TURKISH REPUBLIC OF NORTH CYPRUS
NEAR EAST UNIVERSITY
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MOLECULAR DETECTION OF SOME VIRULENCE GENES IN *Pseudomonas aeruginos*a ISOLATED FROM CLINICAL SOURCE

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MASTER OF SCIENCE THESIS

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MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

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Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis.

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AHMED SHERZAD AWLA

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DEDICATION

THIS THESIS IS DEDICATED TO:

MY BELOVED PARENTS

MY SUPERVISORS

MY DEAR BROTHERS

MY FRIENDS

ALL WHO HELPED ME

ÖZET

Pseudomonas aeruginosa bakterisi, insan hastalıklarını indükleyen en halka açık fırsatçı patojenlerden biridir ve aynı zamanda tüm dünyada hastane ortamlarında enfeksiyon oluşumunda rol oynayan en sık karşılaşılan mikroorganizmalardan biridir. Bu bakteri ile enfeksiyonlar, 144 tür içeren *Pseudomonas* cinsinde en yaygın olanıdır ve bunların yaklaşık yüzde 25'i insan enfeksiyonlarıyla ilişkilidir. Patojenitesinin bir sonucu olarak üriner, pulmoner sistem ve sindirim yolları dahil olmak üzere birçok anatomik bölge; cilt; yumuşak dokular; kan; kemikler ve gözler; ve göz kapaklarının deri ve yumuşak dokuları; *Pseudomonas aeruginosa*, özellikle ciddi yanıklar geçirmiş veya bağışıklığı baskılanmış hastalarda çeşitli sistemik enfeksiyon kaynaklarını indükleyebilir. Antibiyotik direnci özelliklerine sahip izolatların ortaya çıkmasından bu yana, Pseudomonas aeruginosa insanlarda önemli oranda ölümle sonuçlanmıştır. Farklı klinik örneklerden alınan *Pseudomonas aeruginosa* izolatları, 69 bakteri örneğinde virülans genlerinin moleküler tanımını tanımlamaya çalışan bu araştırmada kullanıldı.

Şubat 2021 ve Mayıs 2021 boyunca, Irak'ın Erbil kentindeki çok sayıda özel ve kamu hastanesine kayıtlı çok sayıda bireyden alınan çok sayıda klinik örnekten alınan 149 örnek arasında altmış dokuz *Pseudomonas aeruginosa* izolatı tespit edildi. Örnekler morfolojik, kültürel ve biyokimyasal testler kullanılarak tanımlandı. Ek olarak, fenotipik olarak tanımlanan izolatlar, Polimeraz Zincir Reaksiyonu (PCR) ile doğrulandı. PCR yaklaşımı, rpoB geninin amplifikasyonu yoluyla başlatıldı. PCR ürünü, altmış dokuz izolatın Pseudomonas aeruginosa izolatı olarak doğrulandığı *rpo*B için 759 bp idi.

Amikasin (AK), Aztreonam (ATM), Ceftazidime (CAZ), Siprofloksasin (CIP), Colistin (COL), Gentamisin (CN), Levofloksasin (LEV), Norfloksasin dahil olmak üzere 10 antibiyotik kategorisi gerçekleştiren tüm izolatlar için antibiyotik duyarlılık analizi değerlendirildi. (NOR), Ofloksasin (OFX) ve Tobramisin (TOB). Test edilen *Pseudomonas aeruginosa* suşlarının sadece üç izolatında (%4.35) antibiyotik direnci bulundu. Amikasinin, *Pseudomonas aeruginosa* izolatlarına karşı en etkili antibakteriyel ajan olduğu ortaya çıktı. İzolatların bu antibiyotiklere direnç oranları değişkenlik göstermiş ve seftazidim için yüksek direnç oranı %78.26, amikasine

karşı en düşük direnç oranı %4.35 olarak kaydedilmiştir. *Pseudomonas aeruginosa* izolatları, virülans faktörleri olarak hemolizin aktiviteleri üretme yetenekleri açısından test edilmiştir. Sonuçlar, 51 izolatın (%73.91) ve 18 izolatın (%26.09) sırasıyla β-hemolitik ve α-hemolitik üretici olduğunu gösterdi.

Dört virülans geni, amplikon boyutlu genomik DNA (207, 752, 1281) ile gen varlığını (*exo*A, *pvda*, *protease* ve *plch*) test etmek için geleneksel polimeraz zincir reaksiyonu (PCR) kullanılarak *Pseudomonas aeruginosa*'nın altmış dokuz izolatının tümü için tarandı. ve 307 bp) sırasıyla. Bulgular, izolatların 66'sında (%95.65) temsil edilen *pvda* geninin ve ardından *protease* geninin 56'sında (%81.16) virülans genleri olarak, 54 izolattan (%78.28) ise *plch* geninin eksprese edildiğini göstermiştir. Bununla birlikte, *Pseudomonas aeruginosa*'nın altmış dokuz izolatının tamamı arasında en düşük eksprese edilen gen, 46 izolatta (%66.67) *exo*A geniydi.

SUMMARY

The bacterium *Pseudomonas aeruginosa* is one of the most public opportunistic pathogens that induce human diseases, and it is also one of the more often encountered microorganisms implicated in occurrences of infections in the hospital settings all over the world. Infections with this bacterium are the most common in the genus *Pseudomonas* that contains 144 species, with around 25 percent of them being associated with human infections. As a result of its pathogenicity multiple anatomic sites, including the urinary, pulmonary system, and digestive tracts; skin; soft tissues; blood; bones and eyes; and the skin and soft tissues of the eyelids; *Pseudomonas aeruginosa* could induce a varied sources of systemic infections, particularly in patients who have suffered severe burns or are immunosuppressed. Since the appearance of isolates with antibiotic resistance features, *Pseudomonas aeruginosa* has resulted in significant percentages of death in humans. *Pseudomonas aeruginosa* isolates from different clinical specimens were used in this investigation, which attempted to define the molecular description of virulence genes in 69 samples of the bacteria.

During February 2021 and May 2021, sixty-nine isolates of *Pseudomonas aeruginosa* were identified among 149 specimens, taken from numerous clinical samples of individuals' registered to numerous private and public hospitals in Erbil city, Iraq. Specimens were identified using morphological, cultural, and biochemical tests. In addition, the phenotypic identified isolates were confirmed through Polymerase Chain Reaction (PCR). The PCR approach was initiated through the amplification of *rpoB* gene. The PCR product was 759 bp for *rpoB*, which sixty-nine isolates has been confirmed as *Pseudomonas aeruginosa* isolates.

Antibiotic sensitivity analysis was assessed for all isolates performing 10 categories of antibiotics including Amikacin (AK), Aztreonam (ATM), Ceftazidime (CAZ), Ciprofloxacin (CIP), Colistin (COL), Gentamycin (CN), Levofloxacin (LEV), Norfloxacin (NOR), Ofloxacin (OFX), and Tobramycin (TOB). Antibiotic resistance was found in just three isolates (4.35%) of the *P. aeruginosa* strains tested. Amikacin was revealed to be the most efficient antibacterial agent towards the isolates of *P. aeruginosa*. The resistance rates of the isolates to these antibiotics

varied and the high resistant rate were 78.26% for ceftazidime, and the lowest resistant rate were 4.35% recorded toward amikacin. *Pseudomonas aeruginosa* isolates tested for their ability to produce hemolysin activities as virulence factors. The results showed that 51 isolates (73.91%) and 18 isolates (26.09%) were β-haemolytic and α-haemolytic producers respectively.

Four virulence genes were screened for all sixty-nine isolates of *P. aeruginosa* using conventional polymerase chain reaction (PCR) to test the genes existence (*exo*A, *pvda*, *protease*, and *plch*) with amplicon-sized genomic DNA (207, 752, 1281 and 307 bp) respectively. The findings has been shown that *pvda* gene represented in 66 (95.65%) of the isolates and followed by *protease* gene is expressed as virulence genes in 56 (81.16%), and *plch* gene which expressed among 54 isolates (78.28%). However, the lowest expressed gene among all sixty-nine isolates of *Pseudomonas aeruginosa* were *exo*A gene in 46 isolates (66.67%).

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LIST OF ABBREVIATIONS

Abbreviations	Word details
A_{600}=1	Absorbance at 600 nm
AIDS	Acquired immunodeficiency syndrome
bp	Base pair
CAP	Community-acquired pneumonia
CF	Cystic fibrosis
CFU	Colony forming units
CLSI	Clinical Laboratory Standard Institution
CNS	Central nervous system
D.W	Deionized water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EF-2	Eukaryotic elongation factor 2
EPIs	Efflux pump inhibitors
ETA	Exotoxin A
GIM	German imipenemase
H_2O_2	Hydrogen peroxide
HeLa cells	Henrietta Lacks cells
HP	Human plasma
ICU	Intensive care unit
IM	Interior membrane
IMP	Imipenemase
LB	Laura bertani broth
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide cell surface
Mbp	Mega base pair
MDR	Multidrug-resistant
Mex	Multidrug efflux
MFS	Major facilitator superfamily
MHA	Mueller Hinton Agar
NAD	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCUs	Neonatal care unit
OD	Optical density
OM	Outer membrane
opr	Outer membrane porin
OprD	Outer membrane porin protein
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
QS	Quorum sensing

RNA	Ribonucleic acid
RND	Resistance-nodulation-division
SIM medium	Sulfur, Indole, Motility media
SPM	São Paulo Metallo-β-lactamase
SRC	Scientific Research Center
SRC	Scientific Research Center
SUE	Salahaddin University-Erbil
T3SS	Type III Secretion System
TAE Buffer	Tris-Acetate EDTA buffer
TE buffer	Tris–EDTA buffer
TFP	Type IV pili
Tm	Melting temperature
UV	Ultraviolet radiation
VIM	Verona imipenemase

1. INTRODUCTION

Pseudomonas aeruginosa is a gram-negative aerobic non-spore rod with extraordinary ability to persist and continue under many environmental circumstances (Santajit and Indrawattana, 2016). In both hospitals and communities, *P. aeruginosa* is a common, opportunistic human bacterium (Singh et al., 2014). The pathology of *P. aeruginosa* is associated to different metabolic abilities, numerous resistance mechanisms and a wide range of virulence factors and adaptation, and gene expression organizes all these activities in a very similar way (Nanvazadeh et al., 2013). It is among the most often occurring sources of burn infection (MR and Hajia, 2012).

Burning wound infection is a problem since it delays healing, promotes scarring and can lead to bacteremia, sepsis (or organ failure) syndrome, whereby organ from several systems cannot maintain homeostasis on its own and require immediate medical attention (Héry-Arnaud et al., 2017). The most severe pathogenic burn injuries are bacteria and fungi. Multiple species biofilms are formed on burning injuries in 48 to 72 hours of injury (Meng et al., 2020). Organisms come from the patient's own skin, intestinal and respiratory flora and contact with contaminated environments and health workers (Altaai et al., 2014).

Pseudomonas aeruginosa is a ubiquitous bacteria that can quickly develop resistance to various antibiotics of broad spectrum (Singh et al., 2014). Moreover, in current years resistance to a broad range of antibiotics by these microorganisms has made it difficult to treat infections caused and leads to higher death rates (Fair and Tor, 2014). One of the features of *P. aeruginosa* is the formation of soluble pyocyanin pigment, a water-soluble blue-green chemical that is generated in massive concentrations. Pyocyanin has antimicrobial action towards a varied kind of bacteria and fungi, including pathogenic bacteria (El-Fouly et al., 2015).

Pseudomonas aeruginosa also retains a diverse spectrum of virulence factors, including exotoxin A, exoenzyme S, elastase, and sialidase, all of which are tightly controlled by cell-cell communication. As the predominant virulence factor generated by the majority of *P. aeruginosa* isolates, exotoxin A (ETA) performs a

critical function in the pathogenesis of infections produced by this bacterium. Such exotoxins may contribute to leucopenia, acidosis, blood circulation, and necrosis of the liver, pulmonary oedema, bleeding and kidney tubular necrosis (Tafesse et al., 2014).

The main aims of this study are

Antibiotic susceptibility and resistance are being investigated in this experiment as well as the description of the sources of resistance and resistance pathways, in order to aid in the control of infections associated by *P. aeruginosa*, which are now under investigation. An extensive quantity of research were carried out to test the efficiency of various antibacterial against *P. aeruginosa* isolates. It is possible to analyze the chromosomally encoded resistance pathways between *P. aeruginosa* clinical isolates in a number of different investigations. The exact purposes of this research are:

1. Isolation and identification of *P. aeruginosa* isolated from different clinical specimens of patients by their cultural, morphological, and biochemical tests, in addition to molecular technique assay using polymerase chain reaction (PCR).
2. Using PCR for the recognition of some virulence genes among *P. aeruginosa* isolated from different clinical specimens of patients.
3. Identifying the antibiotic resistance pattern of each isolate was the objective.

2. GENERAL INFORMATION

2.1 *Pseudomonas* genus

Pseudomonas is a genus that contains species that are significant for humanoid healthiness, economics, and the environment. A high level of metabolic complexity is displayed by members of this genus, and they are also extremely adaptable, with the potential to colonize and respond to a wide range of environmental habitats (Moore et al., 2006). Over than 25 species of the genus Pseudomonadaceae are related with human illnesses, and the genus is a member of the Pseudomonadaceae group, which contains over 140 species in total (Scales et al., 2014). *Pseudomonas* is a broad collection of aerobic gram-negative bacterium that have been recovered from a variety of sources, comprising plants, water, soil, and animals, especially humans. There have been a number of species of this genus found to trigger human disease, including *Pseudomonas aeruginosa*, *P. fluorescens, P. putida, P. oleovorans, P. luteola*, and *P. stutzeri*. Species in this genus that have been recognized to induce human disease are commonly related with opportunistic infections and include species such as *Pseudomonas* (Chong, 2009). Solid media particularly "MacConkey agar, blood agar, and chocolate agar" are excellent growth medium for *Pseudomonas* spp. obtained from clinical sources. On various media, the colony features could be nearly colorless, cream-colored, or off-white. Coloration in the form of yellow or blue green pigmentation is generally a typical trait of *P. aeruginosa* strains, while some species create fluorescent colonies when exposed to UV light or using a fluorescent microscope. Species of the *Pseudomonas* are harbor the gene for oxidase enzyme (with the exception of *P. luteola*), and has the hydrogen peroxidase that breakdown the hydrogen peroxide and form water and oxygen bubbles (with the exception of *P. luteola*), citrate consumption assay positive (but not indole positive), and voges-proskaurer screen negative (with the exclusion of *P. luteola*). Certain species of *Pseudomonas* exhibit β-hemolysis on blood agar and produce a bright yellow-green pigment (pyoverdin) when exposed to iron-limiting conditions, whereas others, such as *P. fluorescens*, release thioquinolobactin when exposed to iron-limiting conditions. *P. aeruginosa* isolates release pyoverdine and create siderophores, including such pyocyanin, which are toxic to humans (Al-Daraghi and

Al-Badrwi, 2020). Consequently, *Pseudomonas* species have developed a great level of adaptation to their microenvironment, allowing them to resist a wide range of environmental circumstances, particularly severe little (4°C) and high (43°C) temperature environments, antibacterial treatments, and low ionic gradients. *P. aeruginosa* grows especially at 43°C, which separates it from the other *Pseudomonas* species and correlates to its harmful potential. Approximately 70% of infections caused by the genus *Pseudomonas* arise as nosocomial infections, and colonization increases considerably in individuals who are admitted to a medical facility (Ricker et al., 2018). The genus *Pseudomonas* is also linked with opportunistic infections in immunocompromised patients, including systemic infections, bacteremia, and infections of the gastrointestinal tract (digestive system infection), skin infection, soft tissue infection, urinary tract infection, and respiratory system infection. The primary mode of infection is through transport or contact transfer, in which the bacterium is transmitted and presented into the hospital environment by individuals who have been moved from other hospitals, departments, intensive care unit (ICU), or the operating room. It can also be transmitted through visitors through their shoes and attractive flowers, including through healthcare professionals throughout sanitation, ward rounds, and sample collection, and throughout medical tests such as catheterization, among other methods. Some species of the genus *Pseudomonas* may be present in wet environments, include sanitizers, creams, eye droplets, wipes, lunchtimes, bowls, taps, cosmetics, and also medical devices (e.g. respirators) and contact lens solutions, where they could develop biofilms and cause infections in patients (Raj et al., 2015).

2.2 Historical aspect of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most prevalent pathogens related with healthcare-related infections in hospitalized patients, particularly in the elderly. Healthcare-related infections are most commonly linked with infections including pneumonia, urinary tract, skin and soft-tissue, and other illnesses. This bacterium frequently exhibits multiresistant strains, resulting in increased disease and death in humans (Bassetti et al., 2018). For the therapy of healthcare-linked infections, *P.*

aeruginosa represents a significant therapeutic challenge. The selection of appropriate antipseudomonal drugs for therapy is critical in achieving the best possible therapeutic outcome (Tümmler, 2019)

A variety of issues in *P. aeruginosa* infection started to arise from these studies. It was clear that the organism was mainly connected to very young or very old infections or to chronically damaged individuals. The second is that *P. aeruginosa* infections could be divided into two types: acute diseases that arose unexpectedly and resulted in violet systemic symptoms and death within a couple of days, and chronic infections that often lasted longer, respectively (Moradali et al., 2017). Interestingly *P. aeruginosa* was also stated to be a possible hospital or nosocomial pathogen as early as 1934 when a case of meningitis in an adult woman was suspected to be associated with a spinal injection of contaminated anesthetic (Rodríguez-Lucas et al., 2020).

2.3 General features of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacillus that lives in an aerobic environment (Figure 2.1), which has the extraordinary adaptability to survive and thrive in a broad number of external conditions. This microorganism is typically 1.5-5 microns in length and 0.5-1.0 microns in diameter, and it is motile because to the possession of flagella. Schroeter identified *P. aeruginosa* from a variety of external sources for the first occurrence was in 1872 (Mwinyikombo, 2018). *P. aeruginosa* is a member of the Pseudomonadaceae family, which falls under the classification of Gammaproteobacteria. Possess the cytochrome C oxidase, and has the potential to be grown at the conditions with high temperature as 42°C, and the capabilities to evolve under anaerobic situations through the existence of an appropriate terminal electron acceptor including nitrite or arginine (despite the fact that it is categorized as strictly aerobic) are all characteristics of *P. aeruginosa* isolates. Upwards of eighty chemical compounds are used by the organism as carbon and energy resource substances (Dworkin, 2006).

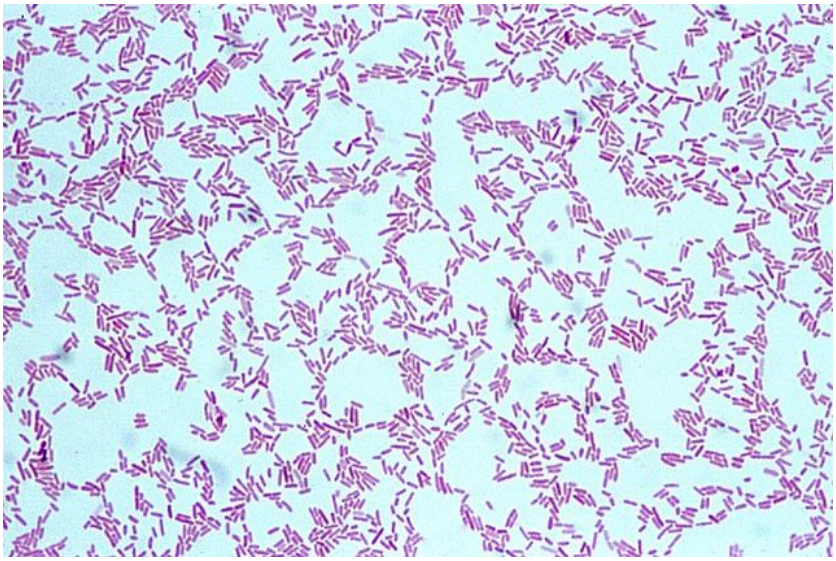


Figure 2.1 Gram staining reaction of *Pseudomonas aeruginosa* cells.

Since revisionist taxonomy on the basis of preserved macromolecules (e.g. 16s ribosomal RNA), *P. aeruginosa* belongs to the family Pseudomonadaceae, the family only comprises *Pseudomonas* members that are subdivided into eight sets and *P. aeruginosa* is member of the group species that comprise twelve additional components (Odoi et al., 2021). *P. aeruginosa* genome, depending upon the strain, is relatively large (6-7 mega bases), encoding approximately 6000 open read frames (ORFs) (Mathee et al., 2008). This bacterial form produces a variety of pigments inhibiting the development of other bacteria, and the most significant pigment being pyocyanin (water-soluble blue) and pyoverdin (green-yellowish pseudobactin) which is toxic in host cells. Other pigments like pyorubin (red) and pyomelanin (brown) are also produced. *P. aeruginosa* is a pathogen with a high genomic flexibility and adaptable potential in different environments. Species are often isolated or colonized from the soil and water on plants, insects, animals and human beings. This bacterium has many factors of virulence and is known to be the principal source of mastitis in dairy livestock (Kaszab et al., 2016).

There are three different categories of colonies that could be produced by *P. aeruginosa* isolates. Small, rough colonies are often produced by natural isolates from soil and water. Medical specimens are often one of two types: smooth with flat edges and an inflated appearance, or rough with raised edges and a raised

appearance. It has a mucoid look, which is thought to be due to the formation of alginate slime in some other type, which is usually derived from respiratory and urinary tract secretions. Several researchers believe that smooth and mucous colonies perform a function in colonization and pathogenicity (Bae et al., 2014). *Pseudomonas* is phenotypically similar to gram negative enteric bacteria and to most enteric bacteria; it is a non-sporulatory anaerobic alternative using motile flagellum (Azuma et al., 2016). One significant difference is that enteric bacteria can ferment glucose to acid but pseudomonas does not ferment glucose to acid (El-Ageery et al., 2016).

Initial research concentrated on rRNA–DNA hybridization hypothesized five RNA classifications in the *Pseudomonas*, with rRNA group I being titled after the genus *Pseudomonas*, which included the type species *P. aeruginosa*. With 202 species of *Pseudomonas* currently recognized in the official bacterial database, the present condition of this genus is unclear. The approved bacterial database is based on a mix of 16S rRNA, cell fatty acid measurement, as well as traditional physiologic and biochemical categorization assays. The genus comprises of a collection of clinically and biotechnologically significant bacteria that could be discovered in a large variety of habitats comprising soil and water habitats, and also plant and animal relationships, among other places. As a result, they are well-known for possessing tremendous metabolic adaptively (Høiby et al., 2010).

In the laboratory, *P. aeruginosa* is capable to grow on a varied types of media, ranging from minimal to complex. Most isolates are easily recognizable on primary isolation media on the basis of colonial morphology, a grape-like odor and production of hydrosoluble pigments such as pyocyanin (blue), pyorubin (red), pyomelanin (brown-black) and pyoverdin (yellow-green or yellow-brown) (Al-Daraghi and Al-Badrwi, 2020). In fact, the name aeruginosa (from Latin aerugo "copper rust") stems from the greenish-blue color of bacterial colonies when pyocyanin and pyoverdin pigments are co-produced. Colonies are typically flat and dispersion and have a serrated edge, but other morphologies can be present, including, among others, the mucoid or the small colony variants. *P. aeruginosa* can metabolize a large array of carbon resources. However, it does not metabolize carbohydrates, but instead produces acid from polysaccharides such as

glucose, fructose, and xylose, but not through lactose or sucrose. Additionally, it is strongly possess the indophenol oxidase, catalase enzymes and arginine utilization. *P. aeruginosa* develops greatest aerobically however may also be grown anaerobically in the occurrence of nitrate as a terminal electron acceptor. As well, although optimal temperature for growing of this bacterium is 37ºC, and it can also grow at 42ºC, which is characteristic that differentiates the *P. aeruginosa* from other infrequently pathogenic fluorescent species such as *P. fluorescens* or *P. putida* (Ali et al., 2021).

2.4 Genetic materials of *Pseudomonas aeruginosa*

Stover and colleagues in 2000 published the entire *P. aeruginosa* strain type PAO1 and since that time many strains have been sequenced by their complete genomes (Winsor et al., 2016). PAO1 initially was isolated in 1955 and has been commonly distinguished since then from wound infection. In many studies, PAO1 is used to equate isolates of wild type to a base-line or a comparator strain (Jeukens et al., 2014). The genome complexity of *P. aeruginosa* (6.3 Mbp; 66.6 percent G+C content) is consists of two constituents, the core genome and the accessory genome. About 90% of the entire genome found in all strains is the extremely controlled and preserved core genome. Several genes inside the core genome have been identified and comprise genes for respiration, antimicrobial resistance and the development of biofilms (Dettman et al., 2013). The rest of the genome consists of a smaller accessory genome and consists of a variety of genetic materials that vary among strains. Specifically, the accessory genome, particularly the gaining or deletion of genomic material via horizontal gene transfers, is the primary propelling force influencing the development of this bacterium. In addition, the accessory genes also contain deletions, mutations and rearrangements. It has been observed that the accessory genome comprises a variety of genes that allow for increased survivability and adaption within various strains of bacteria (Stewart et al., 2014).

2.5 Clinical infection caused by *Pseudomonas aeruginosa*

Despite the fact that it could produce infection in practically any organ of the host body, *P. aeruginosa* does not often induce infection in an otherwise healthy host. When this bacteria infects the respiratory tract, it can also infect the urinary tract, and causing dermatitis and bacteremia. It can also infect soft tissue diseases and also a range of other systematic infections, according to the CDC. Individuals with serious burns, tumor, and AIDS individuals who are immunocompromised are the most likely to transmit the disease. Infestations caused by this bacterium were documented in a number of different situations around the world (Wu et al., 2015). In immunocompromised persons and in individuals with additional disorders like diabetes mellitus, hematological abnormalities, neutropenia, and severe burns, *P. aeruginosa* can produce bacteremia, which is particularly dangerous. The preponderance of *Pseudomonas* bacteremia is obtained in hospital emergency rooms, accounting for 25 percent of all Gram-negative bacteremia obtained in healthcare settings. The bacterium *P. aeruginosa* induces meningitis and brain abscesses when it infiltrates the central nervous system (CNS) from the paranasal sinus or inner ear, when it is inoculated immediately through surgery or invasive detection tests, and when it disperses from a distanced location of the infection, like the urinary tract. *P. aeruginosa* is the bacterial pathogen that produces ear disease in some forms of external otitis, such as swimmer's ear, and is the most common source of ear infection overall. This bacterium could also be present in the normal ear, although it is more commonly present in the external auditory canal when there is inflammation, damage, maceration, or basically when there is a lot of moisture in the air. This has also been identified as the causative component of neonatal ophthalmia, as well as a prevalent source of eye infections such as keratitis, and as the causative factor of septicemia (Bassetti et al., 2018). The most prevalent microbes allegedly participated in osteochondritis following puncture wounds of the foot. *P. aeruginosa* can also cause chronic contiguous osteomyelitis, which is generally the consequence of immediate inoculation of bone. In particular, the oropharynx, rectum, and the entire digestive system from the oropharynx to the rectum are susceptible to *P. aeruginosa* infection (Tada et al., 2019).

Infections produced by *P. aeruginosa* can be different based on whether or not the individual has an underlying disorder or has undergone some form of healthcare procedure (Silva et al., 2013). Among the most prevalent causes of surgical site infection (8.0%), urinary tract infection (7.0%), blood disease (2.0%), and healthcare-related pneumonia (17.0%), *P. aeruginosa* is the fifth highest prevalent pathogen on all locations (9 percent) (Mwinyikombo, 2018). *P. aeruginosa* has recently come to be recognized as a prevalent cause of a wide range of community- and healthcare-related illness. Community-acquired pneumonia (CAP) is caused by the bacteria *P. aeruginosa*, which is more common in individuals with chronic obstructive pulmonary disorder, nursing home patients, and patients who have just been released from the hospital (Restrepo et al., 2018). In natural habitats, it is typically present in soil and water, where it has a broad range of dispersion. Because of the gaseous dissolved nutrients, it can develop in distilled water (Niederman, 2010). It is believed that *P. aeruginosa* can be found in numerous solutions, which include disinfectants, antiseptics, intravenous fluids, and eyewash solutions, among other things. There are many instances of it in medical situations, and it can be found in respiratory apparatus as well as sinks, tubs, and hydropathy baths. Despite the fact that they are present in soil, they are regularly collected from freshly harvested vegetables and plants (Weinstein and Hota, 2004). *P. aeruginosa* infections can vary from a self-limiting folliculitis to a life-threatening bacteremia, depending on the strain. Despite the fact that infections can develop successfully at every anatomical place, these infections primarily happen in the eye, respiratory system, skin infections in the setting of burn injuries, surgical sites, urinary tract, and blood stream. Also common in the ICU is the development of urinary tract infections and ventilator-linked pneumoniae (Mittal et al., 2009). The diseases caused by *P. aeruginosa* could be classified as either acute or chronic in etiology. It was stated that acute infections, particularly ventilator-related pneumonia are invasive and cytotoxic, and that they frequently result in systematic infection, septic shock, and death as a result (Martin and Yost, 2011). On the opposite, chronic respiratory infections related with cystic fibrosis, notwithstanding the high colonization of sputum ($>10^8$ CFU/g), are non-invasive, non-cytotoxic, and only infrequently development to systematic infection. This type of chronic infection can last for years,

eventually resulting in pulmonary damage and death from the disease (Scheetz et al., 2009). When it comes to critical care units, *P. aeruginosa* colonization is important since it possess the potential to colonize in patients. ICUs have been identified as endemic sites for this pathogen (Erbay et al., 2003). It is consequently essential to have a thorough basic considerate and information of the pathways of *P. aeruginosa*. Characteristics associated with the growth of this bacteria in ICUs include the length of residence, the use of mechanical ventilation, the long-term administration of medications, drinking, and the employment of indwelling urine catheters (Altaai et al., 2014). *P. aeruginosa* is only occasionally discovered as a component of the human microbiota of healthy people since this bacterium destruct in the dry skin of healthy humans. *P. aeruginosa* exhibits a diverse array of virulence factors and is found in virtually all environmental habitats. In healthy persons, this bacterium is only infrequently responsible for community-acquired illnesses. However, the occurrence of *P. aeruginosa* related infection is significant in the hospital setting, especially in immunocompromised patients, cystic fibrosis sufferers with compromised epithelium, and patients with severe burns, ulcerations, and mechanical abrasions induced by catheterization, among others. When it comes to cystic fibrosis individuals, *P. aeruginosa* is the most common source of mortality (Moradali et al., 2017). Because of the rising prevalence of multi-drug resistance, infections induced by *P. aeruginosa* not alone become more complicated to cure but also become progressively resistant to one specific drug treatment plan (Hirsch and Tam, 2010). Because of rising percentages of antimicrobial resistance in *P. aeruginosa*, the medication of individuals with risk factors such as severe illness, invasive procedures, a bedridden state, and in hospitalized persons could really result in increased duration of residence and increased treatment costs, and also considerable disease and death, according to the Centers for Disease Control and Prevention (Moore and Flaws, 2011a). In spite of the fact that *P. aeruginosa* was a well-known source of diseases for about 130 years, it is likely to be a major agent of infections in humans for many years to come (Moore and Flaws, 2011b). Patients with chronic illness and immunosuppression over extended periods of time are susceptible hosts for infection with *P. aeruginosa* since the organism is widespread in nature, increasing the likelihood of exposure. Furthermore, *P. aeruginosa* possesses a variety

of resistance pathways to antipseudomonal drugs, which are not often administered appropriately and, whenever overprescribed, contribute to the establishment of resistance in the organism (Altaai et al., 2014).

2.6 Pathogenicity of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is opportunistic bacterium that means that breakdowns in the immune barrier are exploited to cause infection (Andersson et al., 2006). *P. aeruginosa* is a main secondary disease in burnt persons in the hospital (Bayat et al., 2010), and contributes to nosocomial infection, which usually develops late-stay infections (Schechner et al., 2009). The capability of nosocomial pathogenic bacterium to obtain new antimicrobial resistance is a general problematic with nosocomial infections. When the genetic flexibility of *P. aeruginosa* is transformed into the accessibility of a diverse set of features, it allows for the pathogen to be phenotypically adaptable and to survive both outside and inside the host (Figure 2.2).

Multidrug-resistant (MDR) *P. aeruginosa* strains are often isolated among individuals with nosocomial infections, predominantly those in the ICU. Therefore, *P. aeruginosa* infections are mainly hard as the bacterium is naturally resistant to various antibiotic groups (Vatcheva-Dobrevska et al., 2013). Affected individuals are often treated in an ICU, have several invasive instruments, endure surgical operations and, as a consequence of disease and cure, become immunocompromised. Community acquired infection with *P. aeruginosa* is infrequent situation that mostly impacts patients with particular predisposing factors including neutropenia and chronic systemic pulmonary disease (e.g., cystic fibrosis and bronchiectasis) (Strateva et al., 2010).

Pseudomonas aeruginosa endogenous infections that spread through the cutaneous, pulmonary or digestive systems, and can be transferred from one location to another in the event of osteomyelitis or endocarditis. Cystic fibrosis patients account for the majority of those infected with this organism (Neuhaus et al., 2019). Also it cause keratitis, which is one of the most rapidly developing and destructive diseases of the cornea (Munita et al., 2017), also *P. aeruginosa* could be isolated from injuries, sputum, stools and ulcerative keratitis (Azuma et al., 2016), and in

postoperative inflammatory (Su et al., 2020). This microorganism develops a numerous of exoproducts participated in *P. aeruginosa* infection pathogenesis. Amongst the factors that increase their pathogenicity are elastase, proteinase, alginate, exotoxin A, hemolysin, etc. (Jiang et al., 2014).

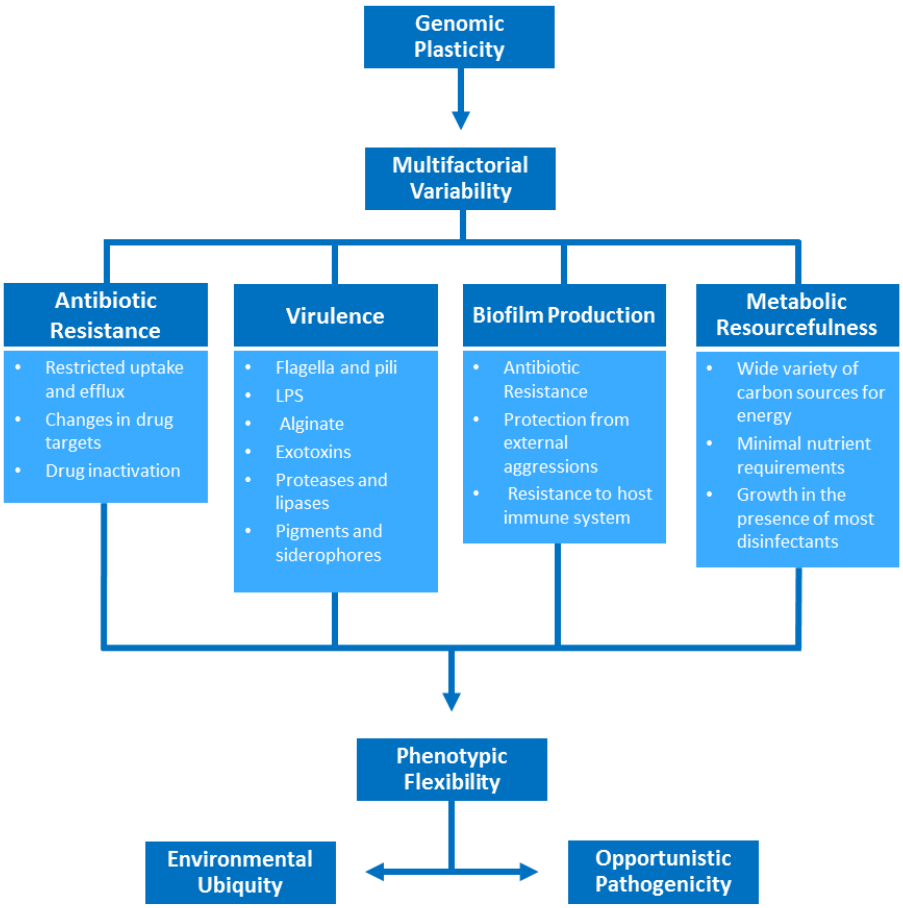


Figure 2.2 Some of the most widely studied characteristics of *Pseudomonas aeruginosa* that play a role to the bacterium's universality, pathogenicity, and general fitness are its virulence, antibiotic resistance, and virulence-resistant resistance.

The virulence factors formed by *P. aeruginosa* are enumerated and shortened in Table 1.1 (Driscoll et al., 2007). All virulence factors employed by *P. aeruginosa* is often synthesized by another microbes, with the exception of pyocyanin, which is only secreted by *P. aeruginosa* and cannot be found in any other bacterium. Many of these variables aid in colonization, whilst others aid in bacterial invasion and proliferation. Numerous components are necessary for forming colonies in bacteria, particularly pili or fimbriae, flagella, and polysaccharides on the surface of the

bacteria themselves. Both the fimbriae and the flagella are considered to be virulence factors, and they are separated into two functional groups: elements that support in the adhesion of the bacterium to the cell, and factors that aid in the invading of the cells and the suppression of the immune reaction. Pili and flagella, which are involved in the earliest stages of movement and adherence to the epithelium, and also the endotoxin lipopolysaccharide (LPS), are examples of virulence factors that can be found in bacteria (Phoon et al., 2018).

Table 2.1 Factors contributing to the virulence of *Pseudomonas aeruginosa* and their effectiveness.

Virulence Factor	Function
Fimbriae	Adhesion to cells and stimulation of pro-inflammatory gene expression are two important aspects of this process.
Polar flagella	Locomotion, adherence to host cells, and stimulation of the interleukin-8 (IL-8) signaling pathway.
Type III secretion system	Introduces toxins (ExoS, ExoT, ExoU, and ExoY) into cells.
ExoS	Activates tumor necrosis factor alpha formation.
ExoT	Stimulates GTPase.
ExoU	Cytotoxin.
ExoY	Adenylate cyclase action.
Quorum-sensing molecules	Organize expression of genes between other pseudomonad cells and stimulates the development of biofilms.
Pyochelin and pyoverdin	Bind iron.
Elastase, proteases, hemolysins, and leukocidin	Support in cell invasion and lyse the cells.
Pyocyanin	Lymphocyte growth and cilia activity are inhibited, and reactive oxygen intermediates (ROI) are formed.
Exotoxin A	Suppress protein production in host cells, which aids in the spread of the microorganism.
Lipopolysaccharide	Endotoxin
Alginate	A free radical scavenger that also prevents phagocytosis, neutrophil chemotaxis, and the stimulation of the complement system.

2.7 Molecular epidemiology

Pseudomonas aeruginosa is an environmental microorganism, and the overwhelming majority of population infections acquired are generally known to be produced through environmental strains. Seven percent of healthy people harbor *P. aeruginosa* in their esophagi. Additional places of colonization involve the skin and gastrointestinal tracts (variable recorded carry rate; to 24 % healthy people) where carriage rate may rely on diet, especially in warm and moist regions, such as axilla. Some cases involve exogenous causes of infections, particularly traumatic implantation of calcaneus osteomyelitis or case of contact colonization, including ears and folliculitis of the whirlpool (Bae et al., 2014, Moradali et al., 2017). The use of narrow-spectrum antimicrobials significantly growths the rate of *P. aeruginosa* colonization and can be chosen for resistant strains. In hospitalized patients, with significantly increased use of antibiotics, intestinal colonization rates have been reported of up to 60% of patients (Horcajada et al., 2019). It is commonly agreed that *P. aeruginosa* strains introduced into hospitals through patients' bowels are the source of their illnesses, which is corroborated by the panmictic (many and different genotypes existent) status of *P. aeruginosa* infections in the overwhelming of healthcare facilities (Fazeli et al., 2012). Nevertheless, *P. aeruginosa* can be transmitted to health workers through environmental sources, such as contaminated water. In some cases, *P. aeruginosa* nosocomial outbreaks are linked to genotypically identical strains. Researches of epidemics of MDR strain in ICU and neonatal care unit (NCUs) have been published (Lila et al., 2018). Often these strains spread quickly in a hospital and could spread across hospitals (Matar et al., 2005).

2.8 Clinical significance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa can easily adapt to changes in new environments because of its wide genome, which encompasses many regulatory genes (Silby et al., 2011). It also has various mechanisms that help to increase antimicrobial resistance and virulence. *P. aeruginosa* has high intrinsically resistant levels and the ability to resist most classes of antibiotics (Llanes et al., 2013). In a sessile community arrangement which provides antibiotic defense, host defense systems, drying up,

ultraviolet (UV) light and disinfectants, *P. aeruginosa* may likewise grow and reproduce (Hogardt and Heesemann, 2011). *P. aeruginosa* is described as an opportunistic bacterium that can lead to acute and chronic infection in humans and animals because of these improved permissibility and virulence behaviors (Kidd et al., 2012). The most prevalent path of infection is thought to be exogenous acquisition, which occurs predominantly as a result of interaction with external sources. Keratitis, skin infections, and otitis media and externa are all examples of community-acquired infections. Nosocomial causes of infection are also significant factors to the spread of infection (Driscoll et al., 2007).

2.9 Antibiotic resistance in *Pseudomonas aeruginosa*

Antibiotics are usually grouped into one of 4 categories: aminoglycosides, beta lactamase, quinolones, and macrolides. All forms of antibiotics must be understood and how they are performed in recent medicine for treating diseases. Antimicrobials operate in one of two mechanisms: by reducing and inhibition the growth of bacteria (Bacteriostatic) or by killing them (Bactericidal). In order to stop pathogens which cause patient infection, both of these functions can be critical. Antibiotics also used in animals feeding as a consequence raising the rate of antibiotic resistance an intentionally (McEachran et al., 2015).

Antimicrobials are either extracted from alive microorganisms or are adapted from derivatives of these drugs. Bacteria or fungi synthesize naturally substances when their environment is controlled in an industrialized manner. Those compounds are then utilized in ways that benefit human health, most of which are primary antimicrobials. Other antibiotics are made from biosynthetic antibiotics (Bayram et al., 2013). Use previous medications (like first generation antibiotics of cephalosporin) as a model will assist as incomes of creating novel medications. It is important to establish new substances that do not harm people but have unique bacterial targets. The true mechanisms had been not known whenever antibiotics first found active toward bacteria. Antibiotics can attack and inactivate a bacterial pathogen in a number of ways. It is important to target specific constructions and pathways of bacteria including bacterial cell walls synthesis, ribosomes and DNA

replication since they constitute a lower risk of causing undesirable human impacts (Turnidge, 2015).

Pseudomonas aeruginosa is not easy to treat as several antibiotic resistance mechanisms have been established. It was documented that its outer membranes are 100-fold resistant to cephalosporin. *P. aeruginosa* possesses the control schemes to defend microorganisms from the effects of antimicrobials. Sensors, porines and proteins are included. Many of them have been described since bacterial efflux pumps were discovered in the 1980s (Pachori et al., 2019). Efflux pumps consist of membrane proteins and are involved in the extrusion in prokaryote and eukaryote cellular of chemical antibiotics and products like organic solvents, dyes, detergents, intermediate molecules in cell communications, metabolic products and biocides (Blanco et al., 2016).

Efflux pump label substrates are oxazolidinones, and tetracyclines. In the case of oxazolidinones, because the efficacy against gram-negative microorganisms fails because of the intrinsic efflux, they are only used in infections produced by gram-positive bacteria. There are great efforts at developing oxazolidinone derivatives that confuse the gram-negative efflux pump (Pu et al., 2016). There are more new compounds in the tetracycline class which differ from the progenitors, because they have less affinity for efflux pumps and the so-called subclass of glycycline. For example, a sequences of tetracycline-specific efflux pumps are confused with gram-negative bacteria by the new tigecycline (Kumar et al., 2016). Some efflux pump inhibitors (EPIs) have recently been shown to increase tigecycline activity (Pu et al., 2016). Most efflux systems are not drug-specific proteins, of course, and extrude a wide range of bacterial compounds (Nas, 2017). While efflux pumps are very successful in the production of multidrug-resistant bacteria, its main function is obviously not the efflux of medicines. Such pumps help with various bacterial pathogenesis processes including the involvement in escape mechanisms for host protection, invasion, and development of biofilms and production of toxins (Fernando and Kumar, 2013). Therefore, a successful road for new antibiotics will be found by gaining a comprehension of the underlying mechanisms of action of pumps and methods for monitoring their pathogenicity (Dreier and Ruggerone, 2015). In

gram-negative and gram-positive bacteria and also in some eukaryotic cells, efflux pumps were identified (Van Bambeke and Lee, 2006).

The majority of efflux pumps have a three-part structure in gram negative bacteria that exceeds both inside and outside membranes. This union extrudes substrates directly from the intracellular to the extracellular and induces drug inefficiency (Abdali et al., 2017). *P. aeruginosa* has specific efflux pumps, including superfamily Resistance-nodulation-division (RND) and major facilitator superfamily (MFS), but the main pump is RND (Adabi et al., 2015). The RND pumps are situated in the interior membrane (IM), which exchange antibiotics. They must communicate with the outer membrane (OM) channel through a periplasmic linker protein (similarly recognized as membrane fusion protein) and therefore create a tripartite complex. OM makes sure the extracted substratum will not remain in the periplasm (Zowawi et al., 2015). These pathways could trigger a protective response to prevent bacteriostatic or bactericidal antibiotics. *P. aeruginosa* sensors could detect an external stressor and drive a signal to a reaction protein that causes the cell to respond (Zaoui et al., 2012).

Microbial pathogens can change from time to time and location, along with their pattern of antibiotic sensitivity. Therefore, in clinical practice, it will be helpful to recognize the current pattern of antibacterial resistance of specific pathogenic bacteria in a particular region. *P. aeruginosa* is unfortunately shown to be resistant to several antibiotics, limiting the availability of appropriate treatment (Shehabi et al., 2019). The increased drug resistance results from the development of resistance in a certain microorganism following exposure to antimicrobials and from the patient's resistant organism spread (Isichei-Ukah and Enabulele, 2018). *Pseudomonas sp*. has a variable antibiotic sensitivity pattern, and it is also the second most prevalent causational organism in post-operative infection in the surgical wound (Yadav et al., 2017). Yadav et al. (2017) have been confirmed that piperacillin-tazobactam was the most powerful antimicrobial with a sensitivity rate of 93%, then come after that imipenem with 91% and levofloxacin with 83.5%. In contrast to gentamicin 53%, amikacin showed better susceptibility 78%; the susceptibility figure was relatively low for cephalosporin and aztreonam. Ilham and Banyan (2011) were revealed that the *P. aeruginosa* isolates were completely resistant (100%) for each these

antibiotics; ampicillin, cefotaxime, chloramphenicol, penicillin, doxycycline, and erythromycin, whereas they showed intermediate resistance to amikacin 19 (39.5%), ciprofloxacin 15 (31.26%) and polymyxin 29 (40 %), and were susceptible to piperacillin, ticarcilline in a proportion rate (20.08%). Likely, Shilba et al. (2015) demonstrated that 58.33% of all isolates of *P. aeruginosa* were resist to imipenem, to meropenem 66.67%, piperacillin 86.67%, ceftazidime 51.67%, aztreonam 43.33%, ciprofloxacin 46.67% and gentamicin 91.67%, and this antibiotic sensitivity examination was established by performing VITEK II compact system. Hassuna et al. (2015) performed antibiotic sensitivity test for 250 isolates of *P. aeruginosa* that were taken from infected burn wounds in children. Result showed strong resistance to ceftazidime (86%), and cefotaxime (72%), and the resistance was not revealed to imipenem, and also twenty-eight isolates (56%) were multidrug resistant.

2.10 Pathways of antimicrobial resistance in the bacterium *Pseudomonas aeruginosa*

Antimicrobial resistance in *P. aeruginosa* has long been recognized as a significant medical risk that affects the whole population of humans, and it continues to grow. To combat this threat, a thorough assessment of antimicrobial sensitivity patterns, antimicrobial resistance genes, and antimicrobial resistance spread is required. When it comes to antimicrobial resistance, the genus *P. aeruginosa* exhibits innate or acquired resistance to a number of antimicrobial classes through a numerous methods. Some strains have inducible chromosomally encoded β-lactamase, biofilm development, mutational genes, plasmids, and transposons, all of these factors contribute to resistance to the predominance of antimicrobials in the environment (Luczkiewicz et al., 2015).

2.10.1 Restricted uptake and efflux pump

A protecting barrier between the bacterial cytoplasm and the surrounding environment, allowing hydrophilic substances to flow through. These chemicals enter the microbial cytoplasm by carrier dependent transport processes or outer

membrane channels. Gram-negative pathogens possess an external membrane barriers that inhibits big hydrophilic substances from passing via them. In the outer membrane are proteins known as porins, which help to build channels that facilitate the transport of numerous hydrophilic chemicals such as antimicrobials through the membrane. A decrease in the quantity of the outer membrane porin protein (OprD) outcomes in a reduction in drug influx into the cell, permitting the microorganisms to acquire resistance to the treatment being administered (Silhavy et al., 2010). Colistin and aminoglycosides are capable of reacting with lipopolysaccharides produced by bacteria and altering the permeability of the microbial cell membrane in order to transfer through, however certain antimicrobials including such β-lactams and quinolones disperse via specific porin channels, like those found in the microbial cell membrane (Meletis and Bagkeri, 2013).

Pseudomonas aeruginosa is known for producing particular porins, which enable only particular compounds to move through. Nevertheless, most microorganisms produce a large number of universal porins that permit practically all hydrophilic molecules to cross through, but only a small number of particular porins that have attaching positions for specific compounds (Meletis and Bagkeri, 2013, Lister et al., 2009). Some Gram-negative pathogens possess membrane proteins that act as exporters, known as efflux pumps, for specific antibacterial drugs, which helps to promote the clearance of antimicrobials from the intracellular division or the membrane space. Because these pumps discharge the antibiotic from the cell at a faster rate than the rest of the cell, the antibiotic concentrations are never high enough to have an antimicrobial impact on the bacterium (Bockstael and Van Aerschot, 2009).

The genus *Pseudomonas* expresses many efflux pumps, which permit antibiotics and other chemicals to be eliminated from the bacterium through the expression of these pumps (Chroma and Kolar, 2010). This has been the most frequent form of efflux pump discovered in Gram-negative pathogens, and it is a type of the resistant nodulation division (RND) superfamily. It is thought to have a function in the increase of the multidrug resistance (MDR) phenotype. In addition to a widespread array of antimicrobials and structurally irrelevant complexes including dyes and bile salts, this sort of pump may also expel detergents and biocides, all of which are

routinely utilized in clinical practices (Kourtesi et al., 2013). As a result, their first two parts are referred to as multidrug efflux (Mex) followed by a letter, for example, mexA and mexB. The outer membrane porin is denoted by the letters opr followed by a letter, for example, oprM. Most antimicrobial agents, with the exception of polymyxins, are excreted by these efflux mechanisms (Lister et al., 2009).

2.10.2 Drug deactivating enzymes

Microorganisms synthesize enzymes including topoisomerase, gyrase, β-lactamases, and aminoglycoside-modifying enzymes, which are capable of irreversibly altering and inactivating antimicrobials. A class of antimicrobial drugs known as the fluoroquinolones targets the microbial DNA synthesis and repair enzymes topoisomerase II/IV (*par*C and *par*E), as well as DNA gyrase (*gyr*A and *gyr*B), which are essential for microbial DNA replication and repair. *P. aeruginosa par*C and *gyr*A each contain subunits that carry antibiotic-resistant changes in the fluoroquinolones resistant defining area of *par*C or *gyr*A; hence, resistance is attributable to changes in these areas, which are frequent in the bacterium (Hooper and Jacoby, 2016).

Antimicrobial classified as beta-lactams are distinguished via the existence of a β-lactam ring in their structure, which is necessary for their activity. The β-lactam ring assists in the deactivation of a group of transpeptidase enzymes that are required for the catalysis of the last cross-linking processes throughout peptidoglycan cell wall formation in bacteria (Zango et al., 2019). Resistance to β-lactam antimicrobials in bacteria is mostly caused by the breakdown of the antimicrobial by β-lactamase enzymes in these bacteria. Cleaving the β-lactam ring via breaking the amide bond is the function of β-lactamases, which are serine protease enzymes. The relevant gene can be found on either chromosomes or plasmids, depending on the situation. Preventing the functions of metallo-β-lactamases is possible with the use of certain chelating chemicals, including EDTA. They are, nevertheless, unaffected by β-lactamase blockers used in therapeutic settings, such as clavulanic acid, and they typically maintain their sensitivity to monobactams (Tooke et al., 2019). The

Imipenemase (IMP) and Verona imipenemase (VIN) are the metallo-β-lactams that are most frequently identified in *P. aeruginosa* (Jabalameli et al., 2018).

Ambler categorized β-lactams depending on their sequence of the amino acid, and subsequently distinguished four groups of β-lactams: A, B, C, and D. Classes A, C, and D had groupings of serine enzymes that were evolutionarily separate from one another, while group B contained zinc-dependent or "EDTA-inhibited" enzymes. Ambler defined metallo-β-lactamase enzymes as belonging to Group B and categorized them into three classes, which include BI, BII, and BIII, according to their activity. Following this, the subclass BI was further subdivided based to their molecular sequences into four groups, which included the IMP and VIM kinds, as well as German imipenemase (GIM) and São Paulo Metallo-β-lactamase (SPM) kinds (Mwinyikombo, 2018). The acquiring or integration of mobile genetic cassettes, SPM, VIM, and IMP on integrons that are coupled with plasmids and transposons of bacterial, has resulted in a rise in the distribution of many antimicrobial resistant determinants. Obtaining resistance genes such as those encoding β-lactamase or aminoglycoside-modifying enzymes in *P. aeruginosa* can be accomplished through horizontal gene transfer or by chromosomal gene mutation of the target fluoroquinolones, especially ciprofloxacin, in the bacteria itself. Metallo-β-lactamase synthesis by *P. aeruginosa* (GIM, VIM, SPM, and IMP) results in the development of antimicrobial resistance in the bacterium (Zarei-Yazdeli et al., 2018).

Bush and Jacoby (2010) were established a functioning categorization approach for β-lactamases basing on the substrate and inhibitor profiles of the enzymes. Cephalosporinases, for example, are among the β-lactamases that are not efficiently blocked by clavulanic acid, and so fall into the first category. The second group is comprised of β-lactamase inhibitors that are active site-directed, including broad-spectrum β-lactamases, penicillinases, and cephalosporinases, and that are frequently inhibited by antibiotics. In the third category, there are metallo-β-lactamases that are inadequately prevented practically through all β-lactam-comprising compounds and which degrade penicillins, cephalosporins, and carbapenems, among other antimicrobials. The penicillinases of the fourth group are those that are not effectively suppressed by clavulanic acid. Second group penicillinases were divided

into subgroups based on the rates at which they hydrolyzed carbenicillin or cloxacillin (oxacillin) (Bush and Bradford, 2016). Bacterial resistance to aminoglycosides is frequently caused by the deactivation of the enzymes acetyltransferase, phosphotransferase, and acetyltransferase by bacteria, which occurs as a consequence of the uptake of plasmid genes that encode for aminoglycoside modifying enzymes. Besides from that, these genes contribute to the spread of antibiotic resistance among different species of bacteria (Doi et al., 2016).

There are several antimicrobials that are potential of defeating the innate barriers of *Pseudomonas* and are effective towards the vast majority of the species in this genus. These medications, on the other hand, are susceptible to being rendered ineffective by mutational resistance displayed by these pathogens. Instances comprise mutations in genes encoding DNA gyrase A and topoisomerases II and IV, suppression of the chromosomal AmpC β-lactamase, up-regulation of MexAB-OprM and efflux pump regulatory genes (Fair and Tor, 2014). In *P. aeruginosa*, the DNA gyrase is one of the most important targeted locations for fluoroquinolones treatment. *P. aeruginosa* strains with mutations in the fluoroquinolone resistance determining region *gyr*A and the *par*C gene had higher rates of resistance to fluoroquinolones than those without mutations in the *gyr*A and *par*C genes (Farahi et al., 2018).

It has been discovered that the regulatory gene MexAB-OprM efflux mechanism provides the natural resistance to *P. aeruginosa* for a broad spectrum of antimicrobials particularly fluoroquinolones, β-lactams, and β-lactamase inhibitors, although the regulatory gene MexXY-OprM promotes the aminoglycoside resistance. Significant concentrations of MexAB-OprM and MexXY-OprM expression might provide substantial numbers of resistance to experimental strains as a consequence of mutations happening mostly in their regulatory genes, MexR and MexZ, which are found in the majority of clinical strains (Beig and Arabestani, 2019). Antimicrobials enhance the amounts of chromosomal *amp*C β-lactamase in the existence of the species *P. aeruginosa*. Because of sporadic alterations on the regulatory genes that occur as a consequence of the constant depression of *amp*C, greater levels of β-lactamase are produced. Three genes, *amp*D, *amp*R, and *amp*G, are associated with peptidoglycan recycling and are thought to be responsible for *amp*C activation.

Inhibition of *amp*D leads in an accumulation of cytoplasmic material and an increase in the expression of *amp*C (Tamma et al., 2019).

2.10.3 Modification in the targets of drug

Pseudomonas aeruginosa isolates that are hypermutable are typically seen in individuals who have had persistent or chronic infections, such as infections of the pulmonary in individuals with cystic fibrosis. Innate resistance in bacteria refers to the intrinsic or passive resistance to antibacterial drugs that is generally produced by the implications of general adaptation mechanisms that are not closely associated to particular antibiotics or a distinct group of antibiotics (Jurado-Martín et al., 2021).

2.11 Virulence factors of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa synthesizes numerous virulence factors, many of which are important in the pathogenesis of infection and toxicity. In some circumstances, *P. aeruginosa* develops virulence factors that are linked with both acute and chronic illnesses, depending on the strain. Exoenzyme S, exotoxin A, phospholipase C, and pili are some of the components that lead to acute infection in the body. In addition to siderophores and alginate pseudo-capsules, other components that defend the cell from antibiotics, phagocytoses, and dehydration are also present. *P. aeruginosa* produces elastase and the type III secretion system, which are responsible for pneumonia (Ben Haj Khalifa et al., 2011). The exotoxin A formed by *P. aeruginosa* prevents the production of proteins in eukaryotic cells. During this cycle, the nicotinamide adenine dinucleotide (NAD) molecule is transported to the eukaryotic elongation factor 2 (EF-2). This element inhibits the activity of EF-2, resulting in the cessation of protein biosynthesis. Exoenzyme S is an ADP-ribosyl transferase that is encoded by the exoS gene. Elastase B is coded by *las*B genes and is a zinc metalloprotease that destroys immunological proteins like the cytokines. It is also encoded by alginate genes, which encode the alginate gene *alg*D, which is necessary for the defense toward biofilm and the host immune response (Michalska and Wolf, 2015).

Adhesions, biofilm development, cyanide generation, synthesis of hemagglutinin, movement, phenazines, pyocyanin, rhamnolipids, siderophores, colonization, and the type III secretion system are just a few of the virulence factors that *P. aeruginosa* is well-known for. Quorum sensing (QS) is contributed in the control of many of these virulence factors, which indicates that effective infections can only begin if the bacterial density reaches a certain level. These virulence factors have a variety of impacts; for example, pyocyanin has an inhibitory influence on cellular respiration. Elastase causes blood vessels to become disorganized and destroys the matrix of epithelial cells (Johnson, 2019), and rhamnolipids damage the cells, allowing *P. aeruginosa* to invade them more easily. Toxins synthesized by *P. aeruginosa* have an effect on the cells of the human immunological response. There are a variety of virulence factors (biofilm synthesis, development of elastase and hemolysis in the host, motility, formation of pyocyanin, and development of rhamnolipids) that are utilized to damage host cells at various stages of infection, as well as the expression of toxins to destroy host cells (Karpagam et al., 2013).

2.11.1 Cell surface virulence factors

Alginate, an exopolysaccharide synthesized by all types of *P. aeruginosa* isolates including mucoid variations, is hypothesized to have a function in the invasion and adhesion of host cells. Moreover, there was no discrepancy in the potential of non-mucoid and mucoid bacteria to adhere to or infiltrate host cells. The purpose of Alginate is to defend towards complementary and phagocytose opsonisation as well as to inhibit the transmission of antimicrobial agents into the cell. In additament to these benefits, *P. aeruginosa* alginates are far more likely than other bacteria to persist in aerosol droplets *in vitro* (Thi et al., 2020). The adherence and consequent internalization of *P. aeruginosa* to host cells are mediated by the lipopolysaccharide cell surface (LPS). *P. aeruginosa* LPS is consists of polysaccharide O and core antigens, as well as lipids A composed of fatty acids, phosphates, and disaccharide-binding glucosamine. Removing off the O polysaccharide side chains increases the likelihood of cells falling to complement-mediated lysis, but it also has the potential to provide a selection advantage by

providing protection to particular antibodies detected by host O specific antigen (Huszczynski et al., 2019).

Type IV pili (TFP) perform a most important function in the attachment of *P. aeruginosa* that composed of single protein to host cell membrane and non-living surface, the most famous example is the ability to stick with stainless steel. Researchers predict that TFP adheres more selectively to CF respiratory epithelial cells (F508 CF) than to normal respiratory epithelium. Glycoproteins on the outer membrane of CF respiratory epithelial cells (F508 CF) have been shown to have reduced sialylation, according to some research. *P. aeruginosa* possesses the ability to generate huge numbers of virulence factors that are able to cause cell damage and death following LPS and TFP adhesion mediated adhesion (Laventie et al., 2019).

2.11.2 Virulence genes in *Pseudomonas aeruginosa*

The intrinsic expression of virulence genes is distinct and contributes to a varying degree of pathogenicity in infected persons (Khattab et al., 2015). These infections are caused mainly by developing patterns of drug resistance, forming of biofilms and producing virulence factors. Biofilm has a high resistance to the immune response and antibacterial agents as a cause of chronic and persistent infection. *P. aeruginosa* growth can increase pigment production in different environments. Their external membrane proteins (OprI and OprL) stimulated the immune responses of patients and became a strong candidate for developing *P. aeruginosa* vaccines (Ingle et al., 2017). Furthermore, environmental fluctuations may improve the expression of elastase in these pathogens. *P. aeruginosa* can generate two forms of phospholipase *plch*, of which Sphingomyelin with phosphatidyllin is hydrolyzed in hemolytic form. Patients who have lung infections show virulence factor *tox*A, whereas *ExoS* is more common in patients with CF. Expression of cellular and extracellular virulence factors in *P. aeruginosa* is controlled through cell signaling mechanisms. The strong link among virulence genes and infection source will aid in community control of those infections (Khattab et al., 2015). Quorum sensing was the most active pathway for controlling their expression, which would enhance therapeutically success in the future. Most research

focuses on the four effector enzymes of the type 3 secretion system (T3SS), *exo*S, *exo*T, *exo*U and *exo*Y. The first T3SS enzymes in *P. aeruginosa* were identified, *exo*T and *exo*S, and share a 75% amino acid homology (Pena et al., 2019).

The *exo*S, the main cytotoxin that have the main role in colonization, invasion and diffusion throughout infection, even though *exo*T preserved cultures from T3SS-dependent lysis *in vitro*. The ADP-ribosyltransferase activity of both enzymes is demonstrated, but *exo*T exhibits only 0.2% of *exo*S action. The *exo*S also target small proteins similar to *Ras* that inhibit internalization, DNA synthesis and apoptosis. The *exo*T is targeted kinases at host phagocytosis and focal adhesion, and was linked to the spread of disease to liver in mouse (Shaver and Hauser, 2004). Expression of *ExoT* alone will lead to death at near-wild rates in *Galleria mellonella*, and induce apoptosis in Henrietta Lacks cells (*HeLa* cells). Exoenzyme S is a ribosyltransferase ADP coded by the *exo*S gene that is directly secreted into the cytosol of epithelial cells via the type III secretion system. Exotoxin injection of *exo*U result of quick death (1-2 h) of host cells (Lee et al., 2005).

This death is marked through the damage of the integrity of the necrotic plasma membrane. The *exo*U also causes the expression of the inflammatory genes in addition to its cytotoxic activity. According to Sawa et al. (2014), the *exo*U is 100 times as cytotoxic as *exo*S. The *lasB* elastase, a zinc metalloprotease encoded by the *lasB* gene, operates on the lungs, targets eukaryotic proteins particularly collagen and elastin and kills cell structural proteins. Moreover, two *plch* and *plcn* encoding phospholipases C (*PLC-H and PLC-N*) can be used to hydrolyze phospholipids found in pulmonary surfactants. The gene known as nan1 codes for sialidase that adheres to the respiratory system (Cotar et al., 2010).

In a study, virulence genes were selected depended on their different modes of action and *P. aeruginosa* cell secretion mechanisms. Alkaline protease (coded by *apr*) is a metal protease generated by T1SS with a varied assortment of substrates, such as collagen and complementary pathway C1q and C3, serum protease suppressors, fibrin, laminen and elastin (Nahar et al., 2017). The phenazines operons (*phzI* and *phzII*) as well as the genes (*phzH*, *phzM* and *phzS*) encode recurrent proteins in the production of three actively secreted *phenazines* compounds in *P.*

aeruginosa: pyocyanin, 1-hydroxyphenazineand-1-carboxamide phenazines. The intracellular redox cycling, reducing agents and oxygen generating superoxide and hydrogen peroxide, increases the intracellular oxidative stress (Higgins et al., 2018).

3. MATERIALS AND METHODS

3.1 Instruments and device

Table (3.1) lists the equipment's, instruments and their manufacturers used in this study.

Table 3.1 Equipment and instruments, company, and origin.

No.	Instruments	Company	Origin
1.	Autoclave	Memmert	Germany
2.	Benchtop UV-Transilluminator	BioTech	USA
3.	Bunsen burner	WLD-TEC	England
4.	Centrifuge	Eppendorf	Germany
5.	Cooled centrifuge	Sigma-Aldrich	USA
6.	Deep freezer	LG	Korea
7.	Electric oven	Memmert	Germany
8.	Electrophoresis system	BioTech	USA
9.	Hood	BioTech	USA
10.	Hot plate	Memmert	Germany
11.	Incubator	Gallenkamp	England
12.	Light microscope	Olympus	Japan
13.	Magnetic stirrer	Memmert	Germany
14.	Microfuge	Eppendorf	Germany
15.	Microwave oven	LG	Korea
16.	NanoDrop spectrophotometer	Thermo	USA
17.	pH meter	Hanna	Holland
18.	Refrigerator	LG	Korea
19.	Sensitive balance	Sartorius	Germany
20.	Shaker incubator	Gallenkamp	England
21.	Shaker water bath	Gallenkamp	England
22.	Thermal cycler PCR	ALPHA	UK
23.	Visible spectrophotometer	EMCLAB	Germany
24.	Vortex	Fisher Scientific	USA

3.2 Chemical materials and bacterial cultural media

3.2.1 Chemicals

Table (3.2) contains a list of the chemicals that were used in the experiments, as well as the chemicals that were declared by their suppliers.

Table 3.2 Chemical materials, company, and origin.

No.	Chemical materials	Company	Origin
1.	Absolute alcohol	Sharlau	Spain
2.	Agarose	GeNet Bio	Korea
3.	EDTA	BDH	England
4.	Glacial acetic acid	Sharlau	Spain
5.	Glycerol	Sharlau	Spain
6.	Hydrogen peroxide	Sharlau	Spain
7.	Methyl green	BDH	England
8.	Peptone	Oxoid	UK
9.	Potassium iodide	BDH	England
10.	Sodium chloride (NaCl)	Sharlau	Spain
11.	Sodium hydroxide (NaOH)	Sharlau	Spain
12.	Tryptone	Oxoid	UK
13.	Urea solution	LAB M	UK
14.	Yeast extract	Oxoid	UK

3.3.2 Bacterial cultural media

3.2.2.1 MacConkey agar

Selective and differential media used to isolate and separate lactose fermentation and non-lactose fermenting, gram-negative enteric bacillus, prepared through melting 36.0 g in 1000 mL of sterilized distilled water, and then autoclaved (Jung and Hoilat, 2021).

3.2.2.2 Nutrient agar

Used to purify bacteria, retain pure slant media and a typical platform process, dissolve 28.0 g in 1000 mL of sterilized distilled water, then autoclave (Power and Johnson, 2009).

3.2.2.3 Nutrient broth medium

This medium was performed for culturing and growth activation of bacteria, and prepared by melting 8.0 g in 1000 mL of sterilized distilled water, then autoclaved (Power and Johnson, 2009).

3.2.2.4 Blood agar

Blood agar base was an enriched medium employed to isolate, identify and differentiate hemolytic bacteria by dissolving 40.0 g of the blood agar base into 1000 mL of sterilized distilled water and sterilized with autoclaving, and then 5% of blood was supplemented after cooling to 50°C. Blood supplemented after autoclaving, plates were incubated at 37°C for 24 hrs. and then tested for hemolysis zone around the colonies (Jorgensen et al., 2015).

3.2.2.5 Luria Bertani (LB) broth

This medium is rich in nutrients and is often employed to cultivate members of the Enterobacteriaceae family of bacteria. LB and related media are used for rapid growth of bacteria to high density for producing plasmid, and prepared by mixing of tryptone 10.0 g, yeast extracts 5.0 g, NaCl 10.0 g. The constituents are melted in 1000 mL of sterilized distilled or deionized water, and the pH was neared to 7 and then autoclaved (MacWilliams and Liao, 2006).

3.2.2.6 DNase agar with methyl green

DNase test agar was used to detect bacterial deoxyribonuclease activity, 42.0 g in 1000 mL of sterilized distilled water were suspended and add 0.005 g of methyl green. Heat to dissolve the medium completely with regular agitation. Autoclaved to sterilize for 15 minutes at a pressure of 15 lbs (118 °C to 121 °C). Later cooled to 45 °C and poured into sterile petriplates (El-Aidie et al., 2017).

3.2.2.7 Simmon citrate agar

The medium was prepared by suspending 24.28 g in 1000 mL sterilized distilled water to detect citrate use by bacteria as sole carbon source. Heated to boiling, and dissolving the medium completely. Mixed well and put it in tubes or flasks. Sterilized at 15 lbs pressure, (121 °C) for 15 minutes by autoclaving (Power and Johnson, 2009).

3.2.2.8 Mueller Hinton agar

Mueller-Hinton agar is recommended media employed for antimicrobial susceptibility test; it was prepared by dissolving 38.0 g in 1000 mL of sterilized distilled water. Boiling to heat to completely dissolving the medium. Autoclaved at 15 lbs of pressure (121 °C) for 15 minutes. Sterilized and cooled to 45-50 °C. Mix well and pour into sterile Petri dishes. Note: This batch performance was tested and standardized in accordance with the current CLSI document (Power and Johnson, 2009).

3.2.2.9 Urea agar

This medium is used to detect bacteria which have urease enzymes and is prepared in 950 mL of sterilized distilled water, by suspecting 24.51 g, and boiling by heat to completely disintegrate the medium. Sterilize at 15 lbs (121 °C) pressure for 15 minutes by autoclaving. Sterile 40% urea solution were added after cooled to 50°C and mix thoroughly. Distribute in sterile tubes and allowed the slanting position to be set (Alfred and Heidi, 2015).

3.2.2.10 Gelatin agar

This agar was used to detect the capacity of a microorganism to form the extracellular proteolytic enzymes-gelatinases which hydrolyze gelatin; it was prepared by suspending 65 g in 1000 mL of preheated water. Brought to warm to completely dissolve the medium. At the pressure of 15 lbs (121 °C), sterilize for 15 minutes by autoclaving. Then mixed well and poured into petri dishes (Alfred and Heidi, 2015).

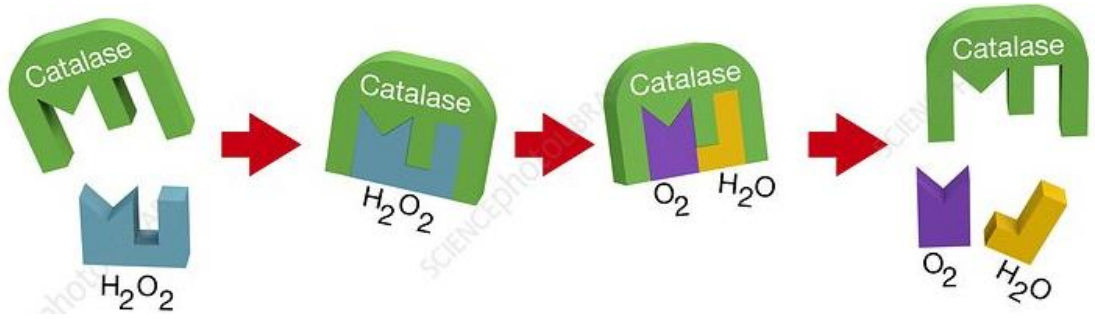
3.2.3 Reagents

3.2.3.1 Oxidase reagent

The formation of oxidase is one of the most important examinations used for distinguishing definite groups of bacteria, then prepared by dissolving 1.0 g of (tetramethyl-p-phenylenediamine dihydrochloride) in 100 mL of sterilized distilled water and used freshly and immediately. The appearance of purple or pink color means the positive result (Suryanto et al., 2011).

3.2.3.2 Catalase reagent

The existence of catalase, an enzyme that catalyzes the generation of oxygen from hydrogen peroxide (H_2O_2), is demonstrated by this assay. Bacterial catalase production is employed to distinguish between bacteria that release catalase and bacteria that do not. Routine cultures are usually carried out using 3 percent hydrogen peroxide (H_2O_2), by taking 1.0 mL from the concentrated hydrogen peroxide (30%) and diluted with sterile distilled water to make 3% concentration (Alfred and Heidi, 2015).



3.3 Specimen's collection

All samples were collected by aseptically microbiological technique. A total of 149 samples were obtained from diverse clinical specimens (including urine, wound, sputum, nasal swap and osteomyelitis soft tissue) from different public and private hospital in Erbil city, during the periods of February 2021 and May 2021 as shown in table (3-3). Sterilized cotton swabs and urine caps were utilized to take samples, in

order to prevent potential cross contamination. The sterilized swabs were moisture with sterile saline before use and the swab samples were inoculated on nutrient agars immediately and transported directly to the microbiology laboratory for incubation at 37°C for 24-48 hr. in order to promote the growth of bacteria.

Table 3.3 Source and number of samples from each source.

Source of isolations	No. of Samples	No. of *P. aeruginosa* isolated	Percent of positive samples
Urine	59	31	20.81
Wound	59	24	16.11
Sputum	19	12	8.05
Nasal swap	6	1	0.67
Osteomyelitis soft tissue	6	1	0.67
Total	**149**	**69**	**46.31**

The study was designed to analyze 149 randomly selected samples from the different clinical samples. The proportion of sex among these samples were revealed in figure (3.1) and the number of male samples comprise 56.38% (*n*=84), while the female samples consists of 43.62% (*n*=65).

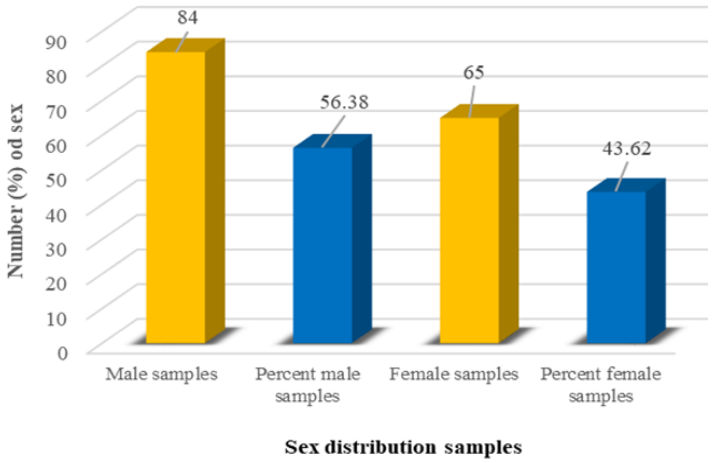


Figure 3.1 Distribution of sex specimens among clinical samples.

3.4 Isolation and identification of *Pseudomonas aeruginosa*

The following requirements were used in the isolation and identification of *P. aeruginosa*:

3.4.1 Bacterial culture

Samples were inoculated on nutrient agar, for this purpose all disposable swabs have been spread on these two media in a disposable petri plates, then incubated them overnight at 37°C.

3.4.2 Microscopic examination

The Gram stain was performed on cultures that had been incubated for 18-24 hours. In order to heat fix a loopful of overnight culture on a glass slide, it was air dried. It was then sufficient to apply crystal violet stain (0.3%) and allow it to sit for one minute. A moderate stream of water was used to rinse away any remaining stain. Grams of iodine (0.4%) were poured and left to leave for 30 seconds before being washed off. The dye was rinsed with ethanol (95%) before being stained for one minute with the counter stain, safranin (0.4%). This was then cleaned for 5 seconds with water to remove the exceed stain. The color of the bacteria under the microscope indicates whether it was Gram-positive or Gram-negative. If the cell was Gram-negative, it would appear pink when seen under a magnifying glass.

3.4.3 Biochemical tests for *Pseudomonas aeruginosa*

Some biochemical assessments of suspected bacteria that were isolated from the clinical specimens were performed, including enzyme catalase, citrate utilization action, hemolysin behavior, oxidase test, urea hydrolysis, motility activity, and gelatinase, among others.

3.4.3.1 Oxidase test

This test was performed by moistening a filter paper with a several drops of newly prepared oxidase reagent, then aseptically a picking up a group of bacterial cells from slant growth with a sterilized wood stick and smearing on the moisten paper, the appearance of violet or purple color within 10-20 sec. indicate a positive

test, this test is a key to differentiate between the genus *P. aeruginosa* (oxidase positive) and Enterobacteriaceae (Wei et al., 2020).

3.4.3.2 Motility test

The biochemical demonstration of bacterial movement is accomplished by making a straight stab through pure colonies of *P. aeruginosa* growing in nutrient broth. This was incubated at 37°C for 24 hrs. SIM medium (Sulfur, Indole, Motility media) used for the detection of movement test, and this medium poured in a test tube, and then inoculate the pure *P. aeruginosa* with a straight wire, making a single stab down the center of the tube to about half the depth of the medium. Incubate at 37°C under the conditions favoring motility (El-Aidie et al., 2017).

3.4.3.3 Hemolysis activity

Inoculating a plate of Blood agar was accomplished by streaking a single line over the surface. The petridishes were incubated at 37ºC for 24 hrs. There were three possibilities for what actually occurred: α-hemolysis (green zones, cell envelopes intact), β-hemolysis (clear, colorless zone, cell envelopes disrupted), or γ-hemolysis (no action on red cells) are all terms used to characterize a negative result for hemolysis activity in red cells.

3.4.3.4 Catalase test

Catalase is an enzyme which reduces hydrogen peroxide to water with the evolution of bubbles of oxygen. A colony was transferred to a clean slide and a 0.1 mL of 3% H_2O_2 was added. When bubbles appear it means positive result (le et al., 2015).

3.4.3.5 Citrate utilization test

The isolates of *P. aeruginosa* were inoculated onto Simmon's citrate agar and incubated at 37°C for 24 hrs. It was discovered that isolates were capable to develop on this medium, indicating that they were able for consuming citrate as a carbon source for their growth (Alfred and Heidi, 2015).

3.4.3.6 Urease test

It was shown that when the isolates colony was cultured on the surface of the urea agar slant and incubated at 37°C for 24 hrs., the enzyme urease catalyzed the analysis of urea, resulting in the change of color to pink, indicating a positive outcome (Alfred and Heidi, 2015).

3.4.3.7 Gelatin hydrolysis activity

The gelatin hydrolysis characteristics of microorganisms inoculated into tubes containing nutritional gelatin medium were investigated. The tubes were incubated at 37°C for 48 hrs. As negative controls, tubes that had not been inoculated were run alongside the inoculated tubes. After leaving the culture tube at 4°C overnight, the liquefaction of the culture media was noticed at the end of the incubation time (Alfred and Heidi, 2015).

3.5 Maintenance and storage of *P. aeruginosa* isolate

A single colony of microorganisms was streaked onto a nutrient agar plate that was then incubated at 37°C until growth appeared. One mL of nutrient broth was introduced to the surface of each plate, and the growth was picked up and transported to 1.5 mL Eppendorf tube containing sterilized 1.0 mL of 20 percent glycerol, which were then stored at –20°C in additament to the slants.

3.6 Antimicrobial disks

According to the guidelines of the Clinical Laboratory Standard Institution (CLSI) standards, a disk diffusion susceptibility test was conducted (CLSI, 2020). Three or four colonies selected from overnight culture incubated on nutrient agar were transferred to 0.5 mL of phosphate buffer saline (PBS). It was necessary to customize the suspension to produce the optical density corresponding to 0.5 McFarland standard range (1.5×10^8 CFU/mL). Within 15 min of setting the optical density (Absorbance), a sterile cotton swab was dipped into the inoculum suspension and rotated several times against the upper side wall of the tube to remove excess liquid. Mueller-Hinton plates were streaked three times, turning the plate 60° between streaks to obtain even inoculation. Inoculated plates could leave for 3 to 15 min before applying the disks. Five antimicrobial agents not closer than 24 mm to each other were applied. All inoculated plates were incubated for a total of 18–24 hrs., at 37°C in an ambient-air incubator. Inhibition zones were determined from the edge of the plate to the closest full millimeter with a ruler as stated in the table (3.4). The CLSI recommended interpretation for zone diameters of inhibition was used.

Table 3.4 Antibiotic disks used for antimicrobial sensitivity in this research.

No.	Antimicrobial Name	Symbol	Disk potency	Company
1.	Amikacin	AK	30	Bioanalysis
2.	Aztreonam	ATM	30	Bioanalysis
3.	Ceftazidime	CAZ	30	Bioanalysis
4.	Ciprofloxacin	CIP	5	Bioanalysis
5.	Colistin	COL	10	Bioanalysis
6.	Gentamicin	G	10	Bioanalysis
7.	Levofloxacin	LEV	5	Bioanalysis
8.	Norfloxacin	NOR	5	Bioanalysis
9.	Ofloxacin	OFX	5	Bioanalysis
10.	Tobramycin	TOB	10	Bioanalysis

3.7 Polymerase chain reaction (PCR) approach

3.7.1 Genomic DNA extraction by kit

3.7.1.1 Sample preparation

Genomic DNA was extracted from pure cultures through the GeneAll® Exgene™ for Clinic Cell SV mini kit (Songpa-gu, Seoul, KOREA). By incubating the culture for 12–24 hours at 37°C with dynamic shaking till the cells enter the log phase, it is acceptable to develop bacterial cells. Bacterial cells collected could be ready to use immediately or kept at -20°C or -80°C for upcoming usage.

1. Harvest cells (up to 2×10^9 CFU/mL) in a 1.5 mL Eppendorf tube by centrifugation for 1 min at *full speed*. Remove supernatant. 1~2 mL of overnight bacterial culture (A_{600}=1) might parallel to 1~2 $\times10^9$ cells.
2. Buffer CL (200 μL) should be used to fully dissolve the cell pellet.
3. Pipette 20 μL of Proteinase K solution (20 mg/mL) into a previous Eppendorf tube. Vortex the mixture rapidly to ensure complete mixing. Incubate at 56°C for 15 min. Upon completion of the lysis process, the lysis mixture will change from turbid to clear. The lysate should be incubated until it is clear and free of particles if it is still cloudy or turbid after this time.
4. Spin the tube down gently to eliminate any drips that have collected on the inside of the lid.
5. Two hundred μL of Buffer BL was added to the Eppendorf tube, and vortex to mix carefully.
6. The tubes was incubated at 70°C for 10 min. Spin down for a short time to eliminate any drops from inside of the lid.
7. Two hundred μL of absolute ethanol was added to the tube, pulse vortex to mix the sample thoroughly, and spin down temporarily to eliminate any drops from inside of the lid. Transfer all the mixture to the SV column carefully, centrifuge for 1 min at >8,000 rpm, and replace the collection tube with new one. If the mixture has not passed completely through the membrane, centrifuge again at full speed (>14,500 rpm) until all the solution has passed through.

8. Six hundred μL of Buffer BW was added, centrifuge for 1 min at >8,000 rpm and change the collection tube with new one. If the SV column has colored deposit after centrifuge, repeat this step till colored deposit disappear.
9. Seven hundred μL of Buffer TW was added. Centrifuge for 1 min at >8,000 rpm. Remove the pass-through and re-insert the SV column into the collection tube, following the manufacturer's instructions. Using full-speed centrifugation will not have any effect on DNA extraction.
10. Centrifuge at 14,500 rpm for 1 min to eliminate remaining wash buffer. Place the SV column into a fresh 1.5 mL Eppendorf tube. Centrifugation necessity be performed at full speed (14,000 ~ 20,000 rpm).
11. One hundred μL of Buffer AE was added. Incubate for 1 min at room temperature. Centrifuge at >14,500 rpm for 1 min.

3.7.2 Determination of DNA concentration

For assessing the concentration and the purity of DNA, after calibration of the machine and setting the initial blank to zero absorption, the NanoDrop spectrophotometer (Scientific Research Center (SRC), SUE) be ready to take the OD of DNA sample and concentration and purity of genomic DNA extraction evaluation was conducted in which 1 μL of the extracted genomic DNA and performed to define concentration and purity. A proportion of 1.8 to 2.0 indicates pure DNA, while a rate of above 2.0 means the existence of RNA in the DNA sample. A proportion of below 1.8 indicates the existence of protein in the extracted genomic DNA.

3.7.3 Primer preparation for PCR

Primers which described in Table (3.5), and used in the present study has been ordered from Macrogen (Korea), were prepared by adding the recommended volume in the datasheet of free nuclease water to produce 100 μM from lyophilized primers (stock solution). Then 10 μM concentration were prepared and used as a workable solution in Polymerase Chain Reaction (PCR) reaction. All primer aliquots were preserved at –20°C.

Table 3.5 The designed oligonucleotide primers for genes amplification.

Gene name	The nucleotide sequence (5'- 3')	Amplicon size (bps)	References
*rpo*B	CAGTTCATGGACCAG AACAACCCG ACGCTGGTTGATGC AGGTGTTC	759	(Benie et al., 2017)
*exo*A	AACCAGCTCAGCCAC ATGTC CGCTGGCCCATTCGCTCC AGCGCT	207	(Holban et al., 2013)
protease	TATTTCGCCGACTCC CTGTA GAATAGACGCCGCTG AAATC	752	(Nitz et al., 2021)
pvda	GACTCAGGCAAC TGCAAC TTCAGGTGCTGG TACAGG	1281	(Fazeli and Momtaz, 2014)
plch	GAAGCCATGGGCTAC TTCAA AGAGTGACGAGGAGC GGTAG	307	(Benie et al., 2017)

3.7.4 DNA amplification

It is an improved ready-to-use 2× PCR mixture composed of Taq DNA polymerase, PCR buffer, deoxynucleotide triphosphates, gel loading dyes, and Novel green dye that produces a fluorescence dye that can be demonstrated immediately after DNA electrophoresis using a blue-light transilluminator or ultraviolet light. The Master Mix contains everything that is PCR competent, with the exception of the primer and DNA template.

3.7.5 Protocol of PCR technique

PCR conducted for all genes were performed in a 25 µL of reaction volume. Master mix tube contain 12.5 µL, forward and reverse primers with 1 µL for each primer, DNA template 1 µL, and lastly sterile (D.W) deionized water 9.5 µL, illustrated in table (3.6).

Table 3.6 Reaction cocktail (25 µL) for PCR used in present study.

Component	Volume (µL)	Final concentration

Master mix	12.5	1x
Forward primer	1	0.1-1 pg
Reverse primer	1	0.1-1 pg
DNA template	1	10-50 ng/µL
Nuclease free water	9.5	
Total	**25**	

3.7.6 PCR technique procedure

The PCR condition and amplicon size for all genes understudy are revealed in table (3.7). For the purpose of identifying *P. aeruginosa*, DNA amplification for the *rpo*B gene was performed out for 35 cycles in a thermal cycler. The procedure was as below:

1. Primary denaturation at 94°C for 5 min (1 of cycle).
2. The total number of cycles were 35 of the following steps
   A. Denaturation 94°C for 1 min.
   B. Annealing 58°C for 1 min.
   C. Extension 72°C for 2 min.
3. Final extension at 72°C for 5 min.

3.7.7 Detection of virulence factors of *Pseudomonas aeruginosa*

All isolates based on biofilm status formation were screened by PCR for detection of some virulence factors and these included four genes (*exo*A, *protease*, *pvda*, *plch*) and the PCR programs of all genes were used in this work were clarifies in Table (3.7).

Table 3.7 PCR program performed for amplifying the virulence genes in *P. aeruginosa*.

Genes	Stages						
	No. of cycles	**Denaturation**		**Annealing**		**Extension**	
		Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
*exo*A	30	96 C	60 sec	55 C	45 sec	72 C	45 sec
protease	35	94 C	45 sec	56 C	40 sec	72 C	45 sec
pvda	35	95 C	40 sec	54 C	45 sec	72 C	45 sec
plch	35	95 C	45 sec	55 C	50 sec	72 C	40 sec

3.7.8 Agarose gel electrophoresis

Agarose was prepared by adding 1.2 g into 250 mL conical flask, which contained 100 mL of 1× TAE buffer swirled to mix well. The mixture melted in a microwave oven. Leaving it to cool slightly and down to about 50–55°C and Red safe dye at 10 µL/100 mL of agarose gel was added. The proper comb inserted into the tray and the agarose poured slowly into the tank to a depth of about 1 cm, the gel allowed to get solidified at room temperature. Carefully the comb removed, and the gel places in the electrophoresis tank with the wells closest to the cathode end. The gel covered with 1× TAE running buffer ensuring that the gel was just submerged. Desired DNA samples to be loaded and pipetting up 0.2 volumes of loading dye, then the sample and loading dye were mixed by filling and emptying the pipette a few times. The ready mixture loaded into a well.

The gel tank closed, the power supply switched on, and the gel run at 5 Volt/cm. Then, the voltage was increased to 75-100 volts, and the electrophoresis could proceed for a sufficient time. The movement of the gel is checked by reference to the marker loading dye. The gel running ceased when the bromophenol blue run 3/4 the length of the gel. The current switched off, DNA bands were pictured by UV illumination at (240-366 nm) wavelength on a UV transilluminator, and the gel photographed with a polaroid photo documentation camera.

4. RESULTS AND DISCUSSION

4.1 Experimental location

All the experiments was conducted between February 2021 and May 2021 in the Microbiology Lab in the Salahaddin University-Erbil, College of Education, and Department of Biology.

4.2 Collection of *Pseudomonas aeruginosa* isolates

Sixty-nine isolates of *P. aeruginosa* were identified among 149 samples were taken and collected from different clinical samples comprising urine, wound, sputum, nasal swap and osteomyelitis soft tissue from private and public hospitals in Erbil city, during the periods of February 2021 and May 2021.

4.3 Dispersal of the *Pseudomonas aeruginosa* isolates

All sixty-nine isolates of *P. aeruginosa* classified according to their source of infection were demonstrated in figure (4.1) that urine isolates (*n*=31) were the most frequent by forming 44.93%, while wound (*n*=24) formed 34.78%, then each of sputum (*n*=12), nasal swap (*n*=1) and osteomyelitis soft tissue (*n*=1) were 17.39%, and 1.45%, respectively (Figure 4.1), however the samples were taken irregularly, but were dependent on the patients who admitted into these hospitals.

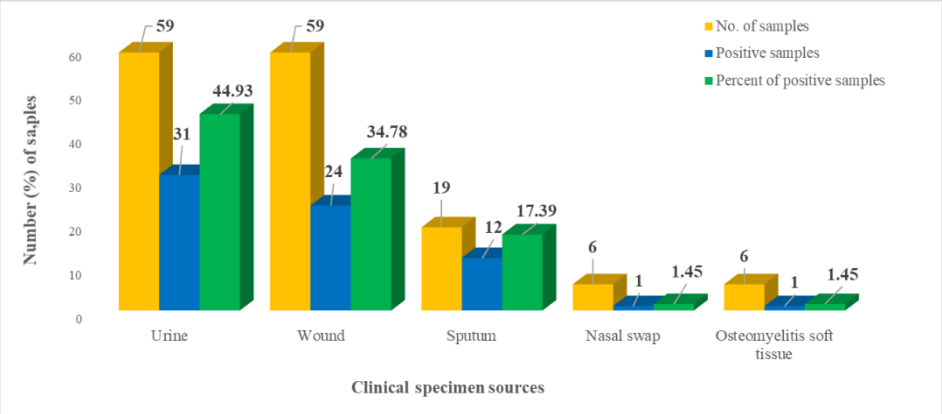


Figure 4.1 The prevalence of *Pseudomonas aeruginosa* samples depending to various origins is depicted in the diagram.

The sex number of our samples were taken for this study varied and the male samples were 84 (57.97%) while the female samples comprise 65 samples (42.03) as revealed in figure 4.2.

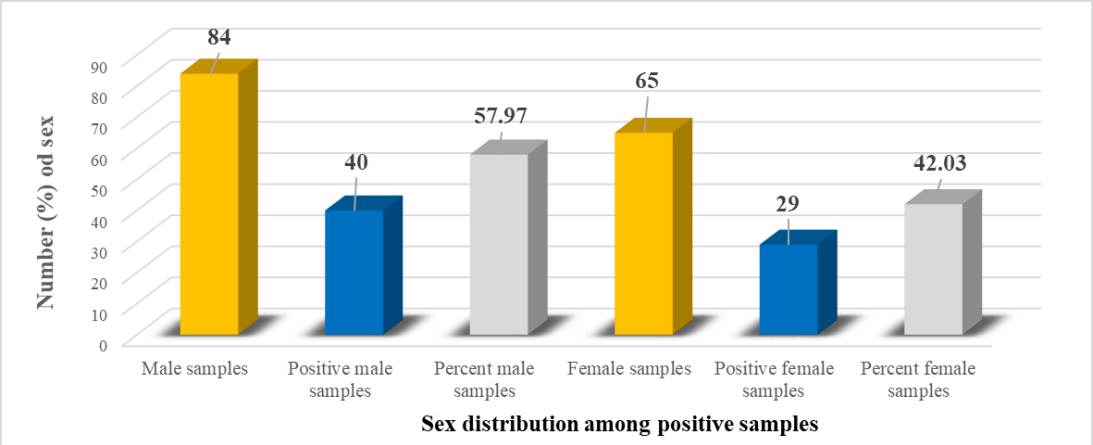


Figure 4.2 Sex distribution among clinical specimens.

4.4 Identification of *Pseudomonas aeruginosa* isolates

An overall number of 149 clinical specimens were obtained from hospitalized patients in a cross-sectional investigation. Standard microbiological and biochemical investigations were performed to identify and approve the presence of *P. aeruginosa* isolates, as detailed in the following methodology:

4.4.1 Morphological characteristics

Following culture on nutrient agar at 37°C, the cell properties were observed. Under the light microscope, all of the strains were recognized as *P. aeruginosa*, which appears as a gram-negative, rod or bacillus, non-spore producing, and motile pathogen.

4.4.2 Cultural identification

Pseudomonas aeruginosa grow more rapidly on most bacteriological media (Nutrient, MacConkey, Blood, Simmon's Citrate, Mueller Hinton agar). Colonies on solid media were small, gray yellow, smooth (in clinical samples), and mucoid (in respiratory and urinary tract secretion), flat edge, appearance of elevation and shiny. The smooth and mucoid colonies are proposed to perform a function in colonization and virulence. The *P. aeruginosa* colonies secrete a diversity of pigments, particularly pyocyanin (blue-green pigment), pyoverdin (fluorescein or yellow-green pigment), and pyorubin (red-brown pigment) as in the figure (4.3).

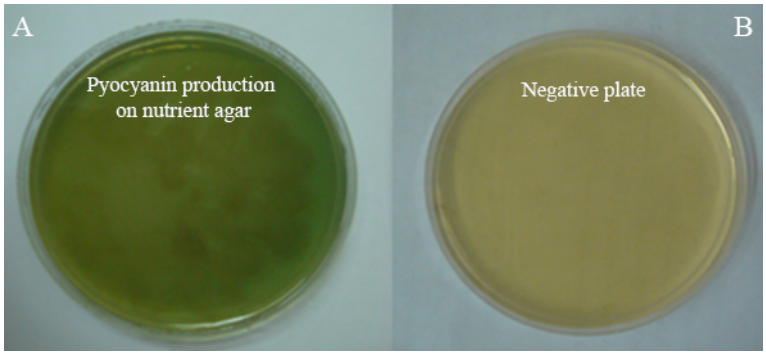


Figure 4. 3 Nutrient agar with and without growth of *Pseudomonas aeruginosa*. (A) Nutrient agar with growth of *Pseudomonas aeruginosa* which produce pyocyanin pigment, (B) Nutrient agar without growth of *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa colonies appeared pale and non-lactose fermenters on MacConkey agar. The colonies of *P. aeruginosa* on 5% blood agar were typically pale colonies and 73.91% of *P. aeruginosa* (*n*=51) exhibited β-hemolytic activity, and also there were 26.06% of *P. aeruginosa* (*n*=18) some isolates revealed α-hemolytic activity.

4.4.3 Biochemical identification

A numerous of biochemical experiments were carried out on all the strains in order to improve their identification and the results are demonstrated in Table 4.1. These investigations were designated as standard phenotypic experiments performed

to identify *P. aeruginosa* isolates among various clinical specimens were obtained and collected for this work. *P. aeruginosa* may be differentiated from other species by its potential to develop and grow at 42°C.

The organism slowly hydrolysis urea and in all times were screened for tested of urease enzyme in *P. aeruginosa* become negative, and has a positive motility test. The potential of a microorganism to create enzymes that digests gelatin is evaluated by the use of the gelatin liquefaction assay, in which differentiate *P. aeruginosa* from other species. Table (4.1) below demonstrate some biochemical tests for *P. aeruginosa*. To test the presence of oxidase enzymes and positive oxidase tests differentiate *P. aeruginosa* from other Enterobacteriaceae and all isolates developed a violet or purple color within 10 seconds which shows a positive test. The reagent (impregnated into strips of filter paper) comprises tetramethyl-p-phenylenediamine, which uses as an alternative substrate for the cytochrome oxidase reaction. To determine whether the isolates of *P. aeruginosa* has the ability of using citrate as sole carbon source for metabolism with resulting alkalinity. If the growth of *P. aeruginosa* on the medium is followed by an elevation in pH that causes the medium to transform from its original green color to a deep blue color, it indicates a positive result. *P. aeruginosa* isolates have been positive for catalase test means it has ability to convert the hydrogen peroxide and produce water with oxygen molecules (forming bubbles). According to these findings, all of the 69 isolates were confirmed as *P. aeruginosa*.

Table 4.1 The diagnosis characterization of *Pseudomonas aeruginosa* strains is accomplished by the use of phenotypic, cultural, biochemical, and molecular investigations.

Biochemical tests	**Results of tested isolates**	
	Positive *n.* (%)	**Negative *n.*** (%)
Oxidase	69 (100)	0 (0.00)
Catalase	69 (100)	0 (0.00)
Citrate utilization	69 (100)	0 (0.00)
Urease	00 (0.00)	69 (100)
Gelatin hydrolysis	69 (0.00)	0 (0.00)
Blood hemolysis	β-hemolysis 51 (73.91)	….
	α-hemolysis 18 (26.09)	….
Motility	69 (100)	0 (0)

rpoB gene	69 (100)	0 (0)

Odu and Akano (2012) were distinguished the isolates of *P. aeruginosa* from other species by its potential to develop and grow at 42°C. Dortet et al. (2012) were identified and differentiated 21 isolates of *P. aeruginosa* by using catalase and oxidase tests and revealed that all isolates demonstrated a positive results for both tests, and revealed that 98% of all isolates has the ability to hydrolyze the gelatin. dela Cruz and Torres (2012) were tested the gelatin liquefaction of *P. aeruginosa* and detects the ability of the organism to produce substances which hydrolyze gelatin, in which differentiate *P. aeruginosa* from other species. The protocol of Kawakami et al. (2010) that performed for identification of *P. aeruginosa* and include some biochemical tests and found that the isolates of *P. aeruginosa* stated positive results for each of citrate, oxidase, motility, and urease tests and this results was confirmed the consequences of the present research. The findings of the current work similar to results of Mwinyikombo (2018), which collected 41 isolates of *P. aeruginosa* and has been identified all isolates through the biochemical tests particularly oxidase, catalase, citrate utilization, hemolysis on blood agar, and secretion of pyocyanin pigment on nutrient agar, and found that all isolates positive for all tests. Likely, Mwinyikombo (2018) revealed that most isolates (n=32) has the ability to cause the β-hemolysis and some of isolates (n=9) produce α-hemolysis. Ali et al. (2021) was performed the hemolysis test for P. aeruginosa isolates on blood agar, and found that all isolates has the potential to produce β-hemolysis.

4.4.4 Molecular identification of *Pseudomonas aeruginosa*

In this research, bacterial genomic DNA was extracted from whole cells by performing the GeneAll® Exgene™ for Clinic Cell SV mini kit (Songpa-gu, Seoul, KOREA). Extracted genomic DNA was run on 0.8% agarose gel using electrophoresis system to approve the successful extraction of the genomic DNA. All isolates of *P. aeruginosa* have been undergone PCR assay for more confirmation to the identity of all isolates, using specific conserved *rpo*B gene (Figure 4.4), unique

designed primers were used for identification of *P. aeruginosa* isolates, and all the isolates were harbor the *rpo*B gene.

All 69 isolates of *P. aeruginosa* were harbours the *rpo*B gene using PCR technique, and this was showed in figures 4.4, by *rpo*B (759 bp) amplified gene. The results of the previous report confirm the findings, which indicate that *rpo*B is ubiquitous. Benie et al. (2017) were used *rpo*B gene to confirm the 112 presumptive isolates of *P. aeruginosa*, which identified by cultural and biochemical assessments, the *rpo*B gene confirmed the 100 isolates of *P. aeruginosa*.

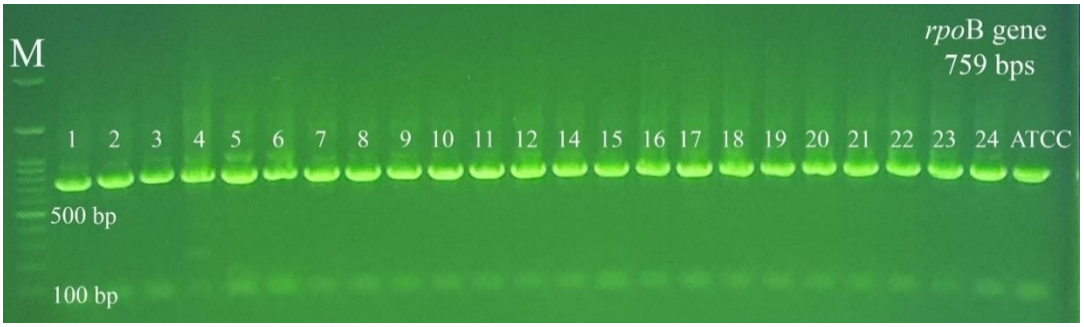


Figure 4.4 Amplification of *rpo*B identification gene of *Pseudomonas aeruginosa* using conventional PCR, lane M: DNA marker 100 base pair, lanes 1-24 with ATCC 29213 represent amplified product (**759** bp) of *Pseudomonas aeruginosa* isolates.

The unique primer for *rpo*B gene was used to identify *P. aeruginosa* by Tayeb et al. (2005), and their results has been revealed that this gene can be used to identify *P. aeruginosa* as a simple and reliable way. In all isolates of *P. aeruginosa*, *rpo*B are revealed to be possess. The significant percentage of molecular identity has revealed that genetic experiments were essential in confirming the accurate taxonomic identification of *P. aeruginosa*. The success of the *rpo*B gene in the identification of *P. aeruginosa* strains may be supported by an observation of the *rpo*B gene, which discriminates between species very similar to *Pseudomonas*. Fazeli et al. (2012) were testified that all *P. aeruginosa* isolated from infected patients were identified through PCR assay shows positive results for *rpo*B gene, which confirmed the outcomes of this research results.

4.5 The assessment of the antibiotic susceptibility of the *Pseudomonas aeruginosa* isolates

The antimicrobial resistant profiles of numerous antimicrobial agents against the clinical isolates of *P. aeruginosa* were assessed by disk diffusion technique according to the CLSI guidelines, and the ability of the isolates to develop in the existence of antibiotics was established. All sixty-nine isolates of *P. aeruginosa* have been tested for resistance to 10 commonly utilized antimicrobials including (Amikacin, Aztreonam, Ceftazidime, Ciprofloxacin, Colistin, Gentamycin, Levofloxacin, Norfloxacin, Penicillin, Ofloxacin, and Tobramycin). The outcomes of antibiotic resistance assay for the all isolates of *P. aeruginosa* understudy are presented in table (4.2). This study clearly demonstrates that the bacterial isolates showed great heterogeneity in their resistance to the antibiotics tested. The high resistance percent of *P. aeruginosa* was 82.61% toward for amikacin, and the lowest resistant percent was 42.03% for gentamycin. Six antibiotics revealed intermediate resistant against all isolates of *P. aeruginosa*.

Table 4.2 Antimicrobial susceptibility patterns of *Pseudomonas aeruginosa* isolate toward antimicrobials.

Antimicrobials	Resistance *n.* (%)	Intermediate *n.* (%)	Sensitivity *n.* (%)
AK	57 (82.61)	9 (13.04)	3 (4.35)
ATM	45 (65.22)	0 (0.00)	24 (34.78)
CAZ	54 (78.26)	15 (21.74)	0 (0.00)
CIP	49 (71.01)	0 (0.00)	20 (28.99)
COL	37 (53.62)	0 (0.00)	32 (46.38)
CN	29 (42.03)	12 (17.39)	28 (40.58)
LEV	30 (43.48)	11 (15.94)	28 (40.58)
NOR	39 (56.52)	2 (8.7)	28 (40.58)
OFX	34 (49.28)	29 (42.03)	2 (8.7)
TOB	36 (52.17)	0 (0.00)	33 (47.83)

*: AK: amikacin, ATM: azithromycin, CAZ: Ceftazidime, CIP: ciprofloxacin, CN: gentamycin, LEV: levofloxacin, NOR: norfloxacin, OFX: ofloxacin, TOB: tobramycin.

Pseudomonas aeruginosa is intrinsically resistant to many antimicrobial agents and adaptive resistance is frequently developed during antibiotic therapy. The mechanisms of resistance consist of chromosomally encoded β-lactamases, multidrug

efflux systems, low outer membrane permeability and LPS change. Antibiotics like piperacillin, aztreonam, ceftazidime, meropenem ciprofloxacin, colistin, azithromycin or tobramycin are usually active against *P. aeruginosa*, but resistance often develops in the case of chronic infected CF individuals (Høiby, 2011). *P. aeruginosa* possesses more than 10 transporters that are members of the resistance-nodulation-division (RND) group, nine of which transport multiple antibiotics. These transporters not only export antibiotics, but they also disseminate biocides, dyes, detergents, metabolic inhibitors, organic solvents, and chemicals required in bacterial cell-cell communication. Regardless of the fact that drug efflux is not a biological behavior of RND pumps, these pumps assist many microorganisms to become resistant to antimicrobial agents, threatening to the therapy of infectious diseases as a result (Poole, 2008). *P. aeruginosa* also produces a chromosomally encoded cephalosporinase designated AmpC. The expression of *amp*C is highly inducible by certain β-lactams, particularly cefoxitin, imipenem or ampicillin. The antipseudomonal penicillins (like piperacillin) and cephalosporins (like ceftazidime) are very weak AmpC inducers, notwithstanding the actuality that this enzyme hydrolyzes them. However, throughout medication with these weak inducers, mutants revealing high expression of *amp*C (*amp*C derepressed mutants) are frequently chosen, causing to the disappointment of antimicrobial treatment. In addition *P. aeruginosa* is able to modify its outer-membrane LPS leading to increased tolerance towards cationic antimicrobial peptides, like colistin, and increased inflammatory responses (Gómez- Lozano et al., 2012).

According to the type and their resistances to antimicrobial under study, *P. aeruginosa* isolates were grouped into 20 groups, as shown in the table (4.3), as antibiogram related to antibiotic resistance and it is clear that the predominant mode which include more resistance isolates were represent mode (1), it was resisting to 80% of antibiotics, and the resistances of other groups ranged between 20-70%.

Olayinka et al. (2009) stated that twenty percent of isolates of *P. aeruginosa* collected from clinical specimens were taken from the patients in the surgical units of Ahmadu Bello University Teaching Hospital in Nigeria were sensitive for ß-lactam antibiotics that possess the broad-spectrum activity against Gram-negative and Gram-positive bacteria. All bacterial isolates displayed a low resistance and the

majority of Enterobacteriaceae isolates showed no resistance. It might be because they are reserve medicines and they are used as the last option in our hospital environment for multidrug-resistant bacteria which agreed with our result. Ebrahimpour et al. (2018) were recorded that 40% of strains of *P. aeruginosa* isolated from burn patients were resist to ceftazidime. In the research of Ali et al. (2017), 75% of *P. aeruginosa* isolates resist Amikacin, 65% for ceftazidime, ciprofloxacin, gentamicin and tobramycin, which is parallel with our findings. Resistance by *P. aeruginosa* can both be due to inducible of beta-lactamases, which can make cephalosporin of broad-spectrum inactive and to beta-lactamases mediated by plasmid, which can lead to several penicillin's and ancient cephalosporin becoming resistant (Bush, 2018). Mechanisms of aminoglycoside resistance in clinical isolates are usually controlled by enzymatic antibiotic inactivation since nine different enzymes that are capable of catalyzing phosphorylation, acetylation, aminoglycosides in bacteria had been described (Krause et al., 2016). The development of the *P. aeruginosa* multi-resistant and its antibiotics mechanisms involves decreased cell permeability, efflux pumps, and changes in target enzymes and antibiotics deactivation (Lambert, 2002). According to our findings, the resistance to third-generation cephalosporins, including ceftazidime was developed, with a final resistance rate of 78.28%.

Table 4.3 Antibiogram groups of *Pseudomonas aeruginosa* isolates and percentage of antimicrobial resistance.

Antibiogram group	No. of isolates	% of resistance	Antibiotics at final concentrations									
			AK	ATM	CAZ	CIP	COL	CN	LEV	NOR	OFX	TOB
1	1	80	S	S	R	R	R	R	R	R	R	R
2	7	70	S	S	I	R	R	R	R	R	R	R
3	9	70	S	R	R	S	R	R	R	R	R	S
4	1	70	S	R	R	R	R	S	S	R	R	R
5	1	60	R	R	R	R	S	S	R	S	S	R
6	1	60	S	R	I	S	R	S	R	R	S	R
7	1	50	S	R	R	R	S	S	S	R	I	R
8	1	50	S	R	R	S	S	R	R	S	R	S
9	6	40	S	R	R	R	S	S	S	R	I	S
10	2	40	S	R	R	S	S	S	S	S	R	R
11	3	40	I	R	R	S	S	R	I	S	I	R
2	6	40	R	R	R	S	S	S	R	I	S	S
13	7	40	S	R	R	S	S	S	R	S	R	S
14	4	40	S	R	R	S	S	S	S	S	R	R
15	1	40	S	R	R	S	R	S	S	S	S	R

16	4	30	S	R	I	S	R	S	S	S	S	R
17	3	30	I	S	R	S	S	R	I	S	I	R
18	2	30	S	R	R	S	S	S	S	S	S	R
19	2	30	S	R	I	S	R	S	S	S	S	R
20	7	20	S	S	R	S	R	I	S	S	I	S

Our findings were revealed that high resistance rates to ciprofloxacin was reported with percentage of 71.01%. This finding was supported with antibiotic susceptibility patterns of Al-Azzawi et al. (2020) and Biswal et al. (2014) who revealed that 91.5 and 79% of all isolates of *P. aeruginosa* were resistance for ciprofloxacin. Ilham and Banyan (2011) stated that *P. aeruginosa* isolated from various clinical specimens were completely resistant for each of amoxicillin, cefotaxime, chloramphenicol, penicillin, whereas exhibit intermediate resistance to amikacin (39.5 %), ciprofloxacin (31.26 %). Furthermore, Nikokar et al. (2013) established that *P. aeruginosa* isolated from burn cases exhibited considerable gentamycin resistance, with a resistant rate of 37.2 percent of the bacteria. Bacterial synthesis of aminoglycoside modifying enzymes, decreases in uptake, membrane permeability, transport or efflux, point mutations at the drug target, and methylation enzymes altering the target, can all contribute to resistance to aminoglycosides (e.g., amikacin and tobramycin) (Bhatt et al., 2015). Increasing antibiotic use results in a selection pressure that works as a motivating factor in the evolution of antimicrobial resistance (Moazami and Eftekhar, 2013). The existence and distribution of plasmids within a heterogeneous bacterial population, that could transmission among genera and species of bacteria and cause to the occurrence of resistance by conjugation and transformation (Yousefi et al., 2010), and also may be associated with the high resistance of the bacterial isolates to various antimicrobial agents (Alvarez-Ortega et al., 2011). In response to a weakened immune function in certain humans as a result of inadequate nutrition or hereditary factors, microorganisms have become more resistant to antibiotics (Kirchner et al., 2012). In the last decades, it have understood an extraordinary rise in the transmission of resistance among *P. aeruginosa* strains, making it more difficult to select an appropriate antibiotic course for the treatment of this microorganism-caused infection. In all nations, *P. aeruginosa* isolates are becoming progressively resistant to a growing number of antimicrobial agents. Additional reason for resistance in *P. aeruginosa* might be linked to a mutation in the

structure of the bacterial cell wall, which results in the absence of porins. This is also considered to be a reason for bacteria's resistance to antibiotics, particularly β-lactam antibiotics (Morales et al., 2012).

Chand et al. (2020) performed a study to isolation and identification of *P. aeruginosa* among 7898 samples that they collected from different clinical samples, and they identified 87 isolates as *P. aeruginosa*. They used 13 antibiotics using disk diffusion for check the antibiotic susceptibility and found that the high resistant rate was 100% toward cefixime and nitrofurantoin, followed by carbenicillin, norfloxacin, ciprofloxacin, ceftazidime, gentamicin, and tobramycin with resistant rate 47.12%, 35.35%, 31.03%, 29.88%, 20.68%, 18.39%, respectively.

The antibiotic sensitivity assessment was performed by Mobaraki et al. (2018) that used disk diffusion method, and the antibiotic disks that used included colistin, amikacin, cefepime, cefotaxime, ceftazidime, tobramycin, gentamicin, and ciprofloxacin, and found that 55% of isolates resist to amikacin, 62.5% to cefepime and ciprofloxacin, 66.5% to ceftazidime, 57.5% to tobramycin, 62% to gentamicin, 3% to colistin, and 74.5% to cefotaxime. The antimicrobial susceptibility profiles of the tested strains of *P. aeruginosa* were determined by Nitz et al. (2021) that used the VITEK II compact system, and when they tested the isolates, they revealed that all of them were resistant to ampicillin, cefoxitin, and tigecycline, however all of them were sensitive to polymyxin B, with the inclusion of a single strain that was colistin resistant (polymyxin E). There was a significant occurrence of strains with resistance to the β-lactam drugs piperacillin/tazobactam (74.7%) and cephalosporins, 70.7% of isolates were resist to ceftazidime (3rd generation cephalosporins) and 61.6% had been resist to cefepime ((4^{th} generation cephalosporins). It is necessary to note that 53.5% of the isolates tested positive for resistance to both cephalosporins. Carbapenem resistance was found in the majority of strains (66.7%), followed by gentamicin resistance (58.6%), carbapenem resistance (57.6%), and meropenem resistance (56.6%).

The antimicrobial susceptibility testing of the 50 isolates of *P. aeruginosa* included in the experiments of Naga et al. (2019), and stated that the highest prevalence of resistance was detected against 3^{rd} and 4^{th} generation cephalosporins

(80%). They found that 40 isolates (80%) of *P. aeruginosa* were resist to ceftazidime, 35 isolates (70%) to aztreonam, 26 isolates (52%) to gentamicin, 28 isolates (56%) to tobramycin, 22 isolates (44%) to amikacin, and 34 isolates (66%) to ciprofloxacin and levofloxacin, while all isolates of *P. aeruginosa* became sensitive to colistin antibiotic. In contrast, they revealed that isolates of *P. aeruginosa* had intermediate resistant toward some antibiotics like ceftazidime, aztreonam, gentamicin, and amikacin.

Tetracycline (32.85%) and ofloxacin (32.85%) were found to have a significant rate of resistance against *P. aeruginosa* isolates, depending to the results of Bahador et al. (2019). For colistin, a low level of resistance was detected (1.42%). One isolate (1.42%) was revealed to be resistant to all of the antibiotics examined. A further finding was that 58.6% of the isolates were susceptible to all of the antimicrobial drugs were assessed. Approximately 24.3% of the isolates demonstrated resistance to at least three separate antimicrobial agent classes and were classified as MDR. The total of 112 *P. aeruginosa* isolates were obtained from various clinical samples by Bahramian et al. (2019), and they performed antibiotic sensitivity approach and discovered that the largest percentage of resistance was associated with cefepime, gentamicin, and ciprofloxacin 99 (*n*=88), and after that come each of meropenem and imipenem with resistant rate 98%.

4.6 Detection of some virulence genes among *Pseudomonas aeruginosa* isolates

The *exo*A, *pvda*, *protease*, and *plch* genes were identified using a PCR technique. The key goals of this investigation are the genotypic assessment of virulence genes (Figure 4.5 to 4.8).

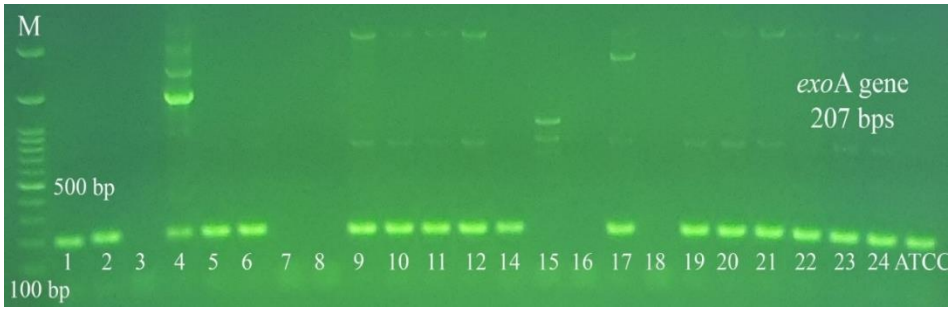


Figure 4.5 PCR amplification of *exo*A virulence genes of *Pseudomonas aeruginosa* using PCR, lane M: representing the DNA marker 100 base pair, lanes 1, 2, 4, 5, 6, 9,

10, 11, 12, 14, 17, 19, 20, 21, 22, 23, 24 and ATCC 29213 represent amplified genes with product size amplicon size (207 bp) of *Pseudomonas aeruginosa* isolates, lane 3, 7, 8, 15, 16, and 18 were negative for *exo*A gene.

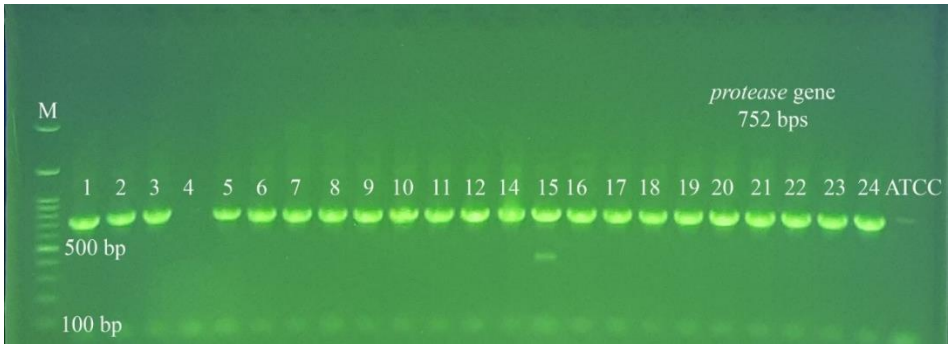


Figure 4.6 PCR amplification of protease virulence genes of *Pseudomonas aeruginosa* using PCR technique, lane M: representing the DNA maker 100 base pair; lanes 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and ATCC 29213 represent amplified genes with product size amplicon size (752 bp) of *Pseudomonas aeruginosa* isolates, lane 4 were negative for *protease* gene.

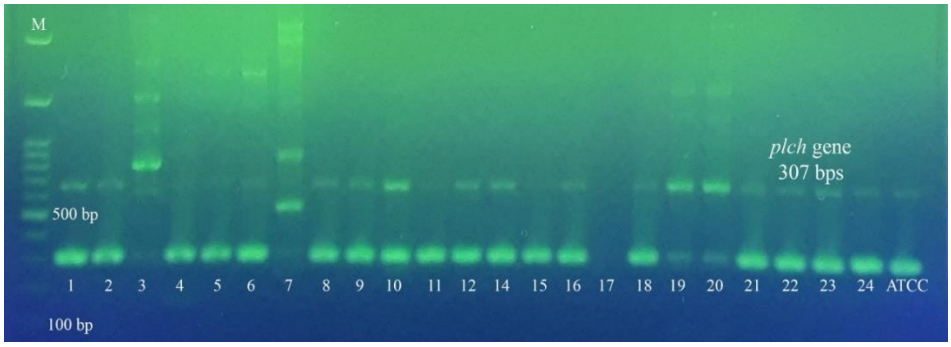


Figure 4.7 PCR amplification of plch virulence genes of *Pseudomonas aeruginosa* using PCR technique, lane M: representing the DNA marker 100 base pair; lanes 1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, and ATCC 29213 strain represent amplified genes with product size amplicon size (307 bp) of *Pseudomonas aeruginosa* isolates; while lane 3, 7, and 17 were negative for *plch* gene.

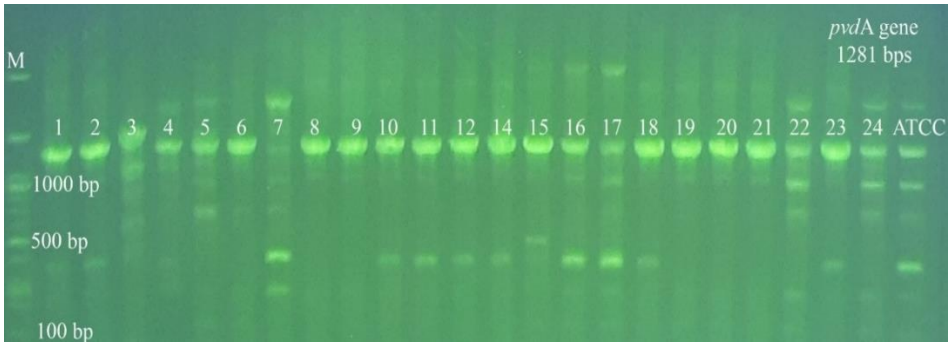


Figure 4.8 PCR amplification of pvda virulence gene of *Pseudomonas aeruginosa* using PCR technique, lane M: representing the DNA marker 100 base pair; lanes 1-

24 with ATCC 29213 strain (except isolate 7 which negative for *pvda* gene) represent amplified genes with product size amplicon size (1281 bp) of *Pseudomonas aeruginosa* isolates.

All the four genes have been found among all isolates with different frequencies, as revealed in table (4.4). Out of 69 total isolates, 66 isolates (95.65%) of *P. aeruginosa* revealed the presence of *pvda* virulence gene however *exo*A, *protease* and *plch* genes were in the different rates among all isolates of *P. aeruginosa* and were established to be 66.67 (*n*=46), 81.16% (*n*=56) and 78.28 (*n*=54), respectively.

Table 4.4 Determining genes contributed in the production of virulence factors in the bacterium *Pseudomonas aeruginosa*.

Isolate no.	*rpo*B ID gene	Virulence factor results				Percent (%) of genes
		*exo*A	*pvda*	*protease*	*plch*	
1	+	+	+	+	-	75
2	+	+	+	+	+	100
3	+	-	+	+	-	50
4	+	+	+	-	+	75
5	+	+	+	+	+	100
6	+	+	+	+	+	100
7	+	-	-	+	-	25
8	+	-	+	+	+	75
9	+	+	+	-	+	75
10	+	+	+	+	+	100
11	+	+	+	+	+	100
12	+	+	+	+	+	100
13	+	+	+	+	+	100
14	+	-	+	+	+	75
15	+	-	+	+	+	75
16	+	+	+	+	-	75
17	+	-	+	+	+	75
18	+	+	+	+	+	100
19	+	+	+	+	+	100
20	+	+	+	+	+	100
21	+	+	+	+	+	100
22	+	-	+	-	-	25
23	+	-	+	-	+	50
24	+	+	+	+	+	100
25	+	+	+	+	-	75
26	+	+	+	+	+	100
27	+	+	+	+	+	100
28	+	-	+	+	-	50
29	+	+	+	-	+	75
30	+	+	+	+	+	100
31	+	+	+	+	+	100

32	+	-	-	+	-	25
33	+	-	+	+	+	75
34	+	+	+	+	+	100
35	+	+	+	+	+	100
36	+	+	+	+	+	100
37	+	+	+	+	-	75
38	+	+	+	+	+	100
39	+	-	+	-	+	50
40	+	-	+	+	+	75
41	+	+	+	-	-	50
42	+	-	+	+	+	75
43	+	+	+	+	+	100
44	+	+	+	+	+	100
45	+	+	+	-	+	75
46	+	+	+	+	+	100
47	+	+	+	+	+	100
48	+	+	+	+	+	100
49	+	-	+	+	+	75
50	+	+	+	-	+	75
51	+	-	+	+	+	75
52	+	+	+	+	+	100
53	+	-	+	+	-	50
54	+	-	+	-	+	50
55	+	+	+	+	+	100
56	+	+	+	+	+	100
57	+	-	-	+	-	25
58	+	-	+	+	+	75
59	+	+	+	+	+	100
60	+	+	+	+	+	100
61	+	+	+	+	+	100
62	+	+	+	+	-	75
63	+	+	+	-	+	75
64	+	-	+	+	+	75
65	+	-	+	+	+	75
66	+	+	+	-	-	50
67	+	-	+	+	+	75
68	+	+	+	+	-	75
69	+	+	+	-	+	75
ATCC	+	+	+	+	+	100
Total positive	**69**	**46**	**66**	**56**	**54**	
Positive percent	**100**	**66.67**	**95.65**	**81.16**	**78.26**	

Benie et al. (2017) were found that the *plch* virulence gene rate among 100 isolates of *P. aeruginosa* was 71%. The PCR results of Al-Dahmoshi et al. (2018) on virulence factor occurrence among 26 isolates of *P. aeruginosa* were taken from burned patients admitted to hospital during a period of 6 months and showed that *exo*A virulence gene was found among 46.15% of isolates (*n*=12).

The genes encoding the exotoxins *exo*S, *exo*T, and *exo*A, which are associated to T3SS, were shown to be dispersed significantly across the isolates studied. Isolates

from burn individuals have the highest percentages of positive T3SS exotoxins codifying genes, followed by isolates from tracheobronchial mucus membranes. The *exo*U, the gene encoding the extremely cytotoxic exoenzyme exoU, and *pvda*, the gene encoding an essential enzyme involved in the manufacture of siderophores pyoverdine, are two of the most significant genes in the genome of *P. aeruginosa* (Park et al., 2017).

It has been confirmed through phenotypic and genotypic studies that *P. aeruginosa* has the ability to modify its virulence when it is the causative agent of infections that arise in a variety of clinical situations in burn patients. Moreover, the expression of several particular virulence genes profiles was shown to be associated with the presence of phenotypic virulence markers, indicating that bacteria may quickly adapt to the microenvironment encountered within the host through regulating the expression of these genes. Isolates from burns of *P. aeruginosa* are recognized to be one of the most virulent strains, demonstrating the whole spectrum of cell-associated and soluble virulence factors and virulence genes that were studied (Holban et al., 2013). As a findings of the research conducted by Holban et al. (2013), it was discovered that the *las*B and *protease* IV genes code for proteases and that they are expressed in the majority of the examined strains. The *plch* gene that codes for the hemolytic phospholipase C enzyme, is found in several *P. aeruginosa* strains, including those obtained from blood cultures (40%) and wound secretions (55%). This finding supports phenotypic data that isolates collected from blood cultures and wound secretions are the most hemolytic.

The prevalence of *exo*A, *exo*U, and *exo*S genes has been reported in a variety of research with varying results. The *exo*A, *exo*U, and *exo*S genes were found to be present in 90.4%, 66.7%, and 65.4 % of clinical isolates of *P. aeruginosa*, respectively, according to Zarei et al. (2018). Eighty-one percent and 61 percent of clinical specimens of *P. aeruginosa* respectively, were found to have the *exo*A and *exo*S strains, according to Amirmozafari et al. (2016). Our findings and other researchers have discovered that *exo*A is a more common cause of viral infection than previously thought. Although it is more common in environmental isolates than clinical isolates. The *exo*U was only discovered in respiratory assistance devices such as a ventilator and a tracheal tube. In the case of ocular infections, no *exo*U was

found. There was no statistically substantial association among virulence genes and the kind of clinical specimen used. All *P. aeruginosa* strains have a genetic structure that is highly preserved and contains genes that are essential for persistence in a range of conditions, and this enables them to produce a wide range of human infections. In the investigation by Elmouaden et al. (2019), the virulence genes (*las*B, *alg*D, *plch*, *exo*A, and *exo*S) were chosen based on their significance in pathogenesis, and PCR assessment was used to screen for these virulence encoding genes. The findings revealed that *las*B (98.7%) and *exo*S (98.7%) were the most frequently found virulence genes in *P. aeruginosa* strains, and after that, *plch* gene prevalence recorded 96.1%, and *alg*D (87.7%), although the minimum normally discovered virulence factor gene was *exo*A (74.2%).

On the basis of the amount of virulence genes found in each type of clinical specimen, Nitz et al. (2021) discovered that all of the virulence genes analyzed were found in isolates obtained from blood culture and in 99.0 percent of the tracheal secretion specimens. The *las*B (89.9 percent) and *exo*Y (87.8%) genes had the highest detecting frequencies for each gene depending to the type of clinical samples, following by *exo*T (82.8%), *tox*A (81.8%), *alg*D (75.7%), *plch* (75.7%), *phz*I (70.7%), and *plch* (70%). The existence of the genes *exo*S (45.4%), *phz*M (34.3%), *pil*B (27.3 percent), *apr* (22.2%), *protease* IV (20.2%), *nan*-1 (14.1%), *pil*A (14.1%), and *apr*A (6.0%) was also discovered, although in a less common manner.

5. CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORKS

5.1 CONCLUSIONS

Pseudomonas aeruginosa is the definition of an opportunistic infection of humans, one that takes benefit of a lack of or a breakdown in the host's defenses. This bacteria is also receiving worldwide interest as a result of its role in the rise of antibiotic resistance. By nature of its widespread occurrence, it emphasizes the significance of selecting appropriate antibiotics as well as utilizing additional techniques to control nosocomial infections and prevent the emergence of drug resistance.

1. Males accounted for the majority of hospitalized patients, suggesting that males are more prone from urine.
2. Despite the fact that *P. aeruginosa* was resistant to nearly all medications when isolated from burn individuals, gentamicin was shown to be the most efficient antibiotic against the bacteria.
3. The use of biochemical methodologies in conjunction with *rpo*B genes increases the confidence in the identification of *P. aeruginosa* using a PCR assay.
4. Following the identification of distinct virulence in *P. aeruginosa* isolates, it has been proposed that they are related with variable levels of innate virulence and pathogenicity. According to the results of the PCR analysis, *pvda* virulence genes were discovered to be more widely dispersed among the four genes that were identified in our research in a high rate of 95.65% of *P. aeruginosa* isolates.

5.2 FUTURE WORKS

1. Establishing fast method for extraction of DNA from a variety of clinical samples (blood, urine, burn, etc.) and environmental samples (water, soil, etc.) subsequent rapid, direct detection and identification of *P. aeruginosa* from them depended on the amplification of the target DNA without culturing the bacteria is recommended.
2. Performing of DNA sequencing for virulence genes in order to detect the type of mutation if found in isolated genes.

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Ministry of Higher Education and Scientific Research
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وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
زانکۆی سەڵاحەددین-هەولێر
کۆلێژی پەروەردە
بەشی بایۆلۆجی

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Date: 23-06-2021

Approval of Research Ethical Committee

Herewith we declare that Ethical Committee-Scientific Research Office in Research Center of Salahaddin University-Erbil has processed the submitted research proposal of (MSc. Student **AHMED SHERZAD AWLA**) master student in (Near East University). His research entitled (**Molecular detection of some virulence genes in *Pseudomanas aeruginosa* isolated from clinical source**) approved to be conducted in the setting of our research center.

ASST. PROF. DR. KAZHAL M. SULAIMAN
Head of Biology Department
23/06/2021

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