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Characterization of Metallo beta lactam-Producing *Pseudomonas aeruginosa* Isolated from Patients with Chronic Suppurative Otitis Media

A thesis

Submitted to the Council of College of Medicine-University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Master in science / Medical Microbiology

By

Alaa Abdullah Rashid Aziz

(B.Sc / College of Science / AL- Mustansiriya University 2007)

Supervised by

Professor **Professor**

Dr . Jawad K. AL-Khafaji **Dr. Safaa S. AL-Ghazali**

2022 A.D **1444 A.H**

بسم الله الرحمن الرحيم

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Professor

Dr.Jawad .K.AL-Khafaji

University of Babylon

College of Medicine

/ / 2022

Professor

Dr. Safaa . S.AL-Ghazali

University of Babylon

College of Medicine

/ / 2022

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

Professor

Dr. Hayam K. Al-Mosudi

Head of Microbiology Department

University of Babylon

College of Medicine

/ / 2022

Decision of Examination Committee

We, the examiners' committee, certify that we have read the thesis entitled (Characterization of Metallo Beta Lactam-Producing Pseudomonas aeruginosa Isolated from Patients with Chronic Suppurative Otitis Media) and have examined the student (**Alaa Abdullah Rashid AL-Bayati**) in its contents and that, in our opinion, it is accepted as a thesis for the degree of Master of Science in Medical Microbiology with (**Excellent**) estimation .

Professor

Dr. Alaa H. AL-Charrakh

College of Medicine
University of Babylon

(Chairman)

Professor.

Dr. Safaa H. AL- Turaihy

College of Medicine

University of Babylon

(Member)

Assistant professor

Dr. Salah M. AL-Saadoon

College of Health and Medical

Technology/Baghdad

(Member)

Professor.

Dr . Jawad K. AL-Khafaji

College of Medicine

University of Babylon

(Member and Supervisor)

Professor.

Dr. Safaa S. AL-Ghazali

College of Medicine

University of Babylon

(Member and supervisor)

Professor

Dr.Mohanned Abass Nori AL-Shalah

Dean of College of Medicine

University of Babylon

Dedication

To those who have

clear souls,, diamond hearts

Who are kind and helpful,, and who sacrifice for lovers

.....To

My devoted mother soul

My endless love father

My lovely wife

My wonderful family

My sweet friends

I dedicated this humble work

□

□

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***Alaa* 2022**

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Summary:

Pseudomonas aeruginosa is an aerobic gram-negative bacterium that has appeared as one of the most problem nosocomial pathogens due to the *P. aeruginosa* strains that are widespread in Iraqi patients. One hundred unilateral specimens from patients suffering from ear infections were collected from patients of various age groups, including four hospitals in (Medical City Hospital) Baghdad from October 2021 to the end of February 2022. The collected samples were cultured on different media and tested biochemically, which included oxidase, catalase, and Indole, Methyl-Red, Voges-Proskaure, Citrate (IMVIC) tests, in order to find out the profile of *P. aeruginosa* colonizing the ear. The results of bacterial culturing revealed that out of 100 samples, 25 samples (25%) were observed to have bacterial growth (positive samples) for *P.aeruginosa*. The percentage of male patients constituted 60% of the total, and females 40%, and their ages ranged from 3–70 years. Antibiotic susceptibility tests were conducted for nine beta-lactams using the Kirby-Bauer method. The isolates showed resistance to cephalosporin generation and amoxacillin-clavulanic acid with a percentage of 100%,then imipenem, meropenem, aztreonam, and piperacillin (60%, 40%, 60%, and 68%), respectively.

The production of bacterial isolates for virulence factors was also conducted, which included protease enzymes on M9 medium, lipase on egg yolk medium, and hemolysis on blood medium, and the formation of biofilms was detected by the micro plate method, where 100% of the isolates showed their ability to form biofilms. Broad-spectrum beta-lactamase enzymes were detected by the Combined disk test and metallic beta-lactamase enzymes by the Combined EDTA disk test

EDTA method. The percentage of isolates produced Extended spectrum beta lactam(ESBL) was Zero%, while the percentage of isolates from Metallo Beta Lactam (MBL) was 96%.Finally, the DNA of bacteria that showed resistance to anti-carbapenem was extracted, and the *NDM*, *GIM*, and KPC genes were detected using polymerase chain reaction (PCR), and the size of the gene was detected by electrophoresis and using a UV source. The isolates carrying the (*NDM*, *GIM* and *KPC*)genes reached 88%, 60%, and 40%, respectively. This study showed several conclusions that isolates of *Pseudomonas aeruginosa* have multiple virulence factors including the formation of biofilms. It is resistant to multi beta lactam antibiotics. And the existence of a relationship between the production of virulence factors and multiple resistance to beta-lactams, and that the majority of Bacterial isolates produce enzymes Metallo beta-lactamases (MBLs) by phenotypic detection.

The greater the ability of bacteria to produce MBL the greater their ability to resist beta-lactam antibiotics, and the higher the proportion of isolates producing the mineral beta-lactam enzyme, which indicates a relationship between multi-resistance to beta lactam antibiotics and the production of MBL enzymes in *Pseudomonas aeruginosa* bacteria.

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List of Abbreviation

Abbreviation	Key
2ME	2-Mercaptoethanol
2MPA	2-Mercapto Propionic Acid
ABC	ATP-binding cassette
AOM	acute otitis media
AST	antibiotic susceptibility test
BaCl2.2H2O	Barium chloride
CD	combined disk
CDC	Control and Prevention
CRPA	carbapenem resistance *P.aeruginosa*
CSOM	chronic suppurative otitis media
DDT	disk diffusion test
EF2	elongation factor 2
EPS	extracellular polymeric material matrix
ET	Elution Buffer
ETA	Exotoxin A
FIM	Florence Imipenemase
GIM	Germany Imipenemase
GNB	Gram negative bacilli
HGT	Horizontal gene transfer
IRPA	imipenem-resistant *P. aeruginosa*
LPS	Lipopolysaccharide
MATE	multidrug and toxic compound extrusion
MFS	major facilitator superfamily
MGEs	mobile genetic elements
MIC	minimal inhibitory concentration
NDM	New Delhi MBL
OME	otitis media with effusion
RND	resistance-nodulation-division
SMA	sodium mercapto acetic acid
SMR	small multidrug resistance
TSI	triple sugar iron
VIM	Verona integrin MBL
WHO	World Health Organization
AMC	Amoxacillin/clavulanic acid
AmpC	Ampicillin class c
ATM	Aztreonam

BaCl2	barium chloride
CAZ	Ceftazidime
CFM	Cefepime
CRO	Ceftriaxon
CTX	Cefotaxiame
ESBL	Extended spectrum beta lactam
IMP	Imipenem
KH2PO4	Potassium dihydrogen phosphate
KPC	*Klebsiella pneumonia* carbapenemases
MBL	Metallo beta lactam
MDR	Multi –drug –resistance
MEM	Meropinem
OprD	outer membrane proteins
PAN	(phenylalanine arginyl -naphthylamide)
PBP	Penicillin binding protein
PIP	Piperacillin
TBE	Tris-Borate EDTA
TCP	Tissue Culture Plate
WGS	Women and Gender study
XDR	Extensively-drug –resistance
T3SS	Type III secretion system

Chapter One

Introduction

and Literatures Review

1.1 Introduction

Pseudomonas aeruginosa is a widespread gram-negative, aerobic, rod-shaped bacterium found in soil, water, plants, animals, and humans, where it is found as part of the regular transitory flora. It's a human opportunistic pathogen that causes serious nosocomial infections in burns, wounds, ear infections, and cystic fibrosis, and it's a major cause of morbidity and mortality (Treepong *et al*., 2018). As a metabolically adaptable bacterium, *P. aeruginosa* can colonize a variety of bodily places (Gellatly and Hancock, 2013).

It also has the ability to change its virulence phenotype depending on the infection site. *P. aeruginosa* infections are becoming increasingly difficult to treat due to the emergence of multi-drug resistant strains and a limited number of effective medications (Pathak, 2012).

It has a number of pathogenicity variables that it uses to get past the host's defenses. In adhesion is facilitated, host cell pathways are modulated or disrupted, and the extracellular matrix is targeted. biofilms can be formed by *P.aeruginosa* to shield it against antibiotics and the host immune system (Alhazmi, 2015).

The majority of *pseudomonas* infections are invasive as well as poisonous. *Pseudomonas aeruginosa* capacity to penetrate tissues is dependent on the synthesis of extracellular enzymes and toxins that break down physical barriers and cause harm to host cells, as well as resistance to phagocytosis and host immune systems. In the invasive stage, *P. aeruginosa* produces two extracellular proteases, elastase and alkaline protease, as well as three soluble proteins, cytotoxin and two hemolysis (Todar, 2008.).

P. aeruginosa represents a phenomenon of resistance to antibiotics, as the difficulty of treatment of injuries caused by drug resistance in this type, as it has resistance Natural ones, including changing the permeability of the outer wall (Breidenstein *et al*.,2011) and having several groups of aeruginosa efflux systems, have several responsible enzymes. Production of manyenzyme, especially beta-lactams such as beta-lactamase enzymes, Extended and broad-spectrum metallo β-lactamases (MβLs) and Extended Spectrum β-Lactamases (ESβLs) (Gellatly and Hancock ,2013).

Pseudomonas aeruginosa has emerged as a significant pathogen. In most hospitals, it accounts for 10% to 20% of all infections. *Pseudomonas* infection is particularly common in patients suffering from burns, cystic fibrosis, acute leukemia, organ transplants, and intravenous drug addiction. *P. aeruginosa* is a common nosocomial contaminant, and epidemics have been linked to a variety of hospital items. Patients who are hospitalized for an extended period of time are frequently colonized by this organism and are at a higher risk of infection. Malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia are among the most serious infections. The likelihood of recovery from pseudomonas infection is proportional to the severity of the underlying disease process in the patient (Bodey *et al*., 1983).

Aim of the study

The purpose of this study was to determine the pattern of antibiotic resistance in *p. aeruginosa* by the antibiotic susceptibility method . In this study , the most effective antibiotic to treat *p. aeruginosa* infections must be identified was achieved through the following objectives :

1. Screening test for β-lactam antibiotic resistance by antibiotics susceptibility test(AST) using disk diffusion test (DDT) .

2. Detection of Extended β-lactamase enzyme (ESBL) by using Double Disc Synergy Test (DDST) .

3. Detection of Metallo β-lactamase enzyme (MBL) by using Combined Disc Synergy Test (CDST).

4. Detection virulence factors (Hemolysins , lipase , protease , Biofilm) .

5. Detection antibiotic resistance genes (*NDM,GIM,KPC*) by molecular technique using polymerase chain reaction(PCR) .

1.2 Literatures Review :

1.2.1 Characterizations of *P. aeruginosa*

Pseudomonas aeruginosa is a rod-shaped gram-negative bacterium (3–1.5) µm in length (0.8–0.5) µm in diameter (Todar, 2006) which is an aerobic obligate bacteria that can grow in the absence of oxygen and emits an odor that is grape-like due to its production of aminoacetophenone) (Al-Araji and Ali, 2017). The optimum temperatures for its growth is 37°C. It grows at 42°C, and it is positive for the oxidase and catalase tests (Adhikari *et al*. 2010).The citrate assay yields a positive result, but the Indole product, Voges-proskauer test, and Methyl red test yield negative results (Liu *et al*., 2021). Some strains obtained from cystic fibrosis patients form capsules and contain a mucous covering. The glycocalyx is responsible for the colonies' mucous appearance; this layer aids bacterium adhesion to respiratory mucous membranes and prevents antibodies from attaching to germs *Pseudomonas aeruginosa* generates two pigments that are significant in clinical and laboratory diagnosis: pyocyanin (the blue-green pigment) and Pyoverdine (the yellowish-green pigment), as well as the red pigment pyorubin or pyomelanin, which is responsible for the appearance of pus in blue wounds (Hassan *et al*.,2019).

It grows on MacConky agar medium, with pale colonies because it does not ferment lactose sugar; it also grows on hemolytic blood agar medium and triple sugar iron (TSI) agar without causing any change in the color of the medium while avoiding the production of H2S gas. The colonies turn a bluish-green color as they grow on cetrimide agar in its electoral (Ludolph *et al*., 2015) medium, showing that they create pigments.

It is an opportunistic pathogen. It is a common bacteria that can be found in a variety of settings, including plants, animals, soil, and humans. This bacteria is found in acute and chronic wound infections, respiratory infections, and on the surfaces of medical devices. *(Hassan et al.*,2019)

Pseudomonas aeruginosa was first classified by Schroeter in 1872 based on phenotypic and genetic characteristics (Ludolph *et al*., 2015), and then pseudomonads were classified on the basis of DNA sequences, r RNA and common planting characteristics *Pseudomonas aeruginosa* taxonomic position (Dimariwu, 2019).

Kingdom	Bacteria
Phylum	Proteobacterea
Class	Proteobactria
Order	Pseudomonadals
Family	Pseudomonadacea
Genus	Pseudomonas
Species	Pseudomonas. aeruginosa

1.2.2 Medical importance of *P. aeruginosa* :

Pseudomonas aeruginosa is one of the most virulent bacteria, and it has been linked to increased morbidity and mortality in patients (Sadikot *et al*., 2005).

The bacteria *Pseudomonas aeruginosa* is one of the most important and harmful organisms in human infections because of the multiple virulence factors it produces. The toxin factor genes (exoA, exoS, and oprI) are thought to be the most productive genes in most isolates. ExoA

is the most prolific gene in most isolates (Bakir *et al*., 2021). Furthermore, *Pseudomonas aeruginosa* survives in low-nutrient conditions where bacterial cells join to produce biomass that extends to biofilms due to its diversified nature and ability to utilize multiple energy sources and connect to different surfaces (Ahmadian-Fard-Fini *et al*. 2020). These membranes prevent antimicrobial chemicals from reaching bacteria, protecting them from both the host immune system and antibiotics (Mulcahy *et al*. 2011).

Pseudomonas aeruginosa is an opportunistics infection that infects only a small percentage of healthy people but colonizes immunocompromised patients often (Zhang *et al*., 2018). *Pseudomonas aeruginosa* can acquire antibiotic resistance, making it harder to eradicate. Folkesson and colleagues (2012) argue that early detection of the bacterium is critical for avoiding long-term infection by *Pseudomonas aeruginosa* (Fleiszig *et al*., 2020).

External Otitis The germ responsible for otitis external malignant external otitis, and this affects a greater percentage. Patients with diabetes, patients with AIDS, the elderly, and purulent otitis media Chronic Suppurative Otitis Media (Qi *et al*., 2022).

Infections caused by multidrug-resistant *Pseudomonas aeruginosa* cause significant mortality and morbidity worldwide (Sreeshma *et al*., 2013). *P. aeruginosa* has numerous intrinsic and acquired drug resistance mechanisms (Buchunde *et al*., 2012). The increase in antibiotic resistance among gram-negative bacteria, including *Klebsiella* species and *P. aeruginosa* especially through the production of metallo-β-lactamases (MBLs) puts the use of the carbapenem for therapeutic purposes at risk. Beta-lactamases are important components of the

antimicrobial resistance mechanism found in gram-negative bacteria, including *P. aeruginosa* (Chika *et al*., 2018).

MBLs are carbapenemases that hydrolyze carbapenems such as imipenem, meropenem, and ertapenem, rendering them ineffective for treatment. They are Ambler's Class B -lactamases that degrade a wide range of β-lactams, including penicillin, cephalosporins, and carbapenem, by hydrolyzing the amide bond of the -lactam ring (Chika *et al*., 2018).

Carbapenem antibiotics are considered last-line therapy for infections caused by multi-resistant gram-negative bacilli. The emergence of carbapenem-resistant organisms such as *Pseudomonas aeruginosa* and *Klebsiella* species has become a significant therapeutic challenge. Carbapenem resistance can be caused by reduced outer membrane permeability, exclusion from the cell by efflux pumps, changes in the penicillin-binding protein, and the production of -lactamase (Ryoo *et al*., 2010). Aztreonam, a monobactam is usually effective against pathogenic bacteria that produce MBLs. However, bacterial organisms that express MBLs and other multidrug resistance enzymes become a significant threat and are clinically significant because these organisms are typically resistant to a wide range of antibiotics, particularly β-lactams, which are important agents used in the treatment of bacterial infections (Chika *et al*., 2016).

It is widely acknowledged that imipenem-resistant *P. aeruginosa* (IRPA) is a current and serious concern, particularly given the pathogen's limited therapeutic options. Given the high likelihood of these carbapenemases spreading among nosocomial isolates, MBL enzymes may play an important role in IRPA (Franco *et al*., 2010). MBL production genes may be chromosomal or plasmid-mediated, and they

pose a threat of horizontal gene transfer to other Gram-negative bacteria in their environment (Zaker *et al*., 2018).

Despite the presence of double disk synergy (DDS) and combined disk (CD) techniques and their widespread use as phenotypic confirmatory tests, there is still a need for more diverse inhibitor combinations. Furthermore, EDTA-based techniques demonstrated high sensitivity but low specificity for detecting carbapenemases. This low specificity of EDTA-based tests is concerning because real-time polymerase chain reaction MBL production genes may be chromosomal or plasmid-mediated, posing a risk of horizontal gene transfer to other Gram-negative bacteria in their environment.

1.2.3 Otitis media infection by *P. aeruginosa* :

Ear infections are a common clinical problem worldwide and the leading cause of hearing loss in developing countries (Monasta *et al*. 2012). The most common types of ear infections are otitis media, otitis external and inner ear infections (Cripps and Kyd, 2003). Otitis external is an inflammation of the external auditory canal whereas Otitis media is a middle ear and mastoid process inflammation. Otitis media, Children are susceptible to acute otitis media (AOM), otitis media with effusion (OME), and chronic suppurative otitis media (CSOM) (Afolabi *et al*., 2012). Ear infections are related, but their symptoms, associated complications, and treatment differ. Hearing loss affects 30 out of every 10,000 people worldwide, and 21,000 people die each year as a result of an otitis media complication(Monasta *et al*.,2012).

The presence of fluid in the middle ear, as well as fever, acute ear pain, and general illness, characterizes a bacterial cause of acute otitis

media (AOM). It is one of the most common reasons for children to take antibiotics . Supportive complications of AOM include acute mastoiditis, brain abscesses and meningitis (Seid *et al*., 2013).

Chronic suppurative otitis media (CSOM) is a two-week-long inflammation of mastoid cavity and middle ear with ear discharge perforated and tympanic membrane (Smith *et al*., 2006), It is distinguished by the presence of an effusion and an intact tympanic membrane in the absence of signs and symptoms of acute inflammation. CSOM in children can result in hearing loss, brain abscesses, or meningitis (Muluye *et al*., 2013).

P. aeruginosa is a common pathogen in hospitalized patients. Otitis is a broad term for inflammation or an opportunistic infection in the human ear that is acquired in a hospital. It is separated into otitis external, which affects the ear canals and outer ear, and otitis media, which affects people who are already sick and immune-suppressed (Muluye *et al*., 2013).

Otitis media is a condition that affects the middle ear and is caused by *P. aeruginosa* infections. The inner ear is affected by otitis media. (Tesfa *et al*., 2020).Acute otitis media is inflammation or an infection of the middle ear that affects people with burns, wounds, cystic fibrosis, leukemia, organ transplants, and intravenous drug users. (Taura, 2021) Infections that cause sore throats, colds, or other respiratory infections travel to the middle ear, causing inflammation. These could be the source of an infection (Lakhdhir *et al*., 2020). Signs and symptoms appear quickly, like the presence of an asymptomatic middle-ear effusion that characterizes otitis media, albeit it can also be coupled with a "blocked ear" sensation. (Cargo-Froom *et al*., 2019).When the ear drum is punctured, the condition is known as chronic suppurative otitis media.

Otitis external is a condition in which the external auditory meatus becomes inflamed over time. Bacteria and fungi can cause it, including *P. aeruginosa, S. aureus, Candida albicans,* and *Aspergillus* species (Bocquet *et al*., 2018) .Otitis external is common in patients were swimming in water pool therefore it called (swimmer's ear) is the most frequent infection, with *P. aeruginosa* accounting for 35–70 percent of cases. Without the use of antibiotics, this can be successfully treated using aural treatment. On the other hand, malignant otitis media and otitis external can arise and, in severe situations, can be fatal (Bocquet *et al*., 2018).

1.2.4 Virulence factors of *Pseudomonas aeruginosa* :

Pseudomonas aeruginosa has a variety of virulence factors that can disrupt host defenses, causing direct tissue injury or boosting the bacterium's competitiveness (Zouhir *et al*., 2022).

Virulence factors cause tissue harm through toxicity and invasiveness, as well as allow germs to establish themselves. *aeruginosa* produces a wide range of extracellular and cell-associated virulence factors. The released virulence factors keep the chronic wound in an inflammatory state for a long time. Exotoxins are released that attach to specific locations on the ribosomes of the host cell, causing protein synthesis to be blocked and cell death (Persson, 2010).

Virulence factors of *P. aeruginosa*, such as elastase production, hem agglutinin, phenazine, pyocyanin, rhamnolipid, phospholipase, exotoxin A, exoenzyme S, hemolysins, rhamnolipids, and siderophore, have different cellular effects. For example, pyocyanin suppresses cellular respiration, elastase destroys blood vessels and degrades the

extracellular matrix of epithelium cells, and rhamnolipids damage cells and promote *P. aeruginosa* invasion (Chadha *et al* .,2021) .

1.2.4.1 Virulence factors associated with cell structure :

1.2.4.1.1. Flagella:

The first step in *P. aeruginosa* infection is pili, flagella, and alginate to bind to the epithelial surface (Cotar *et al*., 2010). Flagella are involved not only in motility but also in the activation of the host inflammatory responses via Toll-like receptors 5 and in the attachment of bacteria to host cell. The flagellin protein, which may be categorized into A and B serotypes and serves as a target antigen for vaccinations, is a key protein constituent of the flagella filament (Campodónico *et al*., 2010) .

1.2.4.1.2 Pili :

Like flagella ,pili play a vital part in the interactions between bacteria and their hosts, including attachment Fimbriae, also known as pili, are surface appendages that are shorter and thinner than flagella and are made up of structural protein subunits called pillins. Ordinary pili are responsible for bacterial cells adhering to each other during conjugation, while sex pili are responsible for bacterial cells adhering to each other during adhesion to host cells (Abiri *et al*., 2021).

1.2.4.1.3 Lipopolysaccharide (LPS) :

LPS plays a significant role in interactions with the bacterium's environment. Both the hydrophobic and polar nature of LPS contribute to a drastic decrease in Pathogens, Gram-Negative bacteria (GNB) has increased membrane permeability; the lipid membrane impedes the

passage of polar solutes, whereas The polar LPS groups repel lipophilic compounds.

LPS is conceptualized as consisting of three distinct domains: lipid A, core oligosaccharide, and O antigen. These regions have both distinct and overlapping functions in bacterial physiology. This review focuses on the role of lipid A, core, and O antigens in the sensing of LPS by host defense systems, targeting by antimicrobials, and the pathogenesis of *P. aeruginosa* **.** Lipopolysaccharide (LPS) is the main component of gram-negative bacteria's outer membrane. The LPS present in their outer cell membrane of Gram Negative bacteria that act as endotoxin (Huszczynski *et al* .,2019).

1.2.4.2 Extracellular virulence factors :

1.2.4.2.1 Hemolysins :

Hemolysins is one of the virulence factors produced by gram-positive and gram-negative bacteria, including *Pseudomonas aeruginosa*, Hemolysins plays a major role in achieving pathogenesis through its analysis of the red blood cells of the host. Therefore, it provides new growth for bacteria as well as leads to skin necrosis. In general, most isolates of *Pseudomonas aeruginosa* are beta-hemolysis-producing (Hinkel and Wargo, 2020).

1.2.4.2.2 Biofilm Formation :

Biofilms are highly structured microbial communities adhering to surfaces and encased in an extracellular polymeric material matrix (EPS). Polysaccharides, proteins, and extracellular DNA are commonly found in EPS (Mandakhalikar, 2019;Vergalito *et al*., 2020).The presence of EPS

permits biofilm to have open water channels for nutrient and waste product movement into and out of biofilm (Hentzer *et al*., 2001).

Pseudomonas aeruginosa, which causes lung infection in cystic fibrosis patients, is a well-studied model organism for biofilm research. The following four steps are involved in the formation of *P. aeruginosa* biofilms: 1. reversible cell adhesion to the surface ; 2. irreversible attachment ; 3. EPS matrix synthesis; and 4. early development of biofilms dispersion (Gonzalez,2021). SEM revealed that *P. aeruginosa* formed bacterial biofilm in vivo on the middle ear mucosal surface, seen only in the infected ear. Interestingly *p.aeruginosa* forms biofilms in the middle ear in CSOM in primates. Such a model lays a foundation for much needed study into the role of biofilms in the pathophysiology of CSOM. Should CSOM be caused by biofilms, the development of novel strategies for treatment and prevention may be possible. (Dohar *et al*., 2005).

1.2.4.2.3 Pigments :

The *P.aeruginosa* produces several pigments like Pyocyanin refer to "blue pus," which is a hallmark of *P. aeruginosa* supportive infection. Pyocyanin is a pigment metabolite of *P. aeruginosa* that has been found to have a variety of pathogenic effects, including raising IL-depressing host-response levels (Jha *et al*., 2022).Pyoverdine when it was shown that pyoverdine modulates the production of additional *P. aeruginosa* virulence factors (exotoxin A, and an end protease) as well as its own secretion, one explanation for this role emerged .Red pigments *P.aeruginosa* are produced by some strains (pyorubin). Pyomelanin, a black pigment, is produced by a small number of *P.aeruginosa* (Lamont *et al*., 2002).

1.2.4.2.4 Enzymes :

Protease IV is another important virulence factor released by *P. aeruginosa;* four forms have been identified: Las A elastase, Las B elastase , alkaline proteas, and protease IV. Theses proteases are linked to virulence because they increase *P. aeruginosa* ability to penetrate tissues while also interfering with host defense mechanisms. Because elastin is found in a variety of organs (lung, vascular, and ocular tissue), the electrolytic activity of these enzymes is critical in pathogenesis (Lamont *et al*., 2002). Protease IV is hypothesized to aid in bacterial adherence by destroying host proteins such as fibrinogen, elastin, and immune system components. phospholipase C is a type II secreted by *P. aeruginosa* that targets eukaryotic membrane phospholipids and has been linked to *P. aeruginosa* pathogenesis in inflammation. Surfactant inactivation may contribute to hemolytic phospholipase C's pathogenicity Furthermore phospholipase has the ability to reduce the oxidative burst response. of neutrophils in the host.

This enzyme is one of the bacterium's virulence factors, and it works to kill tissues by analyzing protein molecules in muscle tissue (Zouhir *et al*., 2022).

Proteins released outside of cells are essential virulence factors in the invasion stage because they contribute to enhanced bacterial pathogenicity and degrade proteins or host peptides, either by decomposition of the enzymes or by tissue injury (Chandra *et al.*, 2022).

1.2.4.3 Toxins :

1.2.4.3.1 Exotoxin A (ETA) :

A single polypeptide chain with a MW of 66.583 Da that is one of the pathogen's most lethal extracellular enzymes similar to diphtheria toxins. It catalyzes the ADP-ribosylation of elongation factor 2 (EF2), causing protein synthesis suppression and cell death (Yates *et al*., 2005).

Local tissue damage, bacterial invasion, and immunological suppression are all caused by ETA, which is the most potent toxic factor. ETA catalyzes ADP ribosylation and EF2 inactivation, an important component of protein synthesis machinery, causing protein production to be inhibited and cell death to occur (Gulyuk *et al*., 2019)**.**

1.2.4. 3.2 Exoenzyme S :

Pseudomonas aeruginosa via the TTSS produces many recognized toxins injected into host cells by *P. aeruginosa*, which are expressed differently in different strains and isolates(Engel and Balachandran, 2009) *Pseudomonas aeruginosa* produces the virulence factor Exo S, which has been shown to have a role in pathogenesis (Pastar *et al*., 2013).

Exo S causes ADP-ribosylation of numerous proteins in the cell and triggers T-cell apoptosis, which is a potent immunological stimulation that activates a substantial proportion of T cells but causes lymphocyte proliferation to be delayed and diminished (Sadikot *et al*., 2005).

Many Type III secretion systems (T3SS) are important for the virulence of many Gram-negative bacterial pathogens because they translocate effector proteins into eukaryotic host cells. Although the

mechanisms of secretion and translocation are highly conserved across species, each pathogen translocate a distinct t set of effectors that disrupt normal host cell physiology to promote pathogenesis. The diversity of mechanisms used to regulate type III secretion system (T3SS) gene expression reflects the uniqueness of each pathogen. Pseudomonas aeruginosa modulates T3SS expression via a complex network of signaling pathways in response to extracellular and intracellular cues. While some pathways are solely dedicated to regulating the T3SS, others co-ordinately regulate T3SS expression with multiple virulence functions on a global scale. T3SS coupling is one of the emerging regulatory themes .

Themes include type T3SS transcriptional coupling with type III secretory activity, global regulatory control via cAMP biosynthesis modulation, stress repression, involvement of multiple two-component regulatory systems, and an inverse relationship between T3SS expression and multicellular behavior. The T3SS activation factors are likely to contribute to the organism's environmental survival as well as the pathogenesis of acute *P. aeruginosa* infections. Active repression of the T3SS, on the other hand, may contribute to the persistence of chronic infections (Timothy *et al*.,2006).

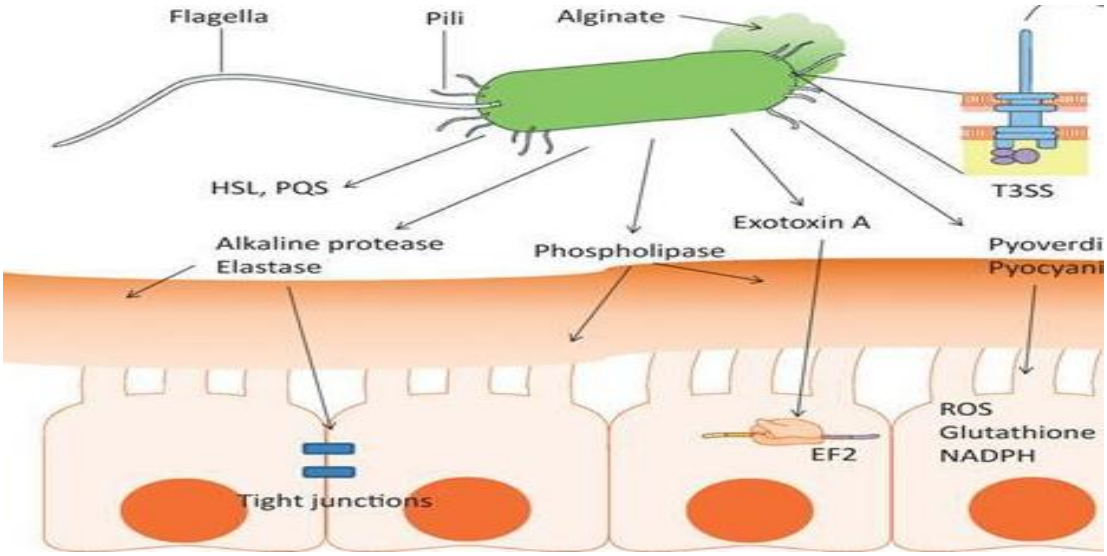


Figure (1-1): **Virulence factors produced by *Pseudomonas aeruginosa***(Abdalhadi *et al*., 2021) **.**

1.2.4.3.3 Other Toxine :

ExoT is a bifunctional protein with an N-terminal GTPase-activating domain that has GAP activity toward Rho, Rac, and Cdc42 and a C-terminal ADP-ribosyltransferase domain. ExoS and exoT are bifunctional proteins with an amino terminal GAP. They are highly homologous. ADP-ribosylation domain and a carboxy-terminal ADP-ribosylation domain. ExoU is a potent cytotoxin with unknown host cell targets and a mechanism of action (Azimi *et al*., 2016).

1.2.5 Epidemiology of *Pseudomonas aeruginosa* Infection :

Pseudomonas aeruginosa is a drug-resistant ubiquitin-opportunistic bacterium. It infects humans, animals, and plants, causing a variety of illnesses. It rarely causes disease in healthy people, but it is one of the most common bacterial species that causes infections in hospitalized patients, especially cancer patients, burns patients, patients with immunodeficiency, and organ transplant recipients. These bacteria were able to live in the community and in healthy environments because of their ability to survive under a limited range of nutrients and their

tolerance to a variety of physical circumstances(Greenwood and Connolly, 2007).

These bacteria are normal flora is at almost 10% in the colon (Hassan *et al*., 2019) and colonizes the skin by 0-2%, the nasal mucosa by 0-3.3 %, and the throat by 0-6.6%, and 24-26% in stool samples (Bachta *et al*., 2020).And it caused 8% of the infections acquired in hospitals and it may be the second cause of pneumonia, and the third most common in urinary tract infections (Gronthoud, 2020).

The increasing prevalence of nosocomial infections caused by MDR and XDR *Pseudomonas aeruginosa* strains is associated with significantly increased morbidity and mortality because it jeopardizes the selection of effective therapies. This growing threat is caused by *P. aeruginosa* extraordinary ability to develop resistance to nearly all available antibiotics through the selection of mutations in chromosomal genes, as well as the increasing prevalence of transferable resistance determinants, particularly those encoding carbapenemases or ESBLs, which are frequently co-transferred with aminoglycoside-modifying enzyme determinants. The spread of MDR/XDR global strains, or high-risk clones, in multiple hospitals around the world adds to the concern. Furthermore, beyond traditional molecular epidemiology and the evaluation of phenotypically targeted resistance mechanisms(del Barrio-Tofiño *et al*., 2019) **.**

1.2.6 Antibiotic treatment of *Pseudomonas aeruginosa* :

Experimental antibiotic therapy for suspected cases of *Pseudomonas aeruginosa* includes mono therapy and combination therapy; this therapy lowers mortality in patients with severe *P. aeruginosa* infections.(El Solh

and Alhajhusain, 2009) However therapy of *P. aeruginosa* This infection Because of this bacterium's ability to resist many antibiotics, it has become a significant challenge (Lister *et al*.,2009) .

The World Health Organization (WHO) has identified three bacterial species and *P.aeruginosa* is one of them that are carbapenem-resistant. The development of new antibiotics to treat infections is critical (Tacconelli *et al*., 2018). Furthermore, overuse of antibiotics during treatment hastens the development of multidrug-resistant *P. aeruginosa* strains, rendering empirical antibiotic therapy ineffective against this microorganism (Hirsch and Tam, 2010) .

1.2.7 Antibiotic Resistance of *Pseudomonas aeruginosa :*

Pseudomonas aeruginosa displays resistance to a variety of antibiotics, including quinolones, β-lactams (Hancock and Speert, 2000). The increasingly common infection caused by multidrug resistance (MDR) and widely used antibiotics drug-resistant (XDR) strain with few treatment options related to high mortality and morbidity (Hasan *et al*., 2019) . *P. aeruginosa* resistance can be classified can as intrinsic, acquired and adaptive *P. aeruginosa* intrinsic resistance includes low outer membrane permeability and resistance is conferred expression efflux pumps, low outer membrane permeability, and the productions of antibiotic-inactivating enzyme. Acquiring resistance can occur because of mutational change or acquisition of resistance genes by horizontal transfer by mobile genetic elements (MGEs) such as integrons, plasmids, or transposons, which are examples of MGEs (Pang *et al*., 2019). In particular, the most disturbing acquired resistance of *P.aeruginosa* is the production of carbapenemases, which confers resistance to most commercially available β-lactams. The class B-carbapenemases, like

Verona integron-encoded metallo(Vim) and Imipenem metallo-β-lactamase (IMP) are the most frequent (Patel and Bonomo, 2011).

The genes encoding VIM and IMP are located on integrons that also carry antibiotic resistance genes that favor Class A carbapenemases. Like *Klebsiella pneumonia* carbapenemases (KPC) (Molina-Mora *et al*., 2021).

Planktonic bacteria exhibit antibiotic sensitivity and low minimal inhibitory concentricity (MIC) values when the biofilm is dispersed. The term "tolerance" distinguishes this type of biofilm-related antibiotic treatment survival from "resistant," which is defined by higher MICs and a resistance phenotype of bacteria dispersed from biofilm. Resistance is caused by acquired mutations and most commonly involves antibiotic-modulated enzymes, efflux pumps, or mutations that remove the antibiotic's molecular target and allow bacteria to survive antibiotic therapy even when not embedded in a biofilm. Antibiotic-tolerant cells in biofilms, on the other hand, can only survive the high antibiotic concentration if they are embedded in the biofilm. Tolerance and resistance are both involved in biofilm resistance to antibiotic treatment(Orazi and O'Toole, 2019).

Because of the low permeability of its outer membrane, the constitutive development of different efflux pumps, and the generation of antibiotic-inactivating enzymes, *P. aeruginosa* is innately resistant to several antibiotics (e.g cephalosporinases). Furthermore, it has a remarkable ability to create or acquire new antibiotic resistance pathways (Mesaros *et al*., 2007) .

For patients whose infections are resistant to conventional antibiotics, the development of new antibiotics or alternative therapeutic strategies

for the treatment of *P. aeruginosa* infections is urgently needed. In recent years, new antibiotics with novel modes of action have been investigated, as have new routes of administration and resistance to modification by bacterial enzymes. When compared to conventional antibiotics, some of these newer antibiotics exhibit excellent in vitro antibacterial activity against *P. aeruginosa* as well as lower minimum inhibitory concentration (Cigana *et al*., 2016).

1.2.7.1 Intrinsic antibiotic resistance :

The intrinsic antibiotic resistance of a bacterial species refers to its innate ability to reduce the efficacy of a specific antibiotic through inherent structural or functional characteristics (Blair *et al*., 2015). *Pseudomonas aeruginosa* has been shown to have a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell, and the production of antibiotic-inactivating enzymes such as –lactamases (Breidenstein *et al*., 2011) .

1.2.7.1.1 Outer membrane permeability :

To reach intracellular targets, most antibiotics used to treat *P. aeruginosa* infections must be able to penetrate the cell membrane (Lambert, 2002) Antibiotics in the aminoglycoside class, for example, such as tobramycin, gentamicin, and amikacin, inhibit bacterial protein synthesis by binding to ribosomal 30S subunits . Quinolone antibiotics like levofloxacin and ciprofloxacin disrupt DNA replication by inhibiting DNA gyrase and topoisomeras IV (Aldred *et al*., 2014).

A beta- lactam ring is found in the molecular structures of beta-lactam antibiotics such as penicillin, carbapenem, cephalosporin and

monobactams. This antibiotic class inhibits bacterial cell wall biosynthesis by binding to penicillin-binding proteins, which are enzymes involved in peptidoglycan synthesis (Poole, 2004). Polymyxins are a class of polypeptide antibiotics that bind to the lipopolysaccharides (LPS) on the surface of bacteria. on Gram-negative bacteria's outer membrane, increasing cell membrane permeability and antibiotic uptake The two polymyxins used in clinical practice are polymyxins B and polymyxins E, also known as colistin, and they kill bacteria by inducing a hydroxyl radical-mediated cell death pathway. (Zavascki *et al*., 2007).

β-lactams and quinolones enter the bacterial cell via porin channels, whereas aminoglycosides and polymyxins promote their own uptake by interacting with bacterial LPS on the outer membrane of Gram-negative bacteria (Hancock and Speert., 2000).

1.2.7.1.2 Efflux systems

Bacterial efflux pumps are classified into five families based on their function in expelling toxic compounds from the cell: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family. Additionally, Efflux pump inhibitors have emerged as a potential therapeutic strategy for *P. aeruginosa* infections. PAN (phenylalanine arginyl -naphthylamide) is a well-studied efflux pump inhibitor that not only reduces antibiotic efflux via competitive inhibition of efflux pumps but also increases bacterial outer membrane permeability (Lamers *et al*., 2013).

This compound has been shown to reduce *P.aeruginosa* virulence, quorum sensing, and increase antibiotic susceptibility.

1.2.7.1.3 Antibiotic-inactivating enzymes :

The production of antibiotic-inactivating enzymes, which break down or modify antibiotics, is one of the major mechanisms of intrinsic resistance in bacteria. Many antibiotics contain chemical bonds, such as amides and esters, that are hydrolyzable by *P. aeruginosa* enzymes such as beta-lactamases and aminoglycoside-modifying enzymes such as nucleotidyltransferases (Wolter and Lister, 2013) .

1.2.7.2 Adaptive antibiotic resistance :

Adaptive resistance increases a bacterium's ability to withstand antibiotic attack as a result of transient changes in gene and/or protein expression in response to an environmental stimulus, and it is reversible when the stimulus is removed.The best-characterized adaptive resistance mechanisms in *P. aeruginosa* are biofilm formation and the generation of persisted cells, which result in persistent infection and a poor prognosis in cystic fibrosis (CF) patients .(Taylor *et al*.,2014)

1.2.7.3 Acquired antibiotic resistance :

Bacteria can acquire antibiotic resistance through mutational changes or through horizontal gene transfer by plasmid ,transposon or another genetic elements (Munita and Arias, 2016). In addition to the high level of intrinsic antibiotic resistance in *P. aeruginosa,* acquired resistance greatly contributes to the development of multidrug-resistant strains, making eradication more difficult and leading to an increase in cases of persistent infections (Henrichfreise *et al*., 2007) **.**

1.2.7.4 Novel therapeutic strategies for *P. aeruginosa* treatment :

Overuse and misuse of antibiotics is a growing public health concern, as it can result in unnecessary side effects and the development of drug-resistant bacterial strains (Ventola, 2015). Furthermore, the development of new antibiotics is both limited and time-consuming. As a result, the development of novel therapeutic approaches to treat *P. aeruginosa* infections is highly desirable and has gotten a lot of attention in the last decade. To combat *P. aeruginosa* infections, these novel therapeutic strategies include the inhibition of quorum sensing and bacterial lectins, as well as the use of iron chelation, phage therapy, vaccine strategy, and the use of nanoparticles, antimicrobial peptides, and electrochemical scaffolds (Pang *et al*., 2019)**.**

1.2.8 Classification of beta-lactam antibiotics :

Beta-lactams are the drugs of choice for treating bacterial infections worldwide. These drugs possess a beta-lactam centric circle and inhibit cell wall structure Various extended spectrum beta-lactamases (ESBLs) in gram negative bacteria have been discovered and classified into four groups, including penicillinases, metallo beta-lactamases, cephalosporinases, and oxacillinase (Khan, 2020).

AmpC is an enzyme in class C. All *P. aeruginosa* strains have an attenuated transcript of the AmpC gene on the chromosome, which could be induced by first generation cephalosporin exposure and produce AmpC enzymes. Furthermore, *Pseudomonas aeruginosa* produces a wide range of beta-lactamases that are carried on plasmids and other mobile genetic elements. (IMP)-1 is a beta-lactamase from Class B that has extended spectrum metallo beta-lactamases (MBL) that hydrolyze all

beta-lactams except aztreonam and are resistant to sulbactam and clavolanic acid. These enzymes are known as carbapenemases because they hydrolyze IMP and meropenem.

IMP was first identified in *P. aeruginosa* as a plasmid-borne gene, and it was later discovered that it could be transported on integrons. As a result, this could explain why imipenem is spreading in some enterobacteraceae genera. The other group of beta-lactamases are oxacillinase. They are susceptible to hydrolyzed ceftazidimem, aztreonam, and clavolanic acid (except for OXA-48). Except for OXA-18, plasmids carry oxacillinase However, due to genetic circulation and gene exchange, oxacillinase and its variants, such as OXA-58, OXA-51, and OXA-23, are more common in entrobacteraceae. *P. aeruginosa* has high genetic transfer (HGT) mechanisms. Mineral beta-lactamase enzymes (MBL) are an important clinical problem that raises concern in all societies in the world because of their analysis and inhibition of almost all antibiotics containing the beta-lactam ring.Possess enzymes Mineral activity in all metallo β lactamase a broad spectrum includes all penicillin's, penicillin's, cephalosporin's, and anti-Rbapinm carbapenem except anti-Monobactams .Its lytic is not inhibited by metallo β lactamase inhibitors such as clavolanic acid, tazobactam, or sulbactam, but it inhibits mediated material: EDTA, Fecl3, and Cucl3, and sodium mercaptoacetic acid (SMA), 2-Mercaptoethanol (2ME), 2-Mercapto Propionic Acid (2MPA), and the water analysis of these enzymes is based on the zinc ions at the bottom.Mineral beta-lactamases belong to the class of metalloenzymes beta-ClassB according to Ambler's Molecular Classification in 1980 and to the third group (3group) according to the functional classification proposed by (Bradford *et al*., 2022).

Pseudomonas aeruginosa, which possesses various types of these

enzymes, including Verona integrin MBL (VIM) and Imipenemase Germany (Imipenemase, IPM) Germany, GIM), and New Delhi MBL (NDM), Florence Imipenemase (FIM), and all of the genes for these enzymes are located on integrons, transposons, or on the chromosome (Ghasemian *et al*., 2018).

1.2.9 *P. aeruginosa* resistance to Carbapenemases :

The most commonly used anticarbapenems are imipenem and meropenem, which belong to one of the beta-lactam groups .They are effective agents for treating infections caused by multi-resistant gram-negative bacilli, especially *P. aeruginosa* (Sader *et al*., 2005), because of their resistance to degradation by beta-lactamase enzymes. This occupies an important place in the fight against infections resulting from these organisms and is the first line of defense for the treatment of infections caused by *P. aeruginosa* (Ding *et al*., 2018).

Excessive use of antibiotics led to the development of the potential of bacteria in their resistance to antibiotics through mutations, and then strains of *Pseudomonas aeruginosa* appeared resistant to carbapenem, so the emergence of such resistant strains significantly limited the effectiveness of treatment with carbapenem.

The resistance of *Pseudomonas aeruginosa* to carbapenem, especially the anti-imipenem, is mainly due to a group of mechanisms, including a decrease in the expression of outer membrane proteins (OprD) (Mac Aogáin *et al*., 2012), or the production of carbapenemases enzymes, especially metallo-β-lactamases and lactamases.), and over-expression of efflux pumps (Bukavaz *et al*.,2018).

The mechanism of action of anticarbapenems is by inhibiting the enzymes that assemble the peptidoglycan layer constituting the bacterial cell wall penicillin-binding protein(PBP), which is located on the outer surface of the cytoplasmic membrane, as these proteins have a specialized target site for beta-lactams. Because they are small and hydrophilic, these antigens can pass effectively through the outer membrane of bacteria, and they enter the cell through pore channels. The main porins for entry of anticarbapenems into *Pseudomonas aeruginosa* is the outer membrane protein OprD (Ocampo-Sosa *et al*., 2012).

1.2.10 Genotyping diversity of *P.aeruginosa* :

1.2.10.1 Types of MBL mineral beta-lactam enzymes :

1.2.10.1.1 Imipenemase (IMP) enzyme:

These enzymes are divided into many sub-groups and the proportion of amino acids is different from 99–95 (Nordmann and Poirel, 2002). These totals have a very seductive activity, and among the 51-known IMP enzymes, 32 of which exist in the pseudo-tangerine identified worldwide.

The majority of carbapenem-hydrolysis enzymes found in *P. aeruginosa*, such as carbapenmase, belong to the Ambler class B-beta lactamase example: IMP enzyme (Hong *et al*., 2015) .

Peptide cross-linking and other peptidase reactions can be inhibited by imipenmase, which acts as a mechanism-based inhibitor of the peptidase domain of PBPs. The ability of carbapenems to bind to multiple PBPs is critical to their efficacy (Papp-Wallace, 2011) .

1.2.10.1.2 Germany's Imipenemase (GIM) enzyme :

The blaGIM-1 gene was discovered *Pseudomonas aeruginosa* clinical isolates from Düsseldorf, Germany, in 2002 on a 24-kb nontransferable plasmid. blaGIM-1 is a gene cassette that is embedded in a class 1 integron and is frequently accompanied by various aminoglycoside resistance genes. blaGIM-1 has now been found in Entrobacteraceae. and It has also been found in various clones of multidrug-resistant *P. aeruginosa* isolates from Germany. GIM-1 belongs to MBL subclass B1 and is most closely related to the IMP enzymes (39 to 43 percent sequence identity), while also being related to the VIM (28 to 31 percent

identity), NDM (28 to 29 percent identity), and SPM-1 (28 percent identity) (Abdeltwab *et al*., 2019) **.**

Peptide cross-linking and other peptidase reactions can be inhibited by imipenmase, which acts as a mechanism-based inhibitor of the peptidase domain of PBPs. The ability of carbapenems to bind to multiple PBPs is critical to their efficacy (Papp-Wallace, 2011).

1.2.10.1.3 New Delhi MBL(NDM) Enzyme :

Multidrug resistance, particularly carbapenem-resistant Gram-negative bacteria, is rapidly spreading around the world. The New Delhi Metallo—lactamase (NDM) is one of the most potent carbapenemases in clinical use . NDM efficiently hydrolyzes-lactams and is the carbapenem of last resort. As a result, the options for treating NDM producer bacteria are limited to a few antibiotics (Ismail and Mahmoud, 2018) .

The NDM-1 gene allows the bacterium to produce an enzyme that prevents these antibiotics from working.

NDM-1 and another similar protein known as KPC have been identified as emerging infectious disease issues by the Centers for Disease Control and Prevention (CDC).

NDM-1 is the protein product of a gene that some bacteria produce. A bacterial strain containing NDM-1 will be resistant to even the most potent antibiotics.Few antibiotics currently available can combat bacteria that carry the NDM-1 gene, making it potentially dangerous.

NDM-1 is abbreviation for New Delhi metallo-ß-lactamase-1. A medical team discovered the gene in a Swedish patient of Indian origin who visited India in 2008. It is unknown what caused NDM-1 to appear in India. This superbug is common in India, and researchers and medical experts discovered it in more than 70 countries by 2015.

Although the NDM-1 protein product does not cause disease, it has the potential to alter the characteristics of bacteria.Antibiotic resistance is conferred by the gene. As a result, it can cause a variety of conditions, including urinary tract, bloodstream, or wound infections, and pneumonia.

Secondary diseases will emerge if NDM-1 crosses over into other bacteria, causing a global health crisis as they spread. According to the WHO, a pregnant woman, for example, could develop a kidney infection that spreads into her bloodstream from a strain containing NDM-1. In this case, there are no treatment options that are safe for a pregnant woman that would be available. The NDM1 gene causes bacteria to produce a carbapenemases enzyme. Carbapenemases renders many commonly used antibiotics inactive, including carbapenem.

A bacterium with a plasmid containing the NDM-1 protein product has the potential to be resistant to many current antibiotics, as well as newer antibiotics that could become available in the near future. e NDM-1 protein product itself does not cause disease, but it has the Scientists believe that secondary NDM-1 genes will spread without any human contact with India.The NDM-1 protein was named after New Delhi, the Indian capital by those whose discovered it .

It has since occurred in drinking water and India's holy rivers, such as the Ganges.Bacteria expressing NDM-1 have been found in patients from the United States, Japan, Australia, and the United Kingdom who spent time in India, traveled through it, or have family members there (Adam ,2018) **.**

1.2.10.1.4 *Klebsiella pneumonia* carbapenemases (KPC) :

It is a molecular class A serine protease that was discovered in North Carolina in 1996.

It is encoded by the bla KPC gene and is almost always found within the Tn4401 Tn3-type transposon, which can insert into the plasmids of a wide range of gram-negative bacteria. despite the fact that it is still most common in *K. pneumonia*.

KPC has also been found in other Entrobacteraceae, such as Escherichia coli, as well as non-fermenters such as *Pseudomonas* and *Acinetobacter species.*

This is noteworthy because the latter frequently contain class B metallo—lactases (e.g., VIM, NDM) and class D-lactases, also known as oxacillinase (OXAs). The first clinical isolate of KPC-producing

Pseudomonas aeruginosa was discovered in a Colombian hospital in 2007,4 and has since spread to other countries such as the United States,5 China6, and Brazil. 7. Surprisingly, there has yet to be a report of P. aeruginosa producing KPC in Europe. As of now, reports indicate that it has arrived in the European Union (Hagemann *et al*. 2018) .

Chapter Two

Materials and Methods

2.1 Materials:

2.1.1 Laboratory Equipment and apparatus:

The laboratory equipment and apparatus that used in present study were listed in table (2-1) .

Table (2-1) Equipment and apparatus :

Equipment	Company	Country
Autoclave	P-selecta/mediclave	Spain
Centrifuge	Hettich	Germany
Compound microscope	Olympus	Japan
Distillater	American	USA
Electronic balance	Mettler AE50	USA
Eppendorf tubes	Eppendorf	Germany
Gel electrophoresis	Mupid_exu	Japan
Nano Drop	Thermo	Japan
Laminar hood	Jeno tech	Korea
Incubator	Sanyo	USA
Micro centrifuge	Eppedorf	Germany
Micropipette various size	Brand	USA
Microwave oven	Sigma	USA
Oven	Memmert	Germany
Polymerase Chain Reaction system	Applied biosystem	Singapore
Refrigerator	Concord	France
Spectrophotometer	Eppendorff	Germany
Vitek compact system	Biomerieurx	France
Vortex	Clay Adams	Germany
Water bath	Precisterm	Germany
Xmax ELISA microplate reader	Bio-rad	Germany
Transilluminator.(U.V light source)	VilberLourmat	France
Tissue culture plate (TCP)	Cosmo Biosciences	China

2.1.2 : Chemicals and Solutions

Table (2-2): The Chemicals and Solutions used in this study

Chemicals	Company	Country
Barium chloride ($BaCl_2.2H_2O$)	BHD	England
DNA ladder (100-1000 bp)	Promega	USA
EDTA	Promega	USA
Ethanol (75 %, 95%)	Fluka	Switzerland
Ethidium Bromide	Promega	USA
Free nuclease distill water	Promega	USA
Glycerol	BHD	England
GoTaq®Green Master Mix	Promega	USA
Hydrogen peroxide H_2O_2 (6%)	BHD	England
Oxidase reagent	Biomerièux	France
Potassium dihydrogen phosphate(KH_2PO_4)	BHD	England
Seder oil	BHD	England
Sodium chloride (Nacl)	BHD	England
Sodium Monohydrogen Phosphate Heptahydrate ($Na_2HPO_4.7H_2O$)	BHD	England
Sulfuric acid H_2SO_4 (98%)	BHD	England
TBE buffer (10x) (Tris-Borate EDTA)	Promega	USA
6X Loading dye	Promega	USA
Crystal violet	BDH	UK
$MgSO_4$,NH4CL,$CaCL_2$,glucose, Gelatin, KOH,α naphthol	Sigma	USA
Tetra methyl-p-phenylene-diamine dihydrochloride	Fluka	Switzerland

2.1.3 Culture Media:

Table (2-3): Culture Media used in this study:

Media	Origin
Blood agar base	Oxide (England)
Brain heart infusion broth	Oxide (England)
Cetrimide agar	Riedel-DeHaen (Germany)
MacConky agar	Oxide (England)
Muller Hinton agar	Mast diagnosis (England)
Nutrient agar	Himedia (India)
Peptone water , Simmon citrate agar	Difco(U.S.A)
M9 medium , Egg-Yolk agar medium	Himedia (India)
Agarose	Promega **(**USA**)**

2.1.4. Kits and materials used in molecular study :

2.1.4.1 PCR Oligonucleotide Primers :

Table (2-4): Primers and their Sequence used in this study:

Gene Name	Primer sequence ¯5 →¯3	Product size	Reference
NDM	**F 5 ACCGCCTGGACCGATGACCA**	264 bP	Scientific Research Co .Ltd
	R 3 GCCAAAGTTGGGCGCGGTTG		
GIM	**F 5 TCGACACACCTTGGTCTGAA**	477 bP	Scientific Research Co .Ltd
	R 3 AACTTCCAACTTTGCCATGC		
KPC	**F 5' ATGTCACTGTATCGCCGTCT**	893 bP	Scientific Research Co . Ltd
	R 3 TTTTCAGAGCCTTACTGCCC		

2.1.4.2 Specific kits used in molecular study .

Table (2-5) kits used in molecular study .

Type of kits	Company	Country
DNA extraction kit	HiGenoMB	India
Green master mix 2X Kit	Bioneer	Korea
DNA ladder 100bp	Bioneer	Korea

2.1.5 Antibiotics Disks :

Antibiotic discs that used in this study are listed in table (2-6)

Table (2-6): Antibiotics discs used in this study.

Antibiotics	Abbreviation	Disc content (µg)	Company/ country
Pipercillin	PIP	30 µg	Bioanalyse/Turkey
Amoxacillin+ clavulanic acid	AMC	20/10 µg	
Ceftriaxone	CRO	30 µg	
Cefataxiam	CTX	30 µg	
Cefepime	CFM	30 µg	
Ceftazidime	CAZ	10 µg	
Imipinem	IMP	10 µg	
Meropinem	MEM	30 µg	
Aztrenum	ATM	30 µg	

2.2 Methods

2.2.1. Preparation of Culture Media:

All media (Nutrient agar, MacConky agar, Brain heart infusion broth, Muller Hinton agar, Nutrient broth, M9agar ,Egg yolk agar), were prepared according to the manufacturing company instruction; they were brought to boil in water bath to dissolve all constituents completely and then sterilized by autoclaving at 121C° for 15 min. at 15 Pound / Inch^2, otherwise the media were incubatedat 37C° for 24 h to ensure sterility.

2.2.1.1 Blood Agar Medium:

were prepared as directed by the manufacturer, autoclaved, and cooled to 45°C before adding 5% fresh human blood. This medium was used to grow bacterial isolates and research the different types of blood hemolysis (Korgaonkar *et al*., 2013) .

2.2.1.2 Cetrimide agar medium :

It was prepared in accordance with manufacturing company instructions by dissolving 46.7 grams in 1000 ml D.W containing 10 ml glycerol,heat to boiling to dissolve the medium complete. Sterilizing by autoclaving at 121°C for 15 min at 15 Pound / Inch^2 pressure and add rehydrated contentsof 1 vial of Nalidixic acid selective supplement. the media were used as a selective media for the isolation of *P. aeruginosa*.

2.2.1.3. MacConky Agar medium:

It was made by combining 51.5 grams of base medium with 1000 milliliters of distilled water, sterilizing it in an autoclave, and pouring it into a petri dish. This medium contains crystal violet, which inhibits the growth of Gram positive bacteria while allowing Gram negative

bacteria to grow. *P.aeruginosa* does not ferment lactose on MacConky agar (Forbes *et al*., 2007) .

2.2.1.4. Nutrient Agar Medium:

This nutrient agar media was made according to the supplier's instructions by dissolving 28gm of nutrient agar powder in 1L of distilled water and sterilizing it at 121°C for 15 minutes. It was used in general experiments such as bacterial isolate cultivation and activation where necessary (MacFaddin, 2000) .

2.2.1.5. Müller-Hinton Agar:

This medium was made according to the supplier's instructions by dissolving 38gm of the medium in 1L of distilled water and sterilizing it in an autoclave at 121°C for 15 minutes. It was used in an antibiotic sensitivity test (MacFaddin, 2000) **.**

2.2.1.6. Brain Heart Infusion (BHI) Broth -Glycerol Medium :

This medium was used to keep the bacterial isolate for a long time. This medium was made by adding 5 mL of glycerol to 95 mL of BHI broth and autoclaving at 121°C for 15 minutes(Forbes *et al*., 2007). It used for maintenance of bacterial isolates.

2.2.1.7. MR-VP broth medium:

By dissolving 17 gm. in 1000 ml of distilled water, it was used to test bacteria's ability to produce and retain stable acid end products from glucose fermentation (Forbes *et al*.,2007) .

2.2.1.8 : Peptone water broth medium

This medium was made by dissolving 8 gm. of peptone in 1000 ml of distilled water, then distributing it into test tubes and

autoclaving them. To show that bacteria can breakdown the amino acid tryptophan to Indole(Forbes *et al*.,2007).

2.2.1.9 : Simmon citrate medium :

A 22.8 grams of medium in 1000 ml of distilled water was used to make this medium. It was used to see if bacteria could use simmon citrate as their sole carbon source and inorganic ammonium salt as their sole nitrogen source(Forbes *et al*., 2007).

2.2.1.10 M9 Medium :

This medium was made according to (Sambrook and Russell, 2001) by dissolving six grams of (NA2HPO4), three grams of (KH2PO4), 0.5 grams of NaCL, and one grams of NH4CL in 95 ml of distilled water with 2 percent agar and then sterilizing in an autoclave at 121 °C for 15 minutes. After cooling the mixture to 50 degrees Celsius, 2 mL of 1 M MgSO4, 0.1 mL of 1 M CaCL2, and 10 mL of 20% glucose (sterilized separately by filtration) were added to the medium, which was then supported by 1% gelatin. This medium was used to detect the production of extracellular protease.

2.2.1.11 Egg-Yolk agar medium :

It was prepared by adding 15 ml of egg yolk suspension to 85 ml of sterile nutrient agar after cooling it to 55 °C. This medium was used to detect the ability of bacteria to produce lipase enzyme (Collee *et al*. 1996).

2.2.3. Preparation of Reagents and Solutions :

2.2.3.1: Catalase Reagent:

The reagent was prepared by adding 3ml H_2O_2 in to 97 ml of DW. It is used for detected of catalase production by bacteria .

2.2.3.2: Oxidase Reagent

The test was based on a specific bacterial oxidase that stimulated electron transport between electron donors in the bacteria and a redox dye (1% Tetra methyl-P-Phenylene-diamine dihydrochloride), which was reduced to a deep purple color A strip of filter paper was soaked in freshly prepared reagent (Forbes *et al*.,2007).

2.2.3.3 Standard Opacity, McFarland Tubes

The McFarland 0.5 standard was made by mixing 0.5 ml of a 1.17 percent (w/v) solution of barium chloride ($BaCl_2$. H_2O) with 99.5 ml of a 1 percent (v/v) solution of pure sulphuric acid (H_2SO_4) to obtain a barium sulphate solution with the specific optical density to provide a turbidity comparable to that of a bacterial suspension containing $1.5 * 10^8$ CFU/ml.

2.2.3.4: Normal saline solution (pH= 7.0):

It was made by dissolving 9 gm. of NaCl in a tiny amount of distilled water and diluting it to 100 ml (9%w/v) .It was also adjusted to a pH of 7.2 and sterilized in an autoclave at 121 °C for 15 minutes, then maintained at 4 °C (MacFaddin, 2000).

2.2.3.5: Kovacs reagent

P-Dimethyl-aminobenzaldehyde was dissolved in isoamyl alcohol with heating in a water bath at 50^oC and acid was added slowly. the reagent

was prepared in small quantities and stored in the refrigerator, this reagent was used for Indole test(Hashim , 2015) .

2.3 Study population :

In present study ,the patients were admitted in Medical City Hospital , all patients were suffering from ear infection.

2.4 Ethical approval:

The appropriate ethics approval was received from the ethical committees at medical city hospital Furthermore, the family and patients must agree to collection and testing**.** We decided to carry out this task .

2.5 Collection of clinical specimens :

A total of one hundred samples (ear swabs) were collected from patients suffering from ear infections in this study. The period of collection extended from October 2021 to January 2022.

2.5.1 Cultivation of specimens

Using the standard closed loop approach, all samples were cultivated on cetrimide agar , MacConky agar, blood agar and nutrient agar medium. The medium was incubated for 24 hours at 37°C.

2.5.2 : Identification of *Pseudomonas aeruginosa* :

2.5.2.1 Cultural characteristics :

The characteristics of morphological colonies were documented in the media that were employed. The pure colonies were used to distinguish *P. aeruginosa* from other bacteria using biochemical assays. Excellent properties of its colonies are pigmented with grape odor.

2.5.2.2 Microscopic Examination :

Gram's stain was used to evaluate the isolated bacteria for examining distinguish gram-negative rods, detecting the form and arrangement of bacterial cells, depending on the microscopically examination*(Carroll et al*., 2016) .

2.5.3 Biochemical Tests :

P. aeruginosa was identified using the following biochemical tests.

2.5.3.1 Catalase Test :

It's an enzyme that encourages hydrogen peroxide to release oxygen. The selected bacterial colonies were marked on brain heart infusion agar and incubated at 37°C for 24 hours. The growth was then transferred to the surface of a clean slide using a wooden stick, and a drop of (3 percent) H_2O_2 was added. If the test was positive, it meant that gas bubbles were forming (Forbes *et al*., 2007).

2.5.3.2 Oxidase Test :

The test was based on a specific bacterial oxidase that stimulated electron transport between electron donors in the bacteria and a redox dye (Tetra methyl-P-Phenylene-diamine dihydrochloride), which was reduced to a deep purple color. A strip of filter paper was soaked in some freshly prepared reagent. A sterile wooden stick was used to pick up the colony to be examined and smear it above the filter paper. When a deep purple color is formed in 5–10 seconds, it indicates a positive result (Forbes *et al*.,2007).

2.5.3.3 Methyl Red Test:

Young bacterial isolates were inoculated into MR-VP broth media and incubated at 37°C for48 hours. The result was read right away after 5 drops of methyl red solution were added. When a bright red is shown, a positive result is reduced. The test shows how bacteria can ferment acid when glucose available (Forbes *et al* .,2007)

2.5.3.4 Voges-Proskaure Test:

Young bacterial isolates were inoculated into MR-VP broth and incubated at 37°C for 24–48 hours. Each tube received one ml of 40% KOH solution and three ml of a 5% naphthol solution. The emergence of pink in 15-20 minutes suggested a positive reaction. This test is based on determining whether an organism produces acetyl methyl carbonyl from glucose fermentation (Forbes *et al* .,2007) .

2.5.3.5 Indole test:

Tubes containing peptone water were inoculated with the colony of tested bacteria and cultured at 37°C for 48 hours, after which several drops of Kovac's reagent were gently added to the broth medium. The appearance of a crimson ring on the liquid medium's surface was considered a positive result. It is a biochemical test performed on a bacterial species to determine the ability of an organism to convert tryptophan to indole (MacFaddin, 2000).

2.5.3.6 Citrate utilization test :

The Simmons citrate medium was inoculated with a colony of the tested bacteria and cultured at 37°C for 1–3 days. The ability to use citrate as a sole carbon source is indicated by the medium's color change from green to blue color mean positive result (MacFaddin, 2000).

2.5.3.7 Growth at 42 °C :

A fresh culture of bacterial isolate was inoculated into a nutrient broth tube, which was then incubated at 42°C. After 24 hours, the growth at this degree of temperature, it is a characteristic of *P. aeruginosa* (Wolska, Grzes, and KuREK, 2012).

2.5.3.8 Vitek compact system :

To identify the isolates, the VITEK-2 System relies on biochemical reactions between the bacterial isolates suspended in their solutions and the media in the VITEK-2 Identification Cards.

The bacterial isolates were inoculated onto MacConky agar plates and then incubated overnight at 37°C. A single colony (1–5) was then taken and suspended in solution. The turbidity of the bacterial suspension was low.0.5 with VITEK Densichek (bioMerieux) to match the McFarland 0.5 standard of 0.45% sodium chloride. Then the VITEK 2 ID-GN (Gram Negative) card and the bacterial suspension tubes were manually loaded into the VITEK-2 system. The following steps in the software were done according to the manufacturer's instructions (BioMerieux, France) **.**

2.6 Detection of Virulence Factors :

2.6.1 Hemolysins production :

A pure colony of bacteria was streaked on blood agar medium and cultured at 37°C for 24 to 48 hours. The presence of a clear zones around the colony denotes β-hemolysis (Baron, 2007).

2.6.2 Lipase production :

An egg-yolk agar medium lipase test was used to determine the ability of the tested bacteria to produce lipase enzyme. Following inoculation with medium agar, the plates were incubated overnight at 37°C. The formation of an opaque pearly coating around the colony indicated a positive outcome (Collee *et al*., 1989).

2.6.3 protease production :

This test was performed using M9 medium with 1 percent gelatin after inoculating the medium with bacterial isolates and incubating it for 24-48 hours at 37°C, . Then 3ml of trichloroacidic acid (5%) was added. A positive result was obtained by observing a translucent area around the colony (Piret *et al*.,1983).

2.6.4 Biofilm production :

This quantitative test described by (Christensen *et al*.,1985). It is considered the gold-standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10 mL of BHI broth with 1% glucose Broths were incubated at 37°C for 24 hrs. The culture was then diluted 1:10 with fresh medium. Individual wells of sterile 96 well- Flat bottom polystyrene tissue culture-treated plates were filled with 200mLof the diluted cultures.

The control isolates were also incubated and diluted and added to the tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24 h. After incubation, the contents of each well were removed by gentle tapping. Then Wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free-floating bacteria. Biofilm formed by bacteria Adherents to

the wells were fixed by 2% sodium acetate and stained by Crystal violet (0.1%) Excess stain was removed by using deionized water. And plates were kept for drying. The wells were full with ethanol (75%) then obtained with micro ELISA reader at wave length 570 nm. The Experiment was performed in triplicate and repeated three times. 200 µl of media without bacteria considered as control repeated in 3 wells. Average of OD values of sterile medium were calculated and subtracted from all test values. Capability of biofilm formation was measured as in the following equation(Bose *et al*. 2009).

biofilm formation= Optical density of test –Optical density of blank

Table (2-7) Tissue Culture Plate Method Result value (TCP)

Mean OD values	Biofilm formation
(1-1.2)	Weak
(1.2-2)	Moderate
(> 2)	High
(< 1)	Non product

Then the efficiency of the isolates on biofilm formation was determined by comparing the obtained readings with the following equations :

- The optical density of the control is greater than or equal to the optical density of the isolate (ODc ≥ OD) mean no product biofilm .
- An isolate is considered poorly biofilm-forming if its average optical density is greater than the control's average optical density and equal to or less than twice the control's optical density (ODc < OD ≤ 2x ODc)
- An isolate is considered medium-forming biofilm if the average optical density of the isolate is greater than two times the optical density of the

control and equal to or less than four times the control ($2xODc < OD \leq 4xODc$)

- An isolate is considered highly membrane-forming if the rate of optical density of the isolate is greater than four times the control rate ($OD > 4xODc$).

2.7 Antibiotics susceptibility test (Kirby-Bauer method):

The Kirby-Bauer method (Vandeppitte *et al*. 1991) . was used as follows:

- By transferring 5 colonies into a tube of 5ml of normal saline to obtain a culture with 1.5 x 10^8 CFU/ml and adjusting to the turbidity standard of McFarland, 0.5 suspensions were used within 30 min. of preparation.
- The MHA plates were inoculated by immersing a sterile swab into the inoculums. The excess was eliminated by compressing and rotating the swab firmly on the side of the tube above the level of the liquid.
- The swab was rubbed three times all over the surface of the medium, rotating the plate at an angle of 60 after each application. The inoculums were left to dry for a few minutes at room temperature with the lid closed.
- Using sterile forceps, the selected antibiotic disc was positioned on the inoculated plate (each plate with 6 discs). Discs should be warmed to room temperature, and then dispensed on the agar surface; they should gently be pressed down with the forceps.
- The plates were prepared for 30 min, then incubated for 18hr at 37°C**.**
- The antibiotic inhibition zones were measured using a ruler millimeter. The zone size was compared to standard zones (CLSI 2021) to determine the susceptibility or resistance of organisms to each antibiotic.

2.7.1 Detection of Beta lactamase enzymes :

2.7.1.1 Detection of Extended spectrum Beta Lactamase (ESBL) :

The ability of *Pseudomonas aeruginosa* isolates to produce broad-spectrum beta-lactamase enzymes was investigated by the double disk synergy test by taking 5 colonies of bacterial isolates grown on a nutrient agar medium at the age of 18hr to test tubes containing 2 ml of physiological saline solution, and the density of the bacterial suspension was adjusted with a tube (0.5) of the McFarland turbidity meter. The cotton swabs were dipped in the bacterial suspension and rotated and pressed against the inner walls of the tube to get rid of the excess inoculum. Then the dishes containing Muller-Hinton agar were inoculated in several directions to ensure the suspension was spread evenly, then the dishes were left to dry.

The antibiotic discs were distributed in the following order. an amoxicillin/clavulanate disc (20/10 µg) was placed in the center of the plate inoculated with bacterial isolates, and the discs of aztreonam (30 µg) piperacillin (100 µg), ceftazidime (30 µg) and cefotaxime (30 µg) were placed around it so that the distance between the edge of the disc in the center and the edge of the discs around it was 15 mm. The appearance of an increase in the diameter of inhibition between the antagonist amoxicillin/clavulanate and any other antagonist is an indication that the isolate produces beta-lactamase enzymes (Jarlier *et al*. 1988) .

2.7.1.2 Detection of Metallo Beta Lactamase (MBL) :

The ability of *Pseudomonas aeruginosa* isolates to produce metallic beta-lactamase enzymes was investigated by the EDTA combined disk test (CDST)-Imipenem by taking 5 colonies of bacterial isolates grown on

the nutrient agar medium at the age of 18 hours into tubes containing 2 ml of physiological salt solution(0.85%), and the density of the bacteria suspension was adjusted with a tube (0.5) of the McFarland turbidity stander, then the cotton swabs were dipped in the bacterial suspension and rotated and pressed on the inner walls of the tube to get rid of the excess inoculum, then the dishes containing Muller-Hinton were inoculated with the suspension. The bacteria were used in different directions to spread the suspension homogeneously, then the dishes were left to dry. Two disks of anti-imipenem (10 µg), one of them containing EDTA and the other without, were placed in Müller-Hinton agar plates, spaced apart.

One of the two discs was incubated in the incubator at 37 °C for 18 hours, and the diameter of the growth inhibiting zone around the two discs was measured. The result is positive if the increase in the diameter of the inhibition zone around the imipenem disc to which EDTA is added is equal to or greater than 7 mm than the diameter of the inhibition zone around the imipenem disc for a unit (Yong *et al*., 2002).

2.8 Molecular methods:

2.8.1 Extraction of genomic DNA from *P.aeruginosa:*

Genomic DNA was extracted from a bacterial isolate using a Genomic DNA purification Kit for Gram Negative Bacteria extraction genomic DNA, purification depending on the instruction of the manufacturing company (HiGenoMB,India), which is summarized as follows:

- Pellet 1.5 ml of overnight bacterial broth culture in a provided 2 ml capped collection tube by centrifuging for two min at 13000 rpm at

room temperature 25°C. Remove the culture medium and discard it. Resuspend the pellet thoroughly in 180 µl of lysis solution .

- A20 µl was added of the proteinase K solution (20 mg /ml) to the sample Mix and incubate for 30 min at 55 °C.
- A 20ml was added of RNase reagent was placed in incubator 5 min at 25°C. Then vortex thoroughly (about 15 sec) and incubate at 55 °C for 10 min .
- A200 µl was added of ethanol (95-100%) to the lysate and mix thoroughly by vortexing for a few seconds.
- the lysate obtained was Transfer to the HiElute Miniprep spin column (capped) and centrifuge at 10000 rpm for 1 min at room temperature. Discard the flow-through liquid and place the spin column in the same 2 mL collection tube.
- Add 500 µl of prewash solution to the column and centrifuge at 10000 rpm for 1 min at room temperature. Discard the flow-through liquid and re-use the same collection tube with the column,then wash with wash solution.
- A500 µl was added of diluted wash solution to the column and centrifuge for 3 min at 13000 rpm at room temperature. Discard the flow through and spin again at the same speed for an additional 1 min to dry the column. The column must be free of ethanol before eluting the DNA
- the HiEute Minipreps spin column (capped) was transfer to a fresh uncapped collection tube and pipette 200 µl of the Elution Buffer (ET) directly into the column without spilling to the sides, incubate for 5 min ,Centrifuge at 10,000 rpm for 4 min at room temperature to elute the DNA.
- the elute was transfer to a freshly capped 2ml collection tube for longer storage.

2.8.2 Estimation of DNA Concentration:

The DNA concentration of samples were estimated by using Nanodrop by putting 1µl of the extracted DNA in the instrument to detect concentration in ng/ µm and purity detected by noticing the ratio of O.D. 260/280 to check the contamination of DNA samples with protein, the accepted 260/280 ratio of pure DNA is between (1.7-1.9)ng/ µm(Randall, 2009) .

2.8.3 Primer Preparation:

Forward and reverse primers for the three genes were lyophilized and suspended in nuclease-free water to give a final concentration of 100 pmol/ µl as a stock solution; to make a 10 Pmol/ µl concentration as a work primer, re-suspended 10 µl in 90 µl of nuclease-free water.

2.8.4 Agarose gel :

The agarose gel was prepared according to the method of (Sambrok and Rusell, 2001), by which 100 ml of 1x TBE buffer had been taken in a beaker. 0.7 gm. of agarose was added to the buffer. The solution was heated to boiling **(**using a water bath) until all the gel particles dissolved. The solution was allowed to cool down at 50-60 °C and mixed with 0.5 mg/ml of ethidium bromide.

2.8.5 PCR Program:

The PCR reactions were conducted in 25 µl of reaction mixture including master mix 12.5µl , D.W 2.5 µL, primer F 2.5 µL, primer R 2.5 µL and DNA 5µL, the technique for detection of the *NDM* gene ,*GIM and KPC* was adapted from (Astbury *et al* .,2020).As indicated in the table(2-9), the PCR technique included an initial denaturation at 94°C for 5 minutes, followed by thirty-six step cycles, which included denaturation

at 94°C for 40 seconds, annealing at 52°C for 50 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. After the amplification process is completed, 5 microliters of the multiply product are transferred to the previously formed holes on a gel.alagarose for the purpose of electrical relay and detection of the presence of the gene.

Table (2-8): The Mixture of Conventional PCR working solution for Detection of, *KPC,NDM and GIM* Genes in *P.aeruginosa*.

Component	**Component of one sample**(µl)
Master mix	12.5 µl
Primer F.	2.5 µl
Primer R.	2.5 µl
DNA template	5 µl
Deionizer water	2.5 µl
Total Volume	25 µl

2.8.6 Detection of antibiotic resistance genes :

The *NDM, GIM, and KPC* in *p. aeruginosa* bacteria were detected using a polymerase chain reaction methodology adapted from (Jalal *et al*., 2000). The PCR reactions were carried out in a 25µl reaction mixture, including 2.5 µl distilled water, PCR master mix12.5 µl , and 2.5 µl of each forward and reverse primer as listed in Tables(2-10), and 5µl of DNA .

After the amplification process is completed, 5 microliters of the multiply product are transferred to the previously formed holes on a

gel.alagarose for the purpose of electrical relay and detection of the presence of the gene.

Table (2-9):PCR Program for *NDM ,KPC and GIM,* Genes Amplification by Conventional Methods.

No	Steps	Temperature(°C)	Time	Cycles
1.	Initialization step	94	5 min	1
2.	Denaturation step	94	40 sec	35
3.	Annealing step	52	50 sec	
4.	Extension step	72	1 min	
5.	Final extension step	72	5 min	1

2.8.7 DNA Loading and Electrophoresis

According to procedure of (Green and Sambrook 2012) the loading and electrophoresis were performing as below:

- The gel preparation was placed in the casting tray, and the comb was placed at one end of the tray.
- After sealing both edges with tape, the agarose solution was dropped into the gel tray and allowed to cool at room temperature for 30 minutes (in the case of low-melting agarose gel, it was left to cool in the refrigerator for more than 30 minutes).
- The comb was carefully removed and the gel replaced in the electrophoresis chamber.
- The chamber was filled with an appropriate amount of TBE buffer, covering 1-2 mm of the gel's surface.
- The sample (7 µl of DNA was mixed with 3µl of 6X loading dye) was

applied to each well of the gel. The cover was placed on the electrophoresis tank.

- ❖ The cathode was connected to one side of the unit and the anode to the other side.
- ❖ It was run for 30 minutes at 100 volt for genomic DNA and for 50 minutes at 100 volt for PCR product.
- ❖ DNA bands were visualized by UV trans-illuminator documentation system, wave length 570 nm 15 min.

2.9 : - Maintenance and Storage of Bacterial isolates :

2.9.1 : Short term storage:

A single pure colony of bacterial isolate was streaked on the nutrient agar as Slants or plates, incubated at 37°C for 24 hrs. Then, kept in refrigerator at 4°C. This method was used to store bacterial isolates for one to three months (Harley, 2002) .

2.9.2 : Long term storage:

For the preservation of bacterial isolates for a long time, sterile tubes containing 3ml of brain heart broth containing 15% glycerol were inoculated with a loopful of bacterial growth and incubated at 37°C for 24 hrs. The tubes were then stored in deep freezing at -20°C .

2.10 Biostatistics Analysis :

Categorical variables were compared using Pearson's chi-square test to compare percentages. A level of significance of α=0.05 was applied to the test. To analyze current data, the SPSS v.23 program was used.

Chapter three

Results and Discussion

3.1 Isolation of *Pseudomonas aeruginosa*

A total of 100 clinical specimens were collected during the period October 2021 to February 2022, from patients presented otitis chronic suppurative otitis media in Medical City Hospital in Baghdad . The material were collected from both genders with an age range from 3 years to 70 years.

The number of patients who took antibiotics was 48, and the number of patients who did not take antibiotics was 52.

All clinical materials of ear infection cases were inoculated on selective media (cetrimide agar media) and incubated at 37℃ for 18 hr.

All grown isolates were submitted to Blood agar , MacConky agar and to biochemical reactions for final identification of *pseudomonas aeruginosa*

3.2 Identification of *pseudomonas aeruginosa* :

3.2.1 Colony morphology :

The isolates were grown on solid cetrimide medium. The appearance of colonies of bright green color and a distinctive odor is evidence that the diagnosed isolates were back to *Pseudomonas aeruginosa.* The colonies on Cetrimide agar were circular, mucous, and smooth. To confirm the diagnosis, all isolates in present study were grown on MacConky medium, they are large convex colonies , had irregular edges, some of them showed stickiness, had a distinctive smell(fruity odor), and were not fermented with lactose sugar. while it appeared on the solid blood medium in the form of colonies of gray color, fully analyzed for blood. They ability to grow at 42°C is a characteristic that distinguishes *P. aeruginosa* from other species of (Carroll and Hobden, 2016) . In this

study, the most common pigments produced by *P. aeruginosa* were pyocyanin and pyoverdine. Cetrimide stimulates the production of *Pseudomonas* pigments such as pyocyanin and pyoverdine, which have distinct blue-green and yellow-green colors, respectively(Owen and Ackerley, 2011; Wu *et al*., 2015)

3.2.2 Microscopic Examination :

The results of the microscopic examination of young bacterial smears stained with the technique of gram-stain ,they are negative rods . It may be present as a single, or a pair (Mahon *et al*., 2018) .

3.2.3 Biochemical tests :

The biochemical tests showed that all isolates of bacteria were positive for catalase, oxidase, and citrate and negative for Indole production tests, methyl red, and Voges-Proskauer products(Adhikari *et al*., 2010; Hassan *et al*., 2012). show in table (3-1).

Table (3-1): Biochemical features for identification of *P.aeruginosa* isolates.

Tests	Results
Gram-stain	G- rods
Catalase test	+
Oxidase test	+
Growth at 42°c	+
Colonies Odor	Grapelike
Pigment production	Blush green
Indole test	-
Methyl-red	-
Voges-Proskauer	-
Simmon's citrate	+
Sugar fermentation	-

3.2.4 Vitek technique:

Using the VITEK 2 system, all clinical isolates were diagnosed. Here is an example of a report generated by this method for identifying these bacteria. This system has been employed in a number of previous studies and has produced positive findings in terms of biochemical test identification and confirmation. The result of Vitek shown in table(3-2).

Table (3-2) Biochemical tests of Vitek System :

Type test	Result	Type test	Result	Type test	Result
APPA	-	SAC	-	BXYL	-
ADO	-	Dtag	-	BAlap	+
PyrA	-	Dtre	-	ProA	+
IARL	-	CIT	+	LIP	+
Dcel	-	MNT	+	PLE	
BGAL	-	5 Kg	-	TyrA	+
H2S	-	ILATK	+	URE	+
BNAG	-	AGLU	-	CMT	+
AGLTp	-	SUCT	+	BGUR	-
Dglu	+	NAGA	-	O129	+
GGT	+	AGAL	-	GGAA	-
OFF	-	PHOS	-	IMLTa	+
BGLU	-	GiyA	-	ELLM	-
Dmal	-	ODC	-	ILATA	+
Dman	-	LDC	-	DSOR	-
Dmne	+	IHISa	-		

After the diagnosis, it was confirmed that 25 isolates included in the present study belonged to the species *P. aeruginosa* .

The presence of 25 isolates of *Pseudomonas aeruginosa* bacteria was confirmed by biochemical test and Vitek system , while the other isolates bacteria species were ignored (37 isolates) , and (38 specimen) did not have growth result Vitek explain in appendix no 1,2 ,3 and no 4.

Table 3-3 Distribution of *Pseudomonas aeruginosa* isolates among 100 samples of ear infection cases .

Sample	No	Percentage %
P.aeruginosa	25	25 %
Other bacteria	37	37 %
No growth	38	38 %
Total sample	100	100 %

The percentage of isolates that obtained from ear infections in the current study was consistent with what was stated by Al-Saeedi and Raheema, (2019) obtained 25.3% of the cases of middle ear infections.

3.3 Age and sex factors :

The age group who had more infections (3-20) years, the result compatible with study work in Iran's result age group of patients from 4–21 years (Hajjartabar, 2004) . The reason is due to the large number of infections at these ages because the children of this age may be more active and their ears more exposed to foreign objects which lead to contamination of the ear and infection with *Pseudomonas aeruginosa* .
Distribution by sex were higher in males, as the number of samples reached 16 male compared to 9 females 64% vs. 36% (P = 0.005) as the

number of samples in the group was 25 samples with a statistically non-significant difference. The reason is that males in this study are more affected than females because males have more movement and activities that they can perform outside the home, in addition to the fact that they often go to swimming places such as rivers and different swimming pools. The results of the current study are similar to those in the study carried out by Shiny and colleagues, the percentage of isolation of *Pseudomonas aeruginosa* from Males (57.75%) and females (42.25%) (Shiny *et al*.,2016).

Table(3-4):*Pseudomonas aeruginosa* infection according to Age and Sex Factors

Age group / Years		Sex		Total
		Male	Female	
1-20	N	8	4	12
	%	32 %	16 %	48 %
21-40	N	1	1	2
	%	4 %	4 %	8 %
41-60	N	2	1	3
	%	8 %	4 %	12%
61-80	N	5	3	8
	%	2 %	12 %	32 %
Total	N	16	9	25
	%	64 %	36 %	100 %
Chi-sequare	1.27			
P-value	0.8 (N.S)			

N.S : Non – significant

3- 4 Virulence Factors :

Bacterial pathogen influence host cells via a variety of released virulence factors, resulting in severe morbidity and mortality.

3.4.1 Hemolysis enzyme :

P.aeruginosa hemolysis production was investigated, and it was founded that 23 (92%) isolates were capable of producing extracellular

hemolysis on blood agar. These findings are consistent with previous findings that hemolysins cause hemolysis to erythrocytes and are poisonous to a variety of host cells, contributing to inflammation, tissue injury, and weakened host defenses. While the results of other study by Marchant and Banat (2012) , was displayed at 100% .

3.4.2 Protease enzyme :

The protease production by *P.aeruginosa* isolates was studied and it was found that in 16 isolates (64%) positive, the enzyme appeared as a zone around the colony when being grown on M9 media after adding 3ml of 5% Triachloro acetic acid and incubation for 24 hrs. This bacterium produces a variety of virulence factors, including proteases, which continue to cause tissue damage. This study compatible with that of Al-Saeedi and Raheema,(2019).

3.4.3 Lipase enzyme :

The ability of *P.aeruginosa* to produce lipase has been examined, and it was discovered that 24 isolates (96%) can produce lipase after 48 hours of incubation on egg yolk agar. These findings are displayed in a table (3–5). The findings were in line with those of (Amara and Salem 2009), who discovered that all isolates tested positive in a lipase production test. Lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester chemical bonds in lipid substrates that are insoluble in water(Nath *et al*., 2018).

Table (3-5) : The status of virulence factors in *P.aeruginosa* isolate strains (N = 25)

No	Virulence factors detection status (N=3)					
	Lipase		Hemolysis		Protease	
	+ve	-ve	+ve	-ve	+ve	-ve
1	+		+		+	
2	+		+			-
3	+		+		+	
4	+		+		+	
5	+		+			-
6	+		+			-
7		-		-		-
8	+			-		-
9	+		+		+	
10	+		+		+	
11	+		+		+	
12	+		+			-
13	+		+			-
14	+		+		+	
15	+		+			-
16	+		+			-
17	+		+		+	
18	+		+		+	
19	+		+		+	
20	+		+		+	
21	+		+		+	
22	+		+		+	
23	+		+		+	
24	+		+		+	
25	+		+		+	
Total N. (%)	**24/25 (96%)**	**1/25 (4%)**	**23/25 (92%)**	**2/25 (8%)**	**16/25 (64%)**	**9/25 (36%)**

3.4.4 Biofilm production :

In present study , 20 isolates (80%) isolates were strong biofilm products, 3 isolates (12%) samples were moderate, and 2 isolates (8%) samples were non product biofilm produce. shown in figure (3-2).

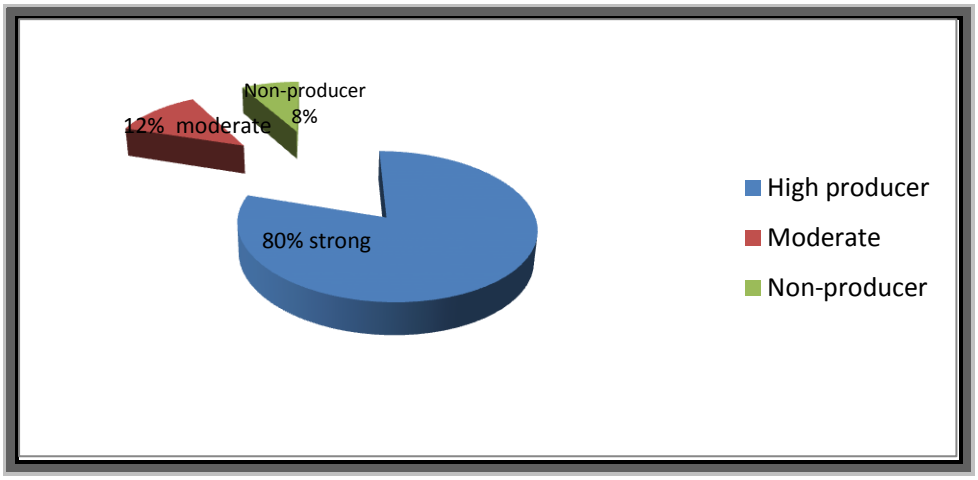


Figure (3-2): This percentage of Biofilm formation in *Pseudomonas aeruginosa* isolates by MTP method.

The findings of this study were in agreement with those of(Moteeb, 2008), who found that 87.5 % of *P. aeruginosa* isolates could form strong biofilms .That is agree with Heydari and Eftekhar (2015), in which 89.47% produced strong biofilm .The present study compatible with study of Abbas *et al*., (2020), after subtract average of negative control from average of optical density of test which read by ELISA reader found that 38 isolates of 50 isolates 76% gave positive result which produces powerful biofilm, but 8 isolates 16% considered as moderate producer isolates, while non-biofilm former performed 4 isolates 8% .

Table (3-6) Biofilm formation status of ***P.aeruginosa*** (N=25) isolates .

Number of *P.aeruginos*	**Result**	***Biofilm status**
Control negative	0.84	
P.aeruginosa -1	0.175	Non
P.aeruginosa -2	0.257	Non
P.aeruginosa -3	1.023	Moderate
P.aeruginosa -4	2.269	Strong
P.aeruginosa -5	3.165	Strong
P.aeruginosa -6	3.408	Strong
P.aeruginosa -7	3.732	Strong

P.aeruginosa -8	1.613	Strong
P.aeruginosa -9	2.575	Strong
P.aeruginosa -10	2.374	Strong
P.aeruginosa 11	2.275	Strong
P.aeruginosa 12	0.875	Moderate
P.aeruginosa 13	2.624	Strong
P.aeruginosa 14	1.220	Moderate
P.aeruginosa 15	2.083	Strong
P.aeruginosa 16	3.326	Strong
P.aeruginosa 17	2.482	Strong
P.aeruginosa 18	2.203	Strong
P.aeruginosa 19	2.606	Strong
P.aeruginosa 20	2.471	Strong
P.aeruginosa 21	2.321	Strong
P.aeruginosa 22	2.012	Strong
P.aeruginosa 23	3.822	Strong
P.aeruginosa 24	3.714	Strong
P.aeruginosa 25	3.231	Strong

Biofilm socre were moderate (1.2-2) ; strong (>2); weak (1-1.2); non product less than (1) .this read repeat three time .

The values of the optical density that we relied on in extracting the values of the biofilm readings were ODc : 0.34411; 2XODc : 0.68821 ; 4XODc : 1.37642

The findings of this investigation study that the majority of the isolates had the potential to build biofilm and were antibiotic resistant. These findings agree with Alwan, (2020) who discovered that 65 % of antibiotic-resistant isolates could form biofilms, whereas isolates that indicate the importance of the biofilm and its role in the emergence of high antibiotic resistance by many of the bacterial species that produce them (Bacalso *et al*., 2011).

That helps it adhere to host cells and gives it protection from external conditions, which helps it stay on hard surfaces, especially in the hospital environment, and this leads to the occurrence of injuries acquired from hospitals (nosocomial infections)(Ramos *et al*., 2013).

Based on the findings of this present study and the findings of other studies can conclude that (micro titer plate) MTP is an important method for detecting biofilm production by *P. aeruginosa* and that it is a great

method because the results were obtained using a very sensitive apparatus such as an ELISA auto reader. Furthermore, based on the findings, this method is an important strategy in studying the early stages of biofilm formation.

3.5 Antimicrobial Susceptibility Test (AST) :

The antibiotic Susceptibility as mentioned in table (3-8) of *P. aeruginosa* isolates under study to nine common β-lactam antibiotics (Meropenem, Imipenem, Ceftriaxone, Cefotaxiam, ceftazidime, cefepime, Aztreonam, Amoxicillin/clavolanic acid and pipracillin) was determined. It uses according to the Kirby-Bauer method using antibiotic disks, and the resistance of these isolates to antibiotics was determined by measuring the diameter of inhibition around the disc.

3.5.1 Resistance *Pseudomonas aeruginosa* to β lactam Antibiotic :

All 25 isolates of *P.aeruginosa* were completely resistant to Amoxicillin/clavulanic acid and cephalosporin antibiotics (ceftriaxone, cefotaxiam, cefepime, and ceftazidime), this result was consistent with findings of (Al-Shwaikh *et al*.,2018).

The present study focused on carbapenem (imipenem, meropenem) to see if bacteria (*P.aeruginosa*) showed resistance to these antibiotics. The study revealed that *P.aeruginosa* was sensitive to imipenem in 40% and resistant in 60%, and sensitive to meropenem in 52% and resisted in 48%, while against aztreonam from the monobactams family, it was sensitive in 20% , resistant in 60% and intermediate 20%.

Nonetheless, the studies are approaching the time of carbapenem resistance, which poses a serious danger because carbapenem are the gold standard for treating multi-drug resistant (MDR) *P. aeruginosa* (Tilahun *et al*.,2021) and this relation explained in appendix No 5 .

Table (3-7): susceptibility tests of *Pseudomonas aeruginosa* against nine β-lactam antibiotic agents .

Antibiotics	Symbol	Percentage β lactam antibiotic of *P.aeruginosa* isolate		
		R	I	S
Piperacillin	PI	17 (68%)	6 (28%)	2 (4%)
Amoxicillin-clavolanic acid	AMC	25 (100%)	0(0.0%)	0(0.0%)
Ceftazidime	CAZ	25 (100%)	0(0.0%)	0(0.0%)
Cefepime	CPM	25 (100%)	0(0.0%)	0(0.0%)
Ceftriaxone	CTR	25 (100%)	0(0.0%)	0(0.0%)
Cefotaxime	CTX	25 (100%)	0(0.0%)	0(0.0%)
Imipenem	IPM	15 (60%)	0(0.0%)	10 (40%)
Meropenem	MEM	12 (48%)	0(0.0%)	13 (52%)
Aztreonam	ATM	15 (60%)	5(20%)	5(20%)

R=resistance **I=intermediate** **S=sensitive**

P.aeruginosa resistance to Piperacillin , Amoxicillin/clavolanic acid , Ceftazidime ,Cefepime , Ceftriaxone , Cefotaxime , Imipenem , Meropenem respectively (68% , 100% , 100% , 100% , 100% , 100% , 60% , 48% , 60%) .

P.aeruginosa sensitive to Piperacillin , Amoxicillin/clavolanic acid , Ceftazidime ,Cefepime , Ceftriaxone , Cefotaxime , Imipenem ,

Meropenem respectively (4%, 0% , 0% , 0% , 0% , 0% , 40% ,52%,20%).

Piperacilline In present study most isolates were tested for piperacillin. The resistance was 68%. The findings of this investigation were close with that of result from that reported by Hussein *et al*., (2018) who reported rates of (67.96%) this result agree with Al-Shwaikh and Alornaaouti, (2018) the resistance was (76.6%) .

Ampicillin+clavulanic acid The results in present study the isolates proved very similar to the results of(Alornaaouti, 2015) which were 100% resistant to each of the Amoxacillin+clavulanicacid .Several studies have shown that *P. aeruginosa* has the ability to resist many different antibiotics, especially beta-lactam antibiotics (Boussoualim *et al*., 2014).

The percentage of resistance to amoxicillin-clavolanic acid was 100%, and this result is consistent with international and local studies, such as those carried out by AL-Shwaikh *et al* (2006), the isolates worked on were resistance 100% Amoxicillin\Clavolanic acid , the results of the current study compatible with the results obtained by (Odumosu *et al*.,2012).

In this study noticed a high rate of resistance of *Pseudomonas aeruginosa* to cephalosporin's (ceftriaxone, cefotaxime, ceftazidimem, cefepime) and penicillin's such as (piperacillin, amoxicillin /clavolanic acid) due to the ability of *Pseudomonas aeruginosa* to produce beta-lactamase enzymes. Like the MBL metallo beta-lactamase enzymes, which break down beta lactam ring of penicillin and cephalosporin, whose genes are carried either on chromosomes or on plasmids in many types of bacteria, which leads to multiple resistance to different antibiotics. In addition to modifying the structure of the penicillin-binding

proteins (PBPs), which are the main target For beta-lactam antibiotic (Ullah *et al*., 2017).

It may also be due to resistance *Pseudomonas aeruginosa* of several generations of the group of cephalosporin to the production of bacterial enzymes Cephalosporinases, which are one of the most important types of β-lactamase enzymes that encode portable genes on chromosomes, are responsible for antimicrobial resistance. First, second and third generation cephalosporin (Bonomo and Szabo,2006).

The widespread and indiscriminate use of these antibiotics by patients (in several cases) may lead to the emergence of resistance due to their availability, ease of oral administration, and cheap price. Antibacterial in general life is a man-made problem that is universal but manifests itself in(Martínez-Solano *et al*., 2008). and is clearly seen in the developing countries of the world compared to the developed countries.

Cefotaxime in this study the isolates were tested for cefotaxime resistance (100%). The findings of this investigation were consistent with that of (AL-Kaisse *et al*., 2015)who showed that 100% of isolates were resistant to cefotaxime. The synthesis of β-lactamase as well as the loss of penicillin binding protein (PBP) due to mutation may provide resistance to cephalosporin resistance in *P. aeruginosa* isolates.

Carbapenem antibiotics (Imipenem, Meropenem) are beta-lactam antibiotics that are primarily used to treat *P. aeruginosa* infections. Carbapenemase enzymes have been found in *P. aeruginosa* strains, similar to those seen in Entrobacteraceae, and are responsible for the bacteria's resistance. Furthermore, the porins of OprD are known to aid in the internalization of Imipenem and, to a lesser extent, Meropenem, but not other-lactams. As a result, modifying the structure of OprD and/or

lowering its expression reduces Imipenem susceptibility. OprD mutations are frequently associated with efflux system overexpression, resulting in high resistance to Imipenem as well as other antibiotic classes such as quinolones and aminoglycosides(Odumosu *et al*., 2012).

Imipenem in this study from all isolates of *P.aeruginosa* were tested for imipenem resistance and the resistance was 60 percent. The findings of this investigation were consistent with the results of Al-Shwaikh and Alornaaouti, (2018) that showed resistance to imipenem was 54%, Imipenem was resist 63.3% compatible with the result of (AL-Kaisse *et al*.,2015) .

Meropenem the resistance percentage was 48%. This finding is consistent with the findings of (Hussein *et al*.,2018) who found that 35% and 37% of isolated bacteria were resistant to Meropenem, respectively. Although Gad *et al*., (2007) reported a lower percentage (22%), and Ochoa *et al*., (2013) reported a higher rate (63%) .

Aztreonam In this study most isolates were tested for aztreonam. The resistance was 60%, sensitive (20%), and intermediate (20%). In the present data, *P. aeruginosa* showed high resistance (68%) to (ATM) antibiotic. This result is compatible the result of the study (Lutz and Lee, 2011), but not compatible with (Banisharif and Momtaz, 2016), which recorded its resistance as 19.4%.

The findings of this investigation compatible with the result of the study of Ochoa *et al*., (2013). The resistance was (63%) lower rates of resistance (54.4%) were documented (Hussein *et al*., 2018) was 48%, as well as a higher rate of resistance of (98.4%) recorded by(Vasco and Trueba, 2021).

3.5.2 The distribution of *Pseudomonas aeruginosa* among treated and non-treated patients with antibiotics .

P. aeruginosa (N = 25) strains were CAZ,CTX ,CFM, and AMC with 14/25 (56%) among patients who had taken antibiotics versus 11/25 (44%) for patients who had not taken antibiotics, which showed resistance for the above mentioned antibiotics respectively.

While the most sensitive antibiotics against *P. aeruginosa* (N = 25) strains were MEM 8 (32%), 5 (20%) for patients who had taken antibiotics, versus patients who had not taken antibiotics. IMP were 5 (20%) and 5 (20%) for patients who had taken antibiotics versus patients who had not taken antibiotics. From this table the present study note that meropenem is the most antibiotic that works well against *Pseudomonas aeruginosa*, as for the rest of the antibiotics in Table 3-8 for the three groups of cephalosporin's, the resistance is very strong. This is why we note that the resistance is 100%, whether those who took the antibiotics or those who did not take the antibiotics. As for imipenem, piperacillin, and aztreonam, the resistance of those who took antibiotics and those who did not take antibiotics is still greater than the sensitivity to *Pseudomonas aeruginosa*.

Table (3-8) The distribution of *Pseudomonas aeruginosa* among treated and non-treated patients with antibiotics .

Name of antibiotic	Susceptibility status	Treatment status		Total
		Yes	No	
Meropenem (MEM)	R	6 (24 %)	6 (24 %)	12 (48%)
	S	8 (32 %)	5 (20 %)	13 (52%)
	I	0 (0.0%)	0 (0.0%)	0 (0.0%)
Imipenem (IMP)	R	9 (36 %)	6 (24 %)	15 (60%)

	S	5 (20 %)	5 (20 %)	10 (40%)
	I	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ceftazideme (CAZ)	R	14 (56 %)	11 (44 %)	25 (100%)
	S	0 (0.0%)	0 (0.0%)	00 (0.0%)
	I	0 (0.0%)	0 (0.0%)	00 (0.0%)
Cefotaxime (CTM)	R	14 (56 %)	11 (44 %)	25 (100%)
	S	0 (0.0%)	0 (0.0%)	00 (0.0%)
	I	0 (0.0%)	0 (0.0%)	00 (0.0%)
Ceftriaxone (CRO)	R	14 (56 %)	11 (44 %)	25 (100%)
	S	0 (0.0%)	0 (0.0%)	00 (0.0%)
	I	0 (0.0%)	0 (0.0%)	00 (0.0%)
Aztreonam (AZM)	R	8 (32 %)	6 (24 %)	14 (56 %)
	S	2 (8 %)	1 (4 %)	3 (36%)
	I	4 (16 %)	4 (16 %)	8 (32 %)
Piperacillin (PIP)	R	9 (36 %)	8 (32 %)	17 (68 %)
	S	1 (4 %)	0 (0.0%)	1 (4 %)
	I	4 (16 %)	3 (12 %)	7 (28 %)
Cefepime (CFM)	R	14 (56 %)	11 (44 %)	25 (100%)
	S	0 (0.0%)	0 (0.0%)	00 (0.0%)
	I	-0 (0.0%)	-0 (0.0%)	00 (0.0%)
Amoxicillin+clavulanic acid (AMC)	R	14 (56 %)	11 (44 %)	25 (100%)
	S	0 (0.0%)	0 (0.0%)	0 (0.0%)0
	I	0 (0.0%)	0 (0.0%)	0 (0.0%)

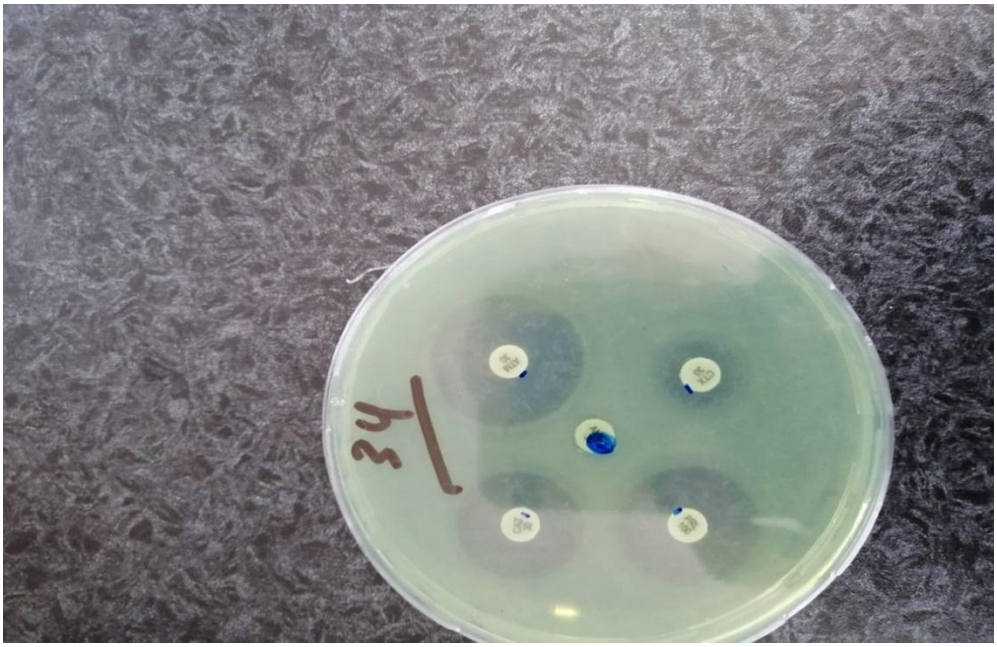
3.5.3 Detection ESBL enzymes.

The present study was focused on *P.aeruginosa* isolated from chronic soppurative otitis media (CSO) cases for detection of β.lactam antibiotics resistance by mechanism of enzyme production .

Using the Combined disk test (CDT) for enzyme detection Beta-lactamases are broad-spectrum, according to what was stated in (CLSI,

2021) and it is one of the easy and accurate methods (Thirapanmethee, 2012) .

In this study, no isolate was able to produce ESBL, was compatible with Jebur,(2018) that all ESBL test were negative results of detecting the production of Extended-spectrum beta-lactamase enzymes ESBLs show in figure (3-4) below.



appearance of figures (3-4) No ESBL enzyme was produced from *P. aeruginosa* sample number 34 .

Pseudomonas aeruginosa has recently begun to produce MBL enzyme in greater quantities than ESBL enzyme because it needs resistance to beta-lactam antibiotics including carbapenem because carbapenem antibiotics have started to be used more recently as a very successful and useful treatment against multi-antibiotic-resistant *Pseudomonas aeruginosa*.

3.5.3.2 :Detection of Metallo beta lactam(MBL) enzyme :

The results of phenotypic detection showed the ability of carbapenem-resistant *Pseudomonas aeruginosa* isolates (25 isolates) to produce metallic beta-lactamases MBLs by the EDTA combined disk test. Imipenem In the present study, most isolates (24/25) produced the MBL enzyme. The results are compatible with those of the study conducted by Jebur (2018) that showed all isolates of Jebur produce the MBL enzyme 100% . Moosavian and Rahimzadeh (2015) reported that 90.2% (110/122) of the resultant isolates isolated from wounds, urine, blood, trachea, and other clinical samples were producing MBLs, which is compatible with the findings of the current study (Tarashi *et al*., 2016). indicated that 64% (178/278) of isolates isolated from burns were MBL-producing. (Heidary *et al*., 2016) found that an average of 57.8% (48/83) of isolates isolated from burns were MBLs. Raouf *et al*. (2018) reported that 85% (17/20) of *Pseudomonas aeruginosa* isolates isolated from wounds were MBL-producing . The MBL enzyme is shown in Figure (3-5) below and this relationship is explained in Appendix No 6 .

Figure (3-5) depicts the appearance of mineral beta-lactamase enzymes (MBLs) produce from *P.aeruginosa* sample no 34.

The most commonly used antibacterial agents are -lactam antibiotics, and rising resistance to these drugs is a concern. Metallo β-lactamase is a group of enzymes that catalyze the hydrolysis of a wide variety of -lactam drugs, including carbapenem.

This variation is reflected in the observation that the enzyme mechanisms differ depending on whether one or two zincs are bound in the active site, which is dependent on the -lactamase subclass. Because of the spread of the genes encoding these enzymes among gram-negative bacteria, they have become a significant source of resistance. Furthermore, no clinically available inhibitors of metallo β-lactamase action are currently available (Palzkill, 2013) .

3.6 : Molecular detection of Metallo beta lactamases .

In the present study the result of molecular detection antibiotic resistance genes reveal NDM 22/25 (88%), GIM 15/25 60%, and KPC 10/25 40%, and which belong to the beta-lactam antibiotic group, especially the carbapenem group, because the focus of the present study is on the spread of resistance to *P. aeruginosa* to antibiotics (beta-lactam) and because *Pseudomonas aeruginosa* bacteria started recently resistance to beta-lactam antibiotics (carbapenem) such as imipenem, meropenem which is considered one of the most powerful beta-lactam antibiotics today, so the study shed light on the genes of these bacteria mentioned above to see the extent of the ability of these bacteria to resistance and sensitivity to these antibiotics. The confirmation of the presence of these genes by the molecular examination is a conclusive confirmation of the Kirby-Bauer examination method that was conducted in the bacteriological examination, which indicated the presence of resistance of *Pseudomonas aeruginosa* to beta-lactam antibiotics, especially imipenem and meropenem. Because of the properties that distinguish this approach,

such as speed and accuracy, and the fact that it requires minimum volume of sample in processing than other techniques, PCR was utilized to confirm the final diagnosis of all isolates (Wellinghausen *et al*., 2009).

The present study used genetic detection of metallo beta-lactamase genes such as NDM, GIM, and KPC to explanation why *P.aeruginosa* resistant to antibiotics especially carbapenem because these genes enable bacteria to defend themselves using as for the mineral beta-lactam enzymes, the NDM gene appeared at a very high rate of22 (88%) followed by GIM gene with a percentage of 15 (60%) and the KPC gene with a percentage of 10 (40%) .

3.6.1 Detection of NDM gene :

Beta lactam antibiotic resistance genes encoded by the NDM gene were detected in *P.aeruginosa* isolates by using specific primers. It was found that 22 (88%) isolates *P. aeruginosa* gave the amplicon for this gene with a molecular length of (264) bp as shown in figure (3-10) our result disagree with the study of Chew *et al*.,(2019) that found only 10.4% of the NDM gene .

In this result a high percentage (88%) of NDM gene compatible with study of (Ismail and Mahmoud, 2018),the presence of the bla NDM-variant among *P.aeruginosa* isolates was reported in this study. The NDM gene was discovered in large quantities in this study because Pseudomonas aeruginosa produces this quantity to be able to resist the antibiotic carbapenem, which is becoming more commonly used in the current period. In order to gain a better understanding of the NDM gene research. In the current study, isolates that carry this gene and are resistant to beta-lactams in particular (IMP, MEM) have biofilm

producers and MBL enzyme producers. It is considered among the local isolates that can be placed in the microbe bank for use in future studies (12,26,53,67,98).

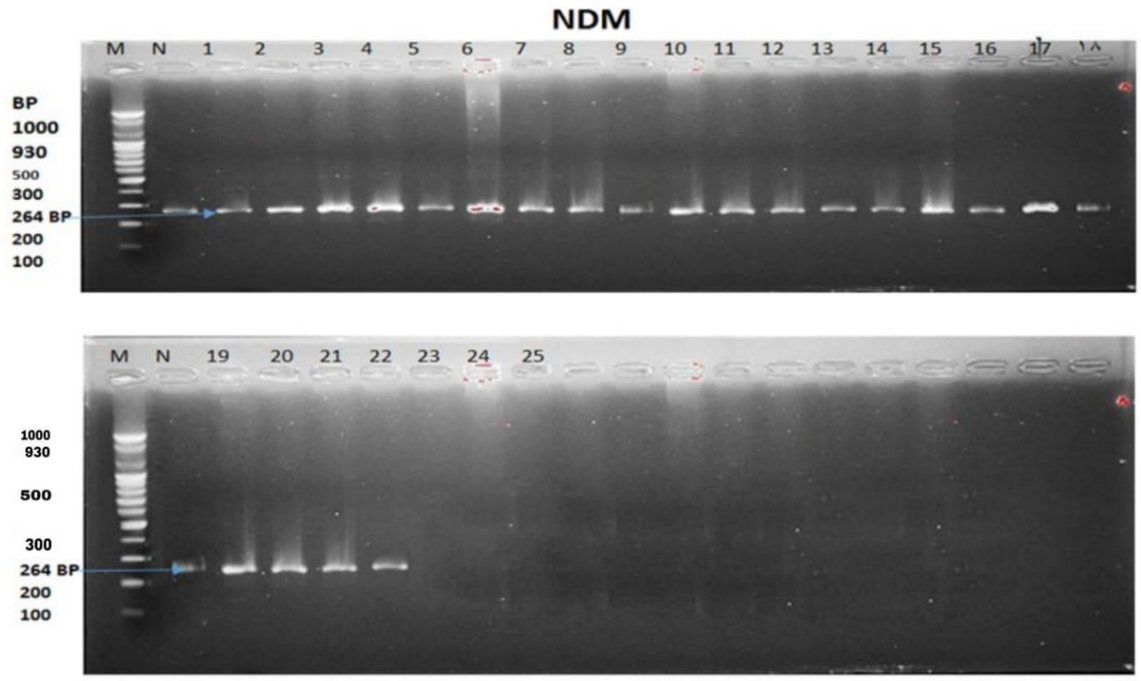


Figure (3-10) Electrophoresis of the NDM gene for *Pseudomonas aeruginosa* using 1% agarose gel containing 0.5 Ethidium bromide dye using a DNA ladder (100-1000 bp) at a voltage of 100 V for 30 min .

This present study agree with (Ismail and Mahmoud, 2018) that describe for first time *P.aeruginosa* resist to MEM,IMP because have NDM gene **,** Most antibiotics were resistant to all carbapenem-susceptible isolates. Although aztreonam is stable to hydrolysis by MBL producers, the current study results showed a moderate level of aztreonam resistance (20%), which is explained in appendix no 7.

3.6.2 Detection of GIM Gene :

Metallo β-lactamas (MBLs) have rapidly spread throughout the world among clinically important gram-negative bacteria, posing a threat to the therapeutic use of beta-lactam antibiotics, particularly carbapenem. The blaGIM-1 gene, which encodes one of these enzymes, was discovered in a *Pseudomonas aeruginosa* isolate in 2002 (Borra *et al*., 2018)**.**

Beta lactam antibiotic resistance genes encoded by the GIM gene were detected in *P. aeruginosa* isolates by using specific primers. It was found that 15/25 (60%) isolates of *P. aeruginosa* gave the amplicon for this gene, with a molecular length of (477) bp, as shown in figure (3-11).

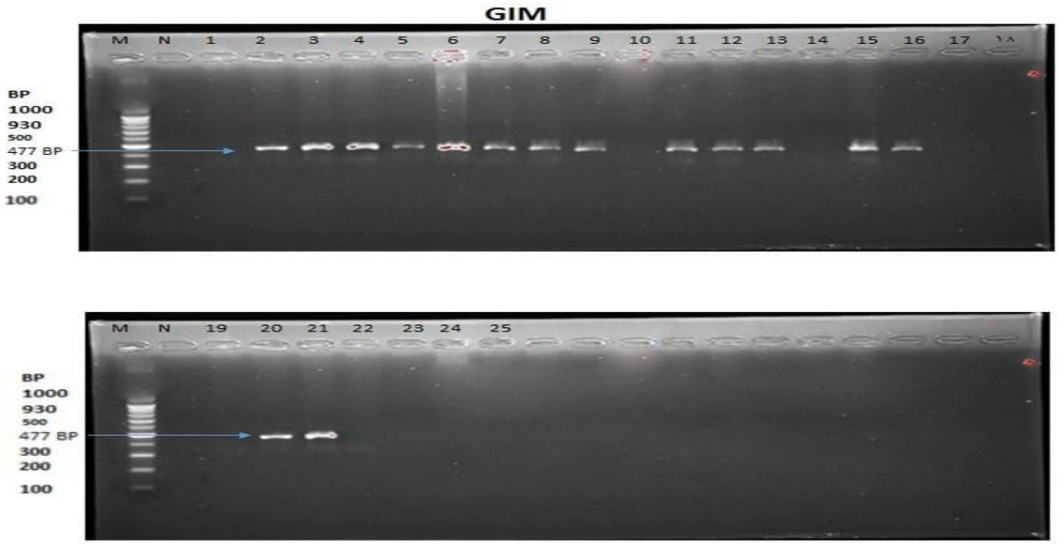


Figure (3-11) Electrophoresis of the *GIM* gene for *Pseudomonas aeruginosa* using

1% agarose gel containing 0.5 Ethidium bromide dye using a DNA ladder (100-1000 bp) at a voltage of 100 V for 30 min .

This present study agrees with the study of (Farhan *et al*. 2021) , have GIM gene 11 / 21(52.3%) isolates . and disagree with the study(Vural *et al*., 2020) they found only 3% of the GIM gene. In this study found that 60% of the clinical isolates contained the GIM gene. In this study found the GIM gene in a large quantity because *Pseudomonas aeruginosa*

produces it in this quantity to be able to resist the antibiotic carbapenem that are starting to be used more often in the current period. In order to be more knowledgeable in this research on the GIM gene, in present study the samples that are carriers of this gene and at the same time resistant to beta-lactams in particular (IMP, MEM) have biofilm producers, and MBL enzyme producers. It is considered one of the local isolates that can be placed in the microbe bank for use in future studies, and it is No. 26.67,92,98) and this relation explain in appendix no 9 .

3.6.3 Detection of *KPC* gene :

In 2007, Colombia (KPC-2) was the first to report producing KPC *Pseudomonas aeruginosa* isolates(Akpaka *et al*., 2009) . By using specific primers, beta-lactam antibiotic resistance genes encoded by the KPC gene were detected in *P. aeruginosa* isolates. It was found that 10/25 (40%) isolates of *P. aeruginosa* gave the amplicon for this gene, with a molecular length of (893) bp, as shown in figure (3-12).

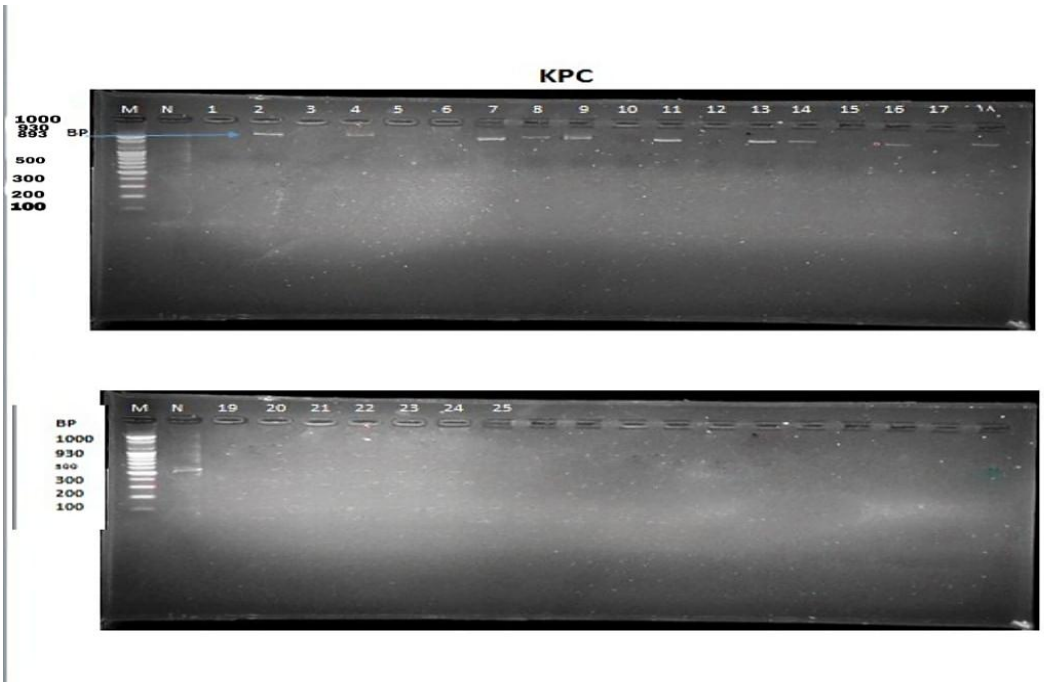


Figure (3-12) Electrophoresis of the KPC gene for *Pseudomonas aeruginosa* using 1% agarose gel containing 0.5 Ethidium bromide dye using a DNA ladder (100-1000 bp) at a voltage of 100 V for 30 min .

In this study, 40% of *Pseudomonas aeruginosa* isolates were found to contain the KPC gene, and this explains the reason for the spread and increase of resistance to *Pseudomonas aeruginosa* with such rapidity and Strong bacterial resistance to beta-lactam antibiotics by 100% for generations of penicillin's and cephalosporin's. In addition to a non-extreme resistance to anti-carbapenem, Carbapenem resistance caused by the production of *Klebsiella pneumonia* carbapenemases (KPC) enzymes in *Pseudomonas aeruginosa* has been linked to various transposon families and plasmids(Galetti *et al*., 2016). The genetic events responsible for the selection and spread of antimicrobial resistance have been identified as chromosomal mutations and horizontal gene transfer (Naas *et al*.,2016).

In this study found the GIM gene produced because *Pseudomonas aeruginosa* produces it to be able to resist the antibiotic carbapenem that is starting to be used more often in the current period. In order to be more knowledgeable in this research on the KPC gene, in present study of isolates that are carriers of this gene and at the same time resistant to beta-lactams in particular (IMP, MEM) have biofilm producers and MBL enzyme producers. It is considered one of the local isolates that can be placed in the microbe bank for use in future studies, and it is No. 26,92,98).

3.7 Detection of Metallo beta lactamase .

Table (3-9): Genotyping status of ***P.aeruginosa*** isolated strains **(N=25)**

No	Genotyping status of genes (N=4)					
	KPC		**GIM**		**NDM**	
	+ve	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**
1		+		+	+	
2	+		+		+	
3		+	+		+	
4	+		+		+	
5		+	+		+	
6		+	+		+	
7	+		+		+	
8	+		+		+	
9	+		+		+	
10		+		+	+	
11	+		+		+	
12		+	+		+	
13	+		+		+	
14	+			+	+	
15		+	+		+	
16	+		+		+	
17		+		+	+	
18	+			+	+	
19		+		+	+	
20		+	+		+	
21		+	+		+	
22		+		+	+	
23		+		+		+
24		+		+		+
25		+		+		+
Total N (%)	**10/25 (40%)**	**15/25 (60%)**	**15/25 (60%)**	**10/25 (40%)**	**22/25 (88%)**	**23/25 (12%)**

Table (3-9) showed that all the isolated *P.aeruginosa* (N=25) strains, while only 10/25 (40%) of *P.aeruginosa* strains carried KPC genes versus 15/25 (60%) not carried this genes. In regarding to the GIM genes only 15 /25 (40%) of *P.aeruginosa* strains not carried his gene versus 10/25 (60%) carried this genes. This table also documented that 23/25 (88%) of *P.aeruginosa* strains carried the NDM genes versus only 3/25 (12%) not carried this genes.

Carbapenem are antimicrobials that are effective against *P. aeruginosa* infections. Carbapenemases enzyme, on the other hand, has emerged as a key mechanism of carbapenem resistance (Goff *et al*., 2017).

In this study, found that isolates carried the *GIM* gene by 60%. This confirms our results when conducting a sensitivity test to beta-lactam antibiotics, as it was found that secret samples containing *Pseudomonas aeruginosa* have multiple resistance to beta-lactam antibiotics, including imipenem and meropenem.

In this study, we found isolates carrying 40% of the *KPC* genes, and this is a clear explanation for why *Pseudomonas aeruginosa* are resistant to many beta-lactams, especially imipenem and meropenem, because these genes for the carbapenemases enzymes production. This study disagree with study that have 1.3% *GIM* gene, while Farhan *et al*., (2019) agree with study that have 52.3% of *GIM* gene.

In this study, when conducting a molecular examination, the isolates carried the *NDM* genes with a large percentage of 88%. This is a clear explanation of the reason why *Pseudomonas aeruginosa* has a strong and multiple resistance to beta-lactams, as these genes encode for the bacterial production of carbapenemases enzymes to resist beta-lactams, especially carbapenem (imipenem, meropenem).

Conclusions and Recommendations

Conclusions

According to the results of the present study the following conclusions could be elucidated

- .the most age effect of *P.aeruginosa* are children and young and the gender are male more than female .
- .The virulence factors with the highest prevalence are Hemolysins , lipase ,biofilm, and protease, respectively
- .It was found that *Pseudomonas aeruginosa* isolates had strong biofilm 80% , moderate 12% and no product 8% biofilm production.
- . *P.aeruginosa* resistance for most antibiotic of beta lactam .
- . It was found that the prevalence of the gene NDM in *P. aeruginosa* isolates was 88% . Other isolates were 40% KPC and 60% GIM genes.
- .The MDR *P. aeruginosa has* become widespread in local hospitals, and the implementation of infection control strategies is a major concern to avoid the spread of this threat.

Recommendations

1-Conducting periodic studies every two years to know the evolution of *Pseudomonas aeruginosa* resistance to modern antibiotics in cooperation with the Hospital Infection Control Program .

2-A molecular study of the disease islands responsible for the production of antibiotic resistance genes for *Pseudomonas aeruginosa* isolates.

3-The use of synergistic antibiotics because they have a significant effect on bacterial resistance as they reduce the speed of production of bacterial resistance against antibiotics, in addition to giving the antibiotic a much greater effectiveness than it is given alone.

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Appendices

Appendices

Appendix (1) : Vitek Diagnosis Results

bioMérieux Customer:

Microbiology Chart Report

Printed January 1, 2022 10:40:35 AM CST

Patient Name:
Location:
Lab ID: 46 alaa abdulla

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	7.82 hours	**Status:**	Final
Selected Organism	96% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	0002041001500200		
ID Analysis Messages				

Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendices

Appendix (2) :Vitek Diagnosis Results

bioMerieux Customer: Microbiology Chart Report Printed January 1, 2022 10:40:40 AM CST

Patient Name: Patient ID:
Location: Physician:
Lab ID: 70 alaa abdulla Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.85 hours	**Status:** Final
Selected Organism	98% Probability	**Pseudomonas aeruginosa**	
	Bionumber:	0003051303500252	
ID Analysis Messages			

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	+	62	ELLM	-	64	ILATa	+			

Appendices

Appendix 3 : Vitek 2

bioMérieux Customer:

Microbiology Chart Report

Printed January 1, 2022 10:40:25 AM CST

Patient Name:
Location:
Lab ID: 34 alaa abdulla

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	8.78 hours	**Status:**	Final
Selected Organism	**Bionumber:**	**Pseudomonas aeruginosa** 0043051341500342		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	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	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	(-)	47	ODC	-	48	LDC	-	53	lHISa	+	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	lMLTa	+	62	ELLM	-	64	lLATa	+			

Appendix 4 : Vitek 2

bioMerieux Customer:

Microbiology Chart Report

Printed January 1, 2022 10:40:39 AM CST

Patient Name:
Location:
Lab ID: 69 alaa abdulla

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:** 5.83 hours	**Status:** Final
Selected Organism	98% Probability **Pseudomonas aeruginosa** **Bionumber:** 0003451303500252	
ID Analysis Messages		

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	+			

Appendices

Appendix(5) :Relation between *P.aeruginosa* with antibiotic β lactam resistance

NO sample	MEM	IMP	CAZ	CTX	CRO	CFM	PIP	AZM	AMC
1.	R	R	R	R	R	R	I	I	R
2.	S	S	R	R	R	R	I	I	R
3.	R	R	R	R	R	R	R	R	R
4.	S	S	R	R	R	R	I	I	R
5.	S	R	R	R	R	R	R	R	R
6.	S	S	R	R	R	R	S	S	R
7.	R	S	R	R	R	R	R	I	R
8.	S	S	R	R	R	R	R	S	R
9.	S	R	R	R	R	R	R	I	R
10.	S	R	R	R	R	R	R	R	R
11.	R	R	R	R	R	R	R	R	R
12.	S	R	R	R	R	R	R	S	R
13.	S	S	R	R	R	R	I	S	R
14.	S	S	R	R	R	R	S	S	R
15.	S	R	R	R	R	R	R	R	R
16.	R	R	R	R	R	R	R	R	R
17.	S	R	R	R	R	R	R	R	R
18.	S	S	R	R	R	R	R	R	R
19.	R	S	R	R	R	R	R	R	R
20.	R	S	R	R	R	R	R	R	R
21.	R	R	R	R	R	R	I	R	R
22.	R	R	R	R	R	R	R	R	R
23.	R	R	R	R	R	R	R	R	R
24.	R	R	R	R	R	R	R	R	R
25.	R	R	R	R	R	R	I	R	R
Total No %	48% R	60% R	100% R	100%R	100%R	100%R	17% R	60%R	100%R

Appendices

Appendix(6) :Relationship between Metallo beta lactam enzyme and IMP,MEM antibiotic

No	MBL test	IMP (S or R)	MEM(S or R)
1.	+	R	R
2.	+	R	R
3.	+	S	S
4.	+	S	S
5.	+	S	S
6.	-	-	-
7.	+	S	S
8.	+	R	R
9.	+	R	R
10.	+	S	S
11.	+	R	S
12.	+	R	R
13.	+	S	S
14.	+	R	R
15.	+	R	S
16.	+	S	R
17.	+	S	R
18.	+	S	R
19.	+	R	R
20.	+	R	R
21.	+	R	R
22.	+	R	R
23.	+	R	R
24.	+	R	R
25	+	R	R
Total No %	24/25 96%	60% R 40% S	64% R 34% S

Appendix(7):Relation between NDM Gene metallo beta lactam enzyme and carbapenem antibiotic

Sample no	NDM	IMP (S or R)	MEM (S or R)
1.	+	R	R
2.	+	S	S
3.	+	S	S
4.	+	S	S
5.	+	R	R
6.	+	R	R
7.	+	S	S
8.	+	-	-
9.	+	R	R
10.	+	S	S
11.	+	S	R
12.	+	S	R
13.	+	R	R
14.	+	R	R
15.	+	R	R
16.	+	R	R
17.	+	R	R
18.	+	R	R
19.	+	R	R
20.	+	R	R
21.	+	R	R
22 .	+	R	R
23 24	+	R	R
	+	R	R
25	+	R	R
Total No %	88%	68% R 28%S	76% R 20% S

Appendices

Appendix(8) :Relationship between KPC gene in *P.aeruginosa* with resistant carbapenem antibiotic

No	Kpc gene	IMP (S or R)	MEM (S orR)
1.	+	R	R
2.	+	S	S
3.	+	R	R
4.	+	S	S
5.	+	R	S
6.	+	S	-
7.	+	S	-
8.	+	R	R
9.	+	R	R
	+	S	S
Total isolate 25	40%	20% R 20% S	16% R 16% S

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Appendix(9)

Relationship between GIM gene in *P.aeruginosa* with resistant of IMP,MEM

NO	GIM gene	IMP (S or R)	MEM(S or R)
1.	+	R	R
2.	+	S	S
3.	+	S	S
4.	+	S	S
5.	+	R	R
6.	+	S	S
7.	+	R	S
8.	+	R	R
9.	+	S	S
10.	+	R	R
11.	+	S	R
12.	+	R	R
13.	+	R	R
14.	+	R	R
15.	+	R	R
Total 25 isolates	60%	36% R 24% S	36% R 24% S

الخلاصة :

*Pseudomonas aeruginosa*هي بكتيريا هوائية سالبة الجرام ظهرت كواحدة من أكثر مسببات الأمراض المستوطنة للمستشفيات والمسببة للمشاكل بسبب سلالات الزائفة الز نجاريه المنتشرة في المرضى العراقيين. تم جمع مائة (100) عينة من المرضى من مختلف الفئات العمرية ، بما في ذلك أربعة مستشفيات في مستشفى مدينة الطب (مستشفى بغداد التعليمي ، ومستشفى غازي الحريري ، والمختبرات التعليمية بالمدينة الطبية ، ومستشفى حماية الطفل بالمدينة الطبية) اعتبارًا من تشرين الأول 2021. حتى نهاية شباط 2022. تم استنبات العينات التي تم جمعها على أوساط مختلفة (وسط الدم الصلب،وسط الماكونكي الصلب،وسط السترمايد الصلب) واختبارها كيميائيًا ، والتي تضمنت اختبارات أوكسيديز ، كاتلاز ، و IMVIC ، من أجل معرفة خصائص الزائفة الزنجارية استعمار (مرضى الأذن). أظهرت نتائج الاستنبات البكتيرية أنه من بين 100 عينة ، لوحظ أن 25 عينة (25٪) لديها نمو بكتيري (عينات موجبة) لبكتريا *الزائفه الزنجاريه*. بلغت نسبة المرضى الذكور 60٪ والإناث 40٪ وتتراوح أعمارهم بين 3 - 70 سنة. أجريت اختبارات الحساسية لتسعة مضادات حيوية من نوع بيتالاكتاميز باستخدام طريقة كريبي بأور. أظهرت العزلات مقاومة للسيفالوسبورين والأموكساسيللين-كلافولانيك بنسبة 100٪ ، ثم الامبنيم ، الميروبينيم ، الأزتريونام ، البيبراسيلين (60٪ ، 40٪ ، 60٪ ، 68٪) على التوالي.

تم إجراء إنتاج العزلات البكتيرية لعوامل الضراوة ، والتي تضمنت إنزيمات البروتيز في وسط M9 ، واللايبيز على وسط صفار البيض ، وانحلال الدم في وسط الدم ، وتم الكشف عن تكوين الأغشية الحيوية بطريقة الصفيحة الدقيقة ، حيث تم التحري عن نسبة 100٪ من العزلات أظهرت قدرتها على تكوين الأغشية الحيوية. تم التحري عن إنزيمات بيتالاكتامايز واسعة الطيف عن طريق اختبار القرص المشترك وإنزيمات بيتالاكتامايز المعدنية بواسطة طريقة EDTA لاختبار

القرص المدمج. كانت جميع العزلات غير منتجة لأنزيمات lactam (ESBL) Extended beta ، بينما كانت نسبة العزلات من Metallo Beta Lactam (MBL) 96%. أخيرًا تم استخلاص الحمض النووي للبكتيريا التي أظهرت مقاومة لمضاد الكاربابنيم. تم التحري عن جينات *NDM* و *GIM* و *KPC* باستخدام تفاعل البلمرة المتسلسل (PCR) ، وتم اكتشاف حجم الجين عن طريق الرحلان الكهربائي واستخدام مصدر للأشعة فوق البنفسجية. بلغت العزلات الحاملة لجينات (*NDM*، GIM, KPC) هي 88٪ و 60٪ و 40٪ على التوالي. أظهرت هذه الدراسة عدة استنتاجات مفادها أن عزلات *P. aeruginosa* لها عوامل ضراوة متعددة بما في ذلك تكوين الأغشية الحيوية. إنها مقاومة للمضادات الحيوية المتعددة. ووجود علاقة بين إنتاج عوامل الضراوة والمقاومة المتعددة للبيتالاكتاميز ، وأن غالبية العزلات البكتيرية تنتج إنزيمات بيتالاكتامايز المعدنية (MBLs) عن طريق الكشف المظهري.

كلما زادت قدرة البكتيريا على إنتاج MBL زادت قدرتها على مقاومة المضادات الحيوية بيتا لاكتام ، وزادت نسبة العزلات المنتجة لإنزيم بيتا لاكتام المعدني ، مما يدل على وجود علاقة بين المقاومة المتعددة لمضادات الميكروبات والإنتاج. من إنزيمات MBL في بكتيريا *P.aeruginosa*

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية الطب

قسم الاحياء المجهرية الطبية

توصيف عزلات بكتريا الزائفة الزنجاريه المنتجة لأنزيمات البيتالاكتاميز المعدنية المعزولة من التهاب الاذن الوسطى القيحي المزمن

رسالة مقدمه الى مجلس كلية الطب جامعة بابل

كجزء من متطلبات نيل درجة الماجستير

في العلوم / الأحياء المجهرية الطبية

من قبل

علاء عبد الله رشيد عزيز

(بكالوريوس علوم حياة / الجامعة المستنصرية 2007)

بإشراف

الاستاذ الدكتور

جواد كاظم الخفاجي

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صفاء صاحب الغزالي

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