REPUBLIC OF TÜRKİYE
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY

MOLECULAR IDENTIFICATION OF EXTENDED-SPECTRUM BETA-LACTAMASE AND AMINOGLYCOSIDE-MODIFYING ENZYME GENES AMONG LACTOSE-FERMENTING ENTEROBACTERIACEAE CLINICAL ISOLATES

Prepared by
Noor Radhwan Abdulkadhim ALBUNSRALLA

Supervisors
Prof. Dr. Abdurrahman AYVAZ
Assoc. Prof. Dr. Pınar SAĞIROĞLU

M.Sc. Thesis

June 2025
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COMPLIANCE WITH SCIENTIFIC ETHICS

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.

Noor Radhwan Abdulkadhim ALBUNSRALLA

This master's thesis write-up on the topic **"Molecular Identification of Extended-Spectrum Beta-Lactamase and Aminoglycoside-Modifying Enzyme Genes Among Lactose-Fermenting Enterobacteriaceae Clinical Isolates**" has been prepared following the Erciyes University Graduate Education and Teaching Institute Thesis Preparation and Writing Guide.

Owner of Thesis
Noor Radhwan Abdulkadhim ALBUNSRALLA

Supervisor
Prof. Dr. Abdurrahman AYVAZ

Co-Advisor
Assoc. Prof. Dr. Pınar SAĞIROĞLU

Head of Department
Prof. Dr. Abdurrahman AYVAZ

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Noor Radhwan Abdulkadhim ALBUNSRALLA

Erciyes University, Department of Biology
M.Sc. Thesis, July 2025
Supervisor: Prof. Dr. Abdurrahman AYVAZ
Co-Advisor: Assoc. Prof. Dr. Pınar SAĞIROĞLU

ABSTRACT

The routine phenotypic methods originally identified the NA22 isolates as *Klebsiella pneumoniae*. Nevertheless, WGS and NCBI annotation have subsequently confirmed that the microorganism is an *Escherichia coli*. The isolate had been recovered from the bloodstream of a patient and was resistant to many of the most important classes of antibiotics. A total of eight contigs >1,000 bp were generated through sequencing on the Oxford Nanopore platform. Three plasmids, NA22_1, NA22_2, and NA22_3, were identified using PlasmidFinder. Among them, NA22_3 harbored significant resistance genes, including *blaNDM-4, blaCTX-M-15,* and *aac(6')-Ib-cr,* along with the tra operon genes that may facilitate plasmid transfer. Functional annotation was performed against the PATRIC, CARD, and VFDB databases, and 76 resistance and over 200 virulence, as well as over 100 metabolism and transport involved subsystems, were found. The antimicrobial susceptibility tests revealed resistance to β-lactams (including carbapenems), fluoroquinolones, aminoglycosides, and sulfonamides, which were consistent with the genome profile. The complete genome sequence is included in BioProject ID: PRJNA1270365. These results highlight the importance of routine genomic surveillance of multidrug-resistant strains within hospitals.

Keywords: genome sequencing, multidrug resistance, plasmids,*blaNDM-4, blaCTX-M-15, aac(6')-Ib-cr*, antimicrobial resistance, virulence genes, hospital infection

LAKTOZ FERMENTE EDEN ENTEROBACTERIACEAE KLINIK İZOLATLARINDA GENIŞ SPEKTRUMLU BETA-LAKTAMAZ VE AMINOGLIKOZID MODIFIYE EDICI ENZIM GENLERININ MOLEKÜLER TANIMLANMASI

Noor Radhwan Abdulkadhim ALBUNSRALLA

Erciyes Üniversitesi, Fen Bilimleri Enstitüsü
Yüksek Lisans Tezi, Temmuz 2025
Danışman: Prof. Dr. Abdurrahman AYVAZ
Eş Danışman: Doç. Dr. Pınar SAĞIROĞLU

ÖZET

Rutin fenotipik yöntemler başlangıçta NA22 izolatlarını *K. pneumoniae* olarak tanımladı. Bununla birlikte, WGS ve NCBI anotasyonu daha sonra mikroorganizmanın *Escherichia coli* olduğunu doğrulamıştır. İzolat, bir hastanın kan dolaşımından elde edilmişti ve en önemli antibiyotik sınıflarının çoğuna dirençliydi. Oxford Nanopore platformunda dizileme yoluyla toplamda 1.000 bp'den büyük sekiz kontig oluşturuldu. Üç plazmid, NA22_1, NA22_2 ve NA22_3, PlasmidFinder kullanılarak tespit edildi. Bunlar arasında, NA22_3, plazmid transferini kolaylaştırabilecek tra operon genleri ile birlikte *blaNDM-4*, *blaCTX-M-15* ve *aac(6')-Ib-cr* gibi önemli direnç genlerini barındırıyordu. Fonksiyonel anotasyon, PATRIC, CARD ve VFDB veritabanlarına karşı gerçekleştirildi ve 76 direnç ve 200'den fazla virülans, ayrıca 100'den fazla metabolizma ve taşıma ile ilgili alt sistemler bulundu. Antimikrobiyal duyarlılık testleri, genoma uygun olarak β-laktamlar (karbapenemler dahil), florokinolonlar, aminoglikozitler ve sülfonamidlerle direnç olduğunu ortaya koydu. Tam genom dizilimi BioProject ID: PRJNA1270365'te yer almaktadır. Bu sonuçlar, hastanelerde çoklu ilaç dirençli suşların rutin genomik izlenmesinin önemini vurgulamaktadır.

Anahtar Kelimeler: genom dizileme, çoklu ilaç direnci, plazmid, *blaNDM-4, blaCTX-M-15, aac(6')-Ib-cr*, antimikrobiyal direnç, virülans genleri, hastane enfeksiyonu

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LIST OF ABBREVIATIONS

CDSs	:	Coding Sequences
CR gene	:	Carbapenem-Resistant Gene
acq.PDR	:	Acquired Pan-Drug Resistance
ARGs	:	Antibiotic-Resistant Genes
AMEs	:	Aminoglycoside-Modifying Enzymes
HAIs	:	Healthcare-Associated Infections
VAP	:	Ventilator-associated pneumonia
AST	:	Antimicrobial Susceptibility Testing
CDC	:	Centers for Disease Control and Prevention
CG	:	Clonal Group
EDTA	:	Ethylenediaminetetraacetic Acid
ESBL	:	Extended-Spectrum β-Lactamases
EUCAST	:	European Committee on Antimicrobial Susceptibility Testing
GC	:	Guanine-Cytosine Content
KPC	:	*K. pneumoniae* Carbapenemase
LB	:	Luria-Bertani
MDR	:	Multidrug Resistant
MIC	:	Minimal Inhibitory Concentration
NDM	:	New Delhi Metallo-β-lactamase
OXA	:	Oxacillinase
QC	:	Quality Control
WGS	:	Whole Genome Sequencing
XDR	:	Extensively Drug-Resistant

INTRODUCTION

In the 21st century, antimicrobial resistance (AMR) has emerged as one of the world's most pressing public health concerns and poses a growing threat to public health, particularly in medical institutions. Hospital patients with invasive procedures, immunocompromised conditions, and those taking broad-spectrum antibiotics are the sites of their reproduction and spread, which results in the spread of resistant organisms. The multidrug-resistant (MDR) organisms *E. coli, Klebsiella pneumoniae,* and Enterobacter species are some of the leading pathogens responsible for healthcare-associated infections (HAIs) such as bloodstream infection, pneumonia, catheter-associated urinary tract infection (CAUTI), and surgical site infection (SSI) [1]. Such an increase in MDR organisms not only complicates the treatment and extends the lengths of stay in the hospitals but also leads to excessive health care costs and morbidity, and mortality. Recent studies and meta-analyses showed that MDR pathogens are widespread in HAIs and HCAIs with a high level of prevalence and spread, and the genus that has been hit the hardest by these is *K. pneumoniae* [2]. Carbapenem-resistant bacteria with extended-spectrum β-lactamases (ESBLs) and carbapenemases, in particular OXA-48-type enzymes, are detected more frequently. The data on KPC- and OXA-48-producing strains isolated since as early as 2012 confirm that carbapenem resistance has taken deep root in the network of health facilities in Türkiye [3]. These resistance mechanisms are often associated with mobile genetic elements that may be horizontally transferable and facilitate the dissemination of resistance traits. Phenotypic techniques for detection of antimicrobial resistance. In-plate susceptibility tests, based on the growth of the bacterial pathogen in the presence of the antibiotic, remain state of the art with traditional diagnostic methods, are not always sensitive enough, and fail to detect resistance mechanisms. In contrast, whole sequencing (WGS) is a hard and pan-genomic molecular method of investigation in bacterial pathogens. WGS also enables the detection of known and new resistance genes, plasmids, transposons, and other mobile genetic elements and

fine-resolution mapping of transmission pathways [4]. As such, WGS quickly became a valuable tool for monitoring AMR and outbreak investigation. Its significance in the context of infection prevention and control has been emphasised locally and globally [5]. Given the clinical and epidemiological importance of MDR Enterobacteriaceae, genome-based surveillance is key to the support of antibacterial stewardship programs and the effective application and assessment of infection prevention activities. In the present study, we employed whole genome sequencing for the analysis of a lactose-fermenting, multi-drug-resistant isolate of the Enterobacteriaceae, NA22, recovered from a nosocomial bacteraemia. The aim is to reveal the genetics behind resistance and to support molecular epidemiology and possibly transmission of this critically important isolate.

CHAPTER 1

GENERAL INFORMATION

1.1. Clinical Significance of Multidrug-Resistant Enterobacteriaceae

Multidrug-resistant Enterobacteriaceae strains pose a threat to hospital environments, especially in intensive care units. Bacteria such as *Escherichia coli* and *K. pneumoniae* are often isolated from hospital environments. These types of bacteria cause infections in the blood, lungs (especially from ventilators), the urinary tract, or even surgical wounds [6]. What makes the situation more dangerous is the development of resistance mechanisms such as the production of ESBLs and carbapenemases. In addition, their ability to survive on hospital instruments and surfaces for extended periods helps them spread easily among patients and staff [7, 8]. All these factors make dealing with these strains increasingly difficult day by day, which increases the length of hospital stays, treatment costs, as well as the difficulty of treatment and the likelihood of complications or death.

1.2. Genomic Features of Multidrug-Resistant Enterobacteriaceae

The Enterobacteriaceae family contains a core genome shared among all strains belonging to this family and an accessory genome that varies among different strains of the Enterobacteriaceae family. Multidrug-resistant intestinal bacteria are highly active and usually contain mobile genetic elements that aid in the spread of resistance traits. Resistance genes, in addition to virulence factors and other adaptive elements, are found in the accessory genome. Plasmids, transposons, and integrons also assist in the horizontal transfer of genes. Plasmids facilitate the horizontal transfer of resistance genes across strains and even species, such as the transfer of resistance genes *bla_CTX-M*, *bla_NDM*, and *aac(6'–Ib).* Insertion sequences (IS) and genomic islands also help in the persistence of foreign DNA in the chromosome [9]. Whole genome sequencing (WGS) significantly

helps in understanding how these parts work together and change over time. Genomic studies have shown that some strains, such as *E. coli* ST131 and *K. pneumoniae* ST258, have spread worldwide because they have acquired numerous resistance and virulence genes. These results demonstrate the importance of genomic studies in monitoring the function of resistance genes at the molecular level and in devising plans to halt infections [10, 11].

1.3. Emergence of Hypervirulent and Multidrug-Resistant Enterobacteriaceae

One of the most concerning issues is that these resistant strains have now become resistant to more than one class of antibiotics and can also carry virulence factors. It was believed that antibiotic resistance and virulence could not occur in the same strain because their evolutionary paths are usually separate. But a recent study has shown that some types of intestinal bacteria have managed to combine these two dangerous traits into a single strain. In 2018, China identified a case of a strain of *K. pneumoniae* that was carbapenem-resistant and highly virulent (CR-hvKp). The isolate was part of the ST11 lineage and contained genes associated with virulence and resistance. This made it a high-risk strain, significantly reducing treatment options [12]. Since then, suspected strains have been found in other places, such as Europe and East Asia [13, 14]. These strains are generally resistant to the last line of antibiotics, including carbapenems, and can make previously healthy individuals fall ill. A combination of hypervirulence genes and multidrug resistance is increasingly considered a serious public health threat.

1.4. Adaptations of MDR Enterobacteriaceae in Clinical Settings

In hospitals, multidrug-resistant Enterobacteriaceae (MDR) bacteria commonly face major selective forces, which are antibiotic treatment and the host's immune system. These factors drive the bacteria into a gradual state of adaptation. Clinical isolates have shown that they acquire mutations that enhance survival and persistence in the host, especially in patients with temporary and/or chronic infections [15]. Biofilm formation is one of the main survival mechanisms. Biofilms are organized communities of bacteria surrounded by a sticky substance, typically forming on medical implants such as catheters or breathing tubes. Such structures limit the penetration of antibiotics and allow bacteria to evade the immune system, making treatment more difficult [16]. Laboratory results indicate that bacteria growing in biofilms acquire resistance more quickly. These

mutations include efflux pump genes, regulatory proteins, and biofilm production [17]. Environmental signals such as temperature changes, nutrient level changes, contact with solid or biotic surfaces, and communication also stimulate distinctive genetic pathways for adhesion. These adaptations are overcome when the more adapted bacterial species to the hospital environment reproduce and spread [18].

1.5. Mechanisms of Antibiotic Resistance in Enterobacteriaceae

1.5.1. β-Lactamase Production

One of the main survival mechanisms for the Enterobacteriaceae in response to antibiotics is the synthesis of β-lactamases. These enzymes inactivate antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems and share the common feature of hydrolysing the β-lactam ring, which is necessary for interaction with penicillin-binding proteins (PBPs), which construct the bacterial cell wall. and when hydrolyzed, it is no longer effective. Based on the molecular classification proposed by Ambler, the β-lactamases belong to one of four classes: A, B, C, and D [19]. Class A enzymes are serine-dependent enzymes such as TEM, SHV, CTX-M, and KPC, many of which can be inhibited by β-lactamase inhibitors clavulanic acid and tazobactam. On the other hand, class B enzymes carbenemases , such as NDM, depend for their enzymatic activity of zinc ions and are immune to inhibition by Kazal-type inhibitors [20]. Among these, the AmpC-type cephalosporinases are classified in Class C, and they are usually of chromosomal origin but can be found plasmid-mediated, and they are not inhibited by clavulanic acid [21]. Class D enzymes such as OXA-48 have particularly narrow-spectrum carbapenemase activity and are frequently misdiagnosed by routine laboratory tests [22]. These β-lactamase genes *bla_TEM, bla_SHV, bla_CTX-M, bla_KPC*, etc, are commonly associated with plasmids or transposons that mediate their transfer between bacterial populations [23]. Their expression could be induced by higher levels of regulators or cellular stresses, for example, the stress response to peptidoglycan degradation is the trigger of the AmpR-AmpC system. Recent years have witnessed a tremendous increase in the prevalence and spread of CTX-M enzymes, particularly CTX-M-15, as the predominant extended-spectrum β-lactamases (ESBLs) worldwide, replacing the previous β-lactamase types, such as TEM and SHV [24]. First identified in India, the spread of NDM is global, and not uncommonly it is found in combination with

resistance determinants such as *mcr-1* and *armA*, heightening multidrug resistance [23]. On the other hand, while subtle in their resistance profile and convenient in their diagnosis, OXA-48-like carbapenemases are still a matter of concern in Türkiye and the wider Middle East [22]. Taken together, β-lactamase production in Enterobacteriaceae, either by narrow-spectrum or powerful carbapenemases, still threatens the utility of even last-line antibiotics and remains a constant threat to infection control in the hospital environment.

1.5.1.1. Extended-Spectrum β-Lactamases (ESBLs)

Extended-spectrum β-lactamases (ESBLs) are enzymes produced by certain Gram-negative bacteria, allowing them to resist third-generation cephalosporins such as cefotaxime, ceftazidime, and ceftriaxone, as well as the monobactam aztreonam. These enzymes mainly evolved from older β-lactamase genes like *bla_TEM* and *bla_SHV* through point mutations that changed amino acids near the active site, expanding their ability to break down antibiotics. A major group within ESBLs, known as CTX-M enzymes, has gained global prominence, especially CTX-M-15, which is now the most frequently detected ESBL variant in clinical settings [25, 26].ESBL genes are usually found on plasmids, which makes them easily transferable between bacteria. These plasmids often carry additional resistance genes to other antibiotic classes, including aminoglycosides and fluoroquinolones. This makes infections harder to treat. Despite this, most ESBL-producing bacteria still respond to carbapenem antibiotics, which remain a reliable treatment. However, the rise of strains that produce both ESBLs and carbapenemases presents an increasing therapeutic challenge that requires close monitoring and molecular surveillance [27].

1.5.1.2. AmpC β-Lactamases

AmpC β-lactamases are a significant group of enzymes that grant resistance to a wide spectrum of β-lactam antibiotics, particularly third-generation cephalosporins (e.g., cefotaxime, ceftriaxone), cephamycins (e.g., cefoxitin), and monobactams. Unlike extended-spectrum β-lactamases (ESBLs), AmpC enzymes are not inhibited by classical β-lactamase inhibitors such as clavulanic acid, sulbactam, or tazobactam, which complicates their detection in clinical laboratories [21]. These enzymes are often encoded chromosomally in organisms such as *Enterobacter* spp., *Citrobacter freundii*, and

Serratia marcescens, but plasmid-mediated AmpC genes (like *bla_CMY, bla_DHA*) have increasingly been found in *E. coli* and *K. pneumoniae*, contributing to community-acquired and nosocomial infections [28, 29]. Plasmid-borne AmpC genes are especially problematic because of their ability to spread horizontally across species. Overproduction of AmpC can result from mutations in the promoter or attenuator regions of the regulatory genes, leading to hyper-resistance. In some cases, resistance is further intensified when these enzymes are combined with porin loss or efflux pump activation, creating multi-mechanistic resistance patterns that render many β-lactams and even carbapenems less effective [30]. Clinically, AmpC producers pose a diagnostic challenge. Many routine phenotypic tests may fail to detect them, making molecular-based approaches essential for accurate identification. The silent spread of these enzymes in hospitals can lead to inappropriate empirical therapy and increased morbidity, which highlights the importance of surveillance and diagnostic refinement [19].

1.5.1.3. Carbapenemases

Carbapenemase production is now recognized as one of the most dangerous mechanisms of antibiotic resistance among Enterobacteriaceae . These enzymes have the ability to inactivate a broad range of β-lactam antibiotics, including carbapenems, which are often used as the last therapeutic option for severe infections. A major concern is that the genes encoding these enzymes are commonly located on mobile genetic elements like plasmids, which allow them to spread rapidly across different bacterial species in both hospital and community settings [20]. Carbapenemases are classified into three major groups based on the Ambler molecular classification: Class A enzymes, such as KPC (*K. pneumoniae* carbapenemase), contain serine residues at the active site and show partial inhibition by β-lactamase inhibitors like clavulanic acid. Class B enzymes such as NDM, VIM, and IMP are metallo-β-lactamases that require zinc ions for their activity and are not affected by "traditional" inhibitors. Class D enzymes, such as OXA-48, also employ a serine mechanism, although they are generally less efficient in the hydrolysis of carbapenems and evade detection in standard laboratory tests [31]. NDM-type beta-lactamase enzymes originated in India and have now spread worldwide. Strains carrying other resistance genes (colistin resistance, aminoglycosides) can also be detected.OXA-48-like enzymes are especially prevalent in Türkiye, the Middle East, and several European countries. Their low-level activity against carbapenems makes them particularly challenging to

identify through standard susceptibility testing [32]. Clinically, infections caused by carbapenemase-producing Enterobacteriaceae (CPE) are associated with high mortality, limited treatment options, and increased healthcare costs. Their detection often requires molecular assays or specialized phenotypic methods, especially when resistance levels are subtle, as seen with OXA-48 [33]. As a result, controlling their spread in healthcare settings demands both molecular surveillance and strict infection control practices.

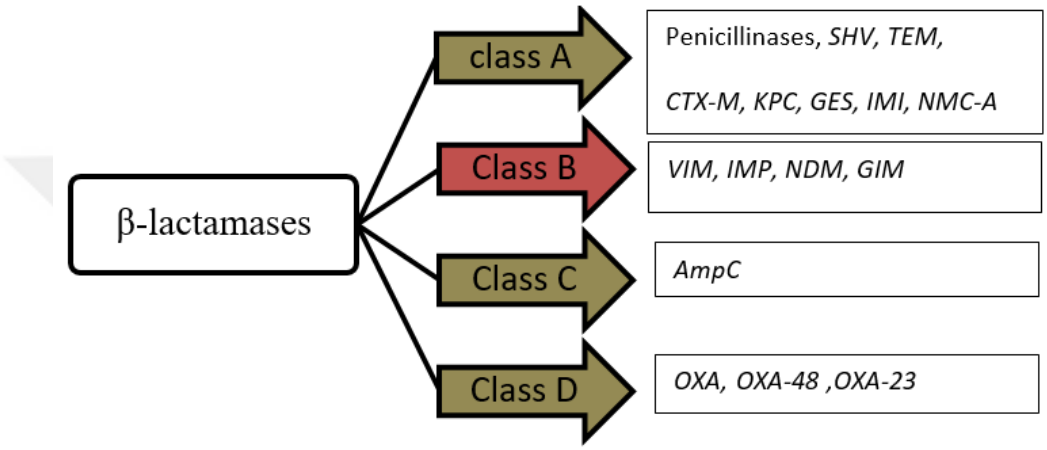

Figure 1.1. Ambler classification of β-lactamases. Classes A, C, and D use serine; Class B requires zinc. Each confers resistance to specific β-lactam antibiotics. Created by the author based on the Ambler classification scheme [19, 34].

Table 1.1. Approximate inhibitory activity of common β-lactamase inhibitor combinations against Ambler classes of β-lactamases.

B-Lactamase inhibitors	Class A			Class B	Class C	Class D
	Narrow	ESBL	KPC	NDM, VIM	AmpC	OXA-48
Clavulanic acid + amoxicillin, ticarcillin						
Sulbactam + ampicillin, piperacillin, cefoperazone						
Tazobactam + piperacillin, ceftolozane						
Avibactam+ceftaroline,ceftazidime, aztreonam						
Relebactam + imipenem						
Vaborbactam + biapenem ,meropenem						

Green = strong inhibition, Yellow = partial/moderate inhibition, Orange = low or no inhibition [35, 107].

1.5.2. Alterations in Membrane Permeability

One of the most important resistance mechanisms developed in the case of Enterobacteriaceae , including loss or changes of the porin proteins, defends the bacterial

cell against penetration of antimicrobials. These porins function as pores that permit the entry of antibiotics into the cell. Similarly, mutations leading to decreased expression or modification of porins such as OmpK35 and OmpK36 greatly reduce antibiotic penetration and have been associated with resistance to a broad spectrum of β-lactams, also to carbapenems and cephalosporines [36, 37]. For example, the loss of OmpK36 in ESBL-producing strains results in high-level resistance to third-generation cephalosporins. These porin changes may additionally interact with the expression of β-lactamase for the expression of such resistance phenotypes in clinical isolates like NA22, confounding the therapeutic strategy. These modifications are frequently acquired in the presence of antibiotics in the hospital setting and are a major obstacle to effective antibiotic treatment.

1.5.3. Efflux Pump Mechanisms

Efflux pump systems are viewed as one of the most important weapons that Gram-negative bacteria deploy to endure antibiotic challenge. These are protein complexes in the bacterial membrane that pump antibiotics out of the cell, decreasing the concentration of antibiotics inside the cell, thereby canceling their treatment response. This process is not only a part of natural defense by the bacteria also in acquired multi-drug resistance. A well-studied representative of the RND family includes the AcrAB-TolC system. This efflux pump has wide-ranging activity for antibiotics such as β-lactams, tetracyclines, fluoroquinolones, and chloramphenicol. It is overexpressed in clinical isolates of Enterobacteriaceae and is associated with reduced antibiotic susceptibility [38]. Efflux pumps may be assembled following exposure to environmental stressors, such as antibiotics. In addition, this resistance mechanism often also works in conjunction with other factors, such as decreased porin expression and β-lactamase production, producing a synergistic effect that enhances resistance as well as treatment complexity [39].

1.5.4. Target Site Modification

Changing the subsites on which antibiotics react is one of the fundamental means by which the Enterobacteriaceae resist antibiotics. This general may result from mutations in chromosomal genes or the acquisition of resistance determinants that cause modifications in bacterial proteins or ribosomal components and, consequently, prevent an efficient binding of the drug. For example, resistance to fluoroquinolones is usually

associated with point mutations in the *gyrA* and *parC* genes, which encode the subunits of DNA gyrase and topoisomerase IV, enzymes necessary for bacterial DNA replication. They alter the stereoscopic structure of the binding pocket so that the antibiotic does not bind properly, and therefore does not function [40, 41]. Methylation of the 16S rRNA in the bacterial ribosome can result in resistance to aminoglycosides. This mechanism, mediated by enzymes like *armA* or *rmtB*, blocks the binding of the antibiotic, resulting in a high resistance to almost all of the molecules from that class of antibiotics [42]. These molecular adaptations present a clinical challenge due to the fact that they usually co-occur with other resistance mechanisms, thus further limiting treatment choices and complicating the design of treatment.

1.5.5. Resistance to Non-β-Lactam Antibiotics

In addition to β-lactam antibiotics, the Enterobacteriaceae family has acquired a range of resistance determinants that enable them to resist other structurally diverse non-β-lactam drugs such as aminoglycosides, fluoroquinolones, polymyxins, tetracyclines, and sulfonamide-trimethoprim combinations. The resistance to aminoglycosides is frequently due to the bacterial production of modifying enzymes, including acetyltransferases (AAC), phosphotransferases (APH), and nucleotidyltransferases (ANT), which modify the structure of the antibiotic and wrongly make it incapable. Moreover, high-level resistance may be mediated by 16S rRNA methylating enzymes encoded by the *armA* or *rmtB* genes, such that the drug cannot bind to its ribosomal target [43]. Resistance to polymyxins, in particular to colistin, is commonly associated with modifications of the lipid A moiety in the outer membrane. This can be achieved by plasmid-mediated *mcr-1* (resulting in addition of a phosphoethanolamine group to the lipid A) or through mutation of *mgrB* or *pmrA/B, pmrC, phoP/Q* genes (inducing bacterial envelope changes decreasing the drug's binding) [44, 45]. The structure and functioning of DNA gyrase and the mechanisms of action of quinolones caused by altered levels of DNA, and are generally due to point mutations in the genes encoding for DNA gyrase and topoisomerase IV (*gyrA* and *parC*, respectively), leading to reduced binding of the antibiotic. Other mechanisms involve efflux pumps such as *AcrAB-TolC* and plasmid-mediated quinolone resistance (PMQR) genes conferring protection to DNA replication enzymes [46]. Tetracycline resistance in general is mediated by efflux systems (*tetA* or *tetB*) or ribosomal protection proteins that outcompete binding of the antibiotic, and

recent findings even describe tetracycline-inactivating enzymes in environmental bacteria [47]. Finally, resistance to inhibitors of folic acid synthesis (sulphonamides and trimethoprim) is commonly facilitated through the production of alternate enzymes mediated by the *sul1* and *sul2*, and *dfrA* genes that are not affected by the drugs [46]. These resistance tools, which usually work concomitantly, markedly complicate the management of multidrug resistant infections.

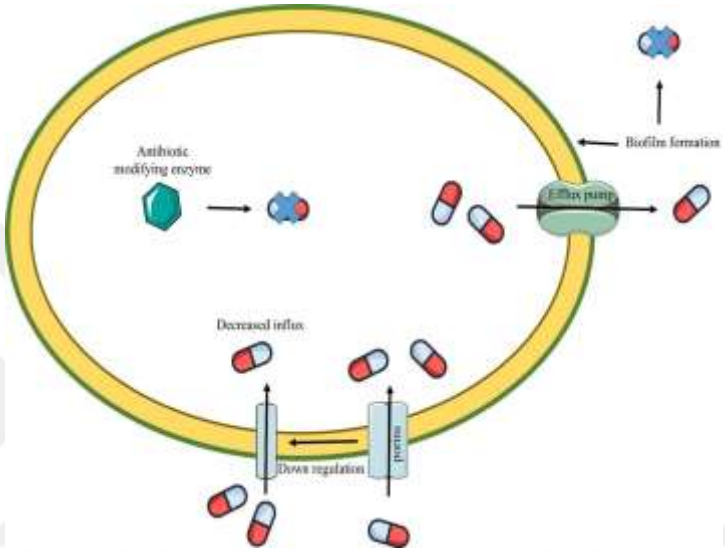

Figure 1.2. Mechanisms of antibiotic resistance in Enterobacteriaceae [48].

Table 1.2. Common Innate Antibiotic Resistance Mechanisms in Enterobacteriaceae .

Resistance Mechanism	Antibiotic Class	Reference
Biofilm formation	Penicillins, aminoglycosides, fluoroquinolones	[49]
Efflux pump	β-lactams, tetracyclines, fluoroquinolones, macrolides	[38]
Enzymatic inactivation or antibiotic-modifying enzymes	β-lactams	[50, 51, 52]
Reduced permeability (porin loss)	β-lactams, fluoroquinolones	[53]

Table 1.3. Acquired Antibiotic Resistance Mechanisms in Enterobacteriaceae .

Gene(s)	Antibiotic Class	Reference
aac(3)-IIa, aph(3')-Ia, ant(2")-Ia	Aminoglycosides	[43]
bla_TEM, bla_SHV, bla_CTX-M, bla_KPC, bla_NDM, bla_OXA-48	β-lactams (ESBLs & carbapenems)	[50] ,[54]
mcr-1	Colistin (Polymyxins)	[55]
FosA	Fosfomycin	[56]
tetA, tetX	Tetracyclines	[57]
16S rRNA methylase (*armA, rmtB*)	Aminoglycosides	[42]
aac(6')-Ib-cr	Quinolones & Aminoglycosides	[58]

1.6. Phenotypic and Genotypic Mechanisms of Antibiotic Resistance in Enterobacteriaceae

Infections with members of the Enterobacteriaceae are increasingly harder to treat because of the increase in antibiotic resistance. Resistance to it can be evaluated by two main methods: phenotypic and genotypic. Phenotypic resistance is determined by observing the behavior of bacteria during laboratory tests, such as automated systems (VITEK 2) and mass spectrometry tools (MALDI-TOF MS). VITEK 2 for tracking the minimum inhibitory concentration (MIC) as changes in bacterial growth over [59], and MALDI-TOF MS in assisting the rapid species identification through the acquisition of protein fingerprinting before performing resistance testing. The tools are useful for capturing resistance to antibiotics such as cephalosporins and carbapenems. On the other hand, genotypic resistance comes from identifying particular genes or mutations that are "known to be associated with resistance", and this has been the case before resistance is evident in the test results. Genes, such as *bla*, *mcr*, and *qnr*, may indicate in advance the potential treatment failure outcomes [60]. Whole-genome sequencing (WGS) has become very important for identifying these genetic factors, which can be silent resistance genes, drug target mutations or overexpression of efflux systems [61]. The combined phenotypic and genotypic approaches complement each other and provide more comprehensive information of the resistance profile, and offer better guidance for treatment and infection control.

1.6.1. Phenotypic Detection of Antimicrobial Resistance

Until now, phenotypic testing is still one of the most feasible and frequently used approaches in detecting AMR to Enterobacteriaceae . The tests rely on watching how bacteria grow when offered antibiotics, and are crucial to choosing treatments that work, not least in hospitals. In contrast to genotypic assays that identify the presence of resistance genes, whether or not they are actively involved in resistance, phenotypic assays provide a direct readout that the resistance genes are, indeed, being expressed and contributing to bacterial tendency [62]. Two widely used phenotypic techniques are disk diffusion and the VITEK 2 system. Both are interpreted according to the EUCAST guidelines, which establish clinical breakpoints that classify bacteria as Susceptible, Intermediate, or Resistant, according to the diameters of the zone of inhibition or MIC

(Minimum Inhibitory Concentration) values. In disc diffusion, Mueller-Hinton agar is inoculated with the bacterial suspension and discs of antibiotics are added to the agar. If the antibiotic has blocked the growth, clear zones will develop around the discs post-incubation. The size of these zones reflects the effectiveness of the antibiotic, resistance being determined by the size of the zone, a larger zone demonstrating higher sensitivity [62]. It provides improved accuracy and efficiency by performing automated analysis of bacterial growth with light and fluorescence sensors. It interprets MIC and provides prompt reports, which can be very useful in resistant organisms that produce ESBLs or carbapenemases [63]. Over the years, MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) has become an important diagnostic technique in clinical microbiology. While it is not an antibiotic resistance test directly, the test quickly and precisely identifies bacteria to the species level through the use of specific protein profiles of bacteria. It is based on the measurement of the mass-to-charge ratio of the cellular proteins, which results in a specific spectrum for each species. Yet some restrictions have been provided in species discrimination; especially when two closely related species as *K. pneumonia* and *E. coli* are compared some erroneous identifications have been reported [64]. Nevertheless, MALDI-TOF is an increasing aid to other phenotypic tests, and it contributes to diagnostic certainty in microbial laboratories.

1.6.2. Genotypic Detection via Whole Genome Sequencing

Use of WGS as the most powerful method to detect ARGs in Enterobacteriaceae, informative data is provided than ever before, with a resolution above phenotypic information that can be gained. In contrast to regular assays that detect only expressed resistance, WGS detects active as well as silent resistance determinants, chromosomal mutations, or plasmid-borne genes. This approach could allow detecting the genes conferring resistance to a broad spectrum of antibiotics, colistin (e.g., the *mcr* genes), aminoglycosides (*aac, armA*), fluoroquinolones (*qnr, gyrA* mutations), and beta-lactams (e.g., *blaCTX-M, blaNDM, blaOXA*) [65, 66]. Additionally, WGS can identify transposable elements and integrons that are associated with horizontal gene transfer and dissemination of resistance. A second benefit could be represented by the identification of efflux pump genes and regulatory mutations, which might not be revealed by phenotypic assays. Moreover, WGS facilitates phylogenetic and comparative genome

analysis used within outbreak investigation and clonal transmission determination. When combined with phenotypic information, WGS provides precise diagnoses and directs targeted antimicrobial stewardship in an era of escalating rates of multidrug-resistant Enterobacteriaceae [67].

1.7. Epidemiological Trends and Resistance Patterns of Enterobacteriaceae

Global spread of antimicrobial-resistant Enterobacteriaceae is a pressing challenge for public health, especially because multidrug-resistant (MDR), and even some extensively drug-resistant (XDR) and occasionally pan-drug resistant (PDR)[1] strains have emerged. This family of bacteria, which includes species such as *K. pneumoniae* and *Escherichia coli*, has been implicated in a variety of hospital-acquired infections, and some are classified as ESKAPE[2] organisms, so-called because they are able to "escape" from the effects of commonly prescribed antimicrobials [68]. There have been concerns regarding hypervirulent and carbapenem-resistant clones, notably the ST23 type [69]. This lineage has been reported in more than 26 countries and represents a rising challenge for the treatment, because of its double risk of resistance and virulence. Moreover, ST340 has a wide distribution in Europe, Asia, and the Americas, suggesting high adaptability and environmental stability [70]. At a high rate have been identified carbapenem-resistant isolates throughout Europe, particularly in Southern countries as Italy, Greece, and Spain, where the most common clones, including ST258 and ST512, frequently harbor *bla_KPC,bla_OXA-48*, and *bla_NDM* genes [71]. Increased reports of ESBL- and carbapenemase-producing strains have come from Middle Eastern countries, such as Iran, Iraq, and SA, and genetic studies found significant contribution of mobilizable genes in resistance transfer [72]. The *bla_CTX-M-1* gene was widely distributed in uropathogenic isolates in Iraq in 2022. There is also a trend in Asia, where such phenotypes have arisen in countries like China and South Korea. Often these are due to chromosomal mutations combined with the acquisition of plasmid-mediated resistance genes [73]. In African local hospitals, MDR Enterobacteriaceae strains are emerging even if proper surveillance is missing. Resistance to last-resort antibiotics, including colistin, has also been observed throughout the Americas, with particular prevalence in patients with extended hospital

[1] PDR is the most dangerous type of resistance more severe than MDR and XDR, as it means resistance to all antibiotics.
[2] ESKAPE : a group of six highly resistant bacteria: E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, and Enterobacter spp.

stays and intensive care unit exposure [74]. These worldwide trends emphasize the need for enhanced surveillance systems, restricted use of antibiotics that are not needed, and genetic typing for containment of resistance.

1.8. Clinical Implications of Multidrug Resistance

Rising prevalence of multidrug (MDR) and extensively drug-resistant (XDR) among Gram-negative pathogens such as *Klebsiella pneumoniae* and *Escherichia coli* has further augmented the burden on healthcare settings. These infections are frequently associated with an increased duration of hospitalization, greater treatment expenses, and high mortality, particularly in intensive care units. Such microorganisms resist first-line antibiotics, such as cephalosporins and carbapenems, and therefore, physicians must use more toxic, for the host, drugs such as colistin and tigecycline, with potential for significant levels of toxicity [75].

1.8.1. Surveillance and Control Measures

Surveillance of resistance mechanisms, *blaNDM*, *bliOXA-48*, and *blaKPC* is very important for infection control. In nations that have invested in robust molecular surveillance, such as the United States, early identification of resistant clones has enhanced outbreak control and infection prevention [76]. But beyond these systems, global coordination is sparse. Warnings from public health institutions such as the WHO have recently emphasized the emergence of highly infectious and resistant *K. pneumoniae* strains that have been detected in multiple countries [77]. To help prevent the spread of such bacteria, hospitals follow the usual infection control procedures: isolating sick patients, encouraging hand hygiene, and avoiding unnecessary antibiotic use. Data from hospitals in southern Europe indicate that such measures, if rigorously applied, can lead to a major fall in the incidence of resistant infections [78]. Meanwhile, genomic tools are enabling scientists to follow how these bacteria evolve and move, yielding a more precise image for regional and international health responses.

1.9. Challenges in Clinical Management of Enterobacteriaceae Infections

Monitoring of resistance genes *blaNDM, blaOXA-48, and blaKPC* is critical for infection control. In the United States and in other countries with established systems for

molecular surveillance, there has been benefit from early detection of resistant strains in outbreak response and infection control [76]. But beyond these systems, global cooperation is scant. Recently, warnings have been made by public health agencies, such as the WHO, with regard to the growing prevalence of highly virulent and resistant *K. pneumoniae* strains, which have been observed in some countries [77]. To curb the spread of these bacteria, hospitals fall back on standard infection control practices: isolating patients who have the infections, promoting hand hygiene, and working to curb all unnecessary use of antibiotics. Perspective Data from hospitals in southern Europe indicate that, if applied consistently, such measures are able to substantially decrease the burden of infections caused by resistant organisms [78]. At the same time, advances in genomic tools are enabling researchers to follow how these bacteria evolve and spread, and the tools are clarifying the picture for both local and global health responses.

1.9.1. Use of Combination Therapy in the Treatment of MDR Enterobacteriaceae

Combined therapy has often been used for treating multidrug-resistant (MDR) Enterobacteriaceae , especially in severe infections where monotherapy is considered suboptimal. Combinations of colistin with carbapenems, tigecycline, or aminoglycosides are frequently employed. However, they are usually associated with inconsistent clinical success. Colistin exhibits considerable nephrotoxicity that hinders its prolonged administration, thereby compromising the utility of the antibiotic; tigecycline has reduced efficacy, including in bloodstream infections, because of its low serum levels [79]. In addition, development of resistance during combined treatment has been documented when complicates therapy and interferes with the effectiveness of these regimens [80]. Although the combination of drugs was introduced initially due to some reasons such as synergistic effects, inhibition of resistant strains and expansion in antimicrobial coverage regime, the clinical outcome has been shown to be inconsistent. Some of the studies have shown that there is short-term benefit in critical ill patients, but overall, for various reasons lies in the reduction of benefit over time, particularly when we are dealing with resistance mechanism, the worst is carbapenemase production (KPC, NDM, OXA). These enzymes can also inactivate combination therapies to disrupt them or develop omp to restore their activity, requiring the development of new anti-microbial and adjunct therapy to save patients' life [79].

1.10. Development of Novel Therapeutic Agents

The increasing prevalence of multidrug-resistant Enterobacteriaceae worldwide has stimulated the discovery of alternative antimicrobials. Beyond that, an interesting step in the right direction is cefiderocol, a siderophore cephalosporin that exploits bacterial iron uptake pathways to enter into the cell and preserve potency against carbapenem-resistant isolates, including NDM-type metallo-β-lactamases producing [81]. Ceftazidime avibactam, a β-lactam/β-lactamase inhibitor combination, is effective against KPC- and OXA-48-producing strains, but is not effective against NDM producers [79]. The meropenem vaborbactam also covers KPC-producers, including some isolates resistant to ceftazidime avibactam; however, it does not cover NDM and OXA-48 carrying organisms [82]. Although progress has been made for all of these agents, they are limited to certain enzymes, highlighting the continued requirement for broader-spectrum therapeutics with activity across a range of resistance mechanisms in Enterobacteriaceae.

1.10.1 Vaccine Development Efforts for Enterobacteriaceae

At the same time, vaccine development against Enterobacteriaceae is becoming established, especially in high-risk patients, including hospitalized and elderly patients. Vaccines, such as the CPS-based vaccines antibodies 17H12 and 8F12, have demonstrated extensive protection in murine models of carbapenem-resistant *K. pneumoniae* through improving immune clearance [83]. Other vaccine strategies are the O-antigen conjugates, the genetically modified outer-membrane vesicles (GMMA), and the multitope subunit vaccines designed by immunoinformatics for cross-species protection of vaccines [84, 85]. Further development is underway for MV-140 (Uromune), which is a sublingual spray of heat-killed *E. coli*, *K. pneumoniae*. Clinical data from randomised and observational studies demonstrate a UTI-free rate of about 58% in vaccinated persons compared to around 25% in placebo recipients during 9 months [86]. None of these vaccines has been approved, but the variety of approaches and continued favorable early results indicate great potential for the prevention of MDR Enterobacteriaceae infections.

1.11. Challenges and Future Directions for Enterobacteriaceae Treatments

The control of infections due *to K. pneumoniae* and other Enterobacteriaceae remains a challenge in spite of recent progress in therapeutics and vaccines. First, there is still a barrier of diagnosis of VGS-associated infections: the diagnosis of VGS is based on traditional culturing which is slow and insensitive, whereas rapid molecular methods including PCR and whole-genome sequencing exist that are sensitive, rapid but are not widely available yet, particularly in low resource settings [87], Secondly, Enterobacteriaceae exhibit genetic diversity in the form of varying capsular types, O-antigens, and plasmid mediated resistance mechanisms, further confounding both treatment options and vaccine design, which are liable to be broad multicomponent affairs [85]. Third, in resource-limited developing nations, lack of laboratory capacity, cold chain, and surveillance systems are serious challenges for successful implementation of diagnostics and prevention [88]. With these challenges in mind, investment in rapid diagnostics, pan-Enterobacteriaceae vaccines, and infrastructure such as well-equipped laboratories in diverse healthcare settings is necessary to ensure comparable levels of progress across the globe.

CHAPTER 2

MATERIAL AND METHODS

2.1. Sample Collection and Processing

A total of 112 clinical isolates were recovered from Erciyes University Hospital and previously identified as *K. pneumoniae*. Isolates were obtained from usually clinical samples such as urine, blood, and sputum. Positive blood culture for bacteremia or sepsis was collected in anticoagulant tubes (EDTA or heparin) from patients who showed clinical signs of bacteremia or sepsis, incubated at 37 °C, and subcultured. Respiratory Samples Sputum samples were obtained from patients with clinical signs of lower respira-tory tract infection and only those which were purulent and mucoid samples were selected for the study [89].

2.2. Bacterial Culture and Identification

The isolates were subcultured to MacConkey and blood agar. Cultures yielded large, pink, mucoid colonies consistent with lactose-fermenting Enterobacteriaceae on MacConkey agar and smooth, gray colonies with no hemolysis on blood [90, 91]. On preliminary identification, they were classified as Gram-negative, non-motile, capsulated rods. Biochemically, they were oxidase- and indole-negative, and they fermented acid and gas in TSI agar [90]. The isolates were finally identified by a VITEK 2 automated system that utilizes a broad spectrum of miniaturized biochemical reactions coupled with algorithms to identify bacterial species and to assess antimicrobial susceptibility testing [92]. Mass spectrometry, more specifically MALDI-TOF MS, has emerged as a reliable method and during the past few years has also become an alternative method for the rapid identification of bacteria. This technique investigates bacterial proteins to produce distinctive spectral fingerprints, which are then compared with reference databases. MALDI-TOF MS is faster than the current phenotyping system, and its accuracy is

frequently superior, particularly in differentiating Enterobacteriaceae with closely related phenotypic characteristics [93].

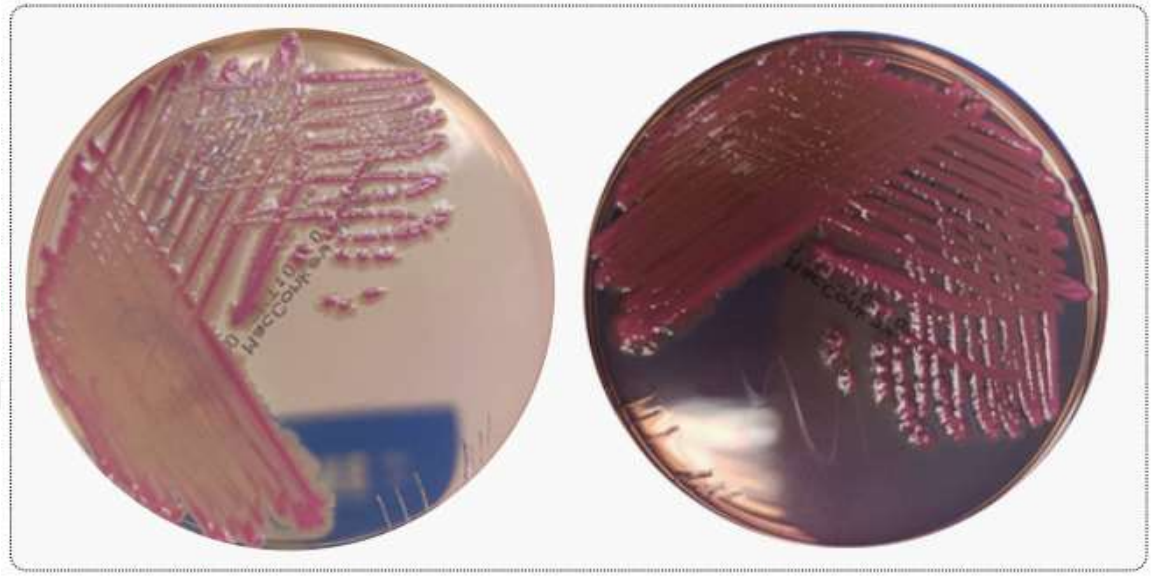

Figure 2.1. Colonies initially identified as *K. pneumoniae* based on morphology on MacConkey agar (Image taken by the researcher as part of routine laboratory work).

2.3 Antibiotic Susceptibility Testing (AST)

Antimicrobial susceptibility of isolates that were preliminarily identified as *K. pneumoniae* was determined by disk diffusion. This technique continues to be used as the standard to identify resistance in clinically important Gram-negative rods, and can be performed easily in the routine laboratory. The analysis was performed according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and zone diameters interpreted using the derived clinical breakpoints [62].

2.3.1. Preparing Culture Media for AST

The culture media were prepared for antibiotics sensitivity tests.Mueller-Hinton agar as directed by the producer was used. The height of agar was set at 4 mm; the following is critical for even distribution of antibiotics. To prevent too high surface moisture plates were dried at room-temperature (20–25°C) and stored at 4–8°C before use [62].

2.3.2. Inoculum Preparation for AST

A few isolated colonies from fresh, non-selective agar plates were selected and suspended in sterile saline. The suspension was adjusted to match the turbidity of a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL. The turbidity was checked both visually and with a photometric device to ensure it remained between 0.4 and 0.6. Standardizing the inoculum is essential to avoid variation in zone sizes and ensure reproducibility [62], [94].The McFarland standard was prepared by mixing 0.5 mL of 0.048 M barium chloride with 99.5 mL of 0.18 M sulfuric acid. The resulting solution was measured at 625 nm using a spectrophotometer with a 1 cm path length. It was stored in tightly sealed glass tubes at room temperature (25°C), protected from light, and vortexed before each use [62].

2.3.3. Agar Plate Inoculation for AST

Mueller-Hinton agar plates were brought to room temperature before inoculation. The standardized bacterial suspension was used within 15 minutes to ensure cell viability. A sterile cotton swab was dipped into the suspension, and excess liquid was removed by pressing it against the inner wall of the tube. The inoculum was then evenly spread across the entire surface of the agar using a zigzag streak method. Antibiotic discs were applied within 15 minutes after inoculation to prevent early diffusion. To avoid overgrowth that may affect the accuracy of zone measurements, the plates were not left exposed to room temperature for long before incubation [62, 94].

2.3.4. Antimicrobial Disc Application for AST

Antibiotic discs were applied carefully and spaced evenly, with no more than 6 discs per plate to avoid overlapping zones. Disc potency was preserved by storing them in sealed containers with desiccants, protected from light, and kept under recommended conditions. Before use, the discs were brought to room temperature to prevent condensation. Discs were applied immediately after opening, and expired or damaged discs were discarded to ensure test reliability. These practices help maintain the accuracy and consistency of susceptibility testing results [62, 94].

2.3.5. Incubating Plates for AST

After placing the antibiotic discs, all inoculated Mueller-Hinton agar plates were promptly transferred to the incubator within 15 minutes. Any delay in incubation could allow uncontrolled bacterial multiplication at room temperature, leading to inaccurate diffusion patterns and altered zone diameters. To minimize such variability, plates were incubated at $35 \pm 1°C$ for 18 ± 2 hours. The plates were inverted during incubation to prevent condensation from affecting the agar surface. Additionally, they were placed without stacking to allow even temperature distribution, maintaining reliable diffusion conditions throughout the incubation process [62].

2.3.6. Determining Bacterial Susceptibility to Antibiotics

Following incubation, the diameters of the inhibition zones around each antibiotic disc were carefully measured in millimeters using a calibrated ruler. Also we used VITEK 2 AST cards and E test for AST. Minimum Inhibitory Concentration (MIC) of various antimicrobial agents against isolates is also tested with Etest. Standardized bacterial suspension poured onto the surface Mueller-Hinton agar, to achieve confluent growth. Following inoculation, an Etest strip, which contains a predefined, continuous gradient of an antimicrobial agent, is gently placed onto the agar surface. After suitable incubation, an elliptical zone of inhibition forms around the strip. The MIC value is then directly read from the numerical scale on the strip at the point where the elliptical zone edge intersects the strip, providing a precise quantitative measure of the isolate's susceptibility to the antibiotic (Figure 2.2). These measurements were interpreted in accordance with EUCAST version 13.0 breakpoints, which categorize bacterial isolates into one of three clinical interpretations: “Susceptible” indicates that standard doses of the antibiotic are expected to be clinically effective; “Intermediate” suggests that the antibiotic may be effective under specific conditions, such as higher dosing or when the drug concentrates at the infection site; and “Resistant” means that treatment with that antibiotic is unlikely to produce therapeutic success. All results were compared with EUCAST reference values and antibiotic concentrations, and ambiguous zones were examined closely to avoid misinterpretation. ATCC-13883 reference strains were used throughout the testing process to ensure result validity and consistency [62, 94].

2.3.7. Quality Control (QC) for AST

To ensure accuracy in antimicrobial susceptibility testing, quality control (QC) strains with defined resistance profiles were used. These reference organisms, which included known β-lactamase and aminoglycoside producers, were obtained from certified sources and preserved at –70°C in glycerol stocks. Both daily-use working stocks and archival stocks were maintained, and subculturing was performed periodically to prevent phenotypic drift. Every testing cycle was verified using EUCAST quality control tables. Additionally, zone diameter reproducibility was assessed by repeating testing ten times on selected isolates. Testing was conducted on Mueller-Hinton agar while carefully monitoring critical variables such as agar depth and cation concentrations, particularly calcium and magnesium, as these factors can influence the activity of certain antibiotics. These quality control practices ensured consistent and accurate results, supporting reliable therapeutic decision-making [62, 94].

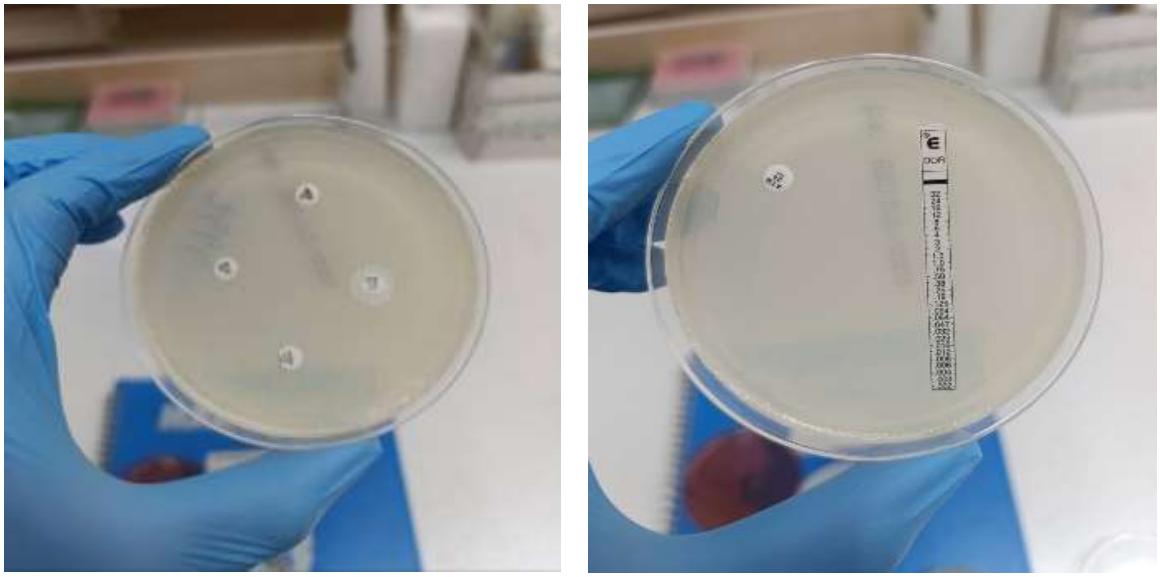

Figure 2.2. Visual representation of antibiotic susceptibility testing methods: Disk diffusion assay (left) and E test strip method (right, both images were taken by the researcher during laboratory procedures).

2.4. DNA Extraction

Genomic DNA (gDNA) was prepared from isolates originally identified as *K. pneumoniae* by using commercially available NucleoGene DNA extraction kits according to the manufacturer's instructions [96]. Colonies were sub-cultured from MacConkey

agar into 5 ml of Luria-Bertani (LB) broth, and the culture was allowed to grow overnight at 37°C with shaking at 150 rpm to promote adequate growth of the bacteria. Following incubation, the culture was decanted into sterile tubes and pelleted by centrifugation to harvest the bacteria. Cell pellets were processed directly or preserved in skim milk at –70°C for future use [95]. For DNA binds, the bacterial pellets were lysed and resuspended in 400 µL of binding buffer and then vortexed for 15 sec. The mixture was then loaded into a pre-equilibrated NucleoGene spin column set in a 2 mL collection tube and, after 2 minutes centrifuging at 10,000 × g, the DNA was adsorbed to the silica membrane. A two-stage washing process was employed for the removal of contaminants. Wash buffer 1 was created by adding 13 mL of buffer concentrate to 13 mL absolute ethanol, and Wash buffer 2 by adding 15 mL concentrate to 60 mL ethanol. The column was washed with 500 µL wash buffer 1, centrifuged, and washed with two washes of 750 µL wash buffer 2. Three more minutes of spinning were performed to make sure that all ethanol was removed. The purified DNA was eluted with 200 µL of elution buffer by incubating the suspended membrane at room temperature for 1 min, followed by centrifugation (10,000 × g for 1 min). Stored DNA was maintained at −20°C until used [96].

2.5. Whole-Genome Sequencing with Oxford Nanopore Technologies

2.5.1. DNA Concentration and Purity Assessment

The concentration and the genomic DNA extracts were determined using a Qubit 4.0 Fluorometer and spectrophotometry. In contrast to the traditional Nanodrop, which scatters in the UV, fluorophore dyes in the Qubit enable a more directed and sensitive determination of nucleic acids [97]. DNA purity was measured by the ratio of A260/A280, of which the range between 1.8 and 2.0 was considered qualified for sequencing.

2.5.2. The whole genome sequencing protocol

The genoman was sequenced with the Oxford Nanopore Technologies (ONT) platform. DNA library preparation was carried out using the SQK-NBD114-24 ligation-based kit following the manufacturer's instructions. Sequencing was performed on the ONT PromethION instrument, in particular the P2 Solo (compact) kit, for small and/or single sample runs. The constructed libraries were loaded on a flow cell andsequential

observations were made by transmission of sequences of DNA molecules entering the pores for decoding of DNA, up to 24 hours. This setting was chosen with the aim of obtaining long reads and high datathroughput for de novo genome assembly [98].

2.5.3. Basecalling and Read Quality Filtering

The raw signal data produced by sequencing were first in the FAST5 format. The signals were basecalled using Guppy basecalling software (version 6.1.7) in high-accuracy mode [99]. FASTQ files generated, comprising raw read sequence information and the quality scores for each base. After that, low-quality and duplicate reads were removed to improve the quality of the data by BBNorm (version 38.90). The software is obtained by read normalization and is used to prevent variability of the enrichment over the genome to be biased, contributing to portraying uniformly all regions of the genome. They increase the accuracy and reliability of a following genome assembly [100].

2.5.4. Genomic Assembly and Polishing

Assembly: The raw reads were assembled *de novo*[3] Flye version 2.9, a genome assembler designed for long-read sequencing technologies , with e.g., Oxford Nanopore [98].Two rounds of polishing were conducted with Racon to correct base-level errors caused by the sequencing. The assembled contigs were further error-corrected with Minimap2, which maps reads to the assembled contigs to remove remaining errors, especially single-base pair mismatches. Contigs less than 1,000 bp and putatively low coverage depth (less than 5×) were filtered out in an effort to guarantee high-quality assembly. Following filtering, the remaining assembly for the isolate consisted of eight contigs.

2.5.5. Assembly Quality Evaluation

The integrity of the genome was evaluated with QUAST v5.2.0 [101]. This software will print important information, including total length of the genome assembly, the number of contigs, genome completeness and N50 (the contig length at which half of the nucleotides of the genome assembly were accounted for). The larger the N50 value, the more continuous the assembly is. These quality measures were employed for validation

[3] De novo means: A reference genome was not used, but the genome was built directly from the reads.

of completeness and quality of the genome assembly for further analyses, such a resistance or virulence genes detection.

2.5.6. Genome annotation

The draft genome of strain NA22 was annotated by PATRIC/BV-BRC using the RASTtk pipeline to infer gene functions and locate the genomic features (coding DNA sequences, CDS; transfer RNAs, tRNA; ribosomal RNAs, rRNA; and other potential virulence or antimicrobial resistance genes). This system gave a functional summary of the genome utilizing the annotated subsystems along with similarity-based predictions [102]. The genome was re-annotated for NCBI standard annotation followed by a submission to GenBank through the NCBI submission portal using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) that included homology-based methods and ab initio prediction approaches to provide the most accurate representation for calling genes and features [103]. In this process structural and non-coding RNAs in addition to CRISPR loci were also identified, giving a complete overview of the genomic content in strain NA22.

2.5.7. Plasmid Detection

Plasmid replicons were identified in the NA22 genome assembly using PlasmidFinder 2.0 [104] to test whether they were present. Default parameters on the local server setup were employed for the analysis, which is designed to compare input sequences with a curated database of the known plasmid replicon types. Plasmids serve as major vehicles to spread antibiotic resistance and virulence determinant genes in clinical and environmental bacteria ; therefore, the discovery of plasmids harbored in this NA22 genome is crucial.

2.5.8. Phylogenetic Placement

Genomic relatedness of strain NA2 2 to reference bacterial genomes was assessed using phylogeny. The genome ofthe bacterium was verified and registered in the BV-BRC portal [105] and parsed using the Codon Tree methodology inorder to produce agenome-wide phylogenetic tree. Here we present a method that relies on alignments of orthologous single copygenes across different genomes to infer phylogenetic

relationships. These quences were aligned with MUSCLE [106] , and trees were inferred using RAxML maximum likelihood. Based on the resulting phylogenetic tree, the identity of strain NA22 was also determined, showing a close relationship between strain NA22 and other closely related bacterial strains.

2.5.9. Genome Submission

The whole genome sequence of the isolate, designated as NA22, was made available at the National Centre for Biotechnology Information (NCBI) under BioProject accession no PRJNA1270365, and (Taxonomy ID: 573). Manuscript submission Submitted to Erciyes University on May 31, 2025.

CHAPTER 3

RESULTS

3.1. Initial Phenotypic Identification and Antimicrobial Resistance Profile

The identification of the strain was mainly phenotypic (MALDI-TOF MS), and the bacterium was finally identified as *K. pneumoniae*. On the basis of this classification, the AST was performed with the VITEK 2 automated system and by the disk diffusion method according to the guidelines of the European Committee on AST(2025). Various classes of antibiotics were tested, e.g., β-lactams, carbapenems, fluoroquinolones, sulfonamides, and aminoglycosides. The isolate was resistant to several antibiotics and had a multidrug-resistant (MDR) profile. Resistance was also detected in some of the isolates even at higher concentrations (≥ 20 μg/mL and ≥ 64 μg/mL), suggesting the poor efficacy of the drug in the treatment. The isolate demonstrated resistance to a broad spectrum of β-lactam antibiotics, including ampicillin, ceftazidime, ceftriaxone, cefepime, and amoxicillin/clavulanic acid, as well as to trimethoprim-sulfamethoxazole [108]. Overall, the antimicrobial profile is that of a common rusk pathogen with multidrug resistance that can interfere in the selection of treatment.

Table 3.1. Antimicrobial susceptibility profile of strain NA22 based on MIC values and EUCAST 2025 interpretation.

Antibiotic	MIC Value	Method	Antibiotic Class	Interpretation
Ampicillin	≥ 32 μg/mL	VITEK 2	Beta-lactams	Resistant
Amoxicillin/Clavulanic acid	≥ 32 μg/mL	VITEK 2	Beta-lactams	Resistant
Ceftriaxone	≥ 64 μg/mL	VITEK 2	Beta-lactams	Resistant
Ceftazidime	≥ 64 μg/mL	VITEK 2	Beta-lactams	Resistant
Cefepime	≥ 64 μg/mL	VITEK 2	Beta-lactams	Resistant
Imipenem	≥ 16 μg/mL	VITEK 2	Carbapenems	Resistant
Meropenem	≥ 16 μg/mL	VITEK 2	Carbapenems	Resistant
Amikacin	≥ 64 μg/mL	VITEK 2	Aminoglycosides	Resistant
Gentamicin	≥ 16 μg/mL	VITEK 2	Aminoglycosides	Resistant
Ciprofloxacin	≥ 4 μg/mL	VITEK 2	Fluoroquinolones	Resistant
Trimethoprim/Sulfamethoxazole	≥ 320 μg/mL	VITEK 2	Sulfonamides	Resistant
Ertapenem	Zone < EUCAST breakpoint	Disk diffusion	Carbapenems	Resistant

3.2. Whole Genome Sequencing and Assembly Overview

To shed light on the genomic structure of the isolate, as well as its resistance-related determinants, whole-genome sequencing was performed. The isolate was labeled as *K. pneumoniae* by phenotypic characteristics and MALDI-TOF MS at the time of sequencing. The quality of assembly was assessed based on *de novo* assembled reads using the QUAST tool [101]. The assembly yielded eight contigs, which suggested a good quality of the assembly. In general, less and longer contigs are better quality for an assembly because short and fragmented assemblies are already indicating issues with sequencing or structure. The assembled genome measured 5.269.304 base pairs, a size that fits within the expected range for members of the Enterobacteriaceae family. The GC content was 50.46% [109]. The largest contig alone spanned 5,120,907 base pairs, and this same value was recorded for both N50 and N90 metrics meaning that over 90% of the genome was represented in a single continuous sequence. These figures suggest minimal fragmentation. In addition, the auN value was 4,979,219.8, offering further confirmation of the assembly's consistency and quality, since this measurement takes into account the overall distribution of contig lengths [101]. Other quality indicators, such as L50 and L90, were both equal to 1. These values reflect the number of contigs needed to cover 50% and 90% of the genome, respectively, and a value of 1 point to a highly contiguous assembly. A total of 26 complete rRNA genes were identified, alongside one

partial gene. The incomplete rRNA gene may be located at the edge of a contig, which could explain why it wasn't fully captured [105]. Ambiguous bases, denoted as "N" in the sequence, represent regions where the exact nucleotide could not be determined.

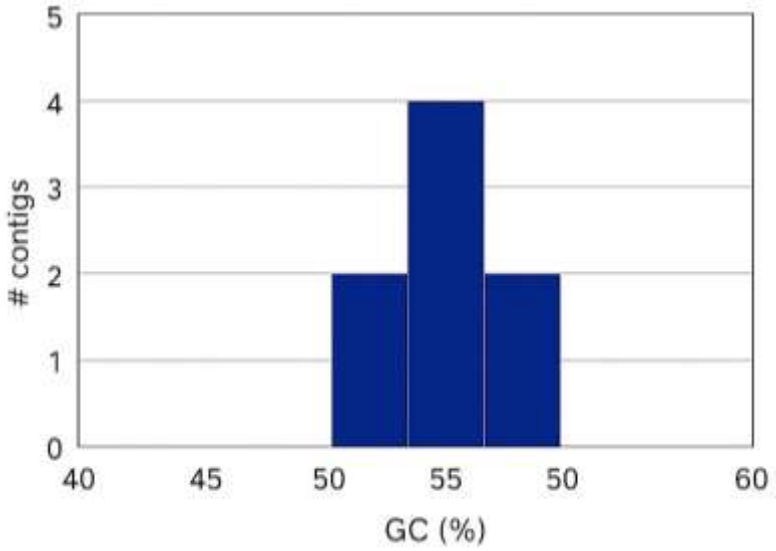

Figure 3.1. GC content of assembled contigs. The GC content across the assembled contigs ranged from approximately 48% to 52%, with a mean value of 50.46%. This is consistent with known values for species within the Enterobacteriaceae family. The analysis was performed using the QUAST tool [101].

Table 3.2. Genomic Assembly Summary of Isolate NA22 Analyzed Using QUAST [101].

Genome Feature	Value	Genome Feature	Value
Number of contigs (≥ 0 bp)	8	N50	5,120,907
Number of contigs(≥1000bp)	8	N90	5,120,907
Total length ≥ 1000 bp	5,269,304	AuN	4,979,219.8
#contigs	8	L50	1
Largest contig	5,269,304	L90	1
#predicted rRNA genes	26+1 part	#N`s per 100 kbp	0

3.3 Genome Annotation

The NA22 isolates were first characterized by phenotypic and MALDI-TOF MS methods; however, WGS revealed more in-depth information. Annotation of the assembled genome on the BV-BRC (PATRIC) platform, using the RASTtk toolkit, indicated that the isolate should be correctly named *Escherichia coli*. This observation points to the limitation of the classical identification technique and the importance of genomic-based analysis to clarify the bacterial taxonomy [105]. The genome was identified as 562.167973, and the taxonomic placement is:

Superkingdom	:	Bacteria
Phylum	:	Pseudomonadota
Class	:	Gammaproteobacteria
Order	:	Enterobacterales
Family	:	Enterobacteriaceae
Genus	:	*Escherichia*
Species	:	*Escherichia coli*

The genome is distributed between 5,215 protein-coding sequences (CDSs), 90 tRNA genes, and 26 rRNA genes, and a full-length and high-quality genome could be assembled. Furthermore, 101 repeat regions were identified, which might function in gene regulation or resistance. Among all annotated proteins, 681 were considered hypothetical and 4,534 had assigned biological functions. Among these, 1,308 proteins were annotated to EC numbers, 1,091 to GO terms, and 919 to KEGG pathways, and were also associated with cellular processes and metabolism. Furthermore, the annotated sequences were assigned to protein families: 5,051 proteins were members of the PATRIC Global Protein Families (PGFam), which are classes of protein families shared by multiple bacterial species, and 4,875 were grouped into the Local Protein Families (PLFam), which are more genus-specific classifications.

Table 3.3. Bioinformatics tools and databases used to analyze the *Escherichia coli* NA22 genome.

Tool	Tool Function	Result Type	Reference
PATRIC (RASTtk)	Genome annotation and subsystem-based functional analysis	CDS, functional proteins, tRNA, rRNA	[105]
QUAST	Quality assessment of genome assembly	GC content, N50, L50, contig stats	[101]
CARD	Detection of antimicrobial resistance genes and mechanisms	Resistance genes and mechanisms	[65]
PATRIC VF	Identification of virulence factors	Virulence-associated genes	[110]
DrugBank	Identification of potential drug targets	Drug–target associations	[111]

Table 3.4. Summary of annotated genomic features in the *Escherichia coli* NA22 genome. Protein features were identified using the RASTtk annotation tool via the PATRIC platform [105].

Feature	Count
Coding sequences (CDSs)	5,215
Repeat regions	101
tRNA genes	90
rRNA genes	26
Partial CDS	0
Other RNA elements (miscellaneous)	0

Table 3.5. Summary of annotated protein features in *Escherichia coli* NA22 genome. Data were obtained from the genome annotation using the RASTtk pipeline on the PATRIC platform [105].

Feature	Count
Hypothetical Proteins	681
Functional Proteins	4,534
Proteins with EC Numbers	1,308
Proteins Assigned Gene Ontology (GO) Terms	1,091
Proteins Associated with KEGG Pathways	919
Genus-Specific Protein Families (PLFam)	4,875
Cross-Genus Protein Families (PGFam)	5,051

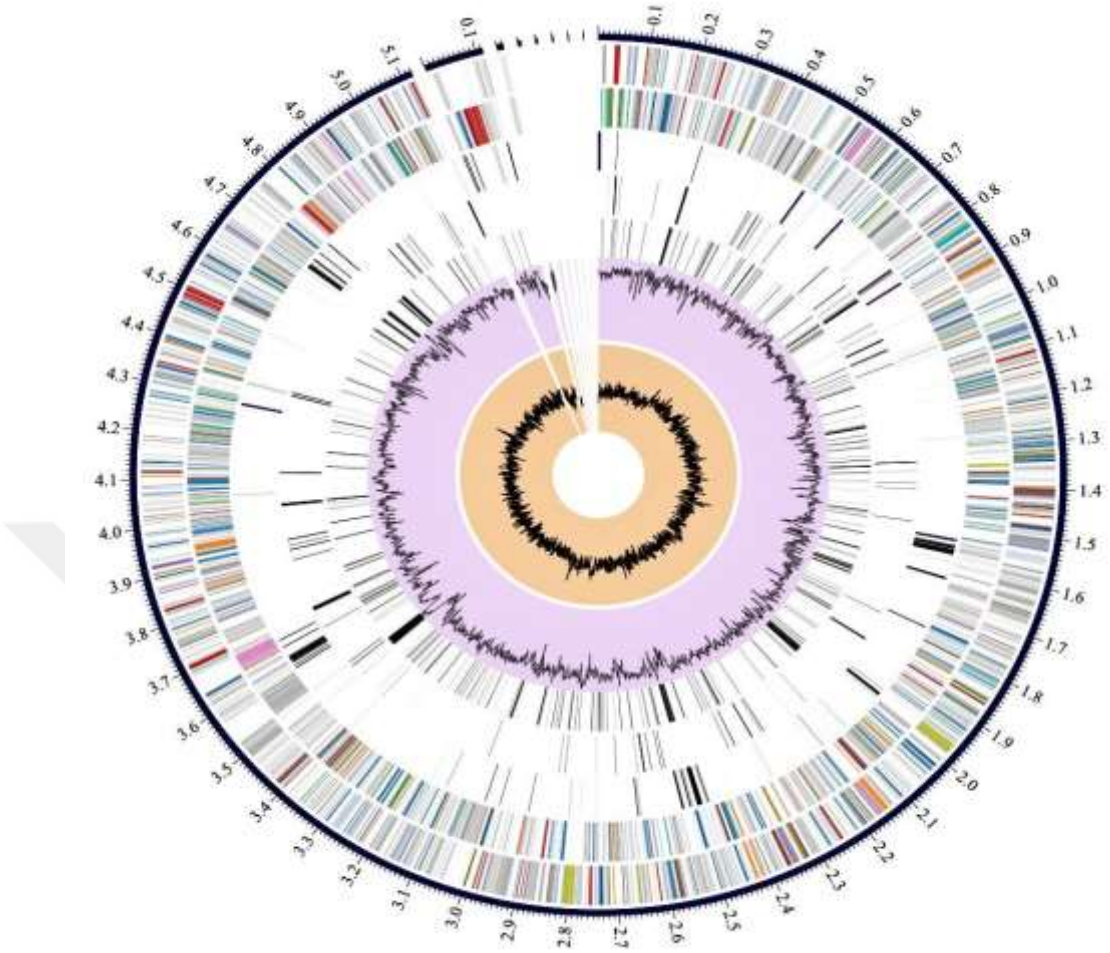

Figure 3.2. Circular representation of the *Escherichia coli* NA22 genome.The outer circles, named 0.1 to 4.8 according to genome size in Megabases, serve gene positions as references. The second ring of this figure represents the concatenated contigs. The third ring represents CDSs on the forward strand and different colors correspond to different functional categories. The fourth ring represents the CDSs on the reverse strand. The fifth circle (short black bars) indicates rRNA and tRNA as RNA genes. A sixth ring (thick, black) depicts antibiotic resistance genes. The seventh (grey coloured) ring denotes virulence effectors. The eighth ring (purple) represents the GC content. The core (the black wavy line) shows the GC skew i. i.e., the content difference that can discriminate regions of either normal or anomalous distribution of GC base pairs. This genome map was produced utilizing the PATRIC Genome Annotation Service [105].

3.4. Subsystem Analysis

Subsystem analysis can give insights into the biological functions of genes by aggregating them into functional units related to cellular functions. Based on annotation results obtained from the PATRIC, a total of 951 genes were found in the *E. coli* NA22

genome associated with metabolism, 115 of which belonged to distinct subsystems. This indicates that the organism has broad metabolic capabilities that could help it adapt to diverse ecological niches. Other significant groups were 265 genes related to protein processing, 202 to virulence and defense, and 213 to membrane transport. Other genes involved in DNA and RNA metabolism, cell wall biosynthesis and energy generation were also identified as being linked to the cluster. Collectively, these characteristics indicate that the organism is genetically endowed with mechanisms for survival as well as for potential genes with pathogenetic set up. The data were retrieved from the PATRIC annotation service [105].

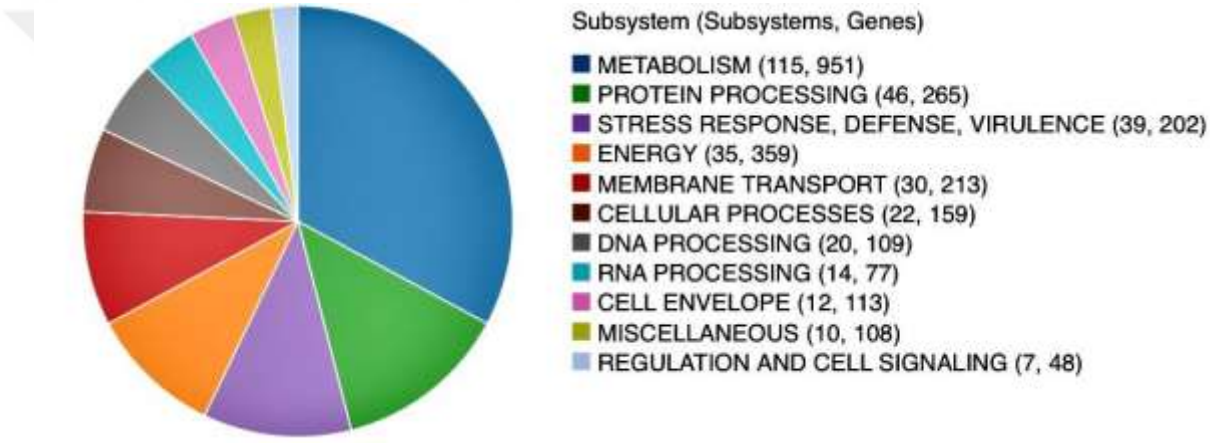

Figure 3.3. Subsystems identified in the *Escherichia coli* NA22 genome using the PATRIC annotation platform. The figure was generated based on subsystem classification data provided by the RASTtk tool on the PATRIC system [105].

3.5. Analysis of Specialty Genes

A number of gene categories associated with antibiotic resistance, transporters, virulence factors, and drug targets were recognized in this strain based on publicly available databases. The resistance-encoding genes were identified using the Comprehensive Antibiotic Resistance Database (CARD) [65], the National Database of Antibiotic-Resistant Organisms (NDARO), and the PATRIC_VF tool [105]. Two resistance factors were found in CARD, one was found in NDARO, and 59 resistance features were found by PATRIC, indicating that the isolate may harbor multiple antibiotic resistance mechanisms. For transporter genes, we detected 888 genes by mapping them to the Transporter Classification Database (TCDB) [112]. This high number demonstrates the

bacterium's ability to move many substances across its membrane, leading to its often being able to thrive in extreme conditions as well as tolerate drugs more effectively. As for virulence, different databases offered overlapping yet different results. PATRIC_VF detected 217 virulence-related genes, whereas 117 and 233 were obtained from VFDB [110] and Victors, respectively. These genes are associated with immune evasion, toxins, and adhesion to the host. Drug targets were also evaluated. The Therapeutic Target Database (TTD) [113] referred to 57 genes, whereas DrugBank, an inclusive database consisting of both pharmacology and genomics sections, recorded 387 genes that could be targeted with drugs [111]. Significantly, a subset of CHP2-Ex-positive genes were also present in TTD and DrugBank, suggesting their potential role as drug targets. Overall, the variety of resistance, virulence, and drug resistance-associated genes in this strain is indicative of its complex genetic background and diversity. These characteristics might explain why it is challenging to eliminate in clinical practice and its ability to escape from therapy.

Table 3.6. Specialized genes detected in *Escherichia coli* NA22 based on reference databases (for full references, see text).

Source	Category	Number of Genes
Victors	Virulence Factor	233
CARD	Antibiotic Resistance	76
NDARO	Antibiotic Resistance	1
PATRIC	Antibiotic Resistance	59
DrugBank	Drug Target	387
TTD	Drug Target	57
TCDB	Transporter	888
PATRIC_VF	Virulence Factor	217
VFDB	Virulence Factor	117

3.6. Antimicrobial Resistance Genes

The antibiotic resistance genes were called with the use of the Platform for the Analysis and Triangulation of Identified Chemicals (PATRIC) which uses a k-mer method for antimicrobial resistance (AMR) gene analysis [105]. This approach applies short-sequence nucleotides (k-mers) to map genome segments to curated resistance gene databases. The system returns rich annotations upon matching: the function of the gene, the type of resistance, the family of antibiotics to which resistance is conferred as well as

in some cases the specific antibiotic involved. But the genetic detection of a resistance gene does not necessarily mean resistance. Their gene expression may be regulated by regulatory elements, the genomic environment or variations, such as SNPs. Such distinctions would generate the presence of a resistance gene either non-functional or mutated not to actually grant resistance in a clinical setting.

Table 3.7. Summary of antimicrobial resistance genes and their mechanisms in the *Escherichia coli* NA22 genome [105].

Resistance Mechanism	**Associated Genes**
Antibiotic activation enzyme	KatG
Antibiotic inactivation enzyme	*blaEC* family
Resistance operons	*marA, marR, marB*
Genes potentially involved in resistance pathways	*Alr, ddl, dtx, EF-G, EF-Tu, folA, dfr, folP, gyrA, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p, gyrB*
Target protection protein	BcrC
Efflux pump proteins	*acrAB-tolC, acrAD-tolC, acrEF-tolC, emrAB-tolC, emrD, emrE, emrKY-tolC, mdfA/cmr, mdtABC-TolC, mdtEF-molC, mdtL, sugE, tolC/OpmH, macB, macA, acrZ*
Loss-of-function mutation	GidB
Modification of membrane surface charge	*gdpD, pgsA*
Regulation of resistance genes	*oxyR, acrAB-tolC, emrAB-tolC, gadE, h-NS*

Table 3.8 Antibiotic resistance genes detected in the *Escherichia coli* NA22 and their corresponding resistant antibiotics.

Genes	**Resistant Antibiotic**	**References**
blaEC family	Beta-lactams (penicillins, cephalosporins)	[19]
marA, marR, marB	Multiple antibiotics, including fluoroquinolones, tetracyclines, and chloramphenicol	[38]
gyrA, gyrB	Fluoroquinolones	[114]
rpoB, rpoC	Rifampicin	[115]
folA, dfr, folP	Trimethoprim	[116]
Efflux pumps *(acrAB-tolC, emrAB, mdtABC,* etc.*)*	Multidrug resistance, including fluoroquinolones, macrolides, tetracyclines	[38, 65]
GidB	Streptomycin (aminoglycoside)	[117]
gdpD, pgsA	Colistin (polymyxins)	[118]

Table 3.9. Correlation between phenotypic resistance (VITEK 2 results) and genotypic mechanisms (WGS) in *Escherichia coli* NA22. Table constructed based on original findings.

Antibiotic	Result	Genes	Mechanism
Cefepime, ceftriaxone, ceftazidime ampicillin, amoxicillin/clavulanic [4]acid	R	*blaEC*	Beta-lactamase (ESBL)
Amikacin	R	*gidB , mdtEF*	Target protection, efflux pump
Gentamicin	R	*gidB, mdtABC*	16S rRNA methylation, efflux pump
Ertapenem	R	*narA, gadE*	Efflux Pump Regulation
Imipenem	R	*acrAB-toIC,marR*	Porin regulation, efflux pump
Meropenem	R	*emrAB-toIC, marA, gadE*	Activity regulation, efflux pump
Trimethoprim\Sulfamethoxazole	R	*foIA,foIP, dfr*	Target modification (folic acid pathway)
Ciprofloxacin	R	*gyrA,gryB*	Target mutation, efflux pump

Note. R = Resistant (based on VITEK 2 results).

3.7. Phylogenetic Analysis Based on Whole-Genome Sequencing

To determine the phylogenetic position of the NA22 isolate (genomically identified as *Escherichia coli*), the genome was analyzed using the BV-BRC platform [105]. Similar genomes were found using the Mash/MinHash algorithm, which provides a quick estimate of genomic similarity [119]. Global protein families (PGFams) were then used to identify shared proteins between the isolate and related genomes [120]. Protein sequences were aligned using MUSCLE, a tool commonly used for accurate multiple sequence alignments [106]. The corresponding nucleotide sequences were also aligned, and a single matrix was generated Phylogenetic placement of the isolate NA22 Based on the genomic sequence, the isolate NA22 was also confirmed as *E. coli* and its evolutionary distance with other nearest neighbors was determined using the BV-BRC tool [105]. The Mash/MinHash program, which provides an estimated measure ofgenomic relatedness [119], returned matches. Shared proteins between the isolate and the related genome were then identified by employing PGFams [120]. Sequence alignments of proteins were conducted with MUSCLE [106] with a high degree of accuracy for the multiple sequence alignment. Pairs of homologous nucleotide sequences were aligned in a CLUSTAL and the combined matrix was analysed to construct the tree, following the application of

[4] Although the *blaEC* gene was detected, it may not fully account for resistance to amoxicillin-clavulanic acid, suggesting the involvement of additional resistance mechanisms.

RAxML with rapid bootstrapping [121, 122] to build the consensus tree shown in Fig. 3.4.to build the final phylogenetic tree .

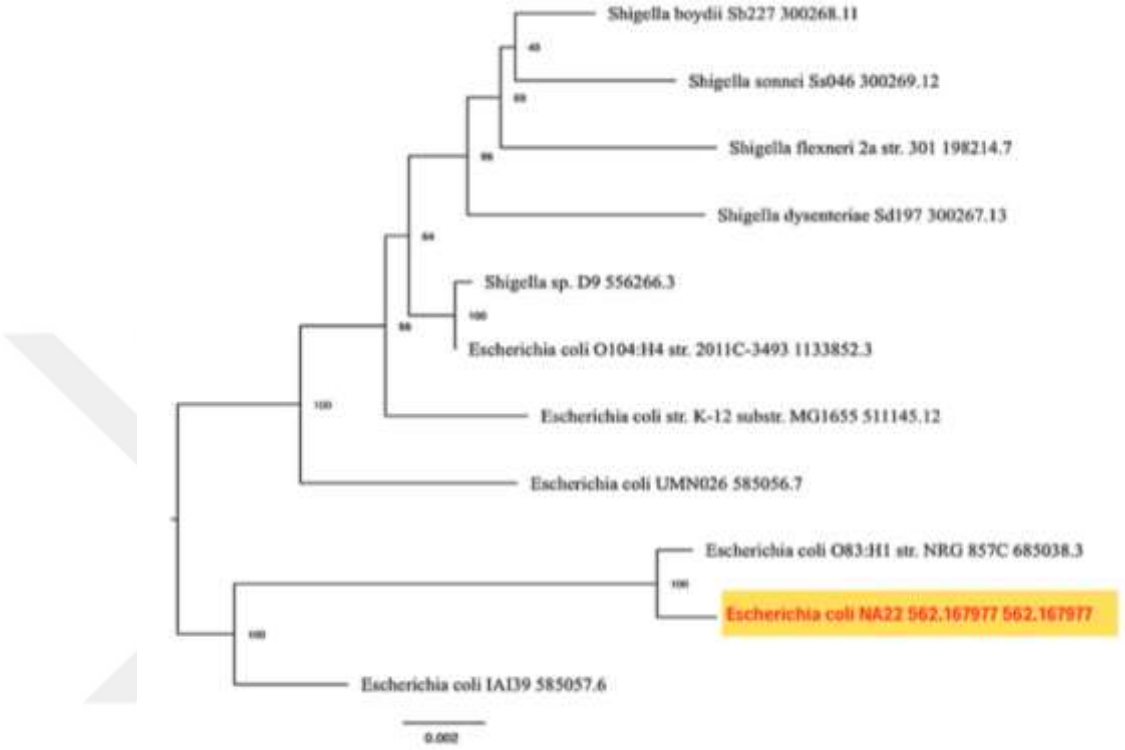

Figure 3.4. Phylogenetic tree showing the position of NA22 strain among reference genomes.This tree shows how the *Escherichia coli* NA222 isolate is related to other *E. coli* and *Shigella* strains based on whole-genome comparison. As seen in red, the NA222 strain clusters very closely with the *E. coli* O83:H1 strain NRG 857C. The bootstrap values reflect the confidence of these relationships The tree was generated using the PATRIC analysis platform [105].

3.8. Plasmid Detection and Similarity Analysis

Through PlasmidFinder 2.0 [104], three plasmids were identified in the *Escherichia coli* NA22 genome, all of which showed high sequence identity to known *E. coli* plasmids. These included Col156 (NA22_1), IncFIB (NA22_2), and IncFII(p) (NA22_3), all located within contig 8. Among them, NA22_3 is particularly noteworthy due to the presence of multiple resistance genes, such as *blaNDM-4*, *blaCTX-M-15*, and *aac(6')-Ib-cr*. This plasmid also carries genes from the *tra* operon, which are involved in conjugative transfer. The combination of antibiotic resistance genes and transfer elements suggests a

significant role in spreading resistance traits, particularly in clinical environments where such plasmids can easily disseminate among bacteria.

Table 3.10. Plasmids Detected in *Escherichia coli* NA22 Genome Based on PlasmidFinder 2.0 Results [104].

Plasmid (study ID)	A similar Plasmid in NCBI	Identity (%)	Query length	Contig	Position in contig	Accession number
NA22_1	Col156	98.7	154 / 154	contig_8	11356..11509	NC_009781
NA22_2	IncFIB(AP001918)	96.63	682 / 682	contig_8	105235..105916	AP001918
NA22_3	IncFII(29)	99.61	259 / 259	contig_8	36127..36385	CP003035

CHAPTER 4

DISCUSSION

4.1. Overview of Findings

The NA22 strain was initially collected as *K. pneumoniae* based on its colony and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) profile. However, whole-genome sequencing indicated that the isolate was an *E. coli*. The dissonance further underscores deficiencies in phenotypic identification and the inability to distinguish between closely related Enterobacteriaceae [123].

Our study, mirroring observations from a 2021 investigation conducted in Sweden, revealed significant MALDI-TOF MS misidentification of *Klebsiella* species. These reinforcing findings consistently highlight the critical limitations of phenotypic methods. Both studies underscore the indispensable role of whole-genome sequencing (WGS) for accurate bacterial species determination [145].

The genome of NA22 was alarming: 76 antimicrobial resistance genes and over 200 virulence-associated genes were detected. The *blaNDM-4* and *blaCTX-M-15* genes, which are two of the most commonly reported clinically relevant β-lactamase genes, were co-located on a plasmid with conjugation-related genes [124]. This suggests that NA22 may be a reservoir of resistance genes transferable by horizontal transfer to other bacteria in the hospital environment. Additionally, as there was good correlation between phenotypical resistance (VITEK 2 and disk diffusion) and genotypic resistance (WGS), the performance of nature test principles along a reference laboratory routine of clinical microbiology seems to be quite robust [125]. In cases where there were discrepancies (i.e., some fluoroquinolones), the overall presence of acquired resistance genes in the genome ought to suggest the potential to select therewith resistance, not yet necessarily phenotypically manifested. Altogether, results from this study may provide an evidence

base for the molecular screen to be readily available as part of a panel of test for routine clinical diagnostics and hospital infection control. The misidentification of a multidrug-resistant strain of *E. coli* as *K. pneumoniae* could have led to erroneous therapy or the inability to identify a mode of transmission. Therefore, NA22 highlights the diagnostic challenge, as well as implications for patient care, of such genotypically characterized multidrug-resistant strains in the hospital.

4.2. Phenotypic Resistance Patterns in NA22 Isolate

The first isolate, NA22, which was initially characterized as *Klebsiella pneumoniae*, was resistant to most antibiotics according to the phenotypic tests. The finding was indicative that the isolate was resistant to multiple β-lactam antibiotics, such as third-generation cephalosporins and carbapenems [126]. Resistant to the antimicrobial agents: aminoglycosides, fluoroquinolones, and sulfonamides were also described. This resistance profile indicates that this isolate is MDR and hard to treat with routine therapeutic agents. Notably is also resistance to carbapenems, which are often used as a last resort when other antibiotics have proven ineffective. Moreover, resistance to fluoroquinolones as well as to aminoglycosides challenges the potential use of these classes of antibiotics, either alone or in combination. This could offer few treatment options, especially in the event of severity. The resistance pattern in this isolate is phenotypically very similar to hospital-acquired isolates [127]. It emphasizes the importance of judicious antibiotic use and surveillance of resistance patterns in the hospital.

4.3. Genotypic Characterization of Resistance

A total of 439 resistance genes of various types were identified in the isolated NA22 using whole-genome sequencing. A total of 76 resistance genes were detected, and they encoded resistance to various classes of antibiotics, such as β-lactams, aminoglycosides, fluoroquinolones, sulfonamides, macrolides, and tetracyclines [128, 129]. Of these, the detection of *blaNDM-4* and *blaCTX-M-15* merit special mention, since they confer resistance towards carbapenems and extended-spectrum cephalosporins which are the drugs of choice for the treatment of severe Gram-negative infections [130, 131]. Finally, the recruitment of both aminoglycoside and fluoroquinolone resistance mechanisms in *aac(6')-Ib-cr* confirms the multiresistant nature of this strain [9]. Other genes identified

were qnr, *sul1/sul2*, and a number of aminoglycosides modifying enzymes, which again reaffirms the multidrug resistant features of NA2_2 [132]. Many of these resistance genes were on plasmid sequences, with a predominant prevalence of genes on a plasmid identified as NA22_3. This plasmid carried also conjugation-related genes like those of the tra operon, indicating the possibility of plasmid transfer to other bacteria [133, 134]. This leads to worries of potential spread of these resistance determinants in a clinical environment, especially in high-risk wards, e.g. intensive care units [135]. In general, results of genotypic data very well supported phenotypic results and contribute to a better understanding of the genetic of the observed resistance. These findings further underscore the importance of genome-based analysis to estimate in a reliable manner the content of resistance genes and the potential for their dissemination [136].

4.4. Correlation Between Phenotypic and Genotypic Resistance

The agreement between phenotypic (VITEK 2 and disk diffusion) and genotypic (WGS) was generally good. Resistance to β-lactam antibiotics (penicillins, cephalosporins, and carbapenems) was well supported by presence of the β-lactamase genes *blaNDM-4* and *blaCTX-M-15*. This is consistent with the functional expression of these resistance genes in the clinical strain [131]. Similarly, detected phenotypic resistance against aminoglycosides and fluoroquinolones also correlated with the presence of genes including *aac(6')-Ib-cr* and different AMEs. These data demonstrate the implication of these genes in the in vitro resistance phenotype. But in some cases, resistance genes were found in the genome that could not be related to a clear phenotypic potential. This could be due to very low gene expression, regulatory elements, or other compensating mechanisms that may control the effectiveness of similar encoded resistance under the screening conditions. Resistance or intermediate susceptibility was also observed among some fluoroquinolones while known resistance determinants were present [137]. These results demonstrate the added value of using complementary phenotypic and genotypic methods. The former, phenotypic testing, describes the true resistance in given conditions, whereas the latter, genotypic approach, provides a more comprehensive and sensitive picture of the resistance possibility [125]. Both combined methods will complement each other for a comprehensive and reliable evaluation of antimicrobial resistance [136].

4.5. Virulence and Pathogenicity Factors

It was determined by genome analyses of NA22 that there were many genes of virulence classified in each functional group. These included genes specific for iron acquisition systems, adhesion, serum resistance and biofilm formation. These traits are often linked to the ability of bacterial strains to colonize the host, to escape the immune response and to produce infections at different body sites. Genes associated with iron acquisition, including siderophore synthesis and export genes, suggest that the isolate is well equipped to search for iron in the iron-deprived human body [138]. Moreover, due to the production of multiple adhesins and fimbrial genes, the capacity to colonize epithelial surfaces and to form biofilms, especially on medical devices, like catheters or endotracheal tubes, might be even higher [139]. Capsular and serum resistance determinants similarly fit with the observation that the species live in the blood and are resistant to killing by complement [140]. These are critical for systemic infection and could well contribute to persistence in either in immunocompromised hosts or long-term patients. In summary, the virulence feature of NA22 indicates that the strain might be a highly invasive and hard-to-cure strain, especially for its multiantibiotics resistance. The multiresistant and virulence characteristics of such strains serve to warrantclinical surveillance of these isolates [141].

4.6. Phylogenetic Relationships

Phylogenetic inference using the NA22 genome positioned the isolate near other medically important strains within the Enterobacteriaceae . The whole-genome-based tree indicated clustering with genomes of comparable resistance and virulence patterns, suggestive of either genetic lineage [142]. Core genome alignment and global protein family analysis were instrumental in gaining more accurate insight into where NA22 stands within the larger genetic context of hospital-associated isolates. Close relationship to isolates containing both resistance and virulence genes indicates that NA22 may have been formed under the force of strong selection pressures, such as repeated antibiotic treatment and host immune mechanisms [104]. These data suggest that mobile genetic elements, such as plasmids and transposons, may have also influenced the genetic structure of this strain [143]. The acquisition of major drug resistance genes and virulence determinants via horizontal gene transfer could have facilitated its adaptation as well as prolonged its existence in a hospital setting[144].In general, the phylogenetic

observations are consistent with the hypothesis that NA22 is part of a group of emerging multidrug-resistant pathogens that are adapting to clinical pressures. This highlights the importance of ongoing genomic surveillance so that the spread can be monitored, and infection control measures targeted accordingly.

4.7. The Role of Plasmids in Multidrug Resistance in the NA22 Isolate

The genomics analysis revealed that a few resistance genes in NA22 were found located in plasmid sequences, especially the plasmid denoted as NA22_3. This plasmid harbored important β-lactamase genes such as *blaNDM-4* and *blaCTX-M-15*, as well as other resistance genes associated with aminoglycosides and fluoroquinolones. Another aspect of this plasmid that makes it particularly alarming is the occurrence of conjugation genes like the *tra* genes. These genes are related to plasmids' transferability among bacteria and lead to the spread of resistance to other strains in the hospital [133]. The combination of different resistance genes on the same MGE may pose a threat of co-selection. In such instances, separate exposure to an antibiotic can sustain indirectly resistance to other antibiotics despite the latter not being used [9]. This may account for why multidrug resistance can remain a clinical problem even with modifications to the prescribing of antibiotics. The results underscore the significance of plasmids in mediating resistance in a single strain as well as in the dissemination of antimicrobial resistance through bacterial communities. Plasmid content and mobility thus require monitoring to understand and control resistance patterns in health care facilities.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

In this study, we characterized the NA22 isolate in comparison to its alignment by antimicrobial resistance and genomic traits. The isolate was initially misidentified phenotypically as *Klebsiella pneumoniae*, while the whole-genome sequencing revealed that it was *E. coli*. Phenotypic analysis, including disk diffusion and VITEK 2 susceptibility testing, demonstrated resistance to several antibiotic families, e.g., β-lactams, carbapenems, aminoglycosides, fluoroquinolones, and sulfonamides, thereby classifying NA22 as a multiresistant strain with potential consequences for medical purposes. Genomic analysis showed 76 resistance genes and several efflux pump systems (*acrAB-tolC, emrAB*, and *mdtEF*) and target modification genes (*gyrA, folA, rpoB*, and *gidB*). Three plasmids were found, with one (NA22_3) carrying the main resistance genes (*blaNDM-4, blaCTX-M-15*, and *aac(6')-Ib-cr*) and conjugation-related genes from the tra operon, suggesting possible plasmid-mediated transfer. The large repertoire of virulence-associated genes and transport systems suggests the ability of NA22 to survive in the hospital environment and eventually contribute to healthcare-associated infections. The analysis based on the phylogenetic position of the isolate fell in the *E. coli* cluster again and confirmed that the isolate was indeed a true *E. coli* isolate and reinforced the limitation of phenotypic traits alone for the classification of bacteria. These results highlight the necessity of developing synergy between genomics and traditional diagnostics to enhance the identification and drug susceptibility profiling. In view of the high-risk plasmids identified and complex resistance mechanisms that were detected, the infection control should be strengthened, genomic monitoring should be performed in an ongoing manner, and the function of hypothetical proteins and new resistance genes identified in the isolate should be further elucidated; this may reveal a new resistance mechanism.

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APPENDICES

Appendix A. Whole-Genome Sequencing Details

Sequencing Platform	**Average Coverage**
Assembly Tool	Flye 2.9 with polishing via Racon and Minimap2
Total Contigs	8 (≥ 1000 bp)
Genome Size	~5.2 Megabases (Mb)
Average Coverage	~60×

Appendix B. Bioinformatics Tools Used

Tool	**Version**	**Purpose**
Flye	2.9	Genome Assembly
Racon	1.4.20	Polishing Reads
Minimap2	2.24	Read Mapping
QUAST	5.2.0	Assembly Quality Check
CARD	2023	Resistance Gene Annotation
VFDB	2022	Virulence Factor Detection
PlasmidFinder	2.0	Plasmid Replicon Typing

Appendix C. NCBI Genome Submission Details

Organism	NA22 Isolate (genotypically Escherichia coli)
BioProject ID	PRJNA1270365
Taxonomy ID	573
Submission Date	31 May 2025
Deposited via	Erciyes University, Türkiye

CURRICULUM VITAE

PROFESSIONAL SUMMARY

A motivated biology graduate with hands-on experience in clinical laboratory techniques and a strong academic background in microbiology and molecular biology. Currently pursuing a master's degree in biology at Erciyes University, with research focused on antibiotic resistance in resistant lactose-fermenting Enterobacteriaceae strains.

PERSONAL INFORMATION

Name Surname : Noor Albunsralal

Nationality : IRAQ

Date of Birth :

Place :

Social Status :

e-mail :

Address :

EDUCATION

Degree	Institution	Date of graduation
Bachelor	Certificated in Biology from Baghdad University	2021

LANGUAGES

Arabic, English, Turkish