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MOLECULAR, BACTERIOLOGICAL, AND BIOCHEMICAL STUDY FOR *LASB* AND *APRA* GENE IN *PSEUDOMONAS AERUGINOSA* BACTERIA

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AHMED ABDULHUSSEIN HILO HILO

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By Ahmed Abdulhussein Hilo HILO

January 2024

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

Advisor **:** Assoc. Prof. Dr. Pınar ARSLAN
Co-Advisor **:** Asst. Prof. Dr. Abdulameer Jasim MOHAMMED

Examining Committee Members:

Chairman **:** Asst. Prof. Dr. Sinem PEHLİVAN
Medical Pharmacology
Ankara Medipol University

Member **:** Asst. Prof. Dr. İlkay ÇORAK ÖCAL
Biology
Çankırı Karatekin University

Member **:** Assoc. Prof. Dr. Pınar ARSLAN
Biology
Çankırı Karatekin University

Approved for the Graduate School of Natural and Applied Sciences

Prof. Dr. Hamit ALYAR
Director of Graduate School

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Ahmed Abdulhussein Hilo HILO

ABSTRACT

MOLECULAR, BACTERIOLOGICAL, AND BIOCHEMICAL STUDY FOR *LASB* AND *APRA* GENE IN *PSEUDOMONAS AERUGINOSA* BACTERIA

Ahmed Abdulhussein Hilo HILO

Master of Science in Biology

Advisor: Asst. Prof. Dr. Pınar ARSLAN

January 2024

Pseudomonas aeruginosa plays an essential role in a broad range of clinical illnesses, and it is also becoming more resistant to the antibiotics that are most widely used. As a result, the purpose of this research was to investigate the distribution of protease genes in Pseudomonas aeruginosa. There have been 225 distinct *P. aeruginosa* strains identified from their respective environments. Samples were collected from multiple disease cases, including burns, urine, blood, wounds, and ears. The isolates were identified using a series of biochemical tests, and the final diagnostic test included genotypic identification of the *16SrRNA* gene through the use of polymerase chain reaction (PCR). After being identified, a total of 58 isolates were found to be positive for *P. aeruginosa*. These 58 isolates comprised 11 from the burn, 13 from the wound, 20 from the ear, and 14 from urinary tract infections. The antibiotic susceptibility test was carried out using the disc diffusion method on seven different antibiotics. The results showed that the majority of the isolates were resistant to Rifambin by a factor of 69%. Additionally, these isolates exhibited high resistance to Amoxicillin (67%), Norofloxacin (55%), Amikacin (43%), Naldixic acid (38%) ,Cephalexin (14 %) and Ceftriaxon (9%). Genotypic detection of some genes responsible for protease production in *P. aeruginosa* was investigated by (PCR) which included elastase (*lasB*) and alkaline protease (*aprA*). The results revealed that 45/58 (78%) isolates were harbor *lasB* gene while 41/58 (71%) were positive for *aprA*. Finally, we conclude that the studied isolates of Pseudomonas aeruginosa bacteria differ in their resistance and sensitivity to antibiotics depending on the type of gene, the source of the isolate, or the

genetic variation of these isolates.It was also shown that the resistance of the majority of bacterial isolates to the studied antibiotics is that they are resistant to more than one type of antibiotic. Therefore, these isolates are also multiresistant.The results of genetic analysis showed that the *aprA* and *lasB* genes are diagnostic genes for these studied isolates and are responsible for the resistance characteristics of these bacteria.

2024, 64 pages

Keywords: *Pseudomonas aeruginosa*, *lasB*, *aprA*, Antibiotic resistance, PCR

ÖZET

PSEUDOMONAS AERUGINOSA BAKTERİLERİNDE *LASB* VE *APRA* GENİ İÇİN MOLEKÜLER ,BAKTERİYOLOJİK, VE BİYOKİMYASAL ÇALIŞMASI

Ahmed Abdulhussein Hilo HILO
Biyoloji, Yüksek Lisans
Tez Danışmanı: Dr. Öğr. Üyesi Pınar ARSLAN
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Pseudomonas aeruginosa çok çeşitli klinik hastalıklarda önemli bir rol oynar ve aynı zamanda en yaygın olarak kullanılan antibiyotiklere karşı daha dirençli hale gelmektedir. Sonuç olarak bu araştırmanın amacı Pseudomonas aeruginosa'da proteaz genlerinin dağılımını araştırmaktı. Kendi ortamlarından tanımlanmış 225 farklı *P. aeruginosa* suşu bulunmaktadır. Yanıklar, idrar, kan, yaralar ve kulaklar dahil olmak üzere birçok hastalık vakasından örnekler toplanmıştır. İzolatlar bir dizi biyokimyasal test kullanılarak tanımlandı ve son teşhis testi, polimeraz zincir reaksiyonu (PCR) kullanılarak 16SrRNA geninin genotipik tanımlanmasını içeriyordu. Tanımlamanın ardından toplam 58 izolatın *P. aeruginosa* açısından pozitif olduğu belirlenmiştir. Bu 58 izolatın 11'i yanık, 13'ü yara, 20'si kulak ve 14'ü idrar yolu enfeksiyonlarından oluşmuştur. Antibiyotik duyarlılık testi yedi farklı antibiyotik üzerinde disk difüzyon yöntemi kullanılarak yapıldı. Sonuçlar izolatların çoğunun Rifambine %69 oranında dirençli olduğunu gösterdi. Ayrıca bu izolatlar Amoksisilin (%67), Norofloksasin (%55), Amikasin (%43), Naldiksik asit (%38), Sefaleksin (%14) ve Seftriakson (%9)'a karşı da yüksek direnç göstermiştir. P. aeruginosa'da proteaz üretiminden sorumlu bazı genlerin genotipik tespiti, elastaz (lasB) ve alkalin proteaz (aprA) içeren (PCR) ile araştırılmıştır. Sonuçlar izolatların 45/58'inin (%78) *lasB* genini barındırdığını, 41/58'inin (%71) *aprA* pozitif olduğunu ortaya çıkardı. Son olarak, incelenen *Pseudomonas aeruginosa* bakterisi izolatlarının, gen tipine, izolatın kaynağına veya bu izolatların genetik varyasyonuna bağlı olarak direnç ve antibiyotiklere duyarlılık açısından farklılık gösterdiği sonucuna vardık. Ayrıca bakteri izolatlarının

çoğunluğunun çalışılan antibiyotiklere karşı direncinin, birden fazla antibiyotiğe dirençli olmaları olduğu da gösterilmiştir. Dolayısıyla bu izolatlar aynı zamanda çoklu dirençlidir. Genetik analiz sonuçları, *aprA* ve *lasB* genlerinin, incelenen bu izolatlar için tanısal genler olduğunu ve bu bakterilerin direnç özelliklerinden sorumlu olduğunu göstermiştir.

2024, 64 sayfa

Anahtar Kelimeler: *Pseudomonas aeruginosa, lasB, aprA*, Antibiyotik direnci, PCR

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I dedicate this work to those whose bodies have left us while they live in our hearts, the people of first virtue, my father and mother, may God have mercy on them.

Ahmed Abdulhussein Hilo HILO

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CONTENTS

LIST OF SYMBOLS

-	Minus
%	Percent
**	Significant
/	Divide
+	Plus
<	Greater than
=	Equal
>	Less than
±	Plus minus
≤	Greater or equal to
≥	Less or equal to
Ca	Calcium
Cu	Copper
dL	Deciliter
g	Gram
kg	Kilogram
L	Liter
m^2	Square meter
µg	Microgram
Mg	Magnesium
mIU	Milli-international units
min	Minute
mL	Milliliter
mmol	Millimole
mol	Mole
ng	Nanogram
nm	Nanometer
NS	Non-significant
rpm	Round per minute
Se	Selenium
Zn	Zinc
µL	Microliter
µm	Micrometer

LIST OF ABBREVIATIONS

CF	Cystic fibrosis
DI	Discrimination index
ESBL	Extended-spectrum beta-lactamases
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
O-Ag	O antigen
PBPs	Penicillin-binding proteins
QS	Quorum sensing
WHO	World health organization

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1. INTRODUCTION

Pseudomonas aeruginosa is characterized by the presence of Gram-negative rods, the absence of fermentation and sporulation, and the status as one of the most significant and opportunistic infections that contribute to a high incidence of death and morbidity in patients who are hospitalized. The purpose of this thesis is to explain the significance of proteases in the virulence of *Pseudomonas aeruginosa* and the risks of increases in bacterial resistance (Bassetti *et al.* 2018).

P. aeruginosa is a penetrating aerobe that may be found on plants and in soil as well as in crucial environments. Patients often report that the tap water in their rooms has been tainted by *P. aeruginosa* gluteus maximus. In spite of everything, it seems that clinical isolates of *P. aeruginosa* are just as susceptible to amoebal infection as environmental isolates. *P. aeruginosa* is known to inflict suffering on healthy individuals on occasion, but in all likelihood, patients whose immune systems are suppressed as a result of other conditions or injuries are more likely to be affected. *P. aeruginosa* is a self-serving pathogen; in order to cause an infection, it looks for various points of weakness in the host's defenses and exploits them. *P. aeruginosa* may cause infections and can also produce toxins (Azam and Khan 2019).

In addition, there are a great deal of proteins that are required for the production of these molecules and the secretion that often takes place in response to certain environmental inducers. These substances assist the microorganism attach to and invade its host by causing damage to the immune responses of the host and building a barrier against antibiotics. This allows the microorganism to do so. Genes on chromosomes or plasmids are responsible for the encoding of cell-associated and secreted virulence factors (Pang *et al.* 2019).

P. aeruginosa's resistance was broken down into two primary categories: the first of these is called intrinsic resistance, and it refers to the natural resistance of the bacteria. Intrinsic resistance refers to genetic characteristics that are responsible for preventing

antimicrobial action, such as, for example, resistance that arises from a low level of permeability in the bacterial envelope (Maqbool *et al.* 2020).

Extrinsic resistance is the second form of *P. aeruginosa* resistance, and it relates to the ease with which *P. aeruginosa* may acquire acquired resistance either through alteration in alleles that are chromosomally expressed or by through horizontal exchange of antibiotic resistance factors. This type of resistance is also known as acquired resistance. This brings us to the second kind of resistant bacteria, which is known as acquired resistance (Chevalier *et al.* 2017).

This Thesis aimed to collect Pseudomonas aeruginosa bacteria from clinical cases and diagnose them biochemically and molecularly, and examine their protease activity, in addition to explaining the importance of proteases in *Pseudomonas aeruginosa virulence*, the risks of increasing bacterial resistance, verifying sensitivity and resistance to a number of antibiotics, and determining the frequency of protease genes in clinical isolates of bacteria.

2. LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

Pseudomonas is a genus of bacteria that is naturally present in the environment, notably in water and soil, and is frequently referred to as a germ. *Pseudomonas* may be found in all environments. *Pseudomonas aeruginosa* is the one that most often causes infections in humans. *Pseudomonas aeruginosa* is a strain of the bacterium that has been linked to post-operative infections in the blood, the lungs (pneumonia), and other human beings' organs. Most probably, these kinds of bacteria are always coming up with new ways to evade the effects of the antibiotics that are used to treat the illnesses that they are responsible for producing. This makes it more difficult to treat the conditions that they are responsible for. Antibiotic resistance is the situation that occurs when bacteria no longer respond to medications that are intended to kill them. These germs may become multidrug-resistant if they develop resistance to multiple classes of medicines at the same time (Wagner *et al.* 2015). Antibiotic resistance is a condition that occurs when microorganisms no longer react to treatments that are designed to kill them.

P. aeruginosa strains are the ones responsible for the production of two distinct types of O antigen (O-Ag): a common substance antigen (A-band) that is composed of up of a homo polymer of d-rhamnose, and an O-specific antigens (B-band) that is made up of a heteropolymer of between three and five distinct sugars in its repeat units. Both of these O antigens are produced by P. The d-rham component may be found in both of these antigens. To this day, *P. aeruginosa* has been classified into a total of 20 distinct serotypes based on its various isolated forms. Because its lipopolysaccharide (LPS) is less hazardous than the LPS of other Gram-negative rods, P. aeruginosa is able to more readily establish chronic infections (Oliver *et al.* 2015). This is because it evokes a lesser inflammatory response from the host than other Gram-negative rods' LPS.

People in healthcare settings may get *P. aeruginosa* from the environment if they come into contact with water or soil that has been polluted with these bacteria. *P. aeruginosa* exists in the environment. In hospital settings, drug-resistant strains of the pathogen

may potentially transmit from one patient to another through contaminated hands, equipment, or surfaces. Antibiotics are often the treatment of choice for *P. aeruginosa* infections. Unfortunately, due to rising rates of antibiotic resistance, *P. aeruginosa* infections are bieng challenging to treat in patients who have been exposed to healthcare environments such as hospitals and nursing homes (Crone *et al.* 2020).

In order to discover whether antibiotics are effective against a particular pathogen, medical professionals will send a sample to a laboratory (this process is often referred to as a culture) and examine any bacteria that develop in response to a group of antibiotics to see which ones kill the pathogen. The healthcare professional will then choose an antibiotic based on the activity of the antibiotic as well as other criteria, such as the possibility of adverse effects or combinations with other medications. Treatment choices may be limited for certain strains of *Pseudomonas aeruginosa* that are resistant to many drugs (Streeter and Katouli 2016).

2.1.1 Causes and diagnosis

P. aeruginosa may be passed from person to person as a result of poor hygiene practices, such as the unwashed hands of healthcare professionals or the use of infected medical equipment that has not been properly sanitized. People who are sick in the hospital are most likely to get these infections, especially those whose immune systems are compromised as a result of having an illness or receiving treatment for a prolonged period of time (Cornelis 2020).

When patients are hospitalized, those who have surgical wounds or burns, they become at a higher risk for a serious. This risk is increased even further for those who have recently undergone surgery. Healthy persons are also susceptible to developing minor *P. aeruginosa* infections when they come into contact with polluted water. For instance, ear infections and skin rashes caused by *P. aeruginosa* may be acquired from hot tubs and swimming pools that have not been sufficiently cleansed (Klockgether and Tümmler 2017).

In addition, they are capable of causing eye infections in those who use contact lenses. In order to make a diagnosis of *P. aeruginosa*, the physician will do a physical examination on the patient and ask questions about the patient's symptoms and medical history. After that, they will test for the bacteria in a laboratory by sending a sample of blood or another body fluid from the patient (Pachori *et al.* 2019).

The prognosis of an illness often relies on the kind of infection a person has as well as how quickly they get antibiotic treatment. Antibiotics are often an effective and simple course of treatment for treating mild *P. aeruginosa* infections linked to water. However, due to the fact that certain bacterial strains demonstrate resistance to practically all kinds of potent antibiotics, it is becoming more difficult to treat serious *P. aeruginosa* infections that are connected with hospitals (Chatterjee *et al.* 2016).

In order to treat these illnesses that are resistant to several treatments, it is necessary to submit samples to a laboratory so that the bacteria may be tested against a variety of antibiotics. The results of these tests will hopefully lead to the discovery of pharmaceuticals that can treat the infection. A person will often get a mix of several antibiotics in order to reduce the risk of the infection becoming resistant to treatment. Patients suffering from cystic fibrosis have an increased risk of developing such infections. In point of fact, roughly half of all individuals diagnosed with cystic fibrosis have a persistent infection with the bacterium. Those who are immunocompromised, which means they have a compromised immune system and are thus very susceptible to *P. aeruginosa* infections (Alhazmi 2015).

2.1.2 Transmission

Acquisition of *P. aeruginosa* may take place either exogenously or endogenously, depending on the circumstances. Acquisition from an exogenous source might take place via the infected hands of healthcare personnel or through the contamination of ambient surfaces. In order to examine the external transmission of multidrug-resistant *P. aeruginosa* (MDR), comprehensive epidemiologic research was carried out in six critical care units. During the course of delivering ordinary patient care, specimens were

collected from the hands, gloves, and gowns of healthcare professionals in order to get cultures. As part of the monitoring approach, cultures were also obtained from patients (Valentini *et al.* 2018).

Molecular typing with the use of pulsed-field gel electrophoresis was carried out in order to determine whether or not a clonal connection exists between the various strains. Acinetobacter species that are resistant to several drugs were the most common infections found on the hands and gloves of healthcare workers. This contamination occurred during 17.4 percent of all contacts that took place between medical personnel and patients. It was determined that the presence of environmental contamination, doing a physical examination on the patient, spending more than five minutes in the patient's room, and having direct contact with the mechanical ventilator were all independent risk factors associated with health care worker pollution. *P. aeruginosa* was discovered in 22% of the rooms, which is evidence that environmental pollution was quite pervasive. In addition, *P. aeruginosa* was discovered in 22% of the rooms. This study, along with a large number of others, highlights the significance of external transmission playing an important part in the nosocomial getting of *P. aeruginosa* and the vital function that contamination of the environment serves in the spread of this bacteria to patients as well as health care personnel. External transmission plays an important part in the nosocomial acquisition of *P. aeruginosa* (Botelho *et al.* 2019).

Colonization with an antimicrobial-susceptible strain of *P. aeruginosa*, which eventually transforms into a strain resistant to antimicrobials predominantly as a result of antimicrobial selective pressure inside the host, is classified as endogenous acquisition of a resistant strain of *P. aeruginosa*. According to the findings of a research on the transmission of imipenem-resistant *P. aeruginosa*, endogenous acquisition was responsible for only 19% of the observed acquisition events, while external acquisition was responsible for 31% of the events (Bhagirath *et al.* 2016).

As was mentioned previously environmental reservoirs are a substantial contributor to the spread of *P. aeruginosa*, the most common places either have a high moisture content, a high humidity level, or are related with water. Other common places have

both of these characteristics. In the setting of hospitals, *P. aeruginosa* outbreaks have been linked to water supplies a significant amount of the time. These water sources consist of potable water as well as showerheads and sinks with running water, all of these sources have the potential to transmit *Haemophilus influenzae* (Salomoni *et al.* 2017).

Finally, it is difficult to remove *P. aeruginosa* because of its capacity to build biofilms on surfaces, which boosts its ability to live on inanimate surfaces and makes it more resistant to disinfectants. Biofilms are colonies of microorganisms that are firmly attached to surfaces and are kept together by structural polysaccharides, sometimes known as slime. The production of biofilms by *P. aeruginosa* leads to antimicrobial tolerance and makes it more difficult for environmental cleaning solutions to eradicate the bacteria (Poulsen *et al.* 2019).

2.1.3 Types

The family that *P. aeruginosa* belongs to also consists of 12 additional species. *P. aeruginosa*, like other members of the genus, is often discovered in a variety of environments, including soil, water, plants, and even human beings. It is considered that *Pseudomonas* bacteria are one of just a few genuine pathogens that may affect plants. It is important to note that *P. aeruginosa* has developed as a new opportunistic pathogen in clinical settings. Recent epidemiological investigations have shown evidence that it is a nosocomial infection, especially with regard to those strains that have gained resistance to antibiotics (Serra *et al.* 2015).

P. aeruginosa is able to establish an infection by targeting vulnerable points in the host's defense system. *P. aeruginosa* is, in all likelihood, the quintessential example of an opportunistic human pathogen. However, the bacteria are able to infiltrate any tissue that is challenged by immunodeficiency and has a very low risk of infecting healthy tissues. The typical incidence of *P. aeruginosa* infections in hospitals in the United States is around 0.4 percent, or four per thousand discharges. *P. aeruginosa* takes the form of a rod that is 0.5–0.8 μm long and 1.5–3.0 μm wide. The bacterial strains are

able to move about thanks to their solitary polar flagellums. However, while having a respiratory metabolism dependent on oxygen, this common bacterium can grow in the presence of nitric oxide but not in the presence of oxygen (Hewer and Smyth 2017).

In nature, the normal *Pseudomonas* may either take the form of a biofilm, which is an attachment to a surface or substrate, or it can take the shape of a planktonic organism, which is a unicellular organism that swims actively utilizing its flagellum. *Pseudomonas* is, in point of fact, a robust bacterium that swims very quickly, as can be observed in samples taken from patients or from the wild. *P. aeruginosa* may not be a typical pseudomonad in natural surroundings. *P. aeruginosa* only requires a limited source of nutrients to thrive and may even do so in water that has been purified. A medium that contains acetate (as a carbon source) and ammonium sulfate is another suitable environment for the growth of *P. aeruginosa* (nitrogen source). *P. aeruginosa* may develop at temperatures as high as 42 °C, however the optimal temperature for growth is 37 °C (Berube *et al.* 2016).

These characteristics of *P. aeruginosa* are important contributors to its success in the environment. Isolates of *P. aeruginosa* have been shown to produce three distinct kinds of colonies. Natural isolates found in soil or water usually take the form of a small, irregular colony, whereas clinical isolates most often take the form of smooth colony types and may occasionally take on the appearance of a fried egg, which is characterized by being large, smooth, having flat edges, and appearing elevated (Pachori *et al.* 2019).

The mucoid appearance may be seen in secretions from the respiratory and urinary tracts (alginate slime). Pyocyanin is a term that describes blue pus, which is a characteristic of suppurative infections that are produced by *P. aeruginosa*. Pyochelin, which is a derivative of pyocyanin, is a siderophore. Siderophores are able to collect iron from their hosts or from low-iron environments in order to continue the proliferation of pathogens. Pyocyanin may disrupt the normal operation of human nose cilia and the respiratory epithelium, which increases the likelihood that it may cause

pro-inflammatory responses in human beings, there is no evidence shown for the luminous pigments having any pathogenicity (Bassetti *et al.* 2018).

2.1.4 Microbiology characteristics

Pseudomonas aeruginosa is a kind of gram-negative bacillus that is widespread throughout the natural world and may be found in a variety of environments, including water and soil. P. aeruginosa is what's known as an opportunistic pathogen, which implies that it only causes sickness in normal hosts very seldom. On the other hand, it is a substantial source of infection among persons who have underlying illnesses or disorders that damage their immune systems. These people are more likely to get infected. P. aeruginosa is able to adapt to environments in which it would be impossible for other species to live since it has a limited set of nutritional requirements. This bacterium does not ferment lactose or any of the other carbohydrates; instead, it oxidizes glucose and xylose. Organisms are able to live in either an aerobic or anaerobic environment if an inorganic electron acceptor such as nitrate is available in the environment. People who have cystic fibrosis (CF) often have a condition similar to this in their lungs. These different patterns are diagnostic of environmental isolates and include motility, piliation, and the generation of numerous exoproducts (Azam and Khan 2019). The gene expression of *P. aeruginosa* responds to environmental settings with various patterns that are indicative of environmental isolates.

Pyoverdine and pyochelin are two examples of the organism's naturally occurring light siderophores that it produces. Iron removal is the major objective of these devices. One example of a redox-active phenazine is the pigment known as pyocyanin, which is responsible for giving *P. aeruginosa* its characteristic blue hue. Pyocyanin and other redox-active phenazines play a crucial function in electron transport, especially in microaerophilic environments. These redox-active phenazines also improve the bioavailability of iron and boost virulence via oxidative stress. *P. aeruginosa* may be identified based on the presence of three biochemical features known as indophenol oxidase activity, citrate activity, and l-arginine dehydrolase activity (Cornelis 2020). These traits can be found in bacteria.

P. aeruginosa is an example of an opportunistic pathogen, which means that it very seldom causes sickness in healthy hosts, but it is a major role in the development of infections in patients who already have preexisting issues. This is because *P. aeruginosa* is able to take advantage of the conditions that healthy hosts already have. A better understanding of the metabolic and pathogenic mechanisms that are responsible for the success of this multipurpose pathogen has been brought about as a result of the sequencing of the genomes of multiple strains (Pang *et al.* 2019).

Due to the fact that it is so massive genome, the creature has an incredible degree of genetic plasticity. *P. aeruginosa* has a low number of dietary needs and has the ability to adapt to situations that other organisms cannot survive. Lactose and other carbohydrates are not fermented by this organism; instead, glucose and xylose are oxidized. In environments where nitrate is present as an inorganic electron acceptor, such as the lungs of people with cystic fibrosis (CF), organisms are able to thrive either aerobically or anaerobically. The environmental circumstances have an effect on the gene expression of *P. aeruginosa*, which displays distinct patterns that are characteristic of environmental isolates. These patterns include motility, piliation, and the development of many exoproducts (Moradali *et al.* 2017).

2.2 Antibiotic Resistance

Alexander Fleming, a Scottish medical doctor and microbiologist, delivered the acceptance speech for the Nobel Prize he received in 1945 for developing the first antibiotic penicillin. In that address, Fleming sounded an early alarm concerning antibiotic resistance. Since then, there has been a rise in worry over antibiotic resistance and the implications that come with it; in fact, the situation has now become a catastrophe on a worldwide scale. The World Health Organization (WHO) and the United Nations have both emphasized that a "post antibiotic age" is a very real picture and that immediate action is necessary all around the world (Frieri *et al.* 2017).

It is common for humans and other animals to have *Escherichia coli* as part of their regular gut flora. Despite this, it is the top source of foodborne infections across the

globe and the most prevalent cause of healthcare- and community-acquired urinary tract infections and blood stream infections. There have been reports of an alarmingly high frequency of antibiotic-resistant *E. coli*. An Indian tertiary care hospital found that 86 percent of the blood *E. coli* isolates there were resistant to cephalosporins of the third generation (MacGowan and Macnaughton 2017).

Antibiotic resistance is a worldwide problem that has been made worse by the discovery of extended-spectrum beta-lactamases (ESBL) in certain strains of Enterobacteriaceae. ESBLs are capable of hydrolyzing betalactam antibiotics and rendering them inactive. This includes cephalosporins of the third generation. In addition, ESBL-producing bacteria have shown cross-resistance to quinolones, aminoglycosides, and sulphonamides, which contributes to the development of MDR. For instance, research conducted at a hospital in Kenya found that 90 percent of the ESBL-producing *E. coli* isolates tested were resistant to fluoroquinolones as well (Larsson *et al.* 2022).

Antibiotic resistance is a contributing factor in the lengthening of diseases and the increased number of fatalities. It is estimated that more than 200,000 babies pass away every year as a result of diseases for which there are no drugs that can effectively treat them. Infection caused by bacteria that are resistant to antibiotics will almost always result in a higher cost burden, both for the person and for the healthcare system as a whole. Last line antibiotics are frequently more costly and less accessible. It was shown that patients in India who had illnesses that were resistant to antibiotic treatment had to spend an additional 700 USD above those who had infections that were treatable by first-line medicines (Munita and Arias 2016).

The capacity of bacteria to alter in ways that resist the effects of medications has been termed as antibiotic resistance, that is, the germs are not destroyed, and their proliferation is not inhibited. Therefore, the development of resistance is a normal process that happens in bacteria when they are exposed to antibiotics. Nevertheless, the improper use of antibiotics speeds up the process, and a variety of variables might contribute to the disease's spread. The level to which these pathways are active may

vary based on the particular bacteria involved, the particular kind of resistance, as well as the specific location and environment (Yelin and Kishony 2018).

The concept of "One Health" is emphasized throughout the WHO worldwide action plan for combating antibiotic resistance. The idea of "One Health" holds that human health, animal health, and environmental health should all be seen as aspects of the same whole because of their interdependence. Because antibiotic resistance may spread across humans, animals, and the environment, the "One Health" concept is very necessary when discussing this issue of antibiotic resistance (Blair *et al.* 2015).

The worldwide community is becoming more concerned about the presence of antibiotics in the environment as a whole, and more specifically in aquatic environments. A significant contributor to the spread of antibiotic-resistant bacteria and antibiotics into the natural environment is the discharge of antibiotics from hospital effluents and even the hospital wastewater treatment facilities itself. Antibiotics are discharged into the environment in a continuous, daily, and constant manner in hospital wastewater (Chokshi *et al.* 2019).

Sulphonamides were the first antibiotics that were developed for widespread use in clinical settings after they were introduced. Because sulphonamides prevent the production of folic acid, they are effective against bacteria in a selective manner. This is the reason for their efficacy. Throughout history, sulphonamides have been used all throughout the globe as reasonably priced and efficient treatments for bacterial infections (Berendonk *et al.* 2015).

However, the use of sulphonamides is becoming less common these days. This is owing to the fact that many individuals have had allergic adverse effects in their skin and blood-forming organs as a result of the treatment. In addition, resistance to sulphonamides has quickly arisen in a wide variety of human diseases (Ventola 2015). In bacteria that originated in the environment, researchers have identified four distinct kinds of *sul* genes: *sul1, sul2, sul3,* and *sulA*. Fluoroquinolones, sometimes known as are a class of synthetic antibiotics with a wide range of activity. They are a by-product

of the commercial manufacture of chloroquine, which is a medication used to treat malaria (Chellat *et al.* 2016).

Ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, and moxifloxacin are the members of this class of antibacterial medication. Because they disrupt the process of DNA synthesis, fluoroquinolones have a bactericidal action that is very selective. The majority of cases of fluoroquinolone resistance are brought on by mutations in the quinolone-resistance-determining areas of the genome, most notably the *gyrA* gene (Friedman *et al.* 2016).

Tetracyclines block peptide synthesis as their primary mode of action because of this mechanism. They prevent the ribosome from being attached to the amino acid that carries the tRNA molecule by attaching to the 70S subunit of the ribosome. Tetracycline, oxytetracycline, doxycycline, and lymecycline are the four tetracycline derivatives that are used in clinical settings with the greatest frequency. Tetracyclines are antibiotics that have been used extensively in both people and animals because to the fact that they are effective, have very few adverse effects, and are inexpensive (Landecker 2016).

More than 22 these genes have been discovered in bacterial isolates that have been obtained from water treatment facilities. It has been shown that the *tetE* gene is able to be transferred across species, namely to *Escherichia coli*. The gene is often found on large plasmids that are capable of horizontal transfer in Aeromonas species. It was also shown that tet(A), tetD, and tetM may be transmitted horizontally by the oxytetracycline resistance plasmid from ambient microbes to a non-resistant *E. coli* strain. This indicates that tet ARGs may represent a risk to the environment (Dodds 2017).

Macrolides are a family of antibiotics that are considered to be therapeutically relevant. They are used to treat illnesses brought on by Gram-positive bacteria. Macrolides are one of the most widely used antibiotic families. One member of the macrolide class that is particularly well-known is erythromycin. Streptomyces erythreus was the organism

that led to the discovery of this antibiotic in the year 1952. Erythromycin is able to selectively restrict the development of bacteria because it binds to the ribosomes of bacteria, where it inhibits the production of protein in a reversible manner (MacLean and Millan 2019).

Resistance to macrolides is often caused by the methylation of a particular adenine residue in 23S rRNA, and it has been linked to the presence of *erm(B)* genes, which are responsible for the encoding of rRNA methylase, it is believed that the *ermB* gene, which is part of the macrolide resistance determinants, is the most common gene found in environmental bacteria, particularly the *Enterococcus* strain. The class of antibiotics known as beta-lactams is one of the most widely used. All antibiotic agents that have a structure that includes a beta-lactam ring are included in the category of antibiotics known as beta-lactam antibiotics, this is a very large class of antibiotics (Podolsky 2018).

The antibacterial properties of beta-lactams come from their ability to suppress the production of cell walls in bacteria. In more specific terms, beta-lactam antibiotics impede the production of cell walls by attaching to penicillin-binding proteins (PBPs), which are found in the membranes of living cells. Because of this inhibition of PBPs, the production of peptidoglycan is also inhibited, which ultimately results in cell death. The production of a beta-lactamase by a bacterium that is resistant to beta-lactams is the most common type of bacterial resistance to these antibiotics. This enzyme cleaves the beta-lactam bond, rendering the beta lactam incapable of interfering with the bacterial cell's ability to produce new proteins (Martens and Demain 2017).

2.3 *LasB* and *AprA*

2.3.1 Background

Pseudomonas aeruginosa is the organism that is responsible for producing a variety of proteases outside of the cell that are used for this purpose. Infections caused by *P.*

aeruginosa are often linked to the presence of two of these proteases, namely the 33-kDa elastase *LasB* and the 50-kDa acidic protease *AprA*. They are able to be detected at many different sites of infection, both in vitro (in test tubes) and in vivo (in living organisms), at a variety of distinct places. Calcium ions are required for zinc metalloproteases like *AprA* and *LasB* to maintain their structure (Casilag *et al.* 2016). Zinc metalloproteases like *AprA* and *LasB* are both instances of zinc metalloproteases.

Pseudolysin is the protease that can be extracted in the greatest quantity from pseudomonal secretomes. It is also known by the acronym *lasB*, which stands for multifunctional elastase B, and it has been the subject of a significant amount of research. The expression of the *lasB* gene is what kicks off the creation of the *LasB* neutral, metallo-type (Zn^2+Ca^2 + cofactors) protease. The production of this protease is subsequently regulated by the quorum sensing (QS) transcription systems, which are las and rhl. In other words, *lasB* is a protease that is of the metallo-type (Zn^2+/Ca^2+ cofactors), and it is produced by the bacterium Lactobacillus acidophilus. In the acute stage of *P. aeruginosa* infections, the molecule *lasB* is very important since it is accountable for the disintegration of the host cells' extracellular components. According to Badamchi et al. (2017), the breakdown of the host cells' external matrix materials is the primary factor that eventually results in the development of tissue injury and bleeding.

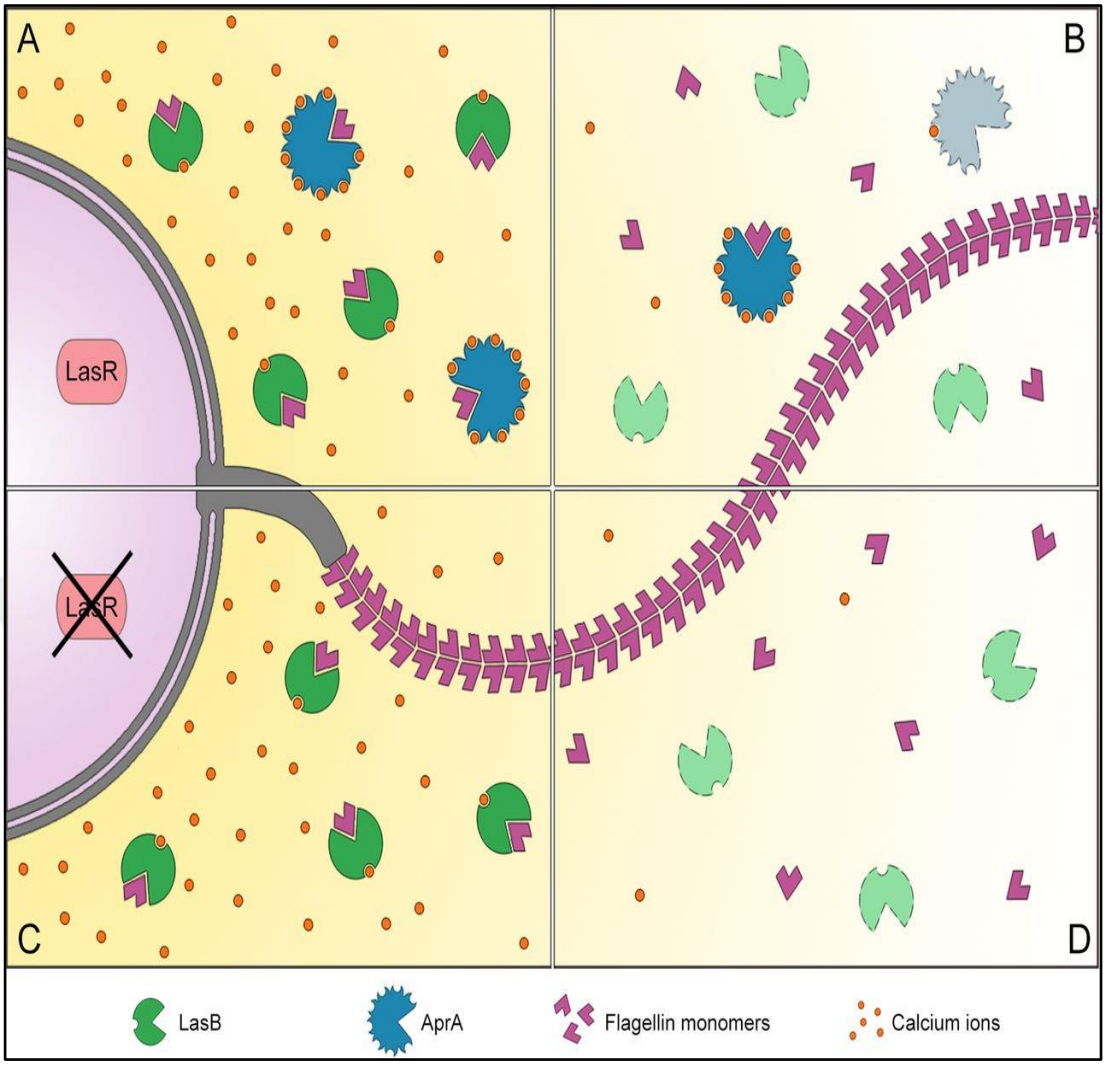

Figure 2.1 *LasB* and *AprA* elements (Rigane *et al.* 2020)

In addition to this, *lasB*'s capacity to regulate host responses reveals crucial activities during persistent infections. Both of these actions can be found in the complement system. It was shown that the degree of corneal ulceration in rabbits that were infected with a *lasB* mutant strain of *P. aeruginosa* was decreased, which is evidence that *lasB* plays a significant role in the pathogenesis of *P. aeruginosa*. When compared to the infection induced by the corresponding wild-type strain (Everett *et al.* 2021).

In order to combat the growing danger posed by multidrug-resistant *P. aeruginosa*, the development of novel chemotherapeutic approaches is an urgent imperative. As a direct result of this, a number of chemists have shifted their emphasis toward the development

and synthesis of new therapeutic compounds based on metals. Because of their reactivity with bacterial cells, metal-based antibiotics have multi-target and flexible modes of action, which gives them tremendous potential as antibacterial agents (Saint *et al.* 2018).

2.3.2 Interaction

In regards to pathogens, the significance of flagella, the components of flagella, and the motility that flagella impart has been demonstrated beyond a reasonable doubt. In spite of this fact, flagellin is nonetheless highly conserved across a number of different bacterial species, which is an intriguing fact. This suggests that the immunogenic effects do not exceed the functional value of the antigen (DehghanZadeh *et al.* 2021).

It is interesting to note that as a defensive mechanism, many different types of bacteria adopt a variety of ways to reduce or even eliminate flagellin-mediated recognition (Niu *et al.* 2016). For instance, it has been proven that *Campylobacter jejuni*, *Helicobacter pylori*, and *Bartonella bacilliformis* are all capable of creating a modified flagellin that TLR5 is unable to identify. It has also been found that flagellin can be modified in bacteria via the process of glycosylation; however, the significance of this finding in the context of host immunological recognition are less obvious (Bastaert *et al.* 2018).

Indicators of virulence in *P. aeruginosa* include both flagellin and extracellular proteases. Quorum sensing is primarily responsible for the majority of the regulation of virulence in this organism (QS). This, in turn, prompts the required modifications in the execution of a variety of cell tasks (Islamieh *et al.* 2019).

Additionally, there is evidence that QS plays a role in the development of biofilms, which are typically connected to chronic *Pseudomonas* infections. *P. aeruginosa* has three primary QS circuits, which are referred to as the Las, Rhl, and PQS systems. The extracellular proteases *AprA* and *LasB* are controlled by the Las system, which is responsible for their synthesis (Camberlein *et al.* 2022).

2.3.3 Structure

It has been shown that in order for the creation of the elastase structural gene, *lasB*, to take place, the *lasR* gene and the *P. aeruginosa* autoinducer both need to be present and functional. The *lasB* gene has been successfully cloned, and its sequence has been determined (PAI). PAI has been determined to be N-(3-oxododecanoyl) homoserine lactone, and it is a chemical that belongs to the N-acylhomoserine lactone family of substances. PAI is one of the N-acylhomoserine lactone compounds that, in conjunction with a regulatory protein (such as *LasR*), is involved in cell density signaling and the activation of genes. PAI is one of the N-acylhomoserine lactone compounds (Gaviard *et al.* 2019).

Two results that were published not long ago point to a complexity in the regulation of the *lasB* gene that goes beyond the requirements of *LasR* and *PAI*. *P. aeruginosa*'s ability to produce rhamnolipids necessitates the presence of a second activator called *RhlR*, which has homology with *LasR*. It was discovered that a *rhlR* mutant strain was lacking not only in rhamnolipid synthesis but also in elastase activity. This finding suggests that the control of elastase production is influenced by a number of different factors (Thanh *et al.* 2021).

It has been determined that the factor 2 component is N-butyrylhomoserine lactone. The authors raise the notion that *lasB* is susceptible to a complicated chain of regulatory processes that include a multitude of autoinducers and activators. However, the information that is now available suggests that *LasR* and *PAI* are sufficient on their own to achieve large levels of *lasB* expression (Santajit *et al.* 2021).

For example, it has been shown that *LasB* can destroy mucins and surfactant proteins, all of which work together to facilitate the removal of germs from the body (Roshani *et al.* 2018). *AprA* has the capacity to degrade flagellin, which is a recognized stimulator of proinflammatory responses that is triggered by the recognition of toll-like receptor 5 (TLR5). This ability is part of *AprA*'s immune-modulating characteristics, in addition to its ability to act against responsive host factors (Kaya *et al.* 2022).

In the majority of instances, it is antibiotic-resistant and is linked to a high fatality rate. It is responsible for around 35% of deaths that are caused by bacteremia and accounts for approximately 10% of urinary tract infections. The diagnosis, treatment, and epidemiological monitoring of bacterial illnesses are all greatly aided by the ability to type bacterial strains. The classification of bacteria may be broken down into two categories: traditional epidemiological typing and DNA-based typing. Methods such as antibiogram, phage typing, and serotyping are examples of conventional forms of epidemiological typing. The amplification of a negligible quantity of a particular DNA sequence is required for the PCR-based typing process (Green and Sambrook 2019).

As a result of its ability to amplify ambiguous sections of the genome, RAPD is a term which refers to a primed PCR arbitrary, or AP-PCR. Primers typically have a rather brief duration (about 10 bp). The ability of RAPD to discriminate between genetic variants is dependent on the number of primers employed, their sequence, and the amplification conditions. The ERIC-PCR fingerprinting method focuses on highly conserved 126-base-pair central inverted repeats that are found in the extragenic regions of bacterial genomes. As a result of the fact that the location of these areas varies across species and strains, we refer to them as genetic markers (Cao *et al.* 2017).

PCR-ribotyping is a method that may be used to identify variation in the intergenic regions of rRNA or tRNA. Intergenic spacer regions, also known as ISRs, are subjected to lower levels of selective pressure than other parts of the genome, and as a result, they exhibit wider genetic variability that are dependent (Ahrberg *et al.* 2016).

When identifying these ISRs, two different PCR-based approaches are used. Primers that amplify the sequences between 16S and 23S genes are used in the first technique known as PCR-ribotyping. This technique is utilized for the purpose of identifying and discriminating between different bacterial strains and species. The second method is called tDNA-PCR, and it is used to amplify intergenic spacers that are found between tRNA genes (Hadidi *et al.* 2017).

These genes have been passed down through the generations and may be found in several copies spread out over the bacterial genome. In addition to this, they are clustered together and separated by spacers of varying lengths and sequences, both of which are prone to considerable change. The effectiveness of the technique of typing may be judged based on a number of criteria, including its readability and its capacity for discriminating. The proportion of bacterial isolates that can be positively typed by the typing marker is referred to as the typeability of the bacterium (Kadri 2019).

It is possible to acquire a rough approximation of the discriminating strength of a certain technique of typing by comparing the number of types generated by this approach to the relative frequencies of those kinds. A high level of polymorphism along with a distribution that is stable throughout the genome are two essential qualities of the ideal marker. In addition to that, it needs to provide a satisfactory resolution of genetic differences. By analyzing a variety of factors, it is possible to determine how useful molecular markers are for determining the genetic diversity of a population (Green and Sambrook 2019).

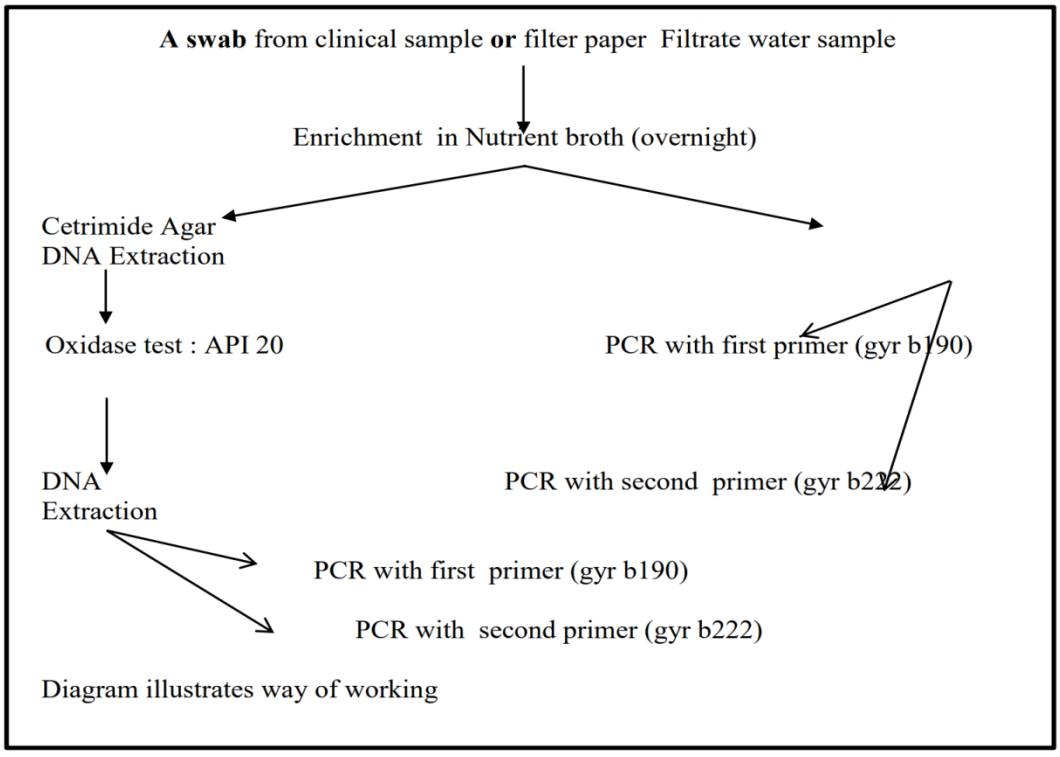

Figure 2.2 PCR steps for identification (Gaňová *et al.* 2021)

3. MATERIALS AND METHODS

3.1 Material

3.1.1 Equipments

Table 3.1 shows some devices and its origins and Table 3.2 shows other laboratory materials.

Table 3.1 The used devices and their origins

No.	Equipment and apparatus	Company	Origin
1	Autoclave	Hirayama	Japan
2	Balance	Kevn	Jordan
3	Compound light microscope	Olympus	Japan
4	Water bath	Gallenkamp	England
5	Cooler ıncubator	Binder	England
6	Distillator	G.F.L	Germany
7	Electric oven	Binder	England
8	Hop plate-magnatic stirrer and bar	Biocote	UK
9	Refrigerator	Fiocchetti	Italia
10	Vortex	Biocote	UK
11	Micro – volume uv/v is spectrophotometer	ACT gene	Korea
12	Electrophoreses	Fischer scientific	Germany
13	Gel Documentation	Difico	USA
14	Thermocycler	Thermo scientific	USA
15	Vitek2	Bio mexieux	France
16	Power supply	Cleaver	UK
17	Densichek	Bio merieux	France
18	Nano drop	Dihan	Korea
19	Centrifuge	GallenKamp	England

Table 3.2 Other medical tools in which used vitro

No.	Other Materials	Company and origin
1	Eppndroff tube	Promeg (USA)
2	Filter papers Whatman (0.2 μm)	Schleicher and Schuel (USA)
3	Forceps	Olympus (Japan)
4	Micropipette (different size).	Brand (Germany)
5	Plane tubes (10 mL), Petri dishes and Sterile swab	China
6	Volumetric flask	Jlassco (India)

3.1.2 Chemicals and biological materials

Tables 3.3 shows a list of chemicals and biological materials used during this study.

Table 3.3 List of chemicals and biological materials

No.	Chemicals and Biological materials	Company (origin)
1	Acetic acid	BDH-England
2	Alcohol	BDH-England
3	Alpha-naphthol	BDH-England
4	Casein	BDH-England
5	Catalase Reagent	Syrbio (Syria)
6	Cholin chloride	BDH-England
7	Crystal violet	Biomerieux -France
8	Ethanol	Merck (Germany)
9	Gelatin	BDH-England
10	Glycerol	Oxide (England)
11	Gram stain	Syrbio (Syria)
12	Hydrocloric acid	GCC/UK
13	Hydrogen peroxide (H_2O_2)	BDH (England)
14	Iodine	Biomerieux -France
15	Isoamyl alcohol	BDH-England
16	KOH	BDH-England
17	McFarland solution	Biomerieux (France)
18	Methanol	BDH-England
19	Methyl red	BDH-England
20	NaCl	BDH-England
21	NaOH	BDH, UK
22	Oxidase Reagent	Syrbio (Syria)
23	Para-dimethyl-amino benzaldehyde	BDH-England
24	Resazurin (Alamer blue)	BDH-England
25	Safranine	Biomerieux -France
26	Tetra methyl-para-phenyl diamine dihydrochloride	BDH-England
27	Trichloroacetic acid (TCA)	BDH-England

Table 3.4 Antibiotic disc

Antibiotics	**code**	**Class**	**Company (origin)**	**Concentration (µg)**
Norfloxacin	NER	Quinolone	Oxoid ™(UK)	10
Nalidixic acid	NAL		Oxoid ™(UK)	30
Ceftriaxone	CRO	Cephalosporins	Oxoid ™(UK)	30
Cephalexin	CEF		Oxoid ™(UK)	30
Rifampin	RIF	Antimycobacterial	Oxoid ™(UK)	5
Amoxicillin	AMX	Penicillin	Oxoid ™(UK)	30
Amikacin	AMK	Aminoglycoside	Oxoid ™(UK)	10

3.2 Methods

3.2.1 The preparation of the media

1. Ready to use media

The preparation of Nutrient agar, Cetrimide agar, MacConkey agar, Muller Hinton, Brain heart infusion agar, Nutrient broth, Peptone water, and Muller Hinton broth was carried out in accordance with the guidelines provided by the manufacturing business.

They were placed in a water bath and heated to a boil in order to thoroughly dissolve all of the ingredients. After bringing the pH up to seven and sterilizing the solution in an autoclave at 121ºC under 15 pounds per square inch for fifteen minutes, it was put onto petri dishes and kept at 4°C until it was needed.

2. Laboratory prepared media

A. Blood agar

We used autoclave to sterilise a blood agar base after it was made in accordance with the instructions provided by the supplier. After bringing the temperature of the medium down to 45°C, 5% of fresh human blood was added to it. This medium was used for the culture of the majority of pathogenic bacteria in order to investigate both their capacity to hemolyze blood and the sort of hemolysis that they caused (Harley and Prescott 2002).

B. Gelatin medium

It was made by dissolving 1.3 grams of nutritional broth and 4 grams of gelatin in 100 mL of deionized water, thoroughly mixing the solution, and then dispensing it into test

tubes before sterilizing it (Breed *et al.* 2014). It was employed as a test to determine whether or not bacteria had the capacity to manufacture the gelatenase enzyme.

C. Broth prepared by infusing the brain and heart

It was produced in accordance with the instructions provided by the provider, and then it was autoclaved to ensure its cleanliness. After waiting for the medium to reach a temperature of 45 °C, 15% sterile glycerol was added to it. After bringing the pH up to 7, the solution was placed onto sterile Petri plates and kept at 4°C until it was needed (Brown and Smith 2005). It was used for the purpose of maintaining bacterial isolates.

3. The preparation of the reactants and solutions

A. A reagent for catalase

This reagent was made by diluting the stock solution of hydrogen peroxide (H2O2) to a concentration of 3%. It was employed as a test to determine whether or not bacteria have the capacity to manufacture the catalase enzyme. According to Collee *et al.* (1996), the reagent was stored in a dark container.

B. Oxidase reagent

In order to make it, one milligram of tetramethyl para phenylene-diamine dihydrochloride was dissolved in 100 mL of distilled water. After that, it was kept in a dark bottle until it was utilized, at which point it was discarded. According to the findings of Collee *et al.* (1996), this test was used to determine whether or not bacteria had the capacity to manufacture the oxidase enzyme.

C. Kovac's reagent

After heating in a water bath at 50 °C to dissolve 10 grams of para-dimethyl-amino benzaldehyde in 150 mL of isoamyl alcohol, 50 mL of (1M) of hydrochloric acid was added slowly. This reagent was used for the Indole test, which was conducted by Collee *et al.* (1996). The reagent was made in minute quantities and then kept in the refrigerator.

D. Vogas-proskauer reagent

This kind of reagent mades during combining of about 40% potassium hydroxide in addition to 5% -naphthol in 96% ethanol, the final product had a purity of 96%. The solution, which was stored in a dark container and required mixing before to use, it served as an indication in the Voges-Proskauer test that was conducted by Collee *et al.* (1996).

4. Solutions

A. Normal saline solution

To make normal saline, 0.85 grams of sodium chloride were dissolved in 90 mL of distilled water, the pH was brought up to 7, the final mixture was brought up to 100 mL, and the mixture was stirred until the salt was completely dissolved. After that, the solution was autoclaved at a temperature of 121 °C and a pressure of 15 pounds per square inch. It was put to use in the technique that called for needed dilutions (Macfaddin 2000).

B.Casein solution 3%

First,3 grams of casein were dissolved in 90 ml of distilled water at a temperature of 90 °C in a water bath. Next, 2 to 5 drops of 1 ml of sodium hydroxide were added, and the volume was brought up to 100 mL by adding more distilled water.

C. Buffer made of acetate (0.6 M, pH = 6)

It was made by combining 3 mL of acetic acid with 50 mL of distilled water, after which the pH was brought down to six using sodium hydroxide, and the volume was brought up to 100 mL (Whitaker 1972).

D. Casein substrate solution

Prepared by combining casein solution, distilled water in addition to the acetate buffer and in the ratio of 1:1:4 correspondingly, in order to get a final level of the casein of 0.5 percent (Whitaker 1972).

E.Trichloroacetic acid 5% (TCA)

The main procedure for the preparation in the vitro in done through the dissolving of about 5 g of TCA, the final mixture distilled in about 100 mL of water.

F.Sodium phosphate buffer (pH = 7.3)

This preperation of this kind of mixture during the dissolving the following salts in one liter of distilled water and the pH was adjusted to 7.3, these salts were: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g (Collee *et al*. 1996).

G. Red blood cell suspension

RBC preperation through the washing human RBC of the group O, the washing about three times by 8.5% sodium chloride, the next step by suspending in 5% of the sodium phosphate solution (Wilson *et al*. 1987).

H. TBE (1X) solution

TBE buffer diluted from 10x to 1x by mixing 10 mL of stock TBE-10x with 90 mL of distilled water, the next step through kept the mixture at 4˚C, the keping of the mixture until the using in DNA gel electrophoresis (Sharpe 2005).

I. Ethidium Bromide dye

It was prepared by dissolving 0.05g of Ethidium bromide in 10 mL distilled water and stirred on magnetic stirrer. Then it was filtrated, and stored in a dark bottle at 4ºC (Sambrook and Russell 2001).

3.2.2 Sample collection

I collected 225 samples for different pathological cases, including (urine, burns, wounds, ear infections, stool) from patients attending Imam Ali Hospital and Fatima al-Zahra Hospital for Women and Children in Sadr City, Baghdad, for the period from April 2022 to November 2022. Samples were collected randomly for Both genders and different age groups of patients.Samples were collected as follows (95 urine) (25 burns) (30 wounds) (30 ear) (45 Feces) Figure (3.1) and each sample was dealt with as follows:

1. Urine samples collection

Samples of midstream urine were collected in sterile glass bottles and brought to the hospital laboratory for cultivation within a period not exceeding one hour after collection.

2. Wound specimen collection

Samples were collected from the wound site by dipping cotton swabs in the normal saline solution, and after rotating them in the wound site, they were transferred to the laboratory directly for cultivation.

3. Burn samples collection

Samples were collected from the burn site by dipping sterile cotton swabs in a normal saline solution and then rotating them at the burn site and then transferred to the laboratory directly for cultivation.

4. Ear infections sample collection

Samples were collected with otitis media by dipping cotton swabs in normal saline solution and then rotating them at the site of otitis media and then transported directly to the laboratory for cultivation.

5. Feces samples collection

Samples were collected from the stool and placed in plastic bottles and then planted in dishes prepared from the agar.

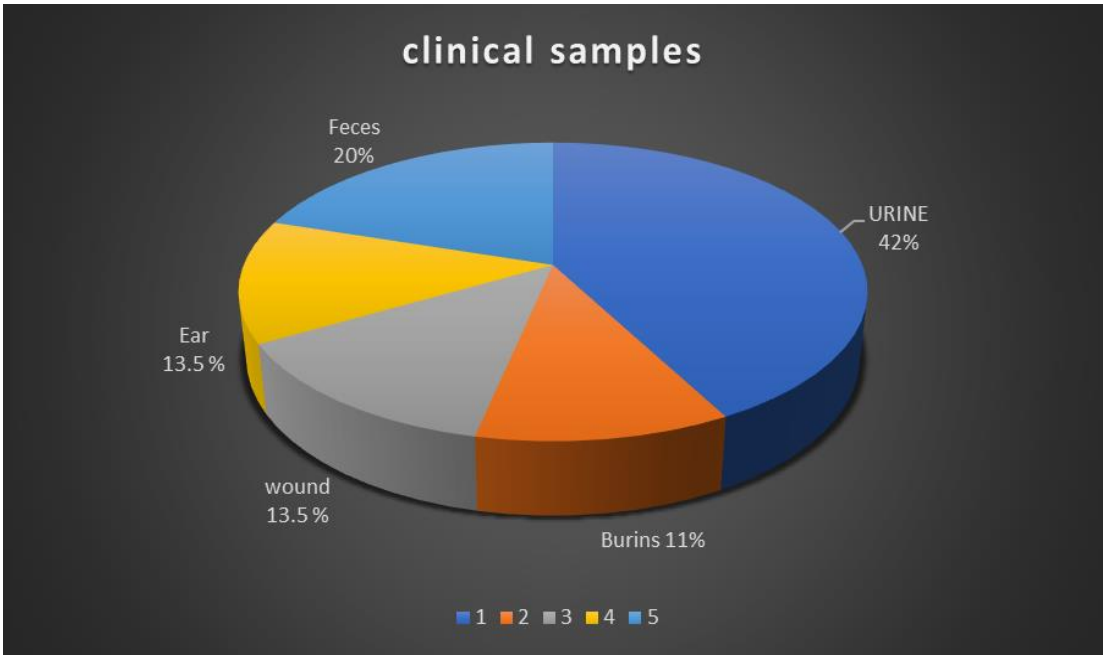

Figure 3.1 Dispersal of gathered clinical samples in accordance with the sources

3.2.3 Isolate of *Pseudomonas aeruginosa* bacteria

After collecting the samples within a period not exceeding one hour, the samples were planted using the Loop conveyor in the planning method on the solid Maconkey agar medium, then the plates were incubated in the incubator at 37 °C for a period of (24) hours, and after the incubation period, the results were read. It is not fermented to the sugar lactose and has a bluish green color.

1. Preserving isolates

Isolates were temporarily maintained on agar-agar nutrient media after inoculation and incubation with pure colonies.At a temperature of 37 °C for 24 hours and then preserved at 4 °C, the isolates were perpetuated by replanting them on the same medium every week for daily work (Collee *et al.* 1996).

2. Identification of isolates

Microscopic and biochemical examinations were conducted based on the scientific sources used globally to diagnose bacteria (Baron and finegold 1990, Holt *et al.* 1994), and the diagnosis included the following steps:

A. The cultural properties

The characteristics of the colonies growing in the medium were noted, including texture, colour, colony height, viscosity, odor, colony diameter and tip.

B. The microscopic test

I made swabs of selected Bacteria isolated from the samples (urinary, burns, wounds, ear and feces) by taking part of the developing colonies by means of the carrier loop on the nutrient medium of the agar to a glass slide and stained directly with a chromium

stain in order to identify the shape of the bacteria and the interaction of chromium stain with it, as well as To know the size of bacteria and ways to collect them.

C. The biochemical test

These tests were used to diagnose the bacterial isolates under study and included the following:

i. Motility test

I inoculated the tubes containing semisolid medium were in a stabbing method and incubated at a temperature of 37 °C for 24 hours, as the presence of a foggy area around the stabbing area and the spread of bacteria indicate a positive interaction and the absence of bacteria around the stabbing area indicates that the bacteria are immobile (Charles *et al.* 2008).

ii. Oxidase test

I immersed a filter paper was in a solution of the oxidase reagent and the colony to be examined was transferred to it by means of a stick, as the appearance of violet color within (10-60) seconds was considered as an indication of the positive reaction (Botes *et al.* 2003).

iii. Catalase test

we transferred a part of the bacteria to be examined was by means of the carrier loop to the surface of a glass slide and a drop of 3% hydrogen peroxide solution was added, as the appearance of bubbles with a sizzling sound indicated the positiveness of the examination (Botes *et al.* 2003).

iv. Indole test

I inoculated the isolates were in peptone water with the bacteria to be tested and incubated at 37 °C for 48 hours for the purpose of assaying indole.I added 0.5 mL of Kovacs reagent solution and I gently shook the mixture. When the red ring appeared on the surface resulting from the metabolism of the amino acid tryptophan, a positive test for indole (Jadallah *et al.* 1994).

v. Voges-proskaure

We cultured the bacteria to be examined were on MR-VP media and we incubated at a temperature of 37°C for 24 hours, then Fuchs Prosquare VP1 + VP2 solution was added to it. Indication of a negative test.

vi. H_2S production test

We inoculated the slanted kligler iron agar medium with the bacteria to be examined by the method of stabbing at the bottom button and incubated at a temperature of 37 °C for a period of 24 hours, where a black precipitate formed at the bottom indicating the positive reaction.

vii. Urease test

I inoculated the urea agar medium by stabbing and streaking method with the bacteria to be examined and we incubated at a temperature of 37 °C for 24 hours. The change in the color of the medium from yellow to pink is an indication of the positive test, while the yellow color of the medium is considered negative.

3.2.4 Studying the genetic content of bacterial isolates (extraction of genomic DNA)

The chromosomal DNA samples of bacterial isolates were extracted using a bacterial chromosomal DNA extraction kit produced by Geneaid Company, and the Table 3.5 shows its components.

Table 3.4 The components of the genomic DNA extraction kit

Volume	Component
30 mL	Gram+Buffer1
30 mL	GT Buffer
40 mL	GB Buffer
45 mL	W1 Buffer
25 mL (100 mL)	Wash Buffer2 (Add Ethanol)
2×11mg (1.1 mL)	Proteinase K_3 (Add ddH_2O)
30 mL	Elution Buffer
100	GD Columns
200	2 mL collection tubes

3.2.5 Sample preparation

1. I grew the bacterial isolates by taking a single colony growing on MacConkey medium (negative isolates) with a culture loop and adding it to a test tube containing 3 ml of Luria broth medium, then I incubating the tubes for 18 hours at 37°C.
2. I then added 1 ml of each suspension of the bacterial isolate growing in Luria Broth medium to a 1.5 ml Eppendorf tube.
3. Then I collected the suspended cells in the culture medium after centrifugation at 14,000 rpm for 2 minutes, then I poured off the upper aqueous layer and the accumulated cells remained at the bottom of a 1.5 ml Eppendorf tube.
4. After the centrifugation and disposal of the filtrate, the precipitate is preserved and 180 microliters of GT Buffer are added, then shaken on the Vortex or by pipette, then the proteinase k 20 µl (which was prepared by adding 1.1 of DW) is added and then I placed in the water bath at a temperature 60 ° C for a quarter of an hour with stirring

during the incubation period every 3 minutes. After that, we move on to the DNA extraction steps, which are:

a. Lysis

1. We added 200 µl of GB Buffer to the bacterial isolates and mixed them with Vortex for 10 seconds.

2. We incubate it in a water bath at a temperature of 70 ° C for 10 minutes., at this time we put the Elution Buffer solution inside the water bath at a temperature of 70 ° C until it is used.

b. DNA binding

I added 200 microliters of ethanol solution are to each sample and stirred quickly by hand from top to bottom until the DNA strands appear.Then we transfer the mixture by pipette to a GD Column with a capacity of 2 mL.It is marked with special numbers for the samples. then I placed The filter tubes inside the GD Column and then we do centrifugation At a speed of 14000-16000 rpm for two minutes after the centrifugation, the GD Column is neglected and another one is also marked.

c. Wash

1. We added 400 µl of W1Buffer were to the GD Column containing the filtered tubes and we centrifuged them at a speed of 14000-16000 round/min for 5 minutes, then we get rid of the filter inside the GD Column and we retrieve it again.
2. We added 600 µl of the prepared Wash Buffer (by adding 100 mL of ethanol), then we centrifuge it for 45 seconds and get rid of the filtrate and keep the precipitate.
3. It was centrifuged for the last time inside the GD Column which is dry without any addition for 3 minutes after which we keep the filter and get rid of the filter, then the filter is placed inside a clean and sterile eppendrof.

D. Elution

We added 100 µl of Elution Buffer to the medium, i.e. the center of the filter, and wait for five minutes, then we centrifuge it for 30 seconds. We get rid of the filter and keep the precipitate, which is the pure DNA. Then we keep the DNA in the refrigerator at -20 degrees until use.

3.2.6 The specific genes detection by PCR methods

1. Primer preparation

To give a final concentration of (100 pM/L) (as stock solution), reverse primers in addition to the lyophilized forward had been suspended with right mixture of deionized water, or (ddH_2O), as suggested by Eurofins Genomics Corporation protocol; to prepare 10 pM concentration as work starting point, re-suspended 10 L of stock solution in 90 l of ddH_2O to reach a final concentration of 10 pM, mixed well. Before being put to use, a vortex was used to mix them to ensure that they were all uniform (Ruppé *et al.* 2009). The Eurofins Genomics Company is responsible for the synthesis of these primers, which may be found in Tables 3.6 and 3.7.

The reason for using these primers is to amplify the DNA sequence. where it is considered Primers are the building blocks from which the rest of the blocks are built , primers consist of a short sequence of nucleotides complementary to the target sequence on the DNA template,whereThe pair of primers is linked at the beginning and end of the region to be amplified.

Table 3.5 Primer preparation

Gene		**Sequences (5'-3')**	**TM (˚C)**	**Product size(bp)**
AprA	F	GTCGACCAGGCGGCGGAGCAGAATA	69.5	973 bp
	R	GCCGAGGCCGCCGTAGAGGATGTC	71.3	
lasB	F	TTCTACCCGAAGGACTGATAC	57.9	153 bp
	R	AACACCCATGATCGCAAC	53.7	

Table 3.6 PCR program for *lasB* gene amplification

No.	Steps	Temperature (°C)	Time	Cycles
1.	Initial denaturation	95	5 min.	1
2.	Denaturation	95	30 sec.	35
3.	Annealing	55	30 sec.	
4.	Extension	72	30 sec.	
5.	Final extension	72	7 min.	1

Table 3.7 PCR program for *aprA* gene amplification

No.	Steps	Temperature (°C)	Time	Cycles
1.	Initial denaturation	95	5 min	1
2.	Denaturation	95	30 sec	30
3.	Annealing	65	1 min	
4.	Extension	72	2 min	
5.	Final extension	72	7min	1

3.2.7 Electrophoresis in agarose gel

1. Reagents

A. Agarose.
B. TBE Buffer (1x).
C. Loading dye (Bromo-phenol blue).
D. Ethidium Bromide (5 mg/mL).
E. DNA Ladder Marker (50 bp).

2. Protocol of gel electrophoresis

In order to determine the integrity and purity of the extracted DNA prior to the PCR reaction, gel electrophoresis was performed on agarose gel at a level of 1%. This was followed by gel electrophoresis at a level of 1.5% of agarose gel to identify the PCR result, as described by Sambrook and Russell (2001) as follows:

A. Agarose with concentrations of 1% and 1.5% was made by dissolving 1 gram and 1.5 grams of agarose powder, respectively, in 100 mL of 1x TBE, and bringing the mixture to a boil while stirring it constantly in a microwave oven. The temperature of the agarose was kept constant at 60 °C.
B. A total of 2 L of a dye containing ethidium bromide at a concentration of 5 mg/mL was applied to the agarose. After the agarose was put into the tray that had been taped, the comb was positioned towards the gel's perimeter.
C. After allowing the gel to harden at room temperature for half an hour, during which time it became opaque, the comb and the tape were carefully removed from the container.
D. The tray was put in the electrophoresis tank in a horizontal position, and then the electrophoresis tank was filled with one volume of TBE buffer. A pipette was used to get rid of the bubbles that were in the wells.
E. During the electrophoresis procedure, five micro grams of the DNA ladder containing 50 base pairs was placed into the first lane so that it could act as a marker.
F. The samples were loaded onto the gel wells after first mixing 5 ul of DNA extracted samples with 2 ul of loading dye. Alternatively, 5 ul of PCR product may be immediately loaded into the wells since it already includes the dye that is required to track the movement of DNA while it is being electrophoresed.
G. The electrophoresis tank was covered, and the power supply was adjusted to provide 70 volts/cm2 during the first 60 minutes of the PCR products and the subsequent 90 minutes of the DNA extraction.
H. Following electrophoresis, the gel was irradiated with ultraviolet light using a UV transilluminator set at 365 nanometers.

3.3 Statistical Analysis

The methodical,structured process of statistical analysis starts with data collection,moves on the classification and tabulation,uses aset of equations created by statisticians to performthe analysis,and ends with a succinct presentationof the findings using tables and graphs. The results of the study were subjected to statistical analysis for

the purpose of knowing the least significant difference, and the differences were considered statistically significant and highly significant at the level (0.05) for the possibility of error. A p-value of less than 0.05 was deemed significant.the results of this study were statistical package for social sciences (SPSS)program version 23 from Microsoft.

4. RESULTS AND DISCUSSION

4.1 Isolation and Identification of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was isolated from 58 bacterial isolates from different disease sites including (wounds, burns, otitis media and urinary tract infection, where the highest infection rate of this bacteria was in otitis media samples by 66.6%, followed by burns samples by 44%, then followed by wound samples with a percentage of 43.3%, then the lowest percentage in UTI with a percentage of 14.7%, but it was absent in the stool samples, and the total percentage of these bacteria was 25.7%.

Table 4.1 Table isolation of *Pseudomonas aeruginosa* bacteria

Source of sample	**Watching**		**Total**	**Expected**		**Total**	**The ratio %**
	+	-		+	-		
Wounds	13	17	30	7,7	22,3	30	%43,3
Burns	11	14	25	6,4	18,6	25	%44
Feces	0	45	45	11,6	33,4	45	0
Ear infection	20	10	30	7,7	22,3	30	%66,6
Inflammation of UTI	14	81	95	24,5	70,5	95	%14,7
Total	58	167	225	57,9	167,1	225	%25,7

The results of the statistical test for chi-square at the level of (0.05) showed that the calculated chi-square value is higher than the tabular value, which means that the incidence rates are related to the cases indicated for the sites from which they were collected, which showed the highest percentage of 66.6% in ear infection, followed by burn sites by 44% then wounds by 43.3%, while the lowest percentage was in UTI by 14.7%.

Through the foregoing, we find that the results of the study were somewhat in agreement with the results of Al-Muhammadawi (2006), who found that the main cause of middle ear infections was the *Pseudomonas aeruginosa* bacteria, whose infection rate reached 48.5%. Al-Sabawi (2017) found in his study to investigate the beta-lactamase

enzymes of clinical isolates of *Pseudomonas aeruginosa* on 37 isolates of this bacteria that the distribution rate was 67.6% for burns.

Whereas, Cristina *et al.* (2013) showed that the highest percentage of *Pseudomonas aeruginosa* bacteria was isolated from urinary tract infections, reaching 31%. For patients lying in hospitals, intensive care wards, and burn wards.

The isolates of otitis media came in the first place, with 29 isolates of *Pseudomonas aeruginosa* bacteria, at a rate of 72.5%. The cause may be that these microbes are opportunistic bacteria, which means that they take advantage of a general or local defect in one of the body's mechanical or immune defenses, or both (Brooks *et al.* 2007). Alternatively, this may be because of to the contamination of explodes from the atmosphere of healthcare facilities, the tools that are used, as well as the hands of hospital workers. As for the cases of infections of wounds with these bacteria, the reason is attributed to the adhesion factors possessed by these bacteria and their resistance to a number of antibiotics.

While (Abdul-Razak 2000) found in his study that *Pseudomonas aeruginosa* bacteria were the main cause of middle ear infections. Comparing the results obtained with the results of other studies reveals that there is both convergence and variation in the percentage of isolating *Pseudomonas aeruginosa* bacteria from different samples.

This is because of a number of factors, including the different places of isolation, such as clinics and intensive care centers, and the discrepancy may be due to the variety of samples collected before Researchers and their types, in addition to the fact that the disparity may be caused by the fact that the distance between the *Pseudomonas aeruginosa* bacteria are resistant to a number of sterilizers and antibiotics, which is the primary reason hospital-acquired infections occur, and other reasons are due to the degree of attention paid to hygiene, the type of cleaners and various disinfectants used in hospitals, and the methods used in disinfection operations. Moreover, other reasons are due to the methods used in sterilization operations (Kiffer *et al.* 2005).

4.2 Microscopic and Biochemical Diagnostic Test

Microscopic testing indicats that it was single cells, Gram-negative, non- spore forming, in addition to the rod shape, these results referred that this isolate may belong to *Pseudomonas spp*. and some biochemical tests were performed for more validation. Results showed that 58 isolates gave a positive result for oxidase, catalase, negative to Gram's stain. *P. aeruginosa* has different colonies types according to the medium and source of infection from small colonies to large mucoid colonies.

From these results, it can be concluded that these isolates (58 samples) primarily is a strain of *P. aeruginosa*, the results mentioned above were in agreement with (Todar 2011).

Table 4.2 Morphological characteristics and biochemical tests for *P. aeruginosa* isolates

Tests	**Result**
The medium of the MacConkey agar	Non-lactose fermented
The medium of the Cetrimide agar	+
The production of the Pigments	+ Pyocyanin
Hemolysis	+ ß-hemolysis
Gelatin liqufication	+
Gram-stain	G – rods
Catalase test	+
Oxidase test	+
The examination of the Indol	-
The examination of the Methyl red	-
The examination of the MR-VP	-
The examination of the Citrate utilization	+
The Growing in tempreture at 42°c	+
The Growing in tempreture at 4°c	-
(+) Positive test, (-) Negative test	

Results indicated that the infections gave excellent outcomes for catalase as the results of the ability for the producetion of the catalase enzyme, positive outcomes for oxidase due to the capability to produce cytochrome C oxidase (that oxidase tetramethyl-p phenylenediamine), enthusiastic results for gelatin vaporization due to the ability to produce gelatinase enzyme, positive results for using citrate.

On blood agar, most isolates produce β-hemolysis and others do not produce hemolysis. Cetrimide agar contains cetrimidal which is considered as toxic and inhibits other microbial flora that grow with *Pseudomonas spp*., Cetrimide agar is considered as selective medium for *P. aeruginosa*, and also all isolates had the ability fot the growing at the tepmreture of 42ºC, while it had been indicated that no growth at tepmreture about 4ºC, this notification appear due to *P. aeruginosa* tolerate high temperatures, and can produce green pigment.

4.3 Susceptibility to Antibiotics

The sensitivity test of bacterial isolates to antibiotics was conducted by the method of diffusion around the discs (Kirby Bauer) to determine the extent of sensitivity or resistance of these bacterial isolates to 7 types of antibiotics, depending on the diameter of the inhibition of the area surrounding the discs of antibiotics and comparing it with the diameters of the standard inhibition contained in (CLSI 2016).

One of the primary sources of infection is *pseudomonas aeruginosa*, Due to the high resistance shown by *Pseudomonas aeruginosa* to some drugs,, treatment for more serious infections including pneumonia and bacteremia is sometimes challenging and has a high death rate. We can be handled illnesses brought on by *P. aeruginosa* Using antibiotics from the following main classes according to their modes of action:"
- aminoglycosides such as amikacin (interference with protein synthesis) .
- Whereas Pencillin such as (Amoxicillin) and related beta- lactams are among the most traditional and extensively utilized antibiotics. Penicillin-binding proteins (PBPs) are enzymes that are known to target and are involved in the construction of the bacterial cell wall They lead to (inhibition of cell wall synthesis) such as first generation cephalosporins (cephalexin) where cephalexin works by causing toxic dysfunction in the target biosynthetic machinery that involves a futile cycle of cell wall synthesis and degradation, This depletes cellular resources and increases their killing activity By preventing bacteria from forming their cell walls, this weakens the walls until they break and the germs are killed.

-The third generation (ciprofloxacin) works on (interference with DNA replication). The current findings suggest that (Nalidixic acid) has an impact on *P. aeruginosa's* shape and protein expression."

- A quinolone called (Norfloxacin)which is made from carboxylic acid works well to treat *Pseudomonas aeruginosa* infections When norfloxacin is introduced to a *P. aeruginosa* culture that is developing logarithmically it instantly stops DNA replication.

- Additionally, RNA-dependent RNA polymerase (DNA)Bacterial is inhibited by Rifampin.

The results showed that most bacterial isolates were resistant to most antibiotics, which is a high rate of resistance and is expected for isolates taken from pathological cases, and this is the result of the random and excessive use of antibiotics without medical advice, in addition to the possibility of these bacterial isolates possessing different resistance mechanisms and developing these mechanisms against most of the antibiotics used in the treatment, where the test results showed 58 isolates of *P. aeruginos* bacteria isolated from pathological conditions (wounds, ear infections, urinary tract infections, burns and discharge) against antibiotics. Rifampin, Naldix acid, Ceftriaxione, Cephalexine, AmiKacin, Amoxicillin, Nerfloxacine.andThis Table (4.3) shows the total number of tested bacterial isolates and the number of sensitive, intermediate and resistant bacterial isolates under study.

Table 4.3 Susceptibility to antibiotics

Antibiotics	**Code**	***P. aeruginosa* isolates**					
		Resistant		**Intermediate**		**Sensitive**	
		No.	**%**	**No.**	**%**	**No.**	**%**
Nerfloxacine	NER	32	55	17	29	9	15
Rifampin	RIF	40	69	18	31	0	0
Amikacin	AMK	25	43	14	24	19	33
Ceftriaxione	CRO	5	9	20	34	33	57
Amoxicillin	AMX	39	67	7	12	12	21
Naldix acid	NAL	22	38	17	29	19	33
Cephalexine	CEF	8	14	6	10	44	76

Where the results of the study showed that the antibiotic test Nerfloxacine, Rifampin, Naldix acid, Amoxicillin for bacterial isolates was resistant to these antibiotics by 90%, and these results were identical to what was reached by the researcher Mohsen (2010).

It indicated that the resistance of *Pseudomonas aeruginosa* bacteria isolated from different disease models to Rifampin, Naldix acid, and Nerfloxacine, was 100% , the resistance of this bacteria is attributed to its possession of the spectrum of beta-lactamase enzymes, which are carried on plasmids, which facilitates their transmission between bacterial generations of this type (Al-Amri 2005). These bacteria also possess 12 systems responsible for pumping antibiotics out of the cell before they reach the target site (Chiu *et al.* 2013).

Some studies indicated the percentage of its resistance to these antibiotics due to its abundant secretion of polysaccharide Alginate, which binds with the positively charged antibiotics belonging to the group of anti-aminoglycosidas and thus prevents their spread inside the cell (Lambert 2002).

The results of the study obtained were that the sensitivity of *Pseudomonas aeruginosa* bacteria to the anti-AmiKacin, Cephalexine and Cefotaxime was 21%,76% 57%, while the resistance of *Pseudomonas aeruginosa* bacteria to Naldixic acid, Nerfloxacine, Rifampin, Amoxicillin was 55%, 55%, 69%, and 67%respectively, and the results of the current study are somewhat similar to what I obtained (Al-Shuwaikh 2006).

It concluded that the resistance of *Pseudomonas aeruginosa* bacteria isolated from hospital infections to the antibiotics, Cephalexine, Nerfloxacine, Rifampin was 45%.

The evolution of bacteria that are resistant to numerous antibiotics is seen as a significant treatment challenge. An explanation for this phenomenon was provided by (Sotto *et al.* 2001), who linked it to the effect of incorrect or excessive antibiotic usage. Because it is resistant to a wide variety of antimicrobial treatments, *P. aeruginosa* has risen to the top of the food chain and assumed a significant role (Brooks *et al.* 2007).

This high resistance of *P. aeruginosa* to this group of antibiotics may be due either to the non-permeability of these antimicrobial agents through the plasma membrane (Brooks *et al.* 2007) or due to the presence of extended-spectrum -lactamase enzymes, which are often expressed through plasmids. Cephalosporins contain the beta-lactam ring, which inhibits the bacterial cell wall synthesis. According to Bush and Jacoby (2010), these enzymes disassemble the beta-lactam ring, which causes it to lose its capacity to connect to the proteins that make up the cell wall.

Or maybe the resistance of *P. aeruginosa* isolates to the third generation of cephalosporins maybe due to the secretion of cephalosporinase enzyme which is expressed through chromosome (Lopez-Yeste *et al.* 1996).

4.4 Molecular Detection of Protease Genes by PCR Technique

4.4.1 Detection of *lasB* gene

PCR was used to look for the *lasB* gene in all of the examined isolates. For this, specific primers were used, each of which was designed to target a particular region within the target gene.After that, the amplified products were run for another 1.5 hours on an agarose gel at a concentration of 1.5%. When the agarose gel was examined using ultraviolet light, one line of bands could be seen running across the wells of the gel. These bands were also found at the same level in the bacterial isolates that carried this gene. This suggests that the primer is successfully interacting with the sequence in the DNA strand that is complementary to it.

The findings of the gel electrophoresis indicated that 45/58 (78%) of the isolates had this gene, and the bands that emerged on the electrophoresis were within the predicted size of the gene (153 base pairs) for all positive isolates. The findings of the reexamination of the isolates that did not display this gene remained the same; following electrophoresis on agarose gel, the bands corresponding to the gene did not emerge.

They are almost matched with those of (Khattab *et al.* 2015, Fadhil *et al.* 2016), who discovered that all of the investigated isolates had the *lasB* gene. They also partly accorded with the findings of (Benie *et al.* 2017), which demonstrated that 89% of isolates tested positive for the *lasB* gene.

Although Sabharwal *et al.* (2014) and Al-Dahmoshi *et al.* (2018) found that 75% and 69.23% of *P. aeruginosa* isolates, respectively, were positive for the presence of this gene, it is important to note that this gene is not universally present.

According to Cathcart *et al.* (2011), lasB is a key factor for P. aeruginosa because it codes for the creation of elastease, which is an important virulence factor that helps P. aeruginosa degrade elastin. This finding was published in the journal Microbiology. According to Bai *et al.* (2011), elastin is one of the primary components of the lung and is responsible for the expansion and contraction of the lungs. Elastin is also an essential component in human blood vessels, where it gives the blood vessels their elasticity and is responsible for the suppleness of the blood vessels themselves.

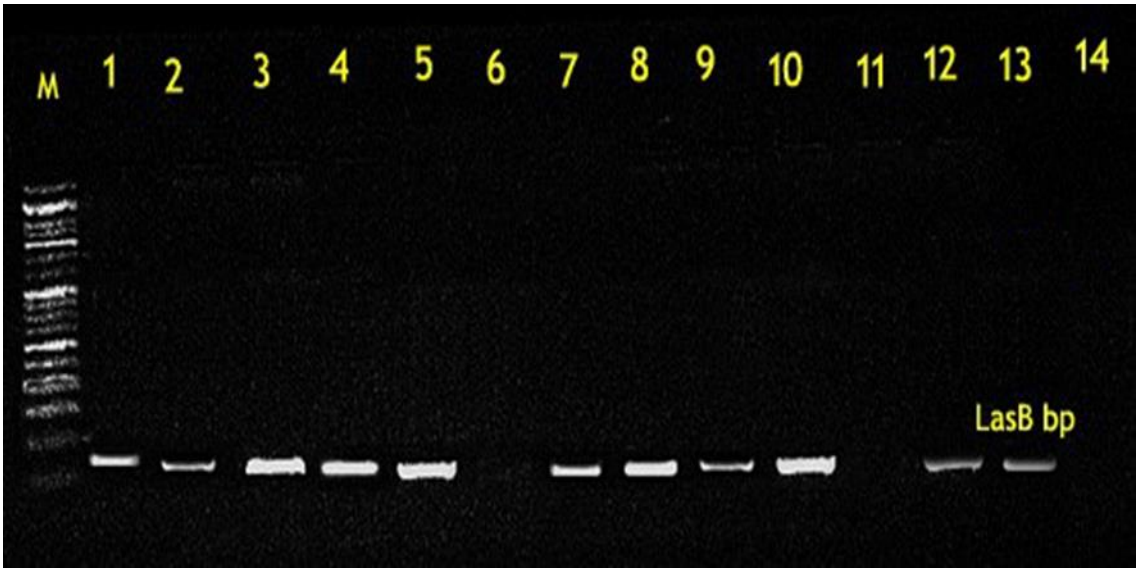

Figure 4.1 Agarose gel electrophoresis (1.5% agarose, 70 V/cm2 for 90 min) of lasB gene (153bp). Lane M represents 1500bp DNA ladder, Lanes1-14 represent bands of P. aeruginosa isolates

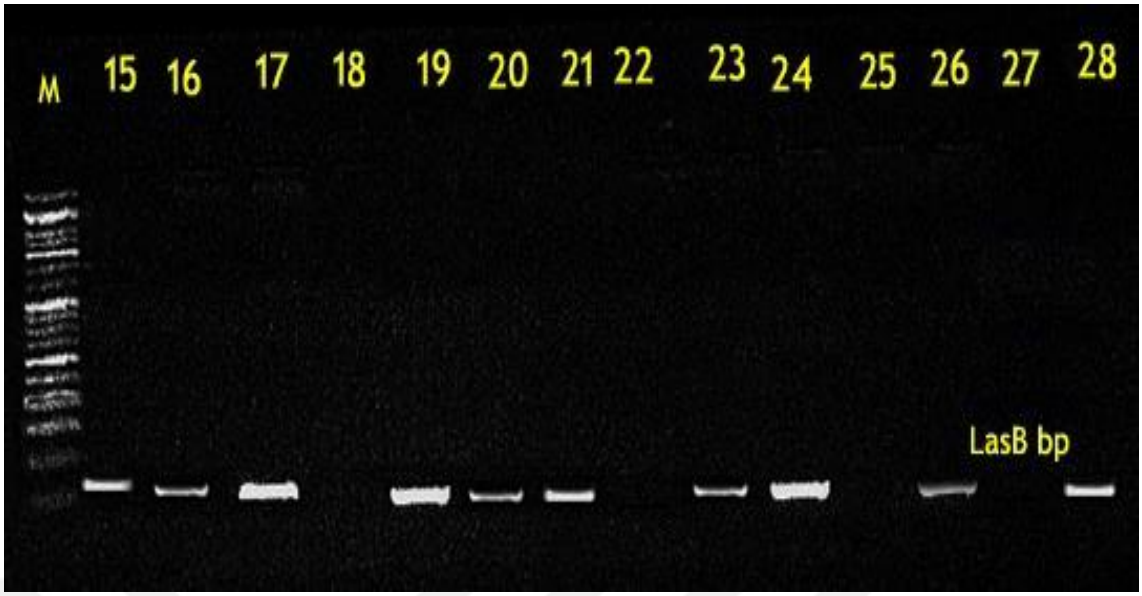

Figure 4.2 Agarose gel electrophoresis (1.5% agarose, 70 V/cm2 for 90 min) of lasB gene (153bp). Lane M represents 1500bp DNA ladder, Lanes15-28 represent bands of P. aeruginosa isolates

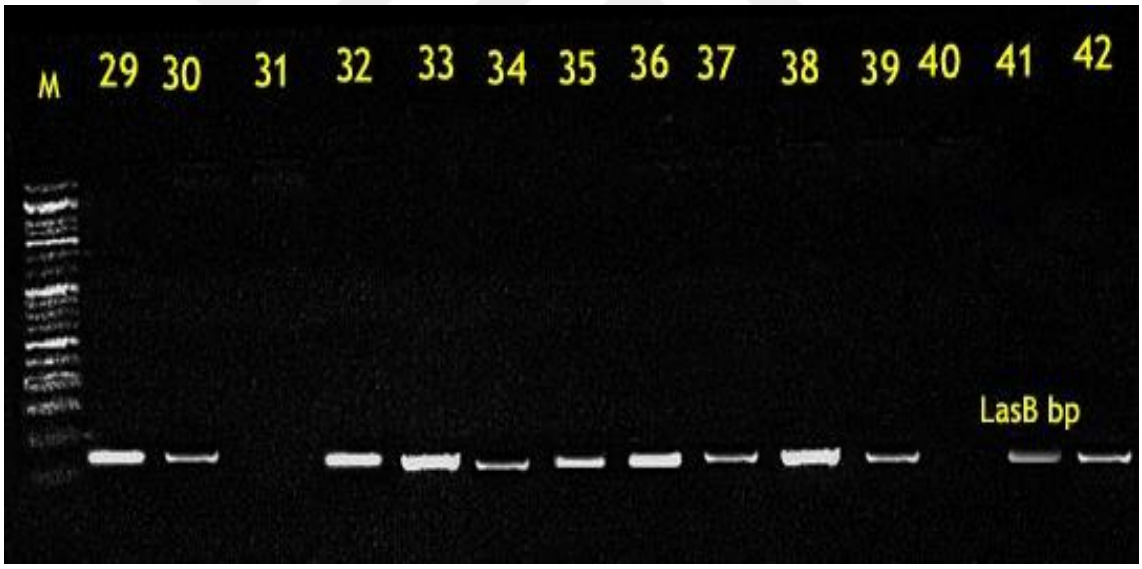

Figure 4.3 Agarose gel electrophoresis (1.5% agarose, 70 V/cm2 for 90 min) of *lasB* gene (153bp). Lane M represents 1500bp DNA ladder, Lanes29-42 represent bands of *P. aeruginosa* isolates

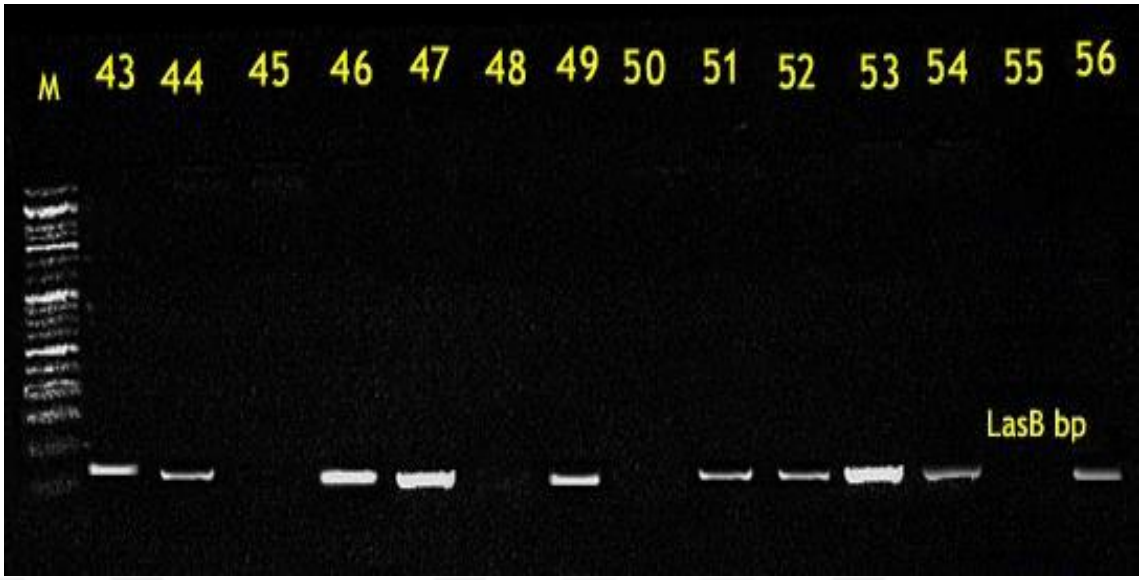

Figure 4.4 Agarose gel electrophoresis (1.5% agarose, 70 V/cm2 for 90 min) of lasB gene (153bp). Lane M represents 1500bp DNA ladder, Lanes43-56 represent bands of P. aeruginosa isolates

Figure 4.5 Agarose gel electrophoresis (1.5% agarose, 70 V/cm^2 for 90 min) of *lasB* gene (153 bp). Lane M represents 1500 bp DNA ladder, Lanes 57-58 represent bands of *P. aeruginosa* isolates

4.4.2 Detection of *aprA* gene

The alkaline protease encoded by the *aprA* gene was the second gene from proteases of *P. aeruginosa* detected in the current study by using a specific primer. Results of *aprA*

gene prevalence revealed that 41/58 (71%) of the isolates were harbored this gene. Figure shows the positive results for some isolates with amplified size approximately equal to (973bp). The isolates that did not show this gene were re-examined. The results were identical where the bands of the gene did not appear after the electrophoresis on agarose gel.

Melican and his team (2011) illustrated that some isolates of *P. aeruginosa* did not expose this characteristic or did not have the ability to produce protease despite the presence of the responsible gene when detected genetically. This is because the genetic variability in the genetic structure where the results of the phenotypic characteristics of *P. aeruginosa* showed the presence of this enzyme in the proportion 75% although the gene results showed that 100% of isolates had this gene.

This maybe due to two main reasons: the presence of the gene that encodes the enzyme which lysis the protein phenotypically, but it is ineffective, therefore the protein lysis did not appear in the phenotype. The second reason may be related to the biosynthesis of the bacteria and genetically expresses this characteristic without the needs to an external effect, or may be because *P. aeruginosa* has other genes responsible for the production of protease. Furthermore, protease production is affected by internal body conditions as well as with presence high levels of Exotoxin A and Exoenzyme S (Obaid and Al-Shwaikh 2022).

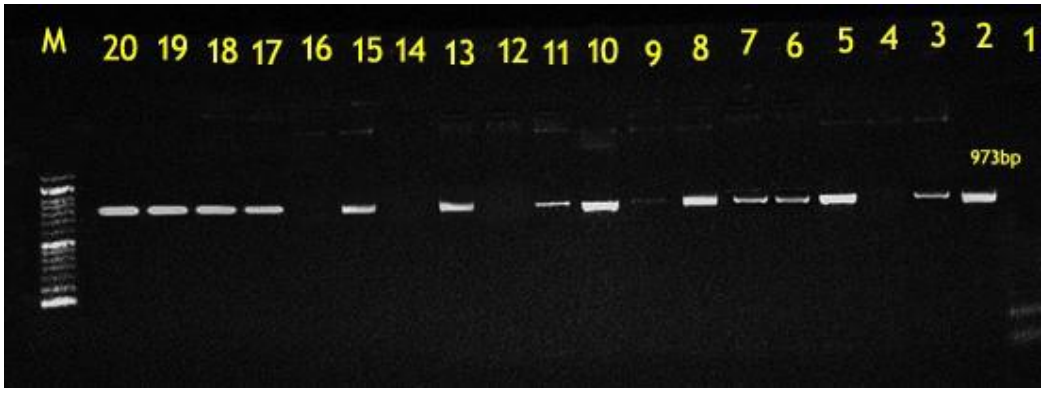

Figure 4.6 Agarose gel electrophoresis (1.5% agarose, 70 V/cm2 for 90 min) of aprA gene (973bp). Lane M represents 1500bp DNA ladder, Lanes1-20 represent bands of P. aeruginosa isolates

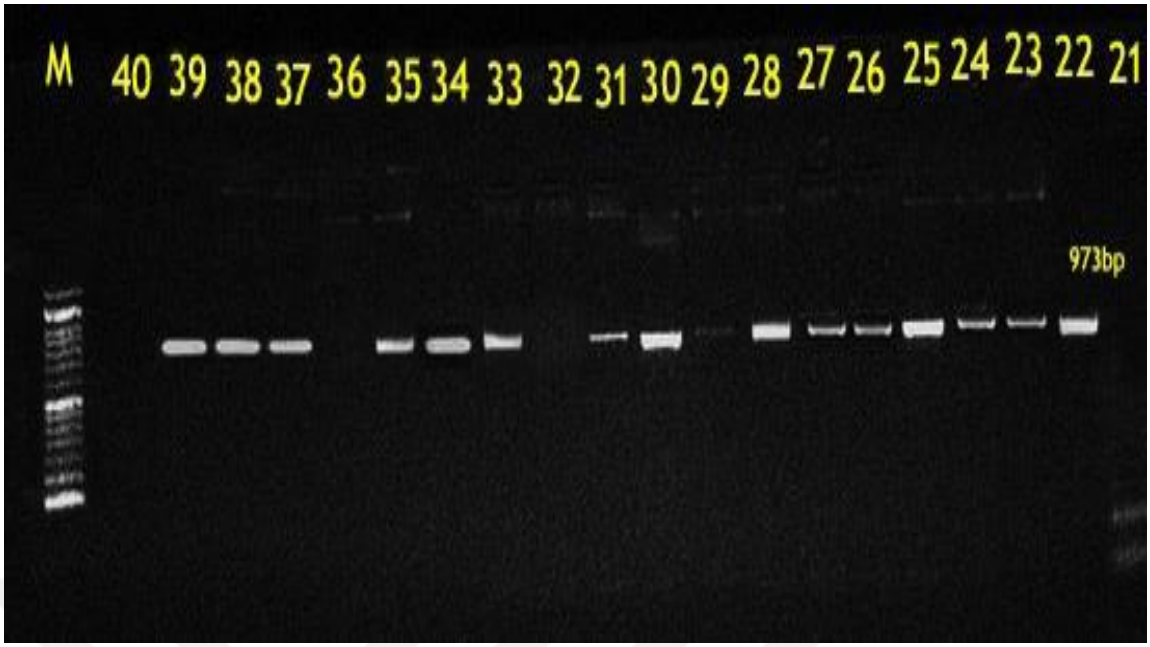

Figure 4.7 Agarose gel electrophoresis (1.5% agarose, 70 V/cm2 for 90 min) of aprA gene (973bp). Lane M represents 1500bp DNA ladder, Lanes21-40 represent bands of P. aeruginosa isolates

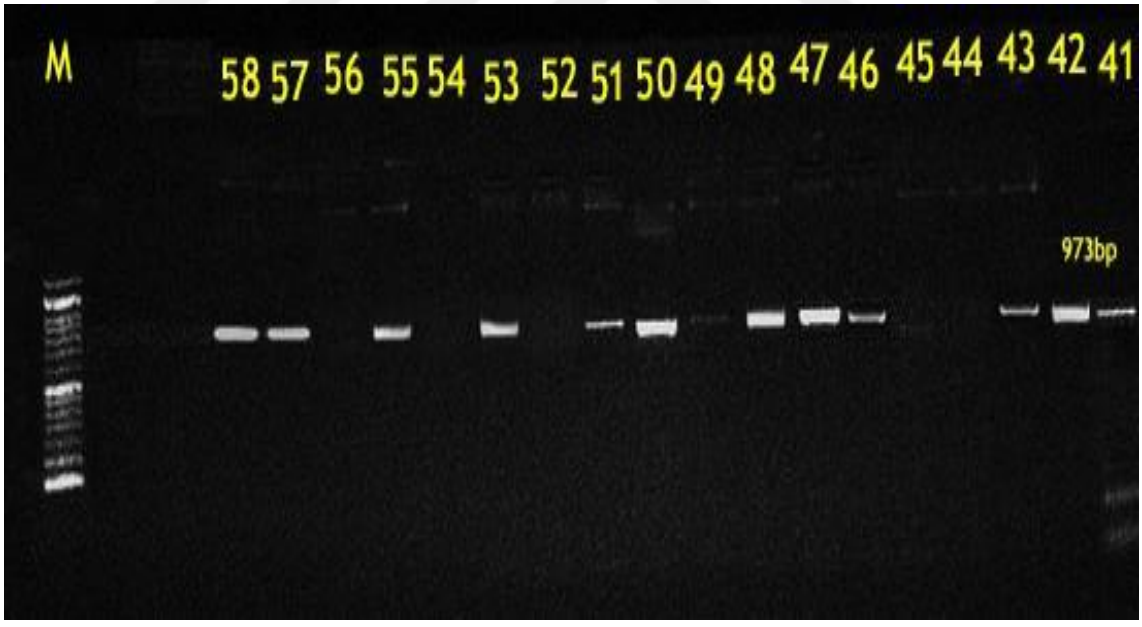

Figure 4.8 Agarose gel electrophoresis (1.5% agarose, 70 V/cm^2 for 90 min) of aprA gene (973bp). Lane M represents 1500bp DNA ladder, Lanes 41-58 represent bands of *P. aeruginosa* isolates

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

1. Variation of the studied isolates in the genera of *Pseudomonas aeruginosa* bacteria in their resistance and sensitivity to antibiotics according to the type of gene and the source of the isolate, and this difference could be due to the source of the isolation or the genetic variation of these alleles.
2. The resistance of the majority of bacterial isolates to the studied antibiotics was relative to the isolates taken from different disease states, as they resisted more than one type of antibiotics. Therefore, these isolates are multi-resistant.
3. The results of the genetic analysis showed that the genes aprA and lasB are diagnostic genes for these studied isolates and are responsible for the resistance traits of these bacteria, or they may be present in these bacteria, but the resistance traits are not apparent.
4. The polymerase chain reaction is the best way to detect genes, as the results showed that the traits of these studied genes are dominant genes, which means that these bacteria possess the gene carrying the genetic code for the character of diagnosis of this bacteria.

5.2 Recommendations

1. Conducting a study on different other genes of the same bacteria to find out the causes of bacterial resistance.
2. Identification of the genes encoding and pathogenicity of *Pseudomonas aeruginosa.*
3. Conducting *aprA* and *lasB* gene transformation using competent cell to detect the possibility of gene expression

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APPENDICES

APPENDIX 1. The number of isolated *Pseudomonas aeruginosa* bacteria and its antibiotic resistance

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No.of sample	Sources of the samples	NER	RIF	AMK	CRO	AMX	NAL	CEF
1	Wound	R	I	S	I	R	S	I
2	Wound	I	R	R	S	R	I	S
3	Wound	R	R	I	R	I	R	S
4	Wound	S	R	R	S	R	S	R
5	Wound	I	I	S	S	R	S	I
6	Wound	R	R	I	S	R	I	S
7	Wound	R	R	R	I	S	I	S
8	Wound	I	R	S	S	R	R	S
9	Wound	S	R	R	S	I	R	S
10	Wound	R	I	S	I	R	S	S
11	Wound	I	R	R	S	R	I	S
12	Wound	R	R	I	S	S	R	R
13	Wound	R	I	R	I	R	R	S
14	Burns	I	R	S	I	R	I	S
15	Burns	R	I	R	S	I	S	I
16	Burns	S	R	S	I	R	I	S
17	Burns	R	R	I	S	R	R	S
18	Burns	R	I	R	S	S	S	R
19	Burns	I	R	R	S	I	S	S
20	Burns	R	R	I	I	R	R	S
21	Burns	R	I	R	S	R	I	S
22	Burns	R	R	I	S	S	R	S
23	Burns	S	R	R	R	R	S	S
24	Burns	R	R	S	I	R	R	S
25	Ear	S	I	R	I	S	I	S
26	Ear	R	R	I	S	R	S	R
27	Ear	I	R	R	S	R	I	S
28	Ear	I	I	S	R	R	R	S
29	Ear	R	R	R	S	S	R	S
30	Ear	R	R	I	I	R	S	I
31	Ear	R	R	I	S	R	I	S
32	Ear	R	I	R	I	R	S	S
33	Ear	S	R	R	S	R	I	S
34	Ear	I	R	S	S	R	R	R

APPENDIX 1. The number of isolated Pseudomonas aeruginosa bacteria and its antibiotic resistance (Continued)

35	Ear	R	R	I	I	S	S	S
36	Ear	I	I	R	S	R	S	I
37	Ear	R	R	S	R	I	R	S
38	Ear	S	R	S	I	R	I	S
39	Ear	R	I	R	S	S	R	S
40	Ear	I	R	S	S	R	R	S
41	Ear	R	R	S	I	R	S	S
42	Ear	I	R	R	S	S	R	R
43	Ear	R	R	I	S	R	R	S
44	Ear	R	I	S	I	R	I	S
45	Urine	R	I	S	S	R	I	S
46	Urine	I	R	R	I	I	S	S
47	Urine	R	R	I	S	R	I	S
48	Urine	R	I	R	S	R	S	R
49	Urine	S	R	R	I	S	R	S
50	Urine	R	R	I	S	R	S	S
51	Urine	I	R	S	S	S	R	R
52	Urine	I	I	R	I	R	S	S
53	Urine	R	I	S	S	R	R	S
54	Urine	R	R	I	S	I	S	S
55	Urine	S	R	R	I	R	I	S
56	Urine	I	I	S	S	R	R	I
57	Urine	R	R	S	I	S	R	S
58	Urine	I	R	R	S	R	I	S

R = Resistant

I = intermediate

S = Sensitive

CURRICULUM VITAE

Personal Information

Name and Surname : Ahmed Abdulhussein Hilo HILO

Education

MSc	Çankırı Karatekin University Graduate School of Natural and Applied Sciences Department of Biology	2021-2024
Undergraduate	Al-Mansour Technical Medical institute / Baghdad Department of pathological Analysis	2002 - 2004
	Baghdad University College of science Department of Biology	2005-2008