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Molecular Study of Metallo-β-lactamases with Integron Class I of *Pseudomonas aeruginosa* Isolates

A dissertation

Submitted to the Council of College of Science at University of Babylon in Partial Fulfillment of Requirement for the Degree of Doctorate of Philosophy of Science in Biology

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I certify that the preparation of this dissertation was prepared by **Ahmed Abdulkareem Hadi Mohammad Almuttairi** under my supervision at University of Babylon, Collage of Sciences, Department of Biology, as a Partial Fulfillment of the Requirement for the Degree Doctorate of Philosophy of Science in Biology. Accordingly, I recommend this study for discussion.

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Dedication

I Dedicate My Humble to Work as Guardian of GOD's Greatest Imam Mahdi (Allah Hasten His Reappearance)

To martyrs of Iraq

To My First Teacher who gives me Encouragement, Happiness, and Inspiring me with Hope;

My Mother

To the Great Person who The Source of Endless Support and Strength;

My Father

To my support and my shadow and who immerses me with his prayers

My Wife

To the Candles which light my life;

My Brothers: Arshed, Ameer and Aadel

To my colleagues in various knowledge fields;

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Summary

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* represents a frequent and challenging nosocomial pathogen. The World Health Organization (WHO) designated carbapenem-resistant *P. aeruginosa* a priority 1 or ‘‘critical’’ pathogen in substantial need of new therapies to counteract this imminent public health crisis of resistance. Integron genes as mobile genetic elements are playing an important role in the spread of *P. aeruginosa* antibiotic resistance. Class I integrons have the most diverse gene cassettes encoding antibiotic resistance genes.

One hundred thirty one of *P. aeruginosa* isolates were recovered from 385 (34.0%) clinical specimens of urine, burns, wounds, ear and sputum collected from both sexes with different ages were isolated from different hospitals and laboratories in Babylon province, during the period of February 2022 to October 2022.

P. aeruginosa was isolated from various clinical specimens 131 (34.0 %), devided to burns comprised103 (78.63%) of all clinical specimens, wounds 7(5.34 %), urine 9 (6.87%), ear 9 (6.87%), and sputum 3 (2.29%). The isolates were diagnosed by routine biochemical tests and VITEK® 2 Compact System then confirmed the species by species-specific PCR assay with *ecfX* gene.

The results revealed resistance to 22 antibiotics at percentages as follows: all *P. aeruginosa* isolates were resistant to Ampicillin, Cefixime, Cefotaxime in 131 (100%); followed by Ceftizoxime 130 (99.23%), Cefepime 123 (93.90%), Tetracycline 120 (91.60%), Doxycycline 119 (90.84%), Cefoperazone 111 (84.73%), Gentamicin 110 (83.97%), Tobramycin and Meropenem in 107 (81.68%), Ticarcillin clavulanic 106 (80.91%), Amikacin 104 (79.39%) Piperacillin 102 (77.86%), Levofloxacin 99 (75.57%), Aztreonam 97 (74.05%), Ciprofloxacin 96 (73.28%), Imipenem 95 (72.52%), Norfloxacin 94 (71.75%),

Piperacillin-tazobactam 84 (64.12%), and Doripenem 71 (54.20%). Azithromycin demonstrated the lowest level of resistance 53 (40.46%). All isolates (100%) displayed multidrug- resistance (MDR) resistance to at least three groups of antibiotics, with some isolates displaying resistance to nearly all classes.

The percentage of β-lactamases production was 33/131 (25.19%) of MDR isolates. The metallo-β-lactamases enzymes were 3 (2.29%) and 10 (7.63%) of MDR isolates by Combined Disk Diffusion Test (CDDT) and Epsilo meter-test (E-test) Imipenem /Imipenem EDTA methods respectively.

Out of 131 *P. aeruginosa* isolates examined for biofilm formation, 112 (85.49%) had biofilm-forming capacity, and were classified as follows: 40 (30.53%) generated strong biofilm, 51 (38.93%) formed moderate biofilm, 21 (16.03%) formed weak biofilm, whereas 19 (14.50%) formed non-biofilm. Antimicrobial resistance was found to be significantly higher in biofilm producing *P. aeruginosa* isolates than in non-biofilm producing *P. aeruginosa* isolates ($p \leq 0.05$).

P. aeruginosa chromosomal DNA and plasmid DNA were extracted and a plasmid has been identified in 63/131(48 %) *P. aeruginosa* isolates.

PCR analysis showed the presence of the genes encoding (integrases) to class 1 (*intI1*) in (100%), while no isolates gave an *intI1* gene carried on a plasmid. Regarding the presence of (*intI2*) 5 (3.81%), and (*intI3*) (0) of MDR isolates, while the genes that harbored on plasmid were not detectable *intI2* gene, and one positive isolation resulted from the genes located on the plasmid DNA for *intI3*.

Statistical analysis of the present study revealed a significant relationship between the presences of the class 1 Integron gene with resistance to different antibiotic classes ($p \leq 0.05$). The analysis of integron gene cassettes indicated that integron I variable regions were amplified in 131 isolates, with each isolate displaying 1 variable region ranging in size from 200 bp to 8000 bp.

The results of molecular detection of virulence genes revealed that the highest rate of *oprL* genes among *P. aeruginosa* isolates was founded in 131 (100%), followed by *Alg* and *oprI* in 129 (98.47%), respectively, and 125 (95.42%) for *oprD*2 of MDR isolates.

The frequency of Metallo -β- lactamases **(**MBLs) - encoding genes: the bla_{IMP-1}, bla_{IMP-2}, bla_{IMP-7}, bla_{IMP-10}, bla_{IMP-13}, bla_{IMP-25}, bla_{VIM-1}, bla_{VIM-2}, bla_{NDM}, bla_{GIM-1}, bla_{SIM-2} and bla_{SPM} genes were determined by PCR in 131 isolates. Three isolates (2.29%) were found to have just the bla_{IMP-2} gene, follow by 3(2.3%), 6(4.58%), 125(95.42%), 7(5.34%), and 12(9.16%) for bla_{VIM-1}, bla_{VIM-2}, bla_{NDM}, bla_{SIM-2} and bla_{SPM} genes respectively carried on chromosomal DNA, with no additional genes detectable. However, it was found that 9 (14.29), 5(7.94), 8(12.70%), 3(4.76%), and 1(1.59%) isolates possessed bla_{IMP-7}, bla_{IMP-13}, bla_{VIM-1}, bla_{VIM-2}, and bla_{SPM} genes respectively, carried on plasmid DNA, respectively, while the other genes were not detected. It is noteworthy that all 131 *P. aeruginosa* isolates harbored *intI1* gene

The present findings of the variable region (5'CS/3'CS) correspond to ten isolates, and they showed genetic homology sequences similarity with NCBI BLAST sequences (99%). The genetic variation study of the *oprD2* gene reveals deletion mutations when compared to NCBI BLAST.

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List of Abbreviations

Abbreviation	Terms
AR	Antibiotic Resistance
bla Genes	β-lactamases Gene
CDDT	Combined Double Disk Test
CFU	Colony Forming Unit
CIs	Chromosomal Integrons
CLSI	Clinical and Laboratory Standards Institute

Abbreviation	Terms
DNA	Deoxyribonucleic Acid
eDNA	Environmental DNA
EDTA	Ethylene Diamine Tetra Acetic Acid
GIM	Germany Imipenemase
GN	Gram Negative
HGT	Horizontal Gene Transfer
IMD	Imipenem + EDTA
IMI/IMD	Imipenem /Imipenem + EDTA
intI1	Integrase Gene of Class 1 Integron
intI2	Integrase Gene of Class 2 Integron
intI3	Integrase Gene of Class 3 Integron
IS	Insertion Sequences
MBL	Metallo-Beta-Lactamase
MDR	Multi Drug Resistance
MGEs	Mobile Genetic Elements
MIs	Mobile Integrons
NDM	New Delhi metallo-β-lactamase
OD	Optical Density
PBPs	Penicillin-Binding Proteins
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
SIM	Seoul Imipenemase

Abbreviation	Terms
SPM	Sao Paulo Imipenemases
TBE	Tris boric acid EDTA buffer
Tns	Transposons
UTI	Urinary Tract Infection
UV	Ultraviolet
VFs	Virulence Factors
VIM	Verona integron-encoded metallo-β- lactamase
WHO	World Health Organization

Units Abbreviations

Abbreviation	Units
A˚	Angstrom
µg	Microgram
Mg	Milligram
G	Gram
µl	Microliter
Ml	Milliliter
µm	Micrometer
Mm	Millimeter
Hr	Hour
Sec	Second
Rpm	Rotations per Minute
Min	Minute
Pmol	Picomol
Bp	Base pair
˚C	Celsius degree

Chapter One

Introduction

1. Introduction

P. aeruginosa, a human opportunistic Gram-negative pathogen, is one of the most important nosocomial pathogens and is a major health problem, primarily in immunocompromised individuals (Çiçek *et al*., 2021). *P. aeruginosa* has several innate or acquired resistance mechanisms, with high rates of resistance to diverse antibiotic classes that proved a multidrug resistance (Spagnolo *et al*., 2021). Dissemination of antibiotic resistance genes by horizontal transfer results in the rapid emergence of antibiotic resistance among clinical bacterial isolates (Khosravi *et al*., 2011).

The gene acquisition by horizontal gene transfer is mediated by mobile genetic elements such as plasmids, transposons and integrons. Integrons are one of the main types of mobile elements known to be the natural gene capture systems in bacteria that are considered responsible for multi-drug resistance. These mobile elements have two conserved regions (integrase and recombination site) divided by a variable region that includes integrated gene cassettes, which frequently include antibiotic resistance genes. Gene cassettes can be mobile and contain only a single gene and a specific recombination site 59 base element; hence, they play important roles in the horizontal dissemination of antibiotic resistance genes among bacteria (Sabbagh *et al*., 2021).

Several typing methods have been used to study the evolution and genetic heterogeneity of *P. aeruginosa* because it is characterized by high genetic diversity. In recent years, the development of Whole Genome Sequencing has given rise to the study of Multi-Drug Resistant (MDR) *P. aeruginosa* molecular epidemiology (Telling *et al*., 2018). However, more than 130 different gene cassettes have been found in class 1 integrons, most of which are the antibiotic-resistance gene cassettes encoding proteins with resistance to all main antibiotic classes. These

include the quaternary ammonium-compound family, erythromycin, aminoglycosides, sulfonamides, quinolones, chloramphenicol, fosfomycin, trimethoprim, β-lactam, and other clinically relevant antibiotics (Liu *et al*., 2020).

Metallo-β-lactamases are metallo enzymes of Ambler class B which are clavulanic acid resistance enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by EDTA as well as other chelating agents of divalent cation (Bahr *et al*., 2021). There are two dominant types of transferable MBL genes among clinical isolates, bla_{IMP} (Beta lactamase Imipenemase) and bla_{VIM} (Verona integron-encoded metallo-β-lactamase), which are frequently present on gene cassettes inserted into integrons located on the chromosome or on plasmids (Anoar *et al*., 2014). Other types of MBLs such as bla_{AIM}, bla_{GIM}, and bla_{SPM} are found only sporadically in some geographic regions (Abdulateef *et al.,* 2014). Most integrons containing gene cassette for MBL also harbor additional gene cassette classes such as aminoglycoside or chloramphenicol, therefore integron transfer might lead to a single step transfer of complex multidrug resistant phenotype and the dissemination of MBL genes among Gram negative pathogens is mediated by mobile elements of DNA which explains why the same gene might be associated with plasmids or interested in chromosome in different strains (Jeon *et al*., 2023).

Bacterial integrons are useful PCR amplification target in epidemiological surveys of bacterial antibiotic resistance, therefore the aim of this study was to investigate the association between class I, II and III integrons and metallo-β-lactamase *P. aeruginosa* isolates with the following objectives:

1. Isolation and identification of *P.aeruginosa* from clinical specimens.

2. Detection of the antibiotic susceptibility profile and biofilm assay of *P. aeruginosa* isolates.
3. Molecular detection of metallo-β-lactamase genes (bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2}, bla_{SPM}, bla_{SIM-2}, bla_{NDM-1} *and* bla_{GIM}) and biofilm related- genes by PCR assay.
4. Survey and detection of the presence of class integron genes (*Intl1*; *Intl2; Intl3*) and gene cassettes (5'CS/3'CS) by PCR assay.
5. Study the interaction between the metallo-β-lactamase and intergons with MDR *P. aeruginosa*.
6. Sequencing of *oprD2* and 5'CS genes in some of *P. aeruginosa* isolates.

Chapter Two

Literature Review

2. Literature Review

2.1. Historical Perspective of *Pseudomonas* species

Pseudomonas was first isolated by a French military surgeon Sedillot in 1850 from a blue-green discharge on surgical dressing with associated infections, the genus *Pseudomonas* was first proposed early in microbiological history by Migulas in 1894. Migulas first illustration of this genus was very brief and, in hindsight, inaccurate. His description stated “Cells with polar organs of motility". Formation of spores occurs in some species, but it is rare. Eventually, the *Pseudomonads* were correctly identified as being non-spore-forming and the “spores” originally visualized by Migula have been postulated to be “refractile granules of reserve materials” (Baxter, 2000; Mahmoudi *et al*., 2021).

2.2 General Characteristics

Pseudomonas aeruginosa is a Gram negative bacteria with rod shape, structure includes a (0.5 – 0.8) μm by (1.5-3.0) μm, and has one flagellum for mobilization. *P. aeruginosa* is positive for an oxidase reaction. Moreover, it is permanently unable to ferment lactose (Minion, 2010). In culture media, there are three different categories of colonies that could be produced by *P. aeruginosa* isolates. Small, rough colonies are often produced by natural isolates from soil and water. Medical specimens are often one of two types: smooth with flat edges and an inflated appearance, or rough with raised edges and a raised appearance. It has a mucoid look, which is thought to be due to the formation of alginate slime in some other type, which is usually derived from respiratory and urinary tract secretions. Several researchers believe that smooth and mucous colonies perform a function in colonization and pathogenicity (Bae *et al*., 2014).

P. aeruginosa is a non-fermentative aerobe that derives its energy from oxidation rather than fermentation of carbohydrates. Although able to use more than 75 different organic compounds, it can grow on media supplying only acetate for carbon and ammonium sulfate for nitrogen. Furthermore, although an aerobe, it can grow anaerobically, using nitrate as an electron acceptor (Ghrissi, 2020). This organism grows well at 25° C to 37° C, but can grow slowly or at least survive at higher and lower temperatures. Indeed, the ability to grow at 42° C distinguishes it from many other *Pseudomonas* species. In addition to its nutritional versatility, *P aeruginosa* resists high concentrations of salt, dyes, weak antiseptics, and many commonly used antibiotics. These properties help explain its ubiquitous nature and contribute to its preeminence as a cause of nosocomial infections (Shrivastava *et al*., 2004).

In the laboratory, *P. aeruginosa* was capable to grow on varied types of media, ranging from minimal to complex. Most isolates were easily recognizable on primary isolation media on the basis of colonial morphology, a grape-like odor and production of hydro soluble pigments such as pyocyanin (blue), pyorubin (red), pyomelanin (brown-black) and pyoverdin (yellow-green or yellow-brown) (Divya, 2013).

2.3. Pathogenicity of *P. aeruginosa*

P. aeruginosa occurs in both abiotic and biotic environments, from soil and aquatic environments to plant and animal tissues. *P. aeruginosa* is an important nosocomial pathogen, invasive and toxigenic, produces infections in patients with abnormal host defenses, including those who have weak immune systems, the elderly, and those who have been hospitalized for long periods of time (Sadikot *et al*.,2005). It is opportunistic pathogens, causing acute or chronic infections including: several nosocomial and life-threatening infections in patients with cystic

fibrosis (CF), wounds, urinary tract infections (UTIs), immunocompromised individuals with chronic obstructive pulmonary disease (COPD), cancer, traumas, burns, sepsis, and ventilator-associated pneumonia (VAP) including those caused by COVID-19, and pulmonary infections; from the medical equipment, such as inhalers, dialysis equipment, respirators, anesthesiology equipment, and vaporizers; and from toilets and sinks (Azam and Khan 2019 and Qin *et al*., 2022).

Wound infection is one of health problems that are caused by the invasion of pathogenic organisms in different part of body and it threat life of large number of people in many countries. *P. aeruginosa* is found as major colonizer of the burn wound because it lives on moist burn wound surface and usually gains access to burn patients through cross contamination. It persists as major nosocomial infection threat to burn patients, arising of resistance against multiple antimicrobial drugs frequently complicates the treatment of *P. aeruginosa* infection. This may lead to serious infection and thus mortality rate in these patients become high (Estahbanati *et al*., 2002; AL-Shamaa *et al*., 2016).

2.4. Epidemiology of *P. aeruginosa*

P. aeruginosa has a well-documented clinical history as associated with burn and wound infections; however, recent manifestations of *P. aeruginosa* include pneumonias in cystic fibrosis patients, endocarditis in drug addicts, postoperative wound infections, urinary tract infections (UTIs), and sepsis (Bennett *et al*.,2015). A true community-acquired *P. aeruginosa* infection among patients without any prior health care exposure is rare, as *P. aeruginosa* is not part of a healthy human's microbiota (Gales *et al*., 2001). Common community-acquired infections include ulcerative keratitis, otitis externa, and skin and soft tissue infections (SSTIs) (Driscoll *et al*., 2007). Community-acquired infections are commonly linked to

recreational water use, contact lens use, home humidifiers, soil, and vegetables (Craun *et al*., 2005).

According to data reported to the National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention (CDC), *P. aeruginosa* is the fifth most common pathogen implicated in all hospital-acquired infections (Sievert *et al*., 2013). *P. aeruginosa* is the most common bacteria implicated in nosocomial pneumonia and the second most common pathogen implicated in ventilator-associated pneumonia (Bennett *et al*., 2015). Common hospital-acquired infections include pneumonias, UTIs, blood stream infections (BSIs), surgical site infections, and skin infections (Driscoll *et al*., 2007). Hospital-acquired infections are estimated to complicate 5 to 10% of hospitalizations in the United States (US) annually, leading to increased health care costs and prolonged hospitalizations (Ledizet *et al*., 2012). The CDC estimates that there are about 51,000 healthcare-associated *P. aeruginosa* infections in the US per year (CDC, 2017). When a microbiological agent is identifiable, 11.3 to 13.8% of all nosocomial infections are caused by *P. aeruginosa*, this percentage increases in infections reported in intensive care units (ICUs) by 13.2 to 22.6% (Driscoll *et al*., 2007).

P. aeruginosa has the ability to develop resistance to an antibiotic during the course of treating the infection (Lister *et al*., 2009) According to the CDC, in 2013 there were 6,700 MDR infections of *P. aeruginosa* that resulted in 440 deaths (CDC, 2017). Infections caused by resistant strains have been found to be associated with a three-fold higher rate of mortality, a nine-fold higher rate of secondary bacteremia, a two-fold increase in the length of hospital stay, and a noticeable increase in healthcare costs (Mesaro *et al*., 2007) It is important, therefore, to monitor *P. aeruginosa* trends and changes in epidemiology on an ongoing basis. This analysis presents surveillance of *P. aeruginosa* infection

burden among Military Health System (MHS) beneficiaries in calendar year (CY) 2015 (Gierhart and Chukwuma, 2017).

2.5. Virulence Factors of *P. aeruginosa*

Several virulences may cause pathogenicity that facilitates adhesion and/or disrupt host cell signaling pathways while targeting the extracellular matrix. *P. aeruginosa* is capable of causing several diseases invading the organism and its immune system leading infections nearly impossible to eradicate (Skariyachan *et al*., 2018) .

P. aeruginosa possesses a variety of virulence factors that may contribute to its pathogenicity, such as adhesions exotoxin A, exoenzyme S, *nan* 1 and *Las* genes. The outer membrane proteins of *P. aeruginosa* OprI and OprL play important roles in the interaction of the bacterium with the environment as well as the inherent resistance of *P. aeruginosa* to antibiotics where the consequence of the presence of these specific outer membrane proteins that have been implicated in efflux transport systems that affect cell permeability (De Vos *et al*.,1997; Mengal *et al*., 2019).

P. aeruginosa can produce some toxic proteins which not only cause extensive tissue damage but also interfere with the human immune system's defense mechanisms. These proteins range from potent toxins that enter and kill host cells at or near the site of colonization to degradative enzymes that permanently disrupt the cell membranes and connective tissues in various organs (Ray *et al*., 2010).

P. aeruginosa also effectively colonizes a variety of surfaces including medical materials (urinary catheters, implants, contact lenses, etc.). It is important therefore to diagnose *P. aeruginosa* infections before biofilm development which could enhance the susceptibility of *P. aeruginosa* towards antimicrobial treatments.

However, the increasing incidence of acute and persisting infections worldwide also highlights the need to develop therapeutic strategies as an alternative to traditional antibiotics, expectedly to disarm and eradicate this Gram-negative bacterium (Thi *et al*., 2020).

2.5.1. Flagellum and Pili

P. aeruginosa initiates infection by virtue of cell adhesion molecules like adhesions, flagella and pili (essential part of bacterial chemotaxis, initiates an inflammatory response and may mediate initial surface interactions by binding with asialylated glycolipid (aGM1) of epithelial cells of the host). Its characteristic allows *P. aeruginosa* to exhibit swimming and swarming motilities (Kazmierczak *et al*., 2015; Chadha *et al*., 2022).

2.5.2. Outer Membrane Components

The Gram-negative bacterial cell membrane is composed of an outer membrane and an inner membrane. The outer membrane is exposed to the outside environment, while the inner membrane envelops the cytoplasm. Contrary to the usual biological membrane, the gram-negative bacterial membrane does not have a phospholipid bilayer but an asymmetric bilayer with LPS on the outside and phospholipid on the inside (Sperandeo *et al*., 2019). Lipopolysaccharide (LPS) is a major component of the outer membrane in Gram-negative bacteria, and can be found in all *P. aeruginosa* strains (Pier, 2007).

2.5.2.1. Lipopolysaccharide (LPS)

The LPS is a predominant component of the outer membrane of *P. aeruginosa*. Bacterial LPS typically consists of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating core oligosaccharide, and a distal polysaccharide (or O-antigen) (Pier and Ramphal, 2010). LPS plays a prominent role in the activation

of the host's innate (TLR4, NLRP1, NLRP2, and NLRP3) and adaptive (or acquired) immune responses; and, eventually causes dysregulated inflammation responses that contribute to morbidity and mortality. LPS may be the most extensively studied bacterial molecule due to its high immunogenicity and surface accessibility (Liao *et al.*, 2022).

2.5.2.2. Outer Membrane Proteins

The outer membrane of Gram- negative bacteria, such as *P. aeruginosa*, which acts as a selective barrier to prevent antibiotic penetration, is an asymmetric bilayer of phospholipid and LPS embedded with porins that form β-barrel protein channels (Delcour, 2009). Generally, the family of porins can be divided into four classes: the non-specific porins, which allow for slow diffusion of most of the small hydrophilic molecules; specific porins, which possess specific sites to bind a particular set of molecules; gated porins, which are ion-regulated outer membrane proteins responsible for uptake gated porins, which are ion-regulated outer membrane proteins responsible for uptake of ion complexes; and efflux porins, which are important components of efflux pumps (Hancock and Brinkman, 2002; Masi *et al*., 2019).

In *P. aeruginosa*, the OprF protein is the major non-specific porin; OprB, OprD, OprE, OprO and OprP are specific porins; and OprC and OprH belong to the class of gated porins. The class of efflux porins includes OprM, OprN and OprJ (Hancock and Brinkman, 2002). The outer membrane permeability of *P. aeruginosa* is extremely restricted; it is about 12-to 100-fold lower than that of *E. coli* (Breidenstein *et al*., 2011).

OprF, a homolog of *E. coli* outer membrane protein A (OmpA), is the predominant porin of *P. aeruginosa* and is responsible for non-specific uptake of ions and saccharides including trisaccharides and tetrasaccharides, but it has low

efficiency for antibiotic permeation (Pang *et al*., 2019). OprF is able to fold into two conformers: the two-domain closed conformer consisting of an N-terminal transmembrane β-barrel and a C-terminal periplasmic globular domain, and the one-domain open-channel conformer containing a single transmembrane domain. The closed conformer is the dominant structure of OprF channels, and only a small fraction of OprF form open channels, representing less than 5% of this protein population (Sugawara *et al*., 2006). The presence of mostly closed OprF channels may explain why the outer membrane permeability of *P. aeruginosa* is much lower than other bacteria. Additionally, absence of the *P. aeruginosa* OprF leads to increased biofilm formation through up regulation of bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), which is an important messenger for controlling biofilm formation (Bouffartigues *et al*., 2015).

As mentioned above, *P. aeruginosa* possesses a number of specific porins, including the carbohydrate-specific porin OprB, the basic amino acid-specific porin OprD, the phosphate- specific porin OprP, and the pyrophosphate-specific porin OprO (Hancock and Brinkman, 2002). OprH is the smallest *P. aeruginosa* porin, and overexpression of OprH as a consequence of Mg^{+2} starvation has been found to be associated with increased resistance to polymyxin B and gentamicin through stabilization of the outer membrane by inducing LPS modification (Macfarlane *et al*., 1999).

OprD is a small, specialized outer membrane porin protein allowing for selective permeation of basic amino acids and other structures including carbapenems. OprD porin consists of 443 amino acids that allows the diffusion of sugars, small peptides, basic amino acids, and carbapenems typically imipenem into the cell (Pirnay *et al.,* 2002; Suelter, 2020), and sixteen-strand transmembrane beta-barrel structure and of eight loop regions (L1–L8) localized on the external

surface. Alterations of the loop 3 region have been showed to increase the imipenem minimum inhibitory concentration (MIC). Resistant strains have lost or have very reduced levels of OprD expression, which prevents carbapenems from destroying invading pathogens (Angeletti *et al.,* 2018). OprD is involved in antibiotic uptake, it contains the binding sites for carbapenems, a class of β- lactam antibiotics, and absence of OprD in *P. aeruginosa* increases the resistance to this class of antibiotic (Li *et al*., 2012). Carbapenem resistance of *P. aeruginosa* is mainly due to a combination of different factors, including low permeability of outer membrane porin and mutations in the gene encoding OprD, the production of the *AmpC* bate-lactamases, overproduction of efflux systems, co-regulation with trace metal resistance mechanisms such as Zinc and Copper, salicylate-mediated reduction, and producing Carbapenemase (Amin *et al*., 2005; Rostami *et al*., 2018).

However, among these mechanisms, the loss or mutation of outer membrane porin (*oprD*) and promoter of this gene appears to be the most common mechanisms of intrinsic resistance to imipenem and a lesser extent to meropenem. This mechanism causes blocking of the entrance of carbapenems particularly imipenem into a bacterium (Amin *et al*., 2005; Shen *et al*., 2015). The typed of mutations in the *oprD* gene and upstream regions and promoters of this gene are various such as nucleotide deletions, insertions, and point mutations that have been recognized to be the major mechanisms leading to inactivation of the *oprD* gene and promoter in imipenem-resistant isolates of *P. aeruginosa* (Pirnay *et al*., 2002; Gutiérrez *et al*., 2007).

Transcription of *oprD* in *P. aeruginosa* PAO1 initiates with equal frequencies from two start sites, located 23 bases (SS1) and 71 bases (SS2) upstream of the structural gene (Kiani *et al*., 2021).

2.5.3. Biofilm Formation

Biofilms are communities of microorganisms that are attached to a surface and play a significant role in the persistence of bacterial infections (Rabin *et al*., 2015). Biofilm formation is one of the key strategies for the survival of species during unexpected changes of living conditions such as temperature fluctuation and nutrient availability. Bacteria within a biofilm can escape host immune responses and resist antimicrobial treatments up to 1000 times more than their planktonic counterparts (Lewis, 2001; Petrova and Sauer, 2016). *P. aeruginosa* is a well-known biofilm former, which makes it an excellent model to study biofilm formation (Ghafoor *et al*., 2011; Crespo *et al*., 2018). A resilient biofilm is a critical weapon for *P. aeruginosa* to compete, survive and dominate in the cystic fibrosis lung polymicrobial environment (Oluyombo *et al*., 2019).

P. aeruginosa also effectively colonizes a variety of surfaces including medical materials (urinary catheters, implants, contact lenses, etc.) (Ghafoor *et al*., 2011), and food industry equipment (mixing tanks, vats and tubing) (Coughlan *et al*., 2016).

The biofilm development is divided into five distinct stages. **Stage 1**: Bacterial cells adhere to a surface via support of cell appendages such as flagella and type IV pili (This adherence is reversible). It is evident through records of the presence of specific bacterial proteins and their altered quantities when *P. aeruginosa* sense and react in response to a given surface. **Stage 2**: Bacterial cells undergo the switch from reversible to irreversible attachment. **Stage 3**: Progressive propagation of attached bacteria into a more structured architecture, termed microcolonies. **Stage 4**: These microcolonies develop further into extensive three-dimensional mushroom-like structures, a hallmark of biofilm maturation. **Stage 5**: In the center of the microcolony, matrix cavity is disrupted through cell autolysis for the

liberation of dispersed cells, followed by the transition from sessile to planktonic growth mode for seeding of uncolonized spaces, which allows the biofilm cycle to repeat as Figure (2.1). It was recently demonstrated that endonuclease EndA is required for dispersion of existing biofilm via eDNA degradation (Rasamiravaka *et al*., 2015; Guilbaud *et al*., 2017 and Cherny and Sauer, 2019).

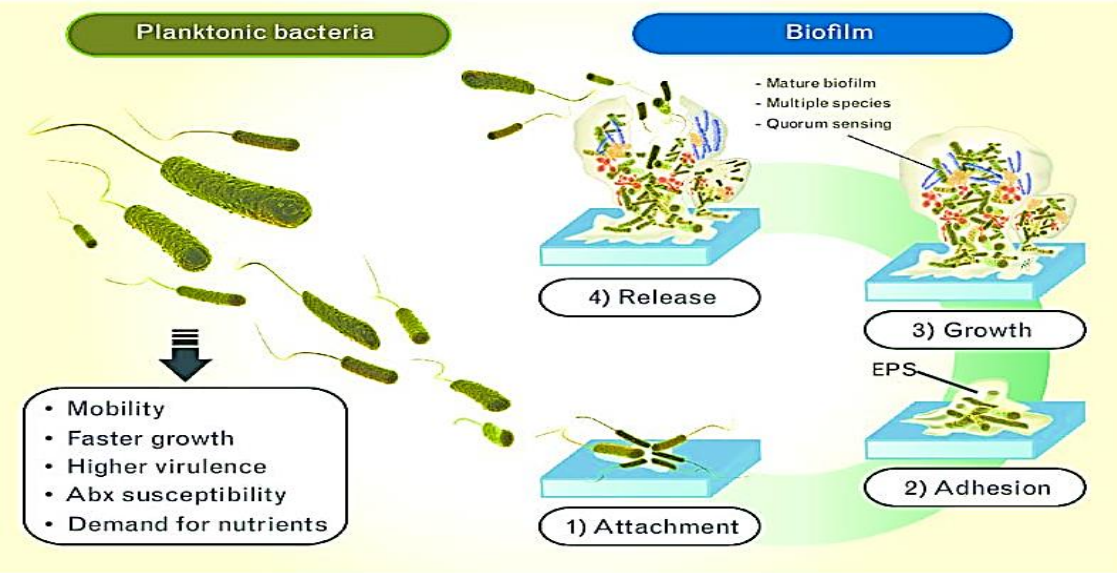


Figure (2.1): Stages of biofilm development in *P. aeruginosa* (Pirrone *et al.*, 2016)

P. aeruginosa also effectively colonizes a variety of surfaces including medical materials (urinary catheters, implants, contact lenses, etc.). It is important therefore to diagnose *P. aeruginosa* infections before biofilm development which could enhance the susceptibility of *P. aeruginosa* towards antimicrobial treatments. However, the increasing incidence of acute and persisting infections worldwide also highlights the need to develop therapeutic strategies as an alternative to traditional antibiotics, expectedly to disarm and eradicate this Gram-negative bacterium. (Crespo *et al*., 2018 ; Oluyombo *et al*., 2019).

Alginate is predominately produced in the biofilm of mucoid *Pseudomonas* strains due to a mutation in *muc*A22 allele. The mucoid phenotypes are found

mostly in CF isolates, signifying the conversion from acute to chronic infection (Ciofu *et al*., 2015). Alginate is a negatively charged acetylated polymer consisting of mannuronic acid and guluronic acid residues. A wide range of important functions of alginate including biofilm maturation, protection from phagocytosis and opsonization, and decreased diffusion of antibiotics through the biofilm has been well-documented (Tseng *et al*., 2013 ; Hay *et al*., 2013).

2.5.4. Exopolysaccharides

Exopolysaccharides are sugar-based extracellular macromolecules secreted by *P. aeruginosa* to enhance bacterial tolerance to harsh survival environments such as desiccation, oxidizing agents, and host defense (Franklin *et al*., 2011). As one of the main compositions of extracellular polymeric substances essential for the functional and structural integrity of biofilms, exopolysaccharides are important for *P. aeruginosa* in biofilm formation, and can also act as adhesins contributing to bacterial persistence in patients (Song *et al*., 2003; Ellis and Kuehn, 2010). At present, alginate, Psl and Pel are three exopolysaccharides discovered in *P. aeruginosa*. Alginate is generally secreted by the strains isolated from cystic fibrosis patients, while Psl and Pel are mainly produced by the strains obtained from the environment (Franklin *et al*., 2011). Although the mechanisms of their action remain poorly understood, the antibodies targeting alginate or Psl have been developed for antivirulence therapy (DiGiandomenico *et al.*, 2014; Loos *et al*., 2019).

2.6. Antibiotic Resistance for *P. aeruginosa*

Infections with this microorganism are challenging to treat due to its natural resistance and the accelerated emergence of strains resistant to almost all antibiotics, including carbapenems (last-resort treatments). Therefore, WHO,

(2017) and Gómez-Martínez *et al*., (2022) included *P. aeruginosa* in the critical-level priority pathogens group, along with *Acinetobacter baumannii* and carbapenem-resistant Enterobacteriaceae.

Infections by this bacterium are hard to treat that due to of high intrinsic resistant to numerous antibiotics or because its ability develops acquired genes of resistance, either by chromosomal encoded mutation genes or by horizontal transfer the gene of antibiotic resistance (Rasool *et al*., 2021). In *P. aeruginosa*, there are three types of resistance mechanisms: innate, acquired, and adaptive, figure (2.2).

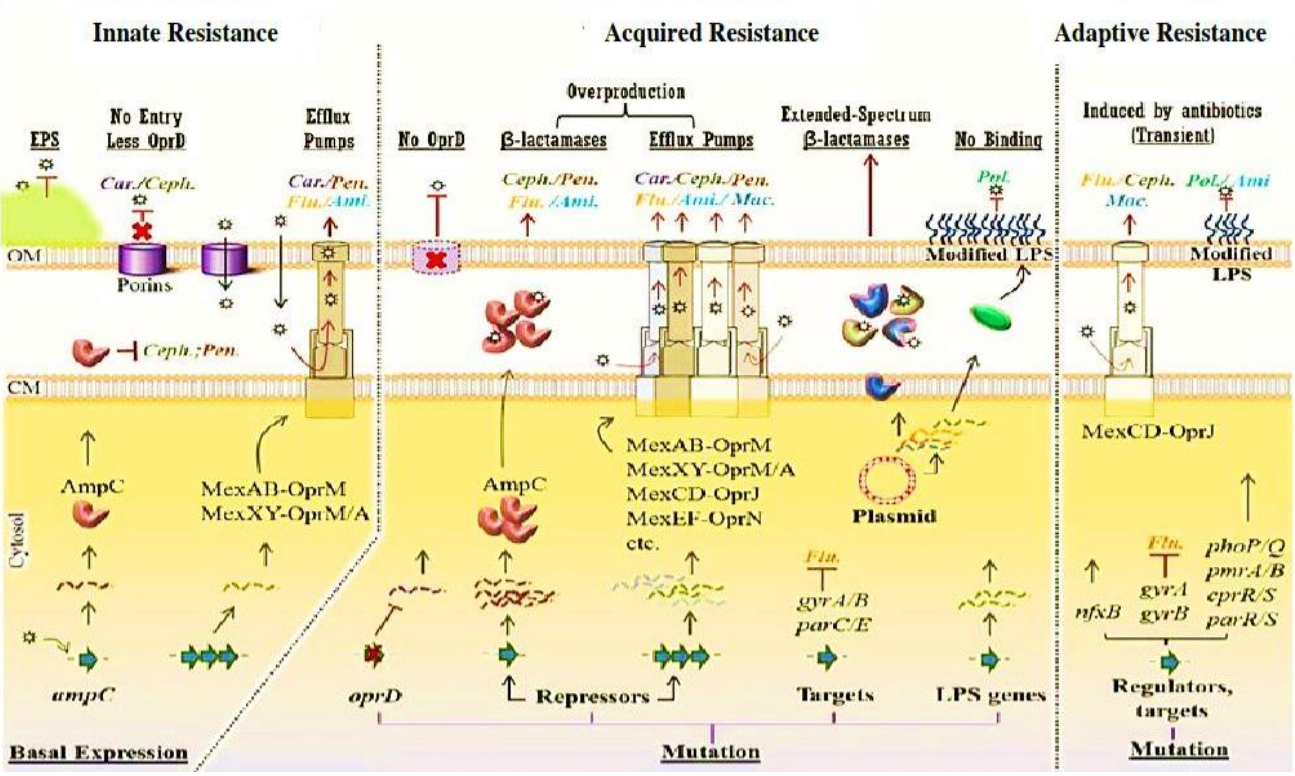


Figure (2.2): Antibiotic resistance in *P. aeruginosa* by innate, acquired, and adaptive mechanisms, (Lodise and Bidell, 2016).

P. aeruginosa easily acquires further resistance mechanisms to multiple classes of antimicrobial agents, even during the course of a treatment. Different

mechanisms of resistance often exist simultaneously, thus conferring combined resistance (Pachori *et al*., 2019**)**

A wide group of *P. aeruginosa* strains are resistance to various classes of antibiotics or antibacterial agents that makes it difficult to control the infection, it has evolved antimicrobial resistance, making it difficult to treat and limiting our therapeutic options. Some *P. aeruginosa* strains are resistant to most of the available antimicrobial agents, from carbapenem to the third-generation cephalosporins, which are the preferred options for treating multidrug-resistant (MDR) bacteria (Azam and Khan, 2019).

2.6.1. Enzymatic Modification

P. aeruginosa consists of elements generally termed as transposons which induce resistance due to the modification of aminoglycoside enzymes. The infection due to the pathogen is usually combated by various class/groups of aminoglycoside antibiotics like kanamycin, gentamicin, streptomycin, amikacin and neomycin (Mohanty *et al*., 2021). Previous studies elucidate that, there are three types of enzymatic conformational change which are accountable for the resistance against the bactericidal compounds. These are phosphorylation of aminoglycoside phosphoryl transferase (APH) (Wright, 2005; Strateva and Yordanov, 2009) adenylation of aminoglycoside nucleotidyl transferase (ANT) and acetylation of aminoglycoside acetyl transferase (AAC) (Bonomo and Szabo, 2006).

The conformational modification and phosphorylation in the 3′-OH group is carried out by the APH enzyme. APH (3′) family of enzymes shows resistance against streptomycin, butirocin, amikacin, kanamycin and neomycin by encoding the genes such as *aphA* and *hpaA* which are involved in the metabolism of 4-

hydroxy-phenylacetic acid (4-HPA). However, APH (2″) shows resistance to tobramycin and gentamycin classes of antibiotics. Due to adenylation of ANT enzymes *P. aeruginosa* increases resistance towards tobramycin, gentamicin, streptomycin and amikacin (Kotra *et al*., 2000; Marvig *et al*., 2015). The family of enzymes such as ANT (2″), (3″) and (4′) also shows a similar type of resistance in different strains of *P. aeruginosa* isolated from hospitals and intensive care unit (ICU) premises (Azucena and Mobashery, 2001). The *N*-terminal positions (1, 2′, 3 and 6′) of the (AAC) shows the enzymatic acetylation. Amongst various families, AAC (3-I), (3-II) and (3-III) are also resistant to gentamicin, tobramycin and kanamycin antibiotics respectively. Apart from that AAC (6′) family of enzymes contributes to the resistance along with akamicin (Ramirez and Tolmasky, 2010; Ruiz-Martínez *et al*., 2011).

2.6.2. Impermeability Resistance

Impermeability to various exocompounds in Gram-negative bacteria is due to lipopolysaccharide (LPS) present in the cell wall. LPS is made up of lipid A, oligosaccharide core and O antigen regions which are linked covalently (Delcour, 2009). The lipid A region is hydrophobic in nature and made up of a disaccharide of glucosamine which is phosphorylated and helps in the anchoring of LPS to the cell membrane (Emptage *et al*., 2012). The core oligosaccharide is accumulation of sugar, ethanolamine, phosphate and amino acids and can be divided into inner and outer core. The O antigen is the outer domain of bacterial LPS made up of repeating glycan polymers and attached with the core region. It has been observed that the deletion of lipid A makes the bacteria susceptible to various classes of hydrophobic antibiotics and degradation of O side chains determine the smoothness and roughness of the LPS (Zhang *et al*., 2000; Crompton *et al*., 2016).

The use of ethylenediaminetetraacetic acid (EDTA), some organic acids like lactic acid and citric acid are found to alter the impermeability of the *Pseudomonas* spp. These chelating agents can neutralize the negatively charged oligosaccharide core by binding with the (Mg^{+2}) cations in the LPS molecule and promotes the removal of LPS molecules (Lambert, 2002).

2.6.3. The Efflux System

The drug efflux system in bacteria includes three major components i.e. outer membrane channel-forming protein (OMF), resistance nodulation division (RND) which helps in drug-protein antiport process and the membrane fusion protein that acts as a periplasmic link between above two components (Stover *et al*., 2000). The *mexXY* operon codes the inner membrane protein (i.e. MexY) and periplasmic protein (i.e. MexX). Resistance nodulation division (RND) involves the *MexXY* efflux system which develops the resistance in *Pseudomonas* spp (Dreier and Ruggerone, 2015). MexAB-OprM shows resistance against ticarcillin, broad-spectrum cephalosporin and β-lactam of clinical isolates, while the combination of MexAB-OprM, MexCD-OprJ and MexXY-OprM shows the carbapenem resistance (Villegas *et al*., 2007). Treatment with ofloxacin and gentamicin increases the level of *MexXY* expression in case of mutants compared to wild-type strains (Jeannot *et al*., 2005).

The wild-type of strains of *Pseudomonas* is resistance to the antibiotic classes like tetracyclines, aminoglycosides, glycylcyclines and erythromycin but the *MexXY* can express in presence of diverse class of antibiotics like macrolides (Saiman *et al*., 2003), fluoroquinolones (Livermore, 2002), β-lactams (Villegas *et al*., 2007), novobiocin (Zavascki *et al*., 2010) along with the wild type of antibiotic classes. In the reduced aminoglycosides condition both adaptive and

impermeability resistance in the *Pseudomonas* spp. is expressed. The expression of *MexXY* gene is regulated by *mexZ* repressor, present in the upstream region of *MexXY* region of the gene and belongs to tetracycline repressor protein (TetR) and AcrR repressor protein family (Poole *et al*., 2018).

2.6.4. Modification in the Outer Membrane

The exoskeleton of the Gram-negative bacteria is present to resist against the adverse environmental conditions. Likewise, the outer membrane of *P. aeruginosa* is designed in such a way that it can permit small hydrophilic molecules and inhibit larger molecules such as antibiotics (Poole, 2011). Due to the crucial arrangement of aquaporin proteins in the cell membrane, the small hydrophilic antibiotics of quinolone and β-lactam classes can pass through the outer membrane. *P. aeruginosa* strains produce four major aquaporins (i.e. oprP, oprD, oprF and oprB) and two minor aquaporins (i.e. oprC, oprE) whereas the mutant strains lack oprF (Lee and Ko, 2012). The oprD is a specialized porin molecule present in bacterial membrane that helps in the process of uptaking positively charged amino acids like arginine and lysine (Hancock and Speert, 2000).

The minimum inhibitory concentration increases due to the loss of *oprD* porin from the outer membrane of the *Pseudomonas* spp. thus increasing the resistance to imipenem class of antibiotics (Fernández and Hancock, 2012). As the porin channels are impermeable to the polymyxin E and aminoglycoside, these molecules bind with the LPS present in the outer membrane, destructs the barrier and allows the antibiotics to enter into the bacterial cells (Breidenstein *et al*., 2011). Through this mechanism the aminoglycosides can enter into the cytoplasm of the bacterial cell and disturb the protein synthesis process in the ribosomes that kills the bacteria simultaneously. But the overexpression of the oprH an outer

membrane protein (Fernández *et al*., 2017), prevents the binding of antibiotics to LPS making it resistant for laboratory strains of *Pseudomonas* spp.

2.7. Carbapenem Resistance

Carbapenems are antibiotics that belong to the β-lactam class and are considered as last-resort antibiotics for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. Carbapenems are being used in clinical use more frequently, the emergence of carbapenem-resistant pathogens now poses a great threat to human health (Sheu *et al*., 2019). Carbapenems, such as imipenem and meropenem, are most commonly used to treat infections caused by Enterobacteriaceae that produce extended-spectrum beta lactamases; hence the development of enzymes capable of inactivating carbapenems would limit treatment options (Ebomah and Okoh, 2020). The types of the mechanisms causing carbapenem resistance (eg, carbapenemase production, porin mutation, or efflux pump upregulation). The key organisms to consider include the order Enterobacteriales (which includes the family Enterobacteriaceae), *P. aeruginosa*, *A. baumannii*, and *Stenotrophomonas maltophilia* (Doi, 2019). Carbapenem resistance in Enterobacteriaceae bacteria is becoming a severe public health issue around the world. Sporadic outbreaks or endemic circumstances of antibiotic resistant enterobacterial isolates (Nordmann and Poirel, 2019). The most important determinants sustaining carbapenem resistance are integrin acquired class A *Klebsiella pneumoniae* carbapenemases (KPC), class B (Imipenemase IMP, Verona Integron-encoded Metallo-ß- lactamase VIM, New Delhi Metallo-β-lactamase NDM), or class D Oxacillinase of type (OXA-48, OXA-181) the related genes are typically found on plasmids and are linked to a variety of mobile genetic structures (insertion sequences, integrons, and transposons), allowing them to spread even more widely (Cheng *et al*., 2018).

Carbapenem-resistance mucoid is one of the most common recognized pathogenesis that causes urinary tract infections, wound infections, and septicemia in immunocompromised people (Muntean *et al*., 2017).

CRE can lead to treatment failure, posing a substantial public health risk (Bush, 2015). Antimicrobial resistance genes (ARGs) can be transferred to other bacterial species, resulting in the production of various enzymes (such as beta-lactamases) that inactivate antimicrobial activities; the types of enzymes produced by carbapenemase-producing carbapenem-resistant Enterobacteriaceae (CP-CRE) include clavulanic-acid-inhibited-lactamases (Ebomah and Okoh, 2020).

P. aeruginosa was the first species in which acquired carbapenem resistance emerged after the introduction of the first carbapenem, imipenem, in the mid-1980s; resistance was due to changes in porin expression, which rendered the outer bacterial membrane impermeable to imipenem. Although meropenem is less prone to this resistance mechanism, *P. aeruginosa* can become resistant to meropenem by upregulation of efflux pumps. Globally, carbapenem resistance due to production of MBLs (in particular the VIM group) appears to be common in *P. aeruginosa*, which has implications when considering treatment options, as most β-lactamase inhibitors (BLIs) are unable to inhibit their activity. MBLs are considered uncommon in *P. aeruginosa* in the US, but outbreaks by VIM producing *P. aeruginosa* have been reported (Doi, 2019).

Carbapenemase producers are currently the most serious clinical issue in antibiotic resistance in Gram negative bacteria, especially in Enterobacteriaceae (Taggar *et al.*, 2020).

Carbapenems have the broadest spectrum of all β-lactam antibiotics and are increasingly used to treat infections caused by otherwise multidrug-

resistant Gram-negative bacteria. Consequently, emerging resistance to carbapenems is the major public health concern, especially when it involves acquired, horizontally transmissible carbapenemases (Queenan and Bush, 2007). Those that hudrolyse imipenem and/or meropenem are classified in either Ambler Classes A, B or D (genetic differences) or in Bush-Jacoby-Medieros groups 2f, 3a or 3b (substrate preference and molecular structure).

The carbapenem resistance determinants carried by *P. aeruginosa* are often encoded on plasmids, such as IncP type; class I integrons, such as those carrying the bla_{VIM} gene; and other mobile genetics elements, such as those associated with insertion sequences with a common region (ISCRs), which enhance the organism's ability to disseminate resistance among multiple species (Yoon, and Jeong, 2021). In addition, these isolates frequently carry additional resistance determinants that diminish the clinical utility of the fluoroquinolones and aminoglycosides. Carbapenemase-producing *P. aeruginosa* (CP-PA) are often resistant to all of these therapeutic options, thus making treatment failure a likely outcome. CPPA has also been associated with nosocomial spread prompting infection prevention interventions (Tenover *et al*., 2022).

According to Ambler molecular classification scheme, which is based on the protein sequence similarity these are classified into four classes A, B, C and D. This classification also is based on conserved and variable amino acid motifs of the proteins. Class A, C, and D include the enzymes that hydrolyze their substrates by forming acyl enzymes via the active site serine, while class B (metalloenzymes) utilizes active site zinc to facilitate β-lactam hydrolysis (Bush and Jacoby, 2010).

This structural classification is based on amino acid homology but lacks detail concerning enzymatic activity (Queenan and Bush, 2007). In this system,

carbapenemases are categorized as classes A, B, and D. Rare carbapenemases belonging to Ambler class C exist. These usually cause reduced susceptibility to carbapenems as a consequence of the low enzyme's catalytic efficiency and a permeability defect in the bacterial cell (Meletis, 2016).

2.7.1. Ambler Class A Carbapenemases

The molecular class A carbapenemases, including the plasmid mediated serine β-lactamases KPC (for *K. pnenmoniae* carbapenemase) and GES (Guiana extended spectrum) and the chromosomally encoded SME (for *Serratia marcescens* enzyme) and IMI/NMC (imipenem hydrolyzing β-lactamase / not metalloenzyme carbapenemase) enzymes, are effective carbapenemases (Forssten, 2009). The class A carbapenemases have the ability to hydrolyze a broad spectrum of antibiotic, including penicillins, early and extended-spectrum cephalosporins, aztreonam as well as carbapenems, and all are inhibited by clavulanic acid and tazobactam (Queenan and Bush, 2007).

2.7.2. Ambler Class B Metallo-β-Lactamases

The molecular class B enzymes "metallo-β-lactamases" (MBLs) were first to distinguish from serine β-lactamases in 1980 (Queenan and Bush, 2007).

Metallo-β-lactamases depend on heavy metals like Zn^{+2} for β-lactam hydrolysis, due to this zinc dependency, chelators such as ethylenediamine tetra-acetic acid (EDTA) inhibit MBL activity (Marsik and Nambiar, 2011). They are resistant to well known β-lactamase inhibitors like clavulanic acid, sulbactam, and tazobactam and confer resistance to all β-lactam antibiotics except monobactam (Mirsa, 2012). The metallo-β-lactamases are subdivided on the basis of sequence alignments into three subclass B1, B2 and B3. Phylogenetic studies suggest that B1

and B2 descend from a common ancestor and subclass B3 share only in structural similarities with these subclasses. B1 and B3 are able to bind one or two zinc ions, B2 are mono-Zn enzymes that have evolved specificity toward carbapenems. The greater importance are the acquired or transferable families of MBLs which include IMP (active on imipenem), VIM (Verona integron encoded metallo-β-lactamase), GIM (German imipenemase), SIM (Seoul imipenemase), SPM (Sao Paulo MBL) and NDM (New Delhei Metallo-β-lactamase) which are located within gene cassettes as a part of integron structures (Dugal and Fernandes, 2011; Mirsa, 2012). The IMP type of MBLs is a transferable type of β-lactamase which confers the property of hydrolyzing imipenems as well as some extended spectrum cephalosporins. They are insensitive to most inhibitory agents but are however susceptible to aztreonam (Dugal and Fernandes, 2011).

The first indication of the IMP type was found in the Japanese region in *P. aeurginosa* strain GN17203 in 1988 (Gupta, 2008; Wang *et al*., 2018). They were subsequently reported in four *S. marcescens* isolates in Japan. The IMP genes are located on transferable conjugated plasmid of about 120KD which could be readily mobilized to other *Pseudomonas* strains. This same gene was then transferred to four *S. marcescens* collected 32 in seven general hospitals in Japan in 1993 (Dugal and Fernandes, 2011).

The VIM class 1 integron associated MBL was first observed in *P.aeruginosa* isolate in Verona, Italy in 1997 (Cornaglia *et al.,* 2000). It is closely related to BCII from *Bacillus cereus* sharing 39% amino acid identity (Yong *et al.,* 2009). VIM enzymes are resistant to a number of β-lactams like piperacillin, cefazidime, imipenem and aztreonam. These enzymes are dependent on metal ions, which are indicated by loss of activity on addition of EDTA and restoration up on addition of (Zn^{+2}) (Dugal and Fernandes, 2011). GIM-1 was isolated in Germany in 2002

from *P. aerugenosa* strain (Castanheira *et al.*, 2004). The enzyme SIM was first detected in *A. baumannii* strains in Korea; SIM-1 exhibited (64 to 69%) amino acid identity to the IMP enzymes (Lee *et al*., 2005). SPM was first detected from the clinical *P. aerugenosa* strain in 1997 in Brazil and designated bla_{SMP-1} (Toleman *et al*., 2002). Since their initial discoveries, SPM, GIM and SIM metallo-β-lactamases have not spread beyond their countries of origin (Queenan and Bush, 2007).

2.7.3. Ambler Class C Carbapenemases

The Ambler class C β-lactamases (AmpC cephalosporinases) confer resistance to penicillin, oxyiminocephalosporins, cephamycins (cefoxitin and cefotetan), and, variably, to aztreonam (Jeon *et al*., 2015). An exceptional member with different properties is ACC-1, a plasmid-encoded class C β-lactamase identified in clinical isolates of *K. pneumoniae*, *P. mirabilis*, *Salmonella enterica*, and *E. coli*. Due to conformational alterations in this enzyme structure, ACC-1-producing bacteria are susceptible to cefoxitin, whereas they are resistant to oxyiminocephalosporins (Bae *et al*., 2019). AmpC cephalosporinases are not significantly inhibited by other β-lactamase inhibitors such as clavulanic acid, but may be inhibited by boronic acid and cloxacillin (Hammoudi *et al*., 2014).

They may be encoded by the chromosome of many *Enterobacteriaceae* like *E. cloacae* and *S. marcescens*, and a few non-enterobacterial organisms like *P. aeruginosa*. In such instances, they are inducible by antibiotics or expressed at high levels by mutation. Nevertheless, AmpC enzymes may also be acquired on transmissible plasmids, which, consequently, makes them appear in bacteria by lacking or poorly expressing a chromosomal *bla*AmpC gene, such as *E. coli*, *K. pneumoniae*, and *P. mirabilis* (Jacoby, 2009).

2.7.4. Ambler Class D OXA Carbapenemases

Class D enzymes are OXA (for oxacillin hydrolyzing) enzymes, which are penicillinases capable of hydrolyzing oxacillin and cloxacillin (Bush, 2018). These serine β-lactamases are plasmid encoded and are found primarily in *P. aeruginosa*, *A. baumannii*, and rarely in isolates of Enterobacteriaceae from the United States (Marsik and Nambiar, 2011). The major concern with OXA carbapenemases is their ability to rapidly mutate and expand their spectrum of activity (Codjoe and Donkor, 2017).

2.8. Integrons

Integrons are genetic elements that allow efficient capture and expression of exogenous genes. They are widely known for their role in the dissemination of antibiotic resistance, particularly among Gram-negative bacterial pathogens. Integrons were first identified because of their central role in assembling and disseminating antibiotic resistance genes in commensal and pathogenic bacteria (Christaki *et al*., 2020). However, these clinically relevant integrons represent only a small proportion of integron diversity. Integrons are now known to be ancient genetic elements that are hotspots for genomic diversity, helping to generate adaptive phenotypes. This perspective examines the diversity, functions, and activities of integrons within both natural and clinical environments (Ghaly *et al.*, 2021). Integrons occur in all environments, are able to move between species and lineages over evolutionary time frames, and have access to a vast pool of novel genes whose functions are largely yet to be determined. Over the last decade, exploration of integron diversity in natural environments has shown that they are more than just a curious feature of antibiotic-resistant pathogens but have a more general and important role in bacterial adaptation and genome evolution (Gillings, 2014).

Integrons are important factor in dissemination of antibacterial resistance among different bacterial species and the association between integrons and drug resistance has been shown. Integrons are not self-mobilizable factors, but contain an integrase (IntI)-encoding gene that allows the insertion of antimicrobials resistance gene cassettes between highly conserved nucleotide sequences. Among the integron classes, class 1 integrons (*intI1*) was the most prevalent among clinical isolates of *P. aeruginosa* (Sharifi *et al*., 2019), figure (2.3).

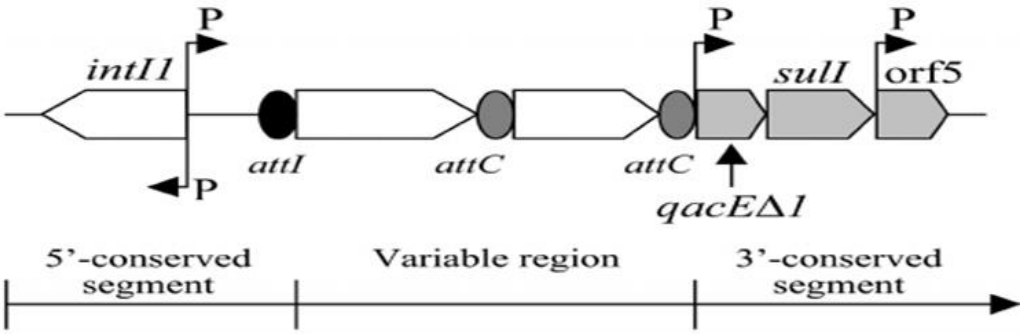


Figure (2-3): General structure of class 1 integrons. Cassettes are inserted in the variable region by the integrase using a site-specific recombination mechanism. The *attI* and *attC* sites are shown by a black and a grey oval, respectively, and promoters are denoted by P. Genes are as follows: *intI1*, integrase gene; *qacE1*, antiseptic resistance gene; *sulI*, sulfonamide resistance gene; *orf5*, gene of unknown function, (Drouin *et al.*, 2002).

2.8.1. Structure of Integrons

In general, 3 key elements are required for integration. **(i)** The integrase gene which belongs to the tyrosine recombinase family that is involved in the integration of the gene cassettes at the attachment site (Mazel, 2006; Davies and Davies, 2010). **(ii)** The recombination site can be of two types depending on its location **(1)** on the integron: *att1*, primary site of attachment of the gene cassettes and **(2)** on the gene cassette itself: *attC*. These sites are recognized by the integrase and are essential in recombination of the cassettes present in the integron. **(iii)** The outward-oriented promoter in the integrase which directs the transcription of

the inserted gene cassettes, since they cannot express independently (Fluit and Schmitz, 2004).

The cassette has a specific recombination site that confer mobility because it is recognized by recombinase encoded by the integron that catalyses its integration into specific site within the integrons (Giedraitiene *et al.*, 2011). These integron structures may then associate with transposons and plasmids which then can be easily transferred between bacteria (Tailor, 2011).

2.8.2. The *attC* sites

The *attC* region contains two simple sites, each composed of a pair of conserved 'core sites' (7 or 8 bp), referred to as R″ and R′, L′ and L″. The R′ and R″ sites are part of the RH consensus sequence, which is more or less equivalent to the RH simple site. The L′ and L″ sites are part of the LH consensus sequence, which is more or less equivalent to the LH simple site (Xu *et al*., 2011). The LH and RH sites in the *attC* are possibly distinguished by the integrase, which might explain the orientation of integration of the gene cassettes. L″ also appears to be significant for orientation (Fluit and Schmitz, 2004). The LH simple site is not only required for orientation but also enhances RH activity. The *attC* sites are generally associated with a single ORF in a structure termed gene cassette, which are not necessarily observed in integrations, but once integrated they become part of the integron (Deng *et al*., 2015).

2.8.3. Gene cassettes

According to previous reports, cassettes located within the variable region of integrons are sometimes absent in the structure of integrons (Nemec *et al*., 2004; Deng *et al*., 2015). Via specific excision and integration, gene cassettes are integrated between two recombination sites (*attI* and *attC*) and thus become part of

the integron, and exist in either the independent circular DNA molecule which is unable for stably maintain during cell division or the linear form which is created by a highly orientation-specific insertion of the free circular element into the integron (Xu *et al*., 2011). Despite possession of a coding sequence, gene cassettes are generally found to be lack of promoters to constitute the mobile component of the system, and most cassettes encode resistance against antibiotics cover a wide range of antibiotics, with up to date more than 130 distinct antibiotic resistance genes characterized via unique *attC* sites (Xu *et al*., 2009). Together, these cassettes confer resistance to most classes of antibiotics containing all known β-lactams, all aminoglycosides, chloramphenicol, streptothricin, trimethoprim, rifampin, erythromycin, quinolones, fosfomycin, lincomycin, and antiseptics of the quaternary ammonium-compound family (Mazel, 2006; Cambray *et al*., 2010).

2.8.4. Mobility

Reported as widely spread and distributed in clinical organisms, the mobility of integrons has been considered to be a major concern of clinically antibiotic resistance, which is defined as being associated with mobile DNA elements (transposons or plasmids) and antibiotic resistance genes in addition to having a small array size and substantial heterogeneity in the sequence of *attC* sites (Boucher *et al*., 2007; Xu *et al*., 2011).

Despite the defectivity of self-transposition, currently existent integrons (mostly class 1 integron) has been considered to be a potentially mobile genetic element and commonly found to be located on plasmids as facilitation of conjugative-mediated transfer, as it contains gene cassettes that are mobile and capable of transferring to other integrons or to secondary sites in the bacterial genome. The integron system is a natural capture system and assembly platform,

which allows microorganisms to incorporate gene cassettes and further convert them to functional proteins via correct expression (Deng *et al*., 2015).

Each unique ORF is conceivably capable of being structured as a novel type of gene cassette and vital to decipher the mechanism governing cassette genesis. As a consequence, with the naturally huge pool of gene cassettes, integron may have the potentially limitless capacity to exchange and stockpile functional gene cassettes which consequently permits rapid adaptation to selective pressure and may ultimately endow increased fitness and advantage to the host (Mazel, 2006; Boucher *et al*., 2007).

In addition, mobile genetic elements, including conjugative plasmids, transposons, insertion sequences and genomic islands, may potentially be the vast reservoirs and massive genetic pool for integron, which will further be shared among bacteria (Deng *et al*., 2015). With mobility from gene cassettes, integrons play key role in the dissemination and spread of resistance genes, responsible for both spread and exchange of resistance genes to a wide range of distinct antibiotics among diverse bacteria (Nemergut *et al.*, 2008). Aside from clinical perspectives, a large number of reports on integrons from environmental microorganisms, as well as the high sequence diversity observed and various functional products other than resistance encoded by such cassettes, strongly indicates integrons are ancient genetic element within the genomes and may have played a critical role in evolution and adaptation for a considerable period (Deng *et al.*, 2015).

2.8.5. Classification of Integron

Integrons are classified based on sequence similarity, there are at least five classes of integrons with class I integrons being the most studied and characterized (Ravi *et al*., 2014). To date, several classes of integron have been described

(Goudarzi *et al.,* 2016). Class 1 integrons are the most common and widely distributed among Gram-negative bacteria and are associated with functional and non-functional transposons derived from Tn*402*, which can be further embedded in larger transposons such as 22 Tn*21* (Koczura *et al*., 2013). Class 1 integron is composed of the 3‟conserved sequence area (3‟CS) and includes two open reading frames (ORFs), the *qacE*Δ1 gene, which confers resistance to quaternary ammonium compounds, and the *sul1* gene which confers resistance to sulphonamides (Domínguez *et al.*, 2019).

Class 2 integrons are found in transposon Tn*7* derivatives, the integrase gene of class 2 integron is *intI2* (Alonso *et al.,* 2018). Its 3‟CS has five *tns* genes that are responsible for the mobility of transposons and also found in *Salmonella*, and *Shigella* (Ravi *et al.*, 2014). The class3 Integrons also have been reported, but the 3‟CS is still not well described (Mirnejad *et al.,* 2013). Class 3 integrons are also thought to be located in a transposon, and are less prevalent than class 2. Class 3 integrons are very similar to the other two classes but are related to the Tn*402* transposon (El Salabi *et al*., 2013).

The class 4 integron is embedded in a subset of the integrative and conjugative element SXT found in *V. cholera*. Class 5 integrons are located in a compound transposon carried on the pRSV1 plasmid of *Vibrio salmonicida* (Cambray *et al.,* 2010).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Instrument and Equipment

Table 3-1: The equipment and Instrument used in this study

Equipment	Company/ Origin
Autoclave	Lab Tech /Korea
Bio drop spectrophotometer	Bio lab/England
Burner	Himedia/ India
Cooling centrifuge	Hettich/Germany
Distillator	GFL (Germany)
ELISA plate	Tarson / India
ELISA reader	Biolab/England
Gel documentation system	Biometra/Germany
Hot plate stirrer	Heiddph/Germany
Incubator	Memmert/Germany
Micro centrifuge tube 1.5ml	POSI-CLICK TUBES/USA
Micropipette set (0.5-1000µl)	Dragon MED/USA
Millipore filter (0.22µm,0.44 µm)	CHM CA syringe filter/China
Thermocycler system	Cleaver Scientific/UK
PCR tubes	Eppendorf /Germany
pH-meter	HANNA/Germany
Sensitive balance	Sartorius/ UK

Equipment	Company/ Origin
Standard loop 0.01 ml	Himedia/India
VITEK2	Biomerieux/France
Vortex	Griffen and George Ltd/UK
Unit DNA Electrophoresis	Cleaver Scientific/UK

3.1.2. Chemicals

Table 3-2: The chemicals used in this study

Chemical	Company/ Origin
Agar-agar	Himedia/India
Agarose	Fisher /Germany
Barium Chloride ($BaCl.\ 2H_2O$)	Thomas Baker/ India
Crystal violet	Thomas Baker / India
Disodium hydrogen phosphate (Na_2HPO_4)	BDH/England
Absolute Ethanol	honeywell /Germany
Ethyenediamine tetra-acetic acid (EDTA)	Thomas Baker/India
Glucose ($C_6H_{12}O_6$)	Difco/USA
Glycerol ($C_3H_8O_3$)	AppliChem /Spain
Hydrogen peroxide (H_2O_2)	Scharlab S.L./Spain
Iodine	Osaka/Japan
Kovac's reagent	Himedia/India
Methyl red	BDH/ England

Chemical	Company/ Origin
N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD)	SCHUAROT/Germany
Pencillin G	MACKLIN /Chain
Potassium Hydroxide (KOH)	Reagent world/USA
Potassium Iodide (KI)	Thomas Baker/ India
Red safe Nucleic Acid Staining	Bioland Scientific
Sodium Acetate (CH_3COONa)	Thomas Baker/ India
Sodium Chloride (NaCl)	BDH/England
Sodium Dihydrogen Phosphate Dihydrate ($NaH_2PO_4.2H_2O$)	BDH/England
Sodium Hydroxide (NaOH)	AppliChem/Germany
Starch	BDH/England
Sulfuric acid (H_2SO_4)	Thomas Baker/ India
Tris-borate-EDTA (TBE)	CONDA/Spain
Urea	BDH/ England
α-Naphthol ($C_{10}H_8O$)	BDH/England

3.1.3. Culture Media

Table 3-3: The culture media used in this study

Media	Company / Origin
Brain heart infusion broth	Himedia / India Liofilchem / Italy
Cetrimide agar	
MacConkey agar	
Methyl red-vogus proskaur medium	
Muller-Hinton agar	
Nutrient agar	
Nutrient broth	
Pepton water broth	
Simmone citrate agar	
Urea agar base	

3.1.4. Kits, Reference strain and Markers

Table 3-4: Kits and Marker used in this study

Name of Kit	Company	Origin
DNA loading dye	Geneaid	Korea
FavorPrep / Genomic DNA Mini Kit	Favorgen	Taiwan
FavorPrep / Plasmid Extraction Mini Kit	Favorgen	Taiwan
Liofilchem MIC test strip	Liofilchem®s.r.l.	Italy

Name of Kit	Company	Origin
P. aeruginosa ATCC 27853	ATCC	USA
Taq Green master mix	Promega	USA
100 bp DNA ladder	Promega	USA
100 bp DNA ladder	Bioneer	Korea
(100, 1000) bp DNA ladder	iNtRON	Korea
100 bp DNA ladder	SolGent	Chain

3.1.5. Antibiotic Disks

Table 3-5: List of antibiotics used in this study according to CLSI, 2022

Class	Antibiotic	Symbol	Concentration (µg/disk)	Company/ Origin
Aminoglycosides	Amikacin	AK	30 µg	Bioanalyse/Turkey Liofilchem/ Italy
	Gentamicin	CN	10 µg	
	Tobramicin	TOB	10 µg	
Beta-Lactam	Piperacillin-tazobactam	PIT	100/10 µg	
	Ticarcillin-clavulanate	TCC	75/10 µg	
Carbepenems	Imipenem	IMP	10 µg	
	Meropenem	MEM	10 µg	
	Doripenem	DOR	10 µg	
Cephems	Ceftizoxime	CZX	30 µg	

Class	Antibiotic	Symbol	Concentration (µg/disk)	Company/ Origin
Cephems	Cefixime	CFM	5 µg	Bioanalyse/Turkey Liofilchem/ Italy
	Cefotaxime	CTX	30 µg	
	Cefoperazone	CFP	75 µg	
	Cefepime	FEP	30 µg	
Macrolides	Azithromycin	AZM	30 µg	
Monobactams	Aztreonam	ATM	30 µg	
Penicillins	Ampicillin	AMP	10 µg	
	Piperacillin	PRL	100 µg	
Tetracyclines	Tetracycline	TE	30 µg	
	Doxycycline	DO	30 µg	
Quinolones and Fluoroquinolones	Levofloxacin	LEV	5 µg	
	Ciprofloxacin	CIP	5 µg	
	Norfloxacin	NOR	10 µg	

3.1.6 Primers

Table 3-6: List of primers used in this study

Primer name	Genes	Oligo Sequences (5'-3')	Produc Size (bp)	Reference
Species specific gene	*ecfX*	F: ATG CCT ATC AGG CGT TCC AT R: GGC GAT CTG GAA AAG AAA TG	146	Colinon *et al.*, 2013
Integrons and gene cassatte	*intI1*	F: GCA TCC TCG GTT TTC TGG R: GGT GTG GCG GGC TTC GTG	457	Azami, 2013
	intI2	F: CAC GGA TAT GCG ACA AAA AGG T R: GTA GCA AAC GAG TGA CGA AAT G	789	Azami, 2013
	intI3	F: ATC TGC CAA ACC TGA CTG R: CGA ATG CCC CAA CAA CTC	922	Azami, 2013
	5′CS/ 3′CS	F: GGC ATC CAA GCA GCA AG R: AAG CAG ACT TGA CC	V	Rizk and El-Mahdy,2017
Virulence genes	*oprD2*	F: CGC CGA CAA GAA GAA CTA GC R: GTC GAT TAC AGG ATC GAC AG	1332	Shen *et al.*, 2015
	oprL	F: ATG GAA ATG CTG AAA TTC GGC R: CTT CTT CAG CTC GAC GCG ACG	504	Mokhtaria and Amini, 2019
	oprI	F:ATG AAC AAC GTT CTG AAA TTC TCT GCT R: CTT GCG GCT GGC TTT TTC CAG	249	Mokhtaria and Amini, 2019
	Alg	F: TTC CCT CGC AGA GAA AAC ATC R: CCT GGT TGA TCA GGT CGA TCT	520	Mokhtaria and Amini, 2019

Primer name	Genes	Oligo sequences (5'-3')	Product size (bp)	Reference
Antibiotic Resistance Genes Metallo-β-lactamases	*bla*$_{NDM}$	F: GCA GCT TGT CGG CCA TGC GGG C R: GGT CGC GAA GCT GAG CAC CGC AT	782	Safarirad *et al*., 2021
	bla$_{SPM}$	F: GCG TTT TGT TTG CTC R: TTG GGG ATG TGA GAC TAC	786	Safarirad *et al*., 2021
	bla$_{GIM-1}$	F: TCG ACA CAC CTT GGT CTG AA R: AAC TTC CAA CTT TGC CAT GC	477	Safarirad *et al*., 2021
	bla$_{SIM-2}$	F: TAC AAG GGA TTC GGC ATC G R: TAA TGG CCT GTT CCC ATG TG	570	Safarirad *et al*., 2021
	bla$_{VIM-1}$	F: AGT GGT GAG TAT CCG ACA G R: ATG AAA GTG CGT GGA GAC	261	Safarirad *et al*., 2021
	bla$_{VIM-2}$	F: ATG TTC AAA CTT TTG AGT AAG R: CTA CTC AAC GAC TGA GCG	801	Azami, 2013
	bla$_{IMP-1}$	F: ACC GCA GCA GAG TCT TTG CC R: ACA ACC AGT TTT GCC TTA CC	587	Azami, 2013
	bla$_{IMP-2}$	F: GTT TTA TGT GTA TGC TTC C R: AGC CTG TTC CCA TGT AC	678	Shibata *et al*., 2003
	bla$_{IMP-7}$	F: AAG GCA GTA TCT CCT CTC ATT TTC R: ACT CTA TGT TCA GGT AGC CAA ACC	243	Sonbol *et al*., 2015
	bla$_{IMP-10}$	F: AAT GCT GAG GCT TAC CTA ATT GAC R: CCA AGC TTC TAT ATT TGC GTC AC	388	Sonbol *et al*., 2015
	bla$_{IMP-13}$	F: AGA CGC CTA TCT AAT TGA CAC TCC R: CCA CTA GGT TAT CTT GAGTGTGACC	311	Sonbol *et al*., 2015

Primer name	Genes	Oligo sequences (5'-3')	Product size (bp)	Reference
	bla_{IMP-25}	F: GCA GTA TTT CCT CAC ATT TCCATAG R: TCA CCC AAA TTA CCT AGA CCG TAG	295	Sonbol *et al*., 2015

V: Variable, F: Forword, R: Reverse

3.2. Methods

3.2.1. Preparation of Reagents

The following reagents were prepared as described in MacFaddin (2000):

3.2.1.1. Catalase Reagent

Hydrogen peroxide (3%) was prepared and used for detection the ability of bacteria to produce catalase enzyme.

3.2.1.2. Oxidase Reagent

A quantity of 0.1g of tetramethyl *p*-phenyl diamine- dihydrochloride was dissolved in 10 ml D.W. This reagent was prepared freshly in a dark bottle.

3.2.1.3. Methyl Red Reagent

It was prepared by dissolving 0.1g of methyl red in 300 ml of (96%) ethanol, and then completed to 500 ml with D.W. This reagent was used as indicator in methyl red test.

3.2.1.4. Voges-Proskauer Reagent

The Voges-Proskauer (VP) test is used to determine if an organism produces acetylmethyl carbinol from glucose fermentation. If present, acetylmethyl carbinol

is converted to diacetyl in the presence of ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen.

The reagents were prepared as follows:

Solution A (α-naphthol): Prepared by dissolving 5g of α-naphthol in 100 ml of ethanol.

Solution B (40% KOH): Prepared by dissolving 40g of KOH in 100 ml D.W.

Two drops of 40% KOH solution and six drops of 5% solution of α-naphthol were added to each test tube.

3.2.2. Preparation of Buffers and Solutions (Collee *et al*., 1996):

Buffers and solutions which require sterilization were autoclaved at 121°C for 15 min. Millipore filters (0.22µm) were used for sterilization of heat-sensitive solutions like antibiotics. pH of the solutions was adjusted using 1M NaOH or 1M HCl.

3.2.2.1. Normal Saline Solution

This solution was prepared by dissolving 0.85g of NaCl in 90ml D.W. and further completed to 100 ml with D.W. Autoclaved at 121°C for 15 min.

3.2.2.2. Phosphate Buffer Solution

This buffer consists of two solutions and was prepared as follows: **Solution A**: A quantity of 3.12 g of $NaH_2PO_4.12H_2O$ was dissolved in 90 ml of D.W and then completed to 100 ml with D.W.

Solution B: A quantity of 2.839g of Na_2HPO_4 was dissolved in 90 ml of D.W and the volume was completed to 100 ml. Then, 87.7 ml of solution A was added to

12.3 ml of solution B and mixed carefully. The pH was adjusted to 6 and the buffer was used for the detection of β-lactamase production. The pH was adjusted to 7.2; this buffer was used in biofilm formation test.

3.2.2.3. Solutions for β-lactamase Detection

These solutions were prepared as follows:

a) **Penicillin G Solution:** It was prepared by dissolving 0.569g of penicillin G in 100 ml PBS. The solution was sterilized, dispensed in small vials and stored at -20˚C.
b) **Starch Solution:** This solution was prepared by dissolving 1g of soluble starch in 100 ml of D.W boiled in the water bath for 10min. and stored in a dark bottle at 4˚C.
c) **Iodine Solution:** Iodine (2.03g) and KI (5.32g) were dissolved in 90 ml of D.W., the volume was completed to 100 ml with D.W and stored in a dark bottle at 4˚C.

3.2.2.4. Crystal Violet Solution

This solution was prepared by dissolving 1g of crystal violet in 90 ml D.W. and further completed to 100 ml with D.W filtered solution by Millipore filter 0.45µm, and stored in dark bottle at room temperature.

3.2.2.5. EDTA Solution for Disks Preparation

A 0.5 M EDTA solution was prepared by dissolving 18.61g of EDTA in 100 ml of distilled water and adjusting it pH 8.0 by using NaOH (Yong *et al*., 2002).

3.2.2.6. McFarland standard solution (tube No. 0.5)

It is the turbidity standard solution which is the most widely used method of inoculums preparation or standardization especially the McFarland 0.5 standard,

which is prepared by adding 0.05 ml of 1% solution of barium chloride (BaCl. $2H_2O$) and 9.95 ml of 1% (v/v) solution of pure sulfuric acid (H_2SO_4) to obtain barium sulfate solution with the specific density to provide a turbidity comparable to that of bacterial suspension containing 1.5×10^8 colony forming unit (CFU)/ml.

3.2.2.7. Primers Dilution

All the primers were synthesized at (macrogen/Korea) these were provided in a lyophilized from, which were re-dissolved with nuclease-free water according to institution of manufacture company to reach to the final concentration (10 Pmol/µl).

3.2.3. Preparation of Culture Media

All culture media were prepared according to the instructions of the manufactures, and then sterilized by autoclaving at 121^{o}C at 15 pound per square inch for 15 min.

3.2.3.1. Urea Agar Medium

This medium was prepared by adding 15 ml of urea solution sterilized by filtration (0.22µm), to 100 ml of urea agar base sterilized by autoclaving at 121^{o}C for 15 min, and cooled at 50^{o}C, and then the medium distributed into sterilized test tubes and allowed to solidify in a slant form. This medium was used to test the ability of bacteria to produce urease enzyme (Mahon *et al.*, 2007).

3.3. Study Design

Steps of the research project were shown in Figure (3.1).

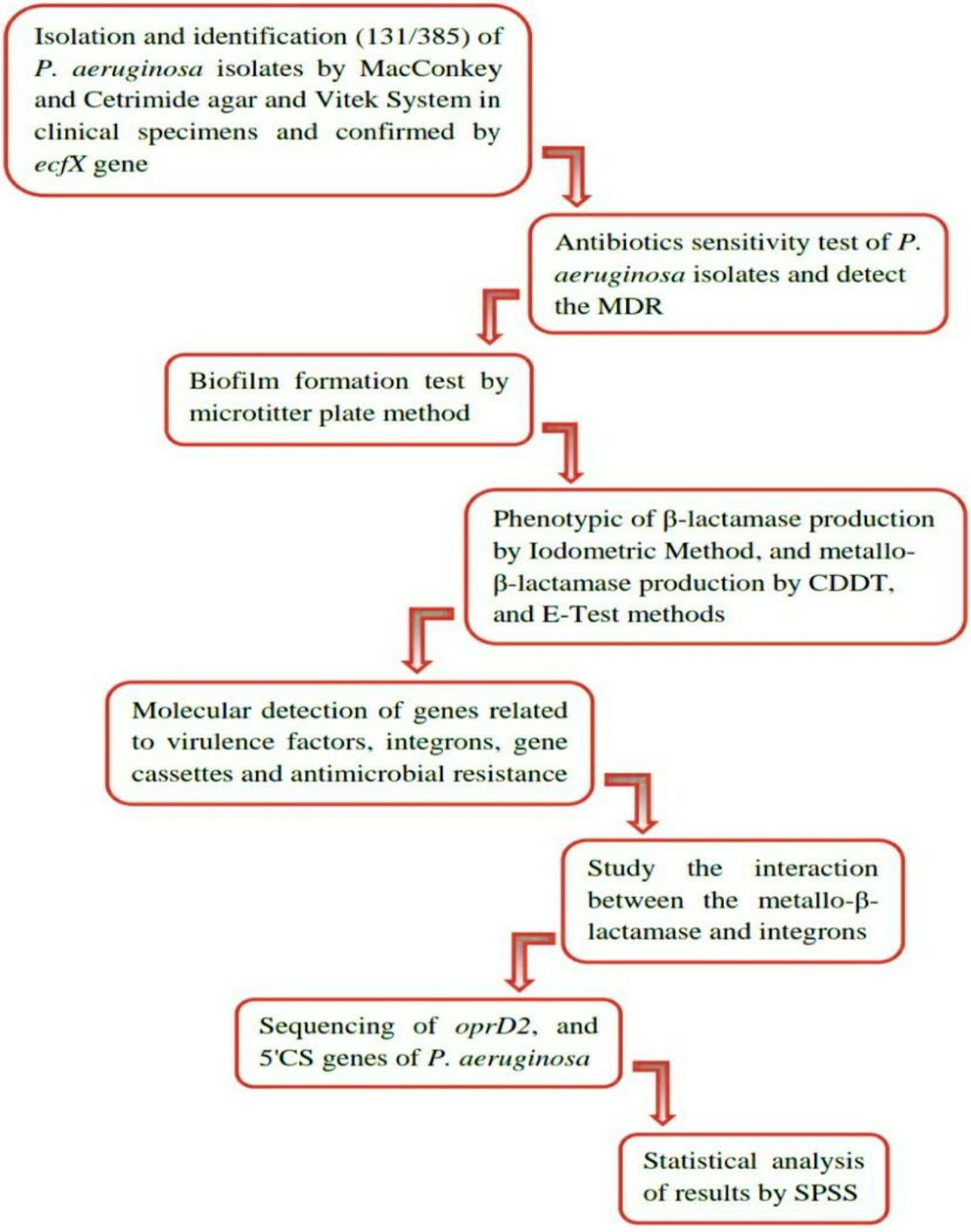


Figure (3.1): Scheme of the research plan

3.4. Collection of Specimens

Three hundred and eighty-five clinical samples of sputum, ear, urine, burns and wounds were collected from patients attended different hospitals and laboratories in the Hilla city/Iraq (Hilla Teaching Hospital, Babil Educational Hospital for Maternity and Children, and Al-Imam Al-Sadiq Teaching Hospital). The samples collect from both sexes males and females, which were isolated between February 2022 and October 2022, and their identification was achieved by routine biochemical tests and Vitek2 System then confirmed species by species-specific PCR assay with *ecfX* gene.

3.5. Colonial Morphology and Microscopic Examination

3.5.1. Colonial Morphology

Samples were cultured on the MacConkey agar by streak plate method and incubated at 37ºC for 24 hrs to observe the colony morphology (shape, size, surface texture, edge elevation, and colour). The organisms showing characteristic colony morphology of *P. aeruginosa* was subsequently subcultured onto cetrimide agar until the pure culture with homogenous colonies were obtained.

3.5.2. Gram-Staining

The organisms revealed Gram negative, pink colored with rod shaped appearance and slime were suspected as *P. aeruginosa* (Holt *et al.*, 1994; Winn, 2006).

3.5.3. Biochemical Tests

3.5.3.1. Oxidase test:

A piece of clean filter paper was saturated with oxidase reagent. Wood stick of referred colony was transferred to the paper. If the color turned rose to purple, the oxidase test would be positive (Tille, 2017).

3.5.3.2. Catalase test:

A colony of organisms was transferred by sterile wooden stick to the surface of a clean, dry glass slide, and one drop of 3% H_2O_2 was added to it. The formation of gas bubbles indicated the positive result (Tille, 2017).

3.5.3.3. Indole test:

It was used for determination of organism's ability to produce indole from deamination of tryptophan by tryptophanase. Peptone water was inoculated with the fresh tested bacteria and incubated at 37°C for 24 hrs. A few drops of Kovac's reagent were added to each tube and shake gently. The formation of red color ring at top of broth indicates for positive reaction while a yellow color ring indicated a negative reaction (MacFaddin, 2000).

3.5.3.4. Methyl Red test:

It was employed to detect the production of sufficient acid during the fermentation of glucose. The change of color to orange was positive reaction (Murray *et al.*, 2009).

3.5.3.5. Voges-Proskauer (acetoin production) Test:

The VP test was used to detect acetone (acetyl-methyl-carbinol), which was produced by certain bacteria during growth in peptone glucose broth (MR-VP

broth) the positive result was changing of the color of the medium to red (MacFaddin, 2000).

3.5.3.6. Simmon's Citrate Test:

The citrate test was used to determine the ability of bacterium to utilize citrate as its only source of carbon. The positive result was changing of color of media from green to blue (Tille, 2017).

3.5.3.7. Urease Test:

The urea base agar was sterilized by autoclave, after that it was cooled to 50°C and urea substrate was added to it and it was poured in sterile tubes; then it was inoculated by bacterial culture, which was incubated for (24-48) hrs at 37°C. When urea was broken down, ammonia was released and the pH of medium increased. This pH change was detected by a pH indicator that turned pink in a basic environment. A pink medium indicated a positive test for urease. Failure of deep pink color to develop indicates a negative reaction (Collee *et al.*, 1996).

3.5.4. Identification Process with VITEK2 System

All isolates of *P. aeruginosa* were identified using the automated VITEK-2 compact system and by GN-ID cards according to the manufacturer's instructions.

3.5.5. Preservation of *P. aeruginosa* (Collee *et al.*, 1996).

3.5.5.1. Short Term Storage

Slants of nutrient agar were inoculated with bacterial growth, incubated for 18-24 hrs. at 37ºC and stored for a period of few weeks at refrigerator.

3.5.5.2. Long Term Storage

Cultures of *P. aeruginosa* isolates were inoculated into 15% glycerol in brain heart infusion broth and stored at -20ºC until required.

3.6. Antibiotic Sensitivity Test

Disk agar diffusion according to Kirby Bauer standardized antimicrobial susceptibility single disk method was carried out (Bauer *et al.*, 1966; CLSI, 2022):

- Preparation of culture media and plates; Muller-Hinton medium was employed and heated to 45-50°C, then poured in Petri dishes on a level surface to a depth of 4mm. when the media was solidified the Petri dishes were placed in the incubator at 37°C for 15-30 min. to let the excess moisture evaporated.

- Bacterial inoculums; with the sterile wire loop, the tops of 4-5 isolated colonies of *P. aeruginosa* were picked from the original culture and introduced in to a test tube containing 2ml of normal saline to produce a bacterial suspension of moderate turbidity. Its turbidity was compared to that of the recommended turbidity standard to 0.5 McFarland turbidity equal to 1.5×10^8 CFU/ml.

- Inoculation of the test plates; within 15 min. of adjusting the density of the inoculums, a sterile cotton swab was dipped in to the standardized bacterial suspension. The swabs then streaked on the upper most surface of Muller-Hinton in three different planes to obtain uneven distribution of the inoculums. The lids were replaced and the inoculated plates were allowed to remain on a flat and level surface undistributed for 3-5 min to allow absorption of excess moisture.

- Disks were listed in table (3-5) placed on the inoculated plate and pressed in to the agar with a sterile forceps. Within 15 min. the inoculated plates were incubated at 37°C for 18 hrs. in an inverted position.

• Reading of the results; after incubation the diameters of the complete zone of the inhibition were noted and measured using reflected light and ruler. The end point measured to the nearest millimeter, was taken as the area showing no visible growth.

• Interpretation of zone size; the diameter of inhibition zone for individual antimicrobial agent was translated in terms of sensitive and resistant, the appearance of the inhibition zone of each antibiotic disk was measured and the results were interpreted by CLSI recommendation according to CLSI, (2022).

• As a control positive, *P. aeruginosa* ATCC 27853(ATCC /USA) was also examined as a reference strain for antibiotic resistance.

3.6.1. Production of β-lactamase

All bacterial isolates that were tested for β-lactamase production by Rapid Iodometric Method. Several colonies of a young bacterial culture on MacConkey agar, were transferred to Eppendorf tube containing 100µl of penicillin G solution, and the tubes were incubated at 37°C for 30 min. Then, 50µl of starch solution was added and mixed well with the content of the tube, 20µl of iodine solution was added to the tube which cause the appearance of dark blue colour, rapid change of this color to white (within few sec to 2 min) indicated a positive result (Collee *et al.*, 1996).

3.6.2. Detection of Metallo-β-lactamases

a) Combined Double Disk Test (CDDT)

Two 10µg Imipenem disks (one impregnated with 10µl of 0.5 M EDTA) were placed on the Mueller Hinton (MH) agar medium inoculated with test organism standardized with 0.5 McFarland standards. After overnight incubation at 37°C, the

zones of inhibition around Imipenem + EDTA disks compared with Imipenem alone was greater than 7mm, was considered as MBL producing (Yong *et al.*,2002).

b) E-Test

Metallo-β-lactamase enzyme and MIC of Imipenem were tested by using E-test which consists of a plastic strip to determine the MIC of Imipenem by using the Liofilchem MIC test strip. One half of the strip was impregnated with imipenem gradient against seven dilution (0.125, 0.19, 0.25, 0.38, 1.0, 1.5, 2, 3, 4, 8µg/ml) and the other end of the strip was impregnated with Imipenem overlapped with constant concentration of EDTA ranging from 0.032-2µg/ml, Imipenem /Imipenem +EDTA (IMI/IMD). Tested colonies from overnight culture were suspended with 0.85% of normal saline (NaCl) to a turbidity of 0.5 McFarland standards according to manufacturer's recommendation (kumar *et al.*, 2012).

A sterile cotton swab was used to produce a uniform layer on a Mueller-Hinton agar plate and the excess moisture was allowed to be absorbed for about 15min. before the E-test MBL strip was applied. The plate was incubated for 16 to 20hr. at 37ºC and the MIC end points were read where the inhibition ellipses intersected the strip (Bashir *et al.*, 2011). MIC ratio of (IMI/IMD) was calculated and a positive metallo-β-lactamase test was decided if the value of (IMI/IMD) is ≥ 8 or if there is zone of deformation insensitive area along the strip or appearance of phantom zone along the strip according to the manufacturer recommendation (kumar *et al.*, 2012).

3.7. Biofilm Formation Assay

Quantitative biofilm assay by spectrophotometric method described by Stepanovic *et al.*, (2007) and Lotfi *et al.*, (2014) used for detection the bacterial

ability for biofilm formation, this method included inoculation 5ml of Brain Heart Infusion broth with particular isolates and incubated for overnight in 37ºC, after that, add 20µl of *P. aeruginosa* of overnight culture were used to inoculate 96-microtiter wells plate containing 180 µl of BHI broth with 2% glucose. Negative control wells contained the broth only. Incubate for 24 hrs in 37ºC. Culture was removed and the wells were rinsed with PBS (pH 7.2), after drying at room temperature for 15 min, adherent organisms forming-biofilms in plate were fixed with sodium acetate (2%) and 200µl per well of crystal violet stain (0.1%) was added to the well for 20 min. The stained biofilms were rinsed three times with PBS (pH 7.2), and allowed to dry at room temperature for 15min., then extracted with 200µl per well of 95% ethanol, and estimate O.D 630 to each well by using automatic microtiter plate reader. All assays were performed in triplicate.

The average O.D values were calculated for all tested isolates and negative controls.

a) The cut-off value (O.Dc) was established, it was defined as

a three standard deviations (SD) above the O.D mean of the negative control, the O.D values of a tested isolates was expressed as average O.D value of the isolate reduced by O.Dc value. O.D = average O.D of isolate – O.Dc

b) The biofilm results of isolates were divided into the following: Non-biofilm producer (O.D $\leq$ O.Dc); weak-biofilm producer (O.Dc < O.D $\leq$ 2xO.Dc); moderate-biofilm producer (2xO.Dc < O.D $\leq$ 4xO.Dc); strong biofilm producer (4xO.Dc < O.D).

3.8. Extraction of DNA

3.8.1. Chromosomal DNA Extraction

Step 1- Preparation of Specimens:

- One milliliter of bacterial culture was transferred to a 1.5 ml microcentrifuge tube, and the tube was centrifuged for one minute at full speed (14,000 rpm). The extra fluid was discarded
- Two hundred µl of FATG Buffer were added, then the pellet was re-suspended by vertexing or pipetting, incubated for 5 min at room temperature.

Step 2 -Cell Lysis

- Two hundred µl of FABG Buffer were added to the sample and vortexed for 5 sec.
- Incubated for 10 min at 70 °C or until the sample lysate is clear. During incubation, the tube was inverted every 3 min.
- Elution Buffer was preheated (for Step 5 DNA Elution) in a 70 ° C water bath.

Step 3 – Binding

- The samples and vortex were both exposed to 200µl of ethanol (96–100%) for 10 sec.
- A FABG Column was attached to a 2 ml collecting tube. The sample mixture was transferred, to the FABG Column. The 2 ml collection tube was discarded after centrifuging at full power (14,000 rpm) for 5 min. A fresh 2 ml collection tube was filled with the FABG Column.

Step 4 - Washing

- FABG Column was washed with 400 µl W1 Buffer, the flow - through was discarded, after Centrifuging for 30 sec at full speed (14,000 rpm)

- The FABG Column back was placed in the 2 ml collection tube. FABG Column was washed with 600 µl Wash Buffer (ethanol added), the flow - through was discarded, after Centrifuging for 30 sec at full speed (14,000 rpm)
- The FABG Column back was placed in the 2 ml Collection tube. To dry the column was centrifuged for an additional 3 min at full speed (14,000 rpm).

Step 5 - Elution

- The dry FARG Column was placed to a new 1.5 ml microcentrifuge tube.
- One hundred µl of Preheated Elution Buffer or TE was added to the membrane center of FABG Column. Stand FAGB Column for 3-5 min or until the buffer was absorbed by the membrane.
- The DNA elute was centrifuged at high speed (14,000 rpm) for 30 sec. Elution was typically done in 100 µl of volume.

3.8.2. Plasmid DNA Extraction

- Three ml of well-grown bacterial culture was transferred to a centrifuge tube. It was centrifuged at 9,000 rpm for 1 min to pellet the cells and the supernatant completely was discarded.
- Two hundred µl of FAPD1 Buffer (RNase A added) was added to the cell pellet and the cells completely were resuspended by pipetting.
- Two hundred µl of FAPD2 Butter was added and gently inverted the tube 5-10 times. The sample mixture was incubated at room temperature for 2-5 min to lyse the cells.
- Three hundred µl of FAPD3 Butter was added and the tube was inverted 5-10 times immediately to neutralize the lysate.

- The lysate was centrifuged at full speed (14,000 rpm) for 5 min. During centrifugation, a FAPD Column was placed in a collection tube.
- The supernatant was transferred carefully to the FAPD Column and centrifuged at 9,000 rpm for 30 sec. The flow-through was discarded and the column back was placed to the collection tube.
- Four hundred µl of WP Buffer was added to the FAPD Column and centrifuged of 9,000 rpm for 30 sec. The flow-through was discarded and the column back was placed to the collection tube.
- Seven hundred µl of Wash Buffer was added to the FAPD Column and centrifuged at 9,000 rpm for 30 sec. The flow-through was discarded and the column was placed to the collection tube (ethanol (96-100%) added).
- The FAPD Column was centrifuged at full speed (14,000 rpm) for an additional 3 min to dry.
- The FAPD Column was placed to a new 1.5 ml microcentrifuge tube.
- Fifty µl -100 µl of Elution Buffer was added to the membrane center of the FAPD Column. Stand the column for 1 minute to ensure the Elution Buffer is absorbed by the matrix.
- Centrifuged at 14,000 rpm for 60 sec to elute the purified DNA.

3.8.3. Assessment of DNA Purity and Concentration

Purity and concentration of DNA solutions were measured by using spectrophotometry with Nanodrop. The quantity and purity of DNA was determined by measuring the absorbance at 260/280nm. With pure DNA having a 260/280 nm, the purity of DNA ranged between 1.8 to 2.

3.9. PCR Assay for Gene Determination

3.9.1. Genetic Identification of *P. aeruginosa*

P. aeruginosa was identified by screened of presence *ecfX* gene by PCR technique to detection the presence *ecfX* encodes, the reaction was carried out by using a 25µl mixture including 12.5µl Taq Green master mix, 1µl of each *ecfX* primers (10 pmol), 2µl of template DNA, and the volume was completed with nuclease free water. The condition program of PCR according to (Talukder *et al*., 2018) was mentioned in table (3-7).

Table 3-7: Program of PCR conditions to *ecfX* gene

Step	Temperature(˚C)	Time	No. of cycles
Initial denaturation	95	5min.	1
Denaturation	98	10sec.	50
Annealing	63	20sec.	
Extension	63	20sec.	
Final extension	72	8min.	1
Cooling	4	4min.	

3.9.2. Prevalence of Mobile Genetic Elements (Integrons) and Variable Region

P. aeruginosa isolates were screened for the presence of classes 1, 2, 3 integrons and 5′CS/3′CS by uniplex-PCR technique to detection the presence of *intI1* (integrase gene of class 1 integron), *intI2* (integrase gene of class 2 integron), *intI3* (integrase gene of class 3 integron) and 5′CS/3′CS. One set of primers were used specifying the *intI1*, *intI2*, *intI3* and 5′CS/3′CS genes table (3-6) among *P.*

aeruginosa isolates. The reaction was carried out by using a 25µl mixture including 12.5µl Taq Green master mix, 1µl of each *intI1*, *intI2*, *intI3* and 5´CS/ 3′CS primers (10 pmol/ µl), 2µl of template DNA, and the volume was completed with nuclease free water.

The PCR was performed with a PCR thermal cycler under the following conditions to genes of integrons classes I, II, and III according to (Azami, 2013), and (5'CS/3'CS) gene according to (Rizk and El-Mahdy, 2017), as in table (3-8)

Table 3-8: Program of PCR conditions to *intI1*, *intI2*, *intI3* and 5´CS/3′CS genes

Genes	Temperature (^{o}C)/Time					No. of Cycles
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
intI1	94°C for 5 min	94°C for 30 sec	55 °C / 45 sec	72 °C for 2 min	72°C for 10 min	30
intI2	94°C for 5 min	94°C for 30 sec	55 °C / 45 sec	72 °C for 2 min	72°C for 10 min	30
intI3	94°C for 5 min	94°C for 30 sec	57 °C / 45 sec	72 °C for 2 min	72°C for 10 min	30
5´CS/3′CS	94 °C for 5 min	94°C for 1 min	55°C /1 min	72 °C for 2 min	72°C for 10 min	35

3.9.3. Determination of Genetic Virulence Profiles

Virulence genes characteristic were detected by PCR assays. The following genes were targeted; *oprD* (outer membrane porin D), *oprL* (peptidoglycan-associated lipoprotein OprL), *oprI* (outer membrane lipoprotein I) and *Alg* (Alginate). Determination of *oprD2, oprL, oprI* and *Alg* genes were detected by uniplex-PCR technique.

Primers were used specifying the *oprD2*, *oprL*, *oprI* and *Alg* genes, table (3-6). The reaction was carried out by using a 25µl mixture including, 12.5µl Taq Green master mix,1µl of each *oprD*, *oprL*, *oprI* and *Alg* primers (10pmol/ µl), 2µl of template DNA, and the volume was completed by nuclease free water.

The PCR was performed with a PCR thermal cycler under the following conditions to genes *oprL*, *oprI* and *Alg* according to (Mokhtaria and Amini, 2019), and *oprD2* gene according to (Shen *et al*., 2015), as in table (3-9).

Table 3-9: Program of PCR conditions to *oprD2*, *oprL*, *oprI* and *Alg* genes

Genes	Temperature (^{o}C)/Time					No. of Cycles
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
oprD2	94°C for 5 min	94°C for 60 sec,	55 °C / 45 sec	72°C /1 min	72°C for 7 min.	30
OprL	94°C for 5 min	94°C for 40 sec	59°C /40 sec	72°C for 45 sec	72°C for 10 min	35
OprI	94 °C for 4 min	94 °C /1 min	61°C /40 sec	72°C for 45 sec	72°C for 45 sec	30
Alg	94°C for 5 min	94°C for 40 sec	59°C/40 sec	72°C for 45 sec	72°C for 10 min	35

3.9.4. Prevalence of Antibiotic Resistance Genes

P. aeruginosa isolates were screened for the presence of metallo-β-lactamases genes bla_{IMP-1}, bla_{IMP-2}, bla_{IMP-7}, bla_{IMP-10}, bla_{IMP-13}, and bla_{IMP-25}, bla_{VIM-1},and bla_{VIM-2} (Verona integron encoded metallo-β-lactamase), bla_{SPM} (Sao Paulo metallo-β-lactamases), bla_{NDM} (New-Delhi metallo-β-lactamase), bla_{SIM-2} (Seul imipenemase) and bla_{GIM-1} (German imipenemase) by Uniplex-PCR technique.

The reaction was carried out by using a 25µl mixture including 12.5µl Taq Green master mix, 1µl of each $bla_{IMP,}$ bla_{VIM}, bla_{NDM}, bla_{GIM-1}, bla_{SIM-2} and bla_{SPM} primers (10 pmol/ µl), 2µl of template DNA, and the volume was completed with nuclease free water.

The PCR was performed with a PCR thermal cycler under the following conditions to bla_{NDM}, bla_{SPM}, bla_{GIM-1}, bla_{SIM-2}, and bla_{VIM-1} genes according to (Safarirad *et al*., 2021), bla_{VIM-2}, and bla_{IMP-1} genes according to (Azami, 2013), bla_{IMP-2} gene according to (Shibata *et al*., 2003), while (bla_{IMP-7}, bla_{IMP-10}, $bla_{IMP-13,}$ and bla_{IMP-25} genes according to (Sonbol *et al*., 2015), as in table (3-10).

Table 3-10: Program of PCR conditions to $bla_{IMP,}$ bla_{VIM}, bla_{NDM}, bla_{GIM-1}, bla_{SIM-2} and bla_{SPM} genes

Genes	Temperature (ºC)/Time					No. of Cycles
	First Denaturation	Condition of cycling			Final Extension	
		Denaturation	Annealing	Extension		
***bla*$_{NDM}$**	95°C / 5 min	95°C /45 sec	59°C/45sec	72°C/1min	72°C / 8 min	35
***bla*$_{SPM}$**	94°C/ 5 min	95°C /45 sec	52°C/45s	72°C/1min	72°C/10 min	35
***bla*$_{GIM-1}$**	94°C /5 min	94°C /30 sec	52°C/40sec	72°C/50sec	72°C/5 min	36
***bla*$_{SIM-2}$**	94°C/ 5 min	95°C /45 sec	52°C/45s	72°C/1min	72°C/6 min	35
***bla*$_{VIM-1}$**	94 °C /5 min	94 °C/25sec	52°C/40sec	72°C/50sec	72°C / 6 min	30
***bla*$_{VIM-2}$**	94°C/ 5 min	94°C/ 40 sec	52°C/40 sec	72°C/45sec	72°C/10 min	35
***bla*$_{IMP-1}$**	94°C/ 5 min	94°C/ 30 sec	55°C/1 min	72°C/ 90sec	72°C/ 10min	30
***bla*$_{IMP-2}$**	94°C/ 5 min	94°C/40 sec	52°C/40 sec	72°C/45 sec	72°C/ 10min	35
***bla*$_{IMP-7}$**	94 °C/4 min	94 °C/1min	55°C/1 min	72 °C/1 min	72°C/ 10min	30

Genes	Temperature (ºC)/Time					No. of Cycles
	First Denaturation	Condition of cycling			Final Extension	
		Denaturation	Annealing	Extension		
***bla*$_{IMP-10}$**	94 °C/4 min	94 °C/1min	55– 60 °C/1 min	72 °C/1 min	72°C/ 10min	30
***bla*$_{IMP-13}$**	94 °C/4 min	94 °C/1min	55°C/1 min	72 °C/1 min	72°C/ 10min	30
***bla*$_{IMP-25}$**	94 °C/4 min	94 °C/1min	55– 60 °C/1 min	72 °C/1 min	72°C/ 10min	30

3.10. Gel Electrophoresis (Sambrook, and Russell, 2001)

3.10.1. Preparation of Agarose Gel

Agarose gel was prepared by adding agarose powder to 1X TBE buffer previously prepared in percent specific for each PCR products. The muddle was placed in microwave until it become clear, allowed to cool to 50°C, and 5µl/100ml Red Safe dye at concentration of 6X was added. The agarose poured kindly in equilibrated gel tray earlier set with its comb. The agarose allowed solidifying at room temperature for 30 min. DNA samples are put into wells formed by a comb.

3.10.2. Agarose Gel Electrophoresis

The amplified PCR products were checked for the expected size on agarose gel electrophoresis and visualized after staining with Red Safe dye under ultraviolet exposure. A DNA molecular weight marker was used to estimate the weight of the fragments. Finally, the results photographed using gel documentation system.

3.11. DNA Sequencing

The 10 isolates chosen at random to detect the variable region (5' CS/3' CS) and the 8 isolates chosen at random to detect the *oprD*2 were sent to sequencing at

Macrogene/ Korea and analyzed using the Basic Local Alignment Search Tool (BLAST) online program from the National Center for Biotechnology Information.

3.12. Statistical Analysis

SPSS version 23.0 statistical software package was used for statistical analysis by using Chi-square test and t-test for all obtained results and test of proportions, p value $\leq$ 0.05 and 95% confidence interval (CI) was considered statistically significant.

Chapter Four

Results and Discussion

4. Results and Discussion

4.1. Isolation and Identification of *P. aeruginosa*

One hundred and thirty one of *P. aeruginosa* isolates were isolated from 385 (34.0%) clinical specimens of urine, burns, wounds, ear and sputum collected from both sexes males were 196 (50.9%), and females were 189 (49.1%), with different ages. Different hospitals and laboratories in Babylon province were included in this isolation. Identification of isolates were accomplished by routine biochemical tests and VITEK® 2 Compact system. The isolates were further molecularly identified using the gene species-specific *ecfX*. Based on morphological and cultural characteristics of *P. aeruginosa*, the results revealed that only 131 isolates (34.0 %) were *P. aeruginosa*, and 160 (41.55%) isolates were identified as other bacterial spp., while 94 (24.41%) of the samples displayed no growth, Figure (4.1).

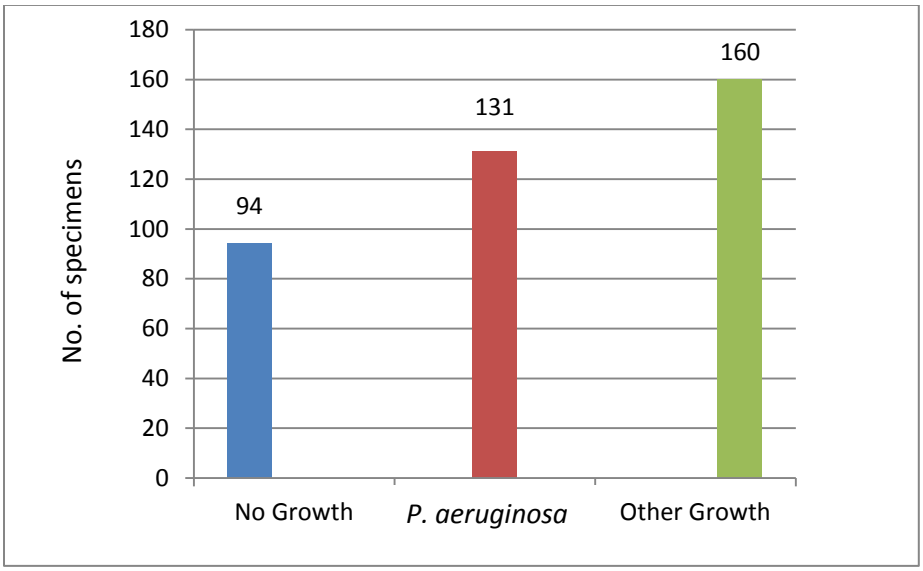


Figure (4-1): Distributoin of various clinical specimens acoording to growth type

The prevalence of *P. aeruginosa* recovered from 385 various clinical specimens was 131 (34.0 %), burns comprised 103 (78.63%) of all clinical

specimens, wounds 7 (5.3 %), urine 9 (6.87%), ear 9 (6.87%) and sputum 3 (2.29%), as show in Table 4-1.

Table 4-1: Numbers and percentages of *P. aeruginosa* distributed by source of clinical specimens

Source of samples	No. of isolates	Percentage of isolates (%)
Burns	103	78.63
Wounds	7	5.34
Urine	9	6.87
Ear	9	6.87
Sputum	3	2.29
Total	**131**	**100**

4.1.1. Cultural Characteristics

Based on suggestive morphological, growth characteristics, and biochemical profiling, 131 isolates were confirmed to be *P. aeruginosa*, including but not limited to: on MacConkey agar, the bacterial colonies of *P. aeruginosa* were smooth, flat, and pale because they had not fermented lactose. *P. aeruginosa* bacterial colonies emerged in greenish-blue color on cetrimide agar because the most of these colonies generate pyocyanin, a greenish-blue dye, Figure 4.2.

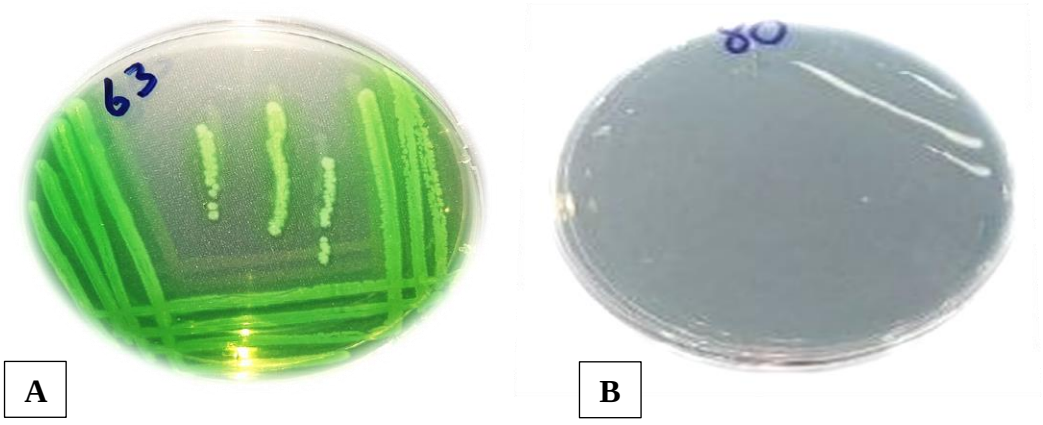


Figure (4.2): *P. aeruginosa* isolates on cetrimide agar. **A:** positive pigment formation. **B:** negative pigment formation.

Cetrimide agar is a selective medium for isolating and identifying of *P. aeruginosa* while limiting the development of other microbes. It is used to test *P. aeruginosa's* capacity to proliferate in the presence of 0.03% cetrimide. It works as a cationic quaternary ammonium molecule, causing nitrogen and phosphorus to be released from bacterial cells other than *P. aeruginosa*. It is producing a range of water-soluble pigments, which are isolated and detected via cetrimide agar (Breed *et al.,* 2014).

Under light microscope, the Gram-negative *P. aeruginosa* cells appeared as red color rods-shaped bacterium. All pure colonies that yielded lactose fermenting Gram negative rods were tested for several biochemical characteristics. Results of the current study revealed that, catalase and oxidase were positive, urease, MR, VP and indole were negative, while citrate positive. These characteristics matched the profile of *P. aeruginosa*, Table (4-2). The current results are consistent with previous studies findings (Todar, 2011; Tille, 2017). Biochemical tests were confirmed by VITEK® 2 Compact system with probability between 94%-99%, as detailed in **Appendix 1**.

Table 4-2: Results of microscopic examination and biochemical tests

Test	Result
Gram stain	-
Catalase	+
Oxidase	+
Urease	-
Citrate utilization	+
Indole	-
Methyl red	-
Voges-proskauer	-

(-) Negative, (+) Positive

4.1.2. Polymerase Chain Reaction Assay for Identification of *P. aeruginosa*

In order to further validate and to identify the isolates at the species level, a PCR technique targeting the *ecfX* gene was conducted to identify species-specific *ecfX* in *P. aeruginosa*. (Figure 4.3).

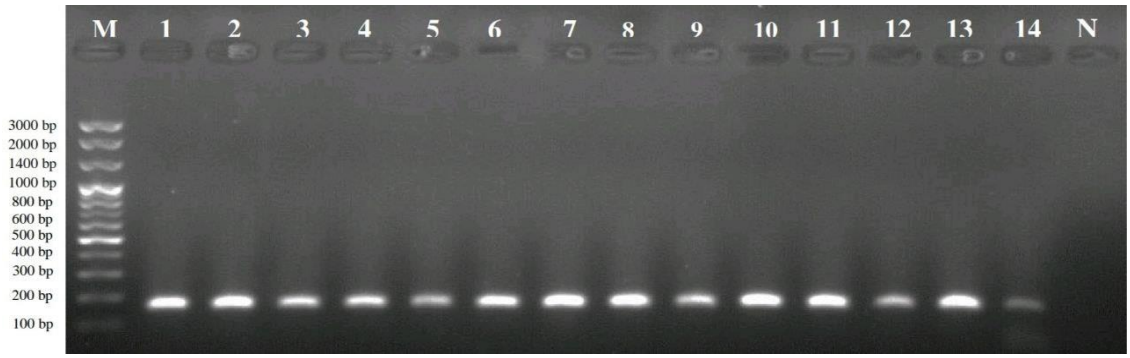


Figure (4.3): PCR amplification of ***ecfX*** gene (**146 bp**) in *P. aeruginosa* isolates, on 2 % agarose at 70 volt for 1 hr. Lane M: 100-bp DNA marker. Lanes (1-14) positive isolates, N :Negative control.

Identification *P. aeruginosa* isolates by PCR, revealed all the *P. aeruginosa* isolates were positive for the species-specific *ecfX* gene with amplification product of approximately 146 bp.

Because false-positive (using *16S rRNA* and *oprI* genes) and false-negative (using *algD* and *toxA* genes) findings have been reported, the *ecfX* gene is a potential target for species specific identification of *P. aeruginosa* isolates (Lavenir *et al*., 2007; Anuj *et al*., 2009; Wang *et al*., 2022). The *ecfX* gene encodes an ecf sigma factor (extracytoplasmic function sigma factor), which is involved in haem uptake and virulence (AL-Shimmary *et al*., 2018). According to findings from earlier studies confirmed that the *ecfX* gene has been shown to be an accurate PCR target for identification of *P. aeruginosa* (Lavenir *et al*., 2007; Anuj *et al*., 2009; Cattoir *et al*., 2010).

4.2. Antibiotic Susceptibility of *P. aeruginosa*

Susceptibility testing was performed on all 131 *P. aeruginosa* isolates against 22 antibiotics belonging to 9 classes, were tested, and the determination of whether an isolate was resistant or sensitive was based on a comparison of the dimensions of the inhibition zone with the standard value of CLSI, (2022). The findings suggested that varying degrees of susceptibility to different antibiotics have been reported among isolates as shown in **Table (4-3).** *P. aeruginosa* ATCC 27853 was also tested as a positive control strain for antibiotic resistance pattern.

Table 4-3: Antibiotic Susceptibility Pattern of *P. aeruginosa* (No=131)

Antibiotics	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
Ampicillin AMP	131(100%)	0	0
Piperacillin PRL	102(77.86%)	11(8.39%)	18(13.74%)
Piperacillin-tazobactam PIT	84(64.12%)	23(17.55%)	24(18.32%)
Ticarcillin clavulanic TCC	106(80.91%)	11(8.39%)	14(10.68%)
Ceftizoxime CZX	130(99.23%)	0	1(0.77%)
Cefixime CFM	131(100%)	0	0
Cefotaxime CTX	131(100%)	0	0
Cefepime FEP	123(93.90%)	4(3.05%)	4(3.05%)
Cefoperazone CFP	111(84.73%)	3(2.29%)	17(12.98%)
Amikacin AK	104(79.39%)	2(1.52%)	25(19.08%)
Gentamicin CN	110(83.97%)	4(3.05%)	17(12.98%)
Tobramycin TOB	107(81.68%)	0	24(18.32%)
Ciprofloxacin CIP	96(73.28%)	0	35(26.72%)
Norfloxacin NOR	94(71.75%)	2(1.52%)	35(26.72%)
Tetracycline TE	120(91.60%)	6(4.58%)	5(3.82%)
Doxycycline DO	119(90.84%)	6(4.58%)	6(4.58%)
Imipenem IMP	95(72.52%)	12(9.16%)	24(18.32%)
Meropenem MEM	107(81.68%)	2(1.52%)	22(16.80%)
Doripenem DOR	71(54.20%)	23(17.55%)	37(28.24%)
Azithromycin AZM	53(40.46%)	0	78(59.54%)
Aztreonam ATM	97(74.05%)	6(4.58%)	28(21.35%)
Levofloxacin LEV	99(75.57%)	3(2.29%)	29(22.14%)

The findings suggested that different isolates' rates of susceptibility to various antibiotics may be observed. Table (4.3) shows that all *P. aeruginosa* isolates were resistant to Ampicillin, Cefixime, Cefotaxime in (100%); followed by Ceftizoxime (99.23%), Cefepime (93.90%), Tetracycline (91.60%), Doxycycline (90.84%), Cefoperazone (84.73%), Gentamicin (83.97%), Tobramycin and Meropenem (81.68%), Ticarcillin clavulanic (80.91%), Amikacin (79.39 %) Piperacillin (77.86%), Levofloxacin (75.57%), Aztreonam (74.05%), Ciprofloxacin (73.28%), Imipenem (72.52%), Norfloxacin (71.75%), Piperacillin-tazobactam (64.12%), and Doripenem (54.20%). Azithromycin demonstrated the lowest level of resistance (40.46%). All isolates (100%) displayed MDR resistance to at least three groups of antibiotics, with some isolates displaying resistance to nearly all classes.

The current findings were relative agreement with previous findings, as described by Azimi *et al*. (2018) the 160 isolates of *P. aeruginosa* were 100% resistant to amoxicillin clavulanate, ciprofloxacin, and cefoxitin, 99.4% resistant to gentamicin, amikacin, cefepime, and aztreonam, 98.8% resistant to imipenem, 98.1% resistant to meropenem, 84.4% resistant to piperacillin/tazobactam, and 80% resistant to ceftazidime. In addition, all these 160 isolates were classified as multidrug resistant (MDR).

The current investigation found a high prevalence of resistant *P. aeruginosa* isolates with MDR rate of (40.90% - 100%), which is higher than those findings reported by Jalil *et al*., (40.8% - 81.6%) (Jalil *et al*., 2017), Al-Mayali and Salman (27.5% - 85%) (Al-Mayali and Salman, 2020), and Abdou *et al*. (20% - 95%) (Abdou *et al*., 2021).

Pseudomonas aeruginosa - MDR isolates have emerged and spread around the world, raising serious concerns that could make it more difficult to choose empirical agents. It is huge significant pathogens producing a variety of diseases in

hospitals and healthcare situations (Gellatly and Hancock, 2013; Leylabadlo *et al*., 2015; Qin *et al*., 2022). Therefore, antibiotics used to treat *P. aeruginosa* infections lose their therapeutic efficiency due to the spread of multi-drug resistance in *P. aeruginosa* strains (Tümmler, 2019; Coyne *et al*., 2022).

According to Ranjan *et al*. (2015), 66 *P. aeruginosa* isolates were screened out as possible MBL producers based on their resistance to carbapenems and ceftazidime. They detected 21.3% resistance to each of imipenem and meropenem, and 26.9% resistance to ceftazidime. While Ratajczak *et al*., (2021) revealed a significant level of carbapenem antibiotic resistance. 41.0% of the isolates tested positive for imipenem, and 61.6% tested positive for meropenem. A 5-year Latin American longitudinal research found that *P. aeruginosa* resistance to carbapenems has increased to 40% (Andrade *et al*., 2003). These data indicate that carbapenem resistance in *P. aeruginosa* is increasing. Resistance to carbapenems in *P. aeruginosa* is frequently caused by the down-regulation of porin channels, the up-regulation of an active efflux pump system, or the synthesis of MBLs.

Azithromycin had the lowest antibiotic resistant rate in the current study, while the majority of *P. aeruginosa* isolates displayed significant antibacterial agent resistance rates. According to the results of the current study, patients are becoming more resistant (100%) to several categories of antibiotics like ampicillin, cefixime, and cefotaxime. Mutations in the class A β-lactamases are the primary source of the resistance to third generation cephalosporins (Bush and Bradford, 2020). In the middle of the 1990s, the fourth-generation Cephalosporin (Cefepime) was released into clinical use (Endimiani *et al*., 2008). Furthermore, 83.97% and 79.39% of our isolates were resistant to aminoglycoside groups; gentamicin and amikacin, respectively. Resistance of *P. aeruginosa* isolates to aminoglycoside

antibiotics in this study was parallel to study of Poonsuk *et al*. (Poonsuk *et al*., 2012).

The antibiotic most successful in treating MDR isolates is carbapenem. However, a current global challenge is the rise in carbapenem-resistant *P. aeruginosa* (Abdeta *et al*., 2023). Ugwuanyi *et al*., (2021) reported all *Pseudomonas* isolates (100%) in their investigation conducted in Nigeria were revealed to be resistant to CZX, CZM, and AMP-clavulanate. Additionally, Odumosu *et al*., (2013) found that *P. aeruginosa* isolates were 100% resistant to amoxicillin-clavulanate in their study in southwestern Nigeria, and 87.1% resistant to ceftriaxone.

P. aeruginosa possesses both intrinsic (natural) and acquired resistance to a variety of antibiotic classes. Natural resistance is shown in β-lactam antibiotics (benzylpenicillin, isoxazolyl penicillins, aminopenicillin and their combinations with β-lactamase inhibitors, first- and second-generation cephalosporins), tetracyclines, chloramphenicol, and trimethoprim. Acquired resistance is mainly related to the generation of enzymes that degrade β-lactam antibiotics (Urbanowicz *et al*., 2017).

A Retrospective Analysis in Addis Ababa, Ethiopia by Abdeta and his coworkers (2023) reported that between 2017 and 2021, the prevalence of carbapenem-resistant *P. aeruginosa* fluctuated. It was 30.3% in 2017 but decreased to 8.8% in 2018, increased to 24.2% in 2019, and peaked at 33.3% in 2020 before falling back to 22.7% in 2021.

4.3. Detection of β-lactamase Production

The rapid iodometric technique was used to screen all MDR *P. aeruginosa* isolates for β-lactamase production. The results revealed that 33/131 (25.19%) of

tested isolates were positive for β-lactamase production, which was less than isolates gave non β-lactamase 98/131 (74.80%), as depicted in Figure (4-4).

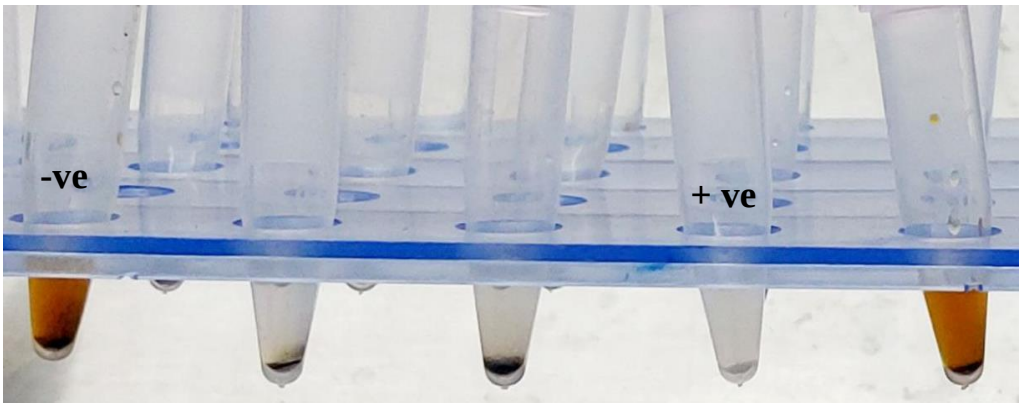


Figure (4.4); Prevelance of positive and negative isolates to β-lactamase production

-ve: Negative isolates; **+ve**: Positive isolates

The study results indicated the remaining 33 isolates β-lactamase-producing *P. aeruginosa* isolates had a positive reaction within seconds to multiple minutes after adding the reagent.

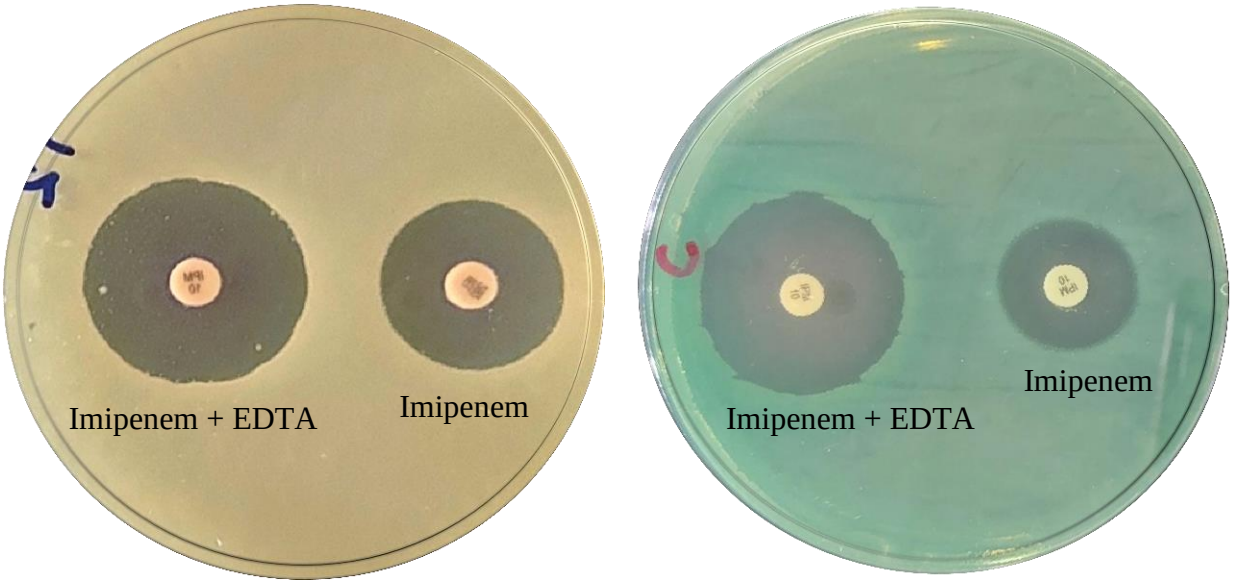
β-lactam antibiotics act by binding to cell wall synthesis enzymes known as penicillin-binding proteins (PBPs), thereby inhibiting peptidoglycan synthesis (Cho *et al*., 2014). Inhibiting PBPs weakens the cell wall, which inhibits cell development and usually results in cell death. β-lactam resistance pathways include decreased access to PBPs, decreased PBP binding affinity, antibiotic degradation via the expression of β-lactamase (enzymes that bind and hydrolyze β-lactams), and alterations in outer membrane permeability (Tooke *et al*., 2019).

Reduced permeability due to porin loss could decrease the steady state of periplasmic drug concentrations and hence PBP inactivation. As a result, lower permeability could act synergistically with the expression of β-lactamase or active

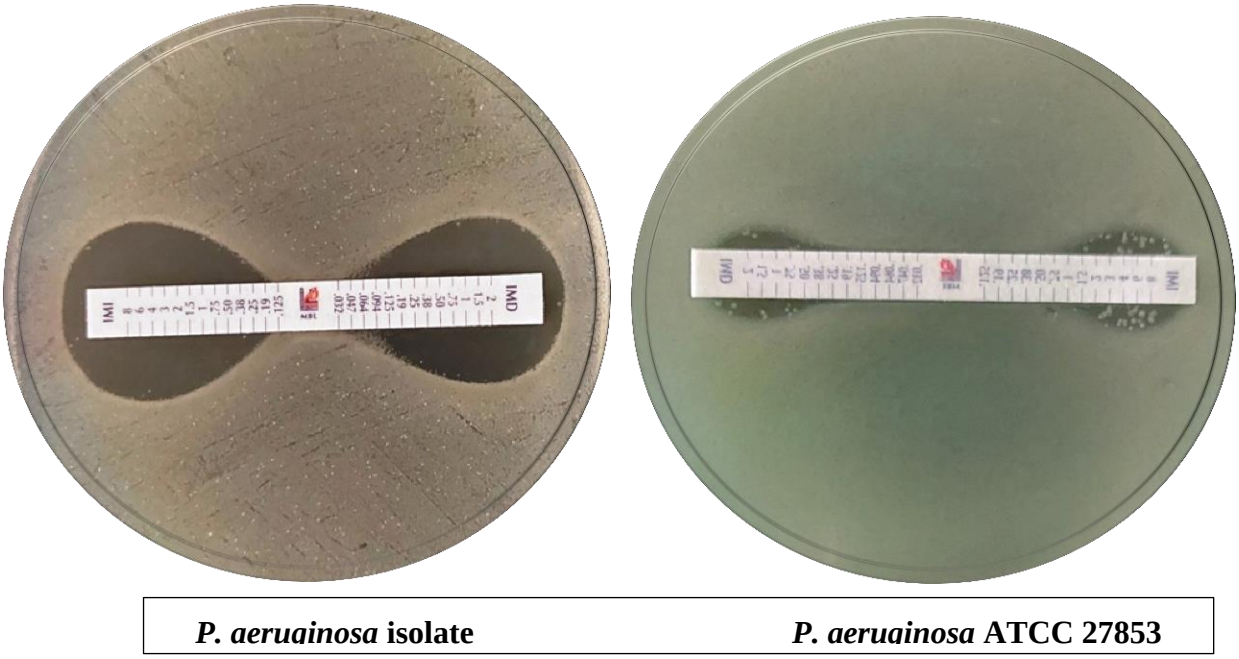
efflux to give increased levels of β-lactam resistance (Livermore and Woodford 2006).

4.4. Phenotypic Detection of Metallo -β -lactamases (MBLs)

All MDR *P. aeruginosa* isolates were tested for metallo -β- lactamases (MBLs) production using two phenotypic confirmatory screening tests: the combined double disk test (CDDT) and the Epsilo meter-test (E-test). The results revealed that the CDDT 3 (2.29%) of isolates that were positive showed an increase in the zone diameter around the IMP-EDTA disk, while 10 (7.63%) of isolates were positive for E-test. In CDDT test, an increase in zone diameter of > 7 mm around the imipenem-EDTA disk compared to that of the imipenem disk alone were considered positive for MBL production, whereas in E-test, MBL imipenem/ imipenem-EDTA, E-test demonstrated enhanced MIC of imipenem in the presence of EDTA IMI / IMD of ≥ 8 for MBL activity, as show in Figure (4-5).



A ***P. aeruginosa*** **isolate** ***P. aeruginosa*** **ATCC 27853**



***P. aeruginosa* isolate** ***P. aeruginosa* ATCC 27853**

Figure (4.5): Phenotypic tests to detect MBL production.

(A) Combined disk test (CDDT) showing enhanced inhibition zone of > 7mm around IMP+EDTA disk indicating MBL positivity.

(B) MBL IMI/IMD E-test demonstrating enhanced MIC of imipenem in the presence of IMI/IMD of ≥ 8 for MBL activity.

The MBL E-test was positive in 24 isolates, and as a gold standard test for MBL detection with 100% sensitivity, the prevalence of MBL generating isolates of *P. aeruginosa* was (24 / 160) 15% (Ranjan *et al*. 2015). As well as Khosravi and Mihani, (2008) revealed that an MBL E-test identified 8 MBL-positive *P. aeruginosa* strains among 41 imipenem resistant isolates recovered from burned individuals in Ahwaz, Iran. However, these results generally consistent with the current study's findings. The sensitivity of the MBL E-test is thought to be 100%, as determined by Khosravi *et al*. (2012) and Walsh *et al*. (2002).

Except for monobactams, MBLs can hydrolyze most β-lactams and give broad-spectrum β-lactams resistance to the bacterial host, which is not reversible

by typical therapeutic β-lactamase inhibitors (Walsh *et al.,* 2005; Boyd *et al*., 2020).

MBL-producing bacteria can be detected using a variety of phenotypic approaches. All of these approaches are based on metal chelatons' capacity to inhibit MBL action, such as EDTA and thiol-based compounds. CDDT, DDST, Hodge test, and MBL-E-test are examples of these tests (Lee *et al*., 2003; Vamsi *et al*., 2021).

Results of the present study using the E-test conducted higher frequency rate of MBL-producers in comparison to the CDDT, as also previously described by Walsh *et al.,* (2002); El-Kholy *et al.,* (2005) and Ranjan *et al.,* (2015). On the other hand, this is unlike Fam *et al.,* (2006) who demonstrated that the CDDT and DDST were superior to E-test for screening MBL production in Enterobacteriaceae.

As a consequence, the MBL E-test was utilized throughout this study to confirm MBL production among positively screened suspected MBL generating *P. aeruginosa* isolates. The MBL-E test verified the presence of MBL in 10 of the 33 putative MBL generating isolates. Thus, among the numerous clinical samples of *P. aeruginosa* gathered in this study, the rate of MBL generating isolates was 33/131 (25.19%). Therefore, early identification of MBL-producing *P. aeruginosa* may aid in effective antibiotic therapy and prevent the formation and spread of these multi-drug resistant strains.

4.5. Biofilm Formation Test

The results revealed that quantification of biofilm formation by microtiter plate method. Out of 131 *P. aeruginosa* isolates examined for biofilm formation, 112 (85.50%) had biofilm-forming capacity, and were classified as follows: 40 (30.53%) generated strong biofilm, 51 (38.93%) formed moderate biofilm, 21

(16.03%) formed weak biofilm, whereas 19 (14.50%) formed non-biofilm (Figure 4.6).

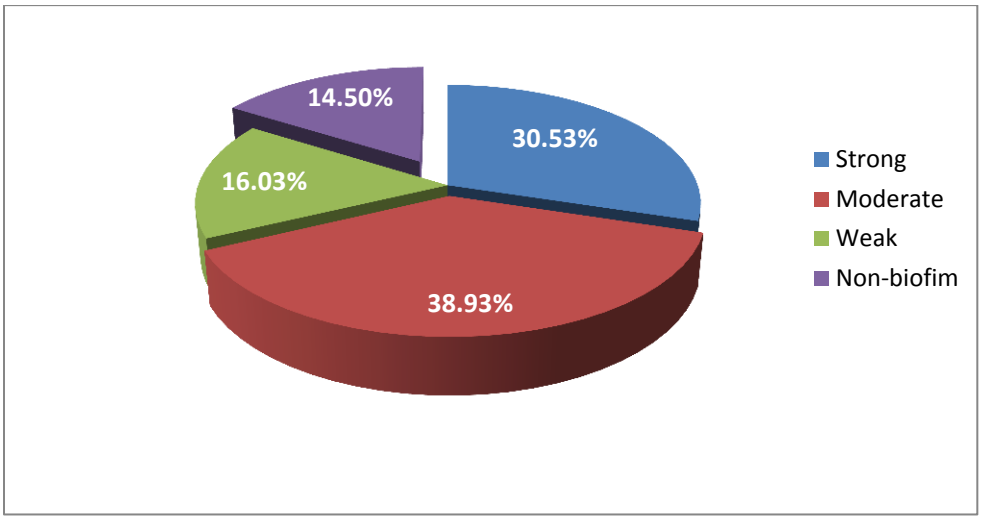


Figure (4.6): Biofilm formation of *P aeruginosa* isolates.

A previous report achieved by Ratajczak *et al*., (2021) demonstrated that the majority (73.6%) of *P. aeruginosa* strains were found to be strong biofilm producers. Only 9.6% of strains showed a weak biofilm-forming ability.

P. aeruginosa may survive in nutrient-poor conditions because of its capacity to utilize multiple energy sources and adhere to varied surfaces. The adhesion of motile bacteria to a surface, followed by division, results in the creation of microcolonies. Bacterial microcolonies grow and join together to produce biofilms (Ghanbari *et al*., 2016).

Bacterial that form biofilms have been demonstrated to be highly resistant to antimicrobial agents (Tyerman *et al*., 2013), it is also bacterial biofilm protects against the host's immune system and antimicrobial agents (Mulcahy *et al*., 2014).

Microorganisms that form biofilms are significantly more resistant to antibiotics than bacteria that develop as single cells (Yekani *et al*., 2017).

Biofilm development is a two-step process in which bacteria first attach to a surface via capsular antigen or flagellar antigen, followed by multiplication to generate a multilayered biofilm linked with exopolysaccharide matrix synthesis (Rather *et al*., 2021).

Chronic infections can be caused by bacteria that adhere to implanted medical devices or damaged tissue. According to Teodosio *et al*. (2012), biofilms are responsible for 65% of microbial infections. *P. aeruginosa* biofilm is regarded to be one of the main causes of therapy failure (Kamali *et al*., 2020).

4.5.1. Association of Antimicrobial Resistance with Formation of Biofilm

Table 4-4 illustrates the antimicrobial resistance trend of *P. aeruginosa* isolates in both biofilm formers and non-formers. Antimicrobial resistance was found to be significantly higher in biofilm producing *P. aeruginosa* isolates than in non-biofilm producing *P. aeruginosa* isolates ($p \leq 0.05$). Antibiotics from different classes, including Piperacillin-tazobactam, Ticarcillin clavulanic, and Levofloxacin, were found to have a statistically significant ($p \leq 0.05$) correlation between biofilm production and antimicrobial resistance. However, the association was not found to be significant in other classes of antibiotics.

Table 4-4: Antimicrobial resistance pattern of *P. aeruginosa* among biofilm formation isolates

Biofilm / Antibiotics	Strong (40)			Moderate (51)			Weak (21)			Non-biofilm (19)			P value
	R no.(%)	S no.(%)	I no.(%)	R no.(%)	S no.(%)	I no.(%)	R no.(%)	S no.(%)	I no.(%)	R no.(%)	S no.(%)	I no.(%)	
Ampicillin AMP	40(100)	-	-	51(100)	-	-	21(100)	-	-	19(100)	-	-	0,967
Piperacillin PRL	29(72.5)	6(15)	5(12.5)	40(78.43)	6(11.76)	5(9.8)	18(85.71)	2(9.52)	1(4.76)	15(78.95)	4(21.05)	-	0,844
Piperacillin-tazobactam PIT	23(57.5)	9(22.5)	8(20)	36(70.5)	7(13.72)	8(15.69	10(47.62)	4(19.05	7(33.33	15(78.25)	3(15.79)	1(5.26)	0,043*
Ticarcillin-clavulanic TTC	31(77.5)	5(12.5)	4(10)	45(88.23)	4(7.84)	2(3.92)	15(71.42)	3(14.29)	3(14.29)	15(78.96)	2(10.52)	2(10.52)	0,036*
Cefuroxime CZX	40(100)	-	-	50(98.04)	1(1.96)	-	21(100)	-	-	19(100)	-	-	0,970
Cefixime CFM	40(100)	-	-	51(100)	-	-	21(100)	-	-	19(100)	-	-	0,967
Cefotaxime CTX	40(100)	-	-	51(100)	-	-	21(100)	-	-	19(100)	-	-	0,967
Cefepime FEP	36(90)	2(5)	2(5)	51(100)	-	-	19(90.48)	1(4.76)	1(4.76)	17(89.47)	1(5.26)	1(5.26)	0,938
Cefoperazone CFP	33(82.5)	6(15)	1(2.5)	45(88.24)	5(9.8)	1(1.96)	18(85.71)	2(9.52)	1(4.76)	15(78.95)	4(21.05)	-	0,869
Amikacin AK	31(77.5)	8(20)	1(2.5)	41(80.4)	9(17.64)	1(1.96)	18(85.71)	3(14.29)	-	14(73.68)	5(26.32)	-	0,946
Gentamicin CN	34(85)	5(12.5)	4(10)	45(88.24)	4(7.84)	2(3.92)	18(85.71)	3(14.29)	-	14(73.68)	4(21.05)	1(5.26)	0,702
Tobramycin TOB	32(80)	8(20)	-	42(82.35)	9(17.65)	-	18(85.71)	3(14.29)	-	15(78.95)	4(21.05)	-	0,980
Ciprofloxacin CIP	28(70)	12(30)	-	40(78.43)	11(21.57)	-	14(66.66)	7(33.33)	-	15(78.95)	4(21.05)	-	0,880
Norfloxacin NOR	25(62.5)	15(37.5)	-	40(78.43)	10(19.6)	1(1.96)	13(61.9)	7(33.33)	1(4.76)	15(78.95)	4(21.05)	-	0,535
Tetracycline TE	36(90)	3(7.5)	1(2.5)	48(94.12)	1(1.96)	2(3.42)	20(95.24)	1(4.76)	-	16(84.21)	2(10.53)	1(5.26)	0,819
Doxycycline DO	37(92.5)	3(7.5)	-	44(86.27)	1(1.96)	6(11.76)	21(100)	-	-	17(89.47)	2(10.53)	-	0,451
Imipenem IPM	31(77.5)	6(15)	3(7.5)	38(74.5)	9(17.65)	4(7.84)	14(66.66)	5(23.81	2(9.52)	11(57.9)	4(21.05)	4(21.05)	0,632
Meropenem MEM	33(82.5)	7(17.5)	-	42(82.35)	8(15.68)	1(1.96)	18(85.71)	3(14.29)	-	13(68.42)	5(26.31)	1(5.26)	0,897
Doripenem DOR	23(57.5)	11(27.5)	6(15)	29(56.86)	15(29.41)	7(13.73)	12(57.14)	6(28.57)	3(14.29)	7(36.84)	5(26.32)	7(36.84)	0,421
Azithromycin AZM	18(45)	22(55)	-	16(31.37)	35(68.63)	-	7(33.33)	14(66.66)	-	13(68.42)	6(31.58)	-	0,684
Aztreonam ATM	26(65)	13(32.5)	1(2.5)	40(78.43)	7(13.73)	4(7.84)	17(80.95)	3(14.29)	1(4.76)	14(73.68)	5(26.32)	-	0,386
Levofloxacin LEV	28(70)	10(25)	2(5)	41(80.39)	9(17.65)	1(1.96)	15(71.43)	6(28.57)	-	15(78.95)	4(21.05)	-	0,029*

Production of biofilms has been identified as a key pathogenicity factor in *P. aeruginosa* infections (Choy *et al*., 2008). According to present findings, 112 (85.50%) of *P. aeruginosa* isolates produced biofilm.

The persistence of delayed infections is caused by two key virulence features of *P. aeruginosa*: biofilm development and antimicrobial resistance. The rise in MDR strains observed in the current investigation may be related to the recent, unrestricted usage of antibiotics in healthcare. The primary cause of MDR, which results in the selection and spread of bacteria resistant to antibiotics in clinical medicine, is the reckless use of antibiotics without antibiotic sensitivity testing.

4.6. Molecular Study

4.6.1. DNA Genomic Extraction (Chromosomal and Plasmid DNA)

P. aeruginosa chromosomal DNA was extracted using the Favor PrepTM Genomic DNA Mini Kit for DNA extraction. Using the Nano drop devise, the DNA had an estimated concentration of 80-210 ng/ µl with a high purity sample of DNA range of (1.68 - 1.85). Additionally, the Favor PrepTM plasmid DNA Mini Kit was used to determine whether a particular bacteria possessed plasmids. A plasmid has been identified in 48 % (63/131) *P. aeruginosa* isolates.

4.6.2. Prevalence of Integrase Genes

The presence of class I, class II and class III integrons was based on the detection of the integrase genes (*intI1, intI2* and *intI3*) by PCR.

The presence of genes encoding integrases to class 1 integron was found in 131 (100 %) of MDR isolates, with an amplification product approximately of (457 bp) on chromosomal DNA, Figure 4.7a. While no isolates gave an *intI1* gene carried on a plasmid, Figure 4.7b.

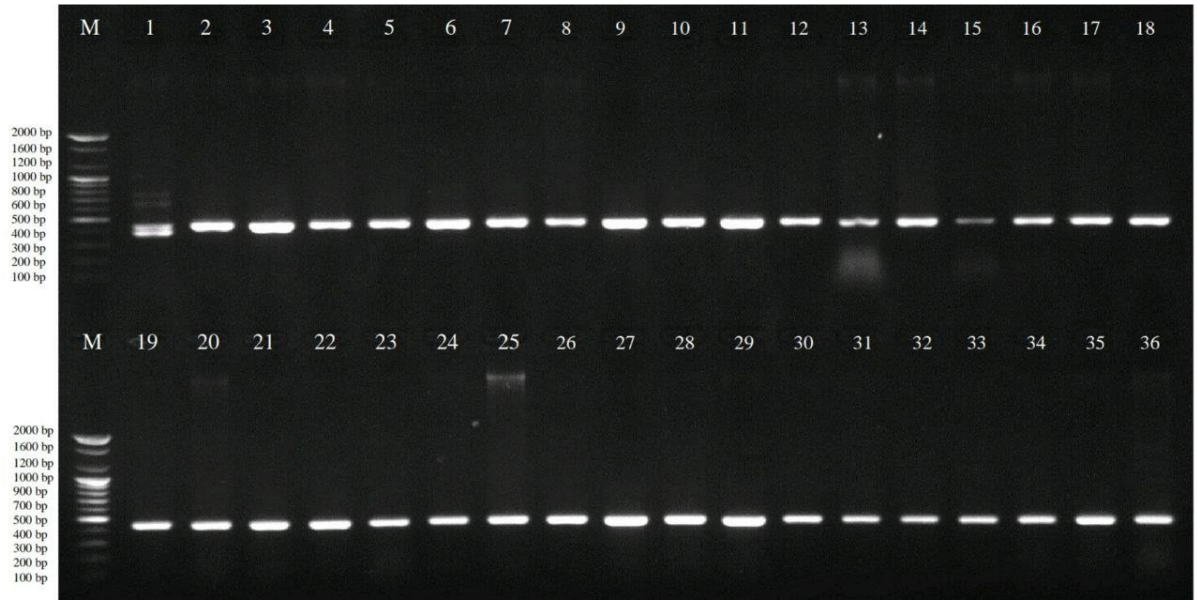


Figure (4.7a): PCR amplification of ***intI1*** gene on chromosome (**457bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were positive.

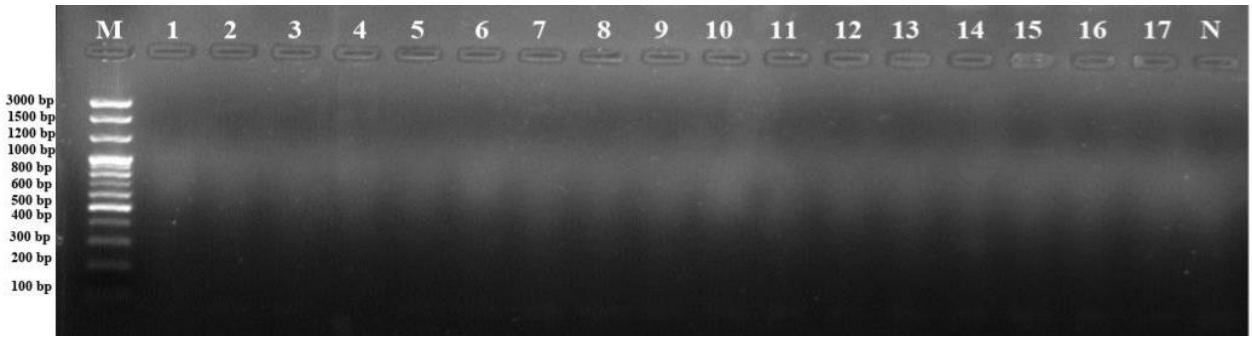


Figure (4.7b): PCR amplification of ***intI1*** gene on plasmid (**457bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative. N: negative control.

Regarding the presence of the genes encoding integrases to class 2 integron **(*intI2***) was identified in 5/131(3.81%) of clinical isolates of *P. aeruginosa*, with an amplification product of (789 bp), as shown in figure (**4.8a**), and not detectable this gene in any isolate located on the plasmid DNA, figure (**4.8b**).

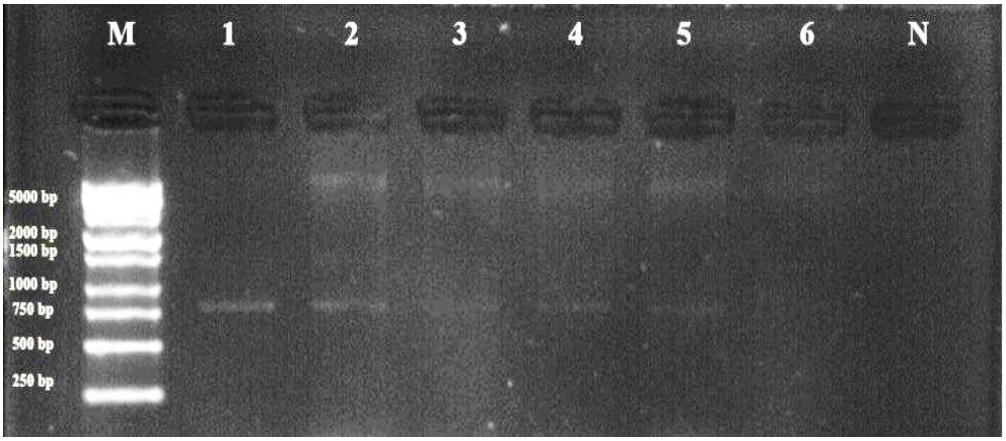


Figure (4.8a): PCR amplification of ***intI2*** gene on chromosome (**789bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 1000-bp DNA marker. Lanes (1-5) positive isolates, lane (6) negative isolates, N: negative control.

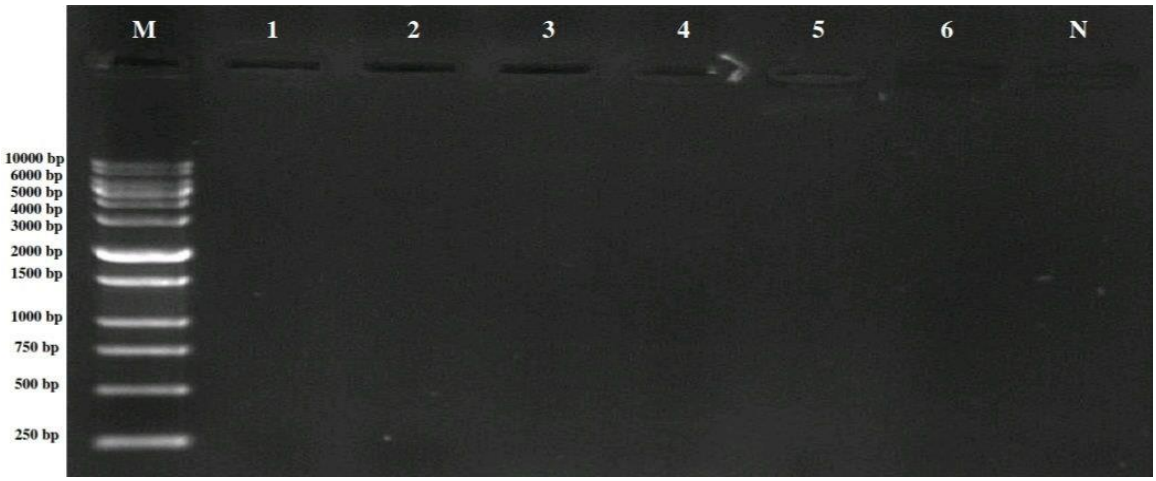


Figure (4.8b): PCR amplification of ***intI2*** gene on plasmid (**789bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 1000-bp DNA marker. All isolates were negative, N: negative control.

As demonstrated in Figure (4.9), all *P. aeruginosa* isolates tested were negative for class 3 integrase, Figure (4.9a). One positive (1.85%) isolate resulted from the genes located on the plasmid DNA, Figure (4.9b).

Figure (4.9a): PCR amplification of ***intI3*** gene on chromosome (**922bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 2 hrs. Lane M: 1000-bp DNA marker. All isolates were negative, N: negative control.

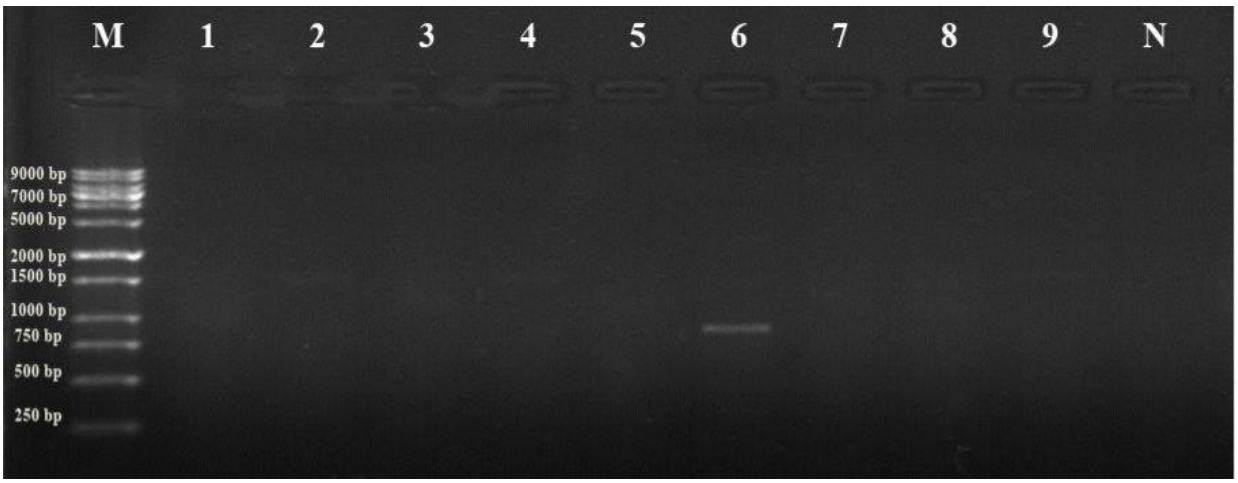


Figure (4.9b): PCR amplification of ***intI3*** gene on plasmid (**922bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 2 hrs. Lane M: 1000-bp DNA marker. Lanes (1-5 and 7, 8, 9) negative isolates, lane (6) positive isolates, N: negative control.

The Prevalence of integrons (I, II, III) genes among *P. aeruginosa* isolates are summarized in figure (4.10).

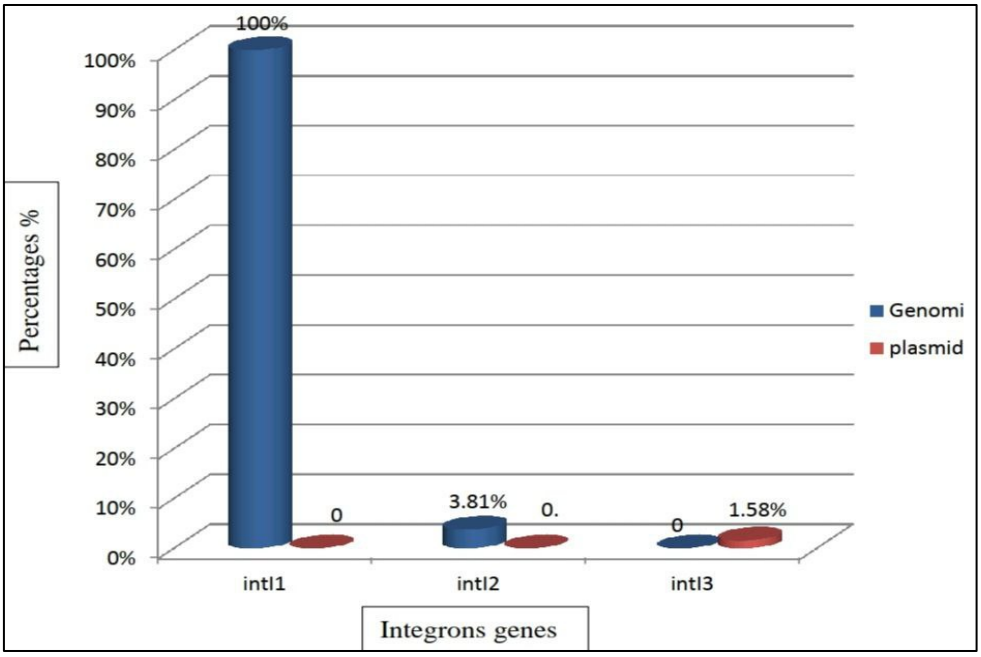


Figure (4.10): Prevalence of integrons (I, II, III) genes among *P. aeruginosa* isolates

Several earlier investigations conducted in Iran found that 43% to 95.7% of *P. aeruginosa* clinical isolates were class 1 integron positive (Nikokar *et al*., 2013; Goli *et al*., 2017; Zarei-Yazdeli *et al*., 2018; Sharifi *et al*., 2019). These results are consistent with the results of the current study.

The current investigation found that MDR *P. aeruginosa* harbored genes encoding integrases to class I, II, and III integrons in 131 (100%), 5 (3.81%), and 0 (0.0%) cases, respectively. However, the presence rate is higher than in previous studies conducted in Iraq, where the incidence of class I integron was 46.73% in *P. aeruginosa* isolated from burn and wound infection (Hammadi *et al*., 2020), Egypt (83%) of carbapenem-resistant *P. aeruginosa* carried *intI1*, and Iran (55.5%) (Mohamed *et al*., 2016; Faghri *et al*., 2018).

MDR *P. aeruginosa* has become resistant to numerous medication classes, including carbapenems, fluoroquinolones, cephalosporins, and aminoglycosides, as a result of the emergence and dissemination of antimicrobial resistant strains

(Magiorakos *et al*., 2012). Despite the fact that they are often acquired and distributed through chromosomal changes. These comprise many mobile DNA segment types, including plasmids, transposons, and integrons (Carattoli, 2001). Integrons have been found as a major source of resistance genes and were thought to act as antimicrobial resistance gene reservoirs in microbial communities. Antibiotic resistance mediated by integrons was reported in *P. aeruginosa* isolates (Poirel *et al*., 2004; Li *et al*., 2006; Pitout *et al*., 2007).

Many researches have reported on existence the *intI1* gene in tested isolates of *P. aeruginosa* (Xu *et al*., 2009; Nikokar *et al*., 2013; Sun *et al*., 2014). The significance of class 1 integrons in relative to highly antibiotic-resistant *P. aeruginosa* isolates was shown in the current investigation, which also highlighted their prevalence and importance.While *intI2* gene's frequency has been verified in several papers (Xu *et al*., 2009; Goudarzi *et al*., 2016). Resistance to several β-lactams, including aminoglycosides, rifamycin, chloramphenicol, and quinolones, is conferred by the class 1 integrons. The *attI* recombination site, 3ewhich is recognized by the integrase and serves as a receptor site for incoming gene cassettes, and the *intI* gene, which codes for a site-specific recombinase, are the two key parts of an integron (Qi *et al*., 2023). Based on the existence of class 1 integrons, the establishment of a high frequency of *intI1* in our area raises severe future concerns and has the potential to increase and spread antibiotic resistance. Previous research found that the *intI2* and *intI3* genes were absent (Poonsuk *et al*., 2012).

The integron is a genetic component that can play a significant part in the spread of multidrug resistance in Gram-negative bacteria, particularly in *Pseudomonas* (Li *et al*., 2006)**.** The integrase genes (*intI2* and *intI3*) are found in class 2 and class 3 integrons, and their corresponding produces are 46% and 61%

identical, to class 1 integrase (Stalder *et al*., 2012). Antimicrobial resistance is attributable to several mechanisms integrons, beta lactamases and efflux pumps (Bialvaei *et al*., 2016). Integrons have a significant role in the spread of antibiotic resistance.

Reduced sensitivity to β-lactams in integron positive bacteria is probably because to the related of β-lactamase genes within integrons or integron-carrying plasmids, some of which have been shown to be integron encoded (Hussain *et al*., 2021). However, genes conferring resistance to extended-spectrum β-lactams, such as cefiazidlme and aztreonam, have never been found to be integron-encoded, revealing a relationship between integrons and extended-spectrum β-lactamase carriage, possibly via a common host plasmid. Quinolone resistance arises from chromosomal point mutations rather than mobile genetic elements (De, 2021).

4.6.2.1. Association between Class 1 Integron Gene and Antibiotics Resistance

Statistical analysis of the present study revealed a significant relationship between the presence of the class 1 Integrons gene with resistance to different antibiotic classes ($p \leq 0.05$), as indicated in Table 4-5.

Table 4-5: Association between Class 1 Integrons and Antibiotics Resistance in *P. aeruginosa* isolates

AB	No. (%) positive isolates of Integron -1 gene			*P* value
	R	I	S	
Ampicillin AMP	131(100%)	0	0	≤0.0001**
Piperacillin PRL	102(77.86%)	11(8.39%)	18(13.74%)	≤0.0001**
Piperacillin-tazobactam PIT	84(64.12%)	23(17.55%)	24(18.32%)	≤0.0001**
Ticarcillin -clavulanic TCC	106(80.91%)	11(8.39%)	14(10.68%)	≤0.0001**
Ceftizoxime CZX	130(99.23%)	0	1(0.77%)	≤0.0001**
Cefixime CFM	131(100%)	0	0	≤0.0001**
Cefotaxime CTX	131(100%)	0	0	≤0.0001**
Cefepime FEP	123(93.90%)	4(3.05%)	4(3.05%)	≤0.0001**

AB	No. (%) positive isolates of Integron -1 gene			*P* value
	R	I	S	
Cefoperazone CFP	111(84.73%)	3(2.29%)	17(12.98%)	≤0.0001**
Amikacin AK	104(79.39%)	2(1.52%)	25(19.08%)	≤0.0001**
Gentamicin CN	110(83.97%)	4(3.05%)	17(12.98%)	≤0.0001**
Tobramycin TOB	107(81.68%)	0	24(18.32%)	≤0.0001**
Ciprofloxacin CIP	96(73.28%)	0	35(26.72%)	≤0.0001**
Norfloxacin NOR	94(71.75%)	2(1.52%)	35(26.72%)	≤0.0001**
Tetracycline TE	120(91.60%)	6(4.58%)	5(3.82%)	≤0.0001**
Doxycycline DO	119(90.84%)	6(4.58%)	6(4.58%)	≤0.0001**
Imipenem IPM	95(72.52%)	12(9.16%)	24(18.32%)	≤0.0001**
Meropenem MEM	107(81.68%)	2(1.52%)	22(16.80%)	≤0.0001**
Doripenem DOR	71(54.20%)	23(17.55%)	37(28.24%)	≤0.0001**
Azithromycin AZM	53(40.46%)	0	78(59.54%)	≤0.0001**
Aztreonam ATM	97(74.05%)	6(4.58%)	28(21.35%)	≤0.0001**
Levofloxacin LEV	99(75.57%)	3(2.29%)	29(22.14%)	≤0.0001**

High sgnificant differences, **R: Resistance, **I**: Intermediate, **S**: Sensitive

The class 1 integron was found to be prevalent in MDR isolates in the current investigation; these findings are consistent with a several previous studies (Chen *et al*., 2009; Yousefi *et al*., 2010). A previous study done in Iran by Zarei-Yazdeli *et al*., (2018) reported that resistance to antibiotics such as aminoglycosides, quinolones, and β-lactam drugs was significantly related with class 1 integron. Furthermore, Dokht Khosravi *et al*., (2017) observed significant antimicrobial resistance among isolates positive for the *int1* gene, including for gentamicin (94.62%), ciprofloxacin (93.54%), and meropenem (90.32%).

This is not unexpected results given that numerous antibiotic resistance gene cassettes encoding resistance to a wide variety of antibiotics in *P. aeruginosa* are carried by class 1 integron. However, resistance to the antibiotics was also reported in other integron-negative isolates (class II and class III). The acquisition of the isolates' antibiotic-resistance genes might be attributed to chromosomal-encoded enzymes or other mobile elements.

4.6.3. Prevalence of Gene Cassettes

Gene cassettes are small mobile elements that consist of a single gene and recombination site and are captured by bigger elements termed integrons. A gene cassette is generally made up of a single promoter-less gene and a recombination site. These recombination sites vary in length and sequence, but they all include conserved regions at the ends and are often incomplete inverted repetitions expected to form stem-loop structures (Partridge *et al*., 2009).

The variable region of integron class 1 indicated 14 distinct product categories, 131 (100%) *P. aeruginosa* isolates were positive to 5'CS/3'CS genes. The highest 38 (29.0%) integron 1 positive isolates had 2500 bp products, followed by 32 (24.43%) integron 1 positive isolates with 3000 bp products, and 24 (18.32%) integron 1 positive isolates with 3500 bp products. The sizes of the integron gene class I cassettes and their distribution among investigated isolates are illustrated in (Table 4-6. and Figure 4.11).

Table 4-6: Sizes of variable regions of integron class I cassettes in *P. aeruginosa* isolates

Band	Variable region size (bp)	No. of isolates (%)
1	8000	1(0.763)
2	5000	2(1.53)
3	4500	3(2.29)
4	4000	4(3.05)
5	3500	24(18.32)
6	3000	32(24.43)
7	2500	38(29.0)
8	2200	9(6.87)
9	2000	2(1.53)
10	1750	2(1.53)
11	1500	1(0.76)
12	1000	3(2.29)

Band	Variable region size (bp)	No. of isolates (%)
13	800	3(2.29)
14	200	7(5.34)
Total		**131(100)**

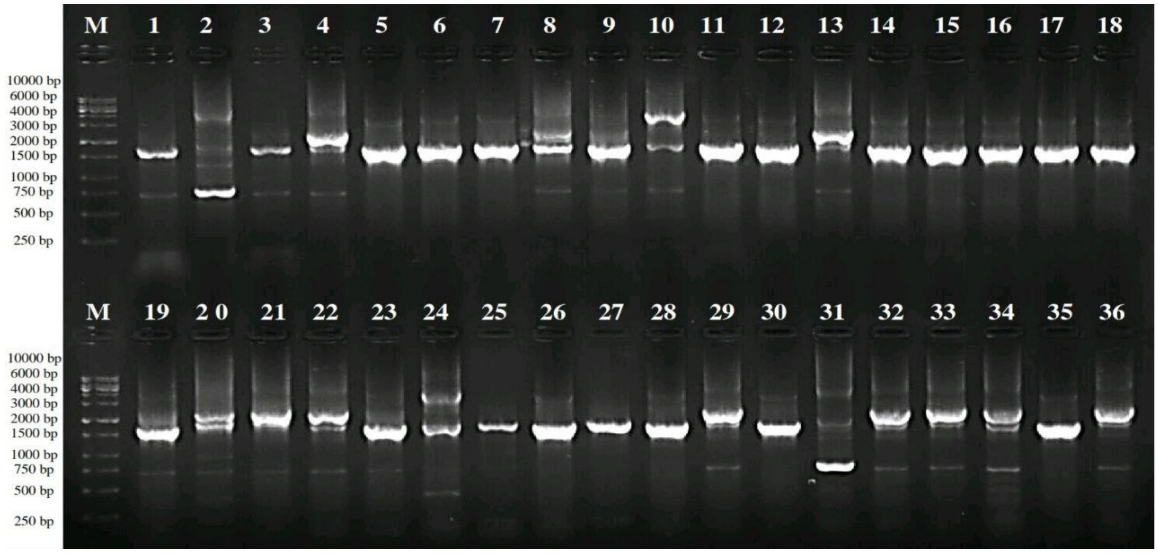


Figure (4.11): PCR amplification of **5'CS/3'CS** gene (**variable size**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 2 hrs. Lane M: 1000-bp DNA marker. All isolates were positive.

The analysis of integron gene cassettes indicated that integron I variable regions were amplified in 131 isolates, with each isolate displaying 1 variable region ranging in size from 200 bp to 8000 bp.

Resistance gene cassette dissemination by class 1 integrons among *P. aeruginosa* clinical isolates leads to treatment failures and a significant mortality rate in immunocompromised individuals (Poonsuk *et al*., 2012).

Integrons have been identified as an acquired resistance mechanism that aids in the dissemination of antibiotic resistance by capturing, excising, and expressing gene cassettes via site-specific recombination (Dehkordi *et al*., 2020).

The most prevalent variant of class 1 integron retains the 5'CS but includes just a portion of the 3'CS and only a portion of the Tn*402 tni* region (Partridge *et al*., 2009).

The variable portions of the class 1 integron were investigated using conserved segment PCR (CS-PCR). Different sized cassettes inserted between CS regions generated among the MDR- *P. aeruginosa* investigated demonstrates the varied nature of these structures, most likely reflecting changes in the number and type of inserted gene cassettes. The size of the variable sections of type I integron indicated 14 distinct size groups. These findings are relative agreement with recent research conducted in Egypt by Abdel-Rhman *et al*. (2021).

4.6.4. Detection of Genes Virulence Factors

The PCR result showed 131(100%) isolates tested positive for the *oprL* with amplification products of 504 bp, (Figures 4.12).

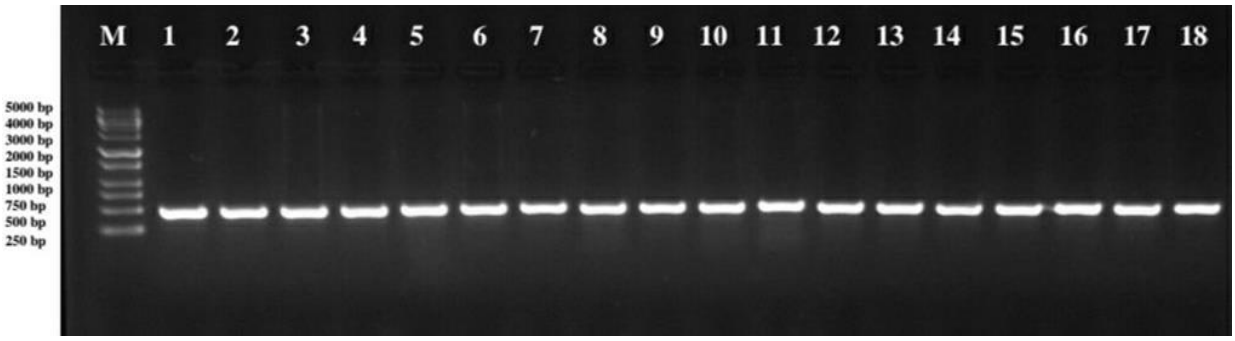


Figure (4.12): PCR amplification of *oprL* gene (**504 bp**) in *P. aeruginosa* isolates, on 1% agarose at 80 volt for 1 hr. Lane M: 1000-bp DNA marker. All isolates were positive isolates.

Whereas presence the genes encode *Alg* and *oprI* were detected in 129 (98.47%) of clinical isolates of *P. aeruginosa* with amplification product of 520 bp and 249 bp respectively, as show in Figure (4.13 and 4.14).

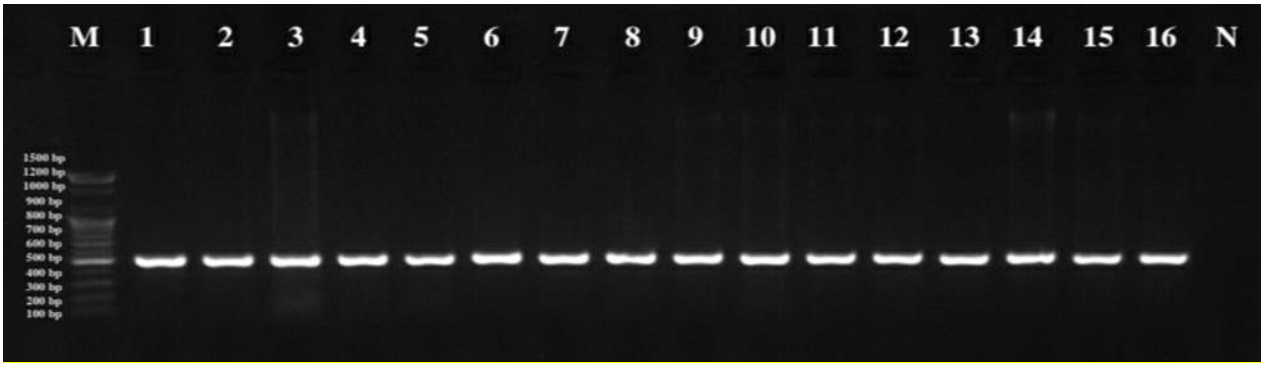


Figure (4.13): PCR amplification of ***Alg*** gene (**520 bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. Lanes (1-16) were positive isolates and N: negative control.

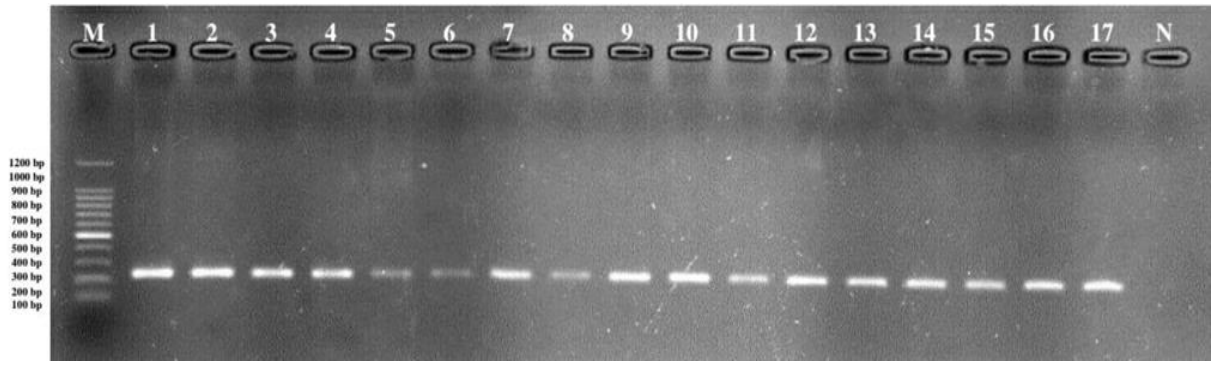


Figure (4.14): PCR amplification of ***oprI*** gene (**249 bp**) in *P. aeruginosa* isolates, on 2%agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. Lane (1-17) positive isolates. N: negative control.

Regarding the prevalence the gene encodes of outer membrane porin gene (*oprD*2) was detected in 125(95.42%) of clinical isolate of *P. aeruginosa* with amplification product of (1332 bp), as show in Figure (4.15).

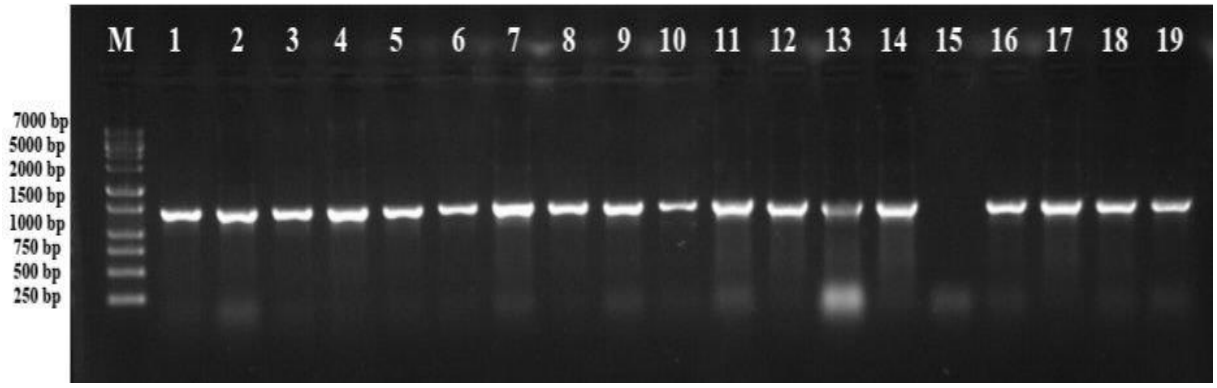


Figure (4.15): PCR amplification of ***oprD2*** gene (**1332 bp**) in *P. aeruginosa* isolates, on 1% agarose at 80 volt for 2 hrs. Lane M: 1000-bp DNA marker. Lanes (1-14 and 16-19) positive isolates. Lane (15) negative isolate.

Figure 4.16 demonstrates that the highest rate of *oprL* genes among *P. aeruginosa* isolates was recorded in 131 (100%), followed by *Alg* and *oprI* in 129 (98.47%) and 125(95.42%) for *oprD2*.

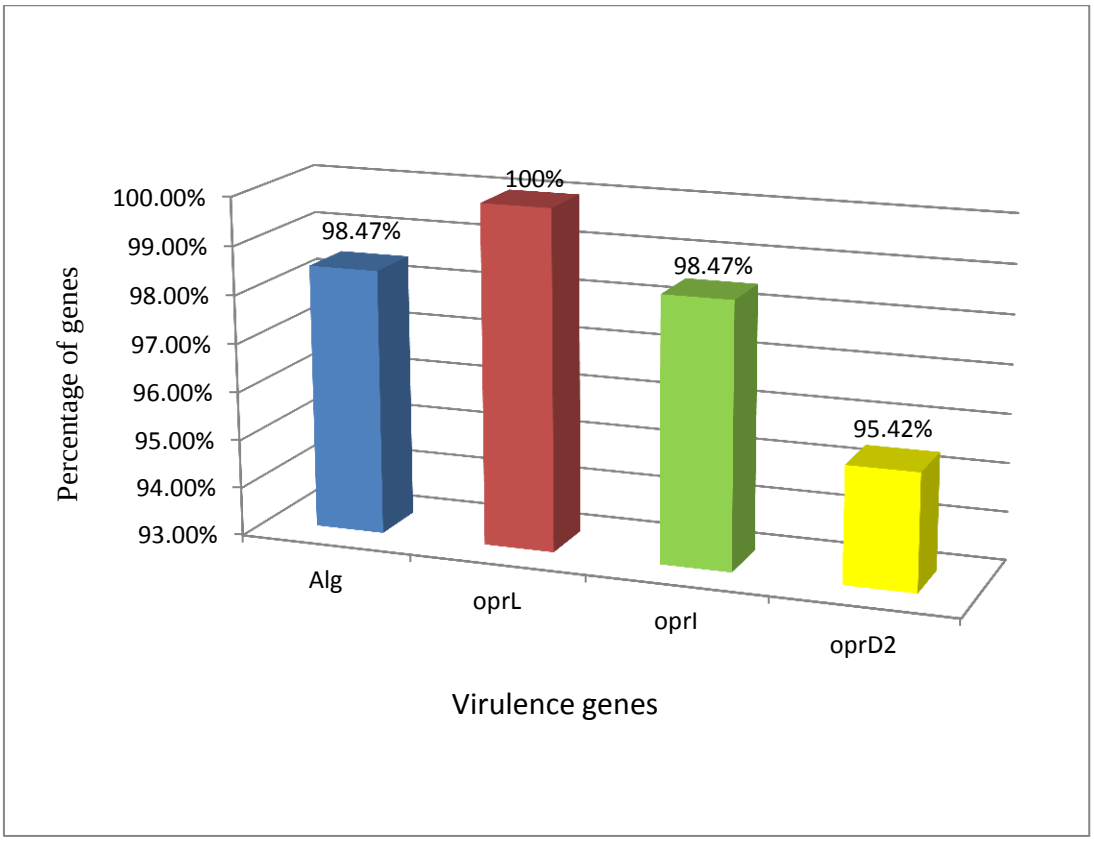


Figure (4.16): Occurrences of virulence factors genes among *P. aeruginosa* isolates

The extracellular and cell-mediated virulence factors of *P. aeruginosa*, including *toxA, exoY, oprL, oprI, lasA, lasB, and oprD*, are also associated to the pathogenesis of the organism. These factors have the ability to invade and propagate throughout the host body and destroy host tissue (Haghi *et al*., 2018). The main components of *P. aeruginosa* outer membrane lipoproteins, which are also utilized as indicators for the detection of *P. aeruginosa*-associated infections, include virulence genes such as *oprL*, *oprI*, and *oprD* (Nikbin *et al*., 2012; Jurado-Martín *et al*., 2021).

P. aeruginosa OprD is a substrate-specific outer membrane porin that permits basic amino acids, short peptides, and imipenem to enter the cell (Chevalier *et al*., 2017). Carbapenems, a category of small molecular weight hydrophilic β-lactam antibiotics, can pass through the bacterial outer membrane porin proteins OprC, OprD2, and OprE, however OprD2 is the imipenem channel specific protein (Shen *et al*., 2015).

In the case of imipenem, OprD loss might raise the MIC over the resistance breakpoint (Fang *et al*., 2014). The majority of carbapenem-resistant *P. aeruginosa* strains lack OprD expression (Naenna *et al*., 2010).

The *oprD*2 gene, as well as its promoter and downstream regions, were sequenced, and the results revealed that the majority of the resistant isolates had insertion mutations in the *oprD*2 gene; additionally, there was a direct relationship between the alteration or loss of oprD and an increase in MIC for imipenem but not meropenem or other carbapenems (Zarei-Yazdeli *et al.*, 2014; Shen *et al.*, 2015).

Another study conducted by Gutiérrez *et al*. (2007) observed several mutations in the *oprD*2 gene, the most common of which were frameshift mutations caused

by single nucleotide insertions or deletions, and point mutations resulting in the formation of a premature stop.

In clinics, at least two forms of imipenem-resistant mutants have been observed. The most common kind includes OprD deletion due to mutation, and such mutants are solely resistant to zwitterionic carbapenem antibiotics (Kiani *et al*., 2021). The genetic investigation of laboratory-derived mutants revealed that the loss of *oprD2* expression is caused by deletions in the *oprD* coding region and the upstream promoter region (Ocampo-Sosa *et al*., 2012). A second prevalent sort of resistance discovered is multiple antibiotic resistances to both imipenem and other unrelated groups of antibiotics (Reygaert, 2018).

OprD is an outer membrane pore protein that is important for antibiotic permeability in *P. aeruginosa*. Imipenem (IPM) binds to a particular location on *oprD2*. Several studies have shown that the absence or low expression of *oprD2* affects the permeability of *P. aeruginosa* to carbapenem, lowering the minimum inhibitory concentration of carbapenem. Furthermore, insertion of OprD2 sequence (IS) elements can result in *oprD2* gene inactivation (Shariati *et al*., 2018). Also, overexpression of efflux pumps has been related to *P. aeruginosa* resistance to carbapenems (Piddock, 2006).

4.6.4.1. Relationship of virulence factors genes with biofilm formation

Among clinical isolates of *P. aeruginosa*, the prevalence of genes; *Alg*, *oprL*, and *oprI* in most isolates whether biofilm forming or non-forming. As demonstrated in table (4-7).

Table 4-7: Biofilm formation and related genes, virulence factors genes among *P. aeruginosa*.

Biofilm formation	*Alg* No. %	*oprL* No. %	*oprI* No. %
Strong	39(97.5%)	40(100%)	39(97.5%)
Moderate	51(100%)	51(100%)	50(98.03%)
Weak	21(100%)	21(100%)	21(100%)
Non-biofilm	18(94.73%)	19(100%)	19(100%)

According to research (Namuq *et al*., 2019) on the relationship between the *AlgD* gene and biofilm production in *P. aeruginosa*, *AlgD* is highly expressed in all biofilm formers and is lacking in non-biofilm formers. Heidari *et al*., (2018) reported 100% of *P.aeruginosa* biofilm producers carried the *AlgD* gene. A variety of methods, such as the formation of multidrug efflux systems, enzymes production, outer membrane protein (porin) loss, and target alterations, can be used by *P. aeruginosa* to mediate MDR (Tavajjohi *et al*., 2011).

4.6.5. Genotypic Detection of Metallo-β-lactamases

Based on their molecular structure, MBLs are classified into six groups: IMP, VIM, SIM, SPM, GIM, and AIM (Sacha *et al*., 2008) have been reported in *P. aeruginosa*. Most of MBLs-encoding genes are found on plasmids and spread to other strains, eventually leading to antibiotic resistance.

The frequency of bla_{VIM-1}, bla_{VIM-2}, bla_{NDM}, bla_{GIM-1}, bla_{SIM-2} and bla_{SPM} genes was determined by PCR on 131 isolates., Three isolates (2.3%) to , follow by 6(4.58%), 125(95.42%), 7(5.34%), and 13(9.92%) for bla_{VIM-1}, bla_{VIM-2}, bla_{NDM}, bla_{SIM-2} and bla_{SPM} genes respectively carried on chromosomal DNA, with no additional genes detectable (bla_{IMP-1} and bla_{GIM-1}) **Appendix3**. However, it was found that 8(12.70%), 3(4.76%) and 1(1.59%) isolates possessed bla_{VIM-1}, bla_{VIM-2}

and *bla*$_{SPM}$ genes respectively, carried on plasmid DNA, while the other genes were not detected. It is noteworthy that all 131 *P. aeruginosa* isolates harbored the *intI1* gene. Figures ((4.17a), (4.17b), (4.18a), (4.18b), (4.19a), (4.19b), (4.20a), (4.20b), (4.21a), and (4.21b)).

Figure (4.17a): PCR amplification of ***bla*$_{VIM-1}$** gene on chromosome (**261bp**) in *P. aeruginosa* isolates, on 2% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. Lanes (1-5) negative isolates, lane (6) positive isolate, N negative control.

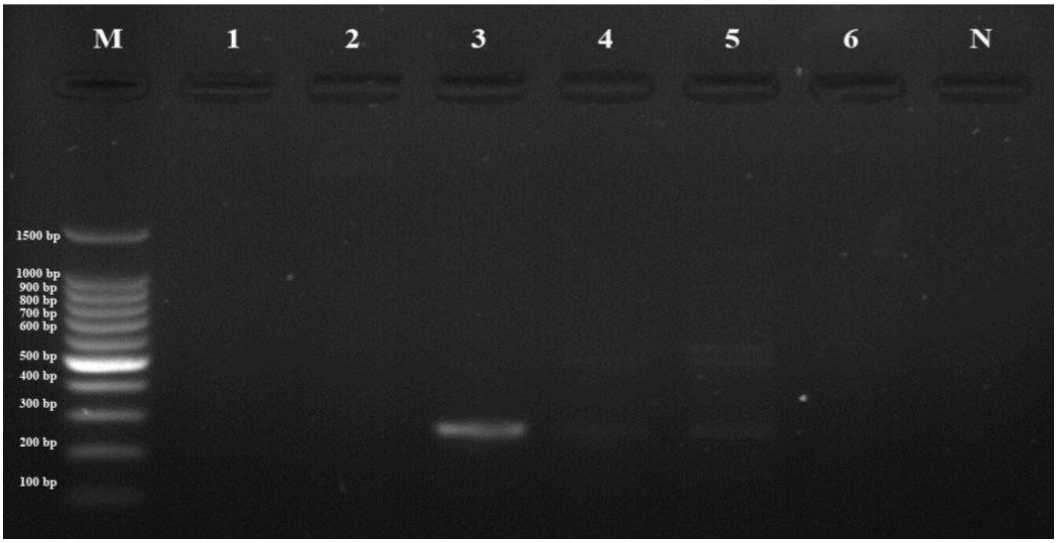


Figure (4.17b): PCR amplification of ***bla*$_{VIM-1}$** gene on plasmid (**261bp**) in *P. aeruginosa* isolates, on 2% agarose at 80 volt for 1hr. Lane M: 100-bp DNA marker. Lanes (1, 2, and 6) negative isolates. Lanes (3-5) positive isolates, N: negative control.

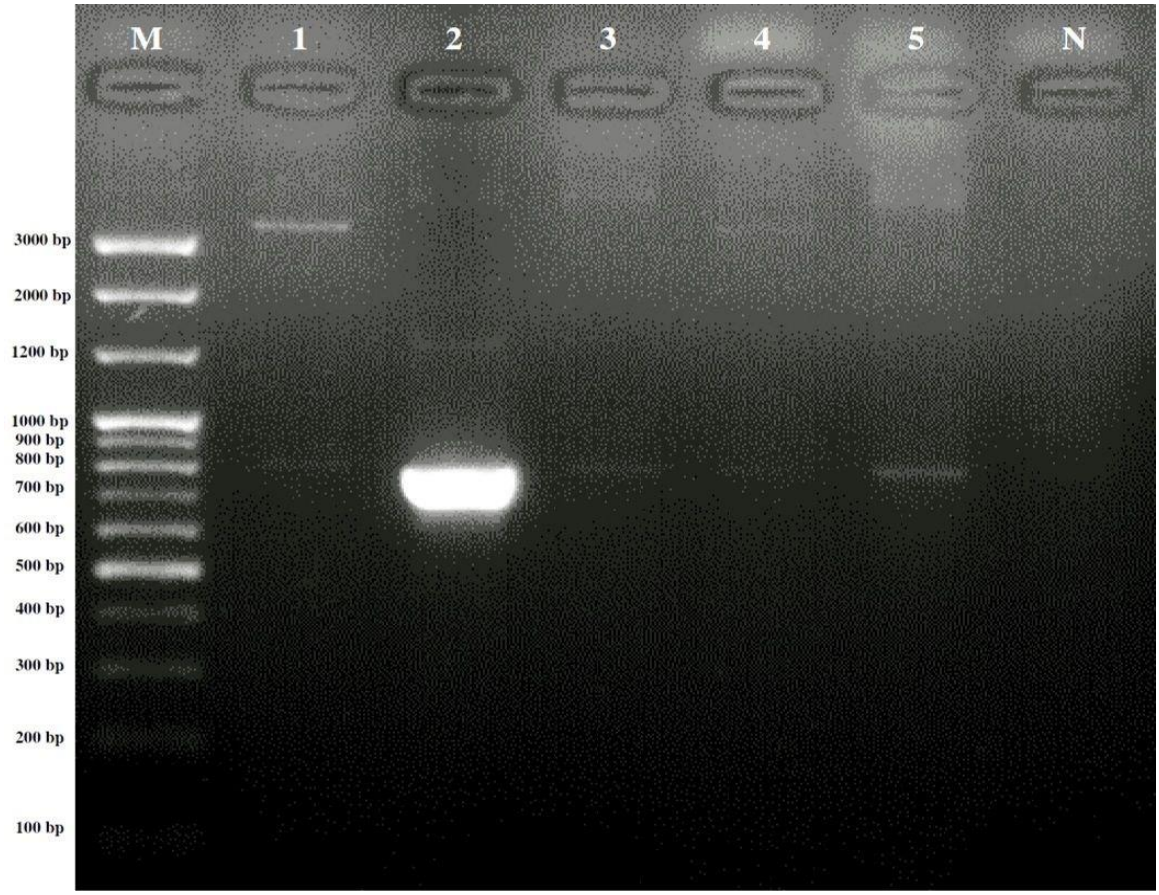


Figure (4.18a): PCR amplification of ***bla*$_{VIM-2}$** gene on chromosome (**801bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 100-bp DNA marker. Lanes (1-5) positive isolates, N: negative control.

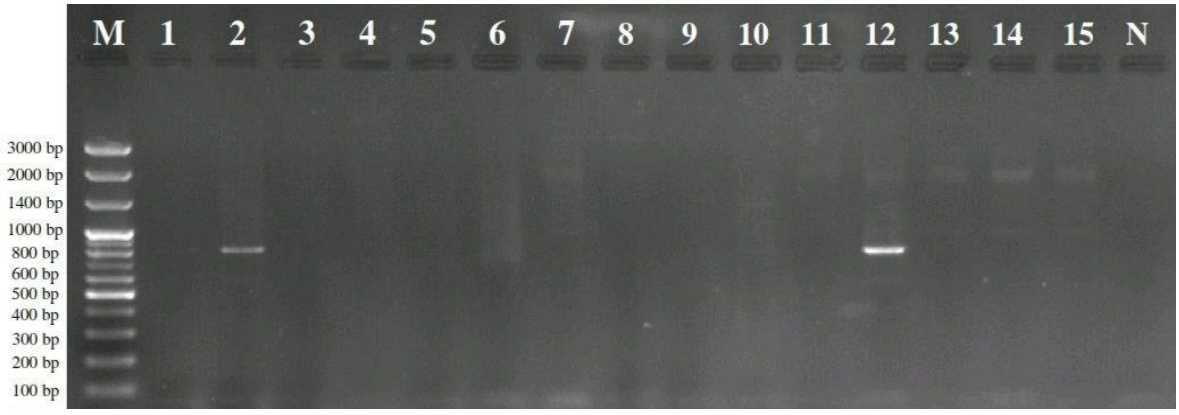


Figure (4.18b): PCR amplification of ***bla*$_{VIM-2}$** gene on plasmid (**801bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 100-bp DNA marker. Lanes (1, 3-11, and 13-15) negative isolates. Lanes (2 and 12) positive isolates, N: negative control.

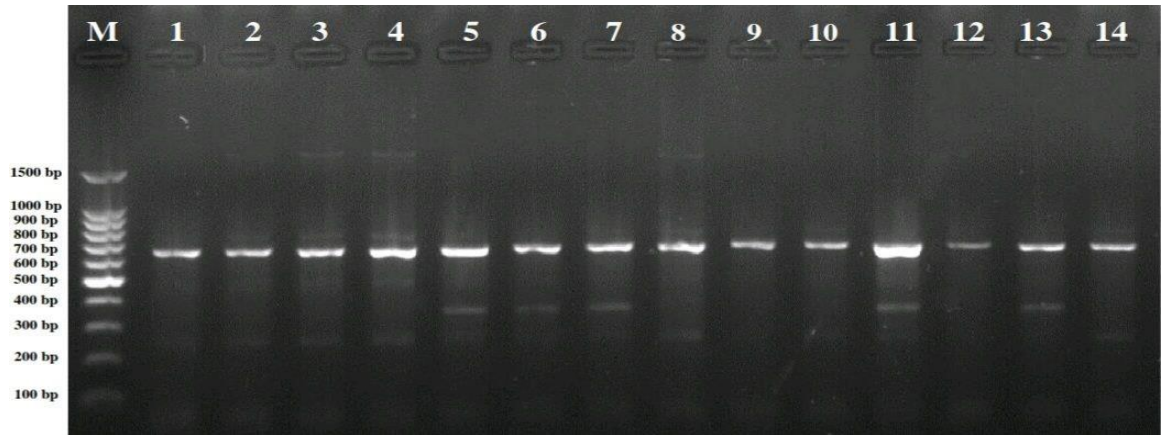


Figure (4.19a): PCR amplification of ***bla$_{NDM}$*** gene on chromosome (**782bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 100-bp DNA marker. All isolates were positive.

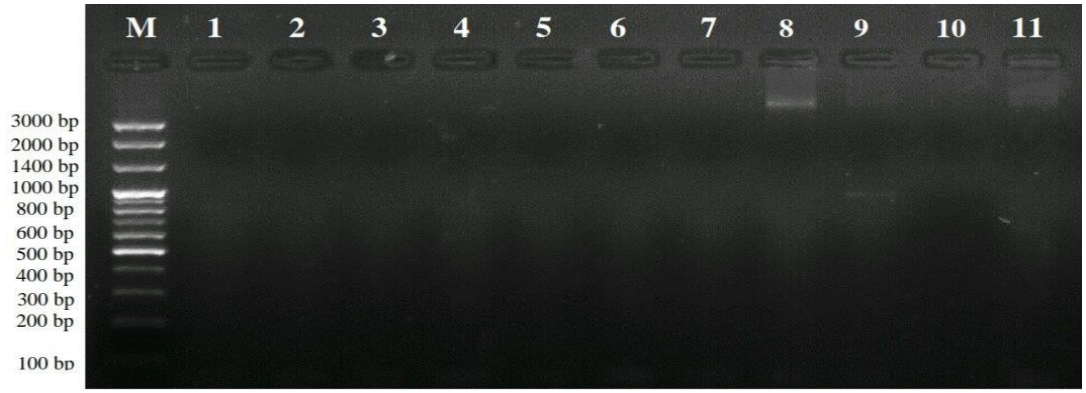


Figure (4.19b): PCR amplification of ***bla$_{NDM}$*** gene on plasmid (**782bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 100-bp DNA marker. All isolates were negative.

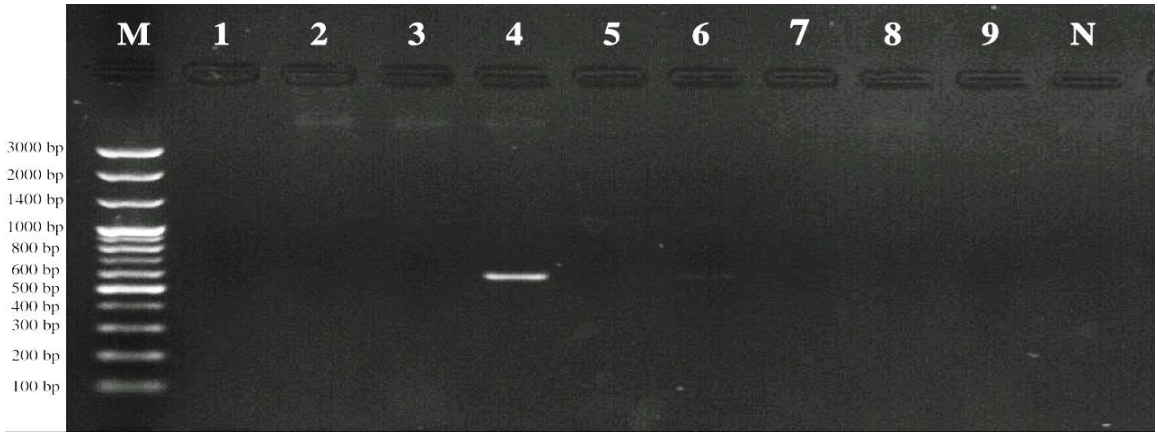


Figure (4.20a): PCR amplification of ***bla$_{SPM}$*** gene on chromosome (**786bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 100-bp DNA marker. Lanes (1-3, 5, 7-9) negative isolates, lanes (4, 6) positive isolates. N: negative control.

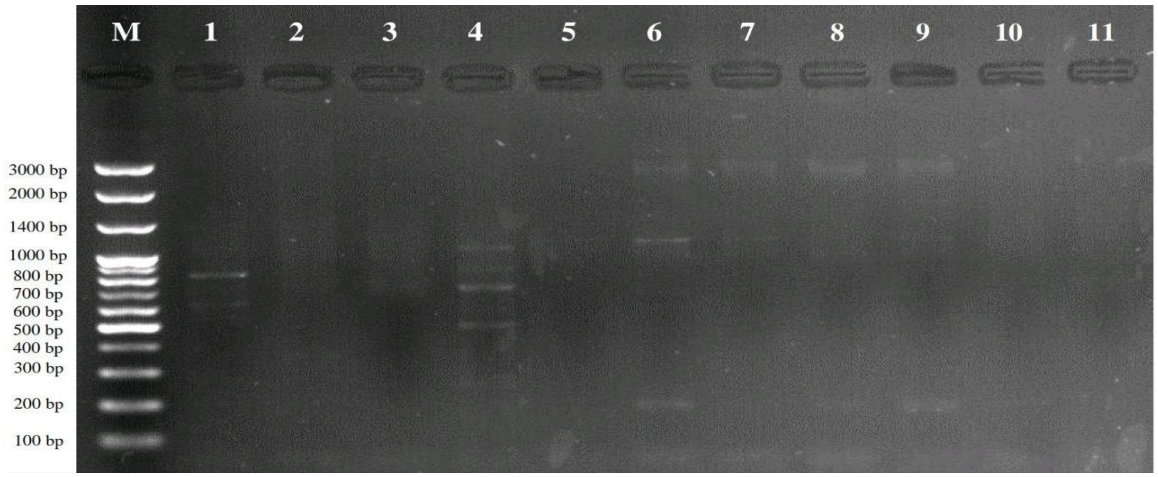


Figure (4.20b): PCR amplification of ***bla*$_{SPM}$** gene on plasmid (**786bp**) in *P. aeruginosa* isolates, on 1% agarose at 70 volt for 90 min. Lane M: 100-bp DNA marker. Lanes (1-3, 5-11) negative isolates. Lane (4) positive isolate.

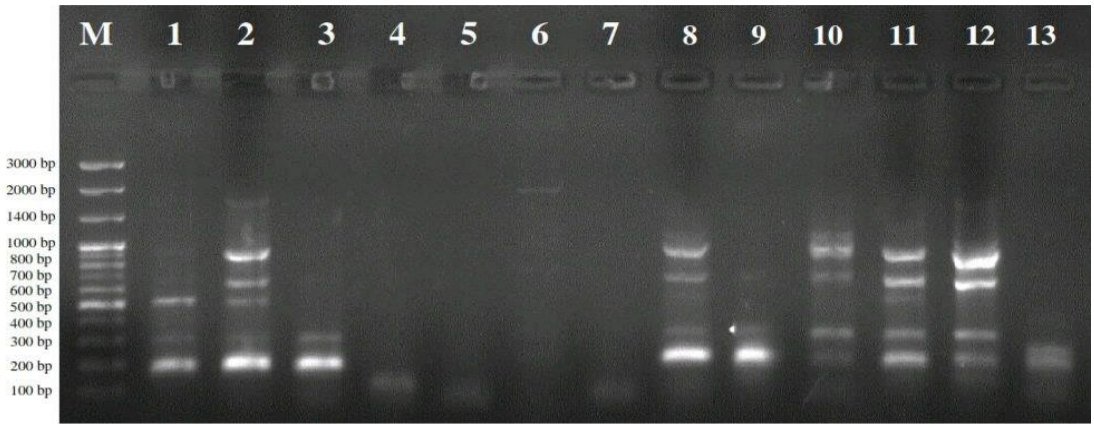


Figure (4.21a): PCR amplification of ***bla*$_{SIM-2}$** gene on chromosome (**570bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1hr. Lane M: 100-bp DNA marker. Lanes (1-2) positive isolates. Lanes (3-13) negative isolates.

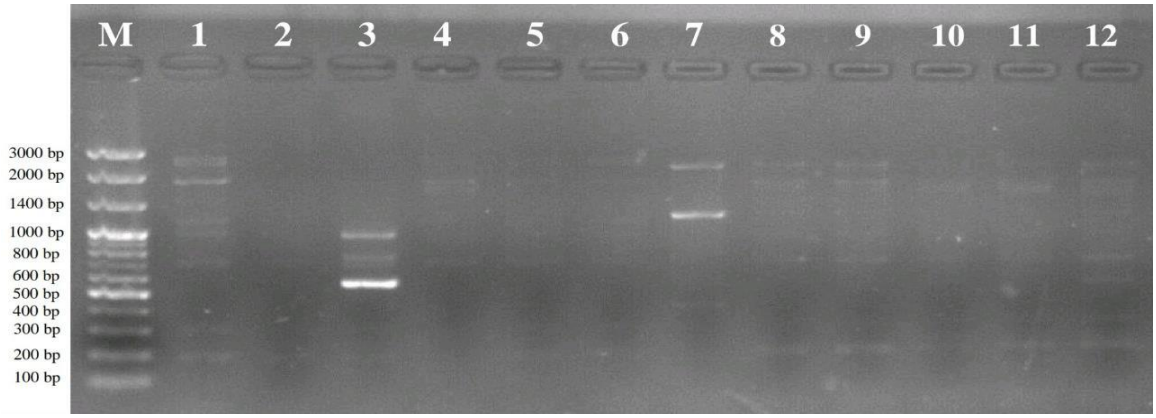


Figure (4.21b): PCR amplification of ***bla*$_{SIM-2}$** gene on plasmid (**570bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. Lanes (1-2, 4-12) negative isolates. Lane (3) positive isolate.

The frequency of *bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{IMP-7}$, *bla*$_{IMP-10}$, *bla*$_{IMP-13}$, and *bla*$_{IMP-25}$, genes was determined by PCR on 131 isolates, three isolates (2.3%) were found to have just the *bla*$_{IMP-2}$ gene carried on chromosomal DNA**,** with no additional genes detectable. However, it was found that 9(14.29%), and 5(7.94%) isolates possessed *bla*$_{IMP-7}$, and *bla*$_{IMP-13}$ genes respectively, carried on plasmid DNA, while the other genes were not detected. Figures ((4.22a), (4.22b), (4.23a),(4.23b), and (4.23c)).

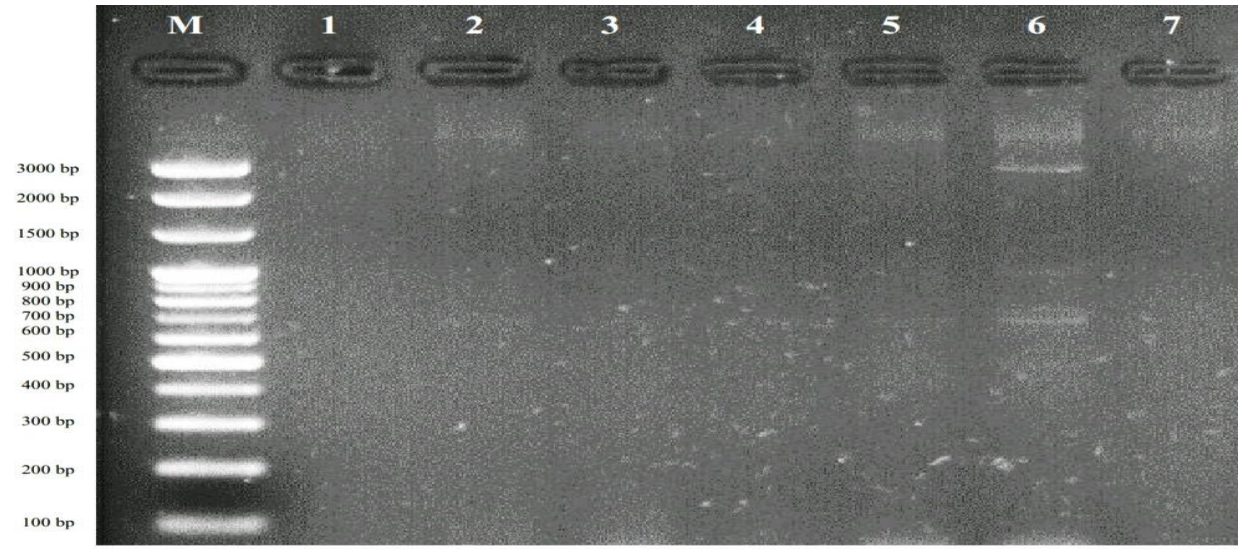


Figure (4.22a): PCR amplification of ***bla*$_{IMP-2}$** gene on chromosome (**678bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. Lane (6) positive isolate, lanes (1-5, 7) negative isolates

Figure (4.22b): PCR amplification of ***bla*$_{IMP-2}$** gene on plasmid (**678bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative.

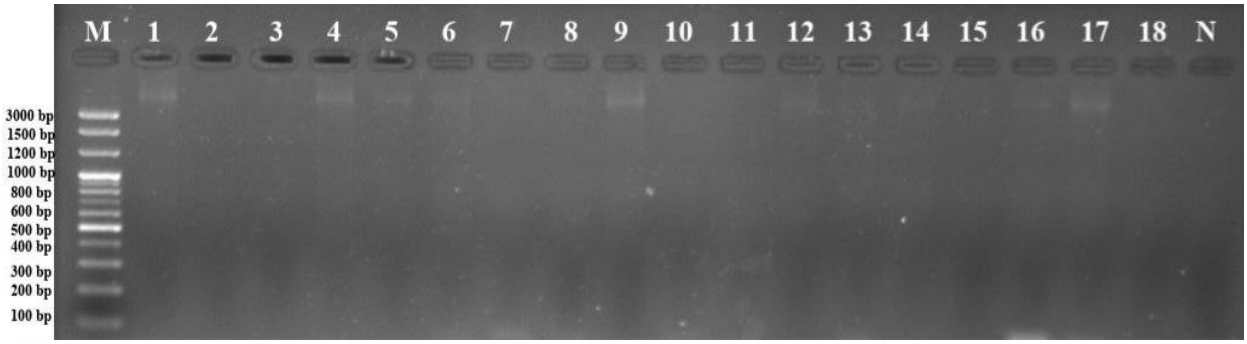


Figure (4.23a): PCR amplification of ***bla*$_{IMP-7}$**, ***bla*$_{IMP-10}$**, ***bla*$_{IMP-13}$** and ***bla*$_{IMP-25}$** on chromosome (**243bp**, **388bp**, **311bp**, and **295bp** respectively) in *P. aeruginosa* isolates, on 2% agarose at 80 volt for 1hr. Lane M: 100-bp DNA marker. Lanes (1-18) negative isolates, N: negative control.

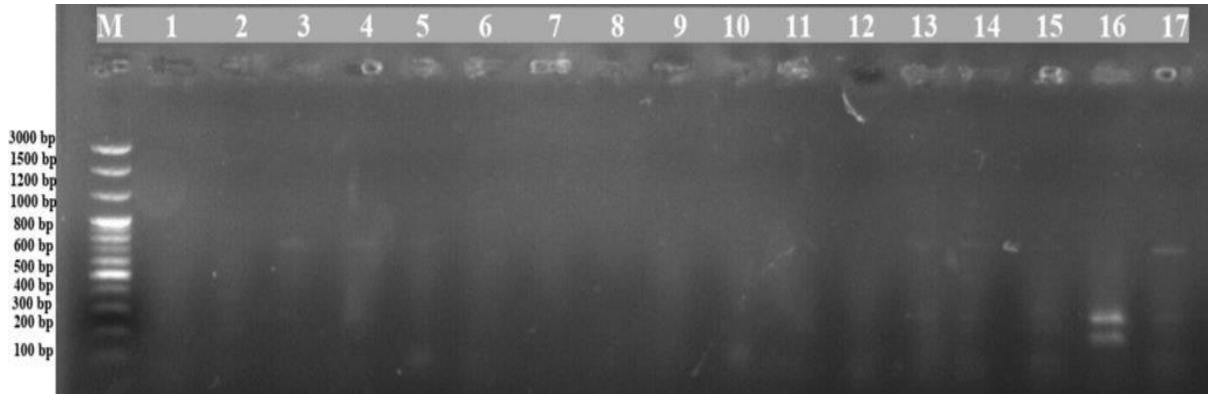


Figure (4.23b): PCR amplification of ***bla*$_{IMP-7}$** and ***bla*$_{IMP-13}$** on plasmid (**243bp**, and **311bp**) in *P. aeruginosa* isolates, on 2% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. Lanes (1-15 and 17) negative isolates, lane (16) positive isolates.

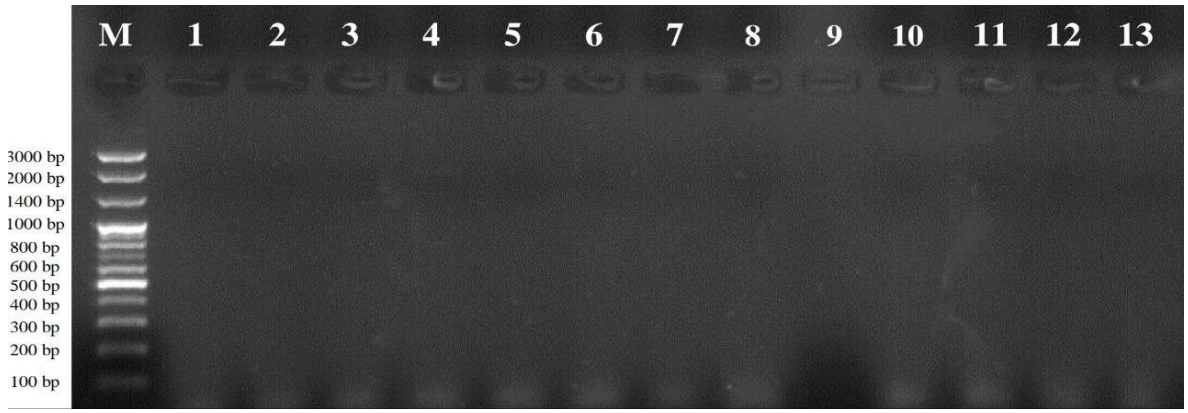
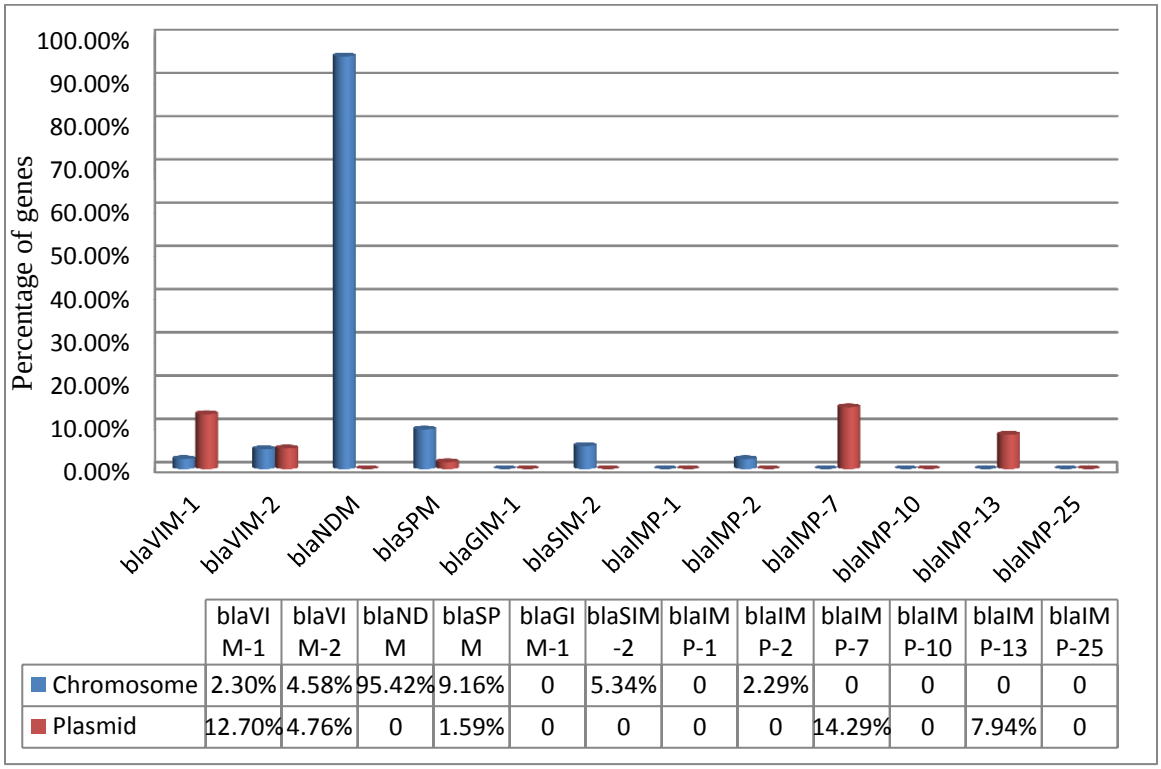


Figure (4.23c): PCR amplification of ***bla*$_{IMP\text{-}10}$** and ***bla*$_{IMP\text{-}25}$** on plasmid (**388bp**, and **295bp**) in *P. aeruginosa* isolates, on 2% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative.

The Prevalence of metallo -β- lactamase antibiotics genes among *P. aeruginosa* isolates are summarized in Figure (4.24).



	blaVIM-1	blaVIM-2	blaNDM	blaSPM	blaGIM-1	blaSIM-2	blaIMP-1	blaIMP-2	blaIMP-7	blaIMP-10	blaIMP-13	blaIMP-25
Chromosome	2.30%	4.58%	95.42%	9.16%	0	5.34%	0	2.29%	0	0	0	0
Plasmid	12.70%	4.76%	0	1.59%	0	0	0	0	14.29%	0	7.94%	0

Figure (4.24): Percentage of metallo -β-lactamase antibiotics genes among *P. aeruginosa* isolates**.**

At least three major families of plasmid-mediated MBLs the bla_{IMP}, bla_{VIM}, and bla_{SPM} types have been identified worldwide, and their genetic determinants are frequently linked with integrons (Sawa *et al*., 2020). Furthermore, genes for bla_{IMP} and bla_{VIM} MBLs are typically found in integrons that effectively accumulate a large number of antibiotic-resistant gene cassettes as a gene cluster (Partridge *et al*., 2018).

In diverse bacterial species, at least nine genetically distinct integrons have been discovered; class 1, class 2, and class 3 integrons are frequently found in pathogenic Gram negative bacilli such as *Pseudomonas aeruginosa*, *Pseudomonas putida, Escherichia coli*, and *Salmonella spp* (Ploy *et al*., 2000; 2003). Among these integrons, those in classes 1 and 3 have been indicated to have MBL genetic determinants.

The development and rapid expansion of carbapenem resistance via the acquisition of MBL genes has been a source of widespread worry, particularly among Gram-negative bacteria (Nordmann and Poirel, 2002; Aurilio *et al*., 2022).

The current study used phenotypic and genotypic approaches to describe the prevalence of carbapenemase producing *P. aeruginosa* isolates. The most important frequency of the bla_{NDM} gene in the collected isolates is one of the most noteworthy findings of the current investigation.

Previously, it was shown that the most of bla_{NDM-1} carrying isolates came from Asian countries, including India, Pakistan, and China (Shahcheraghi *et al*., 2013). β-lactam antibiotics, such as penicillins, cephalosporins, and carbapenems, are effective treatments for a wide range of infectious illnesses caused by clinically relevant *P. aeruginosa.*

The rise of highly resistant *P. aeruginosa* strains, particularly multidrug resistant (MDR) strains, which are responsible for 13% of all hospital-acquired infections, is a global public health concern (Morehead *et al*., 2018).

Resistance to carbapenems arises by impermeability caused by the loss of the OprD porin, the up-regulation of an active efflux pump system of the cytoplasmic membrane, or the formation of metallo-β-lactamases (MBLs). The existence of these mechanisms can cause to treatment failure in carbapenem therapy of *P. aeruginosa* infections (Laupland *et al*., 2005).

4.6.6. Association between Intgron class 1 and MBL genes

The current study's statistical analysis demonstrated a significant association between the existence of the Intgron class 1 and MBL genes ($p \leq 0.05$). As shown in Table (4-7).

Table 4-8: Association between Integron class 1 and MBL genes

MBL genes	Distribution of MBL Genes and Integron 1 positive isolates no. (%) 131(100%)		*P* value
	+ ve MBL genes no. (%)	- ve MBL genes no. (%)	
bla_{IMP-2}	3(2.29%)	128(97.71%)	0.000
bla_{NDM}	125(95.42%)	6(4.58%)	0.000*
bla_{SIM-2}	7(5.34%)	124(94.66%)	0.000
bla_{SPM}	13(9.92%)	118(90.08%)	0.000
bla_{VIM-1}	3(2.3%)	128(97.7%)	0.000
bla_{VIM-2}	6(4.58%)	125(95.42%)	0.000

* Significant differences at $p \leq 0.05$

There was statistically significant relationship between the presence of bla_{NDM} gene and Integron 1 at $p \leq 0.05$.

The *bla*$_{NDM}$ generating bacteria are resistant to a wide range of different antibiotic groups and carry several additional resistance genes such as fluoroquinolone, aminoglycoside, sulfonamide, and macrolide resistance genes. Furthermore, the *bla*$_{NDM-1}$ enzyme is emerging, leading in practically entire antibiotic resistance (Jovčić *et al*., 2014).

Previous investigations found that the acquisition of MBL determinants such as *bla*$_{NDM-1}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *and bla*$_{SPM}$ resulted in the emergence of MDR or XDR *P. aeruginosa* (Flateau *et al*., 2012; Paul *et al*., 2015). The presence of *bla*$_{NDM-1}$ isolates in *P. aeruginosa* poses a significant difficulty in treatment and is concerning for world health.

4.7. DNA Sequencing

A DNA sequencing approach was used to investigate genetic variation in the variable region (5'CS/3'CS) and *oprD2* genes (Macrogen/ Korea/ by AB DNA sequencing system). The genetic homology sequence similarity between these genes and NCBI BLAST genes ranged from (99% - 99.9%). The present findings of the variable region (5'CS/3'CS) correspond to ten isolates, and they show genetic homology sequences similarity with NCBI BLAST sequences (99%), **Appendix 2**.

The present study reported that class 1 gene cassette regions were amplified and sequenced in 10 integron-positive isolates, according to the length of the cassettes, the most frequently found gene cassettes encoded resistance to Class B metallo-lactamases and Aminoglycoside adenylyl transferases.

Integrons can transmit across bacteria via transposons and plasmids, allowing for the horizontal transfer of multidrug resistance genes (Mazel, 2006; Li *et al*., 2022).

Class I integrons can capture and disseminate gene cassettes among other integron classes. The 5'CS of a class 1 integron comprises of the *intI1* gene, the Pc promoter, and the *attI1* site. While the 3'CS contains: *qacE1* (quaternary ammonium compound disinfection), a *sul1* gene for sulphonamide resistance, and an open reading frame (*orf5*) (Gillings, 2014).

The 5′CS of a typical class I integron comprise the *intI1* gene and *attI* only tracked by gene cassettes, forming the integron's variable region.While the 3'CS has only the *qacH* and *sul3* genes as replacements for the *qacE1* and *sul1* genes, respectively, or a total deletion of the 3′CS (Sáenz *et al.,* 2010).

The gene cassettes have been proposed that cassettes from multiple resistance integrons (MRIs) may be relatively ancient structures. Furthermore, it is thought that MRIs developed from super integrons via the recruitment of super-integron gene cassettes (Recchia and Hall, 1997; Hall and Stokes, 2004).

Eventually, this investigation indicated the widespread expansion of class 1 integrons discovered using several gene cassette arrays in our Babylon hospital. This study adds to our understanding of the mechanisms behind the development of various antibiotic resistance genes in clinical isolates from the Babylon community.

When compared to NCBI BLAST, a genetic variation investigation of the *oprD2* gene indicates deletion mutations. The *oprD2* gene from 8 MDR *P. aeruginosa* isolates was amplified, and sent to DNA sequencing showed partial deletions or point mutations at multiple locations in this gene (Figure 4.25).

DNA sequencing revealed that 8 isolates exhibited partial deletions of small portions and point mutations in different sites. The present results were analyzed

by (Bio Edit sequence Alignment Editor, version 7.1, WI, Madison, DNASTAR, USA).

Pseudomonas aeruginosa strain P8W chromosome, complete genome
Sequence ID: CP081477.2 Length: 7080376 Number of Matches: 1

Range 1: 1069562 to 1070656 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1989 bits(1077)	0.0	1090/1095(99%)	5/1095(0%)	Plus/Plus

```
Query  1        AAACCAAAGGAGCAATCACAATGAAAGTGATGAAGTGGAGCGCCATTGCACTGGCGGTTT  60
Sbjct  1069562  ............................................................  1069621

Query  61       CCGCAGGTAGCACTCAGTTCGCCGTGGCCGACGCATTCGTCAGCGATCAGGCCGAAGCGA  120
Sbjct  1069622  ............................................................  1069681

Query  121      AGGGGTTCATCGAAGACAGCAGCCTCGACCTGCTGCTCCGCAACTACTATTTCAACCGTG  180
Sbjct  1069682  ............................................................  1069741

Query  181      ACGGCAAGAGCGGCAGCGGGGACCGCGTCGACTGGA-CCAAGGCTTCCTCACCACCTATG  239
Sbjct  1069742  ..................................C.........................  1069801

Query  240      AATCCGGCTTCACCCAAGGCACCGTGGGCTTCGGCGTCGATGCCTTCGGCTACCTCGGCC  299
Sbjct  1069802  ............................................................  1069861

Query  300      TGAAGCTCGACGGCACCTCGGACAAGACCGGCACCGGCAACCTGCCGGTGATGAACGACG  359
Sbjct  1069862  ............................................................  1069921

Query  360      GCAAGCCGCGCGACGACTACAGCCGCGCTGGCGGCGCCCTGAAGGTGCGCATTTCCAAGA  419
Sbjct  1069922  ............................................................  1069981

Query  420      CCATGCTGAAGTGGGGCGAAATGCAACCTACCGCGCCGGTCTTCGCCGCCGGCGGCAGCC  479
Sbjct  1069982  ............................................................  1070041

Query  480      GCCTGTTCCCGCAGACCGCGACCGGCTTCCAACTGCAGAGCAGTGAATTCGAAGGGCTCG  539
Sbjct  1070042  ............................................................  1070101

Query  540      ATCTCGAAGCGGGCCACTTCACCGAGGGCAAGGAGCCGACCACCGTCAAATCGCGTGGCG  599
Sbjct  1070102  ............................................................  1070161

Query  600      AACTCTATGCCACCTACGCAGGCGAGACCGCCAAGAGCGCCGATTTCATTGGGGGCCGCT  659
Sbjct  1070162  ............................................................  1070221

Query  660      ACGCAATCACCGATAACCTCAGCGCCTCCCTGTACGGCGCCGAACTCGAAGACATCTATC  719
Sbjct  1070222  ............................................................  1070281

Query  720      GCCAGTATTACCTGAACAGCAACTACACCATCCCACTGGCATCCGACCAATCGCTGGGCT  779
Sbjct  1070282  ............................................................  1070341

Query  780      TCGATTTCAACATCTACCGCACAAACGATGAAGGCAAGGCCAAGGCCGGCGACATCAGCA  839
Sbjct  1070342  ............................................................  1070401

Query  840      ACACCACTTGGTCCCTGGCGGCAGCCTACACTCTGGATGCGCACACTTTCACCTTGGCCT  899
Sbjct  1070402  ............................................................  1070461

Query  900      ACCAGAAGGTCCATGGCGATCAGCCGTTTGATTATATCGGCTTCGGCCGCAACGGCTCTG  959
Sbjct  1070462  ............................................................  1070521

Query  960      GCGCAGGTGGCGACTCGATTTTCCTCGCCAACTCTGTCCAGTACTCCGACTTCAACGGCC  1019
Sbjct  1070522  ............................................................  1070581

Query  1020     CTGGCG----AGAAATCCTGGCAGGCTCGCTACGACCTGAACCTAGCCTCCTATGGCGTT  1075
Sbjct  1070582  ......AGAA....................................................  1070641

Query  1076     CCCGGCCTGACTTTC  1090
Sbjct  1070642  ...............  1070656
```

Figure (4.25): Analysis of partial sequence of *oprD2* gene from clinical isolates. BLAST of *oprD2* PCR products against *P. aeruginosa* strain P8W.

Sun *et al*., (2016) identified an insertion sequence element (ISRP10) in 96% of imipenem-resistant *P. aeruginosa* isolates that disrupts the *oprD2* gene. According to the findings of a study conducted by Yan *et al*., (2014), a 4-bp insertion in the *oprD2* gene led in a frameshift in the *oprD2* gene and imipenem resistance.

According to reports, the principal mechanisms that produce imipenem resistance in *P. aeruginosa* strains include mutation and inactivation or deletion of an *oprD2* gene, disruption in the promoter and upstream region of the *oprD2* gene.

Clinical examples of altered antibiotic translocation across the porin channel also involve amino acid substitution at or near the constriction region of the porin channel. To date, two series of OmpC-type porin mutants have been isolated from patients under chemotherapy and characterized (Lou *et al*., 2011; Bajaj *et al*., 2016). Interestingly, all these mutations yield significant conformational changes in the OmpC pore lumen strongly reduced translocation of β-lactams thus bacterial susceptibilities. In parallel, the role of specific amino acid residues has been investigated by site directed mutagenesis for many years and pointed to the importance of the constriction region of the porin channel (Masi *et al*., 2019).

In conclusion, this study indicated that *P. aeruginosa* resistance to most antibiotic classes is the result of a synergistic effect of numerous resistance mechanisms, including but not limited to metallo-β-lactamase synthesis, *oprD2* loss, and the presence of integrons gene cassette. As a result, it is critical to investigate antimicrobial resistance in isolates isolated locally in order to develop specific treatments for infections.

Finally, the findings of this study demonstrated that MBL-producing *P. aeruginosa* isolates were prevalent in our hospitals. MBL-encoding genes are frequently carried by mobile genetic elements, which can move fast across isolates

via horizontal gene transfer. However, early detection of MBL-producing isolates, the implementation of comprehensive guidelines and infection control measures, and the use of an all-inclusive antimicrobial therapy protocol based on laboratory data are all required to significantly reduce the spread of these resistant pathogens in our medical settings.

Conclusions

and

Recommendations

Conclusions

1. All *P. aeruginosa* isolates were appeared high resistance to multiple antibiotic classes among patients.
2. Screening of MBL production using the E-test revealed higher prevalence rate of MBL-producers in comparison to the CDDT.
3. Most of the *P. aeruginosa* isolates tested positive for biofilm production, with the majority being strong to moderate producers.
4. Class1 integron was the principle integron class in the study isolates, and all *P. aeruginosa* isolates were negative for class 3 integron.
5. There is high association between presences of class 1 integron with antibiotics in MDR *P. aeruginosa* isolates.
6. The most common MBL genes were bla_{NDM}, there is high association between presence of class 1 integron and MBL genes particulary bla_{NDM}.
7. The most abundant variable regions were 2500 bp, 3000 bp, and 3500 bp in size of gene cassette.
8. The highest rate of virulence factor genes *was oprL* of MDR isolates.
9. The *oprD2* gene reveals deletion mutations when compared to NCBI BLAST.

Recommendations

1. The nature and mechanism of integrons' relationship with multidrug resistance among clinical isolates obtained from the community might be studied further.
2. More research is needed to focus on intrinsic resistance mechanisms, including Porin modification, which provides considerable imipenem resistance.
3. Further study on *oprD2* gene expression is needed to understand its association with *oprD* mutations in *P. aeruginosa* isolates.

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Appendixes

Appendix1

bioMérieux Customer: Microbiology Chart Report Printed June 16, 2022 1:05:46 PM CDT

Patient Name: 7, ahmed Patient ID: 7620221
Location: Physician:
Lab ID: 7620221 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: * **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.10 hours	**Status:**	Final
Selected Organism	99% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	0003053003500250		
ID Analysis Messages				

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

bioMérieux Customer: Microbiology Chart Report Printed June 16, 2022 1:06:07 PM CDT

Patient Name: 67, ahmed Patient ID: 14620226
Location: Physician:
Lab ID: 14620226 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	4.85 hours	**Status:**	Final
Selected Organism	97% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	0043053143500252		
ID Analysis Messages				

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

bioMérieux Customer: Microbiology Chart Report Printed June 16, 2022 1:06:18 PM CDT

Patient Name: Ahmed, 87 Patient ID: 86202219
Location: Physician:
Lab ID: 86202219 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.80 hours	**Status:**	Final
Selected Organism	95% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	1003453103500250		
ID Analysis Messages				

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

bioMérieux Customer: Microbiology Chart Report Printed June 16, 2022 1:06:28 PM CDT

Patient Name: 101, ahmed Patient ID: 136202215
Location: Physician:
Lab ID: 136202215 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.02 hours	**Status:**	Final
Selected Organism	97% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	0043453303500250		
ID Analysis Messages				

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

Appendix 2

✓ 5'CS Gene Sequencing

Pseudomonas aeruginosa strain 97 chromosome, complete genome

Sequence ID: CP031449.2 **Length:** 6925889 **Number of Matches:** 5

Range 1: 5919055 to 5919960 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1657 bits(897)	0.0	903/906(99%)	0/906(0%)	Plus/Plus

```
Query  1        GGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCCTCGCCG  60
Sbjct  5919055  ............................................................  5919114

Query  61       AAGCGAAAGATCGCTACTTGAAGTGTTGACGCCTTTGTTTTAAAGTTTCGCGTCTATCTT  120
Sbjct  5919115  ............................................................  5919174

Query  121      TCTGATTTTGTTAAAAAATCAGACTTTGATTAATCATGTTAGACGTCAGAGGTATTTTGA  180
Sbjct  5919175  ............................................................  5919234

Query  181      CTTAAACGCCTCTGAGGTCGAATTAACGTTAGCCACCAAGAAGGTACCATGAAAACATTT  240
Sbjct  5919235  ..........................................G.................  5919294

Query  241      GCCGCATATGTAATTATCGCGTGTCTTTCGAGTACGGCATTAGCTGGTTCAATTACAGAA  300
Sbjct  5919295  ............................................................  5919354

Query  301      AATACGTCTTGGAACAAAGAGTTCTCTGCCGAAGCCGTCAATGGTGTCTTCGTGCTTTGT  360
Sbjct  5919355  ............................................................  5919414

Query  361      AAAAGTAGCAGTAAATCCTGCGCTACCAATGACTTAGCTCGTGCATCAAAGGAATATCTT  420
Sbjct  5919415  ............................................................  5919474

Query  421      CCAGCATCAACATTTAAGATCCCCGACGCAATTATCGGCCTAGAAACTGGTGTCATAAAG  480
Sbjct  5919475  ......................A.....................................  5919534

Query  481      AATGAGCATCAGGTTTTCAAATGGGACGGAAAGCCAAGAGCCATGAAGCAATGGGAAAGA  540
Sbjct  5919535  ............................................................  5919594

Query  541      GACTTGACCTTAAGAGGGGCAATACAAGTTTCAGCTTTTCCCGTATTTCAACAAATCGCC  600
Sbjct  5919595  ..................................G.........................  5919654

Query  601      AGAGAAGTTGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTTCCTATGGCAACCAG  660
Sbjct  5919655  ............................................................  5919714

Query  661      AATATCAGTGGTGGCATTGACAAATTCTGGTTGGAAGGCCAGCTTAGAATTTCCGCAGTT  720
Sbjct  5919715  ............................................................  5919774

Query  721      AATCAAGTGGAGTTTCTAGAGTCTCTATATTTAAATAAATTGTCAGCATCTAAAGAAAAC  780
Sbjct  5919775  ............................................................  5919834

Query  781      CAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGGCGGCACCTGAATATCTAGTGCATTCA  840
Sbjct  5919835  ............................................................  5919894

Query  841      AAAACTGGTTTTTCTGGTGTGGGAACTGAGTCAAATCCTGGTGTCGCATGGTGGGTTGGG  900
Sbjct  5919895  ............................................................  5919954

Query  901      TGGGTT  906
Sbjct  5919955  ......  5919960
```

Pseudomonas aeruginosa strain PaLo532 plasmid pPALO532, complete sequence

Sequence ID: CP075772.1 Length: 204064 Number of Matches: 1

Range 1: 105988 to 107674 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
3066 bits(1660)	0.0	1678/1687(99%)	0/1687(0%)	Plus/Plus

```
Query  1       TTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTA  60
Sbjct  105988  ............................................................  106047

Query  61      GGCCTCGCCGAAGCGAAAGATCGCTACTTGAAGTGTTGACGCCTTTGTTTTAAAGTTTCG  120
Sbjct  106048  ............................................................  106107

Query  121     CGTCTATCTTTCTGATTTTGTTAAAAAATCAGACTTTGATTAATCATGTTAGACGTCAGA  180
Sbjct  106108  ............................................................  106167

Query  181     GGTATTTTGACTTAAACGCCTCTGAGGTCGAATTAACGTTAGCCACCAAGAAGGTGCCAT  240
Sbjct  106168  ............................................................  106227

Query  241     GAAAACATTTGCCGCATATGTAATTATCGCGTGTCTTTCGAGTACGGCATTAGCTGGTTC  300
Sbjct  106228  ............................................................  106287

Query  301     AATTACAGAAAATACGTCTTGGAACAAAGAGTTCTCTGCCGAAGCCGTCAATGGTGTCTT  360
Sbjct  106288  ............................................................  106347

Query  361     CGTGCTTTGTAAAAGTAGCAGTAAATCCTGCGCTACCAATGACTTAGCTCGTGCATCAAA  420
Sbjct  106348  ............................................................  106407

Query  421     GGAATATCTTCCAGCATCAACATTTAAGATCCCCGACGCAATTATCGGCCTAGAAACTGG  480
Sbjct  106408  ..................................A.........................  106467

Query  481     TGTCATAAAGAATGAGCATCAGGTTTTCAAATGGGACGGAAAGCCAAGAGCCATGAAGCA  540
Sbjct  106468  ............................................................  106527

Query  541     ATGGGAAAGAGACTTGACCTTAAGAGGGGCAATACAAGTTTCAGCTGTTCCCGTATTTCA  600
Sbjct  106528  ............................................................  106587

Query  601     ACAAATCGCCAGAGAAGTTGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTTCCTA  660
Sbjct  106588  ............................................................  106647

Query  661     TGGCAACCAGAATATCAGTGGTGGCATTGACAAATTCTGGTTGGAAGGCCAGCTTAGAAT  720
Sbjct  106648  ............................................................  106707

Query  721     TTCCGCAGTTAATCAAGTGGAGTTTCTAGAGTCTCTATATTTAAATAAATTGTCAGCATC  780
Sbjct  106708  ............................................................  106767

Query  781     TAAAGAAAACCAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGGCGGCACCTGAATATCT  840
Sbjct  106768  ............................................................  106827

Query  841     AGTGCATTCAAAAACTGGTTTTTCTGGTGTGGGAACTGAGTCAAATCCTGGTGTCGCATG  900
Sbjct  106828  ............................................................  106887

Query  901     GTGGGTTGGGTGGGTTGAGAAGGAGACAGAGGTTTACTTTGTCGCCTTTAACATGGACAT  960
Sbjct  106888  ..........................................T.................T..  106947

Query  961     AGACAACGACAGTAAGTTGCCGCTAAGAAAATCCATTCCCACCAAAATCATGGAAAGTGA  1020
Sbjct  106948  .........A..................................................  107007

Query  1021    GGGCATCATTGGCGTCTAATCCAAAGTTAGGCATCACAAAGTACAGCATCGTGACCAACA  1080
Sbjct  107008  ............T.G....AA.....................................  107067

Query  1081    GCACCGATTCCGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGT  1140
Sbjct  107068  ...A........................................................  107127

Query  1141    GGCTAAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTG  1200
Sbjct  107128  ............................................................  107187

Query  1201    CTGACGTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACA  1260
Sbjct  107188  ............................................................  107247

Query  1261    TTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCG  1320
Sbjct  107248  ............................................................  107307

Query  1321    GGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTCACTGG  1380
Sbjct  107308  ............................................................  107367

Query  1381    CGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCTCTGGTTGAGT  1440
Sbjct  107368  ............................................................  107427

Query  1441    TGCTGTTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGC  1500
Sbjct  107428  ............................................................  107487

Query  1501    GAGCGATCCGATGCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAG  1560
Sbjct  107488  ............................................................  107547

Query  1561    ATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAGTGATG  1620
Sbjct  107548  ............................................................  107607

Query  1621    CCTAACCCTTCCATCGAGGGGGACGTCCAAGGGCTGGCGCCCTTGGCCGCCCCTCATGTC  1680
Sbjct  107608  ............................................................  107667

Query  1681    AAACGTT  1687
Sbjct  107668  .......  107674
```

Pseudomonas aeruginosa strain FDAARGOS_570 plasmid unnamed, complete sequence

Sequence ID: CP033834.1 **Length:** 36032 **Number of Matches:** 2

Range 1: 9954 to 11023 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1971 bits(1067)	0.0	1069/1070(99%)	0/1070(0%)	Plus/Plus

```
Query  1      TGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTTCCTATGGCAACCAGAATATCAG  60
Sbjct  9954   ............................................................  10013

Query  61     TGGTGGCATTGACAAATTCGGGTTGGAAGGCCAGCTTAGAATTTCCGCAGTTAATCAAGT  120
Sbjct  10014  ..................T.........................................  10073

Query  121    GGAGTTTCTAGAGTCTCTATATTTAAATAAATTGTCAGCATCTAAAGAAAACCAGCTAAT  180
Sbjct  10074  ............................................................  10133

Query  181    AGTAAAAGAGGCTTTGGTAACGGAGGCGGCACCTGAATATCTAGTGCATTCAAAAACTGG  240
Sbjct  10134  ............................................................  10193

Query  241    TTTTTCTGGTGTGGGAACTGAGTCAAATCCTGGTGTCGCATGGTGGGTTGGGTGGGTTGA  300
Sbjct  10194  ............................................................  10253

Query  301    GAAGGAGACAGAGGTTTACTTTTTCGCCTTTAACATGGATATAGACAACGAAAGTAAGTT  360
Sbjct  10254  ............................................................  10313

Query  361    GCCGCTAAGAAAATCCATTCCCACCAAAATCATGGAAAGTGAGGGCATCATTGGTGGCTA  420
Sbjct  10314  ............................................................  10373

Query  421    AAACAAAGTTAGGCATCACAAAGTACAGCATCGTGACCAACAGCAACGATTCCGTCACAC  480
Sbjct  10374  ............................................................  10433

Query  481    TGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATA  540
Sbjct  10434  ............................................................  10493

Query  541    TCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGT  600
Sbjct  10494  ............................................................  10553

Query  601    ACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGAG  660
Sbjct  10554  ............................................................  10613

Query  661    AGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTGGGAAG  720
Sbjct  10614  ............................................................  10673

Query  721    AAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTTACTGGCGAATGCATCACAACTGG  780
Sbjct  10674  ............................................................  10733

Query  781    GCAAAGGCTTGGGAACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAATGATCCCG  840
Sbjct  10734  ............................................................  10793

Query  841    AGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACG  900
Sbjct  10794  ............................................................  10853

Query  901    AGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACA  960
Sbjct  10854  ............................................................  10913

Query  961    TGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAGTGATGCCTAACCCTTCCATCGAG  1020
Sbjct  10914  ............................................................  10973

Query  1021   GGGGACGTCCAAGGGCTGGCGCCCTTGGCCGCCCCTCATGTCAAACGTTA  1070
Sbjct  10974  ..................................................  11023
```

Pseudomonas aeruginosa strain 97 chromosome, complete genome

Sequence ID: CP031449.2 Length: 6925889 Number of Matches: 5

Range 1: 5919055 to 5920734 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
3097 bits(1677)	0.0	1679/1680(99%)	0/1680(0%)	Plus/Plus

```
Query  1        GGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCCTCGCCG  60
Sbjct  5919055  ............................................................  5919114

Query  61       AAGCGAAAGATCGCTACTTGAAGTGTTGACGCCTTTGTTTTAAAGTTTCGCGTCTATCTT  120
Sbjct  5919115  ............................................................  5919174

Query  121      TCTGATTTTGTTAAAAAATCAGACTTTGATTAATCATGTTAGACGTCAGAGGTATTTTGA  180
Sbjct  5919175  ............................................................  5919234

Query  181      CTTAAACGCCTCTGAGGTCGAATTAACGTTAGCCACCAAGAAGGTGCCATGAAAACATTT  240
Sbjct  5919235  ............................................................  5919294

Query  241      GCCGCATATGTAATTATCGCGTGTCTTTCGAGTACGGCATTAGCTGGTTCAATTACAGAA  300
Sbjct  5919295  ............................................................  5919354

Query  301      AATACGTCTTGGAACAAAGAGTTCTCTGCCGAAGCCGTCAATGGTGTCTTCGTGCTTTGT  360
Sbjct  5919355  ............................................................  5919414

Query  361      AAAAGTAGCAGTAAATCCTGCGCTACCAATGACTTAGCTCGTGCATCAAAGGAATATCTT  420
Sbjct  5919415  ............................................................  5919474

Query  421      CCAGCATCAACATTTAAGATCCCCAACGCAATTATCGGCCTAGAAACTGGTGTCATAAAG  480
Sbjct  5919475  ............................................................  5919534

Query  481      AATGAGCATCAGGTTTTCAAATGGGACGGAAAGCCAAGAGCCATGAAGCAATGGGAAAGA  540
Sbjct  5919535  ............................................................  5919594

Query  541      GACTTGACCTTAAGAGGGGCAATACAAGTTTCAGCTGCTCCCGTATTTCAACAAATCGCC  600
Sbjct  5919595  ..........................................T.................  5919654

Query  601      AGAGAAGTTGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTTCCTATGGCAACCAG  660
Sbjct  5919655  ............................................................  5919714

Query  661      AATATCAGTGGTGGCATTGACAAATTCTGGTTGGAAGGCCAGCTTAGAATTTCCGCAGTT  720
Sbjct  5919715  ............................................................  5919774

Query  721      AATCAAGTGGAGTTTCTAGAGTCTCTATATTTAAATAAATTGTCAGCATCTAAAGAAAAC  780
Sbjct  5919775  ............................................................  5919834

Query  781      CAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGGCGGCACCTGAATATCTAGTGCATTCA  840
Sbjct  5919835  ............................................................  5919894

Query  841      AAAACTGGTTTTTCTGGTGTGGGAACTGAGTCAAATCCTGGTGTCGCATGGTGGGTTGGG  900
Sbjct  5919895  ............................................................  5919954

Query  901      TGGGTTGAGAAGGAGACAGAGGTTTACTTTTTCGCCTTTAACATGGATATAGACAACGAA  960
Sbjct  5919955  ............................................................  5920014

Query  961      AGTAAGTTGCCGCTAAGAAAATCCATTCCCACCAAAATCATGGAAAGTGAGGGCATCATT  1020
Sbjct  5920015  ............................................................  5920074

Query  1021     GGTGGCTAAAACAAAGTTAGGCATCACAAAGTACAGCATCGTGACCAACAGCAACGATTC  1080
Sbjct  5920075  ............................................................  5920134

Query  1081     CGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCG  1140
Sbjct  5920135  ............................................................  5920194

Query  1141     ATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACA  1200
Sbjct  5920195  ............................................................  5920254

Query  1201     GGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCT  1260
Sbjct  5920255  ............................................................  5920314

Query  1261     GAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATG  1320
Sbjct  5920315  ............................................................  5920374

Query  1321     GTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTTACTGGCGAATGCATC  1380
Sbjct  5920375  ............................................................  5920434

Query  1381     ACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAA  1440
Sbjct  5920435  ............................................................  5920494

Query  1441     TGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCG  1500
Sbjct  5920495  ............................................................  5920554

Query  1501     ATGCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGC  1560
Sbjct  5920555  ............................................................  5920614

Query  1561     CGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAGTGATGCCTAACCCTT  1620
Sbjct  5920615  ............................................................  5920674

Query  1621     CCATCGAGGGGGACGTCCAAGGGCTGGCGCCCTTGGCCGCCCCTCATGTCAAACGTTAGA  1680
Sbjct  5920675  ............................................................  5920734
```

Pseudomonas aeruginosa strain 97 chromosome, complete genome

Sequence ID: CP031449.2 **Length:** 6925889 **Number of Matches:** 5

Range 1: 5919055 to 5919960 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1663 bits(900)	0.0	904/906(99%)	0/906(0%)	Plus/Plus

```
Query  1        GGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCCTCGCCG  60
Sbjct  5919055  ............................................................  5919114

Query  61       AAGCGAAAGATCGCTACTTGAAGTGTTGACGCCTTTGTTTTAAAGTTTCGCGTCTATCTT  120
Sbjct  5919115  ............................................................  5919174

Query  121      TCTGATTTTGTTAAAAAATCAGACTTTGATTAATCATGTTAGACGTCAGAGGTATTTTGA  180
Sbjct  5919175  ............................................................  5919234

Query  181      CTTAAACGCCTCTGAGGTCGAATTAACGTTAGCCACCAAGAAGGTACCATGAAAACATTT  240
Sbjct  5919235  ...........................................G................  5919294

Query  241      GCCGCATATGTAATTATCGCGTGTCTTTCGAGTACGGCATTAGCTGGTTCAATTACAGAA  300
Sbjct  5919295  ............................................................  5919354

Query  301      AATACGTCTTGGAACAAAGAGTTCTCTGCCGAAGCCGTCAATGGTGTCTTCGTGCTTTGT  360
Sbjct  5919355  ............................................................  5919414

Query  361      AAAAGTAGCAGTAAATCCTGCGCTACCAATGACTTAGCTCGTGCATCAAAGGAATATCTT  420
Sbjct  5919415  ............................................................  5919474

Query  421      CCAGCATCAACATTTAAGATCCCCGACGCAATTATCGGCCTAGAAACTGGTGTCATAAAG  480
Sbjct  5919475  ........................A...................................  5919534

Query  481      AATGAGCATCAGGTTTTCAAATGGGACGGAAAGCCAAGAGCCATGAAGCAATGGGAAAGA  540
Sbjct  5919535  ............................................................  5919594

Query  541      GACTTGACCTTAAGAGGGGCAATACAAGTTTCAGCTGTTCCCGTATTTCAACAAATCGCC  600
Sbjct  5919595  ............................................................  5919654

Query  601      AGAGAAGTTGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTTCCTATGGCAACCAG  660
Sbjct  5919655  ............................................................  5919714

Query  661      AATATCAGTGGTGGCATTGACAAATTCTGGTTGGAAGGCCAGCTTAGAATTTCCGCAGTT  720
Sbjct  5919715  ............................................................  5919774

Query  721      AATCAAGTGGAGTTTCTAGAGTCTCTATATTTAAATAAATTGTCAGCATCTAAAGAAAAC  780
Sbjct  5919775  ............................................................  5919834

Query  781      CAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGGCGGCACCTGAATATCTAGTGCATTCA  840
Sbjct  5919835  ............................................................  5919894

Query  841      AAAACTGGTTTTTCTGGTGTGGGAACTGAGTCAAATCCTGGTGTCGCATGGTGGGTTGGG  900
Sbjct  5919895  ............................................................  5919954

Query  901      TGGGTT  906
Sbjct  5919955  ......  5919960
```

Pseudomonas aeruginosa strain GIMC5021:PA52Ts17, complete sequence

Sequence ID: CP051770.1 **Length:** 6850320 **Number of Matches:** 1

Range 1: 155384 to 156158 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1426 bits(772)	0.0	774/775(99%)	0/775(0%)	Plus/Plus

```
Query  1       TTCGCGTCTATCTTTCTGATTTTGTTAAAAAATCAGACTTTGATTAATCATGTTAGACGT  60
Sbjct  155384  ............................................................  155443

Query  61      CAGAGGTATTTTGACTTAAACGCCTCTGAGGTCGAATTAACGTTAGCCACCAAGAAGGTG  120
Sbjct  155444  ............................................................  155503

Query  121     CCATGAAAACATTTGCCGCATATGTAATTATCGCGTGTCTTTCGAGTACGGCATTAGCTG  180
Sbjct  155504  ............................................................  155563

Query  181     GTTCAATTACAGAAAATACGTCTTGGAACAAAGAGTTCTCTGCCGAAGCCGTCAATGGTG  240
Sbjct  155564  ............................................................  155623

Query  241     TCTTCGTGCTTTGTAAAAGTAGCAGTAAATCCTGCGCTACCAATGACTTAGCTCGTGCAT  300
Sbjct  155624  ............................................................  155683

Query  301     CAAAGGAATATCTTCCAGCATCAACATTTAAGATCCCCAACGCAATTATCGGCCTAGAAA  360
Sbjct  155684  ............................................................  155743

Query  361     CTGGTGTCATAAAGAATGAGCATCAGGTTTTCAAATGGGACGGAAAGCCAAGAGCCATGA  420
Sbjct  155744  ............................................................  155803

Query  421     AGCAATGGGAAAGAGACTTGACCTTAAGAGGGGCAATACAAGTTTCAGCTGCTCCCGTAT  480
Sbjct  155804  ...........................................T................  155863

Query  481     TTCAACAAATCGCCAGAGAAGTTGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTT  540
Sbjct  155864  ............................................................  155923

Query  541     CCTATGGCAACCAGAATATCAGTGGTGGCATTGACAAATTCTGGTTGGAAGGCCAGCTTA  600
Sbjct  155924  ............................................................  155983

Query  601     GAATTTCCGCAGTTAATCAAGTGGAGTTTCTAGAGTCTCTATATTTAAATAAATTGTCAG  660
Sbjct  155984  ............................................................  156043

Query  661     CATCTAAAGAAAACCAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGGCGGCACCTGAAT  720
Sbjct  156044  ............................................................  156103

Query  721     ATCTAGTGCATTCAAAAACTGGTTTTTCTGGTGTGGGAACTGAGTCAAATCCTGG  775
Sbjct  156104  ..................................................  156158
```

Appendix 3

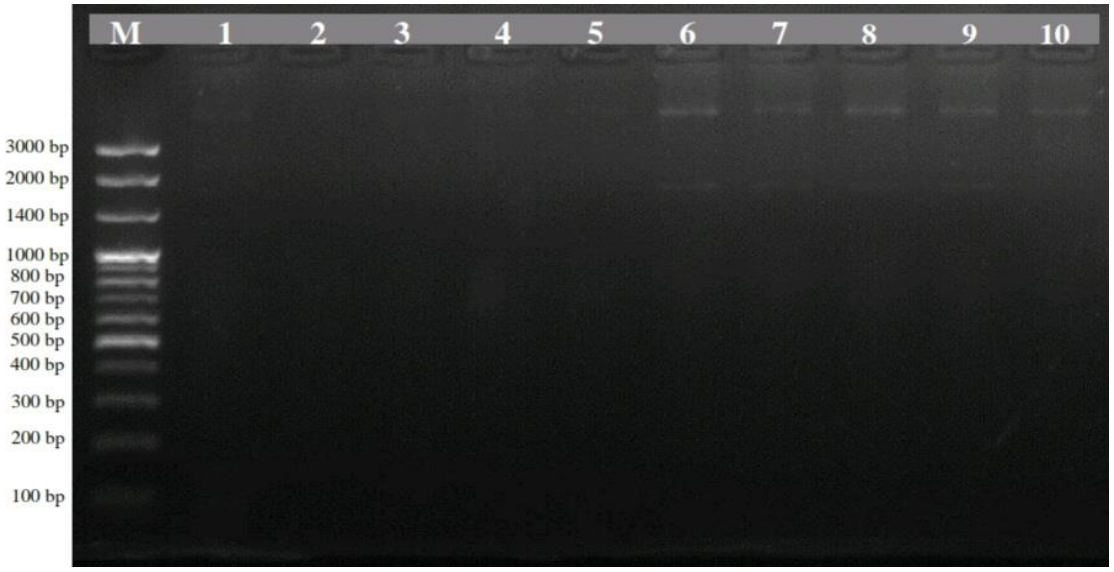

Figure (1): PCR amplification of ***bla*$_{GIM-1}$** gene on chromosome (**477bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative.

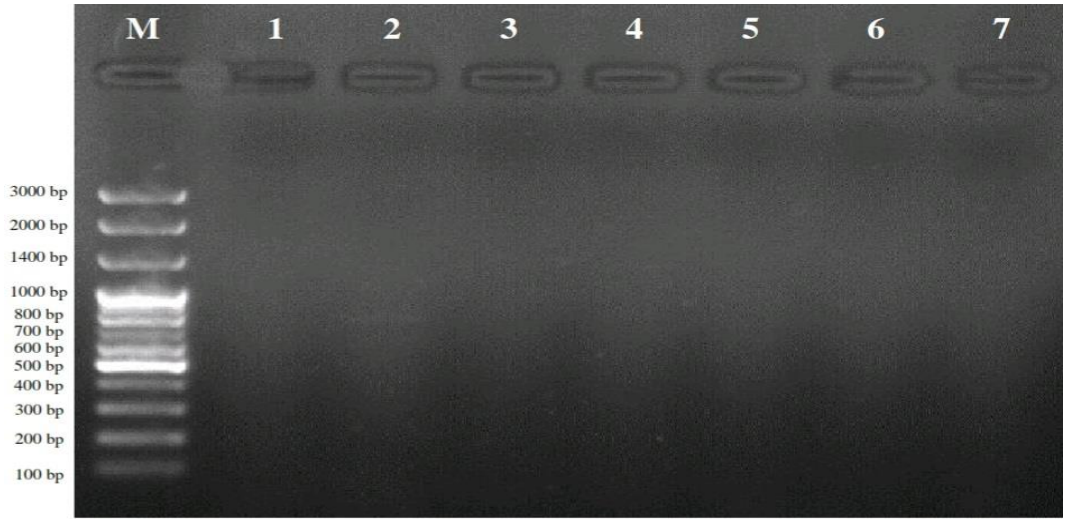

Figure (2): PCR amplification of ***bla*$_{GIM-1}$** gene on plasmid (**477bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative.

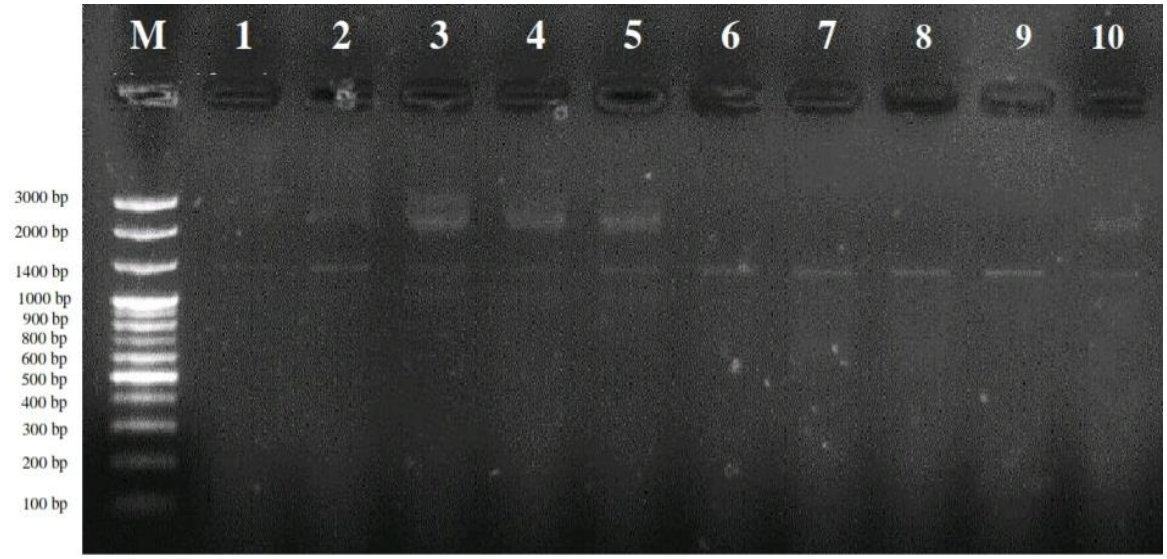

Figure (3): PCR amplification of ***bla*$_{IMP-1}$** gene on chromodome (**587bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative.

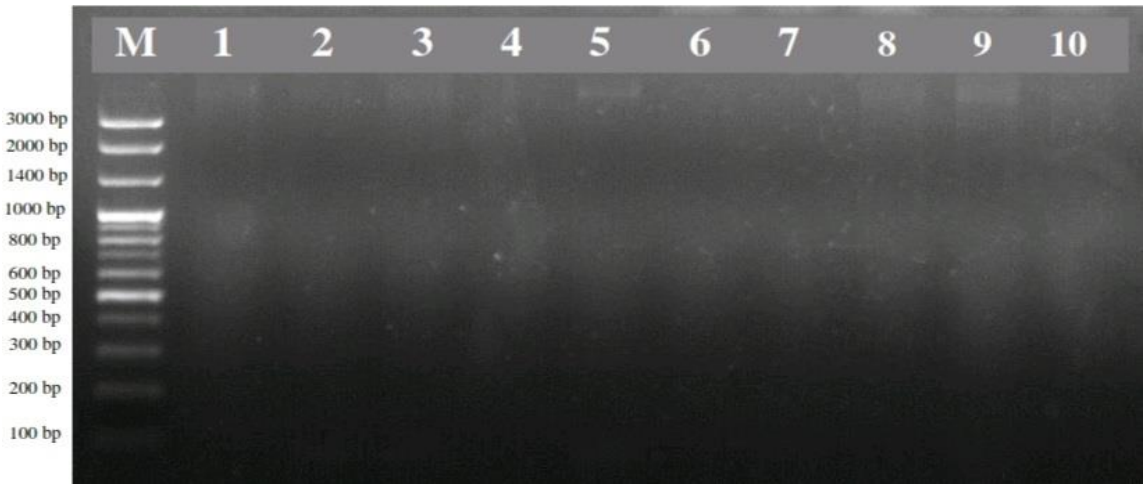

Figure (4): PCR amplification of ***bla*$_{IMP-1}$** gene on plasmid (**587bp**) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lane M: 100-bp DNA marker. All isolates were negative.

الخلاصة

تمثـل الزوائـف الزنجاريــة متعـددة المقاومــة للمضــادات الحيويــة الكـائن الممـرض التحـدي الاول للمستشفيات، حيث اعتبرت منظمة الصحة العالمية الزوائف الزنجارية المقاومة للكاربابينيم كعامل ممرض "حرج" في الحاجة الجوهرية لتصنيع ادوية جديدة من اجل التصدي لاي ازمة صحية وشيكة بسبب مقاومة المضادات الحيوية. تلعب جينات الانتكرون (وحدات جينية انتقالية) دورا مهما في انتشار المقاومة للمضادات الحيوية للزوائف الزنجارية. تتواجد كاسيتات الجينات التي تشفر لجينات المقاومة للمضادات الحيوية في الصنف الاول من الانتكرون.

جمعت ١٣١عزلة من الزائفة الزنجارية من ٣٨٥ (٣٤٪) عينة سريرية من البول والحروق والجروح وألتهاب الاذن والبلغم، لأفراد من كلا الجنسين وبمختلف الأعمار وتم عزلها من مستشفيات ومختبرات مختلفة في محافظة بابل خلال المدة من شهر شباط ٢٠٢٢ الى شهر تشرين الاول ٢٠٢٢.

عزلت ١٣١ (٣٤,٠٪) عزلة لبكتريا الزائفة الزنجارية من مختلف العينات السريرية وكانت الحروق ١٠٣ (٧٨,٦٣٪) من جميع العينات السريرية وكانت الجروح ٧ (٥,٤٪) و البول ٩ (٦,٨٧٪) والاذن ٩ (٦,٨٧٪) والبلغم ٣ (٢,٢٩٪) وشُخصت من خلال الاختبارات الكيموحيوية الاعتيادية و نظام الفايتك. وتمَّ تأكيدها عن طريق تفاعل البلمرة المتسلسل باستخدام الجين *ecfX* الخاص بالنوع .

أظهرت النتائج نسب المقاومة للمضادَّات الحيوية تجاه ٢٢ مضادا حيويا كالآتي: كانت جميع عزلات الزوائف الزنجارية مقاومة لكل من المضادات الامبسيلين والسفيكسيم والسيفوتاكسيم بنسبة ١٣١ (١٠٠٪) و يتبعها المضادات سيفيسوكزيم ١٣٠(٩٩,٢٣٪) و سيفيبيم١٢٣ (٩٣,٩٠٪) وتتراسايكلين١٢٠ (٩١,٦٠٪)

بينما دوكسيسايكلين كانت ١١٩ (٩٠,٨٤٪) و سيفوبيرازون ١١١ (٨٤,٧٣٪) و جنتامايسين ١١٠ (٨٣,٩٧٪) و توبرامايسين و ميروبينيم ١٠٧ (٨١,٦٨٪) و تيكارسيلين كلافولانيت ١٠٦ (٨٠,٩٪) و اميكاسين ١٠٤ (٧٩,٣٩٪) و ببراسلين ١٠٢ (٧٧,٨٦٪) و ليفوفلاكساسين ٩٩ (٧٥,٥٧٪) و ازيترونم ٩٧ (٧٤,٠٥٪) و سبروفلوكساسين ٩٦ (٧٣,٢٨٪) و اميبينم ٩٥ (٧٢,٥٢٪) و نورفلوكساسين ٩٤ (٧١,٧٥٪) و بيبيراسيلين–تازوباكتم ٨٤ (٦٤,١٢٪) و دوريبنم ٧١ (٥٤,٢٠٪) وكان المضاد ازثرومايسين الاقل نسبة مقاومة ٥٣ (٤٠,٤٦٪).

كانت جميع العزلات متعدِّدة المقاومة للمضادات الحيوية بنسبة (١٠٠٪) على الأقل لثلاث مجاميع من المضادات الحيوية واظهرت بعض العزلات مقاومة لكل المجاميع المستخدمة .

أظهرت النتائج أن نسبة إنتاج إنزيم بيتالاكتميز كانت ١٣١/٣٣(٢٥,١٩٪) من نسبة العزلات المتعدِّدة المقاومة للمضادات الحياتية ، ونسبة إنتاج إنزيمات بيتالاكتميز المعدنية كانت (٣ (٢,٢٩٪) و١٠ (٧,٦٣٪)) من نسبة العزلات المتعدِّدة المقاومة للمضادات الحياتية بطرق (اختبار انتشار القرص المزدوج واشرطة E–test IMI/IMD) على التوالي.

كانت نتائج اختبار تكوين الأغشية الحيوية ، من بين ١٣١عزلة من الزوائف الزنجارية ، كانت ١١٢ عزلة بنسبة (٨٥,٥٠٪) لديها القدرة على تكوين الأغشية الحيوية ، وتم تصنيفها على النحو التالي: ٤٠ (٣٠,٥٣٪) كونت غشاء حيوي قوي و ٥١ (٣٨,٩٣٪) كونت غشاء حيوي متوسط و ٢١ (١٦,٠٣٪) كونت غشاء حيوي ضعيف ، في حين أن ١٩ (١٤,٥٠٪) كانت غير مكونه للغشاء الحيوي. وجد أن المقاومة للمضادات الحيوية أعلى بكثير في عزلات الزائفة الزنجارية المنتجة للغشاء الحيوي مقارنة مع عزلات الزائفة الزنجارية غير المنتجة للغشاء الحيوي ($p < 0.05$).

تم استخلاص الدنا الكروموسومي والبلازميدي لجميع عزلات بكتريا الزائفة الزنجارية و تم تحديد البلازميد في ١٣١/٦٣ (٤٨٪) من عزلات الزائفة الزنجارية.

أظهرت تقنية تفاعل البلمرة المتسلسل وجود جين الانتكرون من النوع الاول (*intI1*) في جميع العزلات (١٠٠٪) محمولا على الدنا الكروموسومي ، بينما لم تعط أي عزلة للجين (*intI1*) محمولا على البلازميد. فيما يتعلق بوجود الجينات المشفرة للصنف٢ (*intI2*) كانت ٥ (٣,٨١٪) محمولا على الدنا الكروموسومي و الصنف ٣ (*intI3*) كانت صفر من العزلات متعددة المقاومة للمضادات الحيوية ، بينما لم يحدد وجود الجين المشفر للانتكرون الصنف٢ (*intI2*) وكانت عزلة موجبة واحدة ناتجة عن الجينات المحمولة على الدنا البلازميدي لجين الانتكرون الثالث (*intI3*).

اظهر التحليل الإحصائي لهذه الدراسة وجود علاقة معنوية بين وجود جين الانتكرون من الصنف الاول مع المقاومة للمضادات الحيوية المختلفة ($p \leq 0.05$). أشار تحليل كاسيتات الجين لتضخيم المناطق المتغايرة لانتكرون الصنف الاول في ١٣١ عزلة ، حيث أظهرت كل عزلة منطقة متغايرة واحدة تتراوح في الحجم من ٢٠٠ زوج قاعدي إلى ٨٠٠٠ زوج قاعدي.

أوضحت نتائج الكشف الجزيئي لجينات عوامل الضراوة أن أعلى معدل للجين *oprL* بين عزلات الزائفة الزنجارية سُجل في ١٣١ (١٠٠٪)، يليه *Alg* و *oprI* في ١٢٩(٩٨,٤٧٪) على التوالي و ١٢٥ (٩٥,٤٢٪) للجين *oprD2* من العزلات متعددة المقاومة للمضادات الحيوية. ان تكرار تواجد الجينات المشفرة لانزيمات البيتالاكتميز المعدنية والتي حددت بتقنية تفاعل البلمرة المتسلسل كانت كالاتي: bla_{IMP-1}, bla_{IMP-2}, bla_{IMP-7}, bla_{IMP-10} , bla_{IMP-13} , bla_{IMP-25} , bla_{VIM-1} , bla_{VIM-2} , bla_{NDM} , bla_{GIM-1}, bla_{SIM-2} و bla_{SPM} ثلاث عزلات (٢,٣٪) تحتوي على جين bla_{IMP-2} فقط ، تليها ٣ (٢,٣٪) ، ٦

(٤,٥٨٪) ، ١٢٥ (٩٥,٤٢٪) ، ٧ (٥,٣٤٪) ، و ١٢ (٩,١٦٪). تحمل جينات bla_{VIM-1} و bla_{VIM-2} و bla_{NDM} و bla_{SIM-2} و bla_{SPM} على التوالي على الدنا الكروموسومي ، ولم يحدد وجود جينات إضافية. لكن وجد أن ٩ (١٤,٢٩٪) و ٥ (٧,٩٤٪) و ٨ (١٢,٧٠٪) و ٣ (٤,٧٦٪) و ١(١,٥٩٪) تمتلك جينات bla_{IMP-7} و bla_{IMP-13} و bla_{VIM-1} و bla_{VIM-2} و bla_{SPM} محمولة على الدنا البلازميدي على التوالي ، بينما لم يتم الكشف عن الجينات الأخرى. من الجدير بالذكر أن جميع عزلات الزائفة الزنجارية البالغ عددها ١٣١ تحتوي على الجين *intI1*.

النتائج الحالية المتعلقة بتحليل المنطقة المتغايرة (5'CS) للعزلات العشرة المرسلة لتحليل تسلسل النيوكلوتيدات ، اظهرت تشابه و تماثل جيني بالتسلسل بنسبة (٩٩٪) مع تسلسل NCBI BLAST . كما اظهرت دراسة التباين الجيني لجين *oprD2* عن وجود طفرات الحذف عند مقارنتها مع تسلسل NCBI BLAST .

وزارة التعليم العالي والبحث العلمي
جامعة بابل/ كلية العلوم
قسم علوم الحياة

دراسة جزيئية لأنزيمات البيتالاكتميز المعدنية مع الانتكرون الصنف الاول لعزلات بكتريا الزائفة الزنجارية

اطروحة مقدمة إلى
مجلس كلية العلوم / جامعة بابل وهي جزء من متطلبات
نيل شهادة الدكتوراه فلسفة في العلوم /علوم الحياة

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١٤٤٥هـ ٢٠٢٣م