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Molecular Study of Some β-lactamase Genes among Multidrug Resistance *Pseudomonas aeruginosa* (MDR)

A Thesis

Submitted to the Council of the College of Science, University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا (٨٥)

صَدَقَ الله العَلي العَظيم

سورة الأسراء/الآية ٨٥

Supervisors Certification

We certify that this thesis entitled " **Molecular Study of Some β-lactamase Genes among Multidrug Resistance *Pseudomonas aeruginosa* (MDR)**" was prepared under our supervision at the Department of Biology, College of Science, University of Babylon, in partial fulfillment of the requirements for the degree of master of Science in Biology/ Microbiology.

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Dedication

I dedicated this humble work

To the Prophet of Mercy, Muhammad peace be upon him and his Infallibe progeny

To the martyrs of Iraq in the Ministry of the Interior ... with a tear of sadness.

To our heroes in the Federal Police Force Command….

To whom taught me to withstand the difficulties .. To the one who gave me and still gives me without borders ..To whom Im proud of him (My father) .....

To the person who taught me patience, and pushed me toward success in life and gives me all care and happiness, To the strong and gentle soul who taught me trust in ALLAH (My Mother)

To my brothers and sisters

To all my colleagues who supported me, especially Kadhim Jawad and Ameer Jassim and those who have innocent smiles, clear souls, diamond hearts who are kind and helpful…

Malik

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Summary

Pseudomonas aeruginosa is a Gram-negative aerobic bacteria that has emerged as one of the most problematic pathogens characterizing strains of *P. aeruginosa* circulating in Iraqi. One hundred and ninty-four specimens which were from wound (96), diabetic foot (13) , urine (5) ,pulmonary fluid (2) , gun fire bomb(5) and burn (73) swabs of patients were collected. These patients were hospitalized in Al-Hillah city hospitals (Al-Hillah Teaching Hospital, Mirgian hospital and Imam AL-Sadeiq Hospital),in Kirkuk hospitals (Kirkuk General Hospital, Azadi Teaching Hospital) and also Medical City hospitals in Baghdad (Burns Specialized, AL-Shaheed Ghazi Hariri, Baghdad Teaching, and National Center for Educational Laboratories) and Ibn AL Bitar Hospital of both genders with different ages. The specimens were collected during the period from July/ 2022 to October/ 2022 .

Thirty-six 18.5% isolates from 194 symboled (PsA1 to PsA36) were identified as *Pseudomonas aeruginosa* by using morphological, microscopical properties and biochemical tests.These isolates were cultured on (blood, MacConkey, Nutrient broth ,*pseudomonas* cetrimide and Chromogenic agar), while 111/194 57.2% of specimens were observed to have other bacterial growth and 47/194 24.3% no bacterial growth (unknown) . The DNA was extracted from *Pseudomonas aeruginosa* isolates using bacterial favorgen genomic DNA extraction mini kit and measured its purity using nanodrop (1.8-2 nm). The identification of *Pseudomonas aeruginosa* was confirmed using polymerase chain reaction (PCR) using 16SrRNA ,the result exhibited that all 36/36 (100)% were *Pseudomonas aeruginosa* isolates. The antibiotic susceptibility test was performed against 17 types of antibiotics, using the disc diffusion method according to Clinical Laboratory Pathogenic Bacteria Standard Institute, CLSI-2023. The results showed that all 36 isolates were highly resistant 100% to

Ticarcillin and Piperacillin, Ceftazidime88.9% Cefepime50% ,Aztreonam 58.3%, Ciprofloxacin 41.6%, Levofloxacin 36.1%, Imipenem 52.7%, Meropenem 41.7%, Tobramycin 83.3%,Gentamicin 66.7%, Amikacin 83.3%, Norfloxacin 38.9%, Piperacillin-tazobactam 41.7%, Doripenem 38.9%, Colstin 8.3% and PolymyxinB 8.3%. The extended spectrum β-lactamase ESBLs enzyme screening was performed by Double Disc Synergy test (DDS) on a solid culture medium (Muller Hinton agar) in a Petri dish .The combination disk method based on the inhibitory effect of clavulanic acid was also used according to the (CLSI, 2023).The results showed that 4/36(11.1)% *Pseudomonas aeruginosa* isolates were positive which were (PsA32, PsA33, PsA34 and PsA36).

Polymerase chain reaction has been used to investigate 10 genes for extended-spectrum β-lactamase (ESBLs) in *Pseudomonas aeruginosa* isolates by using a specific primer for each gene of ***bla*** $_{OXA-10}$, ***bla*** $_{OXA-488}$, ***bla*** $_{OXA-145}$, $bla_{OXA-181}$, **PER-1, VEB, PER , GES-2** and **GES**. After performing the electrophoresis, the results showed that *Pseudomonas aeruginosa* has $bla_{OXA-145}$ 34/36 (94.44)% **PER-1** 31/36 (86.1)% bla_{OXA-10} 30/36 (83.33)%, $bla_{OXA-488}$ 24/36 (66.6)%, $bla_{OXA-181}$ 21/36 (58.3)% , and no result of three primer which were **PER , GES-2** and **GES** .

The results of real-time PCR of VEB showed that 33/36 (91.7)% of *Pseudomonas aeruginosa* isolates were positive .

The whole genomic DNA sequences analysis for of 15 isolates (PsA1 to PsA15) of *Pseudomonas aeruginosa* for 3 genes (***bla*** $_{OXA-145}$, ***bla*** $_{OXA-488}$, and ***bla*** **PER-1**) were sent to Macrongen company in Korea , the incorrect sequences were trimmed and the correct sequences were submitted for NCBI BLASTN for similarity, the matching of the current study isolates with NCBI-Gen bank global *Pseudomona s*trains showed the similarity percentage of current isolates ranged from(81 - 98)% .

Finally, in studying the specimens wounds are the most sites for isolating multidrug resistance (MDR) 19/36 (52.8) % *Pseudomonas aeruginosa* followed by different burns, and the of ***bla*** $_{OXA-145}$ gene was the most frequently gene among these isolates followed by **VEB, PER-1,** ***bla*** $_{OXA-10}$ **,** ***bla*** $_{OXA-488}$ gene and ***bla***$_{OXA-181}$ gene.

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List of Abbreviations

NO.	Symbol	Description
1.	A	Acidic
2.	ACC	Ambler class C
3.	AK	Amikacin
4.	AmpC	Ampicillin resistant gene
5.	AMR	Antimicrobial resistance
6.	AST	Antimicrobial susceptibility testing
7.	ATM	Aztreonam
8.	BEL e	Belgium Extended β-Lactamas
9.	BES	Brazil Extended Spectrum
10.	BP	Base Pair
11.	CAZ	Ceftazidime
12.	CF	cystic fibrosis
13.	CIP	Ciprofloxacin
14.	CLSI	Clinical Laboratory Standard Institute
15.	CMY	Cephamycins
16.	CN	Gentamicin

17.	CPO	Carbapenemase-producing Organisms
18.	CT	Colistin
19.	CTX-M	*Cefotaximase* Family
20.	D.W	Distil water
21.	DDS	Double disk synergy
22.	DNA	Deoxyribonucleic acid
23.	DOR	Dorpenem
24.	ESBLs	Extended Spectrum Beta Lactamase
25.	FEP	Cefepime
26.	FOX	Cefoxitin
27.	FQ	Fluoroquinolones
28.	GES	Guiana-extended spectrum
29.	GNB	Gram-negative bacteria
30.	H2O2	Hydrogen peroxide
31.	HAIs	Hospital Acquired Infections
32.	IPM	Imipenem
33.	IRT	inhibitor-Resistant TEM
34.	K	Alkaline
35.	KPC	Klebsiella pneumoniae carbapenemases
36.	LEV	Levofloxacin
37.	LPS	Lipopolysaccharide
38.	MBLs	Metallo Beta Lactamase
39.	MDR	Multidrug-resistant

40.	MEM	Meropenem
41.	MHA	Muller Hinton agar
42.	NCBI	National Center for Biotechnology Information
43.	NOR	Norfloxacin
44.	NS	normal saline
45.	OprD	outer membrane porin protein
46.	OXA	Oxacillinases
47.	OXY	In K. oxytoca
48.	PB	PolymyxinB
49.	PBPs	Penicillin binding protiens
50.	PBS	Phosphate Buffer Saline
51.	PCR	polymerase chain reaction
52.	PER	Pseudomonas Extended Resistant
53.	PIP	Piperacillin
54.	PTZ	Piperacillin-tazobactam
55.	RNA	Ribonucleic acid
56.	SBLs	Serine-β-lactamases
57.	SNPs	Single nucleotide polymorphisms
58.	SFO	In Serratia fonticola
59.	SHV	Sulfhydryl Family
60.	TBE	Tris Borate EDTA
61.	TEM	Temoneira Family
62.	TOB	Tobramycin
63.	TSB	Tryptic soy broth

64.	UV	Ultra violet
65.	VEB	Vietnam Extended Spectrum β-lactamase
66.	WGS	Whole genome sequencing
67.	WHO	World Health Organization

Chapter One

Introduction

1.1: Introduction

One of the most significant Gram-negative opportunistic bacteria in nosocomial infections is *Pseudomonas aeruginosa*, which is commonly seen in burn and wound units. The cornerstone of antimicrobial resistance , notably in Gram-negative bacteria (GNB), has been identified as β -lactamase genes, despite the fact that infections have a variety of methods of resistance (Tooke *et al.,* 2019., Sid Ahmed *et al*., 2022). Gram-negative bacteria have high rates of antibiotic resistance, which is associated with increased disease and death, according to a WHO report. Clinical isolates of *P.aeruginosa* quickly acquire antibiotic resistance and spread across hospitals, putting them on the list of multidrug-resistant (MDR) bacteria by WHO (Athreya, Shareef and Gopinath, 2020., Hashemi *et al*., 2021)

The ability to rapidly develop resistance to current therapies, and innate one-to-many drug classes ,the permeability of outer membrane, excessive expression of efflux systems, and antibiotic-inactivating enzymes are a few of the known mechanisms of PA resistance. Acquired resistance is caused by mutations in the genes encoding efflux pumps, porins, penicellin-binding proteins, and enzymes and horizontal gene transfer. Adaptive resistance is brought on by repeated antibiotic exposure and excessive environmental stress (Karruli *et al.,* 2023).Classic antipseudomonal antibiotics have a variety of mechanisms of action, including inhibition of bacterial cell walls for β-lactam agents like Ceftazidime/Cefepime, Piperacillint-azobactam, Imepenem-cilastatin, Miropenem, Dorepenem, and Aztreonam, which block the synthesis of DNA for Flueroquinolones, and inhibition the synthesis of protein for Aminoglycoside .

Resistance to β-lactamases in *P. aeruginosa* has increased due transmissible antibiotic resistance genes that play a role in promoting resistance. A, B, D, and C are β -lactamases. Class B mechanism is a MBLs (Metallo β -lactamases) , which opposed to A, C, and D . Some microorganisms have the ability to produce enzymes that enhance resistance to beta-blockers. Most of these organisms are *Pseudomonas aeruginosa* bacteria(Nasser, Gayen and Kharat, 2020 , Mohammed Saleh Naji, 2022).

The presence of such a many different groups of β-lactamase genes was noticed in MDR-*P. aeruginosa* isolated form clinical specimens that were phenotypically resistant to Ceftazedime-avibactam and/or Ceftolozane-tazobactam. Resistance to Ceftazedime-avibactam and Ciftolozane-tazobactam was linked to the class B β -lactamases genes (like blaVIM-2) and class D β-lactamases genes (like bla*OXA-10*), whereas ciftolozane-tazobactam resistance was linked to the class A β-lactamases (like blaVEB-9), class (like bl*aOXA-488*). Resistance to these novel therapeutic agents was also associated with the presence of other β -lactamase genes such as blaPDC-35 and bla*OXA-10* (Sid Ahmed *et al.,* 2022).

Resistance to antibiotics in *P. aeruginosa* can be mediated by several different mechanisms, including site-targeted drug modification or outer membrane modification; generation of β-lactamases; and efflux pumps, among others. Antibiotic resistance is on the rise primarily as a result of widespread usage in burn units of antibiotics such as Ciprofloxacin, β-lactamases, and Aminoglycosides, as well as a lack of readily available alternatives and their expensive costs (Ali *et al*., 2020).

Extended-spectrum beta-lactams (ESB), Ampicillin resistant gene (AmpC), carbapenemases, and other forms of β-lactamases can be located

on the chromosome or plasmid, among other places. There has been a rise in the number of *P. aeruginosa* isolates that are resistant to β-lactams, including the ESBL, AmpC β-lactamases, and metalo β-lactamases, all of which have been linked to transmissible genetic factors that promote resistance. A B, C, and D are the four molecular classes of β-lactamases. In contrast to classes A, C, and D, which use serine-based mechanisms, metallo β-lactamases (MBLs) are a class B mechanism . Some microbes, such as *P. aeruginosa*, develop a group of enzymes known as extended-spectrum β-lactamases (ESBLs), which are capable of hydrolyzing antimicrobial drugs such as Penicillins, Cephalosporins, Monobactams, and Carbapenems and causing resistance to them (Nasser *et al*., 2020).

Aim of Study

The Multidrug Resistant (MDR) isolates that are present in the hospital environment represent not only therapeutic problems but also serious concerns for infection control management, so this study aimed to examine the antibiotics susceptibility profile and detection of metallo-β-lactamase gene among *P. aeruginosa* isolates from patients in different hospitals.

Objectives of Study

1. Collection of different clinical specimens (wounds and burns) from some Iraqi hospitals .
2. Isolation and identification of *P. aeruginosa* from these specimens by cultural different media , morphological and biochemical tests.
3. Confirmation of identification by using 16SrRNA
4. Determination of the antibiotic susceptibility tests of the isolates against the common antibiotics.

5. Detection of ***bla*** $_{OXA-10}$, ***bla*** $_{OXA-488}$, ***bla*** $_{OXA-145}$, ***bla*** $_{OXA-181}$, **PER-1, VEB, PER , Ges-2** and **GES** genes using polymerase chain reaction (PCR) .
6. DNA sequencing analysis of these isolates .

Chapter Two

Literature Review

2. Literature Review

2.1 History of *Pseudomonas aeruginosa*

The genus *Pseudomonas* is a large group of Gram-negative bacteria belonging to the family *pseudomonadaceae* within the gammaproteobacteria. Other members of this diverse family include the genera *Xanthomonas*, *Burkholderia*, and*Ralstonia* (Anzai *et al*, 2000). *Pseudomonas* was first isolated by a French military surgeon Sedillot in 1850 from a blue-green discharge on surgical dressing with associated infections, the genus *Pseudomonas* was first proposed early in microbiological history by Migula in 1894.

As Palleroni (2010) describes, Migula first illustration of this genus was very brief and, in hindsight, inaccurate. His description stated "Cells with polar organs of motility. Formation of spores occurs in some species, but it is rare". Eventually, the *Pseudomonads* were correctly identified as being non-spore-forming and the "spores" originally visualized by Migula have been postulated to be "refractile of reserve materials"(Palleroni, 2010).

Later, the *pseudomonads* were split into five rRNA subgroups on the basis of RNA-DNA measurements, and this classification was reported in the 1984 edition of Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). In the 1980s, Woese and his colleagues proposed the investigation of the 16S ribosomal RNA gene sequences for the classification of bacteria, placing the genus *Pseudomonas* in the Gamma Proteobacteria, which caused the greatest shifts in bacterial taxonomy (Woese *et al*., 1984) and Anzai *et al*., (2000) compiled the sequences of the 16S rRNA gene of 128 *Pseudomonas* species and found that several species did not fit inside the *Pseudomonas* sensu stricto cluster, which includes members of the rRNA group I from Palleroni (1984).

Nevertheless, the most relevant of these changes affecting the genus *Pseudomonas*

began from the 2000 year onwards with a first work compiling the sequences of the 16S rRNA gene of 128 Pseudomonas species carried out by(Anzai *et al*., 2000), who showed that many species did not fit within the *Pseudomonas* sensu stricto cluster, which contained the members of the rRNA group I from Palleroni (1984). The members of the remaining rRNA groups were splitted in more than 25 genera belonging to the classes Alpha, Beta, and Gamma-proteobacteria (Peix *et al*., 2009; García-Valdés and Lalucat, 2016). At present the genus comprises more than 220 species (Lalucat *et al*., 2020), havebeen characterized, and their taxonomic names have been validated. These species are present in the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2018).

2.2. Current Classification of *P. aeruginosa*

Pseudomonas aeruginosa was isolated as a pure culture in 1882, when Carle Gessard, a French pharmacist reported in a publication "On the Blue and Green Coloring of Bandages", in 1894 gave the Genus name *Pseudomonas* (Gessard**,** 1984). The word Pseudo means a false unit from Greek, and monas, means a single unit, and aeruginosa means verdigris. *P. aeruginosa* classified as follows:

Kingdom: Bacteria

Phylum: *Proteobacteria*

Class: *Gammaproteobacteria*

Order: *Pseudomonadales*

Family: *Pseudomonadaceae*

Genus*: Pseudomonas*

species: *Pseudomonas aeruginosa*

(Diggle and Whiteley, 2020).

Pseudomonas taxonomy was reviewed in our previous assessment 2.1, which included the species discovered through 2009 (Peix *et al*., 2009). More than 70 new species have been described so far this year 2009, bringing their total to more than 200, several of them have been isolated from human or animal sources, such as *'P. saudiphocaensis*', *'P.saudimassiliensis'*, and *'P. massiliensis'*, which were all isolated from currency notes (Azhar *et al*., 2017), and *'P. massiliensis'*, which was isolated from a woman stool specimen (Bardet *et al.*, 2018).

2.3 Characterization of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacterium, an obligate aerobic respiratory metabolism by oxygen, but in some cases, it uses nitrate as an alternative source (as final electron acceptor) that allows growth anaerobically (Schurek *et al*., 2012; Kahraman and Karaderi, 2021). A rodshaped slightly curved or straight, lactose non-fermenter, has a special, distinctive fruity odor, they are motile by one or two polar flagella, noncapsulated but it has pseudo capsule, slime layers which they play as a barrier against antibiotics, phagocytes and, lymphocytes (Todar, 2011; Lalucat *et al*., 2021).

Non-spore-forming (Wilson and Pandey, 2020; Vázquez *et al*., 2021), and can produce many types of pigments, pyoverdin and pyocyanin give its distinctive blue-green color on solid media as well as this pigment were used as an inhibitor to many gram-negative and gram positive bacteria, fungi and also can damages mammalian cells (Bogiel *et al*., 2021). *P. aeruginosa* has huge virulence factors which cause both extensive tissue damage and interfere with the immune defense weapons (Ratkai *et al*., 2011; Urgancı *et al*., 2022).

2.4. Nosocomial Infection .

Pseudomonas aeruginosa is a life, threatening opportunistic bacterium that is commonly detected in hospitalized patients. *P.aeruginosa* produces nosocomial infections in immunocompromised people due to conditions such as burns, Crohn's disease, and neutrophils (Dlken Gur and Aksoz, 2016). It accounts for almost 77% of burn patient death over the last 25 years. Tissue injury as a result of a thermal energy-induced burn is one of the most painful experiences people have. Thousands of people die each year as a result of burn injuries (Mehta *et al.,* 2017).

Despite advancements in burn treatment, infections continue to be one of the major causes of death in these patients, particularly in the critically burned Burn patients have more skin shortages, longer hospital stays, and repeated invasive operations than other hospitalized patients, making them more susceptible to infection Multi - drug resistant *P. aeruginosa* causes 40-60 percent of hospital - acquired infections in burn patients, resulting in high morbidity and mortality (Dou *et al.,* 2017).

Pseudomonas aeruginosa has become one of the leading causes of nosocomial infections in hospitals in past years due to its natural resistance to a wide spectrum of medicines, with the ability to resist virtually all effective antibiotics (Sorkh *et al.,* 2017) **.**

2.4.1 Burn Infections

Burns damage the skin's barrier and make it easier for bacteria to get in, which slows the healing of burn wounds (Forson *et al*., 2017). Gas, hot water, electricity, heat, and chemicals can all cause burns (Mirmohammadi *et al*., 2013). Based on their severity, depth, and size, burns are categorized as either superficial (first-degree), superficial partial-thickness (second degree), full-thickness (third-degree), or fourth-degree (Jeschke *et al*., 2020)

2.4.2 Wound Infection

In developing countries, wound infections are linked to illness and death. Wound infections are strongly linked to a wound's location near a possible source of contamination, poor management of moisture, exudate, or edema, the way the wound were made, the presence of a predisposing condition, and living in a city (Jeschke *et al*., 2020).

2.5. Epidemiology of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a commensal bacterium which is existed on skin surfaces (few numbers), in nostrils, in the upper respiratory tract, and as normal flora in the intestinal tract (Brook *et al.,* 2013). *P. aeruginosa* isolates are tolerant to a wide variety of harsh conditions, such as high concentrations of dyes, salts and a wide range of antibiotics used in hospitals. It can survive in the presence of some disinfectants and even grow in distilled water and tap water (Wunderink and Mendoza, 2007).

It is a water-borne pathogen, different moist environment sources could be acting as a reservoir (Streeter and Katouli, 2016). *P. aeruginosa* is responsible for so many nosocomial infections, due to its non-fastidious organism, ability to grow and multiply in moist and minimal nutritional environments has resulted in contamination of a wide variety of organic substrates, intravenous tubing, hot tubes, laboratory water baths, and other water-containing vessels drain, sinks, washing machines even distilled water, drug and, food factory (Harvey *et al.*, 2013; Panda *et al*.,2022).

2.6 Pathogenesis of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common nosocomial pathogen responsible for significant morbidity and mortality internationally and infections by these organism are difficult to treat due to a number of antibiotic resistance mechanisms and the

organism's propensity to form multicellular biofilms infections, urinary tract infections and bacteremia (Reynolds and Kollef,2021). Its produces two siderophores , pyoverdine and pyochelin, important for scavenging iron in low-iron environments.

These secondary metabolites important for *P. aeruginosa* fitness in a number of environments (Diggle and Whiteley**,** 2020).*P. aeruginosa* is a common opportunistic organism with a remarked ability for survival in various environmental conditions. QS is the key element that regulates gene expression and virulence behaviors such as proteases, hemolysin, pyocyanin pigment, and biofilm formation(Thornton *et al.*, 2021).

A deep understanding of these virulence mechanisms is crucial for the design of therapeutic strategies and vaccines against this multiresistant pathogen(Martín *et al*.,2021). *Pseudomonas aeruginosa* is a common pathogen causing localized infections such as Ocular Infections, skin, soft tissue infections, urinary tract infections, respiratory tract infections, and disseminated systemic infections, the septic shock, also responsible for 60% of chronic infections . Additionally, it was linked to infectious diarrhea, with confirmed cases often affecting people with hematological malignancies and neutropenia as a result of chemotherapy or infant epidemics (Florman *et al*., 1950; Falcão *et al*., 1972; Mackenzie *et al*.,2021). *P. aeruginosa* is a common cause of Hospital Acquired Infections (HAIs) due to its antimicrobial-resistant and opportunity to survive in low-nutrient environments. *P. aeruginosa* adaptability allows it to infect a wide range of tissues, wide spectrum of tissues tropism and live in both natural and hospitals environment (Caschera ,2021).

Also, contact lens wear is the most common cause of *P. aeruginosa* corneal infections, but other risk factors for keratitis in non-contact lens wearers include ocular trauma, ocular surgery, and previous ocular surface disease (Enzor *et al.*, 2021).

2.7.Virulence factor

2.7.1. Pili

Pili or fimbriae are small expansions of the filamentous surface of *P. aeruginosa*. Several pili are commonly found on the surface. *P. aeruginosa* pili are one of the few prokaryotic pili that participate in bacterial motility. This motility is caused by the retractile characteristics of *P. aeruginosa* pili, which allows *P. aeruginosa* to "spread" rather than "swim" along damp surfaces (Kipnis *et al*., 2006).

2.7.2 Flagella

Flagella are complex protein structures on the surface of *P. aeruginosa* that form a filamentous polar appendage. The major mobile appendage of gram-negative bacteria, flagella, permits *P. aeruginosa* to swim in a propeller or screw-like motion. Flagella play a key part in pathogenesis by connecting and sticking to epithelial cells with a comparable membrane (Kipnis and Sawa, 2006).

2.7.3 Lipopolysaccharide (LPS)

While the inner surface of the outer membrane resembles a traditional phospholipid bilayer, the outer membrane's outer surface is mostly LPS. Lipid A, the hydrophobic domain of LPS, is integrated into the phospholipid bilayer, while the polysaccharide core and polysaccharide O-specific polysaccharide from the hydrophilic tail (Kipnis and Sawa, 2006).

2.7.4 Hemolysis

P. aeruginosa hemolysis causes cytopathic effects in blood and tissue culture cells. Morphological changes have indicated the lysis and destruction of the cell architecture, which includes membrane and cytoplasm. Normal serum and albumin

prevent the hemolytic effect of hemolysis. *P. aeruginosa* hemolysis is also responsible for the colonization of the lungs and other organs, and its cytotoxic effects on eukaryotic cells aid invasion (Tokunaga and Cox, 2000).

2.7.5 Siderophore

It is a low-molecular-mass molecule which have a high chelating or binding iron specificity. It has identified more than 500 different siderophores from micro-organisms. Some bacteria produce over one kind of siderophores. The aerobic bacteria and other living organisms require iron for a variety of biochemical cell reactions. Though iron is the fourth most abundant element in the crust of the Earth (Challis, 2005). *P. aeruginosa* secreted siderophores (pyoverdin and pyochelin), allow the bacteria to multiply in the absence of ferrous ions (Ben *et al*., 2011).

2.7.6 Pigments

Pseudomonas aeruginosa frequently produces the nonfluorescent bluish pigment pyocyanin, which diffuses into the agar. Other species of *Pseudomonas* do not make pyocyanin. Some strains of *P.aeruginosa* produce the dark red pigment pyorubin or the black pigment pyomelanin, while others generate the fluorescent pigment pyoverdin, which gives the agar a greenish hue (Brooks *et al*., 2013).

2.8 Multidrug resistance of antibiotics in *P. aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen that may cause severe over run diseases in grievous ill patients. The frequency of infections caused by them is increasing and multidrug-resistant (MDR) strains, resistant to almost all obtainable antibiotics, are emerging in hospitalized patients. Because of its ubiquitous nature, ability to survive in moist environments, and innate resistance to many antibiotics and antiseptics (Magalhães *et al*., 2020).

Multidrug-resistant(MDR) is when bacteria are non-susceptible to at least one agent in three or more antibiotics categories. Antibiotic resistance and, in particular, MDR are public health threats. Multidrug-resistant infections are associated with poorer clinical outcomes and higher cost of treatment than other infections, and there is concern that the emergence of pan-resistant strains (pathogens resistant to all available antibiotics) will render some infections untreatable (Serra-Burriel *et al.*, 2020). MDR *P. aeruginosa* was frequently isolated from patients with cystic fibrosis (CF) patients with neoplasia and burn wound infections (Nasser and Kharat, 2019).

Pseudomonas aeruginosa can develop antibiotic resistance through site-targeted drug modification, outer membrane modification, β-lactamases production, and efflux pumps. Antibiotic resistance is rising due to the frequent use of antibiotics in burn units, a lack of alternatives, and their high prices (Ali *et al.,* 2020). Some microorganisms, like *P. aeruginosa*, produce ESBLs, which hydrolyze antimicrobial medicines and cause resistance (Nasser *et al.,* 2020).

2.8.1. Intrinsic Resistance

Intrinsic resistance refers to a microbe's ability to resist antimicrobial agents. Antimicrobial resistance in *P. aeruginosa* is quite high. Antibiotic inactivating enzymes are synthesized, MDR efflux pumps are developed, and outer membrane permeability decreases in *P. aeruginosa* all contribute to intrinsic antimicrobial resistance (Hall *et al*., 2018). The permeability barrier of the outer membrane prevents antimicrobials from entering bacterial cells. The carbapenems' major target, the outer membrane porin protein OprD, changes, resulting in a decrease in membrane permeability. Certain chemical moieties or the breakdown of the antibiotic molecule can be added to or removed from antibiotics by enzymes produced by bacteria. antimicrobial resistance is caused by hydrolyzing antibiotics, such as AmpC

β-lactamases, which hydrolyze most β-lactams, and the carbapenem hydrolyzing enzyme PoxB, which is encoded on the chromosomal level (Arzanlou *et al*., 2017).

2.8.2. Acquired Resistance

Resistance in *P. aeruginosa* can be acquired by horizontal gene transfer and chromosomal gene alterations resulting from the acquisition of foreign resistance genes. Many types of antibiotics are resistant to mutation-induced acquired resistance, including lactam, fluoroquinolones (FQ), and aminoglycosides (Hasan and Al-Harmoosh, 2020). Efficient efflux pumps and decreased antibiotic permeability, target site alterations and the formation of antibiotic-modifying enzymes are among the other reasons of resistance. As a result of these processes, antibiotics are mutated or eliminated chemically. Acquired resistance to antibiotics is facilitated by the development of enzymes that modify the chemical structure of antibiotics extended-spectrum β-lactamases, aminoglycoside nucleotidyltransferases, carbapenemases, 16s rRNA methylases, and enzymes that change lipopolysaccharide (LPS) (Poole, 2011)**.**

2.8.3 Adaptive Resistance

Adaptive resistance is an inducible resistance that develops in response to antimicrobial medications (e.g., antibiotics) or other chemical or physical stresses, like a change in medium, pH, temperature, oxygen, or other growth conditions. In contrast to adaptive resistance, intrinsic and acquired resistance are permanent and unaffected by antibiotics and other environmental stressors (Fernández *et al*., 2011). Adaptive resistance can be activated by several environmental variables, including as heat shock, DNA damage, polyamines, nutritional shortages, biocides, anaerobiosis, cation levels, changes in carbon sources, and social activities like as biofilm formation and swarming motility.

2.9 Mechanisms of Antimicrobial Resistance in *P. aeruginosa*

Pseudomonas aeruginosa is resistant to numerous antimicrobials, which is facilitated by different mechanisms that include (Recio *et al.*, 2020) restricting outer membrane permeability, (Kadri *et al.*, 2018) the expression of many efflux systems (sush as : constitutive MexAB-OprM, inducible MexXY-OprM), (Tamma *et al.*, 2021) the production of naturally-occurring antimicrobial-inactivating enzymes such as the hydrolytic β-lactamase enzymes (bla_{AmpC} and $bla_{\text{OXA-50}}$) and the aminoglycoside modifying enzyme (AME) APH(3′)-IIb, and (Horcajada *et al.*, 2019) mutations and enzymes that modify the targets of the antimicrobials (Baylay, Piddock and Webber, 2019; Pang *et al.*, 2019),. *P. aeruginosa* is also capable of developing antimicrobial resistance via horizontal gene transfer and the acquisition of resistance genes. It is important to note that both intrinsic and acquired resistance mechanisms play an important role in the evolution of MDR *P. aeruginosa*. For example, carbapenem resistance mechanisms in *P. aeruginosa* include the overexpression of AmpC enzyme, the acquisition of extended-spectrum β-lactamase (ESBL) and/or carbapenemase encoding genes through horizontal gene transfer, reduction in membrane permeability (like mutations in the outer membrane porin, OprD), overexpression of *mexAB-oprM* efflux pump, and/or modification of penicillin binding proteins (PBPs) (Hirsch *et al.*, 2020) .

2.10 Antibiotics Resistance

Antibiotic as "a chemical produced by a microorganism to kill other microorganisms" and was essential in discovering soil-dwelling filamentous actinomycetales ('actinomycetes') as prolific makers of antimicrobial compounds (Khadayat *et al*., 2020) . Selective toxicity refers to the toxicity of the majority of antibiotics. Inhibitors of cytoplasmic membrane synthesis, inhibitors of protein synthesis, inhibitors of DNA synthesis, inhibitors of RNA synthesis, inhibitors of cell

wall synthesis, and metabolite analogs such as sulfonamide and trimethoprim are divided into five types based on their mode of action (Kapoor *et al*., 2017).

2.10.1 β-Lactam Antibiotics

β-Lactams are the most widely used class of antibacterial agents owing to their broad spectrum and proven safety profiles. Clinical trials in the 1970s and 1980s studied combination therapies with promising results for empirical nonoptimized double β-lactam therapies (Jiao *et al.*, 2019)

The β-lactams are categorized into 4 main subclasses: Penicillin, Cephalosporin, Monobactam, and Carbapenem. Structurally, they consisted of a β-lactam ring, which is consisting of three carbon atoms and one nitrogen atom and is linked to a thiazolidine ring. The β-lactam ring in Penicillins is connected to a five-membered thiazolidine ring and the side chain, R, differentiates the different Penicillins. In Cephalosporins, the β-lactam ring and dihydrothiazine ring are merged, however, in the Carbapenems, the β-lactam ring is joined with a hydroxyethyl side chain, deficiency of oxygen or sulphur atom in the bicyclic nucleus, while Monobactam has no additional ring (Zhang *et al*., 2021).

2.10.2 Mechanism of action of β-Lactams.

β-lactams perform their antibacterial activity by inhibiting bacterial cell wall, peptidoglycan, and synthesis by preventing the precise functioning of the penicillin-binding protein (PBP), also known as transpeptidases. Peptidoglycan is a main structural component of the bacterial cell and the periplasmic part. Apart from rigidity, it protects against high internal osmotic pressure and gives an overall defined shape to a bacterial cell (Walter and Mayer, 2019).

Penicillin binding protiens catalyzes the cross-linking of amino acids in adjacent amino acid chains, which form a network in the periplasmic space between

the inner and outer membranes. Interestingly, β-lactam ring is similar to that of D-Alanine-D-alanine of the N acetylmuramic acid pentapeptide, and thus PBPs "mistakenly" (due to very close shape resemblances) pick these up (β-lactam in fact) to use them as building blocks during cell wall synthesis. The bacterial cell pays for this mistake that leads to acylation of the PBP and thus eventually renders the enzyme (transpeptidases) inactive with inhibition of the transpeptidation reactions resulting in accumulation of cell wall precursor units that trigger activation of the cell wall autocatalytic system, leading to cell lysis (Yao *et al*., 2012). By simultaneously blocking transpeptidases and activating autolysin, β-lactams lead to disruption of the synthesis of the cell wall and initiates its active destruction, ultimately, lysis of the bacterium due to osmotic pressure (Yao *et al.*, 2012).

2.11 Classification of β-lactamases

A, C, and D enzymes, B metallo enzymes that require divalent zinc ions (metal ion) for substrate hydrolysis and use serine for β-lactam hydrolysis according to the first classification (Ambler molecular classification) that is based on conserved motifs, protein sequences and further categorizes β-lactamases (Dehbashi *et al.*,2020).

The second categorization (functional classification) categorizes β-lactamases in accordance with their substrate and inhibitor characteristics. This method links -lactamases with features of clinical isolates. Class A chromosomes in gram-negative bacteria such as *P. aeruginosa* include penicillinase and extended-spectrum β-lactamase. It is comprised of several subtypes of -lactamase *SHV*, *TEM*, and *CTX-M*-based substrate. Oxacillinase of class D (*OXA*) derives from plasmids (Poirel *et al*., 2002).

2.11.1 Serine β-Lactamases

The serine β-lactamases (SBLs) are so-called because, like penicillin-binding proteins, they share nucleophilic serine residues in their active site. Indeed, SBLs are

thought to be evolutionarily derived from PBPs . Based on their sequence identity and substrate profiles, the SBLs are divided into three classes, Ambler classes A, C, and D, corresponding to penicillinases, cephalosporinases, and oxacillinases, respectively (Tooke *et al*., 2019).

2.11.1.1 Class A Serine-β-Lactamases

Class A SBLs is the most widely studied SBLs. Class A enzymes include the Temoneira (the name for a patient) β-lactamase (*TEM*), sulfhydryl reagent variable β-lactamase (*SHV*) enzymes, extended-spectrum SBLs (ESBLs) such as cefotaxime hydrolase from Munich (*CTX-M*) enzymes (Datta and Kontomichalou, 1965; Bauernfeind *et al*., 1990). The class A SBLs exhibit a shared amino acid sequence identity of(40-60)% between class members and a much lower identity with members of other SBLclasses. The class A SBLs are typically inhibited by β-lactam-based SBLs inhibitors such as clavulanic acid, although *KPC* enzymes are an exception, and some resistant *TEM* variants do exist, as well as avibactam (Dehbashi *et al*.,2020).

2.11.1.2 Class C Serine-β-Lactamases

Class C SBLs (*AmpCs*) are usually chromosomally mediated, while plasmidmediated class C enzymes exist and are found primarily in *enterobacteriaceae.* These enzymes can hydrolyze Penicillins but are most active against Cephalosporins and Cephamycins, with some *AmpC* enzymes acting at the diffusion limit during Cephalosporin hydrolysis (Hishinuma *et al*., 2020). Class C enzymes are not usually inhibited by SBLs inhibitors such as clavulanic acid, although some are inhibited by Sulbactam or Tazobactam. hey, are, however, inhibited by Aztreonam because they have a strong affinity for this substrate but a low turnover rate (Dehbashi *et al*., 2020).

Several β-lactamases belonging to this group, including Cephamycins (*CMY),* Ambler class C *(ACC),* and Cefoxitin *(FOX),* have been encoded on the plasmid in

both *Enterobacteriaceae* and non-fermenting organisms, such as *P. aeruginosa*. In *P. aeruginosa*, *AmpC* mutants have been associated with decreased sensitivity to Imipenem, Ceftazidime, and Cefepime. These mutants, including plasmid-coded *CMY-10*, *CMY-19*, and *CMY-37* mutants, are categorized within the Bush-Jacoby functional subgroup 1 (Hishinuma *et al*., 2020).

2.11.1.3 Class D Serine β-Lactamases

Class D β-lactamases, also known as oxacillinases or OXA-type β-lactamases (*OXAs*), are active-serine-site enzymes like Ambler class A and class C β-lactamases, differing from class A and C enzymes in amino acid structure, which can confer resistance to Penicillins, Cephalosporins and, in some cases, Carbapenems. The OXA enzymes may be chromosomal or plasmid-mediated and therefore some OXA variants may be transferred between pathogenic species. OXA enzymes are generally with widely differing sensitivities to inhibitors (Liu *et al.,* 2018) .

2.11.2 Extended Spectrum β-lactamases *P. aeruginosa*

ESBLs are the enzymes responsible for resistance to the majority of -lactam antibiotics (Altayb *et al*., 2021). These enzymes hydrolyze and create resistance to antimicrobial drugs such as Penicillins, Cephalosporins, Monobactams, and Carbapenems. They are generated by some microbes, such as *P. aeruginosa* (Nasser *et al*., 2020). In *P. aeruginosa*, sulfhydryl variable (*SHV*), cefotaxiase (*CTX-M*), and temoneira (*TEM*) types are the most prevalent ESBL genes (Dallenne *et al*., 2010; Lin *et al*., 2012). ESBLs are often not carried on the bacterial chromosome, but rather on a separate DNA fragment known as a plasmid. Plasmids can carry a variety of ESBL genes and have the potential to replicate themselves into other bacteria. This can be really serious for a variety of reasons (Tavajjohi *et al*., 2011).

Infections caused by ESBLs-producing *P. aeruginosa* are becoming increasingly prevalent globally, resulting in high fatality rates, lengthy hospital stays, and growing

medical expenses (Mohajeri *et al*., 2018). The presence of these genes in enteric bacteria increases the potential of these organisms to develop resistance to -lactam antibiotics (Moremi *et al*., 2021).

The *TEM* and *SHV* families of ESBL enzymes are mutations of -lactamases, whereas the *CTX-M* family arose from environmental bacteria. Moreover, multiple variations of bla_{CTX}-M have emerged as a result of point mutations in this gene. More than 450 variations of *CTX-M*, *TEM*, and *SHV* enzymes can be secreted by ESBL-carrying bacteria (Ejaz *et al*., 2021).

2.11.3 Types of ESBLs

ESBLs hydrolyze expanded-spectrum -lactam antibiotics and are inhibited by clavulanate, although their genes are variable and may be classified into multiple groups. TEM and SHV type ESBLs are closely related, with just a few amino acid alterations separating types. CTX-M type ESBLs are genetically heterogeneous. Many ESBL enzymes are derived from the original SHV or TEM β-lactamases and categorized in several groups with different designations Table(2-1)(Bradford, 2001).

The ESBLs phenotype usually evolved as a consequence of point mutations at selected loci. SHV and TEM enzymes are most common in bacterial strains belonging to *Klebsiella pneumoniae* and *Escherichia coli*. As well, they have also been detected inother genera of Enterobacteriaceae like *Providencia* spp. And *Proteus* spp.

Table (2-1) :**Nomenclature origin of the major groups of the Extended-spectrum β-lactamase.**

NO	**Group Designation**	**Designation Origin**
1	TEM	Temoneira, patient name
2	SHV	Sulfhydryl reagent variable
3	CTX- M	Cefotaxime-hydrolyzing β-lactamase from Munich
4	IRT	Inhibitor-Resistant TEM
5	OXA	Active on oxacillin
6	GES	Guiana-extended spectrum
7	VEB	Vietnam Extended Spectrum β-lactamase
8	BEL	Belgium Extended β-Lactamas
9	SFO	In Serratia fonticola
10	OXY	In K. oxytoca
11	TLA	Tlahuicas Indians (Mexican people group)
12	PER	*Pseudomonas* Extended Resistant
13	CME	From Chryseobacterium meningosepticum
14	BES	Brazil Extended Spectrum

2.11.3.1 OXAs family

OXA β-lactamase is a growing group of ESBLs belonging to the functional group 2d and Ambler class D (Bush, Jacoby and Medeiros, 1995) .These enzymes, which display resistance to Cephalothin and Ampicillin, are well known for their specific hydrolysis of Cloxacillin and Oxacillin and poor inhibition by clavulanic acid (Bush and Jacoby, 2010; Bush, Jacoby and Medeiros, 1995) .Generally, OXA enzymes show variability in amino acid sequences and substrate profiles.

Nevertheless, many OXA enzymes have been reported to hydrolyze Cephems, and/or Monobactams as well as Cephalosporins. Therefore, these enzymes are now classified as subgroup 2de (Bush and and Jacoby, 2010) .Whether or not OXA enzymes with expanded-spectrum activity are considered as ESBLs is still questionable (Liu *et al.*, 2018) .Many scientists do not agree with applying the ESBLs designation to oxacillinases because the OXA variants are grouped in the 2de subgroup and not in the 2be, in addition to their resistance to inhibition by clavulanate (Castanheira *et al*.,2021).

2.11.3.2 GES Family

World Health Organization (WHO) has declared that antimicrobial resistance is one of the main threats to global health (World Health Organization (WHO).

Carbapenemase-producing Organisms (CPO) have become the principal reason of carbapenem resistance (Martínez-Martínez and González-López, 2014). KPC-type enzymes are one of the most clinically relevant, and the main class A carbapenemase. Nevertheless, further KPC, exists a wide diversity of "minor class A carbapenemases" including GES-type and its prevalence could be underestimated due to the lack of specific diagnostic tests (Bonnin *et al*., 2020).

GES (Guiana Extended Spectrum) β-lactamase was first described in France in 1998 in a *K. pneumoniae* isolate producing GES-1, an extended-spectrum β-lactamase (ESBL) which confer Penicillin and Cephalosporins resistance, but has no carbapenemase activity (Poirel *et al.*, 2002). GES-genes are horizontally transmissible between genera and species and nowadays, they are reported increasingly in Gram-negative rods, including *Acinetobacter baumannii*, *P. aeruginosa* and enterobacterales (Bonnin *et al.*, 2020).

2.11.3.3 PER family

There are several extended-spectrum β-lactamases have been described that are not part of the well-known groups of β- lactamases Table 2-1.The PER-1 (Pseudomonas Extended Resistant)enzyme was firstly characterized in a *P. aeruginosa* isolate which was resistant to 3rd generation Cephalosporins and inhibited by clavulanate (Nordmann *et al.*, 1993) (Jacoby, 2006) . Furthermore, this enzyme could hydrolyze many Penicillins as well as Cephalosporins including Ceftazidime, Cefoperazone, Cefalotin, Cefuroxime, and Ceftriaxone, but not Oxacillin, Imipenem, and Cephamycins. Soon after, it was also reported among other bacterial strains belonging to *A. baumannii* and *S. enterica Typhimurium*(Vahaboglu *et al.*, 1995) and (Vahaboglu *et al.*, 1996) (Vahaboglu *et al.*, 1997).

This enzyme is most commonly described in Turkey and Mediterranean countries in up to 60% of *A.bauman* (Kolayli *et al.*, 2005) . From isolates that are Ceftazidime-resistant (Vahaboglu *et al.*, 1997) (Ranellou *et al.*, 2012) .Interestingly, the PER-1 enzyme was plasmid-mediatedin several nosocomial strains of *S. enterica Typhimurium*, which might suggest the spread and acquisition of the resistance plasmid in the hospital setting Consequently,PER-2 was characterized in another S. enterica Typhimurium strain from Argentina with 86.4% homology with the originalPER-1 enzyme (Bauernfeind *et al.*, 1996) .

Since after, PER variants have been reported in different species of Enterobacterales as well as *Aeromonas* spp. and *A.baumannii* isolates. PER-1 and PER-2 enzymes are the most common variants of the PER group, they have been characterized by their susceptibility to avibactam in comparison to other class A enzymes (Ortiz de la Rosa *et al.*, 2019)(Ruggiero *et al.*, 2019). Noteworthy, a recent analysis has documented that *A.baumannii* strains expressing PER variants can

show increasedminimum inhibitory concentrations against the siderophore Cephalosporin, Cefiderocol (Kohira *et al.*, 2020)

2.12 DNA Sequencing

The technique of determining the nucleic acid sequence, or the order of nucleotides in DNA, is known as nucleotide sequencing. Any method or technology for determining the order of the four bases, adenine, cytosine, guanine and thymine, is included. RapidDNA sequencing has significantly advanced biological and medical research and discoveries. DNA sequence knowledge is increasingly required for basic biological research as well as a variety of applied applications including medical diagnosis, biotechnology, forensic biology, virology, and biological systematics.

Modem DNA sequencing technology has aided in the sequencing of full DNA sequences, or genomes, of many types and species of life, including the human genome and the genomes of many other animals, plants, and microbial species (EL-Fouly *et al.*, 2015)

The Sanger technique, also known as the dideoxy or chain termination method, is based on the synthesis of DNA chains using didcoxynucleotides that stop DNA amplification at the elongation phase. Elongation is stopped when the polymerase enzyme inserts a nucleotide containing a 3' hydroxyl group into the chain. By separating the PCR products on an acrylamide gel oyaxvrvsis, the dideoxy nucleotide terminated in the chain can be determined (Bekele *et al*., 2011).

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Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1 Laboratory Equipment's and Instruments

The Laboratory equipment and instruments used in the study were listed as shown in Table 3-1.

Table (3-1) Equipment ,Supplies and producers used in this study.

No.	Laboratory Equipment's	Company	Origin
1.	Autoclave	Hirayama	Japan
2.	Benson burner	Membrane	Germany
3.	Centrifuge	Hermle	Germany
4.	Compound Light Microscope	Zeiss	Germany
5.	Digital camera	Sony	Japan
6.	Disposable (Pteri Dish, Syringe and Plane tube)	Afco-Dispo	Jordan
7.	Distiller	Ogawa	Japan
8.	Electric sensitive balance	Kern	USA
9.	Electrical Oven	Memmert	Germany
10.	Electrophoresis system	Fisher Scientific	USA
11.	Eppendorf Centrifuge	Hettich	Germany
12.	Eppendorf rack	HAD	China
13.	Eppendorf tubes	Sigma	U K
14.	Gel Electrophoresis System	Cleaver Scientific	(UK)
15.	Hood	Bio LAB	Korea
16.	Hot plat	Biocote	England
17.	Incubator	Memmert	Germany

18.	Latex Gloves	Broche	Malaysia
19.	Mask	HAD	China
20.	Micropipette	Dragonlab	China
21.	Microtiter plate reader	Memmert	Germany
22.	Microwave	Sanyo electric	Japan
23.	Nanodrop	Implen	Germany
24.	PCR centrifuge	Zip-IQ	USA
25.	PCR Device	Leica	Spain
26.	PCR tube	Eppendorf	Germany
27.	Platinum Wire Loop	Himedia	Indian
28.	Refrigerator	Concord	Germany
29.	Slides and Cover slide	Sail Brand	China
30.	Sterilize Cotton Swab	Afco-Dispo	(Jordan)
31.	Sterilized cotton	Afco-Dispo	(Jordan)
32.	UV light transminator	Cleaver	England
33.	Volumetric flasks	Jlassco	India
34.	Vortex mixer	Gemmy	Taiwan
35.	Water bath	Kottermann	Germany

3.1.2 Biological and Chemical Materials

The biological and chemical materials used in this study are listed in Table 3-2.

Table(3-2): Chemical and biological materials utilized in this study.

No.	Materials	Company	Origin
1.	Acetone	BDH	England
2.	Agarose	Condalab	(Spain)
3.	Catalase reagent	Himedia	(India)
4.	Crystal violet	BDH	England
5.	DNA ladder marker (100-1500) bp	SolGent	Korea
6.	DNA Loading dye	Promega	(USA)
7.	Ethanol (95%)	BHD	(England)
8.	Glucose	Sigma	England
9.	Glycerol	BHD	(England)
10.	Gram stain solution	Fluka	USA
11.	Hydrogen peroxide (H2O2) 30%	BDH	England
12.	Kovac's reagent	HIMEDIA	India
13.	Methyl red	BDH	England
14.	Normal Saline solution	Mehico	(India)
15.	Nuclease Free Water	Bioneer	(Korea)
16.	Oxidase reagent	Himedia	(India)
17.	Peptone water	HIMEDIA	India
18.	Safe Red	Gold View	Korea
19.	Tris-Borate EDTA buffer (TBE)	Promega	USA
20.	Urea Solution	SD-Fine	India

3.1.3 Antimicrobial susceptibility test

The phenotypic detection of extended-spectrum beta-lactamases (ESBL) was performed using the Double-Disk Synergy (DDS) test according to clinical laboratory guidelines (CLSI-2023) (0.5 McFarland tube was used to obtain 1×10^8 CFU/mL bacterial culture). Table(3-3) shows the antimicrobial disks used in this Study.

Table (3-3) : The antibiotic disk used in the study.

Antibiotics	Antimicrobial Class	Abbreviation	µg / disk	Inhibition zone/diameter µm			Company/ origin
				S	I	R	
Aztreonam	Monobactams	ATM	30	≥22	16-21	≤ 15	Biolab /Budapest
Ceftazidime	Cephems	CAZ	30	≥18	15-17	≤14	Bioanalyse (Turkey)
Cefepime		FEP	30	≤18	15-17	≤14	Bioanalyse (Turkey)
Piperacillin tazobactam	β-Lactams combinations	TZP	110	≥21	15-20	≤14	Roseto /Italy
Piperacillin	Penicillins	PIP	100	≥21	15-20	≤14	Liofilchem /Italy
Ticarcillin		TIC	75	≥24	23-16	≤15	Biolab (Budapest)
Gentamicin	Aminoglycosides	CN	10	≥15	13-14	≤12	Bioanalyse (Turkey)
Tobramycin		TOB	30	≥15	13-14	≤12	Liofilchem /Italy
Amikacin		AK	30	≥17	15-16	≤14	Liofilchem /Italy
Ciprofloxacin	Fluoroquinolones	CIP	30	≥25	19-24	≤18	Biolab (Budapest)
Norfloxacin		NOR	10	≥17	13-17	≤12	Liofilchem /Italy
Levofloxacin		LEV	15	≥16	15-21	≤12	Biolab (Budapest)
Imipenem		IPM	10	≥19	16-18	≤15	Bioanalyse (Turkey)

Doripenem	Carbepenem	DOR	10	≥19	16-18	≤15	Liofilchem /Italy
Meropenem		MEM	10	≥19	16-18	≤15	Liofilchem /Italy
Colistin	Polymyxins	CT	40	≥ 4	-	≤ 2	Biolab (Budapest)
Polymyxin B		PB300	300	2≤	-	≥ 4	Condalab-Spain

3.1.4 Screening for extended spectrum β-lactamase

Phenotypic confirmatory disc diffusion test Mueller Hinton agar (MHA) was inoculated with standard inoculum (0.5 McFarland) of the test isolate. It was tested for Ceftazidime (30 µg) and Ceftazidime - clavulanic acid (30 µg/10 µg). An increase in zone diameter of ≥5 mm in the presence of clavulanic acid than Ceftazidime alone was interpreted as ESBL producer Double disc synergy test Mueller Hinton agar was inoculated with the standard (0.5 McFarland) inoculum of the test isolate. Ceftazidime (30 µg) disc was placed on agar 15 mm away from the center of Amoxicillin-clavulanic acid (20 µg/10 µg) disc. Extension of zone of inhibition towards Amoxicillin-clavulanic acid was interpreted as ESBL producer (Sinha *et al*.,.2018)

3.1.5 Diagnostic kits

The commercial kits used in the present study are illustrated in table(3-4).

Table(3-4):Type of Kits used in the study.

No.	Kit	Components of the kit	Company	Origin
1.	Genomic DNA extraction Kit	Solution DS (15 ml) Solution MS(20 ml) Proteinase K(20 mg/ml) Wash BUFFER PS TE Elution Buffer (10 mM Tris–HCL, 1 mM EDTA, PH 8.5)	Favorgen	Taiwan
2.	100bp plusDNA ladder	A ladder consists -double-stranded DNA -Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orangeG, 10 Mm.tris-HCl, pH 7.5 and 50 mM EDTA).	SolGent Bioneer	Korea
3.	Primers	***bla*** $_{OXA-10}$***, bla*** $_{OXA-488}$***, bla*** $_{OXA-145}$ **,** ***bla*** $_{OXA-181}$ **,** PER-1, VEB, PER **,** GES-2 and GES	Macrogen	Korea
4.	*P. aeruginosa* Specific Primer	16Rrna	Macrogen	Korea
5.	PCR master mix		IntronBio	Korea

3.1.6 Master Mix Materials

The Master Mix Materials used in the study were listed in Table (3-5).

Table(3-5): contents of master mix

No.	Materials
1.	DNA polymerase enzyme (Taq)
2.	dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm
3.	$MgCl_2$ (3mM)
4.	Reaction buffer (pH 8.3)

3.1.7 Polymer Chain Reaction (PCR) Mixture

The PCR reaction mixture used in the study were listed in Table (3-6).

Table (3-6): PCR Reaction Mixture

No.	Contents	Volume
1.	Master Mix	12.5µl
2.	Template DNA	3 µl
3.	Forward primer (10 pmol/µl)	1.5 µl
4.	Reverse primer (10 pmol/µl)	1.5 µl
5.	Nuclease free water	4 µl
6.	Stain	2.5 µl
7.	Total volume	25 µl

3.1.8 Real-time PCR

The real -time PCR reaction mixture used in the study were listed in Table (3-7).

Table (3-7): Real-time PCR Reaction Mixture .

No.	Contents	Volume
1.	Master Mix	15µl
2.	Template DNA	2µl
3.	Forward primer (10 pmol/µl)	1.5Ml
4.	Reverse primer (10 pmol/µl)	1.5Ml
5.	Rox	0.4µl
6.	Total volume	20.4 µl

3.1.9 Primers

The commercial Primers used in the present study were illustrated in Table (3-8) .

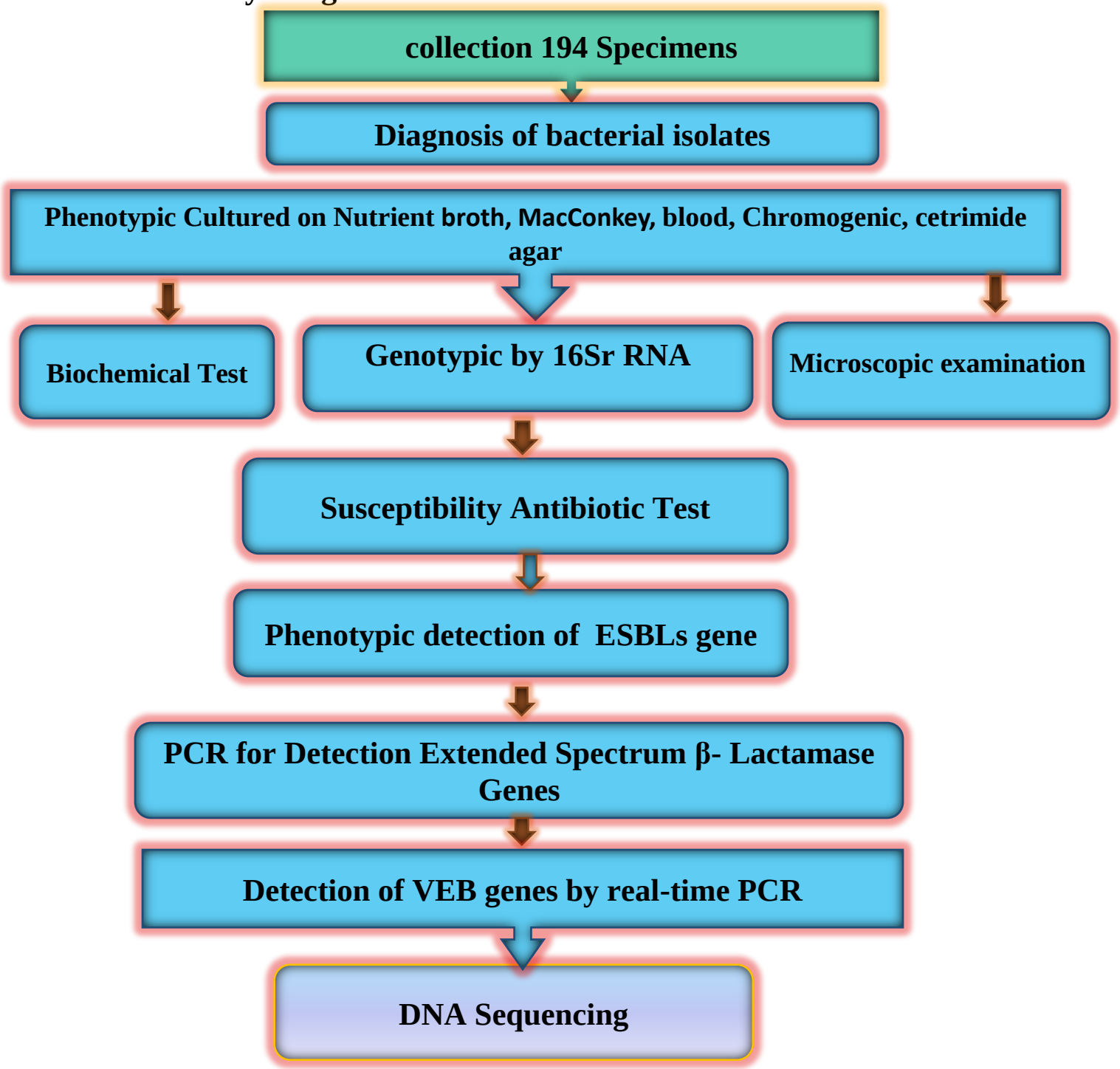
Table (3-8): Commercial Primers used in this study.

Primer		Sequence (5----------›3)	Amplicon size (bp)	Conditions	Cycle No.	Source
				Temp C˚ Tim		
bla $_{OXA-10}$	F	TCATATCGTCGAGTGGTGGG	219	95C˚/5 min 60C˚30 sec / 72C˚/5 min	30	Designed at this study
	R	TGGTCTATTCCGCGTACTCC				
bla $_{OXA-488}$	F	ACTGGTAATGCCGACCAGAC	226	95C˚/ 5min 62C˚/ 30sec 72C˚/5 min	30	
	R	GGTAGTGCGGATCAACCTGT				
bla $_{OXA-145}$	F	AAGCCGTCAATGGTGTTTTC	204	94C˚/5 min 60C˚/30 sec 72C˚/7 min	35	
	R	CCCATTGTTTCATGGCTCTT				
16srRNA	F	TGCCTGGTAGTGGGGGATAA	505	94C˚/5 min 66C˚/30 sec 72C˚/7 min	35	Shaebth,2018
	R	GAGAAGCTAGAGCGCGAAAA				
PER-1	F	ACTGTAGGCGTTGCAGTGTG	198	95C˚/5 min 61C˚/40 sec 72C˚/7 min	35	Designed at this study
	R	CGGAGCCCAGGTATTCTGAT				
VEB	F	GCGGTAATTTAACCAGA	961	95C˚/ 5min 50C˚/ 30sec 72C˚/5 min	30	(Amirkamali *et al*., 2017)
	R	GCCTATGAGCCAGTGTT				
GES-2	F	GAGAAGCTAGAGCGCGAAAA	163	95C˚/ 5min 59C˚/ 30sec 72C˚/5 min	30	Designed at this study
	R	CGGTGCCTGAGTCAATTCTT				

GES	F	ATGCGCTTCATTCACGCAC	846	95C˚/ 5min 63C˚/ 30sec 72C˚/5 min	30	(Amirkamali *et al*., 2017)
	R	CTATTTGTCCGTGCTCAGG				
bla $_{OXA-181}$	F	CAGAAGCAGAAGGAGGTGGA	205	94C˚/ 5min 63C˚/ 30sec 72C˚/7 min	30	Designed at this study
	R	GTGGGGTTGTTTGGCATGAT				
PER	F	AGTCAGCGGCTTAGATA	978	95C˚/ 5min 63C˚/ 30sec 72C˚/5 min	30	(Amirkamali *et al*., 2017)
	R	CGTATGAAAAGGACAATC				

3.2.Methods:

3.2.1. Study design:



Figure(3-1): Study design .

3.2.2 Laboratory Preparation of Culture Media

All media were prepared according to the instructions of the manufacturing company. Sterilization of culture media and solutions were achieved by autoclave at 121C° and pressure (15 pound/inch) for 15 minutes. (Brown and Smith, 2017). After sterilization urea agar base was supplemented with 20%sterile urea solution and blood agar base was supplemented with 5% fresh human blood, then media poured on petri dish or plane tubes, and incubated at 37 C° for 24 hours to ensure their sterility. Sterile media were Stored in the refrigerator to prevent dehydration (Cappuccino and Welsh, 2018). PH was adjusted to 7.0 and the media sterilized by autoclave (Brown and Smith, 2017) (Table 3-9).

Table(3-9)Culture media used in the diagnosis of bacteria with the purposes.

no	Media name	The purpose	Company
1.	MacConkey agar	is a selective and differential media. It is used in the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria (Niederstebruch *et al.*, 2017).	HIMEDIA / India
2.	Nutrient broth	this medium was used in a general experiment such as cultivation and activation of bacterial isolates when it necessary (MacFaddin, 2000).	Rashmi / India
3.	Blood agar	Is an enriched, bacterial growth medium, isolation, identification and determine the type of hemolysis (Niederstebruch *et al*., 2017).	HIMEDIA / India
4.	Cetrimide agar	This medium was used as a selective medium for the isolation of *P. aeruginosa* (Aryal, 2015).	Rashmi / India
5.	Brain heart infusion Broth	This medium used to preserve the bacterial isolated as standard for a long time with 15% glycerol (Forbes *et al*., 2007).	Rashmi / India
6.	MüllerHinton agar	This medium used in the antibiotic sensitivity test (MacFaddin, 2000).	Rashmi / India

7.	Simmonscitrate	It was used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source (Forbes *et al*., 2007).	Rashmi / India
8.	Tryptic Soy broth	it was used for activation of bacteria and for general experiments (MacFaddin, 2000)	HIMEDIA / India
9.	Urea agar	It was used to test the ability of bacteria to produce urease enzyme (MacFadden, 2000).	HIMEDIA / India
10.	Kligler,s iron agar	It was used to determine the ability of bacteria to utilize carbohydrates supplemented with phenol red as the indicator (Forbes *et al*., 2007).	HIMEDIA / India
11.	Voges Proskauer	It was used to detection of specific breakdown products of carbohydrate metabolism by bacteria (MacFaddin, 2000).	Rashmi / India
12.	Chromogenic agar	Solid selective medium for identification of *Pseudomonas aeruginosa*. (MacFaddin, 2000).	Tulip / India

3.2.3 Preparation of Reagents and Solutions

3.2.3.1 Oxidase reagent

It is made by dissolving one gram of tetra methyl-P-phenyl diamine dihydrochloride in 100 ml of distilled water then adding another 100 ml of distilled water. D.W. investigated the capacity of bacteria to produce the oxidase enzyme. (Cappuccino and Welsh , 2018)

3.2.3.2. Catalase reagent

To study bacteria's ability to create the enzyme catalyzes analyzed for hydrogen peroxide, hydrogen peroxide was produced at a concentration of 3%. (Cappuccino and Welsh, 2018).

3.2.3.3. Vogas-Proskauer reagent

This substance consisted of two solutions: α-naphthol solution made by dissolving 5 gm of -naphthol in 100 ml of (95) % ethanol, storing the solution in a dark bottle, and mixing it prior to use. 40 percent Potassium hydroxide solution made by dissolving 40 grams of KOH in 100 milliliters of deionized water and mixing the solution prior to use (MacFaddin, 2000).

3.2.3.4. Methyl red indicator

This solution was prepared by dissolving 0.2 gm of methyl red in 300 ml of (95)% ethanol, and then the volume was completed to 500 ml by D.W. (MacFadden, 2000).

3.2.3.5. Kovacs reagent

Ten grams of dimethyl-amino benzaldehyde were dissolved in 150 milliliters of isoamyl alcohol by heating in a water bath at 50 degrees Celsius, followed by the addition of 50 milliliters of concentrated HCL. Small quantities of the reagent were made and stored in the refrigerator MacFadden, (2000).

3.2.3.6. Gram stains solutions

1- Primary stain: 2 gm Crystal violet, 20ml 95% ethyl alcohol, 0.8gm ammonium oxalate and100 ml distilled water.

2- Stain fixative agent: 2 gm potassium iodide, 1gm iodine crystals and 100 ml distilled water.

3- Decolorize: 70% ethyl alcohol+30% acetone.

4- Counter stain: 4.0 gm safranin, 200 ml 95% ethanol and 800 ml distilled water. (Jawetz *et al*., 2019).

3.2.3.7 Turbidity standard (McFarland)

The turbidity standard (0.5 McFarland solution) was prepared in accordance with Baron and Feingold (1990). In a graduated cylinder, 0.5 ml of 1.175% (w/v) barium chloride dehydrate ($BaC_{12}.H_2O$) dissolved by D.W was added to 99.5 ml of 1% sulfuric acid; then, 10 ml of the mixture was transferred to a sterile test tube and stored in a dark place at room temperature. At a wavelength of 600 nm, a spectrophotometer measured the absorbance. 0.08 - 0.13 nm is the allowable absorbance range for the standard. Before performing an antibiotic susceptibility test on 36 isolates, this solution was used to set the number of bacterial cells.

3.2.3.8 Safe red Stain

Red Safe Nucleic Acid Staining Solution is a new and safe alternative to GoldView (Biosharp) for DNA and RNA identification on agarose gels. Red Safe is as sensitive as GoldView , and the staining procedure is virtually comparable; however, compared to GoldView, which is known to be a powerful mutagen, Red Safe produces much fewer mutations in the Ames test. Importantly, it is non-hazardous, can be disposed of using standard laboratory procedures, and has a long shelf life (Machida and Knowlton, 2012).

3.2.3.9. Preparation Tris-Borate-EDTA (TBE buffer):

A buffer of 1X TBE was prepared from of 10X TBE buffer. To give the final concentration, 1X TBE added 100ml of 10X TBE to 900ml of sterile distal water. This solution was used to dissolving agarose and in electrophoresis (Heintz and Gong, 2019).

3.2.3.10 Agarose gel

According to Green and Sambrook (2012), the agarose gel was made by dissolving 1 gram of agarose in 100 milliliters of 1X TBE buffer (10ml completed

with 90ml distal water). The solution was heated to boiling (using a microwave) until all the gel particles dissolved, the solution was cooled to 50-60C°, and 5ml of melting agarose gel was combined with 5ml of simply safe to achieve a final concentration of 0.5g/ml.

3.2.4 Collection of Specimens

In this study, 194 clinical specimens (including burns ,urine, diabetic foot, gun fire bombs, and wound) were collected were of both gender and various age groups from July to October /2022.These specimens were collected from several hospitals in the Hillah city hospital (Al-Hillah Teaching Hospital, Mirgian Teaching Hospital and Imam AL-Sadiq Teaching) and (Burns Specialized, AL-Shaheed Ghazi Hariri, Baghdad Teaching, Ibn AL Bitar Hospital and National Center for Educational Laboratories) in Baghdad and (Kirkuk General Hospital, Azadi Teaching Hospital) in Kirkuk .

3.2.5 Bacterial diagnosis

3.2.5.1 Culturing

All specimens were cultured on different media for identification of *Pseudomonas* such as blood agar, MacConkey agar, chromogenic agar and cetrimid agar, using sterile loop spread on the surface of agar media and incubated at 37 C° for 24 hr. Purified colonies kept in nutrient broth containing glycerol 15% at -20 C° in (Jawetz *et al*., 2019). After final diagnosis of specimens, 36/194 (18.5)% isolates of *P. aeruginosa* (named PsA1 to PsA36) were obtained were isolated from 194 clinical specimens of burns ,urine, diabetic foot, gun fire bombs, and wound of cites of Iraq which included, Babylon, Baghdad and Kirkuk.

3.2.5.2. Microscopic examination

After the growth of bacteria on MacConkey agar, blood agar, nutrient agar, chromogenic agar and cetrimid agar, their shape, size, texture, and colony arrangement was observed. A single colony was picked up, stained with Gram stain, and examined under the light microscope (100x) using oil emersion (Jawetz *et al*., 2019).

3.2.5.3. Biochemical tests

1- Catalase test

Few drops of catalase reagent were added on slide with single colony of *P. aeruginosa* by using sterile loop. A positive result indicated the formation of bubbles. This test was used to detect the ability of bacteria to produce the catalase enzyme, which broke down the H_2O_2 into oxygen and water (Brown and Smith, 2017; Cappuccino and Welsh, 2018).

2- Oxidase test

The oxidase reagent was added in few drops on filter paper and mixed with single colony of *P. aeruginosa* using sterile wooden stick. A positive reaction was indicated by the development of purple color within 10 second. This test was used to detect the ability of bacteria to produce the oxidase enzyme (Brown and smith, 2017; Cappuccino and Welsh, 2018).

3- IMVC test

As mentioned by MacFaddin (2000), this test was done in the following way.

A- Indole test

Peptone water medium was inoculated with overnight tested bacterial culture and incubated at 37 Cº for 24 hr. After that 10 drops of Kovac's reagent were added directly to the culture tube; the appearance of the red ring at the top of the

broth after gentle shaking indicates a positive result. This test is used to detect the *P. aeruginosa* capacity to produce a tryptophanase enzyme which hydrolyzed tryptophan to indole, pyruvic acid, and ammonia. (Cappuccino and Welsh, 2018).

B- Methyl red test

Methyl red-Voges proskauer medium was inoculated with bacterial culture that was tested and incubated at 37 Cº for 24 hours. Then five drops of the methyl red were added. A positive test changed of medium color from yellow to red. This test was used to detect the bacterial ability to ferment glucose and produce acid as a final product. (Cappuccino and Welsh, 2018).

C- Voges-Proskauer test

Methyl red-Voges proskauer medium was inoculated with bacterial culture that was tested and incubated at 37 Cº for 24 hours, then few drops of α-naphthol, and KOH were added. A positive reaction was indicated by development of a pink color with15 minutes**.** This test was used to detect the bacterial ability to ferment glucose and produce acetoin (Cappuccino and Welsh, 2018)

D- Citrate utilization test

Simmon's citrate slant agar was inoculated with tested bacterial culture by sterile loop and incubated at 37 Cº for 24 hours, a positive result was indicated by changing the color of the medium from green to blue. This test was used to detect the bacterial ability to utilize sodium citrate as carbon source(Cappuccino and Welsh, 2018).

4-Urease test

Urea agar slant was inoculated with tested bacterial culture by sterile loop, and then incubated at 37 Cº for 24 hours; existence of pink color indicates a positive result. This test was used to detect the bacterial capacity to produce

urease enzyme which hydrolyzes urea to ammonia and carbon dioxide (Cappuccino and Welsh, 2018).

5- Motility test

Semisolid mannitol media were stabbed in the center with an inoculated needle and incubated at 37 C° for (24 hours). Spread out growth from the line of inoculation indicates the existence of motile bacteria (MacFaddin, 2000; (Brown and Smith, 2017).

6- Kliglers iron agar test

The cultured isolates were streaked on surface of slope and stabbed into butt, and then incubated for 37C° for (24) hours. The positive result of *P. aeruginosa* was alkaline / no change or alkaline / alkaline with no produce H_2S and gas (Brown and Smith, 2017).

7-Hemolysin production

Hemolysis production was carried out by inoculating the blood agar medium with bacterial isolates at 37C° for (24-48) hrs. An appearance of a clear zone around the colonies referred to complete hemolysis (β-hemolysis) or greenish zone around the colonies referred to partial hemolysis (α-hemolysis), while the no changing, the colonies referred to non-hemolytic (γ-hemolytic) MacFaddin, (2000).

3.2.6 Antibiotic susceptibility test

One of the most common methods used routinely in diagnostic laboratories is based on inoculating the bacteria under test on a solid culture medium (Muller Hinton agar) in a Petri dish. After cultivate of the bacterial isolate using brain heart infusion broth at 37°C for (24) hours, and by adding sterile normal saline

compared with (0.5) a standard McFarland tube (1.5 x 10^8 CFU/ml), then spread on Muller Hinton agar (MHA) using a sterile cotton swab and leave it to dry, different antibiotic tablets were used in different concentrations such as Ticarcillin (75µg), Pipracillin (100µg), Peracillin-Tazobactam (110µg), Ceftazidime (30µg), Cefepime (30µg), Aztreonam (30 µg), Levofloxacin(15µg), Norfloxacin (10µg), Ciprofloxacin (30µg), Imipenem (10µg), Meropenem (10µg), Doripenem(10µg) Tobramycin (30µg), Gentamicin (10µg), Amikacin (30µg), Colstin (40µg), and Polymyxin B(300 µg) . *P. aeruginosa* were considered resistance or sensitive on the basis of zone of inhibition following the criteria of clinical and laboratory standard institute CLSI,2023 . With sterile forceps, the selected antimicrobial disks were placed on the surface of the inoculated medium and incubated at 37°C for 24 h, during the incubation period the antibiotic spread from the disc to the medium. If the organism is sensitive to antibiotics, zones of lack of growth appear around the disc, and the higher the sensitivity, the larger the diameter of the area of inhibition(Grewal *et al.*, 2017). Antibiotic inhibition zones were noted and measured with a ruler or caliper, the antibiotics names and its standard inhibition diameter were used according to the Clinical and Laboratory Standards Institute (CLSI, 2023) for sensitivity or resistance of the organism to each antibiotic .

3.2.7 Maintenance of bacterial Isolates

Maintenance of bacterial isolates was performed as follows:

A- Short- term storage

The separated Bacteria isolate (36 isolate)were preserved by streaking them on Brain Heart Infusion Agar medium, then incubating them at 37° C for 24 hours to get a lot of growth, and finally storing them at 4° C for a few weeks (Jain *et al.,* 2020).

B- Long- term storage

The bacteria can be stored in a screw-capped tube at -20 °C for a long time in Brain Heart Infusion Broth with 15% glycerol (Jain *et al.,* 2020).

3.2.8 Molecular detection methods

3.2.8.1 DNA Extraction

The DNA extraction, was done according to manufacturing origin company protocol (Favorgen, Taiwan).

The Protocol

1. Activation before starting DNA extraction: The culture was inoculated in 10 ml of nutrient agar medium and incubated at 37 C° for overnight.
2. Cell harvesting: *P. aeruginosa* culture was transferred to 1.5 ml micro centrifuge tubes containing 1 ml Normal Saline.
3. Then centrifuged for 1 min. at 14.000 rpm and the supernatant was then discarded.
4. 200 µl of cell lysis FATG was added to the specimen and mixed by the vortex for 5 min.
5. 200 µl of FABG was added to the sample and mixed by the vortex for 5 sec.
6. Incubated at 70 C° for 10 minutes or until the specimen lysate is clear,
7. during incubation, the tubes were inverted 3 times for every 3 minutes.

Heating the elution buffer in the incubator at 70 C° to be completely absorbed.

8. A 200 µl of absolute ethanol was added to the mixture and mixed immediately by vortex for 10 sec.
9. The mixture was transfer to the GD column and centrifuged at 18.000 rpm for 1 min.
10. W1 buffer (400 µl) was added to the GD column and centrifuged at 13.000 rpm for 30 seconds, the flow-through was discarded.

11. Wash buffer (600 µl) was added to the GD column, it was centrifuged at 13.000 rpm for 30 seconds then was discarded the flow-through.
12. Centrifuge at 14.000 rpm for 3 min to dry the column matrix.
13. Place the column in a clean 1.5 ml micro centrifuge tube and add 100 µl of elution buffer directly onto the filter membrane.
14. Incubate at 37 C° for 10 min, and then centrifuge at 14.000 rpm for 1min.
15. Finally, storing the DNA fragment at 4 C° or -20 C°.

3.2.8.2 The protocol PCR

1- Conventional PCR technique was used for amplifying *P. aeruginosa* specific gene 16S rRNA. The mixture reaction was performed in a total volume 12.5 µl of PCR Pre Mix (Bioneer, South Korea) consisting of 1.5 µl from each primer forward and reverse, 3 µl of DNA, stain 2.5 µl and, the volume completed up to 4 µl with free nucleases deionized water according to the instructions of the company and reaction buffer mixed, as in Table(3-6).

2- Detection of ***bla*** $_{OXA-10}$, ***bla*** $_{OXA-488}$, ***bla*** $_{OXA-145}$, ***bla***$_{OXA-181}$, PER-1, VEB, PER , GES-2 and GES, genes were carried out by using a 12.5 µl master mix of Gold conventional PCR (Bioneer, South Korea) including 2.5 µl stain , 3 µl DNA, 1.5 µl from each primer forward and reverse, and the volume was completed up to 4 µl with free nucleases deionized water according to the instructions of the company and reaction buffer mixed.

3.2.8.3 The program PCR thermal controller

PCR cycling thermal program parameters used in this reaction for detection of *P. aeruginosa* specific, ***bla*** $_{OXA-10}$, ***bla*** $_{OXA-488}$, ***bla*** $_{OXA-145}$, ***bla***$_{OXA-181}$, PER-1, VEB, PER , GES-2 and GES genes were shown in Table (3-10)**.**

Table (3-10): PCR thermal cycling program for *P. aeruginosa* genes.

Gene	thermal cycling condations										
	Initial Denaturation		Denaturation		Annealing		Extension		Final Extension		No of cycle
	Tem C°	Time min	Tem C°	Time sec	Tem C°	Time sec	Tem C°	Time sec	Tem C°	Time min	
bla OXA-10	95	30	95	5	60	30	72	30	72	5	30
bla OXA-145	94	30	94	5	60	30	72	30	72	7	35
bla OXA-488	95	30	95	5	62	30	72	30	72	5	30
bla OXA-181	94	30	94	5	63	30	72	30	72	7	30
PER-1	95	30	95	5	61	40	72	30	72	7	35
16srRNA	94	30	94	5	66	30	72	30	72	7	35
VEB	95	30	95	5	50	30	72	30	72	5	30
PER	95	30	95	5	63	30	72	30	72	5	30
Ges-2	95	30	95	5	59	30	72	30	72	5	30
GES	95	30	95	5	63	30	72	30	72	5	30

3.2.8.4 Agarose Gel Electrophoresis

Agarose gel was created by dissolving 1.5 gram of agarose powder in 100 milliliters of 1X TBE buffer. A melting agarose gel was created by mixing 10ml of TBE buffer with 90ml of destilled water. This gel was then melted in a microwave until the solution turned transparent. The amount of agarose that can be dissolved depends on the intended application of the agarose gel. 0.7 percent of agarose gel is utilized for DNA visualization following extraction, while 1.5 percent to 2 percent agarose sheet is used for PCR product visualization (amplicon). The stock solution concentration of simply safe (replacement for

GoldView) was 10 mg/ml. To get a final concentration of 0.5 mg/ml, only 5µl of simply safe stock solution was added to 100ml of agarose gel that was melting (Green and Sambrook, 2012). The agarose was poured into the gel tray with the ends capped, the comb was appropriately positioned, and then it was left to dry. 10 µl of the amplified DNA (the result of the PCR process) is loaded into a second well of the gel, while 5µl of the DNA Ladder marker is loaded into the first well. The electrodes were properly attached, and the run was performed in accordance with the gel percentage and gel size. (The time required for agarose gel electrophoresis is 60 minutes for genomic DNA and one hour for PCR product.

3.2.9 DNA Sequencing analysis

To study the genetic variation of (***bla*** $_{OXA-10}$ **,** ***bla*** $_{OXA-145}$, and PER-1) genes for 15 *P. aeruginosa* isolates, DNA sequencing technique was performed. The PCR products were sent to Macrongen company in Korea in ice bag by DHL. All ABI file opend by BioEdit Sequence Alignment Editor (Hall, 1999). the incorrect sequences were trimmed and the correct sequences were submitted for NCBI BLASTN for similarity. The homology sequence identity and the mutation analysis were conducted using NCBI BLAST analysis. This genes of *P. aeruginosa* isolates of the current study were registered in NCBI-Gen Bank data base with accession numbers. The DNA Sequencing PCR reaction mixture used in the study were listed in Table (3-11).

Table(3-11): DNA Sequencing PCR Reaction Mixture

No.	Contents	Volume
1	Master Mix	25µl
2	Template DNA	5µl
3	Forward primer (10 pmol/µl)	3 µl
4	Reverse primer (10 pmol/µl)	3 µl
5	Nuclease free water	9 µl
6	Stain	5µl
7	Total volume	50 µl

3.3 Ethical approval

1-The study was done and the cases were collected after getting the agreement of the patients (verbal consent).

2- Approval of Babylon Science College Ethical committee.

3- Before starting the study, permission were taken from Babylon health presidency.

Chapter Four

Results and Discussion

Results and Discussion

4.1 Collection of Specimens

The current study included a collection of (194) samples swabs from patients suffering from wounds, diabetic foot, urine ,pulmonary fluid ,gun fire bomb and burns were collected. These were patients hospitalized in Al-Hillah city hospitals , Kirkuk hospitals and also Medical City hospitals in Baghdad in both genders 115 (59.2)% males and 79 (40.8)% females Figure (4-1), with various ages ranging from (4-72) years Table(4-1). The specimens were collected during the period from July / 2022 to October / 2022.

The isolates were identified depending on traditional methods (morphological features of the colonies and the cells and biochemical tests) After that, the confirmation of these isolates was achieved via PCR using 16SrRNA of a *P. aeruginosa* unique gene . The results showed these isolates were as followes : 36/194 (18.5%) isolates of *P. aeruginosa* 111/194 (57.2)% of specimens were observed to have other bacterial growth and 47/194 (24.3)% no bacterial growth (un known), as shown in Figure (4-2) . Thirty-six isolates of *P. aeruginosa*, (symboled PsA1 to PsA36) were isolated from 194 clinical specimens out of these isolates, 96 (49.4)% were isolated from wounds, 73 (37.6)% were isolated from burns, 13 (6.7)% were isolated from diabetics foot 5 (2.5)% were isolated from urine, 5 (2.5) % were isolated from gun fire bomb and 2 (1.3) % were isolated from pulmonary fluid from different cites of Iraq which included Baghdad, Babylon and Kirkuk in Figure (4-3).

Table(4-1): Distribution of the specimens according to gender and Age group (Year) .

Age group (Year)	Male NO. (%)	Female NO. (%)	Total
4-15	21 (10.82)	22(11.34)	43 (22.2)
16-25	24(12.37)	17(8.76)	41 (21.2)
26-35	29(14.94)	7(3.60)	36(18.5)
36-45	18(9.27)	10(5.15)	28 (14.4)
46-55	10(5.15)	10(5.15)	20(10.3)
56-65	11(5.67)	10(5.15)	21(10.8)
66>	2(1.03)	3(1.54)	5 (2.6)
Total	115(59.2)	79 (40.8)	194 (100)

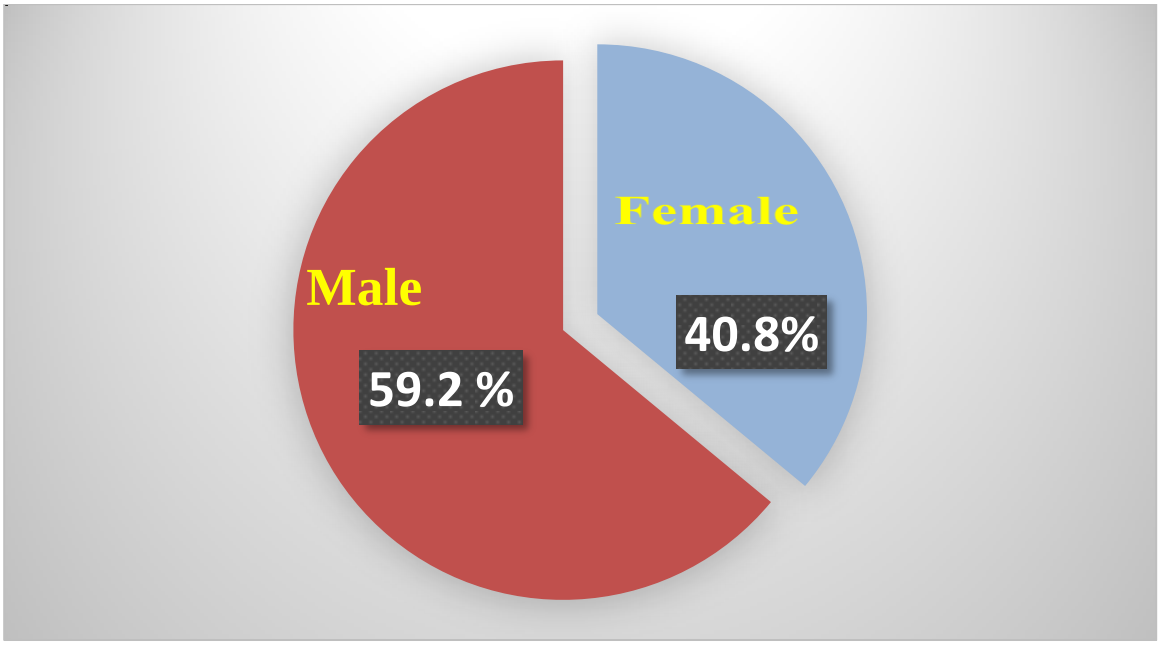


Figure (4-1): Distribution of the specimens according to gender

The results of the percentage distribution of the incidence according to the gender of the patients agree with the findings of Shehab and Jassim (2019). In

contrast, (Shahraki *et al.* 2018) observed a percentage of 43 % males to 57 % females. The possible reasons may be due to the types of populations studied, different geographic locations, types of hospitals. Furthermore, males may have routine outdoor work and are often at risk of infection from infected environments (Manandhar *et al.*, 2017).

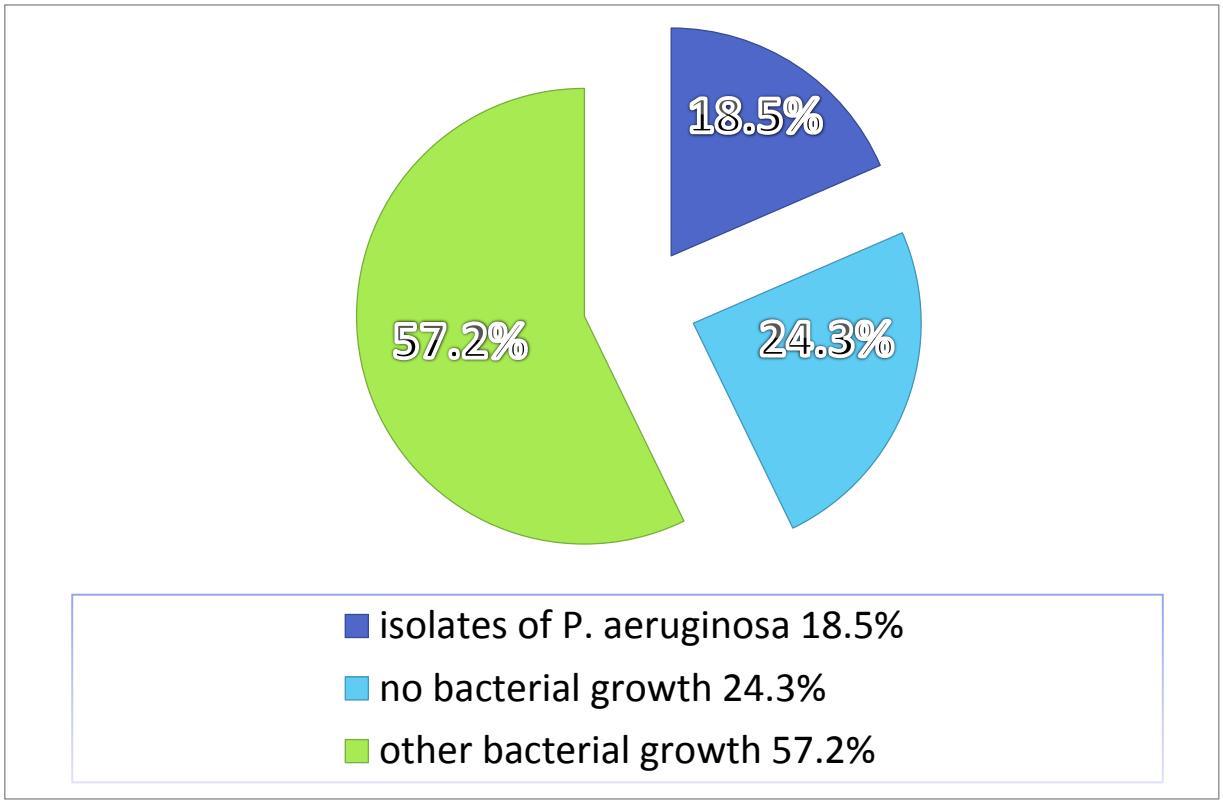


Figure (4-2): Percentage frequencies of all specimens positive and negative for bacterial infection.

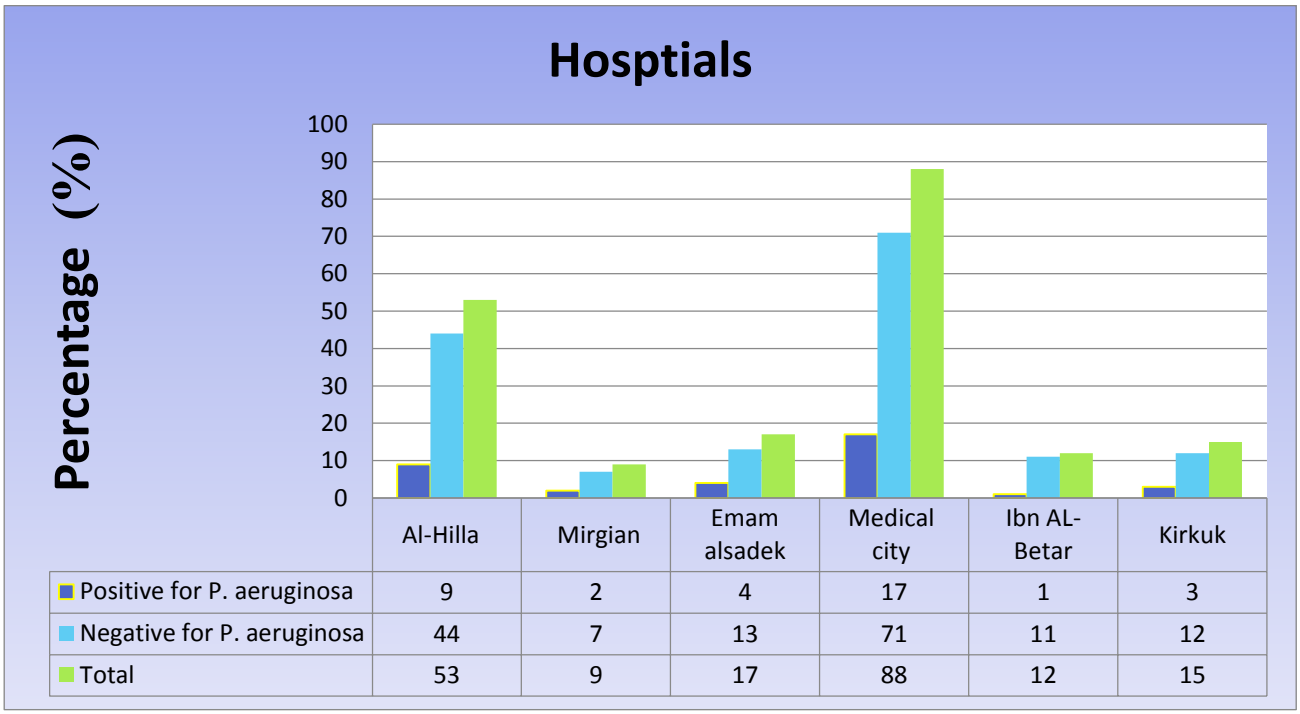


Figure (4-3): Distribution of growth of *P. aeruginosa* isolates according to hospitals.

The highest rate of bacterial infection was within the age group of 26-35 year **(**5.6)% , followed by the age group of 4-15 year **(**4.6)% , followed by 16-25 year (3.6) %, followed by 36-45 year (2.6)% followed by 56-65 year (1.1)% and 46-55 and 66> year were both (0,5)%, as shown in Table (4-2).

Table(4-2): Distribution of patients percentage according to age groups .

Age group (Year)	Patient		Total
	Positive for *P.aeruginosa*	Negative for *P.aeruginosa*	
4-15	9 (4.6) %	34 (21.5)%	43 (22.2) %
16-25	7 (3.6)%	34 (21.5)%	41 (21.2)%
26-35	11 (5.6)%	25 (15.8)%	36(18.5)%
36-45	5 (2.6)%	23(14.5)%	28 (14.4)%
46-55	1 (0.5)%	19(12.1)%	20(10.3)%
56-65	2(1.1)%	19 (12.1)%	21(10.8)%
66>	1(0.5)%	4 (2.5)%	5 (2.6)%
Total	36 (18.5)%	158 (81.5)%	194 (100)%

With age, pathological disorders and procedures such as diabetic foot or cesarean section for women arise. This may be related to the fact that these age groups are very mobile in terms of working indoors or outside the home (such as in bakeries, ovens, and vehicle accidents). Recent studies in Iraq (Jawad, 2016; Oumeri and Yassin, 2021) found that most of the wound infections were occurred in individuals of 5-25 and 31-40 years, respectively

4.2 Diagnosis of bacterial isolates

The diagnosis of these isolates was accomplished through the use of Phenotypic (culture on media) , microscopic examination, biochemical test and Genotypic using (16Sr RNA). A total of 36 isolates of *P. aeruginosa* were isolated from 194 clinical specimens . Out of these, 17 isolates were isolated from injuries, 12 isolates were isolated from burns, 3 isolates were isolated from each (diabetic foot and gun fire, bomb), and 1 isolates were isolated from pulmonary fluid the details of distribution and percentages of the isolates were summarized in Table (4-3).

Table (4-3): Distribution of isolated bacteria with their percentages in the collected specimens .

Specimen type	Positive for *P.aeruginosa* isolate		Negative for *P.aeruginosa* isolate		Total	
	No.	%	No.	%	No.	%
Wound	17	17.7	79	82.3	96	100
diabetic foot	3	23	10	77	13	100
Burns	12	16.4	61	83.6	73	100
urine	0	0	5	100	5	100
gun fire, bomb	3	60	2	40	5	100
pulmonary fluid	1	50	1	50	2	100
Total	36	18.5	158	81.5	194	100

Pseudomonas aeruginosa is an opportunistic bacterium that has the ability to rapidly grow in diverse environmental niches, from different soils to human respiratory tract. It is involved in severe human diseases like meningitis, septicemia or cystic fibrosis and is also a major cause of nosocomial infections due to its high capacity to develop resistances (Issa *et al* ., 2018). The pathogen is also frequently associated with nosocomial infections such as bloodstream, respiratory and urinary tract infections and can cause in patients both acute and chronic infections, characterized by different lifestyles (Auguste *et al*., 2019).

4.2.1 Phenotypic by culture *P. aeruginosa* on different media.

For the purpose of isolation *P. aeruginosa* was inoculated on different cultural media (nutrient agar, MacConky agar ,blood agar, pseudomonas chromogenic agar, and cetrimide agar) . The culture plates were incubated at 37 C° for (24-48) hours .The results as shown in Table (4-4) and Figure (4-4).

Table (4-4): Culture *P. aeruginosa* on different media.

NO	Media type	Appearance
1	MacConkey agar	large smooth elevated colonies and pale because they are non lactose fermenter with irregular edge
2	blood agar	the colonies were black and hemolysis blood
3	cetrimide agar	mucoid, smooth in shape with flat edges and elevated center, creamy or green color colonies, and had a fruity odor
4	Chromogenic agar	greenish blue color
5	nutrient agar	greenish-blue color with greenish colonies, and odor (grape-like odor),

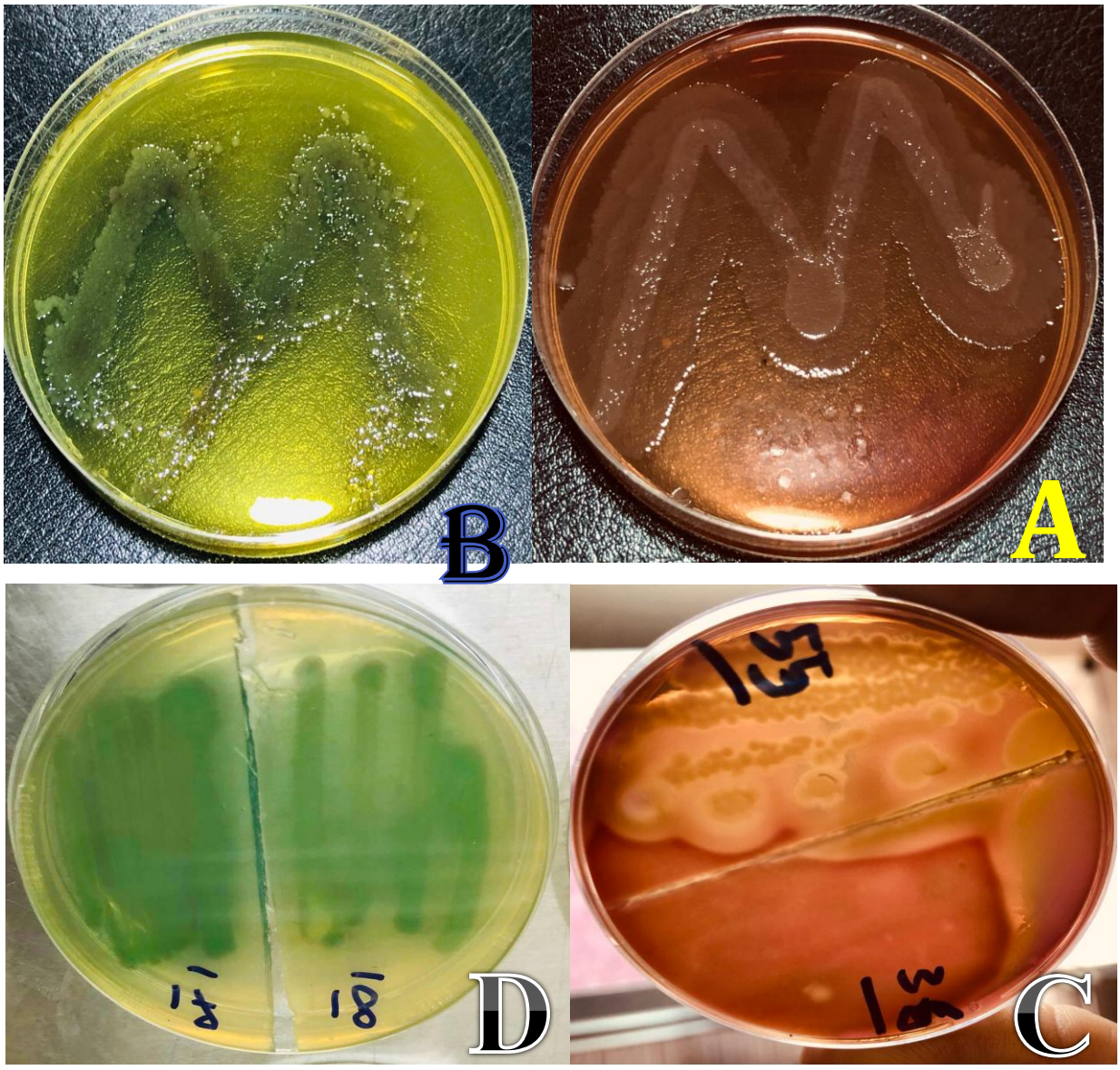


Figure (4-4) : Morphological properties of *P.aeruginosa* colonies on A: MaCconky agar, B: pseudomonase chromogenic agar ,C :Blood agar, D : cetrimide agar grown at 37 C° for 24 hours .

On blood agar, the colonies were black and most had a translucent halo, indicating they could hemolysis red blood cells (Jawetz, *et al*. 2019), When growth on the nutrient agar medium, the bacteria gave a greenish-blue color as an indication of their secretion of the blue pyocyanin pigment. The bacterial cell were appeared under the microscope as a rod shape , gram negative and motile

with single or double arrangement. Also on nutrient agar, *P. aeruginosa* colonies were recognized based on pigments and odor generation (grape-like odor), with greenish colonies. While the colonies of *P. aeruginosa* appeared on chromogenic agar in greenish blue color (DeBritto *et al*., 2020) .

On cetrimide agar *P. aeruginosa* colonies appeared mucoid, smooth, and greenish yellow because most of them produce pyocyanin, a greenish-blue dye, and pyoverdine, a greenish-yellow pigment that shines under ultraviolet (UV) rays . These dyes are soluble in water (Mitra *et al*., 2022).

This bacteria is particularly pigmented on Cetrimide agar (selective medium), so it acts as a detergent that inhibits most other bacteria and reduces the production of two types of pigments, pyocyanin and pyoverdine . It consists of peptone, $MgCl_2$, K_2SO_4, cetrimide, agar, and the rehydrated contents of one vial of nalidixic acid selective supplement (FD130) This finding is similar to research in Iraq (AL-Rubaye *et al*.,2015)

4.2.2 Diagnosis of *P. aeruginosa* using biochemical test

The biochemical tests that needed to diagnose bacterial isolates including (catalase , oxidase , urease , the hemolysis blood production , motility , IMVC) test and the gas production . All *P. aeruginosa* isolates have shown a gram-negative bacilli, and it gave positive result in biochemical tests for catalase tests, oxidase tests, and pigment (blue water-soluble pigment pyocyanin, yellow-green pyoverdine) . IMVC tests have yielded negative results, although isolates have shown positive results for citrate testing .Hemolysis on blood agar was observed in (36) isolate . In Kligler iron agar have given alkaline slant and change the bottom, H_2S negative due to the fact that they are strictly aerobic and negative to Gram's stain as mentioned by Behbahani *et al*. (2019). as shown in Table (4-3).

Table (4-5): Biochemical tests for diagnosis of *P. aeruginosa* isolates .

Test	Results
Gram-stain	G-ve rods
Oxidase test	+
Catalase test	+
Growth at 42°c	+
H_2S production	-
Hemolysis	+
Kligler's iron agar	K/A
Indole test	-
Methyl-red	-
Voges-Proskauer	-
Simmon's citrate	+
Pigments production	+
Motility	+
Urease	+

Abbreviations: (+)= positive test , (-)= Negative test , K = Alkaline , A= Acidic

4.2.3 Genotypic identification by 16SrRNA

Detection of *P. aeruginosa* isolates were confirmed by PCR species-specific primer *P. aeruginosa* specific gene 16S rRNA the result revealed that all isolates were 36/36 (100)% *P. aeruginosa* Figure (4-5). By combining both the chromogenic agar and PCR methods, a practical, cost-effective and reliable method was developed which allowed for the identification and quantification of *P. aeruginosa* within a reduced time.

Many studies found that the cetrimide agar for *P. aeruginosa* is promising medium for direct isolation and identification with high sensitivity and specificity (Laine *et al.*, 2009; Moremi *et al.*, 2021). Cetrimide agar will not only aid routine to detect *P. aeruginosa* rapidly using only one media, but it will also

provide opportunity to conduct such procedures in a cost-effective and reliable manner (Safarirad *et al.*, 2021). cetrimide agar is a promising medium allowing for the isolation and simultaneous identification of *P. aeruginosa* from in burn infection (Al-Dahmoshi *et al.*, 2018).

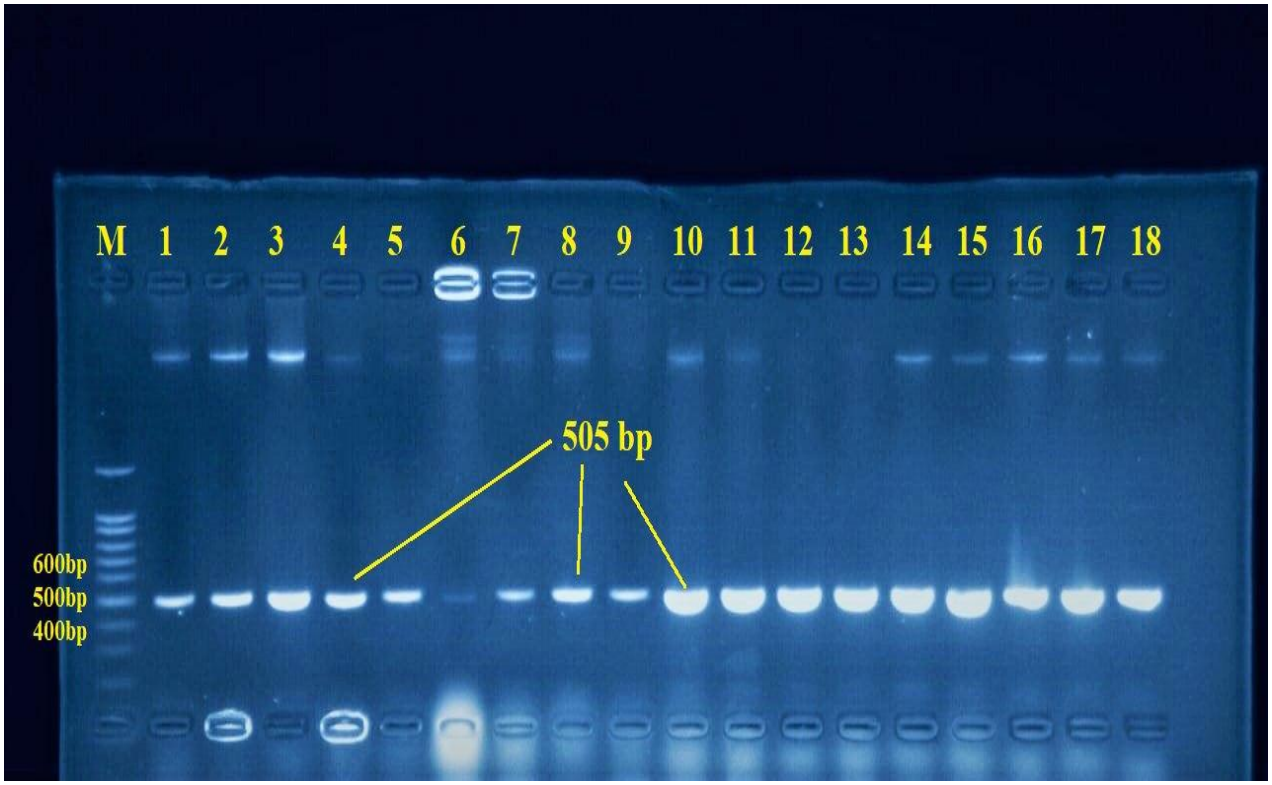


Figure (4-5): Bands were fractionated by electrophoresis agarose 1.5% gel (80 min., 85V/cm) for amplified (505 bp) *P. aeruginosa* specific gene 16S rRNA . Lane(M) represent (DNA ladder marker (100-1500) bp) . isolates: 1-36. positive results.

4.3 Antibiotic Susceptibility Test

Thirty-six identified *P. aeruginosa* isolates (PsA1 to PsA36) were evaluated against 17 common antibiotics, as shown in Figures (4-6).

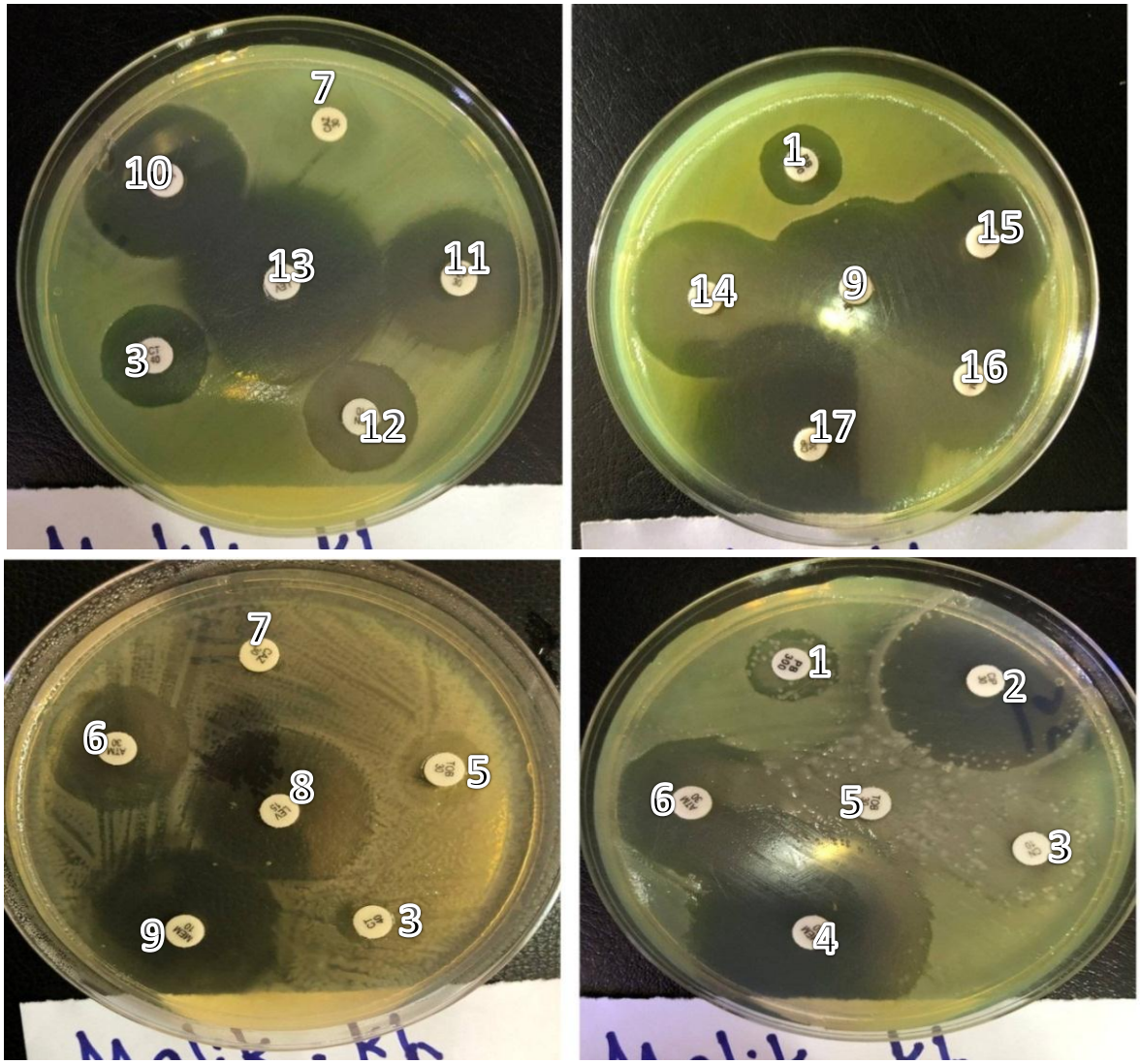


Figure (4-6) :Susceptibility patterns of *P. aeruginosa* to different antibiotics culture on Muller Hinton used in current study.

Abbreviations:**1:PolymyxinB(PB300), 2:Ciprofloxacin(CIP) 3:Colistin(CT) ,4:Meropenem(MEM) , 5:Tobramycin(TOB), 6:Aztreonam(ATM), 7:Ceftazidime(CAZ), 8:Levofloxacin(LEV), 9:Imipenem (IPM), 10:piperacillin-tazobactam (TZP) , 11: Cefepime(FEF), 12:Gentamicin(GM), 13:Amikacin(AK), 14:Dorpenem (DOR), 15:Norfloxacin (NOR), 16:Piperacillin(PIP), 17:Ticarcillin (TIC)**

The majority of isolates exhibited antibiotic resistance,particularly β-lactam antibiotics. All 36 isolates of *P. aeruginosa* test were resistant to, Piperacillin and Tcarcillin. however (PsA10) was sensitive to the majority of antibiotics except for Piperacillin and Ticarcillin as shown in Table (4-6).

Table (4-6): Phenotypic of antibiotic susceptibility of *P. aeruginosa* isolates

Isolate	TIC	PIP	CAZ	FEP	ATM	CIP	LEV	DOR	MEM	IPM	TOB	CN	AK	TZP	NOR	CT	PB3000
PsA1	R	R	R	I	R	S	S	S	S	S	S	S	S	R	S	S	S
PsA2	R	R	R	S	R	S	S	S	S	I	R	R	R	S	I	S	S
PsA3	R	R	R	S	I	S	S	S	S	R	R	R	R	R	S	S	S
PsA4	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA5	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA6	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA7	R	R	R	S	R	I	S	S	S	S	S	S	S	S	I	S	S
PsA8	R	R	R	S	R	S	S	S	S	S	S	S	S	I	I	S	S
PsA9	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA10	R	R	S	S	S	I	S	S	S	S	S	S	S	S	I	S	S
PsA11	R	R	R	S	R	S	S	S	S	R	R	R	R	S	I	S	S
PsA12	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S
PsA13	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA14	R	R	R	S	R	I	S	S	S	S	S	S	S	I	I	S	S
PsA15	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA16	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S
PsA17	R	R	R	S	R	S	S	S	S	S	R	R	R	I	I	S	S
PsA18	R	R	I	R	I	S	S	S	S	S	R	R	R	S	S	S	S
PsA19	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
PsA20	R	R	R	R	R	R	S	S	S	S	R	R	R	S	S	S	S
PsA21	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
PsA22	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	S	S
PsA23	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
PsA24	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S
PsA25	R	R	R	S	R	S	S	S	S	S	R	R	R	R	S	S	S
PsA26	R	R	I	S	I	S	S	S	S	R	R	S	R	S	S	S	S
PsA27	R	R	R	S	S	S	S	S	S	S	R	S	R	R	S	S	S
PsA28	R	R	I	S	R	S	S	S	S	S	S	S	R	I	I	S	S
PsA29	R	R	R	S	R	S	S	S	S	S	R	S	R	S	S	S	S

Isolate	TIC	PIP	CAZ	FEP	ATM	CIP	LEV	DOR	MEM	IPM	TOB	CN	AK	TZP	NOR	CT	PB3000
PsA30	R	R	R	S	R	I	S	S	S	S	R	S	R	S	I	S	S
PsA31	R	R	R	S	R	S	S	S	S	S	R	S	R	S	S	S	S
PsA32	R	R	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R
PsA33	R	R	R	R	R	I	S	S	S	R	R	R	R	S	I	R	R
PsA34	R	R	R	S	S	I	S	S	R	R	R	R	R	S	S	S	S
PsA35	R	R	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
PsA36	R	R	R	R	S	S	S	S	S	R	R	R	R	S	S	R	R

Abbreviations: R, resistance; S, sensitive; I, intermediate;. **Ticarcillin (TIC) ,Piperacillin(PIP) ,Ceftazidime(CAZ) ,Cefepime(FEF) ,Aztreonam(AT) Ciprofloxacin(CIP) ,Levofloxacin(LEV), Meropenem(MEM),Imipenem (IPM), Dorpenem (DOR), piperacillin-tazobactam (TZP) ,Tobramycin(TOB) , Gentamicin(GM),Amikacin(AK), Norfloxacin (NOR), Colistin(CT) ,PolymyxinB(PB300),**

Antibiotic such as the Piperacillin and Ticarcillin all exhibited 100 % resistance, whereas Piperacillin-tazobactam (41.6)% ,Ceftazidime (88.9)% , Cefepime (50)% , Aztreonam (58.3)%, Ciprofloxacin(41.6)%, Levofloxacin (36.1)% Norfloxacin (38.9) % , Meropenem (41.7)%, Imipenem (52.7) %, Tobramycin (83.3) % , Gentamycin (66.7)% ,Amikacin (83.3)%, Doripenem (38.9)% , Colistin (8.3)% and PolymyxinB (8.3) % as shown in Figures (4-7).

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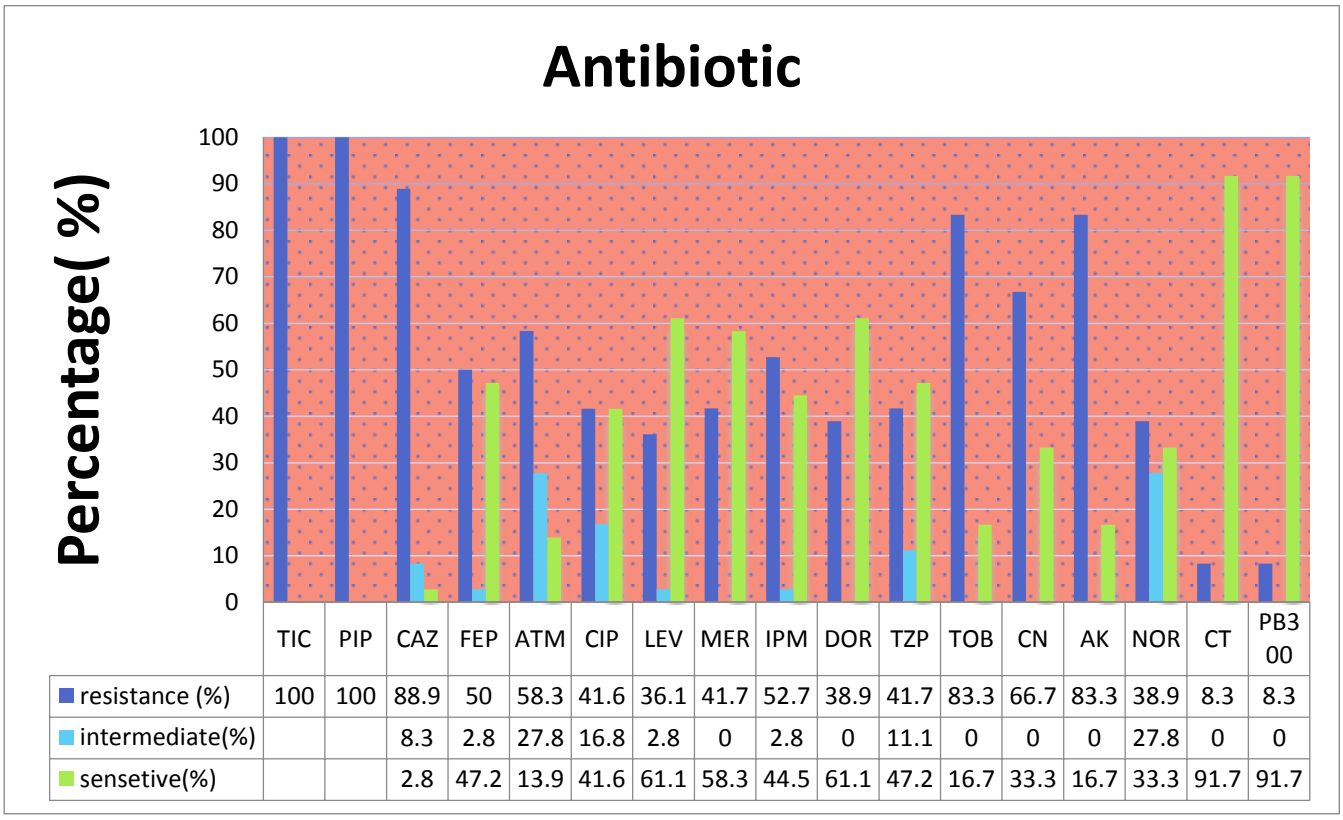


	TIC	PIP	CAZ	FEP	ATM	CIP	LEV	MER	IPM	DOR	TZP	TOB	CN	AK	NOR	CT	PB300
resistance (%)	100	100	88.9	50	58.3	41.6	36.1	41.7	52.7	38.9	41.7	83.3	66.7	83.3	38.9	8.3	8.3
intermediate(%)			8.3	2.8	27.8	16.8	2.8	0	2.8	0	11.1	0	0	0	27.8	0	0
sensetive(%)			2.8	47.2	13.9	41.6	61.1	58.3	44.5	61.1	47.2	16.7	33.3	16.7	33.3	91.7	91.7

Figure (4-7): Antibiotic Susceptibility patterns of *P. aeruginosa* isolates used in current study by disk diffusion method

Ticarcillin (TIC) ,Piperacillin(PIP) ,Ceftazidime(CAZ) ,Cefepime(FEP) ,Aztreonam(ATM) Ciprofloxacin(CIP) ,Levofloxacin(LEV), Meropenem(MEM),Imipenem (IPM), Dorpenem (DOR), piperacillin-tazobactam (TZP) ,Tobramycin(TOB) , Gentamicin(CN),Amikacin(AK), Norfloxacin (NOR), Colistin(CT) ,PolymyxinB(PB300).

The results showed that *P. aeruginosa* resistance to Piperacillin and Ticarcillin 36/36 (100)% . This result is not compatible with Vitkauskienė *et al*., (2010); Senthamarai *et al*., (2016) and Hussein *et al*., (2018) who reported the rates of 37.0%, 59.61% and 67.96% respectively. Although this result compatible or close from the result that reported of Al-Marzoqi (2013) and Corehtash (2015) who reported (100)% and (85.4)% resistance rates respectively .

The resistance to Ceftazidime were 32/36(88.9)% and Cephems were 18/36 (50) % This result was close with the results of Othman *et al.* (2014) who reported a resistant rate of *P. aeruginosa* of ceftazidime which were 73.6%, and compatible to other results obtained by Freitas and Barth (2002) and Hassuna *et al*., (2015) who recorded (87.7 and 86)% resistance rates, respectively. While (Hussein *et al*., 2018)

Reported the resistance which rate were 55.5%.In the present study, the highest resistant percentages toward the antibiotic were found with Tobramycin (83.3)%, while the lowest resistant percentage were found with Levofloxacin (36.1)%. When the β-lactam, Aminoglycoside, or Quinolone is ineffective the Polymyxins, particularly Colistin, remains as the antimicrobial drugs of the last option (Mitra *et al*., 2022).

Beta -lactamase inhibitors combination antibiotics also showed resistance to Piperacillin-tazobactam 15/36 (41.7)%

The results showed high resistance to beta lactams (Ceftazidime, Cefepime, Piperacillin and this is mainly mediated by beta lactamases due to that when use Piperacillin-tazobactam the resistance was dropped from 100% to 41.6%. Beta-lactamases regard as intrinsic mechanism of resistance leading to inactivating of beta lactam rendering them inactive.

Beta lactamase inhibitor like Tazobactam (An irreversible inhibitor of a wide variety of bacterial beta-lactamases) can improve many beta lactams like piperacillin once combined with them. Piperacillin-tazobactam is the most widely used β-lactam-β-Lactamase inhibitor combination for treating *P. aeruginosa* infections (Tannous *et al*., 2020; Al Muqati *et al*., 2021).

The rate resistance to Aztreonam was 21/36 (58.3)%.The result is not the same (81.8)% which was documented by Corehtash *et al*. (2015) , but it disagrees with 48% which was previously documented by Kateete *et al*. (2017) and 54.4% by Hussein *et al*. (2018). Like other gram-negative bacteria, *P. aeruginosa* possesses an inducible ampC gene, encoding the hydrolytic enzyme β-lactamase. This enzyme is able to break the amide bond of a β-lactam ring, leading to inactivation of β-lactam antibiotics which explain the resistance to Aztreonam, Piperacillin, and Ceftazidime (Pang *et al.*, 2019).

Resistace to Carbapenems showed that Imipenem 52.7% , Meropenem 41.7% and Doripenem 38.9% .Imipenem result is not the same to that of Fazeli *et al*., (2017) who reported a rate resistance 98.7%, and also different from Safarirad *et al.*, (2021) and Vitkauskienė (2010) who recorded resistance 22% and 24%, receptivity. Meropenem result is not the same with Gad *et al*., (2007) who reported 22%, and far from Coetzee *et al*. (2013) which reported extremely higher rate 93.4%. Carbapenems (Imipenem and Meropenem) antibiotics are members of a β-lactams family, mainly used to treat *P. aeruginosa* infections.

Similar to enterobacteriaceae, carbapenemase enzymes have been identified in *P. aeruginosa* strains and is responsible for its resistance. In addition, the porin OprD is known to promote the internalization of Imipenem and to some extent, Meropenem but not of other β-lactams. Thus, the modification of OprD structure and/or the reduction of its expression confer reduced susceptibility to Imipenem. The alteration of OprD is often associated with overexpression of efflux systems, thus conferring a high level of resistance to Imipenem, but also to other classes of antibiotics such as Quinolones and Aminoglycosides (Bassetti *et al*., 2018).

Aminoglycosides resistance which icluded,Gentamicin resistance rate recorded in this study was 66.7%, this result is different from that documented by Vitkauskienė *et al*. (2010) who reported 37% and incompatible with Fazeli *et al*. (2017) 91.2%. For Tobramycin resistance rate was 30/36 (83.3)%, this result was close to Aljanaby and Aljanaby (2018) who reported a rate high resistance of 78.8% and Othman *et al*. (2014) who reported a rate of 76.2%. For Amikacin results demonstrated a resistance rate of 30/36 (83.3)% .

This result was close to that of Aljanaby and Aljanaby (2018) 77.4% and similar Corehtash *et al*. (2015) 82%. They differ with the findings of Alramahy and Aladily, (2017) 26% and Juhi *et al*. (2009) 30% . Tobramycin, Amikacin, and Gentamicin are Aminoglycosides antibiotic. Acquired resistance to aminoglycosides is mediated by transferable aminoglycoside-modifying enzymes (AMEs), rRNA methylases and derepression of endogenous efflux systems.

Modification and subsequent inactivation of aminoglycosides are achieved by three deferent mechanisms: (1) acetylation, by aminoglycoside acetyltransferases (AACs), (2) adenylation, by aminoglycoside nucleotidyltransferases (ANTs), and (3) phosphorylation, by aminoglycoside phosphoryl transferases. Methylation of the 16S rRNA of the A site of the 30S ribosomal subunit interferes with aminoglycoside binding and consequently promotes high-level resistance to all aminoglycosides (Meletis and Bagkeri, 2013).

Resistance to Flouroquinolones showed Ciprofloxacin 15/36 (41.6)%, Levofloxacin 13/36 (36.1)%, Norfloxacin 14/36 (38.9)% .For Ciprofloxacin, this result is different with the data reported by Al-derzi. (2012) who recorded

that 23.9% of isolated were resistance to Ciprofloxacin, but disagree with that reported by Othman *et al*. (2014) who recorded 61.3% resistance. For Levofloxacin 39%, this rate is close to the results of Yayan *et al*. (2015) 30.6% and Lila *et al*. (2017) 36.1%, but disagrees with Khadim and Marjani (2019) 57.14 % and Hussein *et al*. (2018) 60.19%. Flouroquinolone antibiotics such as Ciprofloxacin and Levofloxacin interfere with DNA replication by inhibiting DNA gyrase and topoisomerase IV (Pang *et al*., 2019). Ciprofloxacin and Levofloxacin resistance can arise through the acquisition of mutations in genes encoding the target proteins of Ciprofloxacin and regulators of efflux pumps, which leads to overexpression of these pumps leading to increases the expulsion of Ciprofloxacin from *P. aeruginosa* cells and occurs through mutations in regulatory genes of efflux pumps (Rehman *et al*., 2019). The isolate(PsA32) was resistant to all antibiotics except for Levofloxacin, isolates (PsA19,PsA21 ,PsA23) was resistant to all antibiotics except for Colstin and polymyxin B , isolates (PsA12, PsA24) was resistant to all antibiotics except for Piperacillin-tazobactam, Colstin and PolymyxinB and isolates(PsA4 ,PsA5, PsA6,PsA9,PsA13, PsA15 ,PsA16) was resistant to all antibiotics except for Aztreonam, Colstin and Polymyxin B. This study also showed that most of the isolates of *P. aeruginosa* were (52.8 %) multi-drug resistant (resistance to 3 antibiotics up to 17 antibiotics). The details of the distribution of the MDR phenotype among *P. aeruginosa* and percentages of the isolates were summarized in Table (4-7). This result is different with the data reported by Hasan *et al*. (2019) who found 91.6 % of the isolates were MDR, and the resulte was close to the Nasser and Kharat (2019) who found 66.3 % of *P. aeruginosa* isolates were MDR.

Table (4-7): Frequency of multidrug-resistant isolates of *P.aeruginosa*.

Antibiotic	No. MDR isolate
TIC/ PIP../ IPM ./ FEP./TOB /DOR /MEM./ CAZ./NOR./. CN /AK/ CIP/ATM/ TZP/CT / PB/.	PsA32
TIC/ PIP../ IPM ./ FEP./TOB /DOR /MEM./ CAZ./NOR./. CN /AK/ CIP/ATM /LEV	PsA12,PsA24
TIC/ PIP../ IPM ./ FEP./TOB /DOR /MEM./ CAZ./NOR./. CN /AK/ CIP/ATM /LEV/TZP	PsA19,PsA21,PsA23
TIC/PIP/IPM/FEP/TOB/DOR/MEM/CAZ/NOR/CN/AK/ CIP/LEV/ TZP	PsA4, PsA5, PsA6, PsA9, PsA13, PsA15, PsA16
TIC/PIP/FEP/TOB/DOR/MEM/CAZ/NOR/CN/AK/CIP/ATM /LEV	PsA22
TIC/ PIP/IPM./FEP/TOB/CAZ/CN /AK/ATM/CT/PB	PsA33
TIC/ PIP/IPM / FEP./TOB ./ CAZ../. CN /AK /CT / PB	PsA36
TIC/ PIP./ FEP./TOB ./ CAZ./. CN /AK/ CIP/ATM/	PsA20
TIC/ PIP../ CAZ././TOB / IPM /. CN /AK/ TZP/	PsA3
TIC/PIP../IPM/TOB/CAZ../.CN/AK/ATM/	PsA11
TIC/PIP./TOB./CAZ../CN/AK/ATM/TZP	PsA25
TIC/PIP../IPM/TOB/MEM./CAZ./CN/AK/	PsA34
TIC/PIP./TOB/CAZ../.CN/AK/ATM/	PsA2,PsA17
TIC/PIP./FEP./TOB/CN/AK	PsA18
TIC/PIP/TOB/CAZ/TZP/AK	PsA27
TIC/PIP/TOB/CAZ/ATM/AK	PsA29,PsA30,PsA31
TIC/PIP/ATM/CAZ/TZP	PsA1
TIC/PIP/IPM/TOB/AK	PsA26
TIC/PIP/ATM/CAZ	PsA7,PsA8,PsA14
TIC/PIP/ATM/AK	PsA28
TIC/PIP/TOB/CAZ	PsA35

Abbreviations: Ticarcillin (TIC) ,Piperacillin(PIP) ,Ceftazidime(CAZ) ,Cefepime(FEF) ,Aztreonam(AT) Ciprofloxacin(CIP) ,Levofloxacin(LEV), Meropenem(MEM),Imipenem (IPM), Dorpenem (DOR), piperacillin-tazobactam (TZP) ,Tobramycin(TOB) ,Gentamicin(GM),Amikacin(AK), Norfloxacin (NOR), Colistin(CT) ,PolymyxinB(PB300).

The results revealed that 19/36 (52.8)% of *P. aeruginosa* isolates were multidrug resistant (MDR) and 17/36 (47.2)% non MDR Figure(4-8), and *P. aeruginosa* isolates as MDR in this study were resistance (resistance for at least 3 different classes antibiotic) as shown in Table (4-8)The results of (Perez *et al*., 2019) was (69)% . MDR while the results of (Porretta *et al*., 2020) was (32)%.

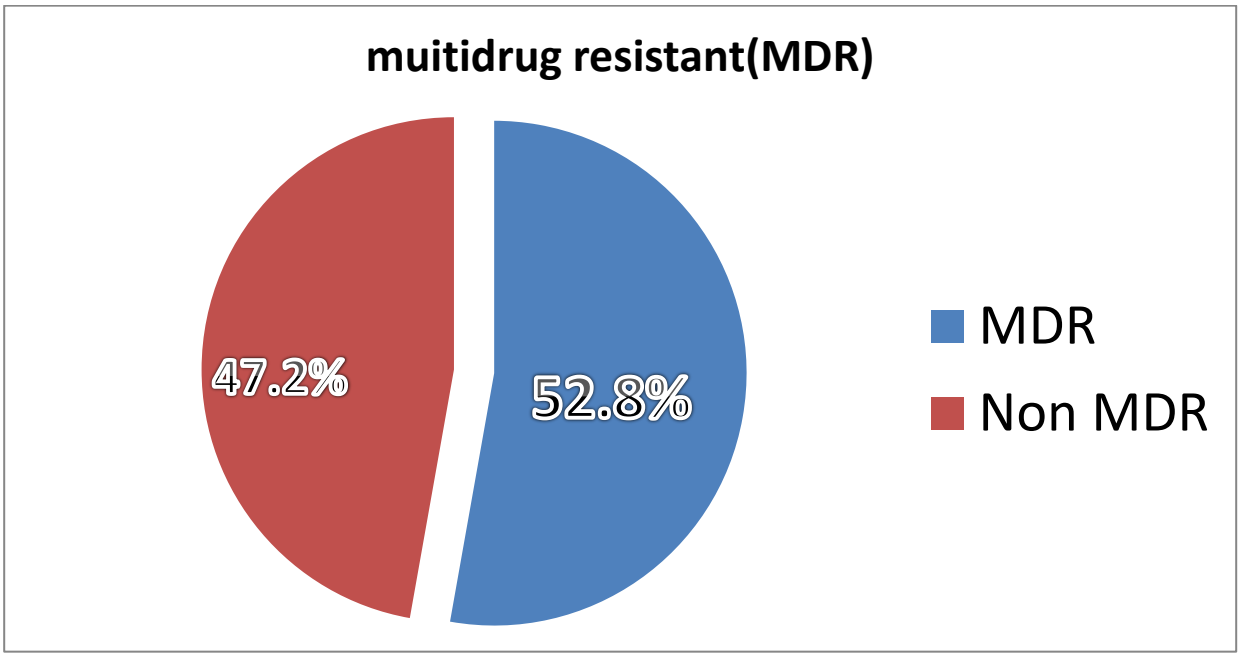


Figure (4-8): Antibioticg resistance patterns among *P. aeruginosa* isolates

Table (4-8): Phenotypic resistance patterns of MDR *P. aeruginosa*

Classes	MDR Phenotype	No	%
7	Penicillin/Monobactam/Carbapenem/Aminoglycoside/Fluoroquinole/ cephem/ β-Lactams combinations	1	5.5
	Penicillin /Carbapenem/Aminoglycoside/Fluoroquinolone/ cephem/ β-Lactams combinations /Polymyxins	1	
6	Penicillin/Carbapenem/Aminoglycoside/Fluoroquinolone/ cephem/ β-Lactams combinations	7	30.6
	Penicillin/Monobactam/Carbapenem/Aminoglycoside/Fluoroquinone / cephem/	4	
5	Penicillin/Monobactam /Aminoglycoside / cephem/ /Polymyxins	1	2.8
4	Penicillin /Aminoglycoside / cephem/ /Polymyxins	1	11.1
	Penicillin/Monobactam/Aminoglycoside/ cephem/	1	
	Penicillin/Monobactam/Aminoglycoside/β-Lactams combinations	1	
	Penicillin/Aminoglycoside/Fluoroquinolone/ cephem	1	
3	Penicillin/Monobactam/Aminoglycoside	1	2.8
Total		19	52.8

The Polymyxins show very little resistance as the Polymyxin B and Colistin, both of which are in the same ratio 3/36 (8.3)%. while Low bacterial resistance was noted against colistin only (Alsadek mohamed, 2020).

When using Piperacillin-tazobactam, beta lactam resistance drops from 46% to 16%. Beta lactamases inactivate β-lactams as an innate resistance mechanism. Beta lactamase inhibitors like Tazobactam enhance several beta lactams like Piperacillin. Piperacillin-tazobactam is the most common β -lactamase inhibitor for *P. aeruginosa* infections (Tannous *et al*., 2020; Al Muqati *et al*., 2021).

4.4 Screening for extended spectrum β-lactamase

The extended spectrum β-lactamase(ESBLs) enzyme were detected by Double Disc Synergy test (DDS) on a solid culture medium (Muller Hinton agar) in a Petri dish .The results showed that 4/36(11.1)% *P. aeruginosa* isolates were positive which were (PsA32, PsA33, PsA34 and PsA36) as shown in figure (4-9)

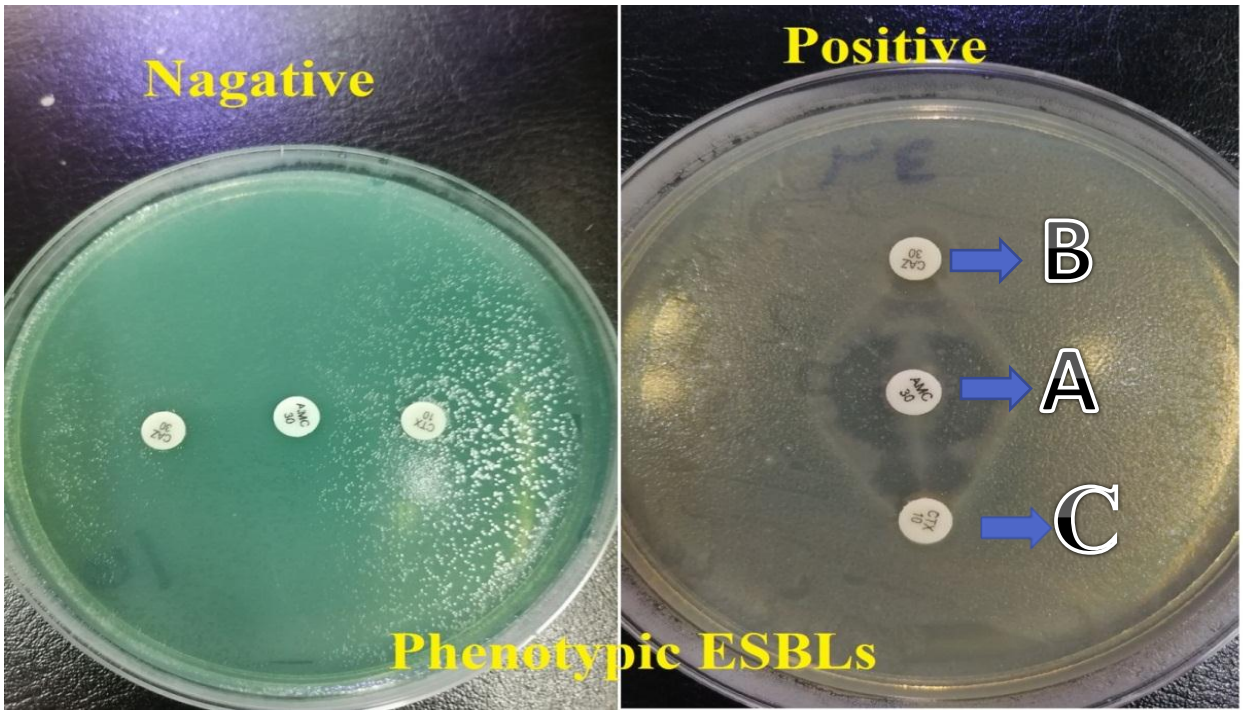


Figure (4-9): Phenotypic ESBLs (extended spectrum β – Lactamase A: Amoxyclav (Amoxillin /Clavulanic acid) AMC (30mg) B:Ceftazidime CAZ (10mg) and C:Cefotaxime CTX(30mg) .

4.5 Detection of β-lactamase genes of *P. aeruginosa* isolates.

PCR detecting sequences of the genes of β-lactamase genes were positive; isolates which were carried genes *bla* $_{OXA-10}$ 30/36 (83.33)%, *bla* $_{OXA-488}$ 24/36 (66.6)%, *bla* $_{OXA-145}$ 34/36 (94.44)% , *bla*$_{OXA-181}$ 21/36 (58.3)% , PER-1 31/36 (86.1)% , VEB 33/36 (91.7)% and no result of three primer which were PER , GES-2 and GES. as shown in Figures (4-10 to 4-14) and Table (4-9).

Table(4-9): Distribution of gene group of *P. aeruginosa* isolates.

Isolate No	*bla* $_{OXA-10}$	*bla* $_{OXA-488}$	*bla* $_{OXA-145}$	*bla*$_{OXA-181}$	VEB	PER-1
PsA1	negtive	Positive	positive	Negative	positive	+ve
PsA2	negtive	Positive	positive	Negative	positive	negtive
PsA3	negtive	Positive	positive	Negative	positive	positive
PsA4	positive	Positive	positive	Negative	positive	positive
PsA5	negtive	Positive	positive	Negative	positive	positive
PsA6	positive	Positive	positive	Negative	positive	- negtive
PsA7	negtive	Positive	positive	Negative	positive	positive
PsA8	positive	Positive	positive	Negative	positive	positive
PsA9	positive	Positive	positive	Negative	negative	- negtive
PsA10	positive	Positive	positive	Negative	positive	negtive
PsA11	positive	Positive	positive	Positive	positive	positive
PsA12	positive	Positive	positive	Positive	positive	positive
PsA13	positive	Positive	positive	Positive	positive	positive
PsA14	positive	Positive	positive	Positive	positive	positive
PsA15	positive	Positive	positive	Positive	positive	positive
PsA16	positive	Positive	positive	Positive	positive	positive
PsA17	positive	Positive	positive	Positive	positive	positive
PsA18	positive	Positive	positive	Positive	positive	positive
PsA19	positive	Positive	positive	Positive	positive	positive
PsA20	positive	Positive	positive	Positive	positive	positive
PsA21	positive	Positive	positive	Positive	positive	positive
PsA22	positive	Positive	positive	Positive	positive	positive
PsA23	positive	Positive	positive	Negative	positive	positive
PsA24	positive	Positive	positive	Positive	positive	positive
PsA25	positive	Negative	positive	Positive	positive	positive
PsA26	positive	Negative	positive	Negative	positive	positive
PsA27	positive	Negative	positive	Positive	positive	positive
PsA28	positive	Negative	positive	Positive	negtive	positive
PsA29	positive	Negative	positive	Positive	positive	positive
PsA30	positive	Negative	positive	Positive	negtive	positive
PsA31	positive	Negative	positive	Positive	positive	positive
PsA32	positive	Negative	positive	Positive	positive	positive
PsA33	positive	Negative	positive	Positive	positive	positive
PsA34	positive	Negative	negtive	Negative	positive	positive
PsA35	positive	Negative	positive	Negative	positive	negtive
PsA36	negtive	Negative	negtive	Negative	positive	positive

Abbreviation: positive: gene possessing., negtive: gene lacking

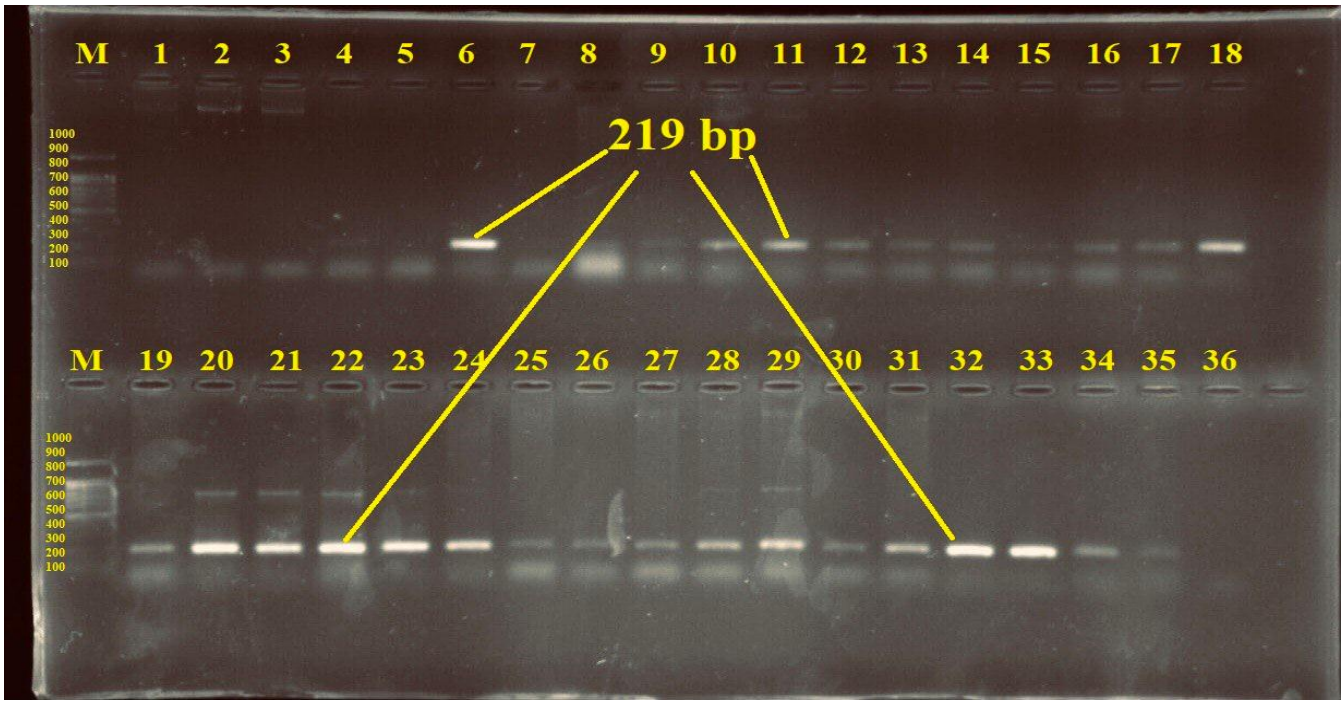
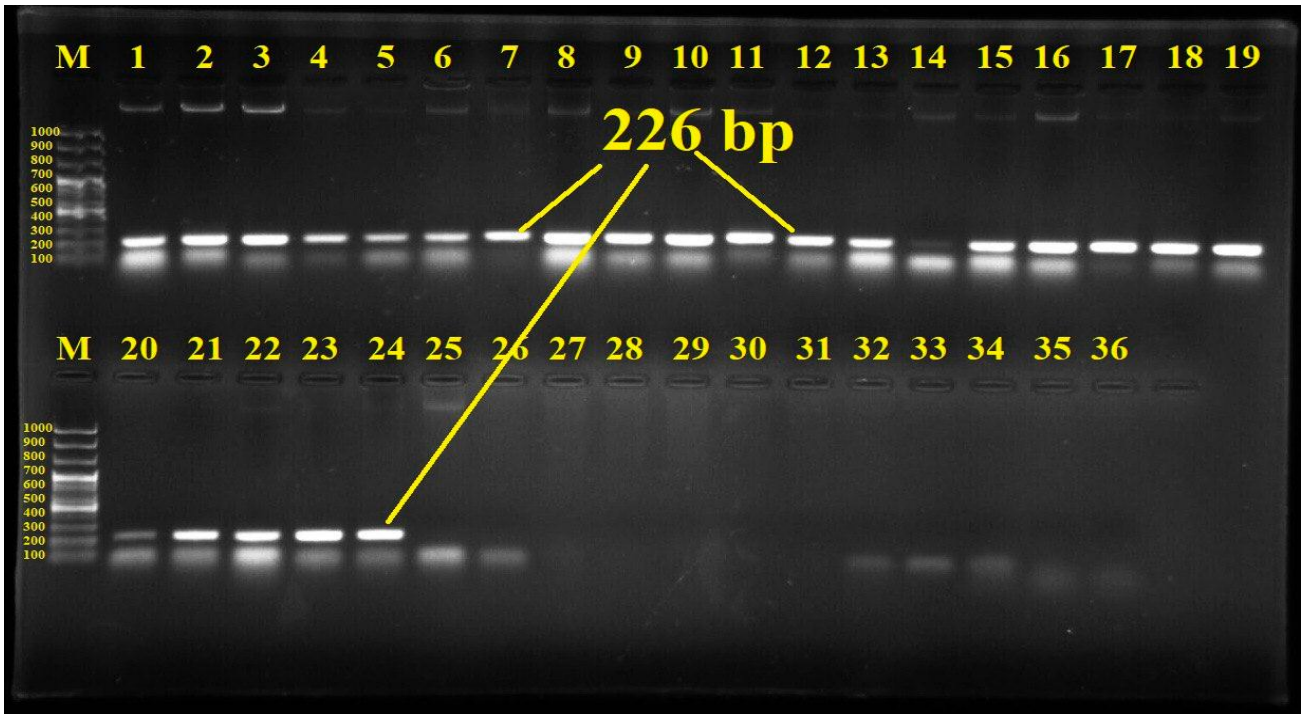


Figure (4-10): The PCR product of bla$_{OXA-10}$ gene (219bp) were separated by electrophoresis in an 1.5% agarose gel, at 80 V\Cm for 60 min. M: DNA ladder marker (100-1500 bp). The absences this gene in some isolated specimens (PsA1, PsA2, PsA3, PsA5, PsA7, and PsA36).



Figure(4-11): The PCR product of bla$_{OXA-488}$ gene (226bp) were separated by electrophoresis in an 1.5% agarose gel, at 80 V\Cm for 60 min. M: DNA ladder marker (100-1500 bp). The absences this gene in some isolated specimens (PsA25, PsA26, PsA27, PsA28, PsA29, PsA30, PsA31, PsA32, PsA33,PsA34,PsA35, and PsA36).

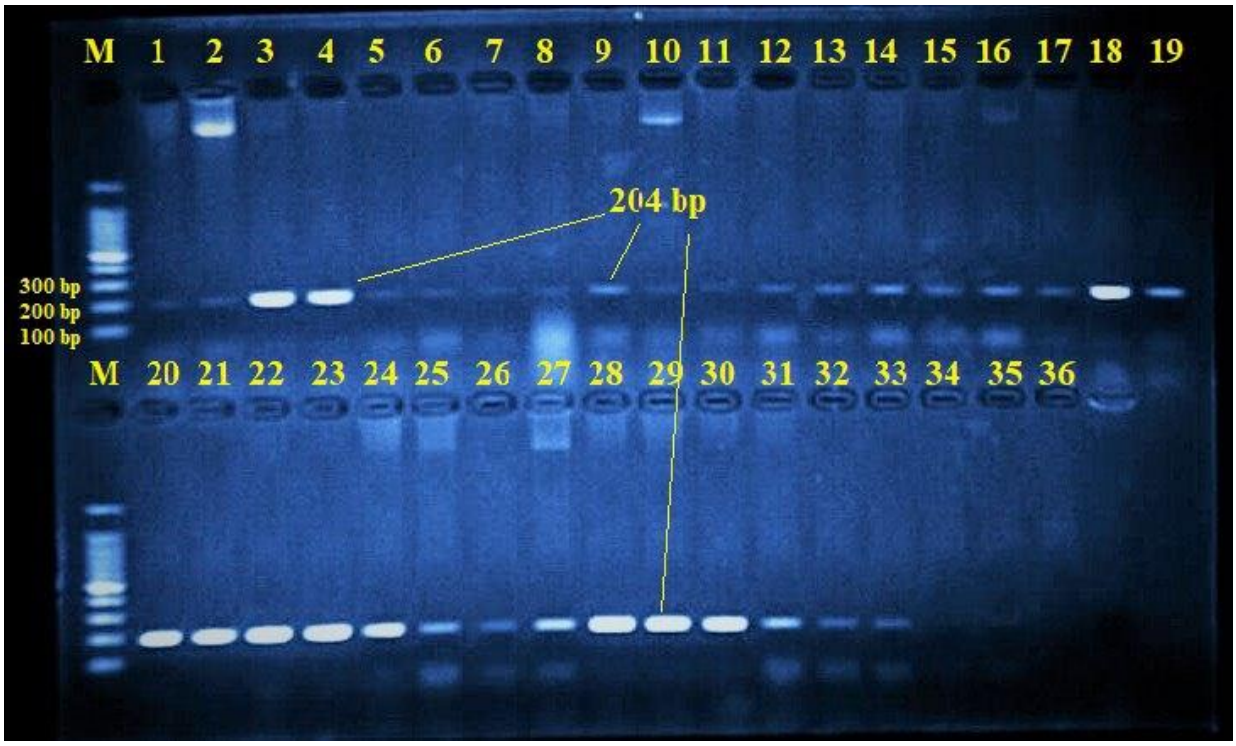


Figure (4-12): The PCR product of bla$_{OXA-145}$ gene (204bp) were separated by electrophoresis in an 1.5% agarose gel, at 80 V\Cm for 60 min. M: DNA ladder marker (100-1500 bp). The absences this gene in some isolated specimens (PsA34, and PsA36).

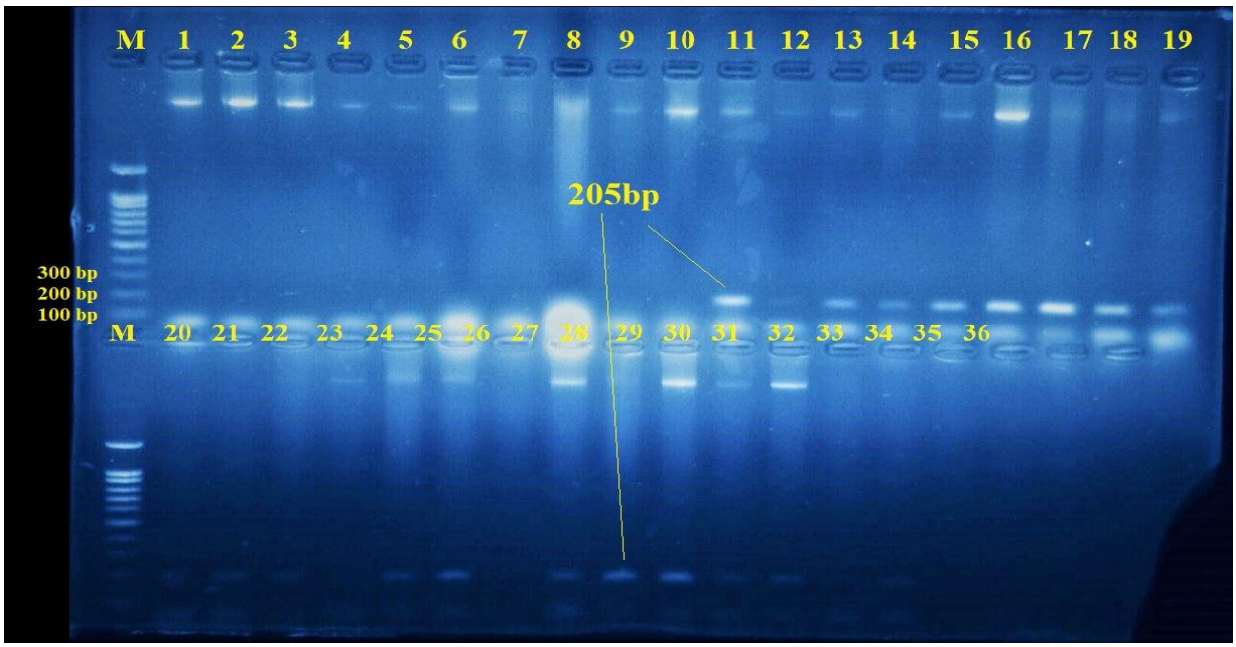


Figure (4-13): The PCR product of bla$_{OXA-181}$ gene (205bp) were separated by electrophoresis in an 1.5% agarose gel, at 80 V\Cm for 60 min. M: DNA ladder marker (100-1500). The absences this gene in some isolated specimens (PsA1, PsA2, PsA3, PsA4, PsA5, PsA6, PsA7, PsA8, PsA9,PsA10,PsA23 , PsA26, ,PsA34, PsA35, and PsA36) .

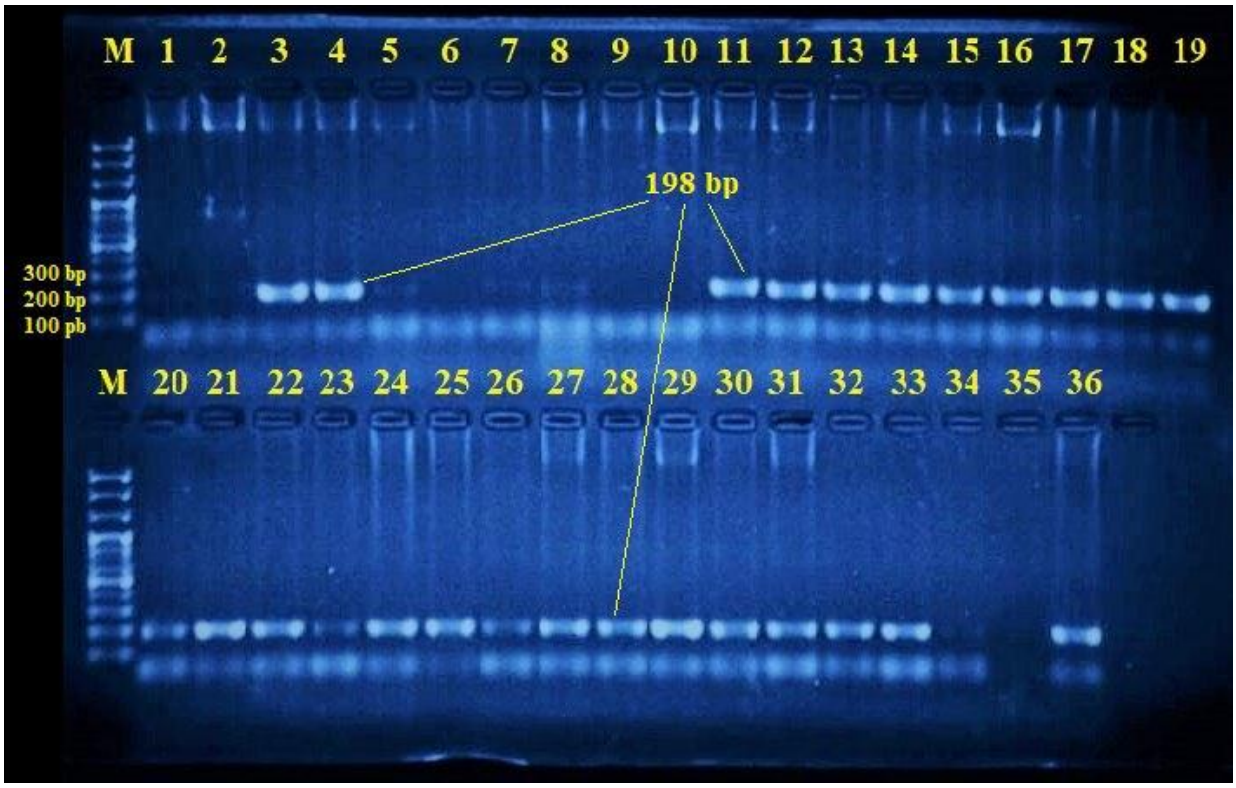


Figure (4-14): The PCR product of PER-1 gene (198bp) were separated by electrophoresis in an 1.5% agarose gel, at 80 V\Cm for 60 min. M: DNA ladder marker (100-1500 bp). The absences this gene in some isolated specimens (PsA2, PsA6, PsA9, PsA10, and PsA35).

Based on these findings, there were 13 isolates PsA11,PsA12, PsA13 , PsA14 PsA15 , PsA16 ,PsA17 , PsA18, PsA19 , PsA20 , PsA21, PsA22, PsA24 (36.1)% possessed *bla*$_{OXA-10}$, bla$_{OXA-488}$, bla$_{OXA-145}$, bla$_{OXA-181}$,VEB and PER-1 genes. the *bla*$_{OXA-10}$, bla$_{OXA-145}$, bla$_{OXA-181}$,VEB and PER-1 genes were identified in 6 (16.7)% isolates PsA25 ,PsA27 , PsA29 , PsA31, PsA32 , PsA33 . 4(11.1)% isolates PsA1,PsA3, PsA5 , PsA7 possessed bla$_{OXA-488}$, bla$_{OXA-145}$ VEB and PER-1 genes. *bla*$_{OXA-10}$,bla$_{OXA-488}$, bla$_{OXA-145}$,VEB and PER-1 genes were identified in 3 (8.3)%isolates PsA4 , PsA8 ,PsA23 . *blaOXA-10,blaOXA-145,blaOXA-181 ,PER-1* genes were identified in 2(5.5)% isolates PsA28 , PsA30 . 2(5.5)% *blaOXA-10,blaOXA488,bla OXA145 ,VEB* isolates PsA6 , PsA10 and one isolate for each gene [(PER-1,VEB PsA36), (*blaOXA-10,blaOXA-145 ,PER-1,VEB* PsA26),

(*blaOXA-10,blaOXA-145 , VEB* PsA35) (bla$_{OXA-488}$, bla$_{OXA-145}$ VEB PsA2) bla$_{OXA-10}$, bla$_{OXA-145}$ and bla$_{OXA-488}$ PsA9), (bla$_{OXA-10}$, VEB and PER-1 PsA26) (2.8)%].The absence of any isolate that does not possess one of the above genes,the distribution of above Genes of *P.aeruginosa* in current study show in Table (4-10).

Table(4-10):Distribution of β-lactamases Genes among *P. aeruginosa* isolates.

Gene	No Isolate.	%
blaOXA-10,bla OXA-48,blaOXA-145,blaOXA-181 ,PER-1,VEB	13	36.1%
blaOXA10,blaOXA145,blaOXA-181 ,PER-1,VEB	6	16.7%
bla OXA-488,bla OXA-145 ,PER-1,VEB	4	11.1%
blaOXA-10,blaOXA488,bla OXA145 ,PER-1,VEB	3	8.3%
blaOXA-10,blaOXA-145,blaOXA-181 ,PER-1	2	5.5%
blaOXA-10,blaOXA488,bla OXA145 ,VEB	2	5.5%
blaOXA-10,blaOXA-488 blaOXA-145	1	2.8%
blaOXA-10,,blaOXA-145,VEB,	1	2.8%
blaOXA-10,blaOXA-145 ,PER-1,VEB	1	2.8%
blaOXA-10,PER-1,VEB	1	2.8%
bla OXA-488,bla OXA-145,VEB	1	2.8%
PER-1,*VEB*	1	2.8%

In the current study, β-lactamases production using antibiotics resistance and molecular detection of *bla $_{OXA-10}$* 30/36 (83.33%), *bla $_{OXA-488}$* 24/36 (66.6)%, *bla $_{OXA-145}$* 34/36 (94.44)% , *bla$_{OXA-181}$* 21/36 (58.3)% ,VEB 33/36 (91.7)% and PER-1 31/36 (86.1)% , genes in *P. aeruginosa* isolates revealed that there was a harmony between results of antibiotic resistance and positive molecular detection of these genes. The highest resistance rate to most antibiotics was observed in the isolates of *bla$_{OXA-145}$* then *PER-1* genes, as shown in Table (4-11).

Table (4-11): Pattern of highest resistance isolates of *P. aeruginosa* isolate to the different type of antibiotcs among these β-lactamases genes.

Gene / Antibiotic	*bla*$_{OXA-10}$		bla$_{OXA-488}$		bla$_{OXA-145}$		bla$_{OXA-181}$		PER-1		*VEB*		*NON*	
	NO	*%*	NO	%	NO	%	NO	%	NO	%	NO	%	NO	%
Ticarcilln	30	100	24	100	34	100	21	100	31	100	33	100	0	0
Piperacillin	30	100	24	100	34	100	21	100	31	100	33	100	0	0
Ceftazidim	26	86.7	22	91.7	30	88.2	19	90.4	27	87	30	90.9	0	0
Piper./Tazo.	12	40	12	50	14	41.1	8	38.1	13	41.9	14	42.4	0	0
Cefepime	16	53.3	15	62.5	17	50	12	57.1	16	51.6	17	51.5	0	0
Aztreonam	18	60	14	58.3	21	61.7	16	76.1	20	64.5	19	57.5	0	0
Ciprofloxacin	14	46.7	14	58.3	15	44.1	10	47.6	13	41.9	14	42.4	0	0
Levofloxacin	12	40	13	54.1	13	38.2	8	38.1	11	35.4	12	36.7	0	0
Meropenem	14	46.7	13	54.1	14	41.1	9	42.8	13	41.9	14	42.4	0	0
Imipinem	16	53.3	14	58.3	17	50	10	47.6	17	54.8	17	51.5	0	0
Tobramycin	26	86.7	18	72	28	82.3	19	90.4	26	83.8	28	84.8	0	0
Gentamicin	20	66.6	19	79.1	22	64.7	15	71.4	21	67.7	23	69.7	0	0
Amikacin	26	86.7	19	79.1	28	82.3	20	95.2	27	87	27	81.8	0	0
Colstin	2	6.7	0	0	2	5.9	2	9.5	3	9.6	3	9.1	0	0
Polymyxin B	2	6.7	0	0	2	5.9	2	9.5	3	9.6	3	9.1	0	0
Doripenem	13	43.3	13	54.1	14	41.1	8	38.1	12	38.7	13	39.4	0	0
Norfloxacin	13	43.3	13	54.1	14	41.1	9	42.8	12	38.7	13	39.4	0	0

Broad-spectrum beta-lactamases (ESBLs), metallo--lactamases (MBLs), and in rare cases AmpC plasmid -lactamases are produced by bacteria that have developed resistance to beta-lactam antibiotics, leading to acquired resistance (Rabiei *et al.*, 2020) . Phylogenetic analysis of beta-lactamases has revealed the presence of genes that can be transmitted through plasmids and integrons, such as blaVEB and blaPER in *P. aeruginosa* (Philippon *et al.*, 2016). Class A beta-lactamases have 100% similarity and are highly conserved, as was found in a study by Ambler (Ambler, 1980) . These enzymes belong to class A2 of the Ambler classification and are primarily responsible for hydrolyzing Cephalosporins like Cephalothin, Ceftazidime, and Cefotaxime, as well as

Aztreonam and Penicillins (Philippon *et al.,* 2016).The result of bacteria that have *Per*-1 31/36 (86.11%) compatible with Haghighi and Goli study which found that the two most frequent PER enzymes, PER-1 and PER-2, are only partially inhibited by avibactam compared to other class A –lactamases (Bonjoch *et al.*, 2013).

However, in our analysis, 93.54 percent of Cefepime-resistant and 64.51 percent of imipenem-resistant isolates harbored the bla PER gene, while 70.27 percent of meropenem-resistant and 78.57 percent of doripenem-resistant isolates did so as well (Haghighi and Goli, 2022). OXA-145 belongs to the OXA-10 family of -lactamases and has a wide spectrum of activity. The hydrolysis spectrum has shifted from Penicillins to third-generation Cephalosporins and Monobactams due to the deletion of Leu-165. A Lys-73 that had been decarboxylated caused Penicillin hydrolysis to be lost (Hocquet *et al.,* 2011).

In a study conducted by Sezadehghani and his group;they found that Among P. aeruginosa isolates, blaOXA-145 was found in 18.3%, blaOXA-224 in 22.0%, blaOXA-539 in 40.3%, and blaOXA-675 in 10.1% (Sezadehghani *et al.*, 2022).

P. aeruginosa NRZ-49259 was discovered to have blaOXA-181 localized on a chromosome. There was a 3153 bp area on a 2.6 Mbp contig that was identical to the *K. pneumoniae* plasmid pKP3-A (GenBank accession number JN205800.1). This area included the ISEcp1 insertion sequence, blaOXA-181, truncated lysR- and er-like genes, and a repA gene that made up the Tn2013 transposon.

However, NRZ-49259 lacked the whole 3' end of the repA gene (744 bp) and the 5' end of ISEcp1 (1008 bp). Intriguingly, a chromosomal position of

blaOXA-181 exhibiting similarity to pKP3-A was earlier discovered for an English isolate of *P. aeruginosa* (Meunier *et al.,* 2016). The high prevalence of MDR *P. aeruginosa* (one hundred percent) demonstrates the critical necessity for epidemiological surveillance, as it indicates an alarmingly high availability of class 1 integrons in our region (Almuttairi and Abdulla, 2022). The presence of blaOXA-181 was confirmed by polymerase chain reactions targeting both common and unusual carbapenemase genes. WGS results verified the gene's chromosomal location, Although *P. aeruginosa* strain from England has been characterized as having the same genetic organization of blaOXA-181, the two isolates had very different sequence types (ST111/ST235) (Schauer *et al.,* 2022).

Baban's research focuses on antimicrobial stewardship to prevent the indiscriminate use of Carbapenem antibiotics and early diagnosis of carbapenem-resistant isolates to prevent cross-transmission among critically ill patients. Active surveillance and strict infection prevention and control may stop carbapenemase resistance, according to Baban's findings (Baban, 2020) .

The GES-2 β-lactamase may contribute in part to the decreased susceptibility of *P. aeruginosa* to Imipenem. However, once expressed from a multicopy vector in *E. coli*, bla_{GES-2} expression did not significantly increase the Imipenem MIC, thus making its clinical detection in enterobacterial isolates by a simple susceptibility study unlikely.

The Guiana-Extended Spectrum (GES-1) ESBL, first discovered in *K. pneumoniae* isolated from a patient in Guyana, has been gradually isolated from Enterobacteriaceae and other bacteria from various geographical areas (Garza-Ramos et al., 2015). Some molecular studies showed that both OXA-10 and GES-1 genes were found in *P.aeruginosa* multidrugresistant strains isolated from nosocomial infections and in a report on the distribution of the prevalence of

ESBLs among burn patients was about 56% and 20% respectively (Tawfik *et al.*, 2012).

The GES gene consider A class enzyme, and are not to be related to any other plasmidmediated β-lactamases. The enzymes show resistance to Penicillin and show less activity against Aztreonam and Imipenem, it also hydrolyzes Carbapenems . The GES gene is difficult to identify in the lab due to nuclear nomenclature and paucity. There are different types of GES which have different hydrolysis properties have been designed by identical names (Snyder, 2001). Class A group of lactamases may be classified into several classes, and the VEB group is one of the smaller subgroups of Class A lactamases with only 12 confirmed variants.

The VEB enzymes tend to be found frequently in non fermenter organisms such as *P.aeruginosa* and *Acinetobacter* as well as in other Enterobacteriaceae spp. and their prevalence is growing (Akinci and Vahaboglu, 2010). (Schauer *et al.*, 2022) reported that blaPER-1, the gene encoding ESBL PER-1, was situated close to the gene of a putative transposase in epidemic strains of *P. aeruginosa*. PER-1, which is responsible for high-level resistance to Ceftazidime, has been found in *Acinetobacter* spp. and *P. aeruginosa* isolates from Turkey, Belgium , Italy and France . However, the genetic structures bearing blaPER-1 in all these bacteria have not been elucidated completely.

4.6 Detection of VEB genes by real-time PCR of *P.aeruginosa* isolates.

The presence of Vietnamese extended-spectrum beta-lactamase gene VEB was investigated by using the RT-PCR technique, as it was 33 specimens out of 36, that contained the gene and the percentage was 91.7% , while 3 specimens out

of 36 specimens did not contain the gene, and the percentage was 8.3%. Table(4-12).

Table (4-12): Distributiong of VEB Genes of *P. aeruginosa*.

Result	No. of samples	%
Positive	33	91.7
Negative	3	8.3
Total	36	100

The researchers Haghighi and Goli, 2022 found that 40% of pathogenic isolates of *P.aeruginosa* bacteria contain the VEB gene. Among the ESBLs, the frequency of distribution of bla VEB-1gene and blaGES-5 gene was 50% and 40 %, respectively. Bacterial isolates simultaneously carrying blaVEB-1 gene with multiple β-lactamases of different classes of biofilm, MDR, PDR, and XDR(Ali *et al*,2021)

4.7 Sequence Analysis

This study include sequences analysis for 15 *P. aeruginosa* isolates for 3 genes ($bla_{OXA-145}$, $bla_{OXA-488,}$ and *PER-1*), the incorrect sequences were trimmed and the correct sequences were submitted for NCBI BLASTN for similarity. The results revealed that, all 5 sequences of $bla_{OXA-145}$ amplicon give similarity (PsA1=82% , PsA2=91% , PsA3=84% , PsA4=87% , PsA5=89%) (Figure 4.15-4.19) , All 5 sequences of $bla_{OXA-488}$ amplicon give similarity (PsA6=90% , PsA7=88% , PsA8=85% , PsA9=89% , PsA10=91%) (Figure 4.20-4.24) and there was 5 sequences which were PER-1amplicon give similarity (PsA11=95% , PsA12=87% , PsA13=88% , PsA14=81% , PsA15=87%) (Figure 4.25 -4.29) as shown in Table (4-13).

Table 4-13: Alignment results of 15 *P. aeruginosa* isolates with reference isolates retired from NCBI .

Isolate No.	Local Isolate	Reference of the isolate with highest percentage similarity(%)		
		Gene	Accession No.	Similarity(%)
1.	*P. aeruginosa* PsA20	$Bla_{OXA-145}$	CP124674.1	82
2.	*P. aeruginosa* PsA21	$Bla_{OXA-145}$	CP124674.1	91
3.	*P.aeruginosa* PsA22	$Bla_{OXA-145}$	CP124674.1	84
4.	*P.aeruginosa* PsA23	$Bla_{OXA-145}$	CP124674.1	87
5.	*P.aeruginosa* PsA24	$Bla_{OXA-145}$	CP124674.1	89
6.	*P. aeruginosa* PsA1	$Bla_{OXA-488}$	CP050323.1	90
7.	*P. aeruginos* PsA2	$Bla_{OXA-488}$	CP050323.1	88
8.	*P.aeruginosa* PsA8	$Bla_{OXA-488}$	CP050323.1	85
9.	*P.aeruginosa* PsA9	$Bla_{OXA-488}$	CP050323.1	89
10.	*P.aeruginosa* PsA12	$Bla_{OXA-488}$	CP054472.1	91
11.	*P.aeruginosa* PsA29	*PER-1*	NG_068210.1	95
12.	*P. aeruginosa* PsA30	*PER-1*	NG_068210.1	87
13.	*P.aeruginosa* PsA31	*PER-1*	NG_068210.1	98
14.	*P.aeruginosa* PsA32	*PER-1*	NG_068210.1	81
15.	*P.aeruginosa* PsA33	*PER-1*	NG_068210.1	87

Conclusions and Recommendation

Conc1usions

1. *P. aeruginosa* isolates showed high levels of resistance toward the antibiotics used in this study were seen, especially toward the Pencillin.
2. the colistin and polymyxin B were the most active antibiotic against this pathogen due to the lack of use of these antibiotics in hospitals.
3. When using combination antibiotics, the resistance of *P. aeruginosa* decreased towards these antibiotics.
4. Presence of MBLs genes among non-MDR isolates may establish real threat of resistance posibility in *P. aeruginosa* isolates documented as sensitive .
5. Resistane to 5, 6 and 7 classes of antibiotics can push alarm for emergence of XDR or even PDR isolates.
6. The specific primers of PCR assay of *P. aeruginosa* were highly sensitive and reliable for molecular identification of *P. aeruginosa* .
7. The following genes were detected (PER , Ges-2 and GES) and were not found in the samples used in this study.
8. The *bla* $_{OXA-10}$, *bla* $_{OXA-488}$, *bla* $_{OXA-145}$, *bla*$_{OXA-181}$,VEB and PER-1 genes of *P. aeruginosa* isolates showed high expressed.

Recommendations:

1- It is need to carry out comprehensive study for OXA-group(*bla* $_{OXA-10}$, *bla* $_{OXA-488}$, *bla* $_{OXA-145}$, *bla*$_{OXA-181}$,) PER-1, VEB, PER , GES-2 and GES group gene sequences for detecting the types and the sites of mutations in these genes .

2- There is a need to control the random use of antibiotics without medical advice to decrease the rate and the severity of infections.

3- Study other genes related with β-lactam resistance .

4- It is important to use the sensitivity test in the hospitals for the purpose of investigation the resistance abilty of *P. aeruginosa* toward antibiotics.

5- Study virulence factor genes of *P. aeruginosa* that related with the Antibiotic resistant.

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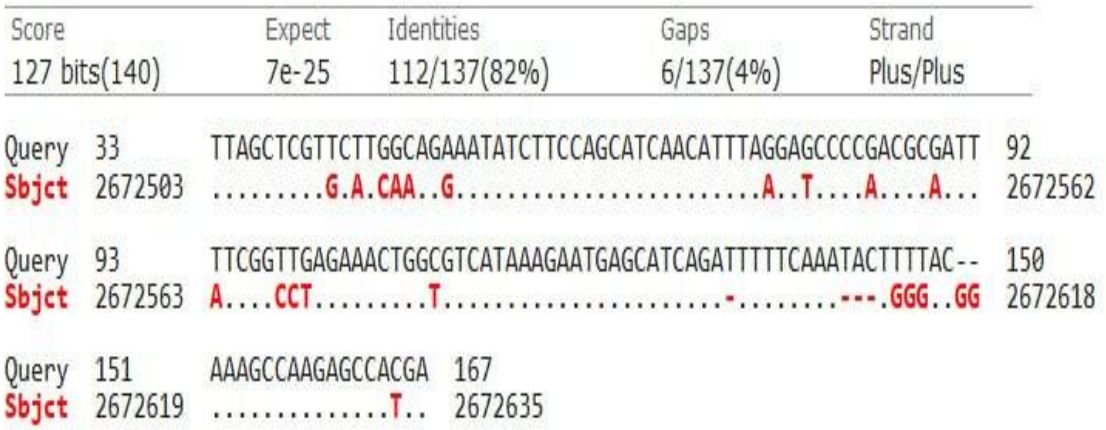

Score	Expect	Identities	Gaps	Strand
127 bits(140)	7e-25	112/137(82%)	6/137(4%)	Plus/Plus

```
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Sbjct  2672503  .........G.A.CAA..G.......................A..T....A....A...  2672562

Query  93       TTCGGTTGAGAAACTGGCGTCATAAAGAATGAGCATCAGATTTTTCAAATACTTTTAC--  150
Sbjct  2672563  A....CCT.........T.....................-........---.GGG..GG  2672618

Query  151      AAAGCCAAGAGCCACGA  167
Sbjct  2672619  ..............T..  2672635
```

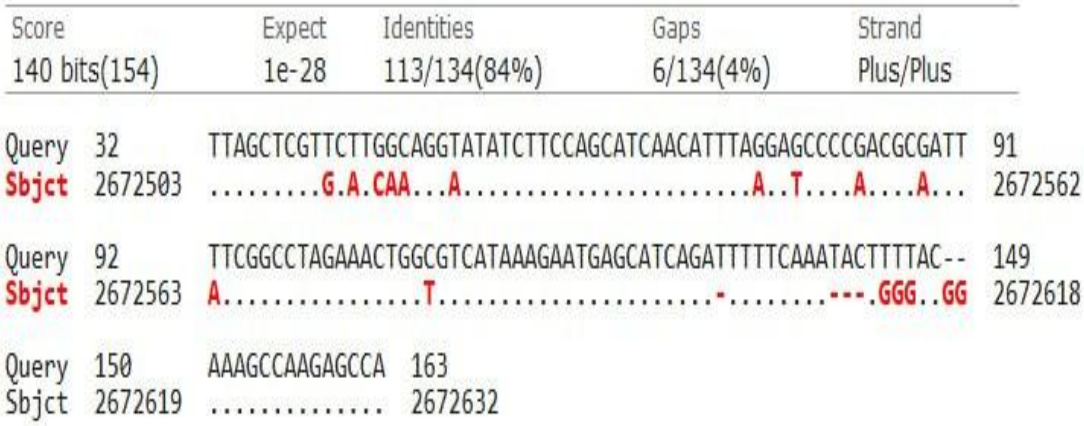
Figure (4-15): DNA sequences alignment of PsA1 genotyped specimens with their corresponding reference sequences of the 204 bp amplicons of the downstream portion of the *Bla*$_{OXA-145}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
121 bits(65)	6e-23	82/90(91%)	1/90(1%)	Plus/Plus

```
Query  52   AAT-TCTTCCAGCATCAACAATAAAGAGCCCCGACGCGATTATCGGTTTAGAAACTGGCG  110
Sbjct  101  ...A................T.T....T.........A.........CC..........T.  160

Query  111  TCATAAAGAATGAGCATCAGGTTTTCAAAT  140
Sbjct  161  .............................  190
```

Figure (4-16): DNA sequences alignment of PsA2 genotyped specimens with their corresponding reference sequences of the 204 bp amplicons of the downstream portion of the *Bla*$_{OXA-145}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .



Score	Expect	Identities	Gaps	Strand
140 bits(154)	1e-28	113/134(84%)	6/134(4%)	Plus/Plus

```
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Sbjct  2672503  .........G.A.CAA...A.....................A..T....A....A...  2672562

Query  92       TTCGGCCTAGAAACTGGCGTCATAAAGAATGAGCATCAGATTTTTCAAATACTTTTAC--  149
Sbjct  2672563  A...............T.....................-........---.GGG..GG  2672618

Query  150      AAAGCCAAGAGCCA  163
Sbjct  2672619  ..............  2672632
```
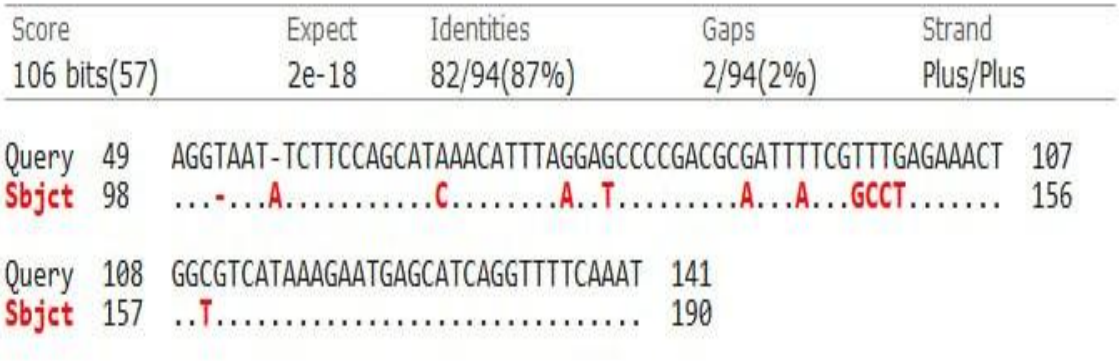

Figure (4-17): DNA sequences alignment of PsA3 genotyped specimens with their corresponding reference sequences of the 204 bp amplicons of the downstream portion of the *Bla*$_{OXA-145}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .



Score	Expect	Identities	Gaps	Strand
106 bits(57)	2e-18	82/94(87%)	2/94(2%)	Plus/Plus

```
Query  49   AGGTAAT-TCTTCCAGCATAAACATTTAGGAGCCCCGACGCGATTTTCGTTTGAGAAACT  107
Sbjct  98   ...-...A...........C........A..T.........A...A...GCCT.......  156

Query  108  GGCGTCATAAAGAATGAGCATCAGGTTTTCAAAT  141
Sbjct  157  ..T.............................  190
```

Figure (4-18): DNA sequences alignment of PsA4 genotyped specimens with their corresponding reference sequences of the 204 bp amplicons of the downstream portion of the *Bla*$_{OXA-145}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
163 bits(88)	9e-36	117/131(89%)	1/131(0%)	Plus/Plus

```
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Sbjct  2672503  .........G.A.CAA.....TA.....................A.......A....A...  2672562

Query  93       TTCGGCCTATAAACTGGTGTCATAAAGAATGAGCATCAGATTTTTCAAATGGGACGGAAA  152
Sbjct  2672563  A........G.............................-.....................  2672621

Query  153      GCAAAGAGCCA  163
Sbjct  2672622  ..C........  2672632
```

Figure (4-19): DNA sequences alignment of PsA5 genotyped specimens with their corresponding reference sequences of the 204 bp amplicons of the downstream portion of the *Bla*$_{OXA-145}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
233 bits(126)	8e-57	163/181(90%)	2/181(1%)	Plus/Minus

```
Query  16     AGC-CCTCCATGCCTTCTGCCTCGCGGA-GTATCTCAGCGACTTTTCGGGCGCTCAGATC  73
Sbjct  14052  ...A............GA.........T..................A....GC......  13993

Query  74     CAGGGGCAGCGACTTTCGGTGAGGGTCCGGCCAATATCCATCAGGATTTGTGAGACCAGC  133
Sbjct  13992  ....C....GC.................................C.....CC.......  13933

Query  134    TTTTCCAGTTCGCTTTCAGACAACTCGGGGAAGCGCTTGGACAGGGTGCTCCACACTACC  193
Sbjct  13932  ............G.....................A.........T..A...G.......  13873

Query  194    T  194
Sbjct  13872  .  13872
```

Figure (4-20): DNA sequences alignment of PsA6 genotyped specimens with their corresponding reference sequences of the 226 bp amplicons of the downstream portion of the *Bla*$_{OXA-488}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
220 bits(119)	6e-53	164/186(88%)	2/186(1%)	Plus/Minus

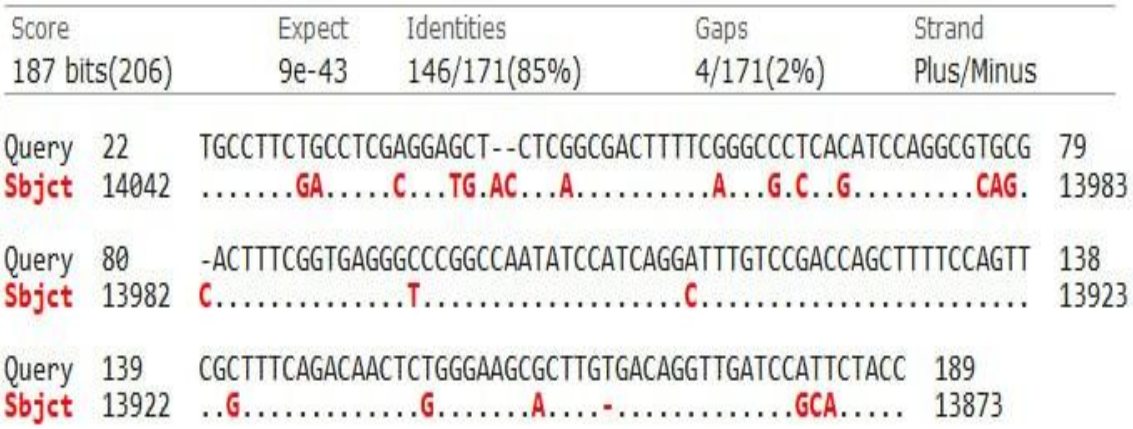
```
Query  14     CTCCAGC-CCTCTGTGCCTTCTGCCTCGCGGATGTATCTCGGCGACTTTTCGGGCCCTCA  72
Sbjct  14056  .......A....CA.......GA.................A..........A...GGC..  13997

Query  73     GATCCAGGGGCAGCGACTTTCGGTGAGGGTCCGGCCAATATCCATCAGGATTTGTGAGAC  132
Sbjct  13996  ........C....GC.................................C.....CC...  13937

Query  133    CAGCTTTTCCAGTTCGCTTTCAGACAACTCGGGGAAGCGCTTGGACAGGTTGCTCAAACA  192
Sbjct  13936  ...............G.....................A.............A..CG-..  13878

Query  193    CTACCT  198
Sbjct  13877  ......  13872
```

Figure (4-21): DNA sequences alignment of PsA7 genotyped specimens with their corresponding reference sequences of the 226 bp amplicons of the downstream portion of the *Bla*$_{OXA-488}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .



Score	Expect	Identities	Gaps	Strand
187 bits(206)	9e-43	146/171(85%)	4/171(2%)	Plus/Minus

```
Query  22     TGCCTTCTGCCTCGAGGAGCT--CTCGGCGACTTTTCGGGCCCTCACATCCAGGCGTGCG  79
Sbjct  14042  .......GA.....C...TG.AC...A..........A...G.C..G.........CAG.  13983

Query  80     -ACTTTCGGTGAGGGCCCGGCCAATATCCATCAGGATTTGTCCGACCAGCTTTTCCAGTT  138
Sbjct  13982  C..............T...................C.........................  13923

Query  139    CGCTTTCAGACAACTCTGGGAAGCGCTTGTGACAGGTTGATCCATTCTACC  189
Sbjct  13922  ..G.............G.......A....-.............GCA.....  13873
```

Figure (4-22): DNA sequences alignment of PsA8 genotyped specimens with their corresponding reference sequences of the 226 bp amplicons of the downstream portion of the *Bla*$_{OXA-488}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
231 bits(255)	9e-56	165/186(89%)	3/186(1%)	Plus/Minus

```
Query  14     CTCCAGCAGTTCTGTGCCTTCTGACCTCGAGGA-GTACGTCAGCGACTTTTCAGGCGCTC  72
Sbjct  14056  ........CC..CA.......-........C...T....C..................C.  13998

Query  73     AGATCCAGGC-CAGGGACTTTCGGTGAGGGCCCGGACAATATCCATCAGGCTTTGTCCAA  131
Sbjct  13997  ..........G....C..............T....C......................G.  13938

Query  132    CCAGATTTTCCAGATCGGTTTCAGAAAACTCGGGGAAGCGCTTGGACAGGTTGAGCCAAA  191
Sbjct  13937  ....C........T...........C.............A..............T..GC.  13878

Query  192    CTACCT  197
Sbjct  13877  ......  13872
```

Figure (4-23): DNA sequences alignment of PsA9 genotyped specimens with their corresponding reference sequences of the 226 bp amplicons of the downstream portion of the *Bla*$_{OXA-488}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
248 bits(134)	3e-61	169/186(91%)	1/186(0%)	Plus/Minus

```
Query  13     CTCCAGCCGTTCTGTGCCTGCTGACCTCGAGGATGTACCTCAGCGACTTTTCAGGCGCTC  72
Sbjct  14549  .......AC...CA.....T.-........C............................C.  14491

Query  73     AGATCAAGGCACAGGGACTTTCGGTGAGGGTACGGAAAATATCCATCAGGCTTTGGCCGA  132
Sbjct  14490  .....C.........C...................CC..................T....  14431

Query  133    CCAGCTTTTCCAGTTCGGTTTCGGACAACTCGGGGAAGCGCTTGGACAGGTTGCTCAACA  192
Sbjct  14430  ....................................A.............A..CG..  14371

Query  193    CTACCT  198
Sbjct  14370  ......  14365
```

Figure (4-24): DNA sequences alignment of PsA10 genotyped specimens with their corresponding reference sequences of the 226 bp amplicons of the downstream portion of the *Bla*$_{OXA-488}$ gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

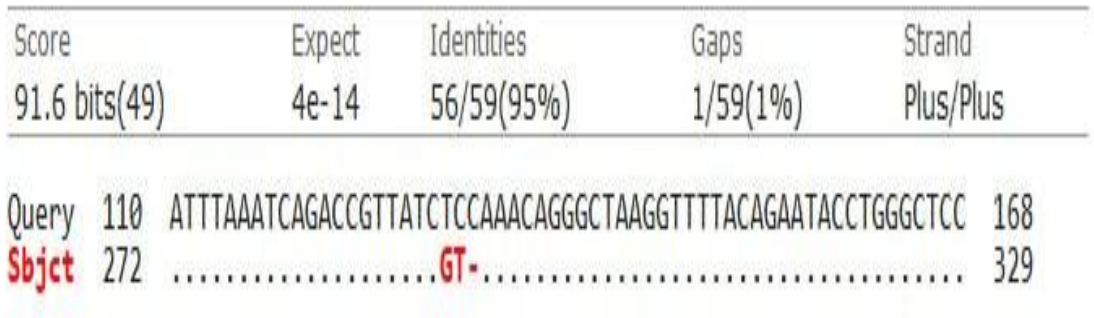


Figure (4-25): DNA sequences alignment of PsA11 genotyped specimens with their corresponding reference sequences of the 198 bp amplicons of the downstream portion of the *PER-1* gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

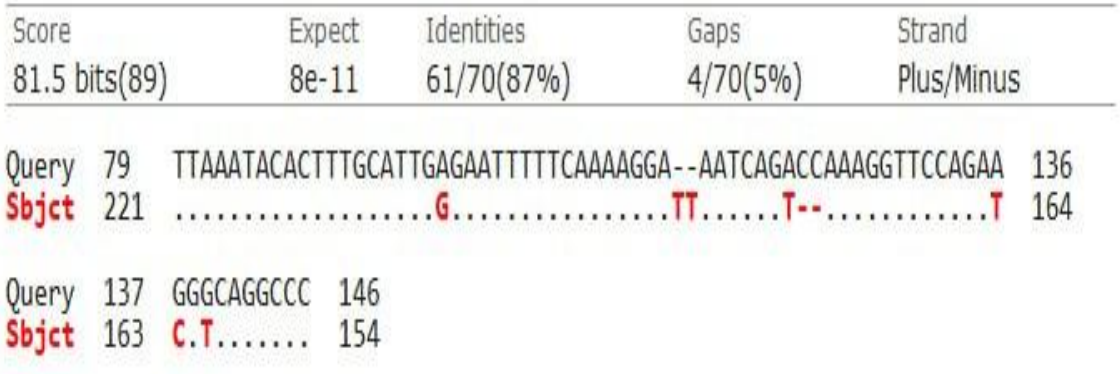


Figure (4-26): DNA sequences alignment of PsA12 genotyped specimens with their corresponding reference sequences of the 198 bp amplicons of the downstream portion of the *PER-1* gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
101 bits(111)	9e-17	57/58(98%)	0/58(0%)	Plus/Plus

```
Query  112  ATTTAAATCAGACCGTTATCGTAAACAGGGCTAAGGTTTTACAGAATACCAGGGCTCC  169
Sbjct  272  ..........................................................T.....  329
```

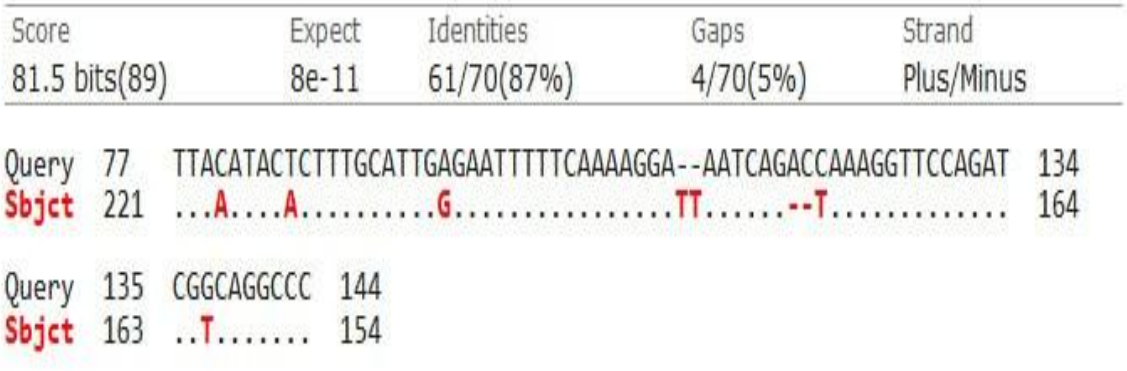
Figure (4-27): DNA sequences alignment of PsA13 genotyped specimens with their corresponding reference sequences of the 198 bp amplicons of the downstream portion of the *PER-1* gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

Score	Expect	Identities	Gaps	Strand
57.2 bits(62)	0.001	52/64(81%)	4/64(6%)	Plus/Minus

```
Query  80   TTACATACTCTTTGCATTGAGAATTTGTCAAATTTA--AATCAGACCAAAGGTTCCAGAT  137
Sbjct  221  ...A....A..........G......T.....AGG.TT......T--.............  164

Query  138  CGTC  141
Sbjct  163  ....  160
```

Figure (4-28): DNA sequences alignment of PsA14 genotyped specimens with their corresponding reference sequences of the 198 bp amplicons of the downstream portion of the *PER-1* gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .



Score	Expect	Identities	Gaps	Strand
81.5 bits(89)	8e-11	61/70(87%)	4/70(5%)	Plus/Minus

```
Query  77   TTACATACTCTTTGCATTGAGAATTTTTCAAAAGGA--AATCAGACCAAAGGTTCCAGAT  134
Sbjct  221  ...A....A..........G.................TT......--T.............  164

Query  135  CGGCAGGCCC  144
Sbjct  163  ..T.......  154
```

Figure (4-29): DNA sequences alignment of PsA15 genotyped specimens with their corresponding reference sequences of the 198 bp amplicons of the downstream portion of the *PER-1* gene. The symbol "Query." refers to the NCBI referring sequence, "Sbjct" refer to the genotyped .

الخلاصة:

الزوائف الزنجارية هي بكتيريا هوائية سالبة الجرام ظهرت كواحدة من أكثر مسببات الأمراض إشكالية التي تميز سلالاتها المنتشرة في العراق. تم جمع ١٩٤ عينة والتي كانت كالاتي من جروح (٩٦) ، قدم سكري (١٣) ، ادرار (٥) ، سائل رئوي (٢) ، طلق ناري وعبوات ناسفة (٥) وحروق (٧٣) مسحة من المرضى. تم نقل العينات من هؤلاء المرضى الراقدين في مستشفيات مدينة الحلة (مستشفى الحلة التعليمي ، مستشفى مرجان ، مستشفى الإمام الصادق) ، في مستشفيات كركوك (مستشفى كركوك العام ، مستشفى آزادي التعليمي) وكذلك مستشفيات مدينة الطب في بغداد (الحروق التخصصية ، الشهيد غازي الحريري ، مركز بغداد التعليمي ، والمركز الوطني للمختبرات التعليمية ومستشفى ابن البيطار) وقد كانت العينات من كلا الجنسين باختلاف الأعمار. تم جمع العينات خلال الفترة من تموز ٢٠٢٢ إلى تشرين الأول ٢٠٢٢.

تم تحديد ٣٦ من اصل ١٩٤ عزلة (١٨.٥٪) برمز (PsA1 إلى PsA36) على أنها الزوائف الزنجارية باستخدام الصفات المظهرية وتم زراعتها على الاوساط (أجار الدم ، أجارماكونكي ، سترومايد أجار وكروموجينك اجار) ، الخصائص المجهرية ، والاختبارات الكيميائية الحيوية ، بينما ١١١ لوحظ أن (٥٧.٢٪) من العينات لديها نمو بكتيري آخر و ٤٧ (٢٤.٣٪) لا يوجد بها نمو بكتيري (غير معروفة).

تم استخلاص الحمض النووي من عزلات الزوائف الزنجارية باستخدام مجموعة صغيرة لاستخلاص DNA الجينومي البكتيرية وقياس نقائها باستخدام(nanodrop (1.8-2 نانومتر. تم تأكيد التعرف على *P. aeruginosa* باستخدام تفاعل البوليميراز المتسلسل (PCR) باستخدام 16SrRNA ، وأظهرت النتيجة أن جميع العزلات الـ ٣٦/٣٦ ١٠٠٪ كانت من عزلات *P. aeruginosa*

تم إجراء اختبار الحساسية للمضادات الحيوية ضد ١٧ نوعًا من المضادات الحيوية ، وذلك باستخدام طريقة انتشار القرص وفقًا لمعهد المعايير السريرية للبكتيريا المسببة للأمراض في المختبر السريري ، CLSI,2023. أظهرت النتائج أن جميع العزلات الـ ٣٦ كانت شديدة المقاومة ١٠٠٪ للتيكارسيلين والبيبراسيلين ، السيفتازيديم ٨٨.٩٪ سيفيبيم ٥٠٪ ، أزتريونام ٥٨.٣٪ سيبروفلوكساسين ٤١.٦٪، الليفوفلوكساسين ٣٦.١٪ ، الإيميبينيم ٥٢.٧٪ ، ميروبينيم ٤١.٧٪ ، توبراميسين ٨٣.٣٪ جنتاميسين ٦٦.٧٪ ، أميكاسين ٨٣.٣٪ ، نورفلوكساسين ٣٨.٩٪ ، بيبيراسيلين-تازوباكتام ٤١.٧٪ ، دوريبينيم ٣٨.٩٪ ، كولستين ٨.٣٪ وبوليميكسين ب ٨،٣٪ .

تم إجراء فحص إنزيم ESBLs β-lactamase ممتد الطيف بواسطة اختبار Double Disc Synergy DDS على وسط استنبات صلب (Muller Hinton agar) في طبق بتري. تم استخدام طريقة القرص المركب بناءً على التأثير المثبط لحمض clavulanic وفقًا إلى (CLSI, 2023).أظهرت النتائج أن عزلات *Pseudomonas aeruginosa* كانت موجبة بنسبة ٣٦/٤(١١.١%) العزلات هي (PsA32, PsA33, PsA34 و PsA36)

. تم استخدام تفاعل البوليميراز المتسلسل لفحص ١٠ جينات للطيف الموسع (ESBLs) β-lactamase في عزلات *P. aeruginosa* باستخدام مادة أولية محددة لكل جين من bla OXA-10 و bla OXA-488 و bla OXA-145 و blaOXA-181 و PER-1 و VEB و PER و Ges-2 و GES. بعد إجراء الرحلان الكهربائي ، أظهرت النتائج أن *P. aeruginosa* تحتوي على bla OXA-145 34/36 (94.44%)، PER-1 31/36 (86.1%) , bla OXA-10 30/36 (83.33%)، bla OXA-488 24/36 (66.6%) ، blaOXA-181 21/36 (58.3%) ، ولا توجد نتيجة لثلاثة برايمر كانت PER و GES-2 و GES.

أظهرت نتائج اختبار تفاعل البوليميراز المتسلسل في الوقت الحقيقيReal-Tim لـ VEB أن ٣٦/٣٣ (٩١.٧٪) من عزلات *P. aeruginosa* كانت إيجابية.

تم إرسال تحليل تسلسل الحمض النووي الجينومي الكامل لـ ١٥ عزلة (PsA1 إلى PsA15) من *P. aeruginosa* لـ ٣ جينات (bla OXA-145 و bla OXA-488 و bla PER-1) إلى شركة Macrongen في كوريا ، التسلسلات غير الصحيحة تم قطعها وتقديم التسلسلات الصحيحة لـ NCBI BLASTN للتشابه ، وأظهرت مطابقة عزلات الدراسة الحالية مع سلالات الزوائف الزنجارية العالمية لبنك NCBI-Gen نسبة التشابه بين العزلات الحالية تتراوح من (٨١٪ - ٩٨٪).

في الختام ، عند دراسة العينات ، كانت الجروح هي أكثر مواقع عزل مقاومة الأدوية المتعددة (MDR) ٣٦/١٩ % ٥٢.٨ *P. aeruginosa* تليها الحروق مختلفة ، وكان جين bla OXA-145 هو الجين الأكثر شيوعًا بين هذه العزلات ، يليه VEB، PER-1 وجين bla OXA-10 و bla OXA-488 وجين blaOXA-181

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

دراسة جزيئية لبعض جينات β-lactamase بين بكتريا *Pseudomonas aeruginosa* ذات المقاومة المتعددة للأدوية (MDR)

رسالة مقدمه الى

مجلس كلية العلوم-جامعة بابل جزء من متطلبات نيل درجة الماجستير في كلية العلوم/قسم علوم الحياة

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APPENDIX