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MEDICAL MICROBIOLOGY DEPARTMENT
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INVESTIGATION OF CARBAPENEMASE PRODUCTION AMONG CARBAPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATES

MASTER'S THESIS

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ÖZET

KARBAPENEM DİRENÇLİ *PSEUDOMONAS AERUGINOSA* IZOLATLARINDA KARBAPENEMAZ ÜRETİMİNİN ARAŞTIRILMASI

SHIRWAN H. DARWEESH

Yüksek Lisans, Tokat Gaziosmanpaşa Üniversitesi Sağlık ve Fen Bilimleri Enstitüsü Tıbbi Mikrobiyoloji Anabilim Dalı

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Giriş: *P. aeruginosa*, özellikle bağışıklığı baskılanmış hastalarda hayatı tehdit eden hastane enfeksiyonlarına neden olan fırsatçı bir patojendir. Karbapenemler de dahil olmak üzere mevcut antibiyotiklerin çoğuna oldukça dirençlidir. Karbapenemaz enziminin üretimi, karbapenem direncinin ana mekanizmasıdır ve bu enzimler yüksek oranda aktarılabilir olduklarından ve terapötik seçenekleri kısıtladıklarından dünya çapında ciddi bir sağlık sorunu haline gelmiştir. Bu çalışmada karbapenem dirençli *P. aeruginosa* izolatlarında karbapenemaz üretiminin fenotipik yöntemlerle araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Tokat-Gaziosmanpaşa Üniversitesi Araştırma ve Uygulama Hastanesi Mikrobiyoloji Laboratuvarına 2016-2019 yılları arasında çeşitli kliniklerden gönderilen, çeşitli örneklerden elde edilen *P. aeruginosa* izolatları retrospektif olarak değerlendirilmiştir. Toplam 172 izolatın 51'i (%29.7) karbapenem dirençli tespit edilmiş ve çalışmaya dahil edilmiştir. İzolatların identifikasyonları ve antibiyotik duyarlılık testleri Vitek 2 (Biomerieux, France) otomatize sistem ile çalışılmıştır. Karbapenem duyarlılıkları ayrıca disk difüzyon yöntemi ile de belirlenmiştir. İzolatlarda karbapenemaz üretimi basitleştirilmiş ve modifiye edilmiş karbapenem inaktivasyon yöntemleri ile araştırılmıştır.

Bulgular: Toplam 51 karbapenem dirençli izolatın 38'i (%74,5) hem imipenem hem de meropeneme dirençli saptanmıştır. Sekiz (%15.7) izolat sadece imipeneme, beş (%9.8) izolat ise meropeneme dirençli bulunmuştur. Tüm izolatların 31'inde (%60.8) modifiye

karbapenem inaktivasyon yöntemi ile karbapenemaz üretimi tespit edilmiştir. Ancak basitleştirilmiş karbapenem inaktivasyon yönteminde izolatlarda karbapenemaz üretimi saptanmamıştır.

Sonuç: Karbapenem dirençli *P. aeruginosa* izolatları arasında fenotipik ve genotipik yaklaşımlar kullanılarak karbapenemaz enzimlerinin hızlı teşhisi, karbapenem dirençli izolatların neden olduğu enfeksiyonun bulaşmasını kontrol altına almak ve bunlara bağlı morbidite ve mortaliteyi kontrol etmek için önemlidir. Bu çalışmada karbapenemaz üretiminin tespitinde modifiye karbapenem inaktivasyon testi kolay ve hızlı uygulanabilmesi yönüyle laboratuvarda tercih edilebilecek bir yöntem olarak görülmüştür.

Anahtar Kelimeler: *P. aeruginosa*; Karbapenemaz; Karbapenem; Antibiyotik direnci; Enfeksiyon.

ABSTRACT

INVESTIGATION OF CARBAPENEMASE PRODUCTION AMONG CARBAPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATES

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Introduction: *P. aeruginosa* is an opportunistic pathogen that causes life-threatening nosocomial infections, especially in immunocompromised patients. It is highly resistant to most current antibiotics, including carbapenems. Production of the carbapenemase enzyme is the main mechanism of carbapenem resistance and has become a serious health concern worldwide as these enzymes are highly transferable and limit therapeutic options. In this study, it was aimed to investigate carbapenemase production in carbapenem-resistant *P. aeruginosa* isolates by phenotypic methods.

Material and Methods: A total of 172 *P. aeruginosa isolates* were obtained from different samples sent from various clinics to Tokat-Gaziosmanpaşa University Research and Application Hospital Microbiology Laboratory between 2016-2019 and were evaluated retrospectively. Of the 172 isolates, 51 (29.7%) were found to be carbapenem-resistant and included in the study. Identification and antibiotic susceptibility tests of the isolates were performed with the Vitek 2 (Biomerieux, France) automated system. Carbapenem sensitivities were also determined by the disc diffusion method. Carbapenemase production in isolates was investigated by simplified and modified carbapenem inactivation methods.

Results: Of the 51 carbapenem-resistant isolates, 38 (74.5%) were found to be resistant to both imipenem and meropenem. Eight (15.7%) isolates were found resistant to imipenem only, and five (9.8%) isolates were found to be resistant to meropenem.

Carbapenemase production was detected in 31 (60.8%) of all isolates by the modified carbapenem inactivation method. However, no carbapenemase production was detected in the isolates in the simplified carbapenem inactivation method.

Conclusion: Rapid identification of carbapenemase enzymes among carbapenem-resistant *P. aeruginosa* isolates using phenotypic and genotypic approaches is important to control the transmission of infection caused by carbapenem-resistant isolates and to control the morbidity and mortality associated with them. In this study, the modified carbapenem inactivation test was seen as a method that can be preferred in the laboratory in terms of its easy and fast application in the detection of carbapenemase production.

Keywords: *P. aeruginosa*; Carbapenemase; Carbapenem; Antibiotic resistance; Infection.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAC Aminoglycoside acetyltransferase
AIDS Acquired immune deficiency syndrome
ATP Adenosine triphosphate
CF Cystic Fibrosis
CFTR Cystic fibrosis transmembrane conductance regulator
CRPA Carbapenem-resistant *P. aeruginosa*
DM Diabetes Mellitus
DNA Deoxyribonucleic acid
E. coli *Escherichia coli*
EPS Extracellular polymeric substances
GAP Guanosine triphosphatase activating protein
GES Guiana extended-spectrum carbapenemase
GIM Germany imipenemase
GIT Gastrointestinal tract
GNS Green nail syndrome
IMI Imipenemase
KPC *Klebsiella pneumoniae* carbapenemase
LecA Lectin A
LecB Lectin B
LPS Lipopolysaccharide
MBL Metallo beta-lactamase
Mbp Million base pairs
MDR Multidrug-resistant
PBP Penicillin-binding protein
Psl Polysaccharide synthesis locus
RNA Ribonucleic acid
SPM Sao Paulo metallo-β-lactamases

TLR5 Toll-like receptor 5

UTI Urinary tract infections

WHO World health organization

γ-INF Gamma interferon

1. INTRODUCTION

1.1 *Pseudomonas aeruginosa*: background

Pseudomonas is a genus of gammaproteobacteria belonging to the Pseudomonadaceae family (Klockgether et al., 2017), which contains around 120 species, including *Pseudomonas aeruginosa* (*P. aeruginosa*), the most prevalent species implicated in nosocomial infection. It is also the most investigated and studied member of this genus (Streeter and Katouli, 2016).

In 1850, a French surgeon, Sedillot, observed a "condition" that caused a bluish-green coloration on surgical dressings that were characterized by a "strange" smell related to infections. Fordos described this coloring for the first time in 1969. When he recovered pyocyanine, a bluish-green pigment (El zowalaty et al., 2015; Williams and Cameron, 1894). In 1882, Carle Gessard identified the bacteria from the pus of injuries that occurred in army forces having dressings had such a similar appearance using Pasteur's cultures. After isolating bacteria that were responsible for the pigmentation, he designated them as Bacillus pyocyaneus (Villavicencio, 1998). In 1894, German botanist Walter Migula used the word "Pseudomonas" to describe a genus of bacteria possessing "polar organs of motility." Although spore formation is possible in certain species, he renamed *Pseudomonas pyocyanea* to *P. aeruginosa*. Afterward, these bacterial species were continuously studied (Palleroni, 2010; Silva et al., 2020).

P. aeruginosa is widespread bacteria that survives in a variety of settings, including water, plants, insects, as well as animals (Chevalier et al., 2018.; El zowalaty et al., 2015). Their capability to colonize these different ecological niches is highly associated with the complexity of their large genomic, which has about 6.3 million base pairs (Mbp). Their genome helps them adapt to harsh, diverse conditions and gives them the ability for utilizing several chemical molecules as organic substrates. It also depends on the capability of this species of bacteria to generate a variety of virulence factors (Igbinosa and Igbinosa., 2015; El zowalaty et al., 2015; Silby et al., 2015).

It is hardly ever found in the human microbiota although it may invade the gastrointestinal system (GIT), Respiratory tract, Genitourinary tract, etc., of patients in hospital settings, especially those who have previously received antibiotic treatments (this will be further clarified in the later sections) (Carroll et al., 2015).

1.2 Microbiology of *P. aeruginosa*

P. aeruginosa is a gram-negative, rod-shaped, strictly aerobic bacterium that may be found singly, in pairings or as small strands. It measures about one to five micrometers long and a half to one micrometer wide (Al-daraghi et al., 2020). It has a flagellum, which is responsible for bacterial motility. Fimbriae are incapable of causing hemagglutination when present on *P. aeruginosa*, whereas the fimbriae of other gram-negative bacilli can cause hemagglutination. Furthermore, *P. aeruginosa* is a non-spore-forming, non-capsulated bacteria. (Al-daraghi et al., 2020; Silva et al., 2020).

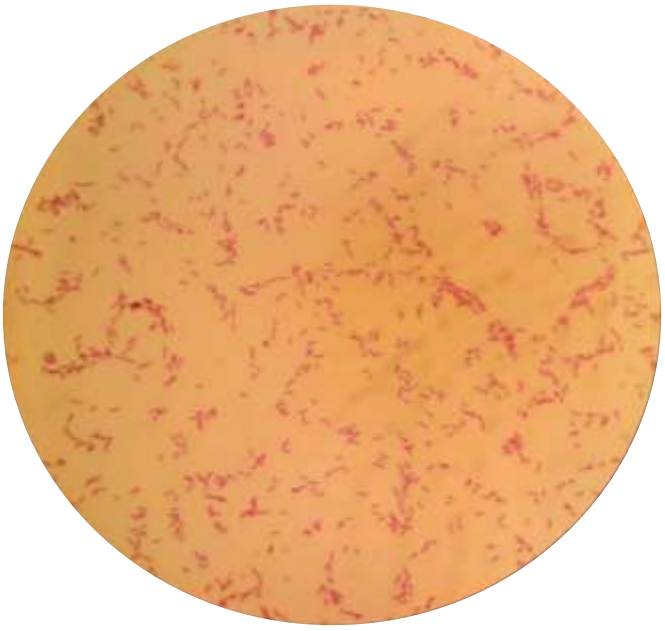

Figure 1.1*: P. aeruginosa* gram-negative rod-shaped bacteria.

Despite the fact that *P. aeruginosa* needs an aerobic environment for proper growth and metabolism. It is able to thrive within oxygen-limited situations if nitrite or nitrates is available as an alternative terminal electron acceptor (Ryan, 2018; Carlson and Ingraham 1983; Davies and Boddy, 1989; Zumft, 1997). In cases where oxygen, nitrate, and nitrite are not available, two types of fermentation processes can promote the growth and survival of *P. aeruginosa*. In this process, arginine converts to ornithine for Adenosine triphosphate (ATP) production. For arginine fermentation, three enzymes are needed: arginase deaminase, carbamate kinase, and catabolic ornithine carbamoyltransferase (Vander Wauven et al., 1984; Lüthi et al., 1990). The second mechanism is pyruvate fermentation, which uses pyruvate as a substrate and converts it to acetate, lactate, and a small amount of succinate. This fermentation just helps the *P. aeruginosa* survive, i.e., it is not useful for growth and proliferation (Schobert and Jahn, 2010; Eschbach et al., 2004).

1.2.1 Cultural characteristic of *P. aeruginosa*

As already mentioned, *P. aeruginosa* survives within a variety of conditions, even under very simple and minimum nutritional requirements. It can grow in a variety of temperatures, but the temperature at which it thrives best is 37°c. At 4°C, growth rates are slower. The ability to grow as high as 42℃ is a helpful feature for distinguishing *P. aeruginosa* from other *Pseudomonas species* that produce fluorescent pigments (Al-daraghi et al., 2020). Hence, it can be grown on almost all types of culture media routinely used in microbiology laboratories, including blood agar, MacConkey agar, and nutrient agar. During growth, *P. aeruginosa* frequently produces a sweet, grape-like odor because of an evaporative aromatic compound called aminoacetophenone (Scott-Thomas et al., 2010).

On MacConkey Agar, it appears as pale, non–lactose-fermenting (colorless) colonies that lie flat and occasionally generate a gelatinous or slimy appearance, especially in areas of higher growth combined with pigmentation and have serrated edges (Sapkota, 2021).

On blood agar medium *P. aeruginosa* forms circular colonies (figure 1.3) that are large, flat, and irregularly edged. Some strains particularly those isolated from clinical

settings show beta-hemolysis by producing hemolysis toxin. However, those isolated from healthy people may or may not show hemolysis (Al-daraghi et al., 2020). In cystic fibrosis patients, mucoid colonies can be observed due to the over-production of alginate (will be more explained in section 1.2.4.9 Respiratory Infections).

Figure 1.2: *P. aeruginosa* cultured on Blood

1.2.2 Pigment production on media

P. aeruginosa can produce several water-soluble pigments which are responsible for the culture media coloration that gives them a characteristic appearance and makes the initial identification easier. These pigments are unnecessary for bacterial growth and proliferation but have a role in pathogenicity and are important for bio-controlling (Rani and Azmi, 2019).

P. aeruginosa can produce four distinct pigments, including pyocyanin, which is a bluish-green toxic pigment that spreads through a medium (figure 1.5). It is the most

frequently produced pigment and its presence is a crucial diagnostic characteristic for *P. aeruginosa* because other non-fermentative gram-negative bacilli do not produce this pigment. Antimicrobial, anticancer, antioxidant, antiinflammatory, and immunosuppression characteristics are all found within pyocyanin pigment (M Tille, 2017; Rani and Azmi, 2019) *P. aeruginosa*, like many other fluorescent Pseudomonas species, produces pyoverdin, or fluorescein. This pigment is water-soluble and gives bacteria colonies a yellow-greenish color. They are best shown in a microscope with an ultraviolet light source. Pyoverdin is a significant siderophore that acts as a powerful iron scavenger and transporter (M Tille,2017; Meyer, 2000). Pyoverdin pigment has a role in bacterial pathogenicity and biofilm formation (Peek et al., 2012). When both pyocyanin and pyoverdin combine, give the culture bright green color, which is a beneficial characteristic in the diagnosis process. Moreover, some strains of *P. aeruginosa* can produce a red-colored pigment known as Pyorubin, some other strains secrete a dark brown pigment called Pyomelanin. (Public Health England, 2014)

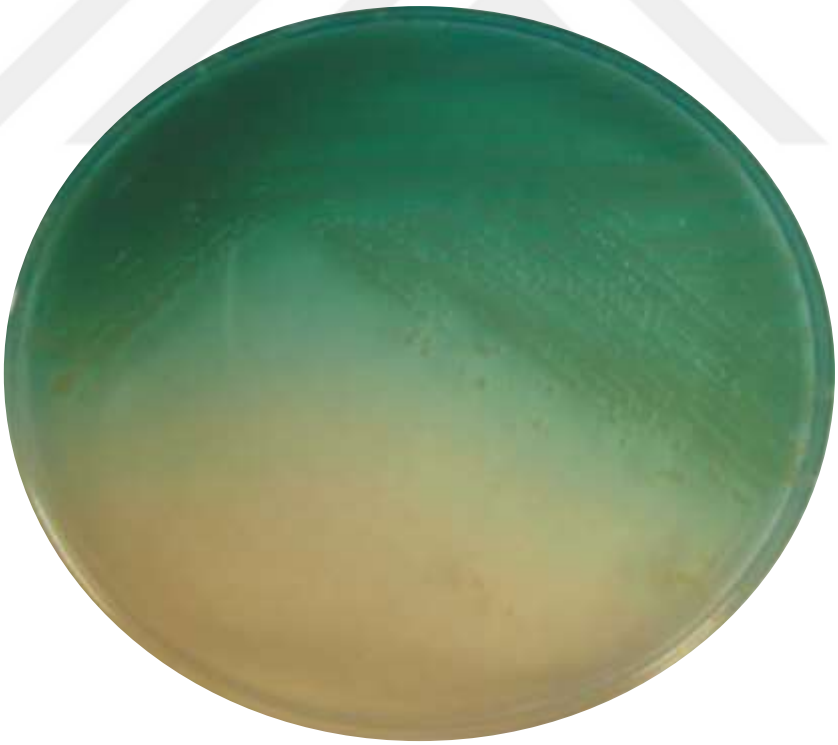

Figure 1.3: *P. aeruginosa* produces blue-green pigment (pyocyanin), which diffuses into the agar.

1.2.3 Biochemical characteristics of *P. aeruginosa*

P. aeruginosa is motile by flagellum (Driscoll et al., 2007). The oxidase test is rapidly positive (Figure 1.6) and it is an important test for their diagnosis. The catalase test is also positive (Figure 1.7) (Al-daraghi et al., 2020). It is a non-fermentative bacterium. Glucose is used oxidatively with oxygen as a terminal electron acceptor. As a result, during the oxidative metabolism of glucose, only acid is produced, which is demonstrated by using special media such as oxidation-fermentation media. As well, xylose and fructose are also used oxidatively. In the nitrate reduction test, nitrates are converted to nitrites and then to gaseous nitrogen. Moreover, arginine dihydrolase is positive. Urea does not undergo hydrolysis. The citrate utilization test is positive. The reactions of methyl red and Voges Proskauer are both negative. The reaction is alkaline on triple sugar iron agar with no changes in growth (Al-daraghi et al., 2020; M Tille, 2017; Mahon et al., 2018).

Figure 1.4: the right-handed stick is positive (Purple) it *is P. aeruginosa*. The left-handed stick is results of negative control (no color changes) *E. coli*.

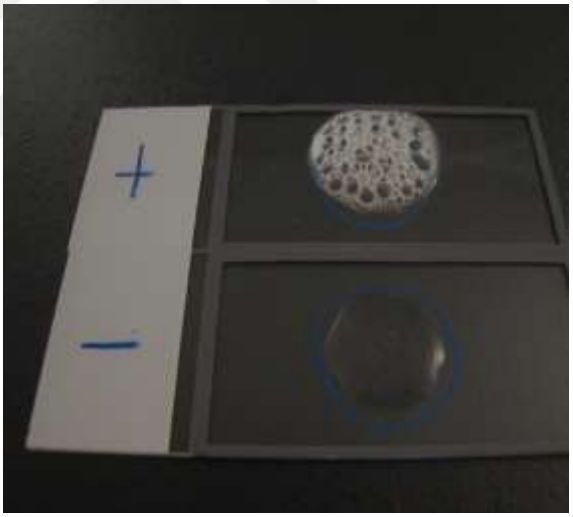

Figure 1.5: catalase test results. *P. aeruginosa* was responsible for the positive reaction (top). The negative reaction was induced by *Strept. Pyogenes* (bottom).

A negative result for a biochemical tests test can be reported only after the test has been performed for a full seven days because it may go through some phenotypic

modifications such as slow growth, alterations in pigmentation, and changes in metabolic activity. (M Tille, 2017).

1.3 Pathogenicity and virulence factors of *P. aeruginosa*

pathogenesis of *P. aeruginosa* is complex and mediated via a several and varied spectrum of virulent determinants (El-mahdy and El-kannishy, 2019; Strateva and Mitov, 2011), which are subdivided into two types cell-associated and extracellular virulence factors (Silva et al., 2020; Strateva and Mitov, 2011).

Virulence factors are regulated by quorum sensing, which is the capability of bacteria to sense information from cells in different populations after they have extended to a significant concentration (Deep et al., 2011). Quorum sensing is made up of intercellular, small, membrane diffusible signaling molecules called autoinducer that is released by bacteria into the environment and has a significant function in pathogenesis (Deep et al., 2011; Veesenmeyer et al., 2009).

Three types of the quorum-sensing system were discovered in *P. aeruginosa.* The rhl and las systems were the first two quorum-sensing systems discovered, then the Pseudomonas Quinolone Signal (PQS), was identified. PQS coordinated by the las system and controls the rhl system. This means it serves as a link between the rhl and las systems (Veesenmeyer et al., 2009). Besides that, quorum-sensing systems are also used by pathogenic bacteria to adapt to metabolic challenges and to thrive in a community (Deep et al., 2011).

1.3.1 Pili

P. aeruginosa has several types of cell surface pili that are principal for bacterial adhesion with cells and other surfaces, as well as mediating motility (Al-Wrafy et al.,2017). Pili are responsible for approximately 90% of *P. aeruginosa* adhesion. The primary component of pili is a helical pilin protein, which promotes adhesion to a wide range of diverse cells by binding to the asialyated glycolipid (asialo-ganglioside M1, aGM1) on host cells (Hahn, 1997).

1.3.2 Flagella

P. aeruginosa has a monopolar flagellum made up of polymerized flagellin and it is encoded and regulated by a complex genetic framework encompassing more than 50 genes (Campodónico et al., 2010). Flagella performs a variety of roles, including motility, chemotaxis, attachment, and can also activate the host inflammatory response through toll-like receptor 5(Al-Wrafy et al., 2017; Campodónico et al., 2010). During the invasion, the bacteria attach to the epithelial cell of the host. TLR5 recognizes flagellin which is the principal constituent of flagella (Garcia et al., 2018). Thus, toll-like receptor 5 is a monitoring mechanism to detect invading *P. aeruginosa* and start an immune response by mediating cytokines secretion of like interleukin-6 and tumor necrosis factor (Sapkota, 2021; Garcia et al., 2018).

1.3.3 Lipopolysaccharide

Lipopolysaccharide (LPS) is a complicated glycolipid that comprises the majority of the cell surface membrane of gram-negative bacteria (Gellatly and Hancock, 2013; king et al., 2009). This molecule acts as a physical barrier between the bacteria and the host's defenses, thus it has a role in antigenicity and inflammatory response, as well as promoting interactions with cell receptors and antibacterials (King et al., 2009). LPS comprises three distinct domains: a membrane-anchored lipid A which causes the immune system to be overstimulated (Gellatly and Hancock, 2013; Sapkota, 2021), a polysaccharide core region is a branching oligosaccharide that acts as a binding site for CFTR receptors and induces epithelial cell internalization, and an extremely diverse O polysaccharide protects bacteria against phagocytosis and complement-mediated destruction (Gellatly and Hancock, 2013; king et al., 2009).

1.3.4 Lectins

Lectins are carbohydrate-binding proteins (Tielker et al., 2005). They are mostly found in the cytoplasm of cells, with minor quantities on the cytoplasmic membrane (Glick and Garber, 1983; Bajolet-laudinat et al., 1994), *P. aeruginosa* synthesized two Lectins: LecA (PA-IL) and LecB (PA-IIL) (Tielker et al., 2005).

The galactophilic molecule LecA inhibits the proliferation of respiratory cells, resulting in their destruction during infections. LecA has also been shown to cause a permeable problem in the intestine, resulting in an enhancement in exotoxin A intake, an essential extracellular virulence factor (Chemani et al., 2009; Tielker et al., 2005; Bajolet-laudinat et al., 1994). Although LecB of *P. aeruginosa* binds with fucose, mannose, and mannose-containing oligosaccharide receptors at the cell surfaces (Passos da Silva et al., 2019) and it plays several roles such as pilus biogenesis, decreasing ciliary beating in the airway epithelium, protease IV activity, and biofilm formation (Passos da Silva et al., 2019; Tielker et al., 2005; Chemani et al., 2009).

1.3.5 Biofilm formation and alginate

During chronic infections, *P. aeruginosa* forms matrix-enveloped, surface-associated communities known as a biofilm (figure 1.7). Biofilm is defined as a population of bacteria that thrive in self-created extracellular polymeric substances attached to a variety of surfaces and/or to one another (Thi et al., 2020; Driscoll et al., 2007). principal constituents of the biofilm matrix are alginate, proteins (e.g., fibrin), cellulose, as well as extracellular RNA and/or DNA (Vestby et al., 2020; Jolivet-gougeon and Bonnaure-mallet, 2014). This matrix provides a home for bacteria to interact and communicate with each other, as well as a store of biochemical compounds, nutrition, and energy to stimulate cell development and protect cells against harmful conditions (Flemming and Wingender, 2010). Bacterial biofilm is known to form on either living or non-living objects. (Cortés et al., 2011).

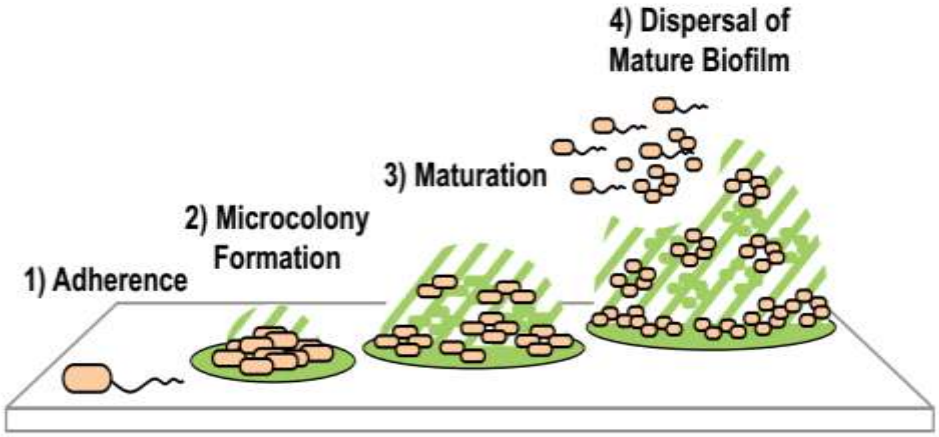

Figure 1.6: The process of bacterial biofilm development progress through four basic stages. 1-Surface attachment. 2- The establishment of Biofilm communities by cell proliferation and matrix development. 3- Maturation of Biofilm structure. 4- Bacteria can actively disintegrate from the biofilm or be left inactively via physical destruction (Taylor et al., 2014).

Furthermore, Biofilm plays a significant role in the pathogenesis, particularly in chronic conditions, as it serves as a protective layer toward antibacterial drugs and also the immunological responses of the host (Moradali et al., 2017; Al-Wrafy et al., 2017; Alhazmi, 2015). It is associated to approximately 80% of bacterial infections (Cortés et al., 2011).

P. aeruginosa, among the most researched bacteria for biofilm development, generates three different exopolysaccharides that aid in biofilm structure: alginate, Pel (pellicle), and Psl (Polysaccharide synthesis locus) (Thi et al., 2020). Alginate is a linear polymer exopolysaccharide consisting of glucuronic acid and mannuronic acid (Fothergill et al., 2012; Al-Wrafy et al., 2017). Overproduction of alginate is mainly due to defects in the MucA gene that encode alternative sigma factor AlgT (Driscoll et al., 2007; Flemming and Wingender, 2010). Alginate is implicated in micro-colony development in the biofilm-forming process. Also, it is important for the stability of the biofilm (Flemming and Wingender, 2010). Moreover, it is involved in *P. aeruginosa* pathogenesis in an array of ways, including adherence to the epithelium, scavenging of free radicals released by macrophages, protecting the bacteria from host defense by

reducing susceptibility to phagocytosis and resistance to antibiotics (Driscoll et al., 2007; Cobb et al., 2004).

In non-mucoid strains of *P. aeruginosa* that do not express alginate biosynthesis genes, the exopolysaccharides Psl and Pel are implicated in the biofilm formation (Flemming and Wingender, 2010). The Psl exopolysaccharide is made up of repeated pentasaccharides that comprise glucose, mannose, rhamnose, galactose, and trace quantities of xylose, also it is critical for bacterial attachment to mucin-coated structures and airway epithelium and the preservation of biofilm (Ma et al., 2019). Whereas, Pel is a type of exopolysaccharide rich in glucose that is necessary for biofilm production (Al-Wrafy et al., 2017; Flemming and Wingender, 2010).

1.3.6 Proteases

P. aeruginosa secretes various kinds of proteases, including two elastases (LasA and LasB), protease IV, and alkaline protease (Alhazmi, 2015). Elastase causes tissue damage and cleaves a wide variety of plasma proteins of the host, like coagulation and complement factors, immunoglobulins, and also inactivates human plasma proteinase inhibitors (Al-Wrafy et al., 2017; Bardoel, et al., 2011). Protease IV maintains *P. aeruginosa* throughout colonization by degrading a variety of components, such as fibrinogen, complement components, immunoglobulin, and antibacterial peptides (Al-Wrafy et al., 2017; Matsumoto, 2004; Engel et al., 1998). The alkaline protease cleaves immune system components including complement C1q and C3, and cytokines like gamma-interferon (γ-INF) and α-TNF. It also helps *P. aeruginosa* in evading identification by inhibiting TLR5 activation as it degrades monomeric flagellin (Laarman et al., 2012; Bardoel, et al., 2011).

1.3.7 Type III secretion system and exoenzymes

Type three secretion system (T3SS) is a double-membrane-embedded nanomachine found in the majority of pathogenic gram-negative bacteria (Al-Wrafy et

al., 2017; Costa et al., 2015). Because T3SSs are usually acquired horizontally, bacteria that are evolutionary distinct may have systems that are closely related, and vice versa (Troisfontaines and Cornelis, 2005). In relation to *P. aeruginosa*, the T3SS is activated after binding the bacteria with host epithelial cells and then directly injecting effector proteins into the cytoplasm or the membrane of human target cells (Driscoll et al., 2007; Costa et al., 2015). *P. aeruginosa* is known to produce four type-III effector proteins including Exoenzyme S, Exoenzyme U, Exoenzyme T, and Exoenzyme Y (Strateva and Mitov 2011).

Exoenzymes S and T are bi-functional toxins with GAP (guanosine triphosphatase activating protein) and ADPRT (adenosine diphosphate ribosyltransferase) activity. These actions have a negative impact on host cells, disrupt actin cytoskeletal rearrangement, inhibit phagocytosis, and induce cell death (Veesenmeyer et al., 2009; Bleves et al., 2010). Exoenzyme Y is an adenylate cyclase that increases the cAMP (cyclic adenosine monophosphate) levels in host cells and promotes actin cytoskeleton rearrangement, while Exoenzyme U leads to epithelial cell and macrophage cytotoxicity (Alhazmi, 2015)

1.3.8 Exotoxin A

Exotoxin A is considered the most important exoproduct generated by pathogenic strains of *P. aeruginosa* and secreted via T3SS (Sapkota, 2021; Alhazmi, 2015). It is encoded by the toxA gene, which is available in almost all strains of *P. aeruginosa* (Alhazmi, 2015). This toxin has been demonstrated to enter the host cells via endocytosis and cause apoptosis in eukaryotic cells by preventing protein synthesis. Exotoxin A also causes non-apoptotic death of airway epithelial cells by causing superoxide formation, mitochondrial malfunction, and DNA destruction (Plotkowski et al., 2009).

Table 1.1: The main virulence factor of *P. aeruginosa* and their roles in pathogenesis.

Virulence factor	Role in pathogenesis
Pili	Adherence to the epithelium.
Flagella	Adhesion, movement.
Lipopolysaccharide	The major antigenic factor on the cell surface; the loss of sugar unit during infection leads to "rough" LPS and serum sensitivity.
Lectins	Adhesion to host cells leads to lung damage and the development of biofilms.
Biofilm and Alginate	Adherence to the epithelium. the barrier to phagocytes and antibiotics. inhibit antibodies and complements.
Proteases	Cytotoxicity, proteolytic activity
Type III Secretion System	Cytotoxic activity
Exotoxin A	Inhibits protein synthesis, is cytotoxic, and causes apoptosis.

1.4 Clinical infections caused by *P. aeruginosa*

Despite a variety of extremely destructive and effective virulence factors, *P. aeruginosa* remains an opportunistic invader pathogen that needs weakened defense systems to introduce infection (Moradali et al., 2017). *P. aeruginosa* can infect various body parts, such as the bloodstream, respiratory tract, heart, central nervous system, bones and joints, gastrointestinal tract, urinary tract, ears, eyes, and skin (Qureshi, 2020). Furthermore, It able to thrive within harsh conditions and inherently resistant to a wide variety of antiseptics and antimicrobial agents (Çiçeket al., 2021), this means it can survive in hospital settings and causes about 10% of hospital-acquired infections with high mortality rates in severely sick and immunocompromised patients as with severe burns, post-surgery, cystic fibrosis, and patients infected by HIV (Khorvash et al., 2017;

Thi et al., 2020; Shaaban et al., 2017). In healthy individuals, pseudomonal infections are usually related to interrupting or escaping the skin barrier (e.g., burns, puncture wounds, using contaminated needles by intravenous drug abusers, and contaminated lenses). Therefore, infections of the skin, bones, heart, and eyes occur (M Tille, 2017).

1.4.1 Bacteremia

P. aeruginosa is the third most prevalent kind of gram-negative bacteria that contributes to infections of the bloodstream, as it causes severe bacteremia in immunocompromised patients and other diseases, namely hematologic malignancies, neutropenia, and serious burns. Pseudomonas bacteremia is mostly gained in healthcare settings (Tumbarello et al., 2011; Martinez-Nadal et al., 2020). The mortality proportion of Pseudomonal bacteremia is between 20-35%, even though it might be higher depending on preexisting illnesses and the inefficiency of empirical antibiotic therapy when the bacteria is resistant to the provided antibiotics (Pelegrin et al., 2021). Despite the development of potent anti-Pseudomonal beta-lactam drugs and the consequent decrease in the fatality rates, the bacteremia caused by *P. aeruginosa* remains one of the most serious hospital-acquired infection (Alhazmi, 2015).

1.4.2 Infection in cystic fibrosis patients

Cystic fibrosis (CF) is a chronic, progressive, and serious genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which provides instructions to cells to produce a protein, also known as CFTR protein (Cystic fibrosis: MedlinePlus Genetics, 2021). Normally, this protein keeps osmotic balance throughout the various epithelial surfaces by serving as a chloride channel on the cell surfaces to regulate the transportation of chloride ions in and out of the cells, but it also transports water, bicarbonates, and sodium (Cystic fibrosis: MedlinePlus Genetics, 2021; Cystic Fibrosis Overview, 2021). In CF, the mutations in the CFTR gene cause the CFTR protein to become dysfunctional, which causes inadequate salt transportation across the cell membrane therefore cells cannot effectively regulate the flow of salt and water, leading to the buildup of abnormally thick and sticky mucus in the lungs, liver, sweat glands, and other mucus-producing organs of the human body (Cystic fibrosis:

MedlinePlus genetics, 2021, (learn about cystic fibrosis | American lung association, n.d.; Staff, 2021).

In the lungs, this thick mucus can restrict the airways, thereby it provides appropriate and beneficial habitat for bacteria (mainly *P. aeruginosa*, *Staphylococcus aureus*, and *Haemophilus influenzae*) to thrive and proliferate, resulting in infectious diseases and further inflammation (Wang et al., 2016).

Moreover, patients with cystic fibrosis almost always have *P. aeruginosa* colonized in their lungs, which is the major health concern and death in this patient population (Moradali et al., 2017; Lamont et al., 2009). The distinguishing characteristic of *P. aeruginosa* strains recovered from the lungs of CF patients is the formation of the mucoid colonies that result from an overproduction of an extracellular polysaccharide referred to as alginate, which helps the bacteria by hindering phagocytosis and giving protection against antimicrobials (Lamont et al., 2009; Cobb et al., 2004).

1.4.3 Hospital-acquired pneumonia

Hospital-acquired pneumonia is the second most prevalent nosocomial infection and the main cause of mortality in severely ill patients from nosocomial infections (Torres et al., 2021). Hospital-acquired pneumonia is mostly associated with mechanical ventilation, in which cases are referred to as ventilator-associated pneumonia (VAP), affects 10 to 20% of patients on ventilators for more than 48 hours and leads to considerable increases in in-hospital stay, costs, duration of mechanical ventilation, and death (Pachori et al., 2019; Parker et al., 2008).

Ventilator-associated pneumonia caused by *P. aeruginosa* is usually related to the destruction of epithelia by mechanical injury during endotracheal intubation, which promotes bacterial adhesion and proliferation. Intubation also allows colonized pathogenic bacteria to penetrate via aspiration of infected oral and gastrointestinal contents (Sadikot et al., 2005). *P. aeruginosa* causes a more severe form of ventilator-associated pneumonia than other pathogenic bacteria, its mortality rate between 34-48%. (Pachori et al., 2019; Sadikot et al., 2005)

1.4.4 Ear infections

The ear is a vital sensory organ that is responsible for hearing and maintaining balance; It is made up of three parts: the outer, middle, and inner ear (Gul et al., 2014). Ear infections (Otitis) can develop in each of these parts, referred to as Otitis Externa, Otitis Media, and Otitis Interna. Over 40% of hearing impairment cases all over the world are because of ear infections, which are mainly caused by bacterial pathogens (Getaneh et al., 2021).

P. aeruginosa is the most common bacterial infection in some cases of external otitis, which may be progressed into malignant otitis externa, especially in patients suffering from chronic health conditions including old people with diabetes mellitus, hypertension, immunocompromised patients, and hematological disorders (Todar, 2015; Sylvester et al., 2017; González et al., 2021). This bacterium is rarely detected in a healthy ear, but it may colonize the external auditory canal because of injury, inflammation, or moist conditions (Todar, 2015).

1.4.5 Urinary Tract Infections

Urinary tract infections (UTI) account for 20-49% of all nosocomial infections, including *P. aeruginosa*, which causes 10% of all UTIs, particularly in immunocompromised patients (Pachori et al., 2019). The infections caused by *P. aeruginosa* are frequently acquired in hospitals and are linked with catheters, surgery, and instrumentation (Fujitani et al., 2017; Todar, 2015). Bacteria use catheters as a route for entering the host because the insertion of the catheter disrupts mucosa and facilitates bacterial colonization (Mittal et al., 2009). *P. aeruginosa* can also enter the circulation through the urinary system, which constitutes about 40% of Pseudomonas bacteremias. (Todar, 2015).

1.4.6 Burn wounds

Infections are the most prevalent concern that burn patients suffer from. Infecting pathogens are not limited to one anatomical area, so they may be implicated in surgical

wound infections and even systemic infections. The lower extremities are the most common locations for surgical burn wound infection (D'abbondanza and shahrokhi, 2021).

P. aeruginosa is rarely recovered from burn patients in the first week of hospitalization, but after that, the probability of positive cultures with *P. aeruginosa* is raised (spernovasilis et al., 2021). The yellow-green pigmentation and Ecthyma gangrenosum of the skin are principal features of *P. aeruginosa* infections in Burn patients (D'abbondanza and Shahrokhi, 2021). Sepsis is the most serious consequence of *P. aeruginosa* in burn wounds and it may be fatal (Tredget et al., 2004).

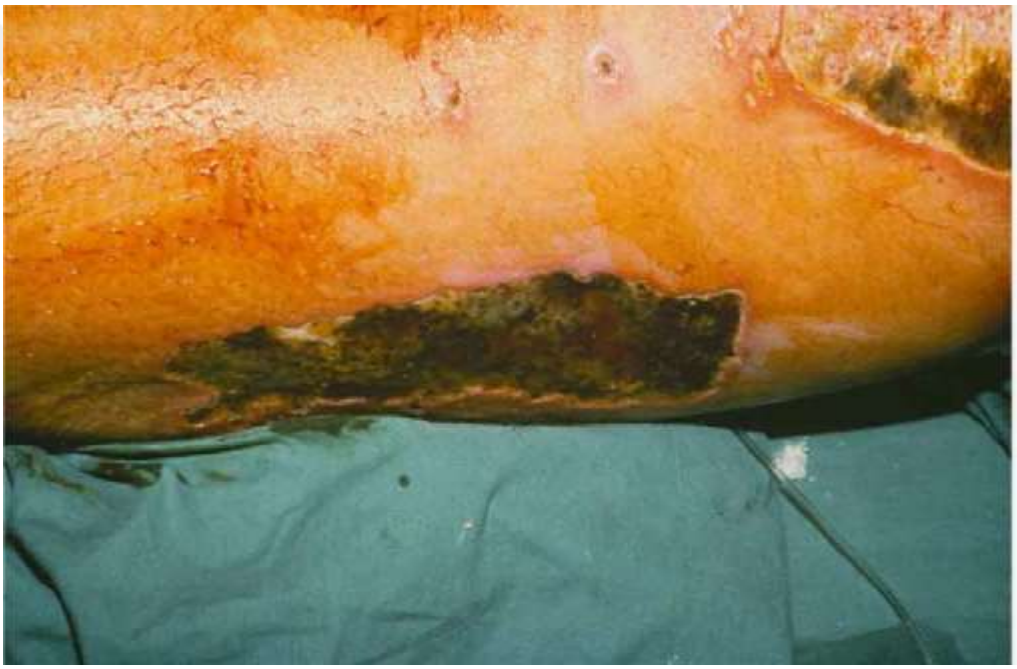

Figure 1.7: Skin burn wound infected by *P. aeruginosa* (Tredget et al., 2004).

1.4.7 Skin and soft tissue infections

P. aeruginosa causes various skin and soft tissue infections (figure1.9) that result from cutaneous colonization or bloodstream infection (Wu et al., 2011). *P. aeruginosa* skin infections are ranging from acute to chronic, benign skin infections to life-

threatening systemic infections, and usually have distinct skin manifestations (Spernovasilis et al., 2021). We herein briefly describe some of these manifestations;

Green Nail Syndrome also called chloronychia, is the classical manifestation of the *P. aeruginosa* infection (Geizhals et al., 2020). The main clinical feature of GNS is the blue-greenish discoloration of the nail plate (figure 1.10) that results from the secretion of pyocyanine and/or pyoverdin pigment(s) (Spernovasilis et al., 2021; Geizhals et al., 2020).

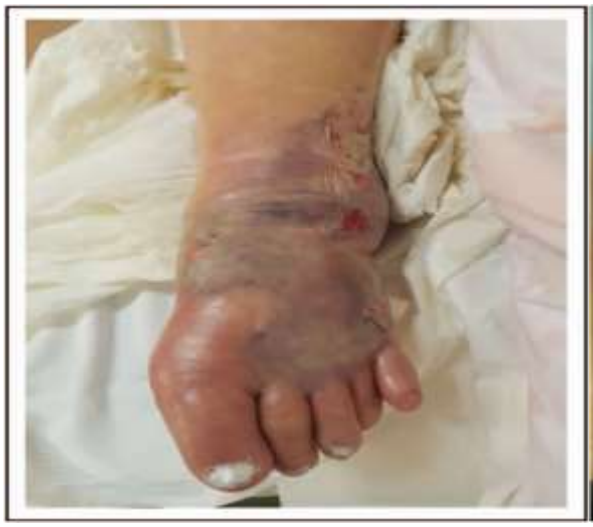

Figure 1.8: *P. aeruginosa* necrotizing skin and soft tissue infection in a diabetic patient with peripheral vascular disease (Spernovasilis et al., 2021).

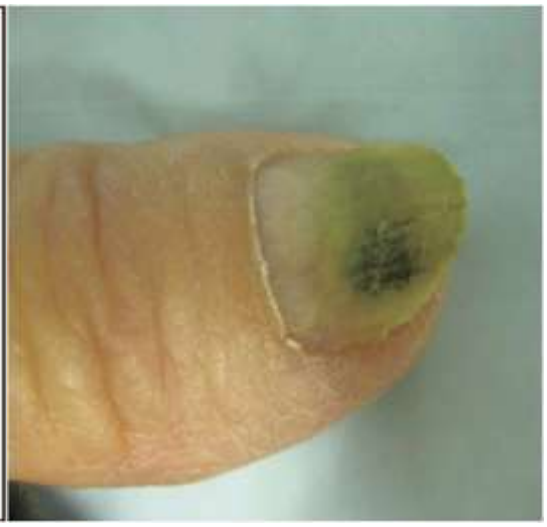

Figure 1.9: Green discoloration of nails caused by *P. aeruginosa* (Wu et al., 2011).

Hot tub folliculitis is one of the most well-known skin manifestations of *P. aeruginosa* infection (Wu et al., 2011). It is generally associated with whirlpools, swimming pools, saunas, and hydrotherapy pools (Yu et al., 2007). This infection commonly occurs in previously healthy people who have been exposed to contaminated water (Wu et al., 2011). Hot tub folliculitis is characterized by the rapid development of multiple monomorphic, painful papules and pustules on the axillae, sides of the thorax, abdomen, or any other body part exposed to contaminated water (Wu et al., 2011; Hott et al., 2019).

Ecthyma gangrenosum is a rare and distinct necrotic skin lesion and a specific manifestation of fatal sepsis, caused most frequently by *P. aeruginosa*. It is more common in immunocompromised patients, particularly those with hematological malignancies (Wuyts et al., 2019; Spernovasilis et al., 2021). Ecthyma gangrenosum is clinically characterized by hemorrhagic pustules that progress to necrotic ulcers (figure 1.11), which have a black scab with a reddish halo (Vaiman et al., 2015).

Figure 1.10: Ecthyma gangrenosum (EG) of the face caused by *P. aeruginosa* (Vaiman et al., 2015).

1.4.8 Other types of infections

In spite of the diseases mentioned above, *P. aeruginosa* can also cause more infections in community and healthcare settings. For example, it is relatively infrequent in central nervous system infections and typically appears as a complication of a trauma or post neurosurgical procedure (Pelegrin et al., 2021). In cases associated with contact lens using infections, *P. aeruginosa* is one of the main causes of bacterial keratitis and endophthalmitis (Hilliam et al., 2020). Pseudomonas infections of bones and joints are caused by either spreading of bacteria through the bloodstream or direct bacterial

inoculation (Todar, 2015). In conclusion, *P. aeruginosa* can invade and infect almost all organs of the human body and does so with a broad of virulence factors.

1.5 Treatment of *P. aeruginosa*

Infections caused by *P. aeruginosa* are typically treated with antibiotics. These antibiotics are bacteriostatic (inhibit bacterial growth) or bactericidal (induce cell death). They interfere with bacterial activities such as cell wall synthesis, DNA replication, RNA transcription, protein synthesis, metabolism, or cell membranes (Wagner et al., 2016). Therapeutic options for *P. aeruginosa* infections are limited and progressively shrinking. Fluoroquinolones, beta-lactams, aminoglycosides, and polymyxins are frequently used to treat *P. aeruginosa* infections. (Kothari et al., 2022).

1.5.1 Aminoglycoside

Aminoglycosides are an important class of antibiotics that are used to treat a wide range of bacterial infection, especially active against pathogenic gram-negative (Semper et al.,2020). The bacterial small ribosomal subunit is the primary target for aminoglycosides and it binds to the 16S ribosomal ribonucleic acid at the transfer ribonucleic acid acceptor A site (Aminoacyl site) causing misreading of codons among messenger-RNA (mRNA) hence it inhibits the microbial protein synthesis (magnet et al., 2005; Pachori et al., 2019). The bactericidal activity of aminoglycosides is concentration-dependent. Therefore, their bactericidal activity becomes rapid when their serum concentration is increased (Young et al., 2013). Antipseudomonal aminoglycosides include tobramycin, gentamicin, and amikacin, but they should not be used as a mono-therapeutic agent unless treating urinary tract infections (Ibrahim et al., 2013). Tobramycin has the highest activity against *P. aeruginosa* and it is two times more active than gentamicin and three to four times more active than amikacin (Mensa et al., 2018)

1.5.2 Fluoroquinolones

Fluoroquinolones are bactericidal and impede bacterial DNA synthesis by interfering with the DNA replication enzymes DNA gyrase and topoisomerase IV (Choi

et al., 2013). This class of antibiotics is recognized for their high gastrointestinal absorption, great penetration into a variety of tissues (including lungs, meninges, and bones), and rapid intracellular diffusion (Choi et al., 2013, Tamma et al., 2012). The main antipseudomonal fluoroquinolones are Ciprofloxacin, levofloxacin, and prulifloxacin. They are the only antipseudomonal agents that are usable in an oral formulation (Ibrahim et al., 2013).

1.5.3 Polymyxins

Polymyxins are a kind of polypeptide antibiotic developed in 1947. They have five different subclasses (polymyxins A–E). Just polymyxin B and polymyxin E (colistin) are utilized in clinical practice (Goli et al., 2016; El solh and Alhajhusain, 2009). Colistin was one of the first antibiotics to have considerable efficacy against gram-negative bacteria, most notably against *P. aeruginosa* (Nation and Li, 2009). It is also the only antibiotic that can be used to treat the infection in certain cases of *P. aeruginosa* resistance to all tested antibiotics, including carbapenem (Azimi and Lari, 2019).

Colistin has a concentration-dependent antibacterial activity. Its primary target is the plasma membrane (Dhariwal et al., 2013). The mechanism of action is initiated by the binding of the cationic polypeptide (Colistin) to the anionic LPS in the outer membrane, resulting in disruption of the cell membrane and displacement of magnesium and calcium, which are stabilizers of LPS. During this process, cell permeability increases, which means intracellular contents leak out, subsequently bacterial death. (Dhariwal et al., 2013; Goli et al., 2016).

1.5.4 β-lactams

β-Lactams are the most effective antibiotics used to treat bacterial infections. This is because they have a broad range of activity, can be taken orally, have good pharmacokinetics, are not toxic, and have bactericidal effects (Foster, 2019). β-lactam antibiotics inhibit the production of bacterial peptidoglycan, which deals with bacterial cell lysis. In particular, beta-lactam antibiotics interact with and acylate the active site of penicillin-binding protein (PBP), the enzyme necessary for bacterial cell wall synthesis (Zeng and Lin, 2013; Letourneau and Calderwood, 2019). There are different kinds of

PBPs that have distinct functions for bacteria. Different β-lactam antibiotics may preferentially bind to or inhibit specific PBPs. So, various β-lactam antibiotics may have varying effects on bacterial morphology as well as varying bacteriostatic and bactericidal capabilities (Letourneau and Calderwood, 2019).

This class of antibiotics consists of several groups of antibacterial agents, including penicillin, cephalosporin, carbapenem, and monobactam (Lima et al., 2020). Among these groups, penicillins, ceftazidime, cefepime, aztreonam, imipenem, meropenem, and doripenem are the highly efficacious beta-lactams that are most frequently prescribed to be used to treat *P. aeruginosa* infections (Pachori et al., 2019).

1.5.5 Combination versus monotherapy for the treatment of *P. aeruginosa*

Treatment of *P. aeruginosa* infection with combination therapy or monotherapy is still an important and controversial issue that needs to be studied and discussed further. When multidrug-resistant *P. aeruginosa* is suspected or when patients are severely and seriously ill, a combination of antipseudomonal medications are preferred over monotherapy (Grossi et al., 2006; Reynolds and Kollef, 2021)

In combination therapy, a β-lactam antibiotic is combined with an antibiotic from the aminoglycoside or fluoroquinolone group (Grossi et al., 2006; Vardakas et al., 2013). Due to their diverse targets and distinct entrance mechanisms to enter the bacterial cell, each of these drugs has better antibacterial activity (Pachori et al., 2019). Several benefits of combination therapy over monotherapy have been identified. These include a higher chance that at least one antibacterial agent will kill the cause of the infection; the ability to kill bacteria in more than one way; a lower chance of the bacteria becoming resistant; and a synergistic effect of the combination (Pachori et al., 2019; El solh and Alhajhusain, 2009). Even with all of these advantages, there is no clear evidence that combination therapy is better than monotherapy for treating a *P. aeruginosa* infection (Paulsson et al., 2017; Hu et al., 2013; Peña et al., 2013).

1.6 Resistance mechanisms in *P. aeruginosa*

Antibiotic resistance refers to the bacterial capability to resist and evade the effects of antibiotics that were formerly successful in treating their infections (Singh et al., 2019). It has developed into various human infections as a consequence of the overuse and misuse of antibiotics (Martinez, 2009; Luyt et al., 2014). Several research studies have found that the therapeutic indication, antibiotic choice, and course of antibiotic therapy are all inappropriate in 30 to 50% of cases (Ventola, 2015; Luyt et al., 2014).

Increasing antibiotic resistance affects infection treatment by reducing the number of active antibiotics, which leads to difficulties in infection management and situations when therapeutic agents are no longer effective (Diallo et al., 2020). In addition, antibiotic resistance is related to increase morbidity and mortality in healthcare settings (Akova, 2016; Fothergill et al., 2012). According to the associations that represent the healthcare sector, it is now a serious issue for the healthcare system all over the world (Public Health England, 2014; WHO, 2015; CDC, 2019).

P. aeruginosa is a notable model of antimicrobial resistance among bacterial pathogens because it has almost all the known mechanisms to be resistant to antibiotics. These include a low-permeability outer membrane, modification of penicillin-binding proteins, a large number of efflux pumps, biofilm, and a variety of antibiotic-degrading enzymes (AL-Fridawy, et al., 2020)

In 2017, The WHO published a list of priorities for the discovery and development of novel antibiotics to treat bacteria that become resistant to commonly used antibiotics., classifying them into 3 groups depending on the need for new antibiotics: priority 1 (critical), priority 2 (high), and priority 3 (medium). *P. aeruginosa* was designated as a critical pathogen because it has developed resistance to virtually all of the antibiotics that are currently in use, including aminoglycosides and carbapenems (Tacconelli et al., 2018).in table 1.3 we summarized the resistance mechanisms of some antibiotics in *P. aeruginosa*.

Table 1.2: Resistance mechanisms of some antibiotics in *P. aeruginosa*.

Antibiotics	**Resistance mechanism**
Aminoglycoside	Reduced permeability
Fosfomycin	Transport system alterations
Quinolones	Modifications in DNA gyrase target and Efflux
Rifampin	Altered DNA polymerase target
Carbapenems	Mutations in OprD, carbapenemase, Efflux
Cephalosporins	Reduced permeability and β-Lactamase inactivation
Penicillin	Modifications in Penicillin-binding proteins

1.6.1 Intrinsic Resistance

Intrinsic antibiotic resistance is the intrinsic capability of bacteria to resist the action of certain antibiotics by inherent functional or structural determinants (Blair et al., 2015). It exists prior to introducing antibiotics; traditionally, this was related to the absence of a susceptible target or decreased bacterial cell permeability for antibiotics. No evolutionary link between a bacterial species and an antibacterial is required for this resistance (Martinez, 2014).

P. aeruginosa is a notable bacteria that intrinsically exhibit a high level of resistance to the frequently used antibiotics via decreasing the permeability of the outer membrane, enzyme production such as Beta-lactamases, and efflux pumps that remove treatments from the cell (Fothergill et al., 2012; Breidenstein et al., 2011).

1.6.1.1 Outer membrane permeability and porin alterations

Low outer membrane permeability is the main characteristic of intrinsic antibiotic resistance in *P. aeruginosa* (Botelho et al., 2019). This mechanism contributes to resistance to several antibiotics, including quinolones, aminoglycosides, and beta-lactams (In order to accomplish their intended effects against *P. aeruginosa*, each of these antibiotic classes must first traverse the bacterial cell membrane) (Pang et al., 2019). As an example, this type of resistance is common in cystic fibrosis isolates that have been exposed repeatedly to antibiotics. Mutations of lipopolysaccharides, changes in membrane proteins involved in absorption, and inhibition of enzyme complexes that are implicated in membrane energy being used for transportation system activation are all mechanisms to make a membrane-less permeable (Bassetti et al., 2018).

However, hydrophilic antibiotics (e.g. β-lactams and quinolone) may enter cells through outer-membrane porin proteins **(**Blair et al., 2015), which produce water-filled pores that spread across the membrane and facilitate the entry of small hydrophilic substances into the bacterial cells (Fernández et al., 2012; Lamont et al., 2019). Alterations of these proteins may be accomplished by three processes: 1) by altering the type of porins expressed, 2) by altering the quantity of porin expression, and 3) by altering porin function (Munita and Arias, 2016; Blair et al., 2015). As one of its own intrinsic strategies to keep antibiotics out, *P. aeruginosa* reduces non-specific porin proteins for nutrient absorption and instead uses more specialized channels. This results in less permeability to harmful substances figure (fernández et al., 2012 p.7/21) (moradali et al., 2017).

1.6.1.2 Overexpression of efflux systems

Antibiotic resistance can also be caused by overproducing complicated bacterial machinery called efflux pumps. These pumps are essential for removing a variety of toxic chemicals from the cell, including dyes, fatty acids, detergents, and antimicrobials (El zowalaty et al., 2015). Five families of efflux systems have been discovered: the resistance-nodulation-division family, the major facilitator superfamily, the ATP-binding

cassette family, the small multidrug resistance family, and the multidrug and toxic compound extrusion family (Mengal et al., 2019; Papp-Wallace et al., 2011).

In particular, efflux systems have an important role in intrinsic and acquired resistance to several antibacterial drugs in *P. aeruginosa* (El zowalaty et al., 2015). It is mostly mediated by members of the resistance-nodulation-division family (Mengal et al., 2019). Four significant efflux systems have been identified: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Bassetti et al., 2018; Tenover et al., 2006). These pumps can efflux all antibiotic classes except polymyxins (Lambert, 2002). For instance, MexEF-OprN is able to pump out quinolones and carbapenems. MexCD-OprJ has the ability to extrude β-lactams. MexAB-OprM is important for β-lactam and quinolone extrusion, while MexXY-OprM removes aminoglycosides (Lambert, 2002; pang et al., 2019).

1.6.1.3 Antibiotic-inactivating enzymes

It has been revealed that *P. aeruginosa* can also intrinsically avoid the inhibitory effect of antipseudomonal agents by producing enzymes that can hydrolyze chemical bonds in their structure (Pang et al., 2019). *P. aeruginosa* has an ampC gene that encodes beta-lactamase, which inactivates beta-lactam antibiotics through cleaving the amide bond of the beta-lactam ring in these antibiotics (Pang et al., 2019; Strateva and Yordanov, 2009).

Additionally, beta-lactamases are classified into four groups according to their amino acid sequences: A, B, C, and D. Beta-lactams are hydrolyzed by enzyme classes A, C, and D through a serine active site. In comparison, class B lactamases are metalloenzymes that need divalent zinc ions to hydrolyze beta-lactams (Glen and Lamont, 2021). Moreover, some isolates of *P. aeruginosa* produce extended-spectrum beta-lactamases (ESBLs), which provide a critical level of resistance to most beta-lactams, including penicillins, aztreonam, and cephalosporins (Pang et al., 2019). Beta-lactamase inhibitors like sulbactam, clavulanate, and tazobactam have been introduced into clinical practice to combat beta-lactamase-mediated resistance. These beta-lactamase inhibitors

have been shown to significantly enhance the effectiveness of beta-lactams when used in combination treatment, which is an important step toward overcoming this resistance (Pang et al., 2019; Glen and Lamont, 2021).

P. aeruginosa also produces an aminoglycoside-modifying enzyme (AME), which is considered the main mechanism of aminoglycoside resistance (el zowalaty et al., 2015). AME inactivates aminoglycosides by attaching acetyl, adenyl, or phosphate groups to amino and hydroxyl substituents in the aminoglycoside molecular structure. These modifications considerably decrease the affinity of aminoglycosides for the 30S ribosomal subunit and thereby inhibit their action (Bassetti et al., 2018; Pachori et al., 2019).

Three kinds of AME are identified: aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC), and aminoglycoside nucleotidyltransferase (ANT) (Lamont et al., 2019; Magnet et al., 2005). These enzymes are different in the aminoglycosides that they can modify and in the segment of the antibiotic molecule that is attacked (Blair et al., 2015). It has been found that APH of *P. aeruginosa* transfers a phosphoryl group to the 3′-hydroxyl of aminoglycosides such as kanamycin, streptomycin, and neomycin thus blocking these antibiotics' action (Poole, 2005). The ANT of *P. aeruginosa* shifts the adenyl group to the amino or hydroxyl group of aminoglycosides. Hence, it inhibits the activities of amikacin, tobramycin, and gentamicin. (Semper et al., 2020). Resistance to kanamycin, gentamicin, amikacin, and tobramycin is conferred by *P. aeruginosa* AACs, which use acetyl coenzyme A (CoA) as a donor substrate to catalyze the acetylation of primary amine groups inside aminoglycoside molecules (Strateva and Yordanov, 2009; Lamont et al., 2019).

1.6.2 Acquired antibiotic resistance

Acquired antibiotic resistance develops when sensitive bacteria acquire the genes that encode a mechanism of resistance. This may happen as a consequence of a mutation in chromosomal genes or as a result of genetic material being gained from other pathogens (Edwards et al., 2009; Martinez, 2014). Acquired resistance of *P. aeruginosa* is a

significant factor in the evolution of multidrug-resistant variants. It makes treatment of *P. aeruginosa* infections more difficult and increases the prevalence of chronic infections to an alarmingly high level (Pang et al., 2019; Lister et al., 2009).

1.6.2.1 Acquisition of resistance genes

Antimicrobial resistance genes may be acquired by conjugation, transformation, or transduction from DNA elements such as plasmids, transposons, integrons, and resistance islands (Edwards et al., 2009; Breidenstein et al., 2011). Gene transfer between bacteria occurs through a process called horizontal gene transfer (HGT). It is a critically important factor contributing to bacterial evolution and is commonly involved in the acquisition of bacterial resistance (Munita and Aria, 2016). This could lead to antibiotic resistance and even multidrug resistance because there are a lot of resistance cassettes on transposons and plasmids (Singh et al., 2019, Breidenstein et al., 2011).

P. aeruginosa has been found to have acquired resistance to a wide range of antibiotics through horizontal gene transfer, including aminoglycoside resistance, which is mostly mediated by aminoglycoside modifying enzymes expressed by class one integrons (Moradali et al., 2017; Horcajada et al., 2019). The transferable genes that encode ESBLs and carbapenemases are also located in the first class of integrons, which are two of the most serious transferable beta-lactamases in the world (Horcajada et al., 2019). Integrons and plasmids have been found to carry genes for some types of *P. aeruginosa* Metallo-beta-lactamases (Pang et al., 2019).

1.6.2.2 Mutational Resistance

Bacteria that are susceptible to an antibiotic can acquire resistance to it via novel mutations (Tenover, 2006). These mutations are often found in gene encoding: antibiotic targets, antibiotic transporters, and those encoding regulators that inhibit the production of transporters or antibiotic-decontaminating elements (mainly efflux systems and enzymes of antibiotic modification that are chromosomally encoded) (Martinez, 2014). Antimicrobial resistance mutations change the activity of antimicrobial drugs through one

of these mechanisms: alteration of the antibacterial target (Antibiotic binding is inhibited by structural changes to the target.), reducing antibiotic uptake, modification of metabolic pathways, or overexpression of efflux systems. Therefore, the resistance that comes from having mutations is more complicated (Munita and Aria, 2016; Tenover, 2006).

P. aeruginosa has shown an extraordinary capability to acquire resistance to antibiotics by obtaining chromosomal mutations (Horcajada et al., 2019; Glen and Lamont, 2021). The main characteristic of mutational resistance in *P. aeruginosa* is the exhibition of a high level of AmpC synthesis that is constant even in the absence of antibiotic inducers. This is especially caused by mutational deactivation of the ampD gene (a repressor of ampC) as well as certain mutations in ampR, both of which encode two essential regulative proteins in the induction of the ampC gene (Moradali et al., 2017). Accordingly, *P. aeruginosa* becomes resistant to a wide range of antibiotics including, Beta-lactams, Fluoroquinolones, and Cephalosporins (Horcajada et al., 2019; Breidenstein et al., 2011). Furthermore, mutations in the gyrase genes (gyrA and gyrB), or even in the topoisomerase IV genes (parC and parE), result in decreased fluoroquinolone binding affinity, which makes them less effective in treating infections (Lister et al., 2009; Pachori et al., 2019)). Antibiotic exposure may cause mutations in the regulatory genes of one of *P. aeruginosa*'s four primary efflux systems, resulting in overexpression of efflux pumps. So if efflux pumps are over-expressed, they significantly extend rates of resistance to previously effective antibiotics (Horcajada et al., 2019; Blair et al., 2015). For instance, MexAB-OprM has the broadest range of substrates, and overexpression of this efflux pump by mutation makes fluoroquinolones less effective and all beta-lactams (except imipenem) less effective (Horcajada et al., 2019).

1.6.3 Adaptive antibiotic resistance

Adaptive resistance is an increase in the bacteria's capability to resist an antibiotic related to changes in gene and/or protein expression as a consequence of antimicrobial or other external stimuli exposure. This mechanism is implicated in *P. aeruginosa*

developing resistance to beta-lactams, aminoglycosides, polymyxins, and fluoroquinolones. When compared to intrinsic and acquired resistance, no significant relationship between adaptive resistance and clinical outcome has been determined, so this event has got not much consideration (Moradali et al., 2017; Breidenstein et al., 2011). The best-known adaptive resistance mechanisms of *P. aeruginosa* are biofilm development and persistent cells, which lead to chronic infections and a poor prognosis for individuals with CF (Pang et al., 2019).

1.6.3.1 Biofilm-mediated resistance

Biofilm serves as a scaffold for holding bacteria together on surfaces and preserving them from external challenges by inhibiting phagocytosis and therefore enhancing the bacteria's ability to colonize and survive for an extended period (Thi et al., 2020; Jolivet-gougeon and Bonnaure-mallet, 2014). Antibiotic resistance is significantly influenced by some aspects of biofilm, including the extracellular biofilm matrix, the development of several genetic variants, and the metabolic versatility of subpopulations within a biofilm. (Taylor et al., 2014). *P. aeruginosa* undergoes several physiological and phenotypic alterations as part of the biofilm developing process. As a consequence, the production of biofilm leads to an increase in antibiotic resistance. For instance, antibacterial agents may be unable to access their targets because the biofilm matrix serves as a protective barrier. Alginate has the capacity to bind to cationic antibiotics such as polymyxins and aminoglycosides, reducing their capability to enter the bacterial cell (Jolivet-gougeon and Bonnaure-mallet, 2014; Fothergill et al., 2012). *P. aeruginosa* is slowly growing in biofilm, so the activity of metabolic therapeutic targets is reduced and the diffusion of antibiotics is decreased, so this may prolong the rate of antibiotic exposure necessary to destroy the organism. (Taylor Et Al., 2014).

1.6.3.2 Persister cells in antibiotic resistance

Persister cells are a small group of bacteria in a biofilm that transiently goes through poor growth or dormant state. They are particularly observed in Chronic UTIs

and the lungs of CF patients. Persister cells are not resistant to antibiotics genetically, but they are tolerant to high dosages of antibiotics (Pang et al., 2019; Jolivet-gougeon and Bonnaure-mallet, 2014). When the therapeutic stress is stopped, these Persister cells can continue to develop and reproduce. Remarkably, the new population is sensitive rather than resistant, indicating that resistance is just a temporary trait. This issue complicates the infection prognosis and production of effective treatments (Fothergill et al., 2012).

1.7 Carbapenem and *P. aeruginosa*

1.7.1 Carbapenem antibiotics

Beta-lactam antibiotics (including penicillins, monobactams, carbapenems, and cephalosporins) are by far the most extensively used class of antibacterial drugs (Meletis, 2016; bush and Bradford, 2016). They all have the same beta-lactam ring in their structure and function by binding to and inactivating the PBP, which is essential for passing through peptidoglycan layer in the cell wall (Zeng and Lin, 2013; Meletis, 2016; Lamont et al., 2019). Inhibition of peptidoglycan production prevents bacteria from reproducing and growing as well as affects the stability of the cell wall, resulting in cell lysis (Glen and Lamont, 2021; Letourneau and Calderwood, 2019).

Carbapenems are very effective beta-lactam antibiotics due to their great penetration into bacteria, high affinity for PBP enzymes, and they also have a particular structure that is comprised of a carbapenem attached to a beta-lactam ring. This structure gives them rigidity against the majority of beta-lactamases (Bedenić and Sardelić, 2018; Codjoe et al., 2017). Typically, carbapenems are recommended over other kinds of drugs in the treatment of serious infections due to the fact that their killing effect on the bacteria responsible for the infection is not reliant on the concentration of the antibiotic (Codjoe et al., 2017).

Carbapenems, which include imipenem, meropenem, ertapenem, and doripenem, are the most recently produced beta-lactams. They are mainly used to treat infections caused by multidrug-resistant (MDR) organisms (Elshamy and Aboshanab, 2020). Imipenem is the first commercially accessible carbapenem and was licensed in 1985 to

be used in the medical field (Sahuquillo-arce et al., 2015). It is used to treat an array of infections, including UTIs and lower respiratory tract infections, especially those caused by cephalosporin-resistant bacteria (Elshamy and Aboshanab, 2020). Meropenem is relatively close to imipenem in terms of its pharmacology and antimicrobial range of activity. The only thing that makes it different from imipenem is that it is not degraded by dipeptidases. It is also less capable of inducing seizures than imipenem (Fernandes et al., 2013). The carbapenem known as ertapenem was first identified in the year 2001. Although it is a bactericidal beta-lactam antibiotic with a wide range of activity, its effectiveness is lowest against non-fermentative gram-negative bacteria (Codjoe et al., 2017; El-gamal et al., 2017). In 2007, another carbapenem was approved by the FDA (Food and Drug Administration) called doripenem, which is particularly resistant to hydrolysis by the vast majority of beta-lactamases, in comparison to other carbapenems, and it has lower minimum inhibitory concentrations for *P. aeruginosa* than meropenem and imipenem (Lima et al., 2020; Codjoe et al., 2017).

1.7.2 Carbapenem-resistance mechanisms in *P. aeruginosa*

P. aeruginosa is a serious opportunistic pathogen that is responsible for a variety of nosocomial outbreaks (Malkoçoğlu et al., 2017). *P. aeruginosa* infections are challenging to control because it is intrinsically resistant to several antipseudomonal agents and their capacity to rapidly acquire resistance to antibiotics (Feng et al., 2021, Malkoçoğlu et al., 2017). Recently, the prevalence of *P. aeruginosa* isolates that are resistant to the majority of currently available antipseudomonal drugs has considerably increased. Hence, the morbidity and mortality rates related to *P. aeruginosa* infection have rapidly increased, and the incidence of nosocomial infections caused by multidrug-resistant (MDR) *P. aeruginosa* has become a global concern (Cho et al., 2018; Çiçek et al., 2021; Muderris et al., 2018).

Carbapenems (especially imipenem and meropenem) are considered to be the last line of treatment against severe infections caused by MDR *P. aeruginosa* (Walsh and Amyes, 2007; Feng et al., 2021). Nevertheless, carbapenem resistance has been reported all over the world, and carbapenem-resistant *P. aeruginosa* is about 10-50% in the

majority of countries. Nevertheless, increased carbapenem resistance has been reported in this pathogen all over the world, with carbapenem-resistant *P. aeruginosa* prevalence varying from 10-50% in the majority of countries (Çiçek et al., 2021). Carbapenem-resistant *P. aeruginosa* is sometimes resistant to nearly all other antibacterials, which makes infections more difficult to treat and increases morbidity and mortality rates among patients that are hospitalized or have compromised immune systems (Shaaban et al., 2017). There are three main mechanisms through which *P. aeruginosa* may develop carbapenem resistance. Including mutations in the outer membrane porin OprD gene, overexpression of efflux pumps, and carbapenemase production (Verma et al., 2019; Feng et al., 2021).

1.7.2.1 Porin-mediated resistance

Gram-negative bacteria have a semi-permeable outer membrane that prevents drugs from penetrating quickly. The outer membrane performs a critical role in transferring metabolically essential compounds (Muderris et al., 2018). OprD is a significant outer membrane protein (OMP) porin that is essential for the intracellular entry of basic amino acids and carbapenems (Mirsalehian et al., 2017). Alterations in the expression of OprD porin or mutations in its encoding genes result in dysfunction or lower expression of the OprD (Hashemizadeh et al., 2020; Muderris et al., 2018). this decreases the outer membrane permeability and inhibits carbapenems from entering and reaching their targets. So, the absence of OprD or decreased OprD protein expression may lead to carbapenem resistance in *P. aeruginosa* (Shaaban et al., 2017).

1.7.2.2 Overproduction of efflux pumps

Efflux pumps are well-known for their ability to remove antibacterial drugs and other medications from bacteria cells before they can reach their target (Fernández et al., 2012; Tenover et al., 2006). Active efflux or overexpression of efflux pumps implicates carbapenem resistance in *P. aeruginosa* (Codjoe et al., 2017; Elshamy and Aboshanab, 2020). The MexAB-OprM and MexXY-OprM pumps are the most critical in facilitating

resistance against carbapenems (Feng et al., 2021; Hashem Zadeh et al., 2020). Overexpression of the MexAB-OprM active efflux pump may result in increased resistance to meropenem and doripenem but not to imipenem because meropenem, ertapenem, and doripenem are substrates for the efflux system, although imipenem is not (Muderris et al., 2018).

1.7.2.3 Carbapenemase-mediated resistance

The synthesis of carbapenemase by *P. aeruginosa* and other gram-negative bacteria is one of the most significant and considerable mechanisms of carbapenem resistance in these bacteria (Malkoçoğlu et al., 2017). Gram-negative bacteria are susceptible to the spread of carbapenemases because their genes are typically found on mobile genetic components such as plasmids, integrons, and transposons, making this a serious issue worldwide (Elshamy and Aboshanab, 2020; Sahuquillo-Arce et al., 2015). Although, increasing the prevalence of carbapenem-resistant isolates that produce carbapenemases is a major issue since carbapenems are one of the last line therapy in the management of infections caused by MDR *P. aeruginosa* (Glen and Lamont, 2021).

Carbapenemases (β-lactamases that hydrolyze carbapenems) are classified into three groups of β-lactamases based on their molecular structures: A, B, and D. Group A and D have a serine residue at the active site to aid ring-opening and are thus referred to as serine β-lactamases (SBLs). Class B enzymes are Metallo beta-lactamase (MBLs), which have an active site that utilize zinc ions to facilitate bond hydrolysis.(Queenan and Bush, 2007; Diene and Rolain, 2014).

Class A carbapenemases:

Class A carbapenemases are a group of monomeric enzymes comprising 265-269 amino acids. These enzymes hydrolyze beta-lactams prior to their binding to PBP sites (Bakhat et al. 2019). It includes *Serratia marcescens* enzyme (SME), not metalloenzyme carbapenemase-A (NMC-A), Imipenem-hydrolyzing β-lactamase Imipenemase (IMI), *Klebsiella pneumoniae* carbapenemase (KPC), Guiana extended-spectrum carbapenemase (GES) (Rao, 2012). Enzymes also including SME, NMC-A, and IMI are

typically chromosomally encoded, while KPC and GES enzymes are plasmid-encoded (Diene and Rolain, 2014). These enzymes hydrolyze penicillins, carbapenems, cephalosporins, and aztreonam, and are commonly blocked by tazobactam and clavulanic acid (Sahuquillo-arce et al., 2015).

SME is one of the carbapenemases that are found on the chromosome. It was identified for the first time in a *Serratia marcescens* isolate from the United Kingdom in 1982. Recently, there are only three distinct categories of SME (SME-1, SME-2, and SME-3) (Rao, 2012). NCM-A enzyme was discovered from an *Enterobacter cloacae* isolate in France in 1990, and it was later found in Argentina and the United States (Codjoe et al., 2017; Queenan and Bush, 2007). IMI was initially identified in an *Enterobacter cloacae* isolates from the United States in 1984 (Rao, 2012). NMC-A and IMI share 97 percent of their amino acid sequences, making them almost similar in structure (Queenan and Bush, 2007). for the reason that SME, NMC-A, and IMI are chromosomally encoded, these enzymes are not broadly distributed across *P. aeruginosa* and other gram-negative bacteria and rarely have an impact on the incidence of carbapenem resistance (Ivanova, 2014).

The KPC carbapenemase is the most prevalent enzyme among plasmid-encoded class A carbapenemases (Codjoe et al., 2017). In 1996, it was firstly identified in a *K. pneumoniae* strain from the United States. To date, thirteen kinds of KPC have been described. KPC-2 and KPC-3 are the most frequently reported (Queenan and Bush, 2007; Elshamy and Aboshanab, 2020). These enzymes provide resistance to all imipenem, penicillins, aztreonam, and cephalosporins, but can be inactivated by clavulanic acid (Rao, 2012). Another considerable serine carbapenemase is GES, which was identified in a *Klebsiella pneumoniae* strain from French Guiana in 2000 and has now gained widespread recognition (Queenan and Bush, 2007). GES enzymes are encoded by genes found in integrons on plasmids, which means that they are transferable. So far, twenty-two variants of GES have been reported. All of the *P. aeruginosa*, *E. coli*, and *K. pneumoniae* isolates have been shown to synthesize these enzymes (Ivanova, 2014; Rao, 2012).

Class B carbapenemases:

Class B beta-lactamases, also known as Metallo-β-lactamases (MBL), are plasmid-encoded and the most diverse class of carbapenemases in terms of molecular diversity. They can hydrolyze almost all beta-lactam antibiotics except aztreonam, but they cannot be inactivated by commonly available beta-lactamase inhibitors, such as clavulanate, tazobactam, and sulbactam (Hashemizadeh et al., 2020; Sahuquillo-arce et al., 2015). The process of hydrolysis is based on interactions between beta-lactams and zinc ions in the active site of the enzyme, which is inhibited by metal ion chelators such as ethylene-diamine-tetra-acetic acid EDTA, a chelator of Zn^{2+} and other divalent cations (Codjoe et al., 2017). Therefore, this class of carbapenemases is a serious issue because of the risk of horizontal gene transfer, a lack of therapeutically relevant inhibitors, as well as a wide hydrolytic feature that degrades all beta-lactams except aztreonam (Ivanova, 2014).

P. aeruginosa MBL-producing isolates were initially discovered in Japan in 1991, and MBLs have been found in carbapenem-resistant *P. aeruginosa* isolates all over the world since then. The production of MBLs is one of the most prevalent and considerable mechanisms of carbapenem resistance in this opportunistic pathogen (Elshamy and Aboshanab, 2020, Phu et al., 2020). MBL-producing *P. aeruginosa* has a significantly higher mortality rate than non–MBL producing *P. aeruginosa* (Khorvash et al., 2017). The most commonly metallo-β-lactamases identified in *P. aeruginosa* are imipenemase metallo-β-lactamase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM), Germany imipenemase (GIM), and Sao Paulo metallo-β-lactamases (SPM) (S. Alsaadi et al., 2020). These enzymes are encoded by genes located on a plasmid and hence may be transmitted from *P. aeruginosa* to other gram-negative bacteria especially the *Enterobacteriales*, increasing the incidence of antibiotic resistance and complicating infection management (Phu et al., 2020).

It is suspected that the frequency of *Pseudomonas species* that generate MBLs varies across different geographical regions, and even across medical centers within the same geographic area. For example, it has been observed that the prevalence of *P. aeruginosa*, which produces MBLs, varies between ten to 56.8 percent in the country of Turkey (Muderris et al., 2018). Among the different kinds of metallo-β-lactamases in *P.*

aeruginosa, the VIM and IMP types are the most prevalent globally, while the VIM and GES types have been the most frequently observed in Turkey (Çekin et al., 2021; Malkoçoğlu et al., 2017). The VIM and IMP families are homologous in that they are both carried on plasmids and integron-associated, hydrolyze all beta-lactams except monobactams, and are both susceptible to all beta-lactam inhibitors (Codjoe et al., 2017).

Class D carbapenemases:

These comprise the oxacillinases (OXA) enzymes, which were named for their ability to critically hydrolyze oxacillin. These enzymes are serine carbapenemases that are mostly identified in *Enterobacteriales* and *P. aeruginosa* (Queenan and Bush, 2007). In 1993, the first OXA enzyme with carbapenemase activity was found in an MDR *A. baumannii*. Most OXA carbapenemases have been identified in this opportunistic pathogen. However, carbapenemases from this class have shown the ability to spread quickly across different gram-negative bacteria because they are plasmid-encoded (Bakhat et al. 2019; Ivanova, 2014). To date, 239 OXA B-lactamases have been identified, at least 9 of which are ESBLs and approximately 37 of which are carbapenemases (Rao, 2012). Among all of these OXA enzymes, in Turkey, resistance is mainly screened for OXA-23, OXA-27, OXA-409, OXA-25, OXA-26, OXA-40, and OXA-48 (Bakhat et al. 2019). The main issues with these enzymes are their ability to rapidly modify, and expand their range of action, and the lack of inhibitors (Codjoe Et Al., 2017; Elshamy and Aboshanab, 2020).

2. MATERIALS AND METHODS

P. aeruginosa isolates were obtained from various samples (blood, wounds, urine, cerebrospinal fluid (CSF), and sputum) that were sent to the Tokat Gaziosmanpaşa University Research and Application Hospital Microbiology Laboratory from various clinics between 2016-2019 and were evaluated retrospectively. 51 (29.7%) of a total of 172 isolates were found to be carbapenem-resistant and included in the study. Identification and antibiotic susceptibility tests of the isolates were performed with the Vitek 2 (Biomerioux, France) automated system. The identified *P. aeruginosa* isolates were stored in skim milk at -80℃ till the study.

2.1 Bacterial reviving

In the first stage, stocks of *P. aeruginosa* isolates were revived by culturing on both blood agar (HiMedia Laboratories Pvt Ltd) and eosin methylene blue (EMB) (HiMedia Laboratories Pvt Ltd) agar, by using the streaking method. then incubated in the incubator at 37℃ for 24 hours. For the result affirmation, gram stain, and oxidase tests were performed.

2.1.1 Gram stain

Smears were prepared and fixed. And slides were stained with Crystal Violet (MOS LAB) for one minute, then washed with tap water. After that, it was flooded for one minute with Gram Iodine (MOS LAB), then rinsed with tap water. Decolorize with 95% ethyl alcohol (MOS LAB) for 15 seconds until the violet color flow disappears, then wash with tap water. Add counterstain with Safranin (MOS LAB) for 0.5 minutes, and wash with tap water. Finally, it was dried and examined microscopically under the oil drop.

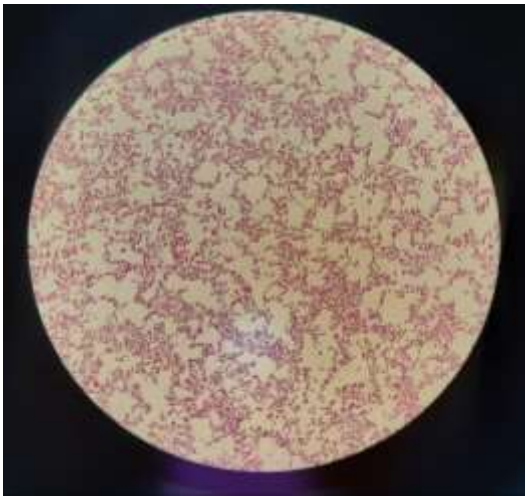

Figure 2.1: *P. aeruginosa* is a gram-negative rod-shaped bacterium.

2.1.2 Oxidase test

Oxidase test sticks (Liofilchem) were used for confirming whether the cultured bacteria are *P. aeruginosa* or not. Cytochrome oxidase, an enzyme produced by many gram-negative bacteria including *P. aeruginosa*, is utilized in oxidase Test Stick as a diagnostic biomarker for microbiological identification and differentiation. This test was done according to Liofilchem standards.

P. aeruginosa colonies were picked up and wiped off with the zone of the stick indicated by the arrows. The injected region was checked for a rapid color change (within 60 seconds) at the location of the inoculation. The appearance of a blue-purple color suggests a positive reaction. An organism that tests negative for cytochrome oxidase does not exhibit any color change, hence the test is considered negative (figure 2.2).

Note: *E. coli ATTC 25922* was used as a negative control.

Figure 2.2: Oxidase test sticks, the right-handed stick (blue-purple) is positive and it was *P. aeruginosa*. The left-handed stick was wiped off by *E. coli* ATTC 25922 which was negative (no color changes).

2.2 Antibiotic sensitivity test (AST)

Antibiotic sensitivity tests were performed for all isolates that were considered carbapenem-resistant via Vitek 2 (Biomerioux France). Also, disk diffusion (Kirby-Bauer) methods were used. Both imipenem 10 µg (Bioanalyse) and meropenem 10 µg (Liofilchem) discs are used as carbapenem antibiotics in this test and it was done as described by Hudzicki with the following steps:

2.2.1 Preparation of inoculum

Isolated colonies of the bacteria are taken with a sterile inoculating loop from blood agar. Suspend the bacteria in 2 mL of saline solution (biomerieux) in a sterile tube. saline tubes were vortexed to make a good suspension. The suspension's turbidity was measured to a 0.5 McFarland (Hudzicki, 2009).

2.2.2 Inoculation of the Muller-Hinton agar (MHA) plate

The plate of MHA (HiMedia Laboratories Pvt Ltd) is inoculated by dipping a sterile swab (WellKang Ltd) into the inoculum tube (which contains the bacteria) and swabbing the entire surface of the MHA medium. After that, antibiotic discs are placed

on the plate using sterile forceps. And it was incubated at 35℃ in the incubator (nuve incubator en 120) for 18 to 24 hours (Hudzicki, 2009).

2.2.3 Measuring zone sizes and reporting the results

Following overnight incubation, the diameter of the inhibition zone was measured (figure 2.3) in millimeters by using a ruler under the surface of the plate without opening the lid. The results of AST have been compared to the guideline of "The European Committee on Antimicrobial Susceptibility Testing." Breakpoint tables for the interpretation of MICs and zone diameters, Version 10.0, 2020. http://www.eucast.org/. to report the results. Isolates were considered resistant to imipenem when the inhibition zone diameter was less than 20 mm, and when the zone diameter was less than 18, it was considered meropenem resistant.

Figure 2.3: Antibiotic sensitivity test, the clear zone around antibiotic discs is a zone of inhibition.

2.3 Carbapenem inactivation method (CIM)

Carbapenemase activity was detected phenotypically by carbapenem inactivation methods according to Kuchibiro et al, it was applied to all carbapenem-resistant isolates to detect the carbapenem-hydrolyzing activity of these enzymes.

2.3.1 Simplified carbapenem inactivation method (sCIM)

A 0.5 McFarland standard suspension (using the direct colony suspension method) of *E. coli ATCC 25922* was diluted 1:10 in saline solution (biomerieux) and inoculated onto the MHA plate, using the standard disk diffusion method. Plates were left to dry for three to ten minutes. After that, overnight colonies of tested isolates of *P. aeruginosa* cultivated on blood agar were smeared onto a disk of imipenem 10 µg (Bioanalyse). The disk was rotated to allow one side of the disk to be evenly coated with the test bacteria; immediately afterward, the side of the disk having bacteria (*P. aeruginosa*) was put on the MHA plate previously inoculated with *E. coli ATCC 25922*. And another imipenem disk is put on MHA as a control (Kuchibiro et al., 2021).

Then all plates were incubated for 16-18 Hours at 35℃. After that, the results were recorded. When the zone of inhibition was more than 26 millimeters, the results were regarded to be negative (Kuchibiro et al., 2021).

Figure 2.4: The results of sCIM.

2.3.2 Modified carbapenem inactivation method (mCIM)

First of all, colonies of *P. aeruginosa* were cultured on MHA. Then emulsified in two ml of trypticase soy broth and the meropenem disk was immersed in the suspension and incubated at 35℃ for four hours. A 0.5 McFarland suspension of *E. coli ATCC 25922* was prepared in saline solution at a concentration of 0.5 McFarland. Subsequently, the *E. coli ATCC 25922* was inoculated on MHA using the standard disk diffusion method. Then, the meropenem disk was removed from the bacterial suspension and placed on an MHA plate previously inoculated with the *E. coli ATCC 25922* (Kuchibiro et al., 2021).

Plates were incubated at 35℃ for 16-20 hours. After that, the diameter of the inhibition zone (figure 2.4) was measured in millimeters by placing a ruler beneath the surface of the plate without trying to open the lid. The diameter of each inhibitory zone, as well as the diameter of the disc, was measured. When the diameter of the inhibition zone is less than 19 mm, it was considered a positive result (Kuchibiro et al., 2021).

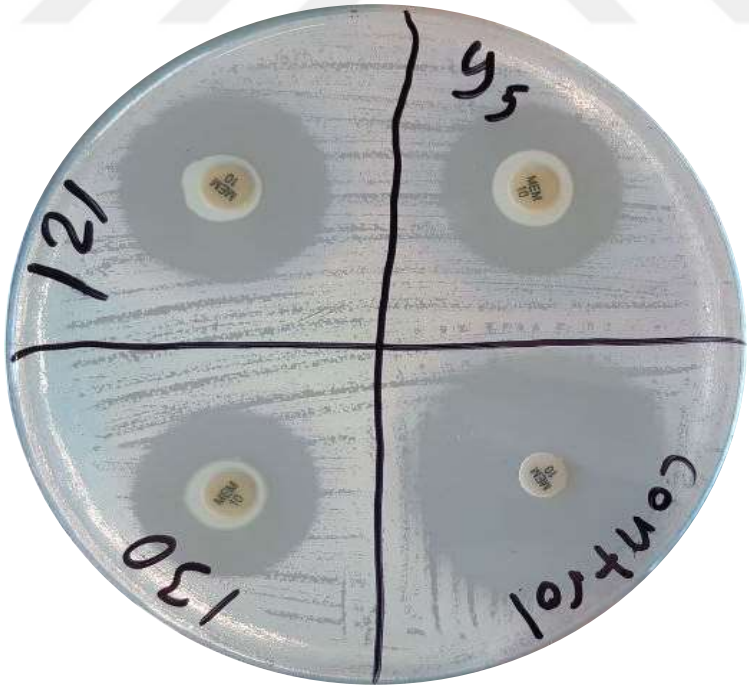

Figure 2.5: Represents the results of mCIM.

3. RESULTS

A total of 172 isolates of *P. aeruginosa* were recovered from different clinical samples of 172 patients from different units. Only 51 (29.7%) samples were resistant to Imipenem and/or Meropenem. These 51 isolates were considered carbapenem-resistant and were included in this study.

The carbapenem-resistant isolates of *P. aeruginosa* were collected from different clinical specimens, which included wounds (n = 17), sputum (n = 15), blood (n = 11), urine (n = 5), and cerebrospinal fluid (n = 3) from different patients. In addition, 32 (62.8%) samples were obtained from male patients, and 19 (37.3%) samples were obtained from female patients. Furthermore, these samples were collected from a variety of clinical units, including neurology (n =10), general surgery (n =8), internal medicine (n =7), and pediatric (n =6), as well as three samples from each gastroenterology service unit, Intensive care unit, oncology, and orthopedic. Anesthesia (n = 2), and one sample were received from each of the cardiology, chest diseases unit, infectious diseases, neurosurgery intensive care unit, oncology service, and plastic surgery (Figure 3.1)

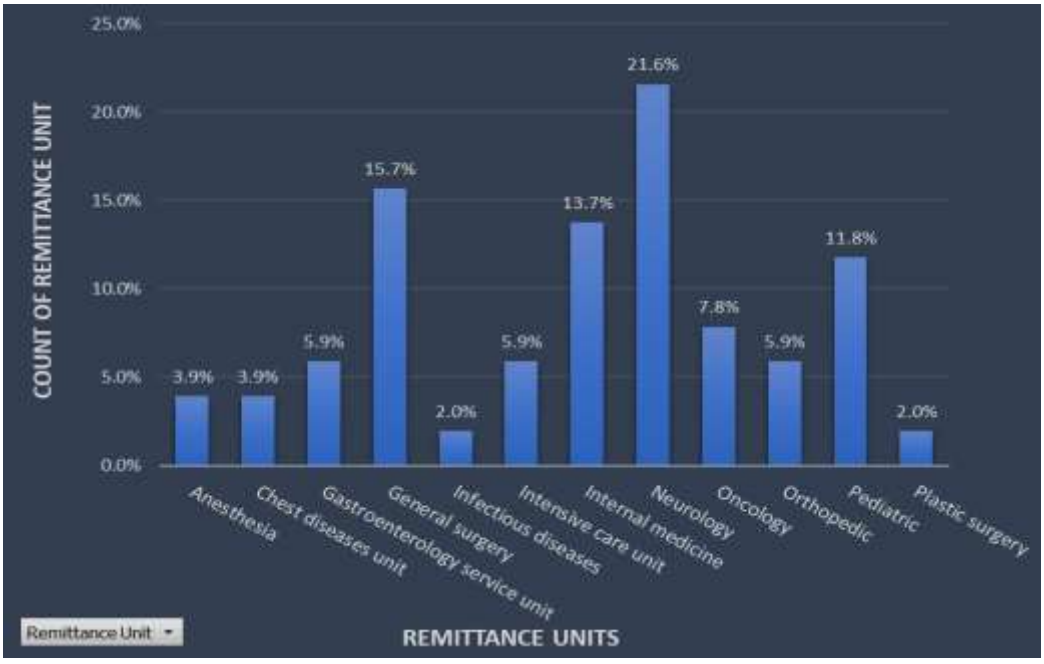

Figure 3.1: Represents the various clinics from which samples were collected.

3.1 Antibiotic sensitivity test (AST)-result

Figure 3.2 shows the AST results of both imipenem and meropenem. 38 (74.5%) isolates were resistant to both imipenem and meropenem. Eight (15.7%) isolates were only resistant to imipenem. And five (9.8%) isolates were resistant to meropenem.

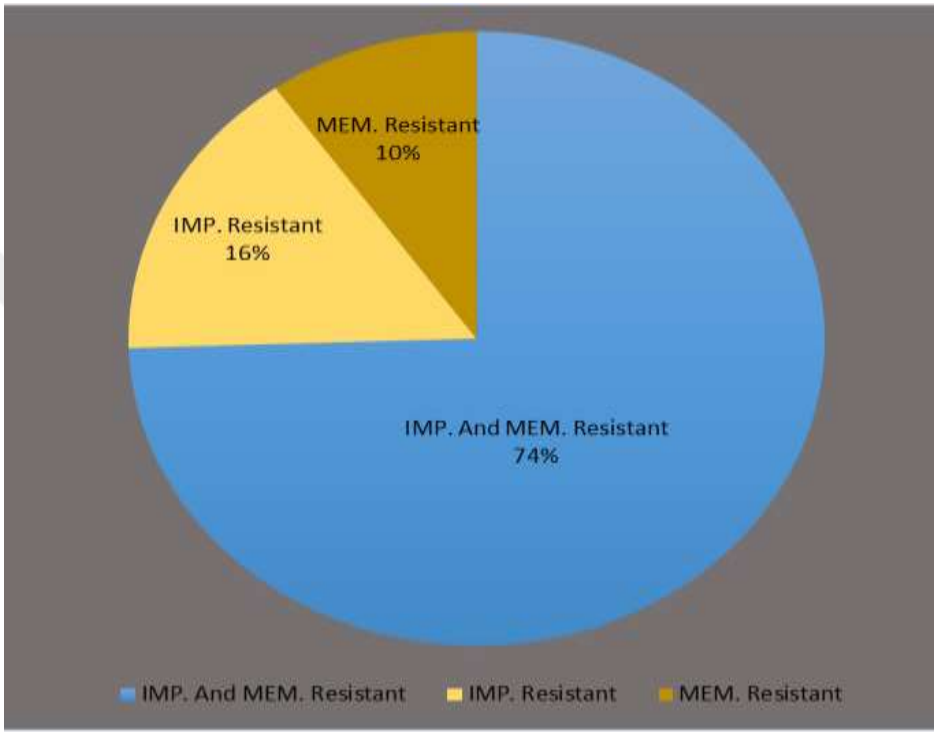

Figure 3.2: Antibiotic sensitivity test results.

3.2 Carbapenem inactivation method-result

3.2.1 sCIM-result

After the incubation period, we recorded the results. As we mentioned before, the isolates were considered to be positive when the zone diameter was less than 26 mm, so all isolates were negative by this method.

3.2.2 mCIM-result

The isolates were considered positive when the diameter of the inhibition zone was less than 19 mm. So according to mCIM 31 (60.8%) isolates were considered to produce the carbapenemase enzyme.

4. DISCUSSION

P. aeruginosa is ubiquitous bacteria that can survive in different environmental conditions. It is an opportunistic pathogen that causes a wide variety of infections that may be fatal, especially in immunocompromised patients (AL-Fridawy et al., 2020). Most antibiotics typically have limited effectiveness against this bacteria due to it is adaptability and high intrinsic antibiotic resistance. Carbapenemase production is a critical factor of carbapenem resistance in *P. aeruginosa*. as a consequence, identifying and monitoring carbapenemase-producing isolates has become critical for the selection of the most effective treatment strategies and the successful execution of infection control measures in hospital settings (Doret et al., 2012; Ivanova, 2014). Therefore, the investigation of carbapenemase enzymes was the focus of the present study.

Carbapenems are the most efficient antibiotic classes for treating pseudomonas infections and are now the last choice for treatment of infections caused by multidrug-resistant *P. aeruginosa* infections (Silva et al., 2020). Unfortunately, improper early antibiotic therapy and/or delay of proper antibiotic therapy are associated with high mortality rates and extended hospital stays in patients with severe *P. aeruginosa* infections, highlighting the significance of early administration of efficient and appropriate antibacterial therapy (Sader et al., 2017; Malkoçoğlu et al., 2017). Hence, the resistance of bacteria to these antibiotics has been broadly studied. For instance, in 2017, Cai et al. retrospectively analyzed the prevalence of infections that caused by carbapenem-resistant gram-negative bacteria in the United States (US), including 56,477 isolates of *P. aeruginosa*. They used data from 206 acute care hospitals in the US over a period of five years. They found that *P. aeruginosa* is one of the leading causes of carbapenem resistance infections in the US and had the second-highest rate of carbapenem resistance, which varied by infection site: 10.3% in the blood, 19.4% in the respiratory tract, 11.7 % in the urine, and 12.6 % in other sites. This survey also indicated that the incidence of infections caused by carbapenem-resistant gram negative pathogens differed depending on the site of the illness. Infections with *P. aeruginosa* were most common in the respiratory system (40.1%). The researchers concluded that patients with

carbapenem-resistant infections cost the healthcare system more since they required longer hospital and intensive-care unit stays, more medications, and a higher fatality rate. (Cai et al.,2017).

Qin et al. examined the antibiotic resistance patterns of *P. aeruginosa* among older patients (65 to 99 years old) in a tertiary hospital over the course of three years. A total of 600 non-duplicated patients with *P. aeruginosa* were studied. They discovered carbapenem resistance in 25.8% (155/600) of clinical isolates of *P. aeruginosa*, which also demonstrated greater resistance to all tested antibacterial agents than carbapenem-susceptible isolates. The study identified multiple independent predictions for infections caused by carbapenem-resistant *P. aeruginosa* in elderly hospitalized patients, including antibiotic use in the past, cerebrovascular disease, indwelling catheters, and an albumin level of less than 35 g/L, and hospitalization for at least 14 days. In addition, they indicated that the use of carbapenems and cephalosporins of the third or fourth generation within the past 30 days is a considerable risk factor for carbapenem-resistant *P. aeruginosa* infection. This increases the risk by seven to thirteen times (Qin et al., 2022).

Because the rate of carbapenem resistance is increasing globally, prompting the WHO to classify these bacteria as critical priority pathogens for new antibiotic discovery and production for antibiotic-resistant bacteria. In 2017, the WHO published a priority list, categorizing them into three groups based on their need for novel antibiotics: priority 1 (critical), priority 2 (high), and priority 3 (medium) *P. aeruginosa* has been called a "critical pathogen" on the priority list of WHO because it has become resistant to almost all currently available antibiotics, including carbapenems (Tacconelli et al., 2018).

In addition, a report on antibiotic resistance threats in the US was issued by the Centers for Disease Control and Prevention (CDC) in 2019. In this report, the CDC classified 18 different types of pathogens into three categories: urgent, serious, or concerning. It was determined that the multidrug-resistant *P. aeruginosa* was classified as a serious threat. Some isolates of multidrug-resistant *P. aeruginosa*, including the carbapenem-resistant strains, are resistant to nearly all antibacterial agents. Two to three percent of *P. aeruginosa* that is resistant to carbapenems have a mobile genetic element that makes the enzyme carbapenemase. They also highlighted that drug development

should be focused on the most dangerous infections, like those caused by carbapenemase-producing bacteria, including carbapenem-resistant *P. aeruginosa* (CDC. 2019)

Carbapenem resistance has also been reported in Turkey and multiple studies have been performed on this issue. Acar et al. performed a meta-analysis in 2019 to determine the resistance prevalence of *P. aeruginosa* to several antibiotics, including carbapenems, over ten years. They discovered that the overall prevalence of meropenem resistance was 30.1% and the overall prevalence of resistance to imipenem was 28.0%. In this meta-analysis, the pooled prevalence of meropenem resistance was found in 30.1% of isolates, whereas imipenem resistance was found in 28.0%. In the first five years of the study, these rates were 25,8% and 25,6%, respectively, but in the second five years, resistance rates critically raised to 36,3% and 32,9%, respectively. The researchers concluded that the prevalence of antibiotic resistance in pathogenic strains of *P. aeruginosa* in Turkey is considerably high and continues to rise compared to previous years. The frequency of carbapenem-resistant *P. aeruginosa* infections has reached an extremely high level. (Acar et al., 2019).

Isik et al. performed a meta-analysis for analyzing the antimicrobial susceptibility of *P. aeruginosa* strains recovered from blood cultures in Turkey over a period of 11 years. They found that the antibiotic resistance rate seriously increased in *P. aeruginosa* particularly carbapenem, imipenem resistance rate in *P. aeruginosa* was 26.8%, according to a meta-analysis of 1421 isolates. The meta-analysis of 637 isolates showed that 25.1% were meropenem resistant. The authors concluded that their meta-analysis demonstrated that the frequency of *P. aeruginosa* strains resistant to carbapenems has dramatically risen in Turkey as a direct result of the extensive usage of these antibiotics. They also mentioned that we should avoid using these antibiotics experimentally and instead prioritize the use of other antibiotics that have anti-pseudomonal activity to treat infections caused by these pathogens (Isik et al.,2021). Similarly, the findings of our study showed that resistance to imipenem and meropenem is critically high. And this means that carbapenem resistance is alarmingly prevalent in Turkey. In order to avoid or minimize the development and further spread of bacterial resistance, infection prevention and control and principled antibiotic utilization strategies need to be improved, and antibiotic resistance profiles should be monitored continuously.

Production of carbapenemase enzymes by *P. aeruginosa* has become a serious health issue globally as these enzymes inactivate almost all beta-lactam antibiotics, including carbapenems. Their production is one of the main resistance mechanisms of *P. aeruginosa*, and it is easily spread between various bacterial species, which frequently carry multiple resistance determinants, thereby limiting treatment options. For these reasons, these enzymes have been studied in numerous studies worldwide, by using different phenotypic and genotypic methods. Finding carbapenemase enzymes by molecular techniques is the gold standard but these methods are more expensive, special equipment is necessary and unable to identify unknown and novel resistance genes (Aktaş et al., 2017). In contrast to that, several phenotypic methods have been developed for the detection of carbapenemase production including the carbapenem inactivation method (CIM). Both simplified carbapenem inactivation method (sCIM) and modified carbapenem inactivation method (mCIM) are among the most commonly used procedures for phenotypically determining carbapenemase production since these methods are simple, reliable, and can be carried out in any laboratory.

CIM has been widely utilized for the identification of carbapenemase enzyme and also it was evaluated in several studies. For example, a research conducted by Akhi et al. evaluated phenotypic methods to investigate carbapenemase production in isolates of carbapenem-resistant *P. aeruginosa*. Carbapenem resistance was found in 121 of the 245 *P. aeruginosa* isolates tested. 35 (28.9%) carbapenem-resistant isolates had positive findings when tested with CIM. Moreover, CIM has been shown to be highly effective considering its sensitivity, specificity, low cost, and simplicity of result interpretation. CIM was suggested to be used routinely in clinical laboratories for the identification of carbapenemase-producing *P. aeruginosa* (Akhi et al., 2017).

A comparative study between the Carbapenem Inactivation Method and the RAPIDEC CARBA NP for the Screening of Carbapenemase-Producing Gram-Negative Bacteria was conducted by Aktaş et al. 84 isolates of carbapenem-resistant *P. aeruginosa* were screened to investigate the presence of the carbapenemase enzyme., but it was detected in only three isolates by CIM. Several advantages of CIM were highlighted, including cost-effectiveness, the use of simple types of equipment that are broadly supplied at laboratories, and interpreting CIM findings is relatively easy. (Aktaş et al.,

2017). CIM was also evaluated in a research caried out by Gutiérrez et al. They highlighted that CIM is highly promising method for routine use because of its sensitivity and specificity. It is also inexpensive and simple to use. Furthermore, utilizing the disks to evaluate the inhibition zones simplifies the interpretation of the results and improves both sensitivity and specificity of the test (Gutiérrez et al., 2019).

in 2021, Beig et al. did a study to determine the carbapenemase production and to assess the efficiency of phenotypic methods, including CIM for quick and reliable identification of carbapenemase enzyme in 97 different strains of *P. aeruginosa* that had been obtained from samples collected in hospitals located in Hamadan, Iran. Their findings show that 49 isolates were resistant to carbapenems. Carbapenem enzyme production was identified in 46 isolates of carbapenem-resistant *P. aeruginosa* by using the carbapenem inactivation method. They demonstrated that CIM methods can be used for fast and accurate identification of carbapenemase enzymes. Additionally, it is suitable to use in medical microbiology labs (Beig et al., 2021).

In 2017, Malkoçoğlu et al. aimed to study the carbapenemase production in 84 isolates of carbapenem-resistant *P. aeruginosa*. The CIM was utilized to identify carbapenemase synthesis phenotypically. But a phenotypic test showed carbapenemase was produced by only three isolates and they noted that the carbapenem resistance in other strains could be due to another mechanism, for example, porin alterations, and efflux pumps. Malkocoglu et al. also emphasized that, because these enzymes are becoming more common, monitoring investigations are continuously required to control the transmission of resistant isolates (Malkoçoğlu et al., 2017). Çekin et al. did a 14-month study in Turkey on the production of carbapenemase enzymes in isolates of carbapenem-resistant *P. aeruginosa*. According to their results, the production of carbapenemase enzyme was discovered in two of the 45 *P. aeruginosa* isolates that were resistant to carbapenems, both of which were multidrug-resistant due to the presence of other resistance determinants (Çekin et al., 2021). Moreover, Carbapenemase production and their encoding genes were screened among 70 isolates *P. aeruginosa* in another research by Çiçek et al. conducted at the medicine faculty of Recep Tayyip Erdoğan University. Their isolates were recovered from different samples and obtained from

different clinical units. They reported that 34.3% of isolates were resistant to carbapenem, also the genes encoding these enzymes were found in 36 isolates (Çiçek et al., 2021).

Tufekci et al. conducted a study at Kastamonu training and research hospital in Turkey to investigate these enzymes in 73 isolates of carbapenem-resistant *P. aeruginosa* and they found that 78% (n =57) of isolates produce these enzymes (Tufekci et al., 2021). The results of the present research followed their findings, as we studied the activity of carbapenemase in 51 isolates that were resistant for carbapenems and 60.8% of isolates were found to be positive for carbapenemase activity by the phenotypic CIM test. The results of this study revealed a noticeably high prevalence of carbapenemase-producing *P. aeruginosa* isolates at our hospital.

Early and further investigation of carbapenemase production in *P. aeruginosa* is urgently needed to prevent carbapenemase spread among gram-negative bacteria in healthcare settings, and reliable and appropriate phenotypic laboratory tests to detect carbapenemase-producing *P. aeruginosa* are available. Based on the results of this study, the modified carbapenem inactivation test could be used more often in the lab because it is a simple and accurate method for the identification of carbapenemase synthesis.

5. CONCLUSION

1- 172 non-duplicate *P. aeruginosa* isolates were recovered from different clinical samples that were submitted to the microbiology department. Only 51 isolates were considered carbapenem-resistant and included in the current study.

2- *P. aeruginosa* carbapenem-resistant isolates were obtained from a variety of clinical specimens, including wounds (33.33%), sputum (29.41%), blood (21.57%), urine (9.80%), and cerebrospinal fluid (5.88%).

3- 62.75% samples were obtained from male patients, and 37.25% samples were obtained from female patients.

4- these samples were collected from different clinical departments, including Neurology, general surgery, internal medicine, pediatric, gastroenterology service unit, Intensive care unit, Oncology, Orthopedic, anesthesia, cardiology, chest diseases unit, infectious diseases, neurosurgery intensive care unit, oncology service, and plastic surgery department.

5- 74.51% of isolates were resistant to both imipenem and meropenem. 15.69% of isolates were only resistant to imipenem. And 9.80% of the isolates were resistant to meropenem.

6- Carbapenemase was found to be produced by 31 (60.8%) of the isolates that were studied.

7- Carbapenemase-mediated carbapenem resistance must be identified in order to facilitate infection prevention and control, patient monitoring, and efforts to improve public health.

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