

**NEAR EAST UNIVERSITY**

**INSTITUTE OF GRADUATE STUDIES**

**DEPARTMENT OF MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY**

**INVESTIGATION OF MULTIDRUG RESISTANCE IN EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *ENTEROBACTERIACEAE* SPECIES**

**M.Sc. THESIS**

**Chinaza Angel UDEOGU**

**Nicosia**

**February, 2022**

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**Supervisor**

**Assoc. Prof. Dr. Emrah RUH**

**Nicosia**

**February, 2022**

**Approval**

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We certify that we have read the thesis submitted by Chinaza Angel Udeogu titled “**Investigation of Multidrug Resistance in Extended-Spectrum Beta-Lactamase Producing *Enterobacteriaceae* Species”** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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**Declaration**

I hereby declare that all the information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Chinaza Angel Udeogu

10/02/2022

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**Özet**

**Genişlemiş Spektrumlu Beta-Laktamaz Üreten *Enterobacteriaceae* Türlerinde Çoklu İlaç Direncinin Araştırılması**

**Udeogu, Chinaza Angel**

**Yüksek Lisans, Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı**

**Danışman: Doç. Dr. Emrah Ruh**

**Şubat 2022, 60 sayfa**

**Amaç:** Bu çalışma hastanede yatan ve toplumdaki bireylerde genişlemiş spektrumlu beta-laktamaz üreten *Enterobacteriaceae* (GSBL-E) türlerindeki çoklu ilaç direncinin, ve bununla ilişkili olabilecek risk faktörlerinin araştırılması için yapılmıştır.

**Gereç ve Yöntem:** Çoklu ilaç direncinin oranlarının saptanması amacıyla çalışmaya 64 GSBL-E izolatı dahil edilmiştir. Bu bakterilerin 49’u Yakın Doğu Üniversitesi Hastanesi’nde yatan hastaların, 15’i ise toplumdaki bireylerin dışkı örneklerinden izole edilmiştir. Duyarlılık testleri ampisilin (10 µg), amoksisilin-klavulanik asit (30 µg), piperasilin-tazobaktam (110 µg), sefotaksim (30 µg), seftazidim (30 µg), sefepim (30 µg), siprofloksasin (5 µg), gentamisin (10 µg), trimetoprim-sulfametoksazol (25 µg) ve tigesiklin (15 µg) antibiyotik diskleri kullanılarak yapılmıştır.

**Bulgular:** Altmış dört izolatın 46 (%71,9)’sı çok ilaca dirençli bulunmuştur. Çoklu ilaç direncinin oranı hasta grubunda 34 (%69,4), kontrol grubunda ise 12 (%80,0) olarak belirlenmiştir. Hasta ve kontrol grupları arasında çok ilaca dirençli *Enterobacteriaceae* türlerinin izolasyonu açısından anlamlı bir fark bulunmamıştır (*p*>0.05). Bu çalışmada, çoklu ilaç direnci ile ilişkili tek anlamlı faktör yaş (*p*=0.021) olmuştur.

**Sonuç:** Bu çalışma GSBL-E izolatlarındaki çoklu ilaç direncinin yüksek oranda olduğunu, bu nedenle Kuzey Kıbrıs’ta antibiyotik direncinin dikkatli şekilde izlenmesi gerektiğini göstermektedir.

***Anahtar kelimeler*:** *Enterobacteriaceae*, antibiyotik, genişlemiş spektrumlu beta-laktamaz, çoklu ilaç direnci.

**Abstract**

**Investigation of Multidrug Resistance in Extended-Spectrum Beta-Lactamase Producing *Enterobacteriaceae* Species**

**Udeogu, Chinaza Angel**

**M.Sc., Department of Medical Microbiology and Clinical Microbiology**

**Supervisor: Assoc. Prof. Dr. Emrah Ruh**

**February 2022, 60 pages**

**Aim:** The present study was carried out to examine the rates of multidrug resistance (MDR) in extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-E) and the possible risk factors in hospitalized patients and individuals in the community.

**Materials and Methods:** A total of 64 ESBL-E isolates were included in order to determine the rates of MDR in the study. These bacteria were isolated from the fecal samples of the patients who were admitted to the Near East University Hospital (n=49) and the individuals from the community (n=15). Susceptibility tests were done using ampicillin (10 µg), amoxicillin-clavulanate (30 µg), piperacillin-tazobactam (110 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg) and tigecycline (15 µg) antibiotic discs.

**Results:** Of the 64 samples, 46 (71.9%) were MDR positive. The rate of MDR was 34 (69.4%) in the patient group and 12 (80.0%) in the control group. There was no statistical significance between the patient and control groups in terms of isolation of MDR bacteria (*p*>0.05). In this study, age (*p*=0.021) was identified as the only significant factor associated with MDR.

**Conclusion:** This study shows that the rate of MDR among ESBL-E isolates is high, therefore, antibiotic resistance should be carefully monitored in Northern Cyprus.

***Keywords*:** *Enterobacteriaceae*, antibiotic, extended-spectrum beta-lactamase, multidrug resistance.

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**List of Abbreviations**

**ABC:** ATP-Binding Cassette

**AM:** Ampicillin

**AMC:** Amoxicillin-clavulanic acid

**ASHP:** American Society of Health-System Pharmacists

**CAZ:** Ceftazidime

**CDC:** Centre for Disease Control and Prevention

**CDT:** Combined Disc Test

**CIP:** Ciprofloxacin

**CLSI:** Clinical and Laboratory Standards Institute

**CN:** Gentamicin

**CTX:** Cefotaxime

**DAEC:** Diffusely-Adherent *E. coli*

**DDST:** Double Disc Synergy Test

**DNA:** Deoxyribonucleic Acid

**EAEC:** Entero-Aggregative *E. coli*

**EHEC:** Entero-Hemorrhagic *E. coli*

**EIEC:** Entero-Invasive *E. coli*

**EMB:** Eosin Methylene Blue

**EPEC:** Enteropathogenic *E. coli*

**ESBL:** Extended Spectrum Beta-Lactamase

**ETEC:** Entero-Toxigenic *E. coli*

**EUCAST:** European Committee on Antimicrobial Susceptibility Testing

**FEP:** Cefepime

**GIS:** Gastrointestinal Syndrome

**HUS:** Hemolytic Uremic Syndrome

**ICU:** Intensive Care Unit

**MBL:** Metallo Beta-Lactamase

**MDR:** Multidrug Resistance

**MIC:** Minimum Inhibitory Concentration

**MNEC:** Meningitis-associated *E. coli*

**MOA:** Mechanism of Action

**mRNA:** Messenger Ribonucleic Acid

**MRP:** Multidrug Resistance Protein

**PABA:** Para aminobenzoic Acid

**PAE:** Post Antibiotic Effect

**PBP:** Penicillin Binding Protein

**PDR:** Pan drug Resistance

**rRNA:** Ribosomal Ribonucleic Acid

**SBL:** Serine Beta-Lactamase

**SXT:** Trimethoprim-sulfamethoxazole

**TGC:** Tigecycline

**TPT:** Piperacillin-tazobactam

**tRNA:** Transfer Ribonucleic Acid

**UDP:** Uridine Diphosphate

**UTI:** Urinary Tract Infection

**WHO:** World Health Organization

**XDR:** Extensively Drug Resistance

 **CHAPTER I**

**Introduction**

When a bacterium is resistant to at least one antibiotic in three or more drug classes, this microorganism is multidrug-resistant. That is why diseases caused by bacteria that are multidrug-resistant are difficult to cure because treatment options are limited (Elizabeth et al., 2016). Antibiotic resistance spreads more easily with MDR because when resistance genes are passed on to other bacteria, they become resistant to a large number of antibiotics simultaneously (Johan et al., 2018). In situations where organisms are exposed to antibiotics on a regular basis, such as hospitals or large production animal farms, multidrug-resistance may become conducive, selected and so, proliferate much farther (Lekita, 2016).

MDR makes attempts to lessen resistance more difficult. When multiple antibiotics are used to treat a resistant bacterium, lowering the use of one type of antibiotic alone is insufficient to decrease resistance to that antibiotic (Carl & Lars, 2014). Gram-negative bacteria such as Escherichia coli and Klebsiella pneumoniae which produces extended-spectrum beta-lactamase (ESBL) are becoming more common all over the world (Nagarajan et al., 2017)*.* Many clinically significant antibiotics are destroyed by enzymes known as ESBLs. ESBL-producing bacterial infections are resistant to treatment and are growing more frequently. A concerning issue is that the number of persons who are asymptomatic carriers of ESBL-producing bacteria is increasing over the world. According to previous studies, ESBL-producing bacteria have colonized more than half of the community populations in several parts of Southeast Asia. Other countries of the world are also witnessing a growth in numbers. This puts many people at risk of developing antibiotic-resistant infections in the future (Woerther et al., 2017).

**Aim of the Study**

The purpose of this study was to examine the multi-drug resistance among ESBL-producing *Enterobacteriaceae* isolates using various antibiotics. Possible risk factors such as the epidemiological factors, demographic/socioeconomic factors, and hospital-related factors were also evaluated in the course of the study.

 **CHAPTER II**

 **Literature Review**

**2.1. General Characteristics**

*Enterobacteriaceae* within the order *Enterobacterales* (Adeolu et al., 2016) is a family of gram-negative, facultatively anaerobic, and non-spore forming bacilli. “Enteric bacteria” refers to members of the *Enterobacteriaceae* family due to the fact that several organisms inhabit the intestines of animals and also humans which makes them a normal part of the intestinal flora. Urinary tract infections (UTIs) are a common occurrence, and some species also cause diarrhea. They can also spread to the bloodstream, posing a serious risk to life (Ronald, 2003).

*Enterobacteriaceae* includes a number of pathogens of which important members include: *Escherichia, Klebsiella, Enterobacter, Citrobacter, Salmonella, Shigella, Proteus, Serratia* and other species. *Klebsiella* is a bacterium that can be found in both nature and humans, and it can be found in parts of the digestive tract where it does not create problems (Quereshi, 2018). Infections with *Klebsiella* are common in hospitals and long-term care homes. *Klebsiella* infections are more common in patients who have a weakened immune system or who have an implanted medical device (such as a urine catheter or an endotracheal tube). *Enterobacter, Citrobacter, Proteus* and *Serratia* are also causes of UTIs while *Salmonella* and *Shigella* are responsible for causing diarrhea in infected patients (Bush et al., 2018).

Just like other bacteria, resistance to antibiotics can be developed by *Enterobacteriaceae*, particularly resistance to carbapenem medicines, which are the last line of antibiotic treatment for resistant organisms. For example, *Klebsiella pneumoniae* strains which are resistant to carbapenem due to the extensive use of antibiotics. Also, *Citrobacter* isolates may be resistant to a wide range of other antibiotics due to plasmid-encoded resistance genes while about 10–20% of *Proteus* *mirabilis* strains are also resistant to first-generation cephalosporin and ampicillin. These infections are potentially more dangerous and difficult to treat (Wang et al., 2014).

*Escherichia coli* is part of the normal microbiota of the gut and is beneficial to their hosts but some strains can be harmful and are one of the most common causes of UTIs. (CDC, 2012). *Escherichia coli* strains are classified into three groups;

**2.1.1.** ***Intestinal/Enteric* E. coli**

There are six types of intestinal pathogens that has been described and they include: Enteropathogenic *E. coli* (EPEC) is the most common cause of infant diarrhea in undeveloped countries and can become fatal (Harry et al., 2004). Entero-Hemorrhagic *E. coli* (EHEC) causes both bloody diarrhea (hemorrhagic colitis) and non-bloody diarrhea, as well as hemolytic uremic syndrome (HUS). Entero-Toxigenic *E. coli* (ETEC) which also causes infant diarrhea in undeveloped countries but is the major cause of traveler’s diarrhea. Entero-Aggregative *E. coli* (EAEC) are becoming more widely recognized as a cause of chronic diarrhea in both developing and developed countries, and have been linked to various outbreaks around the world. Entero-Invasive *E. coli* (EIEC) can induce invasive inflammatory colitis and dysentery, but it usually results in watery diarrhea (Harry et al., 2004). Diffusely-Adherent *E. coli* (DAEC) which causes diarrhea especially in children above 12months of age (CDC, 2020).

**2.1.2. E. coli *that causes Infections of the* *Urinary Tract***

Among the most prevalent sites of bacterial infection is the urinary system, with *E. coli* being the most common pathogen. Infection most likely begins with a uro-pathogenic strain invading the intestine, in addition to the commensal flora (Mukherjee et al., 2013).

**2.1.3.** ***Meningitis/Sepsis-associated* E. coli**

With a patient fatality rate ranging from 15-40% and long-term mental health issues in many survivors (James et al., 2004), this *E. coli* serotype is the most frequent cause of gram-negative newborn meningitis. Though intestinal or urinary tract infections that are caused by *E. coli* strains can readily be transmitted through urine or feces, diseases affecting the peripheral and central nervous system has no visible benefit in terms of virulent MNEC strains selection and transmission (Harry, 2004). Meningitis-causing *E. coli* is transmitted via the bloodstream (CDC, 2012).

**2.2.** **Antibiotics**

Antibiotics have been around since the beginning of time. Antibiotic is a word that basically means "opposing life". Anti "against"; and bios, "life" are two Greek words that are commonly used to describe any substance intended to fight against micro-organisms. Therefore, in medical terms, antibiotics are antimicrobial substances that are effective against bacteria. Ever since antibiotics were discovered and used in medicine, they have surely provided mankind with one of the greatest benefits. Over the next 10-15 years after they were introduced, the population's average lifespan improved dramatically, and infectious diseases became manageable. Antibiotics are the most common form of antibacterial agent been used in the treatment and prevention of infections caused by bacteria, and all antibiotic drugs are commonly used to treat and prevent such diseases (Lee, 2015). Antibiotics have no effect on viruses like the common cold or influenza, although some of them may possess anti-protozoan activity. The vast majority of antibiotics are either (i) microorganism-derived natural products, (ii) semi-synthetically produced natural products, and or (iii) chemically synthesized based on natural product structure (Mahajan et al., 2019).

The majority of antibiotics which are used today are natural products or gotten from natural products, and new antibiotics are being researched using fungal, bacterial, animal and plant extracts. Medicinal plants, for example, are examined for antibacterial chemicals because the plants are employed by traditional healers to treat infections (Uttpal et al., 2019). In addition, soil microorganisms are screened because they have historically been a significant source of antibiotics. Natural products are sometimes tested for their capacity to decrease antibiotic resistance and tolerance, in addition to their direct antibacterial activity. Some secondary metabolites, for example, block drug efflux pumps, allowing antibiotics to reach their cellular targets at higher concentrations and reducing bacterial resistance. The alkaloid lysergol (Cushnie et al., 2014), the flavonoids chrysin and rotenone, and the carotenoids, capsorubin and capsanthin (Quingmei, 2019), have all been reported to be bacterial efflux pump inhibitors (Molnar et al., 2010). It is also possible to use organic materials for their capacity to inhibit the virulence factors of bacteria. Virulence factors are substances, regulatory and cellular components, which enables a bacterium to escape the immune system of the body (e.g., staphyloxanthin, urease), proceeds, binds, or colonizes living tissues (e.g., adhesins, internalins, type IV pili), cause diseases (e.g., exotoxins) and also organize the stimulation of pathogenic strains (e.g., quorum sensing) (Sara, 2021).

Antibiotics that are semi-synthetic are neither entirely natural nor entirely synthetic. Semi-synthetic antibiotics are created by transforming natural starting materials into end compounds through chemical processes. Semi-synthetic antibiotics can also be seen as a halfway house between synthetic and natural antibiotics. They can also be manufactured by altering the active portion of a natural antibiotic chemically to make a single new molecule. A natural product is enhanced and chemically altered to produce new antibiotics with better therapeutic efficacy. They can, for example, act against microorganisms that have developed a resistance to the very first chemical, have a broader spectrum of activity, and have less adverse effects than the original molecule. Penicillins are antibiotics that were first discovered in *Penicillium* molds. The vast majority of penicillins used in medicine are chemically manufactured from penicillin found in nature. Two natural penicillins are currently used in clinical practice: penicillin G and penicillin V.

**2.2.1.** ***Mechanisms of Action of Antibiotics***

The term "mechanism of action" (MOA) in pharmacology refers to the precise biochemical interaction that a drug molecule uses to achieve its pharmacological effect. The exact molecular targets to which the medication interacts, such as an enzyme or receptor, are frequently mentioned in a mechanism of action. Drugs have distinct affinities for receptor sites based on the chemical structure of the medication and the unique action that takes place there. Drugs that don't bind to receptors work by interacting with chemical or physical features in the body to achieve their therapeutic impact. The cytoplasmic membrane of gram-positive bacteria is surrounded by a thick and rigid layer of polysaccharides known as the cell wall. Gram-negative bacteria, on the other hand, have a thin peptidoglycan layer that is covered by a second lipid membrane known as the outer membrane. Periplasm is the gap in-between the cytoplasmic membrane and the outer membrane. In gram-negative bacteria, the outer membrane is an extra protective layer that blocks numerous chemicals from entering the organism. This membrane, on the other hand, contains porins, which allow various compounds, such as medicines, to pass through.

Various antibiotics have different mechanisms of action because of the diversity of their structure and attraction for certain specific locations within bacterial cells. These are:

* Inhibition of cell wall synthesis
* Inhibition of protein synthesis
* Inhibition of nucleic acid synthesis
* Inhibition of folic acid synthesis
* Inhibition of cell membrane function (Vinutha et al., 2020).

**Inhibition of Cell Wall Synthesis.** The peptidoglycan cell wall which is made up of long sugar polymers, covers the bacterial cell wall. Trans-glycosidases crosslink the glycan strands in the peptidoglycan, and the peptide chains extend from the sugars in the polymers, forming cross interconnections from one peptide to the next. The cell wall is made stronger by this cross-linking and the formation is inhibited by β-lactams (penicillin, cephalosporins, carbapenems and monobactams), other antibiotics (Bacitracin and Vancomycin) and glycopeptides.

***Penicillin.***Penicillin binds to the active site of transpeptidase enzyme causing the peptidoglycan fibres to crosslink. Penicillin then permanently blocks the enzyme transpeptidase by interacting with a serine residue in the enzyme. Because this reaction is irreversible, the development of the bacterial cell wall will be inhibited. Penicillins include: penicillin V, penicillin G, ampicillin, amoxicillin, piperacillin, oxacillin, dicloxacillin, ticarcillin and nafcillin. (Petri, 2011).The primary structural component for biological activity is the beta-lactam ring. The loss of all major antibacterial action is caused by metabolic transformation or chemical modification of this component of the molecule. About 30 bacterial enzymes are involved in the manufacture of the peptidoglycan, which can be split into three stages:

* The initial stage is the production of precursors in the cytoplasm. The uridine-diphosphate (UDP)-acetylmuramyl-pentapeptide product is known as a "park nucleotide". The addition of a dipeptide, D-alanyl-D-alanine, is the last step in the synthesis of this molecule.
* UDP-acetylmuramyl-pentapeptide and UDP-acetylglucosamine are joined in the second stage to produce a longer polymer.
* The completion of the cross-link is the third and final stage. A transpeptidation reaction takes place outside the cell membrane to accomplish this. The transpeptidase is a membrane-bound enzyme.

Penicillin binding proteins (PBPs) are a class of proteins defined by their affinity for and binding to the penicillin antibiotics. Many bacteria have them as a natural component. PBPs all take part in the last stages of peptidoglycan production, which is the primary component of bacterial cell walls. In bacteria, cell wall synthesis is required for growth, cell division and cellular structure maintenance (Miyachiro et al., 2020). Inhibition of PBPs causes cell wall deficiencies and abnormalities, such as filamentation, pseudo-multicellular forms, and lesions leading to spheroplast development, and eventually cell death and lysis (Cushnie et al., 2016).

***Ampicillin*.** Ampicillin, often known as a broad-spectrum penicillin, is a kind of aminopenicillin, a semisynthetic category of beta-lactam antibiotics that was created to fight bacteria that were either gram-positive or gram-negative (Catherine et al., 2019; Valerie, 2012). It was also the first "wide spectrum" penicillin with gram-positive bacterial activity. Penicillin was joined to an amino group or side chain to make aminopenicillins. The addition of the side chain changed the drug's efficacy against some bacteria substantially. These antimicrobials worked against *E. coli, Salmonella, Proteus mirabilis, Shigella,* *Neisseria* and *Hemophilus* species at first. Since there has been a change in the susceptibility rate of ampicillin, it is no longer the first-line treatment for infections caused by numerous of these species, including *E. coli* urinary tract infections, unless susceptibility is indicated by culture and susceptibility test results. Ampicillin inhibits cell wall peptidoglycan synthesis and renders inhibitors of autolytic enzymes inactive by interacting to penicillin-binding proteins (PBPs) and interfering with cell wall formation. Therefore, ampicillin is usually bacteriolytic. Co-administration of sulbactam, a medicine that also inhibits beta-lactamase, a bacteria-produced enzyme, broadens its spectrum of efficacy.

***Amoxicillin and its Mechanism of Action.***This is a semi-synthetic derivative of penicillin with a structure similar to ampicillin. It is in the beta-lactam family of antibiotics and is utilized for the management of various infections including urinary tract infections (UTIs) (Deepti & Deepthi, 2010). Beta-lactamase producing bacteria, which have been resistant to most beta-lactam medicines, can degrade amoxicillin. As a result, it can be used in combination with clavulanic acid, a beta-lactamase inhibitor. Amoxicillin works by blocking cross-linking between linear peptidoglycan polymer chains that make up the bacterial cell wall (Bernatova et al., 2013).

 Clavulanic acid can overcome antibiotic resistance in bacteria that release beta-lactamase, which inactivates most penicillin, when used in combination with penicillin-group medicines. Clavulanic acid has a beta-lactam ring in its structure that binds to beta-lactamases in an irreversible manner, preventing beta-lactamases from inactivating certain beta-lactam antibiotics, and is efficient against infections caused by gram-positive and gram-negative bacteria (Townsend, 2002).

***Amoxicillin-Clavulanic Acid and its Mechanism of Action*.** Also known as co-amoxiclav, is the union of potassium clavulanate and amoxicillin. Clavulanic acid is a type of beta-lactamase inhibitor. The clavulanate dose remains constant at 125 mg across the amoxicillin-clavulanate dosage spectrum, however the amoxicillin dose fluctuates between 250mg, 500mg, and 875mg. This combination produces an antibiotic with a broader spectrum of action and improved efficacy against beta-lactamase-producing amoxicillin-resistant bacteria.

The mechanism of action works by inhibiting bacterial development and preventing bacteria from degrading amoxicillin. It provides enhanced antimicrobial defense against beta-lactamase generating strains of *Staphylococcus aureus*, *Haemophilus influenzae*, *Neisseria gonorrhea*, *Escherichia coli*, *Proteus*, *Klebsiella*, *Moraxella catarrhalis*, and *Bacteroides* species. It has limited effect on *Pseudomonas* or methicillin-resistant *Staphylococcus aureus* (Bobak et al., 2021).

***Piperacillin and its Mechanism of Action.***Piperacillin is a ureidopenicillin-class broad-spectrum beta-lactam antibiotic. Piperacillin is ineffective against gram-positive pathogens like *Staphylococcus aureus* when used alone because the beta-lactamase enzyme of bacteria hydrolyzes the beta-lactam ring. The beta-lactamase inhibitor tazobactam (piperacillin/tazobactam) is usually combined with piperacillin (Soroush et al., 2020), which increases the efficiency of piperacillin by blocking several beta lactamases to which it is vulnerable (Hauser et al., 2013).

Piperacillin is a beta-lactam antibiotic that prevents the spread of germs and diseases by inhibiting penicillin-binding proteins (PBPs).

***Tazobactam and its Mechanism of Action*.** Tazobactam is a beta-lactamase inhibitor that prevents other antibiotics from being degraded by bacteria that manufacture beta-lactamase enzymes. When combined with piperacillin and ceftolozane, it is used in the treatment of bacterial illnesses.

Tazobactam broadens the spectrum of piperacillin and ceftolozane by making them active against beta-lactamase-producing pathogens which would ordinarily destroy them. This is accomplished by inhibiting beta-lactamase enzymes in an irreversible manner. Tazobactam may also bind covalently to beta-lactamase enzymes mediated by plasmids and chromosomes (Pagan-Rodriguez et al., 2004).

***Piperacillin-Tazobactam and its Mechanism of Action.*** Since ureidopenicillins and amino-benzylpenicillins operate collaboratively with beta-lactamase inhibitors, the combination of piperacillin and tazobactam boosts their overall bactericidal action.  Piperacillin-tazobactam is one of three drugs used to treat hospital-acquired pneumonia caused by multidrug-resistant bacteria.

Some beta-lactamase enzymes have residue on their binding site, which allows for the hydrolysis of the beta-lactam rings present in antimicrobial drugs. When piperacillin is combined with tazobactam, however, this hydrolytic action is suppressed. Tazobactam forms a stable acyl-enzyme complex with these enzymes, identical to the complex created after the beta-lactam ring is hydrolyzed. As a result, piperacillin is protected against hydrolysis. Resistance to piperacillin-tazobactam is primarily caused by gram-negative bacteria that generate beta-lactamases. Changes in the binding site of PBPs, alterations in the efflux membrane, and bacterium penetrability are all widely recognized mechanisms (Hiyashi et al., 2010).

***Cefepime (Fourth-Generation Cephalosporin)*.** Cefepime is a cephalosporin antibiotic with a wide range of activity that has been used to treat bacteria that cause pneumonia, skin infections, and urinary tract infections. Cefepime works by fighting bacteria that are both gram-positive and gram-negative (Valerie, 2012), and is more effective than third-generation antibiotics in both cases. It is a front-line drug when infection with *Enterobacteriaceae* is known or suspected, as other cephalosporins are destroyed by various plasmid and chromosome-mediated beta-lactamases (Chapman et al., 2003). Cefepime just like penicillin is a bactericidal drug that works by inhibiting the synthesis of bacterial cell wall as its response mechanism.

**Inhibition of Protein Synthesis.** Protein synthesis is a multi-step and multi-enzyme process that involves structural alignment, whereas, the compound that prevents cell growth by altering the processes that result in the production of new proteins is called a protein synthesis inhibitor (Frank, 2021). Antibiotics that inhibit bacterial protein synthesis, on the other hand, disrupts the activities at the 30S or 50S subunits of the 70S bacterial ribosome (Nissen et al., 2000). Antibiotics do not only target the aminoacyl tRNA synthetases that activate each amino acid essential for peptide formation alone, but also the formation of the 30S initiation complex (made up of mRNA, the 30S ribosomal subunit, and formyl methionine tRNA), the formation of the 70S ribosome by the 30S initiation complex and the 50S ribosome, and assembling amino acids into polypeptides. The following are the medications that are protein synthesis inhibitors:

Aminoglycosides

Tetracycline and glycylcycline

Chloramphenicols

Linezolids

Macrolides and ketolides

Streptogramins

***Mechanism of Action*.** The aminoacyl tRNA synthetases that activate each amino acid essential for peptide formation are not targeted by antibiotics, instead, protein synthesis inhibitors generally impede bacterial mRNA translation into proteins at several stages, namely elongation (which includes the entrance of aminoacyl tRNA, transfer of peptidyl, translocation of bacteria and proofreading), initiation and elimination. The assembly of the translation system's components in prokaryotes includes the two ribosomal subunits (big 50S and tiny 30S subunits), the mRNA to be translated, the initial aminoacyl tRNA, and the three initiation elements that helps the initiation complex develop. The A site, the P site, and the E site are the three sites on the ribosome. The point of entry for the aminoacyl tRNA is the A site. In the ribosome, peptide is formed at the P site. The tRNA leaves at the E site after giving its amino acid to the expanding peptide chain. Below is a list of common antibacterial drugs and the stages they target:

***Gentamicin*.** Gentamicin, an aminoglycoside, is a bactericidal antibiotic that works by binding to the ribosome of bacteria which possesses the 30S component. It works by messing with the bacteria’s capacity to produce proteins, which usually results in death. Gentamicin crosses the membrane gram-negative via an active transport system that depends on oxygen. Because anaerobic bacteria require oxygen, aminoglycosides are completely ineffective. Members of the *Enterobacteriaceae* family (e.g., *Klebsiella pneumoniae, Serratia* spp., and *Enterobacter* spp. *Escherichia coli*), *Pseudomonas aeruginosa*, and some strains of the *Neisseria, Moraxella*, and *Haemophilus* genera) are the most common microorganisms in clinical settings that show appropriate therapeutic response (Hathorn et al., 2014). In clinical drug concentrations, gentamicin inhibits a large number of *Staphylococci* that are coagulase-negative and *Staphylococcus aureus* isolates that are susceptible to methicillin, while resistance can develop quickly.

***Tigecycline*.** Tigecycline is a third-generation tetracycline antibiotic with a wide spectrum of activity that is used for the treatment of bacterial infections. Bacteria such as *Staphylococcus* *aureus, Acinetobacter baumannii*, and *E. coli* that are resistant to antibiotics generated due to the increasing rate of antibiotic resistance (Rose et al., 2006). The structural alterations to this tetracycline derivative antibiotic have broadened its curative action to cover gram-positive and gram-negative pathogens, which includes multidrug-resistant organisms. When compared to tetracyclines, tigecycline is demonstrated to enhance MIC against gram-positive and gram-negative pathogens (Nguyen et al., 2014). Tigecycline inhibits the interaction of aminoacyl-tRNA with the A site of the ribosome by binding to the 30S ribosomal subunit of bacteria (Christine et al., 2007).

***Chloramphenicol.*** Chloramphenicol is a bacteriostatic antibiotic that prevents the formation of proteins. It prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. It prevents peptide bond formation by binding to A2451 and A2452 residues particularly (Schiffano et al., 2013) in the 23S rRNA of the 50S ribosomal subunit. In other words, it prevents bacteria from completing the peptidyl transfer stage of elongation on the 50S ribosomal subunit.

***Linezolids.*** Linezolid functions as a protein synthesis inhibitor by inhibiting bacterial protein biosynthesis. Either the bacterial growth stops or the bacteria dies (ASHP, 2016). Although many antibiotics function in this way, linezolid's particular mode of action appears to be unusual in that it prevents protein creation from starting in the first place, rather than at a later stage.

***Macrolides And Ketolides.*** These bind to the 50s ribosomal subunits and impede ribosomal translocation of tRNAs (Gary, 2009).

***Streptogramins.*** These are a class of antibiotics which cause the early release of the peptide chain. (Levinson et al., 2008). They are a unique antibacterial class since each member is made up of two structurally different components: group A streptogramins and group B streptogramins. Both components have bacteriostatic action and hinder bacterial protein synthesis at the ribosome level. Their combination, on the other hand, results in bactericidal activity. Streptogramins include quinupristin, pristinamycin, and virginiamycin. (Dougherty and Pucci, 2012; Schwalbe et al., 2007). Streptogramins act by binding to the bacterial ribosome's 50S subunit. They disrupt protein synthesis by two mechanisms: suppression of aminoacyl tRNA incorporation in the ribosome and inhibition of mRNA translation (Francoise et al., 2017). The synergistic effects of the two components could be owing to a change in ribosome conformation produced by group I component binding, which exposes a fixation site for the group II component.

**Inhibition of Nucleic Acid Synthesis.** A nucleic acid inhibitor is an antibiotic that works by preventing nucleic acid synthesis. DNA inhibitors, such as quinolones, serve as topoisomerase inhibitors by inhibiting DNA gyrase (Gupta, 2009). Anaerobic bacteria are affected by a different class of DNA inhibitors, such as metronidazole (Connor & Jaqueline, 2021). These work by producing metabolites, which are then absorbed into DNA strands, making them more prone to breakage (Denyer et al., 2004).

***Fluoroquinolones*.** Fluoroquinolones are a type of broad-spectrum antimicrobials that are used in the prevention and treatment of a wide range of diseases. Examples of fluoroquinolone antibiotics include ciprofloxacin, gemifloxacin, levofloxacin, moxifloxacin and ofloxacin.

***Ciprofloxacin.*** Ciprofloxacin, a second-generation fluoroquinolone, prevents DNA replication by inhibiting the enzymes topoisomerase (II) and (IV). Topoisomerase (II) reduces the amount of supercoiling of the DNA double-stranded helix during replication, whereas topoisomerase (IV) unlinks the two daughter strands of DNA. As a result, ciprofloxacin is bacteriostatic and bactericidal against gram-positive and gram-negative bacterial infections. *Haemophilus*, *Salmonella*, *Pseudomonas*, and *Enterobacter* are examples of gram-negative bacteria that fluoroquinolones can kill; Gram-positive infections include *Staphylococci* and *Streptococci* (Rita, 2019). It is often used to treat infections of the respiratory, gastrointestinal, urinary, and abdominal tracts. Ciprofloxacin's effect is concentration-dependent; at low concentrations, it simply inhibits topoisomerase (II). It is, however, capable of inhibiting topoisomerase (IV) when taken at higher concentrations (Laponogov et al., 2009). The major target of this medicine is also determined by the infection it is used to combat. If the infection is caused by gram-positive bacteria, topoisomerase (IV) becomes the drug's major target; if the pathogen is gram-negative, topoisomerase (II) becomes the primary target. Ciprofloxacin has a long post-antibiotic effect (PAE) against gram-negative bacteria, which means it continues to work long after the patient has stopped taking it (Tampa, 2018).

**Inhibition of Folic Acid Synthesis.** For humans, folic acid is a vitamin obtained from food. Trimethoprim and sulfonamides both block folate production at two distinct locations. Sulfonamides prevent para-amino benzoic acid (PABA) from being incorporated into dihydropteroic acid. Trimethoprim is an antibiotic made by chemical synthesis and it functions by preventing metabolism in the folic acid production route. Trimethoprim, however, blocks a later metabolic step. The antimetabolite suppresses the synthesis of folic acid to the point that there is a reduction in bacterial growth if administered singly. When the two stages of the metabolic pathway are blocked, the synthesis of folic acid is reduced to a level that is fatal to the microorganism. Trimethoprim inhibits the enzyme dihydrofolate reductase, which prevents dihydrofolate from being converted to tetrahydrofolate. Although this enzyme is found in humans, trimethoprim has a reduced affinity for it. Bacteriostatic prevention of growth against a wide range of gram-positive and gram-negative pathogens (Valerie, 2012) is provided by this mode of action. Sulfonamides and trimethoprim work well together, and they are rarely used alone. Trimethoprim is bacteriostatic when administered alone, but bactericidal when combined with sulfonamides and because their half-lives are similar, sulfamethoxazole is used in combination with trimethoprim (Stephen et al., 2015).

**Inhibition of Cell Membrane Function.** The cell membrane is a biological membrane that separates the interiors of all cells from the external environment. The plasma membrane allows ions and organic molecules to pass through preferentially. It regulates the flow of chemicals into and from cells. The cell membrane essentially guards the bacterium from harsh conditions. It is made up of a lipid bilayer with proteins inserted in it. Ion conductivity, cell signaling and adhesion are among processes that plasma membranes play a role in (Zeidi et al., 2018). Bacteria, fungi and plants all have a cell wall that acts as an exoskeleton for the cell and prevents larger molecules from passing through. By attaching the cytoskeleton to provide the cell shape and adhering to the cell membrane and surrounding cells, the plasma membrane aids in the formation of tissues. Antimicrobial peptides have a target in the bacterial membrane and can bind to gram-negative bacteria's outer membrane, blocking solute transit between the periplasm and the cell exterior, resulting in bacterial toxicity. Antimicrobial medicines that disrupt or injure the plasma membrane fall into various categories. Daptomycin, which has a unique method of operation by disrupting the numerous elements that helps in the functioning of the bacterial cell membrane, is one example. It tends to bind to the membrane, resulting in rapid depletion and a reduction in membrane permeability, which causes protein, DNA, and RNA production to be inhibited, leading to bacterial cell death and it is effective against aerobic gram-positive bacteria such as *Staphylococcus* spp., *Streptococcus* spp., *Clostridium perfringens* among others (Karas et al., 2020). Polymyxin antibiotics, for example, have a typical structure that consists of a circular peptide with a longer hydrophobic tail. They work against *Acinetobacter baumannii* bacteria which is gram negative (Valerie, 2012), *P. aeruginosa*, and *K*. *pneumonia* strains which are multidrug-resistant by interfering with the phospholipids in the bacterial cell membrane, causing it to break down (Audrey et al., 2017).

**2.3.** **Beta-Lactamase Enzymes**

Beta-lactamases are bacteria-produced enzymes that produce resistance to beta-lactam antibiotics. There are two systems that classify beta-lactamases; the functional classification using Bush-Jacoby-Medeiros method (Bush et al., 2013) and the molecular classification using Ambler method (Hall et al., 2005).

**2.3.1.** ***Bush-Jacoby-Medeiros Method***

This method considers substrate and inhibitor characteristics in an attempt to group enzymes in ways that can be linked to their clinical isolate phenotype. The molecular classification system often correlates with the major categories. This classification approach divides beta-lactamases into functional classes (substrate and inhibitor profile). It is broken down into four main categories and various subcategories. ESBLs are characterized as either 2be group or 2d group (OXA-type) enzymes, with the latter having many of the same characteristics as enzymes of 2be group but being inhibitor resistant. The 2be subgroup indicates the enzymes that are generated from group 2b beta-lactamases (such as SHV-1, TEM-1 and TEM-2). Also, the ‘e’ in 2be shows that the beta-lactamases have an extended-spectrum. The ESBLs produced from TEM-2, SHV-1 or TEM-1, can be differentiated by as little as one amino acid from their progenitors. This causes a significant shift in the activity of the ESBLs’ enzyme, allowing for the hydrolyzation of cephalosporins that are third generation or aztreonam, resulting in a wider range of activity than the parent enzymes (Deepti & Deepthi, 2010).

**2.3.2.** ***Ambler System***

This system classifies beta-lactamases into four separate groups: Groups A, B, C, and D, based on distinctive sequence patterns as well as basic distinctions in hydrolytic action. Another important distinction by Larry et al, (2000) is among the three classes of active-site serine enzymes called serine beta-lactamases (SBLs) which include classes A, C, D, then the B class, which includes a diverse collection of zinc metallo-enzymes also known as metallo beta-lactamases (MBLs). Despite the fact that all four classes are found in a wide range of biologically important bacteria and those found in the environment, a small number of families of enzymes from each have spread widely among the most important bacterial pathogens (Catherine et al., 2019). *Enterobacteriaceae* like *K. pneumoniae* and *E*. *coli*, as well as non-fermenting bacteria like *A. baumannii* and *P. aeruginosa*, are bacteria that cause hospital-related infections in vulnerable individuals. SHV, TEM, KPC and CTX-M (A class); VIM and NDM (B class); and ADC and CMY (C class) are some of the most important enzyme families. All D class enzymes are called oxacillinase (OXA) (Catherine et al., 2019).

**Class A Beta-Lactamases.** Among all beta-lactamases, enzymes of class A are the most investigated worldwide. Gram-negative bacteria including *S. aureus, K. pneumonia, E. faecium, A. baumannii, Enterobacter*species and *P. aeruginosa* and are particularly resistant due to A class beta-lactamases (Timothy, 2018). Each A class beta-lactamases have a variety of specific substrate, but they are known for efficiently hydrolyzing first-generation cephalosporins and penicillin as a group. The widespread usage of these antimicrobial drugs, as well as the increase of A class beta-lactamases has resulted in the spread of resistance worldwide (Timothy, 2018). KPC, TEM and CTX-M beta-lactamases are three types of A class beta-lactamases that have emerged in reaction to the widespread use of oxyimino-cephalosporins such as ceftazidime and cefotaxime which are likely causes of gram-negative bacteria that are resistant (Catherine et al., 2019; Timothy, 2018).

**Class B Beta-Lactamases.** The zinc-dependent MBLs of class B are not related to known PBPs and belong to a broad, historic, and widely dispersed superfamily of metallohydrolase which are mostly seen in prokaryotes and function on a wide substrate level, from tiny compounds (thioesters, phosphonates) to nucleic acids, and they are involved in the RNA processing and repair of DNA mechanisms in higher eukaryotes. The His-Xaa-His-Xaa-Asp pattern, which make up the protein's foundation by creating a metallic center at the intersection of both beta-sheets, distinguishes MBLs. The fold is thought to have originated as a result of the duplication of an ancestral alpha-beta domain. Three unique MBL subfamilies are defined by the identities of the residues that make up this core, as well as its chemistry and architecture (termed B1, B2 and B3) (Catherine et al., 2019). The most clinically significant B1 enzymes have a binuclear zinc center with tri-His (Zn1) and Cys-His-Asp (Zn2) metal sites; in B2 enzymes, the initial His of the determining pattern is replaced by Asn, which tends to result in a mononuclear enzyme with only the Zn2 site occupied, and in B3 enzymes, the Zn2-coordinating Cys is supplemented by an extra His residue (Catherine et al., 2019).

Water molecules complete metal coordination in binuclear enzymes, one of which links the two metal ions (so-called "bridging" water) and the other of which is connected to Zn2 (so-called "apical" water). Zn1 is usually defined as tetrahedral, whereas Zn2 is defined as deformed square pyramid. MBLs are zinc enzymes, with the possible exception of IMP enzymes, despite the fact that they are viable when reassembled as various metal forms and other members of the superfamily use a variety of metal ions. The MBLs are notable as beta-lactamases for their extraordinarily wide range of action (Catherine et al., 2019), which includes penicillins, cephalosporins, and carbapenems for the binuclear enzymes, but little or no action against monobactams (Lohans et al., 2017).

**Class C Beta-Lactamases.** This class of beta-lactamases are found in abundance on the genes of most gram-negative bacteria. Most significant gram-negative bacteria have genetic codes for the enzymes of class C, commonly abbreviated as AmpC, that are not expressed under usual circumstances. But the depression of these genes, either by alteration or inducement by certain beta-lactams, could result in elevated levels of expression and, eventually lead to an increase in MICs for susceptible beta-lactams. The presence of specific enzymes such as the DHA, CMY and FOX on transposons in non-fermenting bacteria and *Enterobacteriaceae* species such as *P. aeruginosa*, adds to the therapeutic importance of class C enzymes (Catherine et al., 2019).

**Class D Beta-Lactamases.** Class D OXA enzymes seem to be the most complex and, in many ways, the most poorly comprehended of the beta-lactamases. Originally, the earliest enzymes discovered were only active against penicillin, the OXA family currently includes enzymes that are effective towards carbapenems and cephalosporins and have a wide range of sensitivity to inhibitors (Catherine et al., 2019). The proliferation of enzymes that hydrolyses carbapenem in *A. baumannii* and *Enterobacteriaceae* (especially *K. pneumoniae*) and plasmid-mediated cephalosporinases in *P. aeruginosa* has raised the clinical relevance of this class, despite the fact that most members are hereditary. OXA enzymes have recently been discovered in a variety of gram-positive bacteria, adding to the enzymes' extraordinary diversity and range of distribution. Despite the fact that plasmid-mediated oxacillin resistance caused by OXA enzyme transport was reported in the 1960s and OXA enzymes accountable for a variety of resistant traits were discovered in subsequent years, the first OXA structures were not available until the turn of the millennium. Catherine and colleagues described the preserved active-site lysine (the equivalent of Lys73 in A class enzymes) carboxylation as the critical indicator of interaction as well as proved that it resulted from a reversible reaction with carbon dioxide in the atmosphere, paving the way for a better understanding of the mechanism of OXA (Catherine et al., 2019).

**2.4****. Resistance to Beta-Lactam Antibiotics in *Enterobacteriaceae***

The most important mechanism of beta-lactam antibiotic resistance is the formation of beta-lactamases, particularly ESBLs, which inactivate beta-lactam antibiotics, and this continues to be the most common cause of beta-lactam antibiotic resistance among *Enterobacteriaceae* globally (Dejenie et al., 2019).

In numerous countries, epidemics which occurs as a result of organisms that produces ESBL have been reported in vast numbers. There is much of evidence that ESBL infections are more common in resource-poor countries. Long-term antibiotic treatment, ICU stays, nursing home stays, severe sickness, staying in a facility where ceftazidime and other third-generation cephalosporin are commonly used, and catheterization are all important risk factors for infection with organisms that produce ESBL (Kamlesh et al., 2015). *Escherichia coli*, which can produce ESBLs, has evolved and spread throughout the globe as the main cause of hospital-related and environmental infections, and is now a great risk (Deepti & Deepthi, 2010). The first step in preventing the spread of these microorganisms and avoiding any consequences is to identify potential ESBL carriers early on (Sahuquillo-Arce et al., 2014). Due to the fact that ESBL generation is usually plasmid-mediated, ESBL and non-ESBL producing bacteria strains can be found in a single bacterium (Deepti & Deepthi, 2010; Kamlesh et al., 2015). This implies that many colonies from a primary culture plate should be screened for optimal detection (Chaudhary et al., 2004). It is critical to adequately detect ESBL-producing strains in order to choose the best antibiotic therapy and infection control strategies (Cohen et al., 2010).

ESBL production is associated with bacteria usually found in the gut. ESBL is rapidly spread across *Enterobacteriaceae* membersbecause it is plasmid-mediated. Aside beta-lactams, other regularly used antibiotics such as aminoglycosides, sulfonamides and fluoroquinolones are affected by this type of resistance. As a result, majority individuals require the ‘last resort’ antibiotic treatments like carbapenems. Carbapenem-resistant *Enterobacteriaceae* have emerged rapidly as a result of its use. Only some few antibiotics (for example, tigecycline, colistin and carbapenems) are available today for the treatment of illnesses caused by ESBL-producing bacteria, despite the fact that their efficiency and risk are poorly understood (Dejenie et al., 2019). Antimicrobial resistance mechanisms must be understood in order to logically treat gram-negative bacterial infections, especially because antimicrobial resistance genes are frequently mobile and can be transferred between bacterial species and strains. Antibiotic-resistant *Enterobacteriaceae* are common bacteria that cause infections related to the hospital and community. Understanding the epidemiology and disease severity of ESBL-producing *Enterobacteriaceae*, as well as implementing control methods towards infections gotten from the hospital in order to avoid subsequent incidence and spread of the bacteria (Dejenie et al., 2019).

**2.5. Extended-Spectrum Beta-Lactamases**

Antibiotic-resistant bacteria are rapidly developing new defense mechanisms to combat antibiotics effects. Some *Enterobacteriaceae*, for example, can produce extended-spectrum beta-lactamases. Some consistently used antibiotics, such as penicillin and cephalosporins, are inactivated by ESBL enzymes, rendering them ineffective for treating infections.

As a result of this resistance, there are fewer antibiotic alternatives for treating ESBL-producing *Enterobacteriaceae* infections. Diseases caused by ESBL-producing microorganisms, such as urinary tract infections, can necessitate more sophisticated therapies. It is important to note that ESBLs are often encoded by genes located on large plasmids, which also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulfonamides, and tetracyclines (Deepti & Deepthi, 2010).

**2.5.1. *Methods for ESBL Detection***

Two critical methods can be employed in ESBL testing. Firstly, a screening test that uses a cephalosporin indicator to look for resistance or decreased sensitivity, finding samples that are likely to contain ESBLs. Secondly, distinguishing isolates with ESBLs by searching for interactions between clavulanate and an oxyimino cephalosporin (Deepti & Deepthi, 2010).

**Screening Tests for ESBL-Production.**

***Disk-Diffusion Method*.** By identifying certain zone diameters that are associated with a high possibility of ESBL formation, laboratories adopting the method of disk-diffusion for antibiotic susceptibility testing can also screen for ESBL synthesis. The disks contain ceftazidime, cefpodoxime, cefotaxime, ceftriaxone or aztreonam (Deepti & Deepthi, 2010). Because ESBLs' affinity for different substrates varies, screening with more than one of these compounds improves detection sensitivity (CLSI, 2021). Cefotaxime, always sensitive to CTX-M, and ceftazidime, which is consistently a suitable site for TEM and SHV variants, are both acceptable alternatives (Deepti & Deepthi, 2010).

***Dilution Antimicrobial Susceptibility Test.*** The CLSI has proposed dilution approaches for screening for ESBL generation by *Escherichia coli*, *K. pneumoniae*, *P. mirabilis*, and *K*. *oxytoca*. For *Proteus mirabilis*, a screening concentration of 1µg/mL of cefotaxime, ceftazidime, or cefpodoxime can be used, while ceftazidime, cefotaxime, aztreonam and ceftriaxone uses a dosage of 1µg/mL for the others except cefpodoxime which is used at 4µg/mL. If an organism grows at or above the screening dosage of antibiotics, it indicates that the organism is producing ESBL and should be evaluated with a phenotypic confirmation test (CLSI, 2021).

**Confirmatory Tests for ESBL-Production.**

***Double-Disc Synergy Tests (DDST).*** On inoculated Mueller-Hinton agar, test discs of third-generation cephalosporins and amoxicillin-clavulanate are kept 20mm apart, center to center, according to EUCAST (2017). The cephalosporin inhibitory zone's edge, which extends towards the amoxicillin-clavulanate disc, is marked as a positive for ESBL generation. Studies have indicated that the double-disc diffusion test has sensitivities ranging from 79% to 97% and specificities ranging from 90% to 100%. While the theory behind the double-disc diffusion test is simple, the test's interpretation is very subjective (Deepti & Deepthi, 2010).

***Combined Disc Tests (CDT).*** ESBL confirmation tests based on the combination disc method have been developed by a number of firms. The inhibition zone around a disc of cephalosporin (cefotaxime and ceftazidime) and a disc of the same cephalosporin plus clavulanate is measured using this method. A difference of ≥5mm between the two diameters indicates ESBL production (CLSI, 2021).

 **Commercially Available Methods for ESBL Detection.**

***Vitek ESBL Test.*** The VITEK 2 ESBL test (bioMe'rieux) is an automated antibiotic susceptibility testing method used for the rapid detection of ESBL production (Putra et al., 2020). It is based on a simultaneous evaluation of the inhibitory effects of cefepime (at 1 g/mL), cefotaxime, and ceftazidime, either alone (at 0.5 g/mL) or in combination with clavulanic acid (4 g/mL) (Deepti & Deepthi, 2010). After incubation, cards are placed in the VITEK 2 machine, and turbidity is measured at regular intervals for each antibiotic tested. If a specific reduction in growth in clavulanic acid-containing wells compared to those without clavulanic acid is found, an isolate is said to be ESBL positive (Rahman et al., 2014). Based on the phenotype of susceptibility patterns with various beta-lactam antibiotics, computer algorithms in the VITEK system have been used to categorize beta lactamases present in gram negative clinical isolates. Susceptibility tests using this method are expressed as minimum inhibitory concentration (MIC) values and interpreted as susceptible, intermediate, or resistant with reference to a CLSI (Shah et al., 2016).

***E-Test.*** The E-test strip is a thin, plastic strip with 60mm by 5mm dimensions and has two short gradients in opposite direction but on the same strip. The strip is two sided, which contains a gradient of one of the oxyimino cephalosporins on one end and a gradient of cephalosporin combined with clavulanic acid on the other end (Nitin et al., 2014). After the incubation, the MIC value is read where the edge of the inhibition intersects with the side of the strip.

 ***Becton Dickinson (BD) Phoenix Automated Microbiology System.*** The BD Phoenix system, developed by BD Biosciences is a simple device for the identification of bacteria and sensitivity testing (Karen et al., 2020). The Phoenix ESBL test detects the development of ESBLs by measuring the proliferation reaction to ceftriaxone, cefpodoxime, cefotaxime and ceftazidime, with or without clavulanic acid. Within six hours, the results are usually ready. ESBL production was discovered in more than 90% of genotypically validated ESBL-producing strains using the BD Phoenix ESBL automated detection system. The technique correctly detected ESBL manufacturing by *Proteus, Citrobacter*, and *Enterobacter* spp. in addition to *Klebsiella* and *Escherichia coli* (Deepti & Deepthi, 2010).

**2.6. Multidrug Resistance**

MDR is defined as the antimicrobial resistance shown by a microorganism to at least one antimicrobial drug in three or more antimicrobial categories (Mun & Aimi, 2019). The phrases extensively drug-resistant (XDR) and pan-drug-resistant (PDR) have been proposed to describe different levels of MDR. Extensively drug-resistant (XDR) bacteria are resistant to at least one antimicrobial agent in all but two antimicrobial groups (i.e., bacterial isolates are susceptible to only one or two antimicrobial categories). Nonsusceptibility to all agents in all antimicrobial groups is classified as pan-drug-resistant (Silpi et al., 2016).

**2.6.1. *Mechanism of Multidrug Resistance***

Despite the introduction of various antibiotics, there is continuous development of resistance among pathogenic microorganisms, particularly in patients who have been exposed to drugs for a long time (Jyoti et al., 2014). Antimicrobial medications act on micro-organisms by generally blocking the metabolic pathway such as nucleotide synthesis, which in turn inhibits DNA/RNA synthesis, further protein synthesis, the disruption of the cell membrane, or by competing with the foundation of any other enzyme participating in the synthesis of cell wall (Betemaryam, 2020; Jyoti et al., 2014). Organisms have developed a variety of strategies to overcome the efficacy of medications, allowing them to survive drug exposure (Jyoti et al., 2014).

Firstly, cell wall of bacteria is essential for their existence. As previously stated, antibiotics impede cell wall formation in bacteria by attaching to the peptidoglycan layer, preventing the growth and division of cells. Certain mutations on the chromosomes (Betemaryam, 2020) or the interchange of extrachromosomal DNA elements (horizontal gene transfer) in bacteria (WHO, 2014) might cause changes in the targets of antibiotics. By reducing susceptibility to inhibition, alterations in the genes that encode for the target (Betemaryam, 2020; Jyoti et al., 2014), generate molecular changes and maintain cellular function (Dzidic et al., 2008).

Chemical transformation of antimicrobials, as well as deactivation or enzymatic degradation of antimicrobials by ester or amide bonds hydrolyzation (such as beta-lactam resistance due to beta-lactamases, etc.) have become more widely recognized as MDR causes. Resistant strains of clinical isolates of diverse bacteria have developed the ability to change or diminish antimicrobial drugs so that they do not interact with their targets. (Betemaryam, 2020; Jyoti et al., 2014).

One of the most common types of MDR is MDR facilitated by drug efflux pumps. Overexpression of genes that code for ATP-binding cassette (ABC) transporter membrane proteins (e.g., P-glycoprotein (Pgp)), commonly known as multidrug efflux pumps, which are responsible for the outflow or expulsion of drugs from cells (Betemaryam, 2020; Jyoti et al., 2014), usually results in MDR and allows cellular functions to continue without interruption. P-glycoprotein overexpression in multidrug resistance proteins (MRP) alters fluidity and permeability of the cell membrane, resulting in antimicrobial efflux using ATP as energy source and a decrease in intracellular concentration of the cell (Bansal et al., 2006).

**CHAPTER III**

 **Materials and Methods**

**3.1. Study Design and Participants**

In this study, samples which were collected from the previous study from two groups of participants between March 2019 and July 2019 were used for the microbial analysis. Patients that had been admitted to the Near East University Hospital were the first group (n=49) while the second group (n=15) was the controls who had not been admitted in the last six months before the study. Being over the age of 18 and having lived in Northern Cyprus for at least a year were both requirements. The Near East University Ethics Review Board granted this study ethics approval (No: YDU/2019/65-717). All of the participants signed a written informed consent form.

**3.2. Samples, Participants’ Data and the Bacterial Isolates**

The bacterial strains in this study (n=64) were isolated from the stool samples previously collected from the volunteers (one sample per individual). During the process of collecting the fecal specimen, a survey was taken to examine the factors associated with the carriage of antibiotic resistant bacteria which includes demographic/socioeconomic factors (age, gender, education, marital status), epidemiological factors (hospital stay, GIS, histories of antibiotic use, diarrhea UTI, and travel including other countries) and hospital related factors (ICU stay, surgery, urinary catheter, antibiotic use). The ESBL production was previously determined by the phenotypic confirmation test using combined disc method. The *Enterobacteriaceae* isolates which were also confirmed ESBL-producers were stored at -20ºC in the stock media from the previous study.

**3.3. Purification and Inoculation of Bacterial Isolates**

The ESBL-positive isolates were purified by inoculation into the Eosin Methylene Blue (EMB) medium at 37ºC for 24hours. A standard 0.5 McFarland suspension in sterile 0.9% NaCl was then prepared from the bacterial colonies obtained in the culture plates. Subsequently, each bacterial suspension was transferred to Mueller-Hinton medium by using a sterile swab.

**3.4. Determination of Multidrug Resistance**

 In this study, in order to determine the MDR of the bacterial isolates, antibiotics from different classes were tested. The activities of ampicillin (AMP) (10 µg), amoxicillin-clavulanic acid (AMC) (30 µg), piperacillin-tazobactam (TZP) (110 µg), cefotaxime (CTX) (30 µg), ceftazidime (CAZ) (30 µg), cefepime (FEP) (30 µg), ciprofloxacin (CIP) (5 µg), gentamicin (GEN) (10 µg), trimethoprim-sulfamethoxazole (SXT) (25 µg) and tigecycline (TGC) (15 µg) antibiotics against *Enterobacteriaceae* species were evaluated by using the disc diffusion test. Following the transfer of the bacterial isolates on the Mueller-Hinton agar, the antibiotic-containing paper discs were then placed on the agar and the plate incubated at 37ºC for 24hours. The inhibition zones of ≤13 for AM and AMC; ≤17 for TPT and CAZ; ≤15 for CIP and TGC; ≤22 for CTX; ≤18 for FEP; ≤12 for CN; and ≤10 for SXT were all interpreted as resistant. The diameters of the inhibition zones formed around the antibiotic discs were measured in millimeters (mm) and evaluated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021) except tigecycline which was evaluated according to the Food and Drug Administration guidelines (NDA 21-821/S-016).

**3.5. Statistical Analysis**

Descriptive statistics were obtained for the variables in the survey. For categorical data, information on frequency and percentage was provided, while for continuous variables, arithmetic mean, standard deviation, median, minimum and maximum were calculated. Depending on the sample sizes, the Fisher's exact test or Pearson Chi-square was used to examine the connections between categorical data. The IBM SPSS statistics software for Macintosh (Demo version 22.0; Armonk, NY: IBM Corp.) was used to do all of the statistical calculations. Significance level was set at 0.05.

**CHAPTER IV**

 **Results**

**4.1. The Study Population**

Sixty-four volunteers were included in this study which enrolled 49 patients from the Near East University and 15 participants from the public. Of the total 64 participants, 35 (54.6%) were male while 29 (45.3%) were female. The mean and median age were 51.98 ± 20.66 and 51.50 (19.00-93.00), respectively. From the study population, the age groups in this study were arranged in two groups; age 19-30 was 15 (23.4%), while age 31 and above were 49 (76.6%). Based on education, the high school and below group were 41 (64.1%) while those with university degree and above were 23 (35.9%). Twenty-one (32.8%) participants were single while 43 (67.2%) were married. The participants with low and medium income were 55 (85.9%) while the participants with high income were 9 (14.1%) in number.

At the time of sample collection, 49 (76.6%) of the 64 participants were admitted in the hospital and 42 (65.6%) while 16 (25%) had a case or history of prolonged diarrhea in the last six months. Thirty-seven (57.8%) used antibiotics in the last six months, ten participants (15.6%) suffered from UTI and 34 (53.1%) travelled within the last six months; among these individuals 26 (40.6%) and eight (12.5%) travelled to Asia or Africa and Turkey or Europe, respectively.

**4.2*.* Species Distribution and Antibiotic Susceptibility Results of ESBL-E Isolates**

Among the ESBL-E (n=64) isolates, *E. coli* (n=61/64; 95.3%) was the predominant species which was followed by *K. pneumoniae* (n=1; 1.6%), *Enterobacter* *cloacae* (n=1; 1.6%) and *Citrobacter freundii* (n=1; 1.6%).

Among 64 isolates, none (0.0%) was susceptible to all the antibiotics of different classes tested. Sixty (93.8%) and 56 (87.5%) isolates showed high resistant rates against ceftazidime and cefotaxime respectively while 59 (92.2%) isolates showed a high susceptibility to piperacillin tazobactam as seen in table 1.

Table 1.

*Susceptibility Rates of Antibiotics among the Study Group (n=64).*

|  |  |  |  |
| --- | --- | --- | --- |
| Antibiotics | Susceptiblen (%) | Intermediaten (%) | Resistantn (%) |
| Ampicillin | 0 (0.0) | 0 (0.0) | 64 (100.0) |
| Amoxicillin-clavulanic acid | 30 (46.9) | 11 (17.2) | 23 (35.9) |
| Piperacillin-tazobactam | 59 (92.2) | 4 (6.3) | 1 (1.6) |
| Cefotaxime | 4 (6.3) | 4 (6.3) | 56 (87.5) |
| Ceftazidime | 1 (1.6) | 3 (4.7) | 60 (93.8) |
| Cefepime | 8 (12.5) | 20 (31.3) | 36 (56.3) |
| Ciprofloxacin | 20 (31.3) | 18 (28.1) | 26 (40.6) |
| Gentamicin | 48 (75.0) | 0 (0.0) | 16 (25.0) |
| Trimethoprim-sulfamethoxazole | 28 (43.8) | 8 (12.5) | 28 (43.8) |
| Tigecycline | 22 (34.4) | 40 (62.5) | 2 (3.1) |

**4.3. Prevalence of MDR Bacteria**

In this study, a total of 46 (71.9%) isolates were found to be MDR. The rates of MDR were 69.4% (n=34/49) in the patient group and 80.0% (n=12/15) in the control group. The difference in rates of MDR were not statistically significant (*p*=0.525).

Table 2.

*Distribution of Multidrug Resistant Bacteria among Patients (n=49) and Control (n=15) Groups\**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Participants  | MDR positive | MDR negative | Total  | *p* value |
| Patients  | 34 (69.4) | 15 (30.6) | 49 (100) | 0.525 |
| Controls  | 12 (80.0) | 3 (20.0) | 15 (100) |
| Total | 46 (71.9) | 18 (28.1) | 64 (100) |

\*ESBL-producing *Enterobacteriaceae* species were isolated from all participants (n=64).

**4.4. Risk Factors Associated with Multidrug Resistance**

**4.4.1. *Association of Multidrug Resistance with Demographic and Socioeconomic Factors***

The rate of MDR isolates was significantly higher in the age group of 31 and above (n=39/49; 79.6) (*p*=0.021). In addition, gender did not affect the MDR rate significantly (*p*=0.637). The rate of MDR was not significantly influenced by educational level or marital status (*p*=0.375 and *p*=0.215) respectively, and the statistical analysis found no statistical correlation between socioeconomic status and MDR (*p*=0.052) as seen in table 3.

**4.4.2. *Association of Multidrug Resistance with Epidemiological Factors***

According to the statistical analysis, none of the epidemiological factors significantly affected the MDR rate in the study group. The *p*-value was greater than 0.05 as indicated in table 4.

**4.4.3*. Association of Multidrug Resistance with Hospital-Related Factors***

Among the patient group, 30 individuals were hospitalized for at least 72 hours at the time of sample collection. Hospital-related information of two patients could not be collected. Of the 28 (57.1%) patients, 1 (3.6%) stayed at the intensive care unit (ICU) (*p=*1.000), 9 (32.1%) underwent surgery (*p*=0.165) and 3 (10.7%) patients had urinary catheter (*p*=1.000). Also, 21 (75%) patients used antibiotics during the hospitalization (*p*=0.639). According to the statistical analysis, there was no relationship found between multidrug resistance and hospital-related factors (*p*>0.05) as shown in table 5.

Table 3.

*Association of Multidrug Resistance with Demographic and Socioeconomic Factors in the Study Group (n=64)*

|  |  |  |
| --- | --- | --- |
| Risk factors | MDR positivityn/N (%) | *p* value |
| Age 19-30 31 and above Total  | 7/15 (46.7)39/49 (79.6)46/64 (71.9) | 0.021 |
| Gender Male Female Total | 26/35 (74.3)20/29 (69.0)46/64 (71.9) | 0.637 |
| Education University and higher Lower than university Total | 31/41 (75.6)15/23 (65.2)46/64 (71.9) | 0.375 |
| Marital status Single Married Total | 13/21 (61.9)33/43 (76.7)46/64 (71.9) | 0.215 |
| Socioeconomic status Low and middle  High  Total  | 37/55 (67.3)9/9 (100.0)46 (71.9) | 0.052 |

Table 4.

*Association of Multidrug Resistance with Epidemiological Factors in the Study Group (n=64).*

|  |  |  |
| --- | --- | --- |
|  Risk factors | MDR positiven/N (%) | *p* value |
| Hospital Stay\* Yes  No  Total  | 34/49 (69.4)12/15 (80.0)46/64 (71.9) | 0.525 |
| GIS\* Yes  No  Total | 16/22 (72.7)30/42 (71.4)46/64 (71.9) | 0.913 |
| History of antibiotic use\*\* Yes  No  Total  | 29/37 (78.4)17/27 (63.0)46/64 (71.9) | 0.176 |
| History of diarrhea\*\* Yes  No  Total  | 11/16 (68.8)35/48 (72.9)46/64 (71.9) | 0.756 |
| History of UTI\*\* Yes  No  Total  | 9/10 (90.0)37/54 (68.5)46/64 (71.9) | 0.259 |
| Travel history\*\* Yes No Total  | 22/34 (64.7)24/30 (80.0)46/64 (71.9) | 0.174 |
| Travel to Turkey or Europe\*\* Yes  No  Total  | 17/26 (65.4)5/8 (62.5)22/34 (64.7) | 1.000 |
| Travel to Asia or Africa\*\* Yes  No  Total  | 5/8 (62.5)17/26 (65.4)22/34 (64.7) | 1.000 |

\* Indicates “at the time of sample collection”.

**\*\***Indicates the last six months before the study.

Table 5.

*Association of Multidrug Resistance with Hospital-Related Factors in the Study Group (n=30).*

|  |  |  |
| --- | --- | --- |
| Risk factors | MDR positive | *p* value |
| Stay at ICU\* Yes  No  Total  | 1/1 (100.0)20/27 (74.1)21/28 (75.0) | 1.000 |
| Surgery\* Yes  No  Total  | 5/9 (55.6)16/19 (84.2)21/28 (75.0) | 0.165 |
| Urinary catheter\* Yes  No  Total  | 2/3 (66.7)19/25 (76.0)21/28 (75.0) | 1.000 |
| Antibiotic use\* Yes  No  Total  | 15/21 (71.4)6/7 (85.7)21/28 (75.0) | 0.639 |

**\***Indicates the current hospitalization.

**CHAPTER V**

 **Discussion**

*Enterobacteraiaceae* family, particularly *K. pneumoniae* and *E. coli*, are the primary producers of ESBLs. Bacteria that carry ESBLs can also acquire and develop resistance to other antimicrobial classes, such as quinolones, tetracyclines, trimethoprim and aminoglycosides (Kamlesh et al., 2015), limiting therapy options and posing a therapeutic challenge (Maina et al., 2013). This study aimed to investigate the rate of MDR among ESBL-producing *Enterobacteriaceae* isolates in both hospital and public settings in Northern Cyprus. Also, the risk factors of MDR in ESBL-E were examined.

 In this study, 46 (71.9%) of 64 participants were MDR (Table 2). The greater frequency of MDR reduces the therapeutic options for *Enterobacteriaceae* infections acquired in hospitals. The high prevalence of MDR in isolates of *Enterobacteriaceae* has also been reported by (Malik and Elhag, 2019; Zavala-Cerna et al., 2020; Onduru et al., 2021). The use of an antibiotic from a different class, study time, specimen type and study population could explain the extent of diversity in MDR isolates.

 In this study, *E. coli* was found to be the predominant *Enterobacteriaceae* species among ESBL-positive isolates with a rate of 95.3% which is similar to report made by Ruh et al. (2019) that revealed the prevalence of E. coli species as 94.4%. Also, this present study shows that *K. pneumonia* (1.6%), *C. freundii* (1.6%) and *E. cloacae* (1.6%) were seen at extremely low rates among ESBL-E. Studies by Ruh et al, (2019) also shows *Klebsiella* (2.8%) and *Enterobacter cloacae* (0.9%) at low rates.

 In this present study, a total of 64 ESBL-E isolates were analyzed to determine the rate of MDR. Also, possible factors that are related with the occurrence of MDR were evaluated. The highest resistance rate of all ESBL-E was found to be ampicillin (100.0%), followed by ceftazidime (93.8%), and cefotaxime (87.5%). The high level of resistance against ampicillin, ceftazidime and cefotaxime can be compared with previous studies showing a high rate of resistance for amoxicillin (79%) and ceftazidime (79%) as reported by Gangoue-Pieboji et al. (2006). It was also found in the present study that the highest antimicrobial susceptibility rate was to piperacillin-tazobactam (92.2%), which is among the beta-lactam/beta-lactamase inhibitor cominations and gentamicin (75.0%) when compared with ampicillin (0.0%), amoxicillin-clavulonic acid (46.9%), cefotaxime (6.3%), ceftaxidime (1.6%), cefepime (12.5%), ciprofloxacin (31.3%), trimethoprim-sulfamethoxazole (43.8%) and tigecycline (34.4%). Majority of the isolates (71.9%) were found to be MDR. Thakur et al. (2013) also reported 64.0% MDR *Enterobacteriaceae* in their study.

In our study, demographic and socioeconomic data was analyzed. It was found that the age group of 31 and above had a statistically significant number of MDR isolates than the 19-30 age group (*p*=0.021) (Table 3). In another study, the majority of isolates were from patients aged 40 to 70 years old, 50 to 60 years old and 51 to 70 years old (Kamlesh et al., 2015). This significance can be due to weakened immune system, consistent use of antibiotics or hospitalization among others.

MDR in patients who were in the university or had a higher degree was detected in higher rates than in patients who did not have a university degree, but the result was not found to be significant (Table 3). However, previous studies by Nomamiukor et al. (2015) and See et al. (2017) shows that people from low educational background have a higher chance of self-medication thereby promoting the emergence of MDR.

The data regarding the socioeconomic factors in this study showed no significant correlation with isolation of MDR bacteria (Table 3). Kasim et al. (2020) found suggestive evidence that poverty and material hardship are important factors for MDR. Also, there was no statistically significant correlation between hospital stay and MDR in this study, but other studies prove otherwise. According to Lautenbach et al (2001); there was a 1.7% increase of MDR in patients who had a longer stay at the hospital which was statistically significant.

The presence of GIS was evaluated but it did not have any statistical significance. Also, previous studies done by Katalin et al. (2017) reported the rate of GIS at 3.7% with no significant correlation to MDR.

According to WHO, the use of antibiotics is the first-hand cause of MDR worldwide (WHO, 2014), but in this study, no significant correlation was found between antibiotic use and MDR (Table 4).

Statistical analysis showed that the history of diarrhea, history of UTIs, travel to Turkey/Europe and Asia/Africa were not significant factors for MDR among *Enterobacteriaceae* isolates. Although previous studies reported by Kantele et al, (2015) at 80%; Ruppe et al, (2015) at 72.4% and Arcilla et al (2017) at 95% showed a statistical correlation between MDR and travel to Asia, history of UTIs and history of diarrhea respectively.

Also, in this study, the association of hospital-related factors with MDR in *Enterobacteriaceae* isolates were analyzed and there was no significant relationship between the stay at ICU, surgery, urinary catheter and antibiotic use with MDR (Table 5). However, studies by Grzegorz et al. (2020) shows a relationship between MDR and hospital-related factors.

 **CHAPTER VI**

 **Conclusion**

 In this study, 46 (71.9%) of 64 participants were MDR positive. The rates of MDR were 69.4% (n=34/49) in the patient group and 80.0% (n=12/15) in the control group. The difference in rates of MDR were not statistically significant (*p*>0.05). Among the ESBL-producing *Enterobacteriaceae*, *E. coli* (95.3%) was the predominant species, followed by *K*. *pneumoniae* (1.6%), *Enterobacter cloacae* (1.6%) and *Citrobacter freundii* (1.6%). The risk factors associated with MDR were evaluated and the results showed that only age (*p*=0.021) was the significant factor, while other demographic and socioeconomic factors were not statistically related with MDR (*p*>0.05). Also, the epidemiological and hospital-related factors had no statistical correlation with MDR (*p*>0.05).

 The results of this study indicate that the rate of MDR among ESBL-E isolates is at high levels. This suggests that antibiotic resistance should be carefully monitored in the bacterial isolates in Northern Cyprus.

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