET A1-425, 5: MET A1-426, 6: MET A1-427, 7: MET A1-428, 9: MET A1-429, 10: MET A1-430, 11: MET A1-431, 12: MET A1-432, 13: MET A1-433, 14: MET A1-434

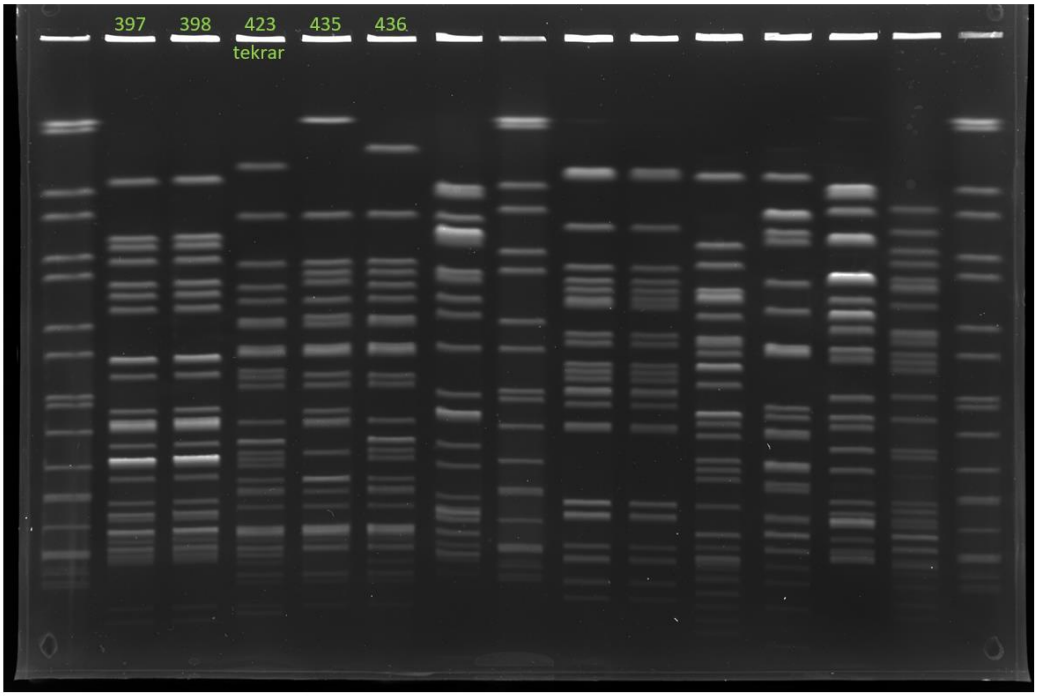

Figure D.6 PFGE gel image of *Escherichia coli* isolates from MET A1-397, MET A1-398, MET A1-423 (repeated), MET A1-435 and MET A1-436.

PFGE gel image of *Escherichia coli* isolates. Lanes 1,8,15: MET S1-713 (*Salmonella* Braenderup) as standard PFGE reference; 2: MET A1-397, 3: MET A1-389, 4: MET A1-423 (repeated), 5: MET A1-435, 6: MET A1-436, rest of the lanes belong to other *E. coli* isolates in the lab database, not isolated in this study