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Effect of Some Inhibitors on Metallo Beta lactamase Genotypically Diagnosed of *Pseudomonas aeruginosa* Isolated from Burn Wound Infections

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Dedication

I’d like to dedicate my work to:

The one who make me love to learn and lighten my ways and does his best to keep me forward, stand steady, stay strong, my power and courage source and my pride my dear father.

The one who I lost during the study, who taught me to chase my dreams, be ambitious, never give up and give the best I can, my source of tenderness and love, symbol of giving, my mother (Mercy upon her soul).

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Summary

Serious burn injury causes damage and skin loss, which is considered as the first line of protector against microbial infections. The opportunistic bacteria including *Pseudomonas aeruginosa* can invade the body of patient through burn areas and infect it with different diseases that could lead to death.

This study aimed to isolate *P. aeruginosa* from hospitalized burn patients and test the effectiveness of some inhibitors to combat Metallo Beta-lactamases (MBLs) produce by *Pseudomonas aeruginosa*, which are responsible for their resistance to the antibiotics.

During the period from December 2021 to April 2022, a total of 249 swabs of burn patients have been collected. All isolates have been diagnosed by culturing on selective media (Cetrimide agar) and confirmed by polymerase chain reaction (PCR) using species specific 16s rDNA for *Pseudomonas aeruginosa*, 100/249 (40.16%) of swabs have been identified as *P. aeruginosa*

Antibiotics susceptibility test according to CLSI 2021 have been done to identify the multidrug resistance bacteria (MDR). The results showed 69/100 (69%) were MDR isolates. As a highest records, the response of 81/100 (81%) of isolates were sensitive to cefepime while 83/100 (83%) were resistant to Piperacillin, however this resistance reduced when using Piperacillin/tazobactam to 27/100 (27%).

The resistant patterns of MDR isolates to the antibiotics showed that the more frequently pattern were Piperacillin, Netilmicin, Gentamicin, Ciprofloxacin and Levofloxacin with repetition of 4/69 isolates (5.79%), and the number of isolates that resist to 5 antibiotics recorded the highest

percentage of 17/69 (24.63%), while the lowest percentage of 1/69 (1.4%) goes to the isolates that resist to 12 antibiotics.

Metallo Beta-lactamase (MBLs) have been phenotypically detected by Combined Disc Synergy Test (CDST), and the results showed that 4/100 (4%) of isolates were positive with MBLs while 96/100 (96%) were negative.

The genotypic detection was carried out to investigate presence of the genes bla_{VIM}, bla_{GIM}, bla_{IMP}, bla_{SIM}, bla_{SPM}, bla_{KPC}, bla_{DIM} and bla_{NDM} that are encoded by *Pseudomonas aeruginosa* to produce MBLs enzymes. The results revealed that 68/100 (68%) of isolates have MBLs genes. The high prevalence gene was bla_{NDM} gene with 38/100 (38%), and the lowest prevalence were bla_{SPM}, and bla_{DIM} genes with 7/100 (7%). The results of the other genes prevalence were 34/100 (34%) for bla_{IMP} gene, 26/100 (26%) for bla_{GIM} gene, 22/100 (22%) for bla_{VIM} gene, 12/100 (12%) for bla_{SIM} gene, and 11/100 (11%) for bla_{KPC} gene. (It's worth to mention that most isolates have more than one gene).

The antibiotics susceptibility test results for all isolates of all genes showed that the highest resistant was against piperacillin and the lower resistant for most of genes was against cefepime.

The results showed the coexistence of Metallo beta-lactamase genes in most of MDR isolates. The high association of genes was 6 genes in the same isolate that revealed resistance to 8 antibiotics.

The inhibitors effect on ability of *Pseudomonas aeruginosa* to produce MBLs enzymes was tested using three different inhibitors Allicin, Sultamicillin tosylate and Diallyl Trisulfide. These inhibitors have been tested individually and in synchronism with antibiotics on MDR and non-

MDR isolates. The results showed no role for all 3 active ingredient of garlic, and haven’t inhibitory effects on MBL enzymes.

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Abbreviations	
16S rRNA	16S Ribosomal RNA
Asp	Aspartic Acid
BAX	B-Cell Associated X
BCL2	B-Cell Lymphoma Protein 2
BLDB	Beta-Lactamase Database
bla$_{DIM}$	Beta-Lactam Dutch Imipenemase
bla$_{GIM}$	Beta-Lactam German impenemase
bla$_{IMP}$	Beta-Lactam Impenemase
bla$_{KPC}$	Beta-Lactam *Klebsiella Pneumoniae* Carbapenemase
bla$_{MDM}$	Beta-Lactam New Delhi
bla$_{SIM}$	Beta-Lactam Seoul impenemase
bla$_{SPM}$	Beta-Lactam Sao Paulo
bla$_{VIM}$	Beta-Lactam Verona integron
Bls	Beta-Lactamases
COX^2	Cyclooxygenase-2
Cys	Cysteinyl Acid
D-Ala-D-Ala	D-Alanyl-D-Alanine
DATS	Diallyl Trisulfide
DIM	Dutch Imipenemase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOTA	1,4,7,10-Tetraazacyclododecane-Tetraacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ESBLs	Extended spectrum β-lactamases
GIM	German Impenemase
H_2S	Hydrogen Sulfide
His	Histidine Acid
HIV	Human Immunodeficiency Virus
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IMP	Impenemase
IP/IPI	Imipenem/Imipenem-Inhibitor
IRPA	Imipenem Resistant *P. aeruginosa*

KPC	*Klebsiella Pneumoniae* Carbapenemase
MBLs	Metallo- Beta-Lactamase
MDR	Multidrug Resistance
MHA	Mueller-Hinton Agar
MHT	Modified Hodge Test
MIC	Minimum Inhibitory Concentration
NDM	New Delhi Metallo-ß-Lactamase
NF-Kb	Nuclear Factor Kappa B
NO	Nitric Oxide
NOTA	1,4,7-Triazacyclononane-Triacetic Acid
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistance
PDGF-B	Platelet-Derived Growth Factor-B
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SIM	Seoul Impenemase
SPM	Sao Paulo Metallo Beta Lactamase
TBE	Tris-Borate- Ethylenediaminetetraacetic Acid
TGF1β	Transforming Growth Factor Beta 1
TNF-A	Tumor Necrosis Factor-Alpha
UTI	Urinary Tract Infection
VIM	Verona Integron Metallo Beta Lactamase
XDR	Extensive Drug Resistance
Zn	Zinc
$ZnSO_4$	Zinc Sulfate

CHAPTER ONE

Introduction

Chapter One

Introduction

1.1 Introduction

Pseudomonas aeruginosa is an aerobic bacterium needs oxygen as a terminal electron acceptor in biochemical pathways. Its non-spore forming, motile by flagella, non-fermentative Gram-negative bacillus usually 1.5 - 5 µm in length and 0.5 - 1.0 µm in width. It can survive with low levels of nutrients and can grow at 42˚C, (Stover *et al*., 2000). Its catalase positive test and oxidase positive test and can hydrolysis of arginine and gelatin. *P. aeruginosa* is an opportunistic human pathogen capable of causing a great threat to health, especially in immunocompromised patients (Farhan, *et al*., 2019). It's the main cause of nosocomial infections and due to their ability to acquire resistance to a wide range of antibiotics making it a major global concern (Kumar, 2021; Jafari-Sales and Khaneshpour, 2020). *P. aeruginosa* has various virulence factors of both cells associated and extracellular that play important role in its pathogenesis which give this pathogenies flexibility. These factors include biofilm formation, protein secretion system, iron acquisition system, quorum sensing, and others (Marshall, *et al*., 2017). It is responsible for a wide range of human infections including urinary tract, respiratory system, burn infection, wound infection and otitis media (Paudel, *et al*., 2021). *P. aeruginosa* is one of the most dangerous burn wound infections pathogens that causes 75% of all deaths in patients, due to easily override the first line of the innate immunity by destruction the skin and considered as a major medical challenge because of difficulty to treat (Farhan, *et al*., 2019).

Pseudomonas aeruginosa has innate resistance to different antibiotics including beta lactam group and has ability to acquire the exchange of genetic material with other bacterial species, such as *Klebsiella pneumoniae*. It can develop its ability of antibiotics resistance by various mechanisms like efflux pumps, biofilm formation, production of beta-lactamase enzymes, and the permeability of the outer membrane. These mechanisms can exist simultaneously or separately (Antonio *et al.*, 2019).

Beta-Lactam antibiotics are the most often used antimicrobial drugs because they have a broad-spectrum antibacterial activity against both gram-positive and gram-negative bacterial pathogens (Palzkill, 2013). The widespread misuse of beta-lactam antibiotics is leading to emergence of resistant strains to it; therefore, this issue becomes a public health concern that threats seriously the antibiotic therapy.

Beta-Lactamase hydrolyze the beta-lactam ring and render the antibiotic inactive before it reaches the penicillin-binding protein (PBP) target. These enzymes are assigned into four groups according to Ambler classification A, B, C and D (Paudel, *et al*., 2021; Vural, *et al*., 2020). The metallo- beta-lactamase (MBLs) belong to class B and efficiently hydrolyze all beta lactams, except aztreonam *in vitro*. MBLs consist of several types of enzymes such as Impenemase (IMP), Verona integron metallo beta lactamase (VIM), Sao Paulo metallo beta lactamase (SPM), German impenemase (GIM), New Delhi metallo-beta-lactamase (NDM), and Seoul impenemase (SIM) (Marshall, *et al*., 2017). *P. aeruginosa* are often difficult to eradicate due to their resistant drug profile. Therefore, detection of MBLs-producing by *P. aeruginosa* is crucial for the optimal treatment of patients particularly in critically ill and hospitalized patients,

and to control the spread of resistance (Safarirad, *et al*., 2021). MBLs require divalent cations (such as zinc ion) as cofactors for enzyme activity, which is inhibited by the action of a metal ion chelator such as Ethylenediaminetetraacetic acid (EDTA) and thiol-based compounds. This behavior is considered as dependent phenotype detection. The toxicity that associated with EDTA makes it unsuitable for therapeutic use in spite of their inhibitory effect (Groundwater *et al*., 2016).

Many beta-lactamases are not inactivated by the available inhibitors (classic inhibitors) and they can resist the inhibitor/antibiotic combination through one or more resistance mechanisms (Danishuddin, 2016). The development of novel inhibitors is hard task because the mechanisms by which beta-lactamases are resistant to classic inhibitors (clavulanic acid, tazobactam and sulbactam) are different even within the same class of enzymes (Papp-Wallace & Bonomo 2016). However, in last decade the attention towards finding novel inhibitors has increased to develop new antimicrobial agent from various sources to face microbial resistance and to overcome the problem of the undesirable toxic effects of high dose of drugs. Greater attention has been paid from the pharmaceutical and scientific communities to screen antimicrobial activity of natural products and its evaluation methods based on the combination of antibiotics with phytochemicals which found that plants are considered as important source of a new antimicrobial agent (Balouiri *et al*., 2016; Savoia, 2012).

1.2. The Aim of The Study

This study aims to investigate the effectiveness of some inhibitors to combat Metallo Beta-lactamases (MBLs) produce by *Pseudomonas aeruginosa*, that responsible of their resistance to antibiotics.

The aim of this study can be achieved by the following steps:

1. Isolation and identification *P. aeruginosa* from burn wounds infection.
2. Antibiotic susceptibility test.
3. Phenotypic detection of Metallo Beta-lactamases.
4. Genotypic detection of Metallo Beta-lactamases genes (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{DIM}$).
5. Antimicrobial effect of inhibitors.
6. Inhibitors Combination with antibiotics

CHAPTER TWO

Literature Review

Chapter Two

Literature Review

2.1 History of *Pseudomonas aeruginosa*

The genus *Pseudomonas* was first proposed by Migula in 1894 as motile cells with polar organs. Sédillot in 1850 was first to observe that the discoloration of surgical wound dressings was associated with a transferable agent. The pigment responsible for the blue coloration was extracted by Fordos in 1860, and in 1862 Lucke was the first to associate this pigment with rod-shaped organisms. *P. aeruginosa* was not successfully isolated in pure culture until 1882, by Carle Gessard.

Between 1889 and 1894, *P. aeruginosa* (*Bacillus pyocyaneus*) was described as the causative agent of blue-green purulence in the wounds of patients (Lister *et al*, 2009). In the 1960s, studies of DNA and RNA hybridisation found at least six main sub-groups have pit sequence homology confirmed by sequencing of 16S rRNA.

The species were referred to by several different names, including *Bacillus pyocyaneus* and *Bacterium aeruginosa*, before finally being classified as *P. aeruginosa* (Villavicencio, 1998). Since 1980s, many types of *Pseudomonas* species have been moved into other new or existing genera, including *Burkholderia, Comamonas, Pandoraea, Ralstonia*, and *Stenotrophomonas* (Peix *et al.*, 2009).

2.2 General Biology

Pseudomonas aeruginosa is grown well in variety of conditions, its encapsulated Gram-negative, facultative aerobic bacillus belonging to the bacterial family *Pseudomonaceae.* Free-living bacterium can survive with

very few nutrients by using a broad range of organic ingredient as vitality power and carbon sources (Juan *et al.*, 2017). Their optimal growing temperature is 37º C, but can also grow at 42ºC, it has a flagellum for motility and pili for adhesion to other cells, it does not ferment lactose and hasn't the ability to form spores, catalase positive and oxidase positive (Gellatly and Hancock, 2013), it's often had mucoid slime layer with extracellular polysaccharide composed of alginate polymers. *P. aeruginosa* produces water-soluble pigments which can diffuse through the medium they are pyocyanin, pyoverdine, and pyorubin which also causes a typical 'sweet' odor in vitro (Vanstokstraeten, 2020).

Colonies are varied in their morphology but, it's often easily recognized, they are large, rough in appearance and round, sometimes be small, smooth described as Coliform-like, although some can be mucoid and very sticky especially isolates from cystic fibrosis patients which have chronic lung infections, exhibition a mucoid colony phenotype (Govan, 2007).

2.3 Pathogenicity

Pseudomonas aeruginosa is the common opportunistic human pathogen cause of bacterial infection in humans, it may vary in severity from a mild to debilitating systemic disease, associated with significant morbidity and mortality (Nguemeleu *et al*., 2020). It's recognized as the causative agent of a remarkable and extremely wide range of diseases in both healthy and immunocompromised patients (Schwendimann *et al*., 2018; Sekiguchi *et al.*, 2007).

Generally, the organism requires a breach in host defences such as a skin abrasion or placing of a urinary catheter to set infection (Percival *et al*., 2015). In healthy patients, bacterial infection is generally limited to

skin and soft tissues. *P. aeruginosa* causes infection in the immunocompromised patient, such as transplant and chemotherapy patients, burns, cancer or human immunodeficiency virus (HIV) (Huang *et al*., 2018). These cases of infection may occur in both nosocomial acquisition or by exposure to unsafe community environment. *P. aeruginosa* has various virulence factors which are encoded by genes that are related with bacteria which causes disease and almost be horizontally acquired usually be expressed during infection, sometimes the factors be inactivated in strains so will not cause disease (Solomon *et al*.,2017; Ali *et al*., 2018).

Also, virulence factors perform a useful function for any organism in their surroundings. Many factors are harmful to humans. In *P. aeruginosa*, virulence factors are divided into dependent specific groups, described as belonging to adhesions and secreted toxins system of 3 types. Secretion system factors of both associated and extracellular cells play important role in its pathogenesis which gives this pathogenies flexibility, those factors include biofilm formation, protein secretion system, iron acquisition system, quorum sensing, and others. Virulence factors produced by *P. aeruginosa* have their roles in contributing to disease and it may vary with the type of infection (Santajit and Indrawattana, 2016).

Pseudomonas aeruginosa is the most pathogen that causes ventilator associated pneumonia causing dysfunctional lung colonisation and chronic infection, it may also be implicated in different infections as bloodstream, Urinary tract infection (UTI), otitis and others (Vincent ,2003).

2.4 Burn Wound

Burns are a very specific type of injury occur when the body is exposed to a heat from many sources such as flame, chemical material, and

electricity (Tolles, 2018). The injuries by burn are vary depending on size of burn area, the location of the skin and how much its deep, the temperature and the duration of exposure (Jeschke *et al*., 2020). Minor burns do not threaten human life as major one but when not treated fairly may threaten the life of the patient and may be so painful and leave marks, so it requires considerably specialized care (Hermans, 2019a).

There are a variety of microorganisms that live on skin as normal flora (Gram-positive bacteria), therefore, if the burn wound patient does not take antibiotics, after few days the skin will be home of microbes) (Forson *et al*., 2017). Gram-negative bacteria causes nosocomial infection, colonize the mucosa of gastrointestinal flora of the patients (Hermans, 2019b), it can invade burn wounds.

P. aeruginosa is one of the most dangerous burn wound pathogens, it is recording a very high mortality rate, so burn wound infected by *P. aeruginosa* is a major concern in burn patient care centre, it causes 75% of all deaths in patients. It is considered as a major medical challenge because it is difficult to treat, so one essential goal of burn care is to prevent colonization and infection by *P. aeruginosa* (de Abreu *et al*, 2014).

In addition, burn patients inhalation injury led to cause edema and sloughing of the respiratory tract mucosa, then failing in normal mucociliary clearance mechanism, so those patients are being more susceptible to upper respiratory tract infections (Alhazmi, 2015).

2.5 Resistance to Antibiotics

A general definition of antimicrobial resistance is the ability of an organism to resist the action of an antimicrobial agent. Nosocomial infections caused by antibiotic resistant *P. aeruginosa*, have emerged as

major concern in clinical care settings as the increasing development of Multidrug resistance (MDR) strains (i.e., resistance to at least three antibiotics) (Magiorakos *et al*., 2012; Dïken and Aksöz, 2016)

Pseudomonas aeruginosa has high innate resistance to various antimicrobial agents in body tissues due to low permeability of its outer membrane (Bouffartigues *et al*., 2015; Livermore *et al*., 2001), which acts as a first barrier reducing the penetration of antibiotic compounds into the bacterial cell (Breidenstein *et al*., 2011). Also, there are other mechanisms responsible for their intrinsic resistance such as production of antibiotic inactivating enzyme and efflux pump system which drive out antibiotic from the bacterial cell (Drenkard, 2003).

In addition to intrinsic resistance, *P. aeruginosa* shows an extraordinary capacity for developing resistance to antibiotics in enzymic and mutational mechanisms by acquiring horizontal resistance determinants through the selection of certain chromosomal mutations that alter antibiotic targets, causing reduced antibiotic uptake and overexpression of antibiotic-inactivating enzymes and efflux pumps and acquisition of resistance genes carried on plasmids, transposons, integrons and prophages (Rampioni *et al*., 2017).

Bacteria can acquire these genes via horizontal gene transfer from the same or different bacterial species (Munita and Arias, 2016; Cavalcanti *et al*., 2015), which can be done by different mechanisms: conjugation, transduction, or transformation. The ability of a bacterium to resist antibiotic can increase due to transient alternations in gene or protein expression in response to an environmental stimulus (Vanstokstraeten, 2020).

2.6. Beta-lactam Antibiotic

It is the most commonly used antimicrobial agents distinguished by the beta-lactam ring in their molecular structure semi-synthetic compounds which originate from fungi and bacteria (Farmer *et.al*, 2007). Some of these antibiotics have a very narrow spectrum, while others have a very broad antimicrobial spectrum for both targets Gram-positive and Gram-negative bacteria.

Most of these antibiotics are block the transpeptidation of the cell wall component peptidoglycan, by inhibition of penicillin binding proteins (Bayles, 2000; Zapun *et al.*, 2008; Fisher and Mobashery, 2009). These antibiotics can be divided into four main groups based on their structure, including penicillin derivatives, cephalosporins, monobactams and carbapenems (Farmer *et al*., 2007). Due to inhibition of penicillin binding proteins by these antibiotics, it becomes to have low toxicity in the cells of the animalia (Soares *et al*., 2020; Fisher and Mobashery, 2009).

2.6.1. Mechanism of Beta-lactam Antibiotics Action

Beta-lactam antibiotics act by frustrating the structure of the peptidoglycan layer of cell wall of the bacteria. The peptidoglycan layer is essential for cell wall structure especially in Gram-positive organisms. In the synthesis of the peptidoglycan, the final transpeptidation step is facilitated by penicillin binding proteins (PBPs). Beta-lactam antibiotics vary in terms of PBPs affinity (Pandey and Cascella, 2019).

Penicillins are the first antibiotics to be commercialised in the 1940's. They are derived from *Penicillium* fungi and divided in sub groups depending on their antimicrobial spectrum and stability against penicillinases. The main feature of the penicillins is the four membered beta-lactam ring structural that essential for their antibacterial activity.

Penicillins are excreted without being metabolised by the kidneys; therefore, the concentration reach high levels in urine. Penicillins are interfered with the synthesis of peptidoglycan in cell wall of bacteria by attaching to binding sites forming penicillin –binding proteins (Weiss and Adkinson, 2005).

Cephalosporins has broad spectrum of activity, it has been the most commonly use class of antimicrobials. These antibiotics are derived from fungus Acremonium, that was known as "Cephalosporium". It's often given in combination with an aminoglycoside or metronidazole for treatment of serious infections such as septicaemia, severe pneumonia or in patients with febrile neutropenia (Paul *et.al.*, 2006; Micek *et.al.*, 2010). Based on antimicrobial properties, these antibiotics are grouped in generations.

There are four recognized class generations of cephalosporins based on their activity spectrum, the fourth generation cephalosporins are known to have true broad-spectrum activity. cephalosporins have similar mechanism to Penicillins (Micek *et.al.*, 2010).

Monobactams (Aztreonam) are monocyclic compound active against gram negative multidrug-resistant including *P. aeruginosa*, *Acinetobacter spp*., and *Burkholderia spp*, by binding to PBPs of bacteria and damage the cell wall synthesis. It is stable towards metallo-beta-lactamases and also has some inhibitory activity towards class C beta-lactamases (Chambers, 2005).

Carbapenems are compounds started to use in the 1980's, it has the widest spectrum of antibacterial activity of all the beta-lactams, including many of the Extended spectrum beta-lactamases (ESBLs) (Tarnberg, 2012). Similar to other antibiotics like penicillin, carbapenems inhibit the bacterial cell wall synthesis by binding to PBP of gram positive and gram-

negative bacteria, leading to cell elongation and lysis. Resistance to carbapenems by imipenem is most commonly due to reduced permeability, while meropenem resistant has a combination of porin loss and increase the rate of efflux pump. These antibiotics show less resistance as compared to other types of beta-lactams because of their stability to be hydrolysed by many ESBLs (Jacoby, 2006).

2.7 Beta-lactamases

Beta-lactamases (BLs) are enzymes produced by some bacteria that provide multi-resistance to beta-lactam antibiotics which have broad spectrum activity against the Gram-positive and Gram-negative bacteria. It inhibits the function of antibiotics which have a common element in their molecular structure: a four-atom ring known as a beta-lactam by breaking down their structure by a hydrolysis reaction in amide bounds that making them further inactive (Persoon *et al.*, 2019).

There are different types of beta-lactamases such as the penicillinases, the extended-spectrum beta-lactamases, the cephalosporinases and the carbapenemases. Carbapenemases are capable to hydrolyze carboxypenicillins, carboxypenicillins + beta-lactamase inhibitor, ureidopenicillins, ureidopenicillins + beta-lactamase inhibitor, ceftazidime, cefepime, aztreonam and imipenem. Therefore, they are known as the most powerful beta-lactamases (Livermore, 1995).

The metallo-beta-lactamases are the most widespread and responsible for the powerful resistance of the beta-lactamases (Bandekar, *et al*., 2011).

2.8 Classification of Beta-Lactamase

Generally, two major classifications for categorizing beta-lactamase enzymes; Ambler and Bush-Jacoby-Medeiros, further to other

classifications based on different standards that place BLs enzymes into classes or groups as in table 2.1.

Table 2.1: Classification of Beta-lactamase

No.	Reference	Mechanism of Action	Year
1	Sawai *et al*	based on the responses of Cephalosporinases and Penicillinases to antisera exposure	1968
2	Richmond and Skye	based on antibiotics profile (only regarding BLs producing by gram negative bacteria)	1973
3	Skyes and Matthew	based on the isoelectric points of plasmid mediated BLs	1976
4	Ambler	based on BLs' structural characteristics and targets molecular and amino acids sequences in BLs	1980
5	Inoue	based on the hydrolyzing activities of three BLs on Cephalosporins, Penicillins and Cefuroxime	1981
6	Bush	based on function and activity of BLs	1989

2.8.1 Ambler Classification of Beta-lactamase

Based on similarity in amino acids, enzymes are classified to four classes A, B, C and D (Vala, *et al*., 2014).

2.8.1.1 Class A of Ambler Classification

Class A is serine beta lactamases, this group can hydrolyse carbapenems, cephalosporins, penicillin, and aztreonam (Merie Queenan, and Bush, 2007). These types of beta lactamases are susceptible to the commercial inhibitors namely, clavulanate, tazobactam, and sulbactam.

The *Klebsiella pneumoniae* Carbapenemase (KPC) is a powerful carbapenem-hydrolysing enzyme which is able to hydrolyse most of the beta-lactams group. Despite its most prevalent in *Klebsiella pneumoniae*, it has been described in other Gram-negative bacteria such as *Escherichia*

coli, *Pseudomonas* and *Acinetobacter* species (Hu, *et al*., 2021). This enzyme encoded by bla_{KPC} gene which is able to transfer into transferable plasmids as a transposon structure and hence enabling it to spread to other bacteria (Weber, *et al*., 2019).

2.8.1.2 Class B of Ambler Classification

It's also known as Metallo-Beta-Lactamase (MBLs) which are Zn^{+2} ions dependent enzymes that follow a different hydrolytic mechanism, usually resistance to penicillin, cephalosporins, carbapenems, and the clinically available beta-lactamase inhibitors. The broad substrate spectrum of these beta-lactamases is of great concern in the medical community.

MBLs use the hydroxy group from a water molecule that is coordinated by Zn^{+2} ions to hydrolyze the amide bond of a beta lactam. MBLs possess either a single Zn^{+2} ion or a pair of Zn^{+2} ions co-ordinated to Histidine/ Cysteinyl/ Aspartic (His/Cys/Asp) residues in the active site. These Zinc ions along with their co-ordinated residues in the active site of the enzyme play an important role in hydrolysing the antibiotics (Watanabe *et al.*, 1991). MBLs require divalent cations as cofactors for enzyme activity, it is inhibited by the action of a metal ion chelator Ethylenediaminetetraacetic acid (EDTA).

The first MBL was described in *Bacillus cereus* in the 1960s and then in different Gram-negative bacteria. Most of these enzymes are produced by chromosomal encode. After that, in 1991, the first reported of plasmid-mediated MBL, Imipenemase (IMP-1), from Japan that is produced by *P. aeruginosa*, followed by verona integron metallo beta lactamase (VIM-1) from Italy in 1999 (Butt *et al.*, 2005).

However, the most common MBLs include the VIM, IMP, Germany imipenemase (GIM), Sao Paulo metallo-beta-lactamase (SPM), Seoul imipenemase (SIM), Dutch imipenemase (DIM), New Delhi metallo-beta-lactamase (NDM), *klebsiella pneumoniae* carbapenemase (KPC). Furthermore, Class B is classified into three subclasses: B1, B2 and B3 according to enzyme structural characteristics in both molecular and functional sides, including Zn^{+2} ions configuration, location and substrates profiles (Kim, *et al*., 2020).

2.8.1.2.1 B1 Subclass of Ambler Classification

These enzymes are monomeric with two Zn^{2+} ions, some types of MBLs such as IMP type bear two different metal ions (Zn^{+2} and Fe^{+3}). The B1 subclass are chromosomal encoded and show broad spectrum activity including IMPs, VIMs, NDMs, GIM-1, DIM-1, SPM-1, and SIM-1. Also, it is recognized as important mobile genetic elements of opportunistic and pathogens in bacteria, therefore, it's the most global clinical concern (Merie Queenan and Bush, 2007).

The B1 is considered as the largest group of MBLs with a wide range of enzymes that hydrolyzing all the beta-lactam antibiotics but not monobactams. The B1 members are considered as critical problems in clinical resistance features (Behzadi, *et al*., 2020).

2.8.1.2.2 B2 Subclass of Ambler Classification

These enzymes are monomeric, it is specific for carbapenems with narrow spectrum activity. This class has one Zn^{+2} ion in their active site that required for hydrolyzing beta-lactam group. The B2 subclass is the smallest MBL category (Bush and Jacoby, 2010).

2.8.1.2.3 B3 Subclass of Ambler Classification

This class is almost produced by environmental bacteria, its enzymes are monomeric have two zinc ions in their active site. The B3 subclass of MBLs encompasses 2 zinc ions of Zn^{+2} (Boschi *et al*.,2000).

2.8.1.3 Class C of Ambler Classification

These types of beta lactamases are located on the bacterial chromosome, and Unlike Class A Extended Spectrum Beta-lactamase (ESBLs), it resist to classical beta-lactamase inhibitors such as clavulanate (Jacoby, 2009).

2.8.1.4 Class D of Ambler Classification

This class of beta-lactamase has ability to hydrolyse oxacillin, and resistance to penicillin, cephalosporins, extended spectrum cephalosporins and carbapenems (Bonomo and Tolmasky, 2007).

2.8.2. Bush-Jacoby-Medeiros Classification of Beta-lactamase

Based on substrate and inhibitor profile, these enzymes are classified into groups from 1 to 4 (Bush and Jacoby, 2010).

In accordance with last updated data on web-based Beta-Lactamase Database (BLDB) (http:// bldb.eu/), the Beta Lactams enzymes involve 4940 members, and these enzymes are divided into four groups: class A (1575 members), class B (707 members), class C (1477 members) and class D (999 members). Class B is classified into three subclasses of B1 (508 members), B2 (22 members) and B3 (177 members) (Vincent, 2003).

2.9. Metallo Beta-lactamase Variants

2.9.1. Imipenemase (IMP)

The first discovery of bla_{IMP-1} genes was in Japan in 1988. The isolates were conferring carbapenem resistance e.g., imipenem, extended-spectrum cephalosporins e.g., a ceftazidime (Watanabe *et al.*, 1991). The resistance allele was borne on a transferable conjugative plasmid that could move to other *Pseudomonas* strains.

Later the same gene was found in other cities and bla_{IMP-6} gene appeared in South Korea (Farmer *et al.*, 2007). bla_{IMP-1} was the first MBL reported in 1991. After that, more than 55 different bla_{IMP} alleles have been described. These enzymes vary in their sequence, but with nearly same effect on their potent of resistance. Most variants are reported in *P. aeruginosa*, and other Gram-negative species (Subedi *et al.*, 2018).

2.9.2. Verona Integron Metallo Beta-lactamase (VIM)

The most dominant MBLs variant is VIM. It was first reported in Verona, Italy, produced by *P. aeruginosa*. Studies have shown that VIMs and resistance to carbapenems may be responsible for increasing the mortality. The isolate with bla_{VIM-1} has been detected in three strains as a source of nosocomial infections, it is resistant to a series of beta-lactams with ability to hydrolyze carbepenems, cephalosporins and penicillins and resistant to the commercially available beta-lactamase inhibitors (Persoon *et al.*, 2019).

Biochemical tests show that a carbapenem-hydrolyzing activity was inhibited by Ethylenediaminetetraacetic acid (EDTA) and restored by addition of Zn^{+2}. In southern France, bla_{VIM-2} was first identified in a blood culture in 1996. This isolate was resistant to most beta-lactams but remained susceptible to aztreonam. The VIM-2 was encoded by a gene

cassette, The gene of *bla*$_{VIM-2}$ was located on a nonconjugative plasmid which can be transferable by electroporation (Liapis, *et al*., 2019).

2.9.3. Sao Paulo Metallo Beta-lactamase (SPM)

The SPM-1 enzyme is discovered at the beginning of the 21st century in isolates of *P. aeruginosa* from Brazil. It has low natural tendency to spread of SPM-1 which could be related to mobile genetic elements, and has affinity to substrate specificity of carbapenems, cephalosporins, and penicillins (Yoon and Jeong, 2021).

2.9.4. German Imipenemase (GIM)

The *bla*$_{GIM-1}$ gene was first found in isolates of *P. aeruginosa* strain in Germany in 2002. The *bla*$_{GIM-1}$ gene was also detected in more than of bacteria including *Pseudomonas putida, Enterobacter cloacae, Serratia marcescens, Escherichia coli, Klebsiella oxytoca, Citrobacter freundii* and *Acinetobacter pittii*. The *bla*$_{GIM-1}$ is associated with MBL-producing bacteria in a German hospital since 2007 (Wendel, et al., 2018).

2.9.5. New Delhi Metallo Beta-lactamase (NDM)

It is the new member and the most important of metallo-beta-lactamase group because it can hydrolyze all beta- lactam group except the monobactam (Aztreonam) antibiotics (Rawson, *et al*., 2022). The enzymes NDM are produced by bacteria strains that represent great health care risk because lacking routine observable characteristics to detect the strains that carry metallo-beta-lactamases. The prevalence for theses enzymes may be associated with another gene cassettes which can be carried on without symptoms. The *bla*$_{NDM}$ gene may be coded on plasmid or transposon with ability to be transferring horizontally between gram negative spp., it may

associate with various types of insertion sequences. The different nature of mobile genetic elements that are associated with acquired metallo-beta-lactamases genes could account their natural tendency to spread. Most integrons containing gene cassettes are carrying resistance determinants for other antibiotic groups e.g., aminoglycosides (Porretta, *et al*., 2020).

2.10. Phenotypic Detection of Metallo Beta-lactamase

Metallo beta lactamase producing bacteria can be detected by many phenotypic methods. These methods are mostly dependant on the ability of metal chelators such as EDTA and thiol-based compounds to block the activity of MBLs **(**Sachdeva *et al.*, 2017)

2.10.1. Carba Nordmann and Poirel Test (Carba NP Test)

It is a rapid biochemical test for detection of metallo beta-lactamase enzymes in Gram-negative bacteria such as *Pseudomonas spp,* Carba NP Test is considered a useful alternative method to detect MBLs. The test principle is based on hydrolysis of imipenem by a bacterial lysate, and when the colour of phenol red indictor changes from red to orange or yellow due to change in pH values, this will represent positive results (Johnson and Woodford, 2013).

2.10.2. Modified Hodge Test

Modified Hodge test (MHT) is a phenotypic test imperative for all bacterial isolates showing intermediate or sensitive zone diameter by disc diffusion. This test is described today as version of Hodge test which was used some years ago. The original Hodge test was evaluated by PCR to confirm the presence of Metallo beta-lactamase (MBL) genes like bla_{IMP-1}

and *bla*$_{VIM-2}$. It is based on the inactivation of a carbapenem by carbapenemase-producing strains (test isolate) that enable a carbapenem-susceptible indicator strain (*E. coli* ATCC® 25922) to extend growth towards a carbapenem-containing disc along the streak of inoculum of the test strain. Positive test result gives cloverleaf-like indentation (Awan, et al., 2019).

Modified Hodge Test is a screening test for carbapenemases production, but concerns for its difficult interpretation and common false-positive results of presence of beta-lactamases have been noted in low-income regions clinical laboratories, the quantification of MHT may be an alternative screening test (CLSI, 2021).

2.10.3. Combined Disc Synergy Test

This test is preformed according to the method suggested by Galani *et al*., (2008) based on inhibition of MBL activity by chelating agents like EDTA. It is recommended to be performed for MBL-producing isolates highly sensitivity and specificity for *Pseudomonas spp* and also for *Acinetobacter spp* (Argimón *et al*., 2020; Kabore *et al*., 2022).

Based on previous studies on *P. aeruginosa* isolates resistant to imipenem that used double-disk synergy test and imipenem/imipenem-inhibitor (IP/IPI) combined disk test, they found that IP/IPI should be taken as positive for MBL where it achieved 100% sensitivity as well as specificity for the Imipenem Resistant *P. aeruginosa* (IRPA), furthermore, it's a viable alternative phenotypic assay to detect MBL production in IRPA in case the Polymerase Chain Reaction (PCR) detection is not a feasible option (Khosravi *et al.*, 2012; Walsh, 2010).

The gold standard for beta-lactamase characterization is the PCR amplification followed by DNA sequencing of the amplicons (Makharita *et al.*, 2020; Garrec *et al.*, 2011).

2.11. Genotypic Detection of Metallo Beta-lactamase

Molecular test methods by PCR have a high specificity in detecting the presence of MBLs genes. It is considered the easiest and fastest method, but the disadvantage of this method is inability to distinguish the variants or identify new variants despite the high specificity of DNA primers. There is another molecular method that used to detect MBLs genes such as isoelectric focusing, nucleotide sequence analysis and polyacrylamide gel electrophoresis (Walsh *et al.*, 2005).

2.12. Problems in Metallo Beta-lactamase Detection Methods

There are no standardized phenotypic methods available or testing criteria to depend on whether the gene is carried by *P. aeruginosa* or for any member of the Enterobacteriaceae. Most Enterobacteriaceae carrying MBL genes appear sensitive, with Imipenem minimum inhibitory concentration (MICs) (1 to 2 μg/ml). The genus of the bacterium is important in this case e.g., *pseudomonads* intrinsically have higher carbapenem MICs than *Enterobacteriaceae*.

The identification by isoelectric focusing technique with counterstaining the gel and chromogenic substrate nitrocefin to determine the MBLs enzyme's isoelectric point is based on the surface charge properties of enzymes, which neutralized with certain pH. MBLs are differ extremely from one to another, so the isoelectric focusing is not recommended to identify them, although it can get useful information of unknown MBLs by using EDTA inhibition as part of the isoelectric

focusing process depended on fact that all MBLs are affected by the removal of zinc from the active site. MBLs possess subtle but significant variations in their active site architecture, and unlike clavulanic acid, which interacts directly with class A enzymes to forms a stable covalent intermediate, MBLs do not form highly populated metastable reaction intermediates (Carcione *et al.*, 2021).

2.13. Inhibitors in Clinical Practice

The integration of beta-lactamase inhibitors with beta-lactam antibiotics has been a very successful strategy for overcoming resistance. There are three famous inhibitors; clavulanic acid, sulbactam and tazobactam that used in combination therapy with different antibiotics. Furthermore, there are two semi-synthetic penicillinate sulfones, were developed by the pharmaceutical industry (tazobactam and sulbactam) (English *et al.,* 1978).

The major difference between beta-lactamase inhibitors is the presence of a leaving group at position C-1 of the five membered rings. These three inhibitors have similar mechanism of inhibition, they are active against class A beta-lactamases, and have no effect on class B, C and D (Philippon *et al.*,1989; Buynak, 2006). Furthermore, those inhibitors are applied in clinical practice for many years in combination with beta-lactam antibiotics. Sulbactam and tazobactam are sulfones, while clavulanate has enol ether oxygen at this position.

Clavulanic acid isolated from *Streptomyces clavuligerus* was the first suicide inhibitor used against the serine beta-lactamases. The structure of clavulic acid was a bicyclic beta-lactam that did not possess a penicillin or a cephalosporin ring. It was showing less potency when used alone, and marked reduction in MICs when used in combination with amoxicillin

against different bacteria such as *Staphylococcus aureus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Escherichia coli* (Reading and Cole, 1977).

2.14. Metallo-Beta-lactamase Inhibitors

Interest in inhibitors of beta-lactamases started in the 1960s when a combination of an antibacterial agent and an enzyme inhibitor acted effectively against certain resistant gram-negative infections, where the addition of an inhibitor to antibiotic extend their utility in a variety of infections (Campoli-Richards and Brogden, 1987).

The beta-lactam antibiotics are the most often used antimicrobial drugs. It had a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacterial pathogens (Palzkill, 2013). Because of increasing the resistance to beta-lactam antibiotics, it become a public health concern. These antibiotics are favoured because of their efficacy and safety (Livermore *et al.*, 2006). Beta-Lactam antibiotics act by inhibiting a group of enzymes called transpeptidases (penicillin binding proteins) which are essential for the synthesis of the bacterial cell wall (Sauvage *et al.*, 2008). Therefore, the growing bacteria will die, and that explains the mechanism of beta-lactam antibiotics.

The resistance of bacteria is serious threat in the antibiotic therapy because of the widespread overuse or wrong way of take the beta-lactam antibiotics (Babic *et al.*, 2006). Beta-Lactam antibiotics have four-membered beta-lactam ring which serves as a substrate for the transpeptidase target enzymes. Transpeptidase enzymes react with the D-Ala-D-Ala terminus of a pentapeptide that is attached to N-acetylmuramic

acid of the peptidoglycan polymer via an active site serine residue to form an acyl-enzyme intermediate (Lee *et al.*, 2001).

A lysine-like residue from another pentapeptide was attack the carbonyl carbon of the resulting intermediate to create a covalent bond between peptides that serves to cross-link the peptidoglycan polymer. The four-membered ring of beta-lactam antibiotics seem like the D-Ala-D-Ala structure where its linkage to the active site of enzymes to form an acyl-enzyme via active site serine. The pentapeptide lysine residue attack the resulting acyl-enzyme, and the covalent bound of beta-lactam ring act as a permanent inhibitor of the transpeptidase, also peptidoglycan cross-linking will block then leads cell to death (Shi *et al.*, 2011).

There are different groups of beta-lactam antibiotics classified depended on their structure (Mahajan & Balachandran, 2012). Among the clinically important beta-lactams are Penicillins, carbapenems, cephalosporins, and monobactams. Penicillins and cephalosporins contain a beta-lactam ring which are joined to five and six rings, that contain a carboxyl group at the C-3 and C-4 positions.

Monobactams do not contain a joined ring and instead linked to sulfonic acid group at the similar position of the carboxylate group that found in penicillins and cephalosporins (Sykes & Bonner, 1985). Carbapenems have structure consist of a beta-lactam ring joined to a penicillin-like five-membered ring that has a carbon replacing the sulfur at C-1 and contain a double bond between C-2 and C-3.

These antibiotics can resist the inactivation by beta-lactamases (Maveyraud *et al.*, 1998), and act as inhibitors for many serine beta-lactamases by forming lasting acyl-enzyme intermediate when react with an active site serine residue (Gebreslasie, 2017). An assortment of

structurally different compounds has been examined as MBL inhibitors (Venkata, *et al*., 2021)

2.14.1. Metal Chelators

A common way for metallo-beta-lactamases inhibition is using metal chelators. Where, metallo-beta-lactamases require zinc ions in their active site for catalytic action. It's found that the use of metal chelators can reduce their activity by segregate the zinc ions in active site of the enzymes (Siemann *et al.*, 2002).

The most common metal chelators that show inhibitory effect against metallo-beta-lactamases is ethylenediaminetetraacetic acid (EDTA). However, the toxicities associated with EDTA make it unsuitable for therapeutic use despite of their inhibitory effect (Groundwater *et al.*, 2016). There is another compound that found to be a novel inhibitor because it's an affinity for zinc ion, its 1, 2-Benzylthiaz-ole-4-carboxlic acid (Chen *et al.*, 2012). Six various compounds were synthesized from 1, 2-benzothiazole-4-carboxylic acid, and tested their inhibitory activity against metallo beta lactamase enzymes (Livermore & Woodford, 2006).

Also, the compound 4, (R)-2-phenyl-4, 5-dihydrothiazole-4-carboxylic acid was to be a good inhibitor 6 times more active than 1 against our enzymes (Chen *et al.*, 2012).

Dipicolinic acid was another zinc chelating agent showed good inhibitory activities too (Horsfall *et al*., 2007).

Another known metal chelating agents were, pyridine 2,4-dicarboxylic acid, 1,4,7-triazacyclononane-triacetic acid (NOTA) and 1,4,7,10-tetraazacyclododecane-tetraacetic acid (DOTA) that restored the activities of meropenem and imipenem against bacteria expressing members of MBLs by acting as zinc chelators and deactivating the MBLs. The main obstacle of the use of these metal chelators as inhibitors is the

expected that it is chelators inhibitors to other metallo enzymes like carboxypeptidases, carbonic anhydrase, and matrix metalloproteinase (Somboro *et al.*, 2015).

Aspergillomarasmine is a fungal natural product related to EDTA in structure and inhibited the MBLs irreversibly after removal metal ion by gel filtration, however, the enzyme activity was restored by the addition of excess Zinc Sulfate ($ZnSO_4$). In the combination with meropenem its successful in stopped lethal dose of the enzyme and found that Aspergillomarasmine toxicity in mice shown low toxicity compared to EDTA (King *et al.*, 2014)).

2.14.2 Thiols and Thiol-carboxylates

Thiols and their carboxylate derivatives are the largest classes of MBL inhibitors (Puerta & Cohen, 2002). Several thiol esters of mercaptoacetic acid are irreversible inhibitors of the metallo-beta-lactamases (Arjomandi *et al.*, 2016).

Thiols and Thiol-Carboxylates have arisen from the hydrolyzing of thiol esters, releasing mercaptoacetic acid, which works to inhibit the enzyme by forming a disulfide bond with cysteine residue of active-site under the aerobic conditions (Mezzetta, 2019).

The metallo-beta-lactamase have inhibitory activity of the mandelic acid and its derivatives while the carboxylate group of these compounds increased potency, the thiol group was essential for activity; replacing the thiol with hydroxyl, bromo or aldoxime inhibits the activity. Racemic thiomandelic acid was shown to be a submicromolar inhibitor of several B1and B3 subclass metallo-beta-lactamase. But this inhibitor was ineffective against B2 subclass of MBL (Mollard *et al.*, 2001).

Several thiol-containing inhibitors show activity against members of all subclasses of metallo-beta-lactamases at low concentrations. Mercaptomethyl benzoic acid mercaptoacetophenone, acylated racemic phenylglycine, acylated unnatural phenylalanine, and acylated unnatural valine (Gebreslasie, 2017).

2.14.3. Dicarboxylic Acids

Disodium salt of 2,3-dimethylmaleic acid inhibits metallo beta lactamase enzymes producing by *P. aeruginosa* (Livermore *et al.*, 2012). The 3-substituted phthalic acids, specially 3-amino and 3-alkoxy derivatives, are potent inhibitors of metallo-beta-lactamase producing strains of *P. aeruginosa*. Both carboxyl groups bound to the two zinc ions in the active site; the piperidine ring extends into a hydrophobic pocket and the hydroxyl group on the piperidine forms a hydrogen bond with a serine residue. The 3,6-bis(4-hydroxypiperidin-1-yl) derivative was the most potent inhibitor (Hiraiwa *et al.*, 2013).

2.14.4 Captopril Derivatives

The ability of L-captopril and its diastereoisomer, and D-captopril were examined to inhibit the metallo-beta-lactamases and found similar inhibitory activities for both (Heinz *et al.*, 2003). D-captopril was potent against NDM-1 enzyme and against IMP-1. When the x-ray crystal structure of D-captopril in complex is solved with beta lactam group B, its found the thiolate group of D-captopril binds to both metal ions in the active site and displacing the bridging hydroxyl (Gebreslasie, 2017).

2.14.5. Boronates

Cyclic boronates were developed as inhibitors of class A serine-beta-lactamases (Hecker *et al.*, 2015). The cyclic boronates were found to be potent inhibitors of several classes B1 metallo-beta-lactamases, and the penicillin-binding protein (PBP). Both boron-bound hydroxyl groups bound to the same zinc ion (Zn 1), while the endocyclic boronate ester oxygen and the carboxyl group of the inhibitor were binding to the other zinc ion (Zn 2) (Brem *et al.*, 2016). This binding makes it broad spectrum lactamase inhibitors, having activity against class A, C and D serine-beta-lactamases (Johnson *et al.*, 2010).

2.14.6. Allicin (Diallylthiosulfinate)

Allicin (diallylthiosulfinate) is an active material extracted from fresh garlic (*Allium sativum*) which has been used since ancient times for its health beneficial properties (Borlinghaus *et al.*, 2014). Allicin has broad antimicrobial activities against Gram-positive and Gram-negative bacteria, and fungi due to their thiosulfinate with two allyl group which inactivate essential enzymes (Cavallito *et al*., 1944).

Allicin is a potent antimicrobial substance, produced by garlic tissues upon wounding as a defence against pathogens, pests, herbivores and parasites. The organosulfur compound responsible of garlic's antibacterial activity was first recognized as allicin (diallylthiosulfinate) by Cavallito in 1944. Allicin is produced upon tissue damage by the enzyme alliinase when cleaves the non-protein amino acid alliin (allylcysteine sulfoxide) into allylsulfenic acid and dehydroalanine, then two molecules of allylsulfenic acid condense spontaneously to form allicin (Borlinghaus *et al*., 2014).

A single garlic clove with 10 gram can produce approximately 5 mg of allicin (Slusarenko *et al*., 2008). However, allicin is unstable at room temperature and its antimicrobial activity may lost within minutes when it's heated up to more than 80 °C (Leontiev *et al.*, 2018). Allicin is volatile so it can kill bacteria via the gas stage. This feature makes it interesting because most lung-pathogenic bacteria are susceptible to it, therefore, tuberculosis is treatment by breathing the vapour of crushed garlic (Minchin, 1912).

2.14.7. Diallyl Trisulfide

Diallyl trisulfide (DATS) is one of the main allyl sulfur compositions that existent in garlic. The reason of DATS biological activity is due to the presence of water-soluble γ-glutamyl S-allyl cysteine and oil-soluble allyl sulfur compounds (Subramanian *et al.*, 2020; Omar and Al-Wabel, 2010).

Diallyl trisulfide has antiplatelet, antithrombotic, and antico-agulant activities (Mikaili *et al.*, 2013). It shows cytoprotective activity with valproate-induced hepatotoxicity by reducing hepatic steatosis and by keeping the integrity of hepatocytes and reducing inflammation-induced necrosis through stopping the production of tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), and Interleukin-1β (IL-1β), as well as by reducing Cyclooxygenase-2 (COX^2) expression and blocking Nuclear factor kappa B (NF-kB) activity (Shaaban and El-Agamy, 2017).

It has protective effects against alcohol-induced oxidative stress, where it improves the generation of cystathionine β-synthase expression and Hydrogen Sulfide (H_2S) through upregulation of cystathionine γ-lyase, and reduces reactive oxygen species (ROS). By reducing the expression of B-cell associated X (BAX) and enhancing the expression of B-cell

lymphoma protein 2 (BCL2). DATS also protects hepatocytes from ethanol-induced apoptosis (Chen *et al.*, 2016).

Diallyl trisulfide increases insulin secretion and improves oral glucose tolerance (Liu *et al.*, 2005). DATS also weakens collagen deposition and blocks hepatic stellate cells by reducing the expression of Transforming Growth Factor Beta 1 (TGFβ1), Platelet-Derived Growth Factor-b (PDGF-b), and Epidermal Growth Factor (EGF) receptors (Zhu *et al.*, 2014). In addition, DATS increases the bioavailability of Nitric Oxide (NO) and releases H_2S as cardioprotective activity (Predmore *et al.*, 2012).

2.14.8. Sultamicillin Tosylate

Sultamicillin is a tosylate salt, oxymethylpenicillinate sulfone ester of ampicillin, linked with the beta-lactamase inhibitor sulbactam by a methylene group (Sabath *et al.*, 1967). Sultamicillin is given for infections caused by microorganisms in respiratory tract infections such as otitis media, bacterial pneumonias, and tonsillitis, also is given for urinary tract infections (UTI), skin and soft tissue infections (Noguchi and Gill, 1988). Sultamicillin is considered the second beta-lactamase inhibitor in the United States (Carcione *et al.*, 2021).

CHAPTER THREE

Materials and Methods

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Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Equipments and Apparatus:

Equipments and Apparatus that used in this study are listed in table 3.1.

Table 3.1. Laboratory equipments and apparatus

Name of apparatus	Company	Origin
Autoclave	HIRAYAMA	Japan
Biological Safety Cabinet	BIOAIR	Italy
Centrifuge	HITTICH	Germany
DensiChek plus Meter	BIOMERIEUX	USA
Dispenser DSP8	Bioanalyse	Turkey
Electrophoresis Equipment	CLEAVER SCIENTIFIC Ltd.	UK
Exispin	BIONEER	Korea
Gel Documentation Imaging	QUANTUM	France
Incubator	MEMMERT	Germany
Magnetic stirrer	IKA RH basic 2	Germany
Micro – centrifuge	HITTICH	Germany
Microscope	OPTIKA	Italy
Microwave Oven	GOSONIC	China
Nanophotometer	IMPLEN	Germany
Prime Thermal Cycler (PCR)	TECHNE	UK
Sensitive Balance	SARTORIUS	Germany
Ultrasonic Device	MEMMERT	Germany
Vortex	HEIDOLPH	Germany

3.1.2. Chemicals and Biological Materials:

The chemicals and biological materials that used in this study are listed in table 3.2.

Table 3.2 Chemicals and biological materials

Chemicals	**Company**	**Origin**
Absolute ethanol	Himedia	India
Agarose	Condalab	Spain
Allicin	MCE	USA
Antibiotic disk	Liofilchem	Italy
Diallyl Trisulfide	MCE	USA
Dimethyl sulphoxide (DMSO)	Himedia	India
DNA Ladder 100–2000 pb	Bioneer	Korea
Glycerol	Merck	England
Gram stain: crystal violet,iodine, acetone and safranin	RBIO	Switzerland
GreenStar^{tm} Nucleic Acid Staining Solution	Bioneer	Korea
Nalidixic Selective Supplement	Himedia	India
Nuclease Free Water	Himedia	India
Oxidase Reagent	Himedia	India
Sultamicillin tosylate	MCE	USA
Tris-Borate-EDTA (TBE)	Himedia	India

3.1.3. Culture Media

All culture media that used in this study are listed in the table 3.3.

Table 3.3 Culture Media

Item name	Company	Origin
Brain heart infusion broth	HIMEDIA	India
Cetrimide agar	HIMEDIA	India
MacConkey agar	HIMEDIA	India
Muller Hinton Agar	HIMEDIA	India
Nutrient agar	HIMEDIA	India

3.1.4. Kits

The Kits that used in this study are listed in table 3.4.

Table 3.4 Laboratory Kits

Kits	Company	Origin
DNA Extraction Kit	FAVORGEN	Austria
Taq Master Mix	PROMEGA	UK
DNA Ladder 100–2000 pb	Bioneer	Korea

3.1.5. Antimicrobial Agents

All antibiotics that used in this study for susceptibility test, phenotypic detection and to check the effect of inhibitors are listed in table 3.5.

Table 3.5 Antimicrobial Agent and their Concentration

Antibiotic Group	Antibiotic Disk	Concentration
Aminoglycosides	Amikacin	10 µg
	Gentamicin	30 µg
	Netilmicin	30 µg
Cephems	Cefepime	30 µg
	Ceftazidime	30 µg
Monobactam	Aztreonam	30 µg
Carbapenem	Imipenem	10 µg
	Imipenem / EDTA	10 µg / 750 µg
	Meropenem	10 µg
Fluoroquinolones	Levofloxacin	5 µg
	Ciprofloxacin	5 µg
Penicillins	piperacillin	100 µg
beta-Lactam Combination Agents	Piperacillin / Tazobactam	100 µg / 10 µg

3.1.6. Inhibitors

The inhibitors that used in this study are listed in the table 3.6.

Table 3.6 The Inhibitors that used in this study

Inhibitors	Company	Original
Allicin	MCE	USA
Diallyl trisulfide	MCE	USA
Sultamicillin tosylate	MCE	USA

3.1.7. Primer Pairs

The primers pairs, sequence, product size and their PCR conditions that used in this study are listed in the table 3.7.

Table 3.7 Primers pairs sequences and their PCR conditions

Primer	Sequence 5 to 3	Product	Annealing	Reference
P. aeruginosa F	GGGGGATCTTCGGACCTCA	956bp	61.0ºC, 30 sec.	Hassan, *et al*., 2012
P. aeruginosa R	TCCTTAGAGTGCCCACCCG			
bla$_{IMP-1}$ F	TGAGCAAGTTATCTGTATTC	740bp	51.1ºC, 30 sec	Moosavian, & Rahimzadeh 2015
bla$_{IMP-1}$ R	TTAGTTGCTTGTTTTGATG			
bla$_{SPM-1}$ F	CCTACAATCTAACGGCGACC	674bp	59.3ºC, 30 sec	Moosavian, & Rahimzadeh 2015
bla$_{SPM-1}$ R	TCGCCGTGTCCAGGTATAAC			
bla$_{VIM-2}$ F	AAAGTTATGCCGCACTCACC	815bp	56.2ºC, 30 sec	Moosavian, & Rahimzadeh 2015
bla$_{VIM-2}$ R	TGCAACTTCATGTTATGCCG			
bla$_{NDM-1}$ F	GGTTTGGCGATCTGGTTTTC	621bp	58.3ºC, 30 sec	Kontopoulou, *et al*., 2021
bla $_{NDM-1}$ R	CGGAATGGCTCATCACGATC			
bla $_{KPC}$ F	CATTCAAGGGCTTTCTTGCTGC	538bp	58.6ºC, 30 sec	Dallenne, *et al*., 2010
bla $_{KPC}$ R	ACGACGGCATAGTCATTTGC			
bla $_{GIM}$ F	TCGACACACCTTGGTCTGAA	477bp	56.2ºC, 30 sec	Poirel, *et al*., 2011
bla $_{GIM}$ R	AACTTCCAACTTTGCCATGC			
bla $_{SIM}$ F	TACAAGGGATTCGGCATCG	570bp	57 ºC, 30 sec	Poirel, *et al*., 2011
bla $_{SIM}$ R	TAATGGCCTGTTCCCATGTG			
bla $_{DIM}$ F	GCTTGTCTTCGCTTGCTAACG	699bp	58.4ºC, 30 sec	Tarashi, *et al*., 2016
bla $_{DIM}$ R	CGTTCGGCTGGATTGATTTG			

3.1.8. Study Plan

The major steps in doing this work can be illustrated in in figure 3.1.

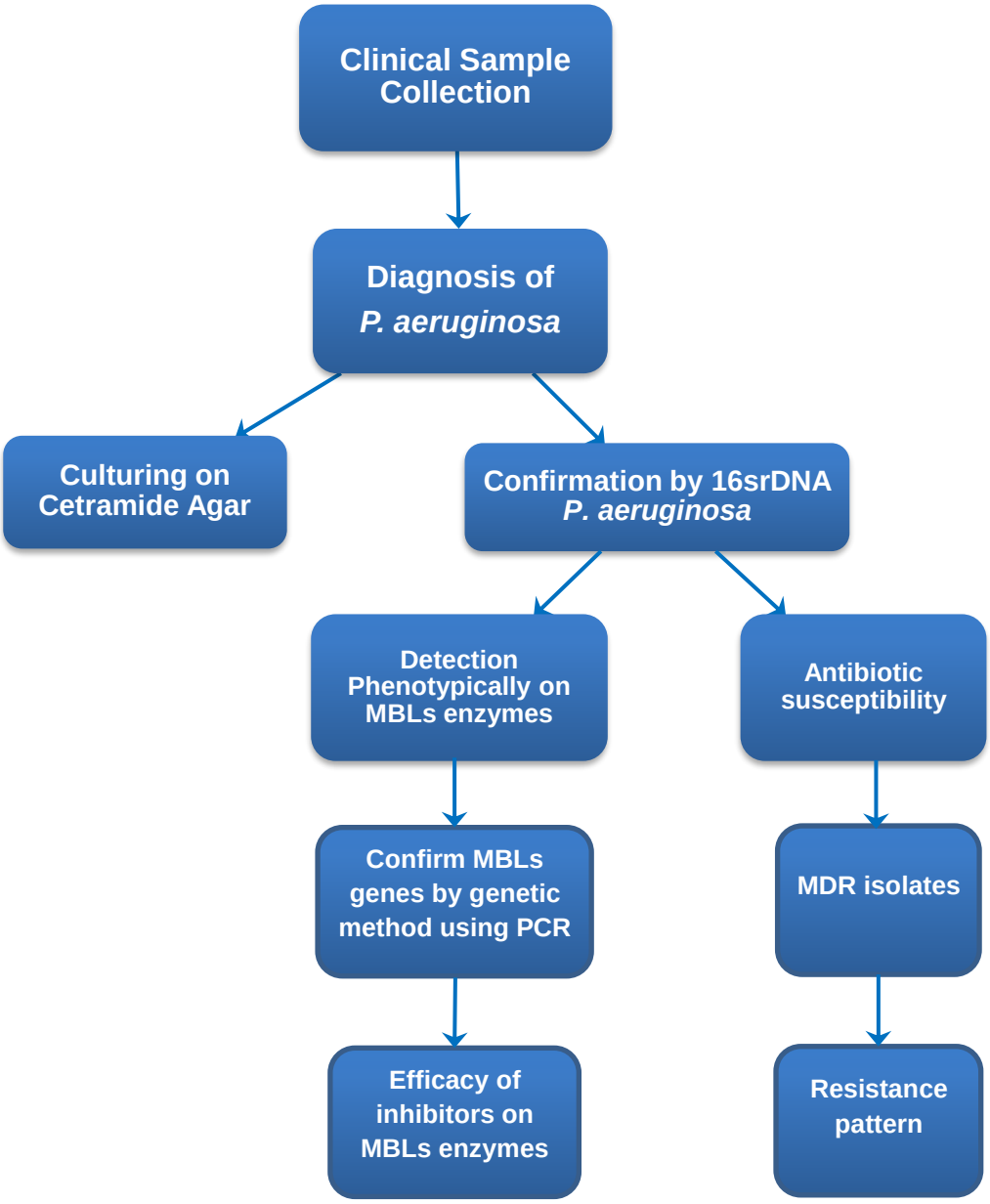

Figure 3.1. Chart flow of study plan.

3.2. Method

3.3. 3.2.1. Sample Collection

During the period from November 2021 to April 2022, 249 specimens were collected from burn patients. All samples were collected from Governmental hospitals in Hilla and around cities in Babylon province.

Specimens collection from burn patients was done by using transporter media cotton swab and under the supervision of the responsible doctor, the burned area is cleaned well by sterile normal saline and a sterile cotton swab is passed gently over the burned area, then, transferred to the laboratory to be incubated at 37˚C for 24 hr before culturing.

All specimens were cultured on MacConkey agar as selective media of Gram-negative bacteria and their ability to nonfermenting of lactose are recognized as differential character from the *Enterobacteriaceae* family, then it cultured on Nutrient agar to show their distinguishing pigments. After that, the isolates were screened by Cetrimide agar which is used as a selective media for *P. aeruginosa*. The incubation for isolates was at 37˚C for 24_48 hr (Bergen and Shelhamer, 1996). Isolates were confirmed by PCR using specific primer pairs for *16S rDNA* gene of *P. aeruginosa*.

3.2.2. Preparation of Culture Media

General culture media is a liquid, solid or semi-solid designed to support the growing of population of microorganisms via the process of cell proliferation. All culture media in this study (MacConkey agar, Nutrient agar, Cetrimide agar, Brain Heart Infusion broth, Mueller-Hinton Agar (MH)) were prepared using the routine methods according to the manufactures instructions as described below:

3.2.2.1. Brain Heart Infusion Broth

It was prepared by dissolving 37 grams of the medium powder in one litre of distilled water and autoclaved at 121°C for 15 minutes, after that its left to be cooled at room temperature to be poured then in plane tubes. This media used for activation of bacteria or to keep it for long time in freezer after adding glycerol of 15 % concentration (Lister *et al.*, 2009).

3.2.2.2. Mueller-Hinton Agar

The medium was prepared according to the manufacturing company by dissolving 38 grams of it in one litre of distilled water and autoclaved at 121°C for 15 minutes, then it's poured into Petri-dishes after being cooled to 40_45°C. This medium was used in antimicrobial susceptibility testing and effectiveness of inhibitors (Lister *et al.*, 2009).

3.2.2.3. MacConkey Agar

The medium was prepared by solving 51 grams of its powder to one litre of distilled water, then sterilized by autoclave at 121°C for 15 minutes. The medium is poured into sterile Petri dishes after being cooled down and then left to solidify at room temperature. This medium is used for detection the family *Enterobacteriaceae* and *Pseudomonas spp*, it is selective for Gram-negative bacteria (when medium contains bile salts as an inhibit Gram-positive bacteria) and helps to recognize the fermenting lactose ability of bacteria indicated by the change of indicator from red to pink (Lister *et al.*, 2009).

3.2.2.4. Nutrient Agar

This medium was prepared by solving 28 grams of Nutrient agar powder in one litre of distilled water, after mixing well it is sterilized by autoclave at 121°C for 15 mins. This medium is used for culturing and supporting growth of a wide range of non-fastidious bacteria (Lister *et al.*, 2009).

3.2.2.5. Cetrimide Agar

This medium is prepared according to the manufacturing company by solving 46.7 grams of its powder in one litre of distilled water, and then adding 10ml of glycerol and boiling them to be dissolved before sterilizing by autoclave at 121°C for 15 mins. When the medium cooled down to 45_50°C, the selective supplement Nalidixic acid is added (prepare by dissolving the antibiotic powder vial contain 15 mg by 5ml of distilled water) and poured into sterile Petri dishes. This medium is used as a selective isolation and presumptive identification of *P. aeruginosa* from clinical specimens (Lister, *et al.*, 2009).

3.2.3. Preparation of Solutions

The following solutions were used in this study:

3.2.3.1. McFarland Turbidity Standard

The McFarland's standard tube (1.5×10^8 CFU/ml) (Colony Forming Units; a unit of measurement used to determine the number of bacterial cells in lab sample) was prepared by adding 0.05 ml of 1.175% Barium chloride ($BaCl_2$) to 1% Sulfuric acid (H_2SO_4) to obtain a Barium sulfate precipitate. The solution was used to visible contrast the turbidity of a suspension of bacteria with the turbidity of the standard. The standard tubes

stored at room temperature in the dark for up to 6 months by sealing with parafilm to prevent evaporation. The accuracy of standard was checked by using a spectrophotometer at 600 nm, where the optical density should be between 0.08 and 0.1 (CLSI, 2021).

3.2.3.2. Tris-Borate-EDTA Buffer

Tris-Borate-EDTA buffer (TBE Buffer) is the most commonly used buffer for DNA and RNA gel electrophoresis. The 10x solution was prepared by dissolving 108 g of Tris base, 55 g of boric acid, and 40 ml of 0.5M EDTA in 1000ml of distilled water. However, the final concentration of working stock solution is prepared by adding 100 ml of 10× TBE buffer to 900 ml of sterile distilled water and stored at room temperature (Fleige and Pfaffl, 2006)

3.2.4. Identification of bacterial isolates

3.2.4.1. Phenotypic methods

The characteristic of *P. aeruginosa* in terms of oxidase reaction, smell and pigment production are enough for laboratory identification. One further test sufficient for identification *P. aeruginosa* is a capacity to grow at 42ºC. Morphology examination is carried by observation the characteristics of colonies of bacteria that grow on MacConkey agar and Nutrient Agar in terms of shape, colour, pigment forming, odour, and fermentation of lactose (Juan *et al*., 2017; Forbes *et al.*, 2007). Oxidase test was done as biochemical test to detect the target bacteria by taking a single colony that grew on Nutrient agar and placing it on the filter paper then a drop of test solution is added on it and hold for 15 seconds to see the result. The positive result when the colony colour turns to violet, while if the colour did not change, the result was recorded as negative (Granato and

Granato, 2011). Also, the isolates were examined under microscope by making a bacterial smear on slid and use Gram stain to identify the shape and colour of the cell (Betsy and Keogh 2005).

3.2.4.2. Genotypic Method

3.2.4.2.1. DNA Extraction of *Pseudomonas aeruginosa*

The Genomic DNA Mini Kit was used for DNA extraction by the following steps.

1. Two hundred microliters of FATG Buffer were added to the sample and resuspend the pellet by vortexing, then incubate it for 5 min at room temperature.
2. Two hundred microliters of FABG Buffer were added to the sample and vortexed for 5 sec. then incubated for 10 min at 70ºC, during incubation the tube was inverting every 3 min.
3. The required Elution Buffer was preheated at 70ºC in water bath.
4. Two hundred microliter of ethanol (96 -100 %) were added to the sample and vortexed for 10 sec.
5. The sample mixture was transferred to FABG Column which already placed to a collection tube and centrifuged at speed 14000 rpm for 1 min then the FABG Column was placed to a new collection tube.
6. Four hundred microliters of W1 Buffer were added to the FABG Column and centrifuge for 30 sec. at speed 14000 rpm, the flow through was discarded and the FABG Column was placed back to the collection tube.
7. The step 6 was repeated with 600 microliters of Wash Buffer.

8. To avoid the subsequent enzymatic reactions from being inhibited by residual liquid, it was centrifuged for an additional 3 min. at speed 14000 rpm to dry the column.
9. The dry FABG Column was placed to a new 1.5 ml microcentrifuge tube (Eppendorf tube).
10. One hundred microliters of preheated Elution Buffer were added to the membrane center of FABG Column. It's important to make sure that the elution solution was dispensed onto the membrane center and absorbed completely.
11. The FABG Column was incubated at 37ºC for 10 min.
12. To elute the DNA, it was centrifuged for 1 min at 14000 rpm. To increase DNA recovery, the DNA elution can be repeated.
13. The microcentrifuge tube which contains eluted DNA was stored at 4ºC.

3.2.4.2.2 Polymerase Chain Reaction (PCR) Protocol

Conventional PCR were used to amplify the target DNA using specific primer pairs. Its typically include three consecutive steps denaturation, annealing, and elongation repeated for specific number of cycles and conditions to get target amplicon which could be visualized after analyzed in agarose gel with TBE buffer and nucleic acid staining solution by electrophoresis equipment. PCR product size was determined by comparison with DNA Ladder 100 - 2000 pb (Bioneer, Korea) as shown in figure 3.2. The primers and PCR cycling conditions mentioned in the table 3.7.

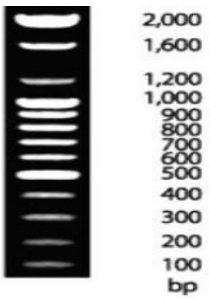

Figure 3.2: DNA Ladder 100 - 2000 pb (Bioneer, Korea)

3.2.4.2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is the best way of separating DNA fragments. To gain good resolution for small fragments of PCR product, the concentration of agarose in a gel is ranging between 0.5% - 2% (Lee *et al.*, 2001). 0.5-2 %weight of agarose was added to 100ml of 1×TBE buffer. To ensure the agarose was dissolved completely, its melted in microwave until the solution became clear. Once the agarose is cooled down to about 50_55ºC, 2oo µl of Green Nucleic Acid staining was added to it, and then, poured in the gel tray and left to be solidified (Green and Sambrook, 2019). The samples were loaded with ladder into gel wells. The time needed to spread DNA fragments was around 60 minutes. After turning on the power supply, DNA fragments will move towards the positive electrode positioned away from the well.

3.3. Phenotypic detection of Metallo Beta Lactamase

3.3.1. Antibiotic Susceptibility Test

The susceptibility of bacterial isolates to 12 antibiotics mentioned in Table 3.5 were determined according to clinical and laboratory standards institute instructions. Activation of isolates were performed using brain heart broth for 18 hours at 37°C and the growth was adjusted to 0.5

McFarland's standard, then spread on Muller Hinton agar (MHA) by a sterile cotton swab. Antibiotic disks were placed onto MHA inoculated with bacteria and incubated for 24 hours. at 37°C and then inhibition zone diameter was read and interpretation as a sensitive, intermediate or resist (CLSI, 2021).

3.3.2. The Imipenem-EDTA combined disc test

The Imipenem-EDTA combined disc test was performed as described by Yong *et al*., (2019). The bacteria were inoculated on plates of Mueller Hinton agar. Two Imipenem discs (10 μg/ml) were placed on the plate in distance 30mm and appropriate amounts of 10 μl of EDTA solution were added to one of them to obtain the desired concentration of 750 μg/ml. The inhibition zone of the Imipenem and Imipenem-EDTA discs were compared after 16-18 hours of incubation at 37 ˚C. The result of combined disc test, IMP-EDTA disc, should be increased in inhibition zone by ≥7 mm than the IMP disc alone to be considered as MBL positive (Chui *et al*., 2014). A Solution of a 0.5 M EDTA was prepared by dissolving 16.8 gm of EDTA in 100 ml of distilled water and its pH was adjusted to 8.0 by using NaOH and sterilized by autoclaving (Agnieszka *et al*., 2017).

3.4. Genotypic method to detect MBLs

3.4.1. Primers Preparation

According to the instruction leaflet of the primers source (Humanizing Genomics Macrogen, Inc. / South Korea), the primers were transported in a lyophilized state, so to prepare stock of primers, it was reconstituted in sterile nuclease-free water. before opening the cap, the tube preferred to spin down, the proper amount of nuclease free water was added according to the oligos manufacturer and vortexed well to obtain the stock. The stock was stored at -20 ºC.

3.5. Preparation of Inhibitors

3.5.1. Allicin and Diallyl trisulfide:

Allicin (cat no. HY-N0315, size: 1 ml with 1.112 g/ml) and Diallyl trisulfide (cat no. HY-117235 size: 1ml with 1.116 mg/ml). 35.8 µl and 35.9 µl have been taken from Allicin and Diallyl trisulfide respectively and each one was dissolved in 10 ml of Dimethyl sulfoxide (DMSO) used an ultrasonic device. Each inhibitor was added to 400 ml of Muller Hinton Agar (MHA) that was previously prepared, then, poured the mixture into petri dish and leaved until solidified according to the preparation instruction by the source (Rahman, 2007; Pu Liu, 2009).

3.5.2. Sultamicillin tosylate

An amount of 0.04 µg of Sultamicillin tosylate (cat no. HY-N7111, amount of 90 mg) was taken and dissolved in 10 ml of Dimethyl sulfoxide (DMSO) by using an ultrasonic device, then the resultant solution was added into 400 ml of Muller Hinton Agar that was already prepared and sterilised by autoclave. The temperature of the Agar was about 45ºC when the solution was added. Then, the mixture solution of inhibitor and muller Hinton Agar was poured into petri dish. It's worth to mention that a 10 ml of the volume of water that is required to prepare the Muller Hinton Agar has been taken out in each time when prepare the agar with all inhibitors to ensure kept the proper condensation of the mixture after adding 10 ml of solution to the agar. Three different concertations were prepared for each inhibitor of 50, 100 and 200 µg/l (Friedel, 1989).

CHAPTER FOUR

Results and Discussion

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4. Results and Discussion

4.1. Isolation and Identification of *Pseudomonas aeruginosa*

A total of 249 swabs were collected from hospitalized burn patients in different hospitals of Babylon province during the study period from December 2021 to April 2022. The samples have been transported by transport swab and then submitted to bacteriological investigation. Male patients were 92/249 (36.94%) with Mean±SD of age (34.56±19.09), while female patients were 157/249 (63.05 %) with Mean±SD of age (36.17±21.23).

Among 249, 100/249 isolates (40.16 %) were diagnosed as *Pseudomonas. aeruginosa* based on phenotypic methods by streaking on different enriched culture media then screening on selective medium (cetrimide agar) and confirming with PCR for species specific primer pairs to amplify *16S rDNA* gene which done for all isolates (Figure 4.1)

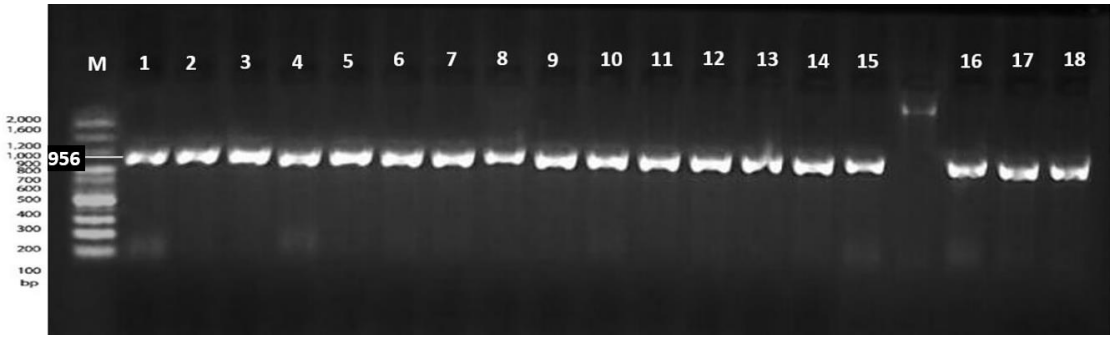


Figure 4.1. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* specific amplicon of *16S rDNA* (956bp), M is 100bp DNA ladder, lanes 1-18 was the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

The prevalence of *P. aeruginosa* in this study showed higher percentages than other published Iraqi results which were (8.2%) (Ulabdeen, 2016), (30.55%) (Khorsheed, 2017), (22.8%) (Al-Dahmoshi *et al*., 2018) and (24.27%) (AL-Rubaye, 2020). This difference in rates may stand to the level of health care, method of antisepsis of burns, the speed arrival of patient to the hospital, the patient had taken antibiotics in advance, or may be because of the long hospitalization in hospital. This percentage may be increased within 72 hours as a result of an infection acquired from hospitals as a result of contact with patients or through using of contaminated instruments that lead to spread these bacteria (AL-Rubaye, 2020).

4.2. Antibiotic susceptibility

All isolates of *pseudomonas aeruginosa* (100) were subjected to antimicrobial susceptibility test by using different antimicrobial agents according to (CLSI, 2021) as shown in (Table 4.1).

Table 4.1. Antimicrobial susceptibility test of *P. aeruginosa* isolates

Antibiotics	Sensitive No./total (%)	Resist No./total (%)
Aztreonam 30mg	46/100 (46)	54/100 (54)
Ceftazidime 30mg	48/100 (48)	52/100 (52)
Piperacillin 100mg	17/100 (17)	83/100 (83)
Cefepime 30mg	81/100 (81)	19/100 (19)
Piperacillin / Tazobactam 100/10mg	73/100 (73)	27/100 (27)
Meropenem 10mg	74/100 (74)	26/100 (26)
Imipenem 10mg	79/100 (79)	21/100 (21)
Amikacin 30mg	68/100 (68)	32/100 (32)
Netilmicin 30mg	60/100 (60)	40/100 (40)
Gentamicin 10mg	42/100 (42)	58/100 (58)
Ciprofloxacin 5mg	35/100 (35)	65/100 (65)
Levofloxacin 5mg	50/100 (50)	50/100 (50)

The results of antibiotics susceptibility revealed that *P. aeruginosa* isolates were highly resistant to beta lactam antibiotics group including Piperacillin 83/100 (83%), Ceftazidime 52/100 (52%), and fluoroquinolone group including Ciprofloxacin 65/100 (65%), Levofloxacin 50/100 (50%), and aminoglycoside group including Gentamicin 58/100 (58%), and monobactam (Aztreonam) 54/100 (54%).

The previous studies of (Hosu, *et al*., 2021; Al-Dahmoshi *et al*., 2018; Khudair and Mahmood, 2021) reported that the highest resistant was Piperacillin with percentages of 64.2%, 67.96% and 72.22% respectively. The ceftazidime resistant results of (Hassuna *et al*., 2015; Hasan, 2019; Khudair and Mahmood, 2021) reported 86%, 94.1% and 68% respectively. Resistance to Aztreonam of this study was (54%) while other studies were (Hosu *et al*., 2021) (57.8%,), (Al-Dahmoshi *et al*., 2018) (54.4%) and (Kateete *et al*., 2017) 48%.

The results also, have indicated that *P. aeruginosa* isolates were highly sensitive to cefepime 81/100 (81%), carbapenem antibiotics including imipenem 79/100 (79%) and meropenem 74/100 (74%), while other results of (Khorsheed *et al.*, 2017; AL-Rubaye 2020) were (17%, 19%) respectively. Aminoglycoside group results including amikacin 68/100 (68%) and netilmicin 60/100 (60%) were compatible with (Aljanaby, 2018; Younus, 2021). Furthermore, fluoroquinolone group (levofloxacin) was 50/100 (50%).

Beta lactam-beta lactamase inhibitors combination antibiotics that represented by Piperacillin-tazobactam revealed excellent effect on *P. aeruginosa* isolates by reducing their resistance to 27/100 (27%) as compared with the high resistance of piperacillin alone 83/100 (83%), and these results agreed with (Tannous *et al*., 2020; Al Muqati *et al*., 2021).

4.3. Co-susceptibility Pattern of Multidrug resistant Isolates

The results revealed that 69/100 (69%) of *P. aeruginosa* isolates were multidrug resistant (MDR) and 31/100 (31%) non MDR (Figure 4.2). The results of (Perez *et al*., 2019) was (69%) MDR while the results of (Mirzaei *et al*., 2020) was (32%).

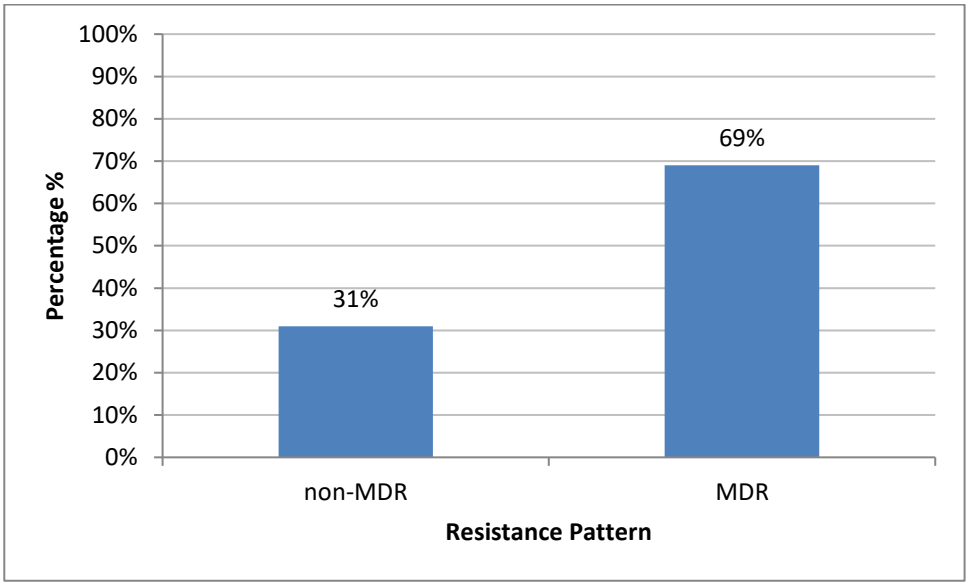


Figure 4.2: resistance patterns among *P. aeruginosa* isolates

Pseudomonas aeruginosa isolates as MDR in this study were resistance to 3 antibiotics up to 12 antibiotics. Its resistant to 4 classes were the highest present with 21/69 (30%) followed by 3 classes MDR 19/69 (27.5%), 5 classes MDR 17/69 (24.6 %), and 6 classes MDR 12/69(17.3 %) (Table 4.2)

Table 4.2: Phenotypic resistance patterns of MDR *P. aeruginosa*

Classes	MDR Phenotype	No.	%
3	Monobactam/Carbapenem/Fluoroquinolone	1	27.5
	Penicillin/Carbapenem/Fluoroquinolone	1	
	Penicillin/Aminoglycoside/Fluoroquinolone	12	
	Penicillin/Monobactam/Fluoroquinolone	3	
	Penicillin/Monobactam/Aminoglycoside	2	
4	Penicillin/Monobactam/Aminoglycoside/Fluoroquinolone	4	30
	Penicillin/BBI/Aminoglycoside/Fluoroquinolone	2	
	Penicillin/Carbapenem/Aminoglycoside/Fluoroquinolone	2	
	Penicillin/Monobactam/Aminoglycoside/Fluoroquinolone	8	
	Penicillin/Monobactam/Carbapenem/Fluoroquinolone	2	
	Penicillin/Monobactam/Carbapenem/Aminoglycoside	1	
	Penicillin/BBI/Monobactam/Fluoroquinolone	1	
	Penicillin/BBI/Carbapenem/Fluoroquinolone	1	
5	Penicillin/Monobactam/Carbapenem/Aminoglycoside/Fluoroquinolone	7	24.6
	Penicillin/BBI/Monobactam/Carbapenem/Fluoroquinolone	2	
	Penicillin/BBI/Carbapenem/Aminoglycoside/Fluoroquinolone	2	
	Penicillin/BBI/Monobactam/Aminoglycoside/Fluoroquinolone	2	
	Penicillin/Monobactam/Carbapenem/Aminoglycoside/Fluoroquinolone	1	
	Penicillin/BBI/Carbapenem/Aminoglycoside/Fluoroquinolone	1	
	Penicillin/BBI/Monobactam/Fluoroquinolone/Aminoglycoside	1	
	Penicillin/BBI/Monobactam/Carbapenem/Aminoglycoside	1	
6	Penicillin/BBI/Monobactam/Carbapenem/Fluoroquinolone/Aminoglycoside	12	17.3
Total		69	100

4.4. Phenotypic Detection of Metallo beta lactamase

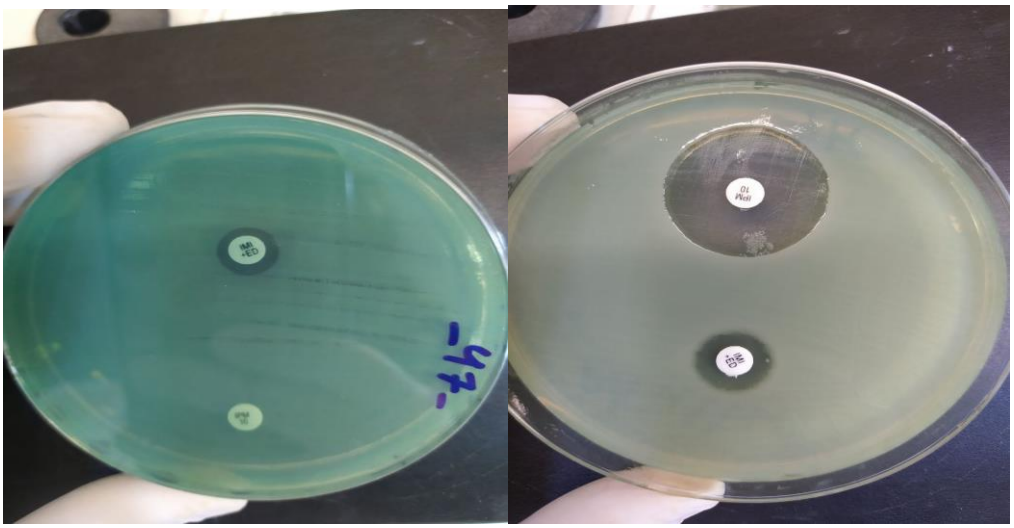
Phenotypic detection of Metallo-beta-lactamase were done by using Combined Disc Synergy Test (CDST). Among 100 isolates of *P.*

aeruginosa, only 4/100 (4%) isolates showed positive results with inhibition zone (Mean±SD) for IMP (10µg) = 9.5±4.72 (mm), and for IMP+EDTA ((10µg+750µg) = 16±5.59 (mm), while 96/100 (96%) appeared negative results with inhibition zone (Mean±SD) for IMP (10µg) = 26.07±6.02 (mm), and for IMP+EDTA ((10µg+750µg) = 17.75±5.68 (mm) as in table 4.3.

The negative results were unexpected because the inhibition zone was diminished upon addition of EDTA to imipenem and it may be explained as this was due to decreasing the uptake of IMP-EDTA combination by porins in somehow not known.

Table 4.3: Combined Disc Synergy Test for *P. aeruginosa* isolates

***P. aeruginosa* strain**	**Inhibition Zone (Mean±SD) (mm)**	
	IMP (10µg)	**IMP+EDTA (10µg+750µg)**
phenotypic negative MBL (n=96)	26.07±6.02	17.75±5.68
phenotypic Positive MBL (n=4)	9.5±4.72	16±5.59



(a) **(b)**

Figure 4.3: Combined Disc Synergy Test for *P. aeruginosa* isolates using IMP and IMP+EDTA: (a) positive results, (b) negative results

4.5. Genotypic Detection of Metallo Beta lactamase

Metallo Beta lactamase enzymes were investigated via detection of MBLs variants genes by PCR using specific primers for each MBLs gene; $bla_{IMP,}$ bla_{VIM}, bla_{NDM}, bla_{KPC}, bla_{GIM}, bla_{SIM}, bla_{SPM} and bla_{DIM} as mentioned in table 3.7 in chapter three. The amplification for all MBLs genes were applied for 100 *P. aeruginosa* isolates, the results revealed that 32/100 (32%) isolates do not possess any gene of MBLs while 68/100 (68%) isolates have at least one gene of MBLs (Figure 4.3). The percentage of MBLs genes among *P. aeruginosa* isolates were (38%) for bla_{NDM}, (34%) for bla_{IMP}, (26%) for bla_{GIM}, (22%) for bla_{VIM}, , (12%) for bla_{SIM}, (11%) for bla_{KPC}, , (7%) for bla_{SPM} and (7%) for bla_{DIM} (Figure 4.4).

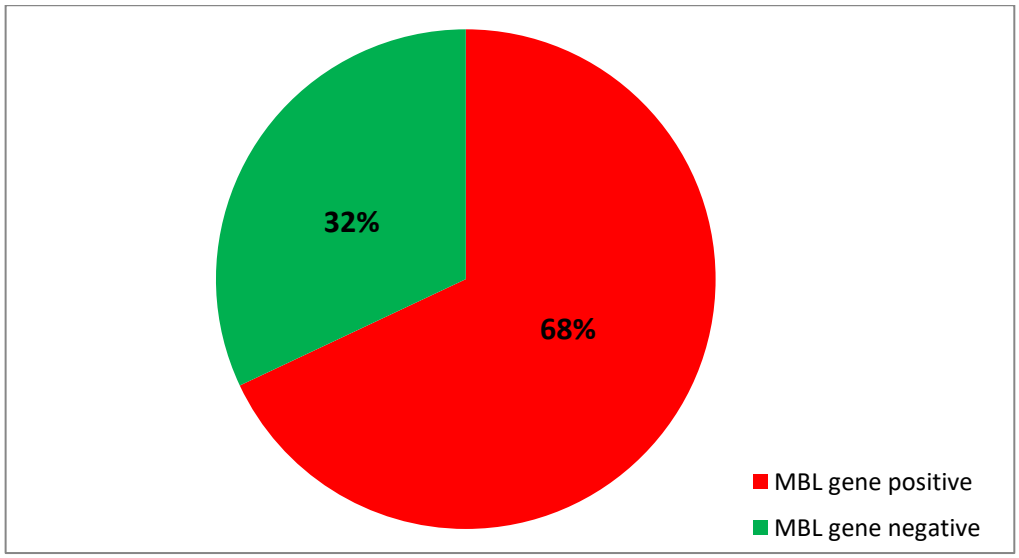


Figure 4.4: Distribution of MBL genes among *P. aeruginosa* isolates

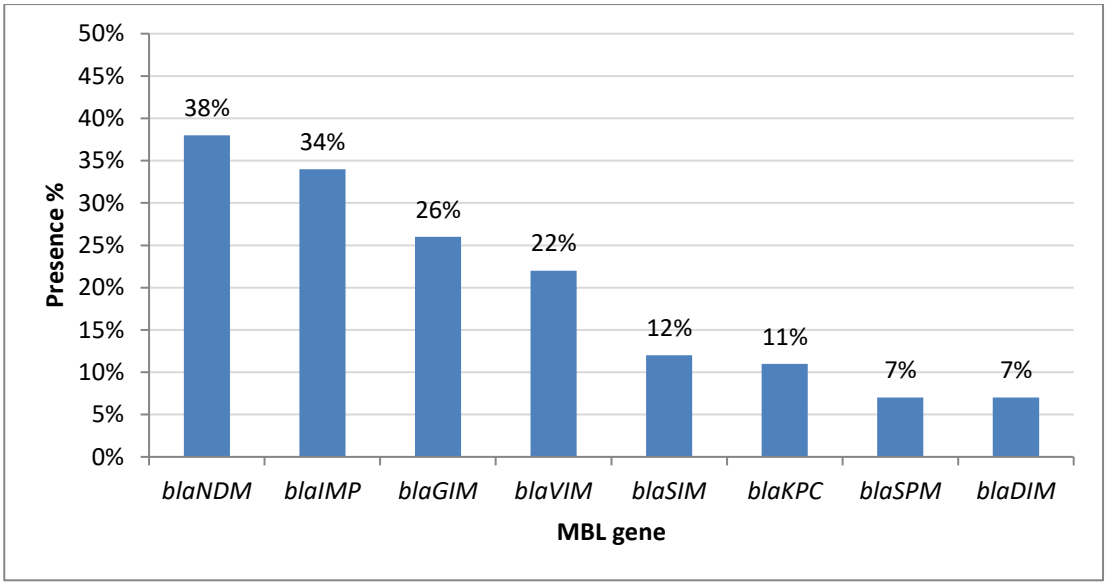


Figure 4.5: The prevalence of MBLs Genes among *P. aeruginosa* isolates

4.5.1 Agarose gel Electrophoresis of MBLs Genes

Results of Agarose gel electrophoresis were illustrated in figures (4.5 to 4.12) for bla_{NDM}, bla_{IMP}, bla_{GIM}, bla_{VIM}, bla_{SIM}, bla_{KPC}, bla_{SPM} and bla_{DIM} genes respectively.

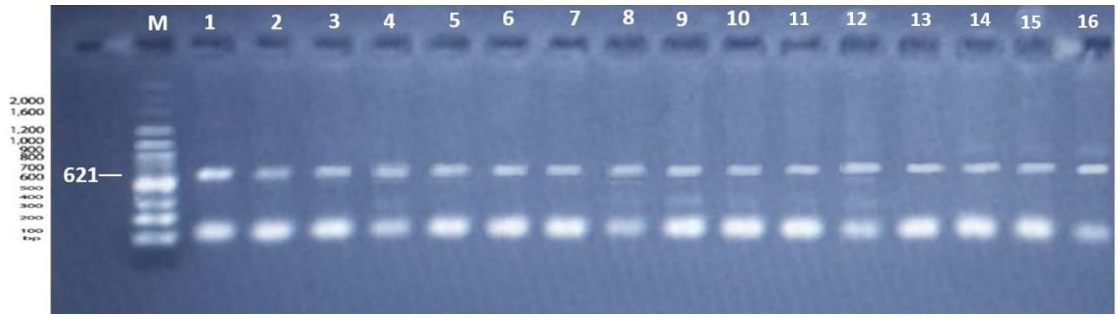


Fig.4.6. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{NDM} amplicon (621bp), M is 100bp DNA ladder, lanes 1-16 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

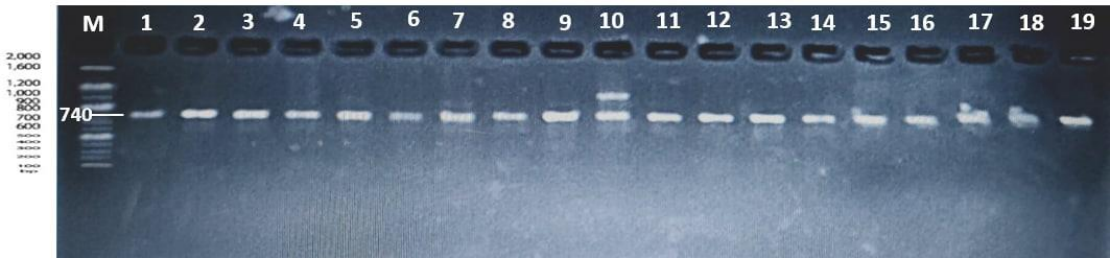


Figure4.7. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{IMP} amplicon (740bp), M is 100bp DNA ladder, lanes 1-19 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

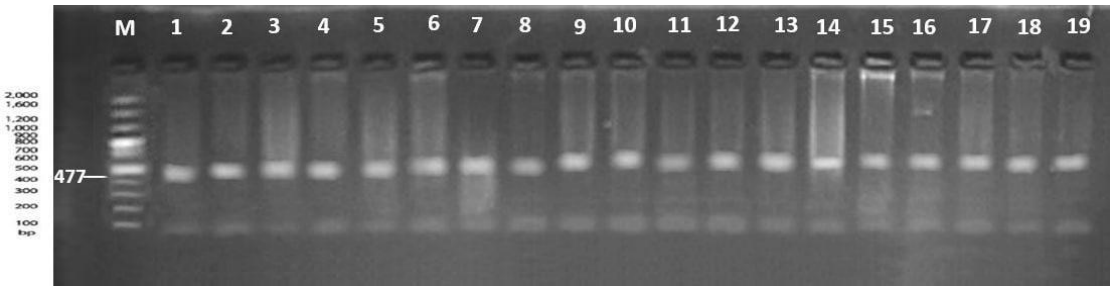


Figure 4.8. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{GIM} amplicon (477bp), M is 100bp DNA ladder, lanes 1-19 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

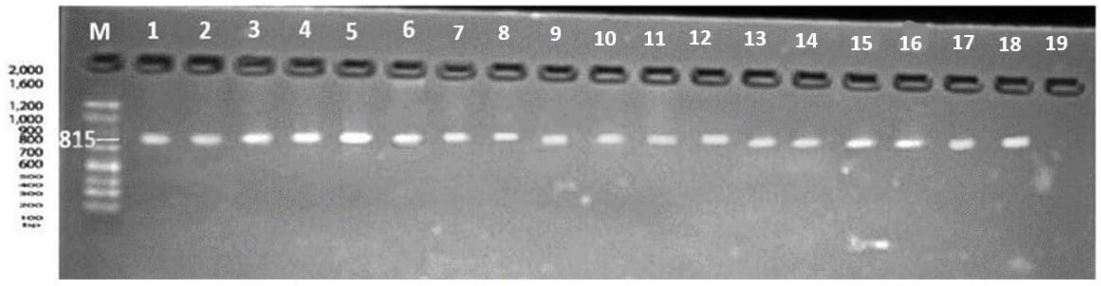


Figure 4.9. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{VIM} amplicon (815 bp), M is 100bp DNA ladder, lanes 1-19 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

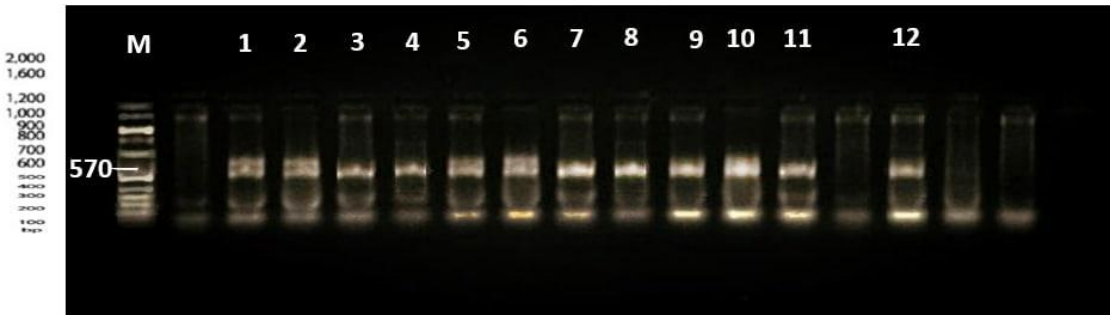


Figure4.10. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{SIM} amplicon (570 bp), M is 100bp DNA ladder, lanes 1-12 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

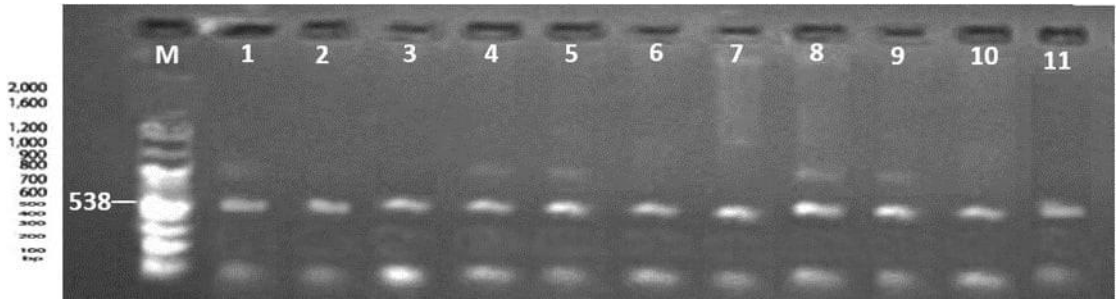


Figure 4.11. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{KPC} amplicon (538 bp), M is 100bp DNA ladder, lane 1-11 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

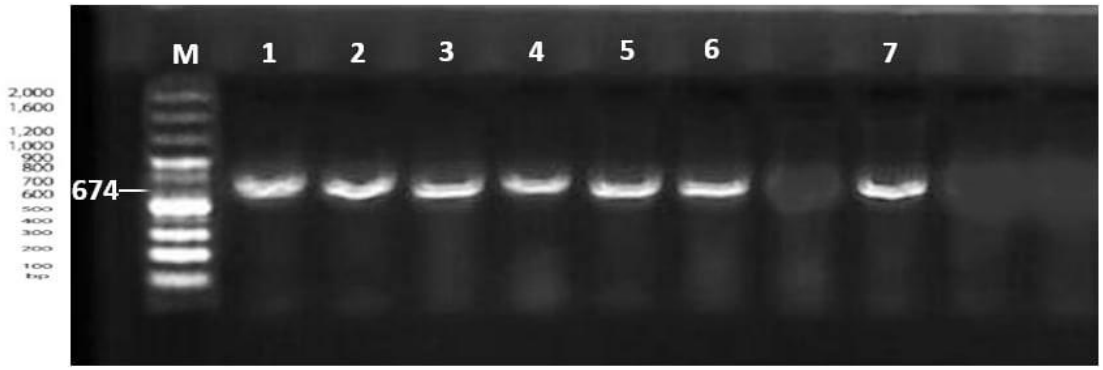


Figure 4.12. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{SPM} amplicon (674bp), M is 100bp DNA ladder, lanes 1-12 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

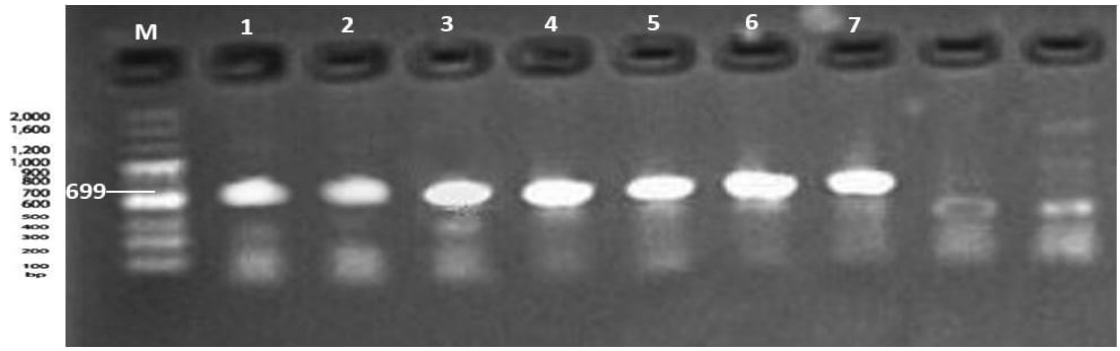


Figure 4.13. Agarose gel (1.5% in TBE) electrophoresis for *P. aeruginosa* bla_{DIM} amplicon (699bp), M is 100bp DNA ladder, lanes 1-7 are the isolates, using GreenStar^{tm} Nucleic Acid Staining Solution, at Voltage 72 volts for 60 min.

The high prevalence gene was bla_{NDM} 38/100 (38%), the results of other studies were (Qureshi, *et al*., 2021) with (35.7%), and (Joji, *et al*.,2019) with (2.5%).

The lower prevalence was of bla_{SPM} and bla_{DIM} with 7/100 (7%) for each one. The other results of (Wang & Wang, 2020) which was (4.0%) for bla_{SPM}, and (Farhan, *et al*., 2019) was (38%) for bla_{SPM}.

Out of 100 of *P. aeruginosa* isolates, 34/100 (34%) were possess bla_{IMP} gene, other studies showed different results, (Hosu, *et al*., 2021) was (1.25%), (Farhan, *et al*., 2019) was (52.3%), and (Jabalameli, *et al*., 2018) was (13.1%).

The prevalence of bla_{GIM} gene was 26/100 (26%), other studies showed (52.3%) from (Farhan, *et al*., 2019), (1.3%) from (Wang & Wang, 2020), and (Alkhudhairy and Al-Shammari 2020) recorded no isolates were carried bla_{GIM} gene.

The prevalence of bla_{VIM} gene were 22/100 (22%) as results showed. (Joji, *et al*., 2019) recorded in their studies no isolated have bla_{VIM} gene, these results identical with (Hosu, *et al*., 2020), (Jabalameli, *et al*., 2018)

was display in his study (21.4%), and (Farhan, 2020) recorded (52.3%) and (Qureshi, *et al*., 2021) was ((52%).

The prevalence of bla_{SIM} gene was 12/100 (12%), other studies showed (6.0%) by (Wang & Wang, 2020), and (Alkhudhairy and Al-Shammari, 2019) recorded no isolates carry bla_{SIM} gene.

The other important gene was detected in this study was the carbapenem resistant gene bla_{KPC}. The results revealed that the prevalence of bla_{KPC} gene was 11/100 (11%), other studied results were 33%, 40.4%, 17% by (Hu, *et al*., 2021), (Zhu, *et al*., 2021), and (Ahmed, *et al*., 2020) respectively, and 5.7% was by (Yassen, *et al*., 2021).

4.5.2 Prevalence of MBLs Genes in Sensitive and Resistant Isolates

The results showed that the MBLs genes can be found in sensitive and resistant isolates as mentioned in tables (4.4 to 4.11) for bla_{NDM}, bla_{IMP}, bla_{GIM}, bla_{VIM}, bla_{SIM}, bla_{KPC}, bla_{SPM} and bla_{DIM} genes respectively.

Table 4.4: Prevalence of bla_{NDM} gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for bla_{NDM}		Resistance Isolates positive for bla_{NDM}		Total
	N	%	N	%	
Ceftazidime 30mg	16	42.1	22	57.89	38
Cefepime 30mg	30	78.94	8	21.05	38
Piperacillin 100mg	7	18.42	31	81.57	38
Piperacillin/tazobactam 10/100mg	28	73.68	10	26.31	38
Aztreonam 30mg	18	47.36	20	52.63	38
Meropenem 10mg	29	76.31	9	23.68	38
Imipenem 10mg	32	84.21	6	15.78	38
Amikacin 30mg	24	63.15	14	36.84	38
Netilmicin 30mg	25	65.78	13	34.21	38
Gentamicin 10mg	19	50	19	50	38
Ciprofloxacin 5mg	22	57.89	16	42.1	38
Levofloxacin 5mg	12	31.57	26	68.42	38

Table 4.5: Prevalence of *bla$_{IMP}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{IMP}$*		Resistance Isolates positive for *bla$_{IMP}$*		Total
	N	%	N	%	
Ceftazidime 30mg	19	55.8	15	44.11	34
Cefepime 30mg	31	91.17	3	8.82	34
Piperacillin 100mg	5	14.7	29	85.29	34
Piperacillin/tazobactam 10/100mg	24	70.58	10	29.41	34
Aztreonam 30mg	21	61.76	13	38.23	34
Meropenem 10mg	25	73.52	9	26.47	34
Imipenem 10mg	28	82.35	6	17.64	34
Amikacin 30mg	23	67.64	11	32.35	34
Netilmicin 30mg	23	67.64	11	32.35	34
Gentamicin 10mg	15	44.11	19	55.88	34
Ciprofloxacin 5mg	15	44.11	19	55.88	34
Levofloxacin 5mg	7	20.58	27	79.41	34

Table 4.6: Prevalence of *bla$_{GIM}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{GIM}$*		Resistance Isolates positive for *bla$_{GIM}$*		Total
	N	%	N	%	
Ceftazidime 30mg	15	57.69	11	42.30	26
Cefepime 30mg	22	84.61	4	15.38	26
Piperacillin 100mg	1	3.84	25	96.15	26
Piperacillin/tazobactam 10/100mg	17	65.38	9	34.61	26
Aztreonam 30mg	14	53.84	12	46.15	26
Meropenem 10mg	15	57.69	11	42.30	26
Imipenem 10mg	18	69.23	8	30.76	26
Amikacin 30mg	18	69.23	8	30.76	26
Netilmicin 30mg	11	42.30	15	57.69	26
Gentamicin 10mg	8	30.76	18	69.23	26
Ciprofloxacin 5mg	8	30.76	18	69.23	26
Levofloxacin 5mg	6	23.07	20	76.92	26

Table 4.7: Prevalence of *bla$_{VIM}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{VIM}$*		Resistance Isolates positive for *bla$_{VIM}$*		Total
	N	%	N	%	
Ceftazidime 30mg	15	68.18	7	31.81	22
Cefepime 30mg	16	72.72	6	27.27	22
Piperacillin 100mg	2	9.09	20	90.90	22
Piperacillin/tazobactam 10/100mg	15	68.18	7	31.81	22
Aztreonam 30mg	14	63.63	8	36.36	22
Meropenem 10mg	13	59.09	9	40.90	22
Imipenem 10mg	18	81.81	4	18.18	22
Amikacin 30mg	16	72.72	6	27.27	22
Netilmicin 30mg	13	59.09	9	40.90	22
Gentamicin 10mg	10	45.45	12	54.54	22
Ciprofloxacin 5mg	8	36.36	14	63.63	22
Levofloxacin 5mg	5	22.72	17	77.27	22

Table 4.8: Prevalence of *bla$_{SIM}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{SIM}$*		Resistance Isolates positive for *bla$_{SIM}$*		Total
	N	%	N	%	
Ceftazidime 30mg	4	33.33	8	66.66	12
Cefepime 30mg	8	66.66	4	33.33	12
Piperacillin 100mg	2	16.66	10	83.33	12
Piperacillin/tazobactam 10/100mg	4	33.33	8	66.66	12
Aztreonam 30mg	6	50	6	50	12
Meropenem 10mg	3	25	9	75	12
Imipenem 10mg	6	50	6	50	12
Amikacin 30mg	5	41.66	7	58.33	12
Netilmicin 30mg	5	41.66	7	58.33	12
Gentamicin 10mg	3	25	9	75	12
Ciprofloxacin 5mg	4	33.33	8	66.66	12
Levofloxacin 5mg	3	25	9	75	12

Table 4.9: Prevalence of *bla$_{KPC}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{KPC}$*		Resistance Isolates positive for *bla$_{KPC}$*		Total
	N	%	N	%	
Ceftazidime 30mg	4	36.36	7	63.63	11
Cefepime 30mg	10	90.90	1	9.09	11
Piperacillin 100mg	0	0	11	100	11
Piperacillin/tazobactam 10/100mg	6	54.54	5	45.45	11
Aztreonam 30mg	3	27.27	8	72.72	11
Meropenem 10mg	6	54.54	5	45.45	11
Imipenem 10mg	6	54.54	5	45.45	11
Amikacin 30mg	6	54.54	5	45.45	11
Netilmicin 30mg	4	36.36	7	63.63	11
Gentamicin 10mg	3	27.27	8	72.72	11
Ciprofloxacin 5mg	4	36.36	7	63.63	11
Levofloxacin 5mg	1	9.09	10	90.90	11

Table 4.10: Prevalence of *bla$_{SPM}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{SPM}$*		Resistance Isolates positive for *bla$_{SPM}$*		Total
	N	%	N	%	
Ceftazidime 30mg	2	28.57	5	71.42	7
Cefepime 30mg	4	57.14	3	42.85	7
Piperacillin 100mg	1	14.28	6	85.71	7
Piperacillin/tazobactam 10/100mg	2	28.57	5	71.42	7
Aztreonam 30mg	3	42.85	4	57.14	7
Meropenem 10mg	3	42.85	4	57.14	7
Imipenem 10mg	3	42.85	4	57.14	7
Amikacin 30mg	3	42.85	4	57.14	7
Netilmicin 30mg	3	42.85	4	57.14	7
Gentamicin 10mg	3	42.85	4	57.14	7
Ciprofloxacin 5mg	4	57.14	3	42.85	7
Levofloxacin 5mg	3	42.85	4	57.14	7

Table 4.11: Prevalence of *bla$_{DIM}$* gene in sensitive and resistance *P. aeruginosa* isolates

Antibiotics	Sensitive Isolates positive for *bla$_{DIM}$*		Resistance Isolates positive for *bla$_{DIM}$*		Total
	N	%	N	%	
Ceftazidime 30mg	4	57.14	3	42.85	7
Cefepime 30mg	6	85.71	1	14.28	7
Piperacillin 100mg	0	0	7	100	7
Piperacillin/tazobactam 10/100mg	3	42.85	4	57.14	7
Aztreonam 30mg	3	42.85	4	57.14	7
Meropenem 10mg	3	42.85	4	57.14	7
Imipenem 10mg	4	57.14	3	42.85	7
Amikacin 30mg	3	42.85	4	57.14	7
Netilmicin 30mg	3	42.85	4	57.14	7
Gentamicin 10mg	2	28.57	5	71.42	7
Ciprofloxacin 5mg	2	28.57	5	71.42	7
Levofloxacin 5mg	0	0	7	100	7

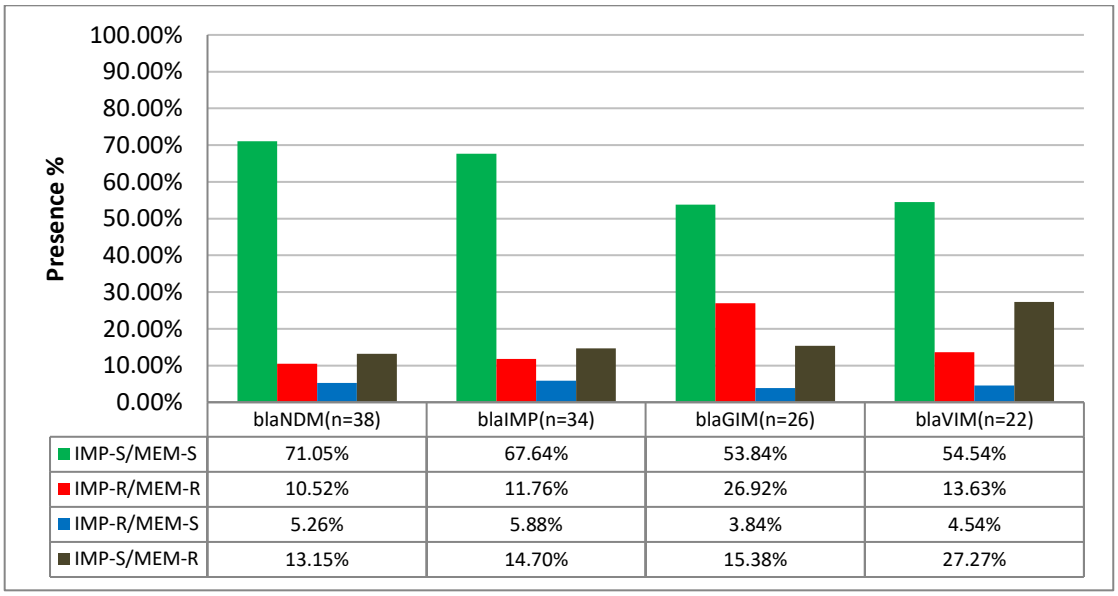
The results of antibiotics susceptibility for *P. aeruginosa* isolates that possess MBLs genes showed the high resistant were to piperacillin with percentage 31/38 (81.57%), 6/7 (85.71%), 7/7 100%, 29/34 (85.29%), 25/26 (96.15%), 20/22 (90.90%), 10/12 (83.33%), and 100% (11/11 isolates) for *bla$_{NDM}$* gene, *bla$_{SPM,}$* gene, *bla$_{DIM}$* gene, *bla$_{IMP}$* gene, *bla$_{GIM}$* gene, *bla$_{VIM}$* gene, *bla$_{SIM}$* gene, and *bla$_{KPC}$* gene respectively.

The results also showed that the lower resistant was for cefepime for isolates that possess *bla$_{SPM,}$* gene, *bla$_{DIM}$* gene, *bla$_{IMP}$* gene, *bla$_{GIM}$* gene, *bla$_{SIM}$* gene, and *bla$_{KPC}$* gene with percentage 3/7 (42.85%), 1/7 (14.28%), 3/34 (8.82%), 4/26 (15.38%), 4/12 (33.33%), and 90.90% (10/11 isolates) respectively.

While the isolates that carried *bla$_{NDM}$* gene, and *bla$_{VIM}$* gene were have low resist to imipenem with 6/38 (15.78%), and 4/22 (18.18%) respectively.

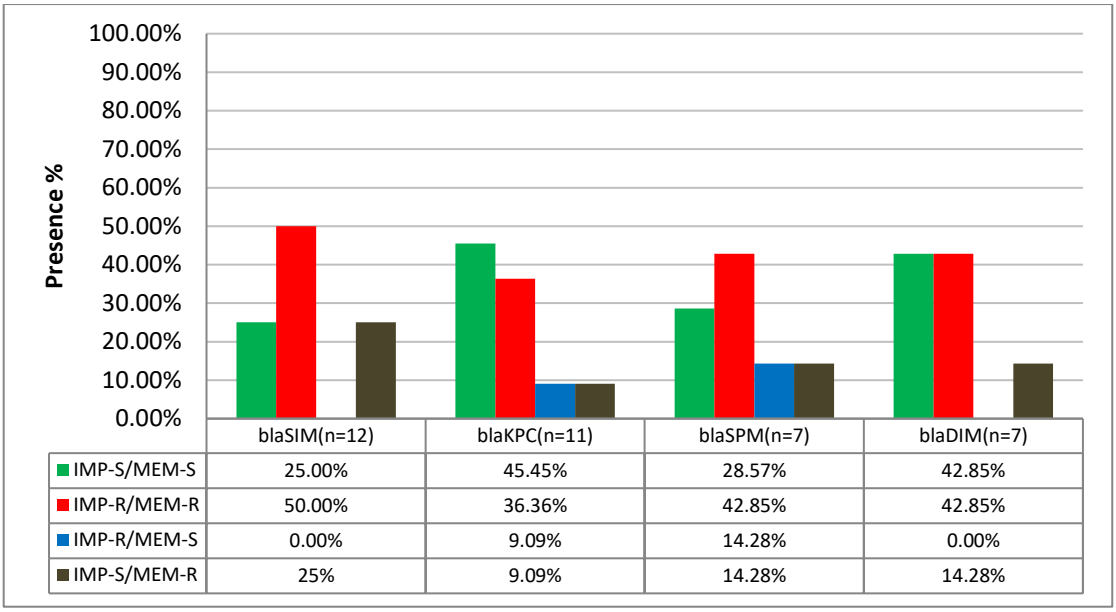
4.6. Carbapenem resistance and MBLs gene content

Phenotypic resistance or sensitive must reflect the resistance gene content and so it is involved in routine work to predict resistance gene presence. The results of relation of genotype to phenotype gave unexpected results when 27/38 (71.05%), 23/34(67.64%), 14/26(53.84%) and 12/22(54.54%) were sensitive for imipenem and meropenem in spite of they have *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{GIM}$ and *bla*$_{VIM}$ respectively (Figure 4.13) and 3/12(25%) for *bla*$_{SIM}$, 5/11(45.45%) for *bla*$_{KPC}$, 2/7(28.57%) for *bla*$_{SPM}$ and 3/7(42.85%) for *bla*$_{DIM}$ (Figure 4.14).



	blaNDM(n=38)	blaIMP(n=34)	blaGIM(n=26)	blaVIM(n=22)
IMP-S/MEM-S	71.05%	67.64%	53.84%	54.54%
IMP-R/MEM-R	10.52%	11.76%	26.92%	13.63%
IMP-R/MEM-S	5.26%	5.88%	3.84%	4.54%
IMP-S/MEM-R	13.15%	14.70%	15.38%	27.27%

Figure 4.14. Presence of *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{GIM}$ and *bla*$_{VIM}$ genes and co-susceptibility among *P. aeruginosa* isolates. IMP-S=imipenem sensitive, IMP-R=imipenem resistant, MEM-S=meropenem sensitive, MEM-R=meropenem resistant.



	blaSIM(n=12)	blaKPC(n=11)	blaSPM(n=7)	blaDIM(n=7)
IMP-S/MEM-S	25.00%	45.45%	28.57%	42.85%
IMP-R/MEM-R	50.00%	36.36%	42.85%	42.85%
IMP-R/MEM-S	0.00%	9.09%	14.28%	0.00%
IMP-S/MEM-R	25%	9.09%	14.28%	14.28%

Figure 4.15: Presence of *bla*$_{SIM}$, *bla*$_{KPC}$, *bla*$_{SPM}$ and *bla*$_{DIM}$ genes and co-susceptibility among *P. aeruginosa* isolates. IMP-S=imipenem sensitive, IMP-R=imipenem resistant, MEM-S=meropenem sensitive, MEM-R=meropenem resistant.

These results considered big issue when phenotypically sensitive isolates have a resistance gene, so it may be explained as gene silencing and no production of enzyme.

4.7. Co-Existence of Metallo Beta Lactamase Gene

Prsence and absence of MBL genes among MDR and non-MDR isoaltes of *P. aeruginosa* were illustrated in (table 4.12). The results reveald that 18/32 (56.25%) of MBL gene-negative isoaltes were phenotypically MDR while 17/68 (25%) of MBL gene-positive isolates were non-MDR. The phenotypically MDR isolates that havent MBL genes can be explained to have another resistance gene other than studied MBL genes. The non-MDR isolates that have at least one of the studied MBL genes may be explaind by gene silencing or non-induced MBL genes at level to express resistance (Stasiak *et al.*, 2021).

Table 4.12: Presence/absence of MBL genes among MDR and non-MDR isolates of *P. aeruginosa*

***P. aeruginosa* isolates with Resistance pattern**	**Positive for MBL genes (n=68)**	**Negative for MBL genes (n=32)**
MDR	51(75%)	18(56.25%)
non-MDR	17(25%)	14(43.75%)
Total	68(100%)	32(100%)

The genotypic detection of MBLs genes of *P. aeruginosa* isolates appeared that most of isolates harboured mor than one gene, the high association of genes was presence of 6 genes on same isolate. This coexistence of gene gave the bacteria potent resistance to antibiotics made the infection by it diffecult to treat. Table (4.13) display the pattren of coexistence of MBL genes.

Table 4.13: Genotypes of MBL genes among MDR and non-MDR isolates of *P. aeruginosa*

No. of Genes	Genotype	MDR	non-MDR	n(%)
	bla$_{KPC}$	2	0	
	bla$_{NDM}$	3	9	
	bla$_{IMP}$	3	2	
	bla$_{SIM}$	2	0	
	bla$_{VIM}$	2	0	
1	*bla*$_{GIM}$	1	0	24(35.29)
	bla$_{IMP}$/*bla*$_{NDM}$	5	3	
	bla$_{NDM}$/*bla*$_{VIM}$	2	0	
	bla$_{IMP}$/*bla*$_{GIM}$	5	0	
	bla$_{IMP}$/*bla*$_{VIM}$	1	0	
	bla$_{GIM}$/*bla*$_{VIM}$	1	0	
	bla$_{NDM}$/*bla*$_{SPM}$	1	0	
2	*bla*$_{SIM}$/*bla*$_{VIM}$	1	0	19(27.94)
	bla$_{NDM}$/*bla*$_{GIM}$/*bla*$_{VIM}$	2	0	
	bla$_{IMP}$/*bla*$_{GIM}$/*bla*$_{VIM}$	2	0	
	bla$_{IMP}$/*bla*$_{NDM}$/*bla*$_{VIM}$	2	0	
	bla$_{SIM}$/*bla*$_{SPM}$/*bla*$_{VIM}$	2	0	
	bla$_{IMP}$/*bla*$_{KPC}$/*bla*$_{NDM}$	1	0	
	bla$_{IMP}$/*bla*$_{KPC}$/*bla*$_{GIM}$	1	0	
	bla$_{IMP}$/*bla*$_{NDM}$/*bla*$_{GIM}$	1	0	
	bla$_{NDM}$/*bla*$_{SIM}$/*bla*$_{SPM}$	1	0	
3	*bla*$_{KPC}$/*bla*$_{NDM}$/*bla*$_{GIM}$	0	1	13(19.11)
	bla$_{IMP}$/*bla*$_{NDM}$/*bla*$_{GIM}$/*bla*$_{VIM}$	2	0	
	bla$_{IMP}$/*bla*$_{KPC}$/*bla*$_{GIM}$/*bla*$_{DIM}$	1	0	
	bla$_{KPC}$/*bla*$_{SIM}$/*bla*$_{GIM}$/*bla*$_{DIM}$	1	0	
	bla$_{KPC}$/*bla*$_{NDM}$/*bla*$_{SIM}$/*bla*$_{GIM}$	1	0	
4	*bla*$_{KPC}$/*bla*$_{SPM}$/*bla*$_{GIM}$/*bla*$_{VIM}$	0	1	6(8.82)
	bla$_{IMP}$/*bla*$_{KPC}$/*bla*$_{NDM}$/*bla*$_{GIM}$/*bla*$_{DIM}$	1	0	
	bla$_{IMP}$/*bla*$_{SIM}$/*bla*$_{GIM}$/*bla*$_{VIM}$/*bla*$_{DIM}$	1	0	
	bla$_{IMP}$/*bla*$_{NDM}$/*bla*$_{GIM}$/*bla*$_{VIM}$/*bla*$_{DIM}$	1	0	
5	*bla*$_{NDM}$/*bla*$_{SIM}$/*bla*$_{SPM}$/*bla*$_{GIM}$/*bla*$_{VIM}$	1	0	4(5.88)
	bla$_{IMP}$/*bla*$_{NDM}$/*bla*$_{SIM}$/*bla*$_{GIM}$/*bla*$_{VIM}$/*bla*$_{DIM}$	1	0	
6	*bla*$_{IMP}$/*bla*$_{KPC}$/*bla*$_{SIM}$/*bla*$_{SPM}$/*bla*$_{GIM}$/*bla*$_{DIM}$	0	1	2(2.94)
		51(75)	17(25)	68(100)

4.8. Bioactivity of Allicin, Sultamicillin tosylate and Diallyl trisulfied against *P. aeruginosa* isolates

Bioactivity of Allicin, Sultamicillin tosylate and Diallyl trisulfied against 10 multidrug resistance (MDR) and 10 non MDR *P. aeruginosa* isolates were tested to detect the antibacterial effects using 3 concentrations (50, 100 and 200 µg/ml) and DMSO was used as negative control. The results reveald that only sultamicillin have antibacterial effect and inhibition zone increased with increased concentration (Table 4.14).

The result of antibiotics and Sultamicillin tosylate/antibiotics showed high P.value for all MDR isolates, and the higher *P-value* was of ciprofloxacine with (0.5> 0.05). The effect on non-MDR isolates were significant for each of levofloxacine and meropenem with (0.04<0.05, and 0.03<0.05) respectively. The *P. value* for non-MDR of piperacillin showed the lower non- significant value with (0.056>0.05). As showen in table 4.14, table 4.15 and table 4.16.

Table 4.14: Antibacterial effects of allicin on *P. aeruginosa* isolates.

Mean±SD Inhibition Zone (mm) of Allicin				
***P. aeruginosa* Isolates**	**200 µg/ml**	**100 µg/ml**	**50 µg/ml**	**DMSO**
MDR isolates (n=10)	0.0±	0.0±	0.0±	0.0±
non-MDR isolates (n=10)	0.0±	0.0±	0.0±	0.0±

Table 4.15: Antibacterial effects of sultamicillin tosylate on *P. aeruginosa* isolates.

Mean±SD Inhibition Zone (mm) of Sultamicilin tosylate				
***P. aeruginosa* Isolates**	**200 µg/ml**	**100 µg/ml**	**50 µg/ml**	**DMSO**
MDR isolates (n=10)	12±5.808	11.5±2.944	7.9±1.677	0.0±
non-MDR isolates (n=10)	11±5.814	9.4±2.909	6.9±1.506	0.0±

Table 4.16: Antibacterial effects of diallyl trisulfied on *P. aeruginosa* isolates.

Mean±SD Inhibition Zone (mm) of Diallyl trisulfide				
***P. aeruginosa* Isolates**	**200 µg/ml**	**100 µg/ml**	**50 µg/ml**	**DMSO**
MDR isolates (n=10)	0.0±	0.0±	0.0±	0.0±
non-MDR isolates (n=10)	0.0±	0.0±	0.0±	0.0±

4.9. Inhibitory effect of Allicin, Sultamicillin tosylate and Diallyl trisulfied MBL enzymes

The Mean of inhibition zone (mm) for antibiotics and antibiotics/inhibitore combination and difference were calculated to asses the inhibitory effect of Allicin, Sultamicillin tosylate and Diallyl trisulfied on MBL enzymes. Actually from Table (4.14) the result showed there were no antibacterial effects of Allicin and Diallyl trisulfied while positive effect was documeneted for Sultamicillin tosylate, so increase (≥5mm) in the zone diameter after addition of Allicin and Diallyl trisulfied will indicate the inhibitory effects on MBL enzymes.

The results revealed non significant (<5mm) increase of inhibition zone for antibiotic+Allicin combination on MDR *P.aeruginosa* isolates and decrease in inhibition zone for antibiotic+Allicin combination on non-MDR isolates when compared with inhibition zone of each antibiotic alone (Table 4.15). For antibiotics+ Sultamicillin tosylate combination the results reveald decrese in inhibition zone diameter for MDR (except for combination with ciprofloxacin and levofloxacin) and increase in inhibition zone diameter for non-MDR isolates (Table 4.16).

Results of antibiotics+ Diallyl trisulfied combination reveald decrease in inhibition zone diameter of combination for both MDR and non-MDR isolates (Table 4.17). The overall results of inhibitory effects of

Allicin, Sultamicillin tosylate and Diallyl trisulfied MBL enzymes reveald no role for all 3 active ingredients of garlic havent inhibitory effects on MBL enzymes.

Table 4.17: Mean of inhibition zone(mm) for antibiotics and antibiotics+Allicin combination on MDR and non-MDR *P. aeruginosa* isoaltes

Isolate	**Mean inhibition zone (mm)**		**diffrent(mm)**
	Gentamycin (10µg)	**Gentamycin(10µg)+Allicin (100µg/ml)**	
MDR (n=10)	9.4	10.1	0.7
non-MDR (n=10)	15.7	13.7	-2
Isolate	**Ciprofloxacin (5µg)**	**Ciprofloxacin(5µg)+Allicin (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	13.7	15.6	1.9
non-MDR (n=10)	30.3	22.5	-7.8
Isolate	**Levofloxacin (5µg)**	**Levofloxacin(5µg)+Allicin (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	12.9	14.7	1.8
non-MDR (n=10)	25.2	19.4	-5.8
Isolate	**Pipracillin(100µg)**	**Pipracilin(100µg)+Allicin (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	11.5	13.7	2.2
non-MDR (n=10)	17.7	14.3	-3.4
Isolate	**Meropenem(10µg)**	**Meropenem(10µg)+Allicin (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	12.9	14.3	1.4
non-MDR (n=10)	32.1	33.8	1.7

Table 4.18: Mean of inhibition zone(mm) for antibiotics and antibiotics+ Sultamicillin tosylate combination on MDR and non-MDR *P. aeruginosa* isoaltes

Isolate	**Mean inhibition zone (mm)**		**diffrent(mm)**
	Gentamycin (10µg)	**Gentamycin(10µg)+Sultamicillin tosylate (100µg/ml)**	
MDR (n=10)	9.4	8.3	-1.1
non-MDR (n=10)	15.7	20.1	4.4
Isolate	**Ciprofloxacin (5µg)**	**Ciprofloxacin(5µg)+Sultamicillin tosylate (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	13.7	19.1	5.4
non-MDR (n=10)	30.3	33.9	3.6
Isolate	**Levofloxacin (5µg)**	**Levofloxacin(5µg)+Sultamicillin tosylate (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	12.9	14.9	2
non-MDR (n=10)	25.2	28	2.8
Isolate	**Pipracillin(100µg)**	**Pipracilin(100µg)+Sultamicillin tosylate (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	11.5	9.9	-1.6
non-MDR (n=10)	17.7	20.1	2.4
Isolate	**Meropenem(10µg)**	**Meropenem(10µg)+Sultamicillin tosylate (100µg/ml)**	**diffrent(mm)**
MDR (n=10)	12.9	10.9	-2
non-MDR (n=10)	32.1	35.9	3.8

Table 4.19: Mean of inhibition zone(mm) for antibiotics and antibiotics+ Diallyl trisulfied combination on MDR and non-MDR *P. aeruginosa* isoaltes

Isolate	**Mean inhibition zone (mm)**		**diffrent(mm)**
	Gentamycin (10µg)	**Gentamycin(10µg) +Diallyl trisulfied(100µg/ml)**	
MDR (n=10)	9.4	8.3	-1.1
non-MDR (n=10)	15.7	11	-4.7
Isolate	**Ciprofloxacin (5µg)**	**Ciprofloxacin(5µg) +Diallyl trisulfied(100µg/ml)**	**diffrent(mm)**
MDR (n=10)	13.7	7.5	-6.2
non-MDR (n=10)	30.3	18.5	-11.8

Isolate	**Levofloxacin (5µg)**	**Levofloxacin(5µg) +Diallyl trisulfied(100µg/ml)**	**diffrent(mm)**
MDR (n=10)	12.9	9	-3.9
non-MDR (n=10)	25.2	16.3	-8.9
Isolate	**Pipracillin(100µg)**	**Pipracilin(100µg) +Diallyl trisulfied(100µg/ml)**	**diffrent(mm)**
MDR (n=10)	11.5	10.1	-1.4
non-MDR (n=10)	17.7	10.9	-6.8
Isolate	**Meropenem(10µg)**	**Meropenem(10µg) +Diallyl trisulfied(100µg/ml)**	**diffrent(mm)**
MDR (n=10)	12.9	11.9	-1
non-MDR (n=10)	32.1	33.8	1.7

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CHAPTER FIVE

Conclusions and Recommendations

Chapter Five

Conclusions and Recommendations

5.1. Conclusions

The conclusions of this study can be summarized in the following.

1-The frequency of Multidrug resistance *P. aeruginosa* in burn wounds still high.

2-Resistane to 5 and 6 classes of antibiotics can push alarm for emergence of XDR or even PDR isolates.

3-The phenotypic assays to ivestigate MBL enzymes may be not enough to enrol or *P. aeruginosa* with MBL.

4-High frequency of MBLs genes among MDR *P. aruginosa* isolates.

5-All studied MBLs genes may present in non-MDR *P. aeruginosa* isolates indicating silencing or no enought induction of MBL genes.

6-Presence of MBLs genes among non-MDR isolates may establish real threat of resistance posibility in *P. aeruginosa* isolates documented as sensitive.

7-Coexsitance of multiple MBLs genes within same isolate and even within non-MDR *P. aeruginosa* isolates

8-Ineffectivity of Garlic active ingredents (Allicin, Sultamicillin tosylate and Diallyl trisulfied as antibacterial or inhibitor for MBLs enzymes.

5.2. Recommendations

1-Studying the gene expression and study MBL genes in MDR and non-MDR *P. aeruginosa* isolates.

2-Studying the group A, C and D β-lactamases gene.

3- Study the effect of new natural products as antimicrobial agent.

4- Study virulence factor genes of *P. aeruginosa* that related with the Antibiotic resistant.

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أظهرت النتائج أن 68٪ من العزلات تحتوي على جينات الإنزيمات المعدنية المحللة للبيتا لاكتام. كان الجين ذو الانتشار العالي هو جين bla_{NDM} بنسبة 100/38 (38٪)، وكان أقل انتشار هو جين bla_{SPM}، وجين bla_{DIM} بنسبة 100/7 (7٪). كانت نتائج انتشار الجينات الأخرى 100/34 (34٪) لجين bla_{IMP}، 100/26 (26٪) لجين bla_{GIM}، 100/22 (22٪) لجين bla_{VIM}، 100/12 (12٪) لجين bla_{SIM} الجين، و 100/11 (11٪) لجين bla_{KPC}.

أظهرت نتائج اختبار الحساسية للمضادات الحيوية لجميع العزلات من جميع الجينات أن أعلى مقاومة كانت ضد البيبراسيلين والأقل مقاومة لمعظم الجينات كانت ضد السيفيبيم.

أظهرت النتائج تعايش جينات الإنزيمات المعدنية المحللة للبيتا لاكتام في معظم عزلات MDR. كان الارتباط العالي للجينات 6 جينات في نفس العزلة التي أظهرت مقاومة لثمانية مضادات حيوية.

تم اختبار تأثير المثبطات على قدرة بكتريا الزائفة الزنجارية على إنتاج الإنزيمات المعدنية المحللة للبيتا لاكتام باستخدام ثلاثة مثبطات مختلفة Allicin و Sultamicillin Tosylate و Diallyl Trisulfide. تم اختبار هذه المثبطات بشكل فردي وبالتزامن مع المضادات الحيوية على عزلات MDR وغير MDR. أظهرت النتائج عدم وجود اي دور لجميع المكونات النشطة الثلاثة وعدم وجود تأثيرات مثبطة للثوم على إنزيمات MBL.

الخلاصة

تتسبب إصابات الحروق الخطيرة في تلف الجلد وفقدانه، وهو ما يعتبر بمثابة الخط الأول لحماية الجسم من العدوى الميكروبية. يمكن للبكتيريا الانتهازية بما في ذلك بكتريا الزائفة الزنجارية أن تغزو جسم المريض من خلال مناطق الحروق وتصيبه بأمراض مختلفة قد تؤدي إلى الوفاة.

عملت هذه الدراسة على عزل بكتيريا الزائفة الزنجارية من أخماج اصابات الحروق واختبار تأثير بعض المثبطات على قدرة البكتيريا على إنتاج الإنزيمات المعدنية المحللة للبيتا لاكتام (MBLs) المسؤولة عن مقاومتها للمضادات الحيوية.

خلال الفترة من كانون الأول 2021 إلى نيسان 2022، تم جمع 249 مسحة من مرضى الحروق، تم تحديد 249/100 (40.16٪) من المسحات على أنها بكتريا الزائفة الزنجارية. تم تشخيص جميع العزلات عن طريق الزرع على وسط انتقائي (سيترمايد أكار) وتم تأكيدها عن طريق تفاعل البوليمر المتسلسل (PCR) باستخدام الجين (16s rDNA) الخاص بجنس الزائفة الزنجارية.

تم إجراء اختبار الحساسية للمضادات الحيوية وفقًا لـ CLSI2021 لتحديد البكتيريا المقاومة للأدوية المتعددة (MDR). أظهرت النتائج أن 100/69 (69٪) كانت عزلات MDR. و كانت استجابة 100/81 (81٪) من العزلات حساسة لمضاد سيفيبيم بينما كانت 100/83 (83٪) مقاومة لعقار بيبراسيلين، ومع ذلك هذه المقاومة قلت عند استخدام بيبراسيلين/تازاباكتام.

أظهرت الأنماط المقاومة لعزلات MDR للمضادات الحيوية أن النمط الأكثر تكرارا كان بيبراسيلين/ نيتلامايسين/ جينتامايسين/ سيبروفلوكساسين/ ليفوفلوكساسين مع تكرار 70/4 عزلة (5.71٪)، وسجل عدد العزلات المقاومة إلى 5 مضادات حيوية أعلى نسبة. نسبة (25.7٪) بينما أقل نسبة (1.4٪) تذهب إلى العزلات المقاومة لـ 12 مضاد حيوي.

تم الكشف عن النمط الظاهري للإنزيمات المعدنية المحللة للبيتا لاكتام بواسطة اختبار تآزر القرص المشترك (CDST)، وأظهرت النتائج أن 4٪ من العزلات كانت إيجابية مع الإنزيمات المعدنية المحللة للبيتا لاكتام بينما كانت 96٪ سلبية.

تم إجراء الكشف عن النمط الجيني للتحقق من وجود الجينات bla_{VIM} وbla_{GIM} و bla_{IMP} و bla_{SIM} و bla_{SPM} و bla_{KPC} و bla_{DIM} و bla_{NDM} التي تم ترميزها بواسطة الزائفة الزنجارية لإنتاج إنزيمات MBLs.

جامعة كربلاء
كلية العلوم
قسم علوم الحياة

تأثير بعض المثبطات على الأنزيمات المعدنية المحلِّلة لِلبيتا لاكتَام والمشخصة جينياً لبكتريا الزائفة الزنجارية المعزولة من أخماج جروح الحروق

رسالة

مقدمة إلى مجلس كلية العلوم ـ جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستير علوم في علوم الحياة

من قبل

أنسام عدنان أحمد الغريري

بإشراف:

الاستاذ المساعد الدكتور
كوكب عبدالله حسين السعدي

الاستاذ الدكتور
حسين عليوي مطلب الدهموشي

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