REPUBLIC OF TÜRKİYE
KIRŞEHİR AHİ EVRAN UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

EFFECT OF NANOPARTICLES ON THE GENE EXPRESSION OF VIRULENCE FACTORS OF *Pseudomonas aeruginosa*

ALI SULTAN MAALA AL-SHAMMARI

MSc THESIS

KIRŞEHİR
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SUPERVISOR
ASST. PROF. DR. LÜTFİ TUTAR

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2023

MSc THESIS APPROVAL

This Master's Thesis was evaluated by the following Jury Members on ..../.../..... and was accepted unanimously / by majority of votes.

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KIRŞEHİR AHİ EVRAN UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

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Student
ALI SULTAN MAALA
AL-SHAMMARI

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October, 2023 ALI SULTAN MAALA AL-SHAMMARI

GENİŞLETİLMİŞ ÖZET

YÜKSEK LİSANS TEZİ

NANOPARTİKÜLLERİN *Pseudomonas aeruginosa*'nın VİRÜLANS FAKTÖRLERİNİN GEN EKSPRESYONU ÜZERİNDEKİ ETKİSİ

ALI SULTAN MAALA AL-SHAMMARI

KIRŞEHİR AHİ EVRAN ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

Danışman: **Dr. Öğr. Üyesi Lütfi TUTAR**
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Prof. Dr. Yusuf TUTAR
Dr. Öğr. Üyesi Sevinç AKÇAY

Fırsatçı bir patojen olan *Pseudomonas aeruginosa*, yanık hastaları ve bağışıklık sistemi zayıf olan kişiler arasında hastalık ve ölüme neden olan birincil faktördür. *P. aeruginosa* enfeksiyonlarıyla mücadelede yeni yollar sunan alternatif tedavi yaklaşımlarının araştırılmasına ve geliştirilmesine yönelik artan bir ihtiyaç vardır. Bu araştırma alanına yönelik ilginin artmasıyla birlikte bu talep sürekli olarak artmaktadır. Bu çalışma, Bağdat'taki dört hastanede farklı enfeksiyonlara sahip hastalardan toplanan ve P. aeruginosa'nın izolasyonu ve tanımlanması için Cetrimide agar, Blood agar ve MacConkey agar plaklarında kültürlenen 120 klinik örneği içermektedir. Morfolojik ve biyokimyasal testlere göre tüm örneklerde 55 adet *Pseudomonas aeruginosa* izolatı (%45,8) tespit edilmiştir. Bu izolatların prevalansı kadın hastalarda 28 (%50,9) iken erkek hastalarda 27 (%49,0) olarak tespit edilmiş; bakteriyel enfeksiyon oranının en yüksek olduğu yaş grubu 31 (20-30 yaş), bunu sırasıyla 12 (31-40 yaş), 10 (41-50 yaş) ve 2 (61-70 yaş) takip etmiştir. Bu çalışmada mikrotitre plak yöntemiyle biyofilm oluşumu sonuçları, 55 izolattan 10'unun (%18,2) güçlü biyofilm üreticisi olduğu bulunmuştur. Ayrıca izolatların 21'inin (%38,2) orta derecede biyofilm üreticisi olduğu, diğer izolatların (n=24; %43,6) biyofilm üretimi açısından zayıf olduğu tespit edilmiştir. Çinko oksit nanopartiküllerinin kullanıma sunulmasından önce, *P. aeruginosa*'nın çinko oksit nanopartiküllerinin sonradan eklenmesiyle karşılaştırıldığında önemli ölçüde daha fazla ($p<0.01$) biyofilm oluşumu gözlenmiştir. Disk difüzyon yöntemi kullanılarak yapılan antibiyotik duyarlılık testi sonuçları, bu çalışmaya dahil edilen tüm *P. aeruginosa* izolatları arasında antibiyotik direncinde farklılıklar olduğunu ortaya çıkarılmıştır. Bu çalışmanın bulgularına göre Levofloksasin, Ceftazidime, İmipenem, Tobramisin ve Aztreonam antibiyotikleri sırasıyla %89, %89, %80, %73 ve %71 ile antibiyotiklere karşı en yüksek dirence sahipti. *P. aeruginosa* izolatları bu çalışmada kullanılan güçlü, orta ve zayıf biyofilmdi ve P. aeruginosa'nın temizlik geni olarak rspL geni kullanılarak PCR tekniği ile tespit edilmiştir. Test edilen *P. aeruginosa*'nın tümü klinik olarak rspL genini içermektedir. Sonuçlar, son genin güçlü biyofilm üreten izolatların %80'inde bulunduğunu, rhlI geninin ise tüm güçlü biyofilm izolatlarında bulunduğunu göstermiştir. 55 izolattan 10 izolat güçlü biyofilm üreticileri olarak ve 8'i her iki lasI genini de (%80) içerirken, tüm bu izolatlarda rhlI bulunmuştur. Kantitatif PCR reaksiyon deneyi, her biri iki biyofilm geni içeren, *Pseudomonas aeruginosa*'nın altı adet oldukça yetkin biyofilm üreticisi izolatını içermektedir. Bu izolatlar,

ZnO-np'ye göre değişen alt MİK değerleri ile bilinçli olarak seçilmiştir. Bu çalışmada, biyofilm genlerinin mRNA ekspresyonu, bakteriyel büyüme sırasında her bir numune için minimum inhibitör konsantrasyonunun (MIC) altındaki konsantrasyonlar kullanılarak ZnO-np ile tedavi edilen numuneler ile tedavi edilmeyen numuneler karşılaştırılarak kantitatif bir RT-PCR tahlili yoluyla incelenmiştir. Sonuçlar, ZnO-np varlığında biyofilm genlerinde önemli bir aşağı regülasyon olduğunu ortaya çıkarmıştır. Sonuçlar, ZnO-np tedavisi öncesinde ve sonrasında test edilen tüm izolatları içeren Pearson korelasyon analizi kullanılarak RSPL, lasI ve rhlI genlerinin gen ekspresyonu ile biyofilm oluşumu arasında anlamlı bir pozitif korelasyon olduğunu göstermiştir.

Anahtar Kelimeler: *Pseudomonas aeruginosa*, nanoparçacık, çinko oksit, virülans, biyofilm.

ABSTRACT

MASTER'S THESIS

EFFECT OF NANOPARTICLES ON THE GENE EXPRESSION OF VIRULENCE FACTORS OF *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, an opportunistic pathogen, is a primary contributor to illness and death among burn patients and individuals with compromised immune systems. There is a growing need for the exploration and advancement of alternative therapeutic approaches that offer fresh avenues to combat *P. aeruginosa* infections. This demand is continuously increasing, with heightened attention directed towards this area of research. This study included One hundred and twenty clinical specimens collected from patients with different infections from four hospitals in Baghdad, which were cultured on Cetrimide agar, Blood agar, and MacConkey agar plates for isolation and identification of P. aeruginosa. According to morphological and biochemical tests, 55 *Pseudomonas aeruginosa* isolates (45.8%) were found in all samples. The prevalence of these isolates was 28 (50.9%) in female patients, compared to 27 (49.0%) in male patients, as shown in table (4-1); the highest rate of bacterial infection was within the age group 31 (20-30 year), followed by 12 (31-40 year), 10 (41-50 year), and 2 (61-70 year) respectively. In the present study, results of biofilm formation by the microtiter plate method showed that ten isolates from 55 isolates (18.2%) were strong biofilm producers. Also, it was found that 21(38.2%) isolates were moderate biofilm producers, and the other isolates (n=24; 43.6%) were weak to produce the biofilm Prior to the introduction of Zinc oxide nanoparticles, *Pseudomonas aeruginosa* exhibited a considerably greater ($p<0.01$) biofilm formation compared to the post-addition of Zinc oxide nanoparticles. The results of the antibiotic susceptibility test, conducted using the disc diffusion method, revealed variations in antibiotic resistance among all *P. aeruginosa* isolates included in this study. According to the findings of this study, the antibiotics Levofloxacin, Ceftazidime, Imipenem, Tobramycin, and Aztreonam had the highest resistance to antibiotics: 89%, 89%, 80%, 73%, and 71% respectively. The isolates of *P. aeruginosa* were the strong, moderate, and weak biofilm used in this study and detected by PCR technique using the rspL gene as the housekeeping gene of *P. aeruginosa*. All the tested *P. aeruginosa* clinically contained the rspL gene. The results showed that the last gene was found in 80% of isolates that produce strong biofilm, while the rhlI gene was found in all potent biofilm isolates. Ten isolates from 55 isolates were potent biofilm producers, and 8 contained both lasI genes (80%), while rhlI was found in all these isolates. The quantitative PCR reaction experiment involved six highly proficient biofilm producer isolates of *Pseudomonas aeruginosa*, each containing two biofilm genes. These isolates were deliberately selected with varying sub-MIC

values to ZnO-np. In this study, the mRNA expression of biofilm genes was examined through a quantitative RT-PCR assay, comparing the samples treated with ZnO-np to those left untreated, using concentrations below the minimum inhibitory concentration (MIC) for each sample during bacterial growth. The results revealed a significant down-regulation in biofilm genes in the present of ZnO-np. The results showed a significant positive correlation between gene expression of RSPL, lasI, and rhlI genes and biofilm formation using Pearson correlation analysis which included all the tested isolates before and after the treatment with ZnO-np.

Keywords: *Pseudomonas aeruginosa*, nanoparticles, zinc oxide, virulence, biofilm.

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LIST OF ICONS AND ABBREVIATIONS

Icons		**Described**
$°C$	**:**	Degrees Celsius
$\%$	**:**	Percentage
μl	**:**	Microliters
Abbreviations		**Described**
AIDS	**:**	Acquired Immune Deficiency Syndrome
AR	**:**	Antibiotic-Resistant
CF	**:**	Cystic Fibrosis
DNA	**:**	Deoxyribonucleic Acid
cDNA	**:**	Complementary Deoxyribonucleic Acid
NHS	**:**	National Health Service
PCR	**:**	Polymerase Chain Reaction
rRNA	**:**	Ribosomal Ribonucleic Acid
SCD	**:**	Special Care Department
ZnO	**:**	Zinc Oxide

1. INTRODUCTION

Pseudomonas aeruginosa is recognized as a significant and versatile pathogen, capable of causing infections in various tissues with varying levels of severity (Lotfpour and Amini, 2020). It is responsible for a wide range of conditions including wounds, burns, urinary tract infections, pneumonia, keratitis, otitis externa, and folliculitis. Furthermore, *P. aeruginosa* poses a considerable threat as a hospital-acquired infection due to its high resistance to antimicrobials and its ability to thrive in nutrient-deprived environments, making eradication challenging (Gellatly and Hancock, 2013). Numerous reports indicate that drug-resistant *P. aeruginosa* infections are associated with significant increases in mortality rates, morbidity, prolonged hospital stays, the need for chronic treatment, and surgical interventions (Ibraheem et al., 2019). *Pseudomonas aeruginosa* is an uncommon disease that primarily affects individuals with multiple underlying conditions such as cancer, AIDS, cystic fibrosis, as well as those with implants or burn injuries. It is a widespread disease that exhibits a propensity for developing antibiotic resistance, thus rendering antibiotic treatments ineffective (Ali et al., 2020). Antibiotic-resistant (AR) infections have become prevalent worldwide, exacerbated by a lack of new antibiotic development in the pharmaceutical industry across many cultures. AR *P. aeruginosa* stands out as a prominent causative agent of healthcare-associated diseases, contributing to global health concerns as multi-drug-resistant strains continue to emerge. The expression of impermeable proteins in the outer membrane is immune to most antibiotics (Samrot et al., 2018). *P. aeruginosa* also uses various mechanisms to survive antibiotics, including seclusion of inactivating enzymes, expression of efflux pumps, and chromosomal mutations (Hemeg, 2017).

Biofilm formation by this species increases the high resistance to antibiotics, including fluoroquinolones, beta-lactams, and carbapenems, and this barrier decreases the possibility of bacteria penetrating the immune cell of biofilms and of antibiotics and acts as adequate protection against the host immune systems and antibiotic agents, which leads to continuous colonization of the organism. Treating burn wound bacterial pathogens is a significant challenge, and new methods of reducing death rates associated with bacterial infections in burn injuries are needed (Shariati et al., 2019). The emergence of resistance in treatment, which is proved to duplicate the time of hospitalization and total cost of patient care, is even more troublesome (Jindal et al., 2015).

Many previous studies exhibited the nanoparticles' applications in the medical field and the treatment of infectious diseases. Higher efficiency was reported on metal oxide nanoparticles resistant strains, such as zinc oxide (ZnO) and silver. Incredible antimicrobial activity and a significant reduction in skin infections and inflammatory function of mice were found in ZnO nanoparticles (Sirelkhatim et al., 2015).

The *Pseudomonas aeruginosa* problem is very severe in local hospitals in Iraq. The current study aims to recognize the effect of ZnO nanoparticles on biofilm formation as a virulence factor of *Pseudomonas aeruginosa* isolates, to eradicate these bacteria by seeking alternative antimicrobial materials.

2. LITERATURE REVIEW

2.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is categorized as a lactose-non-fermenting, gram-negative, non-spore-forming, and motile by one polar flagellum bacillus. The majority of isolates are catalase and oxidase positive. It can use aerobic respiration as its preferred mode of metabolism because it is an obligate respiration. However, it can also breathe anaerobically using nitrate or other alternate electron acceptors; thus, it is the reason why bacteria is common throughout the planet and can be found in water, soil, or sewage as well as in plant, animal, or human hosts (Noomi, 2018). An opportunistic pathogen, *Pseudomonas aeruginosa*, can infect people with acute or chronic illnesses. It frequently infects persons with weak immune systems, and infections like this have a high death rate in burn victims or people who require mechanical breathing. It is also crucial for patients with persistent respiratory infections, such as cystic fibrosis (CF) and other respiratory system illnesses (Schubiger et al., 2020). When burns or wounds occur, the skin acts as a natural barrier, safeguarding the body's tissues. However, this creates an ideal environment for the proliferation of microorganisms, including bacteria such as Pseudomonas spp., posing a significant threat to patients with burns and wounds. These bacteria can infiltrate the bloodstream, leading to bacteremia and septicemia, particularly affecting individuals with leukemia and immunodeficiency (Jalil et al., 2018). Notably, these bacteria possess remarkable resistance to disinfectants, contributing to their role in hospital-acquired infections. They have been found to thrive in solutions, disinfectants, and detergents containing hexachlorophene, thereby further complicating infection control efforts (Sudhakar et al., 2015). It can be challenging to treat this pathogen because of its natural and acquired antibiotic resistance. It has an extraordinary ability to acquire antibiotic-resistance genes, spread from patient to patient, and survive in the hospital setting (Lila et al., 2018).

Pseudomonas aeruginosa can grow in the laboratory on MacConkey agar medium, and their colonies are pale due to their inability to ferment lactose sugar and smell like fermented grapes. On blood agar, colonies appear dark in color and surrounded by a transparent area, indicating their ability to hemolyze blood (type - Hemolytic) due to hemolysin production, as well as their ability to grow at 42 °C (Hossain et al., 2013). *P. aeruginosa* has the cytochrome oxidase enzyme, a phylogenetic taxonomic feature, and many members produce pigments. They do not

form spores and can produce pigments that fluoresce, such as pyocyanine (green-blue) and pyoverdin (yellow-green) (Bunyan et al., 2019). *P. aeruginosa* creates various virulence factors necessary to bypass the host immune system and other natural defenses. One of P. aeruginosa's most crucial virulence factors is the development of biofilms (Pachori et al., 2019).

2.2. *Pseudomonas aeruginosa* Classification

In 1882, Gessard was first isolated from purulent wounds by *Pseudomonas aeruginosa*, after which it became known as *Pseudomonas pyocyanin* and then as *Pseudomonas aeruginosa*, and was called *Bacillus pyocyanin* (Oliveira and Reygaert, 2022). The name of the bacteria, Pseudomonas, originates from the Greek word 'pseudo,' meaning false or fake, and the second Greek term refers to copper rust (Brooks et al., 2010). *Pseudomonas aeruginosa* is part of the Pseudomonadaceae family and includes several species in the Pseudomonas genus (Bailey et al., 2014). The classification of these bactéries is according to the following graph (Slonczewski and Foster, 2014) and is based on DNA sequence, especially Sequence 16S rRNA (Tripathi et al., 2013).

2.3. General Characteristics of *Pseudomonas aeruginosa*

The gram-negative bacteria appear in *Pseudomonas aeruginosa* as a single rod form, pairs, and short chains (Oliveira and Reygaert, 2022). Bacteria of *P. aeruginosa* are slightly or directly curved, stained rods with a diameter of 0.6 to 2.0 μm; motile by a single or more polar flagellum and non-spore-forming (Govan, 2007). *P. aeruginosa* is an obligate aerobic bacterium that demonstrates robust growth on diverse culture media and sometimes releases a distinct odor resembling a sweet grape or corn taco aroma. Blood is hemolyzed by certain strains. *P. aeruginosa* colonies are smooth, round, and pigmented (Oliveira and Reygaert, 2022).

P. aeruginosa is an oxidase-positive, frequently pigmented, and unable to degrade carbohydrates, but many strains oxidize glucose without gas formation (Brooks et al., 2013). In the existence of arginine or nitrates as terminal electron accepters, anaerobic growth from *P. aeruginosa* bacteria has also been proved possible (Todar, 2008). It grows well at 37-42°C, not 4°C, and grows at 42°C; it helps to distinguish *P. aeruginosa* from another fluorescent pigment-producing Pseudomonas. Some Pseudomonas species can be grown at 45°C (Fothergill et al., 2007).

2.4. *Pseudomonas aeruginosa* Pathogenicity

P. aeruginosa is one of the primary nosocomial infections in the hospital setting (Poole, 2011). In patients with severe medical conditions, it may lead to numerous acute opportunistic infections (Gellatly and Hancock, 2013).

Infections are more frequent and diverse during hospitalization for those with immuno-depression, extreme burns, wounds, chemotherapy, and acquired immune deficiency syndrome (AIDS). The most at risk are those with immuno-depressed patients (Park et al., 2014).

P. aeruginosa infections are usually immune to several antibiotics, which can lead to severe and recurrent infections (Doosti et al., 2013); this leads to secondary fungal infections and other complications, a longer stay in the hospital, medication failure, and premature death in cystic fibrosis patients (Tan et al., 2014). *P. aeruginosa* is a pathogen commonly linked to nosocomial pneumonia, nosocomial urinary tract infections, surgical site infections, severe burns, wound infections, external otitis, keratitis, and folliculitis (Gellatly and Hancock, 2013). Either neoplastic disease chemotherapy or broader spectrum antibiotic therapy infections of patients (Shaan and Robert, 2013).

Via pathogenesis of P. aeruginosa, virulence factors may lead the human host to infection at various stages at the same time or act independently (Wolska et al., 2011), i.e., lipopolysaccharide, alginate (exopolysaccharide), which directly influences fever, shock, oliguria or leukocytosis, Leukopenia, adult condition of respirable discomfort and intravascular clotting, pili and flagella, and secreted virulence factors, like toxins, enzymes like protease, phospholipase, elastases and other small molecules such as rhamnolipid, phenazines and cyanide inducing or interacting with the host immune response to trigger infection (Rafie et al.,2014).

When *P. aeruginosa* finds a proper place for colonization, they begin to express the virulence factor genes and eventually activate the infection procedure, followed by the development of virulence factor in the host cells (Rasko and Sperandio, 2010).

The creation of the diversity of pseudomonas different adaptation processes such as diet and metabolic Pathways in addition to gene expression regulation (Riera et al., 2011). Pseudomonas' ability to build biofilms. Aeruginosa contributes significantly to its virulence in the catheter lumen and the lung of cystic fibrosis patients. Pseudomonas Immune to specific antimicrobial agents, aeruginosa becomes dominant as more

susceptible bacteria in the normal microbiota are eliminated. This resistant is due to the capacity of biofilms of bacteria that are embedded in the bacteria in the exopolysaccharide matrix (Salman et al., 2019).

P. aeruginosa provides pathogenesis and establishes quorum sensing multidrug resistance. The presence of MDR pseudomonas has been reported to date. In essential hospital terms such as Burn Unit and Special Care Department (SCD), aero xylic strains are essential in preventing MDR-infected infections (Mahnaie et al., 2020). Longitudinal studies, including longitudinal methodology, provided details on genetic changes subject to *P. aeruginosa* and permitted comparing particular expression genes in various patient periods (Hussien et al., 2012).

Gene expression changes have been identified in multidrug efflux pumps, quorum sensing regulators, and alginate biosynthesis mutations (Rezaie et al., 2018).

2.5. *P. aeruginosa* Infections

2.5.1. Respiratory tract infections

In patients with immunosuppression, chronic lung disease, and heart failure causing Pneumonia, aeruginosa is a primary and joint cause of respiratory tract, and acute respiratory infections have frequent aeruginosa—silver cells. Bronchiectasis is also caused by aeruginosa. Mucoid *P. aeruginosa* strains are common and hard to treat in patients with cystic fibrosis (Wuerth et al., 2019).

2.5.2. Skin infections

P. aeruginosa is a common cause of thin- and hair follicles, skin infections, and damage. This type of infection is transmitted by water using water pools, mineral water baths as well as by skin contact, since it makes it possible to penetrate hair follicles and then begins the production of inflammatory toxins in areas such as rabbits and armpits as well as areas of the uniforms worn by swimmers, as this is the most common type of infection (Domenico et al., 2017). Skin lesions are difficult to treat, although different types of antibiotics with a wide power threaten the patient's treatment (Nagoba et al., 2013).

2.5.3. Ear infections

P. aeruginosa is a common cause of chronic otitis media and other ear inflammations, which also can lead to external otitis, including malignant otitis externa (Roland and Stroman, 2002). Here, the patient develops a hole in the eardrum membrane because of the entry of the pathogen into the water through the pharyngeal nose or the hole when bathing or swimming (Cole et al., 2014).

2.5.4. Urinary tract infections (UTI)

Bacteria entering the urinary tract through catheters, instruments, and irrigation solutions is one of the most frequent *P. aeruginosa* infections. It is the leading cause of UTI in the hospital; it is more common in men, particularly during pregnancy and birth. This injury occurs (Cole et al., 2014; Cole and Lee, 2019).

2.5.5. Infections of burns and wounds

Wound infection poses a significant risk for patients with burns, and burn wounds caused by *P. aeruginosa* are particularly challenging to treat within hospital settings (Jault et al., 2018). Burn injuries are highly debilitating and can have long-lasting impacts on a patient's health. Globally, an estimated 265,000 deaths per year are directly attributed to fire-related burns, with 90% of burn incidents occurring in developing nations where patient mortality rates can reach 100%. These burns often cover more than 40% of the total body surface area. While millions of people worldwide suffer from burn-related conditions, the National Health Service (NHS) in the United Kingdom alone sees approximately 90,000 hospital admissions annually due to burns (Guest et al., 2020).

2.6. *Pseudomonas aeruginosa* Epidemiology

These bacteria spread across the atmosphere and live in the soil, marshes, and aquatic habitats. They live on plant and animal fabrics. These bacteria can thrive in disinfectants, liquid medical products like sabyns drop in the eyes, saline solutions, anesthetics, and many other medical products; these bacteria have their survival skills (Stover et al., 2000).

P. aeruginosa can live in less healthy environments and sustain various physical conditions that allow bacteria to exist in hospital and community settings (Lister et al., 2009). It can replicate and settle efficiently in the tissues affected, leading to systemic sepsis and increasing death (Diggle and Whiteley, 2020). Metabolic flexibility and high

genetic characteristics allow this bacteria to adapt to the most artificial and natural environments worldwide, from medical equipment, animal and plant tissues, water, soil, and even the ISS (Kim et al., 2013).

A high percentage of these bacteria will occur within 72 hours in a hospital. It requires an immune suppressive agent, anti-metabolism, and radiation, which help to raise infections and spread from these bacteria called nosocomial infections in patients with severe burns and wounds. Using infected surgical equipment promotes the spread of this organism and direct and indirect communication between patients (Japion et al., 2009). These *P. aeruginosa* infections also cause death and morbidity because they establish antibiotic resistance rapidly and adapt quickly to different environments and cell-related and extracellular virulence factors (Mitov et al., 2010).

2.7. Virulence Factors of *Pseudomonas aeruginosa*

P. aeruginosa has several factors of virulence that allow it to colonize within the body of its host organism, leading to illnesses, enzymes, and exotoxins, for instance. It is as well a biofilm that defends against phagocytes and environmental stress. (Macin and Akyon, 2017).

It will be necessary to make extracellular enzymes, including elastases, proteases, exotoxin A, and hemolysins, which cause cell death or interfere with the host's immune response to the disease, which are the most.

Significant virulence factors include biofilms and pili, exoenzymes like S, T, U, and Y. (Karen et al.,2016).

Pyocyanin is the most critical, non-fluorescent pigment (blue soluble in water), and pyoverdine is fluoresced pigment (green-yellowish, also known as Pseudobactin), which is toxic to host cells. This kind of bacteria produced a variety of pigments that inhibited the growth of other bacteria. It is also a red pigment, and Pyomelanin is a brown or black pigment, for example, Pyorubin (Lee et al., 2014).

Many antibiotics may become resistant to *P. aeruginosa*, making treatment for their infections more difficult. Biofilm bacteria can create bacterial communities within an exopolysaccharide matrix, which gives them resistance. This environmental bacterium can form biofilms on surfaces such as CF lungs, contact lenses, and infected catheters, both surface and non-living (Hoiby et al., 2010). Protein, polysaccharides, and extracellular DNA are used for exopolysaccharides (Storz et al., 2012). It holds together the cell that is required to interact with the cell. It also enables the formation of 3D

structures, providing bacteria with increased access to nutrients and multicellular livelihood advantages (Sharma et al., 2014).

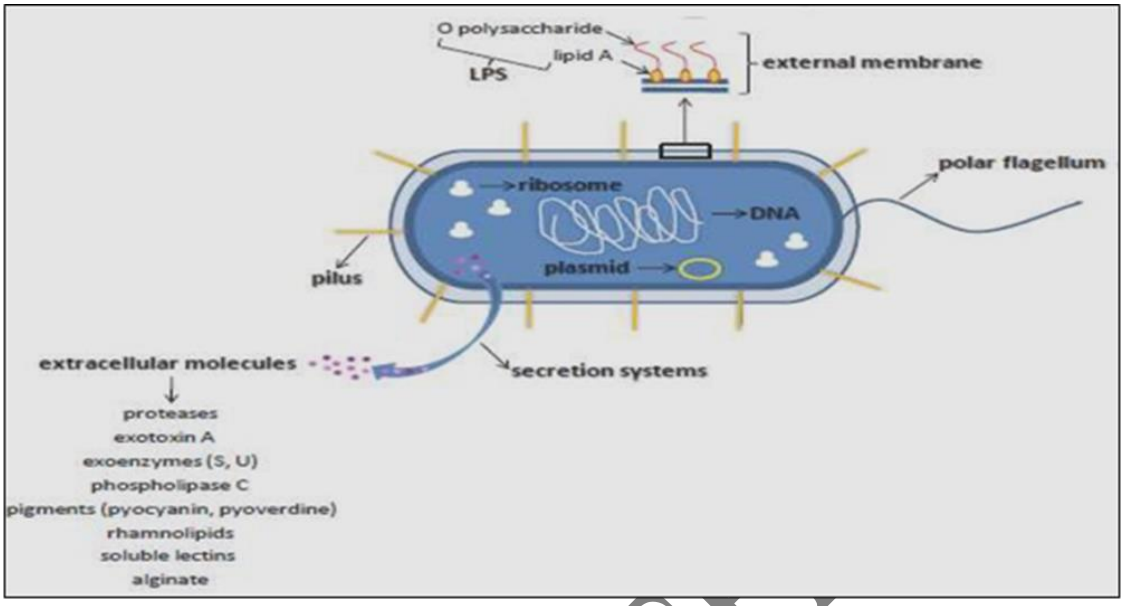

Figure 2.1. *Pseudomonas aeruginosa* virulence factors (Galdino et al., 2017)

2.7.1. Biofilm formation

The biofilm consists of colonies of exopolysaccharide formed by the *P. aeruginosa*, which are surrounded by microorganisms (Tseng et al., 2019). The essence of biofilm is hydrophilic, and water constitutes approximately 97% of membranes. Biofilm has rivers that it uses for the transportation of nutrients. In extreme, challenging environmental conditions, Biofilm is responsible for preserving water and nutrients (Wei and Ma, 2013).

Stages of biofilm formation:

Biofilm training stages:

1. Early-stage substratum attachment.

2. The second level of microcolony development and reproduction and accumulation.

3. Separating the microcolonies from the third stage to the various compositions and maturing.

4. The fourth stage of release, distribution, and colonization of the cells outside to new areas (Harper et al., 2014).

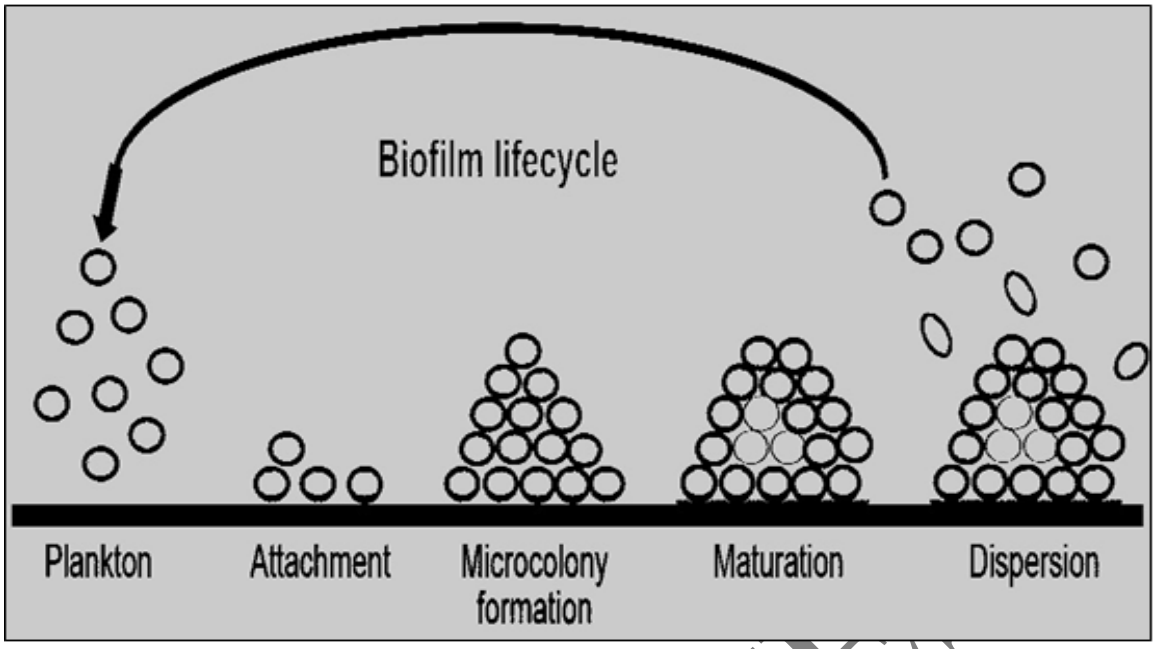

Figure 2.2. Biofilm formation steps (Santos et al., 2018)

However, *P. aeruginosa* 's biofilm formation allows it to remain alive on dry surfaces long. Much research will concentrate on the production phase of biofilm as it is a significant cause of many diseases, such as chronic pneumonia, chronic bladder inflammation, bone inflammation, bacterial cell build-up endocarditis, and toxins (Lanter et al., 2014).

Using Qurum-Sensing genes, the regulation of biopsychological formation can be carried out, and the bacterial cells can thus interact within biofilms (Li and Tian, 2012).

2.8. Antibiotic resistance

P. aeruginosa has become a significant and commonly occasional nosocomial pathogen. This organism has inherent resistance to many antimicrobial types, resulting in difficult-to-treated infections associated with severe disease and mortality (Obritsch et al., 2004).

The pathogen *P. aeruginosa* presents a significant therapeutic challenge when it comes to antibiotic treatment due to its prevalence in both nosocomial infections and community-acquired diseases. Therefore, the careful selection of appropriate antibiotic dosages is crucial to prevent the development of complications (Lister et al., 2009). *P. aeruginosa* is very resistant to numerous different antibiotics intrinsically. In addition, these bacteria can also quickly develop strong resistance under specific selective pressure either by transferring the resistance genes horizontally or by mutating the

chromosomally encoded genes. Bacterial infections are often difficult to treat due to their multidrug resistance phenotype (De Francesco et al., 2013).

P. aeruginosa has multiple resistances to antibacterial agents because of different factors, the lowest of which is the low permeability of the cellular bacterial surrounds genetics. It shows a wide variety of antibiotic resistance mechanisms and a P. aeruginosa's ability to acquire other resistance genes from other bacteria through the conjugation, transformation, and transmission of mobile genes (plasmids, transposons, phages) (Tam et al., 2010).

Several *P. aeruginosa* resistance infections have been commonly found in cancer patients, immunocompromised AIDS, COPD, cystic fibrosis, and even by people with diabetes as secondary infections (Hogardt and Heesemann, 2013), leaving serious, high-mortality, blood-stream infections, and healthcare costs to the patient. Several types remain effective agents of *P. aeruginosa* infections (Ceftazidime, Cefepime, Carbapenems, Tobramycin, and Amikacin). Many other antibiotics related to different groups, like Carbencillin, have become more resistant to *P. aeruginosa* isolates (Black, 2012). Carbapenem classes, on the other hand, have good anti-microbial activity, but the propagation and development of acquired carbapenem class resistance is a challenge for control success and therapeutic efforts. The special imipenem carbapenem classes are used extensively in a clinical setting (Riera et al., 2011).

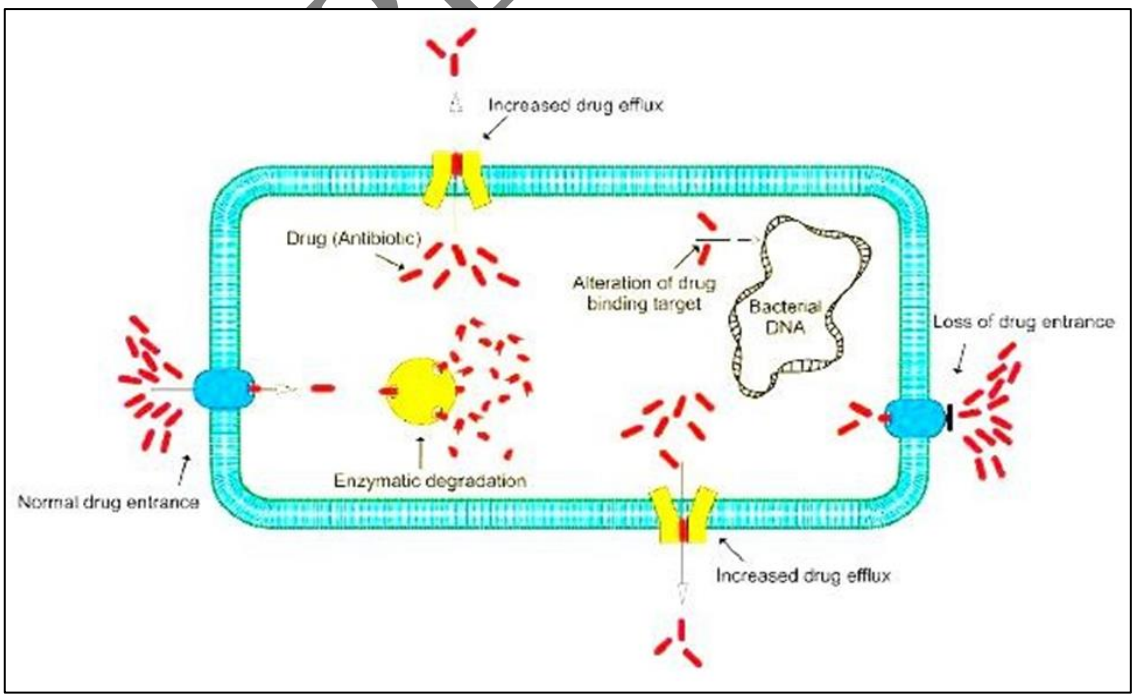

Figure 2.3. *Pseudomonas aeruginosa* mechanism for antibiotic resistance (Rocha et al., 2019).

2.8.1. Carbapenems resistance

Beta-lactam is the most common antibiotic used for carbapenems (meropenem and imipenem) (Morita et al., 2014). It is an effective agent for treating many Pseudomonas infections because of its beta-lactamase resistance (Ding et al., 2018). The mechanisms of carbapenems antibiotics are penicillin-bound protein inhibition, which rests on the plasma membrane's exterior surface. The bacterial external membrane of carbapenems antibiotics may pass through the porine canals (OprD) (Ocampo-Sosa et al., 2012). Pseudomonas. aeruginosa to low-expression carbapenems, especially metal beta-lactamase and extreme AmpC beta-lactamases, or extreme expression efflux pumps, in outer membrane proteins (OprD) or carbapenemase (Patel and Bonomo, 2013).

2.8.2 Extended spectrum beta-lactamase (ESBLs)

Extended-spectrum beta-lactamase in *P. aeruginosa* has already been reported and is remarkably resistant to various antibiotic types, such as penicillins and cephalosporins. ESBLs are new beta-lactamases, which convey a resistance in particular to cephalosporins, some of the latest beta-lactamase antibiotics. Bacterial plasmid genes, which carry genes responsible for resistance to various antimicrobial substances such as Aminoglycosides, Tetracyclines, and Sulfonamides, encode for Extended-Spectrum Beta-Lactamases (ESBLs). These ESBLs have evolved from earlier beta-lactamase enzymes like TEM, SHV, and OXA, with a narrower range of activity in terms of the antibiotics they can degrade. ESBLs contribute to the multidrug resistance observed in gram-negative bacteria. The ESBL enzymes are further categorized into two classes, namely Class A and Class D, based on their structural characteristics (Omer et al., 2020). In *P. aeruginosa* tension, ESBL animatics are mainly observed for the PER classes and PME (class A). The class is called extended-spectrum Class D βlacktamases(ES-OXAs) and are primarily βlactamases from PER, GES, VEB, BEL, and family PME (class A). Antimicrobial resistance is an increasing clinical problem and a recognized global threat to public health.

P. aeruginosa exhibits a particular propensity to resistance development. The development of *P. aeruginosa* resistance also limits future treatment choices and is linked to higher mortality and morbidity rates and increased costs (Kumar et al., 2020).

2.8.3. Metallo beta-lactamases (MBLs)

The synthesis of metallo-beta-lactamase is the prevailing mechanism of carbapenem resistance. Metallo-beta-lactamase, which belongs to Ambler Class B enzymes, is capable of hydrolyzing beta-lactam antibiotics, including carbapenems. Beta-lactamases use serine as an active site so that beta-lactamase inhibitors such as clavulanic acid or sulbactam eight can be easily degraded. However, MBL-producing Pseudomonas now appears as a nightmare for doctors. The MBL-producing Pseudomonas is a doctor's nightmare. In addition, MBL resistance is situated in a highly mobile genetic element, which allows easy dissemination from patient to patient or from patient to the medical provider. Therefore, preventing MBL Pseudomonas is always better than treating them (Mukherjee et al., 2020).

2.9. AmpC Cephalosporins

The wild-type strain of *P. aeruginosa* possesses AmpC cephalosporins that are not inhibited by BLI-inducing inducible molecular class C inhibitors such as clavulanic acid, tazobactam, and sulbactam (Sligl et al., 2015). The expression of AmpC cephalosporins is typically low, providing inherent resistance to aminopenicillins, as well as in combination with BLI, first and second-generation cephalosporins, cephamycins, two third-generation cephalosporins (cefotaxime and ceftriaxone), carbapenems (including ertapenem), and other aminopenicillin-resistant strains, along with reduced membrane permeability and multiple efflux systems (Kumar et al., 2020). However, the wild-type strain of *P. aeruginosa* remains susceptible to carboxypenicillins, ureidopenicillins, C3G ceftazidime, C4G cefepime, aztreonam, imipenem, meropenem, and doripenem, including carbapenems. However, over-expression and point mutation of induced or constitutive AmbCs can offer reduced sensitivity to all β-Lactamine classes except carbapenems. In contrast to the AmpC of Enterobacteriaceae, cefepime can also be affected by AmpC of *P. aeruginosa* and can be produced in serin β-lactamases of Amber Class A of type TEM (Bush 2b), PSE or CARB (carbecillinase) (Lister et al., 2009).

These enzyme substrates are mainly carboxypenicillin and ureidopenicillin and may sometimes be resistant to BLI. These enzymes exhibit different levels of susceptibility to cefepime, cefpirome, and aztreonam, but ceftazidime and carbapenem retain their efficacy against *P. aeruginosa* strains possessing these types of β-lactamases (Gómez et al., 2019).

2.10. Quorum sensing (QS) system

QS Systems found in some bacteria can be defined as a cell-to-cell communications system for chemical mediation to co-ordinate gene expression and community group activities. Dr. Peter Greenberg first found QS in the bioluminescent bacterium Vibrio fischeri 1994 (Yang, 2009). Quorum sensing is significant prevalence of many different Species in the bacterial realm can be considered as "speaking" systems that play a significant role in controlling virulence factors (Yin et al., 2012).

An extensive range of virulence factors, including the corresponding effector protein such as extracellular phospholipases, proteases, and type III secreted toxins (Exo U, S, T, and Y) and secreted type II and III systems, have been identified for *P. aeruginosa*. Moreover, Type IV pili and flagella for adhesion and motility of the host cells (Feinbaum et al., 2012). The factors in the virulence enable and facilitate the invasion of the host by bacteria, The evasion of the host's immune system and suppression of the host's immune response are critical aspects of bacterial pathogenicity. While virulence factors undoubtedly contribute to bacterial growth, they do not solely determine it (Chakravarty and Massé, 2019). Quorum sensing (QS) is responsible for regulating the expression of genes associated with many virulence factors, including those involved in toxin production such as hydrogen cyanide (Feinbaum et al., 2012). It also helps regulate wide-ranging community behaviors, including swimming, twitching, and conjugating (Rutherford and Bassler, 2012). All activities rely on the quorum sensing (QS) system, which involves the synthesis, secretion, and detection of molecules called autoinducers (AI) (Kalia and Purohit, 2011). Quorum sensing enables bacteria to assess the local population and make collective decisions based on cell density, thereby coordinating the behavior of the entire bacterial community. These behaviors are usually always associated, but not involved in bacterial viability, with pathogenicity/virulence (LaSarre and Federle, 2013).

Preventing bacterial adherence to different surfaces was one strategy for inhibiting biofilm formation. The surfaces of antimicrobial

Agents, like metallic nanoparticles, can be covered. Quaternary ammonium salt or other surfactants (e.g., QAS) surfactants (Stone et al., 2020)

The standard QS system includes three components:

·The signal molécules (AI) or the Acyl homocerin lactones (AHL) Signal molecules.

- The AI-producing synthase is capable of activating the receptor through the transcription of specific genes responsible for AI biosynthesis (genes encoding the synthase), (Miller and Bassler, 2001).

-The transcriptional regulator and the receptor.

-The QS controls the manifestation of extracellular and cellular associated VFs by producing QS signal molecules in response to population density (AHLs).

Includes the homoserine lactone ring that differs from the length of the N-linked acyl side chains and C3 substitution. The accumulation of AHLs over a threshold level causes its interaction with the LuxR activator family to allow the transcription of the target genes (Steindler and Venturi, 2007).

In a sparsely populated community, the AHLs (acyl-homoserine lactones) secreted into the surrounding medium become highly diluted through diffusion, resulting in minimal activation of the receptor. However, as the density of bacterial cells increases, the AHLs reach a threshold level, triggering receptor activation (Miller and Bassler et al., 2001).

2.11. Las system

The LasI gene-producing system leads to a synthesis of L-L-3-oxo- dodecanoyl) homoserin lactone (3-Oxo-C12-AHL) (Smith and Iglewski, 2003).This protein can only bind DNA multimerically and regulate the transcript of several genes at high cell density. LasA (lasA protease)

Expression, apr (alkaline protease), toxA (exotoxin A), and lasB (elastase) are regulated in the system (Siechnela et al., 2010).

2.11.1. Rhl system

The rhlI gene is responsible for directing the synthesis of N-(butanol)-L-homoserine (C4-AHL), which interacts with the RhlR regulatory system and activates promoters of target genes (Smith & Iglewski, 2003). The expression of various genes, including alkaline protease, elastase, cyanide, rhamnolipid, and pyocyanin production, is regulated by this system (Dekimpe and Deziel, 2009; Karatuna and Yagci, 2010). Analysis of QS signal molecules may be helpful in various bacterial infections. The detection and identification of QS signals in molecules can provide insight into the density and type of population of the infectious pathogen and its virulence components. Moreover, the new target of developing innovative infection control strategies is the Q

of S regulation mechanisms (Rasmussen and Givskov, 2006). Identifying possible drug target factors to combat *P. aeruginosa* infection of AHLs is also essential in clinical environments (Bjarnsholt and Givskov, 2007).

2.12. Nanomaterials

2.12.1. Background

The word "nano" derives from the Greek word "nano," which means "dwarf," which indicates about 10-9 nm of stuff measuring one trillionth. Due to the broad potential applications such as "nanomedicine" and the unique characteristics of various fields, the nanostructures have attracted more attention and provided simple technology to prepare and synthesize the nano-sized metal particles (Jeevanandam et al., 2018).Nanomaterials (NMs) and nanoparticles (NPs) offer potential solutions to environmental and technological challenges, with their unique characteristics influenced by factors such as composition, size, shape, size range, and distribution (Płaza et al., 2014). The field of nanotechnology is rapidly emerging, finding applications in various scientific and technological domains for creating new substances at the nanoscale. Presently, nanotechnology extends beyond electronics and encompasses diverse areas, including wound healing, anti-inflammatory effects, catalysis, magnetism, and optical and analytical applications (Ali et al., 2020).

2.12.2. The inorganic NPs

This group includes magnetic NPs, noble metal NPs, and semi-conductor NPs (gold and silver NPs) (zinc oxide and titanium oxide). Inorganic NPs are increasingly interested because they offer superior material properties for functional versatility. The most outstanding biomedical agents synthesizing NPs are metallic nPs, e.g., zinc, gold, carbon, silver, titanium, iron, palladium, fullerenes, and copper. For increasing environmental impacts, biological approaches and other green summaries must be developed (Vadlapudi and Kaladhar, 2014). Certain NPs are attractive biological marker samples because:

BETHER Small size from 1-100 nm.

-The wide volume-ratio surface.

-Trees' Biological and chemical characteristics, especially protein components, concerning shape and size, and strong affinity to the target.

-The structural robustness despite the granularity of the atom.

-Enhance or delay aggregation of particles depending on the surface type and photo-emission improvement.

-High heat, electrical conductance, and enhanced catalytic surface activity (Sahayaraj and Rajesh, 2011).

2.12.3. Nanomaterials as antimicrobials

Traditional antibiotic medicines reduce their ability to deal with common infectious diseases and are necessary for many treatments. Antibiotics have paved the way for unexpected medical and societal development in all healthcare systems. Multi-drug resistance among many pathogenic bacteria in the global market has increased considerably, resulting in the most frequently ineffective antibiotics in controlling infectious diseases, creating an issue in healthcare (Laxminarayan et al., 2013).

Developing new antibacterial systems before drug-resistant pathogens thus represents a significant threat to successful microbial disease treatment (Huttner et al., 2013).

In addition, a nanotechnology is an approach to the development of new antibodies known as nano antibiotics which are efficient in the treatment of infectious diseases, and which have many advantages over traditional antibiotics, including lack of adverse effects, increasing drug-resistant species efficiency, and overcoming the development of resistance, interfering by various biological pathway.

Nano antibiotics exhibit antimicrobial activity either on their own or by enhancing the effectiveness and safety of traditional antibiotic administration, leading to elevated local concentrations (Hajipour et al., 2012). Antimicrobial nanoparticles offer clear advantages, including low toxicity, the ability to overcome resistance, and lower costs compared to conventional antibiotics (Huh and Kwon, 2011).

Antimicrobial NPs may cause the bacterial membrane to disturb mechanically and even "unrecognized" cells as a defense treatment for bacteria (Guzman et al., 2012).

2.12.4. Mechanisms for killing bacteria in such NPs include

The production of H2O2, $O2^-$ and OH^- reactive oxygen (ROS) species. The bacterial wall membrane cell disorder. DNA synthesis inhibition and activities of intracellular enzymes. Disrupt energy transduction (Xie et al., 2011). Today many antibacterial NM and NPs are being produced to fill the gap in antibiotic treatment failure with the emergence of nanotechnology (Beyth et al., 2015).

2.12.5. Zinc oxide nanoparticles (ZnO NPs)

ZnO-np, a well-known nanoparticle, belongs to the category of metal oxide nanoparticles. It is an inorganic compound consisting of zinc oxide, appearing as a white powder that is nearly insoluble in water. ZnO-np finds diverse applications, including antimicrobial treatments, wound healing, UV protection, high catalytic and photochemical functioning. What makes it particularly remarkable is its unique combination of features such as minimal impact on human and animal cells, bacterial toxicity, plasma hydrogen stability, and affordability (Xie et al., 2011). The antibacterial effects of ZnO-np involve the production of reactive oxygen species, which can damage bacterial membranes. Additionally, the generation of hydrogen peroxide and Zn2+ ions has also been found to play a significant role in the antibacterial activity of nanoparticles. However, the development of biofilms can provide protective mechanisms for pathogenic microorganisms against inhibitory compounds (Abdelraheem and Mohamed, 2021).In recent years, nanoparticles with metal oxide properties, such as zinc oxide, have garnered significant attention due to their stability and ability to withstand harsh environmental conditions. These nanoparticles can be easily synthesized at low temperatures using a reflux digestion process and are considered safe for both humans and animals. Zinc compounds are now included in the Generally Recognized as Safe (GRAS) list by the US Food and Drug Administration, indicating their recognized safety (Ali et al., 2020).

ZnO NPs (Zinc Oxide Nanoparticles) induce elevated levels of reactive oxygen species (ROS) and malondialdehyde in bacterial cells, leading to lipid peroxidation of the cell membrane. Transmission electron microscopy images of treated bacterial cells confirm that ZnO NPs disrupt the permeable membrane, denature intracellular proteins, cause DNA damage, and result in membrane leakage. These effects are attributed to the action of Zn2+, which mediates broad-spectrum antibacterial activity against β-lactam-resistant Gram-negative food pathogens through oxidative stress, lipid peroxidation, membrane damage, β-lactamase enzyme inhibition, inactivation of intracellular proteins, DNA damage, and ultimately, cell death (Krishnamoorthy et al., 2022). Furthermore, the toxicity mechanism of ZnO NPs varies depending on the medium due to variations in the species of dissolved Zn (Li et al., 2011). The antimicrobial activity of these nanoparticles has been shown to reduce bacterial burden, skin infections, inflammation, and improve the architecture of infected skin in mouse models (Pati et al., 2014).

3. MATERIAL AND METHOD

3.1. Materials

3.1.1. Instruments and equipments

Instruments and equipment used in this study and their manufacturer and suppliers are listed in (Table 3.1).

Table 3.1. Instruments used in the study with their company and origin.

Equipment and Instruments	Company /Origin
Autoclave	Gallenkamp /England
Burner	Amal /Turkey
Centrifuge	Eppendorf/ Germany
Densichek Plus	Biomerieux/France
Disposable Petri dishes	Al-Hani /Lebanon
Distillation	GFL/Germany
Deep Freeze -80	Binder / Germany
electrophoresis system	cleaver/United Kingdom
Gel Documentation	cleaver/United Kingdom
Incubator	Binder / Germany
Laminar air flow	Gallenkamp/England
Light microscope	Olympus/Japan
Metallic loop	Himedia/ India
Micropipettes different sizes	Slammed /Germany
Micro spin Centrifuge	My Fugene / China
pH meter	lab tech/Korea
Quantus Fluorometer	Promega/ USA
Refrigerator	TEKA/Spain
Sensitive balance	Mettler/Switzerland
Serial swabs with transport media	Sterling Ltd /UK
Gradient Thermal Cycler	Eppendorf/Germany
VITEK 2 Compact Instrument	BioMerieux/France
Vortex mixer	Velp/Germany
Water bath	Gel/ Germany
Water distillatory	Gel/ Germany
Gel tube	HiLab/India
Qubit 4	Invitrogen/ USA
Sensitive balance	Denver/ European Union
Thermomixer	Eppendorf/Germany
Autoclave	Hirayama/ Japan
PCR Thermocycler	ThermoFisher/ USA
PCR Workstation	Cleaver / UK
Hot plate with Magnetic stirrer	Gallenkamp/ England
Ependroff tubes	Sterillin/UK
Microtiter plate 96 well	Bio Basic/ Canda
Shaker incubator	GFL/ Germany

3.1.2. Chemicals Materials

Chemicals used in this study and their manufacturer and suppliers are listed in (Table 3.2).

Table 3.2. Chemicals used in the study with their company and origin.

Substances	Company (Origin)
Absolute ethyl alcohol (99.9%)	Diamond (France)
Agar	Himedia (India)
Agarose	Cleaver (England)
Crystal violet	Himedia(India)
Deionized Distillate water	Bioneer (Korea)
DNA marker (100-1000)bp	NEB (England)
Ethanol 96%	Sigma (USA)
Ethidium Bromide	Promega (USA)
Free nuclease water	NEB (England)
Glycerol	Riedel-Dehaeny (Germany)
LL-37 Peptide	Eurogentec (Belgium)
Normal saline	Pioneer (Iraq)
Red safe dye	Intron (south korea)
Resazurin dye	Sigma-Aldrich (Germany)
SYBR green	Promega (USA)
TAE Buffer (50x)	Carl Roth (Germany)
TRIzol Reagent	Thermo Scientific (USA)

3.1.3. Culture media

Cultures media used in this study are listed in (Table 3.3).

Table 3.3. Culture media used in this study.

No.	Media	Company	Origin
1	Brain Heart Infusion Agar	Himedia	India
2	Cetrimide Agar	Himedia	India
3	MacConkey Agar	Himedia	India
4	Muller-Hinton Agar	Oxoid	England
5	Muller-Hinton Broth	Oxoid	England
6	Nutrient Agar	Himedia	India
7	Nutrient Broth	Mast	England
8	Tryptic Soy Broth	Himedia	India
9	Blood Agar	Himedia	India

3.1.4. Antibiotics

Antibiotic discs used in this study are listed in (Table 3.4).

Table 3.4. Antibiotic discs used in this study.

Antibiotics	Abbreviations	Company (origin)
Levofloxacin	LEV	Mast (UK)
Amikacin	AK	
Imipenem	IMP	
Ciprofloxacin	CIP	
Tobramycin	TOB	
Aztreonam	ATM	
Norfloxacin	NOR	
Ceftazidime	CAZ	
Piperacillin	PRL	
Meropenem	MEM	

3.1.5. Primers

Primers used in this study are listed in (Table 3.5).

Table 3.5. Primers utilized in this study (in PCR and gene expression).

Target gene		The nucleotide sequence (5'—3')	Size product (bp)	The reference
last	F	TCG ACG AGA TGG AAA TCG ATG	363	Int et al. (2021)
last	R	GCT CGA TGC CGA TCT TCA G		
rhlI	F	CGA ATT GCT CTC TGA ATC GCT	143	Int et al. (2021)
rhlI	R	GGC TCA TGG CGA CGA TGT A		
rspL	F	GCAACTATCAACCAGCTGGTG	241	Gong et al., (2014)
rspL	R	TCAGCACTACGCTGTGCTCTT		

3.1.6. Kits

All kits used in this study are listed in (Table 3.6).

Table 3.6. Kits used in this study

No.	Kits	Company	Origin
1	Genomic DNA Extraction Kit	iNtron	Korea
2	OneTaq® 2X Master Mix	NEB	England
3	Qubit™ dsDNA HS Assay Kit	ThermoFisher	USA
4	Qubit™ RNA HS Assay Kit	ThermoFisher	USA
5	Luna Universal qPCR MasterMix	NEB	England
6	ProtoScript® First Strand cDNA Synthesis Kit	NEB	England

3.2. Methods

3.2.1. Culture media

3.2.1.1. Laboratory-prepared culture media

All media listed in Table (3-3) were prepared following the instructions provided by the respective manufacturing companies. The constituents were dissolved in distilled

water (D.W.), and the pH was adjusted to 7.2 ± 0.2. The solution was then heated in a water bath to ensure complete dissolution of all constituents. Sterilization of the media was achieved by autoclaving at 121˚C for 15 minutes at a pressure of 15 pounds per square inch. After sterilization, the media were transferred to sterile Petri dishes. In cases where sterility needed to be confirmed, the media were incubated at 37 ºC for 24 hours.

3.2.1.2. Cetrimide Agar preparation

One liter of distilled water was used to dissolve 45.3 g of the medium, and 10 ml of glycerol was added and cooked to finish the process. Sterilize the item by autoclaving it for 15 minutes at 121 °C. The medium was poured into sterile Petri dishes after being chilled to about 50°C. The existence of growth indicates a favorable response. Upon closer inspection, the usual

Yellow-green to blue tint is visible, signifying the production of pyocyanin. *P. aeruginosa* strains generally produce both pyocyanin and fluorescein.

3.2.1.3. Blood agar preparation

The blood agar base was prepared according to the manufacturing company's instructions. Then autoclaved at 121ºC and 15 pounds/inch2 for 15 minutes, cooled to 50ºC; for each 95 ml of the medium, 5 ml of pure human blood was added aseptically, mixing well to homogeneity, poured into sterile Petri dishes. Finally, it cooled to 37ºC and was left to solidify at room temperature, then used for isolating and identifying the morphology and hemolysin-producing isolates.

3.2.2. Resazurin reagent

The resazurin (Alamar Blue) solution was prepared by dissolving 0.015 mg of resazurin in 100 ml sterile distilled water; a vortex mixer was used until well dissolved and stored at 4oC for one week after preparation.

3.2.3. Burn Samples Collection

In order to acquire a moist specimen, the area was first gently cleaned with dry, sterile gauze to remove contamination. The swab was then gently placed into the container and transported immediately to the lab for culturing. Between November 2021 and February 2022, burn swab samples were taken at the Al-Yarmook Teaching Hospital, the AL-Imam Ali Hospital, and the Baghdad Hospital of Medical City.

Swabs were transported from the hospital laboratory using transport media, cultured on MacConkey, Blood agar, and Cetrimide agar in the laboratory, then incubated at 37 °C for 24 hours.

3.2.4. Inoculums preparation

All susceptibility studies' inoculums were created with concentrations according to McFarland standard tube 0.5 (1.5* 108 CFU/ml) to standardize inoculums; this solution was used.

3.2.5. Bacterial identification

3.2.5.1. Cultural characteristics

Isolates were inoculated on various culture mediums, including Cetrimide agar, MacConkey agar, and Nutrient agar. Suspect colonies were recognized visually and biochemically after the media were incubated at 37 °C for 24 hours.

3.2.5.2. Identification of bacteria by VITEK-2 system

Forty-three non-enterobacterial gram-negative taxa were used to identify the VITEK 2 compact system (ID-GNB card). Testing was done as per the manufacturer's instructions. Strains were cultivated on MacConkey agar for 18 to 24 hours at 37°C shortly after the isolate was exposed to analysis. Using the VITEK 2 DensiCheck instrument (BioMe'rieux, France), a suspension of bacteria was calibrated to a McFarland standard of 0.50 to 0.63 in a solution of 0.45% sodium chloride.

The time it took to prepare the solution and fill the card was never more than one hour. Analysis was conducted using the ID-GNB card with 47 fluorescent biochemical tests for gram-negative bacteria. Cards were automatically read every 15 minutes. VT2-R03.1 of the VITEK 2 software was used to examine the data. The highly automated VITEK®2 System detected 39 *P. aeruginosa* isolates; results were available the same day (Brooks et al., 2013).

3.2.6. Maintenance and preservation of *P. aeruginosa*

3.2.6.1. Short term storage

The bacterial isolates were grown in a routine method every two weeks on nutrient agar and MacConkey agar plates at 37°C for 24 hours, and then the cultured plates were stored at 4 °C (WHO, 2003).

3.2.6.2. Medium term storage

Pure bacterial isolates were cultivated on nutrient agar in universal tubes and kept at 4 °C for three months (Prescott and Harley, 1996).

3.2.6.3. Long term storage

Glycerol (20%) was used to preserve P. aeruginosa. A loop of bacteria culture was added to 3 ml of brain heart infusion broth, and the mixture was then incubated for 18 hours between 30 and 37 °C. The chosen culture (2 ml) was then stored in a freezer for up to two years using flasks filled with sterilized glycerol (Rao et al., 2021).

3.2.7. Antibiotic susceptibility testing

The World Health Organization (WHO) recommended the Kirby-Bauer method for susceptibility testing, utilizing 12 different antibiotics. To prepare the bacterial suspension, 1-2 isolated bacterial colonies were selected from the original culture and added to a test tube containing 4 ml of normal saline. The resulting suspension was adjusted to a moderate turbidity level, approximately equivalent to the standard turbidity solution, corresponding to approximately 1.5 × 108 CFU/ml. A volume of the bacterial suspension was carefully and evenly spread over the Mueller-Hinton agar medium using a sterile cotton swab, and it was allowed to sit for 10 minutes. Subsequently, antimicrobial discs were firmly placed on the agar using sterile forceps to ensure contact with the substance.

The plates were then turned over and left to sit at 37°C for 18 to 24 hours. A metric ruler was used to measure the inhibition zones that developed around the discs by the Clinical Laboratories Standards Institute (CLSI, 2021).

The susceptibility of the isolate to specific medications was determined by comparing its inhibitory zones with the typical standards. The antibiotic discs used in this study included Amikacin (AK), Gentamicin (GM), Imipenem (IPM), Meropenem (MEM), Ceftazidime (CAZ), Azteronam (ATM), Ciprofloxacin (CIP), Tetracycline (T), Azithromycin (AZM), Levofloxacin (LEV), Cefepime (FEP), and Piperacillin-tazobactam. After incubating the agar plates at 37 °C for 24 hours, the inhibition zones were measured and interpreted using the CLSI breakpoint interpretation criteria, which determined the percentage of isolates classified as susceptible, intermediate, or resistant (CLSI, 2021).

3.2.8. Assessment of biofilm formation

To quantify *P. aeruginosa* biofilm production, the microtiter plate method was employed as described by Patel et al. (2016). The isolates were cultured overnight in Brain Heart Infusion Broth at 37°C. Each isolate was then added to tryptic soy broth (TSB) containing 1% glucose and thoroughly mixed using a pipette. The suspension of the bacterial isolate was adjusted to McFarland No. 0 turbidity.

Triplicate cultures of each isolate, in a volume of 200 µl, were added to sterile 96-well microtiter plates with U-shaped bottoms. The plates were covered with lids and incubated aerobically at 37°C for 24 hours. After incubation, the plates were rinsed twice with distilled water to remove non-adherent bacteria. The adhering bacterial cells in each well were fixed with 100% methanol (200 µl) at room temperature for 20 minutes. Then, 0.1% crystal violet (200 µl) was added to stain the adherent cells, and the plates were left undisturbed for 15 minutes. After staining, the excess stain was removed by washing the plates with distilled water (2–3 times). The plates were air-dried at room temperature for approximately 30 minutes.

To remove the stain, 33% acetic acid was added. Optical density (OD) readings were measured using an ELISA auto reader at a wavelength of 630 nm. The average OD values of the sterile medium were calculated and subtracted from all test values. A cut-off value (ODc) was determined, which allowed for the classification of isolates as biofilm producers or non-producers. ODc: Average OD of negative control + (3 × standard deviation (SD) of Negative control), OD isolate: Average OD of isolate – ODc. Classification of bacterial adherence is summarized in Table (3-7) based on OD values.

Table 3.7. Classification of bacterial adherence

Mean OD630	**Biofilm intensity**
OD ≤ ODc*	Non-Biofilm
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD > 4ODc	Strong

*Cut off value (ODc) = Average OD of negative control + (3 × standard deviation (SD) of Negative control)

3.2.9. Assay of antibiofilm ZnO-NPs by microtiter plate method

The antibiofilm potential of ZnO-NPs against clinical isolates was characterized following the method described by Abdelghafar et al. (2022). Briefly, overnight cultures of bacteria were diluted to a 1:100 ratio in Tryptic Soy Broth (TSB). Aliquots of approximately 100 µL were added to the wells of a sterile microtiter plate and incubated

for 48 hours to allow biofilm formation. To assess the biofilm eradication activity of ZnO-NPs, aliquots of different concentrations (256, 128, and 64 µg/mL, corresponding to 3.1, 1.57, and 0.78 mM, respectively) of ZnO-NPs were added to the plate. Negative and positive control wells, without bacterial culture and without ZnO-NPs, respectively, were included for each isolate. The plate was then incubated at 37°C for 24 hours, and the impact of ZnO-NPs on bacterial biofilm eradication was evaluated as described previously. Each assay was performed in triplicate, and the percentage of biofilm inhibition was calculated.

3.2.10. Molecular assay

3.2.10.1. Extraction of genomic DNA

A commercial purification method, the Genomic DNA Extraction Mini Kit (iNtron®, Korea), was used to extract DNA from *P. aeruginosa* bacteria. This kit was made to isolate DNA from both Gram-positive and Gram-negative bacteria. Using the bacterial methodology (for Gram-negative bacteria), which can be summed up as follows:

1. To fill a 1.5 ml micro-centrifuge tube, 1 ml of an overnight suspension bacterial cell culture (up to 1×109 CFU/ml) was added.
2. Centrifuge cells in a microcentrifuge tube for 1 minute at 13,000 rpm. by vortexing or repeatedly tapping, collected cells were restored to suspension.
3. G-Buffer solution was aliquoted at 300 µl and thoroughly inverted- mixed.
4. It was incubated at 65°C for 15 min.
5. Binding Buffer was then added to 250 µl and thoroughly mixed by pipetting (at least ten times) or gently vortexing.
6. Cell lysates were loaded onto the column and centrifuged for 1 minute at 13,000 rpm.
7. 500 µl of Washed Buffer A was added to the column, and the centrifuge was run at 13,000 rpm for 1 minute.
8. Add 500 µl of Washing Buffer B to the column and centrifuge for 1 min at 13,000 rpm.
9. Removing solution and centrifuge for 1min at 13,000 rpm.
10. Place the G-spinTM column in a clean 1.5ml micro-centrifuge tube (not supplied) and pour 100 µl of Elution Buffer directly onto the membrane.
11. Incubated at room temperature for 1 min and then centrifuged for 1 min at 13,000 rpm.

12. Until PCR analysis was performed, the DNA was kept in a deep freezer.

3.2.10.2. dsDNA quantitation by qubit 4.0

The assay is accurate for initial sample concentrations of 10 pg/ to 100 ng/µL and is highly selective for double-stranded DNA (dsDNA) over RNA. The test is run at room temperature, and the signal is consistent for three hours. The assay tolerates common impurities, including salts, free nucleotides, solvents, detergents, or proteins.

1. The Qubit® dsDNHS Reagent was diluted 1:200 in the Qubit® dsDNA HS buffer to create the Qubit® working solution.
2. The volume of the Qubit® working solution was added to each standard- sized tube along with 190 µL, followed by 10µL of each standard solution and vortexing.
3. Each tube prepared for the sample was filled with 197 µL of the Qubit® working solution, and then 3 µL of the sample was added one at a time.
4. The mixture has been vortexed and given a 3-minute incubation at room temperature.
5. In order to create a concentration curve, standard tubes have been put into the Qubit device.
6. Tubes for samples have been added individually to read the concentration for dsDNA in each sample.

3.2.10.3. Primer preparation

Al Qubit DNA-USA Company supplied the primers as lyophilized products of different picomoles concentrations. Al Qubit DNA USA company protocol was adopted for primers preparation by adding the demand volume of nuclease-free water to the lyophilized primers to get a stock solution of primers with 100 pmol/µl concentration. A working solution of these primers was prepared by adding 10µl of primer stock solution to 90µl of nuclease-free water to obtain a working primer solution of 10 pmol/µl.

3.2.10.4. Molecular Detection of rspL, last, and rhlI genes by PCR

In order to complete this stage, 12.5 µl of OneTaq (NEB®) master mix, 3 DNA samples, 1µl of each primer containing ten pmol/ µl of DNA, and 7.5 µl of free-nuclease water was added. The reaction was carried out under the best PCR conditions, as indicated in Table (3.8).

Table 3.8. PCR conditions for rspL gene*.

Stage	Temperature	Time	Cycle No.
Initial Denaturation	94 ºC	5 min.	1
Denaturation	94 ºC	45 sec.	35x

Stage	Temperature	Time	Cycle No.
Annealing	60 ºC	45 sec.	
Extension	72 ºC	45 sec.	
Final Extension	72 ºC	7 min.	1

*The exact conditions for other genes lasI and rhlI, except the annealing temperature, were 58 ºC and 56 ºC, respectively.

3.2.10.5. Protocol of Gel Electrophoresis

I- Preparation of 1X TAE Buffer

By adding 200 ml of 50X TAE to 800 ml of deionized distilled water (ddH2O), 200 ml of (TAE) buffer 50x (0.08 M Tris, 0.08 M Acetic acid, and 0.02 M EDTA) was diluted to 10X. This 10X buffer was then reto 1X (working concentration) by adding 100 ml to 900 ml of deionized distilled water (ddH2O).

II-Preparation of Agarose Gel 2%

1. A volumetric cylinder was used to measure 60 microliters of 1X TAE buffer.

2. Powdered agarose 1.2 gm for (2%) and 1.8 gm for (3%) was weighed and placed in a flask

3. 1X TAE buffer has been poured onto agarose to dissolve it.

4. The solution was brought to a boil using a microwave oven, and all gel particles were melted.

5. The solution was let to cool down to 70 °C, and 4 µl RedSafe dye was added as a DNA staining dye.

6. The temperature of the solution was down to 50 °C.

7. In the gel tray, the agarose solution was poured. For 30 minutes, the solution was allowed to solidify at room temperature (20-25ºC).

8. The tank was filled with 400 ml of 1X TAE.

III-Loading of Samples into Gel

1. DNA ladder and ten microliters of PCR product have been added to the gel's wells.

2. For 80 minutes, the power supply's voltage has primarily remained constant at 80V.

3. Gel documentation using a high-definition camera was utilized to take pictures and evaluate the bands after the run.

3.2.11. Gene expression Analysis Using qRT PCR Technique

The evaluation of the gene expression of the two genes was done before and after the treatment with the drugs to determine the impact of the LL-37 peptide alone

and in combination with Ciprofloxacin on the gene expression of las I and las R linked to biofilm formation. The LL-37 peptide alone and combined with Ciprofloxacin were employed at sub-MIC doses to promote bacterial growth.

3.2.11.1. RNA isolation by TRIzol

1. Bacterial cells were collected in a microcentrifuge tube by centrifuging for 1 minute at 13,000 rpm and repeating this process until a sufficient amount of pellet was obtained.

2. 0.5 ml of Trizol was used to resuspension the pellet.

3. The nucleoproteins complex must be completely dissociated after five minutes of incubation.

4. The TRIzol® Reagent used for lysis contained 0.15 ml of chloroform that was added.

5. Incubation for 2–3 minutes.

6. The material was centrifuged at 12,000 g for 15 minutes. The mixture was divided into an upper colorless aqueous phase and a lower red phenol- chloroform phase.

7. The RNA-containing aqueous phase was transferred to a fresh tube.

8. The RNA was precipitated by adding 0.45 ml of isopropanol to the aqueous phase.

9. Ten minutes were spent incubating the mixture.

10. Then centrifuged at 12,000 g for 10 minutes. The pellet of total RNA precipitate at the tube's bottom is white and gel-like.

11. Using a micropipette, the supernatant was discarded.

12. The supernatant gets re-suspended by 0.75 ml of 75% ethanol.

13. The vortex was used to dissolve the pellet, which was then centrifuged for 5 minutes at 7500 g.

14. The supernatant was discarded by micropipette.

15. The tube was left open for 15 minutes to dry the RNA pellet.

16. The pellet was then re-suspended in 20 µl of RNase-free water and heated to 60 C using a thermomixer for 15 minutes.

17. Total RNA samples are stored at -20C until processed to the downstream application.

a) RNA Quantitation by Qubit 4.0

The assay is highly selective for RNA and accurate at concentrations ranging from 10 pg/µL to100 ng/µL. The assay is carried out at room temperature, and the signal remains stable for three hours. The assay tolerates common contaminants such as salts, free nucleotides, solvents, detergents, and proteins.

1. The Qubit® RNA HS Reagent was diluted 1:200 in Qubit® RNA HS buffer to create the Qubit® working solution.

2. A volume of 190 µL of Qubit® working solution was added to each tube designed to be a standard, followed by 10 µL of each provided standard solution, which was vortexed.

3. The Qubit® working solution in 197 µL was added to each sample tube, and then 3 µL of the sample was added individually.

4. All composition was vortexed and incubated at room temperature for 3 mins.

5. In order to create a concentration curve, standard tubes were put into the Qubit device.

6. Sample tubes were introduced one at a time to measure the RNA concentration in each tube.

b) Quantitative Real-time PCR Assay (RT-qPCR) protocol

The primary step in our project was divided into two phases: synthesizing cDNA from RNA using specific primers for lasI, lasR, and 16S rRNA transcripts and proscript cDNA.

This procedure has performed through the steps:

1. Five microliters of total RNA extracted from each sample were added to a new PCR tube.

2. A 10ul proscript reaction mix that contains a buffer, dNTPs, and other necessary ingredients added to each sample.

3. MuLV Enzyme was then added into the reaction as 2ul per sample.

4. Two microliter-specific primers and the volume completed up to 20 µl by adding 1µl.

5. Using a thermocycler, this combination was incubated for 1 hour at 42C before being heated to 80 ºC to inactivate the enzyme. When conducting the second phase, the quantification of the cDNA product was also carried out using Qubit 4.0. (Relative quantitative PCR).

In the second step of this process, samples of bacteria and the control were selected on the same run, and for each sample, three PCR tubes were used, one for each of the genes las I, las R, and 16srRNA, which is regarded as a housekeeping gene in this study. Based on SyberGreen's fluorescence power, amount detection. The table below (3-9) lists the reaction mix components and their corresponding quantities.

Table 3.9. Volumes and concentrations of the qPCR reaction mix

Component	**20ul reaction**
Luna universal qpcr master mix	10ul
Forward primer (10um)	1ul
Reverse primer (10 um)	1ul
Template DNA	5ul
Nuclease-free water	3ul

Quickly spin for PCR tubes to remove the bubbles and collect the liquid (1 minute at 2000g, then the program for Real-Time PCR was setup with indicated thermocycling protocol as shown in Table 3.10.

Table 3.10. RT-PCR cycling program

Cycle Step	**Temperature**	**Time**	**Cycles No.**
Initial Denaturation	95 °C	60 seconds	1
Denaturation	95 °C	15 seconds	40
Annealing	60 °C	30 seconds	-
Extention	60 °C	30 seconds	-
Melt Curve	60-95 °C	40 minutes	1

c) Calculations of delta delta Ct (ΔΔCt) method

The simplest method is this one, which directly compares the Ct values of the target and reference genes. The selection of a calibrator sample is necessary for relative quantification. Any sample you want to compare your unknown to can be used as the calibrator sample, including the untreated and time=0 samples.

First, for each sample, the Ct between the target gene and the reference gene is computed (for the unknown samples and the calibrator sample).

ΔCt = Ct target-Ct reference gene

Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value:

ΔΔCt = (Ct target-Ct reference) sample-(Ct target-Ct reference) calibrator

The normalized target amount in the sample is then equal to $\mathbf{2^{-\Delta\Delta Ct}}$, which can be used to compare expression levels in samples (Schmittgen et al., 2008).

The samples were analyzed in triplicates and standardized against 16S rRNA gene expression. The relative changes in mRNA expression levels were determined using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$). Between the peptide-exposed and antibiotic-non-exposed *P. aeruginosa*.

3.2.12. After adding the ZnO-np

Muller Hinton broth tubes were prepared with the appropriate peptide concentration and incubated at 37°C for 24 hours to monitor the bacterial growth in media.

1. After growth, RNA extraction was done by the same steps by the SV Total RNA Isolation System kit.

2. The same primers, RT master mix, and programs were used as before; add the ZnO-np.

3.2.13. Statistical Analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of different factors on study parameters. To make a significant comparison between means, the T-test was used. This study used the Chi-square test to compare percentages (0.05 and 0.01 probability).

4. RESULT AND DISCUSSIONS

4.1. Description and Distribution of the Study Samples

The current study included the collection of (120) clinical specimens were collected from patients suffering from different infections; (31) urine, (21) burn swabs, (43) wound swabs, (9) blood, (6) sputum, (6) ear swabs and (4) fluids as seen in Figure (4.1). These specimens were collected from patients admitted to four Baghdad hospitals.

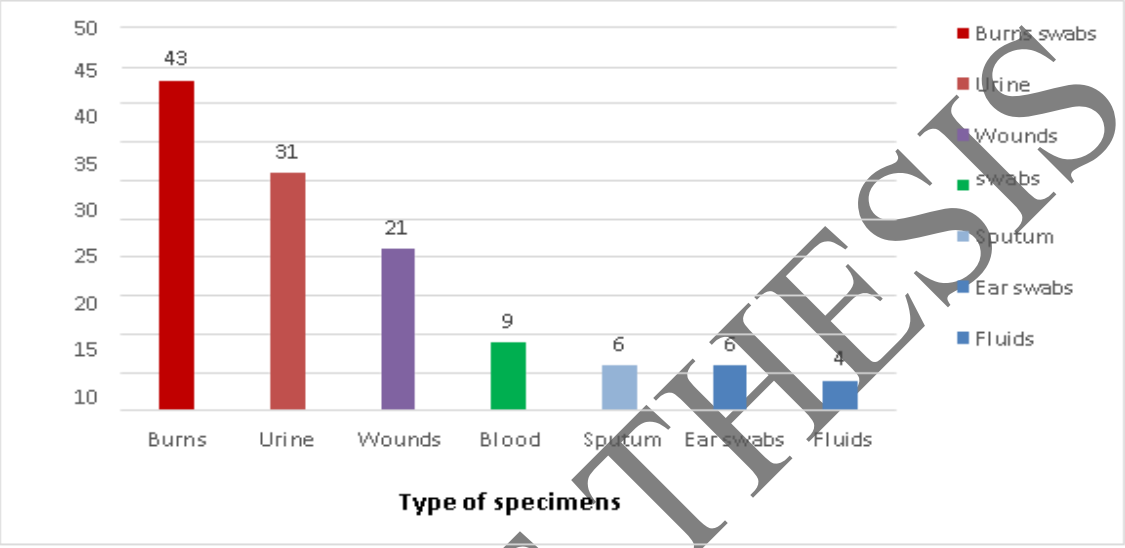

Figure 4.1. Number and prevalence of specimens collected from patients in the current study.

4.2. Isolation and Identification of *Pseudomonas aeruginosa*

4.2.1. Cultural characteristics

All 120 samples were cultured by streaking on Blood, MacConkey, and Cetrimide agar and incubated for 24 hours at 37 °C. The findings showed that the colony on the blood agar is composed of bacteria with sticky textures, a white to a gray hue, and the ability to hemolysis blood, and the type of hemolysis was beta. According to Korgaonkar et al. (2013), *P. aeruginosa* colonies on blood agar frequently display beta hemolysis, a metallic sheen, and can exhibit blue or green pigment. On MacConkey agar, the colonies appear as lactose non-fermenting with small, pale colonies, as depicted in Figure (4.2).

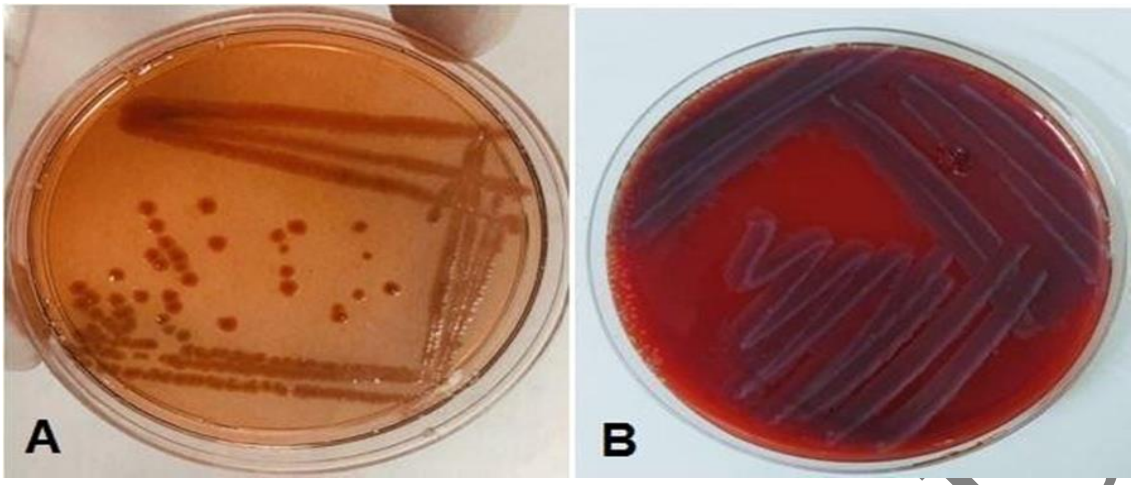

Figure 4.2. Colonies of *P. aeruginosa* on (A) macconkey agar and (B) blood agar after incubation at 37˚C for 24 hrs.

The combination of two metabolites produced by P. aeruginosa, pyocyanin (blue) and pyoverdine (green), gives rise to distinctive coloration in cultures, such as the colonies observed on Cetrimide Agar. Cetrimide Agar is a selective and differential medium utilized for the isolation and identification of *P. aeruginosa* from both clinical and non-clinical specimens. Cetrimide acts as a selective agent, inhibiting the growth of most bacteria by functioning as a detergent (specifically, Cetyltrimethylammonium bromide, a quaternary ammonium cationic detergent) (Priyaja et al., 2014). Positive results for *P. aeruginosa* are indicated by colonies displaying a yellow-green to blue coloration, as illustrated in Figure (4.3).

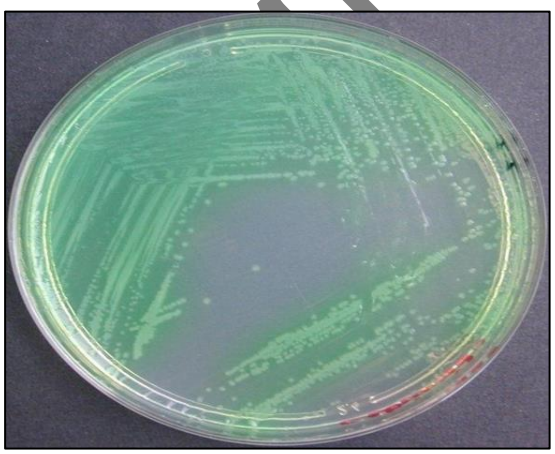

Figure 4.3. Colonies of *P. aeruginosa* on Cetrimide Agar after incubation at 37˚C for 24 hrs.

This outcome is comparable to that of the work conducted by AL- Rubaye et al. (2015), who identified Pseudomonas using cetrimide agar and different media. The Pseudomonas spp. Colonies on this medium have a mucoid, smooth shape with flat

sides and a raised core that has a fruity scent. The growth of *P. aeruginosa* colonies on Nutrient agar was characterized by the development of pigments and a distinctive grape-like odor, with the colonies exhibiting a greenish coloration, consistent with previous studies (DeBritto et al., 2020). These bacterial colonies displayed Beta-hemolysis (β) on blood agar, indicating the production of hemolysin (Salm et al., 2016). They also produced a blue and green pigment known as pyocyanin, while none of the isolates grown on King B agar showed pyocyanin production. This difference is attributed to the presence of adequate concentrations of potassium and magnesium salts in King A medium, which inhibit the development of fluorescein (Pyoverdin) in King B medium. The production of King B agar is a distinctive feature that differentiates *P. aeruginosa* from *P. fluorescens* (Douraghi et al., 2014).

4.2.2. Biochemical Tests

Single greenish colonies were selected from sterile cetrimide agar plates and inoculated into slants for further characterization using various biochemical tests. These tests included the oxidase test, catalase test, nitrate reduction, indole test, methyl red test, Voges-Proskauer test, citrate utilization, and glucose fermentation test, as described by Banerjee et al. (2017). Positive samples were tested twice to ensure accurate results. The confirmation of the identification of all *P. aeruginosa* isolates was performed based on the results obtained from the VITEK 2 System.

4.2.3. The frequency of *Pseudomonas aeruginosa*

According to the results of biochemical tests and the VITEK 2 system, of all 120 clinical specimens, 55 *P. aeruginosa* isolates (45.8%) were found in all samples taken from four hospitals in Baghdad. The prevalence of these isolates was 28 (50.9%) in female patients, compared to 27 (49.0%) in male patients, as shown in table (4- 1). The highest rate of bacterial infection was within the age group 31(20-30year), followed by 12 (31-40year), 10(41-50year), and 2(61- 70year), respectively; a significant difference ($P<0.001$) in the age was observed, as shown in Table (4.1).

Table 4.1. The frequency of *P. aeruginosa* according to the gender

Gender	Number (%)	Chi-square	P. Value
Male	27 (49.0%)	3.8	0.147
Female	28 (50.9%)		

Table 4.2. The frequency of *P. aeruginosa* according to age group.

Group		Number	Frequency
Age (years)	20-30	31	0.6
	31-40	12	0.2
	41-50	10	0.2
	51-60	0	0
	61-70	2	0
Chi-square		53.4	
P. Value		0.001	

The findings of this study align with Hussein et al. (2012), who reported that the highest incidence of burn-related inflammation occurred among individuals aged 21 to 30. Similarly, Muhammad et al. (2014) found that the age group of 11 to 20 years had the highest rate of bacterial infections causing wound infections in Baghdad City, with *P. aeruginosa* accounting for 53% of the infections (36.05%). In another study, 105 burn wound swabs were collected from burn patients admitted to the burn unit of Al-Sadr Teaching Hospital in Misan City, Iraq. Bacteria were identified using the VITEK-2 Compact System, and a total of nine distinct bacterial isolates were identified. Among them, *P. aeruginosa* was the most common pathogen, accounting for 20% of the isolates, followed by Staphylococcus aureus at 17.14% (Rahim, 2021).

In their study, Alshaiki and Toweir (2017) observed that wound swabs yielded the highest number of *P. aeruginosa* isolates. They found that urine isolates were the most common (28.6%), followed by pus isolates (27.5%), pus (14%), blood (7.8%), cerebrospinal fluid (CSF) (5%), ear swabs (4.4%), endotracheal tubes (4.4%), sputum (2.2%), throat swabs (1%), plural fluid (1%), bed sores (1%), and abscess swabs (1%). These findings align with the results of our study.

The high prevalence of *P. aeruginosa* in infected urine suggests it is a common pathogen. *P. aeruginosa* was the primary cause of urological and surgical infections (Reynolds and Kollef, 2021). Our findings are consistent with those reported by Ahmed and Ahmed (1995). The findings of the current study are in line with numerous local, regional, and international studies that have consistently identified *P. aeruginosa* as the most commonly isolated bacteria in various clinical contexts. For example, a study conducted in a hospital in Baghdad by AL-Kaisse et al. (2015) reported that burn injuries and wounds accounted for over 30% of all swabs, with *P. aeruginosa* being isolated in 52.5% of cases. Similarly, in India, *P. aeruginosa* was found in 46% of wound and burn cases, as reported by Augustine et al. (2015). Patients with burn

injuries are particularly susceptible to infectious complications, and infections are a leading cause of mortality, especially beyond the first 72 hours of hospitalization, particularly in tertiary-care burn intensive care units. Of concern is the prevalence of multidrug-resistant (MDR) Gram-negative bacteria (GNB) causing hospital-acquired infections in this population. *P. aeruginosa* colonization is commonly observed in burn patients, and there is evidence of patient-to-patient transmission of highly resistant GNB, as highlighted by Ruegsegger et al. (2022).

4.3. Detection of biofilm formation

This study aimed to quantify the biomass of biofilm attached to the walls of the microtiter plate. The quantification was performed by measuring the amount of biofilm formers in each well after removing the planktonic cells and lightly staining the surface-attached bacterial cells. The present study showed that ten isolates from a total of 55 isolates (18.2%) were strong biofilm producers. Also, it was found that 21(38.2%) isolates were moderate biofilm producers, and the other isolates (n=24; 43.6%) were weak for the production of the biofilm.

Table 4.3. Strong biofilm production among ten *P. aeruginosa* isolates.

Control (negative)	Control (positive)	OD of isolate	Result
0.118	2.01	1.81	strong
0.113	1.188	0.988	strong
0.145	1.243	1.043	strong
0.108	2.143	1.943	strong
0.103	1.098	0.898	strong
0.109	1.067	0.867	strong
0.112	1.714	1.514	strong
0.147	1.088	0.888	strong
0.11	1.247	1.047	strong
0.18	1.039	0.839	Strong
0.1245 (AV.),	0.024771 (SD),	0.198	0.2 (cut off)

According to Kamali et al. (2020), 83.75% of *P. aeruginosa* isolates exhibited biofilm phenotypes, with the distribution as follows: 16.25% produced strong biofilm, 33.75% produced moderate biofilm, 33.75% produced weak biofilm, and 16.25% were identified as non-biofilm producers. Additionally, Mohammad (2013) reported that 68.75% of *P. aeruginosa* isolates had the ability to produce biofilm. Biofilm formation

is a common survival strategy for bacteria in challenging environments, allowing them to form biofilms in water systems and on various abiotic surfaces in natural aquatic environments and water systems (Bronowskil et al., 2014). The ability of *P. aeruginosa* to form biofilms is a significant factor in its virulence and persistence in environments such as burn wounds, leading to chronic infections (De Almeida Silva et al., 2017). Previous studies have demonstrated a correlation between multidrug-resistant phenotypes of *P. aeruginosa* and their ability to form biofilms (Yekani et al., 2017).

In burn infections, *P. aeruginosa* can persist due to its ability to secrete extracellular polymeric substances, including exopolysaccharides, matrix proteins, and extracellular DNA, which contribute to biofilm formation. Biofilm formation confers multiple antibiotic resistances in P. aeruginosa, presenting a significant challenge for conventional single-antibiotic therapeutic approaches (Yin et al., 2022). Asadpour's (2018) study revealed a high frequency of multidrug resistance and a significant presence of virulence-associated genes in *P. aeruginosa* isolates from burn infections. Furthermore, more than 50% of P. aeruginosa isolates exhibited biofilm- and virulence-associated genes, with the toxic and last being the most frequent. All virulence genes were more commonly found in biofilm-forming and multidrug-resistant phenotypes.

4.4. Inhibitory effect of Zinc oxide nanoparticles on biofilm formation

The impact of Zinc oxide nanoparticles on biofilm formation was examined, and the results revealed a significant increase ($p<0.01$) in *P. aeruginosa* biofilm before the addition of Zinc oxide nanoparticles compared to after their addition. This is depicted in Figure 4.4. and Figure 4.5.

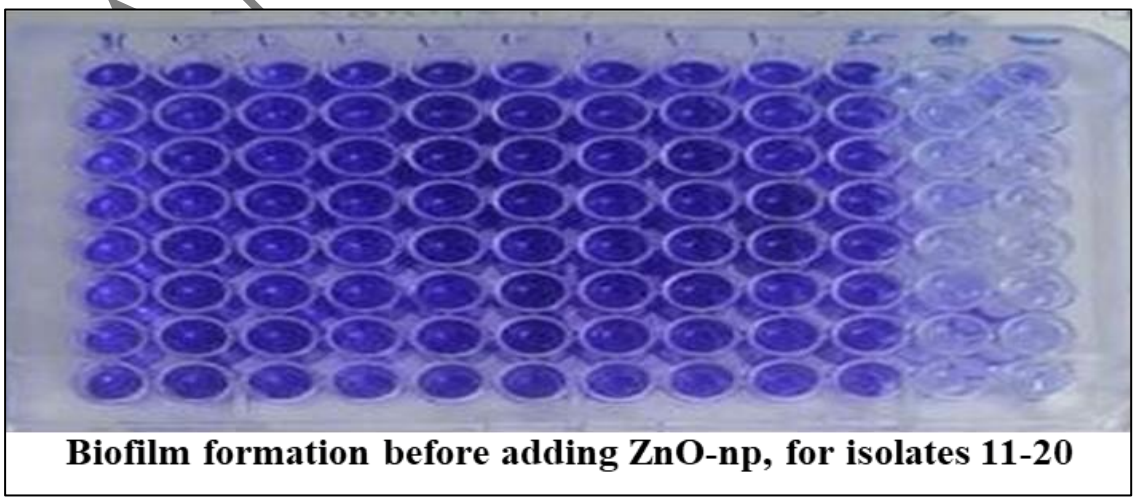

Figure 4.4. Inhibition of biofilm formation by *P. aeruginosa* by zinc oxide nanoparticles

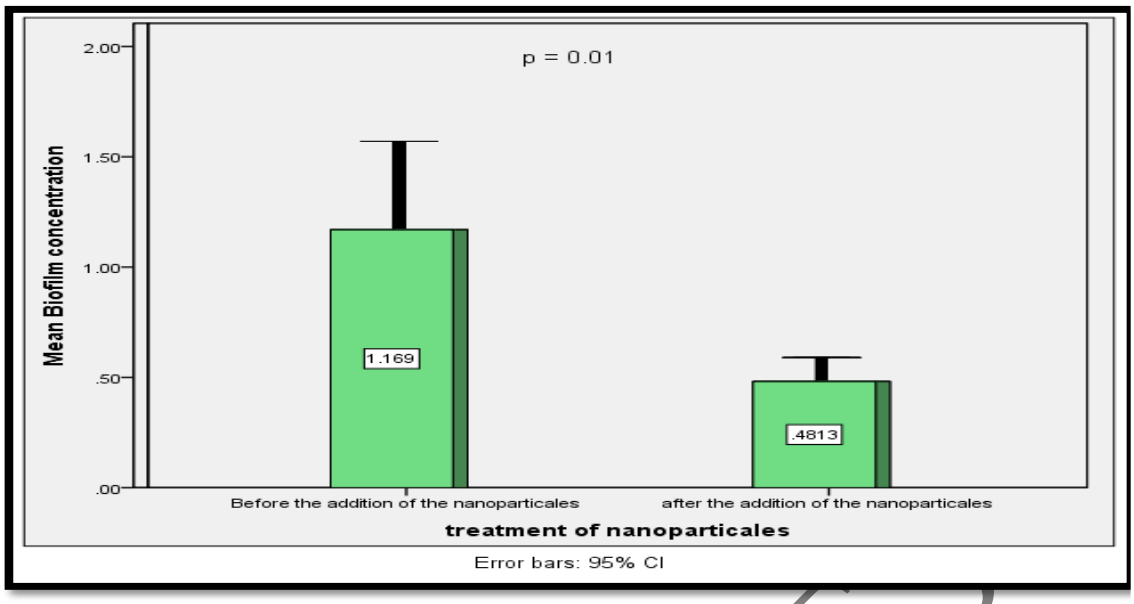

Figure 4.5. Antibiofilm activity of zinc oxide nanoparticles in *P. aeruginosa* isolates

Zinc oxide nanoparticles have been reported to possess superior antibacterial properties, as highlighted in the study by Sangeetha et al. (2012). Aysa et al. (2016) conducted a study to evaluate the antibacterial activity of ZnO nanoparticles against important and pathogenic bacteria such as P. aeruginosa, which aligns with the objectives of our study.

In a study by Sangani et al. (2015), it was observed that the impact of ZnO nanoparticles on *P. aeruginosa* isolates increased steadily with increasing concentrations of ZnO nanoparticles. While Yousef et al. (2012) reported an inhibition zone of 22 mm for nano ZnO at a concentration of 20 g/ml (using a single concentration of ZnO), our study yielded an average inhibition zone of 19.7 mm at the same concentration of nano ZnO for *P. aeruginosa* isolates. It is worth noting that Yousef et al. (2012) used only one sample of *P. aeruginosa* isolates in their study.

4.5. Resistance of *P. aeruginosa* Isolates to Antibiotics

The Modified Kirby-Bauer method, also known as the disk diffusion method, was employed to evaluate the antibacterial susceptibility of *P. aeruginosa* isolates to ten antibiotics. The results of the current study demonstrated significant variations in antibiotic resistance among all *P. aeruginosa* isolates, as indicated in Table 4-4. Notably, the antibiotics Levofloxacin, Ceftazidime, Imipenem, Tobramycin, and Aztreonam exhibited the highest levels of resistance, with rates of 89%, 89%, 80%, 73%, and 71% respectively, as determined by this research.

Table 4.4. The percentages of antibiotic susceptibility of *P. aeruginosa* isolates against 10 antibiotics.

Antibiotics	Abbreviation	Resistant (%)	Sensitive (%)
Levofloxacin	LEV	**49 (89)**	6 (11)
Amikacin	AK	24 (44)	31(56)
Imipenem	IMP	**44 (80)**	11 (20)
Ciprofloxacin	CIP	15 (27)	**40 (73)**
Tobramycin	TOB	40 (73)	15 (27)
Aztreonam	ATM	39 (71)	16 (29)
Norfloxacin	NOR	24 (43)	31(56)
Ceftazidime	CAZ	**49 (89)**	6 (11)
Piperacillin	PRL	15 (27)	**40 (73)**
Meropenem	MEM	15 (27)	**40 (73)**

The findings of the present study align with those of Al-Shwaikh (2018), which reported high resistance rates among *P. aeruginosa* isolates to Ceftazidime (81%), Cefotaxime (78%), Piperacillin (76%), Ciprofloxacin and Tobramycin (74%), Gentamicin (72%), Amikacin and Meropenem (70% each), and Ofloxacin and Gentamicin (65% each). Additionally, in a separate study conducted in four Baghdad hospitals, *P. aeruginosa* was isolated from 31.46% of burn swabs, with 100% of the isolates being resistant to Cefotaxime, Cephalothin, Gentamicin, and Trimethoprim. Furthermore, 55% of the isolates showed resistance to Amikacin and 88% to Ciprofloxacin. These resistance patterns are consistent with the resistance rates observed in the current study of *P. aeruginosa* isolates (Al-Taie et al., 2014).

Carbapenems (meropenem), cephalosporins (like ceftazidime and cefepime), aminoglycosides (tobramycin and amikacin), and fluoroquinolones (like ciprofloxacin and levofloxacin) are often used as an initial treatment before the findings of the clinical specimen culture and antibiotic susceptibility testing are completed in the medical laboratory. However, due to the advent of antibiotic-resistant bacteria, it has become challenging to maintain control over these bacteria and complete patient therapy (Wilson and Pandey, 2020).

A study conducted by Al-Shwaikh et al. (2018) utilized the VITEK 2 system to determine the minimum inhibitory concentration (MIC) values for 69 *P. aeruginosa* isolates obtained from patients with wounds and burns in Baghdad. The study reported resistance rates of 87% for Ticarcillin (8µg/mL), 85.5% for Tazobactam/Piperacillin (8-16µg/mL), Piperacillin (≤4-16µg/mL), and Ticarcillin/Clavulanic acid (16-46µg/mL), and 76.8% for Cefepime (2-8µg/mL). The resistance rate for Ciprofloxacin was 1%

(≤0.25-0.5µg/mL), while Ceftazidime had a resistance rate of 63.8% (2-8µg/mL). In a study conducted on Iranian patients with burn and wound infections, Shilba et al. (2015) found that Gentamicin had the highest resistance rate at 91.67%, followed by Meropenem at 66.67%. These findings are largely consistent with the results of the current study. Regarding Colistin, the current study revealed a resistance rate of 18%, which is similar to the findings reported by Mohanty et al. (2013).

A study conducted by Nasrin et al. (2022) analyzed 413 *P. aeruginosa* isolates obtained from the blood and cerebrospinal fluid of patients from 10 countries between 2005 and 2017. The study found that the most common resistance was observed against aztreonam (56%), followed by levofloxacin (42%). Furthermore, 22% of the strains showed non-susceptibility to meropenem and piperacillin-tazobactam. Among the collected isolates, 27% demonstrated resistance to multiple antibiotics.

Another study by Rashid and Mansour (2022) evaluated the antibiotic sensitivity of 31 *P. aeruginosa* isolates against 17 antimicrobial agents. The findings indicated a high resistance rate to gentamicin, trimethoprim, amikacin, and amoxicillin, while a low resistance rate was observed for ceftazidime, tobramycin, levofloxacin, cotrimoxazole, ciprofloxacin, and aztreonam.

4.6. Molecular Detection of *rspL* Gene and Quorum Sensing Genes (*lasI and rhlI*)

The isolates of *P. aeruginosa* were the strong, moderate, and weak biofilm used in this study and detected by PCR technique using the rspL gene as the housekeeping gene of P. aeruginosa. All the tested *P. aeruginosa* clinical contained the rspL gene (241bp) (Figure 4.5).

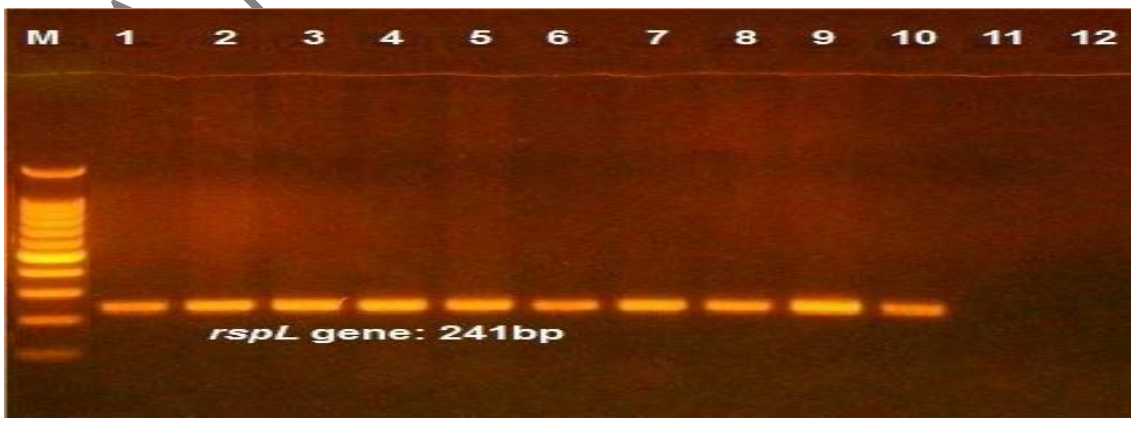

Figure 4.6. The amplification of the rspL gene of *P. aeruginosa* samples was fractionated on 2% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-10 resemble 241bp PCR products.

PCR technique detected two genes, lasI, and rhlI, that seem to be involved in the clinical quorum sensing of P. aeruginosa. The gel electrophoresis of amplified PCR product for lasI is shown in figure (4-6) and rhlI figure (4-7). The results showed that the lasI gene was found in 80% of isolates that produce strong biofilm, while the rhlI gene was found in all potent biofilm isolates.

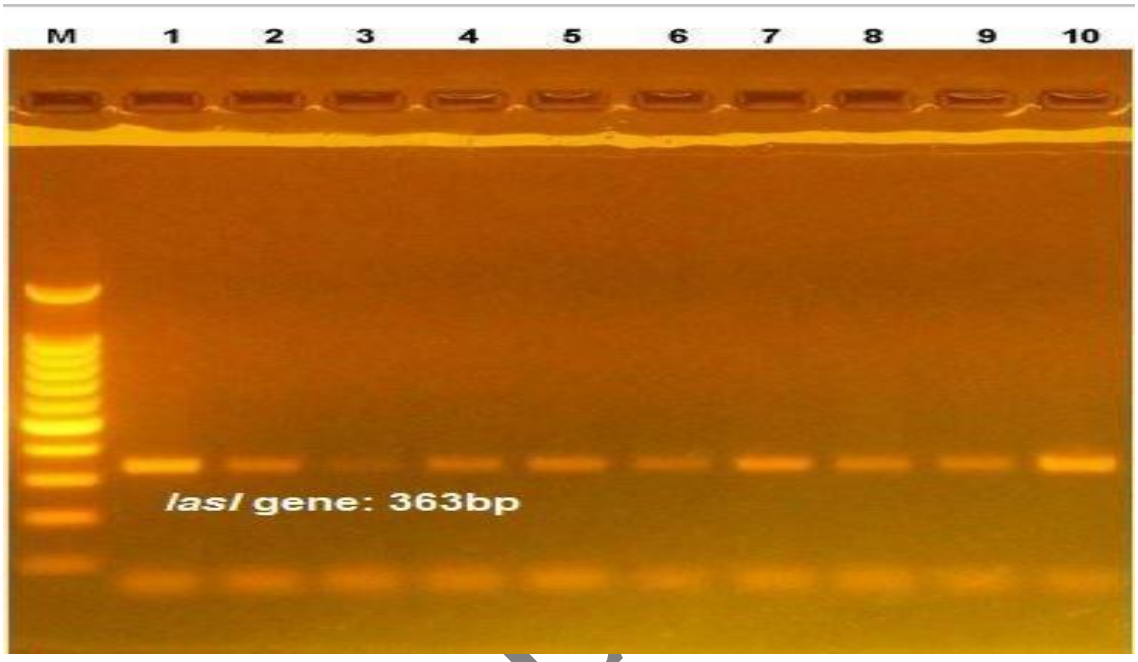

Figure 4.7. Gel electrophoresis amplification of lasI gene of *P. aeruginosa* samples were fractionated on 2% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-32 resemble 363bp PCR products.

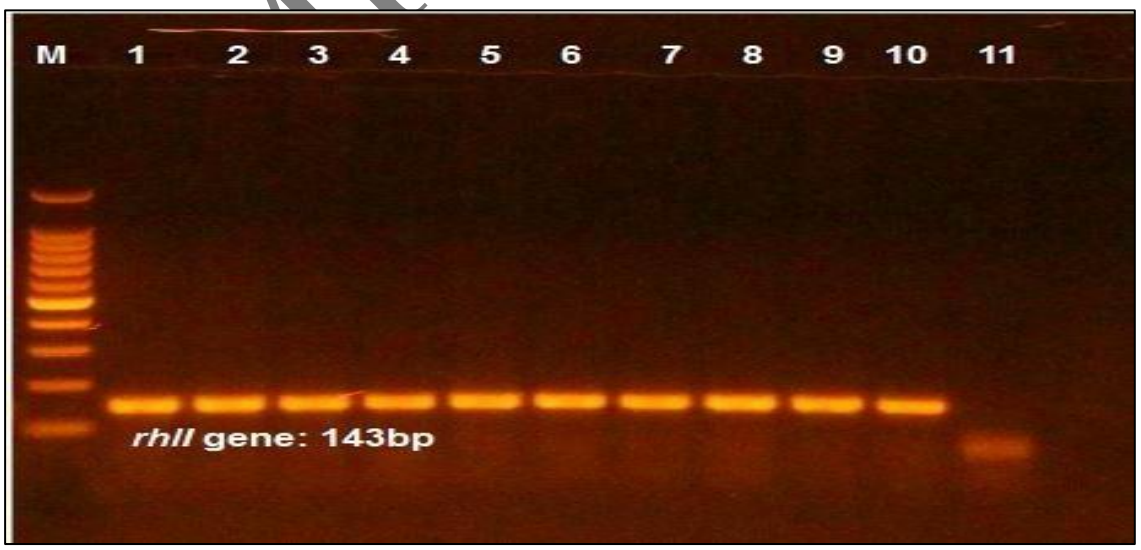

Figure 4.8. Gel electrophoresis amplification of rhlI gene of *P. aeruginosa* isolates were fractionated on 2% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-32 resemble—143bp PCR products.

Ten isolates from 55 isolates were potent biofilm producers, and 8 contained both lasI genes (80%), while rhlI was found in all these isolates, as shown in Table (4.6).

Table 4.5. The existence of last and rhlI genes in potent biofilm isolates

Isolate code	LasI	rhlI
P.1	+	+
P.3	+	+
P.6	-	+
P.12	+	+
P.14	+	+
P.19	+	+
P.22	-	+
P.27	+	+
P.31	+	+
P.33	+	+

(P): *P. aeruginosa* isolate, (+): Present, (-): Absent

In a previous study conducted in Kastamonu, Turkey, 52 carbapenem-*resistant P. aeruginosa* isolates were analyzed. It was found that two QS system genes were detected in 51 isolates (98.1%), with four isolates co-existing. Among the isolates, 41 (78.8%) showed the presence of both lasI/R and rhlI/R genes, which are associated with the QS system. Statistical analyses revealed a significant positive correlation between the las and rhl systems, as well as strong and positive correlations between the rhl system and three virulence genes, slime production, and certain other virulence genes (Başkan et al., 2022). Another study by Aboushleib et al. (2015) also highlighted the importance of QS in *P. aeruginosa* virulence.

In a global study involving 24 *P. aeruginosa* isolates from a culture collection in Turkey, the presence of QS genes (lasI, lasR, rhlI, and rhlR) was identified using PCR. Among the isolates with known biofilm characteristics, 19 showed the presence of lasI, lasR, and rhlI genes, which are associated with biofilm production. These isolates demonstrated moderately strong and robust biofilm formation (Erdal et al., 2020).

A study conducted in Iran included 140 clinical isolates of *P. aeruginosa* collected from hospitals in Tehran and Ilam. The frequency of QS genes (lasIR, rhlIR) was examined, and it was found that most of the isolates were capable of producing biofilm (87.15%), with the majority forming strong biofilms (56.42%). PCR results indicated that the frequency of lasIR and rhlIR genes was 93.57% and 83.57%, respectively. There was a significant correlation between biofilm formation and the

presence of QS system genes in the clinical isolates of *P. aeruginosa* (Hemati et al., 2014)

4.7. Effect of ZnO-np on Gene Expression of Biofilm Formation Genes

In this step, the primary objective was to measure the gene expression of the quorum sensing genes (lasI and rhlI) in *P. aeruginosa* isolates and compare the gene expression in the presence and absence of ZnO nanoparticles (ZnO-np). The aim was to investigate the effect of ZnO-np on biofilm formation by examining its impact on the expression of these genes, particularly in potent biofilm-forming strains. Reverse transcription quantitative PCR (RT-qPCR) was used, which is a method for quantifying gene expression and comparing the expression levels of a specific gene among different samples.

During the experiment, the amplification of the target genes was recorded as Ct values (cycle threshold). The RSPL gene was used as the housekeeping gene, which is commonly used in molecular studies to ensure its consistent expression in the cells or tissues being investigated. The quantitative PCR reaction was performed using six potent biofilm-producing isolates of P. aeruginosa, each with two biofilm genes. These isolates were selected with different sub-MIC values for ZnO-np.

The quantitative RT-PCR assay analyzed the mRNA expression of biofilm genes by comparing the treated and untreated samples of bacterial growth with ZnO-np at concentrations below the minimum inhibitory concentration (MIC) for each sample. Ct values of gene amplification were recorded, and gene expression fold change was calculated using relative quantification (RQ) based on the delta Ct value.

The results of the gene expressions are presented in Tables (4-7) and (4-8). The findings showed that ZnO-np significantly down-regulated the expression levels of all biofilm and virulence genes in *P. aeruginosa* clinical isolates.

Table 4.6. Gene expression (fold change) results for lasI before and after being treated with ZnO-np

Group	Isolate code	RSPL (HKG) *	*lasI*	Δct	ΔΔct	Folding $2^{-\Delta\Delta ct}$	Mean ± SE
After treated	Pl	12.9	15.0	2.2	4.7	0.03	0.293± 0.12
	P2	12.1	9.7	-1.5	0.7	0.61	
	P3	16.6	14.6	-2.1	5.1	0.02	
	P4	15.3	14.2	-1.2	2.2	0.21	
	PS	12.2	12.5	0.3	2.5	0.18	
	P6	20.9	15.1	-5.8	0.5	0.71	
Before treated (control)	C1	16.0	13.5	-2.5	0	1	1.00 ±0.00
	C2	18.6	16.4	-2.2	0	1	
	C3	18.4	11.2	-7.2	0	1	
	C4	21.3	17.0	-3.4	0	1	
	cs	15.2	13.1	-2.1	0	1	
	C6	21.2	14.9	-6.3	0	1	
T-test (P-value)		--	--	_,_	--	0.579 * (0 .045l)	
			* (P≤0.05).				

*HKG: Housekeeping gene

Table 4.7. Gene expression (fold change) results for rhlI before and after being treated with ZnO-np.

Group	Isolate code	RSPL (HKG) *	*rhlI*	Δct	ΔΔct	Folding $2^{-\Delta\Delta ct}$	Mean ± SE
After treated	Pl	15.7	20.9	5.2	1.4	0.39	0.465 ±0.06
	P2	12. 4	17.9	5.4	1.3	0.42	
	P3	18.0	18.6	-0.4	0.6	0.68	
	P4	22.4	25.5	3.1	0.6	0.64	
	P5	11.6	18.4	6.7	1. 4	0.37	
	P6	20.8	20.0	-0.8	1.8	0.29	
Before treated (control)	C1	16.0	19.9	3.9	0	1	1.00 ±0.00
	C2	18.6	22.8	4.2	0	1	
	C3	18.4	17.4	-1	0	1	
	C4	21.3	23.8	2. 5	0	1	
	C5	15.3	20.5	5.3	0	1	
	C6	21.2	18.6	-2.6	0	1	
T-test (P-value)		--	--	--	--	0.0471 * (0.048)	
			* (P≤0.05).				

*HKG: Housekeeping gene

The control of virulence factors and pathogenesis in *P. aeruginosa* through targeting quorum sensing (QS) has shown promise as an alternative to traditional antimicrobial therapy. Anti-virulence therapy, such as QS inhibitors, can be effective in treating microbial diseases as it does not exert pressure on bacterial growth and therefore reduces the likelihood of microbial resistance.

P. aeruginosa produces various virulence factors that contribute to infection establishment, spread, and invasion of host tissues. These factors include pyocyanin, pyoverdin, proteases, elastase, and rhamnolipids. Nanotechnology has been employed to develop nanoparticles that specifically target QS and virulence factors in P. aeruginosa.

The QS systems LasI and RhlI regulate the expression of virulence genes in P. aeruginosa. LasI and RhlI synthesize autoinducers C12-AHL and C4-AHL, respectively. C12-AHL binds to lasR and induces the expression of genes involved in elastase, exotoxin, and protease production, as well as activating the rhlI/R system. Similarly, C4-AHL binds to rhlR and regulates the expression of genes associated with elastase and pyocyanin production. Disrupting the lasI/R and rhlI/R systems can impede the expression of virulence factors.

Studies, such as the one conducted by Saleh et al. (2019), have examined the relative expression of QS-regulatory genes in *P. aeruginosa* using quantitative reverse transcription PCR (qRT-PCR) to explore the potential quorum-quenching effect of ZnO nanoparticles. These studies have demonstrated that ZnO nanoparticles significantly reduce the relative expression of QS regulatory genes, including lasI, lasR, rhlI, rhlR, pqsA, and pqsR, confirming the phenotypic findings. Similar findings have been reported for other nanoparticles, such as silver nanoparticles, which suppressed the expression of lasI, lasR, rhlI, and rhlR genes.

Further research is needed to understand the molecular basis and complete mechanism of nanoparticle-mediated quorum sensing inhibition. In vivo studies have been conducted to investigate the impact of ZnO nanoparticles on *P. aeruginosa* pathogenesis. Synergistic antipseudomonal activity has been observed when combining ZnO nanoparticles with meropenem, along with a reduction in biofilm expression-regulating genes.

Gold nanoparticles (gold-NPs) and selenium nanoparticles (selenium-NPs) have also shown significant inhibition of QS-related virulence factors and the suppression of QS gene expression and toxins in P. aeruginosa. The antimicrobial activity of nanoparticles is believed to involve the generation of free radicals, induction of

oxidative stress, and enhanced microbial cell wall penetration. The specific properties of nanoparticles, including composition, size, surface charge, and shape, play a crucial role in their antimicrobial activity (Taylor, 2011).

Overall, the use of nanoparticles as quorum sensing inhibitors and their impact on virulence traits in *P. aeruginosa* hold promise for the development of novel therapeutic approaches against Pseudomonas infections.

4.8. Correlation Between Gene Expression of LasI, and rhlI Genes And Biofilm

The effect of ZnO nanoparticles (ZnO-np) on gene expression related to biofilm production, specifically the lasI and rhlI genes, was investigated using reverse transcription PCR (RT-PCR). The fold change of each gene was evaluated to assess the impact of ZnO-np on gene expression. The results indicated a significant positive correlation between the gene expression of RSPL, lasI, and rhlI genes and biofilm formation. This correlation was determined using Pearson correlation analysis, which included all the tested isolates before and after treatment with ZnO-np. The findings are presented in Figures 4-8, 4-9, and 4-10. These results suggest that ZnO-np treatment influences the gene expression of RSPL, lasI, and rhlI genes, which are involved in biofilm formation.

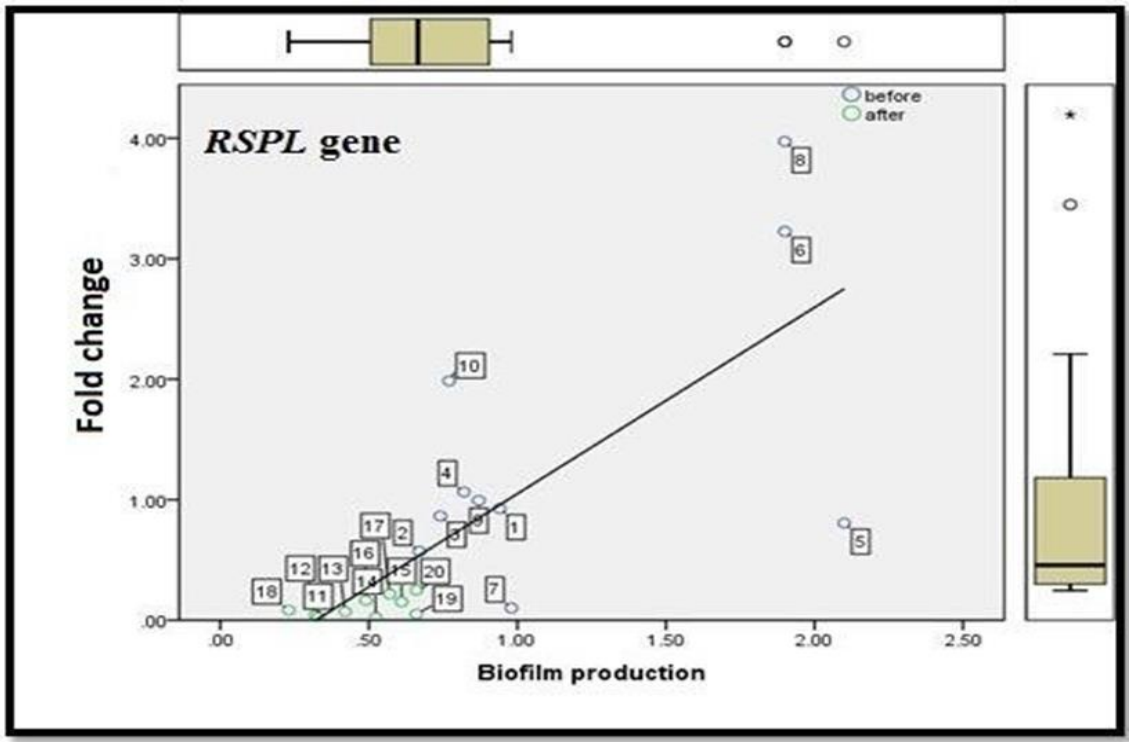

Figure 4.9. Correlation between biofilm formation and gene expression of RSPL gene (r=0.751, 95%, p=0.0001).

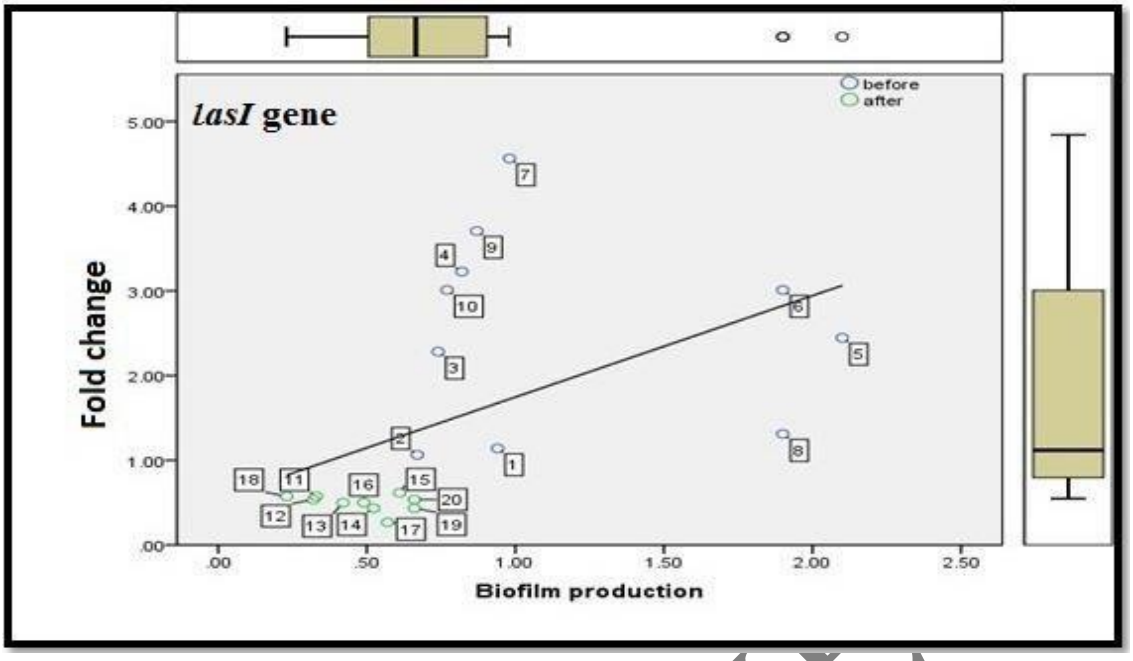

Figure 4.10. Correlation between biofilm formation and gene expression of lasI gene (r=0.479, 95%, p=0.033).

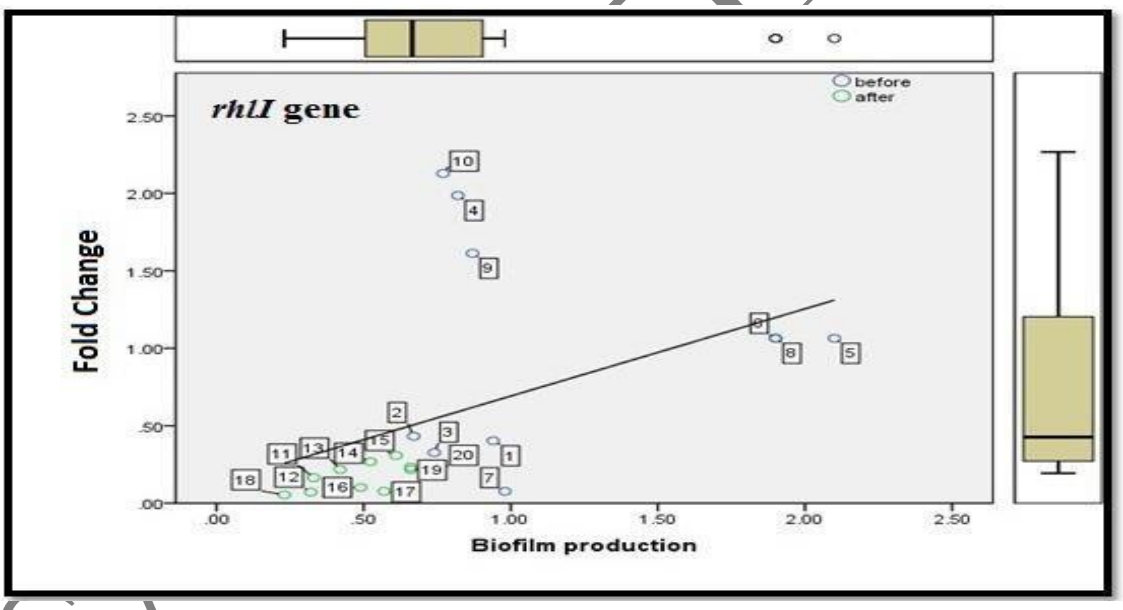

Figure 4.11. Correlation between biofilm formation and gene expression of rhlI gene (r=0.455, 95%, p=0.044).

According to Pati et al. (2014), the use of ZnO nanoparticles (ZnO-np) can disrupt the integrity of bacterial cell membranes and reduce cell surface hydrophobicity. It also inhibits the transcription of oxidative stress resistance genes in bacteria. Lee et al. (2014) showed that ZnO-np with a size of 50 nm can effectively inhibit biofilm formation and the development of virulence factors in P. aeruginosa. Their study demonstrated that ZnO-np at a concentration of 1 mm could reduce biofilm formation

by more than 95% on polystyrene surfaces. Additionally, ZnO-np treatment had a significant regulatory effect on the expression of various virulence genes of P. aeruginosa, as determined by microarray and qRT-PCR analysis. Garcia-Lara et al. (2015) investigated the impact of ZnO-np on the development of virulence factors in clinical and environmental strains of *P. aeruginosa* and observed that ZnO-np had the potential to inhibit the majority of virulence factors produced by these strains.

Kaur et al. (2021) conducted a study that demonstrated the significant antibacterial and anti-biofilm activity of ZnO nanoparticles against *P. aeruginosa* MTCC 4673. The mode of action of ZnO nanoparticles involved changes in the cell membrane permeability, leading to the leakage of nucleic acids from bacterial cells.

In a study by Saleh et al. (2019), qRT-PCR was used to investigate the inhibitory effect of ZnO nanoparticles on quorum sensing genes (lasI, lasR, rhlI, rhlR) related to biofilm production. The results showed a statistically significant reduction in the production of quorum sensing genes and virulence factors such as rhamnolipids and pyocyanin in the presence of ZnO nanoparticles. Similar to the findings of the present study, Saleh et al. observed a significant decrease in the relative expression of quorum sensing genes (slasI, lasR, rhlI, rhlR, pqsA, and pqsR).

Abdelraheem and Mohamed (2021) reported that ZnO nanoparticles exhibited good antibiofilm and antigrowth effects against clinical isolates of P. aeruginosa. They also demonstrated the ability of ZnO nanoparticles to remove pre-formed biofilms produced by *P. aeruginosa* isolates. The study showed that ZnO nanoparticles reduced the expression of genes responsible for biofilm formation and virulence factors production in *P. aeruginosa* clinical isolates.

Furthermore, Krishnamoorthy et al. (2022) found that ZnO nanoparticles have broad-spectrum action against β-lactamase-producing strains. The minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of ZnO nanoparticles ranged from 0.04 to 0.08 mg/mL and 0.12 to 0.24 mg/mL, respectively. These findings indicate the potential of ZnO nanoparticles as effective agents against antibiotic-resistant strains of *P. aeruginosa*.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

1. The effect of ZnO-np on biofilm formation among strong biofilm producer's strains of *P. aeruginosa* demonstrated an obvious inhibition of the biofilm formation.

2. This study indicated the role of ZnO-NPs as adjuvant with the active antibiotics against *P. aeruginosa* to control infection with this pathogen.

3. It was obvious there was a significant antibiofim effect on the multidrug resistant isolates especially on the strong biofilm producers.

4. The results revealed a significant down-regulation in biofilm genes (lasI, and rhlI) in the presence of sub-MIC doses of ZnO-np.

5. The results showed a significant positive correlation between gene expression of, lasI, and rhlI genes and biofilm formation by using Pearson correlation analysis which included all the tested isolates before and after the treatment with ZnO-np.

5.2. Recommendations

1. Study the effect of the combination between nanoparticles and natural products from plants on bacterial biofilm.
2. An *in vivo* experiment complemented this study, especially for the treatment of burn infections.
3. Using other types of nanoparticles for eradication the biofilm formation and studies its effect on the gene expression of related biofilm genes.

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