Republic of Iraq

Ministry of Higher Education

and Scientific Research

University of Babylon, College of Science

Department of Biology

Molecular Characterization of the Extended Spectrum β-Lactamase Genes in *Pseudomonas aeruginosa*

A Thesis

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

By

Mustafa Abed AL-jabber Mohammed Saleh Ali

B. Sc. College of Science / University of Babylon (2019)

Under supervision of

Prof. Dr. Hassan Fadhil Naji

2022 A.D **1444A.H.**

(ذَٰلِكَ ٱلْفَضْلُ مِنَ ٱللَّهِ ۚ وَكَفَىٰ بِٱللَّهِ عَلِيمًا)

صَدَقَ الله العَلي العَظيم

سورة النساء/الآية ٧٠

Supervisor Certification

I certify that this thesis was prepared under my supervision at the department of biology, college of science, university of Babylon, as partial fulfillment of the requirement for the degree of master in Biology/ Biotechnology.

Signature
Prof. Dr. Hassan Fadhil Naji
College of Science / University of Babylon
(Supervisor)
2022

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Signature
Assist Prof. Dr. Adi Jassim Abd AL-Rezzaq
Head of the Department of Biology/
College of Science/University of Babylon
2022

Dedication

To soul of my father…

To my mother who surrounds me with love…

To my wife and love of my life …

To my friend Safaa Jassim and those who have innocent smiles, clear souls, diamond hearts who are kind and helpful…

I dedicated this humble work

Mustafa

Acknowledgments

In the name of "Allah" who deserves all praise and thanks for inspiring me with the willingness, strength, and patience to establish this work, peace and pray upon his messenger. The head of the creatures, Prophet Mohammed, his family, and his companions.

I would like to express my sincere gratitude and appreciation to my brilliant supervisor Prof. Dr. Hassan Fadhil Naji for his guidance, patience, continuous support, and valuable advice during the work, words can never express my thanks to him.

Sincere respect goes to the deanship and its employees, teachers who taught me the right way, and affiliated members of the Department of Biology, College of Science, and the University of Babylon to provide me with an opportunity to confer my M.Sc.

Deepest thanks to Asst. Prof. Dr. Noor Salman Kadhim for her continued assistance during the period of research.

Deep love, respect, and acknowledgment to my father soul, my wife, my mother, my sibling, my friends, the patients, and my colleagues.

Thanks to all I love and all who loved me.

Mustafa

Summary

One hundred fifty specimens of both genders and ages were collected from Babylon hospitals (Al-Hillah Teaching Hospital, Mirgian and Imam AL-Sadiq Teaching Hospital) and from some Baghdad hospitals (Burns Specialized Hospital, AL-Shaheed Ghazi Hariri Hospital, Baghdad Teaching Hospital, and National Center for Educational Laboratories).The specimens were divided into two groups, the first group included fiftyfive swabs obtained from burns and the second group included ninety five swabs obtained from different injuries. These specimens were collected from September 2021 to January 2022.

Forty-six (30%) isolates named (PA1 to PA46) were identified as *Pseudomonas aeruginosa* using morphological, cultureal and microscopical properties, biochemical tests, and Vitek 2 compact, while 54 (36%) isolates were for other bacteria obtained from wounds specimens and 50 (33.3%) of specimens were observed to have no bacterial growth. DNA was extracted from *P. aeruginosa* isolates using bacterial favorgen genomic DNA extraction mini kit and measured its purity using nanodrop (1.96-2.49 nm). The identification of *P. aeruginosa* was confirmed using polymerase chain reaction (PCR) sequencing *P. aeruginosa* specific primer, the sequence analysis confirms the identification 100 % to the results of morphological (cultural and microscopically) properties, biochemical tests and VITEK® 2 Compact. 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-2021. The results showed that all 46 isolates were highly resistant (100%) to tobramycin, piperacillin, cefepime, imipenem, ofloxacin, aztreonam, netilmicin, while for norfloxacin was (39%), piperacillin-tazobactam (26%), levofloxacin (39%), amikacin (74%), meropenem (41%), ciprofloxacin (43%), doripenem (37%), gentamicin (87%), ceftazidime (87%) and gatifloxacin (28%). Bacterial biofilm formation has been

studied using microtiter plates assay, which was considered the most sensitive, and the results showed that 34 isolates were able to produce of biofilm. PCR has been used to investigate 3 genes for extended-spectrum β-lactamase (ESBLs) in *P. aeruginosa* by using a specific primer for each gene of *bla* $_{TEM}$, *bla* $_{SHV}$, and *bla* $_{CTX-M}$. After performing the electrophoresis, the results showed that *P. aeruginosa* has *bla* $_{TEM}$ 26/46 (58.69%), *bla* $_{SHV}$ 28/46 (63%), and *bla* $_{CTX-M}$ 31/46 (67.39%). It was found that all isolates of *P. aeruginosa* were at a rate of 95-100 % identical to the sources of the strains identified in the gene bank (NCBI), and the results showed that the highest matching rate of isolates was 100 % which originated in Egypt. This study revealed that *bla* $_{CTX-M}$ gene was the most frequently gene among these isolates, followed by *bla* $_{SHV}$ gene and then by *bla* $_{TEM}$.

The whole genomic DNA of nine isolates, PA1 to PA9, isolated from different geographic regions of Iraq, was extracted. The sequencing of *bla* $_{TEM}$, *bla* $_{SHV}$, and *bla* $_{CTX-M}$ genes was performed, and the sites of these genes on the genome of the isolates were determinate. The phylogenetic tree analysis, using MEGA X10.2.4 software program was achieved, and the matching of the current study isolates with NCBI-Gen bank global *Pseudomonas* strains, elicited that one isolate (PA1) was originated in UAE, two isolates (PA2 and PA3) were originated in India, three isolates (PA4, PA5 and PA6) were originated in Egypt and also three isolates (PA7, PA8 and PA9) were originated in Iran. Hence, variable frequencies in the sequencing of *bla* $_{TEM}$, *bla* $_{SHV}$, and *bla* $_{CTX-M}$ genes need further studies for creating genetic diversity map of *P. aeruginosa*.

In conclusion, in studying the samples, burns are the common sites for isolating multidrug resistance (MDR) *P. aeruginosa* followed by different wounds. *P. aeruginosa*-specific gene 16S rRNA -based PCR assay is highly accurate and reliable for the identification of *P. aeruginosa*, and the *bla* $_{CTX-M}$ gene was the most frequently gene among these isolates followed by *bla* $_{SHV}$ gene and *bla* $_{TEM}$ gene.

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List of Abbreviations

NO.	Symbol	Description
1.	AK	Amikacin
2.	AST	Antimicrobial susceptibility testing
3.	ATM	Aztreonam
4.	AMEs	Aminoglycoside-modifying enzymes
5.	AACs	Aminoglycoside acetyltransferases
6.	ANTs	Aminoglycoside nucleotidyltransferases
7.	AmpC	Ampicillin resistant gene
8.	ACC	Ambler class C
9.	AMR	Antimicrobial resistance
10.	API	Analytical profile index
11.	BP	Base Pair
12.	CAZ	Ceftazidime
13.	CMY	Cephamycins
14.	CLSI	Clinical Laboratory Standard Institute

15.	DDT	Dichlorodiphenyltrichloroethane
16.	DNA	Deoxyribonucleic acid
17.	ESBLs	Extended Spectrum Beta Lactamase
18.	FQ	Fluoroquinolones
19.	FOX	Cefoxitin
20.	G + C	Guanine + Cytosine
21.	H2O2	Hydrogen peroxide
22.	IMVC	Indole, Methyl red, Voges–Proskauer, Simmone Citrate
23.	KPC	Klebsiella pneumoniae carbapenemases
24.	LPS	Lipopolysaccharide
25.	MBL	Metalo Beta Lactamase
26.	MDR	Multidrug-resistant
27.	MGEs	Mobile genetic elements
28.	ME	Minimal evolution
29.	NCBI	National Center for Biotechnology Information
30.	NJ	Neighbor-Joining
31.	ORF	Open Reading Frame
32.	PCR	Polymerase Chain Reaction
33.	CIP	Ciprofloxacin
34.	CLSI	Clinical and Laboratory Pathogenic Bacteria Standard Institute
35.	CN	Gentamicin
36.	D.W	Distil water
37.	DDS	Double disk diffusion
38.	TEM	*Temoneira* Family
39.	SHV	*Sulfhydryl* Family
40.	CTX-M	*Cefotaximase* Family
41.	RNA	Ribonucleic acid
42.	SBLs	Serine-□-lactamases
43.	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
44.	TBE	Tris Borate
45.	TSB	Tryptic soy broth
46.	TCP	Tissue Calture Plate
47.	UV	Ultra violet
48.	WHO	World Health Organization
49.	WGS	Whole genome sequencing
50.	IPM	Imipenem
51.	LEV	Levofloxacin
52.	LPS	Lipopolysaccharide
53.	MEM	Meropenem
54.	NET	Netilmicin
55.	NS	normal saline

56.	OFX	Ofloxacin
57.	OXA	Oxacillinases
58.	PBPs	Penicillin binding protiens
59.	PBS	Phosphate Buffer Saline
60.	PCR	polymerase chain reaction
61.	PRL	Piperacillin
62.	PTZ	Piperacillin-tazobactam

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Chapter One

Introduction

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 (Crone *et al*., 2020). It is difficult to treat infections of this pathogen in burned patients, especially those that are multidrug-resistant (MDR) (Safaei *et al*., 2017; Salimi *et al*., 2010). The growing incidence of MDR strains (Chauhan *et al*., 2012) is linked to longer hospitalization and a considerable increase in inpatient mortality and morbidity (Cholley *et al*., 2010).

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 (de Almeida *et al.*, 2017). 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). Some of the most common ESBL genes found in *P. aeruginosa* 3 genes of β-lactamases A are the *SHV*, *CTX-M*, and *TEM* kinds, which all belong to the sulfhydryl variable (*SHV*) family. As the name suggests, these enzymes have evolved from the first plasmid-mediated beta-lactamase to be discovered back in the early 1960s, *TEM*-1 (Datta and Kontomichalou, 1965). More than any other class A enzyme in Gram-negative bacteria, the enzyme encoded by *TEM-1* is most well investigated. Gram-negative bacilli's most frequent β-lactam resistance mechanism, they are quickly spreading over the globe (Eiamphungporn *et al.*, 2018; Seyedjavadi *et al*., 2016). Most antibiotics, including penicillin and first-generation cephalosporins like cephaloridine, are resistant to bla_{TEM}. Amino acid substitutions have resulted in the development of newer, more potent, and more effective forms of antibiotic resistance (ESBL) (Peymani *et al*., 2017).

The *SHV*-type β-lactamases (so termed for the sulfhydryl reagent variable) evolved in *Klebsiella pneumonia* as chromosomally encoded enzymes (Livermore, 1995). The first ESBL discovered in 1985 was *SHV*-2, which varied from *SHV*-1 by a single amino acid alteration of Glycine to Serine at position 238 in a single strain of *Klebsiella ozaena* obtained in Germany (Huletsky *et al*., 1993). At some point in the late 1980s, multiple studies documented the emergence of *CTX-M* type-lactamase enzymes. The inaugural usage of *CTX-M* (cefotaximase from Munich) in a German publication was the origin of the name (MMasoud *et al.*, 2022). *CTX-M*-1, *CTX-M*-2, *CTX-M*-8, *CTX-M*-9, and *CTX-M*-25 are the five groups of *CTX-M* kinds based on the amino acid identities of their amino acids (Bonnet, 2004).

Aim of Study

This study aimed to determine the prevalence of extended-spectrum β-lactamases (*bla_{TEM}*, *bla_{CTX-M}*, and *bla_{SHV}*) genes among the isolates of *P. aeruginosa*. and the phylogenic relationships among these isolates which reflect the international origion of these bacteria in Iraq.

Objectives of Study

1. Isolation and identification of *P. aeruginosa* from different clinical cases (burns and wounds).
2. Determination of the antibiotic susceptibility tests of the isolates against the common antibiotics.
3. Detection of *bla_{TEM}*, *bla_{CTX-M}*, *and* *bla_{SHV}* genes using a molecular methodology.
4. Comparative analysis of the data of this study with those in the gene bank of the NCBI to find the DNA variations which reflect the geographic origion of the isolates.

Chapter tow

Literatures Review

2. Literatures Review

2.1 Historical perspective of *P. aeruginosa*

Pseudomonas was described during the end of the nineteenth century (Tiwari *et al*., 2011) when descriptions of genera were based on the macro and microscopic morphologies, a practice universally acknowledged by microbial taxonomists (Tindall and Garrity, 2008). Physiological qualities were offered as taxonomic criteria for bacteria as early as the 20th century (Palleroni, 2003). In the Bergey's Manual released in 1923, additional phenotypic parameters were added to the morphology, Gram-stain, flagellation type, and metabolism with respect to oxigen in an effort to separate the *Pseudomonas* species (Bergey *et al*., 1923).

Genetic approaches made are possible in bacterial taxonomy when techniques based on DNA (Marmur, 1961; Marmur and Doty, 1961; Schildkraut *et al*., 1961). The first techniques applied to *Pseudomonas taxonomy* were DNA base composition (G+C) and DNA-DNA hybridization (Colwell et al., 1965; Colwell and Mandel, 1964). The G+C content of every *Pseudomonas* species was thus included in the Bergey's Manual beginning in 1974 (Peix *et al*., 2009).

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). 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).

Peix *et al*. (2009) and García-Valdés & Lalucat (2016) determined that the members of the remaining rRNA groups were subdivided into more than 25 genera belonging to the classes Alpha, Beta, and Gamma proteobacteria. These modifications were reflected in the 2005 edition of Bergey's Manual of Systematic Bacteriology, which was converted to an online format in 2015, with each genus constituting a separate chapter. This new format will allow for the information to be updated more regularly, which is crucial given that the number of bacterial genera and species is always rising.

2.2. Current taxonomy of *P. aeruginosa*

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).

Several recently discovered species were pathogenic to animals or plants, such as *P. entomophila*, the entomopathogenic *P. entomophila* (Mulet *et al*., 2012), the fish *Dicologlossa cuneata* pathogen *P. baetica* (Lopez *et al*., 2012), and *P. caspiana*, the pathogenic *P. caspiana* (Kauná *et al*., 2016); (Busquets *et al*., 2017). The soil was the primary source of the remaining species, with 30 new *Pseudomonas* species isolated from this habitat.

There are various *in vitro* plant growth promoting pathways in some of the newly discovered *Pseudomonas* species. Thus, siderophores were discovered in *P. sagittaria* and *P. donghuensis* (Gao *et al*., 2015; Liu *et al*., 2013), phosphate could be solubilized in *P. guariconensis* and *P. helmanticensis* (Ramírez-Bahena *et al*.,

2014; Toro *et al*., 2013), *P. endophytica* had many (Ramette *et al.*, 2011; Tambong *et al*., 2017).

Psychrotrophic species were also isolated from extreme environments, such as *P. guguanensis* and *P. yangmingensis* isolated from hot springs (Liu *et al*., 2013.,; Wong and Lee, 2014) and *P. arsenicoxydans* isolated in the Atacama Desert (Campos *et al*., 2010), as well as *P. deceptionensis, P. prosekii, P. yamanorum,* and *P. gregormendel* (Arnau *et al*., 2015; See-Too *et al*., 2017).

2.3. General Characteristics of *P. aeruginosa*

P. aeruginosa is a Gram-negative, uniformly stained rod that is either straight or slightly bent and is about 5 µm long and 0.5–1.0 µm wide. Aerobic bacteria, don't make spores, and move with the help of one or more polar flagella. They either can't use carbohydrates as a source of energy or break them down through a "oxidative pathway" instead of a "fermentative pathway" (Palleroni, 2003); (Jawetz *et al*., 2019).

P. aeruginosa is common in nature and is often found in hospitals where there is a conditions of warmth, moisture, and nutrition for easy colonization of opportunistic pathogens. It can live in different parts of the body (e.g., mucous membrane, respiratory tract, and gastrointestinal tract). It is known to make people sick, especially those with weakened immune systems (like those with neutropenia, chemo, burns, or wounds) (Juan *et al*., 2017). *P. aeruginosa* grows well at temperatures between 25°C and 37°C, and the fact that it can also grow at 42°C sets it apart from many other *Pseudomonas* species (Tang and Sails, 2014).
P. aeruginosa makes a number of pigments, such as pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), pyorubin (red-brown), and pymelanin (black) (black). Pyoverdin is made in large amounts in environments with little

iron, and it could help the bacteria use iron. Pyocyanin, which comes from the word "Pyocyaneus," is a term for "blue pus," which is a sign of *P. aeruginosa* infections (Kang *et al*., 2019).

P. aeruginosa can become resistant to multiple antibiotics through complex mechanisms called intrinsic, adaptive, and acquired (Breidenstein *et al*., 2011) (Pires *et al*., 2015). It is known that *P. aeruginosa* infections that are hard to treat lead to more deaths, longer hospital stays, and higher hospital costs (Nathwani *et al*., 2014).

2.4. Nosocomial Infections

Infections that are acquired during a course of treatment at a healthcare institution are referred to as nosocomial illnesses. These infections are sometimes referred to as healthcare associated infections (Tchouaket Nguemeleu *et al*., 2020). These infections are inextricably linked to the provision of care, and they are ranked among the most prevalent adverse outcomes that may be avoided (Schwendimann *et al*., 2018). Patients who are hospitalized are more likely to suffer from morbidity and death as a result of nosocomial infections. They also drive up the expense of therapy and extend the length of time spent hospitalized. 7–10% of all infections acquired in healthcare settings are considered to be nosocomial (Mitchell *et al*., 2017). *P. aeruginosa* is one of the most common organisms found in nosocomial and ventilator-associated pneumonia, meningitis, abscess, soft tissue infections, urinary tract infections, catheter-associated infections, corneal infections, and conjunctival erythema. It is also one of the most common causes of conjunctival erythema.

P. aeruginosa strains that are resistant to several drugs can live in hospital settings and are readily passed from one patient to another through the hands of medical professionals (Exner *et al*., 2017). *P. aeruginosa* often possesses an innate

resistance to antimicrobial drugs. This resistance manifests itself in a variety of ways, including efflux pump systems, decreased permeability of the outer membrane, enzymatic inactivation, and biofilm development (Ali *et al*., 2018). As a result, it frequently displays resistance to almost all -lactams, aminoglycosides, and quinolones (Bassetti *et al*., 2018).

The presence of nosocomial isolates of *P. aeruginosa* made the treatment of infections more difficult, had a negative impact on clinical outcomes, and led to an increase in the expenses of patient treatment (Solomon *et al*., 2017). In those with compromised immune systems, such as those who have cystic fibrosis or are receiving chemotherapy, *P. aeruginosa* can cause both acute and chronic infections in the lungs, both of which can be fatal (Chatterjee *et al*., 2016).

P. aeruginosa infections are a prevalent kind of infection that can be contracted in hospitals. The identification of this species required the use of colony morphology, characteristic *P. aeruginosa* pigment production, gram staining, and positive oxidase tests. This species has the potential to cause a broad variety of illnesses, particularly in those who have immunological weaknesses (Sayed Zaki *et al*., 2017).

2.5. 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).

2.6. Wound Infections

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 was made, the presence of a predisposing condition, and living in a city (Jeschke *et al*., 2020).

2.7 *P. aeruginosa* Associated Wound and Burn Infection

P. aeruginosa is one of the most common pathogens isolated from burn patients throughout the world (Sousa *et al.*, 2018). *P. aeruginosa* is an opportunistic bacterium associated with healthcare infections in intensive care units, ventilator-associated pneumonia, surgical site infections, and burns (López-Jácome *et al.*, 2019). Burn wounds infection is a great problem because it may lead to death in 75% of patients with injuries (Santucci *et al.*, 2003).

The undamaged human skin surface is vital to the safeguarding of body fluid homeostasis, thermoregulation, and the host's protection against infection. As the first line of defense, the skin is equipped with arange of immune mediators capable of engaging inflammatory cells to support neutralization and clearance of microbes (Steinstraesser *et al.*, 2004). *P. aeruginosa* is one of the most important pathogens involved in burn infections (Rafla and Tredget., 2011). *P. aeruginosa* is a common nosocomial pathogen in burn patients, and rapidly acquires antibiotic resistance; thus, developing an effective therapeutic approach is the most promising strategy for combating infection (Ranjbar *et al.*, 2019). Especially in burn centers, the high prevalence and progressive increasing of MDR *P. aeruginosa* seriously threats the patients with severe burn injure (de Almeida *et al*.,2017; Dou *et al.*, 2017).

Burn wound infections are one of the most important complications that occur after burn injuries and may be associated with serious clinical complications and

increased morbidity and mortality (Turner *et al.,* 2014). Burn injury compromises the primary barrier of the host, the skin, which immediately places the host at risk for infection (Lopez *et al.,* 2017). Burn wounds are major public health problems all over the world. Infection is one of the most complicated issues in burn patients, because the skin, a barrier against microbes, has been destroyed and the immunity agents cannot reach the sites of infection. There is a correlation between the severity of infection and the extent of the burn (Anvarinejad *et al.,* 2014). *P. aeruginosa* is one of the most common bacteria in nosocomial infections, especially in burn units. Burn patients, because of losing the skin barrier, are very vulnerable to infection (Moghoofei *et al.*, 2015).

New therapeutic agents against *P. aeruginosa,* degrading biofilms in burn wounds and improving the efficacy of current antimicrobial agents, are required (Banar *et al.,* 2016). This bacterium causes 75% of death in burned patients, since it can develop a persistent biofilm associated with infections, express several virulence factors, and antibiotic-resistance mechanisms. Some of these virulence factors are proteases such as elastase and alkaline protease, or toxic metabolites such as pyocyanin and is one of the few microorganisms able to produce cyanide, which inhibits the cytochrome oxidase of host cells (López-Jácome *et al.,* 2019). Multiple antibiotic resistant *P. aeruginosa* is a significant cause of burn wound infections and, skin and soft tissue infections. Because of its resistance to commonly used antibiotics and antiseptics, there is a shortage of therapeutic options for effective treatment (Nagoba *et al.,* 2017). *P. aeruginosa* usually attacks the patients with burn and wound infections, where further complicate of the primary condition, may occur and sometimes can cause bacteremia (Inacio *et al.,* 2014).

2.8. Pathogenicity of *P. aeruginosa*

Biofilm formation and the production of numerous membrane and extracellular virulence factors (Khattab *et al*., 2015) have been linked to the pathogenicity of *P. aeruginosa*, which includes initial colonization, immunoevasion, host cell penetration, and the sequestration of nutrients from its host (*P. aeruginosa*); (Johnson, 2018). Lipopolysaccharides, flagella, pili, and alginate are all examples of virulence agents (Fadhil *et al*., 2016), as follow:

2.8.1. 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).

2.8.2 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).

2.8.3 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).

2.8.4 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).

2.8.5 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).

2.8.6 Pigments

P. 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).

2.8.7 Formation of Biofilm

Biofilms are "microbial communities composed of bacterial cells living in close association by encasing themselves in an extracellular matrix made of polymeric substances, adhering to a substratum or each other and display an altered phenotype" (Donlan and Costerton, 2002; Wojtyczka *et al*., 2014). Bacteria in biofilms can be alive and adhered to an infection site. Biofilm-producing microorganisms survive by colonizing the environment and building biofilms on surfaces (Lebeaux and Ghigo, 2012). Microorganisms develop as structured clusters on different surfaces, which is different from planktonic cells (Lerch *et al*., 2017).

Biofilm formation is a complicated and cyclic phenomena involving transport, diffusion, chemical reactions, and ecological factors. It is regulated by bulk transport, adhesion, quorum sensing, detachment, cell death, and dispersal (Fagerlind *et al*., 2012; McCarty *et al*., 2014). Biofilms are microorganism structures that adapt to their environment (Olivares *et al*., 2020). Biofilms can form quickly (Dufour *et al*., 2010). Four steps comprise biofilm formation: Initial attachment of planktonic bacteria to a surface through physical forces and interaction; (b) Adherent cells get attached to the surface irreversibly and encase themselves in extracellular polymeric substances matrix resulting in cell aggregation; (c) Maturation of biofilm by micro colony formation to form a three-dimensional architecture of fully matured biofilm; (d) Release of micro colonies of cells (Jamal *et al*., 2018).

A free-floating bacterium reversibly adheres to a conditioned surface, then the adhering bacteria are irrevocably connected by surface adhesions, followed by the production of an extracellular matrix to build a mature biofilm. Alginate, pel, and psl determine *P. aeruginosa* biofilm stability (Ghafoor *et al*., 2011; Ryder *et al*., 2007). Alginate is a D-mannuronic acid and L-glucuronic acid polymer chain. This polymer protects and stabilizes biofilm.

2.9. Genome of *P. aeruginosa*

The Genome of *P. aeruginosa* is relatively big, ranging between (G+C content: 65-67%, 5.5 and 7 Mb) Figure 1. Analyses of the genomes of distinct strains of *P. aeruginosa* have revealed that the organism's exceptional adaptability can be partially attributed to its genome's extraordinary flexibility (Winsor *et al*., 2016). In addition to genome rearrangements and horizontal gene acquisition, genome reduction has been seen, especially in clinical strains isolated from persistent infections (Didelot *et al*., 2016; Marvig *et al*., 2015).

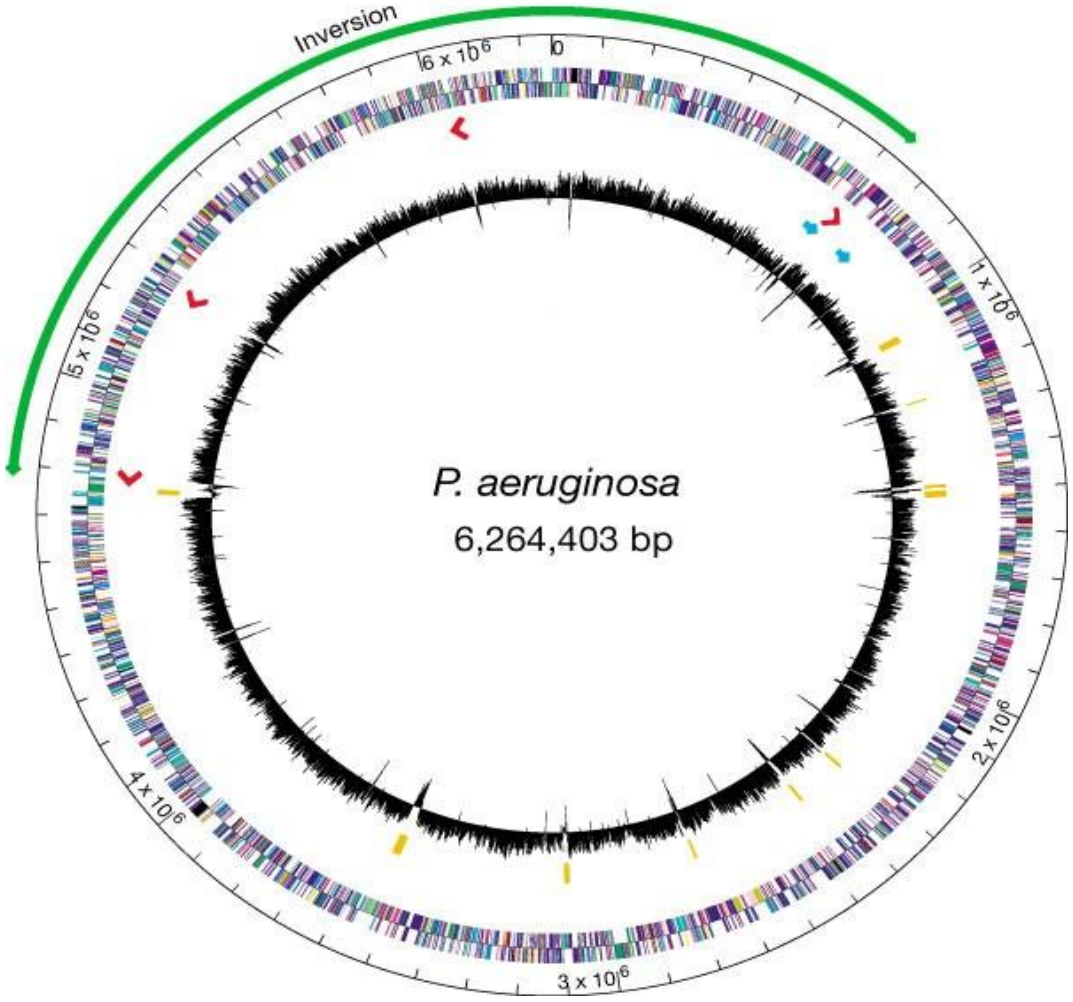


Figure 1. The outermost circle indicates the chromosomal location in base pairs (each tick is 100 kb). The distribution of genes is depicted by coloured boxes according to functional category and direction of transcription (outer band is the plus strand; inner band is the minus strand).

Four clinical *P. aeruginosa* strains were sequenced by Hocquet *et al*., (2016) revealing significant chromosomal deletions (66–270 kb). Using *in vitro* assays, they demonstrated that these strains are highly resistant to two bacterial pyocyanin toxins, which are toxic proteins mediating *P. aeruginosa* inter-strain competition (Ghequire and De Mot, 2014). This may improve their survival in a mixed bacterial population during chronic infection in the host (Hocquet *et al*., 2016).

Smith *et al*., (2006). gathered *P. aeruginosa* strains from a patient over the course of eight years and identified a 188 kb deletion that removed 139 genes (Mena *et al*., 2008). Consequently, core essential genes may be utilized as the fundamental components of minimally controlled cell factories. Individual species, cell types, and development situations necessitate supplementary essential genes. Consequently, accessory essential genes in bacteria are attractive targets for the development of novel antimicrobials (Juhas, 2015).

P. aeruginosa has a larger genome and gene count than other widespread nosocomial infections, including *E. coli* (4.72 Mbp) and S. aureus (2.8 Mbp) (Fredens *et al*., 2019). (Liao *et al*., 2019). Dingmans discovered a clinical strain of *P. aeruginosa* lacking genes encoding the whole type III secretion system and other pathogenic factors (Jurado-Martín *et al*., 2021; Marvig *et al*., 2015). However, the processes responsible for large-scale genomic deletions in *P. aeruginosa* are poorly understood, as are the genetic variables that encourage such reductive evolution.

In general, site-specific deletion and spontaneous deletion are the two most common sources of fragment loss in the genome. The prevalence of site-specific deletions mediated by prophage or other mobile genomic elements is high (Askora *et al*. 2021). The majority of essential genes in P. aeruginosa are involved in fundamental physiological processes such as DNA replication, transcription, and translation, RNA metabolism, protein export, cofactor, amino acid synthesis, and cell wall biogenesis (Lee *et al*., 2015). In the *P. aeruginosa* auxiliary genome,

extrachromosomal components like as plasmids, islands, and DNA blocks are integrated into the chromosome at different locations (Klockgether *et al*., 2011).

2.10. Multidrug resistance of antibiotics in *P. aeruginosa*

P. aeruginosa is an opportunistic pathogen that can cause serious illness in people who are already very sick. Multidrug-resistant (MDR) strains, which are resistant to almost all antibiotics, are showing up in hospitalized patients. This is because they are everywhere, can live in moist places, and are naturally resistant to many antibiotics and antiseptics (Magalhães *et al*., 2020). MDR is when bacteria can't be killed by at least one type of antibiotic in three or more categories. Antibiotics resistance in *P. aeruginosa* may be categorized into inherent resistance, acquired resistance. and adaptive resistance. Because *P. aeruginosa* has both natural and learned ways of being resistant, it is hard to treat any infections it causes (Azam and Khan, 2019).

2.10.1. 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 lactamases, which hydrolyze most b-lactams, and the carbapenem

hydrolyzing enzyme PoxB, which is encoded on the chromosomal level (Arzanlou *et al*., 2017).

2.10.2. 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). Chromosome alterations in the genes for DNA gyrase and topoisomerase IV subunits are one of two FQs (Nouri *et al*., 2016). 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 (Munita and Arias, 2016) extended-spectrum-lactamases, aminoglycoside nucleotidyltransferases, carbapenemases, aminoglycoside nucleotidyltransferases, 16s rRNA methylases, and enzymes that change lipopolysaccharide (LPS) (Poole, 2011)**.**

2.10.3 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.

2.11. Antibiotics Resistance

Waksman (1945) described an 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).

2.11.1 β-Lactam Antibiotics

Among the different classes of antibacterial established, β-lactams are arguably the most important, accounting for around 60% of total antibiotic use worldwide (Versporten *et al*., 2014). 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 (Chaudhry *et al*., 2019).

2.11.2 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).

PBP 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).

2.11.3 Mechanisms of β-lactam Resistance

Antibiotic-resistant *P. aeruginosa* isolates arise from genetic alterations in antibiotic-sensitive bacteria (acquired resistance). Mutations that influence a wide variety of cellular activities can cause acquired resistance. Changes to the PBP3

target protein, reduced antibiotic absorption, increased export, and degradation of antibiotic molecules are the key processes behind the development of β-lactam resistance through mutation (Reygaert 2018). In addition, horizontal gene transfer can cause bacteria to acquire antibiotic-degrading enzymes (β-lactamase) from other bacteria (Munita and Arias 2016).

Changes in metabolism and increased biofilm formation may potentially contribute to resistance (Karballaei Mirzahosseini *et al*. 2020). The mechanisms of resistance are detailed in full below. -lactam resistance can be caused by genetic alterations that diminish antibiotic absorption via porins, enhance β-lactam degradation, modify the PBP3 target protein, or increase antibiotic efflux. This is a common combination of resistance mechanisms. The development of very affordable tools for whole-genome sequencing has substantially sped the identification of genetic variations that contribute to resistance. a number of research have employed experimental evolution to create b-lactam-resistant *P. aeruginosa* from sensitive strains, followed by whole-genome sequencing to discover the mutations responsible for resistance (Huynh and Wood 2021, Wardell *et al*., 2019, Vaillancourt *et al*., 2021, Zhang *et al*., 2022).

Whole-genome sequencing of isolates from chronically infected individuals demonstrates that genes that acquire mutations during in vitro resistance development also acquire alterations that are expected to contribute to resistance throughout infection (Marvig *et al*., 2015). The sequencing of the whole genomes of clinical isolates has also increased our knowledge of the contributions of horizontally acquired genes to b-lactam resistance. These research provide a comprehensive knowledge of the processes of b-lactam resistance in *P. aeruginosa*. Various resistance mechanisms are elaborated about in the next section. In Enterobacteriaceae such as *E. coli* and *P. aeruginosa*, this mechanism model is quite prevalent (Kapil *et al*., 2020).

2.12. β-lactamases

β-lactamases in gram-negative bacteria's primary protection against β-lactam antibiotics. -lactamases are enzymes that hydrolyze the amide bond of the -lactam ring, resulting in medication inactivation and failure of therapy (Agouri, 2014).

β-Lactamases can be generally separated between enzymes with a serine residue in the active site, similar to bacterial penicillin-binding proteins, from which they most likely developed, and metalloenzymes with zinc ion as a cofactor and a distinct ancestry (Abdollahzadeh *et al*., 2012).

Current sequence diversity suggests that the serine group has evolved alongside bacteria during the past 2 billion years. Cephamycins, cephalosporins with an oxyimino side chain, carbapenems, and the monobactam were among the antimicrobials that shared resistance to the then-common -lactamases when they were launched around 20 years ago (Hall and Barlow 2004).

To confer resistance to the most recent -lactam antibiotics, bacteria have developed a plethora of -lactamases, including extended-spectrum β-lactamases, plasmid-mediated AmpC enzymes, and carbapenem-hydrolyzing β-lactamases (carbapenemases) with variable efficacy. The characteristics of these -lactamases, how they may be discovered, their sources, and treatment options for the diseases they cause. In 1940, the isolation of Escherichia led to the production of the first beta-lactamase enzyme, which triggered the penicillin hydrolysis. So far more than 890 beta-lactamase enzymes have been found (Tavakoly *et al*., 2018).

2.12.1 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 (Bush and Bradford, 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*., 2001; Branger *et al*., 2005; Rezaei *et al*., 2018).

2.12.2. 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 (Tooke *et al*., 2019). 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.

2.12.2.1 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; Chaves *et al*., 2001), which can hydrolyze later generation penicillins and cephalosporins, and carbapenemases including imipenamase (IMI) and *Klebsiella pneumoniae* carbapenemase (*KPC*) enzymes (Rapp and Urban, 2012). 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 SBL inhibitors such as clavulanic acid, although *KPC* enzymes are an exception, and some resistant *TEM* variants do exist, as well as avibactam (Bush and Bradford, 2020).

2.13.2.2 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 (Sawa *et al*., 2020). Class C enzymes are not usually inhibited by SBL 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 (Mack *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* (Jacoby, 2009).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 1e (Sawa *et al*., 2020).

2.12.2.3 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 (Evans and Amyes, 2014). 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 (Stojanoski *et al*., 2015).

2.12.3 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). $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV} are the most frequent ESBL-encoding genes (Abrar *et al*., 2019). 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).

Prior to the year 2000, *SHV* and *TEM* were the most prevalent ESBL types; however, *CTX-M* enzymes have replaced them in recent decades (Gruber *et al*., 2013). *P. aeruginosa* species are linked to the global expansion of ESBLs genes, particularly the bla_{CTX-M} genes, which have become more prevalent during the past two decades. bla_{CTX-M} kinds -lactamases-producing bacteria have superseded bla_{TEM} and bla_{SHV} as the most common types of β-lactamases-producing bacteria in recent years (Chong *et al*., 2018).

2.12.4 Extended spectrum β-lactamases Family

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.

2.12.4.1 Temoneira (*TEM*) Family

Temoneira (TEM) was given its name since it was first discovered in an *E. coli* isolate that had been obtained from a blood culture taken from a patient in Greece who went by the name Temoneira (Dallenne *et al*., 2010; Lin *et al*., 2012). It has been shown that *TEM* genes, also known as bla_{TEM}, give resistance to the majority of antibiotics, including first-generation cephalosporins like cephaloridine and penicillin. *TEM*-type ESBLs are produced from *TEM*-1 and *TEM*-2 by making substitutions of amino acids inside the active region (Peymani *et al*., 2017).

This modification did not have any effect on the substrate profile of *TEM*-1; nonetheless, *TEM*-2 was the progenitor of a significant number of the *TEM*-type ESBLs. The *TEM*-3 variation was the first *TEM*-type variant to be reported to have the ESBL phenotype. This occurred in the year 1989. (Sougakoff *et al*., 1988). As of the time this article was written, 243 unique *TEM* variants have been identified;

however, not all of them are ESBLs. Within the *TEM* enzyme, amino acid changes can only take place at a select few different sites (Bradford, 2001).

Gly238 and Glu240, both of which are located on the b3 b-pleated sheet; Arg164, which is located on the neck of the X loop; and Glu104, which is located directly across from Gly238 and Glu240 at the opening of the active-site cavity are the amino acid residues (Ambler numbering) that are most frequently involved in conferring the ESBL phenotype to *TEM*-type enzymes. (Castanheira, Simner and Bradford 2021, Salahuddin, Kumar and Khan 2018). In particular, the gly238Ser and glu240Lys alterations appear to have the most influence on the development of the ESBL phenotype. A few of the more recent variations of *TEM* have nuanced modifications to the substrate profile. For instance, in comparison to ceftazidime and cefotaxime, *TEM*-184 was superior in its ability to hydrolyze aztreonam (Piccirilli *et al*., 2018).

Plasmid-encoded *TEM*-1 -lactamases are the class A enzyme in gram-negative bacteria that has received the most attention from researchers. They are regarded to be the most prevalent beta-lactam resistance mechanism among gram-negative bacilli, and they are quickly spreading around the world (Eiamphungporn *et al*. 2018, Seyedjavadi, Goudarzi and Sabzehali 2016). Even though whole genome sequencing (WGS) is leading to the discovery of a large number of novel variations, only a small fraction of these variants are being phenotypically characterized to identify whether or not they exhibit the characteristics of an ESBL. Computer modeling and network analysis, on the other hand, have made it possible to forecast whether or not a specific sequence is likely to belong to the functional groups 2 b (original wide spectrum), 2be (ESBL), or 2br (inhibitor resistant) (Zeil *et al*., 2016).

When *TEM*-type ESBLs were at the height of their popularity, the incidence of certain of the variations was more prevalent in some geographic areas than others. For instance, *TEM*-3 was quite widespread in France but only seldom observed in the United States (Soilleux et al., 1996). In contrast, *TEM*-10 was the *TEM*-type ESBL that was found the most frequently in the United States (Wiener *et al*., 1999). It's interesting to note that *TEM*-26 was found in samples collected from all around the world (Pitout *et al*., 1998; Soilleux *et al*., 1996; URBAN *et al*., 2000).

2.12.4.2 Sulfhydryl Family

Sulfhydryl (*SHV*) b-lactamases (so termed for sulfhydryl reagent variable) are the enzymes that were first discovered in *Klebsiella pneumoniae* and were chromosomally encoded (Livermore, 1995). In the NCBI Reference Gene Catalog at the moment, there are entries for 182 distinct *SHV* variations (PRJNA313047; request date: 15 January 2021). Their spectrum extends from beta-lactamase, such as bla$_{SHV-4}$, to broad-spectrum beta-lactamase, such as bla$_{SHV-1}$, to extended-spectrum beta-lactamase, such as bla$_{SHV-2}$, all the way up to broad-spectrum ESBL (bla$_{SHV-10}$). *SHV-2* was the first ESBL to be described in 1985. It was discovered in a single strain of *Klebsiella ozaenae* that had been isolated in Germany.

It differed from *SHV-1* in that it had a single amino acid substitution of gly to ser at position 238 (Huletsky *et al*., 1993). *SHV-2* was found in a single strain of Klebsiella ozaenae that had been isolated in Germany. The majority of *SHV*-type ESBLs, like *TEM*-type ESBLs, have mutations at ambler positions 238 (Glycine to Serine) and 240 (Lysine to Glutamine), just like TEM-type ESBLs. These mutations change Glycine to Serine and Lysine to Glutamine, respectively. It would appear that the replacement of serine at position 238 is necessary for the

effective hydrolysis of ceftazidime, whereas the substitution of lysine at residue 240 is critical for the efficient hydrolysis of cefotaxime (Huletsky *et al*., 1993).

Recent research made use of a mathematical model to evaluate the significance of different amino acid alterations in relation to phenotypic changes in substrate profile (Neubauer *et al*., 2020). To this day, 228 sequence variations of *SHV* have been identified; however, not all of these variants have been functionally evaluated to establish if they exhibit the ESBL phenotype. *SHV-5* and *SHV-12* have been the most prevalent ESBL variants reported in Enterobacterales across the world (Perilli *et al*., 2002; Yan *et al*., 2000). SHV-type ESBLs may be discovered in clinical isolates of *K. pneumoniae* the majority of the time; nevertheless, these enzymes have also been found in other genera of Enterobacterales and in *P. aeruginosa* (Coque *et al*., 2008; Perilli *et al*., 2002).

2.12.4.2 Cefotaximase (*CTX-M*) Family

Cefotaximase (CTX-M) -lactamase enzymes were first identified in the late 1980s, appearing simultaneously in a number of different places. The designation *CTX-M*, which stands for cefotaximase from Munich, was first used in a paper that was written in Germany (Bauernfeind *et al*., 1990). However, *CTX-M*-type enzymes found in other locations were given various names, such as FEC-1 (in Japan), Toho-1 (also in Japan), and MEN-1 (in France, in an Italian patient). These names reflect the regions in where the enzymes were found (Bonnet, 2004).

After these early findings, outbreaks were reported in a number of other countries. In subsequent years, the rapid spread of ESBL-carrying isolates around the globe would come to be known as the "*CTX-M* pandemic." Since the early 2000s, *CTX-M*-type enzymes have been acknowledged as the most prevalent category of ESBLs. This means that *TEM* and *SHV* have been supplanted as the predominant kind of ESBL. CTX-M variations have been found in *P. aeruginosa*

and *Acinetobacter* spp (Picão *et al*., 2009; Walther-Rasmussen & Høiby, 2004), two different types of bacteria belonging to the order *Enterobacterales*. Isolates harboring *CTX-M*-encoding genes have been found in nosocomial and community settings, as well as in companion animals, the environment, food items, and cattle.

Additionally, these isolates have been found in contexts where they may have been transmitted to humans (Liu et al., 2018). Based on their similarity in sequence, the majority of *CTX-M* enzymes may be categorized into the following five groups: *CTX-M-1*, *CTX-M-2*, *CTX-M*-8, *CTX-M*-9, and *CTX-M-25*. The *CTX-M*-15 group is by far the most frequent *CTX-M*-1 group, followed by the *CTX-M*-3 group and then the *CTX-M*-1 group (Bonnet, 2004). *CTX-M*-9 and CTX-M-14 were the most prevalent enzymes found in the *CTX-M*-9 group historically; however, in more recent years, *CTX-M*-27 has been often reported (Matsumura *et al*., 2016; Zhang *et al*., 2021). Within their respective groups, *CTX-M*-2, *CTX-M-8*, and *CTX-M*-25 are the variations that are found the most frequently.

2.11.5 Molecular characterization of ESBL-producing isolates

Plasmids, transposons, insertion sequences, integrons, and bacteriophages spread ESBL-encoding genes. Mobile genetic elements can transport themselves and/or genes within a cell through conjugation, transformation, or, in the case of bacteriophages, transduction (Partridge *et al*., 2018). MGEs carry several resistance genes that impart MDR to their hosts (Rodrguez-Bao *et al*., 2013). Below are some of the key ESBL-carrying MGEs. Transposons Tn1-, Tn2-, or Tn3-like carry *TEM*-1, *TEM*-2, and ESBL genes (Partridge and Hall, 2005). These structures were initially designated TnA and have 98% nucleotide homology, with most changes around their resolvase (res) site (Partridge *et al*., 2018). Few investigations describe MGE-carrying, bla$_{TEM}$-encoding ESBL enzymes. In a previous investigation, bla$_{TEM}$-12 was found in Tn841, which is similar to Tn3 (Heritage et al., 1992). *TEM*-

3 gene was stopped on Tn1. Tn2 had bla$_{TEM}$-10, while Tn1 had bla$_{TEM}$-24 (Castanheira *et al*., 2021; Mabilat *et al*., 1992). Marcade's research of conjugative plasmids expressing ESBL genes found that 67% of *TEM*-type plasmids were IncA/C (Wolny-Koładka and Lenart-Boroń, 2018). These plasmids contained blaTEM-3, blaTEM-10, and *bla$_{TEM}$*-21. *bla$_{TEM}$*-encoding ESBLs were verified in IncA/C plasmids (Foley *et al*., 2021; Novais *et al*., 2010; Rozwandowicz, 2020). In the early 1990s, IS26 was found flanking *bla$_{SHV}$*. IS26 was identified as the mobilizing element for several resistance genes and *bla$_{SHV's}$* promoter Figure 2. (Varani *et al*., 2021). Intact copies of IS26 have been found on bacterial plasmids or chromosomes bordering *bla$_{SHV}$*, its 50 proximal termini, or faulty IS26 elements. *SHV* type ESBL genes are on plasmids and chromosomes. IncA/C, IncF, IncHI2, IncI1, IncL/M, IncN, and IncX3 are plasmid replicon types that contain blaSHV-encoding ESBL enzymes (Liakopoulos *et al*., 2016; Poirel *et al*., 2008). Each plasmid type has several bla*SHV* variations, except IncX3, which only has bla$_{SHV-12}$ (Poirel *et al*., 2008). Billard-Pomares described a bla$_{SHV-2}$-carrying *E. coli* with a P1 bacteriophage structure (Billard-Pomares et al., 2014). Lartigue found ISEcp1 upstream of *CTX-M* genes 1, 2, and 9. In another investigation, Eckert (Eckert, Gautier, and Arlet 2006) found ISEcp1 in 23 of 28 isolates bearing 7 bla$_{CTX-M}$ types. ISEcp1 and bla$_{CTX-M}$ sequences revealed hallmark sequences indicating transposition events mobilized bla$_{CTX-M}$. ISEcp1 also boosted the expression of bla$_{CTX-M}$. (Poirel *et al*., 2003). ISEcp1 has been found surrounding various b-lactamase genes, including KLU enzymes in Kluyvera spp. bla$_{CTX-M}$ was found at the 30 end of complex class 1 integrons between two qacED1/sul1 elements (Eckert *et al*., 2006).

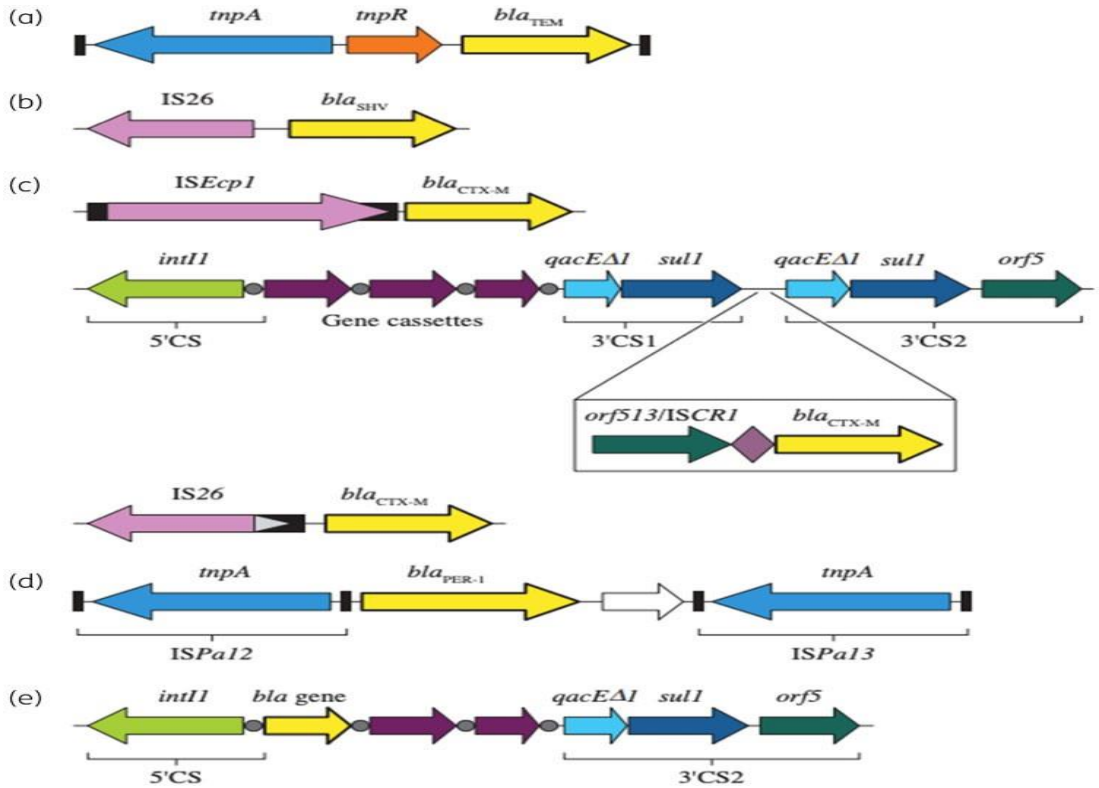


Figure 2. Schematic representations of the genetic structures harbouring genes encoding ESBLs. Genetic structures most commonly reported to harbour (a) bla*TEM,* (b) bla*SHV*, (c) bla*CTX-M*, (d) bla*PER* or (e) class 1 integrons that can carry uncommon ESBL genes were adapted from (Poirel *et al*., 2002; Rossolini *et al*., 2008), (Poirel *et al*., 2012) and. (Poirel *et al*., 2002; Rossolini *et al*., 2008; Diestra *et al*., 2009).

2.12.6 Phenotypic Identification of ESBL

In this procedure, ceftazidime and cefotaxime disks alone and ceftazidime (30µg) and cefotaxime (30µg) +clavulanic acid (10µg) were placed on Muller Hinton agar at a spacing of 2 cm. After 18 hours of incubation at 35 degrees Celsius, ESBL generation was measured by measuring an increase of 5mm or more in the diameter around the disk of ceftazidime/clavulanic acid and/or cefotaxime/clavulanic acid (Ghaffarian *et al*., 2018).

2.13 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. Rapid DNA 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 (Abate *et al*., 2013). 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 (Chmielecki and Meyerson, 2014).

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 (Yildirim *et al*., 2011).

2.13.1 Phylogenetic Tree

A phylogenetic tree, also known as an evolutionary tree or phylogeny, is a tree diagram that depicts the evolutionary histories or relationships of various biological groups or other categories based on physical or genetic similarities and differences (Hu *et al*., 2020). It is used in a branch of biology that analyzes morphological data matrices and molecular sequencing data to identify how various groups of animals have evolved over time. The phylogenetic tree is important because it has been used to explore biodiversity, evolution, genetics, and ecology among groups of organisms. A common ancestor is shown through a single phylogenetic tree that represents all life on earth (Felsenstein, 2004). The phylogenetic tree illustrates phylogeny, or the similarities and differences in genetic makeup and morphology

between several groups of animals (or taxa). It also shows relationships between taxa that suggest evolutionary relatedness. Additionally, it is possible to assume that they be rooted when the ancestral path is mentioned. Phylogenetic trees come in a variety of forms. A rooted phylogenetic tree is one in which the nodes point to the studied taxa's most recent common ancestor: On the other hand, the unrooted tree is a different of phylogenetic tree. This form of tree just considers evolutionary relatedness and makes no assumptions about ancestry (Hodge and Cope, 2000). The ancient beliefs of a ladder-like evolution from lower to higher life forms gave rise to the concept ofa "tree of life" (such as in the Great Chain of Being). In his groundbreaking book "The Origin of Species", Charles Danvin (1859) created one of the first pictures and crucially popularized the idea of an evolutionary "tree." Evolutionary biologists continue to use tree diagrams more than a century after they were first used to represent evolution because they are an efficient way to illustrate the idea that speciation results through the the adaptive and semi random splitting of lineages. The taxonomy of species has' evolved over time to become more dynamic and less static.

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1 Laboratory Equipment's and Apparatus

The Laboratory equipment's and supplies used in the study are listed in Table 3-1.

Table 3-1 Equipment's and Supplies

No.	Equipment's	Company	Origin
1.	Autoclave	Gemmy	Taiwan
2.	Burner	Amal	Turkey
3.	Centrifuge	MSE	England
4.	Disposable (Pteri Dish, Syringe, Plane tube and Latex)	Citro	China
5.	Distiller	Ogawa	Japan
6.	Electric sensitive balance	Sartorius	Germany
7.	Electrophoresis system	Fisher Scientific	USA
8.	ELISA Device	HS-Human Reader	Germany
9.	Electrical Oven	Memmert	Germany
10.	Eppendorf tubes	Eppendorf	Germany
11.	Finn tips with different sizes (20µl, 100 µl, 500 µl, 1000 µl)	Eppendorf	Germany
12.	Hood	Bio LAB	Korea
13.	Hot plat	Biocote	England
14.	Incubator	Binder	Germany
15.	Light microscope	Olympus	Japan

16.	Micropipette (0.5-10 µl , 20-200 µl , 100-1000 µl)	Dragonlab	China
17.	Microtiter plate reader	Memmert	Germany
18.	Millipore Filter Unit 0.80µm	Chm	USA
19.	Microwave	Sanyo electric	Japan
20.	Nanodrop	Optizen	Korea
21.	Oven	Memmert	Germany
22.	Para film	BDH	England
23.	Platinum Wire Loop	Himedia	Indian
24.	PCR Device	Leica	Spain
25.	PCR tube	Eppendorf	Germany
26.	PCR centrifuge	Zip-IQ	USA
27.	Refrigerator	Marubeni	Japan
28.	Slides and Cover slide	Sail Brand	China
29.	Sterilize Swab	ATACO	Brand
30.	Transfer swab	AFCO	Jorden
31.	UV light transminator	wise	USA
32.	Vitek 2 system	Biomerieux	France
33.	Vortex mixer	Eppendorf	Germany
34.	Volumetric flasks	Jlassco	India

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

No.	Biological and Chemical Materials	Company	Origin
1.	Acetone	BDH	England
2.	Agarose	Bio Basic	Canada
3.	Agar-agar	BDH	England
4.	Catalase reagent	Merk	England
5.	Crystal violet	BDH	England
6.	DNA ladder marker (100-1500) bp	Bioneer	Korea
7.	Ethanol 99%	Merck	India
8.	Glycerol (C3H8O3)	Himedia	Switzerland
9.	Gram stain solution	Fluka	USA
10.	Glucose	Sigma	England
11.	Kovac's reagent	HIMEDIA	India
12.	Hydrogen peroxide (H2O2) 3%	Merck	England
13.	Human Blood	Imam Sadiq Teaching Hospital	
14.	Methyl red	BDH	England
15.	Naldixic acid suplement	Mast	U.K
16.	Normal Saline solution	S.D.I	Iraq
17.	Oxidase reagent indicator	BDH	England
18.	Peptone water	HIMEDIA	India
19.	Safe Red	Fisher	USA
20.	Tris-Borate EDTA buffer (TBE)	Promega	USA
21.	Urea Solution	SD-Fine	India

3.1.3 Culture Media

Culture media used in the present study were prepared according to the manufacturer's instruction. The media were sterilized by the autoclave at 121°C for 15 min, and kept at 4°C until use, All the media in this study were perchased from HIMEDIA company/ India. Table 3-3 shows the media used in this study.

Table 3-3 Culture media used in this study

No.	Medium
1.	Agar agar powder
2.	Brain heart infusion agar
3.	Brain heart infusion broth
4.	MacConkey Agar
5.	Muller Hinton Agar
6.	Nutrient Agar
7.	Nutrient Broth
8.	Simmons citrate agar
9.	Cetrimide agar
10.	Kliglers iron agar
11.	Chromogenic agar
12.	Tryptic Soy broth
13.	Urea agar base

3.1.4 Commercial Kits

The commercial kits used in the study are illustrated in Table 3-4.

Table 3-4 Commercial kits used in this study

No.	Kit	Company	Origin
1.	DNA extraction Kit	Favorgen	Taiwan
2.	DNA ladder	IntronBio	Korea
3.	Primers	Macrogen	Korea
4.	*P. aeruginosa* Specific Primer	Macrogen	Korea

5.	PCR master mix	IntronBio	Korea
6.	VITEK® 2 Compact	Biomerieux	France

3.1.5 Polymer Chain Reaction (PCR) Mixture

The PCR reaction mixture used in the study are listed in Table 3-5.

Table 3-5 PCR Reaction Mixture

No.	Contents of reaction mixture	Volume
1.	Master Mix	12µl
2.	Template DNA	3 µl
3.	Forward primer (10 pmol/µl)	2 µl
4.	Reverse primer (10 pmol/µl)	2 µl
5.	Nuclease free water	6 µl
6.	Total volume	25 µl

3.1.6. Go Taq G2 Green Master Mix Materials

The Master Mix Materials used in the study are listed in Table 3-6.

Table 3-6 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. Commercial Primers

The commercial Primers used in the present study are illustrated in Table 3-7.

Table 3-7 Commercial Primers used in this study

Primer		Sequence (5----------›3)	Amplicon size (bp)	Conditions (D,A and E)	Cycle No.	Source
TEM	F	GAGTATTCAACATT CCGTGTC	861	94˚C/30 sec 57˚C/1 min 72˚C/2 min	35	(Bokaeian *et a*l., 2015)
	R	TAATCAGTGAGGCACCTATCTC				
SHV	F	AAGATCCACTATCGCCAGCAG	231	94˚C/30sec 64˚C/1 min 72˚C/1 min	35	
	R	ATTCAGTTCCGTTTCCCAGCGG				
CTX-M	F	GACGATGTCACTGGCTGAGC	499	94˚C/30 sec 57˚C/1 min 72˚C/1 min	35	
	R	AGCCGCCGACGCTAATACA				
P.aeru	F	GGGGGATCTTCGGACCTCA	956	95˚C/30 sec 61˚C/1 min 72˚C/1 min	35	(Spilker *et al*., 2004)
	R	TCCTTAGAGTGCCCACCCG				

3.1.8 Antimicrobial susceptibility test

The phenotypic detection of extended-spectrum beta-lactamases (ESBL) was performed using the double-disk diffusion (DDS) test according to clinical laboratory guidelines (CLSI-2021) (0.5 McFarland tube was used to obtain 1×10^8 CFU/mL bacterial culture). Table 3-8 shows the antimicrobial disks used in this Study.

Table 3-8 Antimicrobial Disks used in this Study

Antibiotics	Antibiotics Classes	Symbol	µg / disk	Inhibition zone/diameter Mm			Company/ origin
				S	IN	R	
Aztreonam	Monobactams	ATM	30	≥22	16-21	≤15	India/Himedia
Ceftazidime	Cephems	CAZ	30	≥18	15-17	≤14	Roseto /Italy
Cefepime		CEP	30	≤18	15-17	≤14	Roseto /Italy
Piperacillin tazobactam	β-Lactams combinations	PTZ	100/10	≥21	15-20	≤14	MAST/U.K
Piperacillin	Penicillins	PRL	100	≥21	15-20	≤14	Roseto /Italy
Gentamicin	Aminoglycosides	CN	10	≥15	13-14	≤12	Condalab/Spain
Tobramycin		TOB	5	≥15	13-14	≤12	Bioanalyse Tur/
Netilmicin		NET	30	≥15	13-14	≤12	Roseto /Italy
Amikacin		AK	30	≥17	15-16	≤14	Roseto /Italy
Ciprofloxacin	Fluoroquinolones	CIP	5	≥25	19-24	≤18	Himedia/ India
Norfloxacin		NX	10	≥17	13-17	≤12	Roseto /Italy
Gatifloxacin		GAT	5	≥18	15-17	≤14	Roseto /Italy

Levofloxacin		LEV	5	≥16	15-21	≤12	Roseto /Italy
Ofloxacin		OFX	5	≥29	13-15	≤28	Bioanalyse /Tur
Imipenem	Carbepenem	IPM	10	≥19	16-18	≤15	Roseto /Italy
Doripenem		DOR	10	≥19	16-18	≤15	Bioanalyse /Tur
Meropenem		MEM	30	≥19	16-18	≤15	Bioanalyse /Tur

3.2. Methods

3.2.1. Laboratory Preparation of Culture Media

All media were preparation according to the instructions of the manufacturing company Sterilization of culture media and solutions were achieved by autoclaving at 121°C／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 for 24 hours to ensure their sterility. Storage of sterile media in the refrigerator to prevent dehydration (Cappuccino and Welsh, 2018). PH was adjusted to 7.0 and the media sterilized by autoclaving (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
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 (Jung and Hoilat, 2020).

2.	Nutrient broth	this medium was used in a general experiment such as cultivation and activation of bacterial isolates when it necessary (MacFaddin, 2000).
3.	Blood agar	Is an enriched, bacterial growth medium, isolation, identification and determine the type of hemolysis (Niederstebruch *et al*., 2017).
4.	Cetrimide agar	This medium was used as a selective medium for the isolation of *P. aeruginosa* (Aryal, 2015).
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).
6.	Müller-Hinton agar	This medium used in the antibiotic sensitivity test (MacFaddin, 2000).
7.	Simmons citrate	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).
8.	Tryptic Soy broth	it was used for activation of bacteria and for general experiments (MacFaddin, 2000)
9.	Urea agar	It was used to test the ability of bacteria to produce urease enzyme (MacFadden, 2000).
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).
11.	Voges Proskauer	It was used to detection of specific breakdown products of carbohydrate metabolism by bacteria

3.2.2. Preparation of Reagents and Solutions

3.2.2.1. Oxidase reagent

It was prepared by dissolving 1 mg of N, N, N, N-tetramethyl-ρ-phenylenediamine dihydrochloride in 100 ml of D.W. Then stored in a darkbottle and used immediately (Forbes *et al*., 2007).

3.2.2.2. Catalase reagent

It was prepared in a dark bottle by using a 3% concentration from hydrogen peroxide (Forbes *et al*., 2007).

3.2.2.3. 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).

3.2.2.4. 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).

3.2.2.5. 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).

3.2.2.6. Gram stains solutions (Jawetz *et al*., 2019).

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.

3.2.2.7. 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 (BaCl2.H2O) 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 46 isolates, this solution was used to set the number of bacterial cells.

3.2.2.8. Safe red

Red Safe Nucleic Acid Staining Solution is a new and safe alternative to ethidium bromide (EtBr) for DNA and RNA identification on agarose gels. Red Safe is as sensitive as EtBr, and the staining procedure is virtually comparable; however, compared to EtBr, 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).

3.2.2.9. Preparation of 1X TBE buffer

1X TBE buffer was prepared by dilution of concentrated 10X TBE buffer, the solution was used to dissolve agarose. Each 10 ml of 10X TBE added to 90 ml of sterile distal water to give final concentration 0.5µg/ml (Sambrook *et al*., 1989).

3.2.2.10 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 50-60oC, and 5ml of melting agarose gel was combined with 5ml of simply safe to achieve a final concentration of 0.5g/ml.

3.2.3 Collection of specimens

In this study, a total of 150 collection of specimens from wound swab and burn swab of patients were hospitalized at: Babylon hospitals (Al-Hillah Teaching Hospital, Mirgian Teaching Hospital and Imam AL-Sadiq Teaching Hospital) and also from Baghdad hospitals (Burns Specialized Hospital, Martyr Ghazi Hariri Hospital, Baghdad Teaching Hospital, and National Center for Educational Laboratories) of both genders with different ages. The specimens collected during the period from September 2021 to January 2022.

3.2.4. Bacterial diagnosis

3.2.4.1. Culturing

All specimens were cultured on different media for identification of *Pseudomonas* such as blood agar, MacConkey agar, and cetrimid agar, using sterile loop spread on the surface of agar media and incubated at 37 C° for 24 hr. (Jawetz *et al*., 2019). Purified colonies kept in nutrient broth containing glycerol at -20 C° in (Jawetz *et al*., 2019). After final diagnosis of samples, 46 (30.6%) isolates of *P. aeruginosa* were obtained. Forty-six isolates of *P. aeruginosa*, (named PA1 to PA46) were isolated from one hundred and fifty clinical specimens of burns, injuries and Diabetic foot of nine cites of Iraq which included Erbil, Ninawa, Kirkuk, Diyala, Baghdad, Babylon, Muthanna, DhiQar and Basra.

3.2.4.2. Microscopic examination

After the growth of bacteria on MacConkey agar, blood agar, cetrimid agar and nutrient 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).

3.2.4.3. 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.

1- 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.

2- 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. Appositive 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.

3- 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.

4- 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.

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; Tille, 2014).

6-Triple sugar iron (TSI) test

The cultured isolates were streaked on surface of slope and stabbed into butt, and then incubated for 37°C for (24) hours. The positive result of *P. aeruginosa* was alkaline / no change or alkaline / alkaline with no produce H2S and gas (Brown and Smith, 2017).

7-Hemolysin production

Hemolysis production was carried out by inoculating the blood agar medium with bacterial isolates at 37°C 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).

8- Detection of biofilm production

Microtiter plates containing 96 wells and trypticase soy broth (Himedia) were used to detect the biofilm production, semi quantitative assessments of biofilm development were determined according to Hemati et al. (2016). Individual wells of 96-well plates were used to cultivate *P. aeruginosa* at 37 C° in trypticase soy broth medium supplemented with 1g of glucose. After 24 hours of growth, the plates were aggressively washed three times with normal saline to eliminate free-floating bacteria. The plates were stained for 15 minutes at room temperature with 100 ml of 0.1% (w/v) crystal violet solution and then washed with normal saline. The crystal violet was then removed from the wells by extracting the crystal violet

solution from the biofilm with 150 µl of 95 percent ethanol and acetone [8:2 (v/v)]. A microplate reader measured the plates at 630 nm and provided the following final results: non, weak, moderate, and strong. The results were interpreted as follows: if OD < ODc had non biofilm formation, if ODc < OD < 2 * ODc the bacteria were weakly adhering; if 2 * ODc < ODc < 4 * ODc, the bacteria were moderately attached; and if 4 * ODc < OD, the bacteria were strong adherent (Hemati et al., 2016).

3.2.5 Antibiotic susceptibility test

One of the most common methods used routinely in diagnostic laboratories and 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 ofloxacin (30µg), levofloxacin(30µg), doripenem(30µg) gatifloxacin (30µg), ceftazidime (30µg), tobramycin (10µg), amikacin (30µg), netilmacin (30µg), ciprofloxacin (5µg), norfloxacin (5 µg), cefepime (30µg), cefotaxime (30µg), imipenem (10µg), and aztreonam (30 µg), meropenem (10µg), Pipracillin (100µg), peracillin-Tazobactam (100g-10µg), gentamicin (10µg). *P. aeruginosa* were considered resistance or sensitive on the basis of zone of inhibition following the criteria of clinical and laboratory standard institute CLSI-2021, (Wi *et al*., 2017). 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. 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 2021) for sensitivity or resistance of the organism to each antibiotic.

3.2.6 Maintenance of bacterial Isolates

Maintenance of bacterial isolates was performed as follows:

A- **Short term storage**

Bacterial isolate (46 isolate) was kept for one month on nutrient agar plates, the plates were tightly covered with Para film and stored at 4°C (Angshumanjana *et al*., 2016).

B- **Long term storage**

A brain heart infusion broth was inoculated by a loop of overnight pure bacterial culture, and incubated at 37 °C after 18 hours, glycerol was added to the inoculate in a final concentration of 15% and stored at (-20C°) for 2-6 months (Green, 2015).

3.2.7 Molecular detection methods

3.2.7.1 Extraction of *P. aeruginosa* genomic DNA

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

3.2.7.1.1 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 13.000 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 specimen 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 13.000 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 13.000 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 13.000 rpm for 1min.
15. Finally, storing the DNA fragment at 4 C° or -20 C°.

3.2.7.2 Estimation of DNA concentration

The DNA concentration of specimens was estimated using nanodrop by putting 1µl of the extracted DNA in the instrument to detect the concentration in ng/µm and purity detected by noticing the ratio of O.D. 260/280 to check the contamination of DNA specimens with protein, the accepted 260/280 ratio of pure DNA was between 1.7-1.9 (Shim *et al*., 2010).

3.2.7.3 PCR protocols for detection of gene technique

1- ConventionalPCR technique was used for amplifying *P. aeruginosa* specific gene 16S rRNA. The mixture reaction was performed in a total volume 12 µl of PCR Pre Mix (Bioneer, South Korea) consisting of 2 µl from each primer forward and reverse, 3 µl of DNA and, the volume completed up to 6 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* $_{TEM}$*, bla* $_{CTX-M}$*, and bla* $_{SHV}$ genes were carried out by using a 12 µl master mix of Gold conventional PCR (Bioneer, South Korea) including 3 µl DNA, 2 µl from each primer forward and reverse, and the volume was completed up to 6 µl with free nucleases deionized water according to the instructions of the company and reaction buffer mixed.

3.2.7.4 PCR program thermal controller

PCR cycling thermal program parameters used in this reaction for detection of *P. aeruginosa* specific, *bla* $_{TEM}$*, bla* $_{CTX-M}$, and *bla* $_{SHV}$ genes were shown in Table 3-10**.**

Table 3-10 PCR thermal cycling program for *P. aeruginosa* specific gene *SHV, TEM* and *CTX-M* genes

No.	Step	Temperature(°C)	Time	No. of cycle
1.	Initial Denaturation	95	5 min	1
2.	Denaturation Annealing Extension	94 57 72	30 sec 1 min 1 min	35
3.	Final Extension	72	5 min	1

***PCR thermal cycling program 64 °C for *SHV* annealing**

3.2.7.5 Agarose Gel Electrophoresis

Agarose gel was created by dissolving 1 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 cold 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 ethidium bromide) 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. 5 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 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 45 minutes for genomic DNA and one hour for PCR product.

3.2.8 DNA sequencing analysis

To study the genetic variation of $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} genes of *P. aeruginosa* isolates, DNA sequencing technique was performed. The PCR products were sent to Macrongen company in Korea in ice bag by DHL. The homology sequence identity and the mutation analysis were conducted using NCBI BLAST analysis. $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} genes of *P. aeruginosa* isolates of the current study were registered in NCBI-Gen Bank data base with accession numbers.

3.2.8.1 Phylogenetic analysis

The phylogenetic tree was designed using the neighbor method with the help

of MEGA4 software program (Tamura *et al*., 2007) and the neighbor-joining phylogeny tree (Saitou and Nei, 1987). The evolutionary distances are computed using the maximum composite likelihood method (Tamura *et al*., 2004) and the reliability of the trees were determined by 1000 data set bootstrap resembling (Felsenstein, 1985).

3.3. Statistical Analysis

SPSS 23 were used to perform the data analysis, and p-values <0.05 were taken as significant. Descriptive statistics are used to describe the frequencies and percentages of all categorical variables. Odds ratios of ESBL-producing *P. aeruginosa* from the burn and wound patient were calculated using binary logistic regression analysis. The Chi-square test was used to see the relationship of isolates with antimicrobial resistance and the presence of bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ genes (Grewal *et al*., 2017).

Chapter Four

Results and Discussion

Results and Discussion

4.1. Description of study specimens

The current study included a collection of (150) swabs from patients suffering from burns and wound injuries in both sexes, (64) males and (36) females, whose ages ranged from 3-73 years Figure (4.1), for the period from September 2021 to January 2022, from some Medical City Hospitals; AL-Shaheed Ghazi Al-Hariri Hospital for Specialized Surgery; Baghdad Teaching Hospital; Burns Hospital Specialized; Educational laboratories Medical City Hospital in Hospital-Baghdad\ Iraq and also from Babylon hospitals at Al-Hillah Teaching Hospital, Mirgian Medical City and Imam AL-Sadiq Teaching Hospital.

After final diagnosis of samples, 46 (30.6%) isolates of *P. aeruginosa* were obtained as shown in Figure (4-2). Forty-six isolates of *P. aeruginosa*, (named PA1 to PA46) were isolated from one hundred and fifty clinical specimens of burns, injuries and Diabetic foot of nine cites of Iraq which included Erbil, Ninawa, Kirkuk, Diyala, Baghdad, Babylon, Muthanna, DhiQar and Basra.

Of these isolates, 23 (50%) were isolated from burns, 17 (37 %) were isolated from injuries, and 6 (13 %) were isolated from feet of diabetics. The isolates were identified depending on traditional methods (morphological features of the colonies and the cells, biochemical tests, Vitek 2 compact) and finally, the confirmation was achieved via PCR-sequencing of a *P. aeruginosa* unique gene.

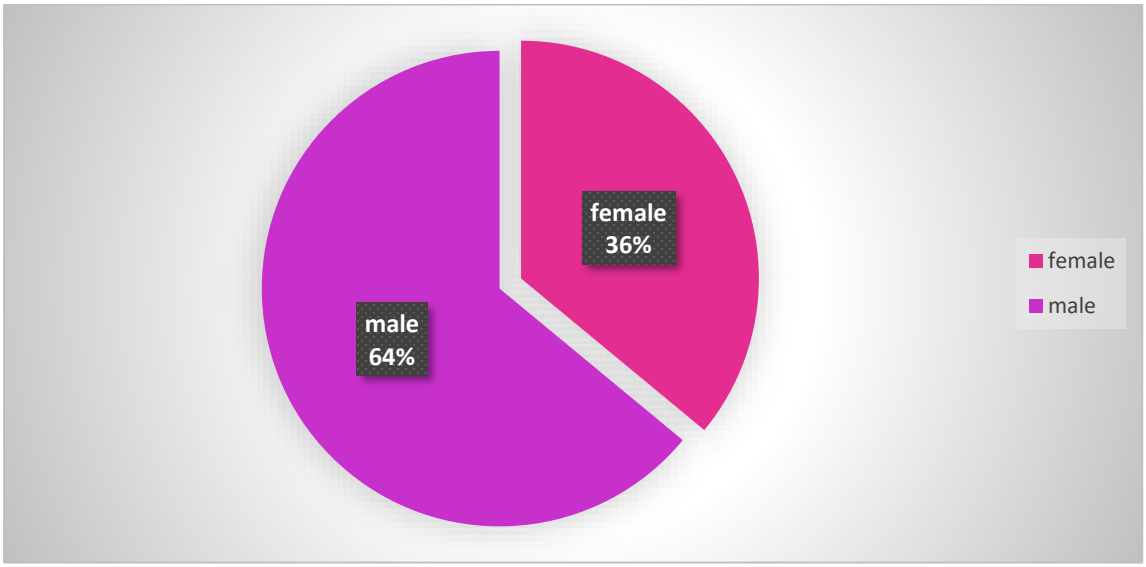
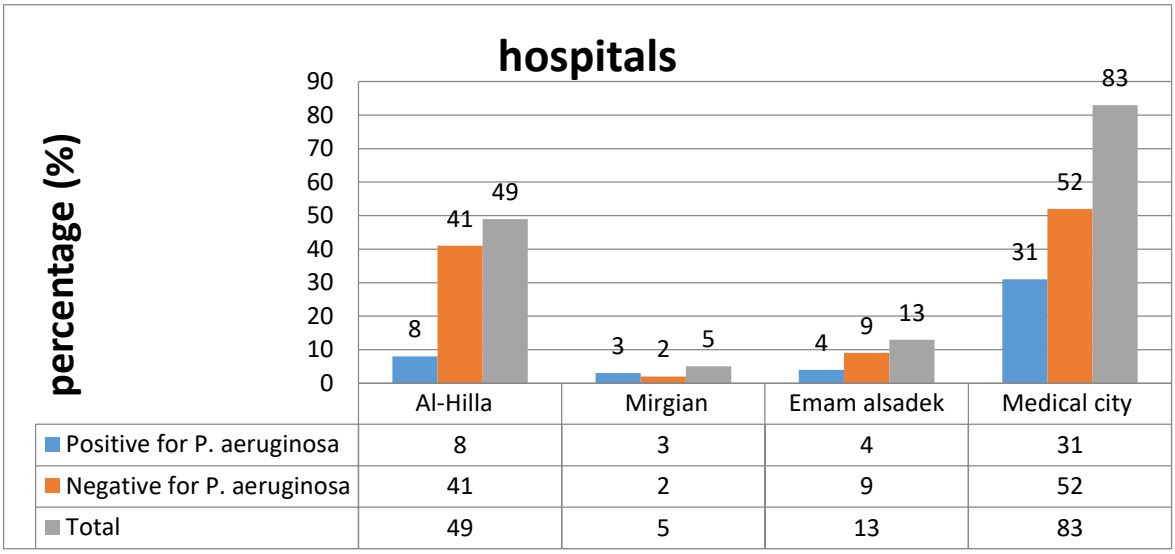


Figure 4.1 Distribution of the specimens according to gender

The results of the percentage distribution of the incidence according to the gender of the patients agreed 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).



	Al-Hilla	Mirgian	Emam alsadek	Medical city
Positive for P. aeruginosa	8	3	4	31
Negative for P. aeruginosa	41	2	9	52
Total	49	5	13	83

Figure (4.2): distribution of growth of *P. aeruginosa* isolates according to hospitals

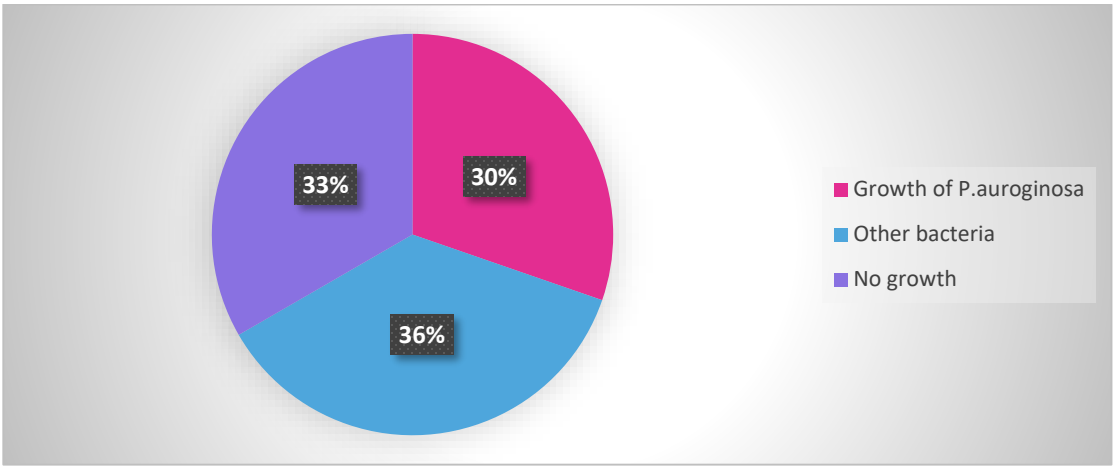


Figure 4.3. Percentage frequencies of all specimens positive and negative for bacterial infection

The highest rate of bacterial infection was within the age group of 18-31 **(**9.5 %) years, followed by the age group of 3-17, 32-45 **(**7.4 %) followed by 60-73 (4.6 %) and 46-59 (2 %), respectively, as shown in Table (4-2).

Table 4.1: Distribution of patients according to age groups

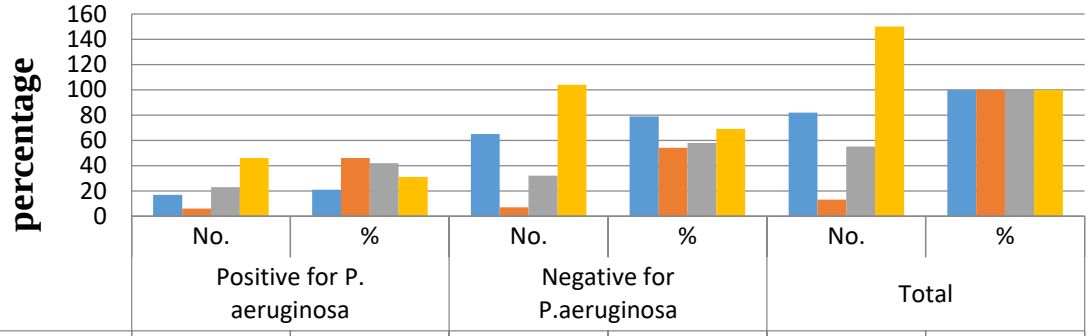
Age group (Year)	Patient		Total	P value (S)*
	Positive for *P. aeruginosa*	Negative for *P. aeruginosa*		
3-17	11 (7.4 %)	20 (13.6 %)	31 (21 %)	0.379
18-31	14 (9.5 %)	28 (18.8 %)	42 (28.3 %)	
32-45	11 (7.4 %)	26 (17.6 %)	37(24 %)	
46-59	3 (2 %)	14(9.5 %)	17 (11.5 %)	
60-73	7 (4.6 %)	16(10.6 %)	23(15.2 %)	
Total	46 (30.6%)	104 (69.4 %)	150 (100 %)	

(S)*: Sig. at P<0.05

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 Detection of *P. aeruginosa*

The identification of these isolates was accomplished through the use of culture and microscopic exams, biochemical tests, the Vitek 2 compact, and finally, confirmation was achieved via PCR-sequencing for a *P. aeruginosa* unique gene. A total of forty-six isolates of *P. aeruginosa* were isolated from 150 clinical specimens. Of these, 17 isolates were isolated from injuries, 23 isolates were isolated from burns and 6 isolates were isolated from diabetic-infected foot, the details of distribution and percentages of the isolates were summarized in figure (4.4).



	Positive for P. aeruginosa		Negative for P.aeruginosa		Total	
	No.	%	No.	%	No.	%
Injuries	17	21	65	79	82	100
diabetic foot	6	46	7	54	13	100
Burns	23	42	32	58	55	100
Total	46	31	104	69	150	100

Figure (4.4): Distribution of growth of *P. aeruginosa* isolates according to specimen types

4.2.1 Identification of *P. aeruginosa* on different media.

MacConkey agar colonies were pale because they couldn't ferment lactose sugar. It smelled like fermented grapes. On blood agar, the colonies were black and most had a translucent halo, indicating they could hemolysis red blood cells (Jawetz, *et al*. 2019), on nutrient agar, *P. aeruginosa* colonies were recognized based on pigments and odor generation (grape-like odor), with greenish colonies (DeBritto *et al*., 2020). While the colonies of *P. aeruginosa* appeared on

chromogenic agar in greenish blue color as depicted in Figure (4.5).

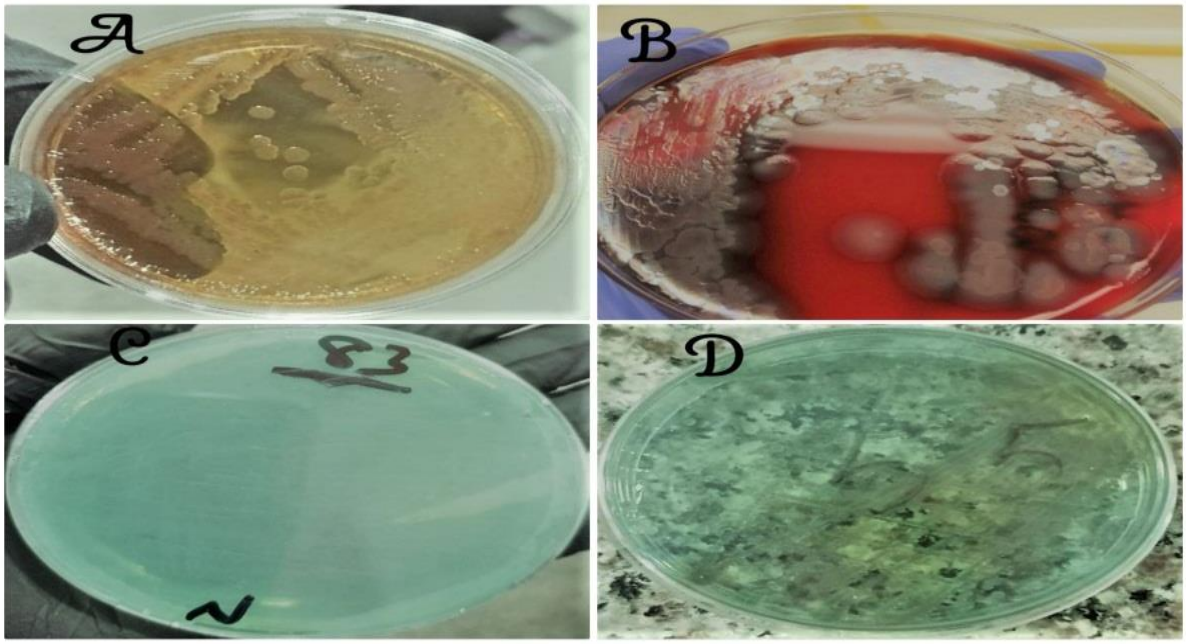


Figure 4.5: *P. aeruginosa* colonies on MaCconky agar(A), Blood agar(B), Nutrient agar (C) and Chromogenic agar (D)

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, as shown in Figure (4.6). These dyes are soluble in water (Sudhakar *et al*., 2015). Cetrimide agar is a selective media for isolation and presumptive identification of *P. aeruginosa*. It consists of peptone, MgCl2, K2SO4, cetrimide, agar, and the rehydrated contents of one vial of nalidixic acid selective supplement (FD130). It inhibits other microbial flora, grows with *Pseudomonas spp*., and produces a blue-green pigment (Aryal, 2015). This finding is similar to research in Iraq (AL-Rubaye *et al*., 2015) that used cetrimide agar and different medium for *P. aeruginosa* diagnosis .

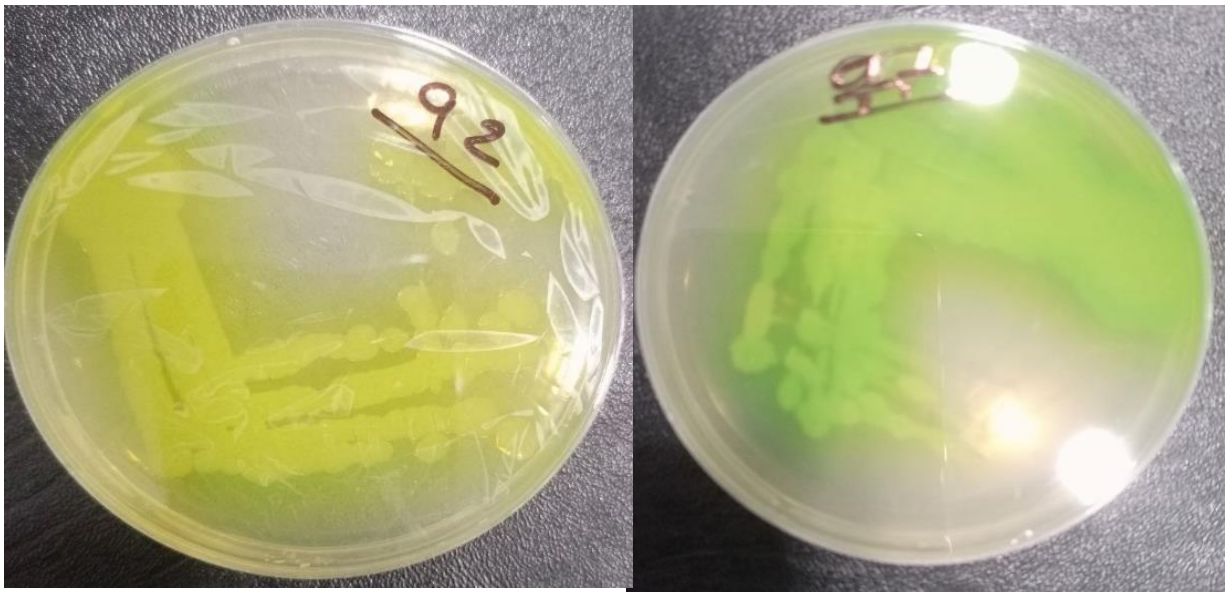


Figure 4.6: *P. aeruginosa* colonies on cetrimide agar

4.2.2 Detection of *P. aeruginosa* using biochemical methods

The Gram stain films of these isolating cultures revealed single or bi arrangement and Gram-negative bacilli. All *P. aeruginosa* isolates have shown a positive result in biochemical tests for oxidase, catalase tests, and pigment (blue water-soluble pigment pyocyanin, yellow-green pyoverdine,) (Barbhaiya and Rao, 1985; El-Fouly *et al*., 2015), and the urease test. IMVIC tests have yielded negative results, although isolates have shown positive results for citrate testing, despite negative results for other tests (C). In Kligler iron agar have given alkaline slant and change the bottom, H2S positive with gas production due to the fact that they are strictly aerobic and negative to Gram's stain as mentioned by Behbahani *et al*. (2019). The results obtained were compared with specifications in 21st edition of Bergey's Manual of Systematic Bacteriology, as shown in Table (4-3).

Table 4.2: Biochemical tests for identification of *P. aeruginosa* isolates

Test	Results
Gram-stain	G-ve rods
Catalase test	+
Oxidase test	+

Growth at 42°c	+
H2S production	+
Indole test	-
Kligler's iron agar	K/A
Methyl-red	-
Voges-Proskauer	-
Pigments production	+
Simmon's citrate	+
Urease	+
Motility	+

Abbreviations: (+), positive test; (-), Negative test

4.2.3. Molecular methods for further identification of *P. aeruginosa*

Detection of *P. aeruginosa* was performed by cetrimide agar (Selective Medium) were confirmed by PCR species-specific primer *P. aeruginosa* specific gene 16S rRNA (Figure 3-7) the result revealed that all isolates were 100% *P. aeruginosa* (Figure 4-7). 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; Momin *et al.*, 2017). 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 (Sivri *et al.*, 2014). 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).

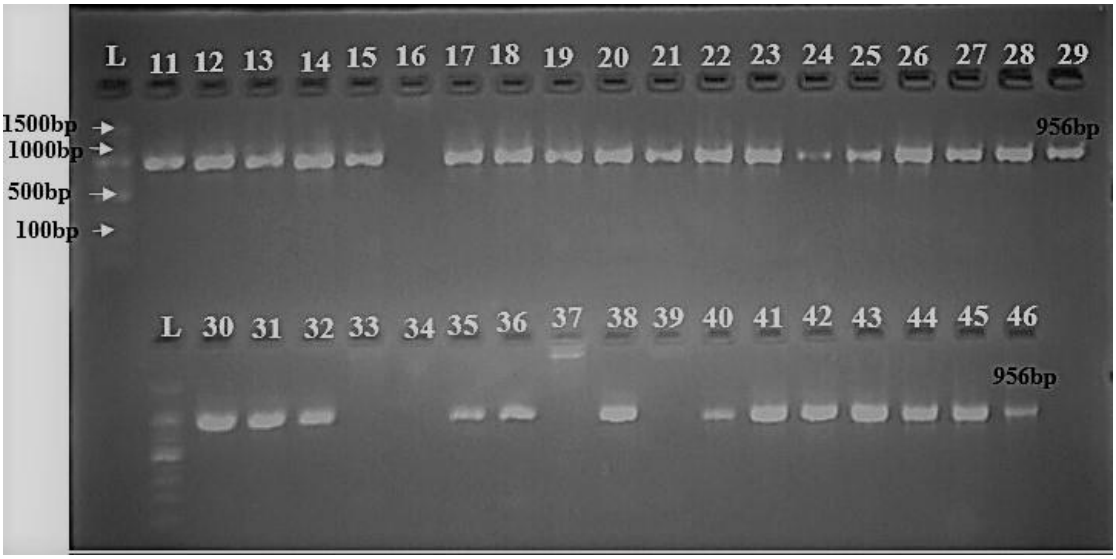
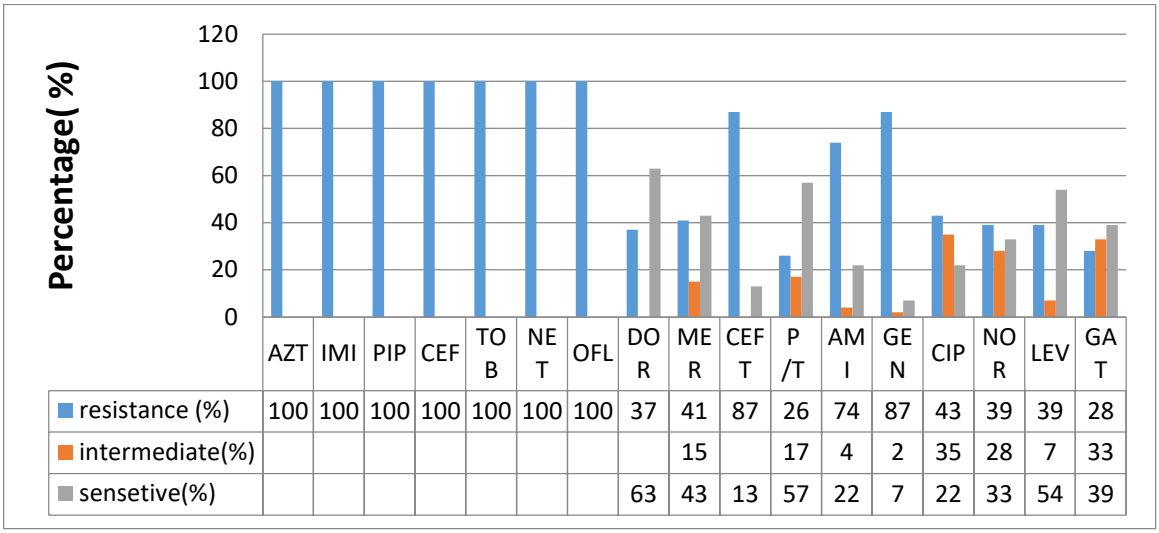


Figure 4.7: Agarose gel electrophoresis for amplified (956 bp) *P. aeruginosa* specific gene 16S rRNA of DM patients. Bands were fractionated by electrophoresis on a 1% gel (1 h., 80V/cm) and visualized under U. V. light after staining with safe red. (L:100 – 1500 bp M: ladder,); Lane: 1-46. positive results.

4.3. Antibiotic Susceptibility Test

Forty-six identified *P. aeruginosa* isolates (PA1 to PA46) were evaluated against 17 common antibiotics, as shown in Figures (4.8). The majority of isolates exhibited antibiotic resistance, particularly β-lactam antibiotics. All 46 isolates of *P. aeruginosa* test were resistant to tobramycin, piperacillin, cefepime, imipenem, ofloxacin, aztreonam, and netilmicin. The isolate PA26 was resistant to all antibiotics except for meropenem and norfloxacin, however, isolate PA2 was responsive to the majority of antibiotics**.** Antibiotic such as the tobramycin, piperacillin, cefepime, imipenem, ofloxacin, aztreonam, and netilmicin all exhibited 100 % resistance, whereas norfloxacin (39 %), piperacillin-tazobactam (26 %), levofloxacin (39 %), amikacin (74 %), meropenem (41 %), ciprofloxacin (43 %), doripenem (37 %). Table (4.4) in Appendex shows the phenotypic of antibiotic susceptibility of bacterial isolates in this study.



	AZT	IMI	PIP	CEF	TOB	NET	OFL	DOR	MER	CEFT	P/T	AMI	GEN	CIP	NOR	LEV	GAT
resistance (%)	100	100	100	100	100	100	100	37	41	87	26	74	87	43	39	39	28
intermediate(%)									15		17	4	2	35	28	7	33
sensetive(%)								63	43	13	57	22	7	22	33	54	39

Figure 4.8 Susceptibility patterns of *P. aeruginosa* to different antibiotics used in current study.

The results showed that *P. aeruginosa* resistance to piperacillin 46/46(100%). This result is not compatible with Vitkauskienė *et al*., (2010); Senthamarai *et al*., (2014) 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.

Beta lactam-beta-lactamase inhibitors combination antibiotics also showed resistance to piperacillin-tazobactam 26/46 (50%) (Figure 3-5). The resistance to cephems were 46/46(100%) and for cefepime and ceftazidime were 40/46(87%). 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 (2005) 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%. The results of the current study are consistent with the results of Al Shwaikh and Alornaaouti (2018) as the isolates of *P. aeruginosa* showed high resistance to ceftazidime, cefotaxime, piperacillin and was resistant to both ciprofloxacin (43%) and tobramycin (100%)

and gentamicin (87%) and resistant to both ofloxacin and imipenem resistant (100%). The majority of isolates exhibited antibiotic resistance., particularly β-lactam antibiotics. All 46 isolates of *P. aeruginosa* in this test were resistant to tobramycin, piperacillin, cefepime, imipenem, ofloxacin, aztreonam, and netilmicin (100%). The isolate PA26 was resistant to all antibiotics except meropenem and norfloxacin, however, isolate PA2 was responsive to the majority of medicines (Table 4-5)**.** In the present study, the highest resistant percentages toward the antibiotics were found with gentamicin (87%), while the lowest resistant percentages were found with doripenem (37%). 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*., 2020). Beta-lactam-beta-lactamase inhibitors combination antibiotics also showed resistance to piperacillin-tazobactam 12/46 (26%) (Figure 4.6). The resistance to cephems were 40/46(87%) for ceftazidime and (100%) for cefepime, (Figure 4.7). This result was very close with the results of Othman *et al.* (2014) who reported a resistant rate of *P. aeruginosa* ceftazidime were 73.6%, and close to other results obtained by Freitas and Barth (2005) and Hassuna *et al*. (2015) who recorded (87.7% and 86%) resistance rates, respectively while Hussein *et al*. (2018) reported resistance rate were 55.5%.

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 26%. 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). In spite of piperacillin antibiotic use rareness in the governmental, private hospitals and daily clinic, but there are high resistance rate (46% resistance and 30% intermediate) in contrast to β-lactam-β-Lactamase inhibitor combination, piperacillin-tazobactam which revealed high susceptibility to *p. aeruginosa* (62%) so, thought that the result due to many mechanism; First: Piperacillin one of β-Lactamse antibiotic bear structural resemblance to a natural substrate of penicillin-binding proteins (PBPs), i.e., the dipeptide D-alanyl-D-alanine, allowing them to effectively bind these enzymes (Goo and Sim, 2011; Sauvage *et al*., 2008). At the PBP active site, a serine residue attacks the carbonyl carbon of the β-lactam, resulting in the formation of a covalent acyl-enzyme complex that is slowly hydrolyzed (Zapun *et al*., 2008). PBP inhibition impairs peptidoglycan cross-linking, thereby leading to deregulation of bacterial cell wall synthesis and activation of cell lysis (Goo and Sim, 2011; Sauvage *et al*., 2008). The determination of PBP inhibition profiles is therefore important for establishing β-lactam activity against a given species. In the case of *P. aeruginosa*, the targets of β-lactams are the PBPs essential for cell viability, namely PBP1b, PBP1c, PBP2, and PBP3 (Moya *et al*., 2010). Also noteworthy is the non-essential PBP and PBP4, whose inhibition triggers a highly efficient and complex β-lactam resistance response and hence serves as a trap target for β-lactams (Moya *et al*., 2009; Moya *et al*., 2010). These resistance mechanisms have resulted in strains resistant to available antipseudomonal agents, including β-lactams, fluoroquinolones and aminoglycosides, and have greatly compromised the clinical efficacy of these agents (Perletti *et al*., 2010; Juan *et al*., 2010). Second: Tazobactam is a sulfone derivative of penicillanic acid (Beale *et al*., 2011) Like other early β-lactamase inhibitors (e.g., clavulanic acid, sulbactam), the moiety at position 1 (a sulfone group in tazobactam) acts as a leaving group that promotes

secondary ring opening at the β-lactamase active site, thereby facilitating covalent bond formation between tazobactam and the enzyme, and subsequently leading to irreversible inhibition (Drawz and Bonomo, 2010). Tazobactam is an inhibitor of most class A β-lactamases including many ESBLs and some class C β-lactamases (cephalosporinases) under the Ambler classification scheme (Drawz and Bonomo, 2010; Beale *et al*., 2011). At the β-lactamase active site, tazobactam forms a stable imine acyl-enzyme complex that undergoes hydrolysis much more slowly than the complex formed by β-lactams to eventually free the enzyme (transient inhibition) (Beale *et al*., 2011). Often referred to as an irreversible or "suicide" β-lactamase inhibitor, tazobactam actually undergoes multiple fates after the formation of this complex: (1) deacylation of the complex to regenerate the active enzyme and an inactive product; (2) tautomerization of the imine to form an enamine, also a reversibly inhibited enzyme; and (3) the formation of an irreversibly inactivated enzyme after a series of degradation reactions (Yang *et al*., 1999). The functional inhibition of the enzyme is determined by the relative rates of each of these pathways (Drawz and Bonomo, 2010). So extended-infusion piperacillin-tazobactam therapy is a suitable alternative to intermittent-infusion piperacillin-tazobactam therapy, and they strongly suggest that improved outcomes may be realized by administering extended-infusion piperacillin-tazobactam therapy to critically ill patients with *P. aeruginosa* infection (Lodise *et al*., 2007).

The rate resistance to aztreonam was 46/46 (100%), (Figure 4.8). A similar result (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 37%, 41% and 100% for doripenem, meropenem and imipenem, receptivity, as shown in Figure (4.9). Imipenem result was close to that of Fazeli *et al*. (2017) who reported a rate resistance 98.7%, but different from Savari (2016) and Vitkauskienė (2010) who recorded resistance 22% and 24%, receptivity. Meropenem result is compatible 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 40/46 (87%), tobramycin 46/46 (100%), amikacin 34/46 (74%), netilmicin 46/46 (100%) as shown in Figure (4.10). Gentamicin resistance rate recorded in this study was (87%), this result is far 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 100%, this result far from 15.9 and 3.3% which found by Al-derzi. (2012) and Coetzee *et al*. (2013), respectively, but high

resistance with Aljanaby and Aljanaby (2018) who reported a rate of 78.8% and Othman *et al*. (2014) who reported a rate of 76.2%. For Amikacin results demonstrated a resistance rate of 74%. This result was close to that of Aljanaby and Aljanaby (2018) (77.4%) and Corehtash *et al*. (2015) (82%). incompatible 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). *P. aeruginosa* shows resistance to a wide range of antibiotics, comprises aminoglycosides, quinolones and β-lactams. The resistance may be intrinsic (low outer membrane permeability, coding for efflux pumps and the making of antibiotic-inactivating enzymes), acquired (either horizontal transport of resistance genes or mutational alteration) and adaptive (involves formation of biofilm which provide as a diffusion barrier to edge antibiotic access to the bacterial cells) resistance (Mulcahy *et al*., 2010; Breidenstein *et al*., 2011). Resistance to flouroquinolones showed 20/46 (43%), 46/46 (100%), 18/46 (39%), to ciprofloxacin, ofloxacin and levofloxacin, respectively, as shown in Figure (4.11). For ciprofloxacin, this result is compatible 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). *P. aeruginosa* respiratory tract infections isolates were 100% resistant to ampicillin, ampicillin/sulbactam, amoxicillin, amoxicillin/clavulanate and chloramphenicol, highly resistant to cefuroxime (89%), tetracycline (89%) and azithromycin (84%), and susceptible to norfloxacin (89%), amikacin (84%) and meropenem (68%). *P. aeruginosa* urinary tract infection isolates were 100% resistant to ampicillin, amoxicillin, chloramphenicol, cefuroxime and tetracycline, highly resistant to amoxicillin/clavulanate (95%), azithromycin (95%), cefalexin (91%) and ampicillin/sulbactam (82%), and susceptible to amikacin (82%), meropenem (73%) and norfloxacin (64%). *P. aeruginosa* skin infection isolates were 100% resistant to ampicillin and amoxicillin, highly resistant to tetracycline (95%), amoxicillin/clavulanate (95%), cefalexin (87%) and azithromycin (84%), and susceptible to amikacin (87%), norfloxacin (71%) and meropenem (68%) (Gad *et al*., 2008). all *P. aeruginosa* isolates were found to be susceptible to Norfloxacin and Ciprofloxacin most isolates were also susceptible to Gentamicin (86.12 %) (Bekele *et al*., 2015). Antibiotic susceptibility testing was performed by using the disc diffusion method. Importantly, present results show that 53% out of *P. aeruginosa* isolates exhibited multi drug resistance (MDR) pattern. *P. aeruginosa*

isolates showed higher resistance to ciprofloxacin, gatifloxacin and meropenem and intermediate resistance to cefoperazone, cefepime, pipracillin, tobramycin, pipracillin-tazobactam, ceftazidime and azetreonem while Low bacterial resistance was noted against colistin only (Alsadek mohamed, 2020). Imipenem was the most effective antibiotic, with 100% of the strains being susceptible, followed by amikacin (87.5%), gentamicin, norfloxacin, gatifloxacin and polymyxin (both with 81.5%of susceptibility) (Leigue *et al*., 2016). This study also showed that most of the isolates of *P. aeruginosa* were 100 % multi-drug resistant (3 different antibiotic resistance). The details of the distribution of the MDR phenotype among *P. aeruginosa* and percentages of the isolates were summarized in Table (4-5). This was slightly more than the finding of Hasan *et al*. (2019) who found 91.6 % of the isolates were MDR, and higher more than the finding of Nasser and Kharat (2019) who found 66.3 % of *P. aeruginosa* isolates were MDR. While, agree with Moazami and Eftekhar (2013) which showed 100 % of *P. aeruginosa* isolates were MDR in the patients burns.

Table 4.4 Frequency of multidrug-resistant isolates of *P. aeruginosa*

Antibiotic	No.of resistant isolate
AZT./IMI./PIP./CEF./TOB./NET./OFL./DOR./MER./CAZ./PPT./GEN./ CIP./NOR.	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./CAZ./GEN./AMI./NOR./GAT./ MER./P/T./DOR	2
AZT./IMI./PIP./CEF./TOB./NET./OFL./CAZ./GEN./AMI./NOR./MER./ P/T./DOR	2
AZT./IMI./PIP./CEF./TOB./NET./OFL./CAZ./GEN./AMI./MER./ PPT./DOR	5
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN./ AMI./ MER./ DOR	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN./ AMI./ DOR	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN./ AMI././DOR./LEV	4
AZT. /IMI. /PIP. /CEF. /TOB. /NET. /OFL. / CAZ	1

AZT. /IMI. /PIP. /CEF. /TOB. /NET. /OFL. /CAZ./GEN./ DOR	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN /CIP/AMI./NOR./GAT	2
AZT./IMI./PIP./CEF./TOB./NET./OFL./CAZ./GEN/CIP/AMI./NOR./GAT./MER./LEV	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN /CIP /AMI NOR	3
AZT. /IMI. /PIP. /CEF. /TOB. /NET. /OFL. /CAZ./GEN /CIP	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./GEN	3
AZT. /IMI. /PIP. /CEF. /TOB. /NET. /OFL. /CAZ. /GEN /CIP. /AMI	5
AZT. /IMI. /PIP. /CEF. /TOB. /NET. /OFL. CAZ./GEN./CIP./AMI./NOR./GAT./MER./ PPT.	2
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN./CIP./AMI./NOR./GAT./MER	5
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN./CIP./AMI./NOR./GAT./MER./PPT./DOR	1
AZT./IMI./PIP./CEF./TOB./NET./OFL./ CAZ./GEN	3

Abbreviations: R, resistance; S, sensitive; I, intermediate; AZT, Aztreonam; IMI, Imipinem; PIP, Piperacillin; CEF, Cefepime; TOB, Tobramycin; NET, Netilmicin; OFL, Ofloxacin; DO, Doripenem, MER, Meropenem; CAZ, Ceftazidim; PPT, Piper. /Tazo.; AMI, Amikacin; GEN, Gentamicin; CIP, Ciprofloxacin; NOR, Norfloxacin; LEV, Levofloxacin; GAT. Gatifloxacin.

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-lactam-β-lactamase inhibitor for *P. aeruginosa* infections (Tannous *et al*., 2020; Al Muqati *et al*., 2021). Despite uncommon usage in government, private hospitals, and day clinics, piperacillin antibiotics have a significant resistance rate 46% and 30% intermediate resistance compared to piperacillin-tazobactam, which showed great susceptibility to *p. aeruginosa* (62 %) thus, considered various mechanisms caused the result; Piperacillin, a β-Lactam antibiotic, resembles a natural substrate of penicillin-binding proteins (PBPs), D-alanyl-D-alanine, allowing it to bind these enzymes (Goo and Sim, 2011; Sauvage *et al*., 2008). At the PBP active site, a serine residue attacks the -lactam carbonyl, forming a covalent acyl-enzyme complex (Zapun *et al*., 2008). PBP inhibition affects peptidoglycan cross-linking,

causing bacterial cell wall production dysregulation and cell lysis (Goo and Sim, 2011; Sauvage *et al*., 2008). Determining PBP inhibition patterns is vital for β-lactam activity against a particular species. In *P. aeruginosa*, β-Lactams target PBP1b, PBP1c, PBP2, and PBP3 (Moya *et al*., 2010). Non-essential PBP4, PBP4, induces a highly efficient and complicated β-lactam resistance response and acts as a trap target for β-Lactams (Moya *et al*., 2009; Moya *et al*., 2010). These resistance mechanisms have created strains resistant to β-lactamase, fluoroquinolones, and aminoglycosides, reducing their therapeutic effectiveness (Perletti *et al*., 2010; Juan *et al*., 2010). Second, Tazobactam is a penicillanic acid sulfone (Beale *et al*., 2011) Like other early β-lactamase inhibitors (e.g., clavulanic acid, sulbactam), tazobactam's position 1 moiety (a sulfone group) stimulates secondary ring opening at the β-lactamase active site, promoting covalent bond formation and permanent inhibition (Drawz and Bonomo, 2010). Under the Ambler categorization approach, tazobactam inhibits most class A β-lactamases (including many ESBLs) and certain class C β-lactamases (cephalosporinases) (Drawz and Bonomo, 2010; Beale *et al*., 2011). At the β-lactamase active site, tazobactam generates a stable imine acyl-enzyme complex that hydrolyzes more slowly than the complex generated by β-lactams (transient inhibition) (Beale *et al*., 2011). Often called an irreversible or "suicide" β-lactamase inhibitor, tazobactam undergoes multiple fates after forming this complex: (1) deacylation to regenerate the active enzyme and an inactive product; (2) tautomerization of the imine to form an enamine, also a reversibly inhibited enzyme; and (3) formation of an irreversibly inactivated enzyme after a series of degradation reactions (Yang *et al*., 1999). Functional inhibition of the enzyme is dictated by these rates (Drawz and Bonomo, 2010). So extended-infusion piperacillin-tazobactam therapy is a good option to intermittent-infusion piperacillin-tazobactam therapy, and they propose improved outcomes by

delivering it to critically sick patients with *P. aeruginosa* infection (Lodise *et al*., 2007).

4.3. Detection of biofilm formation by *P. aeruginosa*

In this study 46 isolates were tested using the microtiter culture plate method (TCP) assay, which Christensen *et al*. (1985) described as it a semi-quantitative microtiter plate test biofilm assay, Jolanta *et al*. (2016) found that biofilms protect against antimicrobial agents very well. The interpretation of biofilm formation was done according to the Stepanovic *et al*. (2007) criteria presented in Table (4-6). The result showed that out of 46 isolates only 7 (%14) isolates (PA3, PA5, PA9, PA12, PA13, PA16, PA19) were moderate positive to form biofilm, the weakly positive isolates were 27 (59%) and the negative isolate were 12 (27 %) as shown in Figure (4.5) in Appendex.

The discrepancy in results between different isolates in this study may be attributed to many factors such as the different cities from which the specimens were collected, the type of clinical specimens from which the isolates were

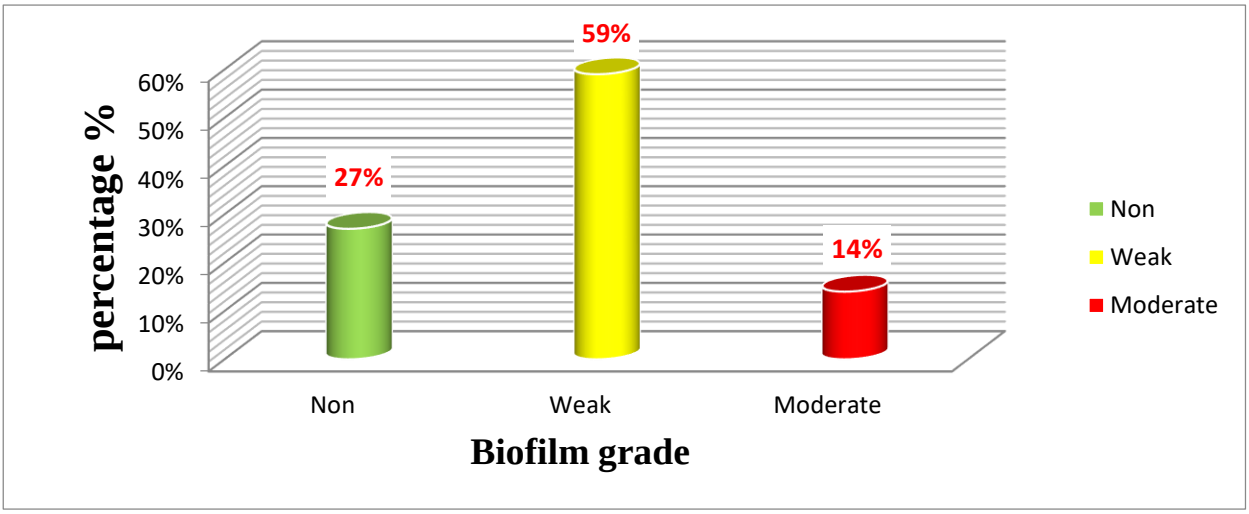


Figure 4.9. Biofilm formation grade of *P. aeruginosa* isolates

obtained and also the differences of isolates capability to form biofilm. The primary number of cells that succeeded in adherence and the differences of quality and quantity of auto inducers (quorum sensing signaling molecules) that were

produced from each isolate may also play an essential and an important role (Maleki *et al*., 2018). Jolanta *et al*. (2016) found that the biofilms were important to protect the bacteria against antimicrobial agents.

It is observed in the present study that a variable activity in biofilm formation was found this result has an agreement with the result obtained by Halstead *et al*., (2015) study, who noted that *P. aeruginosa* isolate exhibited variable biofilm production. The TCP assay was described in the literature as a simple and rapid method to quantify biofilm formation of different bacterial strains. Crystal violet is a basic dye known to bind to negatively charged molecules on the cell surface as well as nucleic acids and polysaccharides, and therefore, gives an overall measure of the whole biofilm. It has been used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of Gram positive and Gram-negative bacteria (MacFaddin, 2000). In the current study regarding to the percentages between biofilm formation and antibiotic resistance, not all isolates were 100 % biofilm formation and not 100 % multi-drug resistant as shown in Table (4-7). The results were agreed with Karami *et al*., (2020) results, as he found that 39 (67.2%) isolates forming biofilm and 19 (32.8%) non-biofilm forming. while low frequency (24%-27%) of biofilm formation stated by another studies (Abootaleb *et al.*, 2020; Abdelraheem *et al.*, 2020). Biofilm style of *P. aeruginosa* represent the aggregates encased in a self-produced extracellular matrix and are difficult or impossible to eradicate with antibiotic treatment. Biofilms are estimated to be responsible for over 65% of nosocomial infections and 60% of all human infections. Additionally, the biofilm risk orginated from the fact that, it is the main driver of persistence of chronic infections (Romling and Balsalobre, 2012; Benhamed *et al*., 2014; Ciofu and Tolker-Nielsen, 2019). Many of *P. aeruginosa* chronic infections have been linked to the biofilm mode of growth. Such infections are difficult to eradicate because bacteria in

biofilms have a higher tolerance against antimicrobial agents than their planktonic counterparts (Rybtke *et al.*, 2015).

Table 4.6 The grade and percentage of biofilm formation by *P. aeruginosa* isolates

Biofilm formation Grade and MDR	Isolate No.	%
Non	13	28
Weak	27	59
Moderate	6	13
Total	46	100

High resistance to all antibiotics with different grades of biofilm formation was recorded, Table 4.8, where the difference between antibiotic resistance and the grade of biofilms was no significant difference. This can be a reason or evidence that once a biofilm exists in any grade, it is sufficient to resist different lifetime antibiotics and this was what distinguishes of *P. aeruginosa* and makes them multi-drug resistant.

Table 4.7: Correlation between biofilm grade and antibiotic resistance.

Antibiotic	**Biofilm grade**						***P*. value**
	Non		**Weak**		**Moderate**		
	No	%	No	%	No	%	
Aztreonam	13	28	27	59	6	13	0.214
Imipinem	13	28	27	59	6	13	0.214
Piperacillin	13	28	27	59	6	13	0.214
Cefepime	13	28	27	59	6	13	0.214
Tobramycin	13	28	27	59	6	13	0.214
Netilmicin	13	28	27	59	6	13	0.214
Ofloxacin	13	28	27	59	6	13	0.214
Doripenem	5	29	10	59	2	12	0.542

Meropenem	6	32	9	47	4	21	0.76
Ceftazidim	12	30	23	57	5	13	0.293
Piper./Tazo.	4	33	8	67	0	0	0.471
Amikacin	9	26	19	56	6	18	0.414
Gentamicin	13	31	23	55	6	14	0.306
Ciprofloxacin	4	20	11	55	5	25	0.605
Norfloxacin	3	17	13	72	2	11	0.401
Levofloxacin	7	39	9	50	2	11	0.601
Gatifloxacin	4	32	7	53	2	15	0.673

There are two key reasons why the use of traditional antibiotic therapy makes biofilm bacteria hard to eliminate. Biofilm polysaccharide which is also referred to as slime, is a polymeric conglomeration generally composed of proteins and polysaccharides (Hall-Stoodley *et al.,* 2004). The extracellular exopolysaccharide of biofilms of *P. aeruginosa* is mainly composed of alginate. It has been stressed that matrix provides a barrier leading to enhance resistance to host defense mechanism as well as to antibiotics causing treatment failure and also promote adherence to epithelial cells. Biofilm formation provides bacteria with a means of persistently colonizing either living or inert surfaces within a human host (Murray *et al.,* 2010).

Another reason is that the biofilm bacteria are either slow-growing or non-growing. Some antibiotics, like β-lactams, require rapid bacterial growth to kill cells (Kaplan *et al*., 2012). Beta-lactam antibiotics have been shown to induce or increase production, biofilm volume and increase alginate production in *P. aeruginosa* biofilms and possible that enhanced biofilm formation by in response to β-lactams may foster genetic exchange and contribute to the spread of antibiotic resistance genes (Kaplan *et al*., 2012). This result agreed with Fricks-Lima *et al*.

(2011) and Kaplan *et al*. (2012) who observed that the greatest increase in biofilm antibiotic resistance occurred with the β-lactam antibiotics.

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

PCR detecting sequences of the genes of β-lactamase genes were positive; 28(63%) isolates carry bla_{SHV} gene, 26 (26.6%) isolates carry bla_{TEM} gene and 31 (67 %) isolates carry $bla_{CTX\text{-}M}$ gene as shown in Figures (4.18 to 4.20) and Table 4.9.

Table 4.8: Distribution of gene group of *P. aeruginosa* isolates.

Isolate No.	*SHV* gen	*TEM* gen	*CTX-M* gen	Genotype
PA1	-ve	+ve	+ve	*TEM and CTX-M*
PA2	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA3	-ve	+ve	+ve	*TEM and CTX-M*
PA4	+ve	+ve	-ve	*SHV and TEM*
PA5	-ve	-ve	+ve	*CTX-M*
PA6	+ve	+ve	-ve	*SHV and TEM*
PA7	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA8	-ve	+ve	+ve	*TEM and CTX-M*
PA9	+ve	-ve	+ve	*SHV and CTX-M*
PA10	-ve	-ve	-ve	None
PA11	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA12	-ve	+ve	+ve	*TEM and CTX-M*
PA13	+ve	-ve	+ve	*SHV and CTX-M*
PA14	+ve	-ve	+ve	*SHV and CTX-M*
PA15	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA16	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA17	+ve	-ve	-ve	*SHV*
PA18	+ve	-ve	+ve	*SHV and CTX-M*
PA19	+ve	-ve	+ve	*SHV and CTX-M*
PA20	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA21	-ve	+ve	-ve	*TEM*
PA22	+ve	-ve	+ve	*SHV and CTX-M*
PA23	-ve	-ve	+ve	*CTX-M*
PA24	-ve	+ve	+ve	*TEM and CTX-M*
PA25	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA26	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA27	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA28	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA29	+ve	-ve	-ve	*SHV*
PA30	+ve	+ve	-ve	*SHV and TEM*
PA31	-ve	+ve	-ve	*TEM*

PA32	+ve	-ve	+ve	*SHV and CTX-M*
PA33	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA34	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA35	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA36	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA37	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA38	+ve	+ve	+ve	*SHV,TEM and CTX-M*
PA39	-ve	-ve	-ve	None
PA40	-ve	-ve	+ve	*CTX-M*
PA41	+ve	-ve	-ve	None
PA42	-ve	-ve	-ve	None
PA43	-ve	-ve	-ve	None
PA44	-ve	-ve	-ve	None
PA45	-ve	-ve	-ve	None
PA46	-ve	-ve	-ve	None

Abbreviation: +ve: gene possessing., -ve: gene lacking

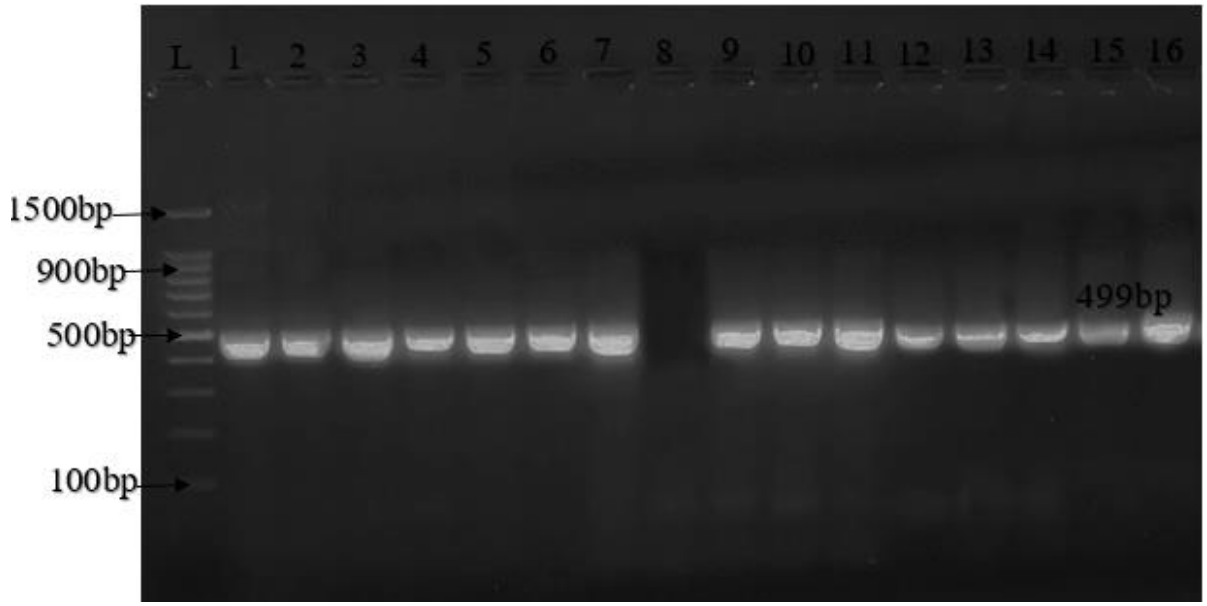


Figure 4.10: The presence and absences of bla$_{CTX-M}$ PCR product (499bp) in some isolated samples. PCR products were separated by electrophoresis in an 1% agarose gel, at 75 V\Cm for 80 min. M: Marker DNA ladder size (1500 bp).

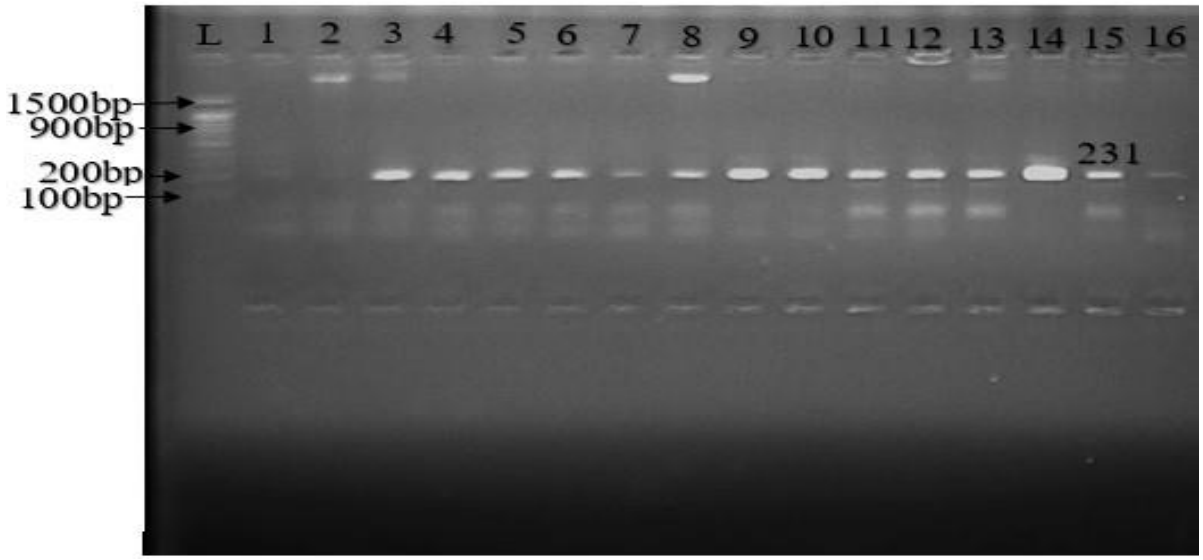


Figure 4.11: The presence and absences of bla$_{SHV}$ PCR product (231bp) in some isolated samples. PCR products were separated by electrophoresis in an 1% agarose gel, at 75 V\Cm for 80 min. M: Marker DNA ladder size (1500 bp).

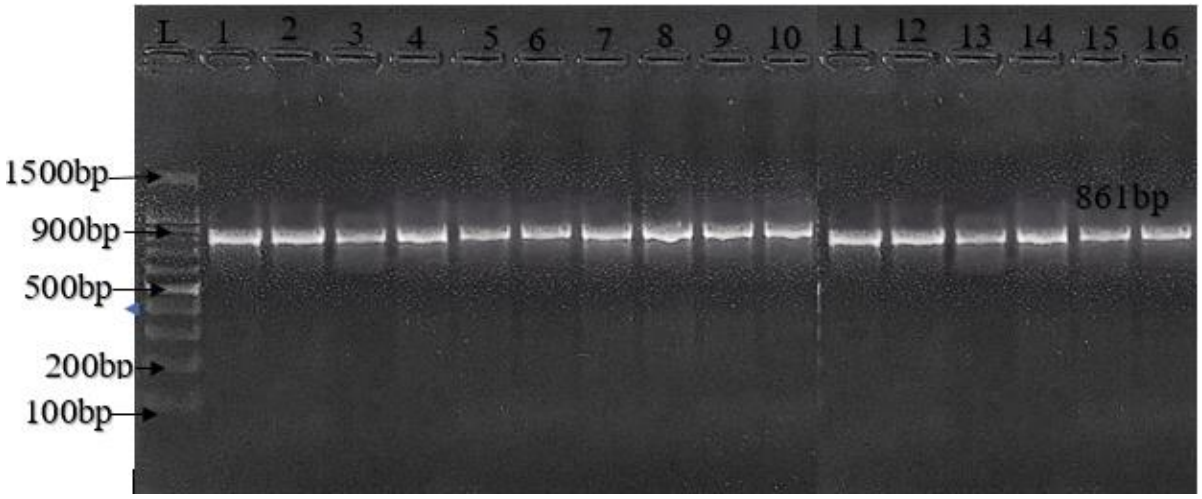


Figure 4.12: The presence and absences of bla$_{TEM}$ PCR product (861bp) in some isolated samples. PCR products were separated by electrophoresis in an 1% agarose gel, at 75 V\Cm for 80 min. M: Marker DNA ladder size (1500bp).

Based on these findings, there were 16 isolates (34.78%) possessed *bla$_{TEM}$*, bla$_{CTX-M}$ and bla$_{SHV}$ genes. the *bla$_{CTX-M}$* and bla$_{SHV}$ genes were identified in 7 (15.21%) isolate, and also *bla$_{CTX-M}$* and bla$_{TEM}$ genes were identified in 5 isolates (10.86%) whereas 7 isolates (PA10, PA39, PA41, PA42, PA43, PA44, PA45, and PA46) lacked of the three genes. the isolates PA5, PA23, and PA40 were found to

have *CTX-M* gene, whereas the PA17 and PA29 isolates were found to possess *SHV* gen, on the other hand, the PA21 and PA31 isolates have *TEM* gene. the distribution of (*SHV, TEM and CTX-M*) Genes of *P. aeruginosa*. the distribution of (*SHV, TEM and CTX-M*) Genes of *P. aeruginosa* in current study show in Table (4.10).

Table 4.9: Distribution of bla_{SHV}, bla_{TEM} and bla_{CTX-M} Genes of *P. aeruginosa*.

Gene	Isolate *P. aeruginosa.* No	%
only *SHV*	2	4.35
only *TEM*	2	4.35
only *CTX-M*	3	6.5
SHV and TEM	3	6.52
SHV and CTX-M	7	15
TEM and CTX-M	5	10.9
SHV,TEM and CTX-M	16	34.7
None	8	17.4

In the current study, β-lactamases production using antibiotics resistance and molecular detection of bla_{SHV}, bla_{TEM} and bla_{CTX-M} 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_{SHV} then bla_{TEM} genes, as shown in Table (4.10).

Table (4.10): Pattern of highest resistance isolates of *P. aeruginosa* isolate to the different type of antibiotcs among these β-lactamases genes

Antibiotic	*SHV*		*TEM*		*CTX-M*		*SHV* and *TEM*		*SHV* and *CTX-M*		*TEM* and *CTX-M*		*SHV, TEM,* and*CTX-M*		None	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Aztreonam	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100
Imipinem	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100
Piperacillin	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100
Cefepime	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100
Tobramycin	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100
Netilmicin	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100
Ofloxacin	2	100	2	100	3	100	3	100	7	100	5	100	16	100	8	100

Doripenem	1	50	0	0	1	33	2	66	2	28	0	0	6	37	5	62
Meropenem	0	0	1	50	1	33	2	66	3	42	2	40	6	37	4	50
Ceftazidim	2	100	2	100	3	100	2	66	5	71	4	80	13	81	8	100
Piper./Tazo.	2	100	1	50	1	33	2	66	1	14	0	0	2	12	2	25
Amikacin	2	100	1	50	2	66	2	66	7	100	5	100	11	68	4	50
Gentamicin	2	100	2	100	3	100	2	66	7	100	4	80	14	87	8	100
Ciprofloxacin	0	0	0	0	0	0	1	33	5	71	3	60	6	37	4	50
Norfloxacin	1	50	1	50	1	33	1	33	0	0	3	60	8	50	3	37
Levofloxacin	0	0	1	50	2	66	1	33	4	57	1	20	4	25	5	62
Gatifloxacin	0	0	1	50	0	0	2	66	2	28	1	20	5	31	2	25

The highest bacterial resistance (100%) was obtained to nalidixic acid (30 µg), followed by tetracycline (30 µg) and amoxicillin (25 µg). The frequencies of bla_{CTX-M}, bla_{SHV}, bla_{TEM} and bla_{OXA-48} genes were 23.95% (23 isolates), 23.08% (26 isolates), 57.29% (55 isolates) and 12.5% (12 isolate), respectively. Sixty isolates resistant to carbapenems (78.9%) carried at least one resistant gene (bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA-48}) and 16 isolates (21.1%) did not have any of these genes (Malek *et al*.,2018). In another study (Peymani *et al*.,2017) of the 75 *P. aeruginosa* isolates with ESBL phenotype, $bla_{\text{TEM-1}}$ (20-26.7%) was the most common gene, followed by $bla_{\text{CTX-M-15}}$ (13- 17.3%), $bla_{\text{SHV-1}}$ (5-6.7%), and $bla_{\text{SHV-12}}$ (3-4%), either alone or in combination. In this study, isolates were, instead, negative for $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, and $bla_{\text{CTX-M-9}}$- group genes, as well as for $bla_{\text{CTX-M-25}}$. In a recent study, it was found that the most prevalent genotype for ESBL production was $bla_{\text{TEM,}}$ which was detected in 65 (79.3%) strains followed by bla_{SHV} (69.5%) and $bla_{\text{CTX-M}}$ (31.7%) (Hosu *et al*., 2021). The antimicrobial resistance profiles of the samples were compared after Shirehjini *et al*. (2017) which found significant levels of antibiotic resistance in *P. aeruginosa* strains and discovered that 34.2% of the bacteria had a *TEM* gene. According to Peymani *et al*. (2017), the most often found gene in *P. aeruginosa* strains was bla_{TEM-1} (26.7%), which was followed by bla_{CTX-M}-15 (17.3%), bla_{SHV}-1 (6.7%), and bla_{SHV}-12 (4%). By using the PCR technique, Bahrami *et al*. (2018) looked into the presence of the beta-lactamase genes bla_{SHV},

bla$_{TEM}$ and *bla*$_{CTX-M}$, in 96 clinical isolates of *P. aeruginosa* in Bandar Abbas. There were 23 isolates with bla$_{CTX-M}$, 23 isolates with bla$_{SHV}$, 26 isolates with bla$_{TEM}$, 55 isolates with bla$_{OXA-48}$, and 12 isolates with bla$_{OXA-48}$, respectively, with prevalence rates of 23.95 %, 23.08 %, 57.29 %, and 12.5%. (19). These results are similar in some aspect to those mentioned in this study. Nosocomial bacterial infections' patterns of resistance can change significantly over time and from one country to another (Prashanth and Badrinath, 2004). The *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-48}$ and *bla*$_{CTX-M}$ genotypes are prevalent in Asian countries (Bahrami *et al*., 2018). In this study, a total of 38 isolates (82.6%) carried at least one of the ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$), and 8 isolates (17.4%) carried no ESBL-producing genes. The most prevalent β-lactamase gene, according to the PCR results, was *bla*$_{CTX-M}$ that was detected in 31 isolates. The frequency of the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were 100% and 66% respectively in the study of Bokaeian *et al*., (2018). Their findings revealed that 6.89% of *P. aeruginosa* MDR isolates tested positive for ESBL. The prevalence of the *bla*$_{TEM}$ gene in study conducted by Mohammed *et al*. (2016) was 25%, which is lower than the comparable prevalence in this study. On the other hand, the association of production of β-lactamases with biofilm formation: All beta-lactamase isolates were positive for the formation of biofilms, as showed in Table 4.6. Although most ESBL producers were biofilm positive, no statistical significance was observed. Then grade of biofilm formation in relation with β-lactamase production among the isolates. The diffusion barrier of biofilm plays a role for resistance of *P. aeruginosa* that overproduce beta-lactamase due to the presence of the biofilm matrix of beta-lactamases which will hydrolyze the beta-lactam antibiotics before reaching the bacterial cells (Hoiby *et al*., 2010). Nichols *et al*. (1989) predicted from mathematical models that the biofilm would not afford protection against diffusion of beta-lactam antibiotics into the bacteria embedded in the biofilm as long as the level of chromosomal beta-lactamase is low. However,

bacteria expressing high level of chromosomal beta-lactamase growing in biofilms would be exposed to reduced concentration of beta-lactam antibiotics due to accumulation of the enzyme in the polysaccharide matrix. The extracellular beta-lactamase would inactivate the antibiotic as it penetrates, thereby protecting the deeper-lying cells (Hoiby *et al*., 2010).

4.5. DNA Sequence and Phylogenetic Tree

4.5.1. DNA amplification of bla$_{CTX-M}$, bla$_{TEM}$ and bla$_{SHV}$ Genes

The DNA amplification was accomplished by the thermo-cycler apparatus under the optimal conditions using specific primers as mentioned in the Tables (3-7). The result of the PCR reaction revealed that the presence of a single band (499 bp) of the target sequence for bla$_{CTX-M}$ gene of *P. aeruginosa* as in Figure (4.9). The result of the PCR reaction revealed that the presence a single band (231 bp) of the target sequence for bla$_{SHV}$ gene of *P. aeruginosa* as in Figure (4.10). The result of the PCR reaction revealed that the presence a single band (848 bp) of the target sequence for bla$_{TEM}$ gene of *P. aeruginosa* as in Figure (4.11).

4.5.2. DNA Sequencing

To obtain a trimmed sequence, each data sequence was trimmed from the beginning to the ending, according to normal waves. When compared to NCBI-Blast, this sequence has a high level of identity to other global sequence data. The waves produced by scanning the sequences indicate the strong and weak regions of the sequences, which are then trimmed, resulting in increased identity with global sequences at NCBI-Blasting. The results of nucleotide sets are checked and confirmed by using NCBI–Basic Local Alignment Search Tool (BLAST analysis)-nucleotide Blast-Search a nucleotide database using a nucleotide query online, which was a perfect program and gave the exact results of identity percentage with other world strains. Sequence alignment must be performed using *bla$_{CTX-M}$, bla$_{SHV}$*

and bla_{TEM} genes of *P. aeruginosa* sequences databases information recorded in Gen Bank to find identity and similarity score degrees of gene and compared with the isolates of this study. The results of sequences alignment of the nine local isolates (PA1 to PA9), isolated from different regions of Iraq, Table 2 and 6 showed identity ranging from 95% to 100% (Figure. 2 to 10 and Table 5), good query cover, and max score with other world strains of *P. aeruginosa*.

Table 4.11: Alignment results of nine *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 (%)	Origin
1.	*P. aeruginosa* PA1	bla_{CTX-M}	KY792758.1	99 %	UAE
2.	*P. aeruginosa* PA2	bla_{CTX-M}	KU139118.1	98 %	India
3.	*P. aeruginosa* PA3	bla_{CTX-M}	KU139120.1	95 %	India
4.	*P. aeruginosa* PA4	bla_{SHV}	KY640504.1	96 %	Egypt
5.	*P. aeruginosa* PA5	bla_{SHV}	KY640504.1	100 %	Egypt
6.	*P. aeruginosa* PA6	bla_{SHV}	KY640504.1	100 %	Egypt
7.	*P. aeruginosa* PA7	bla_{TEM}	MG755406.1	99 %	Iran
8.	*P. aeruginosa* PA8	bla_{TEM}	MG755406.1	99 %	Iran
9.	*P. aeruginosa* PA9	bla_{TEM}	MG755406.1	99 %	Iran

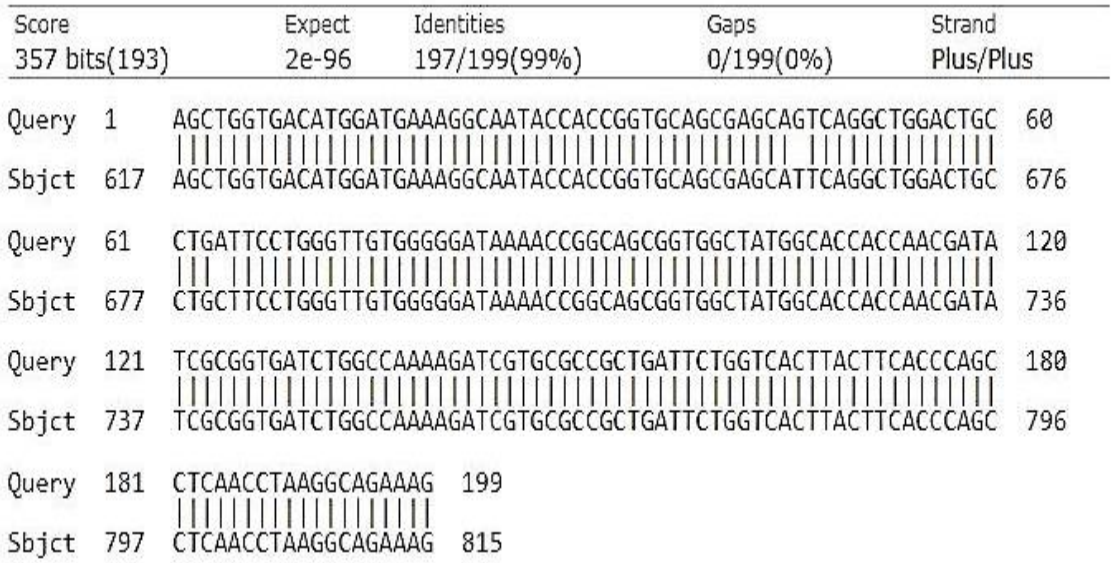


Figure (4.13): Basic Local Alignment of *P. aeruginosa* bla_{CTX-M} gene isolate PA1 with high similarity NCBI-BLAST *P. aeruginosa* strain SKGH_46 beta-lactamase (bla_{CTX-M}) gene, partial sequence (accession number: KY792758.1in GenBank).

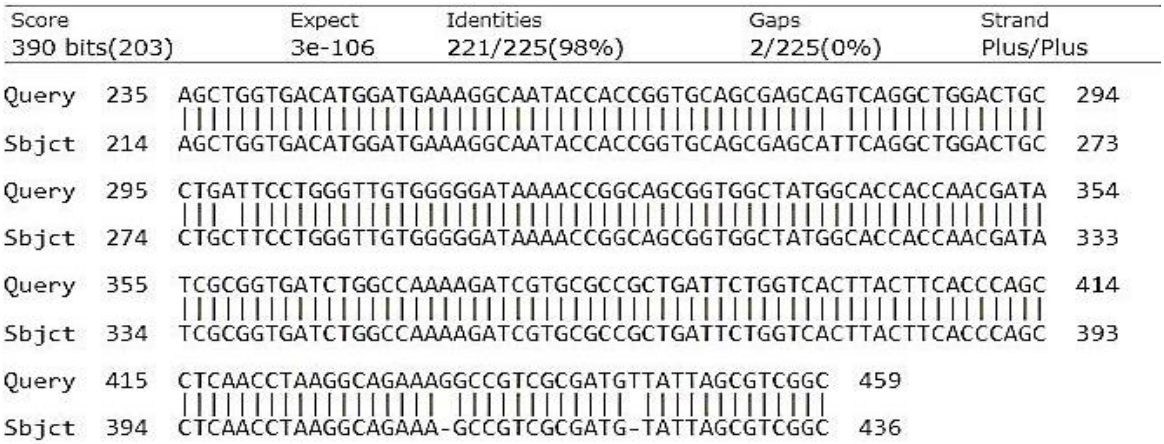


Figure (4.14): Basic Local Alignment of *P. aeruginosa* bla_{CTX-M} gene isolate PA2 with high similarity NCBI-BLAST *P. aeruginosa* strain PA137 beta-lactamase (bla_{CTX-M}) gene, partial sequence (accession number: KU139118.1in GenBank).

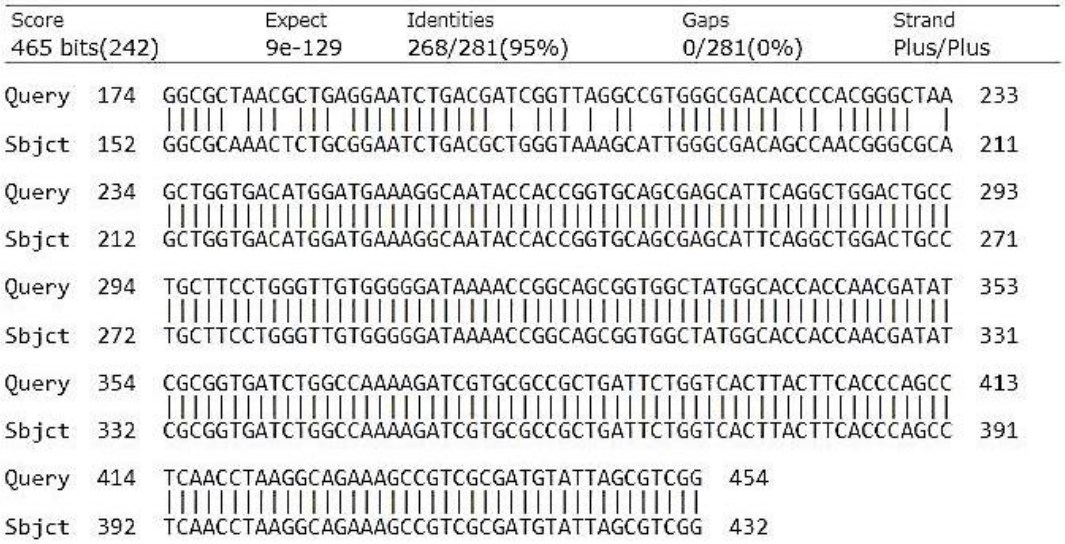


Figure (4.15): Basic Local Alignment of *P. aeruginosa* bla_{CTX-M} gene isolate PA3 with high similarity NCBI-BLAST *P. aeruginosa* strain Palg29 beta-lactamase (bla_{CTX-M}) gene, partial sequence (accession number: KU139120.1 in GenBank).

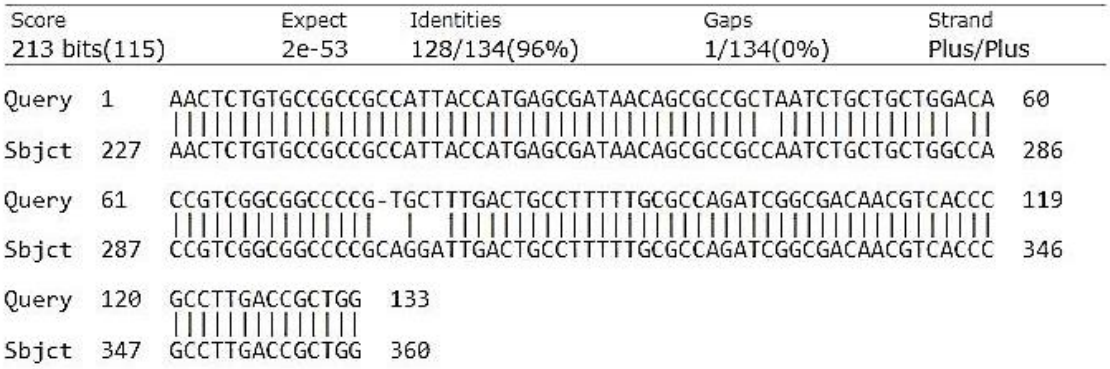


Figure (4.16): Basic Local Alignment of *P. aeruginosa* bla_{SHV} gene isolate PA4 with high similarity NCBI-BLAST *P. aeruginosa* strain E14PAMO beta-lactamase (bla_{SHV-11}) gene, partial sequence (accession number: KY640504.1 in GenBank).

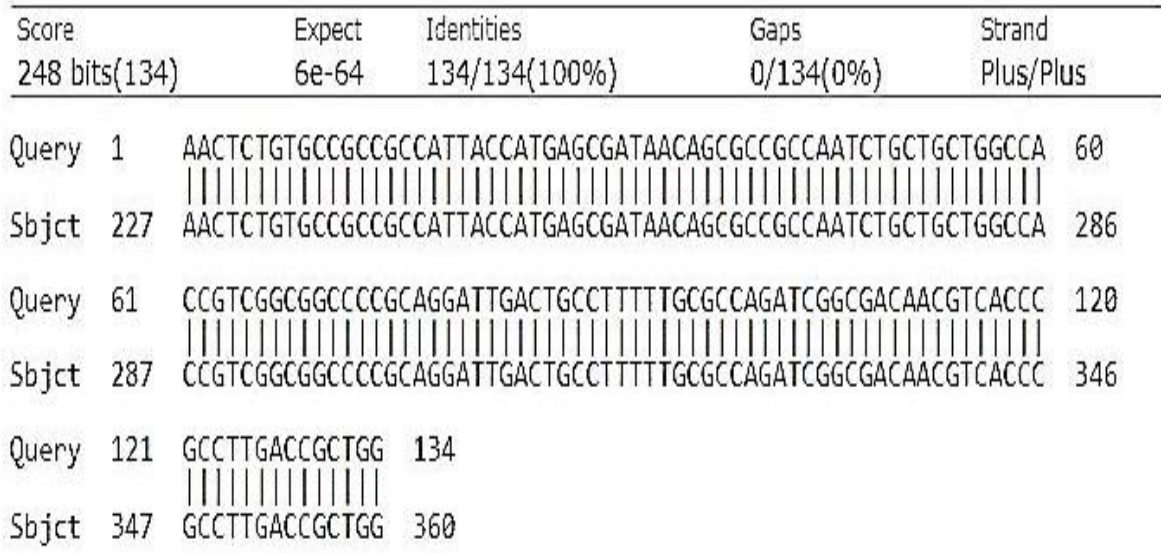


Figure (4.17): Basic Local Alignment of *P. aeruginosa* bla_{SHV} gene isolate PA5 with high similarity NCBI-BLAST *P. aeruginosa* strain E14PAMO beta-lactamase (bla_{SHV-11}) gene, partial sequence (accession number: KY640504.1in GenBank).

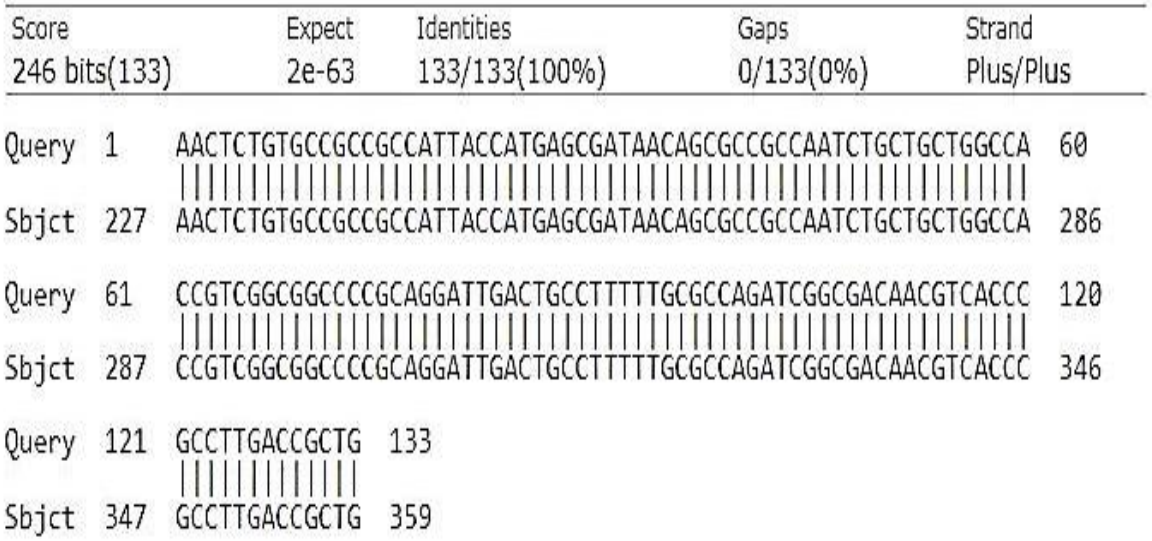


Figure (4.18): Basic Local Alignment of *P. aeruginosa* bla_{SHV} gene isolate PA6 with high similarity NCBI-BLAST *P. aeruginosa* strain E14PAMO beta-lactamase (bla_{SHV-11}) gene, partial sequence (accession number: KY640504.1in GenBank).

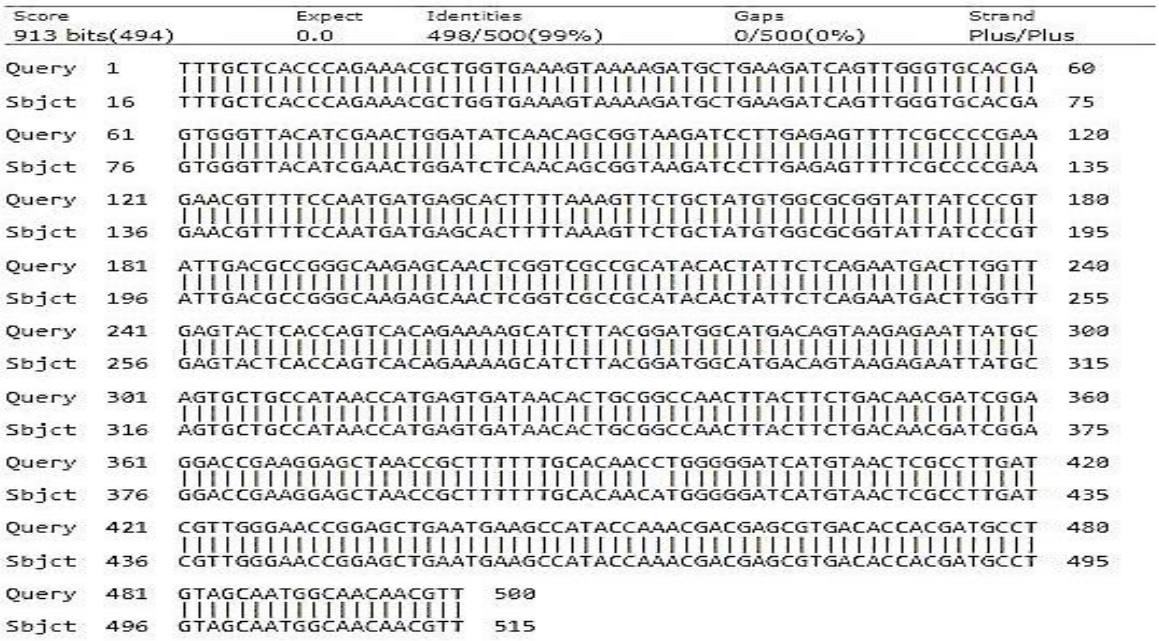


Figure (4.19): Basic Local Alignment of *P. aeruginosa* bla_{TEM} gene isolate PA7 with high similarity NCBI-BLAST *P. aeruginosa* strain F35 beta-lactamase (bla_{TEM}) gene, partial sequence (accession number: MG755406.1in GenBank).

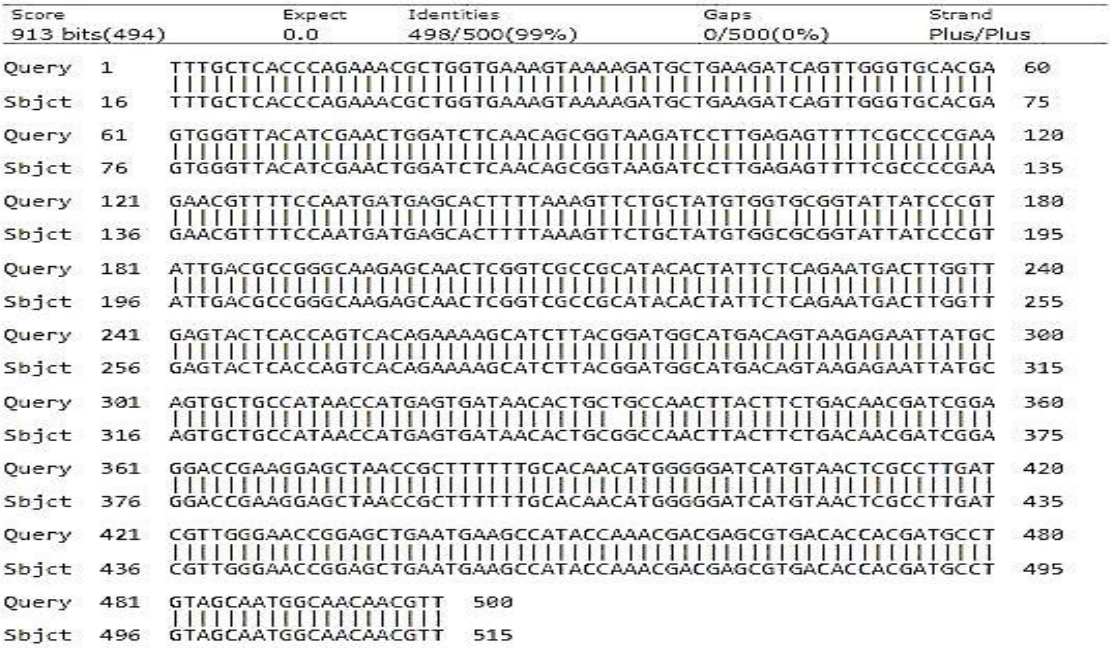


Figure (4.20): Basic Local Alignment of *P. aeruginosa* bla_{TEM} gene isolate PA8 with high similarity NCBI-BLAST *P. aeruginosa* strain F35 beta-lactamase (bla_{TEM}) gene, partial sequence (accession number: MG755406.1in GenBank).

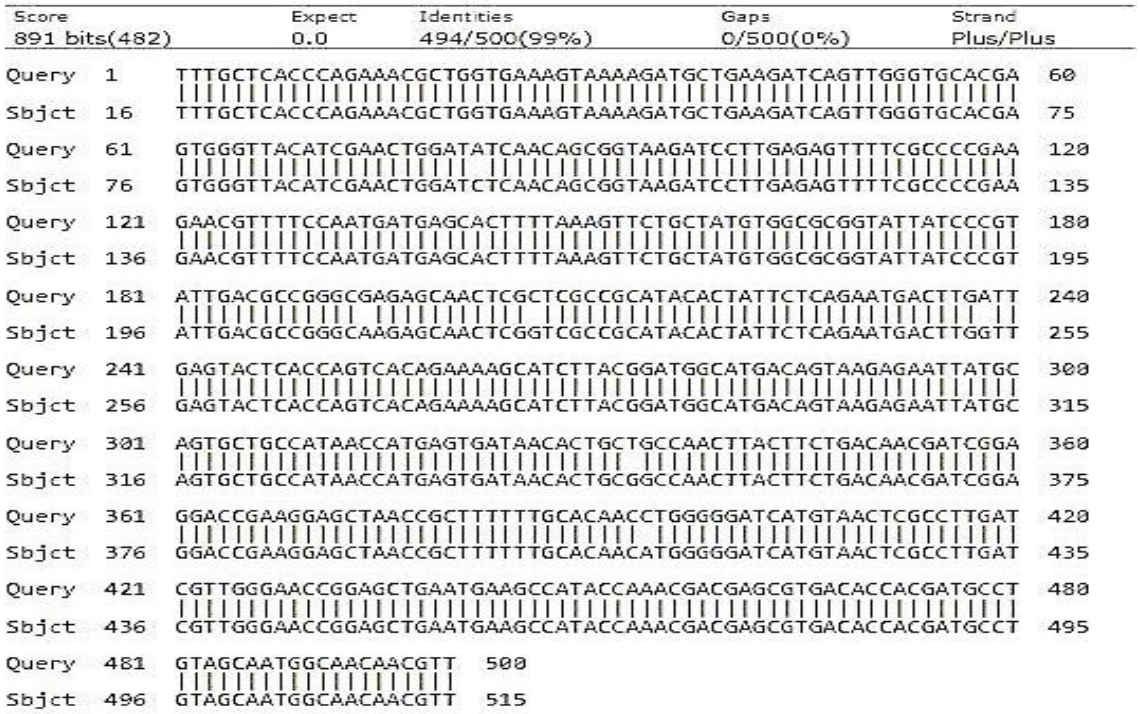


Figure (4.21): Basic Local Alignment of *P. aeruginosa* bla_{TEM} gene isolate PA9 with high similarity NCBI-BLAST *P. aeruginosa* strain F35 beta-lactamase (bla_{TEM}) gene, partial sequence (accession number: MG755406.1in GenBank).

4.5.3. Phylogenetic analysis of local and world strains:

The phylogenetic tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The dataset was cleansed of positions with gaps or missing data (Complete deletion option). MEGA X 10.2.4 is used to perform phylogenetic analysis. There were 13 global taxa about $bla_{CTX\text{-}M}$ gene of *P. aeruginosa* downloaded from NCBI and submitted with 3 local sequences to Mega X 10.2.4 software to obtain the Figure (4.30). There were 10 global taxa about bla_{SHV} gene of *P. aeruginosa* were downloaded from NCBI and submitted with 3 local sequences to Mega X 10.2.4 software to obtain the figure (4.31). There were 11 global taxa about bla_{TEM} gene of *P. aeruginosa* were downloaded from NCBI and submitted with 3 local sequences to Mega X 10.2.4 software to obtain the figure (4.32).

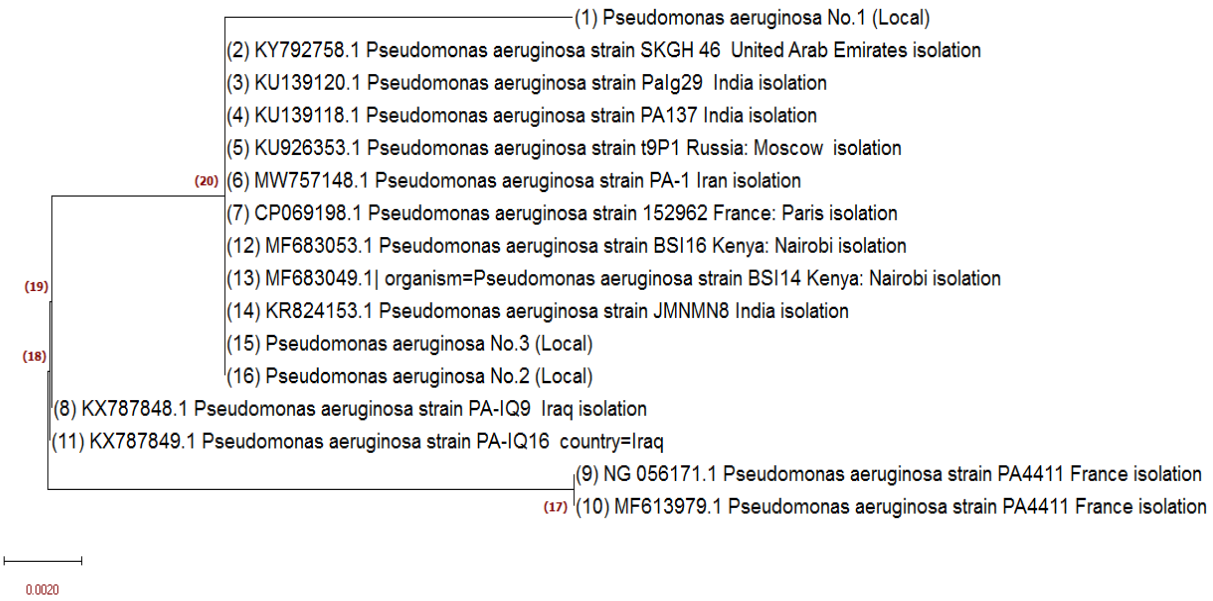


Figure 4.21: Phylogenic tree of *bla*$_{CTX-M}$ gene partial sequences of local and global sequences using neighbor-joining bootstrap 1000 tree figure. Evolutionary relationships of 16 taxa. No.1 to 3 represents local isolates.

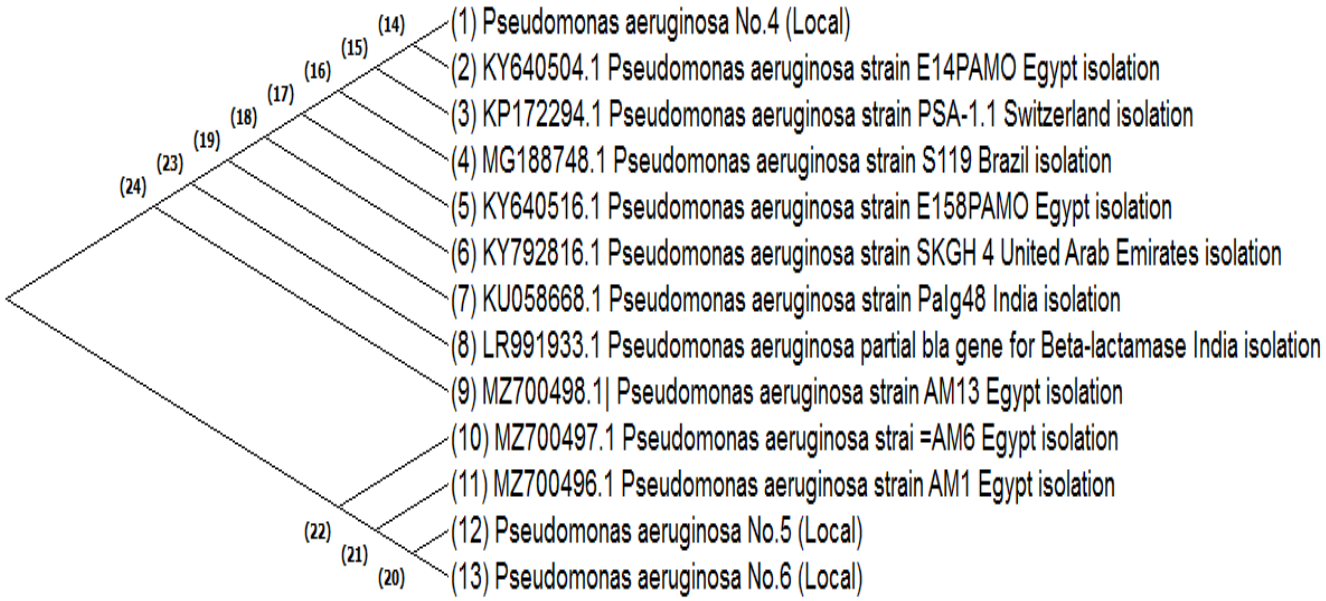


Figure 4.22: Phylogenic tree of blaSHV gene partial sequences of local and global sequences using neighbor-joining bootstrap 1000 tree figure. Evolutionary relationships of 13 taxa. No.1 to 3 represents local isolates.

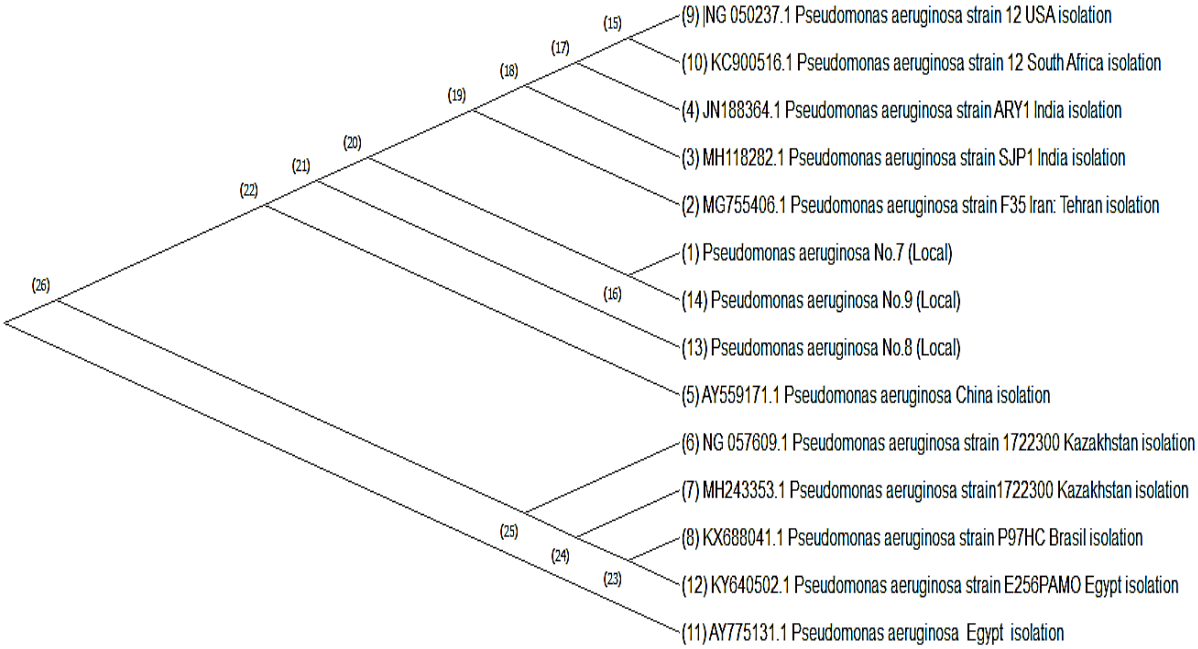


Figure 4.23: Phylogenic tree of *bla$_{TEM}$* gene partial sequences of local and global sequences using neighbor-joining bootstrap 1000 tree figure. Evolutionary relationships of 14 taxa. No.1 to 3 represents local isolates.

The Molecular Evolutionary Genetics Analysis (MEGA) program is a desktop software that allows you to compare homologous gene sequences from various species or multigene families, with a focus on inferring evolutionary links and patterns of DNA and protein evolution. MEGA features a number of useful tools for assembling sequence data sets from files or web-based repositories, as well as tools for visualizing the results in the form of interactive phylogenetic trees and evolutionary distance matrices (Kumar *et al*., 2008). The first stage in the analysis was to align all of the sequences form three genes in this study with other world-wide references using MEGA X 10.2.4 's (Clustal W) program step. This program was shown to have a high degree of similarity with all world sequences, including the sequences used in this study. These (Clustal W) results were significant since they were directly utilized in the phylogenetic tree design.

The Neighbor-Joining (NJ) approach, which is a simplified version of the minimal evolution (ME) method, is used in this study to determine the close relationship between world and local sequences. Because it does not need the assumption of a constant rate of evolution, the NJ method yields an unrooted tree. An out group taxon is needed to find the root (Saitou and Nei, 1987; Rzhetsky and Nei, 1992). A phylogenetic tree, also known as an evolutionary tree, is a branching diagram or "tree" that depicts the assumed evolutionary relationships among distinct biological species or other entities based on physical and/or genetic similarities and differences (Salahuddin and Khan,2014) In bla_{CTX-M} gene phylogeny (Fig. 4.30) , we submitted 16 sequences, 3 sequences belong to local sequences and 13 sequences belong to global sequences obtained by download from NCBI they submitted to a MEGA X 10.2.4 software program for obtaining phylogenic relationship among local and global sequences, after submitting these sequences to MEGA X 10.2.4 at the first time, It was found alignment by Clustal, then use NJ method at bootstrap 1000 , the sequence of *P. aeruginosa* PA1 was near to the sequence KY792758.1 *P. aeruginosa* strain SKGH (UAE) and Indian isolates (KU130118.1 and KU130120.1), while the local sequence of *P. aeruginosa* PA2 and PA3 was closely related to the sequence KR824153.1(Indian strain). All three sequences are far away from Iraqi isolates (KX787848.1 and KX787849.1). In bla_{SHV} gene phylogeny, as shown in Figur 4.31. It was found that both sequences of *P. aeruginosa* PA5 and PA6 were closely related to the sequences of Egyptian strains (MZ700496.1 and MZ700497.1). Also, the local sequences of *P. aeruginosa* PA4 were closely associated with the sequence KY640504.1 strain E14PAMO which is isolated in Egypt.

In the phylogeny of bla_{TEM} gene (Fig.4.32), the local sequences *P. aeruginosa* PA7 and *P. aeruginosa* PA9 were closely related to each other to form

sister sequences, related to sequence MG755406.1 *P. aeruginosa* strain F35 which isolated in Iran. In contrast, the local sequences *P. aeruginosa* PA8 are near to the sequence AY559171.1 which is isolated in china. Thus, Phylogenic relationship among local and world strains provide high information about origin and genetic evolution of local isolates. Mohammed *et al*., (2016) revealed that the phylogenetic tree of *CTX-M-9* gene sequences in *E. coli* strains isolated from ZU Hospitals and published homologous sequences in GenBank revealed varying degrees of dis/similarity between the strains, as well as many unique sequences in the Egyptian strain, which was similar to that of Russia and Australia but not to that of Japan. According to the phylogenetic tree, *SHV* encoded for *K. pneumoniae* showed a compatibility range of 98 %, and *SHV* encoded for *E. coli* showed a compatibility range of 99 percent, whereas *SHV* gene encoded for *P. aeruginosa* isolated showed a variation in the compatibility range with countries (99 %) in Brazil, Egypt, United Arab Emirates, India, Japan, Tunisia, France, and Switzerland, followed by Greece (98%), the USA (74%), Brazil: Belo Horizonte (74%), and Colombia (71%) (Khalaf and Al-Ouqaili ,2018) .

Conclusions and Recommendation

Conc1usion

1. The specific primers of PCR assay of *P. aeruginosa* were highly sensitive and reliable for molecular identification of *P. aeruginosa.*
2. High levels of resistance toward the antibiotics, used in this study were seen, especialy toward the imipenem.
3. The isolates showed ability to produce the biofilm which increased the resistance of *P. aeruginosa* toward the antibiotics.
4. bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes of *P. aeruginosa* isolates showed high expressed.
5. The presence of virulence factors in *P. aeruginosa* vary according to the geographic regions.
6. All isolates of *P. aeruginosa* were similar to the strains of NCBI at a rate of 95-100 % and the highest matching rate of isolates was 100 % which originated in Egypt, 99 % which originated in Iran and UAE, and 99 % which originated in India.

Recommendations

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

2- Further studies for creating genetic diversity map of *P. aeruginosa* was also needed.

3- It is necessary to carry out comprehensive study for $b1a_{TEM}$, $b1a_{SHV}$, and $b1a_{CTX\text{-}M}$ group gene sequences for detecting the types and the sites of mutations in these genes.

4- It is important to use the sensitivity test in the hospitals for checking the resistance abi1ity of *P. aeruginosa* against antibiotics

5- Study the relationship between $b1a_{TEM}$, $b1a_{SHV}$, and $b1a_{CTX\text{-}M}$ genes and other virulence factors.

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Appendix

Table 4.3. Phenotypic of antibiotic susceptibility of *P. aeruginosa* isolates

Isolat	AZ	IM	PIP	CE	TOB	NE	OF	DO	ME	CAZ	PP	AM	GE	CIP	NO	LEV	GA
PA1	R	R	R	R	R	R	R	S	S	R	S	R	I	R	R	S	S
PA2	R	R	R	R	R	R	R	S	I	S	S	S	S	S	S	S	R
PA3	R	R	R	R	R	R	R	S	I	R	S	R	R	R	S	S	S
PA4	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	R
PA5	R	R	R	R	R	R	R	S	S	R	S	R	R	I	S	R	S
PA6	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	S	R
PA7	R	R	R	R	R	R	R	R	I	R	R	S	S	S	R	I	S
PA8	R	R	R	R	R	R	R	S	R	R	S	R	R	I	I	S	I
PA9	R	R	R	R	R	R	R	R	R	R	S	R	R	I	S	R	S
PA10	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	S
PA11	R	R	R	R	R	R	R	I	S	R	S	R	R	R	R	S	I
PA12	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R	S	R
PA13	R	R	R	R	R	R	R	R	S	R	S	R	R	R	I	R	R
PA14	R	R	R	R	R	R	R	S	R	S	S	R	R	R	I	S	R
PA15	R	R	R	R	R	R	R	S	R	S	S	R	R	I	R	R	R
PA16	R	R	R	R	R	R	R	S	R	S	I	R	R	R	R	S	I
PA17	R	R	R	R	R	R	R	S	S	R	R	R	R	I	S	S	I
PA18	R	R	R	R	R	R	R	S	S	R	S	R	R	R	S	R	I
PA19	R	R	R	R	R	R	R	S	S	R	S	R	R	R	S	I	I
PA20	R	R	R	R	R	R	R	S	S	R	S	R	R	I	R	R	S
PA21	R	R	R	R	R	R	R	S	S	R	R	I	R	I	I	R	S
PA22	R	R	R	R	R	R	R	S	S	R	S	R	R	R	S	R	I
PA23	R	R	R	R	R	R	R	S	S	R	S	R	R	I	R	S	S
PA24	R	R	R	R	R	R	R	S	S	R	S	R	R	I	R	R	I
PA25	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	S	I
PA26	R	R	R	R	R	R	R	R	I	R	R	R	R	R	I	R	R
PA27	R	R	R	R	R	R	R	S	S	R	I	S	R	I	I	R	S
PA28	R	R	R	R	R	R	R	S	R	R	I	S	R	R	I	S	I
PA29	R	R	R	R	R	R	R	R	I	R	R	R	R	I	R	S	S
PA30	R	R	R	R	R	R	R	S	S	R	R	R	R	S	R	R	I
PA31	R	R	R	R	R	R	R	S	R	R	I	R	R	S	R	S	R
PA32	R	R	R	R	R	R	R	S	R	R	R	R	R	I	I	S	I
PA33	R	R	R	R	R	R	R	R	I	R	I	R	R	R	R	S	R
PA34	R	R	R	R	R	R	R	S	R	R	I	R	R	R	S	S	S
PA35	R	R	R	R	R	R	R	R	S	R	I	R	R	S	R	S	S
PA36	R	R	R	R	R	R	R	S	R	R	I	R	R	S	I	S	S
PA37	R	R	R	R	R	R	R	S	S	R	R	R	R	I	S	S	S
PA38	R	R	R	R	R	R	R	R	I	R	S	S	R	I	R	S	R
PA39	R	R	R	R	R	R	R	S	R	R	S	S	R	S	S	R	R
PA40	R	R	R	R	R	R	R	R	R	R	R	S	R	I	I	R	I

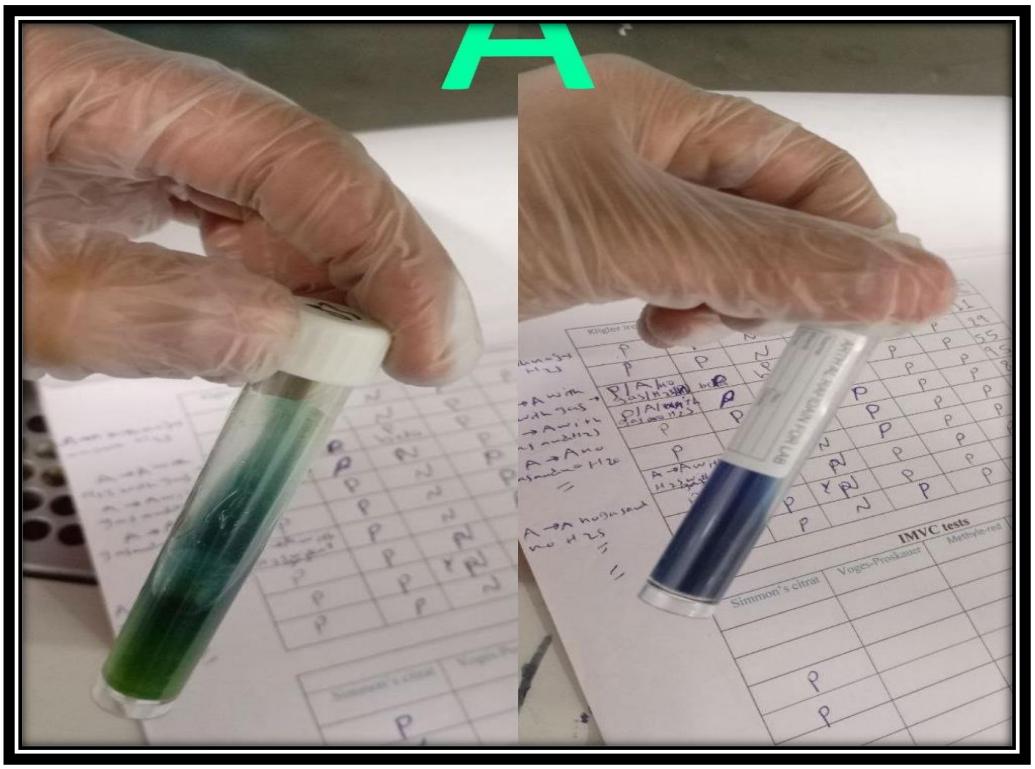
Isolat	AZ	IM	PIP	CE	TOB	NE	OF	DO	ME	CAZ	PP	AM	GE	CIP	NO	LEV	GA
PA41	R	R	R	R	R	R	R	R	R	R	S	S	R	R	I	R	S
PA42	R	R	R	R	R	R	R	R	S	R	S	R	R	R	S	R	R
PA43	R	R	R	R	R	R	R	S	S	R	S	R	R	R	I	R	I
PA44	R	R	R	R	R	R	R	S	S	R	S	R	R	I	I	R	I
PA45	R	R	R	R	R	R	R	R	R	R	S	I	R	I	R	S	S
PA46	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	I	S

Abbreviations: R, resistance; S, sensitive; I, intermediate; AZT, Aztreonam; IMI, Imipinem; PIP, Piperacillin; CEF, Cefepime; TOB, Tobramycin; NET, Netilmicin; OFL, Ofloxacin; DO, Doripenem, MER, Meropenem; CAZ, Ceftazidim; PPT, Piper. /Tazo.; AMI, Amikacin; GEN, Gentamicin; CIP, Ciprofloxacin; NOR, Norfloxacin; LEV, Levofloxacin; GAT. Gatifloxacin.

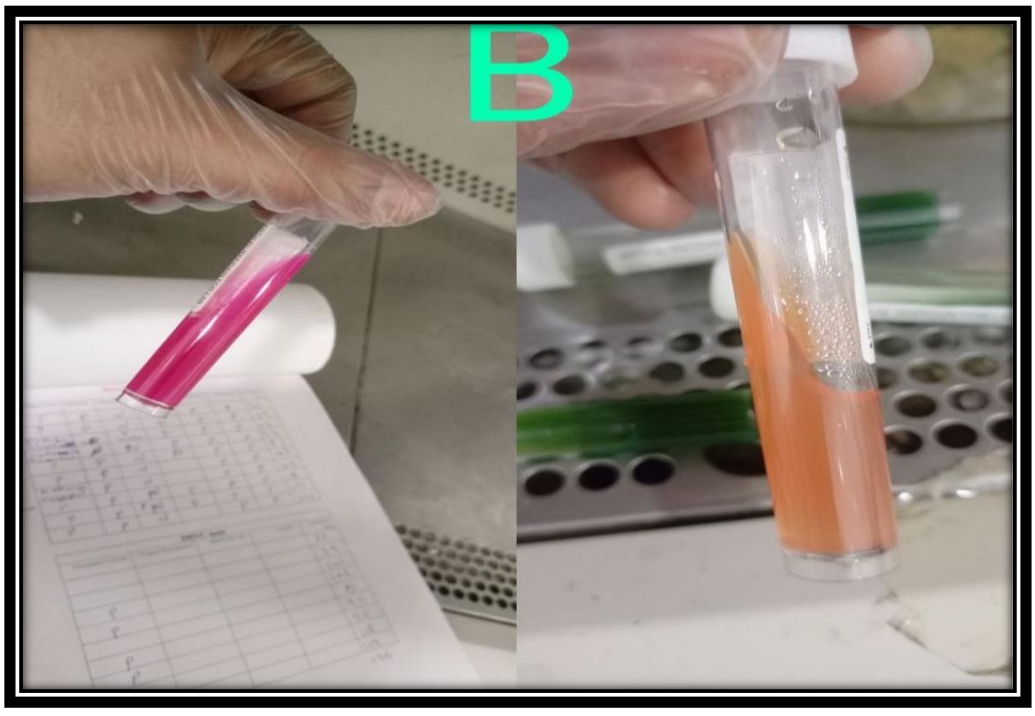
Table 4.5 Biofilm forming capacity of *P. aeruginosa*

Isolate	Specimen type	Geographic region	OD (nm)	Grade
PA1	Burn	Muthanna	0.148	weak
PA2	Injury	Baghdad	0.102	weak
PA3	Diabetic Foot	Ninawa	0.227	moderate
PA4	Burn	DhiQar	0.11	weak
PA5	Injury	Kirkuk	0.247	moderate
PA6	Injury	Diyala	0.204	weak
PA7	Diabetic Foot	Busra	0.107	weak
PA8	Burn	Erbil	0.105	weak
PA9	Injury	Babylon	0.206	moderate
PA10	Burn	Ninawa	0.149	weak
PA11	Diabetic Foot	DhiQar	0.135	weak
PA12	Injury	Diyala	0.269	moderate
PA13	Injury	Baghdad	0.298	moderate
PA14	Injury	Basra	0.132	weak
PA15	Burn	Kirkuk	0.147	weak
PA16	Diabetic Foot	Diyala	0.224	moderate
PA17	Burn	DhiQar	0.103	weak
PA18	Burn	Muthanna	0.112	weak
PA19	Burn	Basra	0.203	moderate
PA20	Burn	Basra	0.095	non
PA21	Injury	Muthanna	0.143	weak
PA22	Burn	Erbil	0.129	weak
PA23	Burn	Baghdad	0.131	weak
PA24	Burn	Baghdad	0.107	weak
PA25	Diabetic Foot	Diyala	0.095	non
PA26	Injury	Baghdad	0.076	non
PA27	Injury	Erbil	0.118	weak
PA28	Burn	Babylon	0.089	non

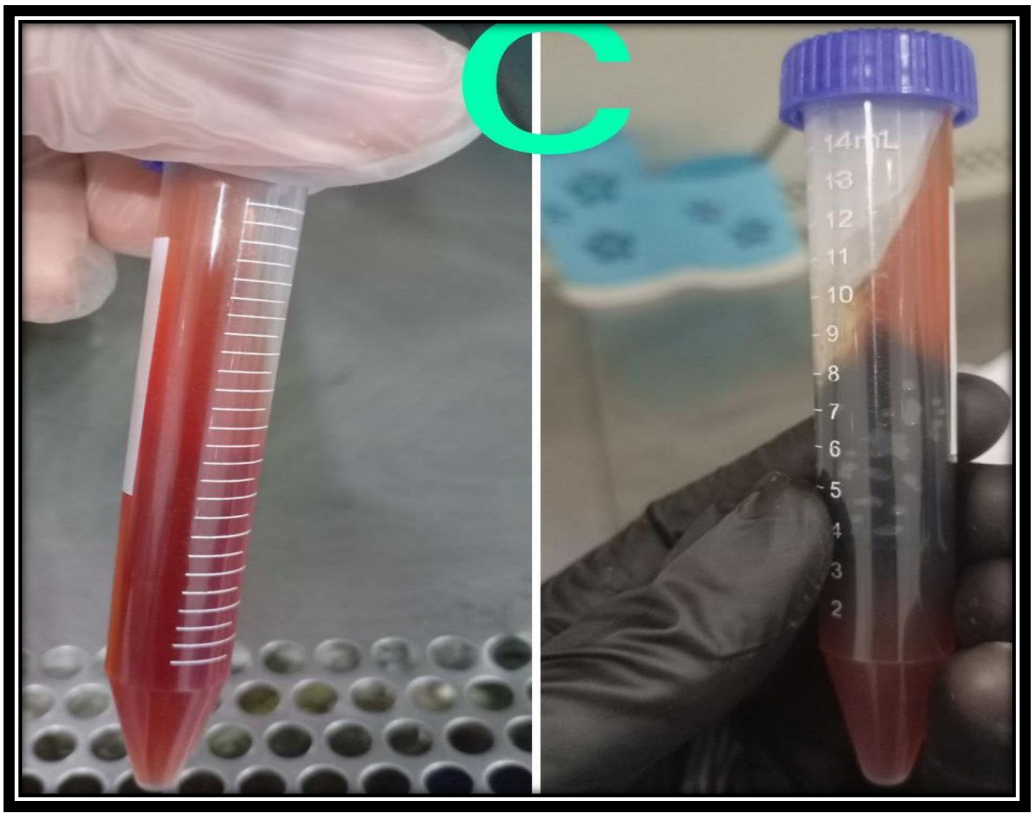
Isolate	Specimen type	Geographic region	OD (nm)	Grade
PA29	Diabetic Foot	Basra	0.103	weak
PA30	Injury	Kirkuk	0.108	weak
PA31	Burn	DhiQar	0.093	non
PA32	Burn	DhiQar	0.09	non
PA33	Burn	Muthanna	0.137	weak
PA34	Burn	Baghdad	0.11	weak
PA35	Burn	Muthanna	0.102	weak
PA36	Burn	Kirkuk	0.11	weak
PA37	Burn	Kirkuk	0.096	non
PA38	Burn	Babylon	0.10	weak
PA39	Injury	Baghdad	0.086	non
PA40	Burn	Babylon	0.09	non
PA41	Burn	DhiQar	0.126	weak
PA42	Burn	Diyala	0.079	non
PA43	Injury	Kirkuk	0.075	non
PA44	Injury	Ninawa	0.114	weak
PA45	Burn	Basra	0.095	non
PA46	Burn	Babylon	0.167	weak



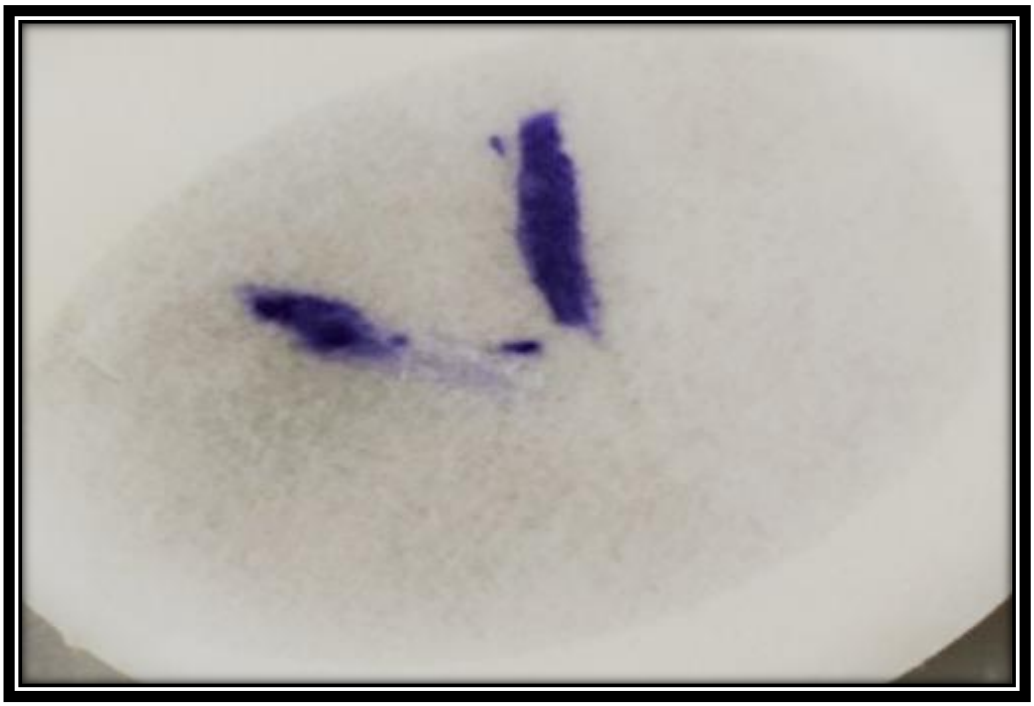
Figuer 1: Citrate utilization positive result



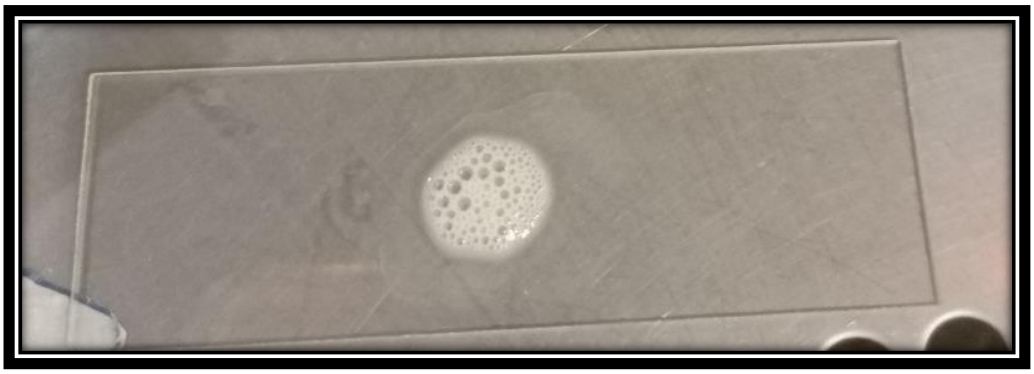
Figuer 2: Urease test positive result



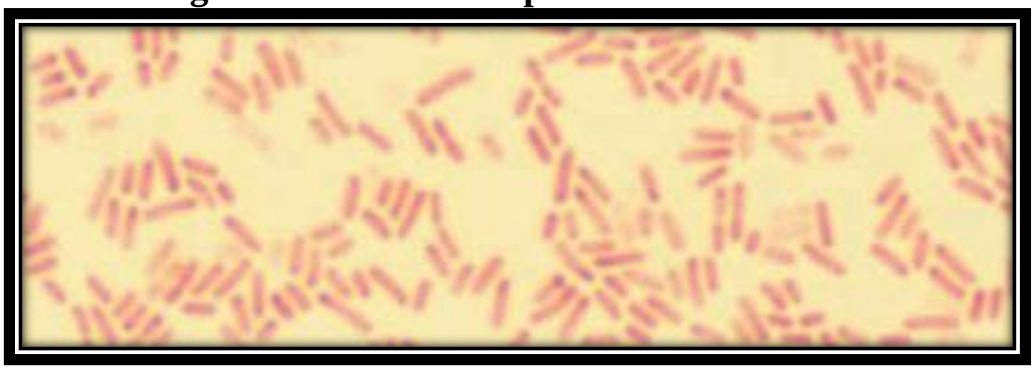
Figuer 3: Kligler′s Iron Agar test positive result



Figuer 4: Oxidase test positive isolates



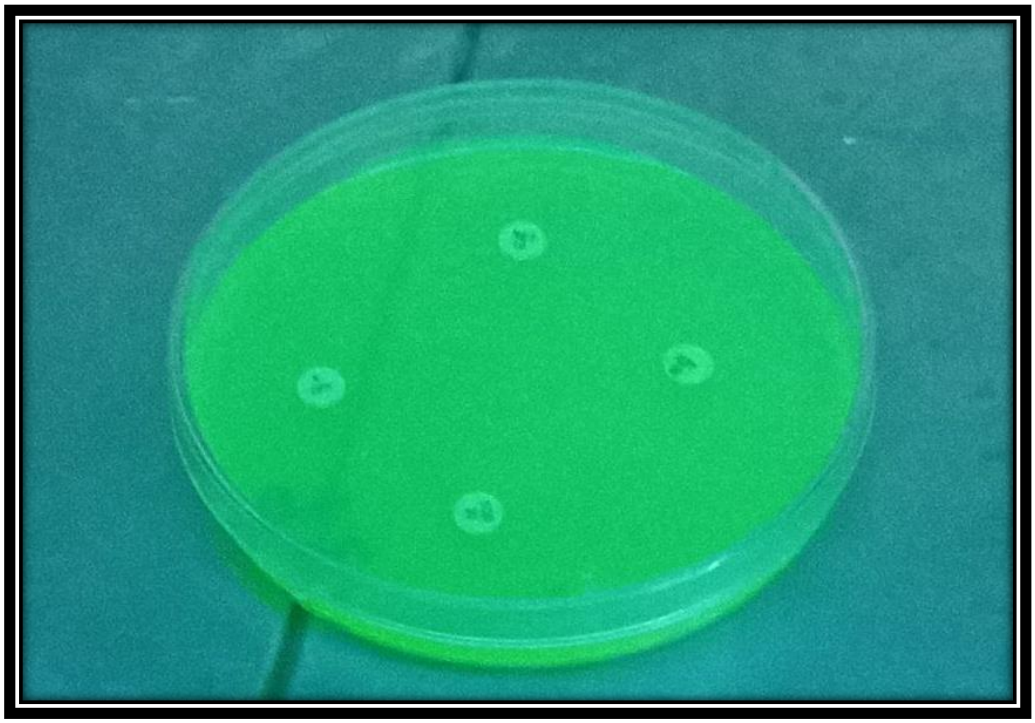
Figuer 5: Catalase test positive isolates



Figuer 6: Gram stain of *Pseudomonas aeruginosa*

Figuer 7: Pigment test positive isolates

Figuer 8: *Pseudomonas aeruginosa* culture on Muller Hinton



Figuer 9: *Pseudomonas aeruginosa* culture under UV- light

الخلاصة

جمعت مائة وخمسين عينة لكلا الجنسين باختلاف الأعمار من مستشفيات بابل (مستشفى الحلة التعليمي ، مستشفى مرجان التعليمي، مستشفى الامام الصادق التعليمي) وكذلك من مستشفيات بغداد (مستشفى الحروق التخصصي ، مستشفى الشهيد غازي الحريري ، مستشفى بغداد التعليمي ، المركز الوطني. للمختبرات التعليمية). قسمت هذه العينات إلى مجموعتين ، المجموعة الأولى تضمنت خمسة وخمسين مسحة تم الحصول عليها من الحروق والمجموعة الثانية تضمنت خمسة وتسعين مسحة جروح تم الحصول عليها من إصابات مختلفة. جمعت هذه العينات من سبتمبر 2021 إلى يناير 2022.

عزلت ستة وأربعين (30%) عزلة من *Pseudomonas aeruginosa* ورمزت بالرمز (PA1 to PA46) وشخصت اعتمادا على الصفات المضهرية، الزرعية، المجهرية، الاختبارات الكيموحيوية و Vitek 2 compact ، بينما لوحظ أن 54 (36%) عزلة لبكتيريا أخرى تم الحصول عليها من عينات الجروح بينما كانت 50 (33.3%) من العينات ليس لها نمو بكتيري. استخلص الحمض النووي من عزلات *P. aeruginosa* باستخدام DNA mini kit وقيست نقاوتها باستخدام جهاز nanodrop (1.96-2.49 nm) اكد تشخيص *P. aeruginosa* باستخدام تقنية تفاعل البلمرة المتسلسل (PCR) للجين النوعي *P. aeruginosa* ، وكانت مطابقة بنسبة % 100 مع النتائج التي تم الحصول عليها من فحوصات الصفات المضهرية، الزرعية، المجهرية، الاختبارات الكيموحيوي

تم فحص حساسية المضادات الحيوية ل 17 نوعًا من المضادات الحيوية ، باستخدام طريقة نشر القرص وفقًا لمعهد المعايير السريرية للبكتيريا المسببة للأمراض في المختبر السريري CLSI-2021. أظهرت النتائج أن جميع العزلات الـ 46 مقاومة للتوبراميسين ، البيبيراسيلين ، السيفبيمي ، الإيميبينيم ، أوفلوكساسين ، الأزتريونام ، النيتيلميسين ، (100%) ، بينما النورفلوكساسين (39%) ، البيبيراسيلين-تازوباكتام (26%) ، الليفوفلوكساسين (39%). أميكاسين (74%) ، ميروبينيم (41%) ، سيبروفلوكساسين (43%) ، دوريبينيم (37%) ، جنتاميسين (87%) ، سيفتازيديم (87%) ، جاتيفلوكساسين (28%). تمت دراسة التكوين البكتيري للغشاء الحيوي باستخدام اختبار طريقة زراعة الانسجة والذي يعد الأكثر حساسية ، وأظهرت النتائج أن 34 عزلة كانت قادرة على إنتاج الغشاء الحيوي.

تم استخدام تفاعل البلمرة المتسلسل لفحص 3 جينات من β-lactamase واسعة الطيف (ESBLs) في *P. aeruginosa* باستخدام المتمم لكل جين: bla_{TEM}, bla_{SHV}, bla_{CTX-m}.

بعد إجراء الترحيل الكهربائي الهلامي ، أظهرت النتائج أن عزلات *P. aeruginosa* حاوية على bla_{TEM} 28/46 (63%), bla_{SHV} 26/46 (58.69%) و 31/46 (67.39%) bla_{CTX-M} وجد أن جميع عزلات *P. aeruginosa* كانت متطابقة بنسبة 95.86-100 % مع مصادر العزلات المحددة في بنك الجينات (NCBI)، وأظهرت النتائج أن أعلى معدل مطابقة للعزلات كان 95-100%. نشأت في مصر. كشفت هذه الدراسة أن مقاومة الأدوية المتعددة (MDR) لبكتيريا *P. aeruginosa* كانت أكثر البكتيريا عزلًا من الحروق ، وأن جين bla_{CTX-M} كان أكثر جينات بيتا لاكتام واسعة الطيف (ESBLs)بين هذه العزلات متبوعة بـ bla_{SHV}. الجين متبوعًا بـ bla_{TEM} .

تم استخراج الحمض النووي الجينومي الكامل لتسع عزلات من PA1 إلى PA9 معزولة من مناطق جغرافية مختلفة في العراق. تم إجراء تسلسل جينات bla_{TEM} و bla_{SHV} و bla_{CTX-M} ، وتم تحديد مواقع هذه الجينات على جينوم العزلات. تم إجراء تحليل شجرة النشوء والتطور باستخدام برنامج MEGA X10.2.4 ، وأظهرت مطابقة عزلات الدراسة الحالية مع سلالات *Pseudomonas* العالمية التابعة لبنك NCBI-Gen ، أن عزلة واحدة (PA1) اصلها في الإمارات العربية المتحدة ، وعزلتان (PA2 و PA3) في الهند ، ثلاث عزلات (PA4 و PA5 PA6) اصلها في مصر و ثلاث عزلات (PA7 و PA8 و PA9) اصلها في إيران. وبالتالي ، فإن الترددات المتغيرة في تسلسل جينات bla_{TEM} و bla_{SHV} و bla_{CTX-M} تحتاج إلى مزيد من الدراسات لإنشاء خريطة التنوع الجيني لـ *P. aeruginosa*.

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

التوصيف الجزيئي لجينات البيتالاكتميز واسعة الطيف في عزلات بكتريا الزائفة الزنجاريه

رسالة

مقدم الى مجلس كلية العلوم-جامعة بابل كجزء من متطلبات نيل درجة الماجستير في العلوم/علوم الحياة

من قبل
مصطفى عبد الجبار محمد صالح علي
بكلوريوس علوم حياة-التقانة الاحيائية
جامعة بابل (٢٠١٨-٢٠١٩)

اشراف

أ.د. حسن فاضل ناجي

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