**Republic of Iraq**

**Ministry of Higher Education**

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**College of Science**

**Department of Biology**

# The inhibition effect of antibiotics on biofilm formation by *Pseudomonas aeruginosa*

A Thesis

Submitted to the College of Science, University of Diyala in Partial Fulfillment of the Requirements for the Master Degree of Science in Biology

**By**

## Neyaf Majid Ali Alageedi

B.Sc. Biology / College of Science / University of Diyala, 2018

**Supervised by**

**Dr. Kareem Ibrahim Mubarak**

Assistant Professor

**2021 A.D.** **1442 A.H.**

بسم الله الرحمن الرحيم

﴿ يَا أَيُّهَا
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صدق الله العظيم

سورة المجادلة: الآية 11

# Certification

I certify that this thesis entitled " **The inhibition effect of antibiotics on biofilm formation by *Pseudomonas aeruginosa* "** was prepared under my supervision at the Department of Biology / College of the Science / University of Diyala, as a partial requirement for the degree of Master of Science in Biology.

Signature**:**

Supervisor

**Dr. Kareem Ibrahim Mubarak**

Assistant Professor

Date: / / 2021

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Signature:

**Dr. Ibrahim H. Mohammed**

Professor

Head of Department of Biology

Date: / / 2021

## Scientific Amendment

I certify that the thesis entitled " **The inhibition effect of antibiotics on biofilm formation by *Pseudomonas aeruginosa*** " presented by (Neyaf Majid Ali Alageedi) has been evaluated scientifically, therefore, it is suitable for debate by the examining committee.

Signature:

Name: Salih Abdul Redha Salih Al-Bakri

Title: Assistant Professor

Date: / / 2021

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I certify that the thesis entitled " **The inhibition effect of antibiotics on biofilm formation by *Pseudomonas aeruginosa*** " presented by (Neyaf Majid Ali Alageedi) has been evaluated scientifically, therefore, it is suitable for debate by the examining committee.

Signature:

Name**:** Nihad Khalawe Tektook

Title: Assistant Professor

Date: / / 2021

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Signature:

Name: Esam Hamid Hameed

Title: Lecturer

Date: / / 2021

## Examining Committee

We certify that we have read this thesis entitled " **The inhibition effect of antibiotics on biofilm formation by *Pseudomonas aeruginosa* "** and we examined the student (Neyaf Majid Ali Alageedi) in its content and in what is related to it, at ( / /2021) and in our opinion it meets the standard of a thesis for the degree of master of science in Biology.

**(Chairman)**

Signature:

Name: Dr. Hadi Rahman Rasheed Al-Taai

Title: Professor

Date: / / 2021

**(Member)**

Signature:

Name: Dr. Alyaa Maan Abdelhameed

Title: Assistant Professor

Date: / / 2021

**(Member)**

Signature:

Name: Dr. Raya Ezat Maroof

Title: Assistant Professor

Date: / / 2021

**(Member/supervisor)**

Signature:

Name: Dr. Kareem Ibrahim Mubarak

Title: Assistant Professor

Date: / / 2021

Approved by the dean of the college of the science / University of Diyala.

**(The Dean)**

Signature:

Name: Dr. Tahseen Hussein Mubarak

Title: Professor

Date: / / 2021

# *Dedication*

*To the decent parents, may God protect them*

*To my brothers and sister*

*To my grandmother, may God protect her*

*To the soul of my grandfather, may God have mercy on him*

*To everyone who thinks and seeks to advance science everywhere*

*Dedicate this humble effort*

***Neyaf***

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*Neyaf*

# *Summary*

# Summary

Two hundred specimens were collected from different sources (urine, wounds, ear, burns, and sputum) from governmental hospitals in Baquba city/ Diyala province, from September 2020 to January 2021. All the specimens were cultured on selective and differential media. Twenty-six isolates of *P.aeruginosa* were identified by colony characteristics, microscopic examination, and biochemical tests. The identification of 26 isolates of *P.aeruginosa* was confirmed by VITEK-2 compact system.

These isolates gave a positive result for oxidase, catalase, and citrate utilization tests, showed an ability to growth on MacConkey agar, Pseudomonas agar, Blood agar, and caused β-hemolysis, growth at 42°C, and ability to pigment production. While they gave negative results to indole test, methyl red test, Voges-Proskauer test, lactose fermentation and growth at 4°C. TSI test, not fermented for any of the three types of sugars (glucose, lactose, sucrose), it does not form CO2 and does not H2S production.

The results of the phenotypic detection for some virulence factors showed all the isolates produced hemolysin, motility, lipase, pigments,protease, gelatinase and urease with percentages of 100%, 100%, 96.15%, 76.9%, 65.38%, 57.69% and 11.53% respectively.

Biofilm formation was detected by Microtiter plate quantitative method with different yields between strongly, moderately, and non-adherent. The result showed 24(92.30%) isolates were produced biofilm among them 38.4% of isolates were strongly biofilm producers and 53.8% moderately biofilm producers. While only two isolates 7.6% represented non-biofilm producers.

# Summary

The antibiotic susceptibility tests profile of 26 isolates were determined against 13 different types of antibiotics by the Kirby Bauer disc diffusion method. The results showed that the lower percentage of antibiotic resistance was against Piperacillin/Tazobactam 3.8% and a higher resistance percentage was seen against Ticarcillin/Clavulanic acid 69.2%. While resistance percentage for all Aztreonam, Cefepime, Meropenem, and Netilmicin was 38.4%. Ceftazidime, Ciprofloxacin, Levofloxacin and Tobramycin 34.6%.While the percentage of antibiotic resistance for Gentamicin and Imipenem was 26.9%, Amikacin 30.7%. Multi-drug resistance (MDR) formed 42.3% of total isolates in the current study.

Serial dilution method was used to determine the minimum inhibitory concentration (MIC), sub-minimum inhibitory concentration (Sub MIC), and minimum bactericidal concentration (MBC) were determined against two antibiotics were Imipenem (IMI) and Ceftazidime (CAZ) for ten isolates selected according to biofilm formation (strong biofilm). The results showed that there were differences in MIC and MBC values. MIC for Ceftazidime ranged from (16–1024 µg/ml) and MBC ranged from (128 - >1024 µg /ml). While MIC for Imipenem ranged (16-512 µg /ml) and MBC was (512- >1024). Sub MIC values were determined by selecting the lowest inhibitory concentration at which the bacteria could grow.

In the current study, each antibiotic was tested at a sub-minimum inhibitory concentration (Sub MIC) to study the change in the ability of *P. aeruginosa* isolates in the biofilm formation. Sub_MIC Ceftazidime and Imipenem affected biofilm by decreased the density of biofilm formation in most isolates after incubating for 24 hours. These effects indicate that sub-MICs Ceftazidime and Imipenem may influence several biofilm formation stages. Investigation of the effects of sub-MIC antibiotics on targeted bacterial

biofilms may lead to the development of antibiotic treatment modalities in the future.

Polymerase Chain Reaction (PCR) assay was carried out for detection of *psl*A, *psl*D, and *pel*A genes which was involved in the formation of biofilm among the clinical isolates. The present results clarified the presence of *psl*A, *psl*D, and *pel*A in all the studied isolates. The results showed 25(96.15%) of the isolates had both *psl*A and *psl*D genes, while 23(88.46%) of the isolates had the *pel*A gene. Almost all *P. aeruginosa* isolates carried *psl*A, *psl*D, and *pel*A genes regardless of the intensity of the biofilm.

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## List of abbreviations

| Abbreviations | key |
|---|---|
| µg/ml | Microgram per milliliter |
| ABC | ATP-binding cassette |
| AIDS | Acquired Immunodeficiency Syndrome |
| AIs | Autoinducers |
| bp | Base pair |
| CF | Cystic fibrosis |
| CFC | Cephalothin, Fucidin, Cetrimide |
| CFU/ml | Colony-Forming units per milliliter |
| CLSI | Clinical and Laboratory Standards Institute |
| DNA | Deoxyribonucleic acid |
| ECMs | Extracellular Matrix |
| eDNA | extracellular DNA |
| EDTA | Ethylenediamine tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EPS | Exopolysaccharide |
| ESBL | Extended-spectrum beta-lactamases |
| Eth. Br. M | Ethidium Bromide |
| ExoS | Exotoxin S |
| ExoT | Exotoxin T |
| ExoU | Exotoxin U |
| ExoY | Exotoxin Y |
| ICU | Intense care unit |
| LPS | Lipopolysaccharide |
| LRTI | Lower respiratory tract infections |
| MATE | Multidrug and toxic compound extrusion |
| MBC | Minimum Bactericidal Concentration |
| MBL | Metallo-β-lactamases |
| MDR | Multidrug-Resistant or Resistance |
| MIC | Minimum Inhibitor Concentration |
| NIH | National Institutes of Health |

| | |
|---|---|
| OD | Optical density |
| OM | Outer membrane |
| *P.aeruginosa* | *Pseudomonas aeruginosa* |
| PCR | Polymerase chain reaction |
| PDR | Pan-drug resistant |
| pH | Potential Hydrogen |
| QS | Quorum Sensing |
| RND | Resistance Nodoulation Division |
| rpm | Revolutions per minute |
| SMR | Small multidrug resistance |
| Sub MIC | Sub Minimum Inhibitory Concentration |
| T3SS | Type III Secretion System |
| TAE | Tris-acetate EDTA Buffer |
| UTI | Urinary tract infections |
| UV | Ultra violate |
| WHO | World Health Organization |
| XDR | Extensively-drug resistant |
| µL | Microliter |

# Chapter One

# Introduction

# 1.Introduction

*Pseudomonas aeruginosa* is an omnipresent gram-negative aerobic bacterium, an opportunistic pathogen widely spread and causes nosocomial infections (Al-Mayali and Salman, 2020) as well as fatal infections in immunocompromised individuals especially those patients with cancer, post-surgery, severe burns or infected by human immunodeficiency virus (HIV) (Gomila *et al*., 2018) despite the advent of newer and stronger antibiotics (Amoon *et al*., 2018).

These bacteria cause several types of infection including wound, gastrointestinal, urinary, and respiratory tract infections. The infections by these bacteria are increasing worldwide due to the survival, adaptation, and resistance mechanisms of different types of antimicrobials (Abdallah and Jabur, 2021). Also, infection with *P.aeruginosa* is particularly difficult to treat due to many intrinsic and acquired mechanisms of antibiotics resistance (Wijaya, 2021).

The wide spectrum of infection caused by bacteria depends on the presence of many virulence factors such as biofilms. Biofilms are defined as sessile and organized communities of mono or multispecies bacteria that adhere to biotic or abiotic surfaces (Costa *et al*., 2021) and their ability to form biofilms on both biotic and abiotic surfaces is an important factor contributing to the pathogenesis of *P.aeruginosa* (Saffari *et al*., 2017). Biofilms own advantage in several infections and greatly enhances the ability of bacteria to resist antibiotics and harsh environmental conditions (Sameet *et al*., 2020). Biofilm formation is an important mechanism for the survival of *P. aeruginosa* and its relationship with antimicrobial resistance represents a challenge for patient therapeutics (Costa – Limaa *et al*., 2018).

The matrix of bacterial biofilm consists of various polymers such as Exopolysaccharides, proteins, and extracellular DNA (eDNA). Exopolysaccharides, pyocyanine, rhamnolipids, and functional proteins are all factors that contribute to the development of *P. aeruginosa* biofilm stability, and protection (Hynen *et al*., 2021and Newman *et al*., 2017).

A core component of biofilm formation in *P.aeruginosa* is the biosynthesis of exopolysaccharides known as polysaccharide encoding locus (pel) and polysaccharide synthesis locus (psl), these are the most essential exogenous polysaccharides exploited in the formation of biofilms in the bacteria (Moradali and Rham, 2019). Past studies have suggested an important role for the psl gene cluster in initiating biofilm formation in *P. aeruginous*. The Psl operon contains 15 genes (pslA-O) involved in the synthesis of an exopolysaccharide (EPS) that is important for the formation of a biofilm for this bacteria. Biofilm formation by *P. aeruginosa* requires or includes multiple gene expressions. Another important gene for the formation of biofilm, development, and maintenance is the Pel gene. This gene is composed of seven gene operons (pelA-G) and is involved in pellicle and biofilm formation (Zimmer *et al*., 2013).

Understanding the effects of antibiotics on biofilms is of paramount importance in clinical practice due to the increased resistance toward antibiotics and dissemination of resistance in biofilms.

## Aims of the study

The study aims to explore the formation of biofilms and the effect of antibiotics on their formation and investigates the genes responsible for biofilm formation in *P. aeruginosa* by the following steps:

1. Isolation and identification of *P.aeruginosa* from clinical specimens and detection of some virulence factors in the isolates
2. Investigate the resistance of the isolates to several antibiotics and determine their minimum inhibitory concentration (MIC) among the clinical isolates
3. Phenotypic and molecular detection of some biofilm formation degree.
4. Study the effect of sub-minimum inhibitory concentration (sub-MIC) of some antibiotics on the biofilm formation in *P.aeruginosa* isolates

# Chapter Two
# Literature Review

# 2.Literature Review

## 2.1 General characters of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a gram-negative bacterium, belongs to the Pseudomonadaceae family, non-fermenting, non-spore-forming, rod-shaped, and aerobic. The bacteria can grows well at 37-42°C, positive for oxidase and catalase, measuring about 1-5 μm long and 0.5-1.0 μm wide, and contains one polar flagellum (monotrichous) essential for movement, chemotaxis, and adhesion ( Garcia *et al*., 2018).

This bacteria can produce various pigments, including bluish-green pyocyanin and yellowish-green fluorescein, as well as the potential for other pigments, such as yellow pyoverdin, dark pyorubin, and dark black pyomelanin, to be produced by certain strains (Cohen *et al*., 2017). Occasionally *P.aeruginosa* strains produce only pyoverdin, which is difficult to distinguish these strains from the other five fluorescent *Pseudomonas* species (Pezzlo *et al*., 2020). *P. aeruginosa* possesses a mucous layer to produce the alginate slime layer which inhibits the process of phagocytosis. As well as, multi-layers of extracellular polysaccharides (Al-Daraghi and Al-Badrwi, 2020).

*Pseudomonas* has minimal nutritional requirements for their growth as a group and can use a wide range of environmental nutrition sources, *P. aeruginosa* often requires only acetate and ammonia as the source of carbon and nitrogen, respectively. Also, *P.aeruginosa* is capable of anaerobic growth and does not ferment, but rather obtains energy from sugar oxidation. In marginal environments such as dry surfaces of hospital operating rooms, hospital rooms, clinics, and medical equipment, as well as sinks and showers, this minimal nutritional requirement enables it to grow and thus has proven to be an important source of nosocomial infection (Farahi *et al*., 2018 ).

## 2.2 Classification of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a member of the Pseudomonadaceae family. Which contains many species with the *Pseudomonas* genus and the Pseudomonadaceae family is divided into 5 groups based on rRNA / DNA homology and common culture characteristics (Riedel *et al*., 2019).

**The scientific classification of *Pseudomonas aeruginosa* shall be as follows:**

**Domine** : Bacteria

**Phylum**: Proteobacteria

**Class**: Gamma Proteobacteria

**Order**: Pseudomonadales

**Family**: Pseudomonadaceae

**Genus**: *Pseudomonas*

**Species**: *aeruginosa*

## 2.3 Pathogenicity of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is an opportunistic human pathogen, especially in patients who are immunocompromised (Al-Mayali and Salman, 2020), often fatal infections (Palavutitotai *et al*., 2018), and can also cause plant disease (Schroth *et al*., 2018). It deems one of the most causative agents of nosocomial contagions in Baghdad (Al-Shimmary *et al*., 2017) In the United States of America (USA), it is among the first six types of bacteria responsible for nosocomial infections (Tümmler, 2019).

As a nosocomial bacterium, *P.aeruginosa* has the ability to colonize wounds and catheters and thus can be promoted among various hospital sectors such as urology units, burns units, and intensive care units (ICUs) (Al-Saeedi and Raheema, 2019).

Urinary tract infections, primary skin infections (burn and wound with blue-green pus), eye infection, ear infection, soft tissue infections, intra-abdominal infections (Tang *et al.*, 2017), bacteremia (Hilliam *et al.*, 2020), and lung infections in cystic fibrosis (CF) patients, it the dominant lung infecting organism (O'Toole, 2018), was communal infections resulted from this bacterium (Al-Dahmoshi, 2017).

*Pseudomonas aeruginosa* is capable of invading tissues and producing toxins that lead to complex infections, colonizes the mucous membrane and skin, and thus causes infections that are stimulated by pili and then by the growth of virulent factors such as toxins and enzymes such as protease, which break down protein fibers to reveal bacterial receptors, and its ability to invade tissues depends on its resistance to phagocytosis and the host immune defenses and its secretion of external enzymes and toxins such as protease, elastase, coagulase, hemolysin, lipase, gelatinase, DNase, as well as alkaline phosphatase, lecithinase which are external virulence agents that break down physical barriers and participate in invasion bacterial (Al-mamari, 2019).

Its infection efficacy and antibiotic resistance capacity have made the organism recognized as a public health threat (Golle *et al.*, 2017). Where *Pseudomonas aeruginosa* has recently been announced by the World Health Organization to be one of the main priority pathogens for which new antibiotics are desperately needed (WHO, 2019 ).

## 2.4 Epidemiology and Spreading of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is widely dispersed in nature and is particularly abundant in soil and water because of its narrow nutritional requirements, which, despite adverse physical and chemical conditions, enhances its distribution, proliferation, and survival (Elshafiee *et al*., 2019). It has the capacity to live in different environments including hospital environments, on medical equipment, such as mechanical ventilators, urinary or dialysis catheters and endoscopes, in sinks, and anesthesia equipment (Azam and Khan, 2019). This bacteria can colonize humid environments and lives preferably in water environments such as rivers, wastewater, and recreational waters (Igbinosa *et al*., 2017), but is rarely isolated from seawater (Schroth *et al*., 2018). This bacterium has a wide metabolic diversity and this enhances its environmental success and potential public health risks (Kordes *et al*., 2019).

It is the most common cause of nosocomial pneumonia 17%; a third most common cause of urinary tract infections (UTI ) 7%; a fourth most common cause of surgical site infections 8%; as well as the fifth common isolate overall from all sites 9% (Fujitani *et al*., 2017). A multicenter cross-sectional analysis from the United States reported *P. aeruginosa* 36.2% to be the most common gram-negative organism isolated from patients with lower respiratory tract infections (LRTI) acquired during a stay in the intensive care unit (ICU) (Claeys *et al*., 2018).

*Pseudomonas aeruginosa* particularly affecting patients with compromised immune defenses or patients with intensive care units (Ruiz-Garbajosa and Cantón, 2017). It is estimated that *P.aeruginosa* in the United States is responsible for more than 50,000 infections associated with health care annually and about 440 deaths (Murray *et al*., 2021). Despite advances in critical care management, *P.aeruginosa* infections are associated with a mortality rate reaching 0%–50% (Bosaeed *et al*., 2020).

## 2.5 Virulence factors of *Pseudomonas aeruginosa*

The bacterial virulence factors are the cell surface and secreted proteins or biological molecules such as exotoxins, flagella, type IV pili, and alginate produced by the pathogenic bacteria that enable them to colonize a place in the host, evade and suppress the host's immune response, destroy the host tissue and also obtain nutrients from the host (Fleitas Martinez *et al*., 2019).

Various types of virulence factors (Ullah *et al*., 2017) are released by *P. aeruginosa*, such as hemolysin, lipopolysaccharide, flagellum, type IV pili, quorum sensing, pigments, siderophores, and a group of enzymes such as proteases, elastase, sialidases (or called neuraminidase), DNAase, gelatinase, as well as several toxins such as exotoxin A and the type III secretion system (T3SS) toxins like exotoxin U, exotoxin Y, exotoxin T, and exotoxin S (Mahdavi *et al*., 2017). These factors enable bacteria to initiate and sustain infections or diseases in various host hosts and tissues (Yeboah, 2021).

These factors are major virulence factors that affect the immune system in different ways, and these factors damage the immune system of their host and represent a barrier to antibiotics that reduce the effectiveness of antibiotics, leading to ineffective and failure treatments. Several virulence factors in *P.aeruginosa*, when targeting the extracellular matrix, may cause pathogenicity that facilitates adhesion and/or disrupts the pathways of host cell signaling and can cause acute and chronic infections (Sánchez-Diener *et al*., 2017). Acute infectious strains are motile and are characterized by the possession of early virulence factors for tissue invasion, tissue necrosis, and epithelial attachment (Moradali *et al*., 2017). Chronic infectious strains are sessile, biofilm-forming, and producing genes (Laventie *et al*., 2019).

### 2.5.1 Lipopolysaccharide

A primary component of the *P.aeruginosa* outer membrane is the lipopolysaccharide (LPS). Usually, bacterial LPS consists of a hydrophobic domain known as lipid A (or endotoxin) an unrepeating core oligosaccharide, and a distal polysaccharide (or O-antigen). The lipid A component of LPS is responsible for endotoxin activity (Murray *et al*., 2021).

Lipopolysaccharide plays a significant role in activating the innate host and adaptive (or acquired) immune responses, and ultimately induces dysregulated inflammatory responses that lead to morbidity and mortality and also plays a direct role in causing fever, shock, oliguria, leukocytosis and leukopenia, disseminated intravascular coagulation, and adult respiratory distress syndrome (Riedel *et al*., 2019).

### 2.5.2 Flagellum

The single uncoated polar flagellum of *P. aeruginosa* is responsible for the swimming motility of this organism. However, its role in virulence goes beyond simple motility. Flagellar proteins have been shown to play important roles in binding, invading, forming biofilms, and mediating inflammatory responses (Alhazmi, 2015)

### 2.5.3 Type IV pili

In response to virulence factors, type IV *P.aeruginosa* plays a role in binding to many cell types, and this is undoubtedly important in processes such as tissue swelling, attachment to specific tissues, and initiation of biofilms, mediated by phagocytic receptors that recognize the relative attachment to microbial surfaces (Persat *et al*., 2015 ).

### 2.5.4 Type III Secretion System

Type III Secretion System (T3SS) is a contact-dependent protein secretion pathway that plays a key role in the pathogenesis of serious *P.aeruginosa* infection and this system secretes effector proteins such as exotoxin S (ExoS) and exotoxin U (ExoU). Exotoxin S is a major cytotoxin required for colonization, invasion, and spread of bacteria during infection. Exotoxin U is a cytotoxin with phospholipase activity that affects epithelial cells and causes lung infection. Furthermore, ExoU has a toxic effect on macrophages ( Raheema and Abed, 2020).

### 2.5.5 Exotoxin A

There are several critical virulence factors secreted by the type II secretion mechanism, including exotoxins A. Exotoxin A (ExoA) is encoded via the toxA gene and is present in most clinical isolates of *P. aeruginosa*, although its role in virulence is not understood, and it causes the destruction of tissues, protein synthesis inhibition, interrupts cell activity and macrophage response (Procop *et al*., 2017).

### 2.5.6 Proteases

Proteases are a large group of enzymes found in a variety of microorganisms. The protease enzyme is one of the most important virulence factors in *P. aeruginosa*, as it works to break down tissues by analyzing protein materials, especially in muscle tissue, and separating close fusion between epithelial cells, as well as analyzing fibronectin and inhibit-antiproteinase. And the enzyme works to stimulate the secretion of mucus. It also interferes with the host's immune response (Murray *et al*., 2021).

### 2.5.7 Alginate

Alginate is a mucoid exopolysaccharide that forms a prominent capsule on the surface of bacteria and protects the organism from phagocytosis and the killing by antibiotics. Alginate is an acetylated random copolymer of β 1-4 bound D-mannuronic acid (poly-M) and L-guluronic acid. The ratios between mannuronic acid and guluronic acid affect the viscoelastic properties of biofilms that lead to impaired lung cough clearance in cystic fibrosis(CF) patients infected with *P. aeruginosa* (Gloag *et al.*, 2018). Alginate is responsible for the mucoid colonies seen in CF patient cultures (Riedel *et al.*, 2019).

### 2.5.8 Haemolysin

It is a potential virulence agent produced by bacteria, which could endanger human health. It causes lysis of red blood cells by disrupting the cell membrane, and hemolysin is believed to be responsible for many different events in the host cell. Since red blood cells (RBCs) are rich in iron-containing heme, the degradation of RBCs releases heme in the surrounding medium, which allows the bacteria to absorb free iron. Hemolysin causes tissue damage, facilitating bacterial proliferation, the release of host nutrients, and may also modulate host signaling pathways that affect many processes, including host cell survival, inflammatory responses, and cytoskeletal dynamic. Most *P. aeruginosa* produces beta-hemolysin, which is the entire blood lysis. Enzyme virulence factors that damage tissue include hemolysin (Reda *et al.*, 2017).

### 2.5.9 Pigment

*Pseudomonas aeruginosa* produces many pigments. The most important of which is the blue-green pigment of pyocyanin pigment, which is observed on the surface of the cultivated plate and is referred to as the blue pus, pyomelanin pigment is black and pyorubin pigment is red (Riedel *et al.*, 2019).

Pyocyanin is a product of secondary metabolism. It belongs to the family of phenazine because it contains the nucleus of phenazine, and in addition to being a virulence agent. It acts as a biosensor signal molecule, participating in a variety of important vital activities including gene expression, and it maintains the vitality of the producing germ cells. It supports the formation of the biofilm and is distinguished by its antibacterial and antifungal activity and cause oxidative damage to tissues, in particular to oxygenated tissues such as the lung and it participates in oxidative stress that promotes the alteration of the host's mitochondrial electron transport (Aykac *et al*., 2017).

### 2.5.10 Quorum Sensing

Quorum sensing (QS) is a cell-to-cell communication mechanism in many bacteria (Whitley *et al*., 2017). That term is used to refer to bacterial gene expression coordinated to function as a population to regulate processes such as virulence factor production, antibiotic exposure, and biofilm formation (Pena *et al*., 2019).

Quorum sensing is mediated by small molecules called autoinducers (AIs) such as Autoinducer-2 (AI-2), so-called global autoinducer, which is responsible for intra- and interspecies bacterial communication (Stotani *et al*., 2018). In *P. aeruginosa*, a quorum-sensing complex regulatory chain comprising N-acyl homoserine lactone and alkyl-quinolone signal molecules was linked to the production of several toxic exoproducts included in virulence in a cell density-dependent manner (Whiteley *et al*., 2017).

Quorum sensing signals in *P. aeruginosa* also control the production of siderophores such as pyoverdine and pyochelin, which are also important for biofilm formation, and modulation or inhibition of QS has emerged as a potential treatment that can control many bacterial virulence factors such as biofilm formation and reduce the ill effect of bacterial infections. QS inhibitors can be

used in combination with other antibiotics to fight antimicrobial resistance (Stotani *et al.*, 2018 ), and QS inhibitors showed activity against biofilm formation and the secretion of virulence factors (Tümmler, 2019).

### 2.5.11 Biofilm formation

Biofilms are communities of microorganisms adhering to the biotic or abiotic surface surrounded by a matrix of exopolysaccharide (EPS) or as microbial communities living in a self-produced matrix essentially composed of polysaccharides, extracellular DNA, and proteins (Ciofu and Tolker-Nielsen, 2019). It is a common cause of chronic infection and is regulated by QS systems (Mukherjee *et al.*, 2018).

Biofilms discovery is attributed to the inventor of the microscope, Anthony van Leeuwenhoek, who observed bacterial clusters on a dental plaque in 1684. Nowadays, it is well known that biofilms play an environmental role and have a major influence in medicine through the development of healthcare-associated infections. Biofilm formation by *P.aeruginosa* is one of the main causes of therapeutic failure and increases morbidity and mortality through its protection against the host's immune system and antibiotic therapy. It is estimated to be involved in 65 % of infectious diseases and more than 80 % of bacterial infections caused by the National Institutes of Health (NIH) (Jamal *et al.*, 2018).

The main stage in the production of biofilms is the synthesis of the extracellular matrix. It contains all components except for bacterial cells. Matrix is the main structural feature of bacterial biofilm by containing up to 90 % of total organic matter. It is strongly hydrated and is mainly composed of exopolysaccharides, proteins, nucleic acids, and minerals. Their composition depends on the type of bacteria and the conditions of growth. Helps to strengthen the structure of biofilms while maintaining high elasticity. It also plays a defensive function as it increases the tolerance of bacteria to antimicrobials by

forming a physical barrier that prevents their spread to other environmental factors (pH, UV rays, changes in osmotic pressure, dehydration) and tolerance mechanisms, for example, contributes to distinct extracellular matrix or anaerobic environments (Brauner *et al*., 2017; Trastoy *et al*., 2018). Thus, when *P. aeruginosa* is presented under stress conditions, biofilm formation is often associated with higher antimicrobial resistance compared to the planktonic form and helps to avoid the host's immune response (Skariyachan *et al*., 2018).

*Pseudomonas aeruginosa* infection associated with the development of biofilms is more common in immunocompromised patients and patients with implanted medical devices in the lungs and middle ear, as well as in patients with contact lenses, catheters, and other implants. The development of these bacteria may be asymptomatic within the human body until the bacteria form a biofilm that overwhelms the immune system. These biofilms can be lethal in the lungs of people with cystic fibrosis and primary ciliary dyskinesia (Gerrard *et al*., 2016). There are different biofilm stages include bacterial physiology and phenotypic responses suggestive of the existence of unique biofilm biology that is not found for planktonic bacteria (Ghannoum *et al*.,2015). The stages include:

### ❖ Reversible attachment

The formation of bacterial biofilms in a few step, the first step in the biofilm formation cycle involves attachment, where free-floating mobile bacteria detect an available conditioned surface through environmental signals such as pH variation, oxygen concentrations, nutrients, temperature, osmolarity, etc., and are transported by physical forces or bacterial appendages (such as flagella). The increased proximity of the support, which is adapted by fluids and flows to the site of its exposure, allows the initial adhesion of bacterial cells by physicochemical and electrostatic interactions. In this stage, the adhesion is the

reversible step (a step wherein a bacterium first contacts a surface) (Ghannoum *et al*., 2015).

### ❖ Irreversible attachment

After this first stage, which can occur a few seconds after initial contact with the surface, the second stage of adhesion occurs, allowing the strengthening of surface bacterial bonds through the inclusion of bacterial compounds, such as type IV pili or generally surface adhesion. The surface attachment becomes irreversible, thus allowing adherent bacteria to multiply, forming microcolonies (Olivares *et al.*,2020).

### ❖ Proliferation

In this stage, bacteria get attached to the surface as well as with each other by secreting EPS (an extracellular polymeric substance) that entraps the cells within a glue-like matrix (Choudhary *et al*., 2020).

### ❖ Maturation of the biofilm

The biofilm environment consists of the nutrient-rich layer which supports the rapid growth of microorganisms. Complex diffusion channels are present in a mature biofilm to transport nutrients, oxygen, and other components required for bacterial growth and remove waste products and dead cells ( Bakar *et al*., 2018).

### ❖ Dispersal of the biofilm

The final stage of biofilm development is dispersion because as long as fresh nutrients are kept providing, biofilm continues to grow and when they get nutrient-deprived, they return to their planktonic mode by detaching themselves from the surface and the dispersion of biofilms can be initiated by various factors including mechanical disturbances (erosion), enzyme secretion (quorum sensing

), or even nutrient deficiency or overpopulation, which results in detachment of biofilm or some parts of it (Zuberi and Nadeem, 2017).

### 2.5.11.1 Extracellular matrix for biofilms

Extracellular Matrix (ECMs) for biofilms are typically composed of exopolysaccharides (EPS), extracellular DNA (eDNA), and proteins, which act as a matrix, adhesive, and protective barrier (Wei and Ma, 2013).

### ❖ Exopolysaccharides

Exopolysaccharides (EPSs) in *P. aeruginosa*: Psl (polysaccharide synthesis locus), Pel ( polysaccharide encoding locus), and Alginate and are among the most important exopolysaccharides that are used in the formation of biofilms. The composition and functions of EPSs in the biofilm of *P. aeruginosa* have been highlighted (Moradali *et al.*, 2017). Which EPSs provide a physical barrier that can be difficult for antibiotics to penetrate, and bacteria within the biofilm often display reduced metabolic activity, which greatly influences their susceptibility to antibiotics, the majority of which depend on active metabolism (Koo *et al.*, 2017).

Psl polysaccharide, a frequent pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose. Named for the polysaccharide synthesis locus identified in 2004 and Psl is an important component of the extracellular matrix (ECM ) for initiation and maintenance of *P. aeruginosa* biofilms by providing cell surface binding and intercellular interactions. In the late stage of biofilm maturation, Psl has been shown to accumulate on the outer surface of structured biofilms. The Psl operon contains 15 genes (*psl*A-O) involved in the synthesis of exopolysaccharide (EPS) which is important for *P. aeruginosa* biofilm formation. The *psl*A gene is usually the first gene of this cluster and *pslA* is the most important role in biofilm formation and on the regulation of the entire Psl operon ( Nader *et al.*, 2017).

Pel polysaccharide is a matrix material rich in glucose, with an unclear formula and an essential component for *P. aeruginosa* to form pellicles at the liquid-air interface and biofilms attached to the solid surface, and the other roles of Pel are to act as a platform for biofilm structure and to provide protection against aminoglycoside antibiotics. Pel is produced by the gene product actions of the *pel*ABCDEFG locus and *Pel*A is a multifunctional enzyme with hydrolase that degrades the Pel polysaccharide (Baker *et al*., 2016).

Alginates are recognized as an agent used to distinguish between mucous and non-mucinous biofilms of *P. aeruginosa*, and alginates play many important roles for biofilms, it contributes to structural stability, biofilm protection, and water and nutrient retention as well (Powell *et al*., 2018).

### ❖ Extracellular DNA

Extracellular DNA (eDNA) is known to play a role in the formation of cation gradients, antibiotic resistance, nutrient source, and early development of biofilm. It is an important component of the *P. aeruginosa* biofilm matrix, which is specifically intervening in the establishment, maintenance, and perpetuation of structured biofilms and is one of the crucial constituents of biofilms (Soler-Arango *et al*., 2019).

### ❖ Protein

Proteins also contribute to the formation of the biofilm matrix, for example, flagella act as an adhesive to aid in the initial bacterial attachment to the surface, and type IV pili also contribute to the formation of mushroom-like biofilm cap structures. CdrA adhesion interacts with Psl and increases biofilm stability. Cup fimbriae is also a proteinaceous component of the extracellular matrix (ECM) and play important roles in cell-to-cell interaction during the initial stage of biofilm formation (Wei and Ma, 2013).

## 2.6 Antibiotics

Any substance that inhibits the growth and reproduction of bacteria or that completely kills them can be called antibiotics. Antibiotics are a type of antimicrobial that kills certain microorganisms or inhibits their growth and are designed to target bacterial infections inside (or on) the body (Grenni *et al*., 2017).

## 2.7 Mechanism of action of the antibiotic

Antimicrobial agents are classified by their specific modes of action against bacterial cells. Mechanisms of action of antibiotic on the bacterial cell can be divided into five categories:

- Cell wall synthesis inhibition (Penicillins e.g. Ticarcillin)
- Protein synthesis inhibition (Aminoglycosides e.g. Amikacin)
- Nucleic acid synthesis inhibition (Fluoroquinolones e.g. Levofloxacin)
- Folate synthesis inhibition (Trimethoprim)
- Disruption structures of the cytoplasmic membrane (Daptomycin) (Etabu and Arikekpar, 2016, Chaudhary *et al*., 2017).

## 2.8 Mechanism of antibiotic resistance in *Pseudomonas aeruginosa*

Antibiotic resistance can be defined as the resistance of microorganisms to a given concentration of the respective antibiotic, or an organism's ability to resist an antimicrobial agent's action to which it was previously susceptible (Pachori *et al*., 2019). *P.aeruginosa* includes several mechanisms to antibiotic resistance, posed a next-level risk by limiting the effectiveness of antibiotics approved for clinical use. These mechanisms are often present simultaneously and thus confer combined resistance to several antibiotics (Papagiannitsis *et al*., 2017).

### 2.8.1 Intrinsic resistance of *Pseudomonas aeruginosa*

In this type of resistance, the use of antibiotics is not related to resistance but rather results from the structural properties of the bacteria (Kadhum and Hasa, 2019). This occurs as a result of intrinsic resistance, or microorganisms that do not follow the target antibiotic structure or antibiotics that do not encounter their target due to their characteristics (Waglechner and Wright, 2017)

*Pseudomonas aeruginosa* has notable intrinsic mechanisms of resistance and is able of acquiring multiple mechanisms of antibiotic resistance, shows inherent resistance to antimicrobial agents by a variety of mechanisms: (1) decreased permeability of the outer membrane, (2) efflux systems that actively pump antibiotics out of the cell, and (3) production of antibiotic-inactivating enzymes (Moore and Flaws,2011).

#### 2.8.1.1 Outer membrane permeability

The outer membrane (OM) of *P. aeruginosa* primarily acts as a permeability barrier and imparts a broad spectrum of intrinsic antibiotic resistance (Purro *et al*., 2018).

The outer membrane (OM) is an asymmetric bilayer consisting of lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet, non-specific porins, and specific uptake channels are embedded. *P. aeruginosa* contains several specific porins, including the carbohydrate-specific porin OprB, basic amino acid-specific porin OprD, phosphate-specific porin OprP, and pyrophosphate-specific porin OprO. OprD pores allow the entry of Carbapenems. When these pores are lost, Carbapenems have to face resistance challenges (Dantas *et al*., 2017). OprF protein is the main nonspecific porin, it is of high importance for *P. aeruginosa* virulence where it was found involved in

quorum sensing, toxin secretion, and cell adhesion, OprF has an N-terminal porin domain and C-terminal peptidoglycan binding domain (Chevalier *et al*., 2017).

Aminoglycosides and Colistin interact with lipopolysaccharides resulting in increased permeability, while beta-lactam and quinolones need to diffuse through certain porin channels. The permeability of OM such as EDTA has been shown to increase the susceptibility to antibiotics, indicating that deficiency of the OprD protein results in a reduction of active antibiotic molecules able to reach the target penicillin-binding proteins, and cationic peptides and small molecule permeabilizes have been shown to increase OM permeability and improve the anti-cellular activity of high-molecular-weight antibiotics such as Erythromycin and Rifampicin against gram-negative pathogens, but these tend to be less effective against *P.aeruginosa* in particular and often have a nonspecific activity that leads to mammalian cell toxicity ( Corbett *et al*., 2017).

### 2.8.1.2 Efflux systems

Among the resistance mechanisms that bacteria use against antibiotics, increasing the activity of the efflux pump, which works by enhancing the efflux of antibiotics from the bacterial cell, is one of the most prominent and thus a potential target of combination therapy and can be classified into five families: resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion (MATE) family. *P.aeruginosa* expresses twelve efflux-pump RND families, four of which (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) contribute to antibiotic resistance (Cepas and Soto, 2020).

Multiple efflux pumps are expressed in drug-resistant *P.aeruginosa*, however, the MexAB-OprM efflux pump exhibits broad antibiotic substrate specificity as well as antibiotic-induced gene expression. The MexAB-OprM

efflux-pump genes, i.e. mexA, mexB and oprM are regulated as an operon responsible for export a spectrum of antibiotics and biocides (Arya *et al*., 2019). Even sub-lethal concentrations of antibiotics in the active site of efflux pumps may predispose the organism to develop high-level target-based resistance (Dzotam and Kuete, 2017). MexAB-OprM efflux pumps are therefore a rational target for the mitigation of antibiotic resistance. MexAB-OprM is responsible for efflux of β-lactams and quinolones. MexCD-OprJ is able to pump out β-lactams . MexEF-OprN is capable of extruding quinolones, while MexXY-OprM is a unique pump in *P. aeruginosa* that provides resistance to the class of antimicrobial aminoglycosides and is inducible to many of its substrate antimicrobials (Singh *et al*., 2017).

### 2.8.1.3 Antimicrobial inactivating enzymes

One of the main mechanisms of intrinsic resistance to bacteria is the production of antibiotic-inactivating enzymes that break down or modify antibiotics, which is the hydrolytic deactivation of the beta-lactam ring in Penicillins and Cephalosporins by a bacterial enzyme called beta-lactamase. Beta-lactamases are enzymes that covalently bind to the lactam ring, hydrolyze it, and render the antibiotic ineffective (Torok *et al*., 2017). In this group, Beta-lactamases, Chloramphenicol, Aminoglycosides, and Erythromycin-modifying enzymes are the most common examples (Sharkey and O'Neill, 2019).

## 2.8.2 Acquired resistance

Acquired resistance occurs when a specific microorganism obtains the ability to resist an antimicrobial agent or because it is not affected by the antibiotics to which it was previously susceptible (Andersson *et al*., 2020). Acquired resistance comes from major chromosome or extrachromosomal structures (plasmids, transposons, etc.) (Aljanaby and Aljanaby, 2018).

Chromosomal resistance results from mutations that randomly change the bacterial chromosome. These mutations can be triggered by certain physical and chemical factors (Majeed and Aljanaby, 2019). This may be due to changes in the composition of the bacterial cells, so that the permeability of the bacterial drug may decrease, or perhaps changes in the drug target in the cell ( Al-Harmoosh *et al*., 2017).

Extrachromosomal resistance depends on extrachromosomal genetic material that can be transmitted via plasmids, transposons, and integrons (Jabuk *et al*., 2017). Usually, the plasmid is responsible for developing inactive antibiotic enzymes (Aljanaby *et al*.,2019). There are major forms of holding the genetic material (resistance genes and plasmids) from bacterial cells, and this form is transduction, transformation, conjugation, and the mechanism of transposition (Al-Labban *et al*., 2019). Genes with antibiotic resistance are intertwined on a chromosome or plasmid and are located at the beginning with different integration groups, or integrons. Integrons are genetic elements that insert mobile gene cassettes into a specific genetic site via site-specific recombination and have been shown to play an important role in spreading antibiotic resistance among strains of *P. aeruginosa* (Khosravi *et al*., 2017). Recombination is very normal in integrons (Adam *et al*., 2019).

### 2.8.3 Adaptive antibiotic resistance

Adaptive resistance refers to a resistance that occurs due to environmental conditions, such as transcriptional changes in genes that determine resistance/susceptibility, and increase the ability of a bacterium to survive an antibiotic attack due to transient changes in gene and/or protein expression in response to an environmental stimulus and is reversible when environmental conditions (for example, complex adaptive growth states such as swarming, biofilm formation, or exposure to stresses, including antibiotics) are reversed or

can be reversed when the stimulus is removed, finally adaptive resistance that is a reflection of the environmental status of the bacterium and includes genetic changes caused by the environment (Schroeder *et al.*, 2017; Pang *et al.*, 2019).

## 2.9 Multidrug resistance *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* can be multidrug-resistant (MDR), extensively drug-resistant (XDR) or pan drug-resistant (PDR). MDR refers to an isolate that is non-susceptible to at least one agent in three antimicrobial categories. XDR refers to an isolate that is non-susceptible to at least one agent in all but two or fewer categories of antimicrobials. PDR refers to an isolate that is non-susceptible to all agents in all categories of antimicrobials (Magiorakos *et al.*, 2012). The increasing resistance of *P. aeruginosa* to numerous antibiotics, due to excessive antibiotics administration, leads to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of multidrug-resistant (MDR) forms of *P. aeruginosa* (Bahador *et al.*, 2019).

The MDR, XDR, and PDR phenotypes detail inactivating enzymes, such as the enzymes of Extended-spectrum-β-lactamases (ESBL) and Metallo-β-lactamases (MBL), which render beta-lactamases and Carbapenems ineffective (Ríos *et al.*, 2018). XDR phenotype may occur at an alarmingly high rate, as in *P. aeruginosa* from burn patients (Safaei *et al.*, 2017).

Prompted the World Health Organization (WHO ) in 2017 to classify MDR gram-negative bacteria, including *P. aeruginosa*, as serious global threats to human health, with greater emphasis on the need for new treatment strategies (independent of antibiotics) (Tacconelli *et al.*, 2018). According to the WHO, *P. aeruginosa* is the second most problematic multidrug-resistant bacteria. If the evolving collateral sensitivity is mutual, it could - in theory - trap the bacteria in a double bind, thus preventing the emergence of multidrug resistance during treatment (Roemhild and Schulenburg, 2019).

Multidrug-resistant resistance (MDR) bacteria have been involved in an intrinsic, acquired, and adaptive antibiotic resistance (Meliani, 2020), and *P. aeruginosa* can develop an MDR phenotype through a complex genome including many intrinsic and acquired mechanisms (Horcajada *et al*., 2019). MDR may be the leading cause of the high mortality rate (Bassetti *et al*., 2018).

## 2.10 Genome of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* has a large and complex genome (6.3 Mb, G + C content 66.6%). Moreover, the proportion of predicted regulatory genes in the genome of *P. aeruginosa* is greater than all other bacterial genomes sequenced (Alayande *et al*., 2018). It consists of two components, the core and accessory genomes, and the highly organized and preserved primary genome makes up approximately 90% of the whole genome present in all strains. The functions of many genes within the core genome have been described, and include the genes responsible for respiration, antibiotic resistance, and formation of biofilm. A comparison of 389 genomes from different strains of *P. aeruginosa* showed that only 17.5% of the genomes are shared. This part of the genome is *P. aeruginosa* core genome (De Smet *et al*., 2017).

The remainder of the genome consists of a smaller accessory genome and includes a set of genetic material it differs between strains. The accessory genome is the main driver behind the evolution of this organism, in particular, the acquisition or loss of genetic material through horizontal gene transfer (Ramsay, 2017).

The first published complete genome of *P. aeruginosa* was for the strain PAO1 and the second *P. aeruginosa* genome sequence of UCBPP-PA14 (University of California Berkeley Plant Pathology) has been published or simply referred to as PA14. This strain was first reported in 1977 from a clinical sample

(Schroth *et al.*, 2018). Thus it has become popular as a reference for pathogenesis research (Mathee, 2018).

## 2.11 Effect of antibiotics on the biofilms

Due to the frequent use of implanted medical devices (eg, pacemakers, prostheses, catheters), the burden of biofilm-related infections has increased in past decades (Del Pozo, 2018; Stewart and Bjarnsholt, 2020). Bacterial biofilms can survive antibiotics due to poor antibiotic diffusion, antibiotic efflux, nutrient and oxygen limitation, selection of resistant mutants, expression of biofilm-specific genetic mechanisms, or tolerant cell survival (Hall and Mah, 2017). nevertheless, antibiotics can have diverse effects on microbial biofilms. These effects can be complex and depend on many factors, such as the concentration of antibiotics to which the organisms are exposed, the growth conditions, and the characteristics of the organism itself. The effect of antibiotics on biofilms in infections can be evaluated roughly as two seemingly opposing effects: disruption of existing biofilms or enhancement of biofilm formation

### 2.11.1 Disrupting biofilms

The biofilm destructive effects of some antibiotics may seem promising for antibiotic therapy. Destabilizing the structure of biofilms can be seen as a means of eliminating the additional protection provided by biofilms, making biofilms more penetrable to antimicrobials, and cells of biofilms more susceptible to antibiotics. However, this strategy is fraught with risks as the destabilization of biofilm structure and disintegration of biofilm matrix could lead to cellular detachment and increase biofilm proliferation, which could lead to increasingly severe and long-term consequences (Penesyan *et al.*, 2020).

In a recent study, Díaz-Pascual *et al.* (2019) examined the effects of antibiotics commonly used to treat cholera on biofilms of *Vibrio cholera*.

Transient exposure to translation-inhibiting antibiotics such as tetracycline caused alterations in cell shape and physiology that resulted in large-scale changes in biofilm architecture and the dismantling of cell-matrix associations. This effect may be considered favourable for *V. cholera* biofilm eradication, as the loosening of biofilm structures may allow antimicrobials better access to the biofilm interior. However, disrupting the biofilm structure may detach cells that can then serve as inocula for new points of infection, and hence cause the spreading of the infectious agent ( Penesyan *et al*., 2020).

### 2.11.2 Enhancing biofilm formation

There is increasing evidence indicating that sub-inhibitory concentrations of many antibiotics can enhance biofilm formation by pathogens. Exposure to concentrations of sub-inhibitory antibiotics can promote the growth of resistant and/or more favorable variants by selecting pre-existing mutations and promoting new mutations (Ahmed *et al*., 2018; Santos-Lopez *et al*., 2019).

The effects of antibiotic concentrations sub-inhibitory on a range of phenotypic outcomes, including enhanced survival, complicate efforts to eliminate biofilms. Rather than achieving biofilm removal, antibiotics may also strengthen and enhance microbial survival by increasing the protection provided by biofilms (Penesyan *et al*., 2019).

## 2.12 Gene screening by Polymerase Chain Reaction technique

DNA polymerase is utilized to synthesize a specific sequence of microbial DNA (target sequence). Two oligonucleotide primers flank the double-stranded DNA to be sequenced by binding to the complementary strands of DNA. The amplification happens by heat up the specimen to separate the double strands of DNA, then cooling the reaction to allow the primers to bind to the two DNA strands. Then extending the sequences from the primers with DNA polymerase.

This cycle of heating, cooling, and polymerization proceeds through several cycles, each time exponentially increasing the number of copies of the target DNA. A polymerase chain reaction (PCR) is designed to make multiple copies of (amplify) the desired gene or another short DNA fragment and is used in clinical laboratories for the detection of pathogens in clinical specimens (Murray *et al.*, 2021). This method is the most popular technique in molecular genetics laboratories around the world and the basis for many studies at the level of DNA due to its specificity in terms of its ability to deal with large numbers of samples also that it does not require large quantities from the DNA (Domingues, 2017).

Three phases of polymerase chain reaction are (1) DNA denaturation or the separation of the two strands of DNA (2) primer annealing and (3) primer extension the portion of the reaction wherein DNA synthesis occurs (Procop *et al.*, 2017)

- Thermal denaturation stage: This is the separation of the double ds-DNA tape into two separate ss-DNA strands, and this phase takes place at a temperature of 90-95°C.
- The primers annealing phase: It represents the association of both primers with the two separate strips at a temperature of 50-68°C.
- Annealed primers extension phase: The enzyme polymerase does this by adding dNTPs, and this phase takes place at a temperature of 68-72°C. This process is performed automatically by a thermocycler programmed by the lab worker.

This three-step cycle is repeated several times, resulting in increased DNA molecules(Domingues, 2017). Due to the danger of the spread of bacterial resistance genes and the economic losses they cause and the length of treatment time for patients, this led to the use of PCR technology for its rapid study and investigation. Was used to detect *pslA, pslD,* and *pelA* genes in *P.aeruginosa* isolated from clinical sources.

# Chapter Three
# Materials and Methods

# 3 Materials and Methods

## 3.1 Materials

### 3.1.1 Equipment and apparatus

The equipment and apparatus used in the current study were presented in table (3-1).

**Table 3-1: Equipment and apparatus**

| NO. | Equipment and apparatus | Company | Origin |
|---|---|---|---|
| 1 | 1.5ml, 0.5ml and 0.2ml Tube | Jet biofil | Singapore |
| 2 | 96 flat-bottom wells | Coastar | USA |
| 3 | Antibiotic disk dispenser | Mast group | UK |
| 4 | Autoclave | Hirayama | Japan |
| 5 | Bunsen burner | Memmert | Korea |
| 6 | Centrifuge | Fisher scientific | USA |
| 7 | Compound light microscope | Olympus | Spain |
| 8 | ELISA reader | Kevin | Germany |
| 9 | Gel imaging system | Major science | Taiwan |
| 10 | Incubator | Memmert | Germany |
| 11 | Inoculating loop | John Bolton | UK |
| 12 | Kardelen hidrophile cotton | MAY | Turkey |
| 13 | Magnetic stirrer with hotplate | FALC | Italy |
| 14 | Micro spin centrifuge | My fugene | China |
| 15 | Micropipettes | Human | Germany |
| 16 | Microwave oven | Gosonic | China |
| 17 | Millipore filters | Millipore corp | Germany |
| 18 | OWL electrophoresis system | Thermo | USA |
| 19 | Quantus florometer | Promega | USA |
| 20 | Refrigerator | TEKA | Spain |
| 21 | Tube plain without additives | AFCO | Jordan |
| 22 | Sensitive balance | OHAUS-PioNEER | USA |
| 23 | Sterile Petri dishes | AFCO | Jordan |
| 24 | Sterile syringes | Hagg | UAE |

| | | | |
|---|---|---|---|
| **25** | Sterile swab stick | Sterellin ltd. | UK |
| **26** | Transport swab | Sterellin ltd. | UK |
| **27** | Thermo cycler | BioRad | USA |
| **28** | Tips | Sterellin ltd. | UK |
| **29** | Vitek 2 | BioMerieux. | France |
| **30** | Vortex | Quality lab system | England |
| **31** | Water bath | Thermo | USA |

### 3.1.2 Chemical and biological materials

Chemical and biological materials used in the current study were presented in tables (3-2).

**Table 3-2: Chemical and biological materials**

| NO. | Chemical and biological materials | Company | Origin |
|---|---|---|---|
| **1** | Absolute ethanol & Methanol | ROMIL pure chemistry | UK |
| **2** | Agar – Agar | Mast group | UK |
| **3** | agarose | Promega | USA |
| **4** | Catalase reagent : 3% Hydrogen peroxide ($H_2O_2$) | BHD | England |
| **5** | CFC supplement | Himedia | India |
| **6** | Gelatin | Oxoid | UK |
| **7** | Glycerol | BDH | England |
| **8** | Human blood | Blood bank | Baquba |
| **9** | Kovac‘s indol reagent : (p-dimethylamino benzaldehyde, isoamyl alcohol) | Bio Mérieux | France |
| **10** | Methyl red reagent | Bio Mérieux | France |
| **11** | Oxidase reagent: [N,N,N,N-tetramethyl-ρ-phenylenediamine dihydrochloride %1] | Bio Mérieux | France |
| **12** | Skim milk | Al-Safii | KSA |
| **13** | Standard MacFarland solution(matching turbidity of $1.5\times10^8$ CFU/ml) | Bio Mérieux | France |
| **14** | Urea solution | BDH | England |
| **15** | Voges-Proskauer reagent :(α –naphthol , KOH) | BDH | England |

### 3.1.3 Culture media

Culture media used in the current study are presented in tables (3-3). The media were prepared according to the instructions of a manufacturing company.

**Table 3-3: Culture media**

| NO. | Media | Company | Origin |
|---|---|---|---|
| 1 | Blood agar base | Mast group | UK |
| 2 | Brain heart infusion broth | Mast group | UK |
| 3 | MacConkey agar | Mast group | UK |
| 4 | Methy red-Voges-Proskauer broth | Mast group | UK |
| 5 | Mueller Hinton agar | Mast group | UK |
| 6 | Mueller Hinton broth | Mast group | UK |
| 7 | Nutrient agar | Mast group | UK |
| 8 | Nutrient broth | Mast group | UK |
| 9 | Peptone water | Mast group | UK |
| 10 | Pseudomonas agar base | Mast group | UK |
| 11 | Simmon citrate agar | Mast group | UK |
| 12 | Triple sugar iron agar | Mast group | UK |
| 13 | Urea agar base | Mast group | UK |

### 3.1.4 Antibiotics discs

Antibiotic discs that were used in the current study were presented in table (3-4) according to (CLSI, 2020) and obtained from (Mast group /UK).

**Table 3-4: Antibiotics discs**

| No | Antimicrobial agent | Codes | Disc potency (µg /Disc) | Diameter of zone inhibition (mm) | | |
|---|---|---|---|---|---|---|
| | | | | Resistant | Intermediate | Sensitive |
| 1 | Piperacillin/Tazobactam | PTZ | 100/10 | ≤14 | 15_20 | ≥21 |
| 2 | Ticarcillin/Clavulanic acid | TIM | 75/10 | ≤15 | 16_23 | ≥24 |
| 3 | Ceftazidime | CAZ | 30 | ≤14 | 15_17 | ≥18 |
| 4 | Cefepime | CPM | 30 | ≤14 | 15_17 | ≥18 |
| 5 | Ciprofloxacin | CIP | 5 | ≤25 | 19_24 | ≥18 |
| 6 | Levofloxacin | LEV | 5 | ≤22 | 15_21 | ≥14 |
| 7 | Gentamicin | CN | 10 | ≤12 | 13_14 | ≥15 |
| 8 | Tobramycin | TN | 10 | ≤12 | 13_14 | ≥ 15 |
| 9 | Amikacin | AK | 30 | ≤14 | 15_16 | ≥17 |
| 10 | Netilmicin | NET | 30 | ≤12 | 13_14 | ≥15 |
| 11 | Aztreonam | ATM | 30 | ≤ 5 | 16_21 | ≥ 22 |
| 12 | Imipenem | IMI | 10 | ≤15 | 16_18 | ≥19 |
| 13 | Meropenem | MEM | 10 | ≤15 | 16_18 | ≥19 |

### 3.1.5 Antibiotics powder

The antibiotics used in the current study as powdered are presented in table (3-5) according to (CLSI, 2020) and obtained from (Direvo industrial biotechnology/Germany).

**Table 3-5: Antibiotic powder**

| Antimicrobial agent | Interpretive categories and MIC breakpoints, µg/mL | | |
|---|---|---|---|
| | Resistant | Intermediate | Sensitive |
| Ceftazidime | ≥32 | 16 | ≤8 |
| Imipenem | ≥8 | 4 | ≤2 |

### 3.1.6 Kits

Kits used in the current study were presented in table (3- 6).

**Table 3-6: Kits**

| NO. | Kits | Company | Origin |
|---|---|---|---|
| 1 | Ethidium bromide solution (10mg/ml), GoTag Green Master Mix, Nuclease free water, TAE10X, Quantifluor dsDNA system | Promega | USA |
| 2 | DNA ladder | Promega | USA |
| 3 | GN ID card (VITEK2) | BioMerieux | France |
| 4 | Gram stain kit | BHD | England |
| 5 | Presto™ Mini gDNA Bacteria Kit | Genaid | Taiwan |
| 6 | Primers | Macrogen | Korea |

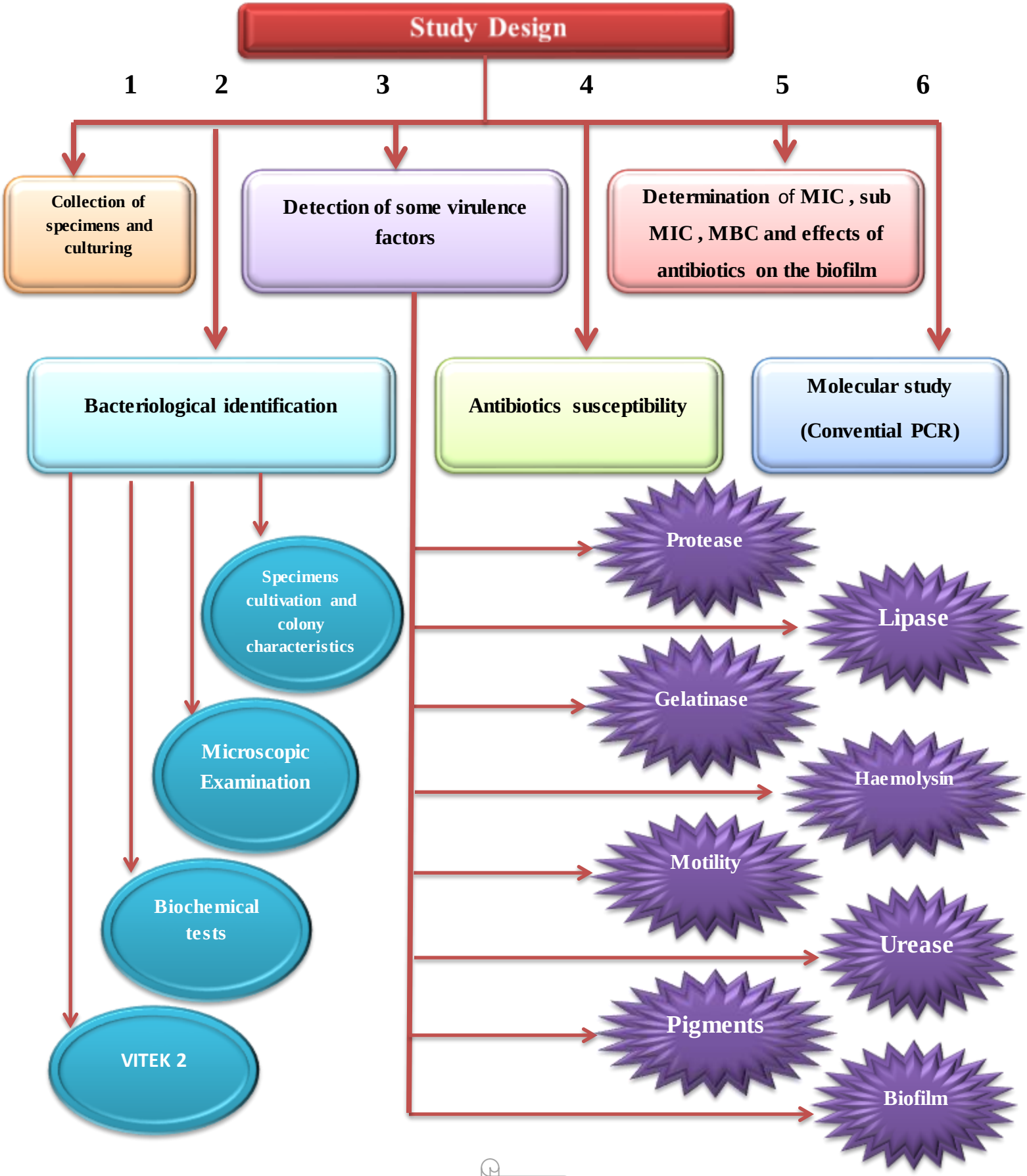
### 3.1.7 Primers

The primers used in the current study for gene detection (from Macrogen, Korea) are presented in tables (3- 7).

**Table 3-7: Primers design that used in the current study**

| Primer Name | Sequences | Ref. | Annealing Temp. (oC) | Size (bp) |
|---|---|---|---|---|
| *pel*A-F | 5'- CCTTCAGCCATCCGTTCTTCT-3' | (Colvin,*et al.*,2011) | 52 | 118 |
| *pel*A-R | 5'- TCGCGTACGAAGTCGACCTT-3' | | | |
| *psl*A-F | 5'- TGGGTCTTCAAGTTCCGCTC -3' | Maita and Boonbumrung ,2014) | | 119 |
| *psl*A-R | 5'- ATGCTGGTCTTGCGGATGAA -3' | | | |
| *psl*D-F | 5'- CTCATGAAACGCACCCTCCT -3' | Maita and Boonbumrung ,2014) | | 295 |
| *psl*D-R | 5'- TGCGACCGATGAACGGATAG -3' | | | |

**3.2 Methods** The steps of the current study illustrated by the following diagram



### 3.2.1 Sterilization methods

For the sterilization of different media, a method of moist heat sterilization was used by autoclave for 15 minutes at 121°C and under a pressure of 15 bar / inch2. Millipore filter (0.22 µm) was used to sterilize the antibiotic solutions which could be damaged due to high temperatures. On the other hand, to prevent any contamination, all glassware was sterilized by dry heat in a microwave oven at 160°C for 2-3 hours (Murray *et al*., 2021).

### 3.2.2 Laboratory prepared of culture media

### 3.2.2.1 Ready-Made media

According to the manufacturer's instructions, the culture medium presented in table (3.3) was prepared by dissolving the medium correctly on a magnetic stirrer with a hot plate. The pH was adjusted to (7.0 ± 0.3) and then sterilized in an autoclave at 121°C and pressure 15 bar / inch2 for 15 minutes. After sterilization and cooling, they poured into sterile Petri dishes and incubated for 24 hours at 37°C to ensure that they are not contaminated. Then they kept at 4°C in the refrigerator until used.

### 3.2.2.2 Laboratory prepared media

### 3.2.2.2.1 Blood agar medium

Blood agar medium was prepared according to the manufacturer company and sterilized by autoclaving at a pressure of 15 bar / inch2 and 121°C for 15 minutes. After cooling to 45°C -50°C, 5 % of fresh human blood (AB group) was then added and poured into sterile Petri dishes. Blood. agar is used for. isolate and. cultivate many. types of fastidious bacteria and it is also used to distinguish bacteria based on hemolytic properties (Tille, 2017 ).

### 3.2.2.2.2 Pseudomonas agar medium

The medium was prepared by dissolving 24 g in 500 ml of distilled water containing 5 ml of glycerol as directed by the manufacturer. Heated to boil until the mixture is completely dissolved and then sterilized for 15 minutes using an autoclave. After cooling to 45-50°C, sterile CFC (Cephalothin, Fucidin, Cetrimide) supplement (FD036) was added, mixed well, and poured into sterile Petri dishes (Salfinger and Tortorello, 2015).

### 3.2.2.2.3 Gelatin liquefaction medium

It was prepared using nutrient broth medium with gelatin, dissolving 1.95 g of nutrient broth with 6 g of gelatin in 150 ml of distilled water, mixing it well and after sterilizing it by an autoclave, it was poured into sterile tube. This medium demonstrates the activity of gelatinase hydrolysis (Cappuccino and Welch, 2020).

### 3.2.2.2.4 Motility test medium

Motility medium was prepared by dissolving 0.6 g of agar-agar and 1 g of nutrient broth medium in 150 ml of distilled water and then sterilized by autoclaving. This medium is used to detect the motility of bacteria (Brooks, 2016).

### 3.2.2.2.5 Egg-yolk agar medium

This medium was prepared using 450 ml of Nutrient agar medium, 50 ml of egg yolk was added to it after sterilization and cooling to 50°C, the volume was completed to 500 ml and then poured into sterile Petri dishes. This medium is used to verify the ability of bacteria to produce the enzyme lipase (Cappuccino and Welch, 2020).

#### 3.2.2.2.6 Skim milk agar medium

The medium was prepared by dissolving 14 g of Nutrient agar in 500 ml of distilled water, after being sterilized and cooled to 45°C, 12 ml of skimmed milk was added to the medium and poured into sterile Petri dishes. This medium is used to determine the protease activity (Macin *et al.*, 2017).

#### 3.2.2.2.7 Urea agar medium

Urea agar slant was prepared according to the manufacturer company by dissolving 7.6 g of urea agar in 150 distilled water and has been sterilized by autoclaving at a pressure of 15 lbs and 121°C for 15 minutes. After cooling to 45-50°C, 7.5 ml of urea solution sterilized with Millipore filters (0.45µm diameter) was added to sterilized urea agar and poured into a sterile tube. It was used for detecting the ability of bacteria to produce urease enzyme (MacFaddin, 2000).

### 3.2.3 Reagents, stains, and solutions that used in isolation and identification of the bacteria

#### 3.2.3.1 Reagents

All reagents used in the current study were obtained ready-to-use from Bio-merieux (France) and BDH (England).

#### 3.2.3.2 Stains and solutions

#### 3.2.3.2.1 Gram stain

The ready-made kit was used, which is a crystal violet stain, Lugol's iodine stain, decolorization solvent, and 0.5% standard safranine stain as counter-stain. The gram stains method is considered the most common method used in the microbiology laboratory, which forms the basis for the classification of bacteria

into two groups (for example, gram-positive and gram-negative) (Murray *et al*., 2021).

### 3.2.3.2.2 Antibiotic stock solutions

A stock solution of antibiotics was prepared by dissolving 1 g of antibiotic (Imipenem and Ceftazidime ) in 90 ml of distilled water, then complete the volume to 100 ml. Sterilized by filtration in millipore with a diameter of 0.22 mm (CLSI, 2017) stores in refrigerator.

### 3.2.3.2.3 Tris-acetate EDTA Buffer

Tris-acetate EDTA Buffer 10X (TAE 10X) is containing a mixture of Tris base, acetic acid and EDTA. Is the most commonly used buffer for agarose DNA electrophoresis. For preparing a 1X TAE working solution, 100 ml of TAE (10X) was added to 900 ml of distilled water.

### 3.2.3.2.4 Ethidium bromide (10mg / ml)

This dye was obtained ready-to-use from Promega /USA concentration 10 mg/ml.

### 3.2.3.2.5 Macfarland standard solution

To calibrate the number of bacterial cells, a prepared solution from the French company Bio Mérieux was used to give an approximate number of bacterial cells equal to $1.5\times10^{8}$CFU/ml.

### 3.2.3.2.6 Bacterial Suspentions

Bacterial suspensions of each isolate were prepared by transferring a single colony grown on MacConkey agar medium to 5 ml of distilled water to obtain a

turbidity suspension of ($1.5\times10^8$) CFU / ml by compared with MacFarland standard.

### 3.2.4 Collection of the specimens

This study started from September 2020 to January 2021, collected 200 pathological specimens taken from different sources included urine (67), wound swab (55), ear swab (41), burn swab (25), sputum (12) from Baquba General Teaching Hospital, Teaching Laboratories, Al-Batoul Teaching Hospital, from males and females with different ages.

### 3.2.5 Bacteriological identification

### 3.2.5.1 Specimens cultivation and colony characteristics

The specimens were cultured on Blood agar, Nutrient agar, and MacConkey agar, the isolates were confirmed on Pseudomonas agar medium and incubated at 37°C for 24 hours. The colony characteristics were studied including colony texture, shape, color, and edges, in addition to its ability to lysis of red blood cells on the medium of blood agar and lactose non-fermentation on the medium of the Mackonkey agar (Tille, 2017 ).

### 3.2.5.2 Microscopic examination

The microscopic examination of the cells of the developing bacterial isolates was carried out by transferring a portion of a young colony by the sterile inoculating loop and mixed with a drop of distilled water on the surface of a clean glass slide, then spread on the surface of the slide, left to dry and fixed by heat on the bunsen burner, then stained in a gram staining method and examined under a compound light microscope (100x) to observe the shape of the cells and the nature of their interaction with gram stain (Procop *et al*., 2017).

### 3.2.5.3 Biochemical tests (Cappuccino and Welsh, 2020)

#### 3.2.5.3.1 IMViC tests

##### 3.2.5.3.1.1 Indole production test

Peptone water medium was inoculated with the bacteria and incubated at 37°C for 24 hours. 5 drops of Kovac's reagent were added after incubation. A positive result was indicated by the formation of a red surface ring. The indole test determines the bacteria that able to producing indole by using the enzyme of tryptophanase.

##### 3.2.5.3.1.2 Methyl red test

Methyred-Voges-Proskauer (MR-VP) medium was inoculated with single colonies of bacteria and incubated at 37°C for 24 hours, 6 drops of methyl red reagent were added to each tube plain and quietly shaken and read reaction immediately, indicating the positive result is the appearance of the red color. The methyl red test determines the bacterial capacity to produce stable acid end products by fermentation of mixed acids of glucose.

##### 3.2.5.3.1.3 Voges-Proskauer test

Methyred-Voges-Proskauer (MR-VP) medium was inoculated with *P. aeruginosa* and incubated at 37°C for 24 hours. After incubation, 6 drops of α - naphthol reagent and 2 drops of KOH reagent were added and shake well after the addition of each reagent. The positive result is red color indicative of acetoin production. The Voges-Proskauer test determines organisms capable to produce acetoin from the degradation of glucose.

### 3.2.5.3.1.4 Citrate utilization test

The simmon citrate agar slant was inoculated with the bacteria and incubated at 37°C for 24 hours. The presence of growth on the surface of the slant, which is accompanied by blue coloration, identifies citrate-positive cultures. The citrate utilization test determines the capacity of an organism to use citrate as a sole source of carbon.

### 3.2.5.3.2 Catalase test

A single colony of the bacterial culture was transferred to a clean glass slide by sterile wooden sticks and mixed with a few drops of H2O2 reagent at a concentration of 3%. We observe bubble formation immediately, which indicates a positive result. The catalase test is used to determine organisms that produce the enzyme.

### 3.2.5.3.3 Oxidase test

A single colony of the bacterial culture was smeared using sterile wooden sticks on filter papers soaked with 1-2 drops oxidase reagent. Within 30 seconds of contact with the oxidase reagent, we observe the appearance of purple color in the organism, which indicates that the test is positive. The oxidase test determines bacteria containing the respiratory enzyme cytochrome c oxidase.

### 3.2.5.3.4 Growth on Pseudomonas agar

Pseudomonas agar as a selective medium for *P. aeruginosa* bacterial isolates. These isolates were cultured on this medium and incubated at 37°C for 24 hours, blue-green pigment indicated the presence of pyocyanin as a positive result.

### 3.2.5.3.5 Growth at 42°C and 4°C

Nutrient agar medium was inoculated with a colony of bacterial isolates and incubated at 42°C and 4°C for 24 hours. Only the *P. aeruginosa* colonies could grow at 42°C.

### 3.2.5.3.6 Triple Sugar–Iron (TSI) test

A small number of bacteria was taken from fresh culture by a sterile straight inoculation needle and inoculated by stabbing the center of the triples sugar–iron medium to the bottom of the tube. The tubes were then incubated at 37°C for 24 hours. Triple sugar–iron (TSI) test is used to determine whether a gram-negative rod ferments glucose, lactose, and sucrose fermentation with or without hydrogen sulfide production.

### 3.2.5.4 Identification of the bacteria by VITEK 2 compact system

The Vitek 2 compact system was used to confirm the diagnosis of the type of the bacterial isolates after they were diagnosed by colony characteristics, microscopic examination, and biochemical test. It is among the automation systems developed for bacteriology diagnostic. Several investigations were performed on Vitek2 and showed that the system was giving reliable results (HernándezDurán *et al.*, 2017). It is an automated method used for bacterial identification and sensitivity testing available from BioMerieux. It uses a growth-based photometric technology in which bacteria use a substrate that alters the color and density that are detected by phototransistor detectors. The system uses calorimetric reagent cards, which are incubated after inoculation with suspect bacteria into Vitek 2 where species identification and antimicrobial susceptibility are automatically interpreted.

The device consists of a cassette holder and a 64-hole reagent card container, each one representing the base material or medium for testing, and plastic tubes, as well as a DensiChek device and an information input and output unit.

The diagnostic process was carried out according to the manufacturer's instructions (BioMerieux, France) following steps :

- A bacterial suspension was made in sterile plastic tubes containing 3 ml of saline solution at a concentration (0.45% NaCl). A number of single colonies were transferred from the culture plate to the tube by a sterile ring and mixed well until the solution was cloudy. The turbidity of the suspension was measured using DensiChek.
- The VITEK 2 GN ID (Gram Negative) card and the bacterial suspension tubes were manually loaded into the VITEK-2 system.
- Test reaction results and analytical techniques: The device works by calculating the results and comparing them with the results stored in the device, which includes many test measurements and a large number of strains developing in different conditions and isolated from various places, and shows the results of the tests in the form of (+), (-). The symbol (-) indicating a negative reaction and the symbol (+) indicating a positive reaction.

### 3.2.6 Preservation of isolates

Two methods were used for the preservation of isolates

#### 3.2.6.1 Preservation for a short period

The bacterial isolates were inoculated after being diagnosed on the slant nutrient agar medium, and incubated at 37 °C for 24 hours, and stored for one to a maximum of 3 months at 4°C in the refrigerator (Fugelsang and Edwards, 2007).

### 3.2.6.2 Preservation for a long period

The bacterial isolates of *P.aeruginosa* were stored for a long period (up to three months) on a brain heart infusion broth medium containing 15% glycerol at -20°C (deep freezing). The medium consisted of 2 mL of glycerol and 8 ml of brain heart infusion broth (final volume is 10 mL) and then autoclaved. After cooling, the medium was inoculated with a pure bacterial isolated colony and incubated at 37°C for 24 hours. The tubes were stored at -20°C in deep freezing (Vandepitte *et al.*, 2003).

## 3.2.7 Detection of virulence factors of *Pseudomonas aeruginosa* isolates

### 3.2.7.1 Protease production

This test was used to investigate the ability of the bacterium to produce the protease enzyme. Bacteria were inoculated on skim milk media and incubated at 37°C for 24 hours. The observation of the formation of a transparent area around the culture line was evidence of the ability of the bacteria to produce the enzyme protease and hydrolyze casein indicate a positive result ( Cappuccino and Welsh, 2018).

### 3.2.7.2 Gelatinase production

Used to determine the ability of the bacteria to produce gelatinases. Gelatin liquefaction medium was inoculated with colonies of *P.aeruginosa* and incubated at 37°C for 24 hours. After incubation, the culture was placed in the refrigerator at 4°C for 30 minutes. Cultures that remain liquefied produce gelatinase and show the hydrolysis of gelatin was referred to as a positive result (Cappuccino and Welsh, 2020).

### 3.2.7.3 Haemolysin production

It was used to detect the production of haemolysin enzyme. The bacteria were inoculated on the blood agar base medium containing 5% human blood and incubated at 37°C for 24 hours. After incubation, total red blood cell lysis (clear zone) formed around the cultured colonies that showed hemolysin positivity (β-hemolysis) ( Najnin *et al.*,2018).

### 3.2.7.4 Lipase production

Used to detect and enumerate lipolytic bacteria. An egg yolk medium was used for this purpose, where the bacteria were inoculated and incubated at 37°C for 24 hours, the appearance of the lipolytic zone refers to a positive result (Okwu1 *et al.*, 2014).

### 3.2.7.5 Urease activity

Urea agar slant was inoculated with bacterial by streaking and incubated at 37°C for 24 hours. The positive results are changes in the color of slant from light orange to magenta (Granato *et al.*, 2019).

### 3.2.7.6 Detection of motility

It was used to detect bacterial motility. The motility medium was inoculated from colonies of *P.aeruginosa* and incubated at 37°C for 24 hours, and motile organisms will spread into the medium from the inoculation site indicating positive results (Tille, 2017).

### 3.2.7.7 Pigments production

Pigment production was examined using Pseudomonas agar and Nutrient agar medium, inoculated with bacterial isolates and incubated at 37°C for 24

hours to visual analysis colony morphology and pigments production (Nader *et al.*, 2017).

### 3.2.7.8 Biofilm formation

The biofilm formation was detected by microtiter plate assay according to (Almeida *et al.*, 2013). The bacteria were inoculated on Nutrient broth medium at 37°C for 24 hours. Thereafter, the broth cultures were compared with a MacFarland standard No. 0.5 using the same medium as the diluent. 200 µl of an isolate suspension were transferred into each of three wells of a 96-well flat-bottomed polystyrene plate and incubated at 37°C for 24 hours. After that, each well was washed three times using distilled water with rough shaking and later dried thoroughly. The adhering bacterial cells were fixed with 200 µl of absolute methanol. After that, each well was stained with 200 µl of 0.5% crystal violet for 15 minutes. Repetitive washing was performed to remove the excess stain. Later, the crystal violet bound to the adherent cells was retained with 200 µl of ethanol per well. The test was made in triplicates, and the absorbance of wells filled with bacteria-free Nutrient broth served as a negative control. The amount of crystal violet removed by 95% ethanol in each well was quantified by measuring the OD 630 nm using an ELISA reader according to what was stated in (Tang *et al.*, 2011). Because of this, the absorbance values represented the intensity of the biofilm formed by well-studied isolates on the surface of the microtiter. The results obtained were categorized into three groups (i.e., Non-biofilm producer, Moderately, and Strongly biofilm producer).

Where the absorption of the cultivated pit was compared with the control pits, as follows: if OD ≤ ODc (Considered non-biofilm producer); if ODc ≤ OD ≤ 2* ODc (Considered moderately biofilm producer); if 2* ODc ≤ OD ( Considered strongly biofilm producer). Where OD (Represent the of tested isolates); ODc (Represent control pits).

### 3.2.8 Antibiotics susceptibility test

The susceptibility test of each isolate against 13 antibiotics mentioned in table (3.8) was carried out using the Kirby-Bauer method on Mueller-Hinton agar as stated in the Clinical and Laboratory Standards Institute (CLSI-2020)

**Table 3-8: The Classes of antibiotics and Concentration**

| Classes of antibiotics | Antibiotics | Concentration (µg / disk) |
|---|---|---|
| **β-Lactam Compination Agents** | Piperacillin/Tozabactam | 100/10 |
| | Ticarcillin/clavulanic acid | 75/10 |
| **Cepheme (parenteral)** | Ceftazidime | 30 |
| | Cefepime | 30 |
| **Fluoroquinolones** | Ciprofloxacin | 5 |
| | Levofloxacin | 5 |
| **Aminoglycosides** | Gentamicim | 10 |
| | Tobramycin | 10 |
| | Amikacin | 30 |
| | Netilmicin | 30 |
| **Monobactams** | Aztreonam | 30 |
| **Carbapenems** | Imipenem | 10 |
| | Meropenem | 10 |

1. Bacterial suspensions of each isolate were prepared by transferring a single colony grown on MacConkey agar medium to 5 mL of distilled water to obtain a turbidity suspension of $(1.5\times10^{8})$ CFU / ml by compared with MacFarland standard.

2. With a sterile swab stick, the bacterial suspension is spread on the surface of the Mueller-Hinton agar medium, the entire surface of the plate is streaked in three directions, then left to dry at room temperature for 15 minutes.

3. Antibiotic discs were transferred by antibiotic disk dispenser to the plates at a rate of 6 discs per dish, and fixed on the surface of the plate and left the plate for 30 minutes and the dishes were incubated at 37°C for 18-24 hours.

The results were read by observing the zones of inhibition formed around the antibiotic disc and the bacteria were considered S-sensitive, I- intermediate, or R-resistant according to (CLSI, 2020).

### 3.2.9 Determination of Minimum, Sub Minimum Inhibitory Concentration (MIC, Sub MIC) and Minimum Bactericidal Concentration (MBC) for two antibiotics (Imipenem and Ceftazidime)

The minimum inhibitory concentration (MIC) was determined for *Pseudomonas aeruginosa* isolates toward Imipenem (IMI) and Ceftazidime (CAZ) by the serial dilution method in Mueller-Hinton broth. Serial dilution of antibiotics between 2–1024 µg/ml of antibiotics. Used ten isolates selected according to biofilm formation (strong biofilm). Bacterial suspension with turbidity equivalent to 0.5 MacFarland was added to the tubes contained a different concentration of antibiotics. After incubation at 37°C for 18-24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antibiotic that inhibits bacterial growth. Sub-minimum inhibitory concentrations (sub-MICs) represent the lowest inhibitory concentration at which bacteria can grow (Andersson and Hughes, 2014 ).After the MIC determination, the values of the minimum bactericidal concentration (MBC) were determined by sub-culturing the content of each tube without any

growth, on Mueller Hinton agar and incubated at 37°C for 24 hours and looking for any bacterial growth.

### 3.2.10 Effects of antibiotics on the biofilm formation

The antibiotics that used in the current study were Imipenem and Ceftazidime. Each antibiotic was tested at sub-MICs to study the change in the ability of *P. aeruginosa* isolates in biofilm formation. Different concentrations of each antibiotic were applied to measure biofilm formation at sub-MIC by microtiter plates as previously mentioned (3.2.9). The microtiter plates were prepared and incubated at 37°C for 24 hours. Control plates were prepared of a free antibiotic-microtiter plate which was dispensed to the wells with 200 µL of Nutrient broth without antibiotics (Hemati *et al*., 2016).

The absorbency was measured in an ELISA reader at 630 nm, according to what was stated in (Tang *et al*., 2011). Where the absorption of the cultivated pit was compared with the control pits, as follows: If OD ≤ ODc (Considered non-biofilm producer); if ODc ≤ OD ≤ 2* ODc (Considered moderately biofilm producer); if 2* ODc ≤ OD ( Considered strongly biofilm producer). Where OD (Represent the of tested isolates); ODc (Represent control pits).

### 3.2.11 Molecular study

### 3.2.11.1 DNA extraction

Genomic DNA was extracted from bacterial growth according to the protocol of Geneaid Extraction as the following steps:

### Suspension and protein digestion

- For pellet cells, 1ml of overnight culture was centrifuged for 3 minutes at 12000 rpm. The supernatant was then discarded.

- For suspension, 180 µl of GT buffer was added to the pellet and mixed by the vortex.
- For protein digestion, 20 µl of proteinase K was added to the suspension.
- All mixtures were incubated at 60ºC for at least 10 minutes.

**Cell lysis**

- For cell lysis, 200 µl of GB buffer was added to the sample and mixed by vortex for 10 seconds.
- The mixture was incubated at 70ºC for at least 10 minutes to ensure the sample lysate is clear.
- After incubation, samples were spanned in a centrifuge for 10 seconds to remove bubbles

**Binding**

- For DNA binding, 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously. Next, the mixture (including any insoluble precipitate) was transferred to the GD column then centrifuged at 12,000 rpm for 1 minute.
- The GD collection tube containing the flow-through was discarded, and the GD column was placed into a new GD collection tube

**Washing**

- For washing, 400 µl of W1buffer was added to the GD column and centrifuged at 12,000 rpm for 30 seconds then the flow-through was discarded.
- The GD column placed back in the 2ml collection tube, and 600 µl of Wash buffer (make sure ethanol was added) was added to the GD column
- Centrifugation at 12,000 rpm for 3 minutes was performed, then the flow-through was discarded, and the GD column was placed back in the 2ml collection tube.

- The empty column matrix was centrifuged at 12,000 rpm for 3 minutes to dry the column matrix.

**Elution**

- The dried GD column was transferred into a clean 1.5 ml micro spin centrifuge tube.
- Aliquot of 100 µl pre-heated elution buffer1 was added into the center of the column matrix. After waiting at least 3minutes (to allow elution buffer to be completely absorbed). Centrifugation at 9,000 rpm for 3 minutes was performed to elute the purified DNA.

### 3.2.11.2 Quantitation of DNA

A quantus fluorometer was used to detect the concentration of extracted DNA to detect the quality of samples. For 1 µl of DNA, 199 µl of diluted quantifluor dye was mixed. After 5 minutes of incubation at room temperature, DNA concentration values were detected.

### 3.2.11.3 Primer preparation

The primers of three genes were used in the current study were supplied by the Macrogen company in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100 pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10 µl of primer stock solution (stored at freezer -20 ºC) to 90 µl of nuclease-free water to obtain a working primer solution of 10 pmol/µl.

### 3.2.11.4 Reaction setup

The PCR reaction was used from GO *Taq* Green Master Mix prepared by Promega USA, PCR mixture was thawed by exposing it to a laboratory temperature, then placed in a centrifuge so that the components collided at the

bottom of the tube. The final volume of the reaction mixture became 20 µl as in table (3-9)

**Table 3-9:Protocol of PCR reaction mixture volumes used in the current study**

| Master mix components | Stock | Volume(µl) |
|---|---|---|
| Master Mix | 2X | 10 |
| Forward primer | 10µM | 1 |
| Reverse primer | 10µM | 1 |
| Nuclease Free Water | - | 6 |
| DNA | ng/µl | 2 |
| Total volume | 20 | |
| Aliquot per single rxn | 18µl of Master mix per tube and add 2 µl of template | |

### 3.2.11.5 Thermal cycling protocol

The PCR tubes containing the mixture were transferred to thermo-cycler and DNA was amplified using the protocol as in table (3-10).

**Table 3-10: Thermal Cycling Protocol**

| Steps | ºC | Minute: Second | Cycle |
|---|---|---|---|
| Initial Denaturation | 95 | 05:00 | 1 |
| Denaturation | 95 | 00:30 | 30 |
| Annealing | 52 | 00:30 | |
| Extension | 72 | 00:30 | |
| Final extension | 72 | 07:00 | 1 |
| Hold | 10 | 10:00 | |

### 3.2.11.6 Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

### 3.2.11.6.1 Preparation of agarose

- 100 ml of 1X TAE was taken in a flask.
- 1.5 gm (for 1.5%) agarose was added to the buffer.
- The solution was heated to boiling (using a Microwave) until all the gel particles were dissolved.
- 1µl of Ethidium Bromide (10mg/ml) was added to the agarose.
- The agarose was stirred to get mixed and to avoid bubbles.
- The solution was left to cool down at 50-60°C.

### 3.2.11.6.2 Casting of the horizontal agarose gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was left to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

### 3.2.11.6.3 DNA loading

Two microliters of loading dye were applied to each 5 µl DNA sample, and samples were carefully added to the individual wells. PCR products were loaded directly. For PCR product, 10 µl was directly loaded to well. Electrical power was turned on at 100 v/m Amp for 75 minutes. DNA moves from cathode to plus

anode poles. The ethidium bromide-stained bands in gel were visualized using Gel imaging system.

### 3.2.12 Statistical analysis

Data were analyzed on SPSS (Statistical Package for Social Science) version 26 software. The chi-square was used to find the significant difference between the number of isolates and according to the source of isolation. T-test was used in evaluating the effect of sub-MIC of antibiotics on biofilm formation. The significance test was accepted when the P-value was $<0.05$(* $P<0.05$, ** $P<0.01$, *** $P<0.001$) (Negi, 2012).

# Chapter Four
# Results and Discussion

# 4. Results and Discussion

## 4.1 Isolation of *Pseudomonas aeruginosa*

Two hundred (200) specimens were collected from different infections included (urine, wounds, ear, burns, and sputum). Results showed that 26 isolates 13% were primarily identified as *P. aeruginosa*. The specimens were collected during the study period from the first of September 2020 ending at the end of January 2021. They were collected from Baquba city / Diyala governmental hospitals.

## 4.2 Identification of *Pseudomonas aeruginosa*

The isolated bacteria were diagnosed based on the colony characteristics, microscopic examination, and biochemical tests presented in table (4-1). And VITEK 2 compact system was dependent to complete the identification of *P.aeruginosa* isolates.

### 4.2.1 Colony characteristics

All the isolates (26) were cultured on Nutrient agar, MacConkey agar and, Blood agar, the isolates were then confirmed on Pseudomonas agar at 37°C for 24 hours. A colony of the bacterium *P. aeruginosa* appeared in the medium of the Nutrient agar a raised appearance and flat edges, smooth in shape, smell like grapes, and most of which produce pyocyanin. On MacConkey agar, the colonies were appeared as pale greenish and lactose non-fermenter this agrees with what he mentioned Al-Daraghi and Al-Badrwi (2020). Blood agar, a differential medium that can differentiate bacteria based on their ability to lyse red blood cells (RBCs), the colonies were round, convex, and surrounded by transparent halo on blood agar indicate complete hemolysis (β-hemolysis) and this agrees with what he mentioned Procop, *et at*. (2017). To confirm the diagnosis, the

specimens were re-cultured on the selective Pseudomonas agar medium of this bacterium, the colonies were mucoid, smooth in shape, and have a fruity odor, and the majority of the bacterial isolates showed their ability to produce pyocyanin (blue-green dye) and pyoverdin dyes (fluorescent pigment ) while in non-pigmented isolates were characterized by their regular circular shape and cream color. Pseudomonas agar a specific selective medium designed to select *P. aeruginosa* from other *Pseudomonas* species. The colonies grown on Mueller-Hinton agar show their ability to produce dyes and especially blue-green dye (pyocyanin). These results agree with Alsaadi, (2020) and Al-Shamaa *et al*. (2016). These pigments play a role in the pathogenesis of *P. aeruginosa* as virulence factors and are also a feature of pigmentation, which remains an important factor among the diagnostic features in the genus *Pseudomonas*. The results also showed the ability of all the isolates to grow at a temperature of 42°C, which is a trait an important prognosis for *P. aeruginosa*. However, not all isolates grow at 4 and this agrees with what he mentioned almamari, (2019).

### 4.2.2 Microscopic examination

The results of the microscopic examination for 26 isolates stained with a gram stain the cells of this bacteria appeared in the form of small bacilli and pink color referred to this bacteria is negative for the gram stain this is consistent with what he mentioned Cappuccino and Welsh, (2018). Gram-negative bacterial walls have a high percentage of lipids in their outer membranes and a thinner layer of peptidoglycan. The lipids are dissolved in a decolorizer and washed with the crystal violet iodine complex. After decolorization, colorless gram-negative organisms take on the red pigment and appear pink (Granato *et al*., 2019).

### 4.2.3 Biochemical tests

Biochemical tests were performed on all 26 isolates, that are included the oxidase test, catalase test, triple sugar iron (TSI) test, and IMVIC tests (indole test, methyl red test, Voges-Proskauer test, and citrate). *P.aeruginosa* was characterized by being positive to oxidase test and dark purple had appeared on the surface of colonies indicates cytochrome oxidase production and used detects the bacteria that produce the cytochrome oxidase enzyme. Bubble formation indicates a positive result for the catalase test and this test is used to identify organisms that produce the enzyme catalase. Positive to citrate test by conversion of the medium to blue the result of consuming citrate. While all the isolates were negative to indol, methyl red and Voges-Proskauer. TSI test, it was found that all isolates under study were not fermented for any of the three types of sugars (glucose, lactose, sucrose), it does not form CO2 and does not H2S production, and this is agreed with what is mentioned almamari, (2019). Table (4-1).

**Table 4-1: The results of diagnostic tests for *Pseudomonas aeruginosa***

| No. | Media and test | Result |
|---|---|---|
| 1 | Growth on MacConkey agar | + |
| 2 | Lactose fermentation | - |
| 3 | β-hemolysis when growth on blood agar | + |
| 4 | Growth on Pseudomonas agar | + |
| 5 | Growth at 42°C and 4°C | +/- |
| 6 | Pigment | + (bluish-green pigmentation) |
| 7 | Gram stain | - |
| 8 | Oxidase | + |
| 9 | Catalase | + |
| 10 | Citrate | + |
| 11 | Indol test | - |
| 12 | Methyl red test | - |
| 13 | Voges-Proskauer | - |
| 14 | Triple sugar iron | Alk / Alk, No $H_2S$, No gas |

**(+) Positive result and (-) Negative result**

### 4.2.4 Biochemical tests by VITEK 2

VITEK2 compact system was employed for the identification of 26 isolates using the identification card (GN ID Card ) to confirm a bacterial diagnosis which gives diagnostic results for bacteria with an accuracy of 99% and contains 64 biochemical tests. All isolates were identified as *P. aeruginosa*. An example report resulting from this system for identifying these bacteria is shown in the appendix (1). Table (4-2) shows the diagnostic results of this device. The device shows the results of weak isolates towards a specific reaction and is denoted by the symbol (-) indicating a negative reaction and the symbol (+) indicating a positive reaction.

**Table 4-2: Biochemical tests results of *Pseudomonas aeruginosa* by VITEK2**

| Test type | Result | Test type | Result | Test type | Result | Test type | Result | Test type | Result | Test type | Result |
|---|---|---|---|---|---|---|---|---|---|---|---|
| APPA | - | ADO | - | PyrA | - | IARL | - | dCEL | - | BGAL | - |
| H2S | - | BNAG | - | AGLTp | - | dGLU | + | GGT | + | OFF | - |
| BGLU | - | dMAL | - | dMAN | + | dMNE | + | BXYL | - | BAIap | + |
| ProA | + | LIP | + | PLE | - | TyrA | + | URE | - | dSOR | - |
| SAC | - | dTAG | - | dTRE | - | CIT | + | MNT | + | 5KG | - |
| ILATK | + | AGLU | - | SUCT | + | NAGA | - | AGAL | - | PHOS | - |
| GLyA | - | ODC | - | LDC | - | IHISa | - | CMT | + | BGUR | - |
| O129R | + | GGAA | - | IMLTa | + | ELLM | - | ILATa | + | | |

## 4.3 Distribution of *Pseudomonas aeruginosa* according to the source

Among 200 clinical specimens, 26 isolates were positive for *P. aeruginosa*. The source of these isolates was as follows: (7) isolates from urine, (7 ) isolates from wounds swab, (6) isolates from ear swab, (4) isolates from burns swab, (2) isolate from sputum taken from patients suffering from respiratory tract infection.

The highest percentage of *P. aeruginosa* was in urine and wound infections 26.92%, followed by ear specimens 23.07%, while the percentage was for burn specimens 15.38%, and the lowest percentage in sputum specimens, which was 7.69%. The results of our study agree with the result of Al-Saadi, (2020), conducted in Baquba city, which was 29.62% wound, 20.98% in urine, 7.40% sputum, and close to the percentage that they found in burn and ear samples, which were 25.92% and 16.04%, respectively. They also were found a highly significant (*P*=0.008) difference between the number of isolates and according to the source of isolation in the current study. Table (4-3).

Our study disagrees with Bakhtiar, (2018) that found the total of 180 different human specimens isolated from Kirkuk general hospital and the general Azadi hospital, the number, and percentage of *P.aeruginosa* isolates were highest in burns 17/41 (41.46%), followed by wound 4/48 (8.33%), urine 3/56 (5.35%) and ear 1/35 (2.85%).

**Table 4-3: The numbers and percentages of *Pseudomonas aeruginosa* among different clinical specimens.**

| Type of specimens | No. of specimens &(%) | No. of *P. aeruginosa* &(%) | Percentage of isolates to specimens |
|---|---|---|---|
| **Urine** | 67 (33.5%) | 7 (26.92%) | 7*** (10.44%) |
| **Wounds swabs** | 55 (27.5%) | 7 (26.92%) | 7 (12.72%) |
| **Ear swab** | 41 (20.5%) | 6 (23.07%) | 6 (14.63%) |
| **Burn swab** | 25 (12.5%) | 4 (15.38%) | 4 (16%) |
| **Sputum** | 12(6%) | 2 (7.69%) | 2 (16.66%) |
| **Total** | 200 (100%) | 26 (100%) | 26 (13%) |

****P*=0.008

The difference in the presence of *P. aeruginosa* among infected isolates is attributed to major factors that include differences in the source of isolation, sampling time, method, sampling season, number of samples collected, and geographical location, in addition to whether the patient is using drugs that inhibit the growth of bacteria or those that the bacteria resist growing, which greatly contributed to their spread. Also, the degree of concern for hygiene and the type of sterilizers and disinfectants used in hospitals. Other restrictions are the lack of easy access to a burns patient compared to other cases. Other cases vary between studies of this type.

## 4.4 Virulence Factors of *Pseudomonas aeruginosa*

The ability of bacterial isolates to cause infections is due to the virulence factors play that a critical role in their infections but does not participate in bacterial growth. In this study, protease, gelatinase, hemolysin, lipase, urease, motility, pigment, and biofilm formation are chosen as representative virulence factors of the *P. aeruginosa*. Figure (4-1).

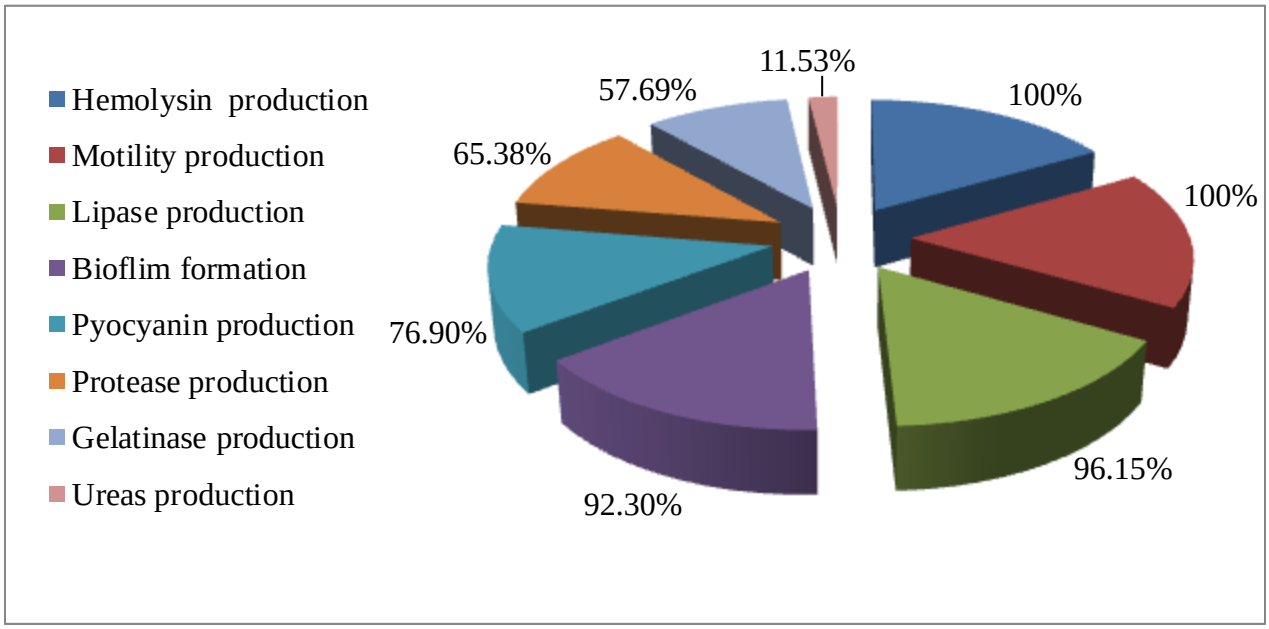



**Figure 4-1: Percentage of virulence factors in 26 *Pseudomonas aeruginosa* isolates**

### 4.4.1 Protease production

The isolates under study were tested for their ability to produce protease enzyme by cultivating them on skim milk agar, as it was found that 17 (65.3%) of isolates produced protease this result agrees with Macin *et al*. (2017) which was 62.3% but this result disagrees with Bakhtiar, (2018) was all isolates 100%, protease producers.

The production of protease enzymes for *P. aeruginosa* varies by sample source, colony type, and severity of the disease, and sample size (Al-Salhi and Hassan, 2015). The isolates producing the pyocyanin dye showed the spread of the dye over this medium, as it is considered one of the important media for observing the production of bacterial pigments, and this result agrees with Al-mamari, (2019). The protease is considered one of the important virulence factors responsible for tissue breakdown and skin necrosis in skin injuries and internal organ hemorrhage in systemic injuries, facilitate bacterial invasion and growth (Al-Yousef *et al*., 2021)

### 4.4.2 Gelatinase production

All the isolates tested for the production of gelatinase were found that only 15 (57.6%) of the isolates produced gelatinase by hydrolysis of gelatin. This result agrees with Iseppi *et al*. (2020) which was 53%, while disagreed with several researchers who reported a high ability of *P. aeruginosa* to produce this enzyme.

A protein found in connective tissue has significance in pathogenesis and allows bacteria to hydrolyze gelatin and metabolize small peptides that arise from their hydrolysis to obtain energy. Gelatinize activity can strengthen their virulence, making it pathogenic, especially for people who are immunocompromised (Iseppi *et al*., 2020)**.**

### 4.4.3 Hemolysin production

The isolates under study were tested for their ability to produce hemolysin by cultivating them on a blood agar base medium containing 5% human blood, as it was found that all isolates 26 (100%) were capable of producing beta-hemolysis around the colonies and this result agrees with Hameed, (2017) and Najeeb, (2020) which was 100% and this result disagrees with Rodulfo, *et al*. (2019) which was 83.3%.

Hemolysin production is the most important virulence factor for *P. aeruginosa*, as its production is associated with neurotoxicity and cytotoxicity of the cell and can destroy red blood cells to extract iron from them and it causes inflict direct cytotoxic effects on the renal epithelium leading to scarring. Also, hemolysins destroy various host tissues and cells including red blood cells, leucocytes, epithelial cells, and endothelial cells. It is a pore-forming toxin capable to destroy cells by lysis (Iseppi *et al*., 2020).

### 4.4.4 Lipase production

The ability of the isolates to produce lipase was tested by cultivating them on egg yolk agar and it was found that 25 (96.1%) of the isolates were lipase-producing and this result agrees with the result of Alim *et al*. (2017) which was 95%.

The production of lipase in these pathogens enables them penetration of the innate immune system of the human being, infecting host cells, and modulate the adaptive immune mechanisms of the human being, thus serving the aim of establishing a systemic or more localized chronic colonization, and thus is associated with increased virulence and also contribute to morbidity and mortality(Rocha *et al*., 2019).

### 4.4.5 Urease production

Regarding urease production, 3(11.53%) isolates were positive to this enzyme, and this result agrees with the result of Al-Salhi and Hassan, (2015) and a close result for Noomi, (2018) which were 11.43% and 19.2% respectively.

Urease is necessary for the colonization of the host organism and due to its enzymatic activity, has a toxic effect on human cells. This enzyme can distract urea into CO2 and NH3 and increase the pH, which promotes bacterial growth (Nile *et al*., 2015).

### 4.4.6 Motility production

All isolates were motile 26(100%) which similar to the result of Noomi, (2018) which was 100%, and disagrees with Macin and Akyon (2017) which was 89%. The isolates gave good evidence that these bacteria are motile by a single polar flagellum, on a semi-solid medium.

Motility is needed for colonization of the host and the establishment of biofilms and is mediate initial surface interactions (Domingo-Calap *et al*., 2016).

### 4.4.7 Pigments production

Pigment production as a virulence factor was tested by using Pseudomonas agar and Nutrient agar. Pigments play an important role in the pathogenicity of *P. aeruginosa*. Which was found 20(76.9%) of isolates production of pyocyanin on both medium this result agree with Nader *et al.* (2019) which was 72.15% and five isolates produced pyoverdin but only one isolate did not produce the pigments.

### 4.4.8 Biofilm formation

Biofilm formation.is another virulence factor due to the inherent resistance of the biofilm to antimicrobial agents and the choice of phenotypic variants. All isolates (26) were evaluated based on their ability to biofilms formation using the microtiter plate method.

The Micro Titer Plate method (MTP) is a method for studying early biofilm formation on abiotic surfaces and it is a colorimetric technique that uses dyes such as crystal violet to stain attached biofilms and to quantify by using an absorbance microtiter plate reader (Jesus and Dedeles, 2020).

The results of the current study presented in table (4-4) showed that the majority of the isolates produced biofilm 24(92.3%) with different yields between strongly and moderately compared to the negative control, while only two isolates (7.6%) represented non-biofilm producer. The absorbency values were ranged from ( 0.041-0. 153) for biofilm-produced isolates and (0.034-0.036) for non-biofilm-produced isolates.

Among biofilm-produced isolates, 38.4% of isolates were strongly biofilm producer this result agrees with Abdulhaq *et al*. (2020) which was 36.5%, and it is noteworthy that the rate of strong biofilm production in urine specimens was higher than other specimens and this result agrees with Bahador *et al*. (2019). While 53.8% moderately biofilm producer this result agrees with Al-Sheikhly *et al*. (2019) which was 56%. The results in the current study disagree with Bahador *et al*. (2019) which was, 60% were strongly biofilm produced and the rates of moderately and weak biofilm produced were 34.3% and 4.3%.

The correlation between biofilm producer and antibiotic resistance was among 38% strongly biofilm produced was 40% multi-drug resistance (MDR) while from 53.8% moderately biofilm produced 50% was MDR.

**Table 4-4: Absorbency values and biofilm pattern by MTP methods**

| NO. of isolates | Isolates source | Absorbency at 630 nm | Biofilm level compared to (ODc=0.038) |
|---|---|---|---|
| **PA 1** | Urine | 0.060 | Moderately |
| **PA 2** | Urine | 0.063 | Moderately |
| **PA 5** | Ear | 0.068 | Moderately |
| **PA 11** | Wound | 0.056 | Moderately |
| **PA 14** | Wound | 0.059 | Moderately |
| **PA 16** | Sputum | 0.069 | Moderately |
| **PA 17** | Burns | 0.061 | Moderately |
| **PA 6** | Ear | 0.056 | Moderately |
| **PA 7** | Wound | 0.048 | Moderately |
| **PA 8** | Wound | 0.049 | Moderately |
| **PA 9** | Urine | 0.045 | Moderately |
| **PA 21** | Burns | 0.069 | Moderately |
| **PA 22** | Ear | 0.041 | Moderately |
| **PA 24** | Burns | 0.049 | Moderately |
| **PA 3** | Urine | 0.096 | Strongly |
| **PA 4** | Urine | 0.105 | Strongly |
| **PA 10** | Wound | 0.077 | Strongly |
| **PA 12** | Wound | 0.131 | Strongly |
| **PA 13** | Wound | 0.081 | Strongly |
| **PA 15** | Sputum | 0.153 | Strongly |
| **PA 25** | Ear | 0.082 | Strongly |
| **PA 18** | Urine | 0.098 | Strongly |
| **PA 19** | Urine | 0.153 | Strongly |
| **PA 20** | Burns | 0.092 | Strongly |
| **PA 23** | Ear | 0.034 | Non-biofilm producer |
| **PA 26** | Ear | 0.036 | Non-biofilm producer |

The process of biofilm formation depends on many factors such as the medium, the detection method used and the incubation conditions as well as the type of surface used for that process, as the difference in the composition of the biofilm on the microtiter plate is due to the quality of the surface composition of the materials on which the biofilm is formed, for example, the membrane formed on the polystyrene surface of microtiter plates is more effective than the silicone surface of the catheter (Wojnicz *et al*., 2015).

The differences in biofilm density between the isolates in this study may be due to several reasons. Differences in the ability of isolates to form biofilms or perhaps differences in the initial number of cells that successfully adhere to and differences in the quality and quantity of quorum-sensing signaling molecules produced from each isolate play important roles (Abdulammer, 2018)

On the other hand, Heydari and Eftekhar (2014) indicated that the variation in the ability of isolates to form biofilm is due to the correlation of production with its ability to produce different types of ß-Lactamase, which leads to the formation of a strong biofilm compared to the isolates that produced one type of enzyme. Conversely, the isolates did not produce this enzyme and were not able to form a biofilm.

This high throughput of biofilm formation may be attributed to the sensitivity of the microtiter plate method to quantify the few quantities formed. Which consider more accurate, easy, and sensitive in the detection of biofilm formation. When studying the early stages in biofilm formation, it is possible to adopt the microtiter plate method because this method uses stable conditions, so it can be used in the study of many factors necessary for the formation of the biofilm, such as flagellum, pili, as well as the genes that play an important role in the production of exopolysaccharides (Obaid, 2019).

Biofilm is representing aggregates encased in a self-produced extracellular matrix that is difficult or impossible to eradicate with antibiotics. Its matrix provides a protective border that allows it to adhere to an environmental substrate. This coating plays a role in bacteria's resistance to antibiotics. Biofilms can provide 10 to 1,000 fold more protection against antibiotic treatment. The risks of biofilm arose from the fact that it is a major driver of the persistence of chronic infection (Ciofu and Tolker-Nielsen, 2019).

The effect of the high percentage of the susceptibility of *P.aeruginosa* isolates in the current study on the production of biofilms demonstrates the high resistance rates for these isolates towards all antibiotics. In this state, bacteria can be up to 1,000-fold more resistant to antibiotics than those in a planktonic state (Cepas *et al.*, 2019).

**Table 4-5: Detection of eight virulence factors of *Pseudomonas aeruginosa* isolates**

| Virulence Factors | NO. of isolates | % |
|---|---|---|
| Protease production | 17 | 65.3% |
| GelatinaseProduction | 15 | 57.6% |
| HemolysinProduction | 26 | 100% |
| Lipase production | 25 | 96.1% |
| Urease production | 3 | 11.5% |
| Motility production | 26 | 100% |
| Pigments production | 20 | 76.9% |
| Biofilm formation | 24 | 92.3% |

## 4.5 Antibiotics susceptibility test

Antibiotic susceptibility test was performed for 26 isolates of *P. aeruginosa* against 13 kinds of antibiotics Piperacillin/Tazobactam (100/10µg), Ticarcillin/Clavulanic acid (75/10 µg), Ceftazidime (30µg), Cefepime (30µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Gentamicin (10µg), Tobramycin

(10µg), Amikacin (30µg), Netilmicin (30µg), Aztreonam (30µg), Imipenem (10µg)and Meropenem (10µg) related to 7 different classes. The standard agar-disk diffusion method known as the Kirby Bauer method was used according to the Clinical and Laboratory Standards Institute (CLSI-2020) guidelines (Performance Standards for Antimicrobial Susceptibility Testing; 30th Informational Supplement).

In the current study, the antibiotic resistance test revealed that a high percentage of resistance was seen against Ticarcillin/Clavulanic acid 69.2% this result agrees with Al-saadi, (2020) who reported Ticarcillin/Clavulanic resistance which was 71.6% and this result disagrees with Mwinyikombo, (2018) which was 100%. While a lower percentage of antibiotic resistance was seen against Piperacillin/Tazobactam 3.8% compared to other antibiotics used in the current study, this result agrees with Hoque *et al*. (2015), safferi *et al*. ( 2017), sala *et al*. (2019), and Namnq *et al*.(2019) who reported Piperacillin/ Tazobactam resistance which was 3.37%, 4.3%, 2%, and 5% respectively and this result disagrees with Elhariri *et al*. (2017) which was 76.2%.

The percentage of resistance for Gentamicin and Imipenem was 26.9%, the resistance percentage to Gentamicin agrees with Hasan *et al*. (2020) and Sameet *et al*. (2020) who reported Gentamicin resistance which was 30% and 29.09% respectively and this result disagrees with Al-Obaidi and Al-Dahmoshi (2020) which was 50%. While the resistance percentage to Imipenem agrees with Macin and Akton (2017), and Shidiki *et al*. (2019) which was 28% and 27.6% respectively, and this result disagree with Osman *et al*. (2018) which was 75%.

For Amikacin the percentage resistance was 30.7%, and this result agrees with Macin *et al*. (2017), and Sameet *et al*. (2020) who reported Amikacin resistance which was 29.5% and 27.2% respectively, while this result, disagrees with Saleh *et al*. (2020) which was 60%.

The percentage of resistance for Ceftazidime, Ciprofloxacin, Levofloxacin, and Tobramycin was 34.6%. The resistance percentage to Ceftazidime agrees with Najnin *et al*. (2018) and Lila *et al*. (2018), who reported Ceftazidime resistance which was 35% and 38.8% respectively, while this result disagrees with Pungcharoenkijkul *et al*. (2021) which was 11.11%. The resistance percentage to Ciprofloxacin agrees with Yang *et al*. (2020) who reported Ciprofloxacin resistance which was 34.2% and this result disagrees with Qayoom *et al*. (2019) which was 72.2%. While the resistance percentage to Levofloxacin agrees with Aziz *et al*. (2019) and Hosu *et al*. (2021) who reported Levofloxacin resistance which was 36.2% and 30.6% respectively and this result disagrees with Ahmad *et al*. (2020 ) which was 16.7 %. The resistance percentage to Tobramycin agrees with Hosu *et al*. (2020) and Emaneini *et al*. (2019) who reported Tobramycin resistance which was 33% and 32.9% respectively and this result disagrees with Asghar and Ahmed (2018) which was 17%.

Finally, The percentage of resistance for Aztreonam, Cefepime, Meropenem, and Netilmicin was 38.4%. The resistance percentage to Aztreonam agrees with Bavasheh and Karmostaji (2017), Aziz *et al*. (2019), and Khadim and AL Marjani (2019) who reported Aztreonam resistance which was 37.5%, 38.7%, and 39.6% respectively, while this result disagrees with Bhuiya *et al*. (2018) which was 11.7%. The resistance percentage to Cefepime agrees with Aziz *et al*. (2019) and Ferman, (2019) who reported Cefepime resistance which was 35% and 38% respectively and this result disagrees with Mohamed *et al*. (2019) which was 57%. The resistance percentage to Meropenem agrees with Namnq *et al*. (2019) and Hosu *et al*. (2021) who reported Meropenem resistance 41.7% and 43% and this result disagrees with Emaneini *et al*. (2019) which was 9.41%. The resistance percentage to Netilmicin agrees with Holbrook and Garneau-Tsodikova (2018) who reported Netilmicin resistance 33.6%. Figure (4-2)

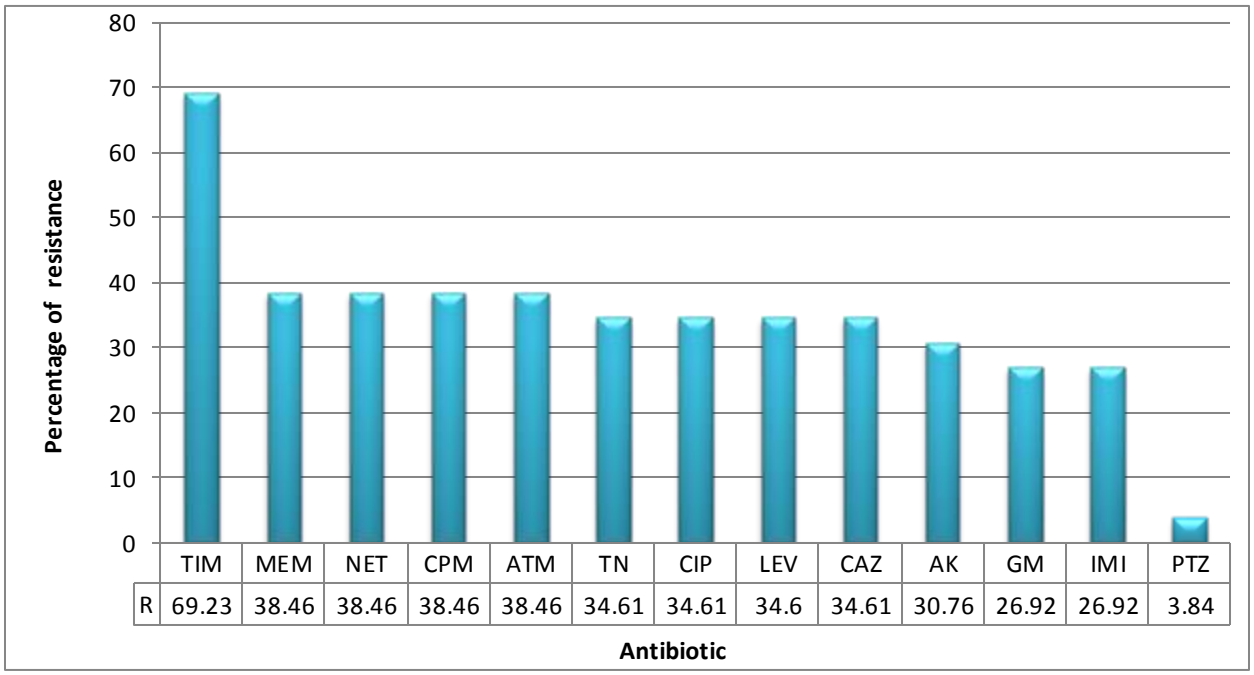



**Figure 4-2: Percentage of antibiotic resistance of *Pseudomonas aeruginosa* isolates.**

TIM = Ticarcillin/clavulanic acid , MEM = Meropenem , NET = Netilmicin , CPM = Cefepime, ATM = Aztreonam , TN = Tobramycin , CIP=ciprofloxacin , LEV= Levofloxacin, CAZ = Ceftazidime, AK= Amikacin , GM = Gentamycin, IMI = Imipenem , PTZ = Piperacillin/Tozabactam .

From the above results, Piperacillin/Tozabactam was the most effective drug compared with others. Whereas, Ticarcillin/clavulanic acid was resisted by the bacteria.

In the current study, a lower prevalence of antibiotic resistance was observed. In contrast to other studies that showed a high prevalence of antibiotic resistance. This could occur due to the differences in strains of the *P. aeruginosa* isolates obtained and may be related to differences in antibiotic use in different environments and selective pressure and this result may be due to the difference in sample size for these bacterial isolates in the current study (Mwinyikombo, 2018).

*Pseudomonas aeruginosa* can produce Cephalosporinase enzymes, which are the most important types of β-lactamase, which encode chromosome-based genes as well as other types of enzymes. These enzymes have been found in *P. aeruginosa* responsible for resistance to Aztreonam, Carbapenems, first, second generation and the third of Cephalosporins and resistance to Cephalosporins are due to the destruction of the antibiotic by beta-lactamases, reduced penetration across the outer membrane of gram-negative bacteria, and enhanced efflux (Torok *et al.*, 2017).

Carbapenems are a large class of beta-lactamase and are often used as a last resort treatment for *Pseudomonas* infection because it exhibits high affinity for penicillin-binding protein, is stable against broad-spectrum beta-lactamases (Lee and Bradley, 2019; Elshamy *et al.*, 2018) and easily passes through the outer membrane (Farhan *et al.*, 2019). Resistance to Carbapenems, including Imipenem 26.9% and Meropenem 38.4%, was also noted in our study. This is completely unexpected, given the fact that Carbapenems represent is one of the most effective and among the best options for treating gram-negative infections and especially multi-drug resistance infections. Multifaceted interactions mediate many of the carbapenem resistance mechanisms and may include loss of outer membrane purine, overexpression of efflux, and production of Carbapenemases. Carbapenem-resistant *P.aeruginosa* isolates are often associated with a higher mortality rate due to the enzyme carbapenemase that mediates resistance and a greater potential for widespread resistance spread through mobile genetic elements (Juayang *et al.*, 2017).

Aminoglycosides are broad-spectrum, high potency antibiotics that are traditionally used to treat serious gram-negative bacteria such as *Pseudomonas* infection (Holbrook and Garneau-Tsodikova, 2018). Aminoglycosides work by inhibiting protein synthesis by binding to 16S rRNA and by disrupting the integrity of the bacterial cell membrane. Aminoglycosides resistance may occur

through a change in membrane permeability thus preventing the antibiotic molecule from entering the bacterial cell (Sameet *et al*., 2020). Amikacin, Gentamicin, and Tobramycin are first or second-line empirical drugs preferred in the antibiotic access group for treatment of common or severe clinical conditions (Sharland *et al*., 2018), in particular, inhaled Tobramycin is used to eliminate the early infection in cystic fibrosis (CF) while Amikacin and Gentamicin are also used in combination therapy with other antibiotics to improve overall efficacy (Ren *et al*., 2019).

Amikacin resistance causes a genetic mutation that disrupts protein synthesis. In this case, the wrong types of amino acids in the polypeptide chain split to form the wrong type of protein, whereas the decrease in the antimicrobial activity of Amikacin was due to modification of enzymes and efflux pumps and increased activity as occurred in 16S rRNA methylation (Apridamayanti *et al*., 2016). The resistance of clinical isolates to Aminoglycoside antibiotics was found to vary with a specific drug, the microorganism, its mechanism of resistance, the geographic area, and many other factors.

Fluoroquinolones resistance is likely to. be the result of a mutation (Farahi *et al*., 2018; Al-Mayali and Salman, 2020 ) due to the selective pressure induced by the use of Fluoroquinolones, and the difference in the rate of Ciprofloxacin resistance is usually related to the frequency of use of Fluoroquinolones and the availability of oral doses(Mohamed *et al*., 2019).

In general, the resistance of different types of antibiotics is attributed to many reasons, including altered cell membrane permeability, alterations in target site structures, and the mechanism of efflux pumps, which reduce antibiotic concentrations (Sheikh *et al*., 2015).

## 4.6 Multi-drug resistance of *Pseudomonas aeruginosa* isolates

Multi-Drug Resistance (MDR) is defining as any isolate of bacteria that resistance against at least 1 antibiotic in 3 or more classes called Multi-Drug Resistance (Magiorakos, 2012).

Among 26 clinical isolates of *P.aeruginosa* that were tested in our study, several strains were found to be MDR.

The following antipseudomonal classes of antimicrobial drugs were tested in the current study: antipseudomonal Cephalosporins (Ceftazidime and Cefepime), antipseudomonal Beta_lactamase inhibitors (Ticarcillin/Clavulanate and Piperacillin/Tazobactam), Monobactams (Aztreonam), antipseudomonal Carbapenems (Imipenem and Meropenem), Aminoglycosides (Gentamicin, Tobramycin, Amikacin, and Netilmicin) and Fluoroquinolones (Ciprofloxacin and Levofloxacin).

The current study results showed that 42.3% of isolates were MDR that agrees with Abdallah and Jabur (2021), who found the percentage of MDR 46% in their isolates, and this result disagrees with Abd El-Baky *et al*. (2020) that found 96% of isolates were MDR. Table (4-6) shows, the most multidrug-resistant isolates came from wounds, followed by burns, and less often in urine, ear, and sputum isolates.

**Table 4-6: Multi-drug resistance of *Pseudomonas aeruginosa* isolates according to the source of infection**

| NO.of antibiotics | NO.of isolates | Total isolates |
|---|---|---|
| 4 | 1(ear) | 1 |
| 7 | 1(burns) | 1 |
| 9 | 1(wounds) | 1 |
| 10 | 1(urine),1(sputum),1(burns) | 3 |
| 11 | 2(Wounds) | 2 |
| 12 | 3(wounds) | 3 |
| Total | 1(ear),1(sputum),1(urine),2(burns),6(wounds) | 11 |

Although the rate of multi-resistance in this study was relatively low, this may be a worrisome condition that reflects a threat that limits treatment options in the treatment centers studied (Kamali *et al*., 2020).

The difference between the results of our study and the other studies attributed to the variation of the antibiotics usage policy applied in each country, and primarily the increased disaster of antibiotic misuse without proper prescriptions (Mohamed *et al*., 2019).

The outbreak of emerging MDR *P.aeruginosa* strains is related to various factors, such as its inherent resistance to a variety of antibiotics, its ability to acquire determinants of antimicrobial resistance, history of surgical interventions and chronic infections, mutation in the genome of *P.aeruginosa*, and environmental conditions of the specific area, irrational use of antibiotics, and abundant use of broad-spectrum antibiotics (Mohammadzadeh *et al*., 2017). Also, the acquisition of resistance genes by horizontal gene transfer further contributes to the emergence of phenotypes of MDR (Moustafa *et al*., 2021).

The increased prevalence of multidrug-resistant strains (MDR) has been recognized as a global problem during the treatment of *P. aeruginosa* infection (Rossi *et al*., 2017).

Infections caused by multidrug-resistant gram-negative bacteria MDR, especially MDR *P. aeruginosa*, are known to be associated with higher morbidity and mortality. Patients with nosocomial infections especially those admitted to various intensive care units usually become infected with MDR strains of *Pseudomonas aeruginosa* (Ahmad *et al*., 2020) and the continued spread of such strains poses serious challenges in infection control management (Murugan *et al*., 2018).

## 4.7 Minimum, Sub Minimum Inhibitory Concentration (MIC, Sub MIC) and Minimum Bactericidal Concentration (MBC) of Imipenem and Ceftazidime against strong biofilm isolates of *P. aeruginosa*

The minimum inhibitory concentration (MIC), sub-minimum inhibitory concentration (Sub MIC), and minimum bactericidal concentration (MBC) were determined against two antibiotics Imipenem and Ceftazidime according to the Clinical and Laboratory Standards Institute (CLSI-2020) guidelines breakpoint for ten isolates selected according to biofilm formation (strong biofilm).

MIC values were determined for each isolate by selecting the lowest concentrations in which no growth by the serial dilution method on Mueller-Hinton broth while the sub MIC values were determined by selecting the lowest inhibitory concentration at which the bacteria could grow. Additionally, MBC values were determined by sub-culturing the content of each tube without any growth and looking for any bacterial growth, on Mueller-Hinton agar. Isolation has been described as resistance if the MIC is greater than the breakpoint as defined by CLSI (2020), whereas it will be sensitive if it is below the breakpoint. All ten isolates of the current study showed high degrees of resistance.

As it is shown in table (4-7), MIC and MBC values of Imipenem which was (16–512 µg /ml ) and (512- 1024 µg /ml), respectively. The result of MIC agrees with the result of Mirsalehian *et al*. (2017) reported that showed high-level resistance to Carbapenems and disagrees with Saffari *et al*. (2019) which was (2-128 µg /ml).While the result of MBC disagrees with Alsaadi, (2020) which was (4-512 µg /ml).

**Table 4-7: The Sub_MIC, MIC & MBC values of Imipenem**

| Isolates NO. | Imipenem(breakpoint µg/ml) ≤2(S) | 4(I) | ≥8(R) |
|---|---|---|---|
| | Sub_MIC | MIC | MBC |
| PA3 | 128 | 256 | 1024 |
| PA4 | 8 | 16 | 512 |
| PA10 | 256 | 512 | 1024 |
| PA12 | 32 | 64 | 1024 |
| PA13 | 32 | 64 | 1024 |
| PA15 | 256 | 512 | 1024 |
| PA18 | 32 | 64 | 1024 |
| PA19 | 64 | 128 | 1024 |
| PA20 | 8 | 16 | 512 |
| PA25 | 8 | 16 | 1024 |

MIC for Ceftazidime was (16-1024 µg /ml ), the breaking point for this antibiotic was ≥32 µg/ml and the lowest MIC was 16 µg/ml for the isolate PA3, but in some cases, the MIC elevated to reach 1024 µg/ml as with two isolates (PA10 and PA13). The research conducted by Najeeb, (2020) explained that the values of MIC for this antibiotic were between (8-512 µg/ml) and the rate of resistance reached 70% and these results are lower than the percentage of this study. While MBC was ranged between ( 128 - >1024 µg /ml) agrees with Alsaadi, (2020). Table ( 4-8)

**Table 4-8: The Sub_MIC, MIC & MBC values of Ceftazidime**

| Isolates NO. | Ceftazidime (breakpoint µg/ml) ≤8(S) | 16(I) | ≥32(R) |
|---|---|---|---|
| | Sub_MIC | MIC | MBC |
| **PA3** | 8 | 16 | 1024 |
| **PA4** | 16 | 32 | 512 |
| **PA10** | 512 | 1024 | ≥1024 |
| **PA12** | 32 | 64 | 512 |
| **PA13** | 512 | 1024 | ≥1024 |
| **PA15** | 16 | 32 | 512 |
| **PA18** | 256 | 512 | 1024 |
| **PA19** | 256 | 512 | 1024 |
| **PA20** | 16 | 32 | 128 |
| **PA25** | 16 | 32 | 1024 |

The reason for the high MIC and MBC value is due to the high production of the enzyme beta-lactamase that breaks the beta-lactam ring and the presence of resistance genes that play a role in inhibiting antibiotic activity (Gonçalves *et al.*, 2017).

AmpC overproducer and mutational inactivation of oprD are known to be the main mechanisms of Carbapenem resistance particularly to Imipenem in the absence of acquired Carbapenemases. It has been shown that the AmpC overproducer, usually when is combined with efflux systems overexpression and/or down-regulation oprD, leads to Carbapenem resistance. AmpC overproduction caused an increase in the MICs of Imipenem (Mirsalehian *et al.*, 2017) and was associated with resistance to Ceftazidime **(**Emaneini *et al.*, 2019). Whereas resistance to Ceftazidime was more commonly associated with AmpC overexpression alone (Hawkey *et al.*, 2018).

## 4.8 Effects of antibiotics on the biofilm formation

Although it is evident that biofilm-producing bacteria are more resistant to antimicrobial agents, their formation can be inhibited or induced when planktonic cells are exposed to sub-minimal inhibitory concentrations (sub-MIC) of some antimicrobial agents. Notably, stimulating biofilm formation is recognized as a major health problem, since bacteria are usually exposed to sub-MICs of antimicrobial agents. Therefore, we examined the effect of antibiotics (e.g., Ceftazidime and Imipenem) on biofilm formation for isolates selected according to biofilm formation (strong biofilm) after MIC determination to her.

The results of the current study showed revealed that Ceftazidime and Imipenem after incubating for 24 hours have decreased the density of biofilm formation in eight isolates for both antibiotics. But, no change in biofilm density was detected in two isolates (PA3, PA20) for Ceftazidime antibiotics and (PA20, PA25) for Imipenem antibiotics. With a highly significant ($P$ = 0.001) difference in the ability of isolates to form biofilms between the non-use of the antibiotic (Free from antibiotic) and the treatment of isolates with MIC for each antibiotic ( After antibiotic treatment).

The Ceftazidime result agrees with Otani *et al.* (2018) noticed that the Ceftazidime at sub-MIC inhibited *P. aeruginosa* biofilm formation and disagrees with Hemati *et al.*(2016) observed that Ceftazidime induced biofilm formation. While the result of Imipenem agrees with (Cirioni *et al.*, 2013). Both the antibiotics reduced biofilm formation**.**

**Table 4-9:The formation of biofilm by *Pseudomonas aeruginosa* treated with Ceftazidime at sub-MIC after incubating for 24 hours.**

| Isolates No. | Free from antibiotic | | After antibiotic treatment | |
|---|---|---|---|---|
| | Absorbency at 630 nm | Biofilm level compared to (ODc=0.038 | Absorbency at 630 nm | Biofilm level compared to (ODc=0.038 |
| **PA3** | 0.096 | Strongly | 0.084 | Strongly |
| **PA4** | 0.105 | Strongly | 0.044 | Moderately |
| **PA10** | 0.077 | Strongly | 0.042 | Moderately |
| **PA12** | 0.131 | Strongly | 0.051 | Moderately |
| **PA13** | 0.081 | Strongly | 0.032 | Non-biofilm producer |
| **PA15** | 0.153 | Strongly | 0.046 | Moderately |
| **PA18** | 0.098 | Strongly | 0.051 | Moderately |
| **PA19** | 0.153 | Strongly | 0.067 | Moderately |
| **PA20** | 0.092 | Strongly | 0.083 | Strongly |
| **PA25** | 0.082 | Strongly | 0.056 | Moderately |

****P*=0.001

**Table 4-10:The formation of biofilm by *Pseudomonas aeruginosa* treated with Imipenem at sub-MIC after incubating for 24 hours**

| Isolates No. | Free from antibiotic | | After antibiotic treatment | |
|---|---|---|---|---|
| | Absorbency at 630 nm | Biofilm level compared to (ODc=0.038 | Absorbency at 630 nm | Biofilm level compared to (ODc=0.038 |
| **PA3** | 0.096 | Strongly | 0.046 | Moderately |
| **PA4** | 0.105 | Strongly | 0.041 | Moderately |
| **PA10** | 0.077 | Strongly | 0.058 | Moderately |
| **PA12** | 0.131 | Strongly | 0.052 | Moderately |
| **PA13** | 0.081 | Strongly | 0.035 | Non-biofilm producer |
| **PA15** | 0.153 | Strongly | 0.061 | Moderately |
| **PA18** | 0.098 | Strongly | 0.032 | Non-biofilm producer |
| **PA19** | 0.153 | Strongly | 0.033 | Non-biofilm producer |
| **PA20** | 0.092 | Strongly | 0.082 | Strongly |
| **PA25** | 0.082 | Strongly | 0.080 | Strongly |

****P*=0.001

Despite the extensive biofilm tolerance to antimicrobials, some conventional antibiotics still show activity against bacterial cells that grow in the state of biofilms.

In a recent study, Otani *et al*. (2018) showed that sub-MICs of ceftazidime reduce biofilm formation after incubating for 24h. Sub-MIC CAZ may inhibit

biofilm formation by altering the effect exerted on the cellular membrane of *P. aeruginosa* (Otani *et al*., 2018).

While another study showed that antibiotics present in lower concentrations than MIC, and can significantly stimulate biofilm formation in a variety of bacterial species in vitro. Such differences between our study and the other studies may be considered normal due to the types of isolates studied and their source in addition to the genetic makeup of the isolates, or the laboratory conditions that accompanied the detection of sub-MIC (Al-Sheikhly *et al*., 2019).

Moreover, biofilm inhibition by *P. aeruginosa* in response to antibiotics is consistent with the hypothesis that metabolic stress is the main signal mediating the response. Disruption of the biofilm structure may result in the separation of cells that can act as a vaccine for new points of infection and thus cause the spread of the infectious agent (Penesyan *et al*., 2020).

Generally, the antibiotics reduced the biofilm formation. However, several studies have shown that antibiotics can significantly induce biofilm formation depending on the antibiotic class and the bacterial strain (Zhou *et al*., 2017).

The study concluded that incubating the *P. aeruginosa* isolates in sub-MIC of antibiotics incubating for 24 hours exhibited reduced the ability of bacteria to the formation of biofilm in most isolates.

## 4.9 Molecular study

### 4.9.1 DNA extraction

Using the Presto™ Mini gDNA Bacteria Kit, the genomic DNA was extracted from *Pseudomonas aeruginosa* isolates. Extraction of genomic DNA from 26 isolates was confirmed as bands by gel electrophoresis. DNA concentration was determined using the Quantus Fluorometer. All isolates have

DNA concentrations between (19.8-35.6 ng /µl). The output depends on the culture method, the bacterial category, the number of particles, and the type of extraction kit. All of these an affect the quality and characteristics of the nucleic acid. Moreover, most practical molecular methods indicated the ease and sufficiency of extraction of DNA from gram-negative than positive and this applies to recent ready extraction kits ( Nader *et al.*, 2017).

### 4.9.2 Detection and association of the *pel*A, *psl*A, and *psl*D genes with biofilm formation in *Pseudomonas aeruginosa* clinical isolates

PCR was carried out for the detection of *psl*A, *psl*D, *and pel*A genes for 26 isolates. All of the genes tested were involved in biofilm formation, which is involved in surface adhesion, micro-colony formation, macrocolony formation, and dispersion.

The results of the genes detection in the current study showed 25 isolates were carried *psl*A and *psl*D genes and the percentage 96.1%. Figure (4-3) showed the bands (119 bp) of DNA for *psl*A and Figure (4-4) showed the bands (295 bp) of DNA for *psl*D. While 23 isolates were carried the *pelA* gene and the percentage was 88.4%. Figure (4-5) showed the bands (118 bp) of DNA.

This result agrees with those of Maita and Boonbumrung, (2014) who stated that the percentage of *psl*A, *psl*D, and *pel*A was 94%, 95.9%, and 97% respectively. which were found in nearly all clinical isolates of *P. aeruginosa*. Table (4-11).

**Table 4-11:The relationship between biofilm formation and the presence of genes for *Pseudomonas aeruginosa* isolate**

| NO. of isolates | Biofilm density | *PslA* | *PslD* | *PelA* |
|---|---|---|---|---|
| 1 | M | + | + | + |
| 2 | M | + | + | + |
| 3 | S | + | + | + |
| 4 | S | + | + | + |
| 5 | M | + | + | + |
| 6 | M | + | + | + |
| 7 | M | + | + | + |
| 8 | M | + | + | + |
| 9 | M | - | - | - |
| 10 | S | + | + | + |
| 11 | M | + | + | + |
| 12 | S | + | + | + |
| 13 | S | + | + | + |
| 14 | M | + | + | + |
| 15 | S | + | + | + |
| 16 | M | + | + | + |
| 17 | M | + | + | + |
| 18 | S | + | + | + |
| 19 | S | + | + | + |
| 20 | S | + | + | - |
| 21 | M | + | + | + |
| 22 | M | + | + | + |
| 23 | Non-biofilm producer | + | + | + |
| 24 | M | + | + | + |
| 25 | S | + | + | - |
| 26 | Non-biofilm producer | + | + | + |

**M(Moderately) S(Strongly)**

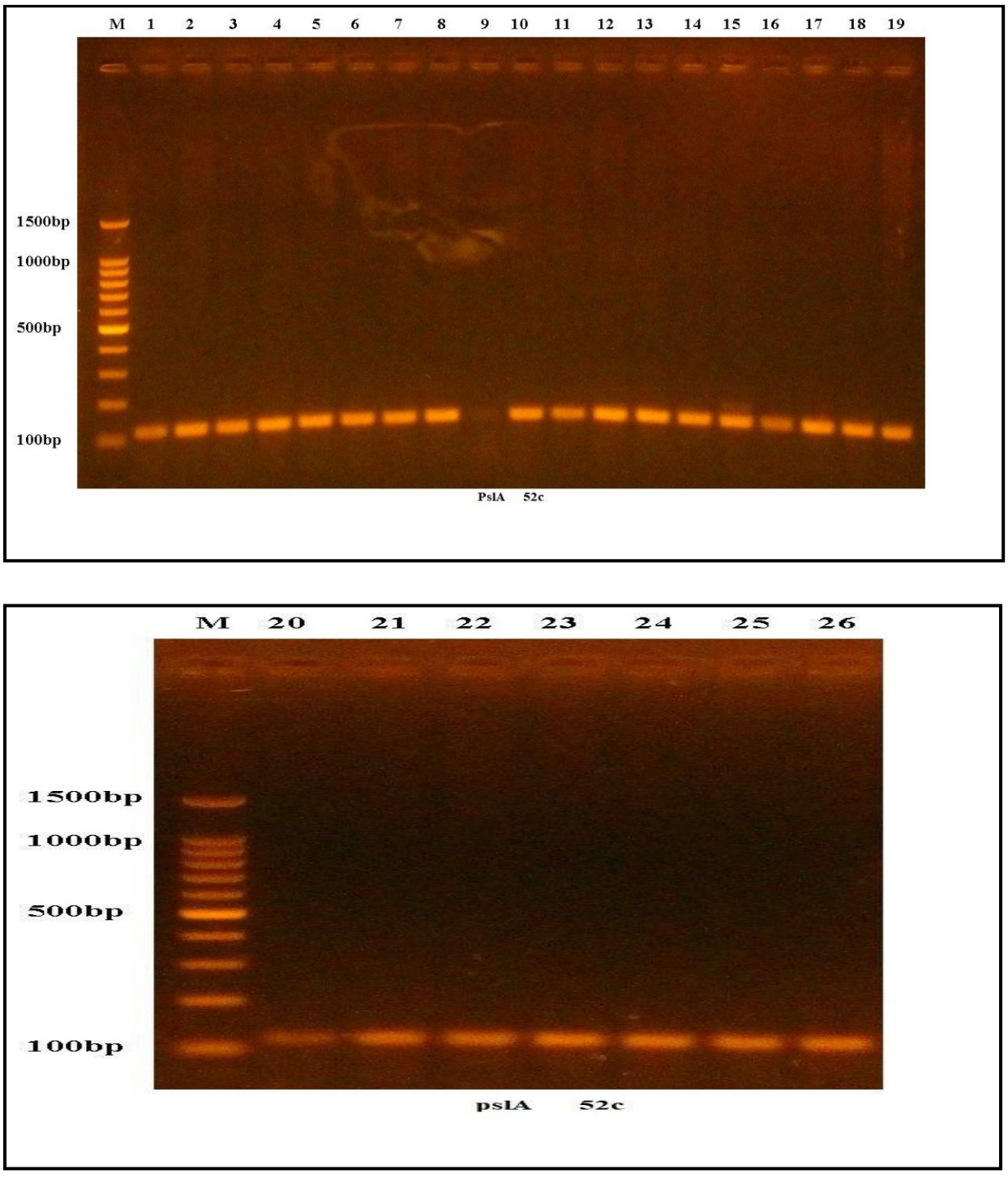



**Figure 4-3: The amplification of *psl*A region in *Pseudomonas aeruginosa* species fractionated on 1.5% agarose (90 min at 100 volts) stained with Eth. Br. M: 100bp ladder marker. Lanes 1-19 resemble 119bp PCR products.**

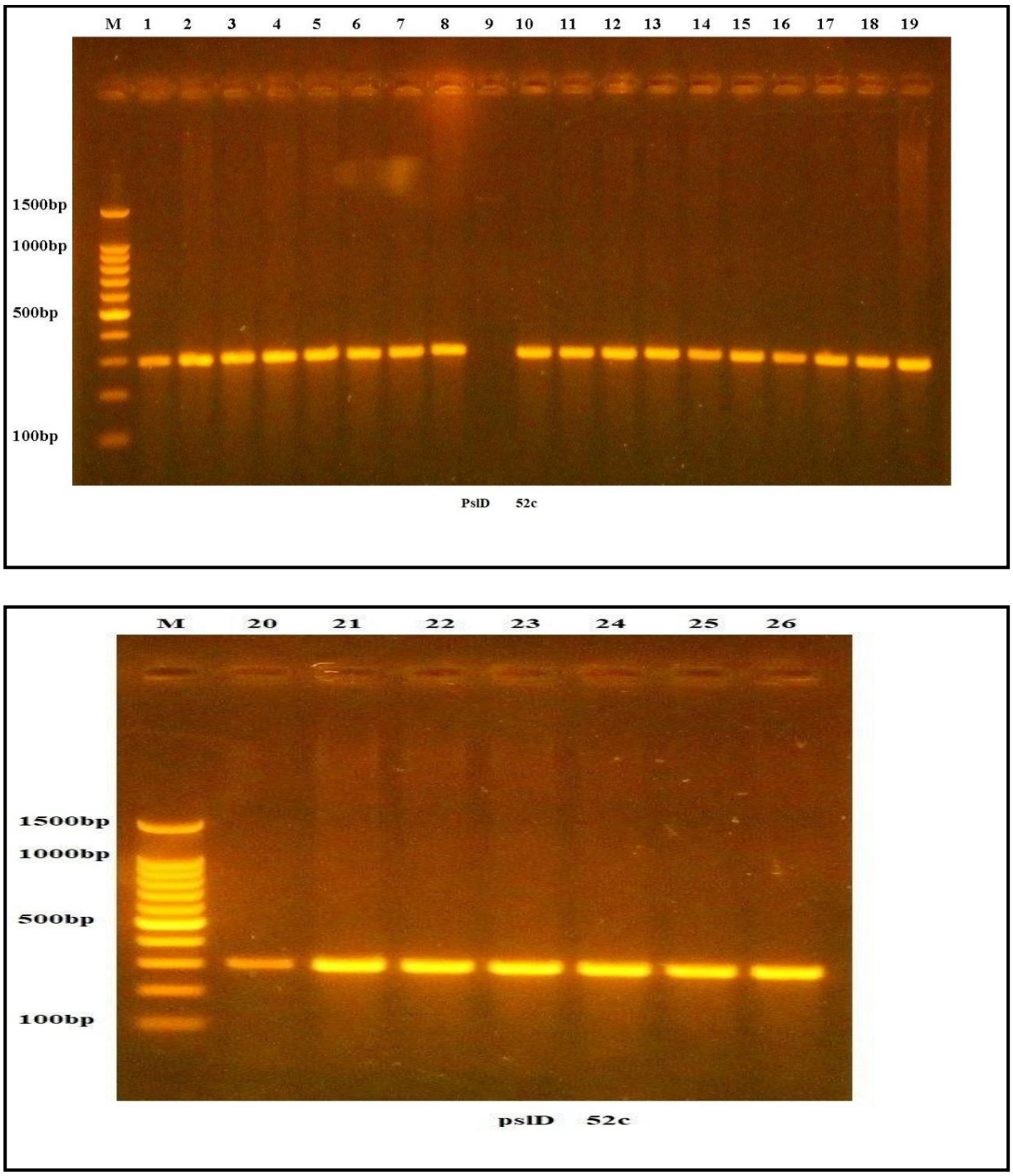



**Figure 4-4: The amplification of *psl*D region in *Pseudomonas aeruginosa* species fractionated on 1.5% agarose (90 min at 100 volts) stained with Eth. Br. M: 100bp ladder marker. Lanes 1-19 resemble 295bp PCR products.**

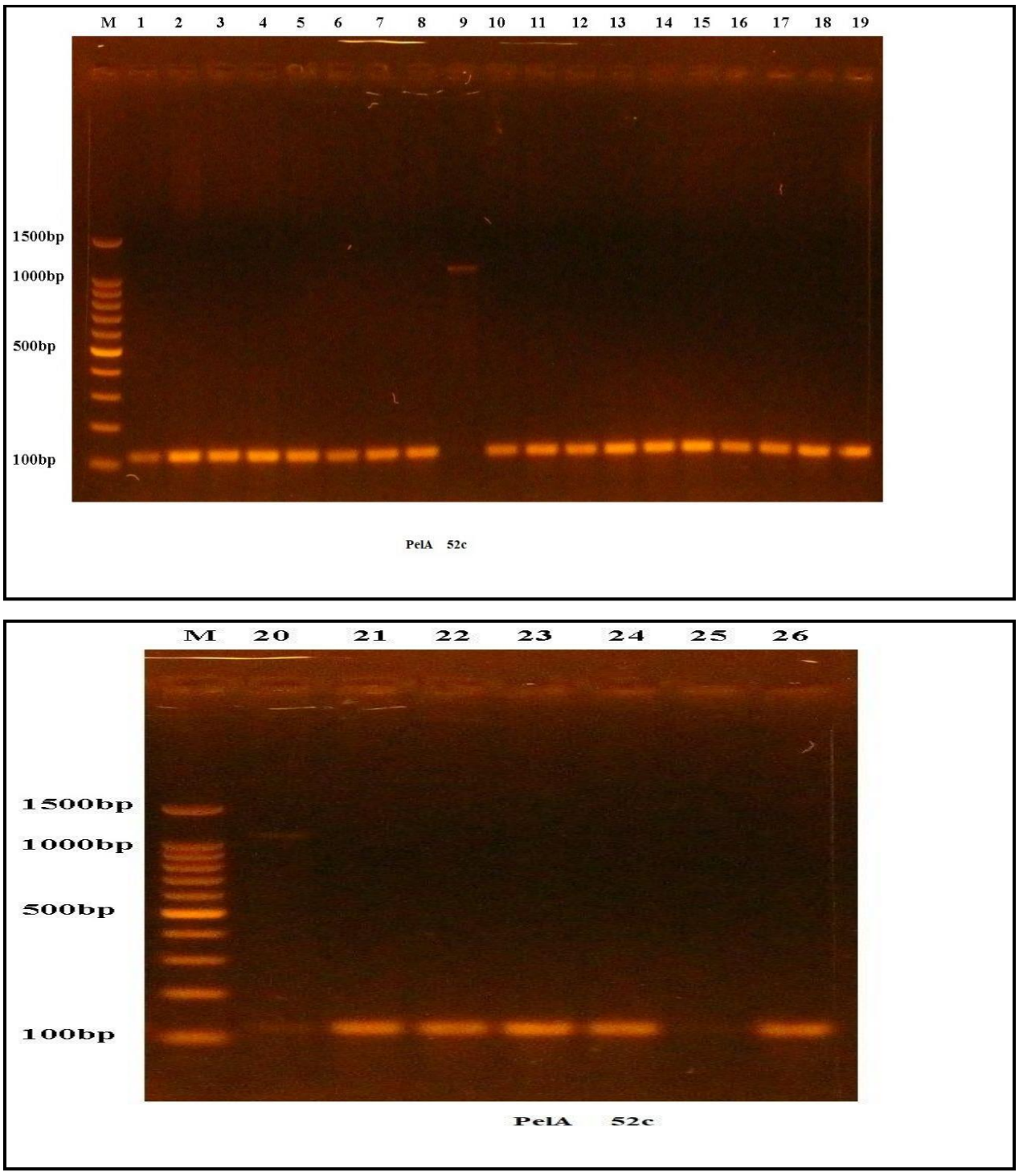



**Figure 4-5: The amplification of *pel*A region in *Pseudomonas aeruginosa* species fractionated on 1.5% agarose (90 min at 100 volts) stained with Eth. Br. M: 100bp ladder marker. Lanes 1-19 resemble 118bp PCR products.**

The genes mentioned above were found in nearly all clinical strains of *P. aeruginosa*, but not all of these genes can contribute to biofilm production, and their presence cannot predict which strains will produce biofilm because many factors influence biofilm formation. And we noticed in the current study that two isolates were not able to form biofilm despite they carrying each of the three genes. This indicates that there are other genes responsible for the formation of biofilms. While one of the isolates did not carry the three genes despite being a producer of the biofilm. The ability to produce the biofilms despite the absence of the biofilm genes studied indicates that other genetic determinants of the biofilm are involved in the matrix formation in *P. aeruginosa*. (Moradali *et al.*, 2017). In contrast, the presence of genes without the production of biofilms may be the result of chromosomal mutations in different regulatory systems, affecting the production of functional proteins associated with the biofilm (Kamali *et al.*, 2020).

The biofilm represents an important virulence factor for *P. aeruginosa* and plays a role in *P. aeruginosa* infection and avoidance of immune defense mechanisms and it protects bacteria from antibiotics (AL-Wrafy *et al.*, 2017). The three exopolysaccharides, i.e., Psl, Pel and alginate, are significantly involved in surface attachment, formation, and the stability of biofilm structure and are the most important exopolysaccharides exploited in the formation of biofilm (da Silva *et al.* 2019). Although preference for Pel or Psl is often breed-specific, many isolates can switch between Pel and Psl synthesis in response to stress to maintain infection in the host and respond to ambient conditions (Thi *et al.*, 2020).

This adaptive mechanism underscores the importance of developing therapies that target exopolysaccharides the relationship between biofilm formation and the presence of genes of *P. aeruginosa*.

# Conclusions and Recommendation

## Conclusions

1. There is a strong relationship between biofilm formation and antibiotic resistance among clinical *Pseudomonas aeruginosa* isolates.
2. The ability of *Pseudomonas aeruginosa* to produce pyocyanin pigment increases with the increasing incubation period and type of medium.
3. The study revealed a high percentage of the presence of virulence factors as hemolysin, motility, lipase, pigments production, and biofilm formation and a low percentage of protease and gelatinase.
4. Although the rate of multiple antibiotic resistance among Pseudomonas aeruginosa isolates was relatively low in this study, judicious use of antimicrobials and high standards for infection prevention and control is essential to prevent further development of resistant strains.
5. Phenotypic detection of the ability of *Pseudomonas aeruginosa* isolates by microtiter plate (MTP) method was compatible with the result of molecular detection by PCR of *psl*A, *Psl*D, and *Pel*A genes that coded the biofilm formation were the two methods showed a high percentage of biofilm formation by isolates.

## Recommendations

Additional and future studies were required for:

1. Isolation and identification of *Pseudomonas aeruginosa* bacteria environments (hospitals and others) and patients to carried comparative studies about virulence factor and antibiotics resistance of isolates from the different source.
2. Investigate the effects of other antibiotics at sub-minimal inhibitory concentrations (sub-MIC) on the biofilm formation in the isolates.
3. Detection of other genes that coded biofilm formation and study the gene expression before and after treatment with antibiotics.
4. In our study, strains are more sensitive to combination drugs like piperacillin+tazobactam. More restricted and rational use of these drugs is necessary.
5. Perhaps some other genes or factors played role in forming a biofilm. Therefore, we should consider other genetic and phenotypic factors as well, which afford for future studies.

# References

# References

## { A }


- **Abdallah**, R. and Gabur, S. (2021). Molecular search of some antibiotic-borne genes of the clinical *Pseudomonas aeruginosa* plasmid. *Systematic Reviews in Pharmacy*,12(1):170-189.
- **AbdEl-Baky**,R., Masoud,S., Mohamed,D., Waly,N.,Shafik,E., Mohareb,D., Elkady,A., Elbadr,M. and Hetta,H.(2020). Prevalence and some possible mechanisms of Colistin resistance among multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa*. *Infection and Drug Resistance*,13: 323–332.
- **Abdulammer**, H. (2018). Detection of quinolones chromosomal resistance in *Pseudomonas aeruginosa* isolated from wounds infection. MSc. Thesis. Biology department. Baghdad University. Iraq.
- **Abdulhaq**,N., Nawaz,Z., Zahoor,M. and Siddique,A.(2020). Association of biofilm formation with multidrug resistance in clinical isolates of *pseudomonas aeruginosa*. *EXCLI Journal*,19:201-208.
- **Adam** R., Al-Labban H., Aljanaby A. and Abbas N.(2019).Synthesis, characterization, and antibacterial activity of some new novel 1,2,3- triazole-chalcone derivatives from N-acetyl- 5Hdibenzo [B, F] azepine-5-carboxamide. *Nano Biomedicine and Engineering*,11 (2): 99-110.
- **Ahmad**,S., Alotaibi,M. and Alamri,M.(2020). Antibiotic Sensitivity Pattern of Clinical Isolates of *Pseudomonas aeruginosa* at a Tertiary Care Hospital in Saudi Arabia. *Dhaka University Journal of Pharmaceutical Sciences*,19(1): 77-82.
- **Ahmed**, M., Porse, A., Sommer, M., Høiby, N., and Ciofu O. (2018). Evolution of antibiotic resistance in biofilm and planktonic *Pseudomonas aeruginosa* populations exposed to subinhibitory levels of ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, 62:e00320-18.

# References


- **Alayande**, A., Aung, M. and Kim, I.(2018 ). Correlation Between Quorum Sensing Signal Molecules and *Pseudomonas aeruginosa's* Biofilm Development and Virulency. *Current Microbiology*, 75(7):787–793.
- **Al-Dahmoshi**, H.(2017). Rapid Detection of Microbial Profile among Women with Vaginitis in Hilla City, Iraq. *Journal of Applied Pharmaceutical Science*, 7(02): 228-232.
- **Al-Daraghi**, W. and Al-Badrwi, M. (2020). Molecular Detection for Nosocomial *Pseudomonas aeruginosa* and its Relationship with multidrug Resistance, Isolated from Hospitals Environment**.** *Medico-legal Update,*20( 1**):**633_635.
- **AL-Harmoosh**, R., Jarallah, E., AL-Shamari, A. and AL-khafaji, H. (2017).Detection of Efflux Pumps Genes in Clinical Isolates of *Acinetobacter baumannii*. *Research Journal of Pharmacy and Technology*, 10(12):4231-4236.
- **Alhazmi**, A.(2015). *Pseudomonas aeruginosa* – Pathogenesis and Pathogenic Mechanisms. *International Journal of Biology*,2(7):44_57.
- **Alim, A.**, Deep, A., Yadav, P. and Chaudhary, U. (2017). Comparison of Antibiotic Resistance and Lipase Production in Extended Spectrum β-lactamases Producing and non-producing Isolates of *Pseudomonas* aeruginosa.*International Journal* of *Current Microbiology* and *Applied* Scie nces,6( 3): 2038-2046.
- **Aljanaby**, A., Witwit, I., Mubark, H. and Al-Labban, H.(2019). synthesis and characterization of new imidazole azo ligand with some of transition metal ions, and their biological effect on two pathogenic bacteria of burn patients. *International Journal of Research in Pharmaceutical Sciences*, 10 (3): 1847-1856.

# References


- **Aljanaby**, A. and Aljanaby, I. (2018). Prevalence of aerobic pathogenic bacteria isolated from patients with burn infection and their antimicrobial susceptibility patterns in Al-Najaf City, Iraq-a three-year cross-sectional study. *F1000Research*., 7: 1157.
- **Al-Labban** H., Adam, R., Aljanaby, A. and Kadhim I.(2019). Synthesis, characterization, and antibacterial activity of some new pyrimidine derivatives from chalcone derivatives. *Drug Invention Today*, 11 (7): 1732-1739.
- **Al-mamari,**N.(2019). Isolation And Identification of *Pseudomonas aeruginosa* from Some for The s Activity, Clinical And Environmental Samples And Study It Production of Pyocyanin And Protease. *Journal of Education and Science*,(28):4.93-107.
- **Al-Mayali**, M. and Salman, E. ( 2020). Bacteriological and Molecular Study of Fluoroquinolones Resistance in *Pseudomonas aeruginosa* Isolated From Different Clinical Sources. *Iraqi Journal of Science*, 61( 9): 2204- 2214.
- **Almeida, C**., Azevedo, N. F., Santos, S., Keevil, C. and Vieira, M. (2013). Correction: Discriminating Multi-Species Populations in Biofilms with Peptide Nucleic Acid Fluorescence In Situ Hybridization (PNA FISH). *PLoS ONE*, 8 (6): 101-371.
- **Al-Obaidi,**R. and Al-Dahmoshi,H.(2020). Biofilm and antibiotic resistance profile among *Pseudomonas aeruginosa* isolated from clinical samples. *EurAsian Journal of BioSciences Eurasia J Biosci*, 14: 1135-1139.
- **Alsaadi**,Lina.(2020). Molecular Detection of Multidrug-Resistant of Some Genes and the Effect of ZnONPs as Alternative to Antibiotics for *Pseudomonas aeruginosa*. PH thesis. Diyala University.iraq.71-112.
- **Al-Saeedi**, R. and Raheema, R., H. (2019). Molecular detection of some virulence genes In Pseudomonas aeruginosa clinical isolates. *Indian Journal of Public Health Research & Development*, 10(4): 769- 776.

# References


- **AL-Salihi**,S. and Hasan,A.(2015). Detection of some virulence factors in *Pseudomonas aeruginosa* associated with diarrhea in Kirkuk City. *Kirkuk University Journal*,(10):1.80-89.
- **AL-Shamaa**, F., Rasmia, A., Abu- Risha and AL-Faham, M. (2016). Virulence Genes Profile of *Pseudomonas aeruginosa* Local Isolates from Burns and Wounds. *Iraqi Journal of Biotechnology*, 15(3), 31–39.
- **AL-Sheikhly**, M., Musleh, L. and Al-Mathkhury, H.(2019). Assessment of pelA-carried *Pseudomonas aeruginosa* isolates in respect to biofilm formation. *Iraqi Journal of Science*, 60(6):1180-1187.
- **AL-Shimmary**, S., Jawad, N. and Wahhab, T.(2017). Detection of toxA gene in clinical isolates of *Pseudomonas aeruginosa* using real-time PCR. *Ann Trop Med Public Health*, 21: SP2043-19.
- **AL-Wrafy**, F., Brzozowska, E., Górska, S. and Gamian, A. (2017). Pathogenic factors of *Pseudomonas aeruginosa* –the role of biofilm in pathogenicity and as a target for phage therapy. *Postepy Hig Med Dosw* (online), 70: 78-91.
- **Al-Yousef**, H., Alam, P., Khanam, Z., Amina, M. and Hassan, W.(2021). Corchorus olitorius aqueous extract attenuates quorum sensing-regulated virulence factor production and biofilm formation. *Asian Pacific Journal of Tropical Biomedicine*, 11(2): 66-73.
- **Amoon**, R., Abdallha, A., Sharif, A., Moglad, E., Altyb, H., Elzaki, S. and Salih, M.( 2018). Molecular characterization of *Pseudomonas aeruginosa* isolates from Sudanese patients: A cross-sectional study A cross-sectional study[version 1; peer review: 1 approved with reservations, 1 not approved], 7: 1135.
- **Andersson**, D., Balaban, N., Baquero, F., Courvalin, P., Glaser, P., Gophna, U. and Tønjum, T.(2020).Antibiotic resistance: turning evolutionary principles into clinical reality. *FEMS Microbiology Reviews*.

# References


- **Andersson**, D. and Hughes,D.(2014).Microbiology effects of sublethal levels of antibiotics. *Nature Reviews Microbiology*,12(7):465_478.
- **Apridamayanti**, P., Meilinasary, K. and Sari, R.(2016)**.** Antibiotic Sensitivity in *Pseudomonas aeruginosa* of Diabetic Patient's Foot Ulcer**.** *Original Article*, (3) :2.81-87.
- **Arya**, S., Sharma, M., Das, R., Rookes, J., Cahill, D. and Lenka, S. (2019). Vanillin mediated green synthesis and application of gold nanoparticles for reversal of antimicrobial resistance in *Pseudomonas aeruginosa* clinical isolates. *Heliyon* ,5(7):e02021.
- **Asghar**, A. and Ahmed, O.(2018). Prevalence of aminoglycoside resistance genes in *Pseudomonas aeruginosa* isolated from a tertiary care hospital in Makkah, KSA. *Clinical practice*,15(2):541-547.
- **Aykac**, K., Ozsurekci, Y. and Tanir, B.(2017). Future directions and molecular basis of ventilator-associated pneumonia. *Can European Respiratory Journal*,2017:2614602.
- **Azam**, M. and Khan, A. (2019). Updates on the pathogenicity status of *Pseudomonas aeruginosa*. *Drug Discov Today*, 24(1): 350– 9.
- **Aziz**,M., Garduno,R., Mirani,Z., Baqai,R., Sheikh,A., Nazir,H., Raza,Y., Ayaz,M. and Kazmi,S.(2019). Determination of the antimicrobial effect of protamine by transmission electron microscopy and SDS PAGE on *Pseudomonas aeruginosa* isolates from a diabetic foot infection. *Basic Medical Sciences*, 22:827-832.

{ ℬ }

- **Bahador**,N., Shoja,S., Faridi,F., Mobarrez,B., Qeshmi,F., Javadpour,S. and Mokhtary,s.(2019). Molecular detection of virulence factors and biofilm formation in *Pseudomonas aeruginosa* obtained from different clinical specimens in Bandar Abbas. *Iranlan Journal of microbiology*,(11):1. 25-30.

# References


- **Bakar**, M., McKimm, J., Haque, S., Majumder, M. and Haque, M. (2018). Chronic tonsillitis and biofilms: a brief overview of treatment modalities. *Journal of Inflammation Research*,11:32.
- **Baker**, P., Hill, J., Snarr, D., Alnabelseya, N., Pestrak, J., Lee, J., Jennings, K., Tam, J., Melnyk, A., Matthew, R., Parsek, R., Sheppard, C., Wozniak, J. and Howell, L.( 2016). Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent *Pseudomonas aeruginosa* biofilms. *Science Advances*,2(5): 1150-1632.
- **Bakhtiar**, C.(2018). Detection of the Exo S gene and some phenotypic factors of the bacterium *Pseudomonas aeruginosa*, clinically isolated, in the city of Kirkuk. MSC thesis. The University of Mosul. Iraq. 90_95.
- **Bassetti**, M., Vena, A., Croxatto, A., Righi, E. and Guery, B. (2018) .How to manage *Pseudomonas aeruginosa* infections.*Drugs Context*,7:212527.
- **Bavasheh**, N. and Karmostaji,A.(2017) . Antibiotic Resistance Pattern and Evaluation of *blaOXA-10*, *blaPER-1*, *blaVEB*, *blaSHV* Genes in Clinical Isolates of *Pseudomonas aeruginosa* Isolated from Hospital in South of Iran in 2014-2015. *Infection Epidemiology Medicine*,3(1): 1-5.
- **Bhuiya**,M., Sarkar,M., Sohag3,M., Ali,H., Roy,C., Akther,L. and Sarker.A.(2018). Enumerating Antibiotic Susceptibility Patterns of *Pseudomonas aeruginosa* Isolated from Different Sources in Dhaka City. *The Open Microbiology Journal*, 12: 172-180.
- **Bosaeed**, M., Ahmad, A., Mahmoud, E., Alsaedy, A., Alswidan, L., Aljuhani, B., Alshamrani, M., Alalwan, S. and Alothman, A.(2020).Experience with Ceftolozane-Tazobactam for the Treatment of Serious *Pseudomonas aeruginosa* Infections in Saudi Tertiary Care Center. *Infectious Diseases: Research and Treatment*,13:1–5.

# References


- **Brauner,** A., Shoresh, N., Fridman, O. and Balaban, N.(2017). An experimental framework for quantifying bacterial tolerance. *Biophysical Journal*,112(12): 2664–2671.
- **Brooks**, G., Butel, J., Carroll, K. and Morse, S. (2016). Jawetz, Melinick, J.L. and Adlebergs Medical Microbiology, 27th ed. A Lange medical book.


{ C }


- **Cappuccino,** J. and Welsh, C. (2018).Microbiology: A Laboratory Manual. 11th ed. England: Pearson Education.
- **Cappuccino**, J. and Welsh, C.(2020).Microbiology A Laboratory Manual. 12 ed. New York: Pearson,155-191.
- **Cepas**, V. and Soto, S.(2020). Relationship between Virulence and Resistance among Gram-Negative Bacteria. *Antibiotics*, 9( 719):1-11.
- **Cepas**,V., López,Y., Muñoz,E., Rolo,D., Ardanuy,C., Martí,S., Xercavins,M., Horcajada,J., Bosch,J. and Soto,S.(2019).Relationship Between Biofilm Formation and Antimicrobial Resistance in Gram-Negative Bacteria. *Microbial Drug Resistance*, 25(1): 72-79.
- **Chaudhary**, R., Thapa, S., Rana, J. and Shah , P. (2017). Surgical site infections and antimicrobial resistance pattern. *Journal Nepal Health Research Counc*il,15(36):120–123.
- **Chevalier**, S., Bouffartigues, E., Bodilis, J., Maillot, O., Lesouhaitier, O., Feuilloley, M., Orange, N., Dufour, A. and Cornelis, P.(2017). Structure, function and regulation of *Pseudomonas aeruginosa* porins. *FEMS Microbiology Reviews*,41(5):698–722.
- **Choudhary**, P., Singh, S., Agarwal, V.(2020). Microbial Biofilms. Dincer,S., Özdenefe, M., Arkut, A. Bacterial Biofilms. 7th. intechopen.415.
- **Ciofu**, O. and Tolker-Nielsen, T. (2019). Tolerance and resistance of *Pseudomonas aeruginosa* Biofilms to antimicrobial agents-how *P. aeruginosa* can escape antibiotics. *Frontiers Microbiology*,10:913.

# References


- **Cirioni**,O., Silvestri,C., Ghiselli,R., Kamysz,W. and Minardi,D.(2013). Effetti in vitro e in vivo di sub-MIC di pexiganane imipenem sull'adesione e sviluppo di biofilm di *Pseudomonas aeruginosa*. *Le Infezioni in Medicina*, 4: 287-295.
- **Claeys**, K., Zasowski, E., Trinh, T., Lagnf, A., Davis, S. and Rybak, M. (2018). Antimicrobial stewardship opportunities in critically ill patients with gram-negative lower respiratory tract infections: a multicenter cross-sectional analysis. *Infectious Diseases and Therapy*, 7( 1): 135-146.
- **Clinical Laboratory Standards Institute** (CLSI). (2017). Performance Standards for Antimicrobial Susceptibility Testing. M100 -S27. Clinical and Laboratory Standards Institute, Wayne, PA.
- **Clinical Laboratory Standards Institute** (CLSI). (2020). Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI document includes updated tables for the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M02, M07, and M11: Wayne Pennsylvania.
- **Cohen**, J., William, G., Opal, P. and Opal, S. (2017). Infectious Diseases. 4th Edition. Brighton: Elsevier,1-2016.
- **Colvin**, K., Irie, Y., Tart, C., Urbano, R., Whitney, J., Ryder, C., Howell, P., Wozniak, D. and Parsek, M.( 2012). The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environ. *Microbiol*, 14(8): 1913–1928.
- **Corbett**, D., Wise, A., Langley, T., Skinner, K., Trimby, E., Birchall, S., Dorali, A., Sandiford, S., Williams, J., Warn, P. and Vaara, M.( 2017) Potentiation of Antibiotic Activity by a Novel Cationic Peptide: Potency and Spectrum of Activity of SPR741. *Antimicrobial Agents Chemother*. 61(8): 2-10.

- **Costa** - Limaa, J., Alves, L., Araújo Jacomé, P., Netoc, J., Maciel,M. and Morais,M. (2018). Biofilm production by clinical isolates of *Pseudomonas aeruginosa* and structural changes in LasR protein of isolates non-biofilm-producing. *Braz. Journal of Infectious Diseases*, 22(2): 129–136.
- **Costa**, D., Exbrayat-Héritier, C., Rambaud, B., Megy, S., Terreux, R., Verrier,B. and Primard,C. (2021). Surface charge modulation of rifampicin-loaded PLA nanoparticles to improve antibiotic delivery in *Staphylococcus aureus* biofilms. *Journal Nanobiotechnol*, 19(12):1-17.


{ $\mathcal{D}$ }


- **Dantas**, R., Silva, R., Ferreira, M., Gonçalves, I., Araújo, B., de Campos, P., Royer, S, da Fonseca Batistão, D., Gontijo- Filho, P. and Ribas, R.(2017). Molecular epidemiological survey of bacteremia by multidrug resistant *Pseudomonas aeruginosa*: the relevance of intrinsic resistance mechanisms. *PloS one*,12(5):e0176774.
- **De Smet**, J., Hendrix, H., Blasdel, B., Danis-Wlodarczyk, K. and Lavigne, R. (2017). "*Pseudomonas predators:* understanding and exploiting phage-host interactions". *Nature Reviews. Microbiology*,15 (9):517-530.
- **Del Pozo**, J. (2018). Biofilm-related disease. *Expert Review of Anti-infective Therapy*,16(1):51-65.
- **Domingo-Calap**,p., Georgel,p. and Bahram,s. (2016). Back to the future: bacteriophages as promising therapeutic tools. *HLA*, 87(3):133-40.
- **Domingues,** L. (2017). PCR-methods and protocols. New York, NY: Humana Press.
- **Dzotam,** J. and Kuete, V.(2017). Antibacterial and antibiotic-modifying activity of methanol extracts from six Cameroonian food plants against multidrug-resistant enteric bacteria. *BioMedResearch International*,2017: 1583510.

# References


{ E

- **Elhariri, M**., Hamza, D., Elhelw, R. and Dorgham, S. (2017). Extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* in camel in Egypt: potential human hazard. *Annals Clinical Microbiology Antimicrobials*, 16:21.
- **Elshafiee**, E., Nader, S., Dorgham, S. and Hamza, D.(2019).Carbapenem-resistant *Pseudomonas aeruginosa* originating from farm animals and people in Egypt. *Journals Vet Res*, 63(3): 333–337.
- **Elshamy**, A., Aboshanab, K., Yassien, M. and Hassouna N. (2018). Prevalence of carbapenem resistance among multidrug-resistant Gram-negative uropathogens. *Archives of Pharmaceutical Sciences Ain Shams University*,2(2):70–77.
- **Emaneini**, M., Kalantar-Neyestanaki, D., Jabalameli, L., Hashemi, M. , Beigverdi,R. and Jabalameli, F. (2019). Molecular analysis and antimicrobial resistance pattern of distinct strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Iran. *Iranlan Journal of microbiology*,11(2):98-107
- **Etebu**, E. and Arikekpar, I.(2016).Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *International Journal of Applied Microbiology and Biotechnology Research*,4: 90-101.

{ F }

- **Farahi**, R., Ali, A. and Gharavi, S. (2018). Characterization of gyrA and parC mutations in ciprofloxacin-resistant *Pseudomonas aeruginosa* isolates from Tehran hospitals in Iran. *Iranian Journal of Microbiology,* 10(4):242-249.
- **Farhan**, S., Ibrahim, R., Mahran, K., Hetta, H. and Abd El-Baky, R. (2019). Antimicrobial resistance pattern and molecular genetic distribution of Metallo-β-lactamases producing *Pseudomonas aeruginosa* isolated from hospitals in Minia, Egypt. *Infection and Drug Resistance*, 12: 2125.

- **Ferman**, F.(2019). Isolation and Identification Bacteriophages of *Pseudomonas aeruginosa* caused Wound and Burn Infections and Study of its Antimicrobial Effective. Msc thesis. Diyala University.iraq.42-43.
- **Fleitas Martinez**, O., Cardoso, M. Ribeiro, S. and Franco, O. (2019). Recent Advances in Anti-virulence Therapeutic Strategies With a Focus on Dismantling Bacterial Membrane Microdomains, Toxin Neutralization, Quorum-Sensing Interference and Biofilm Inhibition. *Frontiers in Cellular and Infection Microbiology*, 9(74):1-24.
- **Fugelsang,** K. and Edwards, C.(2007). Wine microbiology practical applications and procedures. 2nd. ed. New York: Springer.
- **Fujitani**, S., Moffett, K. and Yu, V. (2017).*Pseudomonas aeruginosa*. Antimicrobe – Infectious Disease & Antimicrobial Agents. N.d.; available at http://www.antimicrobe.org/new/b112.asp.


{ G }


- **Garcia**, M., Morello, E., Garnier, J., Barrault, C., Garnier, M. and Burucoa, C.(2018).*Pseudomonas aeruginosa* flagellum is critical for invasion, cutaneous persistence and induction of inflammatory response of skin epidermis. *Virulence*, 9(1):1163–75.
- **Gerard**, T., Funke, B. and Case, C.(2016). Microbiology: An Introduction .12th ed. Pearson Education. 54.
- **Ghannoum**, M., Parsek, M., Whitely, M. and Mukherjee, P.(2015). Microbial Biofilms.2nd.ed. Washington: ASM Press, 216.
- **Gloag**, E., German, G., Stoodley, P. and Wozniak, D.( 2018). Viscoelastic properties of *Pseudomonas aeruginosa* variant biofilms. *Scientific Reports*, 8: 1–11.
- **Golle**, A., Janezic, S. and Rupnik, M.(2017). Low overlap between carbapenem-resistant *Pseudomonas aeruginosa* genotypes isolated from hospitalized patients and wastewater treatment plants. *PLOS One*, 12(10):1-13.

- **Gomila**, A., Carratalà, J. , Badia, J., Camprubí, D., Piriz, M., Shaw, E., Diaz-Brito,V. , Espejo, E., Nicolás, C., Brugués, M. and *et al*. (2018) Preoperative oral antibiotic prophylaxis reduces *Pseudomonas aeruginosa* surgical site infections after elective colorectal surgery: A multicenter prospective cohort study. *BMC Infectious Diseases*, 18: 507.
- **Gonçalves**, I., Dantas, R., Ferreira, M., Batistão, D., Gontijo-Filho, P. and Ribas,R.(2017).Carbapenem-resistant *Pseudomonas aeruginosa:* association with virulence genes and biofilm formation. *brazilian journal of microbiology*, 48(2): 211-217.
- **Granato**, P., Morton,V. and Morello,J. (2019). Laboratory Manual and Workbook in Microbiology .12nd.ed. New York, NY: McGraw-Hill Education.4-337.
- **Grenni**, P., Ancona, V. and Caracciolo, A. (2017) . Ecological effects of antibiotics on natural ecosystems: A review. *Microchemical Journal*, 136:25-39.

{ $\mathcal{H}$ }

- **Hall**, C. and Mah, T.(2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews*,41:276–301.
- **Hameed,** Z. (2017). Detection of CTX-M-1 and CTX-M-3 genes in virulent, Multidrug-resistant *Pseudomonas aeruginosa* isolates. MSc thesis, Diyala University. Iraq.
- **Hasan**, S., Najati, A. and Abass, K.(2020). Prevalence and antibiotic resistance of *"pseudomonas aeruginosa"* isolated from clinical samples in Kirkuk City, Iraq. *EurAsian Journal of BioSciences Eurasia J Biosci*,14: 1821-1825
- **Hawkey**, P., Warren, R., Livermore, D., McNulty, C., Enoch, D., Otter, J. and Wilson, R. (2018). Treatment of infections caused by multidrug-resistant

Gram-negative bacteria: report of the British Society for Antimicrobial Chemotherapy/ Healthcare Infection Society/British Infection AssociationJoint Working Party. *Journal of Antimicrobial Chemotherapy*, 73 (3): 2–78.

- **Hemati**, S., Sadeghifard, N., Ghafurian, S., Maleki, F., Mahdavi, Z., Hassanvand, A., Valadbeigi, H., Hemati, S. and Hatami, V.(2016). The association of biofilm formation and sub-minimal inhibitory concentrations of antimicrobial agents. *Journal of Basic Research in Medical Sciences*, 3(1):26-30.
- **Hernández Durán**, M., López Jácome, L., Colín Castro C., CerónGonzález G., Ortega Peña, S., Vanegas Rodríguez, E. and *et al*. (2017). Comparison of the MicroScan WalkAway and VITEK 2 Compact systems for the identification and susceptibility of clinical Grampositive and Gram-negative bacteria. *Investigaction En Discapacidad*, 6(3): 105-114.
- **Heydari**, S. and Eftekhar, F. (2014). Biofilm Formation and β-Lactamase Production in Burn Isolates of *Pseudomonas aeruginosa*. *Jundishapur Journal of Microbiology*, 8(3): e15514.
- **Hilliam**, Y., Kaye, S. and Winstanley C. (2020). *Pseudomonas aeruginosa* and microbial keratitis. *Journal Medical Microbiology*,69(1): 3-13.
- **Holbrook**, S. and Garneau-Tsodikova, S.(2018). Evaluation of Aminoglycoside and Carbapenem Resistance in a Collection of Drug-Resistant *Pseudomonas aeruginosa* Clinical Isolates. *Microbial drug resistance*,24(7):1020-1030.
- **Hoque**, M., Ahmad, M., Khisa, S., Uddin, M. and Jesmine, R.(2015). Antibiotic Resistance Pattern in *Pseudomonas aeruginosa* Isolated from Different Clinical Specimens. *JAFMC Bangladesh*, 11(1):45-49.
- **Horcajada**, J., Montero, M., Oliver, A., Sorlí, L., Luque, S., Gómez-Zorilla, S., Benito, N. and Grau, S. (2019) . Epidemiology and treatment of multi-drug

resistant and extensively-drug resistant *Pseudomonas aeruginosa* infections. *Clinical Microbiology Reviews*, 32:e00031-19.

- **Hosu**, M., Vasaikar, S., Okuthe, G. and Apalata,T.(2021). Antibiotic-resistant gene of Pseudomonas aeruginosa from non-clinical environment: public health implications in Mthatha, Eastern Cape Province, South Africa. *Research squer*, DOI: https://doi.org/10.21203/rs.3.rs-31330/v1.
- **Hynen**, A., Lazenby, J., Savva, G., McCaughey, L., Turnbull, L., Nolan, L. and Whitchurch, C.(2021). Multiple holins contribute to extracellular DNA release in *Pseudomonas aeruginosa* biofilms. *Microbiology*,10:1-12.

{ *I* }

- **Igbinosa**, I., Beshiru, A. and Igbinosa, E. (2017). Antibiotic resistance profile of Pseudomonas *aeruginosa* isolated from aquaculture and abattoir environments in urban communities. *Asian Pacific Journal of Tropical Disease*,7(1):47–52.
- **Iseppi**, R., Sabia, C., Bondi, M. , Mariani, M. and Messi. P.(2020). Virulence Factors, Drug Resistance and Biofilm Formation in *Pseudomonas* Species Isolated from Healthcare Water Systems. *Current Microbiology*, 1(1):1-9.

{ *J* }

- **Jabuk**, S., jabuk, N., AL-Harmoosh, R. and Hussien, R.(2017).Isolation and Identification of antibiotic-producing bacteria from local soil in Babylon province. *Euphrates Journal of Agriculture Science Second Veterinary Conference*, 304-3010.
- **Jamal,** M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. and *et al*.(2018). Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association*,81(1): 7–11.
- **Jesus**, R. and Dedeles,G.(2020). Data on quantitation of Bacillus cereus sensu lato biofilms by microtiter plate biofilm formation assay. *Data, in brief*,28:104951.

- **Juayang**, A., Lim, J., Bonifacio, A., Lambot, A., Millan, S., Sevilla, V. and Gallega, C. (2017). Five-Year Antimicrobial Susceptibility of *Pseudomonas aeruginosa* from a Local Tertiary Hospital in Bacolod City, Philippines. *Tropical Medicine and Infectious Disease*, 2(3):1-8.

{ K }

- **Kadhum**, H. and Hasan, T.(2019). The Study of Bacillus Subtils Antimicrobial Activity on Some of the Pathological Isolates. *International Journal of Drug Delivery Technology*, 9(2):193-196.
- **Kamali**,E., Jamali,A., Ardebili,A., Ezadi,F. and Mohebbi, A.(2020). Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of *Pseudomonas aeruginosa*. *BMC Research Notes*,13:27.
- **Khadim** ,M. and AL Marjani,M.(2019). Pyocyanin and Biofilm Formation in *Pseudomonas aeruginosa* Isolated from Burn Infections in Baghdad, Iraq. *Jordan Journal of Biological Sciences*, 12(1):31-35.
- **Khosravi**, A., Motahar, M. and Montazeri E. (2017). The frequency of class1 and 2 integrons in *Pseudomonas aeruginosa* strains isolated from burn patients in a burn center of Ahvaz, Iran. *PLoS One*,12(8):e0183061.
- **Koo**, H., Allan, R., Howlin, R., Stoodley, P. and Hall-Stoodley, L. (2017). Targeting microbial biofilms: current and prospective therapeutic strategies. *Nature Reviews Microbiology*,15 740–755.
- **Kordes**, A., Preusse, M., Willger, S., Braubach, P., Jonigk, D., Haverich, A., Warnecke, G., and Häussler S. (2019 ). Genetically diverse *Pseudomonas aeruginosa* populations display similar transcriptomic profiles in cystic fibrosis explanted lung. *Nature Communication*,10(1): 3397.

{ L }

- **Laventie**, B., Sangermani, M., Estermann, F., Manfredi, P., Planes, R., Hug, I., Jaeger, T., Meunier, E., Broz, P. and Jenal, U. (2019). A Surface-Induced

Asymmetric Program Promotes Tissue Colonization by *Pseudomonas aeruginosa*. *Cell Host & Microbe, 25*(1), 140-152.

- **Lee**, Y. and Bradley, N. (2019). Overview and insights into Carbapenem allergy. *Pharmacy,*7(3): 110–116.
- **Lila**,G.,Mulliqi,G., Kurti,A., Bajrami,R. and Azizi,E.(2018). Molecular epidemiology of *Pseudomonas aeruginosa* in University Clinical Center of Kosovo. *Infection and Drug Resistance*,2018(11):2039-2046.

{ M }

- **MacFaddin,** F. (2000). Biochemical Tests for Identification of Medical Bacteria. 3rd ed. The Williams and Wilkins Baltimore. U.S.A.
- **Macin**, S., Akarca, M., Sener, B. and Akyon,Y.(2017). Comparison of virulence factors and antibiotic resistance of *Pseudomonas aeruginosa* strains isolated from patients with and without cystic fibrosis. *Revista Română de Medicină de Laborator,* 25( 4):327-334.
- **Macin**, S. and Akyon, Y.(2017). Phenotypic and Genotypic Virulence Factors in *Pseudomonas aeruginosa* strains according to pigment presence. *Acta Medica Mediterranea*, 33: 1033.
- **Magiorakos**, A., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., Harbarth, S., Hinndler, J. and *et al*. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, 18(3): 269-279.
- **Mahdavi**,M., Salehi,S., Amini,K. and Mobasseri, P.(2017). Frequency of exoY, exoS, exoT and exoU genes among *Pseudomonas aeruginosa* Isolated from patients in Tehran hospitals by Multiplex PCR. *Iran journal Medical Microbiology*,11: 9-17.

# References


- **Maita**, P. and Boonbumrung, K. (2014**).** Association between biofilm formation of *Pseudomonas aeruginosa* clinical isolates versus antibiotic resistance and genes involved with biofilm. *journal Chemical and Pharmaceutical Research*, 6(5): 1022-1028.
- **Majeed**, H. and Aljanaby, A. (2019). Antibiotic Susceptibility Patterns and Prevalence of Some Extended Spectrum Beta-Lactamases Genes in Gram-Negative Bacteria Isolated from Patients Infected with Urinary Tract Infections in Al-Najaf City, Iraq. *Avicenna Journal of medical biotechnology*, 11(2):192.
- **Mathee**, K. (2018). Forensic investigation into the origin of *Pseudomonas aeruginosa* PA14 - old but not lost. *Journal of medical microbiology*, 67(8): 1019-1021.
- **Meliani**, A.(2020). The Multifactorial Resistance of *Pseudomonas aeruginosa*. *EXCLI Journal*,19:813-816.
- **Mirsalehian**,A., Kalantar-Neyestanaki,D., Taherikalani,M., Jabalameli,F. and Emanein,M.(2017). Determination of carbapenem resistance mechanism in clinical isolates of *Pseudomonas aeruginosa* isolated from burn patients, in Tehran, Iran. *Journal of Epidemiology and Global Health*,1-3.
- **Mohamed**, F., Askoura, M. and Shaker, G.(2019). Antibiotic susceptibility of *Pseudomonas aeruginosa* isolated from different clinical sources. *Zagazig Journal Pharmaceutical*, 28(2):10-17.
- **Mohammadzadeh**,A.,Mardaneh,J.,Ahmadi,R.and Adabi, J. (2017). Evaluation of the Virulence Features and Antibiotic Resistance Patterns of Pathogenic *Pseudomonas aeruginosa* Strains Isolated from Hospitalized Patients in Gonabad, Iran. *Archives of Pediatric Infectious Diseases,* 5(3): e41267.
- **Moore**, N. and Flaws, M. (2011). Antimicrobial resistance mechanisms in *Pseudomonas aeruginosa*. *Clinical Laboratory Science*,24: 47-51.

# References


- **Moradali** , M., Ghods, S. and Rehm, B. (2017).*Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Front Cell Infect Microbiology*,7:39.
- **Moradali**, M. and Rehm, B. (2019) .The Role of Alginate in Bacterial Biofilm Formation. InExtracellular Sugar-Based Biopolymers. *Chams*: *Springer*,517-537.
- **Moustafa**, D., Wu, A., Zamora, D., Daly, S., Sturge, C., Pybus, S., Geller, B., Goldberg, J. and Greenberg, D.(2021)Peptide-Conjugated Phosphorodiamidate Morpholino Oligomers Retain Activity against Multidrug-Resistant *Pseudomonas aeruginosa* In Vitro and In Vivo. *mBIO*, 12 (1): e02411-20.
- **Mukherjee,** S., Moustafa, D., Stergioula, V., Smith, C., Goldberg, J. and Bassler, B. (2018). The PqsE and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and biofilm development in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the USA*, 115(40): 9411–9418.
- **Murray**, P., Rosenthal, K. and faller, P.(2021).Medical Microbiology, 9ed, USA: Elsevier, 27-29.
- **Murugan**, N., Malathi, J., Therese, K. and Madhavan, H. (2018). Application of six multiplex PCRs among 200 clinical isolates of *Pseudomonas aeruginosa* for the detection of 20 drug resistance encoding genes. *The Kaohsiung Journal of Medical Sciences*, 34( 2): 79-88.
- **Mwinyikombo**, I.(2018). Isolation, antibiotic susceptibility and molecular characterization of resistance genes in pseudomonas isolates from selected hospitals in mombasa county, kenya. MSc thesis. Kenyatta University of Kenya.1-99.

{ N }


- **Nader**, M, Kareem, A., Rasheed, M. and Issa, M.(2017). Biofilm Formation and Detection of pslÁ Gene in Multidrug-Resistant *Pseudomonas aeruginosa* Isolated from Thi-Qar, Iraq. *Iraqi Journal of Biotechnology,* 16(4): 89-103.
- **Nader**,M., Ibrahim ,J. and AL- Sweidawi,W.(2019). Genetic diagnosis of the Opr I and Opr L genes in *Pseudomonas aeruginosa* Isolated from various local sources. *Anbar University Journal of Pure Sciences*,11(1):26-27.
- **Najeeb**,A.(2020). Genotyping Diversity of *Pseudomonas aeruginosa* Isolated from Clinical specimens in Baquba. MSC thesis, University of Diyala, Iraq, 58-97.
- **Najnin**, A., Farzana, Y., Rashid ,M., Deepthi ,S., Srikumar ,C.,Rashid.S., Nazmul, M. and Fazlul , M. (2018). Detection of virulence factors and β lactamase encoding genes among the clinical isolates of *Pseudomonas aeruginosa*. *Journal of International Pharmaceutical Research*,45(1):190-200.
- **Namuq**,A., Ali, K. and Al-Ani, A.(2019). Correlation between Biofilm Formation, Multi-Drug Resistance and AlgD Gene among *Pseudomonas aeruginosa* Clinical Isolates. *Journal of University of Babylon for Pure and Applied Sciences*, 27 (3): 1-8.
- **Negi**, K.(2012).Methods in biostatistics. 1st ed. INDIA, Delhe: AITBS publishers.
- **Newman**, J., Floyd, R. and Fothergill, J.(2017). The contribution of *Pseudomonas aeruginosa* virulence factors and host factors in the establishment of urinary tract infections. *FEMS Microbiology Letters*, 364(15):1-11.
- **Nile**, H., Hassan, L. and Jabber, E. (2015).Virulence Factors of *Pseudomonas aeruginosa* Isolated from Wound and Burn Infections. *IJCRBP*,2(6):153-162.

- **Noomi**,B.(2018). Detection of virulence factors of *Pseudomonas aeruginosa* in different animals by using bacteriological and molecular methods. *Iraqi Journal of Veterinary Sciences*, 32( 2): 205-210.


{ O }


- **Obaid**, K.(2019). Effect of *Lactobacillus acidophilus* Supernatant on the Biofilm Formation of Some Pathogenic Bacteria. *Journal of University of Babylon for Pure and Applied Sciences*, 27(4):72-90.
- **Okwu1,** M.**,** Mitsan, O. and Okeke, O. (2014). Prevalence and antimicrobial susceptibility profiles of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolate among healthy individuals in Okada, South-South, Nigeria. *Pharmaceutical Biological & Chemical Science Journal*,1(1): 1 - 9.
- **Olivares**, E., Badel-Berchoux, S., Provot, C., Prévost, G., Bernardi, T. and Jehl,F.(2020).Clinical Impact of Antibiotics for the Treatment of *Pseudomonas aeruginosa* Biofilm Infections. *Frontiers in microbiology*, (10): 2894.
- **Osman**,M., El-Hendawy1, H., El-All,S., Hassan,A. and Mahmoud,D. (2018). Correlation between resistance of *Pseudomonas aeruginosa* to benzalkonium chloride and expression of efflux pump genes. *Journal of Applied Pharmaceutical Science*, 8(12):044-052.
- **Otani**, S., Hiramatsu, K., Hashinaga, K., Komiya, K., Umeki, K., Kenji Kishi,K. and Kadota, J.( 2018). Sub-minimum inhibitory concentrations of ceftazidime inhibit *Pseudomonas aeruginosa* biofilm formation. *Journal of Infection & Chemotherapy*, 24(6): 428–433.
- **O'Toole**, G.(2018). Cystic fibrosis airway microbiome: Overturning the old, opening the way for the new. *Journal of Bacteriology*,200: e00561-17.

{ 𝒫 }


- **Pachori**, P., Gothalwal, R. and Gandhi, P.(2019). Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit a critical review. *ScienceDirect,* 6(2) :109-119.
- **Palavutitotai**, N., Jitmuang, A., Tongsai, S., Kiratisin, P. and Angkasekwinai, N. (2018). Epidemiology and risk factors of extensively drug-resistant *Pseudomonas aeruginosa* infections. *PLoS One,* 13 (2): e019343.
- **Pang,** Z., Raudonis, R., Glick, B., Lin, T. and Cheng, Z**.** (2019). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology advances,* 37(1): 177-192.
- **Papagiannitsis**, C. , Medvecky, M., Chudejova, K., Skalova, A., Rotova,V., Spanelova,P., Jakubu,V., Zemlickova,H. and Hrabak ,J .(2017). Molecular Characterization of Carbapenemase-Producing *Pseudomonas aeruginosa* of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-β-Lactamase. *Antimicrobial Agents and Chemotherapy,* 61 (12):1-15.
- **Pena**,R , Blasco,L., Ambroa,A., González-Pedrajo,B., Fernández-García,L., López,M., Bleriot,L., Bou,G., García-Contreras,R., Wood,T., and Tomás,M.(2019).Relationship Between Quorum Sensing and Secretion Systems. *Frontiers in Microbiology*,10:1-14
- **Penesyan**, A., Nagy, S. , Kjelleberg, S., Gillings, M. and Paulsen, I. T. (2019). Rapid microevolution of biofilm cells in response to antibiotics. *NPJ Biofilms Microbiomes,* 5(1):34.
- **Penesyan**, A., Paulsen, I., Gillings, M. , Kjelleberg, S. and Manefield, M. (2020). Secondary Effects of Antibiotics on Microbial Biofilms. *Frontiers in microbiology,* 11:2109.

# References


- **Persat,** A., Inclan, Y., Engel, J., Stone, H. and Gitai, Z. (2015). Type IV pili mechanochemically regulate virulence factors *in Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the *USA*, 112(24): 7563 – 7568.
- **Pezzlo**, M., Peterson, E. , Bittencourt,C. and de la Maza,M.(2020) . Color Atlas of Medical Bacteriology.3th.ed. Hoboken: *American Society for Microbiology and John Wiley & Sons, Inc*, 145- 149.
- **Powell**, L., Pritchard, M., Ferguson, E., Powell, K., Pate, S., Rye, P., Sakellakou, S, Buurma, N., Brilliant, C., Copping, J., Menzies, G., Lewis, P., Hill, K. and Thomas, D.(2018)Targeted disruption of the extracellular polymeric network of *Pseudomonas aeruginosa* biofilms by alginate oligosaccharides. *npj Biofilms and Microbiomes*, 4:13.
- **Procop**, G. ,Church,D., Hall,G., Janda,W., Koneman,E., Schreckenberger,P. and Woods,G.(2017). Color atlas and textbook of diagnostic microbiology. 7th.ed. Philadelphia: Wolters Kluwer Health.
- **Pungcharoenkijkul**, S., Traipattanakul,J., Thunyaharn,S. and Santimaleeworagun,W.(2020). Antimicrobials as Single and Combination Therapy for Colistin-Resistant *Pseudomonas aeruginosa* at a University Hospital in Thailand. *Antibiotics*, 9: 475.
- **Purro**, M., Qiao, J., Liu, Z., Ashcrafta, M. and Xiong, M.(2018). Desferrioxamine:gallium-pluronic micelles increase outer membrane permeability and potentiate antibiotic activity against *Pseudomonas aeruginosa*. *Chemical Communications*,54(1): 13929-13932.

{ Q }

- **Qayoom**, S., Rashid, A., Kohli, A., Masoodi, T. and Amin, M.(2019). Prevalence and antibiotic sensitivity pattern of *Pseudomonas aeruginosa* isolates from respiratory samples, pus samples and body fluids in a tertiary care hospital Kashmir. *Indian Journal of Microbiology Research*,6(4):345–349.

{ ℛ }


- **Raheema**, R. and Abed, K.(2020). A review on Some Virulence factors of *Pseudomonas aeruginosa* Isolates from Iraqi patients.1-15.
- **Ramsay**, K.(2017). Diversity of environmental and clinical *Pseudomonas aeruginosa* isolates. PH thesis. University of Queensland. Australia.17-18.
- **Reda**,F., El-Zawahry,Y. and Omar,A.(2017). Synergistic Effect of Combined Antibiotic and Methanol Extract of *Eucalyptus camaldulensis* leaf Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *J. Applied Sciences and Biotechnology*, 5(4): 486-497.
- **Ren**, H., Liu, Y., Zhou, J., Long, Y., Liu, C., Xia, B., Shi, J., Fan, Z., Liang, Y., Chen, S. and Xu, J.(2019). Combination of Azithromycin and Gentamicin for Efficient Treatment of *Pseudomonas aeruginosa* Infections. Journal of Infectious Diseases,220(10):1667 78.
- **Riedel**, S., Morse, S., Mietzner, T. and Miller, S. (2019). Pseudomonads and Acinetobacter. In: Jawetz, Melinick and Adelbergs Medical Microbiology, 28th. Ed. A lange medical book.
- **Ríos**, P., Rocha, C., Castro, W., Vidal, M., Canal, E., Bernal, M. and Simons, M.(2018). Extensively drug-resistant (XDR) *Pseudomonas aeruginosa* identified in Lima, Peru co-expressing a VIM-2 Metallo-β-lactamase, OXA-1 β-lactamase and GES-1 extended-spectrum β-lactamase. *JMM case reports*, *5*(7).
- **Rocha**, A., Barsottini, M., Rocha, R., Laurindo, M., de Moraes, F. and Rocha, S.(2019). *Pseudomonas Aeruginosa*: Virulence Factors and Antibiotic Resistance Genes. *Brazilian Archives of Biology and Technology*,62: e19180503.

- **Rodulfo**,H., Arcia,A., Hernández,A., Michelli,E. , Martinez,D. , Guzman,M. , Sharma,A. and Donato,M.(2019). Virulence factors and integrons are associated with MDR and XDR phenotypes in nosocomial strains of *Pseudomonas aeruginosa* in a Venezuelan university hospital. *Revista Instituto Medicina Tropical São Paulo*,(16):20.1-11.
- **Roemhild** , R. and Schulenburg,H. (2019). Evolutionary ecology meets the antibiotic crisis: Can we control pathogen adaptation through sequential therapy?. *Evol Med Public Health*,2019(1):37-45.
- **Rossi**, I., Dantas, R., Ferreira, M., Batistao, D., Gontijo-filho, P. and Ribas, R.(2017). Carbapenem-resistant *Pseudomonas aeruginosa*: association with virulence genes and biofilm formation. *Brazilian Journal of Microbiology*, 48(2): 211-217.
- **Ruiz-Garbajosa**, P. and Cantón, R. (2017). Epidemiology of antibiotic resistance in *Pseudomonas aeruginosa*. Implications for empiric and definitive therapy. *Revista Espanola Quimioter*, 30 (1): 8–12.

{ S }

- **Safaei**, H., Moghim, S., Isfahani, B., Fazeli, H., Poursina, F., Yadegari, S., Nasirmoghadas, P. and Hosseininassab Nodoushan, S.(2017). Distribution of the Strains of Multidrug-resistant, Extensively Drug-resistant,and Pandrug-resistant *Pseudomonas aeruginosa* Isolates from Burn Patients. *Adv Biomed*, 6 (74):1-6.
- **Saffari**, E., Khalili, M. and Mehrabadi,J.(2019). Antipseudomonal activity of *Artemisia quettensis* Podlechessential oil and its synergy with imipenem. *Journal of Pharmacy & Pharmacognosy Research*, 7 (3):163-172.
- **Saffari**,M., Karami,S., Firoozeh,F. and Sehat, M.(2017). Evaluation of biofilm-specific antimicrobial resistance genes in *Pseudomonas aeruginosa* isolates in Farabi Hospital. *Journal of Medical Microbiology*,66:905–909.

- **Sala**,A., Ianni,F., Pelizzone,I., Bertocchi,M., Santospirito,D., Rogato,F., Flisi,S. and *et al*.(2019). The prevalence of *Pseudomonas aeruginosa* and multidrug resistant *Pseudomonas aeruginosa* in healthy captive ophidian. *PeerJ*, DOI 10.7717/peerj6706.
- **Saleh**, T., Hashim, S., Al-Obaidi1, R. and AL-Rubaii,B.(2020). A biological study of chitinase produced by clinical isolates of *Pseudomonas aeruginosa* and detection of *ChiA* responsible gene. *International Journal Research Pharmaceutical Sciences*,11(2):1318-1330.
- **Salfinger** Y. and Tortorello M. (2015). Compendium of Methods for the Microbiological Examination of Foods, 5th Ed., *American Public Health Association*, *Washington*, D.C.
- **Sameet**, M., Awadh, H. and Suleiman, A.(2020). Effect of Isolation Source on Virulence Gene Expression, Phenotypic and Antibiotic Resistance Patterns of Clinical Isolate of *Pseudomonas aeruginosa*. *Profile of clinical isolates of Pseudomonas aeruginosa*,23(1):1-6.
- **Sánchez-Diener**, I., Zamorano, L., López-Causapé, C., Cabot, G., Mulet, X., Peña, C. and Martínez-Martínez, L.( 2017). Interplay among resistance profiles, high-risk clones and virulence in the Caenorhabditis elegans *Pseudomonas aeruginosa* infection model. *Antimicrob*. *Agents Chemother*, 61(12):1-11.
- **Santos-Lopez**, A., Marshall, C., Scribner, M., Snyder, D. and Cooper, V. (2019). Evolutionary pathways to antibiotic resistance are dependent upon the environmental structure and bacterial lifestyle. *eLife*, 8:e47612.
- **Schroeder**, M., Brooks, B. and Brooks, A. (2017). The Complex Relationship between Virulence and Antibiotic Resistance. *Genes*, 8(1):39.
- **Schroth,** M., Cho, J., Green, S. and Kominos, S. (2018). Epidemiology of *Pseudomonas aeruginosa* in agricultural areas. *Journal of medical microbiology*, 67(8):1191-1201.

- **Shaikh**, S., Fatima, J., Shakil, S., Rizvi, S. and Kamal, M. (2015). Antibiotic resistance and extended-spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi Journal of Biological Sciences*, 22(1):90–101.
- **Sharkey**, L. and O'Neill, A. (2019). Molecular Mechanisms of Antibiotic Resistance Part II. *Bacterial Resistance to Antibiotics From Molecules to Man*, 27-50.
- **Sharland,** M., Pulcini, C., Harbarth, S., Zeng, M., Gandra, S., Mathur, S. and Magrini, N.( 2018) Classifying antibiotics in the WHO Essential Medicines List for optimal use—be AWaRe. *Lancet Infectious Diseases*,18(1):18–20.
- **Shidiki**.A., Pandit, B. and Vyas,A.(2019). Characterization and Antibiotic profile of *Pseudomonas aeruginosa* isolated from patients visiting National Medical College and Teaching Hospital, Nepal. *Acta Scientific Pharmaceutical Sciences*,3(7):2581-5423.
- **Singh**, M., Yau, Y., Wang, S., Waters, V. and Kumar, A. (2017). MexXY efflux pump overexpression and aminoglycoside resistance in cystic fibrosis isolates of *Pseudomonas aeruginosa* from chronic infections. *Canadian Journal of Microbiology*, 63(12): 929-938.
- **Skariyachan,** S., Sridhar, V., Packirisamy, S., Kumargowda, S. and Challapilli S. (2018). Recent perspectives on the molecular basis of biofilm formation by Pseudomonas aeruginosa and approaches for treatment and biofilm dispersal. *Folia Microbiologica*, 63(4):413–432.
- **Soler-Arango**,J., Figoli,C., Muraca,G., Bosch,A. and Brelles-Mariño, G.(2019). The *Pseudomonas aeruginosa* biofilm matrix and cells are drastically impacted by gas discharge plasma treatment: A comprehensive model explaining plasma-mediated biofilm eradication. *PLoS ONE*,14(6): e0216817.

- **Stewart**, P. and Bjarnsholt, T. (2020). Risk factors for chronic biofilm-related infection associated with implanted medical devices. *Clinical Microbiology and Infection*, 26(8): 1034–1038.
- **Stotani**, S., Gatta, V., Medda, F., Padmanaban, M., Karawajczyk, A., Tammela, P., Giordanetto, F., Tzalis, D. and Collina, S. (2018). Versatile Strategy for the Synthesis of 4,5-Dihydroxy-2,3-Pentanedione (DPD) and Related Compounds as Potential Modulators of Bacterial Quorum Sensing. *Molecules*, 23: 2545.

{ $\mathcal{T}$ }

- **Tacconelli**, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet D. and *et al*. (2018) . Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infectious Disease*, 18:(3)18–27.
- **Tang,** J., Kang, M., Chen, H., Shi, X., Zhou, R., Chen, J. and Du, Y**.** (2011). The Staphylococcal nuclease prevents biofilm formation in *Staphylococcus aureus* and other biofilm-forming bacteria. *Science China Life Science*,54(9):863-9.
- **Tang**, P., Lee, C., Li, C., Li, M., Ko, W. and *et al*.( 2017). Time-to-positivity of blood culture: an independent prognostic factor of monomicrobial *Pseudomonas aeruginosa* bacteremia. *Journal Microbiology-Immunology Infection*,50(4):486–493.
- **Thi**, M., Wibowo, D. and Rehm, B. (2020). *Pseudomonas aeruginosa* Biofilms. *International Journal of Molecular Sciences*, 21( 8671):2-25.
- **Tille**, P. (2017). Bailey and Scott's Diagnostic Microbiology. 14th ed. Louis Missouri: Elsevier Health Sciences. 356_378.
- **Torok**, M., Moran, E. and Cooke, F. (2017). Oxford Handbook of Infectious Diseases and Microbiology.2nd.ed. New York: United States of America.3-111.

- **Trastoy,** R., Manso, T., Fernandez-Garcia, L., Blasco, L., Ambroa, A., Perez Del Molino, M. and *et al***.** (2018). Mechanisms of bacterial tolerance and persistence in the gastrointestinal and respiratory environments. *Clinical Microbiology Reviews,* 31(4): 1-46.
- **Tümmler**,B.(2019). Emerging therapies against infections with *Pseudomonas aeruginosa*. *F1000Research*, *8*, F1000 Faculty Rev-1371.

{ $\mathcal{U}$ }

- **Ullah**, W., Qasim, M., Rahman, H., Jie, Y. and Muhammad, N. (2017). Beta-lactamase-producing *Pseudomonas aeruginosa*: Phenotypic characteristics and molecular identification of virulence genes. *Journal of the Chineses Medical Association*, 80(3): 173-177.

{ $\mathcal{V}$ }

- **Vandepitte**, J., Verhaegen, J., Engbaek, K. , Rohner, P., Piot, P. and Heuck, C. C. (2003). Basic laboratory procedures in clinical bacteriology. (No. Ed. 2). World Health Organization.

{ $\mathcal{W}$ }

- **Waglechner**, N. and Wright, G. ( 2017). Antibiotic resistance. *BMC biology*, 15(1):1-8.
- **Wei**, Q. and Ma, L. (2013). Biofilm matrix and its regulation in Pseudomonas aeruginosa. *International Journal of Molecular Sciences*, 14: 20983-21005.
- **Whiteley**, M., Diggle, S and Greenberg, E. (2017).Progress in and promise of bacterial quorum sensing research. *Nature*,551(7680):313–320.
- **WHO**. (2019). Antibacterial Agents in Clinical Development: an Analysis of the Antibacterial Clinical Development Pipeline, Including Tuberculosis. Geneva: World Health Organization.
- **Wijaya**, A. (2021). Characterization and targeting of the 2-methyl citrate cycle in *Pseudomonas* .https://doi.org/10.17863/CAM.64463.

- **Wojnicz**, D., Tichaczek-Goska, D. and Kicia, M. (2015).Entacyclic triterpenes combined with ciprofloxacin help to eradicate the biofilm formed in vitro by *Escherichia coli. Indian Journal of Medical Research*,141(3): 343–353.

{ Y }

- **Yang**, X., Lai, Y., Yang, J., Jia, M. and Sheng, J.(2020).Molecular epidemiology of *Pseudomonas aeruginosa* isolated from lower respiratory tract of ICU patients. *Brazilian Journal of Biology*,1:1-10.
- **Yeboah**, K.(2021). "Identification of Transcription Regulators of the AlgZ/R Two-Components Regulatory System in *Pseudomonas aeruginosa*" Electronic Theses and Dissertations. MSc.Thesis. Biology department. East Tennessee State University.USA.1-50.

{ Z }

- **Zhou**, L., Li, T., An, J., Liao, C., Li, N. and Wang, X. (2017). Sub-minimal inhibitory concentration (sub-MIC) of antibiotics induces electroactive biofilm formation in bioelectrochemical systems. *Water Research*, 125(15): 280-287.
- **Zimmer**,K., Macedo,A., Nicastro,G., Baldini,R. and Termignoni,C.(2013) *Ticks and Tick-Borne Diseases*, 4(5), 366 -376.
- **Zuberi**,S. and Nadeem, S.(2017). Detection of Biofilm Forming Ability of Bacterial Isolates from Contact Lenses and their Accessories. *Bacteriol Parasitol*, 8(5):1-6.

# Appendix

## Appendix (1): Chart report of VITEK-2 system for identification the results of *Pseudomonas aeruginosa*

مختبر الشمس التخصصي

bioMérieux Customer: M.Sc Manal
System #:

**Laboratory Report**

Printed Sep 15, 2020 04:07 CDT
Printed by: Labadmin

Patient ID: a284

Isolate: a84-1 (Approved)

Card Type: GN Bar Code: 2411143203509200 Testing Instrument: 0000148FFB2A (VK2C8812)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0043453103500270
Organism Quantity:

**Selected Organism: Pseudomonas aeruginosa**

| | |
|---|---|
| **Comments:** | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Identification Information | **Card:** | GN | **Lot Number:** | 2411143203 | **Expires:** | Jan 12, 2021 12:00 CST |
| | **Completed:** | Sep 14, 2020 10:40 CDT | **Status:** | Final | **Analysis Time:** | 5.82 hours |
| **Organism Origin** | VITEK 2 | | | | | |
| Selected Organism | 95% Probability | | **Pseudomonas aeruginosa** | | | |
| | **Bionumber:** | 0043453103500270 | | | **Confidence:** | Very good identification |
| **SRF Organism** | | | | | | |
| **Analysis Organisms and Tests to Separate:** | | | | | | |
| **Analysis Messages:** | | | | | | |
| **Contraindicating Typical Biopattern(s)** Pseudomonas aeruginosa GGAA(1), | | | | | | |

Biochemical Details

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | APPA | - | 3 | ADO | - | 4 | PyrA | - | 5 | IARL | - | 7 | dCEL | - | 9 | BGAL | - |
| 10 | H2S | - | 11 | BNAG | - | 12 | AGLTp | + | 13 | dGLU | + | 14 | GGT | + | 15 | OFF | - |
| 17 | BGLU | - | 18 | dMAL | - | 19 | dMAN | + | 20 | dMNE | + | 21 | BXYL | - | 22 | BAlap | + |
| 23 | ProA | + | 26 | LIP | + | 27 | PLE | - | 29 | TyrA | + | 31 | URE | - | 32 | dSOR | - |
| 33 | SAC | - | 34 | dTAG | - | 35 | dTRE | - | 36 | CIT | + | 37 | MNT | + | 39 | 5KG | - |
| 40 | ILATk | + | 41 | AGLU | - | 42 | SUCT | + | 43 | NAGA | - | 44 | AGAL | - | 45 | PHOS | - |
| 46 | GlyA | - | 47 | ODC | - | 48 | LDC | - | 53 | IHISa | - | 56 | CMT | + | 57 | BGUR | - |
| 58 | O129R | + | 59 | GGAA | + | 61 | IMLTa | + | 62 | ELLM | - | 64 | ILATa | - | | | |

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline: Global CLSI-based
AES Parameter Set Name: Global CLSI-based+Natural Resistance

Therapeutic Interpretation Guideline: NATURAL RESISTANCE
AES Parameter Last Modified: Mar 4, 2018 15:46 CST

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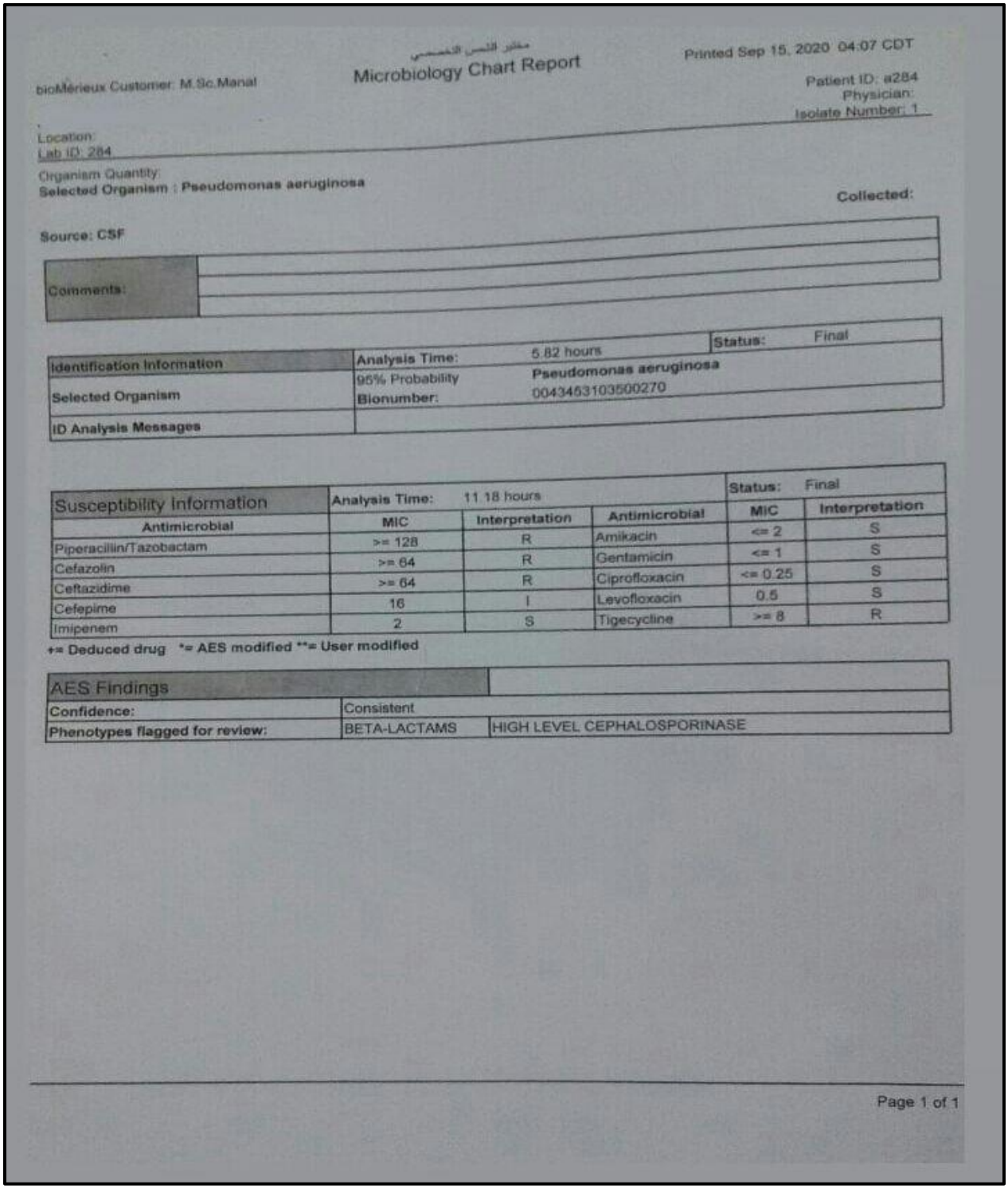

bioMérieux Customer: M.Sc.Manal

مختبر النفس الطبي

Microbiology Chart Report

Printed Sep 15, 2020 04:07 CDT

Patient ID: a284
Physician:
Isolate Number: 1

Location:
Lab ID: 284

Organism Quantity:
**Selected Organism : Pseudomonas aeruginosa**

**Collected:**

**Source: CSF**

| **Comments:** | |
|---|---|

| **Identification Information** | **Analysis Time:** | 5.82 hours | **Status:** | Final |
|---|---|---|---|---|
| **Selected Organism** | 95% Probability<br>**Bionumber:** | **Pseudomonas aeruginosa**<br>0043463103500270 | | |
| **ID Analysis Messages** | | | | |

| Susceptibility Information | **Analysis Time:** | 11.18 hours | | **Status:** | Final |
|---|---|---|---|---|---|
| **Antimicrobial** | **MIC** | **Interpretation** | **Antimicrobial** | **MIC** | **Interpretation** |
| Piperacillin/Tazobactam | >= 128 | R | Amikacin | <= 2 | S |
| Cefazolin | >= 64 | R | Gentamicin | <= 1 | S |
| Ceftazidime | >= 64 | R | Ciprofloxacin | <= 0.25 | S |
| Cefepime | 16 | I | Levofloxacin | 0.5 | S |
| Imipenem | 2 | S | Tigecycline | >= 8 | R |

**+= Deduced drug *= AES modified **= User modified**

| AES Findings | | |
|---|---|---|
| **Confidence:** | Consistent | |
| **Phenotypes flagged for review:** | BETA-LACTAMS | HIGH LEVEL CEPHALOSPORINASE |

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## Appendix(2): Statistical analysis

**T-Test**

**Paired Samples Statistics**

| | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | Free from antibiotic IMI | .10680 | 10 | .028751 | .009092 |
| | After antibiotic treatment (IMI) | .05200 | 10 | .018282 | .005781 |

**Paired Samples Correlations**

| | | N | Correlation | Sig. |
|---|---|---|---|---|
| Pair 1 | Free from antibiotic IMI & After antibiotic treatment (IMI) | 10 | -.228 | .526 |

**Paired Samples Test**

| | | Paired Differences | | | | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference Lower | Upper | | | |
| Pair 1 | Free from antibiotic IMI - After antibiotic treatment (IMI) | .054800 | .037428 | .011836 | .028026 | .081574 | 4.630 | 9 | .001 |

**T-Test**

[DataSet2] C:\Users\Smart\Desktop\Untitled3-NOOF-3.sav

**Paired Samples Statistics**

| | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | Free from antibiotic CAZ | .10680 | 10 | .028751 | .009092 |
| | After antibiotic treatment (CAZ) | .05560 | 10 | .017315 | .005476 |

**Paired Samples Correlations**

| | | N | Correlation | Sig. |
|---|---|---|---|---|
| Pair 1 | Free from antibiotic CAZ & After antibiotic treatment (CAZ) | 10 | .073 | .842 |

**Paired Samples Test**

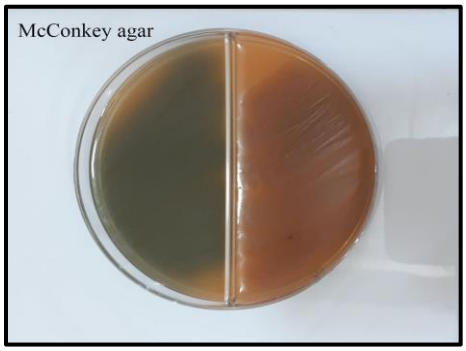
| | | Paired Differences | | | | | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference Lower | Upper | | | |
| Pair 1 | Free from antibiotic CAZ - After antibiotic treatment (CAZ) | .051200 | .032468 | .010267 | .027974 | .074426 | 4.987 | 9 | .001 |

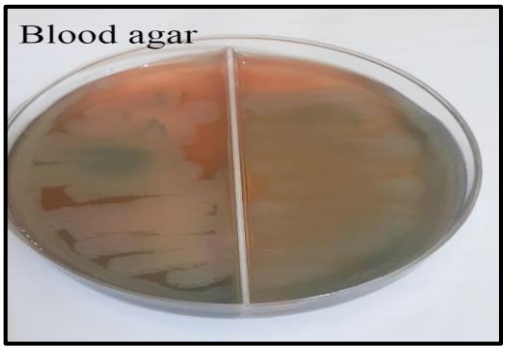
**Test Statistics**

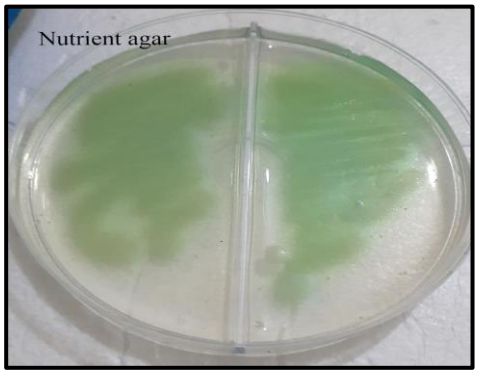
| | Type |
|---|---|
| Chi-Square | 13.800[a] |
| df | 4 |
| Asymp. Sig. | .008 |

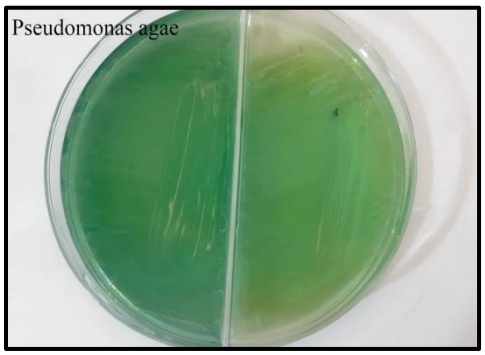
a. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 20.0.

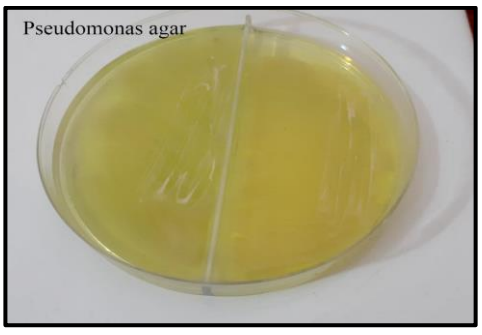
## Appendix(3): *Pseudomonas aeruginosa* cultured (37°C for 24 hours )



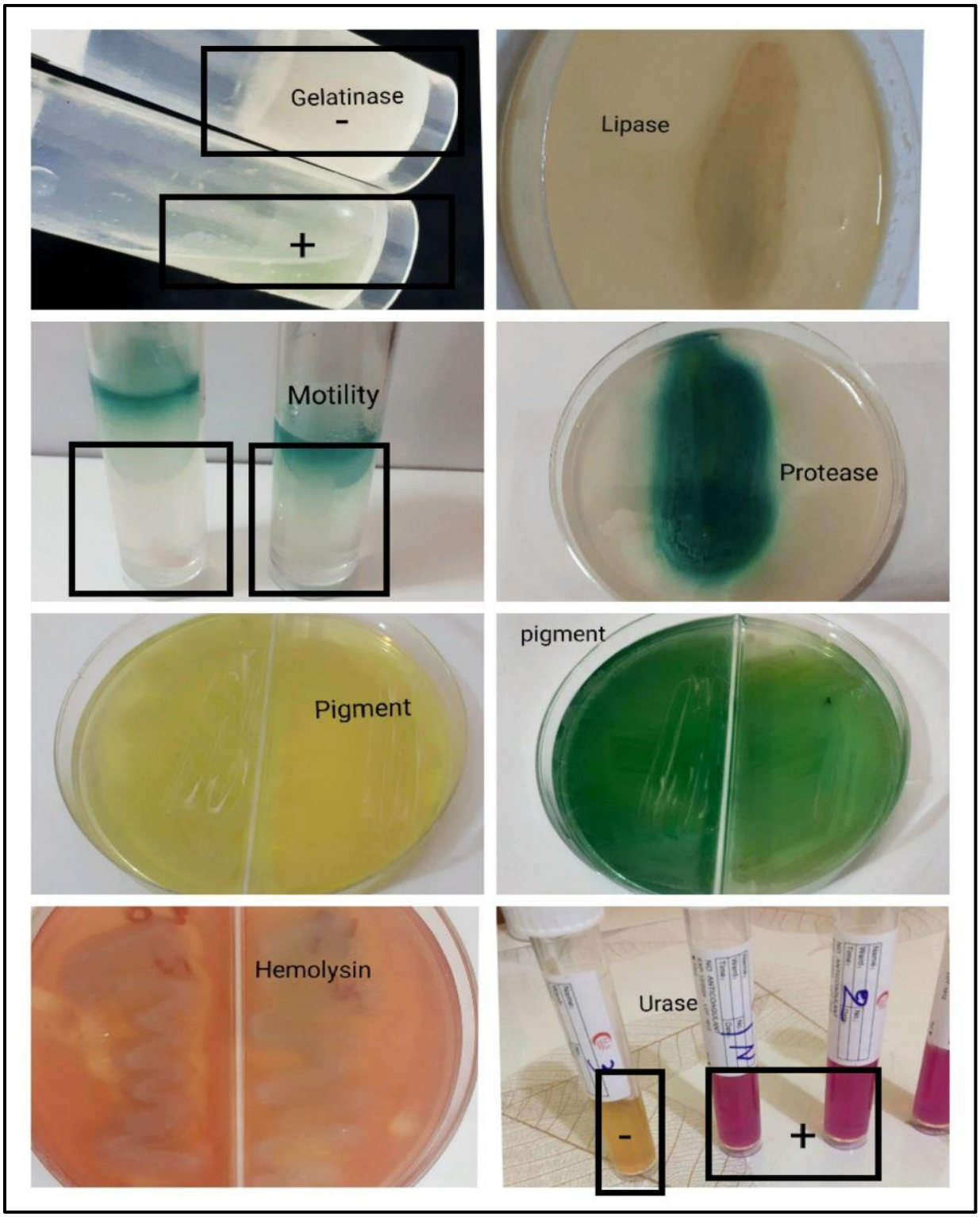



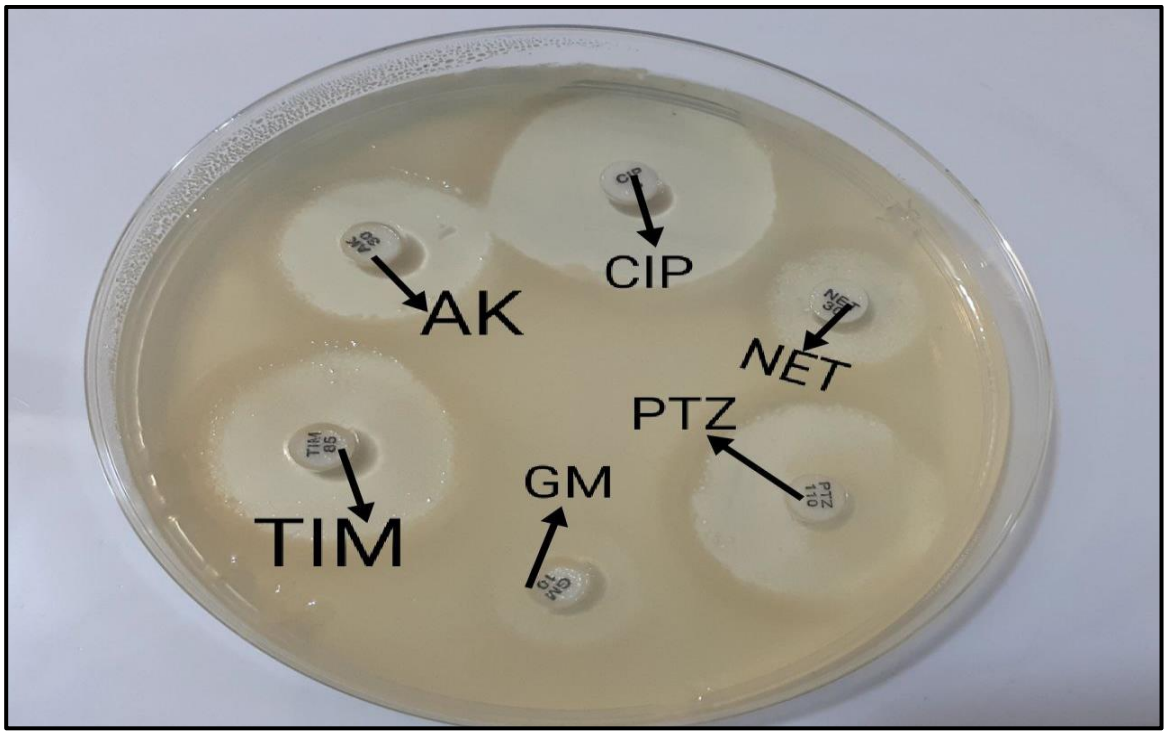



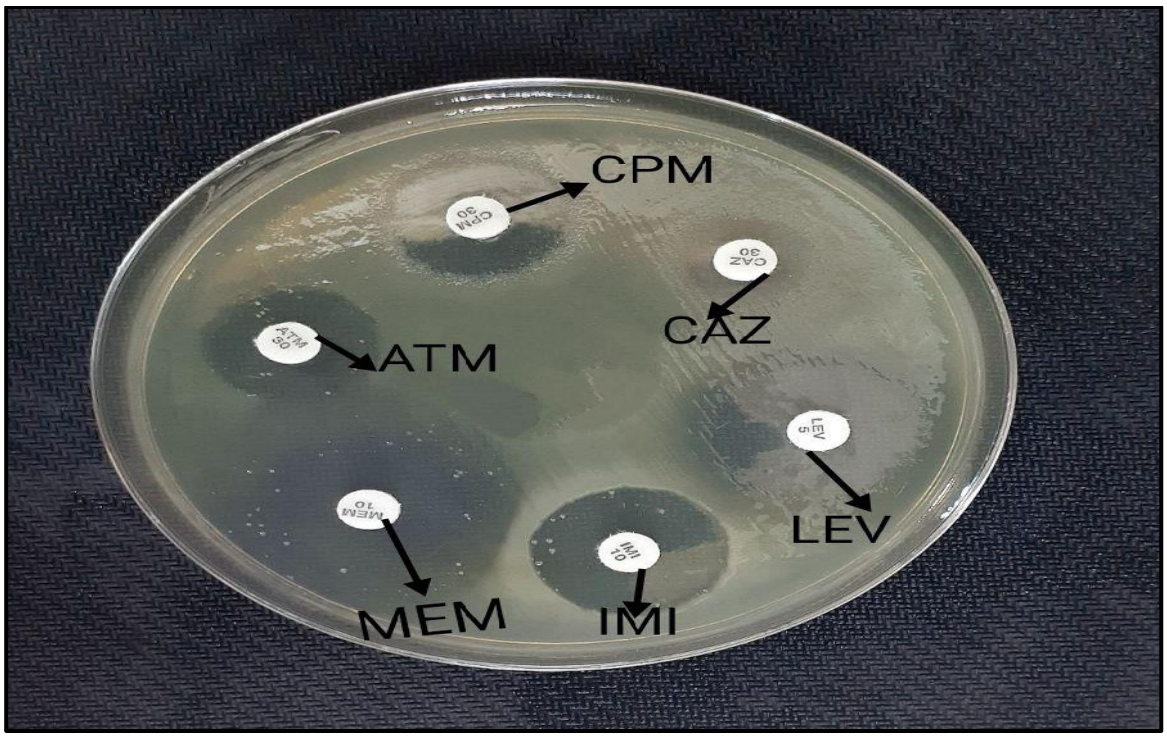
## Appendix(4):Virulence Factors of *Pseudomonas aeruginosa*



**Appendix (5): Micro-titer plate for biofilm formation.**

**Appendix (6): Antibiotics susceptibility test for *Pseudomonas aeruginosa***



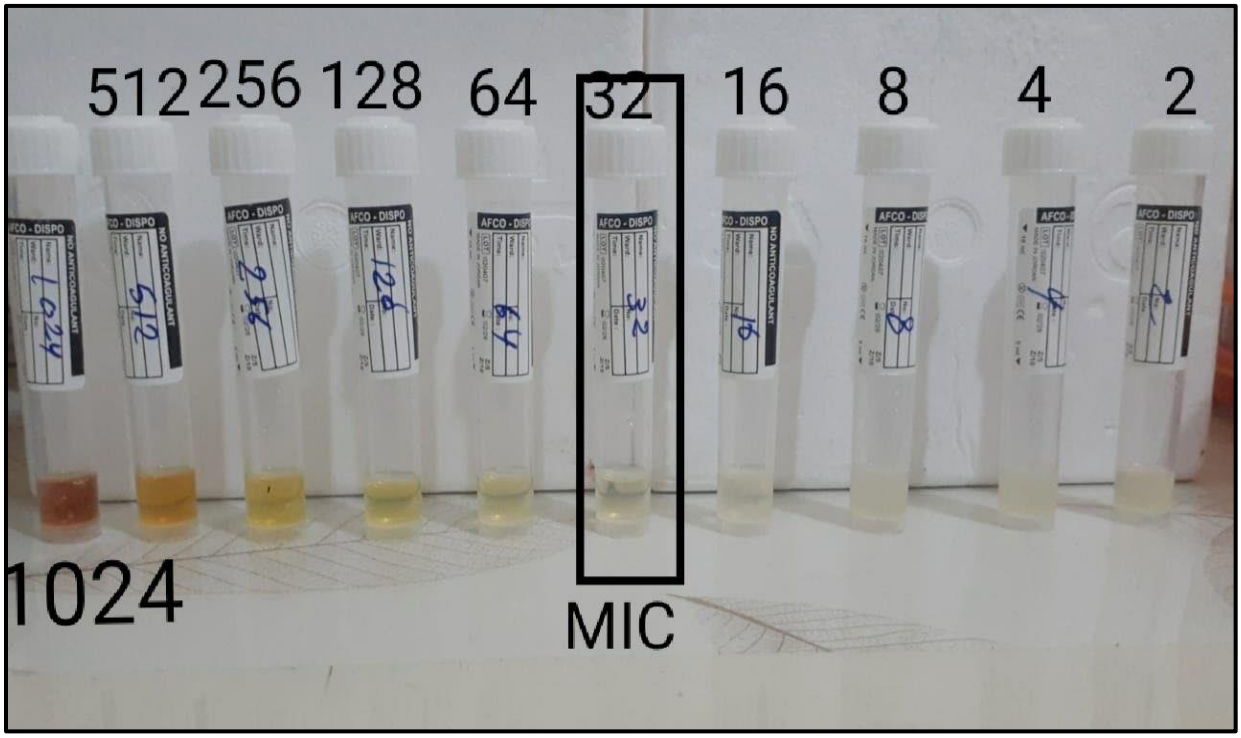



**Appendix(7): The susceptibilities of antibiotics for *Pseudomonas aeruginosa* isolates**

| NO. | TN | NET | GM | PTZ | MEM | IMI | ATM | AK | CPM | CIP | LEV | TIM | CAZ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | S | S | S | S | S | S | S | S | S | S | S | R | S |
| 2 | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 3 | S | S | S | S | S | S | I | S | S | S | S | R | I |
| 4 | S | S | S | S | S | S | S | S | S | S | S | I | S |
| 5 | S | S | S | S | S | S | I | S | S | S | I | I | S |
| 6 | S | S | S | S | R | S | I | S | S | R | R | R | S |
| 7 | R | R | S | S | R | R | R | R | R | R | R | R | R |
| 8 | R | R | R | S | R | R | R | R | R | R | R | R | R |
| 9 | S | R | I | I | R | R | R | R | R | R | R | R | R |
| 10 | R | R | I | I | R | I | R | I | R | R | R | R | R |
| 11 | R | R | R | I | R | R | R | R | R | R | R | R | R |
| 12 | S | S | S | S | S | S | I | S | S | S | S | R | S |
| 13 | R | R | R | I | R | R | R | S | R | R | R | R | R |
| 14 | R | R | R | I | R | R | R | R | R | R | R | R | R |
| 15 | R | R | R | I | R | S | R | R | R | R | R | R | S |
| 16 | S | S | S | S | S | S | S | S | S | S | S | R | S |
| 17 | S | S | S | S | S | S | I | S | S | S | S | R | R |
| 18 | S | S | S | S | S | S | S | S | S | S | S | R | S |
| 19 | S | S | S | S | S | S | I | S | S | S | S | R | S |
| 20 | S | R | R | R | R | R | R | R | R | S | S | R | R |
| 21 | R | R | R | I | S | S | R | R | R | S | S | R | S |
| 22 | R | S | S | S | S | S | I | S | S | S | S | I | S |
| 23 | S | S | S | S | S | S | S | S | S | S | S | I | S |
| 24 | S | S | S | S | S | S | I | S | S | S | S | S | S |
| 25 | S | S | S | S | S | S | S | S | S | I | S | I | S |
| 26 | S | S | I | S | S | S | S | S | S | S | S | I | S |

**Sensitive = S** **Intermediate** = I **Resistant = R**

**Appendix (8): Minimum inhibitory concentration by the serial dilution method on Mueller-Hinton broth.**



جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ديالى

كلية العلوم

قسم علوم الحياة

# التأثيرالتثبيطي لمضادات الحيوية على تكوين الغشاء الحيوي بواسطة بكتريا الزائفة الزنجارية *Pseudomonas aeruginosa*

رسالة مقدمة إلى مجلس كلية العلوم– جامعة ديالى

وهي جزء من متطلبات نيل شهادة الماجستير في علوم الحياة

**من قبل الطالبة**

## نياف ماجد علي العگيدي

بكلوريوس علوم الحياة / كلية العلوم/ جامعة ديالى 2018

**إشراف**

## د. كريم ابراهيم مبارك

(أستاذ مساعد)

**1442هـ** **2021 م**

## الـخلاصة

جمعت مائتي عينة من مصادر مختلفة (بول ، جروح ، اذن ، حروق ، قشع) من المستشفيات الحكومية في بعقوبة / ديالى، خلال فترة الدراسة من ايلول / 2020 الى كانون الثاني / 2021. تم زرع العينات على اوساط انتقائية وتفاضلية ، تم التعرف على 26 عزلة من بكتيريا *P. aeruginosa* من خلال خصائص المستعمرات والفحص المجهري والاختبارات البيوكيميائية. أكد التعرف على 26 عزلة من *P.aeruginosa* بواسطة نظام VITEK-2 المضغوط.

هذه العزلات أعطت نتيجة إيجابية لاختبارات الأوكسيديز والكاتاليز واختبار استهلاك السترات، وأظهرت قدرتها على النمو على وسط MacConkey agar و وسط Pseudomonas agar و وسط Blood agar وتسببت في انحلال الدم (β)، النمو عند 42 درجة مئوية، والقدرة على إنتاج الصباغ. بينما أعطت نتائج سلبية لاختبار الاندول واختبار الميثيل الأحمر واختبار فوجيس-بروسكاور وتخمير اللاكتوز والنمو عند 4 درجات مئوية. اختبار TSI ، غير مخمر لأي نوع من أنواع السكريات الثلاثة (الجلوكوز ، اللاكتوز ، السكروز) ، لا يشكل ثاني أكسيد الكربون ولا ينتج H2S.

نتائج الكشف المظهري لبعض عوامل الضراوة أظهرت ان جميع العزلات أنتجت الهيموليزين ، الحركة ، الليبز ، الصباغ ، البروتيز ، الجيلاتينز واليوريز بنسب 100٪ ، 100٪ ، 96.15٪ ، 76.9٪ ، 65.38٪ ، 57.69٪ و 11.53 ٪ على التوالى.

تكوين الاغشية الحيوية كشفت بالطريقة الكمية باستخدام صفيحة ميكروتيتر بإنتاجية مختلفة بين قوية ومتوسطة وغير ملتصقة ، وأظهرت النتائج أن 26/24 (92.30٪) من العزلات أنتجت الاغشية الحيوية من بينها 38.4٪ من العزلات كانت منتجة للاغشية الحيوية بصورة قوية و 53.8٪ منتجة للاغشية الحيوية بصورة متوسطة، بينما اثنتان فقط من العزلات 7.6٪ غير منتجة للاغشية الحيوية.

تم إجراء اختبارات الحساسية للمضادات الحيوية لـ 26 عزلة مقابل 13 نوعًا مختلفًا من المضادات الحيوية باستخدام طريقة Kirby Bauer Disc Diffusion. أظهرت النتائج أن أقل نسبة مقاومة للمضادات الحيوية كانت ضد Piperacillin / Tazobactam ( 3.8٪) ونسبة مقاومة أعلى ضد Ticarcillin / Clavulanic acid ( ٪ 69.2 ) بينما بلغت نسبة المقاومة لكل من Aztreonam و Cefepime و Meropenem و Netilmicin (38.4٪). Ceftazidime ، Ciprofloxacin ، Levofloxacin وTobramycin (34.6٪). بينما كانت نسبة مقاومة المضادات

الحيوية Gentamicin و Imipenem (26.9٪) ، Amikacin (30.7٪). المقاومة للأدوية المتعددة (MDR) شكلت (42.3٪) من مجموع العزلات في الدراسة الحالية.

تم استخدام طريقة التخفيف التسلسلي لتحديد الحد الأدنى للتركيز المثبط (MIC) والتركيز المثبط الأدنى الفرعي (Sub_ MIC) والحد الأدنى لتركيز مبيد الجراثيم (MBC) ضد اثنين من المضادات الحيوية هما Imipenem و Ceftazidime لعشر عزلات مختارة وفقًا لتكوين الاغشية الحيوية بصورة قوية. أظهرت النتائج وجود اختلافات في قيم MIC و MBC. تراوحت MIC لـ Ceftazidime من (16-1024 ميكروغرام / مل) ، وتراوحت MBC من (128 -> 1024 ميكروغرام / مل). بينما تراوحت MIC لـ Imipenem (16-512 ميكروغرام / مل) وكان MBC (512-> 1024 ميكروغرام / مل). تم تحديد قيم sub_ MIC عن طريق اختيار أقل تركيز مثبط يمكن أن تنمو فيه البكتيريا.

في الدراسة الحالية ، تم اختبار كل مضاد حيوي في sub_ MICs لدراسة التغير في قدرة عزلات *P. aeruginosa* في تكوين الأغشية الحيوية. أثر كل من sub_ MIC لـ CAZ و IMI على الأغشية الحيوية عن طريق تقليل كثافة تكوين الأغشية الحيوية في معظم العزلات بعد الحضانة لمدة 24 ساعة. هذه التأثيرات دلت إلى أن sub_ MICs لـ CAZ و IMI قد تؤثر على عدة مراحل تكوين الأغشية الحيوية. التحقيق في تاثير المضادات الحيوية دون MIC على الأغشية الحيوية البكتيرية المستهدفة إلى تطوير طرق العلاج بالمضادات الحيوية في المستقبل.

تم إجراء اختبار PCR للكشف عن جينات *psl*A و *psl*D و *pel*A التي شاركت في تكوين الأغشية الحيوية بين العزلات السريرية. أوضحت النتائج الحالية وجود *psl*A و *psl*D و *pel*A في جميع العزلات المدروسة. أظهرت النتائج أن 26/25 (96.15٪) من العزلات تحتوي على جينات *psl*A و *psl*D بينما 26/23 عزلة (88.46٪) تحتوي على جين *pel*A. حملت جميع عزلات *P. aeruginosa* جينات *psl*A و *psl*D و *pel*A بغض النظر عن كثافة الغشاء الحيوي.