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Molecular Study of Virulence Factors Genes among Multidrug Resistant *Pseudomonas aeruginosa*

A dissertation

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المجادلة: الآية ١١

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Dedication

Praise be to Allah Almighty , my creator, my strong pillar, my source of inspiration, wisdom, knowledge and understanding. He has been the source of my strength throughout this program and on His wings only have I soared. This dissertation is dedicated to the memory of my father (Hafedh Jameel). Although he was my inspiration to pursue my doctoral degree, he was unable to see my graduation. This is for him.

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Summary

Pseudomonas aeruginosa is known as one of the main causes of nosocomial infections and is repeatedly related to opportunistic infections among hospitalized patients worldwide. In the present study, a total of ٣٨٤ specimens were obtained from Al-imam al-Sadiq hospital, Hilla General Teaching Hospital and Marjan Hospital in Babylon from March / ٢٠٢٢ to November / ٢٠٢٢ were collected from different sources including:(burn, wound, urine, ear and sputum) were recovered from patients. The results showed that ١٣٠ (٣٣.٨ %) of isolates were *P. aeruginosa* isolates, distribution to ١٠٢ burn (٧٨.٥٪), ٩ urine (٦.٩٪), ٩ ear (٦.٩٪), ٧ wound (٥.٤٪) and ٣ sputum (٢.٣٪). Isolates were further identified confirmed as presumptive *P. aeruginosa* using VITEK® ٢ Compact, due to the manufacturer's instructions. Polymerase chain reaction (PCR) screening, targeting the *ecfX* gene was conducted to identify species-specific *ecfX* in *P. aeruginosa*. All the ١٣٠ isolates were positive to *ecfX* gene.

The results revealed that resistance to antibiotics at percentages as follows: Ampicillins (٩٦.٩٪), Piperacillin, (٩٢.٣٪), Piperacillin-Tazobactam (٧٦.٩٪), Ticarcillin (٩٧.٧٪) , Ceftazidime (٩٩.٢٪), Cefepime (٨٧.٧٪), Azetronam (٨٥.٤٪), Doripenem(٩٠.٨٪), Etrapenem (٩٦.٩٪), Meropenem (٧٦.٩٪), Amikacin (٧٧.٧٪), Gentamicin (٧٣.١٪), Tobramycin (٧٦.٢٪), Tetracycline (٩٤.٦٪), Doxycycline (٨٦.٢٪), Erythromycin (٩٦.٩٪), Levofloxacin (٧٢.٣٪) and Ofloxacin (٧٥.٤٪) when compared with its resistance rate to Imipenem and Ciprofloxacin (٦٩.٢٪). Multidrug-resistant *P. aeruginosa* accounted for ١٢٥/١٣٠ (٩٦.١٪). Regarding of biofilm-forming ability according to Congo read agar was ١١١/١٣٠ (٨٥.٤٪) of isolates, being assorted in the following categories: ٤٠ (٣٠.٨٪) formed strong biofilm; ٥١ (٣٩.٢٪) formed moderate biofilm; ٢٠ (١٥.٤٪) formed weak biofilm, while ١٩ (١٤.٦٪) of *P. aeruginosa*

isolates were characterized as non-biofilm former. Among twenty antibiotics investigated, there was no statistically significant (p>٠.٠٥) correlation between biofilm formation positivity and antibiotic resistance patterns, with exception of imipenem (p=٠.٠٠٥٦).

The efflux pump *MexAB-OprM* genes (*MexA*, *MexB* and *OprM*) were screened by PCR technique showed the isolates were positive for the ١٣٠ (١٠٠٪) to *MexA*, ١٢٧ (٩٧.٦٩٪) to *OprM* and ١٢٢ (٩٣.٨٤٪) to *MexB*. Statistical analysis demonstrated a significant association between the existence of the efflux pump genes (*MexA*, *MexB*, and *OprM*) and resistance to various antibiotic classes (P≤ ٠.٠٥), as well as associated significantly with MDR (p< ٠.٠٥).

The current findings display different percentages of virulence factor genes ١٢٧ *apr* (٩٨٪), ١٢٥ *PLCH* (٩٦٪), ١٢٢ both *algD* and *phzS* (٩٤٪), ١٢٠ *PLCN* (٩٢٪), ١١٧ *phzM* (٩٠٪), ١١٥ *lasB* (٨٨٪), ١٠٧ *algU* (٨٢٪), ١٠٦ *pilA* (٨٢٪), ٤١ *lasA* (٣٢٪), and ١٢ *pilB* (٩٪) that were identified in *P. aeruginosa* isolates. Regarding for Type III Secretion System (T٣SS), the PCR results revealed that ١٢٧ (٩٧.٦٩٪) *P. aeruginosa* isolates were positive for the *ToxA* gene, ١٢٤ (٩٥.٣٨٪) *exoS* gene and *exoU* was detected in ١٢١ (٩٣.٠٧٪). Based on virulence genes classification the number of the invasiveness gene-isolates ١٢٤ *exoS* gene was (٩٥٪) which is slightly higher than the cytotoxic gene-isolates ١٢١ *exoU* (٩٣٪). The results revealed a statistically significant (p<٠.٠٥) association between virulence genes and T٣SS genotypes, with exception of *pilB* (p>٠.٠٥). The present findings reported that the presence of *aprA* gene was significantly higher among invasive genotype isolates; however, the presence of *ToxA* gene was significantly higher among the cytotoxic genotype isolates. Statistical analysis of the present study revealed a significant relationship between the presence of the exotoxin genes *exoS*, *exoU*, and *ToxA* with resistance to different antibiotic classes (P≤ ٠.٠٥).

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List of Abbreviations

ABC	Adenosine Triphosphate Binding Cassette
ADPRT	mono-ADP-ribosyl transferase
AIDS	Acquired Immune Deficiency Syndrome
AK	Amikacin
AMP	Ampicillins
AprA	Alkaline Protease
ATM	Aztreonam
ATPases	Adenosine triphosphate
CA	Chorismic Acid
cAMP	cyclic AMP
CAZ	Ceftazidime
CDC	Centers for Disease Control
CF	Cystic fibrosis
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute instructions
CN	Gentamicin
CRA	Congo red agar
DMT	Drug Metabolite Transporter
DNA	Deoxyribonucleic Acid
DOR	Doripenem
DXT	Doxycyclin
E	Erythromycin
ecfX	extracytoplasmic function
EDTA	Ethylenediaminetetraacetic acid
ETP	Ertapenem

exo	Exoenzyme
FEP	Cefepime
GAP	GTPase Activating Protein
GMP	Guanosine monophosphate synthetase
HAIs	Hospital Acquired Infections
ICU	Intensive Care Unit
ICUAP	Intensive care unit-acquired pneumonia
IgG	immunoglobulin G
IPM	Imipenem
LEV	Levofloxacin
LPS	Lipopolysaccharide
MATE	Multidrug and Toxic Compound Extrusion
Mbp	Mega base pair
MDR	Multi Drug Resistance
MEM	Meropenem
MFS	Major Facilitator Super Family
MGEs	Mobile Genetic Elements
MPCBA	Methylphenazine-١-Carboxylic Acid Betaine
NADH	Nicotinamide adenine dinucleotide
OFX	Ofloxacin
PCA	Phenazine-١-Carboxylic Acid
PCR	Polymerase chain reaction
PE	*Pseudomonas* elastase
PLA٢	Phospholipase A٢
PLC	phospholipase C
PLCH	hemolytic *PLC*
PLCN	non-hemolytic *PLC*

PRL	Piperacillin
QS	Quorum scene
RNAP	RNA polymerase
RND	Resistance Nodulation division
rRNA	Ribosomal Ribonucleic acid
TBE	Tris-Borate-EDTA
TE	Tetracyclin
TFP	Type IV pili
TIC	Ticarcillin
TLR۵	Toll like receptor ۵
TOB	Tobramycin
TTSS/T۳SS	Type III Secretion System
TZP	Piperacillin-Tazobactam
UTI	Urinary Tract Infection
UV-Vis	Ultraviolet–Visible Spectrophotometry
WHO	World Health Organization

Chapter One

Introduction

١. Introduction

١.١. Introduction

Pseudomonas aeruginosa is considered one of the major etiological agents of both acute and chronic human infections ranging from minor skin infections to continuous and often life-threatening diseases in healthcare-associated infections (Rao *et al*., ٢٠١١; Morin *et al*., ٢٠٢١) .Treatment of infectious diseases becomes more demanding each year. This is principally true for infections caused by the opportunistic pathogen *P. aeruginosa* (Parsa *et al*., ٢٠٢٠). *P. aeruginosa* exhibits most of these known resistance mechanisms through both intrinsic chromosomally encoded or genetically imported resistance determinants affecting the major classes of antibiotics such as β-lactams, aminoglycosides, quinolones. Eight categories of antibiotics are mainly used to treat *P. aeruginosa* infections including aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams, fosfomycin and polymyxins (colistin, polymyxin B) (Bassetti *et al*., ٢٠١٨).

Infections relating to this bacterium are difficult to cure due to the ability of *P. aeruginosa* to develop resistant classes of antibiotics (Derakhshan and Hosseinzadeh, ٢٠٢٠). In addition, the *P. aeruginosa* pathogenicity is mainly caused by various bacterial virulence factors and genetic flexibility, enabling it to survive in varied environments (Pachori *et al*., ٢٠١٩). Many of these factors aid colonization, and assist bacterial invasion, including *ExoT*oxin A, cytotoxic pigment pyocyanin, siderophores, alkaline protease, elastase, and type III secretion system (T٣SS) effector proteins (Driscoll *et al*., ٢٠٠٧; El-Mahdy and El-Kannishy, ٢٠١٩; Eladawy *et al*., ٢٠٢١).

The T٣SS system is essential for the export of effector proteins through a needle-like structure directly inside target host cells (Hauser, ٢٠٠٩). Four effector proteins have been notified in *P. aeruginosa* including, *ExoU*, *ExoS*, *ExoT* and *ExoY*. The *ExoT* and *ExoY* proteins have scarce contribution in pathogenesis, whereas *ExoU* and *ExoS* have much more contribution, and are considered the most important virulence factors of *P. aeruginosa* (Shi *et al*., ٢٠١٢; Abozahra *et al*., ٢٠٢١). Based on the T٣SS secreted toxin, *P. aeruginosa* isolates are categorized into invasive or cytotoxic genotypes, and this is called virulence genotyping or T٣SS genotyping. Invasive genotype strains are those carrying *ExoS* gene, whilst cytotoxic genotype strains are carrying *ExoU* gene (Shen *et al*., ٢٠١٤).

Therefore, it is pivotal that we amend our understanding of some aspects of the *P. aeruginosa* virulence factors that are related to disease development in healthcare-associated infections and resistance to antibiotics. Previous studies were not comprehensive in their analysis of the *P. aeruginosa* pathogenicity (Saleh *et al*., ٢٠١٢; Al-Hamawandi, ٢٠١٤). Furthermore, the data are about seven or more years old; thus, it is crucial to have a continuous diagnosis and monitoring of this bacterium. It is worth noting that the present study imparts to the comprehension of antibiotic susceptibility profiles and genotypic characterization of virulence factors to assist in the management of infections caused by *P. aeruginosa*.

١.٢. Aim of the Study

The aim of the present study is to analyze the prevalence of antibiotic susceptibility profile, dissemination of virulence genes and

T٣SS genotypes in *P. aeruginosa* isolates recovered from patients. The specific objectives of this study will be achieved by the following:

١. Isolation and identification *P. aeruginosa* isolates from patients.

٢. Confirmation of identification using species-specific *ecfX* gene

٣. Determination the antibiotic susceptibility profile of *P. aeruginosa* isolates.

٤. Classification of *P. aeruginosa* isolates based on the T٣SS genotyping into either invasive isolates (carrying *ExoS* gene) or cytotoxic strains (carrying *ExoU* gene).

٥. To investigate the prevalence of the virulence genes: Alginate (*algDU*), Pilli (*pilA*B), *ExoT*oxin (*ToxA*), Phenazine (*phzMS*), elastases (*(lasAB*), phospholipases C (*PLCHN*), alkaline protease (*AprA*), and *mexAB-oprM* using PCR technique.

Chapter Two

Literature Review

٢. Literature Review

٢.١. History of *Pseudomonas aeruginosa*

Due to the characteristic blue-green color produced during growth, *P. aeruginosa* has been given various names throughout its history. Se'dillot noticed in ١٨٥٠ that discoloration of surgical wound dressings was caused by a transferable agent(Pereira, ٢٠١٥). Fordos extracted the pigment responsible for the blue coloration in ١٨٦٠, and Lucke found that this pigment was caused by rod-shaped organisms in ١٨٦٢. *P. aeruginosa* was not isolated as pure culture until ١٨٨٢(Zamirian & Kariz, ٢٠٢٢) . In ١٨٨٢, Carle Gessard identified the rod-shaped organism that caused the infection as *Bacillus pyocyaneus(*Wood *et al*., ٢٠٢٣). However, in ١٨٩٤, Migula determined that the bacterial species was different from the Bacillus genus in his detailed studies and defined this bacterium as *P. aeruginosa*. In ١٩١٦, Freeman conducted a more thorough investigation on the invasion and spread of *P. aeruginosa* that causes acute or chronic illness (Lister *et al*., ٢٠٠٩). According to their rRNA, Palleroni divided *Pseudomonas* bacteria into five homologous groups in ١٩٧٣. In ١٩٨٣, De Vos and De Ley performed in-depth research on these five homologous groups, they determined the affinities between species and reclassified the groups according to their phylogenetic affinities. *P. aeruginosa* is in homology group I (Palleroni, ١٩٩٣).

٢.٢. Characterization of *Pseudomonas aeruginosa*

P. aeruginosa belongs to the Pseudomonadaceae family of proteobacteria(Mhana, ٢٠٢٢). Gram-negative bacterium with dimensions (٠.٥-٠.٨) μm diameter and (١.٥-٣) μm length rod form with one flagellum for mobilization(Yanez *et al*., ٢٠١٠). *P. aeruginosa* distinguishes itself from most Gram-negative bacteria by being positive in an oxidase

response. Furthermore, *P. aeruginosa* is unable to ferment lactose indefinitely (Diggle and Whiteley, ٢٠٢٠). Non-forming spores that are similar to members of the intestinal family Enterobacteriaceae but differ in that they are obligate aerobes that obtain energy from carbohydrates through oxidation rather than fermentation, some time it uses nitrate as an alternative source (as final electron acceptor) that allows growth anaerobically (Schurek *et al*., ٢٠١٢). Non-capsulated but it has pseudo capsule, slime layers which they play as a barrier against antibiotics, phagocytes and, lymphocytes (Todar, ٢٠١١). *P. aeruginosa* can be identified by its specific odor In vitro and by the color of the producing colonies which is mostly blue greenish. The optimum temperature for the growth of *P. aeruginosa* is ٣٧°C, while maximum temperature of its growth is ٤٢°C. Usually, the strains of *P. aeruginosa* are motile by means of a single polar flagellum (Brooks *et al*., ٢٠١٦). It is often had mucoid slime layer with extracellular polysaccharide composed of alginate polymers. *Pseudomonas aeruginosa* produces water-soluble pigments which can diffuse through the medium they are pyocyanin, pyoverdine, and pyorubin which also causes a typical 'sweet' odor *in vitro* (Vanstokstraeten, ٢٠٢٠).

In culture media, it forms large and small colonies with flat edges and slightly higher than the media, and it also produces mucus colonies in isolates taken from the secretions of the respiratory system and urinary system, where it was first observed by Sonnenshein (١٩٢٧) due to their production of alginate slime (Todar, ٢٠١٢).

P. aeruginosa may be found in a variety of environments, including water, plants, soil, and animal epidermis (Batrich *et al*., ٢٠١٩). It is usually seen in nature as plankton moving through water or as a biofilm, which is a collection of bacteria with similar phenotypes and chemical

features. *P. aeruginosa* is unique in that it can flourish and live in a wide range of temperatures and nutritional conditions (Diggle & Whiteley, ٢٠٢٠). Many researches has shown that *P. aeruginosa* can thrive in distilled water, providing it an edge in adapting to changing settings (Jenny & Kingsbury, ٢٠١٨). *P. aeruginosa* is also an important clinical agent, as this bacterium is an opportunistic pathogen that can cause wide range of acute and chronic injures and diseases in humans (Weiner *et al*., ٢٠١٦). *P.aeruginosa* has huge virulence factors which cause both extensive tissue damage and interfere with the immune defense weapons (Urgancı *et al*., ٢٠٢٢).

٢.٣. Pathogenicity of *Pseudomonas aeruginosa*

P.aeruginosa is the common opportunistic human pathogen, which is the cause of bacterial infection in humans and it may vary in severity from a mild to debilitating systemic disease, associated with significant morbidity and mortality (Nguemeleu *et al*., ٢٠٢٠). During hospitalization, infections are more common and varied (Kumar *et al*., ٢٠١٦). Individuals at most risk include those with immuno suppressed patients such as those with severe burns, cancer and acquired immune deficiency syndrome (AIDS) (Pang *et al*., ٢٠١٩). It was linked to infectious diarrhea, with confirmed cases often affecting people with hematological malignancies and neutropenia as a result of chemotherapy or infant epidemics (Mackenzie *et al*., ٢٠٢١). *P. aeruginosa* is a common cause of Hospital Acquired Infections (HAIs) due to its antibiotic-resistant and opportunity to survive in low-nutrient environments. *P.aeruginosa* adaptability allows it to infect a wide range of tissues, wide spectrum of tissues tropism and live in both natural and hospitals environment (Caschera , ٢٠٢١). This pathogen has various virulence factors which are encoded by genes that

are related with bacteria which causes disease and almost be horizontally acquired usually be expressed during infection, sometimes the factors be inactivated in strains so will not cause disease (Solomon *et al*., ٢٠١٧; Ali *et al*., ٢٠١٨).

Steps for an infection include some disruptions that take place in the primary physical barriers (skin or mucous membrane), or by passing them (by invasive devices) as well as an underlying dysfunction of the immune defense mechanism which is necessary as a complementary reason to accelerate the infections (Ratkai, ٢٠١١). A prior antibiotic therapy that eliminates normal flora can also provide *P. aeruginosa* to access tissue colonizing (Kumar *et al*., ٢٠١٦). World Health Organization (WHO) classified *P. aeruginosa* as one of the critical pathogens in its first published list of antibiotic-resistant priority pathogens based on the urgency of need for new antibiotics (Tacconelli, ٢٠١٧). It is incidence found in approximately ٥٠٪ to ٦٠٪ of the persons with tenuous or immunodeficiency (Migiyama *et al*., ٢٠١٦). It is highly capable to invade tissues and producing toxins resulting in a complex infection (Sawa, ٢٠١٤).

The bacteria also create an enzyme called lyase, which may break down polysaccharide into smaller oligosaccharides units. Both the biosynthesis and breakdown processes have been found to be important in the infection process (Gellatly and Hancock, ٢٠١٣; Alhazmi, ٢٠١٥). It has been classified as a major species associated with multiple antimicrobial resistance of urgent public health concern by the Infectious Diseases Society of America Centers for Disease Control and Prevention and the World Health Organization. It is a leading cause of severe, life-threatening nosocomial human infections and the major pathogen associated with lung infections in patients with cystic fibrosis due to the

pathogens various and successful virulence mechanisms (Amsalu *et al.*, ٢٠٢٠). *P. aeruginosa* also produces infection of wounds and burns, giving rise to blue-green pus; inflammation of the brain membranes (meningitis) when presented by lumbar puncture or during a neurosurgical procedure; and urinary tract infection when presented by catheters and instruments or in irrigating solutions (Chalmers *et al*., ٢٠١٤; Banar *et al*., ٢٠١٦).

٢.٤. Clinical Importance of *Pseudomonas aeruginosa*

P. aeruginosa is a well-known opportunistic pathogen that has been extensively studied due to its obvious clinical importance. The World Health Organization (WHO) ranked *P. aeruginosa* as the highest priority pathogen in its ٢٠١٧ global priority list of pathogens (Tacconelli , ٢٠١٧). Patients infected with *P. aeruginosa* can also serve as sources of new infections in hospitals (Phillips *et al*., ١٩٨٩). *P. aeruginosa* is a leading cause of nosocomial infections and is frequently antibiotic resistant (Rice,٢٠٠٨).

According to the Centers for Disease Control and Prevention (CDC), MDR *P. aeruginosa* infected ٣٢,٦٠٠ hospitalized patients in ٢٠١٧ (CDC, ٢٠١٧). *P. aeruginosa* is cause significant morbidity, mortality, and cost to healthcare infrastructures worldwide (Founou *et al*., ٢٠١٧).

٢.٥. Infections Caused by *Pseudomonas aeruginosa*

P. aeruginosa is associated with a broad spectrum of clinical diseases of varying duration and severity, *P. aeruginosa* is well-known for causing respiratory infections, most notably in the lungs of cystic fibrosis or chronic obstructive pulmonary disease patients, but it also causes bacteremia, osteomyelitis, endocarditis, otitis, and keratitis

(Stapleton *et al*., ١٩٩٥; Carek *et al*., ٢٠٠١; Sadikot *et al*., ٢٠٠٥; Lin *et al*., ٢٠١٦). According to reports, individuals who have HIV have a ١٠ times higher incidence of *P. aeruginosa*-related bacteremia because of their immunocompromised state (Vidal *et al*., ١٩٩٩). Chronic infections can persist for months, years, or even decades. For example, the vast majority of cystic fibrosis patients carry infections throughout the entirety of their lives (Gaspar *et al*.,٢٠١٣).

P. aeruginosa must first enter and colonize the host before it can cause infection, the organism is frequently acquired through inhalation into the respiratory tract, but because it is so widespread, it is difficult to say exactly how the organism is acquired in all cases (Moore and Flaws, ٢٠١١). Two distinct lifestyles of *P. aeruginosa*, it can be found as planktonic, motile bacteria or attached to a surface, forming communities known as biofilms. In biofilms, which are matrices made of exopolysaccharides, proteins, and extracellular DNA, bacteria are hidden from the host immune system and antibiotic treatments (Høiby *et al*., ٢٠١٠).

P. aeruginosa infections are difficult to eradicate because of their elevated intrinsic resistance as well as their ability to acquire resistance to various antibiotics (Breidenstein *et al*., ٢٠١١). Antibiotic administration and/or prior hospital/ICU stay raises the risk of acquiring MDR *P. aeruginosa* (Raman *et al*., ٢٠١٨). MDR is defined as resistance of the microorganism and insensitivity to more than three classes of antibiotics during the period of infection treatment exposure (Popęda *et al*., ٢٠١٤). The extent of antibiotic resistance of infecting *P. aeruginosa* strains (such as MDR clinical isolates) or specific virulence factors such as the type ٣ secretion system (T٣SS) in clinical isolates have been related to increased mortality rates (Ramírez-Estrada *et al*., ٢٠١٦; Duszynska *et al*., ٢٠٢٢).

P. aeruginosa is also the third most prevalent cause of UTIs, contributing for ٧-١٥٪ of reported infections (Bitsori *et al.*, ٢٠١٢). *P. aeruginosa* is a dangerous pathogen in complicated UTIs, especially in people who use catheters, and can cause life-threatening pyelonephritis (Newman *et al.*, ٢٠١٧). Wounds are highly susceptible to *P. aeruginosa* infections, and it is regarded as a dominating colony of burn wounds for its ability to spread promptly within destroyed tissues cause significant diseases and chronic and acute infections (Gjodsbol *et al.*, ٢٠٠٦). According to a study conducted by Chen and his researchers in (٢٠٢١) reported that ١٧١١٩ patients at a burn ward in China between ٢٠٠٦ and ٢٠١٩, *S. aureus* and *P. aeruginosa* were the main clinical pathogens responsible for bacterial infections in these individuals (Chen *et al.*, ٢٠٢١).

٢.٦. Genome of *Pseudomonas aeruginosa*

The first sequenced genome was the opportunistic pathogenic strain *P. aeruginosa* PAO١ (isolated from a wound) which was published by Stover and his researchers in ٢٠٠٠, and this work had an essential importance for understanding the physiology and virulence capabilities of this pathogen (Stover *et al.*, ٢٠٠٠). Since then, the complete genome of many other species of the genus likes (*P. fluorescens* PfO-١, *P. putida* KT٢٤٤٠, and others) have been published (Vodovar *et al.*, ٢٠٠٦). The versatility and adaptability encoded in the genome of *P. aeruginosa* contribute significantly to its success as an opportunistic pathogen. *P. aeruginosa* has a genome with ٥.٥–٧ Mb is relatively large compared to other sequenced bacteria such as *Bacillus subtilis* (٤.٢ Mbp), *Escherichia coli* (٤.٦ Mbp) and *Mycobacterium tuberculosis* (٤.٤ Mbp), and encodes a large proportion of regulatory enzymes important for metabolism,

transportation and efflux of organic compounds (Zhu *et al.*, ٢٠١٨). This enhanced coding capability of the *P. aeruginosa* genome allows for great metabolic versatility and high adaptability to environmental changes (Stover *et al.*, ٢٠٠٠; Suenaga *et al.*, ٢٠١٧). This genetic repertoire has a conserved core genome of about ٤ Mbp, and the remaining genetic material is made up of different collections of uncommon genes and gene islands. The large size of the *P. aeruginosa* genome reflects the presence of numerous and distinct gene families (Rumbaugh, ٢٠١٤).

The genome of *P. aeruginosa* resembles a classical "secretor" genome, which contains a high percentage of regulatory genes (such as efflux pumps and other transport proteins, motility, and chemotaxis), genes controlling metabolic pathways (which enables adaptation to different metabolic states), and genes encoding a variety of virulence factors and antibiotic resistance determinants (Stover *et al.*, ٢٠٠٠; Bao *et al.*, ٢٠١٤).

The introduction of mobile genetic elements via horizontal gene transfer increases the diversity of the *P. aeruginosa* genome (such as conjugative transposons, insertion sequences, and genomic islands) (Bonomo & Szabó, ٢٠٠٦).

Pan genomics is a novel tool for explaining pangenome construction across species, which is resolved using comparative genomics, among others. *P. aeruginosa* pangenome is composed of two components: a core genome and an accessory genome (Mosquera-Rendón *et al.*, ٢٠١٦). The core genome of *P. aeruginosa* is defined as the genes that are present in nearly all strains of *P. aeruginosa* and encode a set of metabolic and pathogenic factors shared by all *P. aeruginosa* strains, irrespective of origin (environmental, clinical, or laboratory). The core genome constitutes approximately ٩٠٪ of the total genome and is highly

conserved from strain to strain. In contrast, the accessory genome encompasses genes that are found in some *P. aeruginosa* strains but not others (Kung *et al*., ٢٠١٠). However, accessory genetic elements also confer specific phenotypes that are advantageous under certain selective conditions. The accessory genome is made up of DNA elements ranging in size from a few hundred bases to more than ٢٠٠ kbp (Pohl *et al*., ٢٠١٤) . It is a source not only of genes that promote *P. aeruginosa* persistence within various host species by encoding virulence factors but also of genes encoding resistance to multiple classes of antibiotics (Mesaros *et al*., ٢٠٠٧). In microbial genomes, mobile genetic elements (MGEs) including phages, plasmids, and transposons are widely found, These MGEs can be important in processes as pathogenesis and antibiotic resistance (Jackson *et al*., ٢٠١١). MGEs have been found in all *P.* species studied. Phages, transposons, and insertion sequence elements are frequently found within chromosomally integrated islands, indicating that the majority of the accessory genome is derived from mobile DNA elements acquired and maintained by the host strain (Klockgether *et al*., ٢٠١١). Thus, *P. aeruginosa* chromosome is commonly described as a mosaic structure with a conserved core genome that is regularly disrupted by the inserted portions of the accessory genome.

٢.٧. Virulence Factors

٢.٧.١. Cell Surface Virulence Factors

٢.٧.١.١. Biofilm Formation

Biofilms are communities of microorganisms protected by a self-synthesized layer of complex polysaccharides, proteins, lipids, and extracellular DNA, collectively called the extracellular polymeric substance (Fleming & Rumbaugh, ٢٠١٧). Being in a biofilm, microbes

are covered by a lot of advantages, including, but not limited to physical protection from the host immune system and antimicrobials/antibiotics, retention of water and tolerance to desiccation, nutrient sorption and storage, high extracellular enzymatic activity, adhesion to the infection site, and cell aggregation leading to coordination of virulence factor expression via Quorum sensing (QS) . Particularly troubling to the medical field, it has been estimated that as much as ٨٠.٠٪ of all human bacterial infections are biofilm-associated, including more than ٩٠.٠٪ of all chronic wound infections (Romling & Balsalbre, ٢٠١٢). *P. aeruginosa* can form a biofilm in various environments. Biofilms have been known to have a rather complex structure with differentiated bacterial populations and increased resistance against hostile environmental factors, including host immune mechanisms and treatments such as antibiotics (Gebreyohannes *et al*., ٢٠١٩). Evidence indicates that *P. aeruginosa* forms a biofilm in CF lungs where the bacterium lives in an anaerobic environment, as opposed to the aerobic biofilm formed in laboratory conditions. The biofilm mode of growth is recognized as an important bacterial trait that is relevant to infections (Perlin *et al*., ٢٠١٧). The successive stages of the evolution of biofilms include the sticking of the bacteria on the surface, adhesion, growth, cell aggregation within tiny colonies and finally the maturation stage and separation of the original cells to form new colonies of biofilms, (Patricia, ٢٠١٤), (Fig ٢.١).

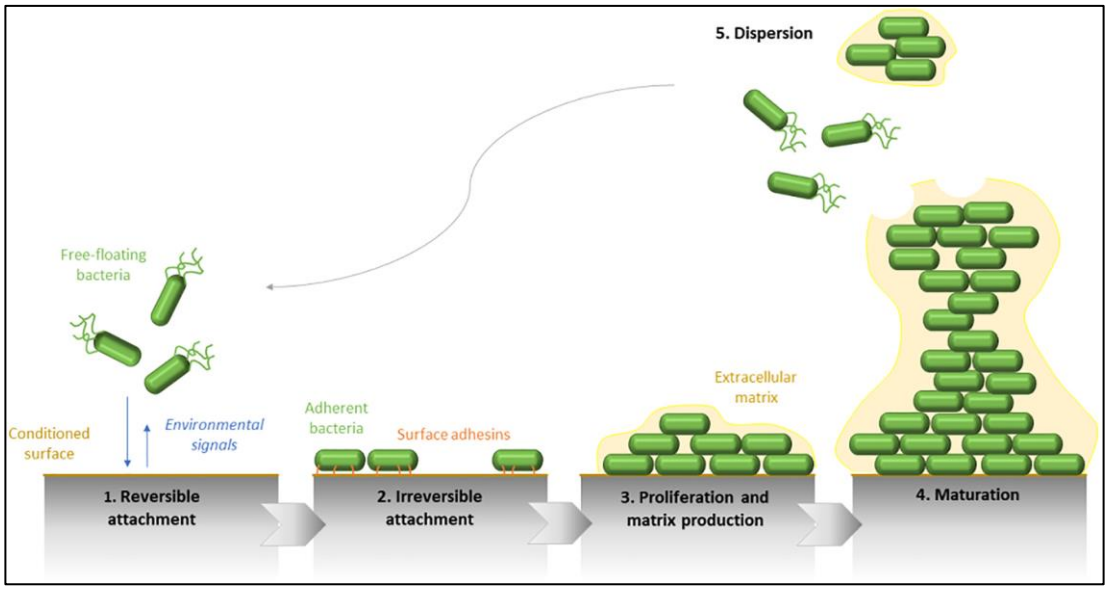

Figure ٢.١: Schematic representation of the five main steps defining the *P. aeruginosa* biofilm development. The biofilm formation begins by the initial attachment of mobile bacterial cells to the surface and is followed by the irreversible adhesion of bacteria, which form a monolayer along the surface. Therefore, biofilm maturation is characterized by the matrix production and the formation of three-dimensional structures. Finally, the biofilm dispersion reflects its life end, (Olivares *et al*., ٢٠٢٠).

Biofilm may form as a result of several factors such as pH depletion, nutrient deficiencies and also to provide necessary protection against host defenses (Sharma *et al*., ٢٠١٤). The ability of microorganisms to form biofilms is an essential survival strategy as it can protect the bacterium from environmental stresses such as extreme pH, metal toxicity and host immune responses (Chen *et al*., ٢٠١٨). In addition to this, biofilms can enhance resistance to killing mechanisms such as antimicrobials or traditional disinfectants (Lineback *et al*., ٢٠١٨). Due to the increased expression of virulence factors, the protection of the bacteria from the host and increased antibiotic resistance within biofilms, *P. aeruginosa* biofilms become almost impossible to eradicate with traditional methods such as the use of antibiotics or other bactericidal products (Koo *et al*., ٢٠١٧).

Many *P. aeruginosa* infections are often associated with biofilm formation which causes persistent colonisation leading to chronic infection (Rasamiravaka *et al*., ٢٠١٥). *P. aeruginosa* biofilms have been found on a variety of medical devices and surfaces within clinical environments including water systems, venous catheters and urinary catheters (Walker and Moore, ٢٠١٥).

Biofilm is one of the most important virulence factors that arise on the surface of bacteria which are embedded within the extracellular matrix (Coulon *et al*., ٢٠١٠; Wei and Ma, ٢٠١٣), and it can be formed on a variety of surfaces such as natural, industrial and hospital niches (Wei and Ma, ٢٠١٣). Biofilm acts as a protective mode of bacteria that allows them to survive in hostile environments and to colonize under desirable conditions (Yang *et al*., ٢٠١١; Wei and Ma, ٢٠١٣), as a protective barrier to antimicrobials and the host immune system (Colvin *et al*., ٢٠١٢). *P. aeruginosa* is an avid biofilm former that is implicated in both chronic and acute infections. It can cause particularly devastating chronic infections or enable life-threatening nosocomial infections in short time courses (Billings *et al*., ٢٠١٣). Three polysaccharides in *P. aeruginosa* are responsible for antibiotic resistance and structure maintenance, such as Psl, Pel, and alginate (Wang *et al*., ٢٠١٥).

Bacterial cells in the biofilm undergo several genetic, metabolic, physiological and phenotypic changes. In *P. aeruginosa*, colonies obtained from biofilms can appear as dwarf, mucoid, hyperpiliated, LPS-deficient, rough, wrinkled, or antibiotic-resistant colonies (Webb *et al*., ٢٠٠٤). Exopolysaccharide Pel overproduction is associated with the wrinkled colony formation, whereas overproduction of alginate is associated with finger-like microcolony formation. Alginate production reduces biofilm susceptibility to antibiotics as well as to human immune

defense mechanisms (Azam & Khan, ٢٠١٩). Genes involved in this process are usually found on operon *algD- algA* PA٣٥٤٠- PA٣٥٥١ that is controlled by *algD* promoter (Wiens *et al*., ٢٠١٤). To trigger inducing and enhancing transcription of this promoter, *AlgT* (*AlgU* or σ٢٢) is used, and to deregulate *AlgT* and convert the strains to mucoid type, *mucA* and *mucB* genes produce inhibitors that inactivate *algT* action. *AlgR* plays a crucial role in regulating *algC* as it binds in three positions downstream and (- ٤٧٩; - ٤٥٧) bp upstream of the transcription starting point. This property resembles enhancers in eukaryotic cells (Aziz *et al*., ٢٠٢١).

Two distinct lifestyles, planktonic and sessile cells, can be adopted by *P. aeruginosa*. The planktonic state can be encountered in a liquid culture suspension, whereas on natural or synthetic surfaces, *P. aeruginosa* can form sticky clusters in permanent rearrangements characterized by the secretion of an adhesive and protective matrix (Filloux and Vallet, ٢٠٠٣). Alginate is an exopolysaccharide and one of the major components of the biofilm in mucoid strains of *P. aeruginosa*. The high molecular weight of this molecule is composed of D-mannuronic and L-guluronic acids, _١-٤ linked and O-acetylated. These enzymes are coded by *algD* operon for alginate synthesis; their expression is regulated by *AlgT* _-factor (Mann and Wozniak, ٢٠١٢).

Alginate attaches to mucin found in the respiratory tract, acting as an adhesin, and its acetyl groups increase the viscosity, which accumulate water and nutrients in the biofilm (Mann and Wozniak, ٢٠١٢).

٢.٧.١.٢. Pili

Pili or fimbriae are the tiny surface appendages filaments of *P. aeruginosa*. Pilli helps colonization because, like flagella, pili are important for colonization adhesion by connecting to the epithelial cell

membrane (Hahn, ١٩٩٧). Type ٤ pili facilitate twitching motility to help the adherence and aggregation of bacterium on target tissue which allows microcolonies of bacteria to protect themselves against the host immune system and antibiotics. Pilin deficient mutants have demonstrated defective *P. aeruginosa* in twitching motility and reduce virulence ability (Laventie *et al*., ٢٠١٩).

The T٤P family is generally subdivided into two groups, type IVa pili (T٤aP) and type IVb pili (T٤bP). This subdivision is based on the sequence of the pilins, the length of the leader peptide, and minor differences in their assembly (Craig *et al*., ٢٠٠٤). The organization of the genes required for pilus formation are different between T٤aP and T٤bP systems. T٤aP genes are spread throughout the genome in several operons, whereas the T٤bP genes are clustered in a single operon (Pelicic, ٢٠٠٨). T٤bP are involved in biofilm formation, bacterial colonization and cell adhesion (Roux *et al*.,٢٠١٢). T٤aP have been identified as the molecular framework behind bacterial twitching motility and other functions such as DNA uptake (Piepenbrink, ٢٠١٩). Their flexibility, elongation and retraction allow them to ensure these different functions. (Craig *et al*., ٢٠٠٤).

The type IV pili of *P. aeruginosa* has a role in adhesion to many cell types and this is likely important in such phenomena as tissue tropism and attachment to particular tissues (Raetz and Whitfield, ٢٠٠٢). Type IV pili of *P. aeruginosa* is a motorized fimbriae composed of repeated copies of a ١٥-kDa protein termed pilin, with three subtypes named Type IVa pili, Type IVb pili and Type IVb-Tad pili. It is associated with bacterial twitching and swarming motility and adhesion on various surfaces, and plays an important role in biofilm formation, regulation of virulence factors, and bacterial exchange of antibiotic resistance genes (Talà *et al*.,

٢٠١٩). Repeated extension and retraction of type IV pili are driven by cytoplasmic ATPases, facilitating bacterial motility, taxis, and attachment, which in turn contribute to self-organization of microcolonies, formation of biofilms, and uptake of DNAs (Craig *et al*., ٢٠١٩). As an important adhesin, type IV pili also enables bacteria to be in intimate contact with surfaces and to influence biofilm formation by regulating cyclic-di-GMP levels (Webster *et al*., ٢٠٢١). Surface-specific behaviors require intimate contact between cells and substrate. In *P. aeruginosa*, contact is mediated by several adhesins, particularly type IV pili (TFP). TFP are long, motorized fimbriae that also provide cells with surface-specific twitching motility and are essential to virulence and biofilm formation. Successive TFP extension, attachment, and retraction promote intimate association with surfaces and motility along them. Because TFP dynamically interact with the substrate, they mechanically couple cells with surfaces. Consequently, although TFP have been viewed as adhesion and motility structures, TFP could also potentially function as mechanical sensors to rapidly signal surface contact (Persat *et al*., ٢٠١٥).

In *P. aeruginosa*, type IV pili are believed to be a major virulence-associated adhesin. Recent models suggest that the pilin subunits from *P. aeruginosa* are organized along either a right-handed one-start helix with a ٤١ Å pitch and four subunits per turn or a left-handed three start helix with also four subunits per turn. Because of its extension from the cell surface, the pilus is believed to be responsible for the initial contact between the bacterium and the epithelial cell surface. Type IV pili bind to the glycolipids asialo-GM١ and asialo-GM٢ on epithelial cell surfaces. The pilin receptor-binding site is only exposed at the tip of the pilus filaments. Pili represents potential vaccine targets because specific antibodies could potentially block the attachment of *P. aeruginosa* to the

host cell receptors (Beaussart *et al*., ٢٠١٤). Type IV pili interact with apical region, on the epithelial surface of the cells (Bucior *et al*., ٢٠١٢).

They are built from *pilA*, a protein subunit that is transported outside the cell by pilQ, a secretin, to produce a fimbrial strand (Laverty *et al*., ٢٠١٤). The most striking structural feature of T٤P is the pilus, which primarily consists of major pilin subunits. This major pilin subunit is called *PilA* in the T٤P model organism *P. aeruginosa*, the major pilins are synthesized as prepilins and targeted to the membrane by an αN-terminal sequence motif, the class III signal peptide. This positively charged signal peptide ensures proper orientation of *PilA* in the inner membrane, The major pilins of T٤aP and T٤bP are distinct, with the Type IVa pili (T٤aP): a very homogeneous and widespread subtype of type IV pili. Their major pilins are characterized by a shorter leader sequence (five or six amino acids) and shorter mature major pilin (~١٥٠ amino acids).

٢.٧.٢. Secreted Virulence Factors

٢.٧.٢.١. Pigments

٢.٧.٢.١.١. Pyocyanin

The genus *P.* can produce a variety of pigments as secondary metabolites, which play a very important role in the pathogenesis of these bacteria (Sismaet *et al*., ٢٠١٧). *P. aeruginosa* may synthesize at least six different pigments: fluorescein, pyoverdine, pyomelanin, aeruginosin A, aeruginosin B, and pyocyanin , Phenazines are the final three pigments on the list (Viana *et al*., ٢٠١٧).

Pyocyanin is a compound soluble in chloroform and diffusible in water, produced at an optimum pH ranging from ٧.٤ to ٨.٤

(Kurashi,١٩٥٨). Pyocyanin, enhances the expression of virulence factors and other phenotypes that converge on *P. aeruginosa*. and are widely known for their capacity for iron uptake from the extracellular medium (Little *et al*., ٢٠١٨). Pyocyanin, a greenish-blue color, comes from the family of the phenazines and plays an important role in the metabolism of iron, being able to participate in reduction mechanisms and releasing iron from transferrins (Jayaseelan *et al*., ٢٠١٤).

The *phzM* gene is responsible for the conversion of phenazine-١-carboxylic acid to pyocyanin (Shi *et al*., ٢٠١٢). At the same time, pyocyanin has been shown to have numerous antagonistic effects, both in vivo and in vitro, due mainly to the pathogenic relation between *P. aeruginosa* and their host organism involving cell damage as the result of pro-inflammatory effects and the release of free radicals (Hall *et al*., ٢٠١٦).

Pyocyanin is a zwitterion with a molecular weight of ٢١٠.٢٣ g/mol (Hall *et al*., ٢٠١٦). Since it is a phenazine, the core structure of the molecule is a pyrazine ring (١,٤-diazabenzene). Pyocyanin is composed of two subunits of N-methyl-١-hydroxyphenazine and easily crosses biological membranes, serving as an electron carrier for *P. aeruginosa*. Preferably, the pigment donates an electron to molecular oxygen, resulting in a blue colour. In addition, the greatest role of pyocyanin in *P. aeruginosa* occurs under anaerobiosis or microaerophilia.

In these cases, pyocyanin accepts electrons generated from NADH during the oxidation of assimilated carbon sources (El Feghali and Nawas, ٢٠١٨). Phenazines (i.e., pyocyanin, PCN) are pigmented bacterial metabolites that have a function on microbial competition and virulence and are secreted in high amounts during the early colonization phase to ensure the establishment of the infection. It exerts a large number of

effects on host cells, including direct damage mainly triggered through formation of reactive oxygen species, alteration of cytokine production, ciliary motion inhibition in human nasal ciliated epithelium and interruption of cell signaling. In addition, they have a role on apoptosis (Bell *et al*., ٢٠١٥) cell cycle arrest (Mossine *et al*., ٢٠١٦) and they induce premature senescence (Muller and Maitz, ٢٠٠٩). Thus, phenazines play an important role on *P. aeruginosa* virulence (Lau *et al*., ٢٠٠٤; Hall *et al*., ٢٠١٦). Pyocyanin is excreted by *P. aeruginosa* to the local climate by secretion system type II (Van Delden and Iglewski, ١٩٩٨). Considerable levels of pyocyanin were detected in sputum (up to ١٣٠ μM), excretion from ear (up to ٢.٧ μM), wounds and urine (up to ٨.١ μM) after *P. aeruginosa* chronic infection (Reimer, ٢٠٠٠).

Two steps have been suggested the synthesis of pyocyanin from phenazine-١-carboxylic acid (PCA), which is the common precursor for many different species-specific phenazines. There are two reaction strategies by which PCA can be converted to pyocyanin. The first, PCA is first acted upon by the enzyme *PhzM*, S-adenosyl methionine (SAM) dependent methyltransferase and gets converted to ٥-methylphenazine-١-carboxylic acid betaine transfer of a methyl group to an N atom of the phenazine-ring moiety, (Fig ٢.٢). This is followed by the action of the enzyme *PhzS*, a FAD-dependent monooxygenase, which involves the hydroxylative decarboxylation of ٥-methylphenazine-١-carboxylic acid betaine to pyocyanin (Mavrodi *et al*., ٢٠٠١).

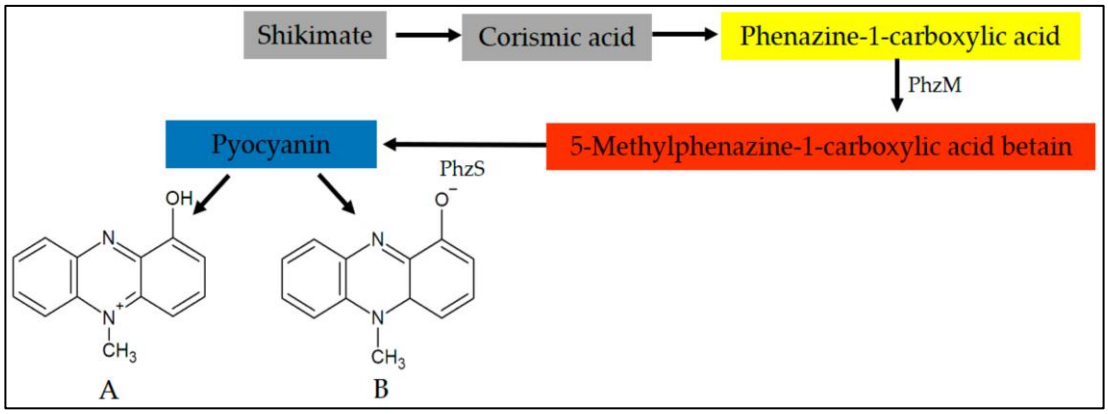

Figure ٢.٢ Steps of the biosynthesis of pyocyanin by *P. aeruginosa* and its oxidated (A) and reduced (B) states, (Gonçalves and Vasconcelos,٢٠٢١).

On the other hand, in the other reaction strategy leading to the biosynthesis of pyocyanin, PCA first gets converted to hydroxyphenazine in presence of the enzyme *PhzS* followed by its methylation to pyocyanin in presence of the SAM dependent methyltransferase, *PhzM* (Parsons *et al*., ٢٠٠٧). In *P. aeruginosa* PAO١, in addition to phzH, two other phenazine-modifying enzymes, *phzM* and *phzS*, were characterized. The *phzM* gene is located upstream of the phzA١B١C١D١E١F١G١ operon and transcribed divergently. It encodes a ٣٣٤ amino acids protein that most closely resembles O-demethylpuromycin-O-methyltransferases with a methyltransferase motif and a S-adenosyl-L-methionine (SAM)- binding domain (Mavrodi *et al*., ٢٠٠١). Functional analysis revealed that the *phzM* gene product is involved in the production of pyocyanin.

The *phzS* gene is located downstream from phzG١ and encodes a ٤٠٢-residue protein similar to bacterial monooxygenases. *PhzS* is thought to be involved in pyocyanin production as well as in the biosynthesis of ١-hydroxyphenazine in *P. aeruginosa* PAO١ (Mavrodi *et al*., ٢٠٠١). The conversion of chorismic acid (CA) to phenazine-١-carboxylic acid (PCA) is controlled by seven genes, encoded by two operons (phzA١B١C١D١E١F١G١ and phzA٢B٢C٢D٢E٢F٢G٢). Then, the synthesis of pyocyanin appears two steps from the PCA . These steps are

regulated by two main genes, *phzM* and *phzS* (El Feghali and Nawas, ٢٠١٨). PCA is converted to ٥- methylphenazine-١-carboxylic acid betaine (MPCBA), by means of a phenazine-specific methyltransferase (*PhzM*). In the second step, MPCBA is catalysed by flavin-dependent monooxygenase (*PhzS*), involving the hydroxylation of the MPCBA betaine to ١-hydroxy- ٥-methyl phenazine, i.e., pyocyanin (Jayaseelan *et al*., ٢٠١٤).

٢.٧.٢.٢. Enzymes

٢.٧.٢.٢.١. Proteases

Proteases are considered an important virulence factor that is capable of damaging host tissues and interfering with host antibacterial defense mechanisms (Oldak and Trafny, ٢٠٠٥). Proteases are enzymes that hydrolyze peptide bonds and can, therefore, degrade proteins and peptides like various plasma proteins such as complement and coagulation factors (Wretlind and Pavlovskis, ١٩٨١). Proteases of *P. aeruginosa* help to establish and maintain infections and thereby controlling and modifying the environment according to the needs of the bacterium within the host tissue (Vidaillac and Chotirmall, ٢٠٢١).

Several potent proteases have been isolated and characterized in *P. aeruginosa* infections such as *P.* elastase (PE), alkaline protease (*AprA*), and protease IV (Caballero *et al*., ٢٠٠١).

P. aeruginosa produces two types elastases: Elastase A (*LasA*) and B (*LasB*). *LasB* (Pseudolysin) and *LasA* (Staphylolysin) are very important virulence factors since they degrade host tissues and immune components such as collagen, immunoglobulin G (IgG), complementary proteins and surfactant proteins A and D. Both enzymes are secreted via type ٢ secretion systems (Le Berre *et al*., ٢٠١١). *LasA*, is a serine protease has

high staphylolytic activity as it can hydrolyze the pentaglycine bridge required for peptidoglycan stabilization in the cell wall of staphylococci. Whereas, it has a low elastolytic ability compared with that of *LasB* and rather it is thought to enhance the proteolytic activity of *LasB*. (Li and Lee, ٢٠١٩). *LasB*, a metalloprotease, cleaves elastin, the components of complement system such as fluid-phase and cell-bound C١ and C٣ and fluid-phase C٥, C٨ and C٩, serum α١-proteinase inhibitor, immunoglobulin A (IgA), IgG, mucins, fibrin, collagen, surfactant proteins A and D. In addition, it increases the permeability of the epithelium by breaking the tight junctions in the respiratory tract epithelium and causes an increase in the number of neutrophils in the infection area.

Elastase exerts a pro-inflammatory effect by stimulating IL-٨ production (Kuang *et al*., ٢٠١١). Mutations in elastase A and B genes have been shown to disarm *P. aeruginosa*, making it impossible for the bacterium to invade host tissues (Cowell *et al*., ٢٠٠٣).

Alkaline protease (*AprA*) is a virulence factor secreted by type I secretion system and controlled by the (QS) circuit (Liao *et al*., ٢٠٢٢). *AprA* is a zinc metalloprotease that has been shown to degrade host complement proteins and host fibronectin (Laarman *et al*., ٢٠١٢). It has a direct function in invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa*. Moreover, it can interfere with flagellin signaling through host Toll like receptor ٥ (TLR٥) by degrading free flagellin monomers and thereby helping *P. aeruginosa* to avoid immune detection (Bardoel *et al*., ٢٠١١). In addition, *AprA* can cleaves interleukin-٦ very efficiently (Matheson *et al*., ٢٠٠٦).

٢.٧.٢.٢.٢. Phospholipase C

Phospholipase C is secreted from the outer membrane of *P. aeruginosa* by the type II secretion system. *P. aeruginosa* produces two types of phospholipase C (*PLC*), hemolytic *PLC* (*PLCH*) and non-hemolytic *PLC* (*PLCN*). Phospholipase C is cytotoxic and has been reported to have a modifying effect on signaling processes in various eukaryotic cells (Barker *et al*., ٢٠٠٤). Elevated levels of *PLCH* were detected in ١٠٠٪ of cystic fibrosis patients with chronic *P. aeruginosa* infections. *PLC* levels in vitro were higher in *P. aeruginosa* isolates from UTIs, when compared to isolates from burns, wounds, CF sputum, pneumonia sputum and blood. However, in a study limited to isolates from tracheal, urinary tract and wound infections, *PLC* was highly produced at every site and a more recent study rarely observed *PLC*. While it is unclear whether there is a definitive role for *PLC* during UTI, it seems plausible that *PLC*-mediated red blood cell lysis and liberation of haem may provide a route for increased iron acquisition by bacteria, though further study is needed (Newman *et al*., ٢٠١٧).

Low concentrations of hemolytic *PLC* can increase IL-٨ expression, and cause excessive neutrophil recruitment involved in pulmonary inflammation and tissue destruction. In addition, hemolytic *PLC* inhibits neutrophil respiratory burst by interfering with a protein kinase C (PKC)-specific, non-p٣٨ kinase-dependent signaling pathway, contributing to *P. aeruginosa* survival in an immune environment rich in neutrophils and, thus, chronic bacterial infection (Terada *et al*., ١٩٩٩). *PLC*-H is hemolytic and acts on substrates like sphingomyelin and phosphatidylcholine. Moreover, *PLC*-H is known to hamper phagocytosis by inhibiting the neutrophil respiratory burst (Terada *et al*., ١٩٩٩). Another study demonstrated that clinical isolates of *Pseudomonas* from patients with urinary tract infections produced high levels of hemolysin (Mittal *et al*.,

٢٠٠٩). While *PLC*-N is non-hemolytic towards human and sheep RBCs, it readily hydrolyzes phosphatidylserine and phosphatidylcholine present in the eukaryotic cell membrane.

The other phospholipase A٢ has been shown to undergo phosphorylation in A٥٤٩ epithelial cells and regulate *P. aeruginosa*-induced inflammation via p٣٨MAPK stimulation in vitro (Bratton *et al*., ٢٠٢١).

٢.٧.٢.٣. Toxins

٢.٧.٢.٣.١. *ExoToxin A*

P. aeruginosa produce an extracellular enzyme known as *ExoT*oxin A. This enzyme belongs to the mono-ADP-ribosyltransferase (ADPRT) family. It is considered one of the most important virulence factors of *P. aeruginosa*. It enables this pathogenic bacterium to survive and initiate infectious responses in host cells (Zahran *et al*., ٢٠٢١). *ExoT*oxin A plays an important role in host pathogenicity by inhibiting host elongation factor ٢ (EF٢) which inhibits proteins synthesis leading ultimately to the death of the host cells (Michalska and Wolf, ٢٠١٥). Additionally, it has direct cytopathic effects and can interfere with cellular immune functions of the host (Dieffenbach and Pastan, ٢٠٢٠).

The studies conducted on strains producing *ExoT*oxin A showed increased virulence compared with deficient mutant strains of *ExoT*oxin A (Zahran *et al*., ٢٠٢١). Toxin A (*ToxA*)–A.K.A., *ExoT*oxin A (*ExoA*), or *Pseudomonas ExoT*oxin (PE)—is an AB toxin secreted by the T٢SS. AB toxins are composed of A and B components where the A component encodes the active enzymatic domain and the B component is responsible for the transport of the A component across the cytoplasmic membrane of target host cells. *ToxA* -induced apoptosis exhibits feature of both intrinsic

and extrinsic apoptosis. *ToxA* -induced apoptosis would be expected to be anti-inflammatory, *ToxA* impacts on immune responses have not been directly investigated.

In one study involving the keratitis model of infection, it was shown that *ToxA* deficient mutant bacteria were rapidly cleared from the eye, with a reduced sign of inflammation at the site of infection. Whether reduced inflammatory responses in the eye were due to reduced bacterial burden or the absence of *ToxA* remains unknown. Due to its potent cytotoxicity, *ToxA* has also been extensively evaluated as a potential anti-cancer immunotoxin therapy. (Wood *et al*., ٢٠٢٣). It has ADP-ribosylation activity and affects the protein synthesis processes in host cells. *ExoA* has been shown to delay wound repair in the animal cutaneious injury model through its effects on cytoskeleton remodeling. Treatment with *ExoA* reduces TJs proteins ZO-١ and ZO-٢ and increases paracellular permeability in type II pneumocyte cultures (Wagener *et al*., ٢٠٢١).

٢.٧.٢.٣.٢. Exoenzyme *(Exo S)*

Exoenzyme S is produced by bacteria in burned tissue and is detectable in the bloodstream even before the bacteria are present. Exoenzyme S may act as an inhibitor of phagocytic cell function in internal organs and the bloodstream, providing protection against *P. aeruginosa* invasion (Engel and Balachandran, ٢٠٠٩).

ExoS is a ٤٨.٣ kDa protein containing ٤٥٣ amino acid length. It has been reported early that *ExoS* participates in host cell apoptosis via its GAP region or ADP-ribosyltransferase (ADPr) activity (Kaminski *et al*., ٢٠١٨). Furthermore, the *ExoS* possesses ADPRT activity, which induces *P. aeruginosa*-afflicted host cell apoptosis by targeting a variety of Ras

proteins (Jia *et al*., ٢٠٠٦). *ExoS* is a bifunctional protein consisting of an N-terminal GTPase Activating Protein (GAP) domain and a C-terminal ADP-ribosyltransferase (ADPRT) domain that is directly translocated into host cytoplasm through the T٣SS (Barbieri, ٢٠٠٠) using SpcS chaperone protein(Da-kang *et al* .,٢٠٠٨). Through these two domains, *ExoS* targets cytoskeletal components in different host cell types, including neutrophils, leukocytes, and epithelial cells (Rangel *et al*., ٢٠١٥). The N-terminal Rho GTPases are critical for actin polymerization and cytoskeletal dynamics (Wagener *et al*., ٢٠٢١). The N terminal domain of *ExoS* is a mimic of eukaryotic RhoGAP domain so that it can prevent small GTPases Rho, Rac, and Cdc٤٢ from activation by keeping them in inactivate GDP bound form (Wagener *et al*., ٢٠٢١). The C-terminus of *ExoS* encodes an ADP-ribosyltransferase (ADPRT) domain which becomes activated after binding to a eukaryotic cofactor (FAS, factor activating *ExoS*)(Nobes *et al* .,١٩٩٥). This domain is able to ADP-ribosylate numerous substrates(Fu *et al* ., ١٩٩٣). ADP-ribosylation of Ras and Rab proteins causes a disruption of the actin cytoskeleton, endocytosis, and vesicular trafficking (Fraylick *et al*.,٢٠٠١).

The requirement for a ١٤-٣-٣ protein – a eukaryotic cofactor – protects the bacteria against self‑damage until *ExoS* is secreted into the host. Once injected, ADPRT activity of *ExoS* becomes active and disrupts the host's actin cytoskeleton, which then interferes with vesicular trafficking and endocytosis, ultimately leading to cell death with the features of apoptosis or necrosis (Rolsma and Frank ٢٠١٤). The disruption of the host's cytoskeleton can also reduce cell–cell adherence, facilitating *P. aeruginosa* invasion through epithelial barriers (Hauser, ٢٠٠٩). *ExoS* is GTPase triggering protein domain, in addition to ADP ribosyl transferase (ADPRT) action. It can stimulate a cytotoxic activity

and is associated with the ability to produce lung impairment and depraved result from infection with this bacterium. The domain ADP ribosyl transferase (ADPRT) in *ExoS* responsible for the ability to obstruct the synthesis of DNA in cultured cells. (Fadhil *et al*.,٢٠١٦).

As for *ExoS*'s impact on host immune responses, *ExoS* has been shown to either dampen or trigger immune responses during infection. In toxication of PBMCs, monocytes, and T cells, with *ExoS*, or recombinant *ExoS* (r*ExoS*), strongly induced transcription of pro-inflammatory cytokines and chemokines, namely, IL-١_, IL-١_, IL-٦, IL-٨, MIP-١_, MIP-١_, MCP-١, RANTES (Wood *et al*., ٢٠٢٣).

٢.٧.٢.٣.٣. *ExoToxin U*

ExoU is the most acutely cytotoxic. It was identified in ١٩٩٧ as an effector secreted by *P. aeruginosa* strain PA١٠٣. The gene encoding *ExoU* was identified as part of a mutant library screen and verified by peptide sequence analyses (Hardy *et al*., ٢٠٢١).

ExoU is the longest *P. aeruginosa* effector (٦٨٧ amino acids), having a molecular size of ٧٣.٩ KDa (Winsor *et al*., ٢٠١٦). The *ExoU* gene is found within an operon downstream of the spcU gene, which, as commented above, encodes its chaperone. *ExoU* is considered the least prevalent of T٣SS effectors in *P. aeruginosa*. Thus, despite having a prevalence of ٩٠ % in a study performed in *Mex*ico (Morales-Espinosa *et al*., ٢٠١٧), in most studies the prevalence varies from ٢٥ to ٥٠ % (Bel Hadj Ahmed *et al*., ٢٠٢٠). *ExoU* is the only *P. aeruginosa* T٣SS effector encoded within a Genomic Island environment (Harrison *et al*., ٢٠١٠). *ExoU* dysregulates the host's innate inflammatory response by poisoning and killing immune cells, including macrophages, neutrophils, epithelial cells, and endothelial cells, allowing bacteria to persist, proliferate, and

spread, and ultimately leading to sepsis, Alzheimer's disease, acute respiratory distress syndrome, etc. (Kaminski *et al*., ٢٠١٨). *ExoU* is associated with carrying phospholipase action playing a role in the hydrolyzing of the ester bond in cell membrane phospholipids resulting in membrane disturbance, elaboration of fatty acid (Kadim and Abed ٢٠٢٢). Analysis of its protein sequence reveals a patatin‐like domain with phospholipase A٢ (PLA٢) activity between residues ١٠٧ and ٣٥٧. PLA٢ enzymes hydrolyze the ester bond of phospholipids at the SN٢ position, resulting in the release of free fatty acids and lysophospholipids. To facilitate host cell lysis, *ExoU* contains a membrane localization domain between residues ٥٥٠–٦٨٧ that directs *ExoU* to the phospholipid plasma membrane of the host cell. To ensure that this potent weapon is not turned against the bacteria itself, *ExoU* requires activation by a eukaryotic cofactor: Cu^{2+}, Zn^{2+}‐ superoxide dismutase ١.

Taken altogether, *ExoU* may be used to kill the host's epithelial cells and immune cells, thereby promoting bacterial invasion and dissemination (Yang *et al*., ٢٠٢٢). In epithelial cells, *ExoU* expression is associated with rapid cellular lysis that occurs within ٣ h of co-culture with *P. aeruginosa*. Cytotoxicity was so rapid that to mechanistically understand how *ExoU* functioned intracellularly, a system using *Saccharomyces cerevisiae* as a surrogate expression host was developed. *ExoU* synthesis could be controlled in yeast experimental model and the yeast cell wall protected the cell long enough to facilitate visualization of *ExoU*-induced damage to intracellular membranes and vacuolar fragmentation. *ExoU* was postulated to function as a phospholipase hydrolyzing neutral lipids and phospholipids. This model reconciled the rapid cytotoxicity in vitro and significant tissue destruction mediated by

ExoU producing strains in animal models of infection (Hardy *et al*., ٢٠٢١).

٢.٧.٢.٤. Secretion Systems

The *P. aeruginosa* has six distinct types that can be distinguished by the characteristics of the proteins/components that make up the secretion machine. These are known as type I to type VI secretion systems (T١SS–T٦SS) (Durand *et al*., ٢٠٠٩). It is also possible to divide the secretion systems into two categories. Those that transport proteins at once across the bacterial cell envelope, thus directly from the cytoplasm to the cell surface, are known as one-step secretion mechanism. Alternatively, the secreted proteins could transit through the periplasm before crossing the outer membrane. These systems are known as two-step secretion mechanism (Filloux, ٢٠١١). Among the known secretion systems of *P. aeruginosa*, the type III secretion system (T٣SS) is the most important in human pathogenesis and is involved in host invasion by injecting toxins directly into eukaryotic cells.

٢.٧.٢.٤.١. Structure of T٣SS

The T٣SS of *P. aeruginosa* is a complex machinery composed of a needle complex, a translocon, effectors, chaperones, and a regulatory system, (Fig ٢.٣) (Hauser, ٢٠٠٩). The Type-III secretion systems (T٣SS) has been identified as a major virulence determinant for poor clinical outcomes in intensive care unit (ICU)-acquired pneumonia (ICUAP), keratitis, and otitis externa (Park *et al*., ٢٠١٧). The T٣SS comprises the needle complex, it consists of a multi-ring base and a needle-like filament, membrane-anchored, multi-component complexes on certain pathogenic bacteria—including *Pseudomonas*—that inject e_ectors from

the bacteria's cytosol directly into the cytoplasm of the eukaryotic host cell (Foulkes *et al*., ٢٠١٩). The function of T٣SS is regulated by various regulators, including four main regulators genes (*exsA*, *exsC*, *exsD*, and *exsE*), which is involved in the transcription activation of the aforementioned classical effectors (*ExoS*, *ExoT*, *ExoU*, and *ExoY*) (Qin *et al*., ٢٠٢٢).

The secretion system that has been identified in Gram-negative bacteria is a third (type III) which inject Pseudomonal toxins directly into the adjacent host cells by T٣SS to begin the infection. This system secrets four identified effector proteins (Exoenzymes): *ExoA*, *ExoT*, *ExoS*, *ExoY* and *ExoU* (Naher *et al*.,٢٠١٤).

The regulation of T٣SS is complex and involves a variety of players. *ExsA* is the general transcriptional regulator binding promoter of T٣SS genes, consisting of its own promoter. Three additional proteins, *ExsC*, *ExsD*, and *ExsE*, control *ExsA* activity through a "catch and release" mechanism depending on whether *P. aeruginosa* is in contact or not with host cells (Hauser, ٢٠٠٩).

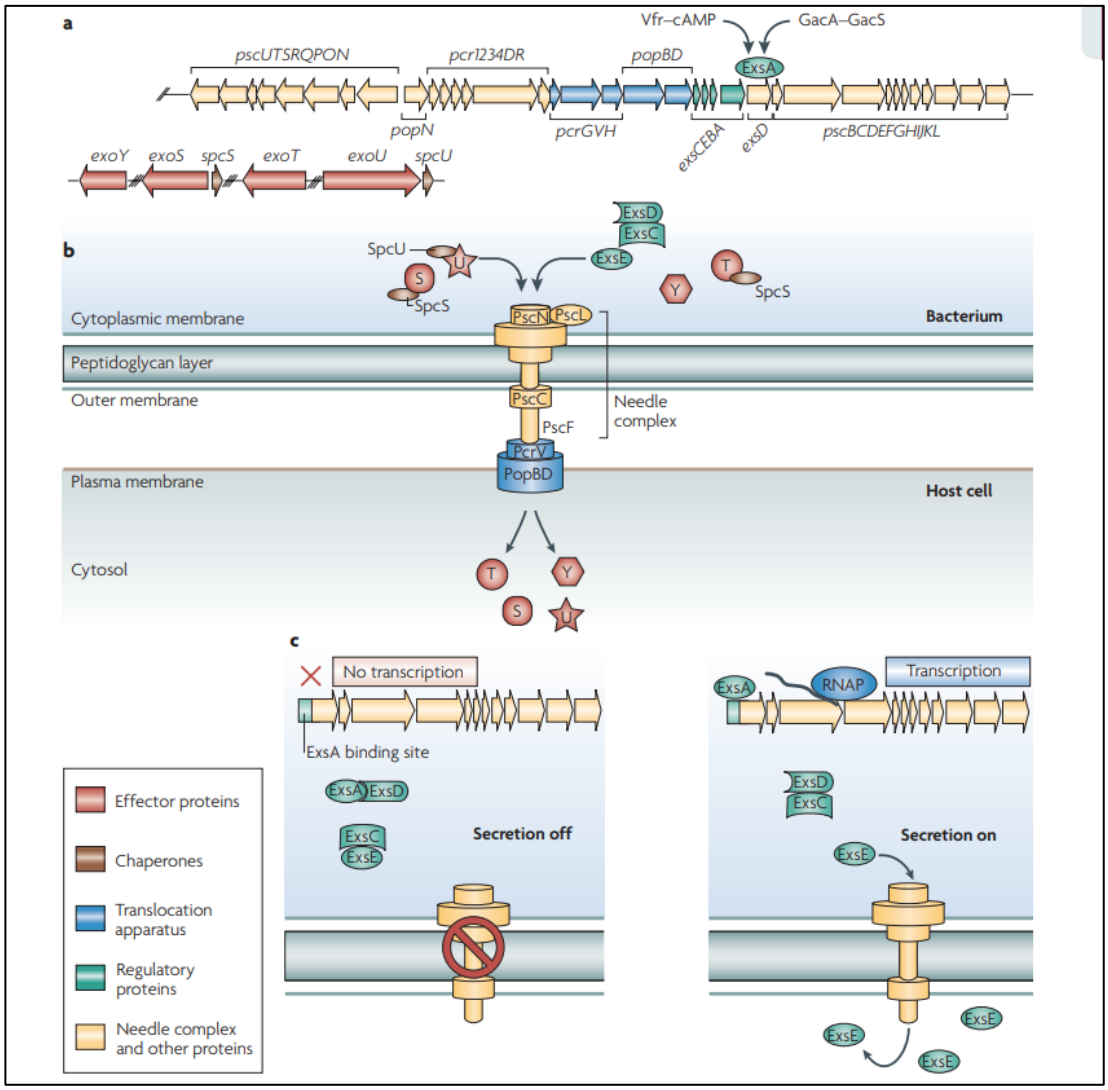

Figure ٢.٣: An overview of *P. aeruginosa* type III secretion. a: Thirty-six genes encoded in five clustered operons, together with at least six other genes scattered around the chromosome, are involved in the type III secretion system. b: The type III secretion system (T٣SS) can be functionally divided into five components: the needle complex, the translocation apparatus, the regulatory proteins, the effector proteins and the chaperones. These five parts work together to inject effector proteins into host cells in a highly regulated manner. c: The linkage of T٣SS transcription to protein secretion is achieved through the interactions of four proteins: *ExsA*, *ExsC*, *ExsD* and *ExsE*. When secretion is turned off, *ExsE* accumulates in the bacterium and binds *ExsC*, allowing *ExsD* to bind *ExsA* and thus preventing the transcription of the type III secretion genes. When secretion is activated, the regulatory protein *ExsE* is secreted from the cell, allowing *ExsC* to bind *ExsD*. This sequestration of *ExsD* frees the transcriptional activator *ExsA* and results in the unimpeded transcription of type III secretion genes. cAMP, cyclic AMP; *exo*, exoenzyme; RNAP, RNA polymerase; S, *ExoS*; T, *ExoT*; U, *ExoU*; Y, *ExoY*, (Hauser, ٢٠٠٩).

The T٣SS is a crucial mechanism for the release of invasion factors by Gram-negative bacteria. It may also help the bacteria evade host

immune responses and initiate invasion, colonization, replication, and spread. Although almost all *P. aeruginosa* strains harbour the genes that encode the type III secretion machinery, most strains do not carry a complete set of the genes that encode the effectors (Iglewski *et al*.,١٩٧٨; Fleiszig *et al*., ١٩٩٧).

٢.٧.٢.٥. Efflux Pump

The *P. aeruginosa* efflux pumps are classified into five families with the Resistance Nodulation division (RND) family playing a key role in antibiotic resistance via *MexAB-OprM*, *MexCD-OprJ*, *MexEF-OprN*, and *MexXY-OprM* (Goli *et al*., ٢٠١٨). *P. aeruginosa* exploits efflux pumps to achieve high degree of resistance in coordination with outer membrane barriers and biofilms (Nikaido, ٢٠٠٣; Li *et al*., ٢٠١٥). Efflux pumps are the main cause of MDR rendering existing antibiotics ineffective and insisting for new therapeutic options (Roberts and Simpson, ٢٠٠٨). These efflux pumps are directly or indirectly responsible for the development of superbugs because these pumps not only make the antibiotics inefficient but also narrow down treatment option against GNB (Draenert *et al*., ٢٠١٥).

Multidrug efflux systems of the RND family are wide spread among Gram-negative bacteria and catalyze the\aQ active efflux of a large diversity of molecules, including antibiotics, and thus significantly contribute to intrinsic and acquired resistance to these molecules (Li *et al*., ٢٠١٥). Despite their significance as determinants of antibiotic resistance, RND multidrug exporters also accommodate biocides, detergents (Poole, ٢٠٠٥), organic solvents (Ramos *et al*., ٢٠٠٢), human or animal metabolic compounds such as bile salts (Thanassi *et al*., ١٩٩٧), toxic fatty acids (Shafer *et al*., ١٩٩٥), plant-derived antimicrobials like

phytoalexins (Burse *et al*., ٢٠٠٤), (QS) effector molecules (Evans *et al*., ١٩٩٨), and virulence factors (Hirakata *et al*., ٢٠٠٢). *P. aeruginosa* possesses at least ١٢ RND efflux pumps, of which four are of clinical relevance and are the main contributors to antibiotic resistance through efflux (Stover *et al*., ٢٠٠٠; Alcalde- Rico *et al*., ٢٠١٨).

All RND efflux pumps cross the cytoplasmic membrane, the periplasm and the outer membrane (Nikaido, ٢٠١٠). The RND efflux pump complex is comprised of the secondary active efflux pump protein embedded in the inner membrane, the outer membrane factor protein, and a membrane fusion protein that extends through the periplasm and connects the two (Daury *et al*., ٢٠١٦; Hernando-Amado *et al*., ٢٠١٦).

Bacterial efflux pumps are categorized into five families, the two ancient super families that are made up of the adenosine triphosphate binding cassette (ABC) and the major facilitator super family (MFS), and three smaller families that consist of the multidrug and toxic compound extrusion family (MATE), the small MDR family (SMR) and the resistance nodulation-cell division family (RND) (Marquez, ٢٠٠٥). RND efflux pumps are the main multidrug efflux systems responsible for antibiotic extrusion and resistance in *P. aeruginosa* (Poole, ٢٠٠١). Efflux pumps are membrane proteins that are involved in the export of noxious substances from the bacterial cell into the external environment. According to their composition, the number of transmembrane spanning regions, energy sources and substrates, *P. aeruginosa* has a significant number of efflux pumps, including four potent RND-type multidrug resistance efflux pumps (*Mex*) that remove harmful chemicals from the periplasm and cytoplasm (Dwivedi *et al*., ٢٠١٧).

The up-regulation of multidrug efflux systems is one of the most important mechanisms leading to multidrug resistant (MDR) phenotype.

Efflux pumps are transport proteins involved in the extrusion process of toxic substances from the cell, resulting in antibiotic resistance (Puzari & Chetia, ٢٠١٧). RND efflux pumps are common in Gram-negative bacteria and are almost always chromosomally encoded (Colclough *et al*., ٢٠٢٠). A number of ١٢ systems belonging to the RND family have been described in *P. aeruginosa* (Poole, ٢٠٠٧). Among these systems, the overexpression of *MexAB-OprM*, *MexCD-OprJ*, *MexEF-OprN*, or *MexXY* (-*OprA*) has been reported to play a relevant role in reduced susceptibility towards antibiotics (Poole, ٢٠٠٥; Jeannot *et al*., ٢٠٠٨).

٢.٧.٢.٥.١. *MexAB-OprM*

The *MexAB-OprM* efflux pump is constitutively active in *P. aeruginosa* wild-type strains and, along with other RND efflux systems, causes low level resistance but can also significantly contribute to the development of multi-drug resistance (Goli *et al*., ٢٠١٦).

When the drug concentration increases near the pump, *MexB* goes through a conformational change and can eject active molecules toward a tunnel formed by *MexA* and *OprM* across the periplasm and outer membrane (Tsutsumi *et al*., ٢٠١٩). *MexAB-OprM* is constitutively expressed in wild-type strains, and its expression is controlled mainly by the repressor genes *MexR* (Poole *et al*., ١٩٩٦), *nalc* (Cao *et al*., ٢٠٠٤), and *nalD* (Morita *et al*., ٢٠٠٦). The use of antibiotics selects *P. aeruginosa* strains with increased *MexAB-OprM* expression (Ziha *et al*., ١٩٩٩; Tafti *et al*., ٢٠٢٠). *MexAB-OprM* extrudes carbapenems, chloramphenicol, fluoroquinolones, lincomycin, macrolides, novobiocin, penicillins, tetracyclines and all b-lactams (except imipenem and biapenem), as well as the antiseptic triclosan and the surfactant sodium dodecyl sulfate. *MexAB-OprM* null mutants are markedly susceptible to

all of the aforementioned antimicrobials, whilst mutants that overproduce *MexAB-OprM* show high levels of resistance (Masuda and Ohya, ١٩٩٢; Li *et al*., ٢٠١٩; Srikumar *et al*., ١٩٩٨; Köhler *et al*., ١٩٩٩; Chen *et al*., ٢٠١٨).

Overexpression of this efflux pump is associated with resistance to most antipseudomonal antibiotics, except for colistin (Rahbar *et al*.,٢٠٢١), and carbapenemase -producing carbapenem resistant *P. aeruginosa* strains often show an increased *MexAB-OprM* expression, which could be contributing to its resistance to carbapenems (Beig *et al*.,٢٠٢٠). Moreover, it has been shown that overexpression of *MexAB-OprM* and *AmpC*, a chromosomally encoded class C _- lactamase that contributes to the resistance to many penicillins (Lodge *et al*., ١٩٩٠; Glen and Lamont, ٢٠٢١). The *MexA*B–*OprM* system in the clinical isolates of *P. aeruginosa* is highly conserved (Tian *et al*.,٢٠١٦), and different regulatory loci, such as the regulatory genes *nalD*, *nalc*, and *MexR*, influence the expression of this operon. Such regulatory genes negatively regulate the expression of *MexA-MexB-OprM* operon in clinical strains (Pan *et al*.,٢٠١٦).

Loss of a functioning *MexR* causes hyperexpression of *MexAB-OprM*. Mutations in *MexR* found in clinical *P. aeruginosa* isolates can lead to the expression of *MexR* protein that is no longer able to dimerize or bind to DNA to repress *MexAB-OprM* expression. In addition, mutations leading to premature termination of the *MexR* peptide resulting in complete absence of the production of a functioning *MexR* have been identified in clinical isolates (Choudhury *et al*., ٢٠١٥; Pan *et al*., ٢٠١٦).

The chromosomal RND-type efflux systems play a significant role in the initiation and progress of clinical antibiotic resistance, bacterial pathogenesis, virulence, and biofilm maturation in Gram-negative

bacteria, particularly *P. aeruginosa*. *MexAB-OprM* system is a multidrug resistant efflux pump with high levels of expression and the first finding caused a significant increase in the uptake of ٨-anilino-١-naphthylenesulfonic acid (a fluorescent hydrophobic probe) and sensitized *P. aeruginosa* to bulky antibiotics (e.g. vancomycin) that are typically incapable of crossing the outer membrane, as well as to detergent-like bile salts (Lamers *et al*., ٢٠١٣).

٢.٨. Antibiotic Resistance in *Pseudomonas aeruginosa*

Antimicrobial resistance is defined broadly as an organism's ability to resist the action of an antimicrobial agent to which it was previously susceptible (Bagge *et al*., ٢٠٠٤). Antibiotic resistance mechanisms are classified into three types: intrinsic, acquired, and adaptive. Intrinsic resistance mechanisms are those that are genetically encoded in the organism's core genome, whereas adaptive resistance mechanisms are those that are induced by environmental stimuli, and acquired resistance results from the acquisition of resistance genes from other organisms or from the selection of advantageous mutations (Tenover, ٢٠٠٦).

The number of bacterial strains that are resistant to treatment has increased. MDR is defined as resistance of the microorganism and insensitivity to more than three classes of antibiotics during the period of infection treatment exposure (Popęda *et al*., ٢٠١٤). Antibiotics work in two ways: either by inhibiting the metabolic pathway or by competing with the base material of enzymes involved in cell wall synthesis. where it has four major targets, the most important of which are DNA replication and repair, metabolic pathways, and cell wall and protein synthesis (Chethana *et al*., ٢٠١٣).

P. aeruginosa is intrinsically resistant and has numerous mechanisms that lead to antibiotic resistance. These mechanisms serve as a means of defending antibiotic-resistant strains and ensuring their survival by inhibiting the effectiveness of pharmaceutical compounds. Because these organisms are not affected by the drug, they develop multiple resistances (Pang *et al*., ٢٠١٩).

P. aeruginosa has been shown to have a high level of intrinsic resistance to most antibiotics via restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell, and the production of antibiotic-inactivating enzymes such as *β*-lactamases (Breidenstein *et al*., ٢٠١١). The majority of antibiotics used to treat *P. aeruginosa* infections must be able to enter cells and reach intracellular targets. A *β*-lactam ring is found in the molecular structures of *β* -lactam antibiotics such as penicillin, cephalosporin, carbapenem, and monobactam. This antibiotic class blocks bacterial cell wall biosynthesis by targeting penicillin binding proteins, which are enzymes involved in peptidoglycan synthesis (Poole, ٢٠٠٤). *β* -lactams and quinolones enter the bacterial cell via porin channels, whereas aminoglycosides and polymyxins promote their own uptake by interacting with bacterial LPS on the outer membrane of Gram-negative bacteria (Lambert, ٢٠٠٢). Gram-negative bacteria's outer membrane, which serves as a selective barrier to prevent antibiotic penetration, is an asymmetric bilayer of phospholipid and LPS embedded with porins that form -barrel protein channels (Delcour, ٢٠٠٩).

P. aeruginosa expresses twelve Resistance Nodulation division family efflux pumps, four of which contribute to antibiotic resistance (*MexAB-OprM*, *MexCD-OprJ*, *MexEF-OprN*, and *MexXY-OprM*). *MexAB-OprM* is responsible for efflux of *β*-lactams and quinolones (Dupont *et al*., ٢٠٠٥). The effectiveness of nonenzymatic mechanical

efflux pumps is important in the development of many other mechanisms and plays a role in resistance (Livermore, ٢٠٠١). Multiple efflux pump overexpression has been observed in some clinical strains of *P. aeruginosa,* broadening bacterial antibiotic resistance and contributing to the development of multidrug resistance (Shigemura *et al*., ٢٠١٥).

Bacteria can acquire antibiotic resistance through mutational changes or through horizontal gene transfer. When exposed to a variety of environmental stimuli, bacteria develop adaptive resistance to increase antibiotic resistance through transient changes in gene and/or protein expression via a variety of methods (Motta *et al*., ٢٠١٥). Acquired resistance resulting from mutations imparts resistance against several classes of antibiotic, including *β*-lactams, fluoroquinolones, aminoglycosides, and polycationic antimicrobials (Munita and Arias ٢٠١٦). Spontaneous mutations can affect the expression or function of a specific porin, thereby reducing bacterial membrane permeability and increasing antibiotic resistance (Fernandez and Hancock, ٢٠١٢). Porins form small water-filled channels within membranes that mediate the diffusion of hydrophilic antibiotics, up to a certain size exclusion limit (Welte *et al*., ١٩٩٥).

The formation of biofilms is the most common strategy used by *P. aeruginosa* to acquire adaptive antibiotic resistance (Maurice *et al*., ٢٠١٨). In addition to *P. aeruginosa*'s high level of intrinsic antibiotic resistance, acquired resistance greatly contributes to the development of multidrug-resistant strains, making eradication more difficult and leading to more cases of persistent infections (Henrichfreise *et al*., ٢٠٠٧).

Chapter Three

Materials and Methods

٣.١. MATERIALS

٣.١.١. Equipment and Apparatus

Equipment and apparatus used in the present study are listed in Table (٣.١).

Table (٣.١): List of laboratory equipment and supplies

Laboratory Equipment	Manufacturer/Origin
Autoclave	Hirayama (Japan)
Benson Burner	Satorins (Germany)
Calibrated Loop ٠.٠١	Himedia (India)
Disposable and Glassware	Cito (China)
Eppendorf Tube	Sigma (UK)
Gel Documentation System	Vilber (France)
Gel Electrophoresis System	Cleaver Scientific (UK)
High Speed Cooling Centrifuge, Eppendorf Centrifuge	Hettich (Germany)
Laboratory Distillation Unit	GFL (Germany)
Laminar Flow cabinet	Cryste (Korea)
Latex Gloves	Broche (Malaysia)
Micropipette (١٠ µl,٢٠٠µl,١٠٠٠µl)	Sartorius (Germany)
NanoDrop	Implen (Germany)
Oven, Incubator	Memmert (Germany)
PCR Thermal Cycler	Techne (UK)
PCR tube ٢٠٠µl	Biobasic (Canada)
Refrigerator	Concord (Lebanon)
Sensitive Balance	Denver (USA)
UV-Vis Spectrophotometer	Analytic Jena (Germany)
Vortex	Gemmy (Taiwan)

٣.١.٢. Biological and Chemical Materials

The biological and chemical materials used in this study are listed in Table (٣.٢).

Table (٣.٢): Biological and chemical materials with their suppliers

Biological and Chemical Materials	**Manufacturer/Origin**
Agarose	Condalab (spain)
Congo Red	Himedia (india)
Absolut Alcohol	Sharlab (Spain)
Glycerol	Fluka (Switzerland)
Normal Saline	Mehico (India)
Nuclease Free Water	Bioneer (Korea)
Primer	Macrogen (Korea)
Simple Safe (Red Safe) Stain	Eurx (Poland)
Tris-Borate-EDTA (TBE) buffer	Himedia (India)

٣.١.٣. Antibiotic Disks

The antibiotic discs used in this study are listed in Table (٣.٣).

Table (٣.٣): The antibiotic discs used in this study

Antibiotic Group	**Antibiotic Disk**	**Concentration (µg/disk)**	**Assembly**	**Company / Origin**
Penicillins	Ampicillins	١٠	AMP	Himedia /India
	Piperacillin	١٠٠	PRL	Bioanalyse/Turkey
	Piperacillin-Tazobactam	١٠٠/١٠	TZP	Liofilchem/Italy
	Ticarcillin	٧٥	TIC	Biolab Zrt./Hungary
Cephalosporins	Ceftazidime	٣٠	CAZ	Bioanalyse/Turkey
	Cefepime	٣٠	FEP	Liofilchem/Italy
Monobactam	Aztreonam	٣٠	ATM	Bioanalyse/Turkey
Carbapenems	Doripenem	١٠	DOR	Liofilchem/Italy
	Ertapenem	١٠	ETP	Biolab Zrt./Hungary
	Imipenem	١٠	IPM	Bioanalyse/Turkey
	Meropenem	١٠	MEM	Bioanalyse/Turkey
Aminoglycosides	Amikacin	٣٠	AK	Liofilchem/Italy
	Gentamicin	١٠	CN	Bioanalyse/Turkey

	Tobramycin	١٠	TOB	Bioanalyse/Turkey
Tetracyclins	Tetracyclin	٣٠	TE	Liofilchem/Italy
	Doxycyclin	٣٠	DXT	Liofilchem/Italy
Macrolides	Erythromycin	١٥	E	Liofilchem/Italy
Fluoroquinolones	Ciprofloxacin	٥	CIP	Himedia /India
	Levofloxacin	٥	LEV	Bioanalyse/Turkey
	Ofloxacin	٥	OFX	Bioanalyse/Turkey

٣.١.٤. Primers

Table (٣.٤): List of primers sets and amplicon size

Gene	Sequence (٥′–٣′)	Amplicon size (bp)	Reference
exoU	F: CCAACACATTAGCAG CGAGA	٩٤	Shi *et al*., ٢٠١٢
	R: TGGGAGTACATTGAGCAGCA		
ecfX	F: ATGCCTATCAGGCGTTCCAT	١٤٦	Colinon *et al.,* ٢٠١٣
	R GGCGATCTGGAAAAGAAATG		
exoS	F: CATCCTCAGGCGTACATCCT	٢٤٠	Shi *et al*., ٢٠١٢
	R: ATCGATGTCAGCGGGATATC		
ToxA	F: ATGGTGTAGATCGGCGACAT	٣٩٧	Shi *et al*., ٢٠١٢
	R: AAGCCTTCGACCTCTGGAAC		
pilB	F: TCGAACTGATGATCGTGG	٤٠٨	Fazeli and Momtaz,٢٠١٤
	R: CTTTCGGAGTGAACATCG		
apr	F: TGTCCAGCAATTCTCTTGC	١٠١٧	Fazeli and Momtaz,٢٠١٤
	R: CGTTTTCCACGGTGACC		
lasA	F: GCAGCACAAAAGATCCC	١٠٧٥	Fazeli and Momtaz,٢٠١٤
	R: GAAATGCAGGTGCGGTC		
lasB	F: ACAGGTAGAACGCACGGTTG	١٢٢٠	Finnan *et al.*, ٢٠٠٤
	R: GATCGACGTGTCCAAACTCC		
pilA	F: ACAGCATCCAACTGAGCG	١٦٧٥	Fazeli and Momtaz,٢٠١٤
	R: TTGACTTCCTCCAGGCTG		
phzS	F: TCGCCATGACCGATACGCTC	١٧٥٢	Fazeli and

	R: ACAACCTGAGCCAGCCTTCC		Momtaz,٢٠١٤
phzM	F: ATGGAGAGCGGGATCGACAG	٨٧٥	Fazeli and Momtaz,٢٠١٤
	R: ATGCGGGTTTCCATCGGCAG		
PLCN	F: TCCGTTATCGCAACCAGCCCTACG	٤٨١	Fazeli and Momtaz,٢٠١٤
	R: TCGCTGTCGAGCAGGTCGAAC		
PLCH	F: GCACGTGGTCATCCTGATGC	٦٠٨	Fazeli and Momtaz,٢٠١٤
	R: TCCGTAGGCGTCGACGTAC		
algD	F: AAGGCGGAAATGCCATCTCC	٢٩٩	Wozniak and Ohman,١٩٩٤
	R AGGGAAGTTCCGGGCGTTTG		
algU	F: CGCGAACCGCACCATCGCTC	١٥٤٧	Schurr *et al*., ١٩٩٤
	R: GCCGCACGTCACGAGC		
mexA	F: CTCGACCCGATCTACGTC	٥٠٣	Al-Grawi *et al*,٢٠١٢
	R: GTCTTCACCTCGACACCC		
mexB	F: TGTCGAAGTTTTTCATTGAG	٢٨٠	Su and Wang ,٢٠١٨
	R: AAGGTCAC GGTGATGGT		
oprM	F: GATCCCCGACTACCAGCGCCCCG	٢٤٧	Su and Wang ,٢٠١٨
	R: ATGCGGTACTGCGCCCGGAAGGC		

٣.١.٥. Kits, Marker and Reference strain

Kits and markers used in this study are listed in Table (٣.٥)

Table (٣.٥): Kits and marker

Name of Kit	Manufacturer/Origin
١٠٠ bp DNA Ladder	Bioneer (Korea)
١٠٠ bp DNA Ladder	SolGent (Chain)
FavorPrep Genomic DNA Mini Kit	Favorgen (Taiwan)
P. aeruginosa ATCC ٢٧٨٥٣	ATCC(USA)
Taq Green Master Mix	Promega (USA)
Vitek ٢ System	Biomerieux (France)

٣.٢. METHODS

٣.٢.١. Sample Collection

A total of ٣٨٤ specimens were obtained from various hospitals, in Babylon province from March /٢٠٢٢ to November/ ٢٠٢٢. One hundred thirty *P. aeruginosa* isolates from different sources including: (burn, wound, urine, ear and sputum) were recovered from patients. Specimens were labeled and transported to the laboratory of Biology Department/ College of Science/ university of Babylon by transport medium to preserve the sample, for additional examination using common microbiological and biochemical characterization.

٣.٢.٢. McFarland's Turbidity Standard No. ٠.٥

The ٠.٥ McFarland**'s** standard (١.٥×١٠^{٨} CFU/ml) was prepared by adding ٠.٠٥ ml of ١.١٧٥٪ barium chloride [$BaCl_{٢}.٢H_{٢}O$: H٢O (w/v, ١.١٧٥g: ٩٨.٨٢٥ ml)] to ١٪ sulfuric acid [$H_{٢}SO_{٤}$: $H_{٢}O$ (v/v, ١ ml: ٩٩ ml)] in order to obtain a barium sulfate precipitate. The solution was used to visually compare the turbidity of a suspension of bacteria with the turbidity of the ٠.٥ McFarland**'s** standard.

٣.٢.٣. Primers Dilution

All the primers were synthesized at (Macrogen/Korea) these were provided in a lyophilized form , which were re-dissolved with nuclease free water according to instructions of manufacturing company to reach to the final concentration (١٠ Pmol/µl).

٣.٢.٤. Preparation of Culture Media

All culture media were prepared according to the instructions of the manufacturing instractions, and then sterilized by autoclaving at ١٢١^{o}C at ١٥ pis per square inch for ١٥ min as in table (٣.٦).

Table (٣.٦): Culture media

Media	Purpose and Preparation	Manufacturer /

		Origin
Cetrimide Agar	Cetrimide Agar is used as a selective medium for the isolation of *Pseudomonas aeruginosa,* also used for determining the ability of an organism to produce fluorescein and pyocyanin. Dissolve ٤٦.٧ g in ٩٩٠ ml distilled water and add ١٠ ml glycerol	Himedia (India)
Nutrient Agar (NA)	NA is a general-purpose medium which can be used for cultivation of a non-highly nutritious bacteria (McFadden, ٢٠٠٠). The NA was prepared by dissolving ٢٨ g in ١٠٠٠ ml.	
Brain Heart Infusion Broth (BHIB)	BHIB is a highly nutritious general-purpose growth medium recommended for the cultivation (McFadden, ٢٠٠٠). The BHI Agar was prepared by dissolving ٣٧ g in ١٠٠٠ ml D.W.	
Nutrient Broth (NB)	NB is a general-purpose medium which supports the growth of bacteria that are not very nutritionally demanding (McFadden, ٢٠٠٠). The NB was prepared by dissolving ٢٥ g in ١٠٠٠ ml D.W.	
Mueller Hinton Agar (MHA)	MHA is used to determine the susceptibility of microorganisms to antimicrobial agents by either the disk diffusion or well diffusion methods. The MHA was prepared by dissolving ٣٨ g in ١٠٠٠ ml D.W.	
Brain Heart Infusion Agar (BHIA)	is a solid medium rich in nutrients, suitable for the cultivation of several fastidious strains of bacteria, Suspend ٥٢ grams of the medium in one liter of distilled water	

٣.٢.٥. Microbiology Experiments

٣.٢.٥.١. Morphological Examination

Primary identification tests included morphological features of bacterial growth on previously inoculated medium such as MacConkey agar, Cetrimide agar, and Nutrient agar at ٣٧ C° and in an aerobic condition for about ١٨-٢٤ hours. Colony shape, colony texture, edges, color, and pigment production were all tested.

٣.٢.٥.٢. Identification by VITEK® ٢ Compact System

All isolates of *P. aeruginosa* were identified using the automated VITEK-٢ compact system and by GN-ID cards according to the manufacturer's instructions, (Appendix ١)

٣.٢.٦. Preservation of Bacterial Isolates (Harley and Prescott, ٢٠٠٢)

Cultures of *P. aeruginosa* isolates were inoculated into ١٥٪ glycerol in brain heart infusion broth and kept at -٢٠ºC until required.

٣.٢.٧. Determination of Antibacterial Susceptibility Test

The *in vitro* susceptibility of ١٣٠ *P. aeruginosa* isolates to ٢٠ antibiotic agents, belonging to ٨ classes, were determined via disk diffusion method according to Clinical and Laboratory Standards Institute instructions (CLSI, ٢٠٢٢). The Kirby Bauer method was utilized to detect the antibiotic resistance profiles among the *P. aeruginosa* isolates. Pure cultures (٢٤ h old) were cultured in a nutrient broth and then sub-cultured on nutrient agar for ٢٤ h at ٣٧ °C. Thereafter, turbidity was adjusted in ٠.٨٥٪ sterile normal saline solution to ٠.٥ McFarland's standard (١.٥×١٠^{٨} CFU/mL) and then spread on Muller-Hinton agar with a sterile cotton swab. Antibiotic disks were positioned onto the MHA inoculated with the *P. aeruginosa* and the cultured plates were incubated for ٢٤ h at ٣٧°C. The *P. aeruginosa* isolates were assigned as resistant, intermediate, and

susceptible as recommended by the CLSI (CLSI, ٢٠٢٢). Resistant and intermediate isolates of *P. aeruginosa* were considered as resistant whereas sensitive isolates were considered as susceptible. Multiple drug resistance (MDR; resistance to ≥١ agent in≥٣ antibiotic classes). In additionally, *P. aeruginosa* ATCC ٢٧٨٥٣ was used as a positive control and as a reference strain for antibiotic resistance.

٣.٢.٨. Biofilm Formation Assay

The biofilm-forming ability of *P. aeruginosa* isolates (١٣٠) were examined by Congo red agar (CRA) modified method as described by Freeman *et al*., (١٩٨٩) and Tajbakhsh *et al*., (٢٠١٦). CRA was prepared by mixing brain heart infusion broth (٣٧ g/L), sucrose (٥٠ g/L), agar No. ١ (١٠ g/L), and Congo red indicator (٨ g/L). The aqueous solution of Congo red was prepared, autoclaved at ١٢١°C for ١٥ min, and added to the autoclaved brain heart infusion agar with sucrose, then cooled down at ٥٥°C. CRA plates were later inoculated with *P. aeruginosa* isolates and incubated at ٣٧°C for ٢٤ h aerobically. A positive finding was indicated by black colonies with a dry crystalline consistency. Intermediate biofilm-formers were indicated by the darkness of the colonies without a dry crystalline colony while, weak biofilm producers were stayed pink with the darkness at the center of colonies. non-biofilm producers o stayed pink crystalline colonies (Tajbakhsh *et al*., ٢٠١٦).

٣.٢.٩. Molecular Analysis

٣.٢.٩.١. Genomic DNA Extraction

The Favor Prep™ Genomic DNA Mini Kit was used to extract genomic DNA from clinical *P. aeruginosa* isolates after ٢٤ h of incubation. The following steps described in are the manufacturer's instructions:

Step ١- Preparation of Specimens:

- One hundred µl of bacterial culture was transferred to a ١.٥ milliliter microcentrifuge tube (Eppendorf), and the tube was centrifuged for one minute at full speed (١٤,٠٠٠ rpm), supernatant was discarded.

- Two hundred µl of FATG Buffer were added, then the pellet was re-suspended by vortexing or pipetting, incubated for ٥ minutes at room temperature.

Step ٢ -Cell Lysis

- To lyse the cells, ٢٠٠ µl of FABG Buffer was added to the sample and vortexed for ٥ seconds. The specimen lysate was then incubated at ٧٠°C for ١٠ minutes, or until it was clear. The tube was inverted every ٣ minutes during the incubation period.

Step ٣ – Binding

- A FABG Column is attached to a ٢ ml collecting tube. The sample mixture was transferred, to the FABG Column. The ٢ ml collection tube was discarded after centrifuging at full power (١٤,٠٠٠ rpm) for ٥ minutes. A fresh ٢ml Collection tube was filled with the FABG Column.

Step ٤ - Washing

- FABG Column was washed with ٤٠٠ ml W١ Buffer. Then centrifuged for ١ min at full speed (١٤,٠٠٠ rpm) and the flow-through was discarded.

- The FABG Column was placed back in the ٢ mL collection tube. FABG column was washed with ٦٠٠ ml wash buffer (ethanol added) and centrifuge for ١ min at full speed (١٤,٠٠٠ rpm) and the flow-through was discarded.

- The FABG Column was placed back in the ٢ mL collection tube, and centrifuge for an additional ٣ min at full speed (١٤,٠٠٠ rpm) to dry the column.

Step ٥ - Elution

- The dry FABG Column was placed to a new ١.٥ mL microcentrifuge tube. ١٠٠ ml of preheated elution buffer were added.

- The FAGB column was incubated at ٣٧ ºC for ١٠ min. After that, it was centrifuged for ١ min at full speed (١٤,٠٠٠ rpm) to elute the DNA.

- The genomic DNA fragment was stored at -٢٠ ºC until further analysis.

٣.٢.١٠. 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 were determined by measuring the absorbance at ٢٦٠/٢٨٠nm. With pure DNA having a ٢٦٠/٢٨٠ nm ratio between ١.٧ and ١.٩.

l.٣.١١. Agarose gel Electrophoresis

٣.٢.١١.١. Solutions and Agarose gel preparation

- Tris-Borate-EDTA (TBE) buffer: ١٠ xTBE (pH: ٨.٣) buffer was diluted to ٠.٥x with distilled water.
- Agarose ١٪: one gram of agarose was dissolved in ١٠٠ml ٠.٥x TBE buffer by heating until the solution became clear. Allowed to cool below ٥٠°Cand ٥µl/١٠٠ml red safe dye at concentration of ٦ x was added. The agarose poured kindly in equilibrated gel tray earlier set

with its comb. The agarose allowed to solidify at room temperature for ٣٠ min. The comb made wells used for loading DNA samples (Sambrook, and Russell, ٢٠٠١).

٣.٢.١١.٢. Agarose Gel Electrophoresis

Agarose gel electrophoresis is the most effective way of separating DNA fragments. The concentration of agarose in a gel depends on the sizes of the DNA fragments need to be separated, ranging between ٠.٥٪-٢٪ (Lee *et al*., ٢٠١٢). A ٠.٧٪ gel was used to obtain good separation of genomic DNA (٥-١٠ kb) after extraction while ١.٥٪-٢٪ was used to gain good resolution for small fragments of PCR product (٠.٢-١ kb). However, the specific weight of agarose was added to ١٠٠ ml of ٠.٥×TBE buffer and then melted in microwave until the solution becomes clear. The samples were loaded in a separate well of the gel, with marker in one well. Electrodes were connected correctly and the run was applied according to the gel percentage and size of gel.

٣.٢.١٢. PCR Assay for Genes Determination

٣.٢.١٢.١. PCR Components

PCR was carried out for both primers' groups in a total volume of ٢٥µl. The reaction components, is described in table (٣.٧).

Table (٣.٧): PCR components for genes amplification employed in this study.

Component	Amount (µl)	Concentration
Master mix	١٢.٥	١ x
Forward primer	١.٥	١٠ pmole/ µl
Reverse primer	١.٥	١٠ pmole/ µl
DNA sample	٣	٠.٠٥-٠.١٥ µg/µl
Nuclease free water	٦.٥	
Total volume	٢٥	

٣.٢.١٢.٢. Molecular identification of *P. aeruginosa*

P. aeruginosa was identified by screened of presence *ecfX* gene by PCR technique. The condition program of PCR was mentioned in table (٣.٨).

Table (٣.٨): Program of PCR conditions to amplify *ecfX* gene

Step	**Temperature (˚C)**	**Time**	**No. of cycles**
Initial denaturation	٩٥	٥min.	١
Denaturation	٩٨	١٠sec.	٥٠
Annealing	٦٣	٢٠sec.	
Extension	٦٣	٢٠sec.	
Final extension	٧٢	٨min.	١
Cooling	٤	٤min.	

٣.٢.١٢.٣. Virulence Factors

Virulence genes characteristic for *P. aeruginosa* were detected by PCR . The following genes were targeted; *pilB*, *pilA*, *apr*, *(lasA*, *lasB*, *phzS*, *phzM*, *PLCN*, *PLCH*, *algD*, and *algU*. The uniplex PCR technique were used to detect fragments of genes encoding selected *P. aeruginosa* virulence factors. The PCR programs were achieved under the following conditions to genes mentioned in Table (٣.٩).

Table (٣.٩): Conditions program of PCR to amplify virulence genes

Gene name	Temperature (°C)/Time					Cycle Number
	Initial denaturation	Cycling condition			Final Extension	
		Denaturation	Annealing	Extension		
pilB	٩٦/٥ min	٩٤/٣٠ sec	٥٥/٣٠ sec	٧٢/١ min	٧٢/ ٥ min	٣٠
pilA	٩٦/٥ min	٩٤/٣٠ sec	٥٩/٣٠ sec	٧٢/١ min	٧٢/٥ min	٣٠
apr	٩٦/٥ min	٩٤/٣٠ sec	٥٩/٣٠ sec	٧٢/١ min	٧٢/٥ min	٣٠
lasA	٩٦/٥ min	٩٤/٣٠ sec	٥٦/٣٠ sec	٧٢/١ min	٧٢/٥ min	٣٠
lasB	٩٦/٥ min	٩٤/٣٠ sec	٥٩/٣٠ sec	٧٢/١ min	٧٢/٥ min	٣٠
phzS	٩٦c/٥ min	٩٤c/٣٠ sec	٥٩/٣٠ sec	٧٢/١ min	٧٢/٥ min	٣٠
phzM	٩٦/٥ min	٩٤/٣٠ sec	٥٩/٣٠ sec	٧٢/١ min	٧٢/٥ min	٣٠
PLCN	٩٤/٣ min	٩٤/٣٠ sec	٥٥/١ min	٧٢ /١ min	٧٢/٥ min	٣٠
PLCH	٩٤/٣ min	٩٤/٣٠ sec	٥٥/١ min	٧٢/١ min	٧٢/٥ min	٣٠
algD	٩٥/٢ min	٩٤/٣٠ sec	٥٨/٣٠ sec	٧٢/١ min	٧٢/٧ min	٣٠
algU	٩٥/٢ min	٩٤/٣٠ sec	٥٨/٣٠ sec	٧٢/١ min	٧٢/٧ min	٣٠

٣.٢.١٢.٤. Type III Secretion System (T٣SS) Genes

A single set of primers was utilized to target the T٣SS genes (*exoS, exoU,* and *ToxA*). Table (٣.١٠) describes a PCR program for detecting T٣SS genes.

Table (٣.١٠): PCR program to amplify T٣SS genes

Gene Name	Temperature (°C)/Time					Cycle Number
	Initial Denaturation	Cycling condition			Final Extension	
		Denaturation	Annealing	Extension		
exoS	٩٥/٣ min	٩٥/٣٠ sec	٥٣/٣٠ sec	٧٢/٥٠ sec	٧٢/ ٥ min	٣٠
exoU	٩٥/٣ min	٩٥/٣٠ sec	٥٣/٣٠ sec	٧٢/٥٠ sec	٧٢/٥ min	٣٠
ToxA	٩٥/٥ min	٩٥/٣٠ sec	٥٣/٣٠ sec	٧٢/٥٠ sec	٧٢ /٥ min	٣٠

٣.٢.١٢.٥. Efflux Pump Genes (*MexAB-OprM*)

A single set of primers was used to target the efflux pump genes (*mexA, mexB,* and *oprM*). Table (٣.١١) describes a PCR program for detecting these genes.

Table (٣.١١): PCR program to amplify efflux pump genes

Gene Name	Temperature (°C)/Time					Cycle Number
	Initial Denaturation	Cycling condition			Final Extension	
		Denaturation	Annealing	Extension		
mexA	٩٤/٣ min	٩٤/٣٠ sec	٥٧/٤٥ sec	٧٢/١ min	٧٢/٧ min	٣٢
mexB	٩٤/٣ min	٩٤/٣٠ sec	٥٧/٤٥ sec	٧٢/١ min	٧٢/٧ min	٣٢
oprM	٩٥/٥ min	٩٥/١min	٥٩/١min	٧٢/٤٥sec	٧٢/٥ min	٣٠

٣.٣. Statistical Analysis

GraphPad Prism version ٨ software package was used for Data analysis. Chi-square test was applied to analyse for all obtained results, and a value $P \leq$ ٠.٠٥ was deemed statistically significant.

٣.٤. Study Design

Steps of the research project were shown in Figure (٣.١).

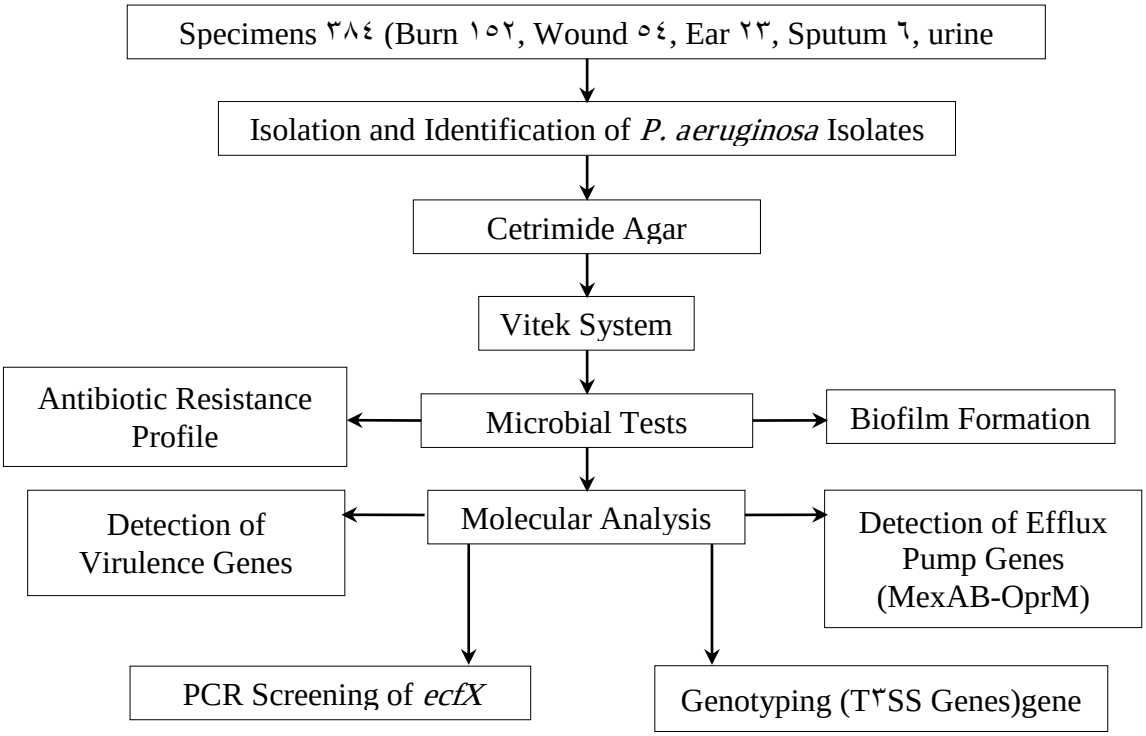

Figure (٣.١): Schematic diagram representing the study project

٣.٥. Ethical Approval

All participants involved in this study have been informed, and verbal consent will be provided by each one prior to sample collection. The committee on publishing ethics of the College of Science, University of Babylon, Iraq, approved this work according to document number M٢٢٠١١٠ in ٣٠/١/٢٠٢٢.

Chapter Four

Results and Discussion

٤. Results and Discussion

٤.١. Isolation and Identification of Bacterial Isolates

One hundred thirty *P. aeruginosa* isolates were obtained from ٣٨٤ (٣٣.٩٪) clinical specimens of burn, wounds, urine, ear and sputum collected from both sex with different ages were isolated from different hospitals and laboratories in Babylon province. All pure colonies that showed non-lactose fermenting Gram negative rods. Identification of Isolates were confirmed by VITEK® ٢ System with probability between ٩٤٪-٩٩٪, as detailed in appendix ١, then confirmed by molecular identification of *P. aeruginosa* isolates using gene species-specific *ecfX*. The prevalence of *P. aeruginosa* recovered from ٣٨٤ various clinical specimens was ١٣٠ (٣٣.٩ %), including burn١٠٢ (٧٨.٥٪), ear and urine ٩(٦.٩٪) for each whereas ٧(٥.٤٪) from wounds and ٣(٢.٣٪) from sputum, as shown in Figure (٤.١).

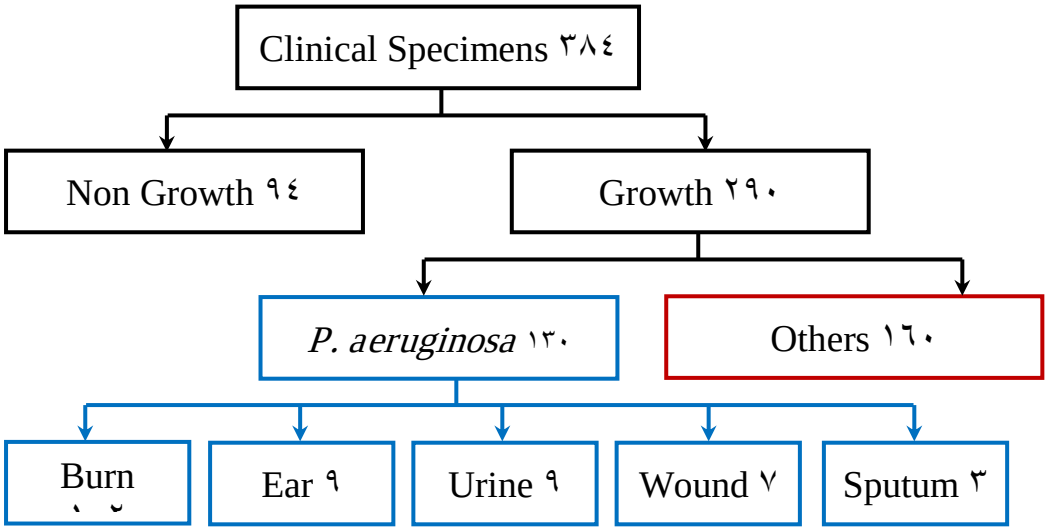

Figure ٤.١: Distribution of *P. aeruginosa* isolation from different clinical specimens.

All isolates were cultured on cetrimide agar. On cetrimide agar, the bacterial colonies of *P. aeruginosa* appeared as greenish-blue because the majority of these colonies produce pyocyanin, a greenish-blue dye (Figure ٤.٢).

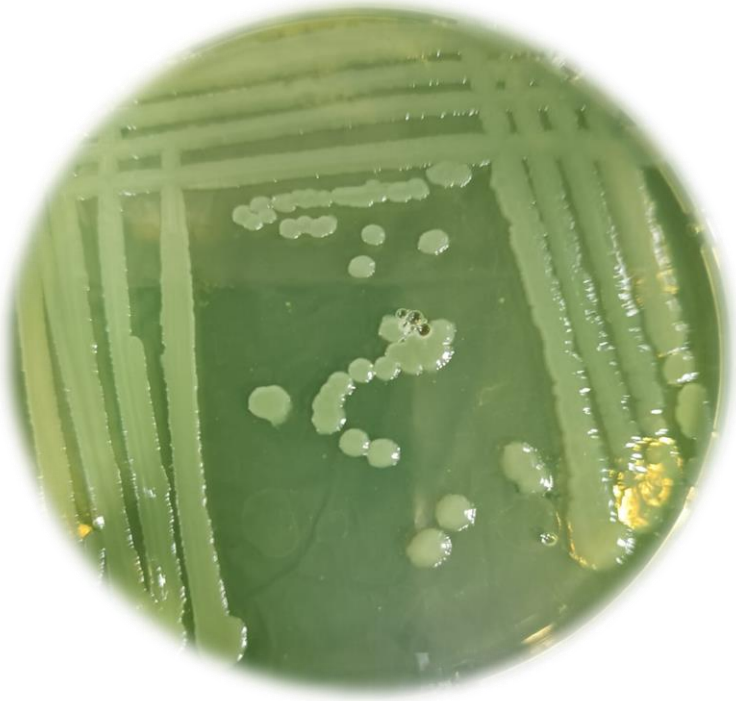

Figure ٤.٢: *P. aeruginosa* colonies on cetrimide agar

Cetrimide agar is a selective medium for the isolation and identification of *P. aeruginosa* while supposedly preventing the growth of other microorganisms. It is used to evaluate the ability of *P. aeruginosa* to grow in the presence of ٠.٠٣٪ cetrimide. It acts as a quaternary ammonium compound, cationic detergent which causes nitrogen and phosphorus to be released from bacterial cells other than *P. aeruginosa* (Brown and Lowbury,١٩٦٥). Cetrimide agar is used to detect pigment production by *P. aeruginosa* which produce a variety of water-soluble pigments.

The present study reported clinical *P. aeruginosa* growth rate in (١٣٠/٣٨٤) ٣٣.٩٪ of total clinical specimens. Earlier studies performed in Iraq documented the presence of *P. aeruginosa* growth rate of ٢٢-٣٤.٤٪ in various clinical specimens (Ismail and Mahmoud, ٢٠١٨; Al-Khudhairy and Al-Shammari, ٢٠٢٠). However, the presence rate of *P. aeruginosa* is higher than reported in the earlier studies conducted in Iran (٧.٣٪) and Nepal (٢١.٤٪) (Sales *et al.*, ٢٠١٧; Bhandari *et al.*, ٢٠٢٢). The prevalence

rate of *P. aeruginosa* in diverse clinical specimens varies with the geographic region, number of specimens, site of infections, condition of patients, and duration of the study.

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

In order to further validate the identification of the isolates at the species level, a PCR technique screening, targeting the *ecfX* gene was conducted to identify species-specific *ecfX* in *P. aeruginosa* (Figure ٤.٣).

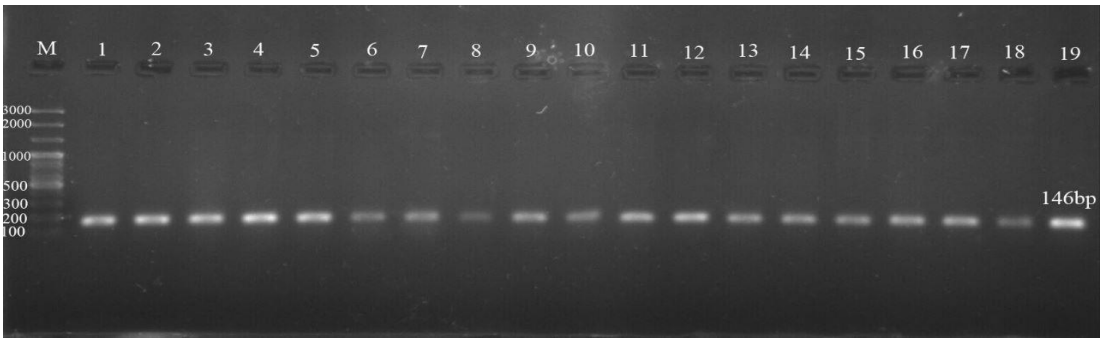

Figure ٤.٣: PCR amplification of *ecfX* (١٤٦ bp) in *P. aeruginosa* isolates, on ٢ % agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-١٩) Positive isolates.

Identification *P. aeruginosa* isolates by PCR, revealed that all the *P. aeruginosa* isolates were positive for the species-specific *ecfX* gene with amplification product of approximately ١٤٦ bp. Many studies reported that the best target for identifying *P. aeruginosa* isolates particularly is the *ecfX* gene (Lavenir *et al.*, ٢٠٠٧; Anuj *et al.*, ٢٠٠٩). The *ecfX* gene encodes an extracytoplasmic function sigma factor that may be involved in haem uptake or virulence (Salih Almohaidi & AL-Shimmary, ٢٠١٩). According to findings from earlier studies, reported that the *ecfX* gene is an accurate PCR target for *P. aeruginosa* detection (Qin *et al.*, ٢٠٠٣; Lavenir *et al.*, ٢٠٠٧).

٤.٢. Antibiotic Susceptibility of *P. aeruginosa* Isolates

The disk diffusion test (Kirby-Bauer method) was used to assess antibiotic resistance patterns in comparison to CLSI (CLSI, ٢٠٢٢) for *P. aeruginosa* isolates. The present study revealed that Ceftazidime resistance was highest (٩٩.٢٪); followed by ticarcillin (٩٧.٧٪); ampicillin, ertapenem, and erythromycin (٩٦.٩٪). The resistance pattern of *P. aeruginosa* isolates displayed also high resistance to tetracyclines (٩٤.٦٪), piperacillin (٩٢.٣٪), and doripenem (٩٠.٨٪). A high proportion of resistance levels to the various antibiotic classes found in the majority of *P. aeruginosa* isolates as depicted in Table (٤.١). *P. aeruginosa* ATCC ٢٧٨٥٣ was also tested as a positive control strain for antibiotic resistance pattern.

Table ٤.١: Antibiotic Susceptibility Pattern of *P. aeruginosa* (N=١٣٠)

Antibiotic Group	Antibiotics	Sensitivity No (%)	Resistant No (%)
Penicillins	Ampicillins	٤(٣.١)	١٢٦(٩٦.٩)
	Piperacillin	١٠(٧.٧)	١٢٠(٩٢.٣)
	Piperacillin-Tazobactam	٣٠(٢٣.١)	١٠٠(٧٦.٩)
	Ticarcillin	٣(٢.٣)	١٢٧(٩٧.٧)
Cephalosporins	Ceftazidime	١(٠.٨)	١٢٩(٩٩.٢)
	Cefepime	١٦(١٢.٣)	١١٤(٨٧.٧)
Monobactam	Aztreonam	١٩(١٤.٦)	١١١(٨٥.٤)
Carbapenems	Doripenem	١٢(٩.٢)	١١٨(٩٠.٨)
	Ertapenem	٤(٣.١)	١٢٦(٩٦.٩)
	Imipenem	٤٠(٣٠.٨)	٩٠(٦٩.٢)
	Meropenem	٣٠(٢٣.١)	١٠٠(٧٦.٩)
Aminoglycosides	Amikacin	٢٩(٢٢.٣)	١٠١(٧٧.٧)

	Gentamicin	٣٥(٢٦.٩)	٩٥(٧٣.١)
	Tobramycin	٣١(٢٣.٨)	٩٩(٧٦.٢)
Tetracyclins	Doxycycline	١٨(١٣.٨)	١١٢(٨٦.٢)
	Tetracycline	٧(٥.٤)	١٢٣(٩٤.٦)
Macrolides	Erythromycin	٤(٣.١)	١٢٦(٩٦.٩)
Fluoroquinolones	Ciprofloxacin	٤٠(٣٠.٨)	٩٠(٦٩.٢)
	Levofloxacin	٣٦(٢٧.٧)	٩٤(٧٢.٣)
	Ofloxacin	٣٢(٢٤.٦)	٩٨(٧٥.٤)

Resistance to both Imipenem and Ciprofloxacin was low (٦٩.٢%). Out of ١٣٠ *P. aeruginosa* isolates, ١٢٥ (٩٦.١٪) were addressed to be MDR. Only ٥ (٣.٨ %) isolates were sensitive to the tested antibiotics (Figure ٤.٤).

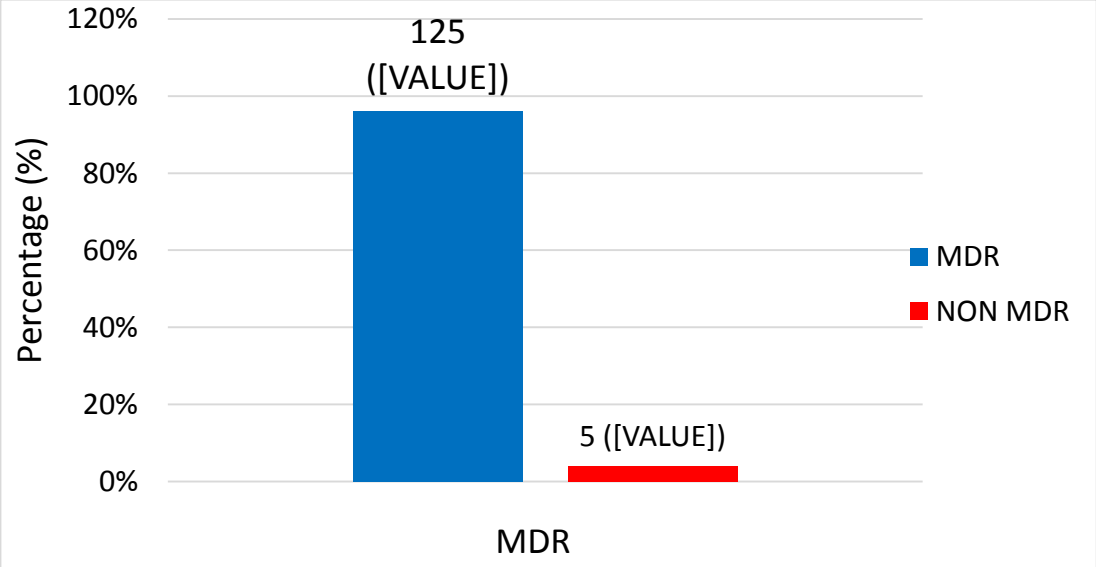

Figure ٤.٤: Frequency of MDR *P. aeruginosa* isolates.

Evolution of MDR caused by *P. aeruginosa* to several antibiotic classes (resistance to at least one antibiotic in three or more different antibiotic classes) is a great threat in handling its infections (Tümmler, ٢٠١٩; Kamali *et al.*, ٢٠٢٠). The prevalence of MDR *P. aeruginosa* isolates is causing a major concern in health care settings attributable to

increased cost of therapy, clinic visits, and hospitalization severe complications (Nathwani *et al*., ٢٠١٤). The finding of the current study displayed a high prevalence of resistant *P. aeruginosa* isolates with MDR ranging between ٦٩.٢-٩٩.٢٪, which is higher than those findings reported by Alhusseini *et al*. (١٤.٨-٩٨.٦٪) (Alhusseini *et al*., ٢٠١٩), and Khadim & Marjani (١١.١١-٨٧.٣) (Khadim & Marjani, ٢٠١٩). The high resistance rate in the present study might indicate that the *P. aeruginosa* isolates manifest several mechanisms of resistance such as production of hydrolytic enzymes. The variance in the prevalence of MDR among earlier reports might be associated with the dissimilar antibiotic usage, different controlling policy, and various site of the infections. Furthermore, the high prevalence of MDR isolates is a critical indicator and reflects the need for routine monitoring of antibiotic resistance in Iraq.

A previous locally study performed by Abd AL-Zwaid and Al-Dahmoshi, (٢٠٢٢) reported that the isolates of *P. aeruginosa* expressed the highest resistance to ceftazidime (٩٣.٦ %) and cefepime (٧٧.٢ %), followed by tobramycin (٦٠٪), amikacin (٥٦٪), ofloxacin (٥٢٪), netilmicin (٥٠٪), imipenem (٢٠٪), and meropenem (٤٤٪). Several studies reported that Ceftazidime was found to be the least effective drug used for treatment in Nepal (Dahal *et al*., ٢٠١٨; Pokharel *et al*., ٢٠١٩). Ceftazidime (one of third generation Cephalosporins) usually used for treating urinary tract, respiratory tract and burn wound infections. Mutations in the class A *β*-lactamases are the primary source of the resistance to third-generation cephalosporins (Bush *et al*., ١٩٩٥).

In the middle of the ١٩٩٠s, the fourth-generation Cephalosporin / Cefepime was released into clinical use **(**Endimiani *et al*., ٢٠٠٨**).** It is known that MDR bacteria colonize wounds in burn patients and are a

source of hospital-acquired infection epidemics in burn units (Agnihotri *et al*., ٢٠٠٤). Two hundred fifty isolates of *P. aeruginosa* that were collected from burn wounds in children were subjected to an antibiotic sensitivity test by Hassuna *et al*. (٢٠١٥) revealed significant resistance to ceftazidime (٨٦٪), cefotaxime (٧٢٪), and imipenem resistance was not detected. Moreover, ٢٨ isolates (٥٦٪) were multidrug resistant. A diverse bacterial population's presence and distribution of plasmids, which can spread between bacterial genera and species and result in the emergence of resistance through conjugation and transformation (Yousefi *et al*., ٢٠١٠).

٤. ٣. Association between Biofilm Formation and Antibiotic Resistance

All *P. aeruginosa* isolates were assayed for biofilm formation by Congo Red Agar method. Strong biofilm former was indicated by dark colonies and crystalline colonies at the CRA, isolates did not display dry crystalline black colonies were characterised as moderate biofilm formers. Weak biofilm formers generally exhibited pink colour with darkness at the centre of colonies and non-biofilm formers remained pink colonies (Figure ٤.٥).

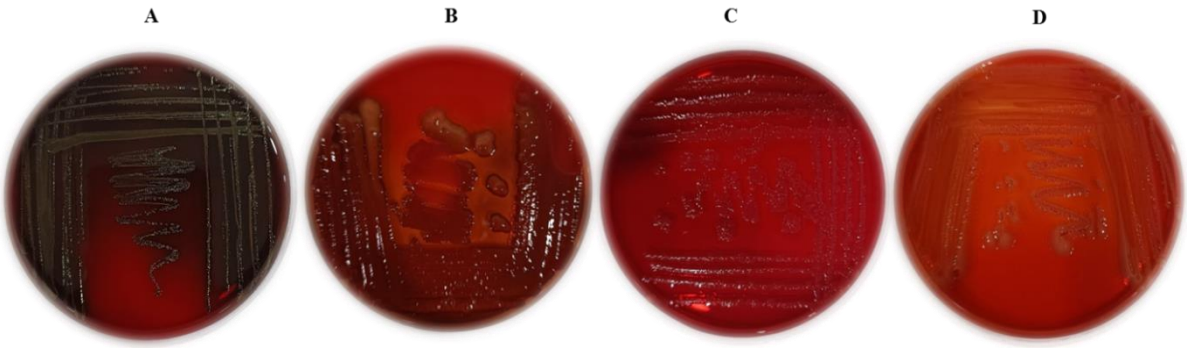

Figure ٤.٥: Congo Red Agar plates showing A) Strong biofilm formation, B) Moderate biofilm formation, C) Weak biofilm formation, D) Non-biofilm formation.

Out of ١٣٠ isolates of *P. aeruginosa* subjected to biofilm formation, ١١١ (٨٥.٤٪) displayed biofilm-forming ability, being assorted in the following categories: ٤٠ (٣٠.٨٪) formed strong biofilm; ٥١ (٣٩.٢٪) formed moderate biofilm; ٢٠ (١٥.٤٪) formed weak biofilm, while ١٩ (١٤.٦%) of *P. aeruginosa* isolates were characterised as non-biofilm former (Figure ٤.٥ and Figure ٤.٦). Based on biofilm-forming ability, the *P. aeruginosa* isolates were categorized into biofilm-forming and non-biofilm-forming groups. Among twenty antibiotics investigated, there was no statistically significant (p>٠.٠٥) correlation between biofilm formation positivity and antibiotic resistance patterns, with exception of imipenem (p=٠.٠٠٥٦**) (Table ٤.٢).

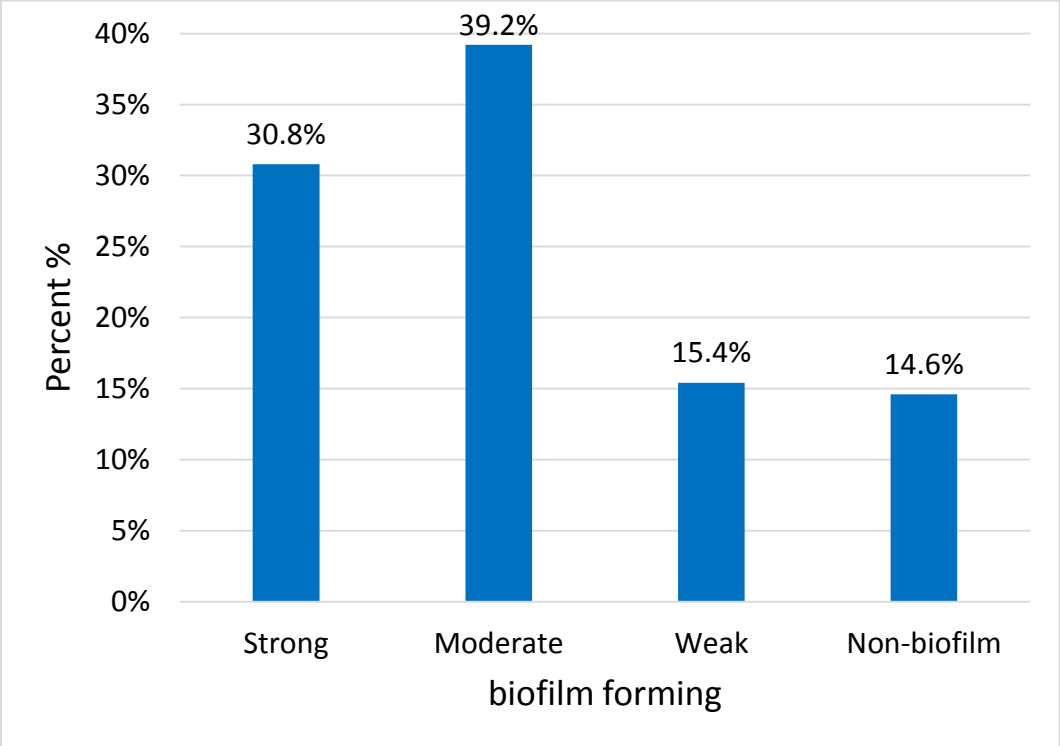

Figure ٤.٦ Distribution of biofilm-forming ability among *P. aeruginosa* isolates (N ١٣٠)

Table ٤.٢: Antibiotic resistance pattern based on biofilm-forming and non-biofilm ability in *P. aeruginosa* isolates.

Antibiotic	Biofilm former		Non biofilm former		*P-value*
	Resistance No. (%)	Sensitive No. (%)	Resistance No. (%)	Sensitive No. (%)	
Ampicillin	١٠٦(٩٥.٥)	٥(٤.٥)	١٩(١٠٠)	٠(٠)	٠.٣٤٥٥
Piperacillin	١٠٤(٩٣.٧)	٧(٦.٣)	١٦(٨٤.٢)	٣(١٥.٨)	٠.١٥١٧
Piperacillin Tazobactam	٨٨(٧٩.٣)	٢٣(٢٠.٧)	١٢(٦٣.٢)	٧(٣٦.٨)	٠.١٢٣٣
Ticarcillin	١٠٧(٩٦.٤)	٤(٣.٦)	١٩(١٠٠)	٠(٠)	٠.٤٠٠٦
Ceftazidime	١٠٩(٩٨.٢)	٢(١.٨)	١٩(١٠٠)	٠(٠)	٠.٥٥٥٤
Cefepime	٩٦(٨٦.٥)	١٥(١٣.٥)	١٨(٩٤.٧)	١(٥.٣)	٠.٣١١٨
Aztreonam	٩٦(٨٦.٥)	١٥(١٣.٥)	١٥(٧٨.٩)	٤(٢١.١)	٠.٣٩
Doripenem	١٠٠(٩٠.١)	١١(٩.٩)	١٨(٩٤.٧)	١(٥.٣)	٠.٥١٧٩
Ertapenem	١٠٦(٩٥.٥)	٥(٤.٥)	١٩(١٠٠)	٠(٠)	٠.٣٤٥٥
Imipenem	٨٢(٧٣.٩)	٢٩(٢٦.١)	٨(٤٢.١)	١١(٥٧.٩)	**٠.٠٠٥٦**
Meropenem	٨٧(٧٨.٤)	٢٤(٢١.٦)	١٣(٦٨.٤)	٦(٣١.٦)	٠.٣٤١١
Gentamicin	٨٦(٧٧.٥)	٢٥(٢٢.٥)	١٥(٧٨.٩)	٤(٢١.١)	٠.٨٨٦٩
Tobramycin	٨٣(٧٤.٨)	٢٨(٢٥.٢)	١٢(٦٣.٢)	٧(٣٦.٨)	٠.٢٩١٥
Amikacin	٨٥(٧٦.٦)	٢٦(٢٣.٤)	١٤(٧٣.٧)	٥(٢٦.٣)	٠.٧٨٤٦
Tetracycline	١٠٣(٩٢.٨)	٨(٧.٢)	١٩(١٠٠)	٠(٠)	٠.٢٢٧١
Doxycycline	٩٣(٨٣.٨)	١٨(١٦.٢)	١٨(٩٤.٧)	١(٥.٣)	٠.٢١١٧
Erythromycin	١٠٦(٩٥.٥)	٥(٤.٥)	١٩(١٠٠)	٠(٠)	٠.٣٤٥٥
Ciprofloxacin	٧٦(٦٨.٥)	٣٥(٣١.٥)	١٤(٧٣.٧)	٥(٢٦.٣)	٠.٦٤٩

Ofloxacin	٧٩(٧١.٢)	٣٢(٢٨.٨)	١٥(٧٨.٩)	٤(٢١.١)	٠.٤٨٤
Levofloxacin	٨٤(٧٥.٧)	٢٧(٢٤.٣)	١٤(٧٣.٧)	٥(٢٦.٣)	٠.٨٥٢٣

Biofilms are communities of microorganisms protected by a self-synthesized layer of complex polysaccharides, proteins, lipids, and extracellular DNA, collectively called the extracellular polymeric substance (Fleming and Rumbaugh, ٢٠١٧). *P. aeruginosa* pathogenicity is mainly attributed to the ability of this bacterium to form biofilm that poses a massive challenge to antibiotic therapy (Tuon *et al*., ٢٠٢٢). In the present study, screening of phenotypic biofilm formation among *P. aeruginosa* isolates exhibited that biofilm-forming ability was noted in ٨٥.٤٪ (١١١/١٣٠), including ٣٠.٨٪, ٣٩.٢٪, and ١٥.٤٪ being assigned as strong biofilm, moderate biofilm, and weak biofilm formers respectively. The finding is consistent with the local studies conducted by Khadim & Marjani, ٢٠١٩ (١٠٠٪) (Khadim & Marjani, ٢٠١٩) and Alhusseini *et al*., (٩٠.٧٪) (Alhusseini *et al*., ٢٠١٩). Furthermore, this study is attempting to recognize the correlation between biofilm-forming ability and antibiotic resistance among clinical *P. aeruginosa* isolates using CRA (qualitative technique). Even though the high rate of resistance observed in both biofilm-forming and non-biofilm-forming *P. aeruginosa* isolates, there was non-statistically significant correlation (p>٠.٠٥) between biofilm formation positivity and antibiotic resistance patterns, with exception of imipenem. These results were in agreement with previous reports that documented a non-statistically significant correlation between biofilm-forming ability and resistance to various antibiotics (Davarzani *et al*., ٢٠٢١; Gajdács *et al*., ٢٠٢١; Yang *et al*., ٢٠٢١), indicating that other antibiotic's resistance mechanisms including expression of efflux pumps, low outer membrane permeability, and expression of the inactivating

enzymes genes are involved (Pang *et al*., ٢٠١٩). However, a significant correlation between biofilm formation and antibiotic resistance was found in earlier reports (El-sayed and Fahmy,٢٠٢١; Heidari *et al*., ٢٠٢٢).

٤.٤. Molecular Analysis

٤.٤.١. Extraction of DNA

Isolation of the genomic DNA of *P. aeruginosa* was conducted using the Favor Prep™ Genomic DNA Mini Kit for DNA extraction. The DNA had an approximate concentration of ٦٥-٢٠٠ ng/µl with high purity sample of DNA range was between (١.٧–١.٨) by using Nano drop devise.

٤.٤.٢. Determination of Genetic Virulence Profiles

Amplification of genes with specific primers listed in Table (٣.٦) by uniplex-PCR technique were done for ١٣٠ isolates of *P. aeruginosa* to detect virulence factors. The PCR result showed ١٢٧ (٩٧.٦٩٪) *P. aeruginosa* isolates, were positive for the *apr* gene with amplification product approximately ١٠١٧ bp (Figure ٤.٧).

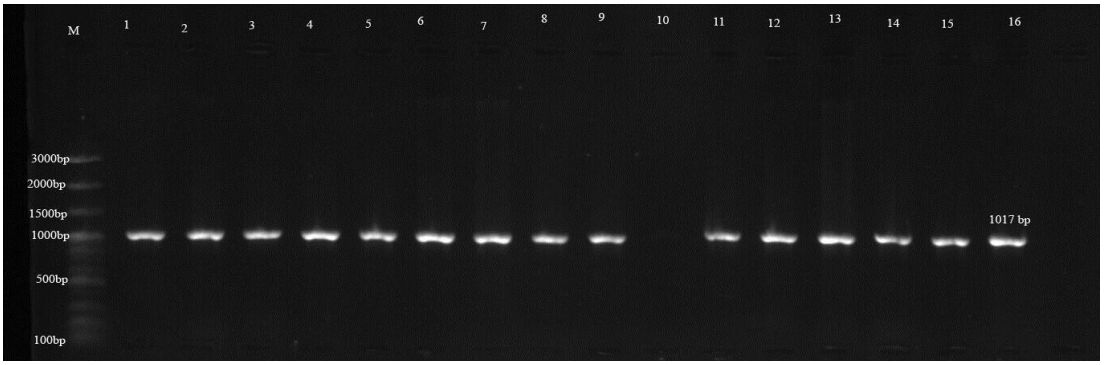

Figure ٤.٧: PCR amplification of *apr* gene (١٠١٧ bp) in *P. aeruginosa* isolates, on ١ % agarose at ٧٠ volt for ١.٥ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١- ٩ and ١١-١٦) Positive isolates. Lane (١٠) negative isolates.

AprA inhibits bacterial clearance by cleaving the C٢ component of the complement system, thus preventing complement-mediated phagocytosis (Laarman *et al*., ٢٠١٢). Also, it degrades flagellin, a known activator of proinflammatory responses (Pel *et al*., ٢٠١٤). *AprA* degrades

laminin which is a biologically active part of the basal lamina, therefore, the alkaline protease may have a function in invasion and hemorrhagic tissue necrosis in *P. aeruginosa* infection (Hoge *et al.*, ٢٠١٠).

Out of ١٣٠ isolates the presence gene encode to heamolytic phospholipase C (*PLCH*) was detected in ١٢٥(٩٦.١٥٪) of *P. aeruginosa* isolates showing amplification product of ٦٠٨ bp (Figure ٤.٨).

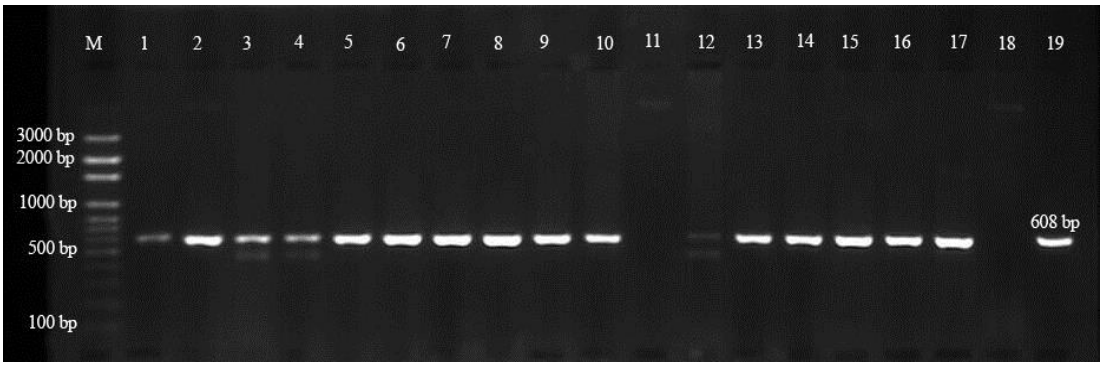

Figure ٤.٨: PCR amplification of *PLCH* gene (٦٠٨ bp) in *P. aeruginosa* isolates, on ١ % agarose at ٧٠ volt for ١.٥ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١- ١٠, ١٢- ١٧ and ١٩) Positive isolates. Lane (١١and ١٨) negative isolates.

While the gene encode to non heamolytic phospholipase C (*PLCN*)was detected in ١٢٠(٩٢.٣٠٪) of clinical isolates of *P. aeruginosa* with amplification product of ٤٨١ bp, as shown in Figure (٤.٩).

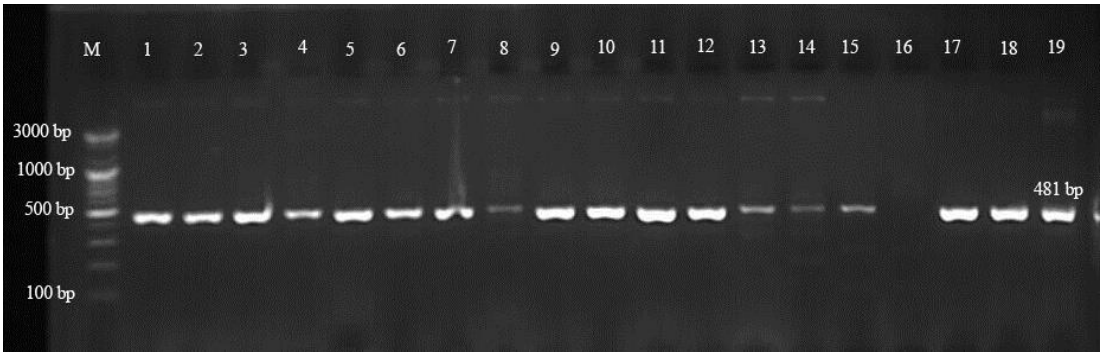

Figure ٤.٩: PCR amplification of *PLCN* gene (٤٨١ bp) in *P. aeruginosa* isolates, on ١.٥ % agarose at٧٠ volt for ١.٥ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١- ١٥, ١٧- ١٩) Positive isolates. Lane (١٦) negative isolates.

The synergistic action of *PLC*s and rhamnolipids is cytotoxic and hemolytic, causing hydrolysis of lipids and lecithin (Van Delden and Iglewski, ١٩٩٨). The mucus layer and cell membranes are specifically

targeted to facilitate bacterial invasion for the acquisition of nutrients. Rhamnolipids act as biosurfactants, solubilizing the phospholipids of alveolar surfactants, rendering them accessible to degradation by *PLC*-H (Chadha *et al.*,٢٠٢٢).

Whereas presence the genes encodes to phenazine (*phzS*) was detected in ١٢٢(٩٣.٨٤٪) of clinical isolates of *P. aeruginosa* with amplification product of ١٧٥٢ bp, as shown in Figure (٤.١٠).

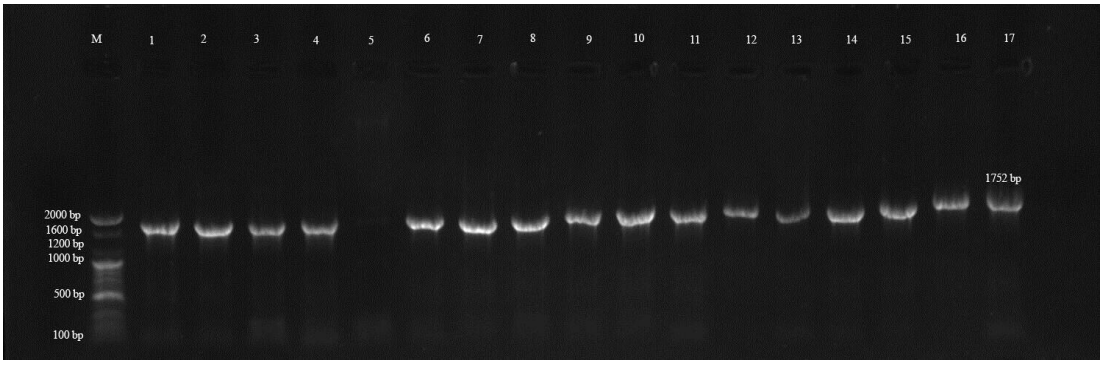

Figure ٤.١٠: PCR amplification of *phzS* gene (١٧٥٢ bp) in *P. aeruginosa* isolates, on ٠.٨ % agarose at ٧٠ volt for ١ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١- ٤, ٦-١٧) Positive isolates. Lane (٥) negative isolates.

Regarding the gene encode to phenazine (*phzM*) was detected in ١١٧(٩٠٪) of *P. aeruginosa* isolates with amplification product of ٨٧٥ bp, as shown in Figure (٤.١١).

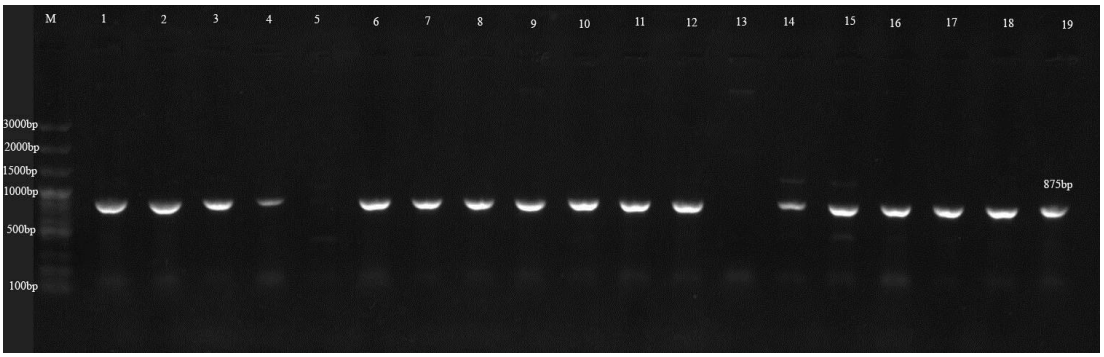

Figure ٤.١١: PCR amplification of *phzM* gene (٨٧٥ bp) in *P. aeruginosa* isolates, on ١ % agarose at ٧٠ volt for ١.٥.٥hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-٤, ٦- ١٢, ١٤- ١٩) Positive isolates. Lane (٥ and ١٣) negative isolates

PhzM is only active in the presence of *PhzS*, suggesting that a protein-protein interaction is involved in the formation of pyocyanin.

PhzS displays disorder near the binding site and this can be part of the substrate recognition process, which allows *PhzM* to form the *PhzM-PhzS* complex (Greenhagen *et al*., ٢٠٠٨). Moreover, *PhzM* has no activity on PCA. Pyocyanin is only produced when *PhzS* and NADH are present because a transient physical interaction is required to activate pyocyanin production (Mavrodi *et al*., ٢٠١٠).

The gene encode to alginate (*algD*) was detected in ١٢٢(٩٣.٨٤٪) of clinical isolates of *P. aeruginosa* with amplification product of ٢٩٩ bp, as shown in Figure (٤.١٢).

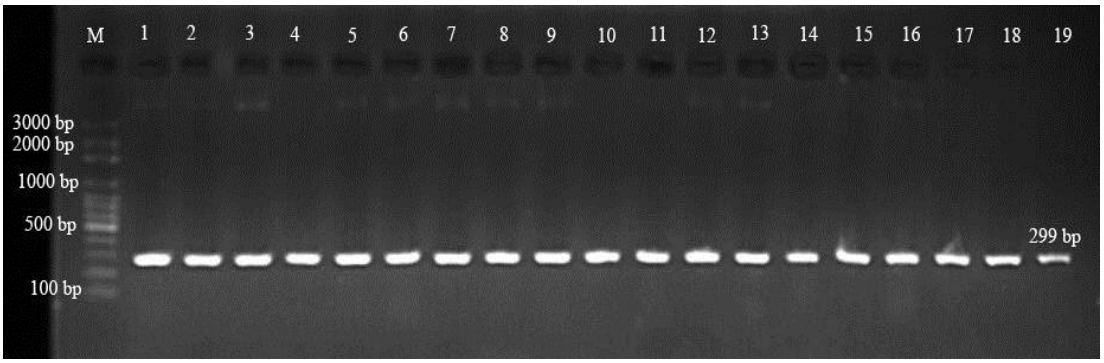

Figure ٤.١٢: PCR amplification of *algD* gene (٢٩٩bp) in *P. aeruginosa* isolates, on ١.٥٪ agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-١٩) Positive isolates.

The gene encode to alginate (*algU*) was detected in ١٠٧(٨٢.٣٠٪) of *P. aeruginosa* isolates with amplification product of ١٥٧٤ bp, as shown in Figure ٤.١٣.

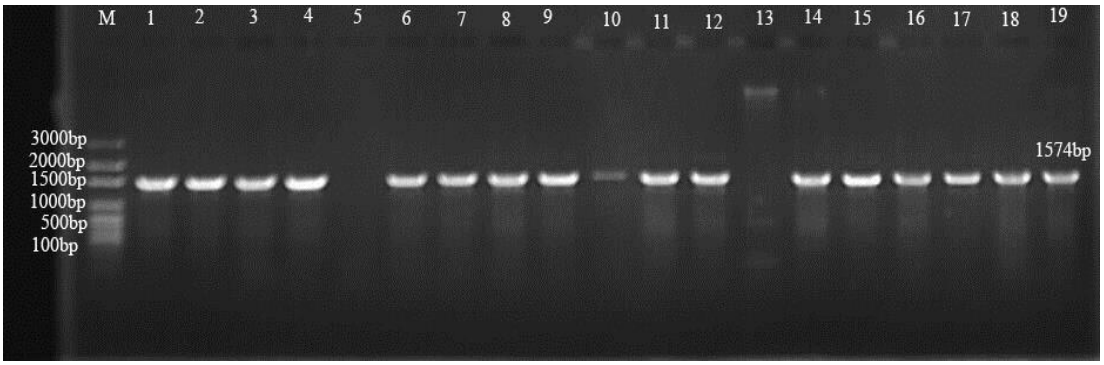

Figure ٤.١٣: PCR amplification of *algU* gene (١٥٧٤ bp) in *P. aeruginosa* isolates, on ٠.٨٪ agarose at ٧٠ volt for ١ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١, ٢, ٣, ٤, ٦, ٧, ٨, ٩, ١٠, ١١, ١٢, ١٤, ١٥, ١٦, ١٧, ١٨, and ١٩) Positive isolates. Lane (٥ and ١٣) negative isolates.

Alginate is overproduced by mucoid *P. aeruginosa* strains, and despite not being necessary for biofilm formation, is important in its maturation and stability (Ryder *et al*., ٢٠٠٧; Ghafoor *et al*., ٢٠١١; Orgad *et al*., ٢٠١١). Alginate also contributes to persistence of the bacterium by protecting *P. aeruginosa* against phagocytosis and scavenging radical oxygen species released by activated macrophages (Leid *et al*., ٢٠٠٥). Furthermore, alginate elicit a strong leukocyte response, the release of radical oxygen species, which contributes to lung inflammation (Rybtke *et al*.,٢٠٢٠).

Figure (٤.١٤) shows that the gene encoding pilin A (*pilA*) was found in ١٠٦ (٨١.٥٣٪) of *P. aeruginosa* isolates, with an amplification product of ١٦٧٥ bp.

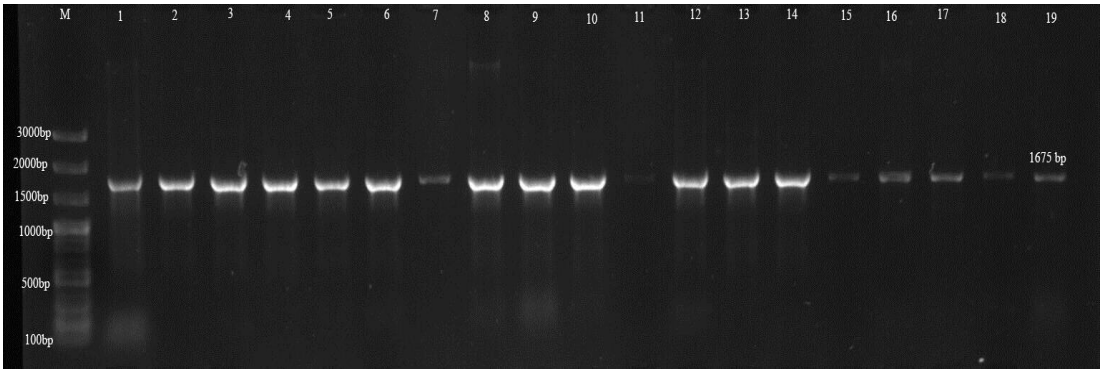

Figure ٤.١٤: PCR amplification of *pilA* gene (١٦٧٥ bp) in *P. aeruginosa* isolates, on ٠.٨ % agarose at ٧٠ volt for ١ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-١٩) Positive isolates.

However, the pilin B (*pilB*) gene was found in ١٢ (٩.٢٪) of *P. aeruginosa* isolates, with an amplification product of ٤٠٨ bp, as depicted in Figure (٤.١٥).

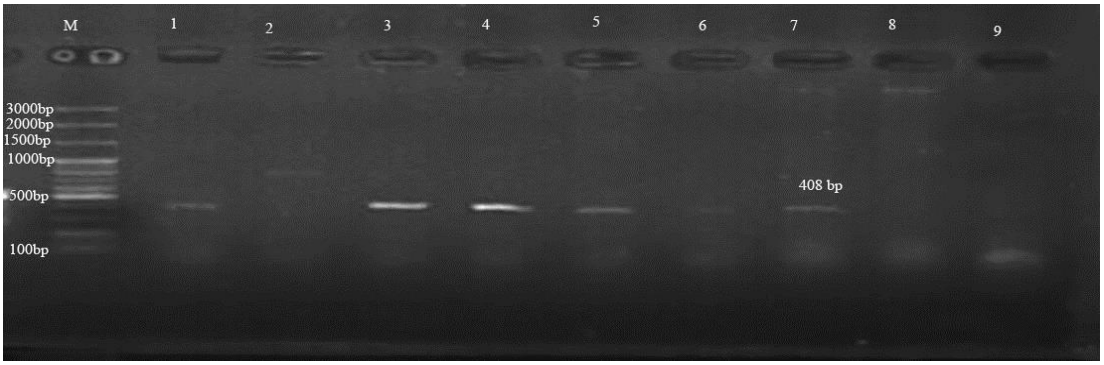

Figure ٤.١٥: PCR amplification of *pilB* gene (٤٠٨ bp) in *P. aeruginosa* isolates, on ١.٥ % agarose at ٧٠ volt for ١.٥ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١, ٣, ٤, ٥, ٦ and ٧) Positive isolates. Lane (٢,٨ and ٩) negative isolates.

Type IV pili (T٤P) are protein fibers produced on the bacterial cell surface. Pilins are small structural proteins with a protein interaction domain and a transmembrane domain (Giltner *et al*., ٢٠١٢). Type IV pili are involved in various processes such as colonisation during infection, twitching motility (bacterial translocation), biofilm formation, bacteriophage infection, DNA uptake and natural transformation. A specialized feature of pilin is the ability to reversibly produce polymeric fibers and aggregate to form ordered bundles (Hazes *et al*., ٢٠٠٠).

Whereas the genes encoding elastase (*(lasA* and *lasB*) were identified in ٤١ (٣١.٥٣٪) and ١٥ (١١.٥٣٪) of *P. aeruginosa* isolates, respectively, with amplification products of ١٠٧٥ bp and ١٢٢٠ bp, as shown in Figures (٤.١٦ and ٤.١٧).

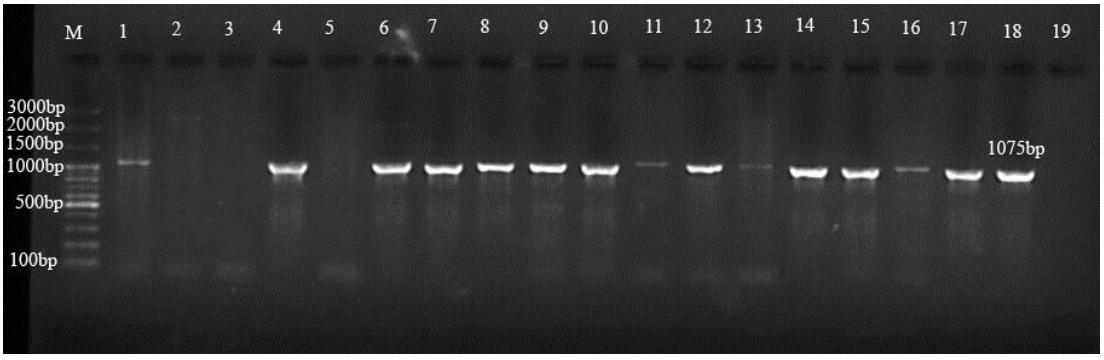

Figure ٤.١٦: PCR amplification of *(lasA* gene (١٠٧٥ bp) in *P. aeruginosa* isolates, on ١ % agarose at ٧٠ volt for ١.٥ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١, ٤, ٦, ٧, ٨, ٩, ١٠, ١١, ١٢, ١٣, ١٤, ١٥, ١٦, ١٧ and ١٨) Positive isolates. Lane (٢, ٣, ٥ and ١٩) negative isolates.

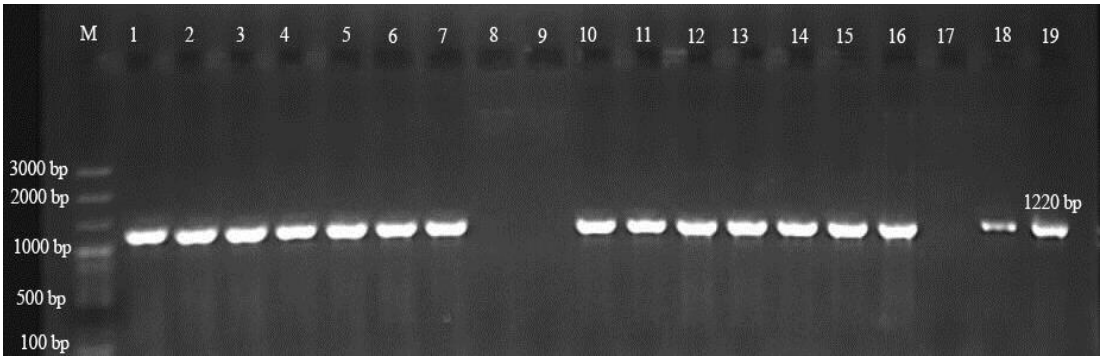

Figure ٤.١٧: PCR amplification of *lasB* gene (١٢٢٠ bp) in *P. aeruginosa* isolates, on ١ % agarose at ٧٠ volt for ١.٥ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-٧,١٠- ١٦, ١٨ and ١٩) Positive isolates. Lane (٨, ٩, and ١٧) negative isolates.

Elastase A and B have been shown to drive host invasion by reducing the action of *ExoT*oxins, which delay the latter process. Elastase B acts against the host immune system by degrading complement proteins, immunoglobulins A and G, and inhibiting phagocytosis (Andrejko and Dudka, ٢٠١٢).

Figure (٤.١٨) summarizes all of the above findings and displays different percentages of virulence factor genes (*apr, PLCH, phzS, algD, PLCN, phzM, lasB, algU, pilA, (lasA*, and *pilB*) that were identified in *P. aeruginosa* isolates.

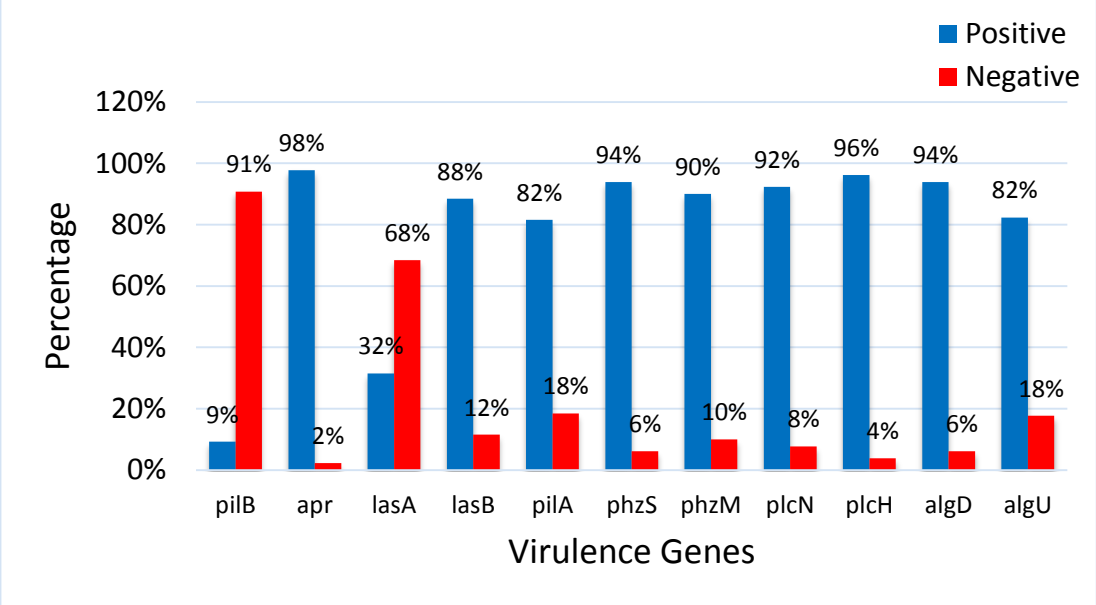

Figure ٤.١٨: Distribution of virulence genes in *P. aeruginosa* isolates

Figure (٤.١٨) shows that these isolates were positive for all virulence factor genes in the current study with different percentages. The most common genes of virulence factors observed in these isolates were *apr* (٩٧.٦٩٪), followed by *PLCH* (٩٦.١٥٪); *phzS* (٩٣.٨٤٪) and *algD* (٩٣.٨٤٪), whereas the least common genes were *(lasA* ٤١(٣١.٥٣٪) and *pilB* (٩.٢٪).

The global threat is significant because the combination of antibiotic resistance mechanisms and virulence factors usually determines the severity of the infection (Miyoshi-Akiyama *et al*., ٢٠١٧). *P. aeruginosa* produces a variety of proteinaceous virulence factors, including cytotoxins, elastase B, and alkaline protease (*AprA*) (Choy *et al*., ٢٠٠٨). Fazeli and Momtaz (٢٠١٤), revealed that all the *exo, apr, phz, las, pvd, pil, tox, alg, PLC,* and *nan* virulence genes are dominant in *P. aeruginosa*-caused human infections. Alkaline protease (*AprA*) is a key virulence factor in *P. aeruginosa*, and its overexpression promoted the production of another virulence factor, such as pyocyanin, a phenomenon known as "crosstalk between virulence factors" (Iiyama *et al*., ٢٠١٧). It is cleaving a variety of proteins, including human interferon- γ, tumor necrosis factor- α, and interleukin-٦ (Hoge *et al*., ٢٠١٠). As a result, *AprA*-mediated protein degradation may impair host immune response (Lomholt *et al*., ٢٠٠١).

In a previous study achieved by Lomholt *et al*., (٢٠٠١) reported that all *P. aeruginosa* isolates examined were harbored *lasB* gene. These findings contrary to the present study, it was (٨٨.٤٦٪). The *lasB* gene encodes a zinc metalloelastase that has elastolytic activity on lung tissue and attacks eukaryotic proteins such as collagen and elastin, eventually destroying the cell's structural proteins (Jaffar-Bandjee *et al*., ١٩٩٥). In addition, it acts synergistically with alkaline protease to inactivate the human cytokines gamma interferon and tumor necrosis factor alpha (Rust

et al., ١٩٦٦). When Lomholt *et al*., (٢٠٠١) analyzed ١٤٥ samples of *P. aeruginosa*, their findings revealed that the *apr* and *lasB* genes were present in all isolates. A recent study conducted in Iran on the incidence of virulence genes of *P. aeruginosa* isolates by Bogiel *et al*., (٢٠٢٣) reported that the *lasB* and *PLCN* genes were found in all of the tested MDR isolates, however, the incidence rate is higher than that found in the present study of *lasB* (٨٨.٤٦٪) and *PLCN* (٩٢.٣٠٪). While the incidence rate of *pilA* (٥.٠٪) and *pilB* (٢.٠٪) genes had a lower frequency compared to the results of the current study of *pilA* (٨١.٥٣٪) and *pilB* (٩.٢٪).

A recent study conducted on *P. aeruginosa* virulence genes in west of Iran by Derakhshan and Hosseinzadeh, (٢٠٢٠) reported that *PLCH* was present in ٤٩ (٩٨٪) of the ٥٠ isolates, and *algD* was present in only ٣ (٦٪) of the isolates, however, compared to the present study's findings in which *PLCH* was detected in ١٢٥ (٩٦.١٥٪) vs ٤٩ (٩٨٪) but for *algD* only in٣ (٦٪) vs ١٢٢ (٩٣.٨٤٪) in this result. Additionally, the prevalence rate of the present study is higher than previous studies performed in Iran (٣٨.١٪) and India (٧٥٪) for *PLCH* and *algD* respectively (Sabharwal *et al*., ٢٠١٤; Badamchi *et al*., ٢٠١٧).

The *PLCH*, a hemolytic protein, is involved in pro-inflammatory activity as well as neutrophil respiratory burst inhibition (Wieland *et al*., ٢٠٠٢). It has also been reported that there is an association between elevated levels of hemolytic activity and the ability to cause significant tissue damage (Mittal *et al*., ٢٠٠٩). Several previous studies conducted the prevalence of alginate (*algD*) have been reported ٠٪,٦٪,٦٠٪, and ٦٣٪, (Fazeli and Momtaz, ٢٠١٤; Derakhshan and Hosseinzadeh, ٢٠٢٠; Badamchi *et al*., ٢٠١٧; Heidary *et al*., ٢٠١٦) respectively. However, these findings are lower than the results of the current study of *algD* (٩٣.٨٤٪).

Nonetheless, the current finding is remarkably similar to those of previous research conducted in Poland (٩٠.٩٪), (Wolska and Szweda, ٢٠٠٩).

One of the factors that affects pathogenesis of *P. aeruginosa* is its ability to form biofilm and the most essential component of *P. aeruginosa* biofilms is an exopolysaccharide known as alginate (Tielen *et al*., ٢٠١١). The GDP-mannose ٦-dehydrogenase (*algD*) enzyme is one of three proteins involved in the synthesis of alginate (Gholami *et al*., ٢٠١٧).

٤.٤.٣. Genotypic Detection of Type III Secretion System (T٣SS) Genes

The PCR and specific primers were used to amplify T٣SS-encoded toxins genes. The PCR results revealed that ١٢٤ (٩٥.٣٨٪) *P. aeruginosa* isolates were positive for the *exoS* gene, with an amplification product of about ٢٤٠ bp (Figure ٤.١٩).

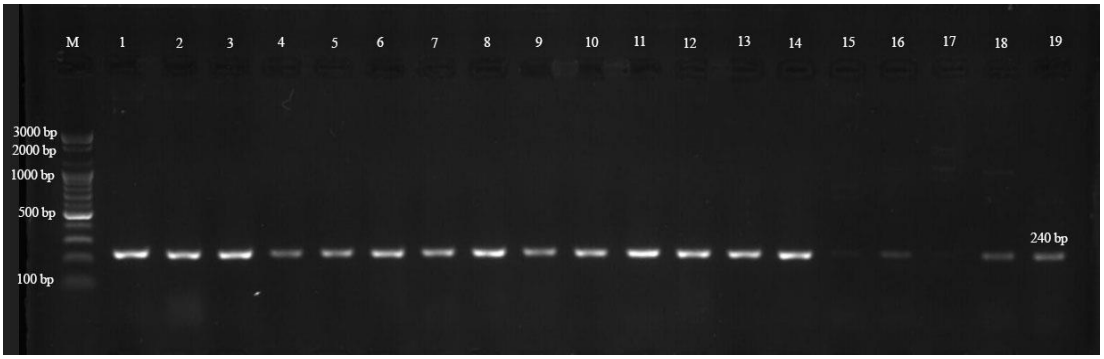

Figure ٤.١٩: PCR amplification of *exoS* gene (٢٤٠bp) in *P. aeruginosa* isolates, on ١.٥٪ agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-١٩) Positive isolates.

Invasive strains producing *ExoS* encoded by the *ExoS* gene can invade corneal epithelial cells (Suzuki and Inoue, ٢٠٢٢). Injection of *ExoS* into the target cell also alters the cell cytoskeleton, resulting in cell rounding, inhibition of phagocytosis, which eventually leads to apoptotic cell death (Foulkes *et al*., ٢٠١٩).

As shown in Figure ٤.٢٠, the presence of the gene encode *exoU* was detected in ١٢١ (٩٣.٠٧٪) of clinical isolates of *P. aeruginosa*, with an amplification product of approximately ٩٤ bp.

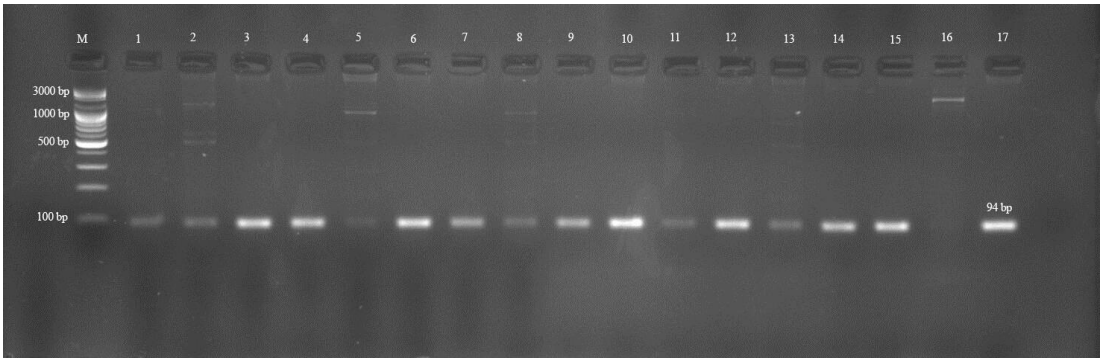

Figure ٤.٢٠: PCR amplification of *exoU* gene (٩٤bp) in *P. aeruginosa* isolates, on ٢ % agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١- ١٥ and ١٧) Positive isolates. Lane (١٦) negative isolates.

The presence of *ExoU* is higher in *P. aeruginosa* recovered from specific sources (Rutherford *et al*., ٢٠١٨). Regarding human infections, a higher prevalence of *ExoU* has been observed in samples from burn units or intensive care units (ICUs) (Horna *et al*., ٢٠١٩ a), or as a cause of chronic otitis media (Park *et al*., ٢٠١٧).

In regard to the presence of the *ToxA* gene, ١٢٧ (٩٧.٦٩٪) *P. aeruginosa* isolates were positive, with an amplification product of approximately ٣٩٧ bp, as shown in Figure (٤.٢١).

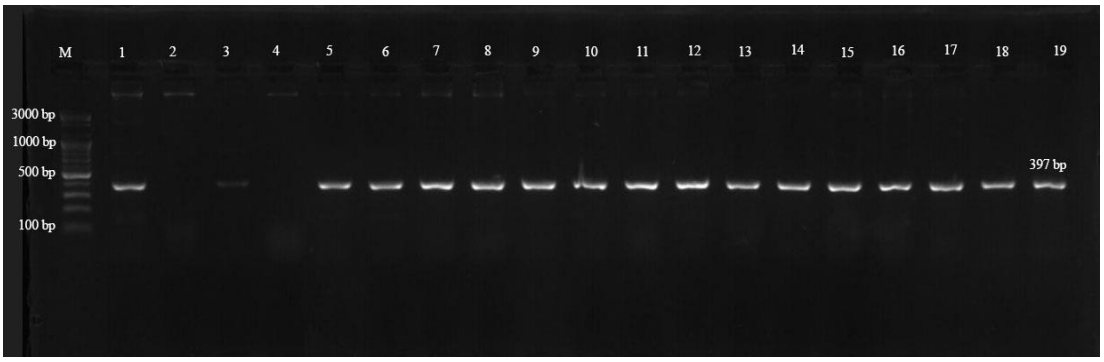

Figure ٤.٢١: PCR amplification of *ToxA* gene (٣٩٧ bp) in *P. aeruginosa* isolates, on ١.٥ % agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١,٣,٥,٦,٧,٨,٩,١٠,١١,١٢,١٣,١٤,١٥,١٦,١٧,١٨, and ١٩) Positive isolates. Lane (٢ and ٤) negative isolates.

P. aeruginosa produces a highly toxic virulence factor *ExoT*oxin A (*ExoA*) which is released into extracellular medium by type ٢ secretion system (T٢SS) (Mazor and Pastan, ٢٠٢٠). *ExoT*oxin A (*ExoA*) is encoded via the *ToxA* gene, obstruct synthesis of protein in the host cells. As well

as, produces phenazines which is redox-active and are toxic to the human cell. (Naher *et al*., ٢٠١٤).

P. aeruginosa has a virulence mechanism called T٣SS, which is a syringe-like structure that allows the introduction of toxins (such as *ExoS* and *ExoU*) into target cell cytoplasm (Engel and Balachandran, ٢٠٠٩).

The results of present study reported that most of *P. aeruginosa* isolates producing the T٣SS exotoxins; *ToxA* ١٢٧(٩٧.٦٩٪), *exoS* ١٢٤(٩٥.٣٨٪), and *exoU* ١٢١(٩٣.٠٧٪) (Figure ٤.٢٢).

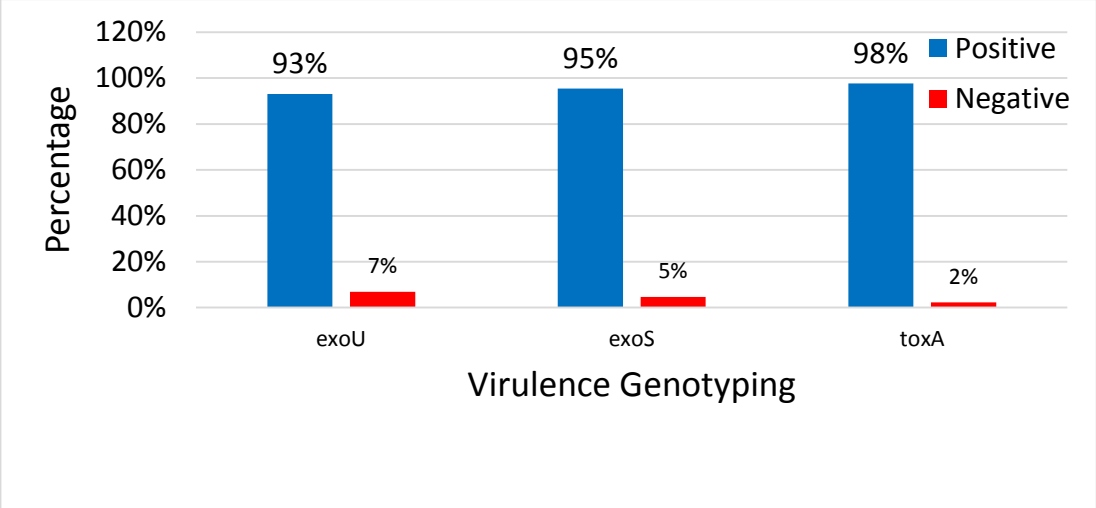

Figure ٤.٢٢: Distribution of T٣SS exotoxins of *P. aeruginosa* isolates

Prevalence and the presence of *exo* genes that encode type T٣SS proteins (*exoS*, *exoU*, and *ToxA*) were detected in all *P. aeruginosa* isolates, the higher percentage of *ToxA* was (٩٧.٦٩٪) followed by *exoS* (٩٥.٤٪) and *exoU* (٩٣.١٪).

The *ToxA* gene was found in ١٢٧ (٩٧.٦٩٪) of the isolates studied. However, the presence rate is higher than in a recent investigation carried out in Iraq (٣٥٪) (Alabdali, ٢٠٢١), and convenient with another study conducted in Sanandaj, west of Iran ٤٧ (٩٤٪) of the ٥٠ isolates (Derakhshan and Hosseinzadeh, ٢٠٢٠).

In clinical infections, exotoxin A is a key virulence factor of *P. aeruginosa*. It is a cytotoxic agent that, like diphtheria toxin, inhibits protein biosynthesis at the level polypeptide chain elongation factor ٢, causing significant tissue and organ damage (Jenkins *et al*., ٢٠٠٤). Exotoxin A (*ToxA*), which is highly conserved in *P. aeruginosa*, is a common target for *P. aeruginosa* detection in PCR and RT-PCR methods (Xu *et al*., ٢٠٠٤). In the present study, *ExoS* toxin was found to be more prevalent than *ExoU* toxin. This is consistent with previous studies' findings (Rumbaugh *et al*., ١٩٩٩; Mitov *et al*., ٢٠١٠). However, estimates of the actual prevalence of genes *exoS* and *exoU* production varied significantly. However, lower percentages were reported by Elnagar *et al*., (٢٠٢٢) ٢٨ (٦٢.٢٢٪) *exoS* and ١٩ (٤٢.٢٢٪) *exoU*, out of ٤٥ clinical isolates.

According to the studies listed and compared to the current study's findings, the observed discrepancies in the prevalence of *exoS* and *exoU* genes may be related to variation in specimen type, strain type, and geographical area of survey.

Regarding the virulence genotyping, the results of the present study, revealed ١٢٤ (٩٥.٣٨٪) of the ١٣٠ isolates belonged to the invasive genotype (positive for the *exoS* gene); however, ١٢١ (٩٣.٠٧٪) isolates belonged to the cytotoxic genotype (positive for the *exoU* gene). Furthermore, ١١٦ (٨٩.٢٣٪) isolates represented the $exoS^{+}/exoU^{+}$ genotype, while ١ (٠.٧٪) isolate was negative for both genes. Although the *exoS* and *exoU* genes are found in different regions of the *P. aeruginosa* genome, the occurrence of both genes in the same strain is rare, according to literature. However, a few recent investigations have revealed a significant incidence of isolates containing both *exoS* and *exoU* (Morales-Espinosa *et al*., ٢٠١٧; Mohamad *et al*., ٢٠١٨; Horna *et al*.,

٢٠١٩a). A previous study done by Feltman *et al*., (٢٠٠١) observed that the distribution of *exoS* and *exoU* is not random and that most isolates bearing *exoS* sequences lack *exoU* and vice versa.

One possible explanation is that each *ExoU* and *ExoS* protein has a various activity and plays a different role depending on the environmental conditions. As a result, the T٣SS genotype of a clinical isolate may be beneficial for predicting the pathogen's specific environmental reservoir (Feltman *et al*., ٢٠٠١; Maatallah *et al*., ٢٠١١).

In most strains, *ExoU* and *ExoS* are present as variable features and are mutually exclusive, the more common of the two genotypes is *exoS* (Agnello and Wong-Beringer, ٢٠١٢). However, this result is consistent with the findings of the current study, and contradicted another study that found *exoU* genotypes were more common than *exoS* genotypes in clinical *P. aeruginosa* isolates (Azimi *et al*.,٢٠١٦). Several studies reported that the *ExoU* is more harmful than *ExoS* toxin and is known to cause cytotoxicity, whereas *ExoS* is known to modulate bacterial invasion (Feltman *et al*., ٢٠٠١; Rabin and Hauser, ٢٠٠٣). Therefore, the presence of isolates producing both toxins observed in the present study could reflect the emergence of highly virulent isolates with a mixed invasive-cytotoxic genotype.

Moreover, in this study, eight *P. aeruginosa* isolates had the *exoS* gene but not the *exoU* gene, five had the *exoU* gene but not the *exoS* gene, and one isolate had both genes. Previous studies found that *P. aeruginosa* isolates contained either the *exoS* or *exoU* gene (Finck-Barbancon *et al*., ١٩٩٧; Fleiszig *et al*., ١٩٩٧). There is no clear explanation for this unusual association. One probability is that *ExoU* and *ExoS* perform important but excessive functions. However, beyond the first six amino acids, there is no sequence similarity between *ExoS* and *ExoU*. Moreover, *ExoS* damage

cell cytoskeletal elements and inhibits DNA replication (Pederson *et al*., ١٩٩٩; Henriksson *et al*., ٢٠٠٠).

٤.٤.٣.١. Association between Virulence Genes and T٣SS Genotypes

Regarding the distribution of virulence genes (*pilB, apr, (lasA, lasB, pilA, phzS, phzM, PLCN, PLCH, algD, algU,*and *ToxA*) among the T٣SS genotypes (invasive and cytotoxic genotypes). Table (٤.٤) revealed a statistically significant (p<٠.٠٥) association between virulence genes and T٣SS genotypes, with exception of *pilB* (p>٠.٠٥).

The present findings reported that the presence of *apr* and *ToxA* genes were significantly higher among invasive genotype isolates; however, the presence of *ToxA* gene was significantly higher among the cytotoxic genotype isolates, (Table ٤.٣).

Table (٤.٣): Association of occurrence of virulence genes among T٣SS genotypes

Virulence genes	Virulence genotypes (T٣SS)				p-value
	No. (%)				
	The invasive genotype (*exoS*$^+$)	**The cytotoxic genotype (*exoU*+)**	**The *exoS*$^+$/*exoU*$^+$ genotype**	**The *exoS*$^-$ /*exoU*$^-$ genotype**	
pilB	١٢ (٩.٢٣)	١١(٨.٤٦)	١١(٨.٤٦)	٠	٠.٩٧١
apr	١٢١(٩٣.٠٨)	١١٨(٩٠.٧٧)	١١٣(٨٦.٩٢)	١(٠.٧٧)	≤٠.٠٠٠١**
lasA	٣٨(٢٩.٢٣)	٣٧(٢٨.٤٦)	٣٥(٢٩.٩٢)	١(٠.٧٧)	٠.٠٠١**
lasB	١١١(٨٥.٣٨)	١٠٨(٨٣.٠٨)	١٠٥(٨٠.٧٧)	١(٠.٧٧)	≤٠.٠٠٠١**
pilA	١٠٠(٧٦.٩٢)	٩٩(٧٦.١٥)	٩٤(٧٢.٣١)	١(٠.٧٧)	≤٠.٠٠٠١**
phzS	١١٦(٨٩.٢٣)	١١٥(٨٨.٤٦)	١١٠(٨٤.٦٢)	١(٠.٧٧)	≤٠.٠٠٠١**
phzM	١١١(٨٥.٣٨)	١١٠(٨٤.٦٢)	١٠٥(٨٠.٧٧)	١(٠.٧٧)	≤٠.٠٠٠١**
PLCN	١١٥(٨٨.٤٦)	١١٥(٨٨.٤٦)	١١١(٨٥.٣٨)	١(٠.٧٧)	≤٠.٠٠٠١**
PLCH	١١٩(٩١.٥٤)	١١٦(٨٩.٢٣)	١١١(٨٥.٣٨)	١(٠.٧٧)	≤٠.٠٠٠١**
algD	١١٧(٩٠)	١١٤(٨٧.٦٩)	١١٠(٨٤.٦٢)	١(٠.٧٧)	≤٠.٠٠٠١**

algU	١٠١(٧٧.٦٩)	٩٩(٧٦.١٥)	٩٤(٧٢.٣١)	١(٠.٧٧)	≤٠.٠٠٠١**
ToxA	١٢١(٩٣.٠٨)	١١٩(٩١.٥٤)	١١٤(٨٧.٦٩)	١(٠.٧٧)	≤٠.٠٠٠١**

** significance differences ($p \leq ٠.٠١$).

According to Table ٤.٤, the invasive and cytotoxic genotypes are related with different virulence factors, and both genotypes may be present in the same isolates.

The type III secretion system (T٣SS) is a key determinant of cytotoxicity and invasion in which *P. aeruginosa* directly distributes multiple effector proteins into the host cell's cytoplasm (Roy-Burman *et al.*, ٢٠٠١). It has been found in the late stages of bacteremia and septicemia. The T٣SS enables the injection of toxins into the cytoplasm of target eukaryotic cells, where they damage host cell defense and signaling systems, causing fast call necrosis or altering the actin cytoskeleton (Sawa *et al.*, ٢٠١٤).

٤.٤.٤. Detection of Efflux Pump Genes (*MexAB-OprM*)

The PCR result showed ١٣٠ (١٠٠٪) *P. aeruginosa* isolates, were positive for the *MexA* gene with amplification product approximately ٥٠٣ bp, Figure (٤.٢٣).

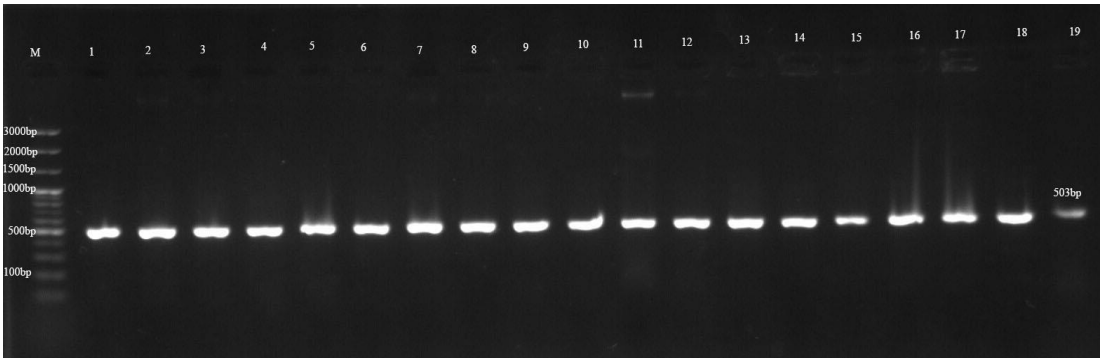

Figure (٤.٢٣): PCR amplification of *MexA* gene (٥٠٣ bp) in *P. aeruginosa* isolates, on ١.٥٪ agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-١٩) Positive isolates.

As indicated in Figure (٤.٢٤), the presence of the gene encoding *MexB* was identified in ١٢٢ (٩٣.٨٤٪) of clinical isolates of *P. aeruginosa* with an amplification product of ٢٨٠bp.

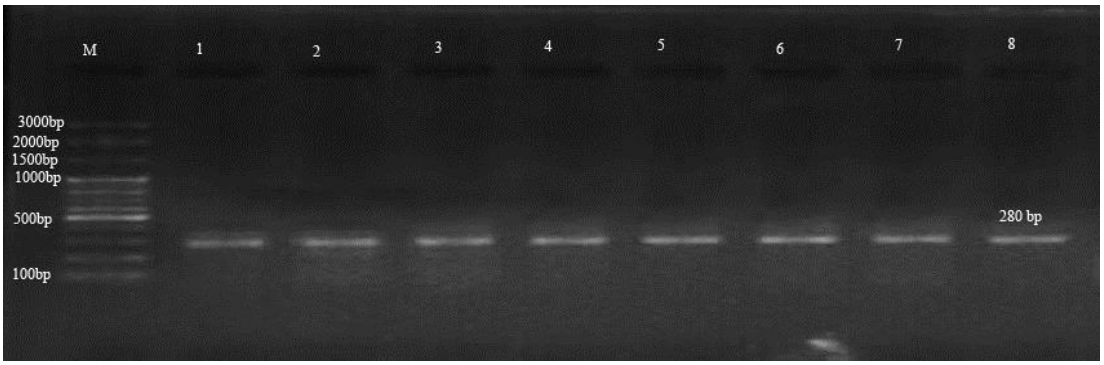

Figure (٤.٢٤): PCR amplification of *MexB* gene (٢٨٠ bp) in *P. aeruginosa* isolates, on ١.٥٪ agarose at ٧٠ volt for ٢ hrs. Lane M: ١٠٠-bp DNA marker. Lane (١-٨) Positive isolates.

Regarding the presence of *OprM* gene, ١٢٧(٩٧.٦٩٪) *P. aeruginosa* isolates, were positive and ٣(٢.٣٠ %) were negative isolates with amplification product approximately ٢٤٧ bp (Figure ٤.٢٥).

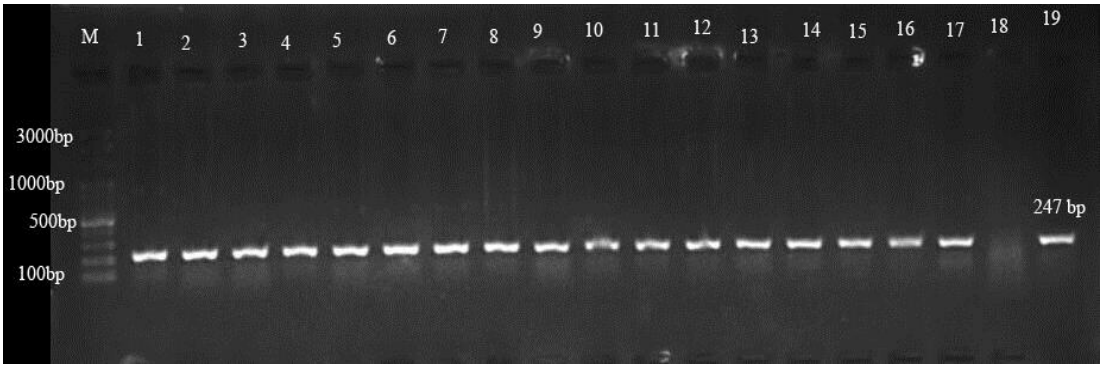

Figure (٤.٢٥): PCR amplification of *OprM* gene (٢٤٧ bp) in *P. aeruginosa* isolates, on ١.٥٪ agarose at ٧٠ volt for ٢ hrs. Lane M: ١Kb DNA marker. Lane (١- ١٧ and ١٩) Positive isolates. Lane (١٨) negative isolates.

The operon *MexAB-OprM* was the first multidrug efflux pump reported in *P. aeruginosa* (Poole *et al*., ١٩٩٣) and is considered the main contributor to antibiotic resistance, with *MexA* as the PMFP, *MexB* as the RNDt, and *OprM* as the OMF (López *et al*., ٢٠١٧).

All of the isolates of *P. aeruginosa* in this study have *MexA* ١٣٠ (١٠٠٪), *OprM* ١٢٧ (٩٧.٦٩٪), and *MexB* ١٢٢ (٩٣.٨٤٪), as shown in Figure (٤.٢٦).

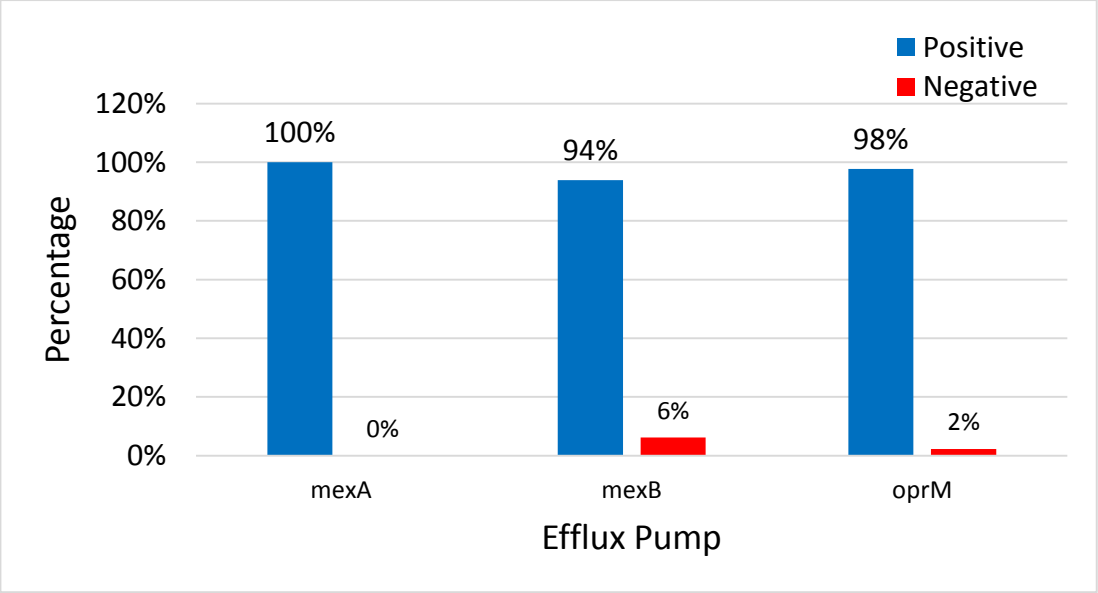

Figure ٤.٢٦: Distribution of Efflux Pump Genes of *P. aeruginosa* isolates

One of the most clinically significant resistance mechanisms is efflux mechanisms in Gram negative bacteria which is the resistance-nodulation-cell division (RND) family. The RND mainly comprises of *MexAB-OprM*, *MexCD-OprJ*, *MexEF-OprN*, and *MexXY-OprM* (Suresh *et al*., ٢٠١٨). *P. aeruginosa* RND efflux pumps (MexAB-OprM) are transport proteins that could be involved in the extrusion of all classes of therapeutically useful antimicrobial medicines into the surrounding environment (Pan *et al*., ٢٠١٦), where it contributes to intrinsic resistance to a variety of antibiotics such as fluoroquinolones, *β*-lactams, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim, and sulphonamides (Srikumar *et al*., ١٩٩٨).

Efflux pump genes are found in all strains but are not highly expressed. However, increased expression can be caused by a mutation in a regulatory gene, such as *mexR*, which regulates the *mexAB-oprM* genes (Azimi *et al*., ٢٠١٦; Schindler and Kaatz, ٢٠١٦). The *OprM* and *MexB*

system are connected by *MexA*, which serves as a connecting bridge, located in the bacterial outer membrane, *OprM* is a lipoprotein that plays a key role in mediating the antibiotic's final evacuation (Ibtesam Ghadban Auda *et al*., ٢٠١٢). Comparable findings reported by Alsaadi, (٢٠٢٢) demonstrated that the (٩٤.٧ %) of resistant *P. aeruginosa* isolates have *MexB* gene, and it also supports a recent study conducted in ALNajaf city, Iraq indicated that (٩٣.٧٥٪) of isolates had the *MexB* gene (Al-Absawe and Tuwaij,٢٠٢٢). While the results of the present study are higher than from another study done by Pourakbari *et al*., (٢٠١٦) reported that *MexB* gene was (٥٣.٣ %) of *P. aeruginosa* isolates.

In a study performed by Abdallah *et al*., (٢٠٢١), they noted that ٧٨ (٢٩.٤٪) *P. aeruginosa* isolates were recovered from various clinical sources, and all of these isolates carried the efflux pump genes *mexA, mexB,* and *oprM*. Another study was conducted by Abbas *et al*., (٢٠١٨) identified *mexA* and *mexB* genes in all *P. aeruginosa* isolates were ١٠٠٪.

According to earlier studies, the efflux pump can actively pump antibiotics out of bacterial cells to decrease the concentration of the drugs, which leads to drug resistance (Adabi *et al*., ٢٠١٥; Cunrath *et al*., ٢٠١٩). Overexpression of *MexA* has been associated to mutations in several genes, including *mexR, nalc*, and *nalD* (Pan *et al*., ٢٠١٦). This was confirmed by (Daigle et al., ٢٠٠٧) reported that mutations in *mexR*, *nalc* and *nalD* can result in increased *MexAB-OprM* expression and multidrug resistance. Additionally, by Choudhury *et al*., (٢٠١٥) and Suresh *et al*., (٢٠١٨) demonstrated that *MexAB-OprM* expression is negatively regulated by the regulatory genes *mexR, nalD,* and *nalc*, and any type of mutant may result in *MexAB-OprM* upregulation, and increasing drug resistance in *P. aeruginosa*.

٤.٤.٤.١. Association between Antibiotics and Efflux Pump Genes

The current study's statistical analysis demonstrated a significant association between the existence of the efflux pump genes (*mexA, mexB,* and *oprM*) and resistance to various antibiotic classes (P≤ ٠.٠٥). As shown in Appendix ٣, there was no statistically significant relationship between the presence of efflux pump genes and resistance to piperacillin-Tazobactam, Doripenem, and Tetracyclin (P > ٠.٠٥). In addition to the MexAB-OprM efflux pump system, three other efflux pump systems (*MexCDOprJ*, *MexEF-OprN*, and *MexXY-OprM*) contribute to antibiotic resistance. Biofilm and efflux pumps are not the only mechanisms that contribute to an organism's antibiotic resistance; numerous other factors also play a role in the development of a drug-resistant organism (Bhandari *et al*., (٢٠٢٢). The findings reported that *mexA*, *mexB* and *oprM* genes were more prevalent in isolates that were resistant to levofloxacin (p≤ ٠.٠٥). These findings are in line with a previous study by Dupont *et al*., (٢٠٠٥). While the current findings are distinct from those of Bhandari *et al*., (٢٠٢٢). The majority of MDR isolates harbored the *MexA, MexB* and *OprM* genes (p≤ ٠.٠٥). Additionally, the abundance of the *MexA, MexB* and *OprM* genes was higher in isolates resistant to different classes of antibiotics (p≤٠.٠٥) as exhibited in Appendix ٣.

Conclusions and Recommendations

Conclusions

- The rate of resistance to multiple antibiotic classes among the *P. aeruginosa* isolates was high in the present study.
- The analysis of the antibiotic resistance of *P. aeruginosa* isolates assayed in this study showed a high level of resistance ranged from ٩٢-١٠٠٪ to ampicillin, ticarcillin, ceftazidime, doripenem, ertapenem, tetracycline, and erythromycin.
- All the *P. aeruginosa* isolates assayed exhibited biofilm-forming ability with majority being strong and moderate producers.
- Based on chi-square, there is no significant association between biofilm-forming ability and multi-drug resistance ($p>٠.٠٥$).
- The most common virulence factor gene was *apr*

- Based on virulence genes classification the number of the invasiveness gene- isolates (*exoS* gene) was slightly higher than the cytotoxic gene-isolates (*exoU*).

Recommendations

- Futur studies to determine the percentage of clinical isolates capable of secreting specific effector proteins and whether secretion correlates with disease outcomes will further elucidate the role of type III secretion in *P. aeruginosa* pathogenesis.
- Further molecular studies looking for subvariants within the two genotypes, invasive and cytotoxic genotypic are warranted.

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Appendices

Appendix١

bioMérieux Customer:

Microbiology Chart Report

Printed June 23, 2022 12:26:43 PM CDT

Patient Name: 132, 132
Location:
Lab ID: 22620223

Patient ID: 22620223
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:** 6.05 hours	**Status:** Final
Selected Organism	99% Probability **Pseudomonas aeruginosa** **Bionumber:** 0043053003500252	
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 23, 2022 12:26:44 PM CDT

Patient Name: 133, 133
Location:
Lab ID: 22620224

Patient ID: 22620224
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:** 4.82 hours	**Status:** Final
Selected Organism	97% Probability **Pseudomonas aeruginosa** **Bionumber:** 0043451303500050	
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 ٢

Association of occurrence of T٣SS genes and antibiotics

Virulence genes / Antibiotics		*exoU*		*ExoS*		*ToxA*		p-value
		+	-	+	-	+	-	
Penicillins								
Ampicillins	**R**	١١٦ (٨٩.٢٣)	٩ (٦.٩٢)	١١٩ (٩١.٥٤)	٦ (٤.٦٢)	١٢٢ (٩٣.٨٥)	٣ (٢.٣١)	٠.٠٢٢*
	S	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	
Piperacillin	**R**	١١٢ (٨٦.١٥)	٨ (٦.١٥)	١١٦ (٨٩.٢٣)	٤ (٣.٠٨)	١١٧ (٩٠.٠)	٣ (٢.٣١)	٠.٠٢٩*
	S	٩ (٦.٩٢)	١ (٠.٧٧)	٨ (٦.١٥)	٢ (١.٥٤)	١٠ (٧.٦٩)	٠ (٠)	
piperacillin-Tazobactam	**R**	٩٢ (٧٠.٧٧)	٨ (٦.١٥)	٩٨ (٧٥.٣٨)	٢ (١.٥٤)	٩٨ (٧٥.٣٨)	٢ (١.٥٤)	٠.٠٤٨*
	S	٢٩ (٢٢.٣١)	١ (٠.٧٧)	٢٦ (٢٠.٠)	٤ (٣.٠٨)	٢٩	١ (٠.٧٧)	
Ticarcillin	**R**	١١٨ (٩٠.٧٧)	٨ (٦.١٥)	١٢٠ (٩٢.٣١)	٦ (٤.٦٢)	١٢٣ (٩٤.٦٢)	٣ (٢.٣١)	٠.٠١١*
	S	٣ (٢.٣١)	١ (٠.٧٧)	٤ (٣.٠٨)	٠ (٠)	٤ (٣.٠٨)	٠ (٠)	
Cephalosporins								
Ceftazidime	**R**	١١٩ (٩١.٥٤)	٩ (٦.٩٢)	١٢٢ (٩٣.٨٥)	٦ (٤.٦٢)	١٢٥ (٩٦.١٥)	٣ (٢.٣١)	٠.٠٣٦*
	S	٢ (١.٥٤)	٠ (٠)	٢ (١.٥٤)	٠ (٠)	٢ (١.٥٤)	٠ (٠)	
Cefepime	**R**	١٠٦ (٨١.٥٤)	٨ (٦.١٥)	١٠٩ (٨٣.٨٥)	٥ (٣.٨٥)	١١١ (٨٥.٣٨)	٣ (٢.٣١)	٠.٠٤٤*
	S	١٥ (١١.٥٤)	١ (٠.٧٧)	١٥ (١١.٥٤)	١ (٠.٧٧)	١٦ (١٢.٣١)	٠ (٠)	

Monobactam								
Azetroname	**R**	١٠٤ (٨٠.٠)	٧ (٥.٣٨)	١٠٨ (٨٣.٠٨)	٣ (٢.٣١)	١٠٨ (٨٣.٠٨)	٣ (٢.٣١)	٠.٠١٩*
	S	١٧ (١٣.٠٨)	٢ (١.٥٤)	١٦ (١٢.٣١)	٣ (٢.٣١)	١٩ (١٤.٦٢)	٠ (٠)	
Carbapenems								
Doripenem	**R**	١١٠ (٨٤.٦٢)	٨ (٦.١٥)	١١٢ (٨٦.١٥)	٦ (٤.٦٢)	١١٥ (٨٨.٤٦)	٣ (٢.٣١)	٠.٠٢٧*
	S	١١ (٨.٤٦)	١ (٠.٧٧)	١٢ (٩.٢٣)	٠ (٠)	١٢ (٩.٢٣)	٠ (٠)	
Etrapenem	**R**	١١٦ (٨٩.٢٣)	٩ (٦.٩٢)	١١٩ (٩١.٥٤)	٦ (٤.٦٢)	١٢٢ (٩٣.٨٥)	٣ (٢.٣١)	٠.٠١١*
	S	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	
Imipenem	**R**	٨٦ (٦٦.١٥)	٤ (٣.٠٨)	٨٨ (٦٧.٦٩)	٢ (١.٥٤)	٨٧ (٦٦.٩٢)	٣ (٢.٣١)	٠.٠٥٢
	S	٣٥ (٢٦.٩٢)	٥ (٣.٨٥)	٣٦ (٢٧.٦٩)	٤ (٣.٠٨)	٤٠ (٣٠.٧٧)	٠ (٠)	
Meropenem	**R**	٩٣ (٧١.٥٤)	٧ (٥.٣٨)	٩٨ (٧٥.٣٨)	٢ (١.٥٤)	٩٧ (٧٤.٦٢)	٣ (٢.٣١)	٠.٠٥٥
	S	٢٩ (٢٢.٣١)	٢ (١.٥٤)	٢٦ (٢٠.٠)	٤ (٣.٠٨)	٣٠ (٢٣.٠٨)	٠ (٠)	
Aminoglycosides								
Gentamicin	**R**	٩٤ (٧٢.٣١)	٧ (٥.٣٨)	٩٧ (٧٤.٦٢)	٤ (٣.٠٨)	٩٨ (٧٥.٣٨)	٣ (٢.٣١)	٠.٠٤٨*
	S	٢٧ (٢٠.٧٧)	٢ (١.٥٤)	٢٧ (٢٠.٧٧)	٢ (١.٥٤)	٢٩ (٢٢.٣١)	٠ (٠)	
Tobramycin	**R**	٨٨ (٦٧.٦٩)	٧ (٥.٣٨)	٩٣ (٧١.٥٤)	٢ (١.٥٤)	٩٣ (٧١.٥٤)	٢ (١.٥٤)	٠.٠٤٩*
	S	٣٣ (٢٥.٣٨)	٢ (١.٥٤)	٣١ (٢٣.٨٥)	٤ (٣.٠٨)	٣٤ (٢٦.١٥)	١ (٠.٧٧)	
Amikacin	**R**	٩٢	٧	٩٦	٣	٩٧	٢	٠.٠٤٤*

		(٧٠.٧٧)	(٥.٣٨)	(٧٣.٨٥)	(٢.٣١)	(٧٤.٦٢)	(١.٥٤)	
	S	٢٩ (٢٢.٣١)	٢ (١.٥٤)	٢٨ (٢١.٥٤)	٣ (٢.٣١)	٣٠ (٢٣.٠٨)	١ (٠.٧٧)	
Tetracyclins								
Tetracyclin	**R**	١١٥ (٨٨.٤٦)	٧ (٥.٣٨)	١١٦ (٨٩.٢٣)	٦ (٤.٦٢)	١١٩ (٩١.٥٤)	٣ (٢.٣١)	٠.٠٢٨*
	S	٦ (٤.٦٢)	٢ (١.٥٤)	٨ (٦.١٥)	٠ (٠)	٨ (٦.١٥)	٠ (٠)	
Doxycyclin	**R**	١٠٤ (٨٠.٠)	٧ (٥.٣٨)	١٠٥ (٨٠.٧٧)	٦ (٤.٦٢)	١٠٨ (٨٣.٠٨)	٣ (٢.٣١)	٠.٠٤٧*
	S	١٧ (١٣.٠٨)	٢ (١.٥٤)	١٩ (١٤.٦٢)	٠ (٠)	١٩ (١٤.٦٢)	٠ (٠)	
Macrolides								
Erythromycin	**R**	١١٧ (٩٠.٠)	٨ (٦.١٥)	١١٩ (٩١.٥٤)	٦ (٤.٦٢)	١٢٢ (٩٣.٨٥)	٣ (٢.٣١)	٠.٠٢٢*
	S	٤ (٣.٠٨)	١ (٠.٧٧)	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	
Fluoroquinolones								
Ciprofloxacin	**R**	٨٤ (٦٤.٦٢)	٦ (٤.٦٢)	٨٨ (٦٧.٦٩)	٢ (١.٥٤)	٨٩ (٦٨.٤٦)	١ (٠.٧٧)	٠.٠٥٤
	S	٣٧ (٢٨.٤٦)	٣ (٢.٣١)	٣٦ (٢٧.٦٩)	٤ (٣.٠٨)	٣٨ (٢٩.٢٣)	٢ (١.٥٤)	
Ofloxacin	**R**	٨٨ (٦٧.٦٩)	٦ (٤.٦٢)	٩٢ (٧٠.٧٧)	٢ (١.٥٤)	٩٢ (٧٠.٧٧)	٢ (١.٥٤)	٠.٠٦٢
	S	٣٣ (٢٥.٣٨)	٣ (٢.٣١)	٣٢ (٢٤.٦٢)	٤ (٣.٠٨)	٣٥ (٢٦.٩٢)	١ (٠.٧٧)	
Levofloxacin	**R**	٩٢ (٧٠.٧٧)	٦ (٤.٦٢)	٩٦ (٧٣.٨٥)	٢ (١.٥٤)	٩٧ (٧٤.٦٢)	١ (٠.٧٧)	٠.٠٤٦*
	S	٢٩ (٢٢.٣١)	٣ (٢.٣١)	٢٨ (٢١.٥٤)	٤ (٣.٠٨)	٣٠ (٢٣.٠٨)	٢ (١.٥٤)	

Appendix ٣

Association of occurrence of Efflux pump genes and antibiotics

Virulence genes / Antibiotics		*MexA*		*MexB*		*OprM*		p-value
		+	-	+	-	+	-	
Penicillins								
Ampicillins	**R**	١٢٥ (٩٦.١٥)	٠ (٠)	١١٧ (٩٠.٠)	٨ (٦.١٥)	١٢٢ (٩٣.٨٥)	٣ (٢.٣١)	٠.٠٤١*
	S	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	
Piperacillin	**R**	١٢٠ (٩٢.٣١)	٠ (٠)	١١٢ (٨٦.١٥)	٨ (٦.١٥)	١١٧ (٩٠.٠)	٣ (٢.٣١)	٠.٠٣٢*
	S	١٠ (٧.٦٩)	٠ (٠)	١٠ (٧.٦٩)	٠ (٠)	١٠ (٧.٦٩)	٠ (٠)	
piperacillin-Tazobactam	**R**	١٠٠ (٧٦.٩٢)	٠ (٠)	٩٣ (٧١.٥٤)	٧ (٥.٣٨)	٩٧ (٧٤.٦٢)	٣ (٢.٣١)	٠.١٣٩
	S	٣٠ (٢٣.٠٨)	٠ (٠)	٢٩ (٢٢.٣١)	١ (٠.٧٧)	٣٠ (٢٣.٠٨)	٠ (٠)	
Ticarcillin	**R**	١٢٦ (٩٦.٩٢)	٠ (٠)	١١٨ (٩٠.٧٧)	٨ (٦.١٥)	١٢٣ (٩٤.٦٢)	٣ (٢.٣١)	٠.٠٣١*
	S	٤ (٣.٠٨)	٠ (٠)	٤ (٣.٠٨)	٠ (٠)	٤ (٣.٠٨)	٠ (٠)	
Cephalosporins								
Ceftazidime	**R**	١٢٨ (٩٨.٤٦)	٠ (٠)	١٢٠ (٩٢.٣١)	٨ (٦.١٥)	١٢٥ (٩٦.١٥)	٣ (٢.٣١)	٠.٠٤٧*
	S	٢ (١.٥٤)	٠ (٠)	٢ (١.٥٤)	٠ (٠)	٢ (١.٥٤)	٠ (٠)	
Cefepime	**R**	١١٤ (٨٧.٦٩)	٠ (٠)	١٠٦ (٨١.٥٤)	٨ (٦.١٥)	١١١ (٨٥.٣٨)	٣ (٢.٣١)	٠.٠٤٤*
	S	١٦ (١٢.٣١)	٠ (٠)	١٦ (١٢.٣١)	٠ (٠)	١٦ (١٢.٣١)	٠ (٠)	

Monobactam								
Azetroname	**R**	١١١ (٨٥.٣٨)	٠ (٠)	١٠٣ (٧٩.٢٣)	٨ (٦.١٥)	١٠٨ (٨٣.٠٨)	٣ (٢.٣١)	٠.٠٤٦*
	S	١٩ (١٤.٦٢)	٠ (٠)	١٩ (١٤.٦٢)	٠ (٠)	١٩ (١٤.٦٢)	٠ (٠)	
Carbapenems								
Doripenem	**R**	١١٨ (٩٠.٧٧)	٠ (٠)	١١٠ (٨٤.٦٢)	٨ (٦.١٥)	١١٥ (٨٨.٤٦)	٣ (٢.٣١)	٠.٠٥٢
	S	١٢ (٩.٢٣)	٠ (٠)	١٢ (٩.٢٣)	٠ (٠)	١٢ (٩.٢٣)	٠ (٠)	
Ertapenem	**R**	١٢٥ (٩٦.١٥)	٠ (٠)	١١٧ (٩٠.٠)	٨ (٦.١٥)	١٢٢ (٩٣.٨٥)	٣ (٢.٣١)	٠.٠٤٩*
	S	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	
Imipenem	**R**	٩٠ (٦٩.٢٣)	٠ (٠)	٨٢ (٦٣.٠٨)	٨ (٦.١٥)	٨٧ (٦٦.٩٢)	٣ (٢.٣١)	٠.٠١٧*
	S	٤٠ (٣٠.٧٧)	٠ (٠)	٤٠ (٣٠.٧٧)	٠ (٠)	٤٠ (٣٠.٧٧)	٠ (٠)	
Meropenem	**R**	١٠٠ (٧٦.٩٢)	٠ (٠)	٩٢ (٧٠.٧٧)	٨ (٦.١٥)	٩٧ (٧٤.٦٢)	٣ (٢.٣١)	٠.٠٤٢*
	S	٣٠ (٢٣.٠٨)	٠ (٠)	٣٠ (٢٣.٠٨)	٠ (٠)	٣٠ (٢٣.٠٨)	٠ (٠)	
Aminoglycosides								
Gentamicin	**R**	١٠١ (٧٧.٦٩)	٠ (٠)	٩٣ (٧١.٥٤)	٨ (٦.١٥)	٩٨ (٧٥.٣٨)	٣ (٢.٣١)	٠.٠٤٤*
	S	٢٩ (٢٢.٣١)	٠ (٠)	٢٩ (٢٢.٣١)	٠ (٠)	٢٩ (٢٢.٣١)	٠ (٠)	
Tobramycin	**R**	٩٥ (٧٣.٠٨)	٠ (٠)	٨٧ (٦٦.٩٢)	٨ (٦.١٥)	٩٢ (٧٠.٧٧)	٣ (٢.٣١)	٠.٠٤٧*
	S	٣٥ (٢٦.٩٢)	٠ (٠)	٣٥ (٢٦.٩٢)	٠ (٠)	٣٥ (٢٦.٩٢)	٠ (٠)	
Amikacin	**R**	٩٩	٠	٩١	٨	٩٦	٣	٠.٠٤٨*

		(٧٦.١٥)	(٠)	(٧٠.٠)	(٦.١٥)	(٧٣.٨٥)	(٢.٣١)	
	S	٣١ (٢٣.٨٥)	٠ (٠)	٣١ (٢٣.٨٥)	٠ (٠)	٣١ (٢٣.٨٥)	٠ (٠)	
Tetracyclins								
Tetracyclin	**R**	١٢٢ (٩٣.٨٥)	٠ (٠)	١١٤ (٨٧.٦٩)	٨ (٦.١٥)	١١٩ (٩١.٥٤)	٣ (٢.٣١)	٠.٠٥١
	S	٨ (٦.١٥)	٠ (٠)	٨ (٦.١٥)	٠ (٠)	٨ (٦.١٥)	٠ (٠)	
Doxycyclin	**R**	١١١ (٨٥.٣٨)	٠ (٠)	١٠٣ (٧٩.٢٣)	٨ (٦.١٥)	١٠٨ (٨٣.٠٨)	٣ (٢.٣١)	٠.٠٣٣*
	S	١٩ (١٤.٦٢)	٠ (٠)	١٩ (١٤.٦٢)	٠ (٠)	١٩ (١٤.٦٢)	٠ (٠)	
Macrolides								
Erythromycin	**R**	١٢٥ (٩٦.١٥)	٠ (٠)	١١٧ (٩٠.٠)	٨ (٦.١٥)	١٢٢ (٩٣.٨٥)	٣ (٢.٣١)	٠.٠٣٩*
	S	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	٥ (٣.٨٥)	٠ (٠)	
Fluoroquinolones								
Ciprofloxacin	**R**	٩٠ (٦٩.٢٣)	٠ (٠)	٨٦ (٦٦.١٥)	٤ (٣.٠٨)	٨٨ (٦٧.٦٩)	٢ (١.٥٤)	٠.٠٥٠*
	S	٤٠ (٣٠.٧٧)	٠ (٠)	٣٦ (٢٧.٦٩)	٤ (٣.٠٨)	٣٩ (٣٠.٠)	١ (٠.٧٧)	
Ofloxacin	**R**	٩٤ (٧٢.٣١)	٠ (٠)	٨٨ (٦٧.٦٩)	٦ (٤.٦٢)	٩١ (٧٠.٠)	٣ (٢.٣١)	٠.٠١٨*
	S	٣٦ (٢٧.٦٩)	٠ (٠)	٢٤ (١٨.٤٦)	٢ (١.٥٤)	٣٦ (٢٧.٦٩)	٠ (٠)	
Levofloxacin	**R**	٩٨ (٧٥.٣٨)	٠ (٠)	٩٢ (٧٠.٧٧)	٦ (٤.٦٢)	٩٥ (٧٣.٠٨)	٣ (٢.٣١)	٠.٠٠٨**
	S	٣٢ (٢٤.٦٢)	٠ (٠)	٣٠ (٢٣.٠٨)	٢ (١.٥٤)	٣٢ (٢٤.٦٢)	٠ (٠)	

الخلاصة

تُعرف *الزائفة الزنجارية* بأنه أحد الأسباب الرئيسية لعدوى المستشفيات ويرتبط مرارًا وتكرارًا بالعدوى الانتهازية بين المرضى في المستشفيات في جميع أنحاء العالم. في الدراسة الحالية، تم الحصول على ٣٨٤ عينة من مستشفى الامام الصادق ومستشفى الحلة التعليمي ومستشفى مرجان من اذار/ ٢٠٢٢ إلى تشرين الثاني /٢٠٢٢ تم جمعها من مصادر مختلفة بما في ذلك مرضى (الحروق، الجروح، التهاب المجاري البولية، التهاب الاذن والبلغم). أظهرت النتائج أن ١٣٠ (٣٣.٨٪) من العزلات كانت *الزوائف الزنجارية* موزعة كالتالي مرضى الحروق ١٠٢(٧٨.٥٪) والبلغم ٣ (٢.٣٪) والتهاب الاذن ٩ (٦.٩٪) والجروح ٧ (٥.٤٪) ومرضى التهاب المجاري البولية ٩ (٦.٩٪). بالإضافة الى ذلك تم تحديد هذه العزلات على أنها *زوائف زنجارية* باستخدام VITEK® ٢ Compact نظرًا لتعليمات الشركة المصنعة. تم استخدام فحص تفاعل البوليميراز المتسلسل (PCR) ، لجين *ecfX* المستهدف و هو الجين التشخيصي الخاص ب *الزائفه الزنجارية واظهرات ان جميع ١٣٠ عزلة موجبة لجين ecfX.*

أظهرت النتائج مقاومة للمضادات الحيوية بنسب مئوية كالتالي: الأمبيسلين (٩٦.٩٪)، بيبيراسيلين (٩٢.٣٪)، بيبيراسيلين-تازوباكتام (٧٦.٩٪)، تيكارسيلين (٩٧.٧٪)، سيفتازيديم (٩٩.٢٪)، سيفيبيم (٨٧.٧٪)، أزيترونام (٨٥.٤٪)، دوريبينيم (٩٠.٨٪)، إيترابينيم (٩٦.٩٪)، ميروبينيم (٧٦.٩٪)، أميكاسين (٧٧.٧٪)، جنتاميسين (٧٣.١٪)، توبراميسين (٧٦.٢٪)، تتراسيكلين (٩٤.٦٪)، دوكسيسيكلين (٨٦.٢٪)، وإريثروميسين (٩٦.٩٪) وليفوفلوكساسين (٧٢.٣٪) وأوفلوكساسين (٧٥.٤٪) عند مقارنتها بمقاومة إيميبينيم وسيبروفلوكساسين (٦٩.٢٪) نتيجة لذلك أظهر بان الزوائف الزنجارية مقاومة للعديد من المضادات الحيوية وكانت نسبته (٩٦.١٪) ١٣٠/١٢٥.

فيما يتعلق بقدرتها على تكوين الأغشية الحيوية وفقًا لطريقة Congo read agar ، كانت ١٣٠/١١١ (٨٥.٤٪) من العزلات، حيث تم تصنيفها في الفئات التالية: ٤٠ (٣٠.٨٪) شكلت غشاء حيوي قويًا؛ ٥١ (٣٩.٢٪) شكلت غشاء حيوي معتدل؛ ٢٠ (١٥.٤%) شكلت غشاء حيوي ضعيف، بينما ١٩ (١٤.٦%) من عزلات *الزوائف الزنجارية* تم وصفها بأنها غير مكونة الاغشية الحيوية. من بين عشرين مضادًا حيويًا تم فحصها، لا يوجد فرق معنوي (P>٠.٠٥) بين قابلية هذه العزلات على تكوين الأغشية الحيوية وأنماط مقاومة المضادات الحيوية، باستثناء imipenem (P=٠.٠٠٥٦). جينات مضخة التدفق (*MexA, MexB* and *OprM*) *MexAB-OprM* تم فحصها بتقنية PCR و أظهرت النتيجة ١٣٠ (١٠٠٪) عزلة

موجبة *MexA* و ١٢٧ (٩٧.٦٩٪) إلى *OprM*. و ١٢٢ (٩٣.٨٤٪) إلى *MexB* أظهر التحليل الإحصائي ارتباطًا معنويًا بين وجود جينات مضخة التدفق(*MexA ,MexB OprM*) ومقاومة لفئات المضادات الحيوية المختلفة (P≤ ٠.٠٥) ،وكذالك مرتبطة بشكل معنوي مع MDR (p <٠.٠٥).

اظهرت النتائج الحالية نسبًا مختلفة من جينات عامل الضراوة ١٢٧ *apr* (٩٨%)، ١٢٥ *PLCH*(٩٦%)، ١٢٢ كلًا من *algD* و *phzS* (٩٤%)، ١٢٠ *PLCN* (٩٢%) ، ١١٧ *phzM* (٩٠%)، ١١٥ *lasB* (٨٨%)، ١٠٧ *algU* (٨٢%)، ١٠٦ *pilA* (٨٢%)، ٤١ *lasA* (٣٢%)، و١٢ *pilB* (٩%) التي تم تحديدها في عزلات *الزوائف الزنجارية*. اما بالنسبة لنظام إلافرازي من النوع الثالث (T٣SS)، فقد اوضحت نتائج PCR أن ١٢٧ (٩٧.٦٩٪) من عزلات *الزوائف الزنجارية* كانت إيجابية لجين *ToxA*، وتم اكتشاف ١٢٤ (٩٥.٣٨٪) من جين *exoS* و*exoU* في ١٢١ (٩٣.٠٧٪). اعتمادا على تصنيف جينات الضراوة الى جينات غازية وجينات سامة، العزلات الحاملة لجين *exoS* تعتبر غازية ١٢٤ (٩٥٪) وهو أعلى قليلاً من العزلات السامة لجين *exoU* ١٢١ (٩٣٪). أظهرت النتائج وجود علاقة ذات دلالة إحصائية (P<٠.٠٥) بين جينات الضراوة والأنماط الجينية T٣SS، باستثناء (P>٠.٠٥) *pilB*. اظهرت النتائج الحالية أن وجود جين *aprA* كان أعلى بكثير بين العزلات الغازي؛ وظهر ارتفاع ملحوظ لجين *ToxA* بين العزلات السامة. كشف التحليل الإحصائي لهذه الدراسة عن وجود علاقة معنوية بين وجود جينات السموم الخارجية *exoS* و*exoU* و*ToxA* مع مقاومة لفئات المضادات الحيوية المختلفة (P ≤ ٠.٠٥).

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

دراسة جزيئية لمورثات عوامل الضراوة في الزوائف الزنجارية متعددة المقاومة للمضادات الحياتية

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

من قبل

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