

The Republic of Iraq
Ministry of Higher Education and
Scientific Research
University of Mosul / College of Science
Department of Biology



Inhibition of quorum sensing production in
***Pseudomonas aeruginosa* and investigate its role in**
immune response modulation

Ahmed Yousif Saeed Salih AL-Badrany

Ph.D. Thesis

Biology /Microbiology

Supervised By

Prof. Dr. Hiyam Adel Ibrahim Altaii

144٦ A.H.

202٤ A.D.

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Ph.D. Thesis Submitted By

Ahmed Yousif Saeed Salih AL-Badrany

To The Council of College of Sciences / University of Mosul in partial
fulfillment of the requirements for the degree of

Doctor of Philosophy

In

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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Ahmed

Abstract

This study was conducted to evaluate the effectiveness, the inhibition of Quorum sensing (QS) produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) and its role in immune modulation response in immune cell culture prepared from mice organ. The pathogenicity of *P. aeruginosa* is associated with virulence factors. Various QS systems in bacteria regulate virulence gene expression.

In the current study , a total of (245) urine specimen were collected from both sexes of patient with Urinary Tract Infection (UTI), from different ages. At Mosul's Ibn Senna Teaching Hospital, they received no antibiotic treatment. This study will take place between March and September of 2023. Depending on the culture, colony morphology, and microscopic analysis of the wet film, staining with Gram's stain, biochemical identification and Analysis Profile Index 20E (API). Eight (4%) isolates of *P. aeruginosa* were obtained out of (200) positive growth . DNA of 8 isolates of *P. aeruginosa* were extracted with high purity and all isolates were diagnosed by 16S rRNA gene sequencing. Four of them were submitted in the National Center for Biotechnology Information (NCBI) as new strains.

The ability of *P. aeruginosa* to produce QS molecules were detected by phenotypic and genotypic methods. In phenotypic we selected virulence factor related with QS, hemolysin, antibiotic resistance, biofilm formation and pyocyanin production. All isolates of *P. aeruginosa* produced hemolysin through their abilities to hemolyse RBC when growth on blood agar. Kirby-Bauer method was used to perform the antibiotics susceptibility test against different antibiotics. All isolates of *P. aeruginosa* were multi drug resistance (MDR) The high prevalent resistance to antibacterial drugs, was obviously observed with ceftriaxone (100 %) while only 12% resistance rate was recorded towards to meropenem .

The biofilm formation in our study was determined in eight isolates of *P. aeruginosa* was determined using three methods :Tissue Culture Plate Method (TCPM), Tube method (TM) and Congo red agar method(CRA). The results from the TCPM showed that 6/8(75%) of the isolates were strong biofilm produces and 2/8(25%) of the isolates were moderate biofilm produces,while, TM showed that 4/8(50%) of the isolates were strong biofilm produces, 3/8(37%) of the isolates were moderate biofilm produces and 1/8(13%) no detected biofilm formation by this method. CRA was not beneficial to detect the biofilm in all isolates. In general, all *P. aeruginosa* isolates had the ability to produce pyocynin with different degrees which was detected using a method similar to TCPM .

Genotypic methods included detection of QS virulence genes. Results highlighted that *las* I and *las* R genes are important because the Las System regulates the *P. aeruginosa* QS network as well as biofilm formation and pyocyanin production in *P. aeruginosa* isolates.

One of the important work in this study was the biofilm formation inhibition and pyocynin production by utilizing three materials :Vitamin C (vit C), Salicylic acid (SA) and human multi serra (paraoxonase containing). Inhibition of biofilm formation by vit C, SA and multi serra were 76, 37, 28 % respectively Vit C showed the highest inhibitory, while in inhibition pyocynin production SA showed the highest inhibition for Pyocyanin production (77%) compared with multi serra and vit C that produced 56 and 38 % respectively.

The QS crude was extracted with manual methods. Then Fourier- transform infrared spectroscopy (FT-IR) and High-pressure Liquid Chromatography (HPLC) was used to confirm the presence of QS molecules in the extract. Results showed the presence of the homoserine lactone signal (HSLs) molecules as the only member of QS molecules.

Finally, another important work in this study evaluated the role of QS molecules in immune modulation which appeared through downregulation for pro-inflammatory cytokines genes expression were revealed when the immune cell cultures were treated with QS crude at different concentrations (5, 12.5 and 25)% and compared to the housekeeping gene. These experiment conducted in immune cell culture prepared from spleen, lymph node and bone marrow for mice. All concentrations caused downregulation for (IL-1 β , IL-6, IL-2, TNF α and NF-KB) genes expression, but in different degrees when compare with (GAPDH) Housekeeping geen. The highest immune modulation was in concentration (25%) in three organs . Through the results, we concluded that there is a major role for QS in the pathogenicity of *P. aeruginosa* by regulating virulence factors and reducing the host immune response. The significant aspect of this study is extraction of QS for the first time locally and investigate its role in immune modulation of the host for genetic level, this is considered a world first.

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List of Abbreviations

Abbreviation	Key
ACN	Acetonitrile
AHLs	N-acyl-homoserine lactones
AI-2	autoinducer factor-2
AIPs	autoinducer peptides
AMR	antimicrobial resistance
API	Analysis Profile Index
B.H.I	Brain Heart Infusion
BAMPs	biofilm-associated molecular patterns
C4-HSL/BHL	N-butyryl-l-homoserine lactone
CD	cluster of differentiation
cDNA	complementary DNA
CLSI	Clinical and Laboratory Standards Institute
CRA	Congo red agar
DCs	dendritic cells
eDNA	extracellular DNA
ELISA	Enzyme Linked Immune Sorbent Assay
ENCODE	Encyclopedia of DNA Elements
EPS	Extracellular Polymeric Substance
FBS	Fetal Bovine Serum
FT-IR	Fourier-transform infrared spectroscopy
G +ve	Gram positive bacteria
G -ve	Gram negative bacteria
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	gas chromatography-mass spectrometry
HDL	High Density Lipoprotein
HPLC	High-pressure Liquid Chromatography

Abbreviation	Key
HSLs	Homoserine lactone signal
3-oxo-C12-HSL/OdDHL	N-3-oxo-dodecanoyl-l-homoserine lactone
IL	Interleukin
LB	Lauria Bertani
LC–MS	liquid chromatography–mass spectrometry
LPS	lipopolysaccharide
Mbp	million base pairs
MDR	Multi Drug Resistance
MvfR	multiple virulence-factor regulator
NCBI	National Center for Biotechnology Information
NF- κ B	Nuclear factor kappa B
NHGRI	National Human Genome Research Institute
NKT	natural killer T
NO	Nitric oxide
OD	Optical Densities
ORFs	Open reading frames
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMNs	Polymorph nuclears
PON	Paraoxonase
pqs	<i>Psuedomonas</i> quinolone signal
PRRs	Pattern recognition receptors
qPCR	Quantitative PCR

Abbreviation	Key
QQ	Quorum quenching
QS	Quorum sensing
QSIs	Quorum-sensing inhibitors
RBC	Red Blood Cell
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute media
RT-PCR	Real Time -PCR
SA	Salicylic acid
SCFM	Synthetic cystic fibrosis medium
STAT3	Signal transducer and activator of transcription 3
TAE	Tris acetate-EDTA buffer
TCPM	Tissue Culture Plate Method
TLR	Toll-like receptors
T _m	Melting temperature
TM	Tube method
TNF- α	Tumor Necrotic Factor Alpha
TSB	Trypticase Soy Broth
UTI	Urinary Tract Infection
VAP	Ventilator-associated pneumonia
vit c	Vitamin C

Chapter One

Introduction

Introduction

P. aeruginosa Gram-negative bacteria, motile bacillus that can grow at 42°C as well as 37°C in aerobic circumstances, is a significant cause of global morbidity and mortality. Although it is often regarded as an extracellular pathogen toward human cells, numerous investigations report its ability to survive and replicate within host cells. Various studies demonstrated specific mechanisms enabling it to adopt an intracellular lifestyle (Resko *et al.*,2024). *P. aeruginosa* is an opportunistic pathogen that causes severe UTIs which are difficult to eradicate due to high intrinsic antimicrobial resistance (AMR) and the bacterium's ability to develop new resistances during antibiotic treatment (Eladawy *et al.*,2023).

P. aeruginosa has a number of virulence factors that play a role in pathogenesis in the host such as biofilm , pyocyanin, toxins and enzymes .UTIs are infections of the genitourinary tract which extends from the renal cortex of the kidney to the urethral meatus. UTIs are one of the most common bacterial infections that affect people of all ages and stages of life (AL-Khikani and Ayit , 2022).

Host colonization relies on a well-coordinated synthesis of virulence factors on a community level to ensure the host invasion is successful. *P. aeruginosa* achieves this by using cell-to-cell communication known as QS, which utilizes chemical signaling to ensure regulation of the pathogenicity in a cell density-dependent manner. QS discovered firstly in the bacterium *Vibrio fischeri* (Kristensen *et al.*,2024). Depending on the signaling molecules in use, QS molecules chemical structure can be classified into several types, N-acyl-homoserine lactones (AHLs), autoinducer peptides (AIPs), and autoinducer factor-2 (AI-2) (Verbeke *et al.*, 2017).

The *P. aeruginosa* QS systems encompass three signaling mechanisms: las, rhl, and pseudomonas quinolone signal (pqs) .The las system consists of the *lasI* protease, which produces the signaling molecule N-3-oxo-dodecanoyl-l-

homoserine lactone (3-oxo-C12-HSL/OdDHL) The *lasR* protease which acts as a receptor protein (Chadha *et al.*, 2022a). The *rhl* system consists of the *RhlI* protease, the *RhlR* protein receptor, and the signaling molecule N-butyryl-L-homoserine lactone (C4-HSL/BHL). The *pqs* system employs 2-heptyl-3-hydroxy-4-quinolone as a signaling molecule closely related to two AHL based systems (He *et al.*, 2024).

The QS controls the virulence factors and the formation of biofilms, so the search for anti-QS strategies could be a potential objective to prevent infection by *P. aeruginosa*. Inhibition of QS, and attenuation of virulence factors in *P. aeruginosa*, have been reported for a wide variety of extracts, essential oils, and chemical constituents (Qais and Ahmad, 2022). By interfering with the QS system of specific microorganisms, hindering the exchange of information between microorganisms. It also reduces the expression level of hazard factors, this phenomenon is called quorum quenching (QQ) (Wu *et al.*, 2020).

Three materials Vit C, SA and Human multi sera, Noteworthy the plasma mammalian paraoxonase (PON) possess a similar AHL lactonase activity, participating to host protection against microbial infections throw Inhibition biofilm formation and pyocyanin production (Pandit *et al.*, 2017; Ahmed *et al.*, 2019).

The properties of QQ molecules are chemical compounds and enzymes, modes of action (competition, inhibition, QS signal interdict, etc.), and targets are diverse. They all are major steps in the QS pathway, including from synthesis to diffusion to, and sensing of a portion of the QS signal may be affected (Wang *et al.*, 2019).

The QS has been extensively studied for decades. Accordingly, the development of methods to detect QS signals has been a key issue in the field of QS. Various methods, such as bioassay, HPLC, liquid chromatography–mass spectrometry (LC–MS), and gas chromatography–mass spectrometry (GC–MS)

(Huang *et al.*,2020), have been developed for the analysis of AHLs, demonstrating that the detection of these molecules is relatively easy.

Polymerase Chain Reaction (PCR) technology is used to detect the genes responsible for QS synthesis and virulence factor genes (Shravani *et al.*,2023).It is also used to detect the gene expression of the genes responsible for the up or down regulation of the immune response to QS, such as the genes responsible secretion cytokine in immune system (Levkovich *et al.*,2023).

The AHL can affect the host innate immune system at varying degrees. For macrophages ,in general, HSL decreases their inflammatory response in a diverse manner, leading to chronic infection with *P. aeruginosa*. It has been shown that *P. aeruginosa* produced AHL exerted an anti-inflammatory response in RAW264.7 mouse macrophages in a dose-dependent manner. At the same time, AHL induced a decrease in the concentration of tumor necrotic factor alpha (TNF- α) and an increase in the concentration of interleukin-10 (IL-10) secreted by RAW264.7 cells (Glucksam-Galnoy *et al.*,2013).

The pqs has an immune modulating activity that is different from that of 3-oxo-C12-HSL in cell proliferation and in the production of IL-2 and TNF- α . (Hooi *et al.*, 2004). One of the methods to study immune modulating activity by QS molecules is cell culture. It is defined as an isolating and processing the cells from their natural environment (*In vivo*) to a controlled artificial environment (Baust *et al.*,2017).

Given the lack of studies that have isolated quorum sensing, especially at the local level, as well as to demonstrate its role in modifying the immune response, we have taken it upon ourselves through the current study to achieve some aims at :

- 1) Isolation and identifying of *P. aeruginosa* from UTI patients.
- 2) Phenotypic and genotypic diagnosis of QS molecules production in *P. aeruginosa*.
- 3) Extracting QS crude extract from bacterial isolates and confirm the presence of QS molecules using FT-IR and HPLC.
- 4) Evaluating the effect of vitamin C, salicylic acid and multi Serra (containing paraoxonase) on inhibition of QS .
- 5) Detecting the effect of QS crude extract on the gene expression of some pro-inflammatory genes.

Chapter Two

Literature review

2 . Literature Review

2. 1. History

Charles-Emmanuel Sédillot, a French military physician, was the first to reference an infection involving *P. aeruginosa* in 1850. Sédillot described how surgical dressings of patients both in the field and hospital beds often became colored with a blue-green substance having a grape-like sweet odor. It is now known as the diagnostic hallmarks of *P. aeruginosa* infections, due to pyocyanin and 2-aminoacetophenone productions, respectively (Pitt, 1998). Thirty years later in 1882, a French pharmacist , Carle Gessard reported the successful isolation of *P. aeruginosa* which he initially termed *Bacillis pyocyaneus*, in a publication entitled “On the blue and green coloration of bandages”(Gessard, 1984).

2. 2. *Pseudomonas aeruginosa* Characteristics and pathogenisty

Bacteria *P. aeruginosa* is a Gram-negative aerobic rod-shaped that can be isolated from most environments including soil, plants, and mammal tissue (Stover *et al.*,2000). This bacterium can survive on water, different surfaces, and medical devices using its influential binding factors, such as flagella, pili, and biofilms. Hence, *P. aeruginosa* is abundant in natural and artificial environments including lakes, hospitals, and household sink drains (Remold *et al.*,2011). *P. aeruginosa* is an opportunistic pathogen that causes several infections in humans Figure (2-1). It has become an important cause of nosocomial infections and antibiotic resistance (Shariati *et al.*,2018). *P. aeruginosa* can be specified as one of the opportunistic bacteria related to healthcare infections including ventilator-associated pneumonia (VAP),intensive care unit infections, central line-related blood stream infections, surgical site infections, urinary tract infections, burn wound infections, keratitis, and otitis media (Ito *et al.*,2021). *P. aeruginosa* is an organism capable of adapting to changes in the environment rapidly developing resistance to antibiotics, and

producing a variety of virulence factors. This pathogen can affect immunocompromised patients in part due to its ability to evade both innate and acquired immune defenses through adhesion, colonization, and biofilm formation. This leads to produce various virulence factors that cause significant tissue damage (Turkina and Vikström, 2019).

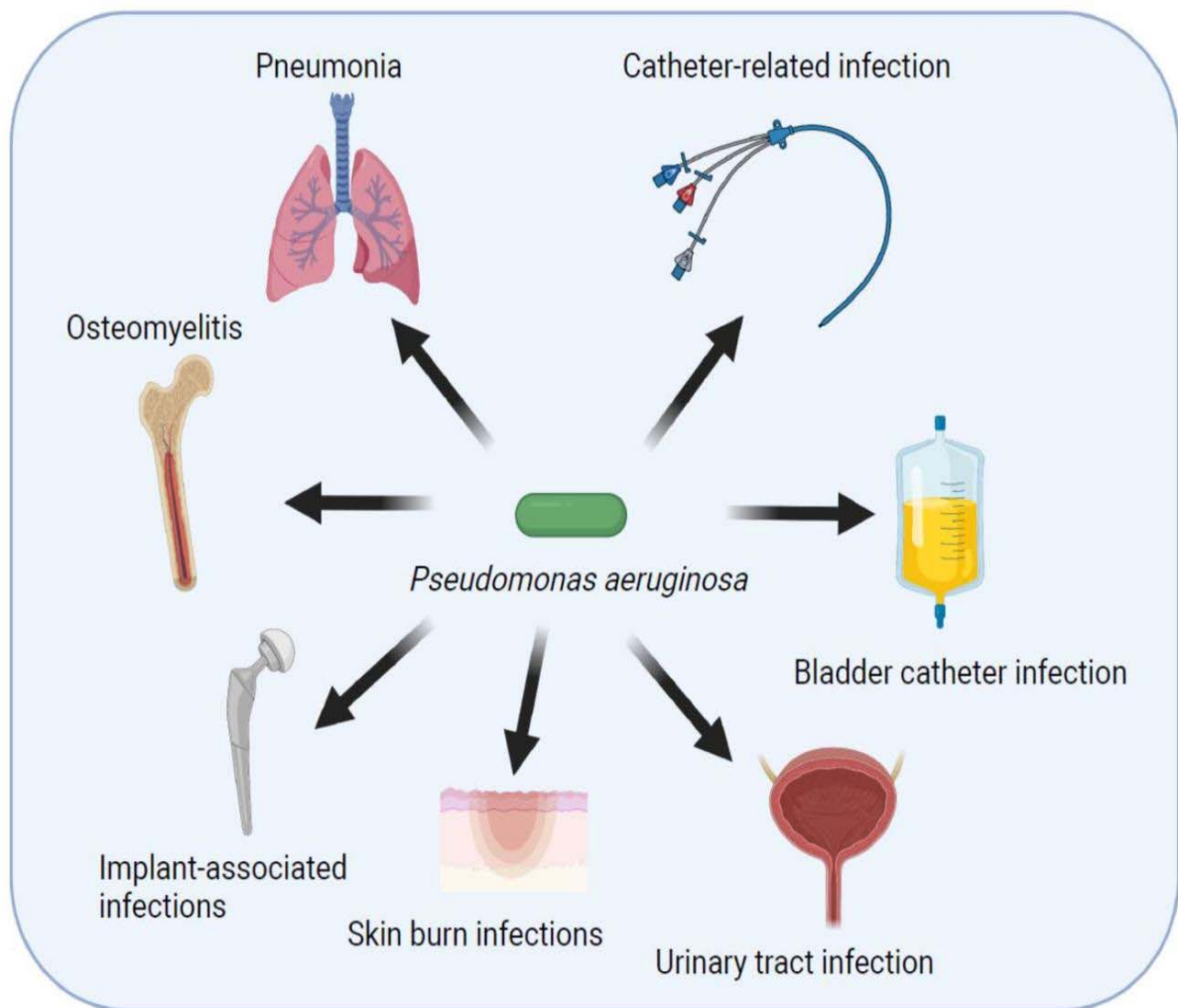


Figure2-1 Main infections caused by *P. aeruginosa*(Tuon *et al.*,2022).

2.3. Classification of *Pseudomonas aeruginosa*

P. aeruginosa is classified based on the sequences of nitrogen bases of nucleic acid and the taxonomic locations (Kahlon, 2016) as the following:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *Pseudomonas aeruginosa*

2.4. Epidemiology

Gessard isolated *P. aeruginosa* from green pus for the first time in 1882. *P. aeruginosa* widespread lifestyle permits it to contribute to human illnesses on a regular basis. It is present in normal intestinal flora. However, it does not adhere well to normal intact epithelium (Pachori *et al.*, 2019). *P. aeruginosa* is a common cause of hospitalized infections in immune-compromised patients. The major source of infection is medical equipment and cross colonization from other patients. This bacterium contaminates the medical devices and forms biofilm which poses a serious problem to the patients. For instance, patients may develop catheter-associated with UTIs as *P. aeruginosa* forms a biofilm on the facets of indwelling catheters. The colonial pathogen causes direct damage to the host tissue and increases the bacterium's competitiveness (Al-Wrafiy *et al.*, 2017). *P. aeruginosa* can infect frequently the respiratory tract, blood cells, urinary tract, ear, and soft skin tissues. However, eyes, heart, central nervous system, bones, and joints are among the sites where the chances of infection are rare. The main cause of infection at these sites is due to trauma following

surgery or by the over usage of a drug or any other thing that makes the tissue vulnerable at an immune-compromised state (Bhardwaj *et al.*,2021).

2.5. Virulence Factors of *Pseudomonas aeruginosa*

The *P. aeruginosa* has a number of virulence factors that play a role in pathogenesis in the host .The most significant pathogenic characteristic of *P. aeruginosa* is the development of biofilms, which allows this bacterium to cause recurrent and chronic UTIs by evading host immune defense systems . Virulence Factors of *P. aeruginosa* elaborated in Figure (1-2) (AL-Khikani and Ayit , 2022)

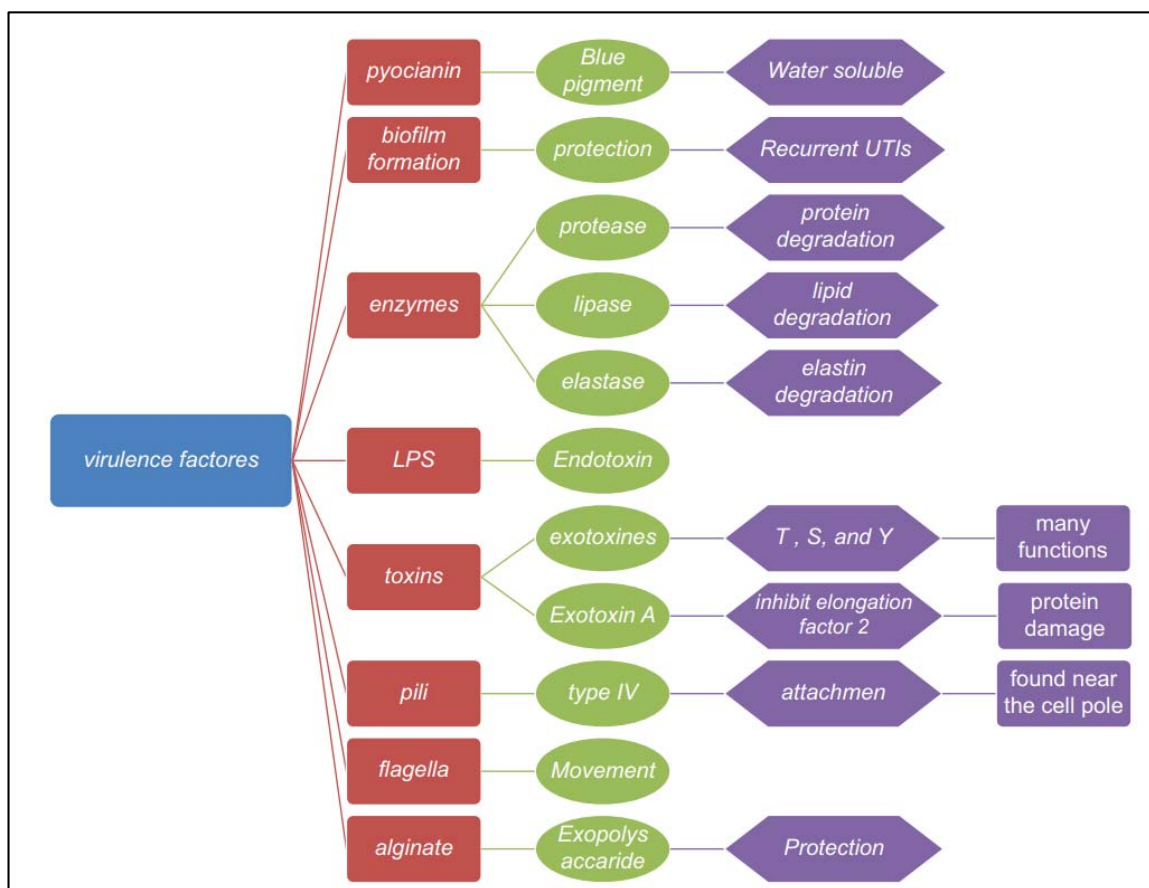


Figure 2-2: Virulence factors of *P. aeruginosa*(AL-Khikani and Ayit , 2022)

2.6. Urinary tract infections (UTIs)

The UTI is a bacterial infection affecting urinary tract. When bacteria from the rectal area enter the urinary tract via the urethra to the bladder and multiply in the urine, thus an infection occurs. In many cases bacteria first travel to the urethra. When bacteria multiply an infection can occur. An infection limited to

the urethra is called urethritis. If bacteria move to the bladder and multiply, a bladder infection called cystitis. If the infection is not treated promptly, bacteria may then travel further up the ureters to multiply and infect the kidneys, called pyelonephritis (Mane and Bhosale , 2023).

2.6.1. Symptoms of UTIs

- Urge to urinate frequently, often in small amounts
- Burning with urination
- Cloudy urine
- Strong unpleasant smell of urine (not as reliable in children)
- Dark or bloody urine , abdominal pain , fever , vomiting and pelvic pain
- Rectal pain (kidney infection)
- Flank or back pain (kidney infection). (Mane and Bhosale , 2023).

2.6.2. *Pseudomonas aeruginosa* as a causative agent for UTIs

Bacteria *P. aeruginosa* is an important uropathogen , associated with 7% – 12% of all nosocomial urinary tract infections .The bacterium usually causes UTIs following catheterization or surgery . UTI is the second most common type of infection in the body (Mittal *et al.*,2009). *P. aeruginosa* was found to be the third most common Gram-negative pathogen causing 7.1% of all nosocomial urinary tract infections in surveillance studies from the Asia-Pacific region in 2009 to 2010(Lu *et al.*,2012). *P. aeruginosa* urinary infection may cause complications such as sepsis that can be fatal in older patients, immunocompromised patients and individuals with diabetes (Shigemura *et al.*,2006). Disruption of the epithelial layer during application of a catheter promotes bacterial colonization (Streeter and Katouli , 2016). The formation of biofilm , Catheter-associated UTI accounts for 20% to 49% of all nosocomial infections; the pathogen enters via extra luminal or by intra luminal route of the catheter (Mittal *et al.*,2009). *P. aeruginosa* can survive intracellularly in bladder

epithelial cells, sets a stable infection and become tolerant to antibiotics in *vivo* and in *vitro* model (Penaranda, *et al.*,2021).

P. aeruginosa causes 1%–4% UTIs after flexible cystoscopy reached to 10-18% after urinary bladder exploration with contaminated cystoscope. (Sorbets *et al.*,2019).

2.7. Genome of *Pseudomonas aeruginosa*

Genome of *P. aeruginosa* is the largest, contain approximately 7 million base pairs (Mbp) representing the higher end of the genome size (Poulsen *et al.*, 2019). All *P. aeruginosa* strains possess a highly conserved core genome (accounting for approximately 90% of the total genome) and an accessory genome (Kandasamy *et al.*, 2020). The determined number of core genes has varied between studies due to variations in gene annotations, strain sets, and core gene definitions. Recently determined that 5,109 protein-coding genes comprised the core genome of all *P. aeruginosa* strains (Poulsen *et al.*, 2019).The *P. aeruginosa* accessory genome holds significant therapeutic interest due to its ability to acquire genes that improve virulence, antibiotic resistance, and general bacterial fitness through horizontal gene transfer (Dangla-Pélissier *et al.*, 2021).

The genome of *P. aeruginosa* was fully sequenced in 2000 and is now known to be 6.3 Mbp (66.6% G + C content) in size, with 5,700 genes. Of these, 5,584 are predicted open reading frames (ORFs). In comparison to other bacterial genomes, *P. aeruginosa* contains a substantially higher 150 genes encoding outer membrane proteins that have virulence, antibiotic, adhesion, and movement functions (de Sousa *et al.*, 2021).

2.8. Immune responses to *P. aeruginosa*

The *P. aeruginosa* generally possesses a number of factors that antagonize host immunity. Flagella and lipopolysaccharide (LPS) are two antagonists to the innate immune system receptors or Toll-like receptors (TLR5 and TLR4),

polymorph nuclears (PMNs) and macrophages is mediated by pattern recognition receptors (PRRs). Binding of BAMPs and PAMPs to PRRs stimulates the PMNs and macrophages resulting in consumption of O₂ for liberation of tissue-toxic reactive oxygen species (ROS) and nitric oxide (NO) (Kolpen *et al.*, 2010; Hansen *et al.*, 2014). Additional responses by the PMNs include secretion of proteases that may cause proteolytic tissue lesions while the macrophage may further enhance the inflammation by emitting pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8, and IL-12. The effector cells of the adaptive immune response mainly reside distantly such as the T-cells and the B-cells in the secondary lymphoid organs and the plasma cells in the bone marrow (Kawai and Akira, 2010). Activated T-cells may release cytokines that further reinforces the inflammation by stimulating the accumulation and activation of PMNs and production of IgG. The contribution of the increased accumulation of activated PMNs to the local inflammation is further accelerated by binding of antigens to IgG, leading to immune complex mediated stimulation of the PMNs and activation of the classical complement pathway, as elaborated in figure 2-3 (Moser *et al.*, 2021).

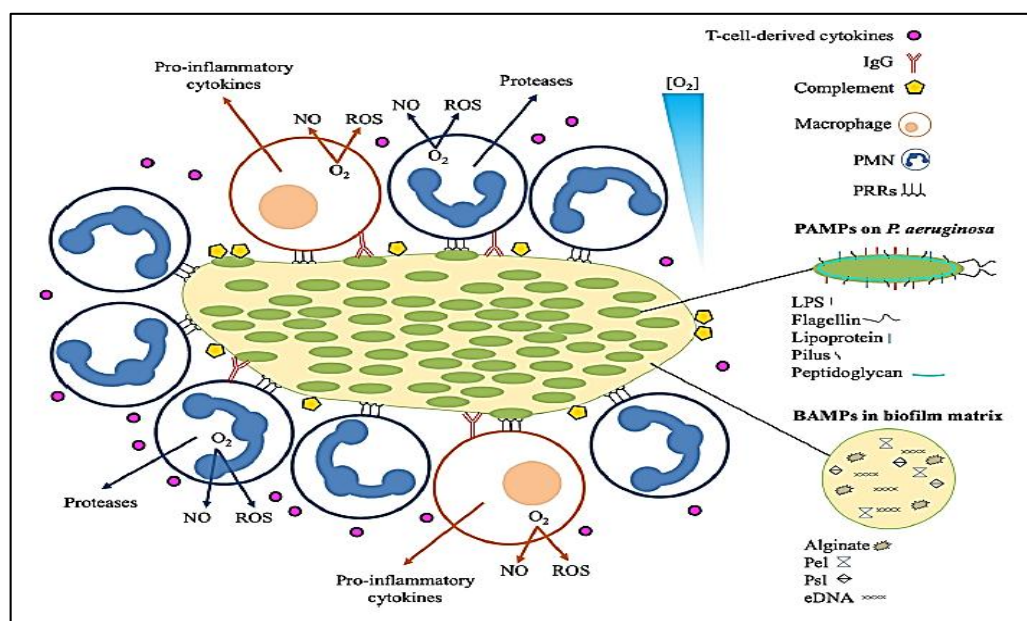


Figure (2-3): host immune response to infectious *P. aeruginosa*(Moser *et al.*, 2021).

2.9. Quorum Sensing .

The QS was first discovered in the marine bioluminescent bacterium *Vibrio fischeri* in the period early 1970 . QS is widely spread in the bacteria, and considered as a “speaking” systems (Lahij *et al* .,2021). QS is also called density sensing, which controls a variety of physiological behaviors in bacteria. Whether in Gram-negative or Gram-positive bacteria, QS mechanisms exist, but the signal molecules they use to transmit information are different. Bacteria control the behavior of the entire bacterial population by synthesizing and secreting signal molecules also known as AIs .(Zhao and Ding , 2020). When the concentration of signal molecules reaches a certain threshold with the bacterial population density, the expression of certain specific genes can be started to regulate the bacterial population adaptation. The QS system regulates various cellular processes, which mainly involve the regulation of virulence factors, such as spore formation, toxin production, motility, biofilm formation, and drug resistance that showed in Figure (2-4) (Naga *et al* ., 2023).

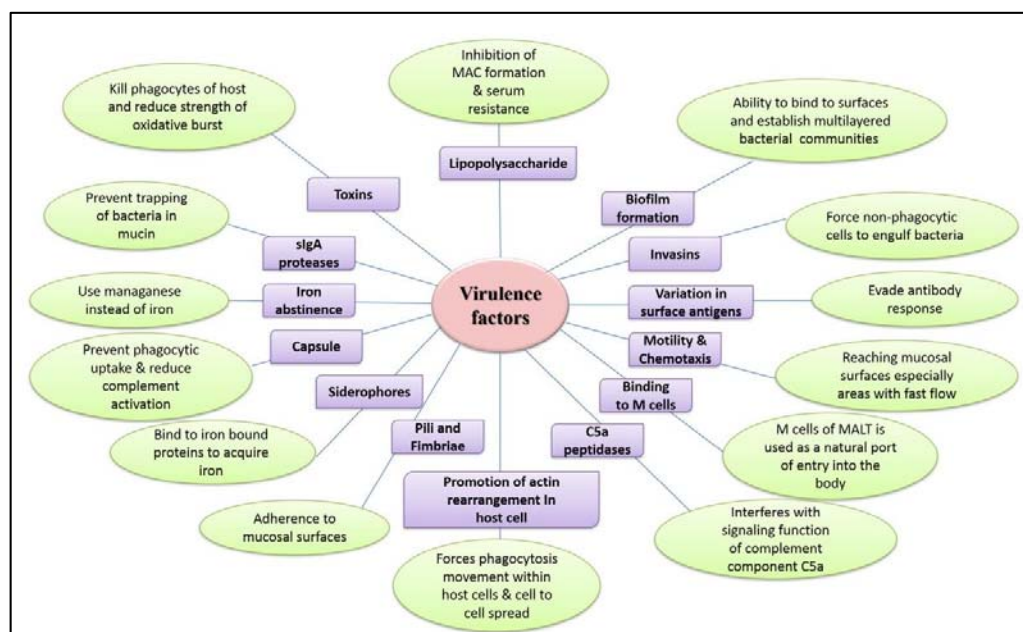
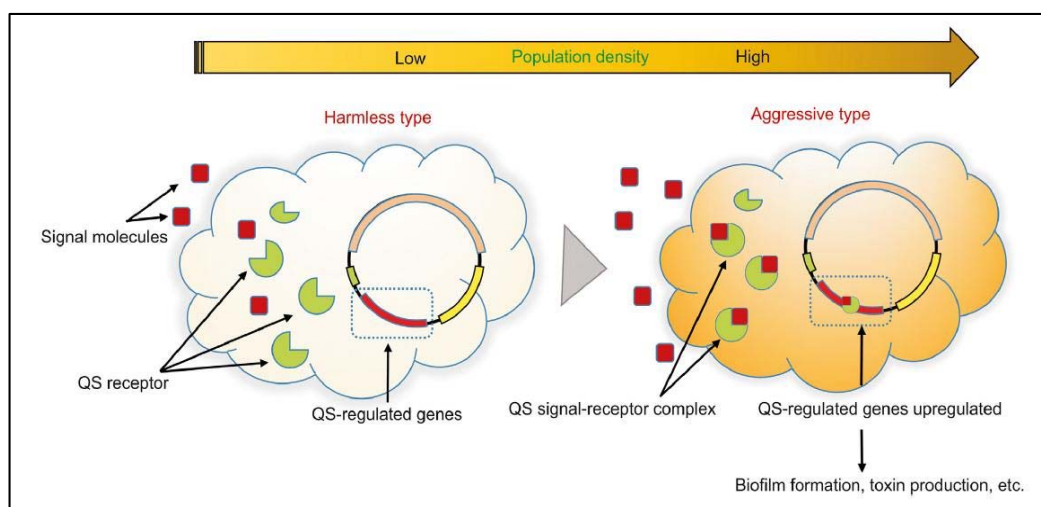


Figure (2-4): Various virulence factors of pathogenic bacteria are regulated by QS(Naga *et al* ., 2023).

The signal molecules related to clinical pathogens have been reported 1) AHLs, which exist in most Gram negative bacteria except *Vibrio harringtonensis* and *Mucoccus flavus* . 2) AIPs, which are produced by most Gram positive bacteria. 3) AI-2 furanones, which are utilized by more than 40 species of Gram positive and Gram negative bacteria for communication and transmission (Verbeke *et al.*, 2017). 4) AI-3, which is used by enterohemorrhagic *Escherichia coli*. 5) other signal molecules such as the Pqs and ketopyridazines, which also play important roles in intraspecific QS. Some biosynthetic pathways regulating the generation of important QS signal molecules such as AHL and AI-2 involve numerous receptors and enzymes, which are summarized as: S-adenosyl methionine dependent methyltransferase, LuxS, AHL synthase (LuxI) , LuxR receptor, histidine kinase response regulated LuxN protein, and AI-2 (LuxP and LsrB) receptor (Lu *et al.*,2022). For example, LuxI/LuxR are key regulatory factors in AHL-mediated QS signaling pathways. LuxI synthesizes AHL derivatives to generate QS signal molecules. The synthesized AHL entities subsequently diffuse in the adjacent environment and accumulate in a cell-density-dependent manner Figure 2-5 (Welsh and Blackwell , 2016). When the AHL level reaches the threshold, the AHL interacts with the LuxR-type receptor to activate target gene transcription in QS signaling pathways (Kumar *et al* ., 2020).



Figure(2-5): Cyclic process of signal molecules QS(Welsh and Blackwell , 2016)

2.9.1. The Quorum Sensing in *P. aeruginosa*

The behavior of *P. aeruginosa* is monitored by a complex regulatory mechanism called QS in acute and chronic infections. The coordination of specific gene expression in the community involves the interaction of diffused molecular signals (Bhardwaj *et al.*, 2021). Generally, QS in *P. aeruginosa* is governed by three systems *las*, *rhl*, and *Pqs* that have been elegantly reviewed (Chadha *et al.*, 2022a). All the quorum-sensing systems are closely related to each other and do not exist independently. These three systems together form an intricate hierarchical QS circuitry. Among the three QS systems, *las* is at the top of the QS hierarchy and is required for the optimal activation of the *rhl* and *pqs* QS systems (Lim *et al.*, 2022).

2.9.1.1. *las* System

The *las* System plays a fundamental role in the regulation of the QS network of *P. aeruginosa*, the signaling molecule 3-oxo-C12-HSL is synthesized by LasI. When the signal concentration reaches the threshold, it binds to the receptor protein LasR to form a complex and initiates the expression of downstream target genes (Maisuria *et al.*, 2016). The *las* signaling system regulates virulence factors such as exotoxin A and the expression of LasA staphylolytic protease, LasB elastase, and Apr alkaline protease as well as biofilm formation (Ali *et al.*, 2017). LasA and LasB play a vital role in regulating protease activity; they are in charge of the degradation of elastin, fibrin, complement, interferon, and collagen during infection. They can also influence toxin levels (Pangastuti *et al.*, 2020).

2.9.1.2. *rhl* System

The *rhl* System belonging to the LuxR-type receptor family in the QS system of *P. aeruginosa* is *rhl*, which is similar to *las*. The difference is that the AHL signaling molecule of *rhl* is C4-HSL, which is produced by RhII, and the signal molecule binds to its receptor and activates transcriptional regulator RhIR (Chang *et al.*, 2014). *Rhl* is related to the production of some virulence

factors such as rhamnolipids , toxic exo-factors hydrogen cyanide and pyocyanin (Boursier et al.,2018). Among these, rhamnolipids are biodegradable surfactants that contain two fatty acid molecules and rhamnose residues (Li *et al.*,2019). They play a vital role in protecting cells from oxidative stress and participating in biofilm growth and maturation (Zhou *et al.*,2019).

2.9.1.3. pqs System

The third QS system in *P. aeruginosa* is called pqs, and it is closely related to two AHL based systems. It puts 2-heptyl-3-hydroxy-4-quinolone (PQS) as its signal molecules, and the precursor of PQS, 2-heptyl-4-hydroxyquinoline (HHQ), is commonly associated with QS along with pqs (Lin and Cheng ,2019). The biosynthesis of these two signal molecules requires a transcriptional regulator PqsR multiple virulence-factor regulator (MvfR) and pqs ABCDE operon, whose expression is controlled by the former (Sahner *et al.*,2015). PqsR plays a key role in pqs and regulates the expression of rhII as well as some virulence functions such as hydrogen cyanide, pyocyanin, bacterial motility, and biofilm formation, of which the expression is activated by LasR but inhibited by RhIR (Aleksic *et al.*,2019).

We have augmented the findings based on pre-existing and recent literature and illustrated the QS circuitry of *P. aeruginosa* in Figure 2-6 (Chadha *et al.*,2022b).

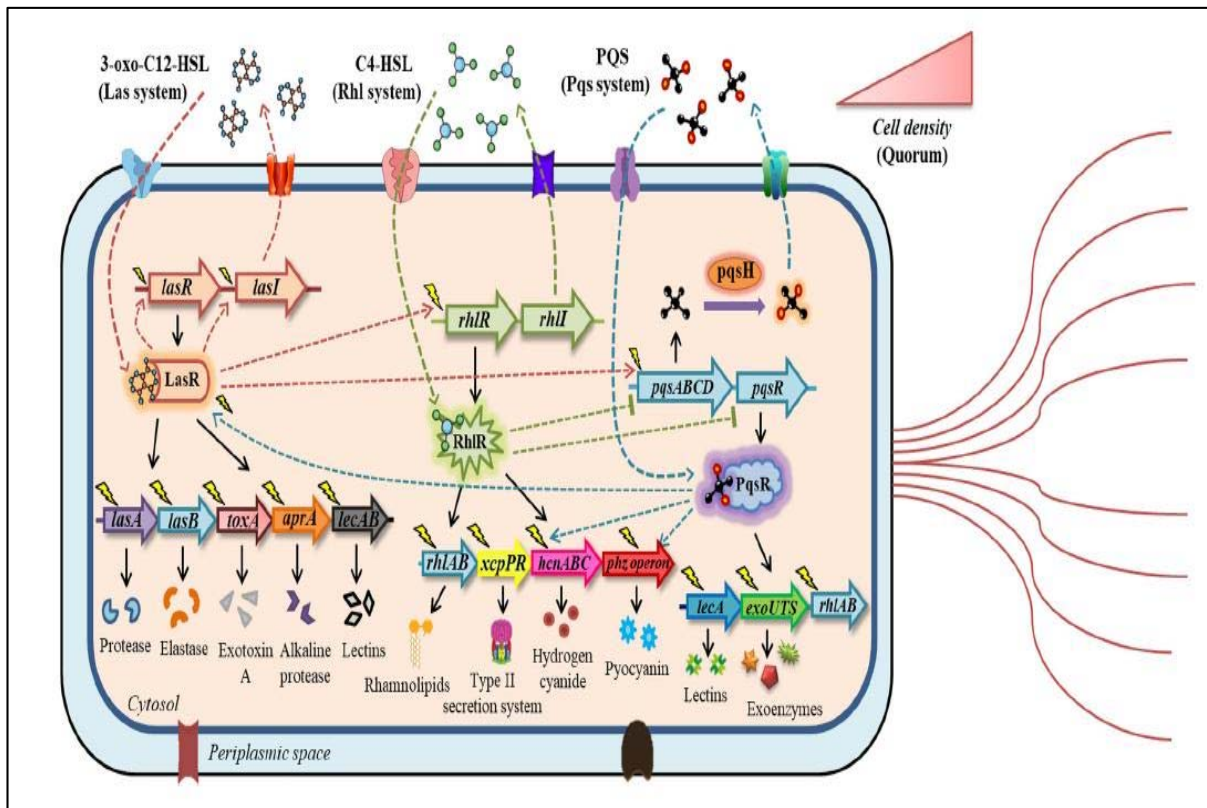


Figure (2-6): Quorum sensing circuitry of *P. aeruginosa* (Chadha *et al.*,2022b).

2.9.2. Virulence factor regulate by QS

Intercellular communication in bacteria stimulates coordinated responses to display a multitude of virulence traits that are not possible to achieve for individual cells. The signaling network in *P. aeruginosa* is perhaps one of the most complex yet profoundly studied examples among all the QS systems. It consists of multiple concatenated signaling pathways that synergistically regulate bacterial virulence, making *P. aeruginosa* a highly notorious and deadly human pathogen (El Zowalaty *et al.*, 2015). The QS circuitry in *P. aeruginosa* represents a global regulatory system that directly or indirectly controls the expression of more than 10% of its genome and about 20% of the bacterial proteome. QS in *P. aeruginosa* is mediated through three main systems, termed Las, Rhl and Pqs (Chadha *et al.*,2022).

2.9.2.1. Hemolysin

Hemolysins are lipoprotein molecules that cause lysis of red blood cells by disrupting the cell membrane. Although the lytic activity of some microbe-derived hemolysins on red blood cells may be of great importance for nutrient acquisition, many hemolysins produced by pathogens do not cause significant destruction of red blood cells during infection. However, hemolysins are often capable of lysing red blood cells *in vitro* (Stipcevic *et al.*, 2005). Many bacteria produce hemolysins that can be detected in the laboratory. *P. aeruginosa* produces two extracellular hemolytic agents, one a heat-labile phospholipase and the other a heat-stable glycolipid. Hemolysin could not be detected until 12 h, well into the stationary phase of growth (Johnson and Boese-Marrazzo, 1980). Hemolysins can be identified by their ability to lyse red blood cells *in vitro*. One way hemolysin lyses erythrocytes is by forming pores in phospholipid bilayers (Chalmeau *et al.*, 2011).

Other hemolysins lyse erythrocytes by hydrolyzing the phospholipids in the bilayer, which are able to cause the lysis of erythrocytes, leukocytes, and platelets by producing pores on the cytoplasmic membrane. Hemolysin is normally secreted by the bacteria in a water-soluble way. These monomers diffuse to the target cells and are attached to them by specific receptors (Thompson *et al.*, 2011).

2.9.2.2. Antibacterial Resistance

Antimicrobial resistance is defined as an organism's capacity to withstand the action of an antimicrobial agent to which it was previously susceptible (Al-Khikani, 2020). The emergence of MDR strains has increased, nosocomial infection caused by antibiotic-resistant *P. aeruginosa* has become a serious problem in clinical care settings, resistance to at least three antibiotics. (Juan *et al.*, 2005). Other virulence factors generated by *P. aeruginosa* might be the source of resistance to most antibacterial drugs. Restricted uptake, efflux, drug inactivation, and changes in targets are three fundamental processes that render

bacteria resistant to the action of antimicrobial drugs(Al-Janabi and Al-Khikani , 2021).

The restricted uptake represented by the innate resistance of *P. aeruginosa* to all classes of antibiotics by lowering the permeability of such agents through bacterial cell wall (Breidenstein *et al.*,2011).Efflux systems of bacteria are composed of three protein components working by eliminating the antibiotic molecules outside the bacterial cell (Sadeghifard *et al.*,2012).Third mechanism of drug inactivation and changes in targets is mainly resulted from mutational changes in target bacterial enzymes to become resistance to the action of selective inhibition of antibiotic(Figure 2-7)(AL-Khikani and Ayit 2022).

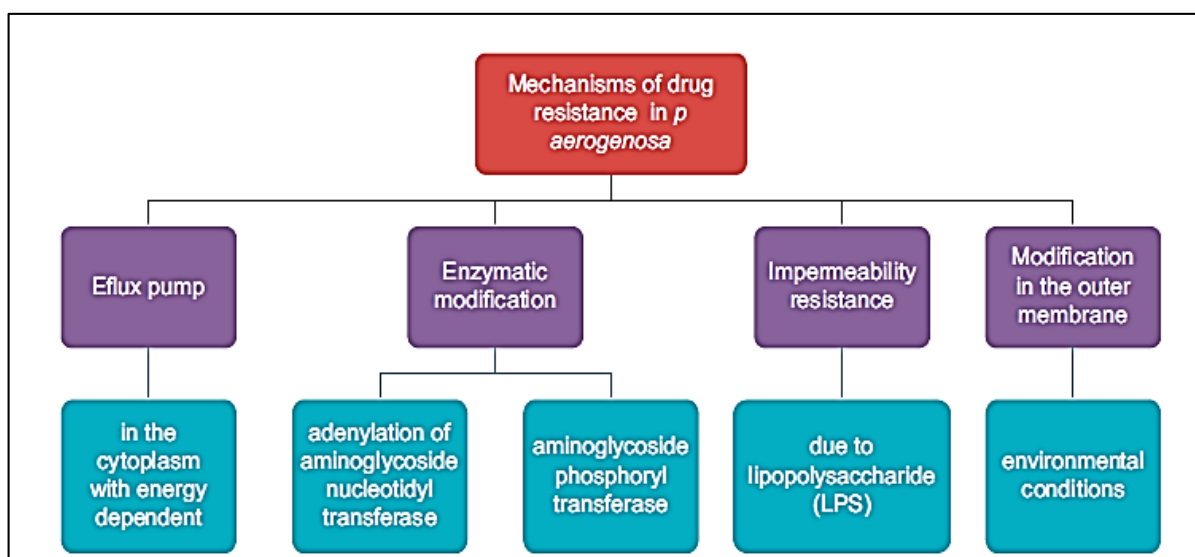


Figure (2-7): Some mechanisms of antibiotic resistance in *P. aeruginosa*

The formation of biofilm by *P. aeruginosa* can potentially contribute to bacterial protection by increasing antibiotic resistance(Chiang *et al.* ,2013). Antibiotics that are often used to treat *P. aeruginosa* infections include aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with-lac (colistin, polymyxin B) (Bassetti *et al.*, 2018). High-level resistance seen in *P. aeruginosa*, because of its poor permeability outer membrane (1/100 that of *E. coli*), *P. aeruginosa* has intrinsic resistance to a variety of antimicrobial drugs

(b-lactam and penem group of antibiotics)(Martin-Loeches *et al.*,2013).The frequent use of antimicrobial drugs is another factor linked to the rise in MDR *P. aeruginosa* . This acquired resistance can occur during antibiotic therapy as a result of a mutational event or the acquisition of a resistance gene through horizontal gene transfer(AL-Khikani and Ayit 2022). A mutational event can result in overexpression of endogenous beta-lactamases or efflux pumps as well as the expression of specific porins (Pachori *et al.*,2019). *P. aeruginosa* can import additional resistance mechanisms by horizontal gene transfer making it resistant to a variety of anti-pseudomonal drugs. As a result, there are limited choices for treating severe nosocomial *pseudomonas* infection. The ever-changing microbial genome poses a greater hazard than infection itself. Horizontal gene transfer, transposon-mediated genomic changes, and the promiscuous nature of gene and genomic products are some of the mechanisms bacteria use to change their genome (Pachori *et al.*,2019).

Most resistant bacteria species, including *P. aeruginosa*, encode enzymes that preferentially target beta-lactam medicines such as penicillin and cephalosporin. Because of the accumulating evidence of beta lactamase's promiscuous nature, the enzyme will acquire affinity for new generation beta lactum antibiotics, eliminating the need for bacteria to have extra genes for new generation antibiotics (AL-Khikani ,2020).

2.9.2.3. Biofilm formation

Bacterial biofilms are defined as structured microbial communities encapsulated in a self-synthesizing extracellular polymeric substance (EPS) and attached to a tissue or surface (Yin *et al.*,2022), that include exopolysaccharides, matrix proteins, and extracellular DNA(eDNA) (Wang *et al.*,2020). Clinically, the long-term colonization of bacteria in humans causes chronic infections, mainly because the bacteria in biofilms are resistant to host immune responses

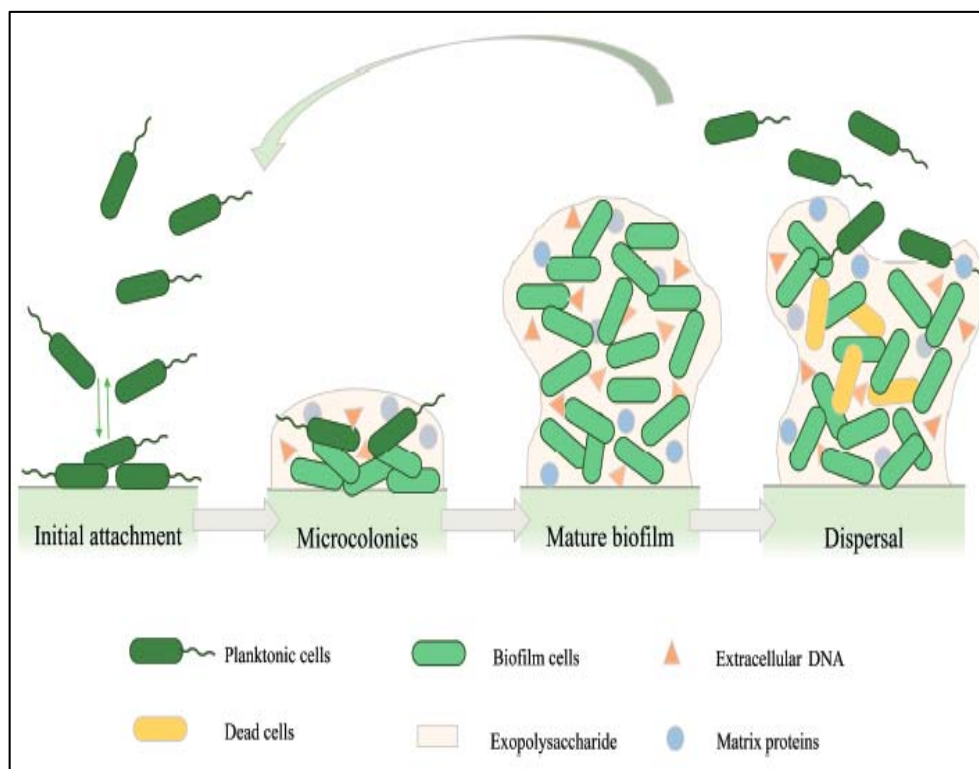


Figure (2-8): Cycle of *P. aeruginosa* biofilm development(Saxena *et al.*, 2019).

2.9.2.4. pyocyanin production

Bacteria *P. aeruginosa* generates a substantial diversity of extracellular pigments, where phenazines are the most important (El-Fouly *et al.*,2015). Therefore, it has gained significant attention as one of the most commercially valuable microbial species recognized for its ability to biosynthesize redox-active phenazine derivatives, specifically: pyocyanin, pyomelanin, pyorubrin, and fluorescein (Hamad *et al.*,2020). Pyocyanin is a redoxactive secondary metabolite with a molecular structure of 5-methyl-1-hydroxyphenazine. It is synthesized during the late stationary phase imparting a distinct blue hue to the culture (Marrez and Mohamad,2020). Pyocyanin is considered a virulence factor for *P. aeruginosa* strains. It has the capability to acquire iron from the extracellular environment (Little *et al.*,2018). It plays a significant role in iron metabolism by actively engaging in reduction mechanisms facilitating the liberation of iron from transferrin(Jayaseelan *et al.*,2014). Pyocyanin, the water soluble pigment, has been shown to exert toxic effects on multiple organs via the

production of ROS (Shouman *et al.*,2023). Its toxicity arises from its ability to alter the electron transfer pathways leading to the generation of excess intracellular oxygen reduction products and subsequent cell death. These findings highlight the complex molecular mechanisms underlying the deleterious effects of pyocyanin on cellular physiology and pathogenesis (Shouman *et al.*,2023). Due to its pharmacological impact on both eukaryotic and prokaryotic cells as well as phyto pathogens, pyocyanin holds potential for use as a biocontrol agent (Narsing Rao *et al.*,2017). Moreover, the anti-biofilm properties of pyocyanin on both established and developing biofilms of different microorganisms have been recorded(Saleem *et al.*,2021). In the field of medicine, pyocyanin has shown promising potential as an anticancer agent due to its ability to exert cytotoxic effects on tumor cells. Additionally, it has been demonstrated to possess antioxidant and radical scavenging properties at low concentrations (Bavithra and Sathiyamurthy, 2019). Therefore, Pyocyanin is a pigment that has a diverse array of prospective applications in areas including medicine, food, pharmaceuticals, biocontrol, nanotechnology, textiles, and physio chemistry. This versatility and multi-functionality make pyocyanin a highly promising substance in various fields (Ozdal , 2019).

2.9.3. Quorum quenching(QQ)

The increase in bacterial resistance has aggravated the difficulty of disease prevention, and the side effects caused by excessive use of drugs may also endanger human health. The discovery of control strategies for quorum quenching diseases in recent years has provided new possibilities for overcoming and solving the problem of microbial resistance (Ćirić *et al.*,2019).

By interfering with the QS system of specific microorganisms, hindering the exchange of information between microorganisms, and reducing the expression level of hazard factors, this phenomenon is called QQ (Wu *et al.*, 2020). The properties of QQ molecular roles (chemical compounds, enzymes), modes of action (competition, inhibition, QS signal interdict, etc.), and targets

are diverse and are all major steps in the QS pathway, including from synthesis to diffusion to, and sensing of a portion of the QS signal may be affected. Normally accumulation, the enzymes that inactivate the QS signal are called QQ enzymes, while the chemicals that disrupt the QS pathway are called QS inhibitors (Wang *et al.*,2019).

The active substances with quorum quenching are collectively referred to as quorum-sensing inhibitors (QSIs). Unlike currently commonly used antibiotics, quorum-quenching agents reduce microbial infections by inhibiting microbial quorum induction, and they generally do not affect microbial growth. There are three main ways of QQ : (1) inhibition of signal molecule production, (2) degradation of signal molecule, and (3) inhibition of signal molecule conduction or binding to receptors, as we showed in(Figure 2-9) (Zhao *et al* .,2020).

The QSIs could contain the following characteristics, QSIs could be very definite for the QS regulators , QSIs could not be toxic or causing side effects to the hosts where bacteria are targeted or pathogenic infections are treated, QSIs could be of low molecular mass so that they effectively suppress the QS related genes expression, QSIs could be chemically stable compounds and could be able to withstand host metabolism and QSIs could not involve in the basic metabolic functions of the bacterial cell like DNA metabolism, cell wall synthesis and protein synthesis which are the drug targets for emerging drug resistance (Shamshad and Rajagopal ,2023).

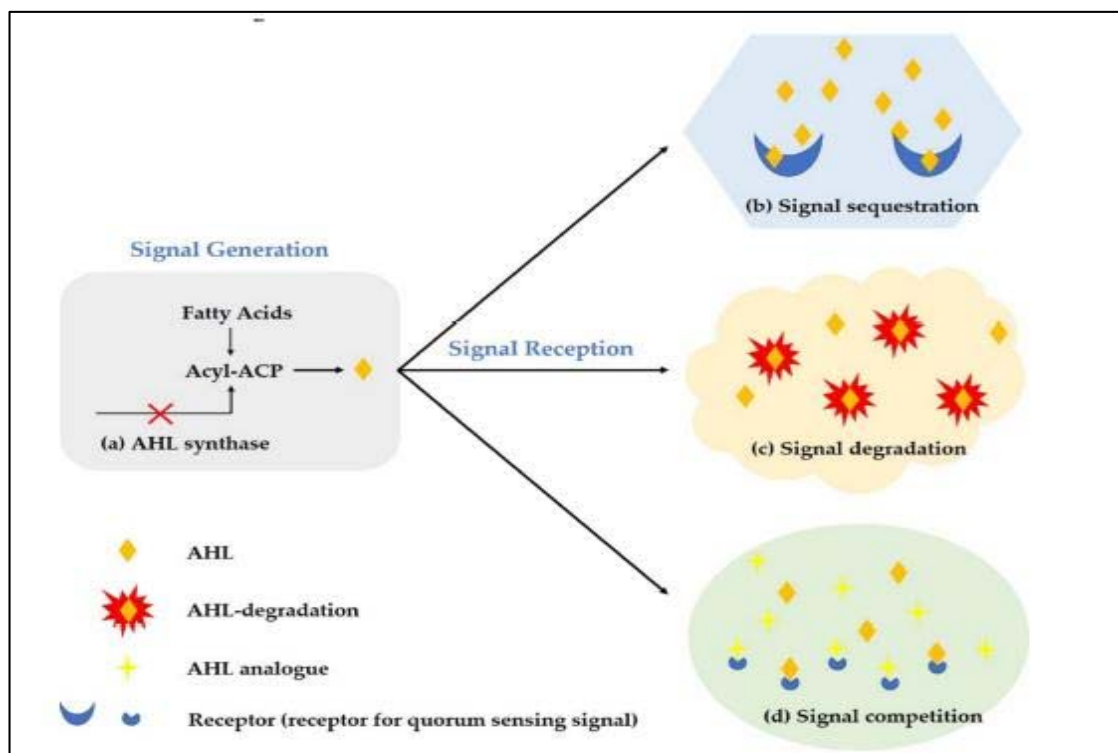


Figure (2-9): Quorum-sensing inhibitor mechanism(Zhao *et al* .,2020).

The differentiation in inhibition of QQ molecules depends on the structure and chemical composition of the compound. For example halogenated furanones, produced by the *Delisea pulchra* marine macroalga, were the first anti-QS compound identified which activity was based on competitive binding to LuxR-type proteins. This mechanism of action leads to increased proteolytic degradation of the LuxR type proteins (Asfour 2018).

Compared with the traditional prevention and control methods, which mainly aim at inhibiting and killing microorganisms, QQ will not pressure the growth of microorganisms, nor will it induce the development of microbial resistance. Therefore, the research and development of QQ-based QSI has gradually attracted the attention of researchers and has become a new strategy for controlling harmful microorganisms (Zhao *et al* .,2020).

2.9.3.1. Inhibition of virulence factor in *P. aeruginosa* by QQ

The QS control the virulence factors and the formation of biofilms, so the search for anti-QS strategies could be a potential objective to prevent infection by *P. aeruginosa*. Regulation of biofilm formation, Inhibition of QS and attenuation of virulence factors in *P. aeruginosa*, have been reported for a wide variety of extracts, essential oils, and chemical constituents (Qais and Ahmad, 2022). From these chemical compound was vit C(Ascorbic acid) is a water-soluble antioxidant that scavenges ROS and terminates peroxidative processes (Pehlivan, 2017). Nonlethal concentrations of vit C inhibit bacterial QS and other regulatory mechanisms underpinning biofilm development (Pandit *et al.*,2017). Plants contain chemical compounds that act as antibacterial and have recently been shown to inhibit bacterial QS, such as SA . These natural compounds can inhibit the gene expression of regulated genes. QS and involved in bacterial virulence, which leads to reduced biofilm formation, pigment production, and proteases (Kenoosh *et al.*,2022)

The QQ phenomenon consists in the enzymatic degradation of signal molecules of the QS system in order to avoid their accumulation in the environment and inhibit the change in gene expression. These enzymes, due to inhibition of the production of auto inducers, are able to prevent the production of virulence factors, including biofilms produced by drug-resistant bacteria (Rajesh and Rai ,2016). Production of virulence factors such as pyocyanin or pyoverdine in *P. aeruginosa* may be inhibited by different compounds. Such activity was proved for some quaternary ammonium salts, especially those with more than one hydrophilic group (Piecuch *et al.*, 2016).The AHL-lactonase enzyme produced by *Bacillus cereus* VT96 directly controls the production of virulence factors such as exopolysaccharide production, biofilm formation, and pyocyanin production in *Pseudomonas aeruginosa* (Rajesh and Rai , 2016). Paraoxonases present in several human cell lines and mammalian serum are also extremely important, as they also have the ability to degrade AHL molecules

(Chang *et al.*,2019). Extract from the *Terminalia bellerica* plant effectively inhibited the production of pyocyanin and EPS in *P. aeruginosa*, whose production is under the control of the QS communication system (Ganesh and Rai ,2018). peppermint oil and menthol also resulted in inhibition of pyocyanin, elastase, protease, EPS production and biofilm production by *P. aeruginosa* (Husain *et al.*2015).

Flavonoids are compounds found in plants that can interfere with communication between microorganisms and have an anti-biofilm effect. Isolated flavonoids from *Centella asiatica* showed inhibitory effects on swarming and twitching motility, and production of pyocyanin and biofilm in *P. aeruginosa* (Vasavi *et al.*, 2016).

Synthetic QQ molecules such as Norspirmidine, a type of polyamine, has reduced the expression of *lasI*, *lasR*, *rhII*, *rhIR*, *mvfR* genes that are involved in the QS system in *P. aeruginosa*. This led to reduced attachment to the surface. A smaller number of cells attached to the surface resulted in limited biofilm production and easier eradication (Qu *et al.*2016).

2.9.4. Measurement of QS Molecules

The quantitative and qualitative QS measurement may be performed by variety of different techniques that may be classified as direct or indirect methods . Most of them are based on detection of signaling molecules that possess functional groups . The techniques, which are now commonly used for quantitative and qualitative analysis of QS and QQ molecules involve colorimetry, bioluminescence, chemiluminescence, fluorescence, chromatography-mass spectroscopy, and electrochemistry (Van der Meer and Belkin, 2010). Techniques based on enzymatic activity measurement may also be applied for AHL detection. The most common QQ inducing enzymes are AHL-acylase, AHL-lactonase, oxidoreductase, and paraoxonase. They may be active only against specific QS molecules or have broad spectrum of activity (Chen *et al.*, 2013).

2.9.4.1. Fourier-transform infrared spectroscopy(FTIR)

The FTIR is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid, or gas. An FTIR spectrometer simultaneously collects high-resolution spectral data over a wide spectral range. This confers a significant advantage over a dispersive spectrometer, which measures intensity over a narrow range of wavelengths at a time. FTIR Principle and Analysis Process demonstrated in (Figure 2-10) (Mohamed *et al.*,2017).

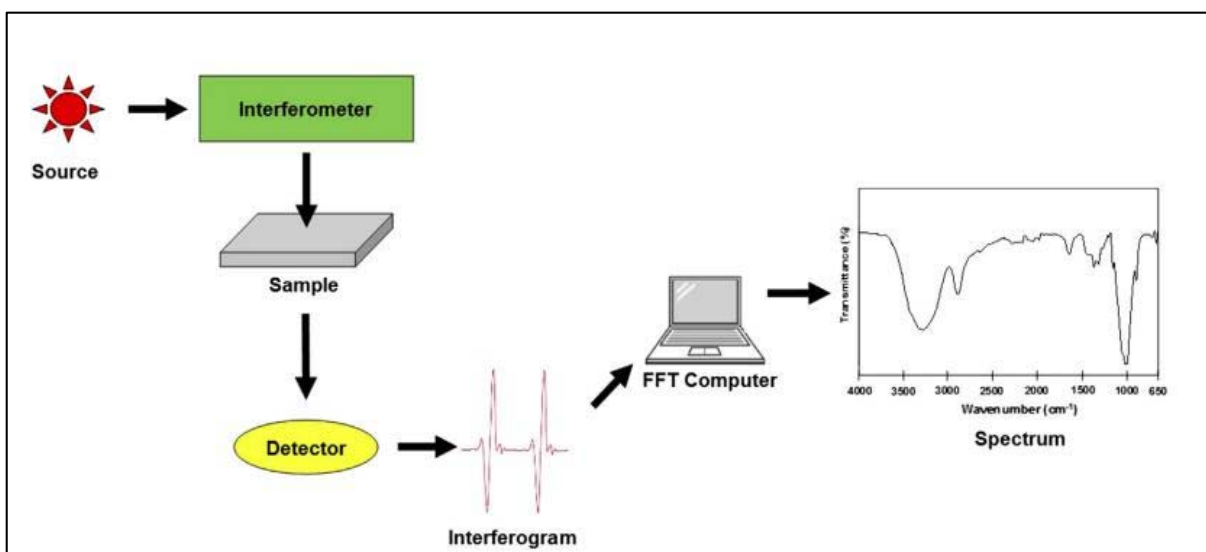


Figure (2-10): Basic component in FTIR (Mohamed *et al.*,2017).

The benefit of IR spectroscopy is identification of the functional groups in a molecule. FTIR instruments can provide good quality IR spectrum from extremely small quantities of a material. The technique also usefully provides a 'fingerprint' of the molecule and its comparison with authentic specimen is confirmatory of the structure of that molecule. IR spectra of AHLs show characteristic absorption peaks at 1780, 1710, 1650 cm⁻¹ arising from the presence of the lactone ring, 3-OXO (when present), and amide carbonyl, respectively (Camara *et al.*, 1998).

2.9.4.2. High-pressure Liquid Chromatography (HPLC)

The HPLC, formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify specific components in mixtures. The mixtures can originate from food, chemicals, pharmaceuticals, biological, environmental and agriculture, etc, which have been dissolved into liquid solutions (Kazakevich and Lobrutto ,2007). It relies on high pressure pumps, which deliver mixtures of various solvents, called the mobile phase, which flows through the system, collecting the sample mixture on the way, delivering it into a cylinder, called the column, filled with solid particles, made of adsorbent material, called the stationary phase. Each component in the sample interacts differently with the adsorbent material, causing different migration rates for each component (Levin ,2004). These different rates lead to separation as the species flow out of the column into a specific detector such as UV detectors. The output of the detector is a graph, called a chromatogram. Chromatograms are graphical representations of the signal intensity versus time or volume, showing peaks, which represent components of the sample. Each sample appears in its respective time, called its retention time, having area proportional to its amount. HPLC is widely used for manufacturing (e.g., during the production process of pharmaceutical and biological products)(Gerber *et al.*,2004). legal (e.g., detecting performance enhancement drugs in urine)(Bayne and Carlin,2017). research (e.g., separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (e.g., detecting vitamin D levels in blood serum) purposes (Seger and Salzmann,2020). HPLC Principle and Analysis Process demonstrated in (Figure 2-11) (Ghodke *et al.*,2023).

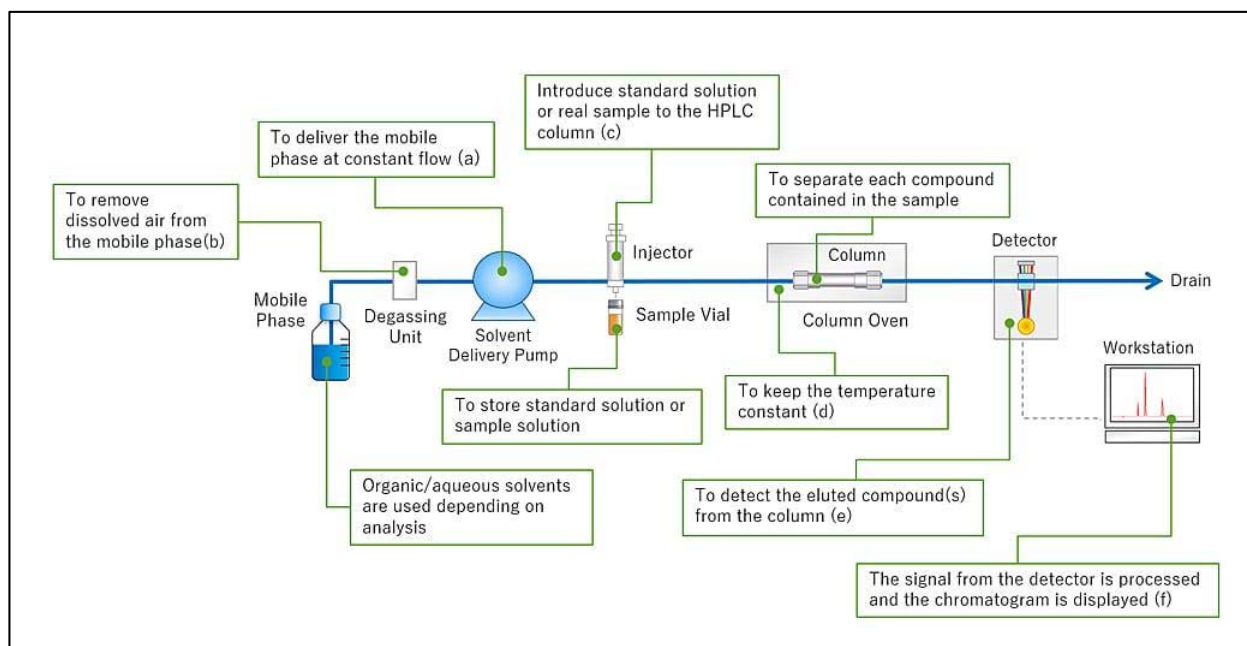


Figure (2-11): HPLC flow diagram and the role of each component (Ghodke *et al.*,2023).

2.9.5. Impact of QS on immunity.

Depending on the acyl chain length, double bonds, and concentration, AHL can affect the host innate immune system at varying degrees. For macrophages, in general, AHL decreases their inflammatory response in a diverse manner, leading to chronic infection with *P. aeruginosa*. It has been shown that *P. aeruginosa* produced AHL exerted an anti-inflammatory response in RAW264.7 mouse macrophages in a dose-dependent manner. At the same time, AHL induced a decrease in the concentration of TNF- α and an increase in the concentration of IL-10 secreted by RAW264.7 cells (Glucksam-Galnoy *et al.*,2013). In addition, it has been reported that macrophages have stronger phagocytic activity in the presence of AHL (Holm *et al.*,2015).

For dendritic cells (DCs), the effects are different and depend on the cell type. In the presence of AHL, the stimulation with lipopolysaccharides was inhibited the secretion of pro-inflammatory cytokines IL-12 and interferon- γ in both human and mouse DCs. However, the same concentrations of AHL and LPS only enhanced the ability of human DCs, but not mouse DCs, to secrete IL-10 (Guo *et al.*, 2020). AHL also has a certain degree of influence on host

adaptive immunity. T lymphocyte proliferation and function, as well as regulating antibody production by B lymphocytes. Furthermore, AHL can induce apoptosis in Jurkat cell lines via the mitochondrial pathway, thus inhibiting the activation and proliferation of DCs and T cells and downregulating the expression of costimulatory molecules on DCs (Xiao *et al.*,2022). While PQS that known produced in the lungs of cystic fibrosis patients infected with *P. aeruginosa*, but it is not clear whether PQS is required for virulence (Kim *et al.*,2010). In a previous study using human peripheral blood mononuclear cells (PBMC), have shown that PQS has an immune modulating activity that is different from that of 3-oxo-C12-HSL in cell proliferation and in the production of IL-2 and TNF- α (Hooi *et al.*,2004). PQS has been revealed to subdue cell proliferation and IL-2 liberation in (PBMC) stimulated with a panactivating lectin (concanavalin A) In addition, PQS can prompt TNF- α liberation from (PBMC)after stimulation with LPS at concentrations over 10 μ M(Hooi *et al.*, 2004).

2.9.6. Inflammation.

Inflammation reaction is a stress response by which a number of activities and signaling including cytokines, chemokines, biogenic amines and eicosanoids that induce diverse biological processes affecting the local or systemic homeostasis (Kotas and Medzhitov , 2015). Inflammation can be caused by different reasons such as bacterial, viral, physical injury , cancer and other condition and that inflammation may be far causing different consequences such as cancer and autoimmune disease as the frequent or chronic inflammation may potentially assist (Roe, 2021). Understanding the Inflammation is considered very important so we can understand the target pathway in order to achieve the meaningful therapies(Praveen *et al.*,2022).

2.9.7. Cell Culture Technique

Cell culture refers to cultures derived from dissociated cells taken from the original tissue ('primary cell culture'). Cells are dispersed (mechanically and/or enzymatically) into a cell suspension which may then be cultured as a monolayer on a solid substrate, or as a suspension in the culture medium. These cultures have lost their histotypic architecture and often some of the biochemical properties associated with it. However, they can be propagated and hence expanded and divided to give rise to replicate cultures (Unchern, 1999). Cell cultures can be characterized as a defined population that can be preserved by freezing. The most obvious advantage of cell culture, and of dissociated cell culture in particular, is that it makes individual living cells accessible. All in all, primary dissociated cell cultures are particularly amenable to study using morphological and physiological techniques, which can be applied on a cell by cell basis (Seegeritz and Vallier, 2017).

2.9.8. Some Immune Related Organ.

Spleen is one of the immune organs which playing an important role in the cellular and molecular levels as it regulates innate and adaptive immune response, control antigen tolerance as protected the host as well as contributed to the disease response (Bronte and Pittet, 2013).

Mesenteric lymph node is considered the key of the immune tolerance and it is the most organ that assault by the antigens as it has been that most of the body's immune cells are actually in the gut (Macpherson and Smith, 2006).

On the other side, evidences are growing up by the time showing that bone marrow is the site where the active function and trafficking of all immune cells including T cells, B cells, dendritic cells, natural killer T (NKT) cells, neutrophils, myeloid-derived suppressor cells and mesenchymal stem cells, are observed in the bone marrow (Zhao *et al.*, 2012).

2.9.9. Some Immune Marker Related to Inflammation.

1-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):

The GAPDH gene, traditionally known as a housekeeping gene which plays a role in glycolysis, has been identified as an internal reference gene to quantitate DNA, RNA and proteins in usual biological experiments. An important reason is that GAPDH is highly and constantly expressed in almost all tissues. However, A study of GAPDH protein in mouse discovered a new side (Shen *et al.*,2023). It might take part in several additional nuclear functions such as nitrosylation of nuclear proteins and the regulation of mRNA stability due to its nitrosylase activity. Subsequently, more and more studies have found that GAPDH is unstable in some pathological stages including aging and cancer (Yang *et al.*,2021).

2-Interleukin-6(IL-6):

Pro-inflammatory cytokines such as IL-1 β , TNF α and IL-6 play crucial roles in inflammation. Among them, IL-6 is a major player in chronic inflammatory diseases, autoimmune diseases, cancer and cytokine storm(Jones and Jenkins ,2018). IL-6, which was discovered in 1986 , is a pleiotropic cytokine involved not only in immune responses but also in inflammation, hematopoiesis, bone metabolism, embryonic development and other fundamental processes (Hasegawa *et al.*,2016). Notably, IL-6 is involved in cell senescence, is produced by senescent cells and is critically involved in senescence-induced inflammation and age-dependent pathologies and cancer . IL-6 is also a key factor in inflammation, autoimmunity and cancer, with its effect mainly exerted through the IL-6–STAT3pathway (Jones and Jenkins ,2018).The IL-6 amplifier is an amplification mechanism for the production of IL-6 and a variety of other cytokines and chemokines through a synergic interaction between STAT3 and nuclear factor-kappa B (NF- κ B). It also plays a key role in inflammatory diseases including cytokine storm syndromes, autoimmune diseases and cancer (Hirano and Murakami,2020).

3-Interleukin-1 β :

Interleukin-1 β is a member of the interleukin-1 family and exists as one of the major agonistic IL-1 ligands, along with IL-1 α . Another ligand in the family, IL-1RA, acts antagonistically. NF- κ B can activate all three ligands. Both IL-1 α and IL-1 β have been found to play dual roles in tumor progression, sometimes promoting it and at other times suppressing it. The IL-1 family has been associated with cancer progression through interactions with inflammatory cytokines and downstream pathways, while certain members exhibit anti-tumorigenic effects (Tong *et al.*, 2022). IL-1 β is generated and discharged by a variety of cell categories, including immune cells, fibroblasts, and cancer cells. The initiation of the IL1 β gene occurs subsequent to cellular "priming," followed by a secondary signal that triggers the maturation of IL-1 β through caspase-1 activation within the inflammasome. Once matured, IL-1 β is liberated, subsequently instigating the transcription of target genes by binding with IL-1R1 receptors on the intended cells. The expression and maturation of IL-1 β are subject to the influence of gene polymorphisms as well as the specific cellular milieu (Rébé and Ghiringhelli, 2020).

4-Nuclear factor kappa B:

The NF- κ B is a family of transcription factors that control the expression of essential regulatory genes involved in immunity, inflammation, cell death, and cell proliferation. Normally located in the cytoplasm, NF- κ B can be activated by various cellular stimuli. It follows two activation pathways, the canonical and non-canonical pathways involving molecular interactions with adapter proteins, phosphorylation, and ubiquitinase enzymes. This results in NF- κ B translocation to the nucleus, regulating gene expression (Zinatizadeh *et al.*, 2020). NF- κ B serves as a central facilitator of the initial trigger for activating the NLRP3 inflammasome. It achieves this by prompting the transcriptional manifestation of NLRP3 and pro-IL-1 β , which occurs in reaction to different ligands of pattern recognition receptors (PRR) and cytokines. Similar to the pro-IL-1 β gene, the

NLRP3 gene is under the direct influence of NF- κ B. Within its promoter region, there exist specific sites where NF- κ B can bind (Sutterwala *et al.*, 2014).

5-Tumor Necrotic Factor- α (TNF- α):

The TNF- α is a pleiotropic pro inflammatory cytokine implicated in a wide range of cellular processes including cell proliferation, survival and death. In addition, TNF- α signaling is associated with the regulation of several inflammatory pathways including the cyclooxygenase-2 and inducible nitric oxide synthase pathways (Li *et al.*, 2018). Hence, TNF- α is a key mediator in the inflammatory response. TNF- α is predominantly secreted by monocytes, macrophages and natural killer cells. TNF- α is first synthesized as a transmembrane protein and can induce immunological responses in effector cells, but also transduce reverse signaling by contact-dependent cell interactions (Delgado and Brunner 2019). In addition, transmembrane TNF- α can be enzymatically cleaved by TNF- α -converting enzyme resulting in soluble TNF- α . Upon distribution in the extracellular space or systemic circulation, soluble TNF- α may exert immunological effects at distant sites. Therefore, both forms are active cytokines that share similar as well as distinctive immunological effects. TNF- α activation of effector cells under physiological conditions generally leads to a pro inflammatory response or apoptosis and aids in the defense against infections and localized tissue injury (Delgado and Brunner 2019). However, the elevated TNF- α tissue levels in the mucosa and lamina propria of inflammatory bowel diseases patients result in an aberrant pro inflammatory response that is associated with the dysregulation of mucosal immune cells and tissue damage (Friedrich *et al.*, 2019).

6- Interleukin-2(IL-2):

The IL-2 was first described as an immune stimulatory factor that supports the expansion of activated effector T cells. A layer of sophistication arose when regulatory CD4⁺ T lymphocytes (Tregs) were shown to require IL-2 for their development, homeostasis, and immunosuppressive functions. (Valle-Mendiola

et al., 2016). Fundamental distinctions in the nature and spatiotemporal expression patterns of IL-2 receptor subunits on naïve memory effector T cells versus Tregs are now being exploited to manipulate the immunomodulatory effects of IL-2 for therapeutic purposes. Although high-dose IL-2 administration has yielded discrete clinical responses, low-dose IL-2 as well as innovative strategies based on IL-2 derivatives, including “muteins,” immune complexes, and immune cytokines, are being explored to therapeutically enhance or inhibit the immune response (Pol *et al.*, 2020).

2.9.10. polymerase chain reaction (PCR), and it’s role to detect QS.

A Nobel Prize-worthy development of the PCR by Kary Mullis in 1992 revolutionized the field of molecular biology into a whole new era. PCR is a technique used to amplify a segment of DNA containing the gene of interest with a wide range of application, from mapping the human genome to the detection of bacteria and viruses, as well as diagnosis of hereditary disorders and investigation of gene transcription profile (Kadri , 2019). The terms PCR, quantitative PCR (qPCR), real time - PCR (RT-PCR), and RT-qPCR are often mistakenly and interchangeably used with lack of attention to what the terms described. In traditional PCR, the amplified DNA segment is qualitatively assessed by gel-aided visualization. qPCR on the other hand, denotes real-time quantification of DNA amplification and measures the amplified DNA segment via fluorescent probe or intercalating dye (Adams ,2020). The acronym RT -PCR denotes reverse transcription PCR where generation of single-stranded complementary DNA (cDNA) using reverse transcriptase enzyme is coupled to a PCR while RT-qPCR combined previously described techniques in just one reaction tube for rapid quantitative detection of gene expression level (Adams ,2020).

A precise, sensitive, and robust qPCR assay relies on several key components, namely, the primer, a purely intact sample, the PCR reagent, and the thermal cycler (Svec *et al.*,2015).With the qPCR assay condition remains mostly constant, together with a wide selection of PCR reagents, the latter two components are conveniently worked out. On the other hand, careful and precise consideration should be put into the primer design and the isolation process of the sensitive genetic material. Between these two components, the primer essentially dictates the success of a qPCR assay (Bustin and Huggett ,2017). An efficient primer would specifically binds to the target molecule at its annealing temperature and remains hybridized at higher temperature to allow the extension by DNA polymerase to take place . Primer's melting temperature (T_m), the G-C content and its size, as well as the targeted amplicon's size are among the parameters that should be considered when designing an optimal primer (Sedek *et al.*,2023).

PCR technology is used to detect the genes responsible for QS synthesis and virulence factor genes (Shravani *et al.*,2023).It also used to detect the gene expression of the genes responsible for the up or down regulation of the immune response to QS such as the genes responsible about secretion cytokine during the work of the immune system (Levkovich *et al.*,2023).

2.9.11.Gene Expression

Understanding the underlying mechanisms of disease, especially cancer, is a challenging task due to the complexity of genomic profiles, expression patterns, and interactions within the tumor microenvironment. The existence of intratumoral heterogeneity, characterized by significant genetic diversity within tumors, further adds to this complexity, as cancer cells exhibit distinct molecular and phenotypic features driven by various genetic alterations and environmental influences (Mohammed *et al.*, 2022).

Gene expression plays a pivotal role in converting the information encoded in genes into functional outcomes. This process involves the transcription of RNA molecules that code for proteins or non-coding RNA molecules with other essential functions. Essentially, gene expression acts as an "on/off switch," controlling when and where RNA molecules and proteins are synthesized, and as a "volume control," determining the quantity of these products. The regulation of gene expression is highly intricate and varies significantly under different conditions and in different cell types (Mattick *et al.*, 2023).

Current technologies allow us to measure mRNA expression of every gene in the entire genome. Traditional approaches also involve observing phenotypes or traits as indicators of gene expression, such as measuring protein activity (Gandellini et al., 2015).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Equipment and instruments

All devices and instruments used in this study are listed in Table (3-1).

Table (3-1): Equipment utilized and instruments used in this study.

Item	Company	Origin
Autoclave	Hirayama	Japan
Burner	Satorins	Germany
Cell culture plate	Biobase	china
Centrifuge	Kokusan	Japan
CO ₂ incubator	Biobase	china
Cool Centrifuge	Hettich	Germany
Cylinder, separate funnel, round bottom flask.	HAD	China
Deep freezer	Biobase	china
Electric balance	Denver	Canada
Electrophoresis	Major science	USA
ELISA system	Awareness	USA
Eppendorf tubes	Sigma-Aldrich	Germany
Evaporator	Griffin & George	England
Filter paper	HAD	China
FTIR apparatus	Bruker	Germany
Homogenizer	Biobase	china
Hot Magnetic Stirrer	Gallenkamp	England
HPLC apparatus	Sykam	Germany
Incubator	Memmert	Germany
Light microscope	Optika	Italy

Item	Company	Origin
Loop	Hi-media	India
Micro pipette	Dragon-med	Spain
Millipore Filter 0.22	BHD	England
Nano drop	Bio Drop	England
Oven	Memmert	Germany
Petri dishes 9 cm	Afco-Dispo	Jordan
pH meter	Basic20	Germany
Q PCR system	Analytikjena	Germany
Refrigerator	Craft	Saudi Arabia
Shaker incubator	Memmert	Germany
Spectrophotometer	Cecil CE 7200	England
Thermo cycler (PCR)	Humburg	Germany
Tips Sterellin	HAD	China
Tissue culture Micro titer plate 96	Coastar	USA
UV Trans-illuminator	MUV21-312	Taiwan
Vortex	Heidolph	Germany
Water bath	Gallenkamp	England
Water distiller	Kottermann	Germany

3.1.2. Culture media

All the culture media used in the study are listed in Table (3-2).

Table (3-2): Cultural media used in this study

Name of Media	Company	Origin
Blood Agar	LAB	England
MacConkey agar	Himedia	India
Brain heart infusion broth	Neogen	England
Mueller-Hinton agar	Himedia	India
Cetrimide agar	Neogen	England
Luria-Bertani broth and agar	Pronadisa	Spain
Trypticase Soy Broth (TSB)	Pronadisa	Spain

3.1.3. Chemical Compounds and Kits

Table (3-3) shows the most important chemical compounds and biological kits used in the current study .

Table (3-3): The major chemicals used in this study.

Chemical Compound and kit	Company	Origin
1kb DNA Ladder	Promega coporation	USA
Acetone	Solvochem	UK
Agarose	Promega	USA
API KIT	BioMérieux	France
Ascorbic acid	Avonchem	UK
Congo red	BDH	Uk
Crystal violate	BDH	Uk
DNA extraction Kit No.GBB100	Geneaid	Taiwan
Dulbecco's phosphate-buffered saline	Euroclone	Italy
Ethanol	BDH	UK

Chemical Compound and kit	Company	Origin
Ethyl acetate 1%	BDH	UK
Fetal bovine serum	Cytiva	Austria
Gentamicin 2ml vial	United	Jorden
Glycerol	BDH	UK
Gram Stain	Arcomex	Jorden
H ₂ O ₂	Oxoid	Uk
Loading Dye	Introne	Korea
Lysozyme	Geneaid	Taiwan
Master Mix	Promega coporation	USA
Midori Green Advance DNA stain	Nippon Genetics	Europe
Multi - Serra	Randox	USA
Normal Saline	Qatar pharma	Qatar
Oxidase Strip	Bioanalyse	Turkey
PBS	Euroclone	Italy
PCR clean-up system	Promega coporation	USA
Pencillin-streptomycin sol.	Euroclone	Italy
Red blood cell lysis	Euroclone	Italy
RPMI 1640	Euroclone	Italy
Salicylic acid	Avonchem	UK
Standard McFarland solution	BioMérieux	France
Triazole	Euroclone	Italy

3.1.4. Antibiotic discs

Antibiotic discs utilized in this study for disc diffusion method are listed in Table (3-4), the antibiotics were chosen according to clinical and laboratory standard institute (CLSI) (Humphries *et al* .,2021).

Table (3-4): Antibiotic discs utilized in this study

Antibiotic	Symbol	Content (µg)	Origin
Amikacin	AK	10	Bioanyalyse
Gentamicin	CN	10	Bioanyalyse
Meropenem	MEM	10	Bioanyalyse
Levofloxacin	LEV	5	Himedia
Norfloxacin	NOR	30	Himedia
Ciprofloxacin	CIP	10	Himedia
Ceftazidime	CAZ	30	Bioanyalyse
Piperacillin	PRL	100	Bioanyalyse
Ceftriaxone	CRO	10	Bioanyalyse

3.2. Methods

The practical aspect of the current study was explained through Figure (3-1).

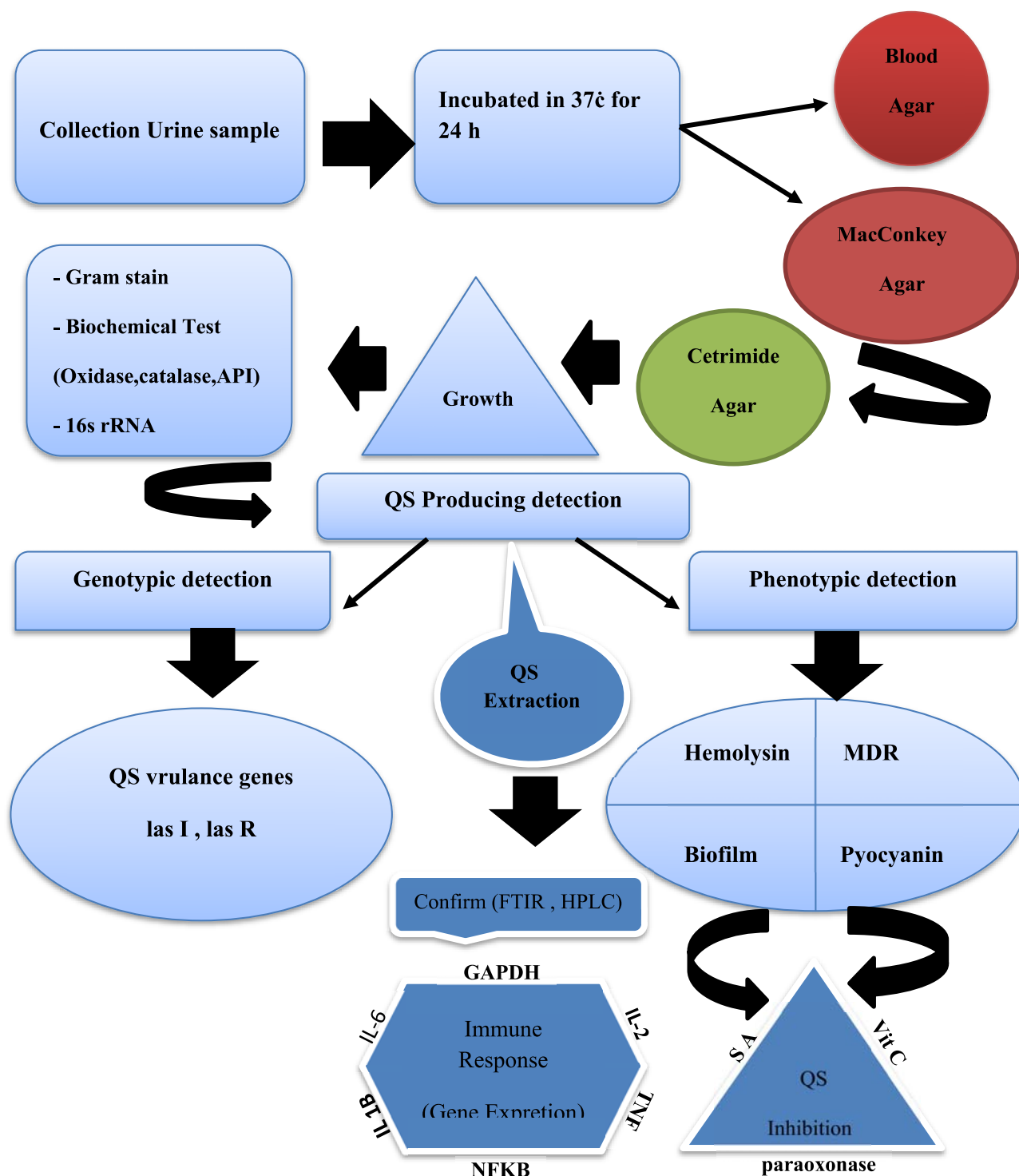


Figure (3-1): Protocol of Methods in Current Study

3.2.1. Sterilization Methods

3.2.1.1. Moist-Heat Sterilization: This method was used to sterilize a number of culture media, solutions, and materials that cannot tolerate dry heat sterilization. In this method, an autoclave was used at a temperature of (121 °C) and under pressure (15 pounds /inch²) for 15 minutes (Presterl *et al.*, 2019).

3.2.1.2. Dry-Heat Sterilization: The oven was used at a temperature of (180°C) for approximately 30 minutes to sterilize a number of metal tools and heat-resistant glassware (Presterl *et al.*, 2019).

3.2.1.3. Direct flame Sterilization: Sterilization of a number of laboratory instruments, such as platinum handles (Loop) and frequently used metal instruments, using a Bunsen lamp (Govindaraj and Muthuraman, 2015).

3.2.1.4. Filtration Method: Millipore filter (0.22µm diameter) was used for sterilization of solutions was used to separate (QS) from the other materials present in the Supernatant at the (QS) extraction method (Vivero-Gomez *et al.*, 2021).

3.2.2. Media Preparation

All media which used in this study were prepared according to the manufacturing company instruction; they were dissolve all constituents completely and then sterilized by autoclaving at 121°C for 15 min. at 15 pounds / inch², otherwise the media were incubated at 37C° for 24 h to ensure sterility.

3.2.2.1. Blood Agar Medium

Forty grams of the Blood agar base were dissolved in one liter of distilled water and the pH was adjusted to (7.2) ±0.2, then sterilized in an autoclave , after that medium was left to cool at a temperature of (45-50 °C), then (5-10%) of pure human blood was added, mixed well, distributed in sterile Petri dishes and kept in the refrigerator until use (Levinson, 2016).

3.2.2.2. MacConkey's Agar Medium

Fifty two grams of the MacConkey's agar medium was dissolved in one liter of distilled water and the pH was adjusted at $(7.2) \pm 0.2$, then sterilized in an autoclave, after that medium was left to cool at a temperature of $(45-50\text{ }^{\circ}\text{C})$, distributed in sterile Petri dishes, and kept in the refrigerator until use (Levinson, 2016).

3.2.2.3. Muller Hinton Agar Medium

Thirty eight grams of the Muller Hinton Agar medium were dissolved in one liter of distilled water and the pH was adjusted at $(7.2) \pm 0.2$, then sterilized in an autoclave. After that medium was left to cool at a temperature of $(50-45\text{ }^{\circ}\text{C})$, distributed in sterile Petri dishes, and kept in the refrigerator until use (Igbeneghu and Lamikanra, 2014).

3.2.2.4. Cetrimide Agar Medium

Forty-five and a half grams of the cetrimide Agar medium was dissolved in one liter of distilled water , then sterilized in an autoclave , after that medium was left to cool at a temperature of $(45-50)^{\circ}\text{C}$, adding 10ml of glycerol and the pH was adjusted at $(7.2) \pm 0.2$,distributed in sterile Petri dishes, and kept in the refrigerator until use. This medium was also used for determining the ability of an organism to produce pyoverdinin and pyocyanin, Cetrimide (N-Acetyl-N, N, N tri methyl ammonium bromide) is incorporated in the medium to inhibit bacteria other than *P. aeruginosa* (Shanthi and Dauraiswamy, 2010).

3.2.2.5. Brain Heart Infusion (B.H.I)

Thirty-seven grams of the B.H.I medium were dissolved in one liter of distilled water and the pH was adjusted at $(7.2) \pm 0.2$, then sterilized in an autoclave , after that medium was left to cool at a temperature of $(45-50)^{\circ}\text{C}$, distributed in sterile Tubes , and kept in the refrigerator until use (Babapour *et al.*, 2016).

3.2.2.6. Lauria Bertani Broth (LB)

Twenty grams of the LB medium were dissolved in one liter of distilled water and the pH was adjusted at $(7.2) \pm 0.2$, then sterilized in an autoclave, after that the medium was left to cool at a temperature of $(45-50)^{\circ}\text{C}$, distributed in sterile tubes, and kept in the refrigerator until use (Russell and Sambrook, 2001).

3.2.2.7. Lauria Bertani Agar

This media was prepared according to the paragraph (3.2.3.6) with the addition of 15g/L agar-agar. These components were dissolved in 1 L of distilled water and autoclaved. The media then was dispensed into sterile Petri dishes and kept at 4°C until used (Russell and Sambrook, 2001).

3.2.2.8. Trypticase Soy Broth (TSB) with 1% glucose

Thirty grams of the (TSB) medium were dissolved in one liter of distilled water and the pH was adjusted at $(7.2) \pm 0.2$. Then sterilized in an autoclave. The medium was left to cool at a temperature of $(45-50)^{\circ}\text{C}$, then adding 1% glucose. After the glucose is pasteurized in a water bath at 63°C for half an hour, kept in the refrigerator until use (Cappuccino and Welsh, 2018).

3.2.2.9. Congo Red Agar Medium (CRA)

The CRA is a specially prepared medium composed of brain heart infusion (BHI) broth (37g/l) supplemented with sucrose (50 g/l), agar agar (10 g/l) and Congo red stain (0.8 g/l). A concentrated aqueous solution of the Congo red stain then sterilized in an autoclave. Finally it was added into the autoclaved BHI agar with sucrose at 55°C . (Ruchi *et al.*, 2015).

3.2.3. Preparation of Reagents and Solutions

3.2.3.1. Phosphate Buffer Saline (PBS).

PBS was prepared by dissolving the ready tablet (Euroclone / Italy) in 100 ml distilled water (D.W), and PH was adjusted at 7.2 according to the manufacturer recommendation.

3.2.3.2. Crystal Violate (1%) Solution.

This solution was prepared by dissolving 1 g of crystal violet powder into 99 ml of D.W and sterilized by using Millipore filters (0.22µm diameter) then kept at 25 °C until used in the detection of biofilm (Babapour *et al.*,2016).

3.2.3.3. Ethanol (70%).

To prepare this solution,70 ml of absolute ethanol (99%) was completed to100 ml with D.W.

3.2.3.4. Preparation of Tris acetate-EDTA buffer (TAE)

In order to make one-liter stock solution of TAE (50X), 242g of Tris base were dissolved in 342.9 ml DW. Then, 100ml of 0.5M EDTA (pH 8.0) and 57.1ml of glacial acetic acid were added.The final volume was completed until the total volume reached one liter. TAE was used for electrophoresis at a concentration of 1X as a running buffer and agarose gel as well (Sambrook *et al.*, 1989).

3.2.4. Sample Collection

Two hundred forty-five clean catch mid-stream, urine samples were collected from both sexes of patient with UTI attending Ibn Senna teaching hospitals (Mosul-Iraq) from different ages whome didn't admister antibiotic. Collection of sample was carried out from March to September 2023.

3.2.5.Sample culturing and isolation.

Two hundred and forty-five urine samples were collected from patients with UTIs. The samples, clean catch med stream ,were placed in sterile plastic containers then transferred to the hospital laboratory within less than half an hour to be cultured by streaking on the Blood agar plates and MacConkey agar ,then incubated at 37 °C overnight aerobically, the bacterial colonies which appeared as non lactose fermenter on MacConkey agar and reacted positive with were oxidase, then selected and sub cultured on cetrimide agar and incubated in the same condition. (Jawetz *et al.*, 2019).

3.2.6. Identification of Bacterial Isolates.

3.2.6.1. Morphological Examination.

Colony shape, colony texture, color, blood hemolysis pattern, pigment production and edges were examined for the primary identification of isolated *P. aeruginosa* (Al-Bayati *et al.*,2021).

3.2.6.2. Microscopic Examination.

Slide smears were prepared for all suspected isolates and stained with Gram stain to observe the color, shape, and arrangement by using light microscope (Al-Bayati *et al.*,2021).

3.2.6.3. Biochemical tests.

3.2.6.3.1. Oxidase test.

A single colony of fresh pure bacterial isolates from MacConkey agar was transferred to strip contain Oxidase reagent (Tetramethyl-p-phenylene diamine dihydrochloride) by wooden applicator sticks. Positive results were showed by the emergence of a dark purple hue within 10 seconds, whereas negative results were indicated by the lack of a color change. The purpose of this test was to determine whether bacteria could manufacture the oxidase enzyme (Al-obadi , 2022).

3.2.6.3.2. Catalase test.

A single colony was transported by woody stick from freshly culture medium into a clean slide. A few drops of 3% hydrogen peroxide were dropped on colonies; the relief of oxygen bubbles designated the presence of catalase (Al-obadi , 2022).

3.2.6.3.3. Analysis Profile Index.

According to API, T. F. (2017). Reference guide . The API reading Scale (color chart) by marking each test as positive or negative on the lid of the tray.

3.2.7. Molecular diagnosis.

The identification of bacterial isolates of *P. aeruginosa* was confirmed by using the PCR technique to detect 16S rRNA gene. The following steps carried out for the molecular diagnosis were detection of certain genes responsible for QS.

3.2.7.1. Detection of 16S rRNA Gene.

1- DNA Extraction.

The Genomic DNA was extracted from the bacterial isolates using a specialized kit from Geneaid company. Genomic DNA was extracted by taking bacterial colonies grown on ceftrimide agar plates for 18-24 hours following the manufacturer's instructions. The extracted DNA were stored at -20°C until further used (Khaleel *et al.*, 2023).

2- Determination of DNA Concentration and Purity.

A Nano drop device operating at a wavelength of 260/280 nm was used to evaluate the concentration and purity of DNA extracted from bacterial isolates. Two microliters of extracted DNA sample was applied to the Nano drop pad. The absorbance of DNA was measured.

3- Preparation of agarose gel.

Two different concentrations of agarose gels were prepared: 1% for running the genomic DNA and 2% for running the PCR products. The agarose powder was dissolved in 1X TAE buffer and heated with a hot magnetic stirrer until the mixture was fully clear. The gel solution was gently mixed with (2–5 µl per 100ml) Midori Green DNA stain. The gel was cast into the gel tray using combs after being cooled to 45°C. The comb was gently removed when the gel had set, and then positioned horizontally in the tank. Agarose gel was covered by 1 cm with 1X TAE, then DNA samples were loaded into the wells (Sambrook *et al.*, 1989).

4- Electrophoresis.

The 1000ng of genomic DNA was mixed with 6X loading dye provided by Introne (Korea) and loaded into agarose wells. When necessary, a quick purple 100 bp DNA ladder (trans Gene) was put in the first well as a size indicator. To allow DNA to move, 80-100 volt electric current was set for 45 minutes. After that, the gel was visualized and photographed using a UV transilluminator.

5- Polymerase Chain Reaction .

PCR for the 16S rRNA detection was conducted in a 20 μ l volume reaction using GoTaq G2 Green Master Mix supplied by Promega (USA), which contains G2 DNA polymerase (a variant of Taq polymerase), dNTPs, MgCl₂, and the polymerase buffer. This master mix contains two dyes (blue and yellow) that are pre-mixed to help you follow the PCR bands. Ingredients for each 20 μ l PCR reaction were as follows: 10 μ l Master mix, 2 μ l of 10 p mol/ μ l forward primer, 2 μ l of 10 p mol/ μ l reverse primer, a variable amount of DNA (up to 250 ng), and a variable amount of nuclease-free water. The elements were combined and put in a thermocycler device along with a 0.2 ml Eppendorf tube.

The universal primers 27F: **AGAGTTTGATCMTGGCTCAG** and 1492 R: **CGGTTACCTTGTTACGACTT** at 1465 bp (Khan *et al.*, 2023), were used to amplify nearly the full region of the 16SrRNA gene. The concentration of primers and the total amount of template DNA were added as recommended by the manufacturer. The PCR program for the 16SrRNA gene was set as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 60 sec in 35 cycles, 45 sec at 53 °C annealing temperature for 35 cycles and elongation step at 68 °C for 2 min in 35 cycles reaction. The final extension for the reaction was at 72 °C for 10 min and held at 4 °C. The PCR product was separated on 2% agarose gel and stained with Midori Green Advance DNA stain. A 100bp DNA marker (Trans Gene) was used as a molecular weight marker. The PCR product's expected size with primer is 1465 bp (Khan *et al.*, 2023).

6- DNA sequencing of 16S rRNA gene.

The PCR products for the 16SrRNA gene were purified and sent for sequencing at Psomagene sequencing company (USA). PCR products were sequenced using Sanger DNA sequencing, then retrieved sequences were searched for homology against published genes submitted in Gene Bank using the BLAST tool at NCBI.

3.2.8. Maintenance of Bacterial Isolates.

After ensuring their purity and completing laboratory diagnosis, the bacterial isolates were cultured in slanted cetrimide agar medium and incubated at a temperature of 37 °C for a period of 24h. Then placed in the refrigerator at a temperature of 4 °C in anticipation of the need for them, taking into account their activation approximately every month, for their preservation. For a longer period, preservation in the BHI is used, and the mechanism of the previous method is used, except that after incubation, sterile glycerol was added to it at a concentration of 15% and it is kept at a temperature of -80°C (Procop *et al.*, 2019).

3.3. Quorum Sensing Production Detection.

It is known that *P. aeruginosa* bacteria produce QS molecules. It was confirmed in this study that the isolated bacteria produce QS through two ways phenotypic and genotypic methods (Hamza *et al.*,2023; Shravani *et al.*,2023).

3.3.1. Phenotypic Methods.

The QS molecules control the regulation of the virulence factors production. Therefore, the presence or detection of these factors is indirect evidence that these bacteria have the ability to form and produce QS molecules (Peña-Díaz *et al.*,2023).

1. Haemolysin Production Detection.

Fresh bacterial colony from cetrimide plate was inoculated on blood agar and incubated at 37°C for 24 hours. The appearance of total lysis of red blood cells (clear zone) formed surrounding the cultured colonies indicated as a positive result (Rashid *et al.*,2019).

2. Antibiotics Susceptibility Tests.

The Kirby-Bauer method was used to perform the antibiotics susceptibility test for different antibiotics as shown in Table 3-4. As described by (CLSI,2022), the inhibition zones around the antibiotic discs were measured by using a metric ruler and were compared with antibiotic profile data prescribed by (CLSI,2022).

3. Detection of Biofilm Formation.

Biofilm formation test was done by CRA, TM and TCPM as described by (Sultan and Nabel,2019). Prepared CRA plates were inoculated with the isolated *p. aeruginosa* and aerobically incubated at 37°C for 24 hours.

The TM method, a loopful of the isolated bacteria from overnight cultured media was inoculated in each glass tube containing 10 ml of trypticase soy broth with 1% glucose. The inoculated tubes were then incubated at 37°C. After incubation for 24 hours, tubes were emptied and washed with phosphate buffer saline and left to dry. Crystal violet (0.1%) was used to stain the dried tubes for 15 minutes. Excess stain was then removed by washing the tubes with deionized water. The tubes were then dried in inverted position and examined for biofilm production.

In the current study we used TCPM as the gold standard test for detection of biofilm formation (Hassan *et al.*,2011). A loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose. The inoculated broth was then kept in the incubator at 37°C for 24 hours. Bacterial suspensions were further diluted 1:100 with fresh medium. Separate wells of a sterile polystyrene tissue culture plate, were filled by 200 µl of the prepared bacterial

suspension. Similarly, control bacteria (Any *p. aeruginosa* is already known to be able to produce biofilm), were put in the tissue culture plate. In addition, only sterile broth was used to ensure sterility and to identify non-biofilm production. After incubation at 37°C for 24 hours, the plate was gently tapped to remove the content of the wells followed by washing with 200 µl of phosphate buffer saline.

The washing step was repeated four times to remove any free bacteria present in the wells. Sodium acetate (2%) were added to the wells and kept for 30 minutes in order to fix the biofilms formed by bacteria attached to the wells. Staining of the fixed biofilms was conducted using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed by deionized water to remove any extra stain. After drying, a micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms. Test was carried out in triplicate and average of three OD values was taken. Optical densities values indicated bacterial adherence to the wells and biofilm formation.

The positive result of biofilm production is the appearance of black colonies in the CRA method while the positive result TM through the formation of a blue ring on the walls of the glass tube. Finally the positive result in TCPM according Table (3-5).

Table (3-5):Grading of biofilm formation by TCPM

Optical densities values	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120- 0.240	Moderate	Moderate
> 0.240	Strong	Strong

4. Pyocyanin production.

Pyocyanin pigment was estimated by measuring the absorbance in Enzyme Linked Immune Sorbent Assay (ELISA) reader equipment (Awareness microplate reader, USA) at 690nm, Pyocyanin production determined according to (Lahij *et al.*, 2021).

A loopful of freshly cultured isolates was inoculated in 10 ml of LB medium broth. The inoculated broth was then incubated at 37°C for 24 hours. Bacterial suspensions were further diluted 1:100 with fresh medium. Separate wells of a sterile polystyrene tissue culture plate, were filled by 200 µl of the prepared bacterial suspension. Similarly, control bacteria (Any *p. aeruginosa* is already known to be able to produce pyocanin) were put in the tissue culture plate. In addition, only sterile broth was used to ensure sterility and to identify non-pyocanin production. After incubation at 37°C for 72 hours, the plates were shaken gently and read the OD for *p. aeruginosa* isolates at 690nm and compared with the OD result for bacterial control and sterile broth.

3.3.2. Genotypic Methods.

In this part of the current study, some genes responsible for the production of the QS were investigated.

3.3.2.1. Detection of *lasI* and *lasR* genes.

We used amplification program to detect the genes that were involved in QS (*lasI*, *lasR*) for *P. aeruginosa* isolates by using the same steps in paragraph (3.2.7.1). The total reaction mixture for PCR was 20µl, with 2µl of purified DNA, 10µl of master mix, and 4µl of the primer mix and complete the total volume to 20µl by added 4µl nuclease free water. Oligonucleotide primers obtained from HELINI Biomolecules, for *lasI* and *lasR* (Shravani *et al.*, 2023), were used as illustrate in Table (3-6). PCR programming was done with an initial denaturation at 95 °C for 5 min, followed by denaturation, annealing, and extension at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec for 35

Table (3-6):PCR primers and sequences

Target gene	Primer sequence (5' - 3')	PCR Product (bp)	Reference
<i>lasI</i>	F: GGCGCGAAGAGTTCGATAAA	311	(Shravani <i>et al.</i> ,2023)
	R: TCCAGAGTTGATGGCGAAAC		
<i>lasR</i>	F: GATCCTGTTCGGCCTGTT	378	
	R: TGCAGTGCGTAGTCCTTG		

3.3.3.1. Inhibition of biofilm formation and pyocyanin production

(%) Inhibition = $[(AC - AS) * 100] / AC$ (Mansuri *et al*.,2020). Where Ac and As is the absorbance of the control and sample respectively.

3.3.3.2. Paraoxonase activity increase with elevating HDL concentration.

The PON is a circulating esterase and lactonase mostly carried on HDL, which is considered one the important protein carriers in the human body, as it transports fats from the blood vessels to the tissues. When its concentration increases, the risk of strokes and heart attacks decreases and the percentage of paraoxonase increases also , which is important in inhibiting the QS (Gugliucci and Menini , 2015). To confirm PON inhibitory effectiveness which increase with elevating HDL concentration, quantitative experiment was done in the current study by using two sterile test tubes containing LB broth medium. It is conducted by inoculating with *P. aeruginosa*, which produce biofilm and pyocyanin. Added 200 µl from serum for healthy person (HDL 30 mg /dl), The concentration of HDL was measured in the laboratory after taking a blood sample from him, to one tube and 200 µL from Multi-Sera- Level 3(39 mg /dl).This experiment was conducted on Biofilm formation and Pyocyanin production.

3.4. Extraction of QS from culture supernatants.

The extraction of QS molecules was conducted in the College of Science Central Laboratory at the University of Mosul carried out using the method previously mentioned by (Vivero-Gomez *et al.*, 2021) that include briefly separating each stationary phase culture, 10 mL(LB media) broth was centrifuged at 10000 rpm for 10 minutes at 4°C cool centrifuge method. As previously described, QS molecules were recovered from the cell-free supernatant. Supernatants were filtered via sterile 0.22 M.M filters and placed in a 50-mL falcon tube. The filtered supernatant was then mixed with 10 mL of acidified Ethyl acetate (0.01% acetic acid) was vortexed for 30 seconds to mix the aqueous and organic phases. The mixture was transferred to separate funnel that had previously been cleaned with acetone and left alone until the two phases were separated. The organic phase was then transferred to another falcon tube .The extraction procedure was done twice or more until a total volume of 30 mL

was reached. The supernatant extracts were separated into 50-mL round-bottom flasks and rotated evaporated until the liquid phase was entirely dry.

3.4.1. Confirming the presence of QS molecules in crude extract

After extraction we confirmed the presence of QS molecules in the crude by using two technique.

3.4.1.1. Fourier-transform infrared spectroscopy

We used FT-IR technique in the College of Science Central Laboratory at the University of Mosul to identification of QS signaling molecules that possess functional groups such as (C=O, C-O, N=H, OH), functional groups were discovered by FT-IR as stated (Taghadosi *et al.*, 2015). Briefly, a drop of the QS crude extract was put on a KBr plate, followed by a second plate. The samples were evaluated. Basically, the IR spectrum obtained from FTIR spectrometer lies in the mid-IR region 2.5-15 mm between 4000 and 666 cm^{-1} . Transition energies corresponding to changes in vibrational energy state for many functional groups are located in the mid-IR region (4000-400 cm^{-1}) and hence the appearance of an absorption band in this region can be used to determine whether specific functional groups exist within the molecule (Errico *et al.*, 2023).

3.4.1.2. High-pressure Liquid Chromatography.

Another validated experiment was performed in private lab by using an HPLC equipment. The QS crude extract was placed into an HPLC 1200 infinite configuration, which pumps molecules by weight through a solvent (mobile phase) and a solid (stationary phase). Molecules collide and adsorb onto the strong substance at that place, eluting at variable rates (Laj *et al.*, 2022). Section dimensions were 2.1×100 mm, with an estimated molecular size of 1.8 mm. The stationary stage employed C18-reinforced silica, whereas the versatile stage used acetonitrile. Non-polar atoms was separated into pH groups ranging from (2 to 9). We changed the draw and discharge rates to 2000 $\text{mL}/\text{min}^{-1}$, the infused volume to 5 mL, the suction temperature to 30 $^{\circ}\text{C}$. The stream rate changed to (

0.4 mL/min⁻¹. The low pressure limitation was set to zero, while the high pressure limit was set to 900 bars. At (1) minute, the solvent included (95%) soluble A (water, 5 mM ammonium formate, and 0.1% formic acid) and (5%) soluble B (ACN). After (10) minutes, the solvent gradient changed to 5% solvent A and 95% solvent B. (Laj *et al.*, 2022). It is worth mentioning that similar recent studies were relied upon in separating and extracting QS molecules to determine the retention time for each molecules.

3.5. Role of QS molecules in immune modulation

3.5.1. Mice

A mouse with age 8-10 weeks was brought (*Mus musculus*, which was obtained from the Animals House in the College of Veterinary Medicine at the University of Mosul.

3.5.2. Preparation of Cell Culture Media (Complete Medium)

The complete medium was prepared by Roswell Park Memorial Institute media (RPMI) from (Euroclone, Italy) with a volume of 9 ml which was taken were added to 1 ml of Fetal Bovine Serum (FBS) which the last represent 10 % of the whole mix , then 0.5 ml of the whole volume of penicillin-streptomycin solution was added to the final volume . The mixture was mixed well before using.

3.5.3. Isolation of immune cells from Mice Organs

The immune cells were collected from different organs, including spleen lymph nodes and bone marrow. All obtained through dissection by a specialized veterinarian.

1- Isolation of splenocytes : After euthanizing the mouse, spleen was obtained under aseptic conditions. In brief, the splenocytes was removed then single cell suspension was prepared by smashing the tissue through a Stomacher blender in complete media .Then cells were filtered through 70 µm filter after that RBC lysis solution from (Euroclone, Italy) was added to get rid

of the erythrocytes. The cells were washed twice with Phosphate Buffer Saline (PBS) at 1300 rpm for 5 minutes in a complete medium. The cells were then seeded in a 96-well plate with 500,000 cells/well (Busbee *et al.*, 2015; Sultan *et al.*, 2021b).

2- Isolation of Mesenteric Lymph node: Several Mesenteric lymph nodes were obtained from the abdominal area of the mouse then isolated as described by (Sultan *et al.*, 2021a). In brief single cells were prepared in a complete medium by smashing the tissue through a Stomacher Blender. The cells were then filtered using a 70 μ m filter and centrifuged at 1300 rpm for 10 minutes. The pellets were then resuspended in a complete RPMI media.

3- Isolation of Bone Marrow Immune cells : Bone Marrow was obtained from the femur bone of the mice where the cells collected by pushing syringe with 2mm gauge inside the whole of the femur bone then the cells were washed with PBS and centrifuged with 1300rpm/min for 7 minutes then the pellets were re suspended in RPMI complete media (Liu and Quant, 2015).

3.5.4. Specimen Preparation Procedure.

Both spleen and lymph nodes were homogenized separately then homogenized, put in a separate tubes and washed by adding 5 ml of the preservative media. They centrifuged at 1300 rpm for 7 minutes. After the supernatant were discarded the pellets then washed with 600 μ l of RBC lysis for one minute then 10 ml of PBS was added for washing and the mix, then was filtered and centrifuged at 1300 rpm for 10 minutes was done then the supernatant was discarded. The RPMI complete media at 1 ml was added which then these 1 ml volume were vortexed and count it by using cell counter then a different amount of sedimented cells were distributed per each well in 96 well plate. A different volumes, 5 %, 12.5 % and 25% were added to these wells. In addition to the untreated groups which contain only immune cells without QS crude, then the 96 well plate were incubated in CO₂ incubator for 24 hours.

After the incubation and under aseptic conditions , the cells were then transferred to separate Eppendorf tubes then centrifuged at 10,000 rpm for 10 minutes then the supernatant was removed . Then 700 µl of Triazole were added and stored in deep freezer at -80 for RT-qPCR reaction (Sultan *et al.*, 2021a, b).

3.6. Gene Expression for pro-inflammatory Cytokines Genes

For the analysis of mRNA expression and corresponding genes, Quantitative PCR (qPCR) was carried out using cDNA synthesized from both total RNA and mRNA samples. The Triazole up plus RNA Kit from a transgene biotech company was utilized to isolate total RNA and qPCR was used to assess the fold change in expression. qPCR reactions was conducted using the primers listed in Table (3-7).

Table (3-7) primer sequences used for qPCR

Gene	Primer	Sequence	References
GAPDH Housekeeping	F	AGGTCGGTGTGAACGGATTTG	Sultan <i>etal.</i> ,2021b
	R	TGTAGACCATGTAGTTGAGGTCA	
IL-1 β	F	GAAATGCCACCTTTTGACAGTG	Han <i>etal.</i> ,2020
	R	TGGATGCTCTCATCAGGACAG	
IL- 6	F	GAACAACGATGATGCACTTGC	Bustos- Salgado <i>etal.</i> ,2021
	R	TCCAGGTAGCTATGGTACTCC	
IL-2	F	AACCTGAAACTCCCCAGGAT	Seetaraman <i>etal .</i> , 2020
	R	CATCATCGAATTGGCACTCA	
TNFα	F	AGAGGCACTCCCCCAAAGA	Zhang <i>etal.</i> ,2021
	R	CGATCACCCCGAAGTTCCATT	
NF-KB	F	CGCAAAAGGACCTACGAGAC	Rasooli <i>etal.</i> , 2018
	R	TGGGGGAAAACATCAAAG	

3.6.1. RNA Extraction and cDNA Synthesis.

In order to isolate total RNA from frozen samples, the Tri Reagent (Transzol up plus RNA Kit) was employed following the manufacturer's instructions. The elution of RNA was done in 20 μ L of RNase-free water, adjusted according to the size of the precipitate. The quality and concentration of the isolated RNA were assessed using Nano Drop (Thermo Fisher Scientific, United States). Subsequently, 500 ng of the extracted RNA was used to synthesize the complementary strand (cDNA) using Trans Script First-Strand cDNA Synthesis Super Mix Kit (Beijing, China) and following the steps recommended by the company.

3.6.2. Quantitative PCR Amplification .

For the quantitative real-time PCR, Trans Start Green qPCR Super mix, a ready-to-use qPCR master mix containing Trans Start Taq DNA Polymerase SYBR Green I, dNTPs, PCR enhancer and stabilizer was utilized. Relative gene expression was quantified based on the expression of housekeeping genes and the genes studied from the treated and control groups. The Light Cycler 480 was used according to the manufacturer's instructions for the PCR process. The cycling conditions were as follows: 45 cycles consisting of three steps at 98 °C for 30 seconds, 98 °C for 10 seconds, and 60 °C for 30 seconds. The detected mRNA concentrations were determined in triplicates and were normalized to the housekeeping gene in each sample. The comparison between various specimens was performed using the Δ Ct method (Kuang *et al.*, 2018 ; Mohammed *et al.*, 2020).

3.6.3. Calculating the level of gene expression.

For calculating and estimating the gene expression, the following equations were used:

$$\text{Folding} = 2^{-\Delta\Delta\text{CT}}$$

$$\Delta\text{CT} = \text{CT (gene)} - \text{CT (housekeeping gene)}$$

$$\Delta\Delta\text{CT} = \text{dCT (treated)} - \text{dCT (control)}$$

CT gene: cycles threshold value of each gene

CT housekeeping gene : cycle threshold value of reference gene

ΔCT treat : studied specimen gene

ΔCT control : control specimen gene

Folding : the inverted value of CT represented by 2 raised to the negative value of $\Delta\Delta\text{CT}$ (Livak and Schmittgen, 2001).

All practical part related to paragraph 3.6 was conducted in the private lab.

3.7. Statistical Analysis.

One of the objectives of this study was not to compare patients in terms of age or sex , nor to compare one medical sample with another, let alone compare one technical evidence to another. Rather, it was an applied and survey study, so there was no data or need to conduct statistical analysis (Lee, 2022).

Chapter Four

Results and Discussion

Isolation and identification were carried out at the Ibn Senna Teaching Hospital for the period from March 2023 to September 2023, Out of 245 urine specimens, 200 specimens were grown on general and selective media (Blood agar, MacConkey agar and cetrimide agar). As mentioned in paragraph (3.2.6), Colonies morphology, microscopic examination, cells staining with Gram's stain and biochemical tests were done Table (4-1). Gram-negative bacteria (G-ve) accounted for 39% of the 200 culture growths, whereas Gram-positive bacteria (G+ve) accounted for 42% and One *Candida* isolate were removed based on Gram's stain, leaving just the G-ve. The findings revealed that 8 isolates (4%) were recognised as *P. aeruginosa* from 200 positive growth cultures using the assays specified by the Health Protection Agency UK (2015), as shown in Figure (4-1). These results are consistent with (Farhadi and Bahador , 2018; AL-Khikani and Ayit , 2019). In a local study in the city of Mosul that was conducted by (Al-obadi , 2022), the results of his study were that (G +ve) more than (G -ve) that consistent with our study. While *P. aeruginosa* constituted of 2% of the total bacterial growth which is less than what we obtained. This is perhaps due to the fact that the number of samples in the (Al-obadi , 2022) study about half of the samples of the current study.

Table (4-1): General index for Identification of *Pseudomonas aeruginosa*

No.	Test	Result
1	Blood hemolysis on blood agar	β- Hemolysis
2	Lactose fermentation on MacConkey agar	Negative
3	Blue- greenish (Pyocynine) on cetrimide agar	Positive
4	Gram 's stain	Negative
5	Catalase	Positive
6	Oxidase	Positive
7	API	Cod. 22020..

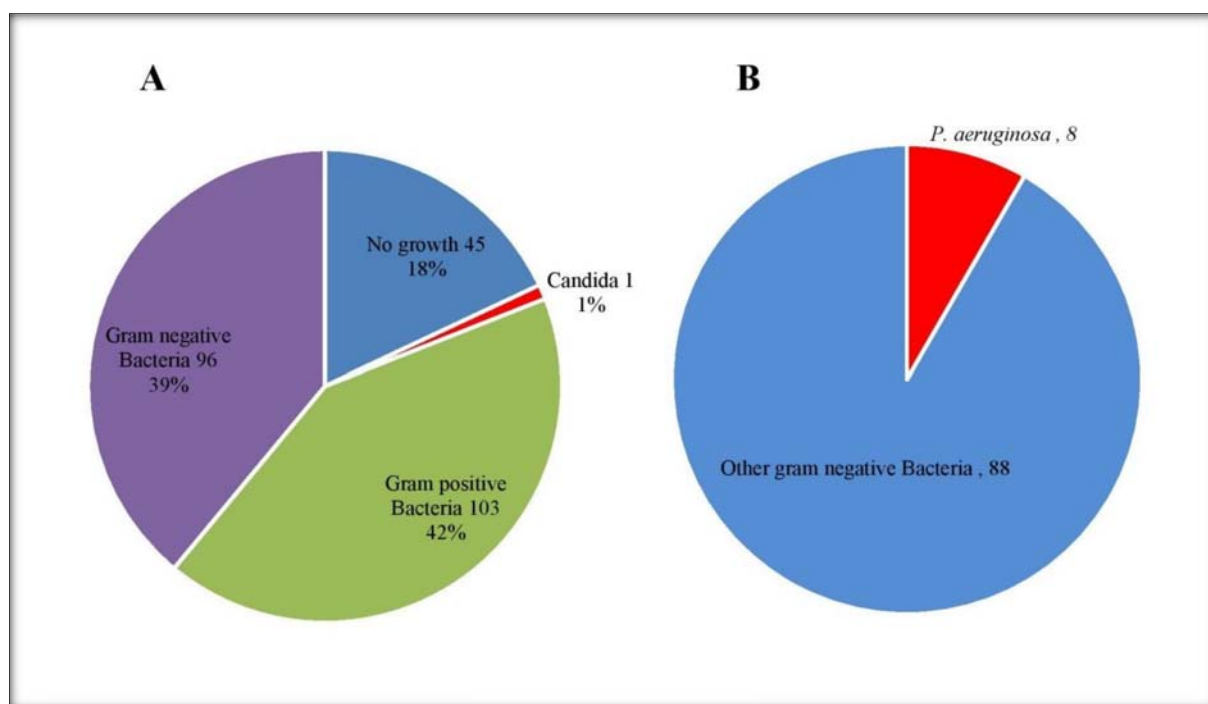


Figure (4-1): Culture Results (A) Culture Results and Percentage,(B) *P. aeruginosa* isolation Number.

The morphology of *P. aeruginosa* colonies for all 8 isolates were surrounded by a clear zone of β -hemolysis on blood agar. Non lactose - fermenting with pale yellowish color on MacConkey agar which considered as a selective inhibited the most G+ve bacteria allow to grow G-ve bacteria also it helped in the differentiation between lactose fermenting G-ve and non-lactose ferment G-ve bacteria and producing pyocyanin pigment in cetrimide agar as a specific medium for *P. aeruginosa* bacterium that recognized. This species from other *Pseudomonas* spp., these pigments produced by *P. aeruginosa* conduct two roles. First, their role in pathogenicity as virulence factors second, they are considered as a significant factors among the diagnostic traits in the genus of *Pseudomonas* (Macin and Akyon, 2017). These culture results are consistent with (Al-kazrage,2021) and more obvious in the Appendix(4-1).

Also the study which conducted by (Al-kazrage,2021) was consistent with our study. All 8 isolates were +ve oxidase by developing a violet color due to the activity of the cytochrome C enzyme. Also, these isolates were positive for catalase test by producing bubbles over the colonies when treated with H_2O_2 solution because of the activity of the catalase enzyme which leads to the decomposition of H_2O_2 into H_2O and O_2 . Microscopically examination was carried out for selected isolates.They were G-ve bacteria. Also they appeared as straight or rods shapes. These results were agreement with (Chevalier *et al.*, 2017).

The API 20E was done according to the instructions of BioMerieux. All positive and negative test results were compiled to obtain a profile number, which is then compared with profile numbers in a commercial codebook (or online) to determine the identification of the bacterial species. All 8 isolates of *P. aeruginosa* gave the same result by using API 20E diagnostic kit. According to current study result of API agreed with a previous study in Wasit, Iraq conducted by (Al-Saeedi and Raheema,2019). The results were further clarified through the Figure in the Appendix(4-2).

4.2. Molecular Identification of *P. aeruginosa*

To know the ability of the bacteria under study to produce quorum sensing, it was necessary to confirm that the bacteria were genetically diagnosed as *P. aeruginosa* by detecting universal gene (16S rRNA). Recently, 16S rRNA gene sequences of known QS species was obtained from NCBI in comparison with new QS dataset to determine bacteria ability to produce QS (Liu *et al.*, 2024).

According to work in paragraph (3.2.7), amplification of the 16SrRNA gene from the genomic DNA isolated directly from bacterial growth on cetrinide agar. Molecular identification analysis showed positive results for *P. aeruginosa* by using specific primers with an amplified size of 1465 bp as in Figure (4-2) via the investigation of DNA segment multiplication of the gene and performance of agarose gel electrophoresis showed single band for each type of bacteria indicating the amplification of the target gene (Khan *et al.*, 2023).

The 16SrRNA sequencing in the current study showed as new four strains recorded in NCBI, Coded (AhmHiy) Accession No. (OR186518, OR186519, OR186520 and OR186521) Appendix(4-4 A,B,C,D). The 16SrRNA gene plays an important role in the accurate diagnosis of bacterial isolates and discovery of new strains in the field of microbiology as its sequences Molecular diagnosis was performed by PCR technique depending on 16SrRNA gene via the investigation of the results of DNA segment multiplication of the gene and performance of agarose gel electrophoresis. Electrophoresis results of the 16SrRNA gene showed a band indicating the amplification of the target gene (Khaleel *et al.*, 2023).

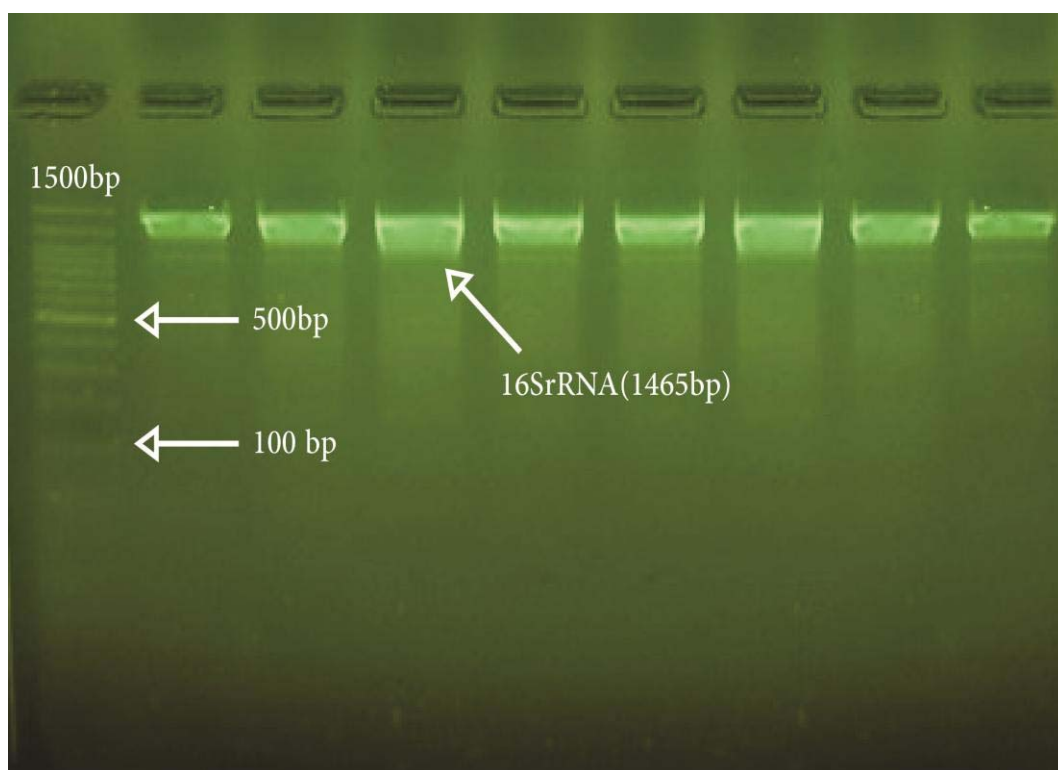


Figure (4-2):Gel electrophoresis of *P. aeruginosa* 16S rRNA gene(1465bp), 100bp DNA ladder, gel electrophoresis was performed by using 2% agarose gel, and the run lasted for 50 min /100V.

4.3. QS Production Detection

The QS molecules produced by *P. aeruginosa*, like many other bacteria, these chemical signals have a functional communication between cells (Hossain and German, 2021). QS produced by *P. aeruginosa* has been known of their controversial effect as an immune modulator (Kariminik *et al.*.,2017).

Many studies have been presented especially in the city of Mosul, Which was one of the first studies about QS, such (AL-Taee, 2010) highlighted the virulence factors related to quorum sensing ,other study (AL-Juboury,2011) focused about production QS by *P. aeruginosa* throw used Biosensor bacteria. In the current study used two ways to detect QS production.

4.3.1. Phenotypic Detection.

As mentioned in paragraph (3.3.1), we tested the ability of *P. aeruginosa* isolates to form and produce QS molecules.

4.3.1.1. Hemolysis Enzyme Production.

All 8 isolates of *P. aeruginosa* were positive for hemolysin enzyme. Colony surrounded by a clear zone of β -hemolysis on blood agar as shown in Appendix(4-1). These results were similar to study done by (Al-kazrage,2021).

The hemolysis enzyme in *P. aeruginosa* plays a significant role in its virulence by contributing to its pathogenicity. This enzyme is regulated by QS systems, specifically by the QS regulatory sRNA AmiL, which influences diverse QS-mediated virulence factors, including hemolysis activity (Pu *et al.*,2022) . In a study conducted by (Sonbol *et al.* ,2022), PCR analysis revealed lacked QS genes, then QS deficient isolates produced less than 50% hemolysis. This gives indication that QS deficient isolates produce hemolysin less than non-QS deficient isolates. These results stand as evidence that hemolysin regulate by QS.

4.3.1.2. Antibacterial Resistance.

All the isolates of *P. aeruginosa* were subjected to antimicrobial susceptibility testing as we showed in paragraph (3.3.1 no. 2) .The highest resistance was found against Ceftriaxone (100 %), while the highest sensitivity was appeared against Meropenem (88%). All the isolates, MDR *P. aeruginosa* against all antibiotics which used in the current study as shown in Table (4-2) and Appendix(4-3).

Table (4-2): Antibiotic susceptibility test of *P. aeruginosa* isolates

NO. bacterial strain	9	23	25	47	72	101	102	170	S %	R %	MS %
Antibiotic											
Amikacin	MS	MS	S	MS	R	R	R	MS	12	38	50
Gentamicin	MS	S	R	R	S	R	R	MS	25	50	25
Meropenem	S	S	S	S	S	S	R	S	88	12	0
Levofloxacin	R	S	S	S	S	S	R	S	75	25	0
Norfloxacin	R	S	S	S	S	S	R	S	75	25	0
Ciprofloxacin	R	S	S	S	S	S	R	S	75	25	0
Ceftazidime	R	R	R	R	R	R	R	S	12	88	0
Piperacillin	MS	S	R	R	R	R	R	S	38	50	12
Ceftriaxone	R	R	R	R	R	R	R	R	0	100	0

S : Sensitive MS : Moderate Sensitive R: Resistance

In the current study, *P. aeruginosa* isolates were described to be multiple antibiotics resistant. The QS system regulates various cellular processes which mainly involve the regulation of virulence factors, such as biofilm formation and drug resistance (Naga *et al.* , 2023). *P. aeruginosa* is one of the most important bacterial pathogens which critically contributing in the hospital infection diseases that requires an urgent attention (Kassob and Hummadi ,2023). The high antimicrobial resistance of *P. aeruginosa* may stem from indiscriminate use of these antibiotics. The development of resistance is attributed to the use of sub-therapeutic antibiotic doses which contributed to the emergence of mutant isolates, the high prevalence resistance to antibacterial drugs, was obviously observed with ceftriaxone to reach 100% (Kassob and Hummadi ,2023). The present research reveals a high compatibility with the recent study that reported 100 % resistance to ceftriaxone. More than published paper simultaneously reported the same findings to give an evidence about the increasing of bacterial resistance to antibiotics (Abbas *et al.* ,2022). In the United States, 10–30% of *P.*

aeruginosa isolates were carbapenem-resistant (Tenover *et al.* , 2022). This percentage was relatively close to our results showing 12% resistance rate to meropenem which significantly reflected on the meropenem sensitivity to reach the high percentage 88%.

To compare with the previous years, it was found that meropenem sensitivity is higher than today as a consequence of the same reasons which led to high resistance to ceftriaxone with somewhat of a lower rate. It was observed that Meropenem consumption is less common among individuals than other antibiotics as a result of random use. The continuous increase in *P. aeruginosa* resistance to meropenem over time was recorded by (Stultz *etal.*,2019), which indicated that using of meropenem over 10 years where the study showed that the rate of meropenem sensitivity at first use in 2008 was more than 90%, to gradually increase to 98% by the year 2011. However, this sensitivity was slightly decreased to 88% in 2017. The rest of used antibiotics in our work were distributed between the highest and least efficacy.

4.3.1.3. Biofilm Formation.

Biofilms formed by adherent cells on cellular or inert substrata , are tangled in 60% of all infections that are characterized by symptoms of moderate severity, chronic development and antimicrobial resistance (DiPasquale *et al.*,2019) Previous reports depicted that biofilm formation is controlled by QS(Pena *et al.* ,2019) *P. aeruginosa* QS is used to initiate growth and produce biofilms(De Kievit *et al.* ,2001). Another study indicated that the amount of biofilm formation that was produced by *P. aeruginosa* QS deficient strains was lower than that of the non-QS deficient strains (Sonbol *et al.* ,2022). Based on the above, the ability of bacteria to form biofilm was used as a detector of the ability of the same bacteria to produce QS. The biofilm formation in our study was determined in eight isolates of *P. aeruginosa* by three methods TCPM, TM and CRA according paragraph (3.3.1 no. 3). By comparing the absorbance with the table (3-5) the results of the TCPM were Strong formation 75% and

Moderate formation 25%, while, TM appeared that Strong formation 50% , Moderate 37% . One isolate showed no detecting of biofilm formation by this method. CRA was not beneficial to detect the biofilm in all isolates as shown in Table (4-3).

Table (4-3): Percentage of Biofilm Formation by Different Methods

Biofilm formation	TCPM	TM	CRA
Strong	6 (75 %)	4 (50 %)	0 (0 %)
Moderate	2 (25%)	3 (37 %)	0 (0 %)
Non/weak	0(0 %)	1 (13%)	8 (100 %)
Total	8 (100 %)	8 (100 %)	8 (100 %)

It was noticed that TCPM method consider efficient to determine the highest rate of biofilm detection, less that in TM method, while the CRA was disabled to show any response of biofilm detection. These findings were identical to the results obtained by previous studies (Halim *et al* ., 2018; Mohsenzadeh *et al.*, 2021). On the contrary, our results were incompatible with a study conducted by (Sultan and Nabel,2019), where TM showed highest rate of biofilm formation by considering TCPM as the gold standard method to detect biofilm formation, whereas, CRA method was reported to be less efficient to produce a little correlation than other method for biofilm detection (Siddhiqui *et al.*, 2018). In our study, the CRA method was not able to detect biofilm producers to present no signal (0 %)perhaps due to the small number of bacteria examined. In addition, some studies used this method to detect the slim layer(Salih and AL-Rawi,2023) where this result comes consistent with the record (Halim *et al* ., 2018). The biofilm detection rate based on the CRA method was 1.3%, This is a slight percentage in comparison to the number tested of bacterial isolates which was 111.

Differences in biofilm development compared to other research are due to variations in culture medium components employed in biofilm formation tests (e.g., TSA or LB medium). The number of bacterial cells in the culture varies, as does the duration of the incubation period. Furthermore, the cell density in biofilm rises with the duration of the incubation period (Kassob and Hummadi, 2023). The concentration of dye employed as an indicator in the test might influence the outcome differences, with 5% (w/v) of dye producing better results than 1%. The results may be affected by the type of microplate used in the assay. It was approved that a 96-microplate made of polystyrene is more efficient in biofilm sticking than a microplate manufactured using other materials such as glass, steel, or granite (Kassob and Hummadi ,2023).

4.3.1.4. Pyocyanin Production.

Eight isolates of *P. aeruginosa* were subjected to pyocyanin production screening as we showed in paragraph (3.3.1 no. 4). All of these isolates were positive for their capacity to create pyocyanin at varied intensities, as demonstrated by comparing the colour intensity produced by pyocyanin dye to the control (culture broth containing bacteria that did not make pyocyanin) using an ELISA device as a reader only. The amount of pyocyanin produced by *P. aeruginosa* QS deficient isolates was less than the isolates that possess QS genes (Sonbol *et al.* ,2022). Las and Rhl are two interconnected QS systems that control production of pyocyanin (Girard and Bloemberg , 2008). Based on the above, we used pyocyanin production as a detector of the ability to produce QS. Result of our study is correspond with local study conducted by (Kassob and Hummadi,2023). In the same time the current study did not agree with the results obtained by (Shouman *et al.* , 2023), and this may be due to the large number of isolates they used, in comparison with only eight isolates in our study or some of the isolates which used in their studies don't have QS gene . The differences in the amounts of pyocyanin pigment production for our isolates due to the presence of regulators of the QS system named QteE. The overexpression of this

regulator will reduce the accumulation of homoserine lactone signals affecting the production of pyocyanin (El-Fouly *et al* .,2015).

4.3.1.5. Inhibition of Biofilm Formation and Pyocyanin Production.

Biofilm formation and pyocyanin production were inhibited using three materials: vit C, SA, and Multi Serra, as described in paragraph (3.3.3.1), where we obtained several values for the absorbance intensity compared to the absorbance value of the control (culture broth untreated with vit C, SA, and Multi Serra), as shown in table (4-4), and then calculated using the equation (%) Inhibition = $[(AC - AS) * 100] / AC$, the percentage of inhibitory capability for each of the three compounds utilised was known, along with their respective concentrations. The findings of Vit C demonstrated the largest inhibitory impact compared to the other components, as shown in Figure (4-3). Inhibition biofilm formation by vit C, SA and Multi sera were implemented (76, 37, 28)% respectively.

Table (4-4): Absorbance values for biofilm formation inhibitor materials

Concentration materials	Control Ab	Vit C Ab	SA Ab	Multi sera Ab
50	0.409	0.327	0.400	0.708
100		0.169	0.319	0.547
150		0.100	0.256	0.294

Ab = Absorbance

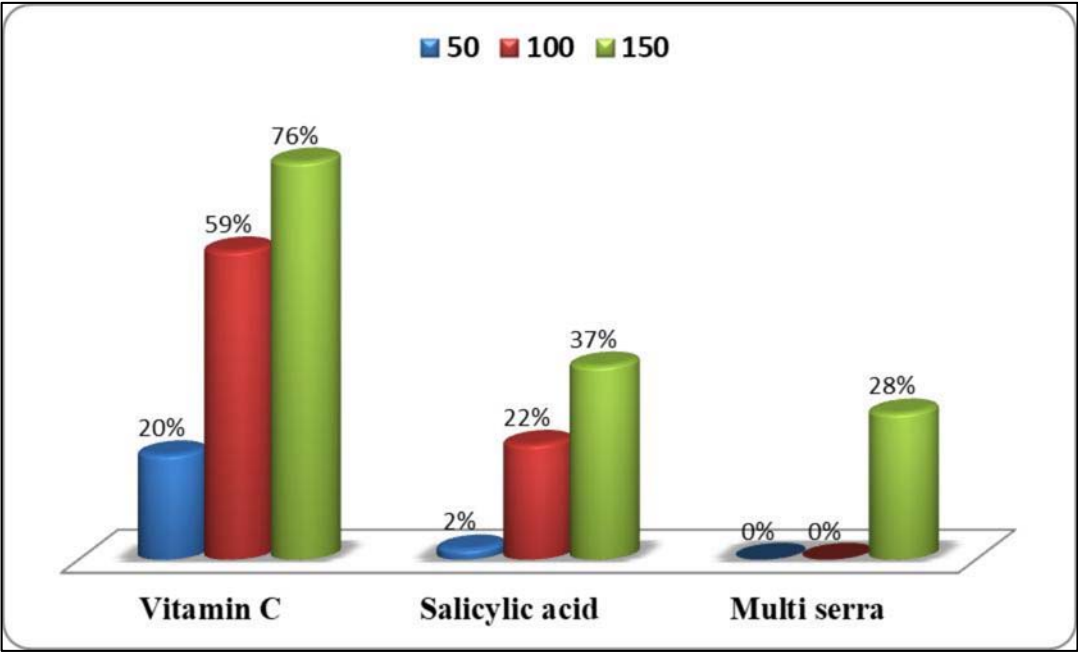


Figure (4-3) : Biofilm formation inhibition by three different materials

The results' findings SA had the greatest inhibitory effect on pyocyanin production compared to the other components. We obtained several values for the absorbance intensity compared to the absorbance value of the control (culture broth untreated with vit C, SA, and Multi Serra), as shown in tables (4-5), and then calculated using the equation $(\%) \text{Inhibition} = [(AC - AS) * 100] / AC$, the percentage of inhibitory capacity for each of the three chemicals used was known, as were their concentrations. Inhibition of pyocynin synthesis SA inhibited pyocyanin production by 77%, whereas MultiSera and Vit C produced (56 and 38%) respectively, as indicated in Figure (4-4).

Table (4-5): Absorbance values for pyocynin production inhibitor materials

Concentration materials	Control Ab	Vit C Ab	SA Ab	Multi sera Ab
50	0.34	0.23	0.19	0.19
100		0.23	0.13	0.17
150		0.21	0.08	0.15

Ab = Absorbance

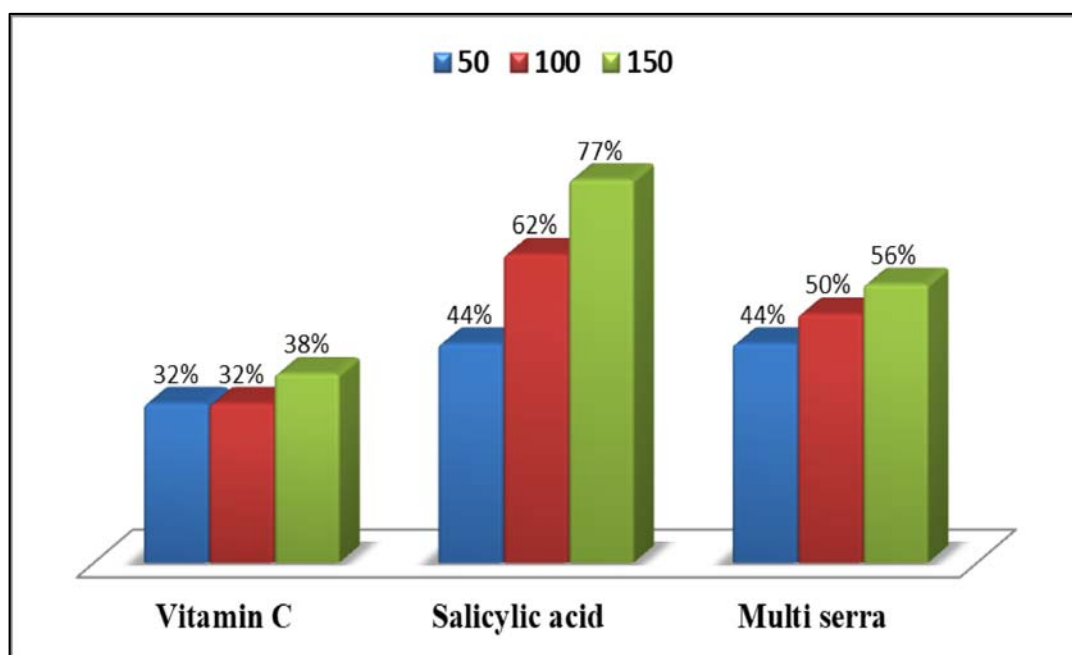


Figure (4-4): Pyocyanin production inhibition by three different materials

The vit C is a reducing agent that rapidly undergoes two consecutive one-electron donation processes forming the ascorbate radical and dehydroascorbic acid (Padayatty and Levine ,2016). The low concentrations of vit C inhibited the expression of several proteins involved directly in the synthesis and export of extracellular polysaccharides. Inhibiting EPS production is a viable strategy for fighting bacterial pathogens. Vit C at low concentrations can be used to disrupt bacterial biofilm formation by inhibiting the EPS production (Pandit *et al.*,2017) . The current study also proved this on the other hand, as the inhibitory effect of vit C on the biofilm was greater than that of pyocyanin when using the same concentrations .This due to that vit C antioxidant properties enable it to interact with pyocyanin causing structural modifications that disrupt biofilm formation and reduce bacterial virulence (Das *et al .*, 2023).

In *P. aeruginosa*, population-dependent QS is activated through the secretion of AHLs and binding of AHLs to regulatory receptors, initially, LasR and RhlA to promote further biosynthesis of LasR, RhlA and PqsR receptors. They motivate the complete QS system activating virulence factor production and biofilm formation (Lee and Zhang, 2015). The structure of

ascorbic acid (five-membered lactone ring) is analogous to AHLs which leads to competition in binding to the AHL receptor(s) and inhibition of the QS system (Han *et al.*.,2020). This leads to inhibition of Pyocyanin production and biofilm formation. This was proven in the current study and the results are consistent with other studies (Pandit *et al.*,2017; Das *et al.*., 2023).

Secondly, SA is the major phenolic acid plant product. It affects the motility and reduces other virulence factors in *P. aeruginosa* (Da *et al.*, 2010). SA can reduce biofilm formation and Pyocyanin production in *P. aeruginosa*. SA suppressed the expression of genes associated with the *las/rhl* QS homoserine lactone-based signaling system in *P. aeruginosa* (Gerner *et al.*,2020). Our obtained result reported in Figure (4-3 and 4-4) show that SA reduced the biofilm formation in *P. aeruginosa* in comparison with the untreated control. Also the effect of SA on pyocyanin production was tested where the results showed a decreasing in Pyocyanin production in comparison with the untreated control. These results were similar to the work of two studies (Aicha *et al.*.,2017; Kenoosh and Shehan, 2022). SA have the ability to kill microorganisms, but at certain concentrations, they can reduce virulence factors without affecting on bacterial growth (Kenoosh and Shehan, 2022). This was observed in the current study where the higher the concentration of salicylic acid, the greater the inhibition. In addition to that, the low concentration of SA was more inhibition to Pyocyanin production than biofilm formation. This result was identical to the study conducted by (Aicha *et al.*.,2017).

Finally, no biofilm inhibition occurred at 50 μ L and 100 μ L due to the low concentration of paraoxonase in this volume. However, it was found that at 150 μ L, showed an inhibition of biofilm formation 28% Figure (4-3). While in pyocyanin production the three-volume at (50, 100, and 150) μ L showed an inhibition at (44 %, 50 %, 56 %) respectively as in Figure (4-4). This provides an evidence that pyocyanin is more sensitive to the paraoxonase enzyme than biofilm. Our results were agreed with many studies which indicated that the

paraoxonase enzyme had an anti-virulence agent by reducing the biofilm formation and pyocyanin production (Aybey *et al.*,2016a) and (Aybey *et al.*,2016 b).The PON was originally described based on its ability to degrade organophosphates. PON enzymes also exhibited lactonase activities were capable of hydrolyzing and inactivating AHLs, used for the disruption of biofilm formation in bacterial pathogens (Khatun *et al.*.,2023).

Multi Sera (level 3) was used, which is a standard blood serum containing all the substances found in human serum at a high concentration especially HDL, because PON residing almost exclusively on HDL (Durrington *et al.*., 2023) . To prove this fact, we conducted a quantitative experiment proving the increased effectiveness of the PON enzyme in inhibiting it as the HDL concentration increased as shown in the Figure (4-5). This experiment showed that the effect of paraoxonase enzyme in inhibition biofilm formation and pyocyanin production is greater when the HDL value was greater al so.

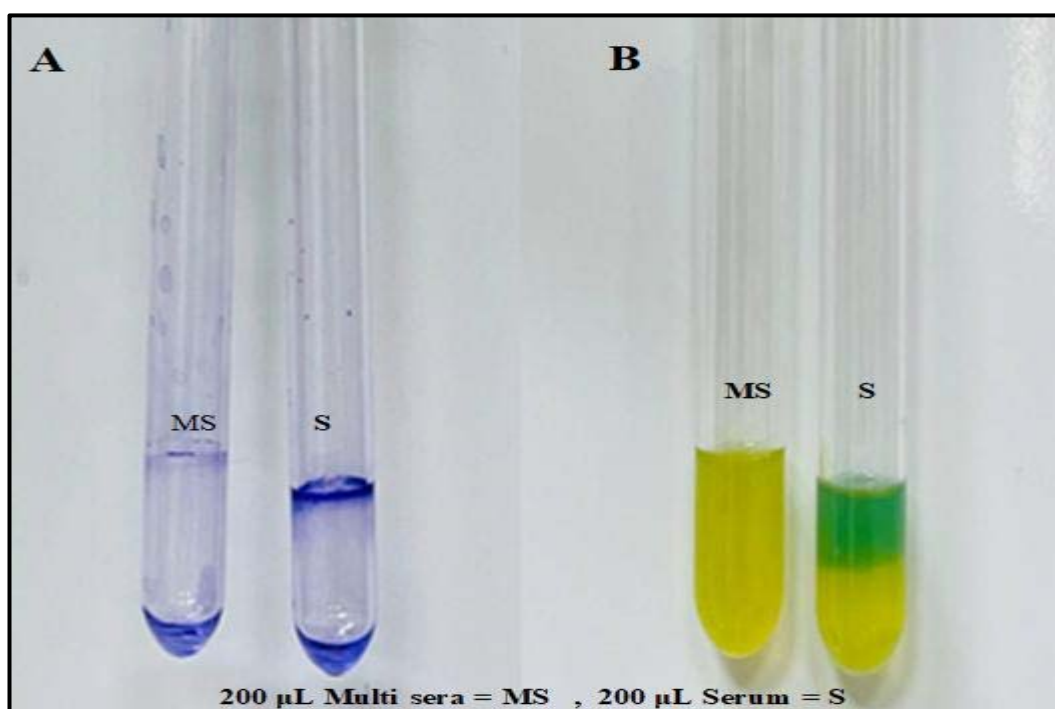


Figure (4-5): Relation between paraoxonase inhibition and HDL concentration (A) Biofilm formation inhibition (B) Pyocyanin production inhibition

According to what was presented, why we have selected these material because *P. aeruginosa* can be consider as specified as one of the opportunistic bacteria related to healthcare infections, blood stream infections, surgical site infections, UTI, burn wound infections, keratitis, and otitis media (Ito *et al.*,2021) . These materials can be used as safe drugs or nutritional supplements systemically or locally during treatment these infection in addition to being cheap that do not require manufacturing or lengthy validity tests.

4.3.2. Genotypic Detection.

4.3.2.1. Detection of QS genes (*lasI* and *lasR*).

Las System regulates *P. aeruginosa* QS network, also regulate biofilm formation and pyocyanin production, *las I* produces the signaling 3-oxo-C12-HSL (Ali *et al.*, 2017). When the signal concentration arrived above the threshold concentration, it interacts to the receptor protein LasR, creating a complex that promotes the expression of downstream target genes. (Maisuria *et al.*, 2016). The results of the current study agreed with (Lima *et al.*, 2018), which showed that all clinical isolates were biofilm produced and contained lasR, lasI and another QS genes. The QS Virulence factors gene (*lasI* and *lasR*) detection was done for two isolates one MDR and another full resistance (pan) same as in paragraph(3.3.2.2). Molecular identification analysis showed positive results for *lasI* and *lasR* by using specific primers with an amplified size of 311 bp and 378 bp respectively. Two isolates had lasI and lasR gens. The results of gel electrophoresis for amplification PCR product was elaborated in Figure (4-6).

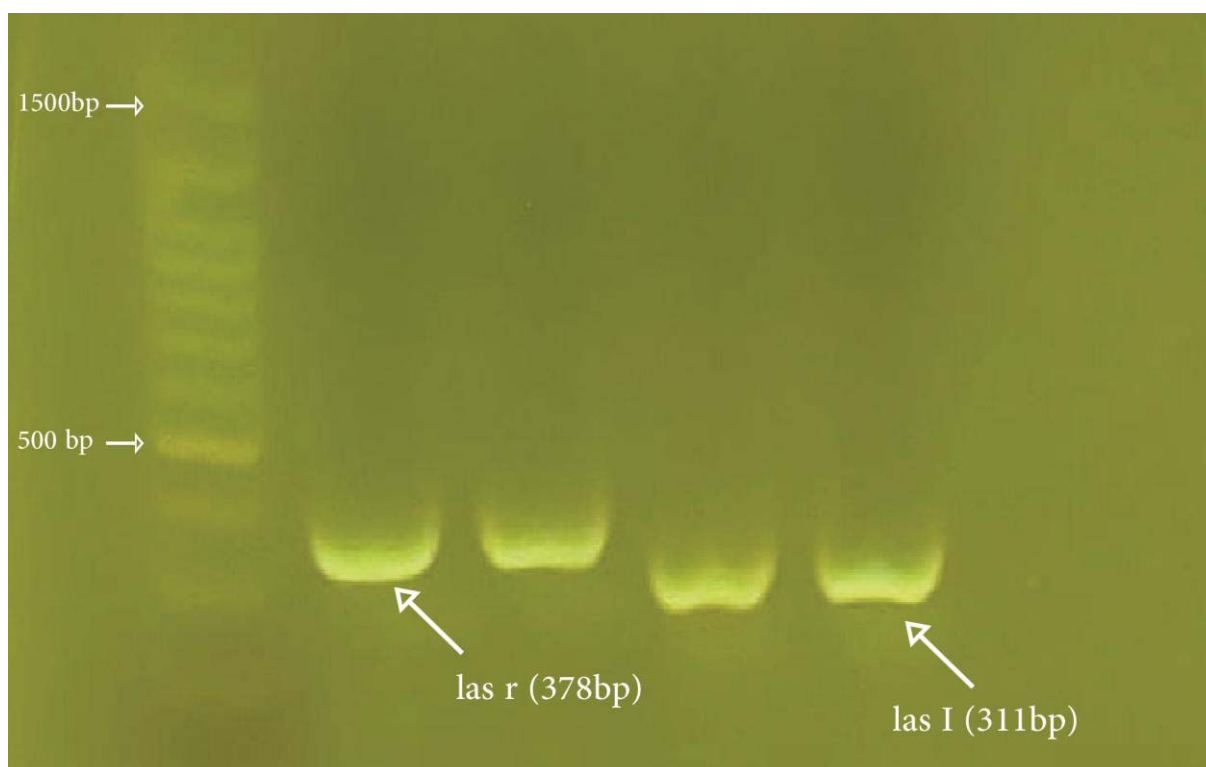


Figure (4-6): Gel electrophoresis of *P. aeruginosa* QS genes, *lasI* (311bp) and *lasR* (378bp), 100bp DNA ladder, gel electrophoresis was performed by using 2% agarose gel, and the run lasted for 50 min /100V.

4.4. QS crude Extraction .

The QS crude extraction was carried out using the method previously reported by (Vivero-Gomez *et al.*, 2021) as it was shown in paragraph (3.4). It is used the strain No, 102 which have ability to blood hemolysis, full resistance to all antibiotic that used in the current study, biofilm formation and pyocyanin production. In addition to possessing the QS Virulence factors gene (*lasI* and *lasR*) that were detected in current study. Bacterial growth was taken to a stationary phase. During the stationary phase, bacterial quorum sensing allows for graded and bimodal cellular responses to variations in population density, influencing gene expression and cooperative behaviors (Rattray *et al.*, 2022). QS in *P. aeruginosa* forms a bistable switch, regulating gene expression in response to cell density, impacting collective behaviors, including during the stationary phase (Schuster *et al.*, 2023). Through present study, we were able to extract QS for the first time locally as shown in Figure (4-7).

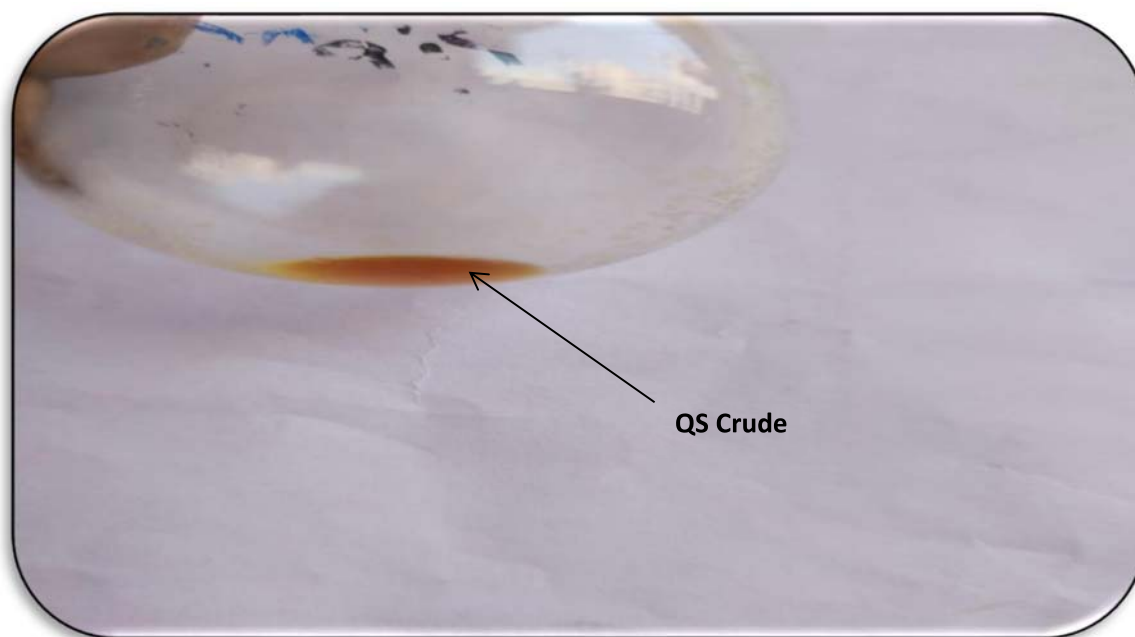


Figure (4-7): QS Extracted Crude

4.4.1. Confirmation of QS molecules presence in Crude Extraction.

The QS Crude extract had been examined to detect the presence of (AHL and pqs) according to paragraph (3.4.1.1), which was done by using FT-IR to identify functional groups are found in the mid-IR range ($4000 - 400 \text{ cm}^{-1}$). Hence the development of an absorption bands in this area may be utilized to assess whether or not particular functional groups were present in the QS molecule. The IR spectra of AHLs showed unique absorption peaks at 1650 , 1710 , and 1780 cm^{-1} due to the presence of the amide carbonyl, 3-OXO, and lactone ring respectively (Camara *et al.*, 1998; Taghadosi *et al.*, 2015). These results agreed with what we obtained in the current study as in Figure (4-8).

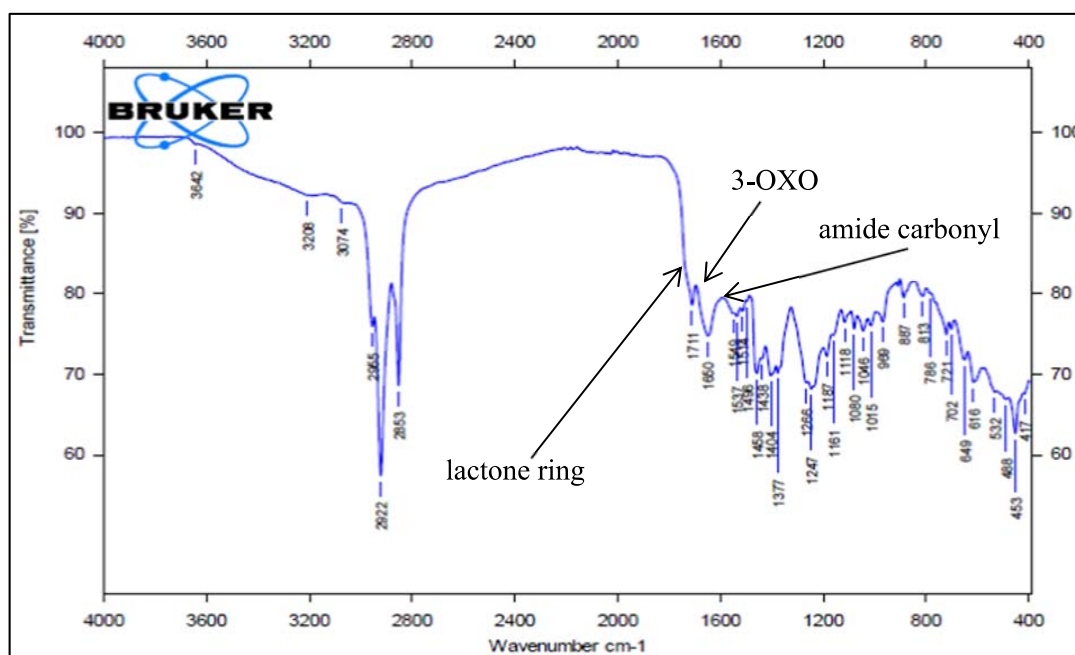
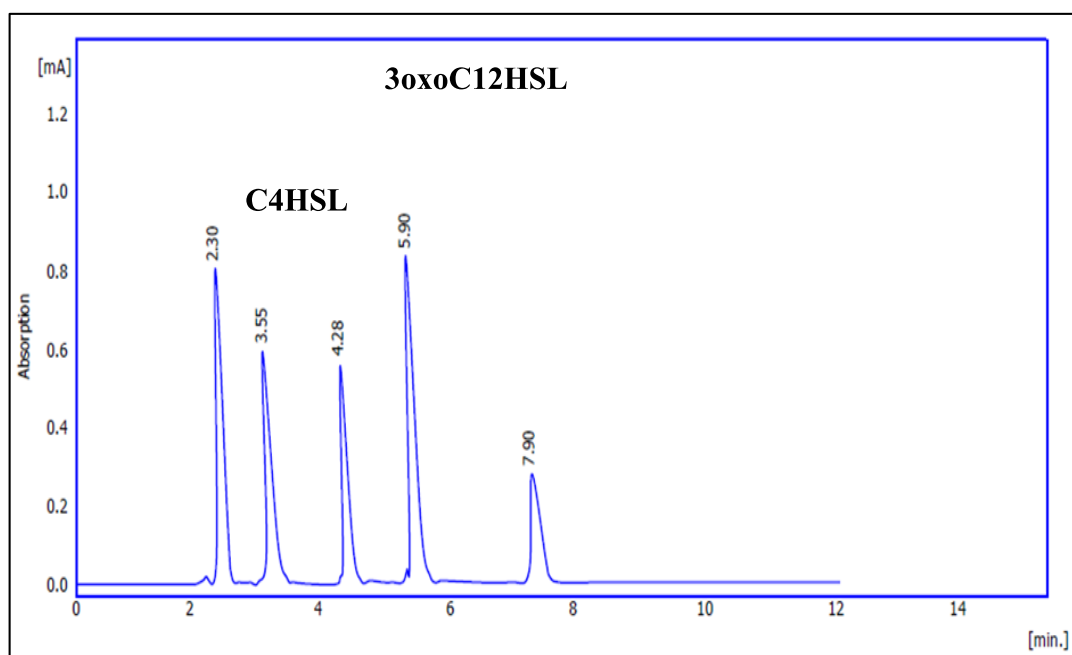


Figure (4-8): FT-IR spectra of QS functional groups in the QS crude extract.

Another confirmation was done by using the HPLC as it is shown in paragraph (3.4.1.2), which determined (Retention Time) for each type of QS molecules, then comparing HPLC results with several studies like (Teiber and Draganov, 2011; Laj *et al.*, 2022). It appeared that QS crude component from QS types (3oxoC12HSL and C4HSL) in the retention time 3.55 min and 5.9 min respectively as shown in Figure (4-9). The third type of QS which is pqs did not detected in the crude extract.

The reason for the absence of pqs in the QS crude extract may be due to the pqs that is known produced in the lungs of cystic fibrosis patients infected with *P. aeruginosa*, but it was not clear whether pqs was required for virulence (Kim *et al.*, 2010; Abdalla *et al.*, 2017) explained that may be detect pqs in sputum samples from cystic fibrosis patients infected with *P. aeruginosa*, and on the other hand pqs has an immune modulating activity that is different from that of AHL in cell proliferation and in the production of IL-2 and TNF- α (Hooi *et al.*, 2004).



AHL Signals (3oxoC12HSL and C4HSL)

Figure (4-9): HPLC chromatograms showing product peaks of QS crude extract

4.5. QS Immune Modulation Detection.

The QS in immune cells regulates population behavior and responses to pathogens potentially impacting immune reactions and the development of immunopathologies (Postat and Bousso,2019). QS regulates immune cell populations and responses by coordinating gene expression (Antonioli and Haskó ,2022). The role of QS in modulation the immune response was highlighted in the current study through cell culture experiments.

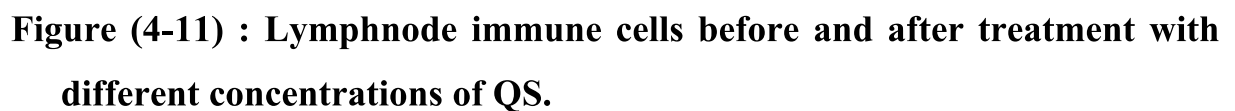
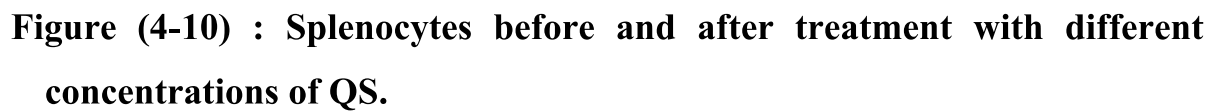
4.5.1. Immune Cell Culture.

Cell culture is defined as an isolation and processing the cells from their natural environment (*In vivo*) to a controlled artificial environment (Baust *et al.*,2017). Immune cell culture was prepared from lymphoid organs(Spleen, lymph node and bone marrow) taken from a laboratory animal (mice) according to work in paragraph(3.5.3). Why the mouse and no other laboratory animals. *Mouse mus* tissue has been found that the genetic mice identical with human genome with more than 85% (Chen *et al.*,2022).Mus musculus have well known

to be used to test the hypothesis and treatments related to understanding the mechanisms of diseases in human as the recent data of human genome project suggested a significant genetic homology between human and mice as that has been verified by the (ENCODE) as well as (NHGRI) revealed that a huge similarity in protein coding, non-protein coding as well as regulatory elements that control gene expression which was achieved by using a number of genomic approaches such as RNA seq , DNA seq as well as Chip seq (Rydell-Törmänen and Johnson, 2019).

4.5.2. The role of QS in immune response and inflammation related genes in different immune cells.

Gene expression was studied for five different markers including NFκB, IL-2, IL-6 , IL1β and TNFα, in addition to GAPDH which showed in paragraph (3.6,3.6.1 and 3.6.2). The housekeeping gene used as a source of normalization for the calculations. The results of our study indicated a sharp downregulation in the gene expression of the inflammatory cytokines NFκB, IL-2, IL-6, IL1-β and TNFα in different immune cells treated with different concentrations of QS crude extracted isolated from *P. aeruginosa* in comparison with the GAPDH (Control-untreated group) as listed below in Figure (4-10, 4-11 and 4-12) in details.



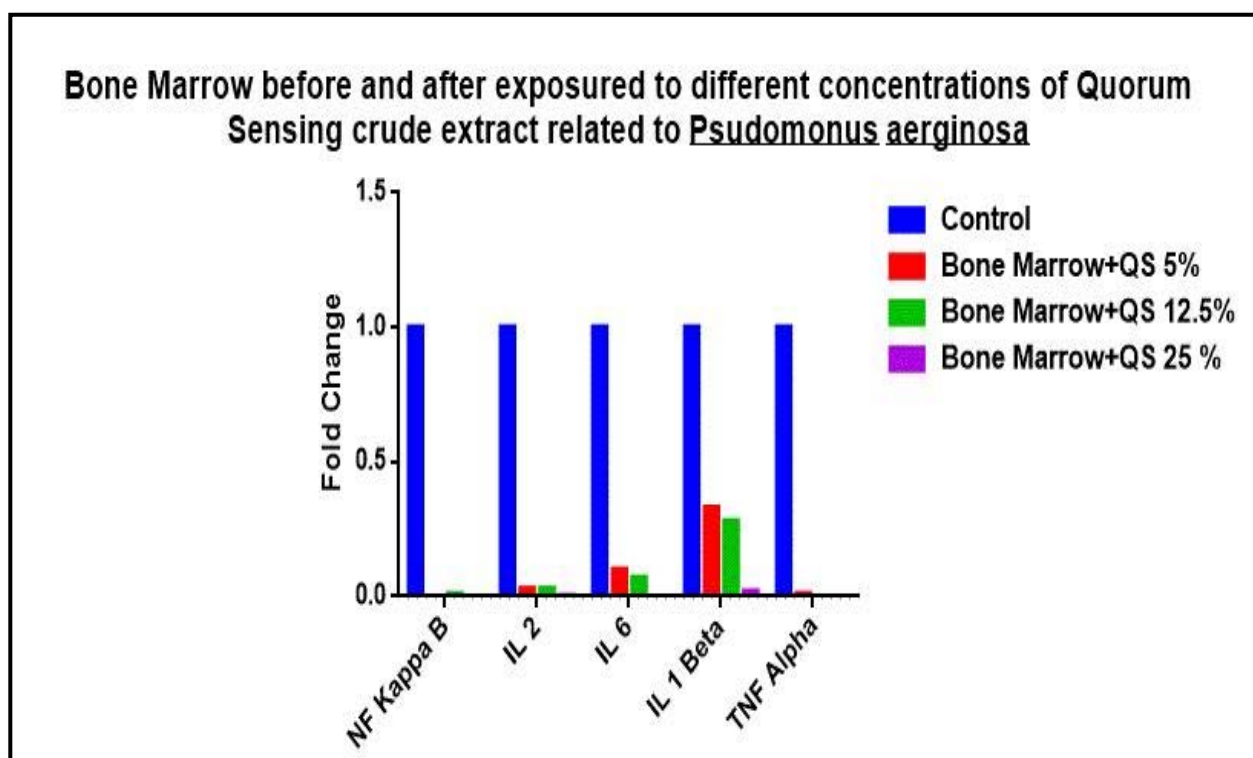


Figure (4-12) : Bone marrow immune cells before and after treatment with different concentrations of QS.

The results of our study in all Figures (4-10, 4-11) of spleen, lymph node indicated a huge downregulation in all of mRNA gene markers (NFKB, IL-2, IL-6, IL1 β and TNF α). On the other hand, our results showed that the downregulation was undetectable in the bone marrow immune cells Figure (4-12), which require further investigation in the future.

Interlingually, our study showed that downregulation of mRNA gene expression started at the lowest amount 5%, in all the three different organs then continued with a volume of QS crude of 12.5% and 25%. These results have been supported with another study that showed that quorum sensing related to *P. aeruginosa* modulated and suppressed the inflammatory response in mammalian cell culture (Kim *et al.*,2010).

While the modulation of immune response is mediated by the transcription factor NFKB by translocation to the nucleus where it binds to such specific target genes. Then regulates some transcription factors. Our results identified with (Kopp and Ghosh, 1995). This study have been suggested the role of QS

crude as it has been decreased the gene expression of NF κ B suggesting the inhibitory role of QS crude against inflammation.

Another biomarker, IL-2, which is on one side, has been responsible for the proliferation of immune cells (Ross and Cantrell ,2018).On the other hand,it has been found that IL2 in a combination with CD28 costimulatory signaling that both are responsible for the proliferation and activation of T cells. However, both these molecules were inhibited in human T cells in vitro after treatment with QS crude molecules (Hooi *et al* .,2004).

These results suggest the inhibitory role of QS crude is identified with the results of our study as we have found that the fold expression of mRNA IL-2 has been severely downregulated in QS crude treated group compared with control group (untreated group) in spleen and lymph node in (Figure 4-10) and (Figure 4-11) suggesting the inhibitory role of QS crude which is identified also in the previous study that we have mentioned previously. Another study which suggested the immune modulatory role of QS crude against IL-2 (Hooi *et al* .,2004).

IL-2 was also severely downregulated in the bone marrow immune cells compared with untreated group (Figure 4-12).Our results identified and supported with another study which found that QS plays such an important role in decreasing the IL-2 gene expression in bone marrow derived cells, while decreasing the T cell proliferation (Skindersoe *et al*.,2009).

IL6 is one of the interleukins which plays an important role in cell signaling of chronic inflammation and autoimmunity (Tanaka *et al*., 2014). At the same time, another inflammatory signals such as IL1 β , which is considered a potent inflammatory cytokine and a key mediator of inflammatory response (Lopez-Castejon and Brough ,2011). Along with that TNF α also is considered a pro inflammatory cytokine as well (van Loo and Bertrand ,2023).

All these studies suggested the inflammatory role of these cytokines. However, it has been well investigated that the immune suppressive role of QS

related to *P. aeruginosa*. The results of our study has been showed a significant decrease in the gene expression of the immune cell culture of splenocytes and lymphocytes and bone marrow in all of the three different concentration 5%, 12.5% and 25% in comparison with control group which these studies are totally supported. Previous studies also indicated the immune modulatory and anti-inflammatory role of QS in human endothelial cells which showed a sharp downregulation in the expression of the genes of IL-6, TNF α and IL1 β (Curutiu *et al.*,2018).

Our results indicated that there was such a significant decrease in the fold change of the gene expression of each inflammatory gene signaling including NFK B ,IL2, IL6, 1L 1 β and TNF α , as listed below in different treated concentrations including 5%, 12.5 % and 25 %, of QS. Even though the down regulation of the mRNA gene expression was at the concentration of 12.5% was less than 5% and 25 % respectively in all the genes of the three organs. They are spleen, lymph node and bone marrow which suggested further investigations and more mechanistic studies which may needed for this purpose in the future. Meanwhile, the downregulation overall for the inflammatory genes was with the highest concentration of 25 % of QS in compare with the control group. Our results identified with another study that have mentioned the potential immune modulatory of QS related to *Pseudomonas aeruginosa*.

The concentration of 25 % was the most suppressor dose that led to downregulation of NF-KB gene expression. This result was identified with results from other study suggesting that Nuclear Factor kappa B (NF- κ B) transcription factor regulates over 500 genes involved in inflammation, cell proliferation and apoptosis (Pancer *et al.*, 1999).

In general, the results of our study indicated that there were significant changes in the fold change of the gene expression which happened in 100 % of the genes of interest in our study specifically in the groups of the immune cells including Splenocytes, Lymphocytes and Bone Marrow immune cells treated

with QS compared with the control group. Thus suggested primarily that QS has a significant effect on gene expression in all cultured immune cells of different organs which may explain the future role of the QS as a target therapy for inflammatory diseases and tremendous immune modulatory and suppressor role of QS crude against the inflammation and the pretreatment with QS as a preventive immune supportive natural compounds may prevent possible inflammation and infection in the future.

In UTI cases, in particular, Demirel *et al*, (2018) referred to the IL-1 β as among the first cytokines to be described in urine samples following infection, suggesting early host activation of inflammasomes during UTI. The studies considered IL-1 β as a major cytokines during UTI infection .

The immune modulation in study conducted by(AL-Ghazal and Musto , 2019) was up regulation for cytokines gene expression in patient with Rota virus infection from these cytokines are IL-1 β ,TNF α and NF- κ B. Same genes showed downregulation in our study . Al so in a recent study by Dinatale *et al*, (2022), they mentioned that IL-1 β supports disease progression of cancer in general including colon cancer by inducing epithelial–mesenchymal transition and promotes the emergence of a cancer stem cell phenotype. It has been found that the tumor cells of preclinical models of pancreatic cancer was expressing IL1 β promoting the tumor microenvironment.The current study showed that downregulation in the gene expression of the inflammatory cytokine IL1 β in all immune cells prepared from the three organs compared with the GAPDH (Control-untreated group). It is possible to benefit from this inhibition cytokine IL-1 β production by using QS crude extract in the future for cancer treatment.

Conclusions & Recommendations

Conclusions and Recommendations

Conclusions

The study has come up with the following conclusions :

- 1) *P. aeruginosa* is one of the causes of UTI which isolated in our study.
- 2) New strain of *P. aeruginosa* in NCBI has been recorded.
- 3) Ability of these isolates to produce QS molecules, by using phenotypic and genotypic detection.
- 4) All isolates were multi drug resistance.
- 5) The study revealed possibility of inhibition QS by using vit C , SA and multi Serra.
- 6) Vit C appeared more effective on inhibition biofilm formation than SA and multi Serra, while in pyocyanin production it was the opposite.
- 7) As the amount of HDL in the blood serum increases, the amount of the paraoxnase enzyme also increases. Then effectiveness of QS decreases.
- 8) Successful isolation and identification QS crude, which contain (3oxo C12HSL and C4HSL).
- 9) The study revealed the role of QS crude in immune modulation through downregulation of pro-inflammatory cytokines gene expression, possibility using the QS crude extract to treat the pathological effects of pro-inflammatory cytokines, such as the damage resulting from the cytokine storm and the cause of some types of cancer.

Recommendations

From our study we recommend that:

- 1) Conducting similar studies on bacteria *P. aeruginosa* isolated from medical samples other than urine and making a comparison with the current study.
- 2) Studying the synergistic effect between vit C , SA and multi Serra .with antibiotics used in our study.
- 3) Conducting study on inhibition action of vit C , SA and multi Serra on inhibition biofilm formation and pyocyanin production at the genetic level.
- 4) Recommending the separation of the two types of HSL signals (3oxo C12HSL and C4HSL) and studying the immune response to each one independently.
- 5) An in-depth study of second concentration of QS crude treatment and finding out the reasons for its incompatibility with the rest of the concentrations.
- 6) Using the QS crude extract to treat the pathological effects of pro-inflammatory cytokines in *vivo*.

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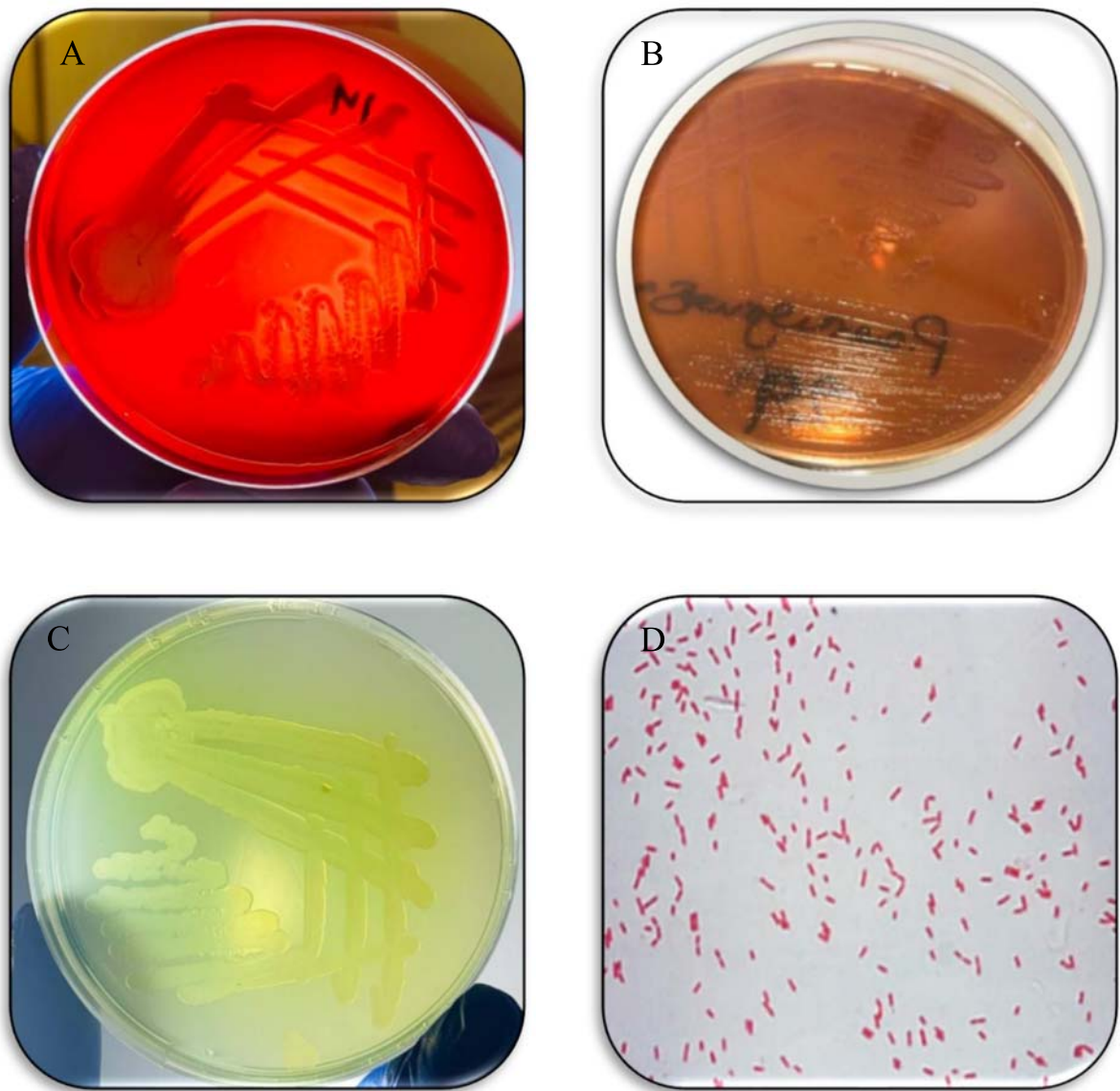
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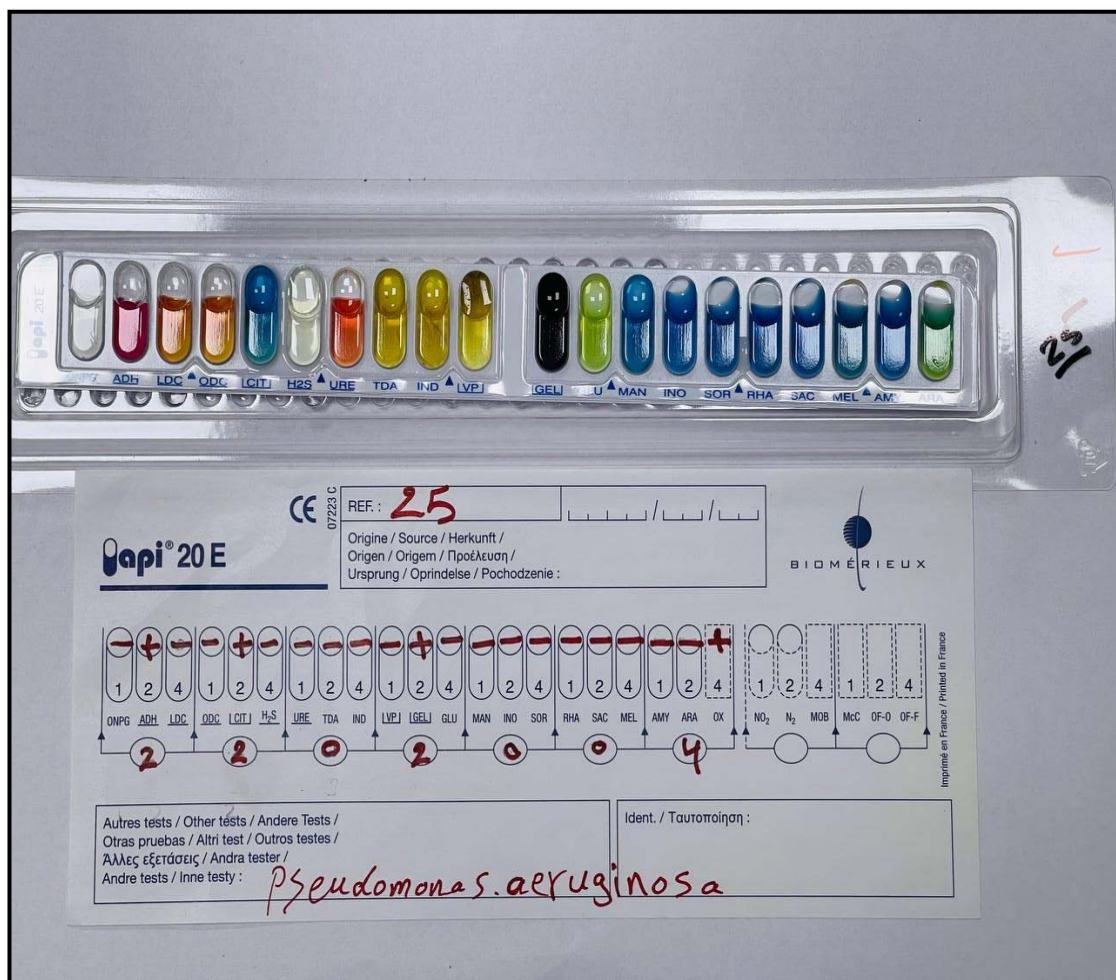
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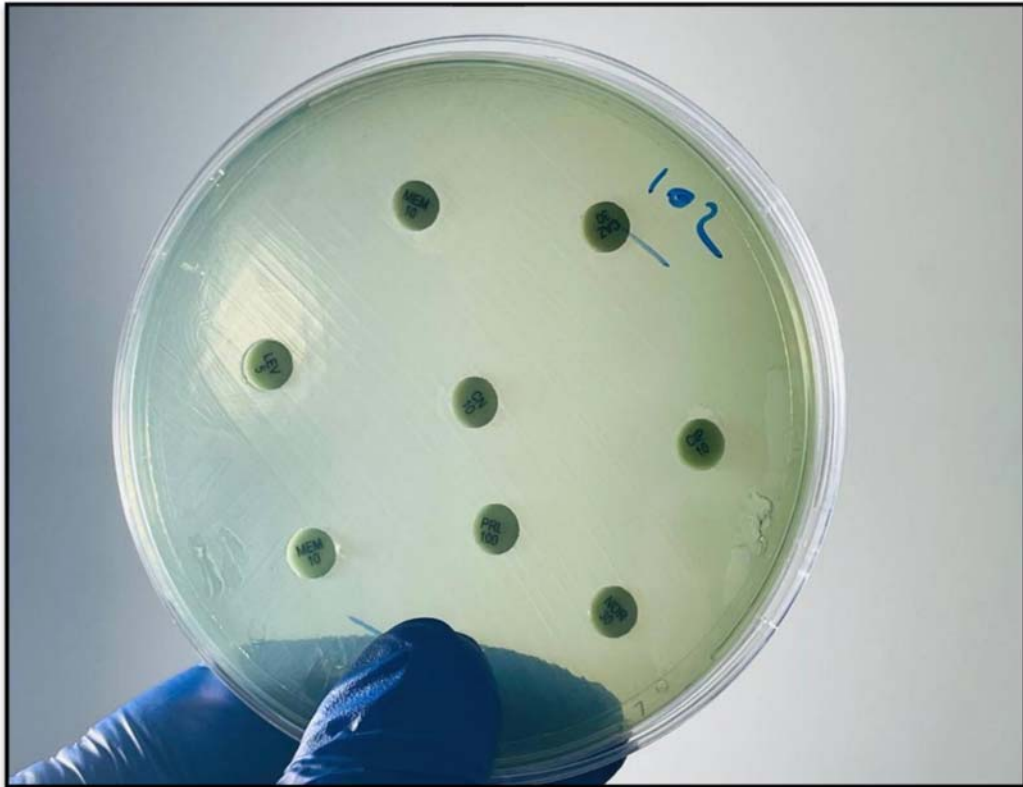
Appendices



Appendix (4-1) : *P. aeruginosa*. (A) Growth on Blood agar (B) Growth on MacConkey agar (C) Growth on Cetrinide agar (D) Gram Stain.



Appendix (4-2) : API for *P. aeruginosa*



Appendix (4-3): *P. aeruginosa* drug full resistance (pan).


National Library of Medicine
National Center for Biotechnology Information

[GenBank](#)

Pseudomonas aeruginosa strain AhmHiy(12) 16S ribosomal RNA gene, partial sequence

GenBank: OR186518.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS OR186518 1059 bp DNA linear BCT 30-JUN-2023
DEFINITION Pseudomonas aeruginosa strain AhmHiy(12) 16S ribosomal RNA gene, partial sequence.
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VERSION OR186518.1
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ORGANISM [Pseudomonas aeruginosa](#)
Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 1059)
AUTHORS ALShaar, A.Y. and ALtaii, H.A.
TITLE Direct Submission
JOURNAL Submitted (25-JUN-2023) Biology Department, Mosul University
/College of Science - Nineveh Health Directorate, Al Majmoua,
Mosul, Nineveh 41001, Iraq
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
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Appendix (4-4A): Pseudomonas aeruginosa strain AhmHiy (OR186518)


National Library of Medicine
National Center for Biotechnology Information

GenBank

Pseudomonas aeruginosa strain AhmHiy(14) 16S ribosomal RNA gene, partial sequence

GenBank: OR186519.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS OR186519 1040 bp DNA linear BCT 30-JUN-2023
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ACCESSION OR186519
VERSION OR186519.1
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SOURCE Pseudomonas aeruginosa
ORGANISM [Pseudomonas aeruginosa](#)
Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 1040)
AUTHORS ALShaar,A.Y. and ALtail,H.A.
TITLE Direct Submission
JOURNAL Submitted (25-JUN-2023) Biology Department, Mosul University /College of Science - Nineveh Health Directorate, Al Majmoua, Mosul, Nineveh 41001, Iraq
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
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Appendix (4-4B): Pseudomonas aeruginosa strain AhmHiy (OR186519)


National Library of Medicine
National Center for Biotechnology Information

GenBank

Pseudomonas aeruginosa strain AhmHiy(25) 16S ribosomal RNA gene, partial sequence

GenBank: OR186520.1

[FASTA](#) [Graphics](#)

[Go to:](#)

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VERSION OR186520.1
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ORGANISM [Pseudomonas aeruginosa](#)
Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 1034)
AUTHORS ALShaar,A.Y. and ALtaii,H.A.
TITLE Direct Submission
JOURNAL Submitted (25-JUN-2023) Biology Department, Mosul University /College of Science - Nineveh Health Directorate, Al Majmoua, Mosul, Nineveh 41001, Iraq
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Sequencing Technology :: Sanger dideoxy sequencing
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Appendix (4-4C): Pseudomonas aeruginosa strain AhmHiy (OR186520)


National Library of Medicine
National Center for Biotechnology Information

Nucleotide

GenBank

Pseudomonas aeruginosa strain AhmHiy(102) 16S ribosomal RNA gene, partial sequence

GenBank: OR186521.1

[FASTA](#) [Graphics](#)

LOCUS OR186521 1034 bp DNA linear BCT 30-JUN-2023

DEFINITION Pseudomonas aeruginosa strain AhmHiy(102) 16S ribosomal RNA gene, partial sequence.

ACCESSION OR186521

VERSION OR186521.1

KEYWORDS .

SOURCE Pseudomonas aeruginosa

ORGANISM [Pseudomonas aeruginosa](#)
Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.

REFERENCE 1 (bases 1 to 1034)

AUTHORS ALShaar,A.Y. and Altafi,H.A.

TITLE Direct Submission

JOURNAL Submitted (25-JUN-2023) Biology Department, Mosul University /College of Science - Nineveh Health Directorate, Al Majmoua, Mosul, Nineveh 41001, Iraq

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Appendix (4-4D): Pseudomonas aeruginosa strain AhmHiy (OR186521)

الخلاصة:-

من خلال هذه الدراسة تم اجراء تقييم لفعالية تثبيط جزيئات استشعار النصاب المنتجة من قبل جرثومة الزائفة الزنجارية، ودورها في تحويل الاستجابة المناعية في مزارع الخلايا المناعية المحضرة مختبريا من اعضاء الفئران .ترتبط إمرضيه جرثومة الزائفة الزنجارية بعوامل الفوعة التي تنظم التعبير الجيني لها من خلال أنظمة استشعار النصاب المختلفة في البكتريا .

في الدراسة الحالية تم جمع (٢٤٥) عينة ادرار من كلا الجنسين من المرضى المصابين بالتهاب المسالك البولية من مختلف الأعمار في مستشفى ابن سينا التعليمي بالموصل، ولم يتلقوا اي علاج بالمضادات الحيوية، ، حيث تم جمع العينات على مدى سبعة أشهر من اذار إلى ايلول ٢٠٢٣. واعتمادًا على نتيجة الزرع ، وشكل المستعمرات، والفحص المجهرى للمسحة الرطبة المصبوغة بصبغة جرام، واختبارات الكيمياء الحيوية ومؤشر التحليل (٢٠E API) . تم الحصول على ٨ (٤٪) عزلات من جرثومة الزائفة الزنجارية من أصل (٢٠٠) عينة ذات نمو جرثومي ايجابي. وتم استخلاص الحمض النووي DNA للعزلات الثمانية من الزائفة الزنجارية بدرجة نقاء عالية وتم تشخيصها جميعا بواسطة تسلسل جين 16S rRNA وتم تقديم أربعة منها إلى المركز الوطني لمعلومات التكنولوجيا الحيوية كسلالات جديدة.

تم الكشف عن قدرة الزائفة الزنجارية على إنتاج جزيئات استشعار النصاب بالطرق المظهرية والجينية، في الطرق المظهرية تم اختيار عوامل ضراوة متعلقة باستشعار النصاب ،الانزيم المحلل للدم، مقاومة المضادات الحيوية، تكوين الأغشية الحيوية وإنتاج صبغة البيوسيانين. جميع عزلات الزائفة الزنجارية التي تم عزلها كانت تمتلك هذا الانزيم حيث قامت بتحليل كرات الدم الحمراء عند نموها على أجار الدم وظهور مناطق شفافة حول المستعمرات النامية. تم استخدام طريقة كيربي باور لإجراء اختبار الحساسية للمضادات الحيوية لمختلف المضادات الحيوية، جميع عزلات جرثومة الزائفة الزنجارية كانت تمتلك مقاومة لأكثر من نوع من المضادات الحيوية التي استخدمت في الدراسة الحالية، وكانت اعلى نسبة مقاومة ابدتها هذه الجرثومة تجاه المضاد الحيوي السيفترياكسون لتصل إلى ١٠٠٪ بينما تم تسجيل معدل مقاومة ١٢٪ فقط تجاه الميروبينيم.

تم تحديد قدرة جرثومة الزائفة الزنجارية على تكوين الغشاء الحيوي في دراستنا لجميع العزلات بثلاث طرق، طريقة الصفيحة المعيارية، طريقة الأنبوب الزجاجي وأجار الكونغو الأحمر. كانت نتائج طريقة الصفيحة المعيارية أن ٨/٦ (٧٥٪) من العزلات كانت منتجات أغشية حيوية قوية و ٨/٢ (٢٥٪) من العزلات كانت منتجات أغشية حيوية معتدلة، بينما أظهرت طريقة الأنبوب الزجاجي أن ٨/٤ (٥٠٪) من العزلات كانت منتجات أغشية حيوية قوية و ٨/٣ (٣٧٪) من العزلات كانت منتجات أغشية حيوية معتدلة و ٨/١ (١٣٪) لم يتم اكتشاف تكوين أغشية حيوية بهذه الطريقة. وأخيراً، لم تكن طريقة أجار

الكونغو الأحمر مفيدة للكشف عن الأغشية الحيوية في جميع العزلات. بشكل عام إن جميع عزلات الزائفة الزنجارية لديها القدرة على إنتاج صبغة البايوسينين بدرجات مختلفة والتي تم اكتشافها باستخدام طريقة مشابهة لطريقة الصفيحة المعيارية .

الطرق الجينية شملت على الكشف عن جينات استشعار النصاب *las I* و *las R* لأن نظام Las ينظم شبكة وإشارات استشعار النصاب في جراثيم الزائفة الزنجارية وكذلك ينظم تكوين الأغشية الحيوية وإنتاج البايوسينين، وتم الكشف عن وجد هذه الجينات في العزلة التي كانت مقاومة لجميع المضادات الحيوية وكذلك التي تمتلك مقاومة لأكثر من نوع من المضادات الحيوية التي استخدمت في الدراسة الحالية.

واحد من أهم الأعمال في هذه الدراسة هو تثبيط تكوين الأغشية الحيوية وإنتاج البايوسينين، وذلك باستخدام ثلاث مواد: فيتامين ث، حمض الساليسيليك والمصل المتعدد (الحاوي على الباروكسونيز). كانت نتائج تثبيط تكوين الأغشية الحيوية بواسطة فيتامين ث ، حمض الساليسيليك و المصل المتعدد هي (76 ، 37 ، 28) % على التوالي، حيث أظهر فيتامين ث أعلى تثبيط ، بينما في تثبيط إنتاج صبغة البايوسينين أظهر حمض الساليسيليك أعلى تثبيط (77 %) مقارنة مع المصل المتعدد و فيتامين ث الذي أنتج (56 و 38 %) على التوالي.

تم استخلاص خام استشعار النصاب بالطرق اليدوية ومن ثم استخدمنا مطياف الأشعة تحت الحمراء وتقنية الكروماتوغرافي السائلة ذات الضغط العالي للتأكد من وجود جزيئات استشعار النصاب في المستخلص، حيث أظهرت النتائج احتوائه *homoserine lactone signal* فقط.

أخيرًا، هناك عمل مهم آخر في هذه الدراسة وهو تقييم دور جزيئات استشعار النصاب في تعديل المناعة، والذي يظهر من خلال التنظيم السلبي للتعبير الجيني للسايتوكينات البادئة للالتهابات عندما تمت معاملة مزارع الخلايا المناعية بمستخلص استشعار النصاب بتركيز مختلفة (5، 12.5 و 25) % ومقارنتها بجين ال *housekeeping*. أجريت هذه التجارب على مزارع الخلايا المناعية والمحضرة في المختبر من الطحال والعقد الليمفاوية ونخاع العظم للفئران. تسببت جميع التركيز في التنظيم السلبي لتعبير جينات $IL-1\beta$ و $IL-6$ و $IL-2$ و $TNF\alpha$ و $NF-KB$ ولكن بدرجات مختلفة عند مقارنتها بجين ال *housekeeping* (GAPDH). كان أعلى تعديل مناعي في التركيز (25 %) في الاعضاء الثلاثة. من هذه النتائج خلصنا إلى أن هناك دور رئيسي لجزيئات استشعار النصاب في إمرضيه الزائفة الزنجارية من خلال تنظيم عوامل الضراوة وتقليل الاستجابة المناعية للمضيف. الجانب المهم في هذه الدراسة هو استخلاص جزيئات استشعار النصاب لأول مرة محليًا واختبار دوره في تعديل المناعة لدى المضيف على المستوى الجيني، وهذا يعتبر الأول من نوعه في العالم.



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تنشيط انتاج استشعار النصاب في جرثومة *Pseudomonas aeruginosa*
والتحري عن دوره في تعديل الاستجابة المناعية

أطروحة دكتوراه تقدم بها الطالب

أحمد يوسف سعيد صالح البدراني

إلى

مجلس كلية العلوم جامعة الموصل وهي جزء من متطلبات

نيل شهادة الدكتوراه فلسفة في اختصاص

علوم الحياة / احياء مجهرية

بإشراف

الاستاذ الدكتور

هيام عادل الطائي

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