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Genotyping Efflux Pump-Mediated Resistance Among Clinical *Pseudomonas aeruginosa* Isolates

A Thesis

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Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy of Science in Biology

By

Afrah Jawad Abd Zwaid

B. Sc. College of Science / University of Babylon (2004)

M.Sc. College of Science for Women / University of Babylon (2016)

Supervised By

Prof. Dr. Hussein Oleiwi Muttaleb Al-Dahmoshi

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بسم الله الرحمن الرحيم

(قَالُواْ سُبْحَٰنَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ)

صدق الله العلي العظيم

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Supervision Certificate

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 requirements for the degree of Doctor of Philosophy of Science in Biology-Microbiology.

Supervisor

Prof. Dr. Hussein Oleiwi Muttaleb Al-Dahmoshi

College of Science

University of Babylon

/ /2022

In view of the available recommendation, I present this thesis for evaluation by the Examining Committee.

Asst. Prof. Dr. Adi Jassim Abd AL-Rezzaq

Head of Biology Department

College of Science

University of Babylon

/ /2022

Decision of Examination Committee

We, the examination committee, certify that we have read the thesis entitled (**Genotyping Efflux Pump-Mediated Resistance Among Clinical *Pseudomonas aeruginosa* Isolates**) and have examined the student (**Afrah Jawad Abd Zwaid**) in its contents, and that in our opinion it is accepted as a thesis for the Degree of Doctorate of Philosophy of Science in Biology with excellent estimation.

Signature:
Prof. Dr. Eman M. Jarallah
College of Science/University of Babylon
(Chairman)

Signature:
Prof. Dr. Ali H. AL-Marzoqi
College of Science for women/University of Babylon
(Member)

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Prof Dr. Mourouge Saadi Abbas Alwash
College of Science/University of Babylon
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Prof. Dr. Baydaa A. Hassan
College of Science/ University of Kufa
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Assist. Prof. Dr. Marwa Fadhil Alsaffar
Al-Mustaqbal University College
(Member)

Signature:
Prof. Dr. Hussein Oleiwi Muttaleb Al-Dahmoshi
College of Science/University of Babylon
(Member and Supervisor)

Signature:
Prof. Dr. Mohammed Mansour Kadhum Alkafaji
(Dean of the College of Science – University of Babylon)
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Dedication

To My Father (Jawad Al-Shemmary)

To My Mother (Fawzeiah Al-Sahaf)

To My Husband (Asim Alaa Balakit)

To My Daughters (Usur, Fatima and Zainab)

To My Brother and Sisters

I sincerely dedicate this thesis

Afrah

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Summary

Pseudomonas aeruginosa is a major cause of hospital-acquired infections. These infections represent a very difficult therapeutic challenge and are associated with morbidity and mortality.

Overexpression of multidrug efflux pumps (EPs) is one of the most important underlying mechanisms. These efflux pumps can extrude antibiotics from the cytoplasm into the extracellular milieu and consequently loss of drug efficacy. The aim of the present study is to investigate the prevalence of efflux pumps profile among clinical isolates of *P. aeruginosa* and its contribution in MDR phenotype. Another aim is to measure the impact of using an efflux pump inhibitor (EPI); phenylalanine-arginine-β-naphthylamide (PAβN) on the minimal inhibitory concentration (MIC) of some antibiotics and study the gene expression of the efflux pump outer membrane coded gene (*oprM*) in MDR and sensitive isolates.

The results of the present study reveal a high percentage of *P. aeruginosa* 79 isolates distributed as urinary tract infections (UTIs) patients 35.4%, lower respiratory tract infection (RTIs) patients 29.1%, wounds and burn infections 18.9%, and 8.8% for otitis media, 2.5% for bacteremia, 3.7% for bacterial vaginitis, and 1.2% for meningitis.

For antibiotic susceptibility testing, the resistance values of *P. aeruginosa* isolates towards ceftazidime (CAZ) and cefepime (FEP) were recorded at 93.6% and 77.2%, respectively, while 65% represented piperacillin (PRL), and 40.5% represented gentamycin (CN). Ciprofloxacin (CIP) was detected in just 37.9% of isolates. On the other hand, 41.7% was recorded for tobramycin (TOB), 39.2% for aztreonam (ATM), 45.5% for amikacin (AK), 43% for ofloxacine (OFX), 37.9%

for netilmicine, 13.9% for imipenem (IPM), and 25.3% for meropenem (MEM). Antibiotic resistance patterns investigation showed that 59% (47 isolates) of isolates being multidrug resistance and 41% (32 isolates) being non-multidrug resistance.

A PCR assay for coding genes was performed by conventional PCR for all isolates (79 isolates). The pump mexAB-oprM with *MexA*, *mexB*, and *oprM* genes were detected in 82.2% (65 strains), 63.29% (50 strains), and 48.1% (38 strains) of the isolates, respectively. The pump mexXY-oprM revealed *mexX* 41.7% (33 strains), *mexY* 50.6% (40 strains), and *oprM* 48.1% (38 strains). The mexPQ-opmE pump revealed *mexP* 36.70% (29 strains), *mexQ* 46.83% (37 strains) and *opmE* 50.6% (40 strains) for muxABC-opmB genes, with 59.4% (47 strains) for *muxA*, followed by 37.9% (30 strains) for *muxB*, *muxC* 48.1% (38 strains) and 62.02% (49 strains) for the *opmB* gene. The detection of mexCD-oprJ efflux revealed *mexC* 41.77% (33 strains), *mexD* 48.1% (38 strains) and *oprJ* 81.1% (64 strains), for mexGHI-opmD genes with 68.35% (54 strains) for *mexG* followed by 49.36% (39 strains) for *mexH*, 37.9% (30 strains) for *mexI* and 20.2% (16 strains) for the *opmD* gene.

The detection of mexJK-oprM efflux revealed *mexJ* 37.9% (30 strains), *mexK* 34.17% (27 strains) and *oprM* 48.1 (38 strains). The detection of mexMN-oprM efflux revealed *mexM* 69.62% (55 strains), *mexN* 50.63% (40 strains) and *oprM* 48.1% (38 strains). The molecular detection of mexVW-oprM efflux revealed *mexV* 92.40% (73 strains), *mexW* 91.13% (72 strains) and *oprM* 48.1% (38 strains) for triABC-opmH genes, with 55.69% (44 strains) for *triA*, followed by 16.4% (13 strains) for *triB*, 50.63% (40 strains) for *triC* and 49.36% (39 strains) for the *opmH* gene. PCR assay for mexEF-oprN genes with 41.77% (33 strains) for *mexE* followed by 39.24% (31 strains) for *mexF*,

Overexpression of *oprM* gene was significantly higher in MDR isolates than non MDR isolates. Addition of the EPI, PAβN, resulted in significant reduction in the MIC of LEV and PIP. Efflux pump-mediated resistance was a significant mechanism contributing to multidrug resistance in clinical isolates of *P. aeruginosa*. However, other drug resistance mechanisms should be considered. Although the addition of EPI; PAβN resulted in a lowering of the MIC in MDR strains. It is, therefore, important to continue the exploration of more effective EPIs, and large-scale surveillance studies are recommended to detect the prevalence of efflux pump overexpression as well as other possible mechanisms among *P. aeruginosa* isolates and their link with multidrug resistance.

List of Contents

List of Tables

List of Figures

List of Abbreviations

Symbol	Description
AK	Amikacin
ATM	Aztreonam
Bp	Base pair
COPD	Chronic Obstructive Pulmonary Disease
CF	Cystic Fibrosis
CAUTI	Catheter Associated Urinary Tract Infections
CAZ	Ceftazidime
CDC	Center for Disease Control and prevention
CEF	Cefepime
CIP	Ciprofloxacin
CLSI	The Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
CN	Gentamicin
DNA	Deoxyribonucleic acid
Eps	Efflux pumps
EDTA	Ethylene Diamine Tetra Acetic Acid
IMP	Imipenem
ICU	Intensive Care unit
IU	International Unit
LEV	Levofloxacin
LPS	Lipopolysaccharide
MFP	Membrane Fusion Protein
MDR	Multi-Drug Resistance

MEM	Meropenem
MHA	Muller-Hinton Agar
MHB	Muller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MEM	Meropenem
NET	Netilmicin
NS	Normal Saline
OFX	Ofloxacin
PaβN	Phenylalanine-arginine beta-naphthylamide
PBP	Penicillin-Binding Protein
PCR	Polymerase chain reaction
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
QS	Quorum Sensing
PRL	Piperacillin
RNA	Ribonucleic Acid
RND	Resistance-Nodulation-cell Division
RTI	Respiratory tract infection
T3SS	Type three secretion system
TBE	Tris Borate – EDTA
UTIs	Urinary Tract Infections
UV	Ultraviolet
WHO	World Health Organization

Chapter One
Introduction & Literature Review

1.1: Introduction:

Pseudomonas aeruginosa a nosocomial opportunistic pathogen that can dominate any niche. They may be able to adapt to various environments since they have several virulence factors that are used for survival (Al-Dahmoshi *et al.*, 2018). It results in chronic lung infections in people with cystic fibrosis and nosocomial infections in immunocompromised patients. Numerous conditions can result in nosocomial infections, including ventilator-associated pneumonia (Greenwald and Wolfgang, 2022), catheter-associated urinary tract infections, wounds, bone, and joint infections in patients with severe burns, gastrointestinal tract infections, and septicemia (Monteiro *et al.*, 2016).

Many different types of resistance mechanisms have been identified among *P. aeruginosa*, including the development of mutations in targets, antibiotic hydrolysis by β-lactamases, target modification by aminoglycoside-modifying enzymes, the up-regulation of efflux systems reflex antibiotics like quinolones, cephalosporins, carbapenem, and aminoglycosides (Pachori *et al.*, 2019; El-Far *et al.*, 2021; Sulaiman and Lam, 2022). According to recent data, antimicrobial resistance is growing, even against first-line antimicrobials, which could result in treatment failure and chronic infection with difficult to treat *P. aeruginosa* (Sid Ahmed *et al.*, 2022; Tamma *et al.*, 2022).

The development of MDR *P. aeruginosa* relies heavily on efflux pump systems like those of nodulation-cell division (RND) family (Seukep *et al.*, 2022). The RND multidrug efflux systems function as tripartite systems made up of an outer membrane protein, a periplasmic membrane fusion protein, and an RND transporter that is connected to the cytoplasmic membrane. Due to their ability to extrude numerous structurally unrelated components, the pumps, especially the RND family including MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY, have attracted special interest in recent times and playing a significant

role in multidrug resistance (Fujiwara *et al.*, 2022; Khalili *et al.*, 2022). Quinolones, antipseudomonal penicillins, cephalosporins, aminoglycosides, and carbapenems are typical substrates for these efflux pumps (Solé *et al.*, 2015). For the treatment of *P. aeruginosa* infection, efflux pump inhibitors (EPIs) have been researched as an alternative to the creation of novel antibiotics (Tambat *et al.*, 2022).

1.2: Aim of the Study:

Antibacterial resistance is still a major worldwide health issue that can reduce the performance of antibacterial agents and waste efforts to create new therapies. The goal of this study was to look into efflux pumps profile of MDR *P. aeruginosa* with possibility of augmentation between anti pseudomonas drug and efflux pumps inhibitors via the following objectives:

1. Isolating, identifying and detection the antibiotic resistance profile of *P. aeruginosa* from hospitals by chromogenic agar and molecular diagnosis
2. Molecular detection of efflux pumps genes among bacterial isolates.
3. Evaluating the efflux pump inhibitor's effect on selected MDR isolates.
4. Estimating of the gene expression of the *oprM* gene in MDR and non-MDR isolates

1.3: Literature Review

1.3.1. Characterization of *Pseudomonas aeruginosa* :

Pseudomonas aeruginosa is an ubiquitous gram-negative, rod-shaped, mono-flagellated, aerobic chemoorganotrophic bacterium. It can inhabit various environments such as soil, vegetation, and surfaces in aqueous habitats and can cause infections in humans (Gellatly & Hancock, 2013). This type of bacteria is an opportunistic pathogen. It contributes to about 10% of all hospital acquired infections and is identified as one of the top five pathogens causing hospital acquired infections (Emori and Gaynes, 1993, Rosenthal *et al.*, 2016).

Pseudomonas aeruginosa can transform into an opportunistic pathogen and cause both acute and chronic infections in humans and animals (Rutherford *et al*., 2012). It is able to cause both acute and chronic infections in immune-compromised individuals (Faure *et al.*, 2018).

The infections caused by *P. aeruginosa* are often hospital-acquired and involved in neutropenia, severe burns, or pneumonia in cystic fibrosis patients (Gellatly & Hancock, 2013; Daikos *et al*., 2021). Multidrug resistant *P. aeruginosa* is classified as a severe threat, with 6,700 infections and 440 deaths per year in the U.S. It is highly adaptable and possesses intrinsic resistance to antimicrobials, which allows it to survive in both natural and artificial environments, including surfaces in medical facilities (Gellatly & Hancock, 2013; Wang *et al*., 2021).

Pseudomonas aeruginosa possesses a variety of mechanisms that contribute to its intrinsic and acquired resistance to antimicrobials. It produces β-lactamases, which are responsible for resistance to β-lactam antibiotics such as penicillin, cephalosporin, carbapenems, and monobactams (Singh *et al*., 2020). *P. aeruginosa* is highly adaptable and ubiquitous in the environment; it can colonize

nearly all major body sites; it is naturally resistant to many antimicrobials such as (cefeme, ceftazidime, pipraciline); it is capable of acquiring antibiotic resistance genes; it is equipped with a large supply of pathogenic factors; and it is thus frequently difficult to treat (Khan *et al*., 2015; Holmes *et al*., 2021).

1.3.2. Pathogenicity of *P. aeruginosa*:

P. aeruginosa pathogenesis is multifactorial and is dependent on various determinants such as adhesions, exotoxins, proteases, hemolysins, and a type III secretion system (Kaszab *et al.*, 2011). *P. aeruginosa* infections are a frequent nosocomial infection that causes significant morbidity and mortality in hospitals around the world. After being exposed to polluted sources within the hospital environment, patients may get colonized or infected with *P. aeruginosa* (Quick *et al*., 2014).

Colonization by *P. aeruginosa* usually occurs before infection (Gómez-Zorrilla *et al.*, 2015). Colonization can be endogenous (originating from the patient's own microbial repertoire) or exogenous (originating from the hospital environment or via cross infection with other patients) (Venier *et al.*, 2014). At extremely low concentrations, *P. aeruginosa* can cause diseases. As a result, for treating *P. aeruginosa* infection, early diagnosis is crucial (Rüger *et al.*, 2014). This bacteria's infection can take three forms: bacterial attachment and colonization; local infection; bloodstream spread and systemic infection (Scotland *et al.*, 2019). *P. aeruginosa* uses a variety of adhesions, including the capsule, pili, flagella, and outer membrane proteins to mediate attachment to surfaces (Mann and Wozniak, 2012). When flagella interact with the mucosal epithelial surface, it causes considerable cell signaling and the release of a number of proinflammatory cytokines (Rehm, 2008). The bacteria also create an enzyme called lyase, which may break down polysaccharide into smaller oligosaccharides units. Both the

biosynthesis and breakdown processes have been found to be important in the infection process (Gellatly and Hancock, 2013; Alhazmi, 2015).

P. aeruginosa pathogenicity is aided by the presence of extracellular virulence factors and cell surface related structures (De Bentzmann & Plésiat, 2011). It can cause pneumonia, urinary tract infections, and bacteremia, as well as a high rate of morbidity and mortality in cystic fibrosis patients due to chronic infections that lead to pulmonary damage and respiratory failure (Diggle and Whiteley, 2020), as shown in figure (1-1).

Patients in intensive care units who are on mechanical ventilation are at the greatest risk of contracting the bacteria. Bloodstream infections in intensive care units (ICU), burn and chronic cutaneous wound infections, surgical site infections, hospital-acquired pneumonia, and respiratory and urinary tract infections are all caused by *P. aeruginosa* (Workentine *et al.*, 2013; Bhatta *et al.*, 2019).

Bacterial outbreaks in neonatal intensive care units (NICUs) have also been linked to *P. aeruginosa* (Gladstone *et al.*, 2011). All of these infections are more common in patients who have other diseases or injuries, such as severe burn wounds, AIDS, lung cancer, chronic obstructive pulmonary disease (COPD), bronchiectasis, and cystic fibrosis (CF) (Malhotra *et al.*, 2019), or nosocomial infections caused by biofilm contamination of medical devices (catheter-related infections) (Olejnickova *et al.*, 2014).

Even though the distinction between *P. aeruginosa* infections is commonly classed as acute or chronic, despite the fact that the line between the two is not always clear, but they are frequently categorized as such clinically (Rodriguez *et al.*, 2021).

P. aeruginosa is the most frequent causative organism in acute otitis externa (swimmer's ear). Perichondritis of the auricle is a consequence of the injured ear caused by this bacteria and may leave a persistent defect. Injured cartilage appears

to be particularly appealing to *P. aeruginosa* (Marais, 2015). Necrotizing enterocolitis (NEC) is the most prevalent gastrointestinal emergency in premature babies, and *P. aeruginosa* is one of the bacteria that causes it (Coggins *et al.*, 2015). On the other hand, *P. aeruginosa* infections of the central nervous system are quite rare. They are frequently connected with neurosurgery or head trauma, as well as bacteremia on rare occasions, and are associated with a high death rate (Pai *et al.*, 2015; Azam and Khan, 2019).

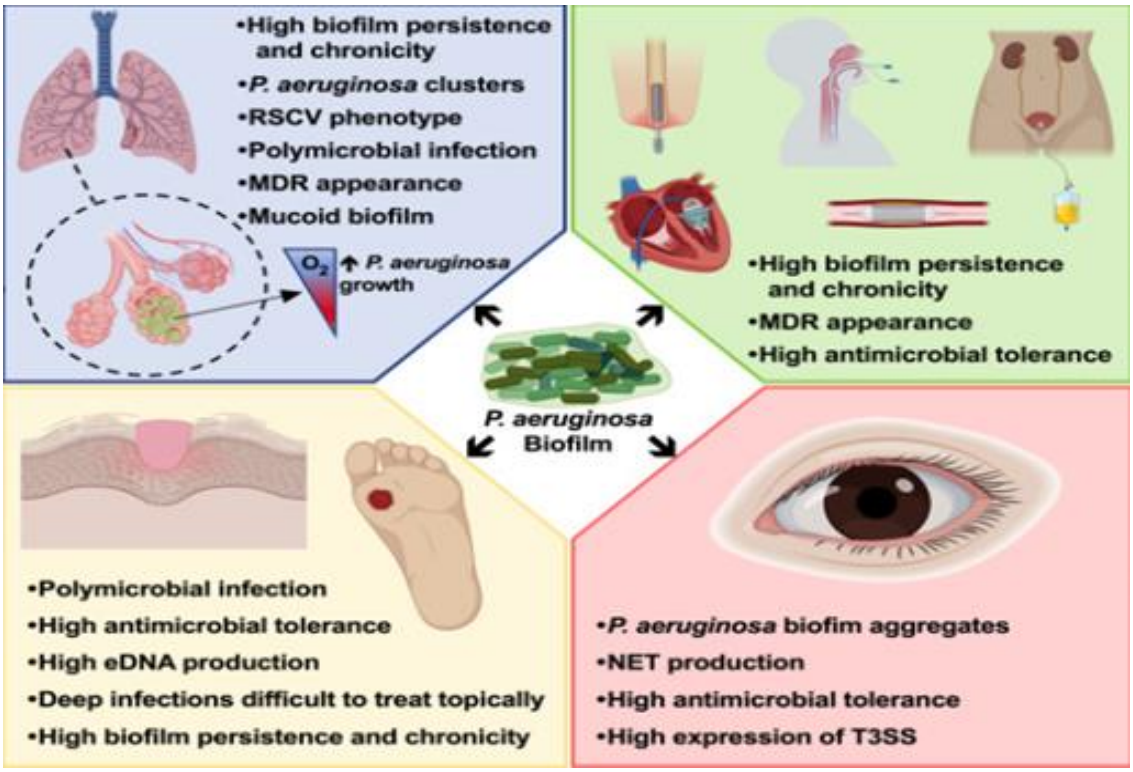

Figure (1-1) A schematic representation of the main features of *P. aeruginosa* infection (del Mar *et al.*, (2021))

1.3.2. 1. *P. aeruginosa* Associated Urogenital Infection:

One of the major factors contributing to nosocomial infections is urinary tract infections. Among the most common microbiological illnesses are urinary

tract infections (UTIs). The medical system is heavily impacted by UTIs (Brusch and Bronze, 2020).

P. aeruginosa should receive special consideration in UTI patients since it can have a negative impact on those with serious underlying diseases (Gomila *et al.,* 2018) and it is a major nosocomial, *P.aeruginosa* is the third most common pathogen causing hospital acquired UTI (Litwin *et al.,* 2021). The rise in multidrug resistance in bacterial uropathogens is a significant and newly developing public health issue in isolates of *P. aeruginosa*, which can tolerate a wide range of physical conditions and various medications by different resistance mechanisms. (Dabbousi *et al.,* 2022). Catheter associated urinary tract infections accounts for 40% of nosocomial infections in hospitalized patients (Tellis *et al.,* 2017).

1.3.2.2 *P. aeruginosa* Associated Respiratory Tract Infection:

Bronchitis is a chronic airway infection that develops irreversibly enlarged and scarred bronchi, which frequently results in bronchial sepsis episodes. Poor mucus clearance and a vicious cycle of chronic bacterial colonization, airway blockage, inflammation, and gradually deteriorating tissue are the effects of this (Keir and Chalmers, 2022).

P. aeruginosa is the most common pathogen linked with cystic fibrosis (CF) lung disease, and it is usually isolated from individuals with ventilator-related pneumonia, healthcare-associated pneumonia, or hospital-acquired pneumonia. *P. aeruginosa* in CF airways is strongly associated with decreased lung function, which increases patient morbidity and death (Greenwald and Wolfgang, 2022). *P. aeruginosa* infections persist in CF lungs despite the inflammatory response and lengthy, intensive antibiotic therapy. Once *P. aeruginosa* enters the CF airways, it is virtually impossible to eradicate due to its remarkable genome

plasticity that enables it to quickly adapt to the extremely stressful CF environment (Folkesson *et al.*, 2012).

This bacteria has a much greater death risk for ventilator-associated pneumonia than other infections (Jongers *et al.*, 2022). Burn patients frequently experience inhalation injury, which causes edema, sloughing of the respiratory tract mucosa, and impairment of the usual mucosal clearance process, rendering these patients more susceptible to *P. aeruginosa* pneumonia and upper respiratory tract infections (Kelly *et al.*,2022).

1.3.2.3. *P. aeruginosa* Associated Wound and Burn Infection:

The high microbial infection of severe burn wounds is currently a major medical challenge and the incidence and steadily rising rates of MDR make *P. aeruginosa* a common nosocomial infection in burn patients, particularly in burn centers. *P. aeruginosa* poses a substantial concern to people who have sustained severe burn injuries (Douzi *et al.*, 2017). One of the most significant post-burn injury sequelae is burn wound infection, which has been linked to substantial clinical problems as well as higher morbidity and death (Turner *et al.*, 2014).

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 (López-Jácome *et al.*, 2019). Skin and soft tissue infections, as well as burn wound infections, are frequently caused by *P. aeruginosa*. The bacteria typically targets patients who have burn and wound infections, when additional complications from the primary condition may arise and occasionally even induce bacteremia (Căpățînă *et al.*, 2022).

The prevalence of MDR is dramatically increasing among *P. aeruginosa* isolates from burn and other hospitalized patients in Iran (Farshadzadeh *et al.*, 2014), while the infections by bacteria able to colonize such injuries, those by *P.*

aeruginosa are among the most severe, causing major delays in burn patient recovery or leading to critical issues. Despite varying degrees of burns, infection continues to be the leading cause of death in burn patients, particularly in those with open, big, necrotic burn wounds who are more vulnerable to *P. aeruginosa* infection (Nguyen *et al.*, 2022).

1.3.2.4. *P. aeruginosa* Associated Otitis Media:

A major chronic suppurative otitis media pathogen is *P. aeruginosa*. This stage of ear infection is marked by a persistent, chronic infection of the middle ear, it is an important chronic suppurative otitis media pathogen that exhibits multiple resistances to antibiotics with increasing frequency, making patient treatment more difficult (Behailu *et al.*, 2022).

The recurrent and chronic nature of chronic suppurative otitis media may be explained by the dispersal of bacteria from a biofilm acting as a bacterial reservoir in the middle ear (Ali *et al.*, 2020). Human middle ear epithelial cells are invaded by *P. aeruginosa,* which causes cytoskeletal changes (Mittal *et al.*, 2015). This pathogen exhibits multiple resistances to antibiotics with increasing frequency and the treatment of Otitis media effusion in this regard is a major concern (Sahu *et al.*, 2019).

1.3.2.5. *P. aeruginosa* Blood Stream Infection:

An extremely dangerous and potentially lethal condition is *Pseudomonas aeruginosa* bacteremia. When Gram-negative sepsis in a community-onset infection is suspected, determining the likelihood of *P. aeruginosa* bacteremia is crucial (Cheong *et al,*. 2008). Blood stream infection by this pathogen is related

with simple mortality rates of 39 % in all patients and 49 % in intensive care unit patients (Bassetti and Carnelutti, 2016).

The outcome of *P.aeruginosa* bacteremia has been shown to be associated to microbial factor and host factors and also treatment (Kim *et al.*, 2014).

Additionally, *P. aeruginosa* primarily affects people with impaired immune systems when it produces bacteremia. Hematologic cancers, AIDS-related immunodeficiency, neutropenia, diabetes mellitus, and severe burns are among the risk factors (Joao *et al*., 2020). As a consequently, *P. aeruginosa* bacteremia has become a serious worry for both adults and children who have underlying medical conditions and are at a high risk of contracting an infection from a healthcare provider (Ciofi *et al.,* 2014).

1.3.2.6. *P. aeruginosa* Associated Meningitis:

P. aeruginosa is a rare cause of community-acquired meningitis, which has a very high death and morbidity rate (Pai *et al*., 2015). Prior neurosurgery surgeries and hospital-related onset are linked to it. Intracerebral hemorrhage is a rare complication of bacterial meningitis, but other cerebrovascular complications are known to occur (Saradna *et al*., 2018).

Usually caused by intraventricular catheters, *P. aeruginosa* neurosurgical meningitis is a rare condition with a significant death rate (Rodrguez-Lucas *et al*., 2020). Adult bacterial meningitis (ABM) is a type of ABM brought on by *P. aeruginosa* infection that is typically brought on by a nosocomial infection and is most frequently encountered in people who have recently had neurosurgery (Pai *et al*., 2015). A key issue in choosing antibiotics is the rise of meningitis brought on by bacteria that are highly drug-resistant (Ye *et al*., 2020).

1.3.3. Virulence Factors

The colonization by *P. aeruginosa* of different host tissues using various patterns such as flagellum, type IV pili, lipopolysaccharide antigen, and fimbriae. This bacterium has a single polar flagellum and several pili, which also play a critical role in its motility. The type IV pili are important adhesive factors which also provide twitching motility independent of the flagellum on solid surfaces (Persat *et al.*, 2015).

Pili deficient strains of *P. aeruginosa* show less adherence to the respiratory surface (Qi *et al.*, 2019). The combination of pili and other factors also plays a critical role in biofilm formation by aggregating bacterial cells on living or non-living surfaces leading to antibiotic resistance (Haiko and Westerlund, 2013; Gellatly and Hancock, 2013).

P. aeruginosa LPS is another virulence factor of *P. aeruginosa* and represents a major component of the outer membrane of the cell envelope. It is also involved in the bacterium's interaction with outer environments, including host cells, and hence plays a role in adhesion (Al-Wrafy *et al.*, 2017).

This component consists of three parts; lipid A, a core oligosaccharide and an O antigen. Lipid A is an endotoxin and is responsible for the inflammatory reactions associated with LPS, which can lead to septic shock (Monteiro *et al*., 2016). The core oligosaccharides are linked between lipid A and the O-antigen and are essential for association and entry of *P. aeruginosa* into respiratory epithelia (Huszczynski and Khursigara, 2019).

The third component, O-antigen, is an important part of LPS due to its immunogenicity. During chronic infections such as in cystic fibrosis patients, the O-antigen is lost from LPS, which probably helps *P. aeruginosa* to persist in the lung by protecting against immune cells (Perry, 2017). Further enhancing the adhesion process of *P. aeruginosa* to abiotic surfaces is by the fimbriae factor,

which are rod-shaped surface structures that are also critical for biofilm formation and in pathogenesis (Toyofuku *et al*., 2016).

Alginate is another factor of biofilm formation. It is an extracellular polysaccharide that is produced by *P. aeruginosa*, which may help in nonspecific adhesion Production of alginate is significantly higher in isolates associated with chronic infections (Gellatly and Hancock, 2013).

Consequently, they form mucoid colonies and are more prone to biofilm. Alginate confers resistance by helping the organism to form microcolonies, persist in the body sites, and prevent killing by phagocytes (Yung *et al*., 2021).

Many Gram-negative bacteria use T3SS, including extracellular pathogens such as *Escherichia coli*, *Citrobacter* spp., and P. *aeruginosa*, intracellular pathogens such as *Salmonella*, *Shigella, and Chlamydia* species, symbionts such as *Rhizobium* spp., and plant pathogens such as *Pseudomonas* that use T3SS to inject. Even though the T3SS machinery is substantially conserved, secretion system proteins distinguish themselves from bacteria by being diverse in their function and number (Deng *et al*., 2017).

Exotoxin A one of the important virulence factor in *P. aeruginosa* which features with its high toxicity to the host cells by its action modifies translation elongation factor 2 leads to cell death by inhibition of protein biosynthesis (Sauvage and Hardouin, 2020). *P. aeruginosa* utilizes a type II secretion system to secrete Exotoxin A into host cells (Jyot *et al.,* 2011). This toxin represses the host immune response by inhibiting the release of cytokines (Anantharajah *et al*., 2016). Tissue damage in host cells may arise from the action of proteases, one of the virulence factors that are increased by *P. aeruginosa* and cause disease. Three types of proteases have been found in *P. aeruginosa* and shown to cause increased virulence in various animal models of infection (Yahr and Parsek, 2006).

Additionally, *P. aeruginosa* secretes a type of protease termed protease IV, which has been shown to be a critical pathogenic (Thibodeaux *et al.*, 2005).

P. aeruginosa secretes a blue redox-active secondary metabolite known as pyocyanin. This pigment gives the blue-green color of bacterial colonies (Yahr and Parsek, 2006). This pigment can cause oxidative stress, induce neutrophil apoptosis, inhibit phagocytosis and modulate the expression of cytokines in respiratory epithelial cells (Gellatly and Hancock, 2013). Pyoverdine is a substance produced by *P. aeruginosa* to acquire iron for cell growth. It is responsible for the greenish-yellow colonies of this organism (Bhardwaj *et al*., 2021).Pyoverdine is an essential virulence factor since *P. aeruginosa* is required to obtain iron to grow on ocular surfaces (Suzuki *et al.*, 2018).

Bacteria release regulatory molecules in response to environmental changes including cell-population density, and these molecules, when they reach a threshold concentration, control the bacterial response to environmental changes such as cell density. When these molecules reach a critical concentration, they control the transcription of numerous genes. This method is known as quorum sensing (Chadha and Harjai, 2022).

These auto-inducer signal molecules bind to their cognate proteins to regulate the transcription of a variety of genes, including virulence factors. Quorum sensing is also important for biofilm formation, Quorum sensing is required for this bacterium to colonize the lung, as in CF patients (Gellatly and Hancock, 2013). Furthermore, quorum sensing signaling molecules can lead to a decrease in immune modulator production and may increase the pathogen's infectivity (Skindersoe *et al.*, 2009; Cooley *et al.*, 2010).

P. aeruginosa has the ability to form biofilms, which are related to quorum sensing (Bjarnsholt *et al.*, 2010). Hall-Stoodley *et al*. (2004) describe biofilms as highly organized surface microcolonies encased in a dense extracellular matrix of

polysaccharides, nucleic acids, lipids, and proteins (Hall-Stoodley *et al.*, 2004). Biofilms can form on non-biological (e.g., medical implants) as well as biological (e.g., human tissue) surfaces. Biofilms are thought to contribute to virulence by providing mechanical resistance to prevent cells from being washed away, a protective environment by reducing antimicrobial penetration and a barrier against host immune defenses (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004).

Biofilm formation begins with strong cell attachment, which is aided by type IV pili, flagella, and cup fimbriae (Mikkelsen *et al.*, 2009). Following firm attachment, the organisms in the biofilm multiply as microcolonies and produce a polysaccharide matrix.

1.3.4. Epidemiology of *P. aeruginosa*

P.aeruginosa can adapt to a number of conditions, including surface waters, disinfectants, and respirator humidifiers, and can be found anywhere in nature. It can multiply in distilled water, presumably by exploiting gaseous dissolved nutrients, but it is rarely separated from sea water (unless in contaminated river estuaries and sewage outfalls) (Fonseca *et al.*, 2007; Lima, 2016). *P.aeruginosa* is almost always found in hospital sinks, taps, and drains. The organism may live in water traps and is rarely polluted in the residential setting (Slama *et al.*, 2011). Normal human carriage is uncommon; faecal carriage rates in healthy patients range from 2% to 10%; faecal colonization appears to be passing in healthy people; and strain types change quickly. On dry, healthy skin, *P. aeruginosa* dies quickly, but under conditions of extreme moisture, such as in divers doing long-term saturation dives, the frequency of skin colonization increases, and infections, particularly otitis externa, are more common (Brooks *et al.*,2007; Scano, 2019).

In spite of the apparent ubiquity of *P.aeruginosa* in the natural environment and the vast array of potential virulence factors, in the healthy people, the

prevalence of community-acquired infections is minimal. The percentage of patients and healthy controls is about the same (about 5%), whereas in burns patients can reach 80 % by the ninth day after the burn (Pirnay *et al.*,2003).

In the United States, *P.aeruginosa* was the most commonly isolated Gram-negative bacterium for nosocomial pneumonia (18.1%) and the second most commonly isolated Gram-negative bacteria for nosocomial urinary tract infection (16.3%) in 2003. (Tam *et al.*,2007).

Approximately any form of hospital tool or device, including disinfectants, antiseptics, intravenous fluids, and eyewash solutions, has been identified as a reservoir for *P.aeruginosa*. In common source outbreaks, these sources may act as foci for the spread of the organism, which is frequently the result of poor sterilization (Brooks *et al.*, 2007; Bédard *et al*., 2016)

1.3.5. Antibiotic Resistance of *P.aeruginosa*:

P. aeruginosa has several mechanisms for intrinsic antibiotic resistance, including constrained outer-membrane permeability; efflux systems that expel drugs from the cells; and the generation of antibiotic-inactive via porin channels. By interacting with *P. aeruginosa* LPS on the enzymes that function on the outer membrane, aminoglycosides and polymyxins increase their own absorption. Cell membranes are penetrated by quinolones and β-lactams (Pang *et al*., 2019). Antibiotic resistance, which has been a major threat to global public health, has been a top priority for several national and international organizations, including the World Health Organization (WHO), the European Centre for Disease Prevention and Control (ECDC), the National Institutes of Health (NIH), and the Centers for Disease Control and Prevention (CDCP) (CDC). The CDC declared the human race to be in the "post-antibiotic age" in 2013, while the WHO warned

in 2014 that the antibiotic resistance situation was getting serious (Ventola, 2015); (WHO, 2018), (Centers for Disease Control and Prevention, 2019).

In many ways, the serious bacterial infection outbreaks that are becoming more common are costly. Extended-spectrum β-lactamase-producing, carbapenemase-producing, and vancomycin-resistant are among the multidrug-resistant bacteria (Hugo and Russell, 2011).

Antibiotic resistance in *P.aeruginosa* can be identified using microbiologic or clinical testing. Microbiological resistance is defined as the presence of a genetically determined resistance mechanism (acquired or mutated) that allows a phenotypic laboratory test to categorize a *P.aeruginosa* as resistant or susceptible, clinical resistance is an antimicrobial activity level linked to a high risk of therapeutic failure (MacGowan, 2008).

Antibiotic resistance is claimed to be innate, and this is thought to be due to differences in the structure of their cell membrane. Resistance or susceptibility reduction can also be phenotypic, arising from adaptation to growth in a particular environment. Horizontal acquisition of resistance genes, deployed via insertion sequences, transposons, and conjugative plasmids, by recombination of foreign DNA into the chromosome, or mutations in distinct chromosomal loci can all be used to achieve resistance (MacGowan and Macnaughton, 2017).

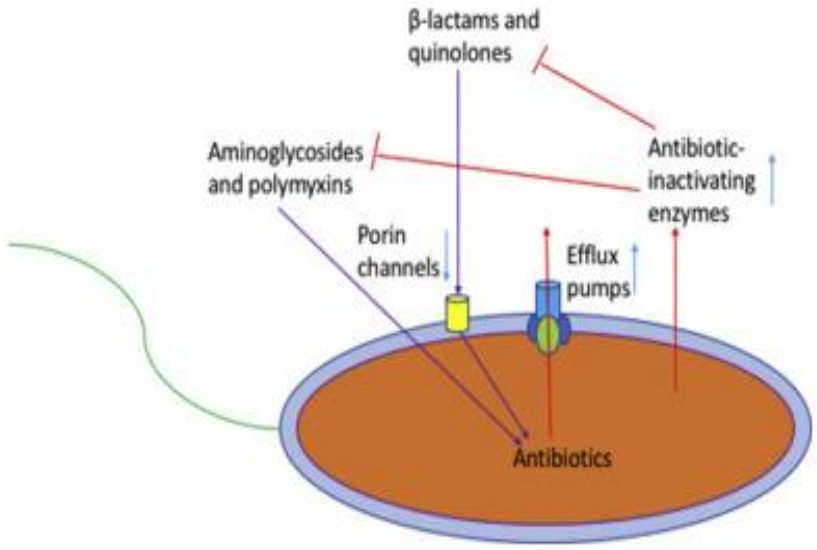

Figure (1-2) A schematic representation of the mechanisms of *P.aeruginosa* (Pang *et al.*, (2019))

The studies indicated that resistance to antibiotic are classified into three major types: phenotypic, intrinsic, and acquired. Intrinsic resistance involve all innate features that are naturally encoded by pathogenic bacteria to limit antimicrobial impact (Ventola, 2015).The term acquired resistance refers to susceptible bacteria that become resistant to antibiotics either by genetic material such as mutation or by integrating plasmids, transposons and others, in comparisons with a above phenotypic resistance is an unstable rise in the ability of bacteria to tolerate the antibiotics as a result of modifications of gene expression in response to an ecological affect, such as growth factor and nutrient conditions (Slama *et al.*, 2011).

Both intrinsic and acquired mechanisms of resistance can be transmitted to the next generations whereas the adaptive one is transitory and efficiency generally regress once the resistance-inducing conditions are removed (Blanco *et al.*, 2016). The higher levels of resistance may result from the overexpression of

efflux pumps which can be unstable when either specific inducers involve in phenotypic resistance or constitutive inducers rendering acquired one are found (Amieva *et al.*, 2022).

1.3.6. Efflux Pumps in *P. aeruginosa*:

Since the 1960s, the recorded observations made by researchers on MDR phenotypes of *P. aeruginosa* have been characterized by their resistance triggered by aminoglycosides, chloramphenicol, penicillin, sulfonamides, and tetracycline (Spratt, 1978; Moore *et al.,* 1986 and Foudraine *et al.,* 2021). Additionally, resistant bacteria are capable of producing drug-inactivating enzymes(e.g., β-lactamases and aminoglycoside-modifying enzymes) (Leive, 1974; Moore *et al.*, 1986; Coetzee *et al.,* 2013 and Othman *et al.,* 2014).

The mechanisms used by *P. aeruginosa* are different mechanisms, and they offer no satisfactory explanation of the cause of resistance, as shown in figure (1-3). A number of causes have been recognized, among which the outer barrier is one. Most gram-negative bacteria are less susceptible to drugs than gram-positive bacteria (Hancock *et al.*, 1979; Terzi *et al.,* 2014 and Suresh *et al.,* 2018).

On the other hand, *P. aeruginosa* is a species characterized by low outer membrane permeability (Sugawara *et al.*, 2006), in detail because of the presence of its closed channel porin OprF mainly (Sugawara *et al.,* 2010). Also, *P. aeruginosa* and *Escherichia coli* share the same similarities in their existing low permeable lipid bilayer (Maccarini *et al*., 2017), which leads to easy drug passage through the outer membrane of mutant *P. aeruginosa* with a deficiency in outer membrane and efflux pump activity (Ramalingam *et al*., 2016; Piselli and Benz, 2021).

Previously , the usage of broad-spectrum β-lactams and quinolones showed an increase in the number of multidrug resistant strains in *vivo* and in *vitro* under

laboratory conditions. (Al-Derzi, 2012; Lila *et al.,* 2017). Quinolone-resistant isolates with the MDR-associated OM protein specifically showed a reduction in ciprofloxacin uptake and active extrusion of ofloxacin (Bassetti *et al*., 2018).

However, it became clear that both mechanisms of OM permeability and β-lactamase activity cannot fully demonstrate MDR phenotypes, prompting researchers to investigate *P. aeruginosa* intrinsic and acquired resistance (Cabassi *et al*., 2017).

Other studies demonstrated the predominant function of the efflux pump mechanism in intrinsic and acquired MDR *P. aeruginosa* as reported in a study (Talebi *et al.,* 2016) which characterized the components of the mexAB-oprM operon of this pathogen. In detail, the efflux encodes a three-component system involved in MDR and expression of multiple drug efflux pumps in *P. aeruginosa* (Li and Plésiat, 2016).

Subsequently, the report showed the three homologue effluxes for MexAB-OprM as mentioned: MexCD-OprJ, MexEF-OprN, and MexXY (Westbrock *et al.,* 1999; Tseng *et al.,* 1999). All of these pumps belong to the RND family of secondary active transporters (Lee *et al.,* 2006). (Jeannot *et al*., 2008; Stickland *et al*., 2010).

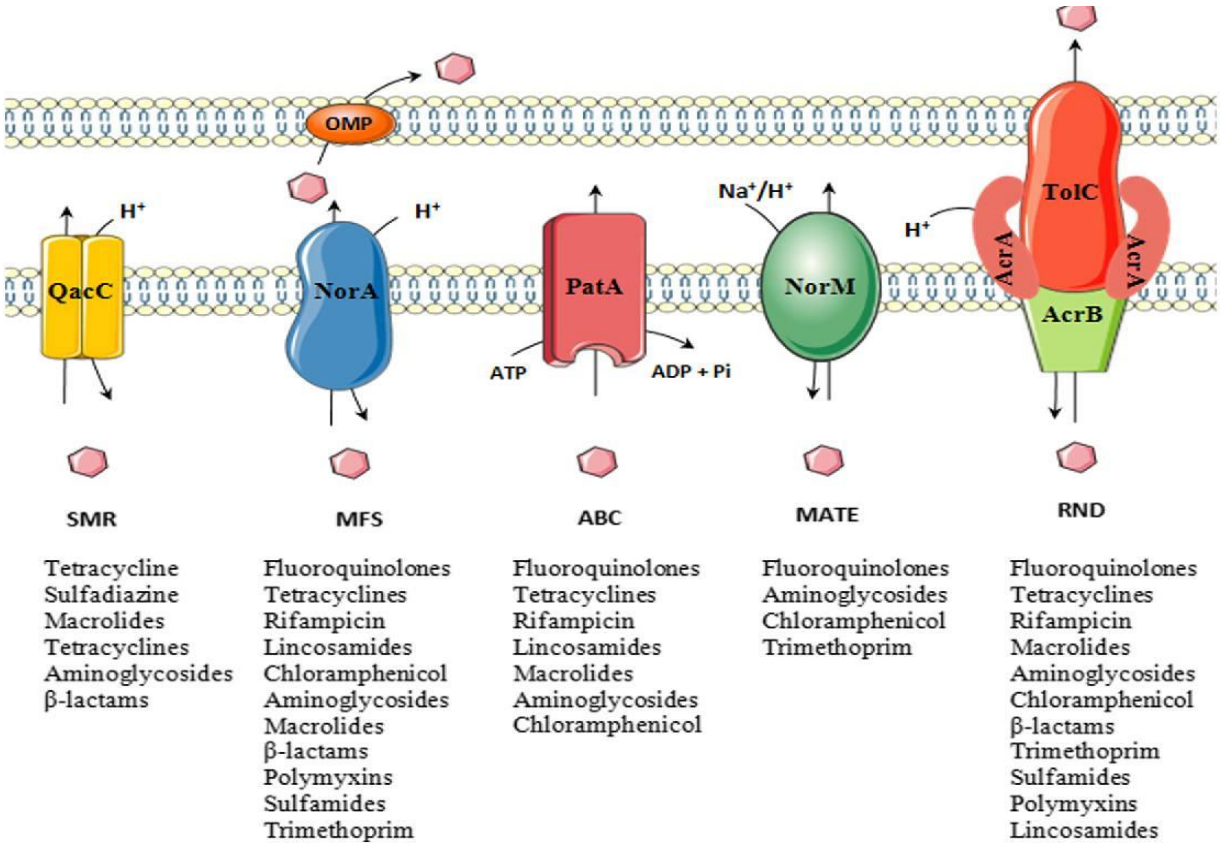

Figure (1-3) Diagram of the Five Multi-Drug Efflux Pump Families. ABC; MATE; MFS; RND; and SMR Adopted from (Yılmaz and Özcengiz, (2017))

1.3.7. The Effectiveness of Antimicrobial Drug Efflux Pumps and Their Clinical Significance in *P. aeruginosa*:

The sequences of *P. aeruginosa* genome show the existence of a number of primary and secondary active transporters. As of now, 12 RND efflux pumps have been characterized for their substrate profiles (Angus *et al.*, 1982; Scoffone *et al.*, 2021). *P. aeruginosa* mutants lacked outer membrane lipopolysaccharide in the presence of readily available medication. Drug efflux activity was later discovered to be lacking in the mutant isolates (Rundell *et al.*, 2020).

On the other hand, drug uptake in *P. aeruginosa* isolates resistant to aminoglycosides or carbapenems may be reduced by quantitative or qualitative changes in the porin (OprD) content of the outer membrane (Assembly , 2016; Ropponen *et al*.,2021). Additionally, the identification of multidrug resistant isolates in vivo following medication administration increased in the 1980s as a result of the usage of fluoroquinolones and improved wide spectrum β-lactamases, drugs have also been shown to generate multi-drug resistance microorganisms easily in vitro, or in a laboratory environment (Masuda and Ohya, 1992; Sartelli *et al*., 2016).

The OM protein profiles of *P. aeruginosa* isolates were examined in multiple investigations while examining the molecular mechanisms of MDR or fluoroquinolone resistance. These revealed overproduction of OM proteins that were related to various gene loci (Masuda and Ohya, 1992; Li and Plésiat, 2016). The Masuda and Ohya study agreed with the study that showed the MDR-associated OM protein *oprM* Importantly, quinolone-resistant isolates had lower ciprofloxacin uptake and active ofloxacin's drug extrusion (Pham *et al*., 2019). In any case of these findings, it became obvious that the permeability barrier of the outer membrane and periplasmic β-lactamase activity could not completely explain MDR phenotypes prompting us to investigate innate and acquired MDR in *P. aeruginosa* (Stover *et al*,. 2000; Dik *et al*., 2017; Jasim *et al*., 2017).

P. aeruginosa has identified the mexAB–oprM operon from *P. aeruginosa*, and it encodes a three-component efflux mechanism implicated in MDR. These investigations appeared to show that drug efflux mechanisms play a major role in intrinsic and acquired MDR in *P. aeruginosa*, as well as the expression of numerous drug efflux pumps (Munita and Arias, 2016; Pelegrin *et al*., 2021).

Before the availability of the first full genome sequence information about *P. aeruginosa* (Kohler *et al.*, 1997), three MexAB-OprM homologues, MexCD-

OprJ, MexEF-OprN, and MexXY (originally referred to as MexGH or AmrAB) were also found to be involved in *P. aeruginosa*. All of these Mex pumps are members of the RND superfamily of secondary active transporters, indicating that they are all secondary active transporters (Masuda *et al.*, 2000), that normally in Gram negative bacteria, need several components to construct an energy-dependent functional extrusion complex across the entire cytoplasmic and outer membranes (Wolloscheck, 2017; Iman *et al*., 2018).

1.3.7.1. MexAB-OprM:

P. aeruginosa's intrinsic drug resistance is considerably aided by many mechanisms, such as the efflux mechanism, which has a constitutive pattern of growth phase-dependent expression in wild-type strains (da Silva, 2016; Shigemura *et al.*, 2016). The wild-type strains become exceedingly susceptible when any component of MexAB-OprM is inactivated, resulting in an 8-fold drop in the values of the minimum inhibitory concentrations (MICs) for several antimicrobial drugs (Kanagaratnam *et al.*, 2017; Poudyal and Sauer, 2018).

Overproduction of MexAB-OprM contributes to acquired MDR and has been found in clinical isolates of numerous mutant types (Li *et al*,. 2003). After being exposed to β-lactams, quinolones, chloramphenicol, macrolides, tetracyclines, biocides, and organic solvents in vitro, MexAB-OprM overproducers were founded (Wand, 2017; Yung *et al*., 2021). Among the known multidrug efflux pumps of *P. aeruginosa*, the MexAB-OprM efflux system has the broadest substrate profile. These antimicrobial agents that have been proven to be substrates comprised of antibiotics included β-lactams, chloramphenicol, quinolones, macrolides, novobiocin, sulfonamides, trimethoprim, tetracyclines, cerulenin, pacidamycin, and thiolactomycin, also extend to non-antibiotics, such

as dyes, detergents, triclosan, organic solvents, tea tree oils, and quorum-sensing molecules (Fruci and Poole, 2018).

It is worth mentioning that the action of imipenem against *P. aeruginosa* does not appear to be a substrate of the MexAB-OprM pump.

In terms of its importance, MexAB-OprM when overproduced decreases the susceptibility of clinical isolates to antimicrobials by two- to eight fold in MIC values when compared with the baseline levels in the absence of other resistance mechanisms such as enzymatic drug inactivation and drug targ*et al*terations (Adamson *et al.,* 2015). Studies linked with MexAB-OprM overproducers are likely to necessitate higher medication dosages or antimicrobial-efflux pump inhibitor combos (Rahbar *et al.,* 2021). Furthermore, increased MexAB-OprM expression promotes the establishment of additional resistance mechanisms (Tetard *et al*., 2019).

1.3.7.2. MexXY-OprM / MexXY-OprA:

The mexXY system, which is encoded by a two-gene operon that lacks an OM protein gene, uses OprM to generate a functional efflux pump in most *P. aeruginosa* strains (Morita *et al.*, 2012), and MexXY can work with either OprM or OprA, *P. aeruginosa* MexXY pump is inducibly expressed and confers resistance to aminoglycosides (Goli *et al*., 2016; Singh and Malik, 2020).

The MIC values of aminoglycosides are reduced four to eight fold in wild-type strains when meXY is inactivated. The mexXY overproduction causes aminoglycoside resistance in clinical isolates of the so-called "impermeability-type mexXY, while increased mexXY expression confers a 2- to 16-fold increase in pump substrate resistance. The mexXY causes fluoroquinolone resistance in *P. aeruginosa* when overexpressed from plasmid vectors (Fraud and Poole, 2011).

The mexXY overproducers are found in abundance in clinical isolates from cystic fibrosis and non-cystic fibrosis patients (Khanam *et al*., 2017). The presence of a lot of reactive oxygen species in the cystic fibrosis lung environment could explain why resistance is so high. Long-term exposure of *P. aeruginosa* to hydrogen peroxide has been found to help MexXY overexpression (Srikumar *et al.,* 1997). The wide distribution and overlapping functions of MDR efflux pumps in bacteria hint at their probable role in physiological functions in addition to mediating intrinsic and acquired MDR (Piddock, 2006; Ramaswamy *et al*., 2017).

1.3.7.3. MexCD-OprJ:

This sort of efflux system appears to be dormant in wild-type strains because chromosomal disruption of this efflux operon has no effect on wild-type cells' antimicrobial sensitivity (Fraud *et al.,* 2008). Many compounds that are considered to be membrane-damaging toxicants are acriflavine, ethidium bromide, rhodamine 6G, chlorhexidine, and tetraphenylphosphonium, inducible MexCD-OprJ expression (Schwartz *et al.,* 2015). These substances are also MexCD-OprJ substrates. The exposure of *P. aeruginosa* to waste water was one of the causes of overexpression in MexCDOprJ (Terzi *et al.,* 2014; Zhao *et al.*, 2020). Resistance to fourth-generation cephalosporins (cefepime and cefpirome), quinolones/fluoroquinolones, chloramphenicol, cerulenin, pacidamycin, and tetracycline appears to be caused by mutation-mediated overexpression of this operon (De *et al.,* 2015). Other cytotoxic chemicals, such as acriflavine, ethidium bromide, quaternary ammonium compounds, rhodamine 6G, triclosan, and organic solvents, are also substrates for MexCD-OprJ (Colinon *et al.*, 2010).
Fluoroquinolone resistance is linked to MexCD-OprJ overproducers, although fluoroquinolone-resistant isolates may also overexpress other efflux pumps (e.g., MexAB-OprM, MexXY, or MexEF-OprN) and/or have quinolone-target

alterations (Sanz-García *et al*., 2022). Indeed, as with MexAB-OprM or MexXY, overexpression of MexCD-OprJ has been documented in a substantial proportion of fluoroquinolone-and/or carbapenem-resistant clinical isolates (Hernando *et al.*, 2016).

1.3.7.4. MexEF-OprN:

Previous research has shown that the expression of this efflux mechanism is not as high as it is in wild-type *P. aeruginosa* cells and that inactivation has no effect on antimicrobial susceptibility (Li and Plésiat, 2016). They've been found in cystic fibrosis and other patients' clinical isolates (Castanheira *et al.*, 2014).
The prevalence varies from one study to another. While many studies showed low frequencies or even no detection of mutants among clinical isolates (Terzi *et al.*, 2014), more recent studies have revealed a higher prevalence of MexEF-OprF overproducers (Bubonja *et al.*, 2015). For example, about 30% of 62 isolates (mostly obtained from intensive care unit patients and with reduced carbapenem susceptibility) were MexEF-OprF overproducers (Yang *et al.*, 2011).

1.3.7.5. MexJK-OprM/OpmH:

In wild-type cells, this efflux mechanism is expressed at low levels (Morita *et al.*, 2012). MexJK requires an outer membrane channel protein for drug efflux despite the lack of an OM protein gene in its producing operon. While OprM is involved in the extrusion of ciprofloxacin, erythromycin, and tetracycline by MexJK, MexJK is reliant on OpmH, another OM protein, for triclosan resistance (Hocquet *et al.*, 2006; Scoffone *et al.*, 2021).

This pump's clinical significance is unknown. Nonetheless, MexJK overproduction was identified in two cefepime-resistant MexXY-hyperexpressing

bacteria isolates (Poonsuk *et al.,* 2014) as well as in a MexXY-/MexVW-overproducing isolate (Stover *et al.,* 2000).

1.3.7.6. MexGHI-OpmD:

MexGHI-OpmD is a four-gene operon that mediates intrinsic resistance, mexG is a protein with an uncertain function, while MexH and MexI are the cytoplasmic membrane exporter and auxiliary membrane fusion protein, respectively (Aendekerk *et al.,* 2002; Aendekerk *et al.,* 2005).

In *P. aeruginosa*, this mechanism is engaged in pseudomonas quinolone signal homeostasis and is linked to quorum sensing (Aendekerk *et al.,* 2012). Its inactivation leads to a decrease in the production of numerous virulence factors, establishing a connection between antimicrobial susceptibility and pathogenicity (Minagawa *et al.*, 2012). Surprisingly, the mexGHI-opmD hypersusceptibility is complemented by increased sensitivity. *Pseudomonas aeruginosa* MexAB-OprM and MexEF-OprN, according to data, convey intercellular signals and/or intermediates produced during their synthesis (Sekiya *et al.,* 2003).

Xenobiotics, such as the antibiotic norfloxacin and the heterocyclic dye acriflavine(Aendekerk *et al.,* 2005), a precursor or derivative of the Pseudomonas quinolone signal (PQS), and a precursor or derivative of the Pseudomonas quinolone signal (PQS) (Sakhtah *et al.,* 2016).

1.3.7.7. MuxABC-OpmB:

Introducing *muxA-muxB-muxC-opmB* into *P. aeruginosa* cells increased the MICs of many antibiotics, the RND-type efflux pump requires an OMP component for function (Adamiak *et al.,* 2021). The downstream region of muxABC contains the opmB gene, which codes for an OMP component and this

reveals that OpmB gene is essential for MuxABC also its functions connect with other multidrug efflux pumps that extrude antibiotics (Zgurskaya *et al*., 2022). Otherwise OpmB gene cannot be replaced by OprM for the function of the MuxABC system. Introduction of muxABC-opmB into *P. aeruginosa*, a drug-hypersusceptible strain, led to elevated MICs of aztreonam, macrolides, novobiocin, and tetracycline. Since muxB and muxC, both of which encode RND components, are essential for function, MuxABC-OpmB is thought to be a drug efflux pump with four components(Rundell *et al.,* 2020). MuxABC-OpmB pump contributes to the intrinsic resistance of *P. aeruginosa* against novobiocin and to the macrolides (Adamiak *et al*., 2021; Castanheira *et al*., 2022).

1.3.7.8. TriABC–OpmH:

The RND-type transporter TriABC-OpmH was linked to triclosan resistance in *Pseudomonas aeruginosa* (Tikhonova *et al*., 2011).TriABC-OpmH is structurally and functionally distinct from other RND-type transporters. Unlike other RND-type efflux complexes that are known to assemble with a single hexameric MFP(Mikolosko *et al.,* 2006), the TriC transporter engages two distinct, TriA and TriB, which are essential for sophisticated functionality. Furthermore, TriABC-OpmH has an extremely tight substrate specificity, with triclosan being the only known substrate for this pump (Fabre *et al*., 2021).

The TriABC-OpmH complex is an appealing tool for determining the molecular mechanism of RND-dependent multidrug efflux in gram-negative bacteria because of its structural complexity and functional specialization. TriA and TriB are that share just 36% of their identity but retain MFP-specific characteristics. Similarly to other MFPs (Jensen *et al.,* 2004; Alav *et al*., 2021).

The pump TriABC overexpression caused by a promoter-up mutation raises *P. aeruginosa* MICs for triclosan. TriABC is thus produced constitutively in *P.*

aeruginosa cells and is thought to contribute to intrinsic triclosan resistance levels(Tikhonova *et al.*, 2011).

1.3.7.9. MexMN-OprM and MexPQ-OpmE:

Multidrug efflux pumps are known to extrude many structurally unrelated compounds and are thus involved in multidrug resistance. Once a bacterial cell acquires a gene for multidrug efflux pump function in the cell, or a silent gene for a multidrug efflux pump turns on, the cell becomes multidrug resistant. The genome project of *P. aeruginosa* revealed the presence of 34 genes or operons for putative multidrug efflux pumps (Heacock *et al.*, 2018).

In *P. aeruginosa* Mex pumps and is amplified in the presence of antimicrobial drugs in the growth medium (Sekiya *et al.*, 2003). Fluoroquinolone is a substrate for all of *P. aeruginosa*'s RND-type multidrug efflux pumps that have been identified thus far. Fluoroquinolone is a substrate for MexPQ-OpmE, but it does not appear to be a substrate for MexMN-OprM. Fluoroquinolones, on the other hand, could be substrates for MexMN-OprM when produced at high levels. The level of MexMN expression may be insufficient for accurate characterization(Zgurskaya *et al.*, 2022).

It's possible that more MexMN and OprM overproduction is required for characterization, when these operons are produced, MexPQ-OpmE and MexMN-OprM can operate as multidrug efflux pumps even though they were silent or had

very modest expression in wild-type *P. aeruginosa*. These operons may be expressed by mutations in their promoter regions or regulatory genes, if present, or by the presence of relevant inducers. *P. aeruginosa* could develop resistance to a variety of antimicrobial drugs as a result of such mutations or inducers (Andersen *et al.*, 2021).

1.3.8 *P. aeruginosa* RND Efflux Pumps Inhibitor phenylalanine-arginine beta-naphthylamide:

Inhibition of efflux pumps appears to be an attractive approach to improvement of the clinical efficacies of antibiotics that are substrates of such pumps. It is expected to decrease the level of intrinsic resistance, significantly reverse acquired resistance, and decrease the frequency of emergence of *P. aeruginosa* mutants highly resistant to antibiotics (Eleftheriadou *et al.*, 2021).

Among the *P. aeruginosa* efflux pump inhibitors, the most studied is Phe-Arg-β- naphthylamide (PAβN), a broad spectrum compound. These inhibitors act with a competitive inhibition approach and are recognized instead of the target antibiotics by MexAB-OprM. Until the pumps extrude these inhibitors outside the cell, the antibiotic stays and increases intracellular concentration.

It was also revealed that PAβN can increase the potency of other antibiotics therefore, it is considered as a broad spectrum (Iman *et al.*, 2018). PaβN was shown to interfere with the RND systems of *P. aeruginosa* as MexAB-OprM,

MexCDOprJ, MexEFOprN, MexXY-OprM. The association of chloramphenicol, fluoroquinolones, macrolides, oxazolidinones, and rifampicin with PAβN increases their effects, the inhibitor PAβN functions as a substrate of Mex efflux pumps and competes with antibiotics, preventing their extrusion, PAβN and its derivatives showed adverse toxicity (Scoffone *et al.,* 2021). Efflux pump inhibitor like PAbN have significant promise as adjuvants for antibiotics that can lower the effective doses of current medications.

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Chapter Two
Materials & Methods

2.1 The Materials:

2.1.1 Equipment and Instruments:

The instruments and equipment which were used in the present study are listed in Table (2-1).

Table (2-1): Laboratory Equipment's and Instruments.

No.	Equipment /Instruments	Company	Origin
1	Autoclave		Korea
2	Benchtop centrifuge	Memmert	Germany
3	Centrifuge	Hettich	Germany
4	DNA extraction tubes 100 µl, P.C.R tubes (50µl)	Capp	Germany
5	Eppendorf tubes	Eppendorf	Germany
6	Freezer	Kelon	China
7	Gel electrophoresis system	Clever	USA
8	Incubator, oven	Memmert	Germany
9	Laboratory distillation unit	Cryste	Korea
10	Laminar flow cabinet	Capp	Germany
11	Micropipettes	Capp	Germany
12	Microwave	Panasonic	Japan
13	Millipore filter (0.45mm)	Satori's membrane	Germany
14	Parafilm	Citotest LaB ware	Pakistan
15	PCR thermo cycler	Technolab	UK
16	Petri dishes (9 cm)	Afco-Dispo	Japan
17	Plain tube (10 ml or 15 ml)	Afco-Dispo	Japan
18	Platinum Wire Loop	Himedia	Indian
19	Premium quality petri dishes divided 3 chambers	Citotest LaB ware	Lebanon
20	Refrigerator	Concord	Italy
21	Sensitive balance	Denver	USA
22	Transport collection swabs	Citotest Lab ware	China
23	UV-trans illuminator	Clever	USA

2.1.2 Chemical Materials:

The main stains and chemical materials used in the present study are listed in Table (2-2).

Table (2-2): Chemical Material.

No	Chemicals	Company / Origin
1	Ethanol (C_2H_5OH) (95%)	BDH/ England
2	Alcohol (70%)	Fluka chemical/
3	Glycerol ($C_3H_8O_3$)	Switzerland
4	Nuclease free water	Bioneer (Korea)
5	Simple safe (Red safe) stain	Promega / USA
6	100 bp DNA ladder (100 bp)	
7	Loading dye (bromophenol blue)	
8	Tris-EDTA buffer (TE)	Promega / USA
9	Tris-Borate-EDTA (TBE) buffer	

2.1.3 Biological Materials:

The main biological materials used throughout the present study are listed in Table (2-3).

Table (2-3): biological materials.

Media	Company / Origin
Pseudomonas chromogenic agar	Condalab/Spain
Müller- Hinton agar	
Brain heart infusion broth	Himedia/India
Nutrient broth	
Agarose	Promega/ USA
Inhibitor Phenylalanine-Arginine Beta-Naphthylamide (PAbN)	Promega/ USA

2.1.4 Antibiotics Disks:

Antibiotic disk diffusion was performed according to CLSI, (2020)

Table (2-4): Antibiotics used in the present this study.

No.	Group	Antimicrobial Agent	Assembly	Content (mg)	Company/Origin
1	Quinolons	Levofloxacin	LEV	250	Sigma/UK
2	Penicillins	Piperacillin	PRL		
1	Penicillins	Piperacillin	PRL	100	Condalab/Spain
2	Cephalosporins	Ceftazidime	CAZ	30	Condalab/Spain
		Cefepime	FEP	30	Condalab/Spain
3	Monobactams	Aztreonam	ATM	30	Condalab/Spain
4	Carbapenem	Imipenem	IPM	10	Condalab/Spain
		Meropenem	MEM	10	
5	Aminoglycosides	Gentamicin	CN	10	Condalab/Spain
		Tobramycin	TOB	10	Condalab/Spain
		Amikacin	AK	30	Condalab/Spain
		Netilmicin	NET	30	Condalab/Spain
6	Quinolons	Ciprofloxacin	CIP	5	Condalab/Spain
		Levofloxacin	LEV	5	Condalab/Spain
		Ofloxacin	OFX	5	Bioanalyse/Turkey

2.1.5. Primer Pairs

The Primer used in present study with condition are listed in table (2-5).

Table (2-5): Sequencing and PCR conditions designed in the present study.

No.	Gene	Sequence 5-3	bp	Conditions
1	*muxA*-F	GAATGGTCAGCACGCCTTTG	500	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	muxA-R	CTCGATAGCCAAGCAGACCC		
2	*muxB*-F	GTCCTGCAAGGTGAACTGGT	489	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 2 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	muxB-R	GATGGCCTGATCGCACAGTA		
3	*muxC*-F	AACACCGGTTCGTTCTTCGT	545	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	muxC-R	CTCGGTTCGTAGTGGCTGAA		

4	*opmB*-F	GAACGTTCCAACCAGACCCT	447	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58ºC, 30 sec. Step 4: 72ºC, 70.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	opmB-R	TTTGTTCTCGGCCACCTTCA		
5	*mexA*-F	GACGGTGACCCTGAATACCG	620	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 70.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexA-R	CGACGGAAACCTCGGAGAAT		
6	*mexB*-F	GTCTACCCGTACGACACCAC	600	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC, forever
	mexB-R	GGTGGAAAGGAACATCCGGT		
7	*oprM*-F	GGTAGCCCAGGACCAGAATG	520	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 61.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times
	oprM-R	GAGCTGGTAGTACTCGTCGC		

				Step 6: 72ºC, 5 min. Step 7: 4ºC, forever
8	*mexC*-F	GGATCGCCTGGTTGTCGAT	500	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.1ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexC-R	CAGCCAGCAGGACTTCGATA		
9	*mexD*-F	TTCCCATTTCACGCTGACGA	549	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexD-R	CTGGCCTTCCCAACCTTCAA		
10	*oprJ*-F	GCGTTGAATGCCTGCTGTTT	597	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	oprJ-R	GATCGACACGCTGGATTGGA		
11	*mexE*-F	CCGAAGTCATCGAACAACCG	555	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.5ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-
	mexE-R	GCTCGACGTACTTGAGGAACA		

				4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
12	*mexF*-F	TTCAACTCGCTGACCCTGTC	562	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexF-R	TGTAGGCGTCCTGAATGTCG		
13	*oprN*-F	TTGACAGTGGACCTTTCGCC	506	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	oprN-R	AGGAAATCGGTGGTGCCTTC		
14	*mexG*-F	CTCGAAAGCAACTGGCTCTG	423	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
15	*mexH*-F	ATGCAGAAACCCGTCCTGAT	607	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 57.2ºC, 30 sec. Step 4: 72ºC, 70.0 sec.
	mexH-R	AATTGCTTTTCAGGGTCCGC		

				Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
16	*mexI*-F	ATGCAAGGCTTCATCACCCA	570	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 57.2ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexI-R	ACGGTTGCTGATCACCATGT		
17	*opmD*-F	CTCCTACCCGAACCTTTCGC	558	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	opmD-R	TGGTAGCCCTGGATCTCGAA		
18	*mexJ*-F	GGTAAAGAAGGACCAGCCCC	633	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 70.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexJ-R	GCCGACAAGGGAACCGATAA		

19	*mexK*-F	GTTCGCACGTGAGCTTCTTC	463	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexK-R	GGTGCGGTGAGGAATACCAT		
20	*oprM*-F	GGTAGCCCAGGACCAGAATG	520	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 61.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	oprM-R	GAGCTGGTAGTACTCGTCGC		
21	*mexM*-F	TGCAGGGAGAAGACTACGGA	489	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC,
	mexM-R	CATCGGTACGGTGACTTCGT		
22	*mexN*-F	AAACTCTCGTAGAGCACGCC	563	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times
	mexN-R	GGGCTGATGGATGTGTCCAA		

				Step 6: 72ºC, 5 min. Step 7: 4ºC
23	*oprM*-F	GGTAGCCCAGGACCAGAATG	520	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 61.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	oprM-R	GAGCTGGTAGTACTCGTCGC		
24	*mexP*-F	ACATCCAGGACGTTACGGTG	528	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexP-R	CATAGGACTCGTCGGTGAGC		
25	*mexQ*-F	CTGGCTCTGGTGGTGTATGG	631	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 70.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexQ-R	GCAATGCCTCGAACACATCG		
26	*opmE*-F	TGTATCCGCAGGTCGAGGTA	515	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-
	opmE-R	AGAGGTATCGTCGGTAGCCA		

				4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC,
27	*mexV*-F	AGGCCATCTCGAAAAGCGAA	554	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexV-R	GCCCTTGTCATCCTTCGACA		
28	*mexW*-F	CGCAGCTTCCCGGAGTATTA	598	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexW-R	GTTCGTGGGTCTCGATCAGG		
29	*oprM*-F	GGTAGCCCAGGACCAGAATG	520	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 61.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	oprM-R	GAGCTGGTAGTACTCGTCGC		
30	*mexX*-F	CATCAGCGAACGCGAGTACA	576	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec.
	mexX-R	TGTGGGTTGACCACCTTGAC		

				Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
31	*mexY*-F	CCGTACGGTGTATGCGATGA	502	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	mexY-R	CTCGAGGTTGAACGAGGGAT		
32	*oprM*-F	GGTAGCCCAGGACCAGAATG	520	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 61.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	oprM-R	GAGCTGGTAGTACTCGTCGC		
33	*triA*-F	GCCAGAGGCGCTTTTCATTC	532	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 59.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC,
	triA-R	CACGCAGCTCGGTATAGGAA		
34	*triB*-F	GGGACCAGCTTTCCTACACG	559	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 61.3ºC, 30 sec.

	triB-R	GGCTTGAGGCTGTTGACTCC		Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
35	*triC*-F	AAGCACAAGTCGGAGCAGAA	597	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58.3ºC, 30 sec. Step 4: 72ºC, 60.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	triC-R	GATATCGCCGATGTTCGGGT		
36	*opmH*-F	AAGGAAGCCGTCGACAACAA	448	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 58.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72ºC, 5 min. Step 7: 4ºC
	opmH-R	GTACGTCGGTCTTGTCGGAA		

2.1.6. The Kits used for diagnosis:

Table (2-6) includes a list of the molecular assay and diagnostic kits used in the current study.

Table (2-6): The materials and Diagnostic Kit Used in Molecular Study.

No.	Types of kits	Company/Country
1	DNA extraction kit	Favorgen/Taiwan
2	Master mix	Promega/USA
3	DNA ladder 100bp	
4	Primers of *muxA, muxB, muxC, opmB, mexA, mexB, oprM, mexX, mexY, mexC, mexD, oprJ, mexE, mexF, oprN, mexG, mexH, mexI, opmD, mexJ, mexK, mexM, mexN, mexP, mexQ, opmE, mexV, mexW, triA, triB, triC, opmH, Ps.spp*	Macrogen/Korea
5	DNase I enzyme kit DNase I enzyme 10x buffer Free nuclease water	Promega/ USA
6	TransScript® II Green One-Step qRT-PCR SuperMix (200 u) X Reaction Buffer (1 x 5) DTT (0.25 Mm dNTP (250) µM each RNase Inhibitor (1 u)	Bioneer /Korea
7	GENEzol TM TriRNA Pure kit	Geneaid /Taiwan

2.1.6.1 Content DNA extraction kit:

1 Ethanol alcohol (96-100%)
2 Proteinase K solution for precipitation proteins
3 Wash buffer, W1 buffer solution
4 FABG buffer
5 ddH_2O
6 Elution buffer
7 RNase solution.

2.1.6.2 Master Mix:

Table (2-7): Contents of Master Mix

No.	Materials
1	DNA polymerase enzyme (Taq)
2	dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm
3	MgCl2 (3mM)
4	Reaction buffer (pH 8.3)

Table (2-8): Contents of PCR Reaction Mixture

Contents of reaction mixture	Volume
Master Mix	12.5 µl
Template DNA	2 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
Nuclease free water	8.5µl
Total volume	25 µl

2.1.6.3 DNA Ladder:

Table (2-9): Composition of Ladder and Loading Dye

No.	Materials
1	Ladder consists of 11 double-stranded DNA with size 100- 1500bp
2	Loading Dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA)

Table (2-10) qRT-PCR Primers for Housekeeping Genes and their Sequence

Primers	Sequence 5'-3'	Amplicon
rpoS gene F	CTCCCCGGGCAACTCCAAAAG	198 bp
rpoS geneR	CGATCATCCGCTTCCGACCAG	

2.2 Methods

2.2.1. Sample Collection and Diagnosis

A total of 186 specimens were obtained from Marjan teaching hospital, Al-Hilla teaching and Babylon Hospital for Paediatrics in Babylon between December 2020 and march 2021. Seventy nine *Pseudomonas aeruginosa* isolates were obtained from seven different types of sample by patients (CSF, high vaginal swabs, blood stream infections, ear swabs, wound burn swabs, bronchoalveolar lavage, and midstream urine).

Isolation was carried out at both private and public hospitals, samples were labeled and transported to the University of Babylon Laboratory for processing and before starting this study. Ethics approval were achieved from University of Babylon/ College of Science, Ministry of Health, in addition to, the research ethics committees at hospital. All 79 isolates were screened on Pseudomonas chromogenic agar, which was employed as a selective medium for the isolation of *P. aeruginosa*. The plates incubated aerobically at 37 C for 24 hours, then the culture media were examined for the presence of *P.aeruginosa* growth and validated by PCR using specific primer pairs gene of Pseudomonas spp. as shown in table (2-11).

Table (2-11): Primer pair sequences and PCR conditions for the identification of *P. aeruginosa*.

Primer	Sequence (5′ to 3′)	Product (bp)	Annealing temp. (ºC)	Conditions
Ps.spp-F Ps.spp-R	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	56.0ºC	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 60.3ºC, 30 sec. Step 4: 72ºC, 50.0 sec. Step 5: Repeat steps 35 Step 6: 72ºC, 5 min. Step 7: 4ºC, forever

2.2.2. Preparation of Reagents and Solutions

2.2.2.1. Standard Solution No. 0.5 by McFarland:

McFarland turbidity standards are prepared by mixing various volumes of 1% sulfuric acid and 1% barium chloride to obtain solutions with specific optical densities. By adjusting the volume of these two chemical reagents.

McFarland standards of varying degrees of turbidity can be prepared which represent different bacterial density or cell count. 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension with a 1.5 x 10^8 colony forming units (CFU/ml).

2.2.2.2. TBE (Tris-Borate-EDTA) Buffer:

TBE was prepared and stored as a 10× stock solution. The 10× working solution was prepared by dissolving 108 g of Tris base, 55 g of boric acid, and 40 ml of 0.5 M EDTA in 1000 ml of D.W. However, final concentration of 1× TBE solution was prepared by adding 100 ml of 10× TBE buffer to 900 ml of sterile D.W.

2.2.3. Preparation of Culture Media

2.2.3.1. Brain Heart Infusion Broth:

According to the manufacturer's instructions, this medium was made. It was used to activate microorganisms and conduct general tests after suspending 37 g of the medium in one liter of distilled water (MacFaddin, 2000).

2.2.3.2. Agar Mueller-Hinton:

The Muller-Hinton agar medium was made according to the instructions provided by the manufacturer. It was employed in antimicrobial susceptibility testing after suspending 38g of the medium in one liter of distilled water (MacFaddin, 2000).

2.2.3.3. The Basic Principle of Chromogenic Agar:

The Pseudomonas chromogenic agar medium was prepared according to the manufacturer's instructions. It was used as a selective medium.

2.2.4. Antibiotic Susceptibility Test

2.2.4.1. Disc Diffusion Method:

Using the disc diffusion method by Kirby-Bauer traditional susceptibility tests of *P. aeruginosa* have been done to detect antibiotic resistance (Muller Hinton agar). Antimicrobial susceptibility testing was done with 13 different antibiotics using the disk diffusion method, including Pipracillin (100 µg)), Ceftazidime (30 µg), cefepime (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), Tobramycin (10 µg), Amikacin (30 µg), Netilmacin (30 µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Ofloxacin. After adjusting the inoculum to 0.5 Macfarland, as per the Clinical and Labrotary Standards Instituite 2021 (CLSI-2021), a table lists the antibiotics utilized in the style, diffusion of antibiotics (the Kirby-Bauer susceptibility test).

The 79 isolates used in this test was created by combining 5 ml of broth with growth from 5 isolated colonies grown on Pseudomonas chromogenic agar plates; the culture was then incubated for 24 hours at 37℃ to generate a mild turbidity 0.5 Macfarland bacterial suspension. An inoculum was obtained from the controlled culture using a sterile swab, which was then switched onto a Müeller–Hinton plate. The antibiotic discs were placed on the surface of the medium with flamed forceps at evenly spaced intervals, and incubation was normally done overnight at 37℃. Antibiotic inhibition zones were measured (from the CLSI 2021) to find the susceptibility of organisms to each antibiotic (Cockerill *et al* ., 2010).

2.2.4.2. MIC Determination by Microtitre Broth Dilution Method:

1. The test isolates was grown in the Mueller–Hinton broth to the right A_{600}. have antibiotics solutions and plates ready before the cultures reach the desired growth phase.

2. The antibiotics (Levofloxacin (LEV) and Piperacillin (PRL)) were weighted and dissolved, then diluted in the test Mueller–Hinton broth to 2x the top concentration desired and kept on ice until use.

3. Then 100 ml of Mueller–Hinton broth was dispensed by multipipettor into all wells of a microtitre plate and labeled the plate and lid.

4. About 100 ml of appropriate 2x antibiotic solutions was transferred into the wells in column 1 (far left of plate).

5. In column 1 antibiotic was mixed into the wells by sucking up and down 6-8 times using multipipettor.

6. To make column 2 a two fold dilution of column 1 about 100 ml was withdrawn from column 1 and added to column 2 then transferred 100 ml to column 3. The procedure was repeated down to column 10.

7. Then100 ml was discarded from column 10 rather than putting it in column.

8. the right A_{600} of bacteria was poured into a sterile petri dish.

9. Then about 5 ml of bacteria was dispensed into wells in columns 11 to 1 in that order by using smaller multipipettor, column 12 was stilled sterility control and blank for the plate scanner.

10. The plates were incubated at 37^{o}C and streaked the bacterial cultures on plates to check their purity

11. After growth is obtained (18-36) h the plates was scanned with an ELISA reader, column 12 was scanned as the blank.

12.MIC was taken as the lowest concentration of drug that reduces, by more than 50% or 90% for MIC_{50} or MIC_{90} respectively.

13. The procedure was repeated two times for desired antibiotics.

2.2.4.3. Using the Inhibitor Phenylalanine-Arginine Betanaphthylamide (PAbN):

Levofloxacin (LEV) and piperacillin's minimal inhibitory concentrations (MIC) were measured in order to assess the effects of utilizing an efflux pump inhibitor (EPI), phenylalanine-arginine-naphthylamide (PAbN).

The previous experiment was repeated in the same way except adding the inhibitor (PAbN) at a concentration of 50 mg/mL to the used culture medium (Mueller–Hinton broth) and recording the results.

2.2.5. Detection of Antibiotic Resistance Genes by Polymerase Chain Reaction (PCR):

A PCR assay was conducted as a confirmatory detection test. *P. aeruginosa* isolates resistance genes. This assay was accepted out in the manner designated by Weissensteiner *et al.*, (2003).

2.2.5.1. Genomic DNA Extraction:

The genomic DNA was extracted from *P. aeruginosa* isolates using FavorPrep™ Genomic DNA Kit.

Preparation of Specimens:

The following procedure was used to prepare an inoculum of selected bacteria at a density of up to 10^9, Gram-negative bacteria should be treated as follows:

a. 1.5 µl micro centrifuge tube was filled with up to 10^9 bacterial cells and spun at full speed for 1 minute (14,000 rpm). After that, the supernatant was discarded. The pellet was resuspended in 200 µl of FATG Buffer by vortexing or pipetting. After that, it was incubated at room temperature for 5 min.

b. To lyse the cells, 200 µl of FABG Buffer was added to the sample and vortexed for 5 seconds. The specimen lysate was then incubated at 70°C for 10 minutes, or until it was clear. The tube was inverted every 3 minutes during the incubation period.

c. Binding: The specimen was immersed in 200 ml of ethanol (96-100% concentration) for 10 seconds and vortexed. In a 200 µl collection tube, an FABG column was placed. The specimen mixture (including any precipitate) was carefully deposited on the FABG column and centrifuged for 5 min at full speed (14,000 rpm) before discarding the collection tube. In a new 2 mL collection tube, the FABG column was put in.

d. Washing: FABG Column was washed with 400 ml W1 Buffer. Then centrifuged for 1 min at full speed (14,000 rpm) and the flow-through was discarded. The FABG Column was placed back in the 2 mL collection tube. FABG column was washed with 600 ml wash buffer (ethanol added) and centrifuge for 1 min at full speed (14,000 rpm) and the flow-through was discarded. The FABG Column was placed back in the 2 mL collection tube, and centrifuge for an additional 3 min at full speed (14,000 rpm) to dry the column.

e. Elution: The dry FABG Column was placed to a new 1.5 mL microcentrifuge tube. 100 ml of preheated elution buffer were added. The FAGB column was incubated at 37 ºC for 10 min. After that, it was centrifuged for 1 min at full speed (14,000 rpm) to elute the DNA.

f. Pure DNA: The genomic DNA fragment was stored at -20 ºC until further analysis.

2.2.5.2. Primers Preparation:

The primers stock tube (100 pmol/µl) was prepared and then the working solution (10 pmol/µl) was prepared from primer stock tube according to the instruction provided by primer manufacturer (Macrogen/ Korea).

2.2.5.3. The Reaction Mixture:

Amplification of DNA was carried out in the final volume of 25µl reaction mixture.

Table (2-12) Contents of Reaction Mixture and its Volume

No	Contents of reaction mixture	Volume
1	master mix	12 µl
2	Upstream primer (10 pmol.µl)	1 µl
3	Downstream primer (10 pmol.µl)	1 µl
4	DNA template	2 µl
5	Nuclease free water	9 µl
Total volume		25 µl

2.2.5.4. Technique of Polymerase Chain Reaction (PCR):

Using specified primer pairs, conventional PCR was performed to amplify the target DNA. It consisted of three processes that were repeated for a certain number of cycles in order to obtain a PCR result (amplicon) that could then be detected after agarose gel electrophoresis.

2.2.5.5. Agarose Gel Electrophoresis:

After extraction, a number of 0.7 % gel was utilized to separate genomic DNA (5–10 kb), whereas 1.2 gels were employed to achieve good resolution for small fragments of PCR product (0.2-1 kb). However, 100ml of 1xTBE buffer was added to the specified weight of agarose, which was then heated in the microwave until the solution became clear. After cooling the agarose to 50–55°C, 5 µl of simply safe dye (10 mg/ml) were added to 100 ml of melting agarose gel to achieve the final concentration of 0.5 g/ml (Sambrook and Russel, 2006). The agarose was poured into the gel tray, sealed at the ends, and the comb was appropriately inserted before drying. The samples were loaded into their own wells on the gel, while the marker was loaded into one well. The electrodes were properly connected, and the run was carried out according to the gel percentage and size.

2.2.6.1. Total Ribonucleic Acid (RNA) Extraction:

Extraction of total RNA of *P. aeruginosa* cells were done by using (GENEzol ™ TriRNA Pure kit) according to instruction of manufacturing company as steps below:

Sample Lysis:

1. Bacteria cells (up to 1 x 10^9) was transfered to a 1.5 ml microcentrifuge tube (RNase-free).

2. The centrifugation was done at 12-16,000 x g for 2 minutes then remove the supernatant completely.
3. Lysis buffer (1 ml) was added to the microcentrifuge tube containing 10 mg of lysozyme.
4. Dissolving the lysozyme powder by vortex the tube until it is completely dissolved.
5. The volume of 100 µl of bacteria lysis buffer that containing lysozyme was added to the bacteria cell pellet.
6. Vortex the cell pellet to make a suspension.
7. The sample was incubated for 5 minutes at room temperature.
8. Incubate at room temperature for 5 minutes after adding 700 µl of GENEzol™ Reagent and thoroughly mixing.

RNA Binding:

1. The sample was centrifuged at 12–16,000 g for 1 min to remove cell debris before transferring the clear supernatant to a new 1.5 ml microcentrifuge tube (RNase-free).
2. In GENEzol™ Reagent, add 1 volume of absolute ethanol to 1 volume of sample mixture (1:1).
3. Place an RB Column in a 2 ml collection tube after thoroughly mixing by vortexing.
4. Transfer 700 µl of the sample mixture to the RB Column then centrifuge at 14-16,000 g for 1 min after that discard the flow-through.

5. Transfer the remaining sample mixture to the RB Column, then centrifuge for 1 min at 14-16,000 g, discard the flow-through, and place the RB Column in a new 2 ml collection tube.

RNA Wash:

1. Pre-Wash Buffer (400 µl) was added then centrifuge at 14-16,000 x g for 30 seconds.
2. Remove the flow-through and replace the RB Column in the 2 ml Collection Tube.
3. Wash Buffer (600 µl) was added to the RB Column.
4. The centrifuge was run at 14-16,000 x g for 30 seconds and then discarded. Return the RB Column to the 2 mL collection tube.
5. Wash Buffer (600 µl) was added to the RB Column.
6. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through.
7. Place the RB Column back in the 2 ml Collection Tube.

RNA Elution:

1. RB Column was put in a clean 1.5 ml microcentrifuge tube (RNase-free).
2. RNase-free Watere (25-50 µl) was added into the CENTER of the column matrix.
3. At least 3 minutes of waiting time to ensure the RNase-free water is completely absorbed by the matrix.

4. Centrifugation was done at 14-16,000 x g for 1 minute to gain the purified RNA.

2.2.6.2. Gene Expression Study for *OprM* Gene By qRT-PCR:

Quantitative reverse transcriptase Real-Time PCR was performed for measurement of relative quantification (gene expression analysis). The experiment was designed to compare the gene expression between two groups of isolates belong to *P. aeruginosa* bacteria, the first group represents resistant isolates (nine MDR strains) and the second group represents susceptible isolates (five strains considered as control group) showed resistance to 13 antibiotics used in previous experiment according to CLSI 2021, both groups contain the same gene according to PCR result.

In this experiment screened gene expression of *oprM* gene that represented the outer membrane for five efflux pumps in RND family (*mexABoprM, mexMNoprM, mexVWoprM, mexJKoprM, mexXYoprM*) of *P. aeruginosa* cells (14 isolates) grown overnight in brain-heart infusion broth at 37°C were sub cultured in the same medium and incubated at 37°C until cultures reached an optical density at 600 nm (OD600) of 0.6–0.8.

Total RNA was isolated, purified and reverse transcribed into cDNA as described previously. Where specified, the antimicrobials piperacillin (PAP; 1024 μg/ml) and levofloxacin (LEV; 16 μg/ml) were added at their respective MICs 30 minutes prior to harvesting cells. The primers used in quantitative real-time PCR (qPCR) were designed to amplify gene fragments with lengths of 520 bp (*oprM*; Forward: GGTAGCCCAGGACCAGAATG; Reverse: GAGCTGGTAGTACTCGTCGC), housekeeping gene 198 bp (rpoS; Forward: CTCCCCGGGCAACTCCAAAAG;

Reverse: CGATCATCCGCTTCCGACCAG). The rpoS reference gene was amplified as described previously. The qRT-PCR reaction mixtures, amplification parameters, and melt curve analyses were performed as previously described. The expression levels of oprM was normalized to that of the reference gene rpoS using the ΔΔC(t) method and $2^{-\Delta\Delta C}$ (fold change).

2.2.6.3. Quantitative Real-Time PCR (qPCR):

Relative quantification by Real-Time PCR was performed for determination of gene expression (mRNA transcript levels) of *oprM* gene for tested bacteria that normalization by *rpoS* (housekeeping gene). This method was carried out by *TransScript*® II Green One-Step qRT-PCR SuperMix for the genes and the components' reaction and their volumes as in Table (3.16)

Table 2-9: master mix component used to prepare qRT-PCR reaction

Component (Concentration)	**Volume**
RNA Template	1µl
Forward primer (10 pmol/µl)	2µl
Reverse primer (10 pmol/µl)	2µl
2x perfect star ™ Green One-Step qRT-PCR SuperMix	10µl
TransScript® Green One-Step qRT-PCR SuperMix	0.4µl
RNase-Free Water	6.5µl

2.2.6.4. qPCR protocol:

Relative quantification by Real-Time PCR was performed for determination of gene expression (mRNA transcript levels) of MDR and non MDR genes for tested bacteria that normalization by housekeeping gene. This method was carried out by qRT-PCR SuperMix for bacterial genes, and housekeeping genes and the components' reaction and their volumes.

After that, these qPCR master mix components mentioned above qPCR Supermixed standard plate tubes that contain the SYBR Green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 min, then placed in QLAGEN Real-Time PCR system: After that, the qPCR plate was loaded with and the following thermocycler condition.

Table 2-10: The program used in the qPCR for *oprM* gene and *rpoS* (housekeeping gene) (Mitchell *et al.*, 2008).

Step	Temperature (°C)	Time	Repeat cycle
Denaturation	95	10 sec	40 cycle
Annealing	55	20 sec	
Extension	72	40 sec	
Final extension	60-95	5 sec	
Hold 1	45	10 min	
Hold 2	94	30 sec	

2.2.6.5. Data Analysis of qRT-PCR:

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) (The ΔCt Method Using a reference gene) described by Livak and Schmittgen (2001) as the

following equation 3.1 ΔCt (contorl) = Ct (ref,control) – Ct (target,control) 3.1 Where Ct is the cycle threshold, control means the control measured Ct, ref refers to reference gene measured Ct. $2^{-\Delta\Delta C}$ (fold change).

Statistical analysis of the results by using computer program (SPSS), Version 23 one-way analysis of variance (ANOVA), the difference was considered significant at ($P \leq 0.05$) (Zar, 1984).

Chapter Three
Results & Discussion

3. Results and Discussion

3.1: Distribution of *P. aeruginosa*:

The distribution of *P. aeruginosa* isolates among collected samples types illustrated in Table (3-1). The results of isolation of *P. aeruginosa* revealed high percentage of *P. aeruginosa* 79 isolates distributed as urinary tract infections (UTIs) patients 35.4% (28 isolates), lower respiratory tract infection (RTIs) patients 29.1% (23 isolates), wounds and Burn infection 18.9% (15 isolates), 8.8% (7 isolates) for otitis media, 2.5% (2 isolates) for Bacteremia, 3.7% (3 isolates) for bacterial vaginitis and 1.2% (1 isolate) for meningitis as showed in (table 3-1).

Table (3-1): Distribution of *P. aeruginosa* isolates among Diseases.

Infection type	Specimen	Sample n=186	*P. aeruginosa*	
			n=79	%
Urinary tract infections	Midstream Urine	75	28	35.4%
Respiratory tract infections	Broncoalveolar Lavage	47	23	29.1%
Wound and burn infections	Wound and Burn Swab	22	15	18.9%
Otitis Media	Ear Swab	15	7	8.8%
Bacteremia	Blood Stream	5	2	2.5%
Vaginitis	High Vaginal Swab	17	3	3.7%
Meningitis	Cerebrospinal Fluid	5	1	1.2%
Total		**186**	**79**	**100%**

P. aeruginosa stands as a relevant pathogen, with a high prevalence at hospitals, particularly in intensive care units, due to its intrinsic resistance to numerous antibiotics and antiseptics, the capacity to develop more resistance mechanisms to various classes of antibiotics, and its ability to persist in damp settings.

Endocarditis, septicemia, urinary tract infections, cystitis, pneumonia, and surgical wound infections are all examples of life-threatening infections implicated by *P. aeruginosa* (Diggle and Whiteley, 2020). *P. aeruginosa* responsible for 10-15% of the nosocomial infections worldwide, often these infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents (Labovská, 2021).

The data from previous studies showed that the most prevalent infection in patients hospitalized to the intensive care unit (ICU) was respiratory tract infections (RTIs) caused by *P. aeruginosa* (Bhatta *et al.*, 2019). Another study conducted by Kamali *et al*,. (2020) founded that the most isolates of *P. aeruginosa* 36.25% came from endotracheal secretions, followed by urine (32.5%), blood (13.75%), wound (10%), CSF (5%), and ear (5%). Results of study conducted by (Motbainor *et al.*,2020) found that, *P. aeruginosa* constitute (8.9%), (8.3%), and (6.3%) in the bloodstream, urinary tract, and surgical site infections respectively. The results of present study was in accordance with results of Iranian study which found that *P. aeruginosa* recovery percentage from high to low: UTIs, RTIs, wound and burn infections, Bacteremia, and meningitides (Mirzaei *et al.*, 2020)

3.2. *P. aeruginosa* Identification:

Pseudomonas chromogenic agar was used to as screening medium for *P. aeruginosa*, and the results were confirmed by PCR using *P. aeruginosa* species-specific primers (Figure 3-1). The results revealed that 79 isolates were *P. aeruginosa* using a combination of chromogenic agar and PCR techniques.

Many studies found that the chromogenic agar for *P. aeruginosa* is promising medium for direct isolation and identification with high sensitivity and specificity (Perry, 2017). This media are based on their enzyme activity and majority of chromogenic media are therefore both selective and differential, accommodating the inhibition of non-target organisms while enabling target pathogens to grow as colored colonies (Momin *et al.*, 2017). It is an efficient method to simultaneously isolate and recognize *P. aeruginosa* from burn infections (Al-Dahmoshi *et al.*, 2018). In contrast with the use of conventional culture media, this may contribute to quicker confirmation of pathogens and reduce the overall time required (Sivri *et al.*, 2014). Other study stated that chromogenic medium is promising medium allowing for the isolation and simultaneous identification of *P. aeruginosa* (Căpățînă *et al.*, 2022). The gene sequencing species specific amplification were a useful methods for bacterial classification, in which the nucleotide sequences of the this region are determined and compared with sequences available from databases figure (3-1) to yield homology matches, thereby allowing bacterial identification of the target samples (Al-Thabhawee and Al-Dahmoshi., 2022).

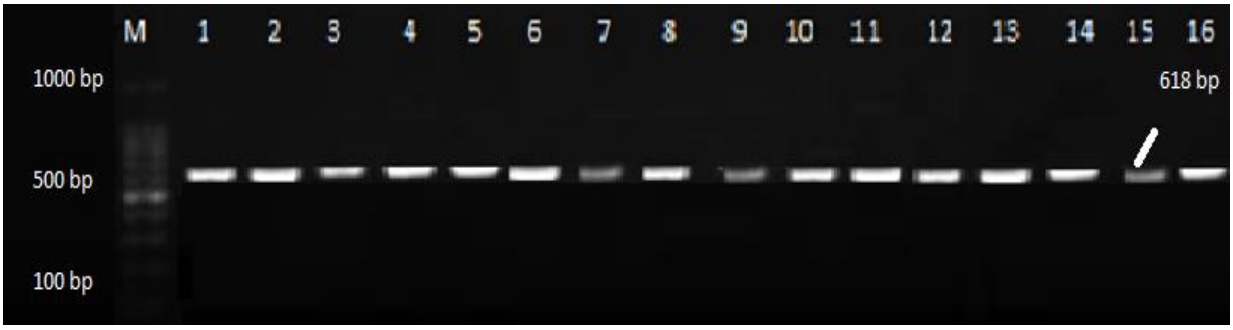
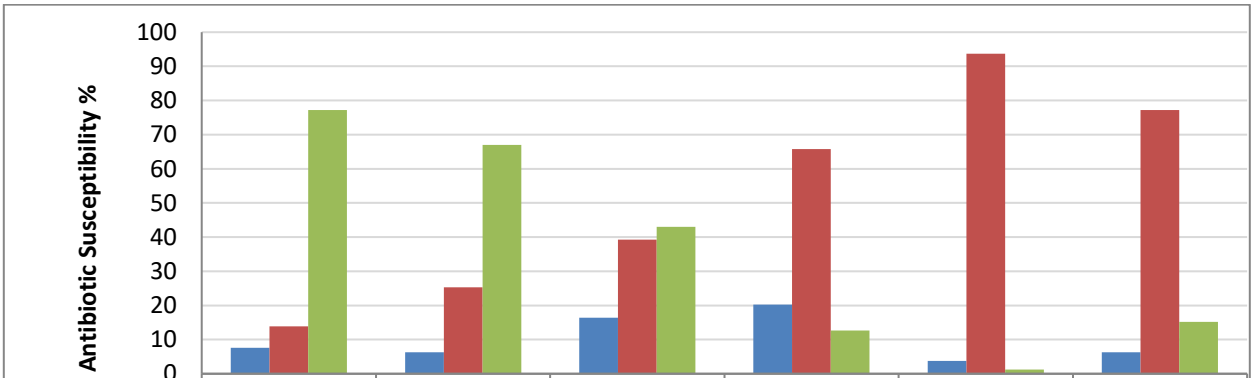


Figure (3-1): 1.5% Agarose gel electrophoresis of Ps.spp. for *P. aeruginosa* amplicon (618 bp). M represents the 100 bp DNA ladder, lanes 1-16 represent the isolates, TBE 1x, at a voltage of 110 volts for 50 min.

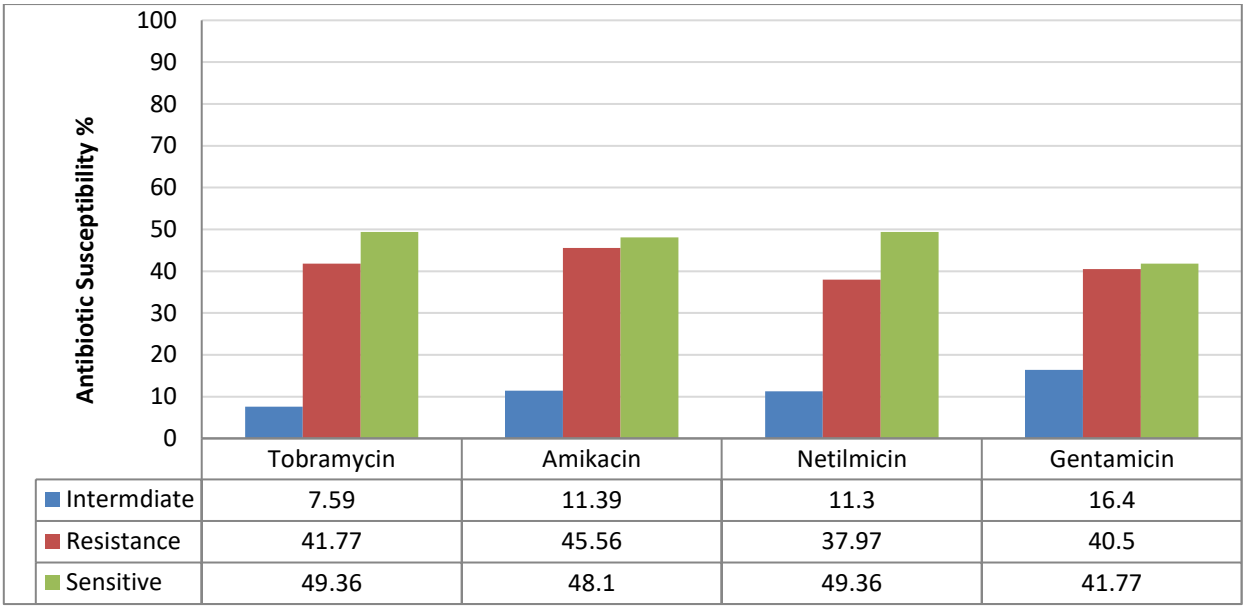
3.3. Antibiotic susceptibility:

In the present study, including the use of 13 antibiotics for antibiotic susceptibility testing (AST) depending on CLSI, (2021), the resistance values of *P. aeruginosa* isolates towards ceftazidime (CAZ), cefepime (FEP) were recorded at 93.6% and 77.2% respectively, while 65% represented piperacillin (PRL), and 40.5% represented gentamycin (CN). Ciprofloxacin resistance (CIP) was detected in just 37.9% of isolates. On the other hand, 41.7% was recorded for tobramycin (TOB), 39.2% for aztreonam (ATM), 45.5% for amikacin (AK), 43% for ofloxacine (OFX) and levofloxacin (LEV), 37.9% for netilmicine (NET), 13.9% for imipenem (IPM) and 25.3% for meropenem (MEM). In contrast, high sensitive values appeared toward IPM and MEM with 77.2%, 67 % respectively, as shown in Figures (from 3-2 to 3-4).



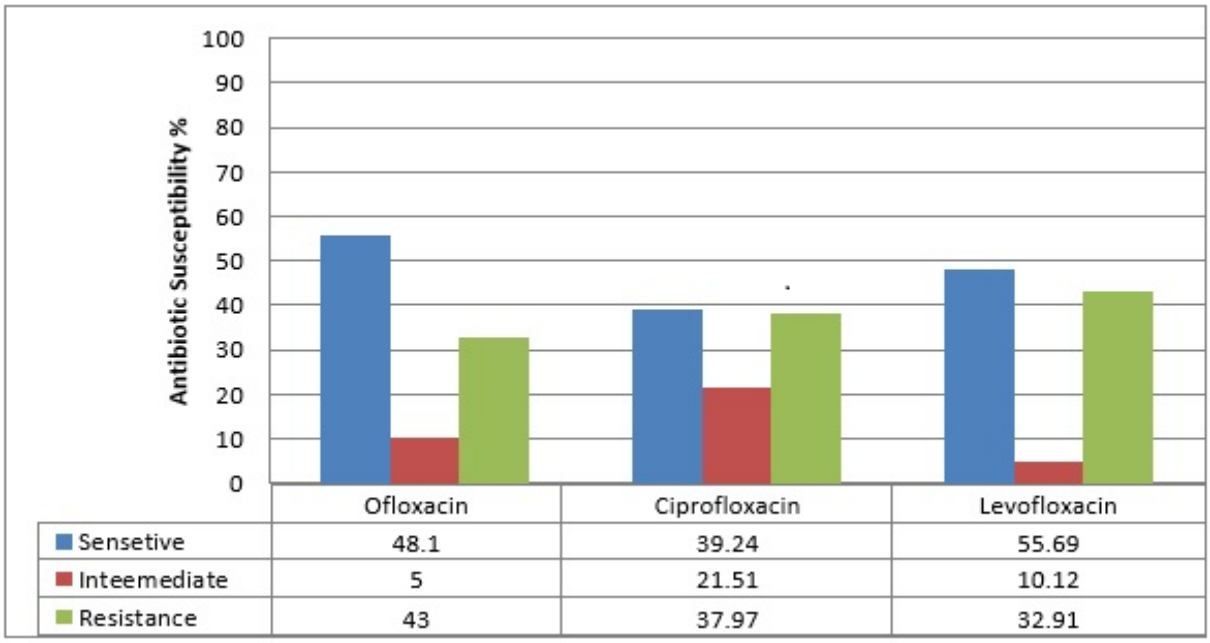
	Imipenem	Meropenem	Aztreonam	Piperacillin	Ceftazidime	Cefepime
Intermdiate	7.59	6.32	16.45	20.25	3.79	6.32
Resistance	13.92	25.31	39.24	65.82	93.67	77.21
Sensitive	77.2	67	43	12.65	1.26	15.18

Figure (3-2): Antibiotic susceptibility of of *P. aeruginosa* to β-lactams (piperacillin (PRL), ceftazidime (CAZ), cefepime (FEP), Aztreonam (ATM), imipenem (IPM) and meropene (MEM)).



	Tobramycin	Amikacin	Netilmicin	Gentamicin
Intermdiate	7.59	11.39	11.3	16.4
Resistance	41.77	45.56	37.97	40.5
Sensitive	49.36	48.1	49.36	41.77

Figure (3-3): Antibiotic susceptibility of of *P. aeruginosa* for Aminoglycosides (Tobramycin (TOB), Amikacin (AK), Netilmicin (NET) and Gentamicin (CN)).



	Ofloxacin	Ciprofloxacin	Levofloxacin
Sensetive	48.1	39.24	55.69
Inteemediate	5	21.51	10.12
Resistance	43	37.97	32.91

Figure (3-4): Antibiotic susceptibility of *P. aeruginosa* for Quinolones (levofloxacin (LEV), ciprofloxacin (CIP), ofloxacin (OFX)).

The present results revealed high percentages of resistance to the B-etalactams Ceftazidime, Cefepime, and Piperacillin. β-lactamases considered an

innate resistance mechanism, lead to disabling β-lactam activity (Tannous *et al.*, 2020; Al Muqati *et al.*, 2021).

The activation of β-lactamase enzymes, which represents one of the fundamental processes leading to bacterial resistance, causes high levels of resistance to β-lactam antibiotics such as ceftazidime (CAZ), cefepime (FEP), and piperacillin (PRL) (Alhusseini *et al.*, 2019). Resistance mechanisms, such as β-lactams, fluoroquinolones, and aminoglycosides, significantly impair the clinical efficacy of these drugs (Foudraine *et al.*, 2021).

For fluoroquinolones antibiotics ciprofloxacin result is compatible with the data reported in a previous study 23.9% of *P. aeruginosa* isolates were resistant to ciprofloxacin (Coetzee *et al.*, 2013) but disagrees with another study in which the results showed 61.3% resistance (Othman *et al.*, 2014). Levofloxacin 32.9% rate was close to the results of the study with 30.6% and 36.1%, respectively (Al-Derzi, 2012; Lila *et al.*, 2017), but disagrees with the study of 60.19% (Bassetti *et al.*, 2018).

P. aeruginosa is resistant to a variety of antibiotics, including aminoglycosides, quinolones and β-lactam classes. Innate resistance can be generated by a variety of factors, including low outer membrane permeability, overexpression of pumps, and the production of enzymes that inactivate drugs.

The other type of acquired resistance was caused by either horizontal transfer of genes or the change that happened by mutations, and the third type of adaptive resistance included the production of a layer of biofilm that acts as a diffusion barrier to reduce antibiotic entrance inside bacteria (Mulcahy *et al.*, 2010; Breidenstein *et al.*, 2011). This class of antibiotics has an important place and is widely used compared to other classes (Konaklieva, 2014). The current results showed high resistance of *P. aeruginosa* to this class, and that belonged to the high

frequency and random use of these antibiotics as a result, leading to the appearance of MDR new bacterial strains (Ali *et al.*, 2015).

3.4. Antibiotic Resistance Phenotypes:

The results revealed that 59% of current isolates were MDR and 41% were non-MDR and none was XDR or PDR as shown in (figure 3-5).

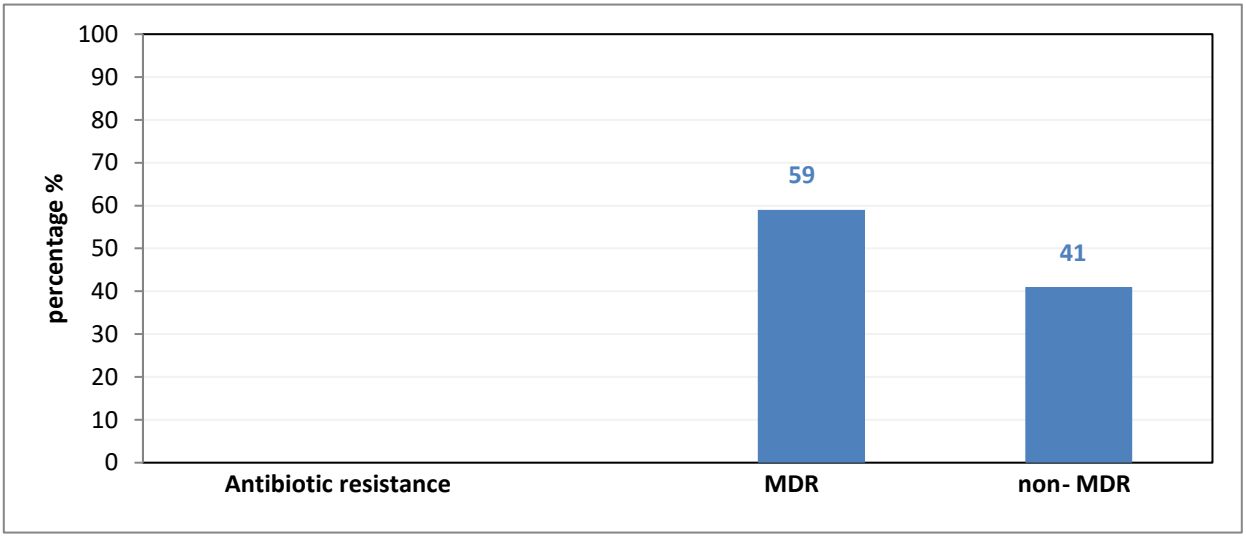


Figure (3-5): Antibiotic resistance patterns distribution among *P. aeruginosa* isolates.

The emergence of MDR is considered as a major public health concern (Driscoll *et al.*, 2007). The prevalence of antibiotic-resistant *P. aeruginosa* should be closely monitored, particularly in patients admitted to the ICU with RTIs because of the high genetic variability and resistance patterns, such as the predominant of *P. aeruginosa* isolates, 46.8% were resistant to amikacin (Yung *et al.*, 2021). From a previous study, MDR isolates were shown to be more prevalent in the burns unit at 54.8%, followed by isolates in surgical wards at 31.7%. *P. aeruginosa* is the most common pathogen responsible for burn infections, and it is also a prominent colonizer of burn wounds due to the moist surfaces of burn

wounds providing a favorable environment for its growth, as well as its capacity to survive in hospital settings (Tsutsui *et al.*, 2011).

Numerous *P. aeruginosa* isolates in this investigation displayed MDR for the antibiotics utilized in the Kirby-Bauer disc diffusion method. Antibiotic-resistant bacteria are becoming more common in community settings due to a number of factors. The misuse of antibiotics in general medications, which allows the selection and dissemination of drug-resistant strains, is the most significant contributor to the rise in resistance (Gawad *et al.*, 2018).

Our findings revealed a significant occurrence of MDR among isolated strains. This result was consistent with the previous studies with 32% and 36% of isolates being MDR (Rehman *et al.*, 2019; Mirzaei *et al.*, 2020), and another study, 69% of *P. aeruginosa* isolates were MDR. The Multi Drug Resistance (MDR) phenotype of *P. aeruginosa* is a key source of concern.

In addition to traditional drug resistance mechanisms, *P. aeruginosa* can develop resistance to antibiotics as the infection progresses, efflux pumps in the Resistance-Nodulation-cell Division (RND) family are able to translocate various compounds (including antibiotics) out of the bacterial cell in an atypical manner, boosting bacteria's resistance to a wide range of therapies (Nikaido, 2018).

3.5. Molecular Characterization of Efflux Pump Coding Genes in *P. aeruginosa* Isolates:

The results of molecular detection of efflux pump profiles in *P. aeruginosa* isolates confirmed the presence of efflux pumps that belong to the RND family through isolates with different values of genes that make up the pumps, as shown in table (3-2).

Table (3-2): Prevalence study of efflux pumps profile in *P. aeruginosa* isolates.

No.	Efflux pumps	Gene	%
1	MexAB-OprM	*mexA*	82.27%
		mexB	63.29%
		oprM	48.1%
2	MexXY-OprM	mexX	41.77%
		mexY	50.63%
		oprM	48.1%
3	MexPQ-OpmE	*mexP*	36.70%
		mexQ	46.83%
		opmE	50.63%
	MuxABC-OpmB	*muxA*	59.49%
		muxB	39.24%
		muxC	48.1%
		opmB	62.02%
5	MexCD-OprJ	*mexC*	41.77%
		mexD	48.1%
		oprJ	81.01%
6	MexGHI-OpmD	*mexG*	68.35%
		mexH	49.36%
		mexI	37.97%
		opmD	20.25%
7	MexJK-OprM	*mexJ*	37.97%
		mexK	34.17%
		oprM	48.1%
8	MexMN-OprM	*mexM*	69.62%
		mexN	50.63%
		oprM	48.1%
9	MexVW-OprM	*mexV*	92.40%

		mexW	91.13%
		oprM	48.1%
10	TriABC-OpmH	*triA*	55.69%
		triB	16.45%
		triC	50.63%
		opmH	49.36
11	MexEF-OprN	*mexE*	41.77%
		mexF	39.24%

The present study showed that RND efflux pumps are widely distributed in our isolates of *P. aeruginosa* isolates. The most common efflux pump genes in both MDR and non-MDR isolates are *mexV*, *mexW*, *mexA,* and *oprM*, whereas the out-membrane protein encoded gene *OprJ* is the one that our isolates are most likely to contain, while other genes are present in varying proportions in our isolates.

According to Li *et al*. (2003), it seemed that the *mexV* and *mexW* genes were silent or expression was weak in wild-type *P. aeruginosa*. Since 2003, research has not attached any importance independently from the pump's *mexV* and *mexW* genes due to their weak gene expression in the wild type, whereas our results showed a high presence of the encoded *mexV* and *mexW* genes, which may explain the extent of development in antibiotic resistance, as the high presence of these genes may give a greater chance for the development of acquired resistance in bacteria through other mechanisms (Colque *et al.*,2020; Tang *et al.*,2021 and Langendonk *et al.*,2021).

On the other hand, the highest incidence of the *oprJ* gene was 81.1% among the isolates, according to the table (3-2), as this efflux pump is known to extrude cefepime (Gomis *et al.*, 2021), and this is consistent with our results, which

showed a high rate of resistance to cefepime of 77.21%, especially in UTI patients (35.4%). However, MexCD-OprJ is often silent in *P. aeruginosa* wild-type isolates that possess the pump (Shigemura *et al*., 2015). This reveals how far antibiotic resistance has progressed in time and demonstrates how to turn on pumps that were previously inactive, and this is supported by the earlier studies that have demonstrated that acquisition of antibiotic resistance due to constitutive overexpression of efflux pump mexCD-oprJ correlates with a decrease in the production of several virulence factors (Sanchez *et al*., 2002; Linares *et al*., 2005; Jeannot *et al*., 2008; Stickland *et al*., 2010).

Also, previous clinical reports showed that the mechanism contributing to resistance in *P. aeruginosa* involved the expression of efflux pump genes, one of them being the overexpression of MexCD-OprJ. These results provided confirmation of our results (Terzi *et al.*, 2014; Shigemura *et al.*, 2015; Zhao *et al.*, 2020). On the other hand, the special importance within the pump MexAB-OprM for its constitutive expression and attribution of resistance to most antibiotics, and this is consistent with the study (Aguilar *et al.*, 2022), these studies proved the impact of efflux MexAB-OprM genes on increasing resistance to antibiotics and found that the high rate of resistance to β-lactams may confer and/or develop resistance among different classes of antibiotics. Other studies confirmed our results, which clarified that MexAB-OprM efflux pump is kept at basal levels by constitutive expression stimulating the overexpression of the MexAB-OprM efflux pump, which led to a rise in bacterial resistance (Terzi *et al.*, 2014; Suresh *et al.*, 2018).

The development and progression of clinical antibiotic resistance, bacterial pathogenicity, virulence, and biofilm maturation in Gram-negative bacteria,

particularly *P. aeruginosa*, are all significantly influenced by the RND efflux systems (Al Rashed *et al.,* 2020). Most of *P. aeruginosa* isolates exhibited multidrug resistance to two or three tested antibiotic classes. These antibiotics and other chemicals that are frequently substrates of pumps relevant for drug resistance, including as bile salts, fatty acids, and ethanol, produce multiple cellular stressors that MDR pumps respond to either as a preexisting mechanism or an active resource, part of these multidrug cross-resistances among *P. aeruginosa* are caused by overexpression of multidrug efflux pumps. Ultimately, improper use of antibiotics could cause resistance to other classes by triggering the overexpression of efflux pumps (Talebi *et al.,* 2016).

The current findings revealed that isolates with a prevalence of one or more efflux pump genes were resistant to different classes of antibiotics this finding is consistent with a previous study that found high rates of resistance with overexpressed of efflux pump in *P. aeruginosa* isolates from ICU and non-ICU patients' bloodstreams (Vitkauskienė *et al.,* 2010), as the same finding in another study found that 85.4 % of multi drug resistant *P. aeruginosa* isolates with present of efflux pump genes belong to RND family were resistant to β-lactams antibiotics (Ugwuanyi *et al.,* 2021).

A PCR assay for coding genes was done by conventional PCR for all isolates (79 isolates). The pump mexAB-oprM with MexA, mexB, and oprM genes were detected in 82.27% (65 isolates), 63.29% (50 isolates), and 48.1% (38 isolates) of the samples, respectively, as shown in (Figures 3-6 to 3-8).

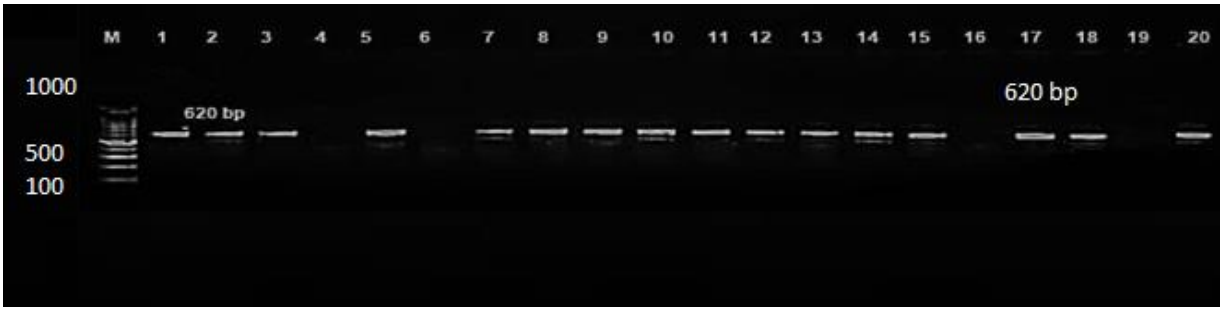


Figure (3-6): 1.5% Agarose gel electrophoresis of *mexA* gene amplicon (620 bp). M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

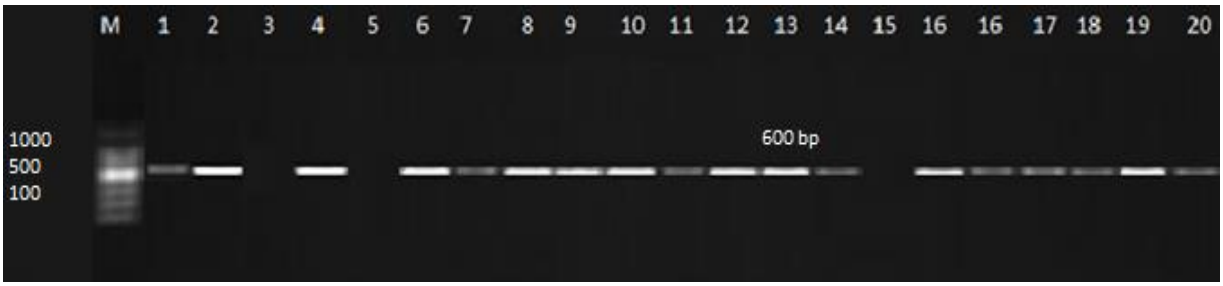


Figure (3-7): 1.5% Agarose gel electrophoresis of *mexB* gene amplicon (600 bp). M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

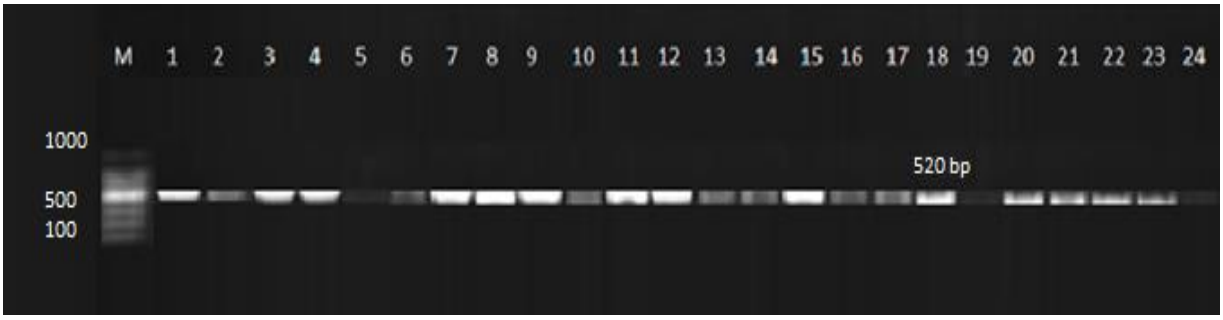


Figure (3-8): 1.5% Agarose gel electrophoresis of oprM gene amplicon (520 bp). M represent 100bp DNA ladder, lane 1-24 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

As shown in (Figures 3-9 and 3-10), the pump mexXY-oprM revealed mexX 41.77% (33 isolates), mexY 50.63% (40 isolates), and oprM 48.1% (38 isolates).

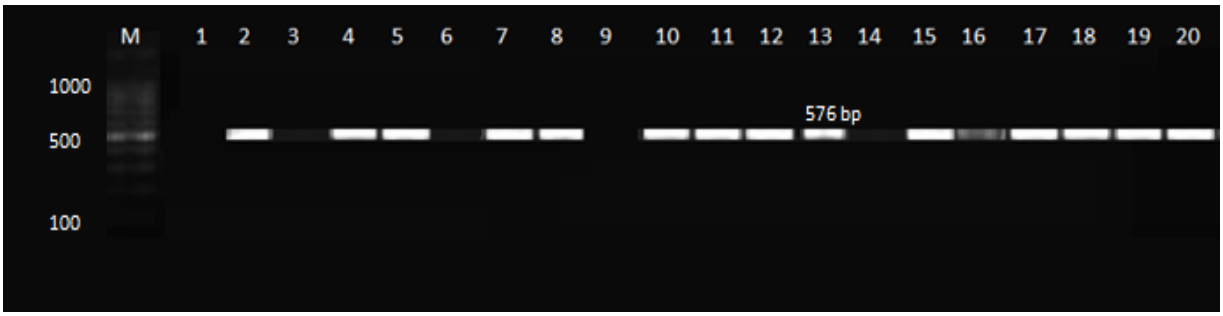


Figure (3-9):1.5% Agarose gel electrophoresis of *mexX* gene amplicon (576 bp). M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

Figure (3-10):1.5% agarose gel electrophoresis of *mexY* gene amplicon (502bp). M represent 100bp DNA ladder, lane 1-33 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

The molecular detection of the mexPQ-opmE pump revealed mexP 36.70% (29 isolates), mexQ 46.83% (37 isolates) and opmE 50.63% (40 isolates) (Figure 3-11 to 3-13).

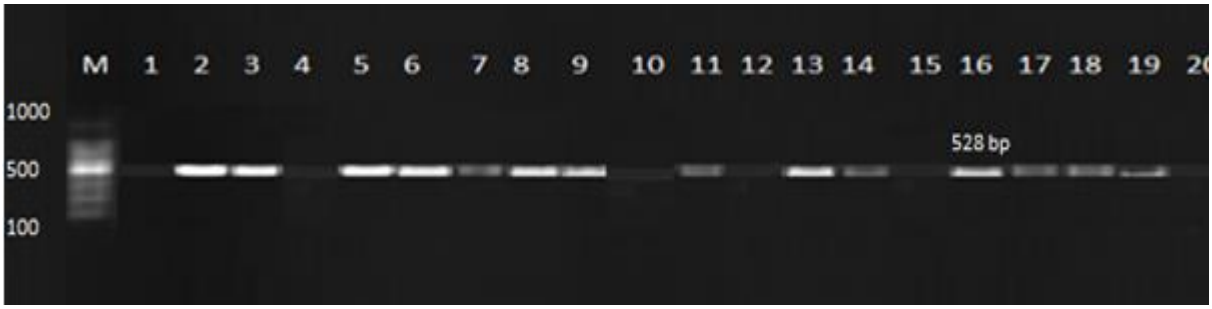


Figure (3-11):1.5% agarose gel electrophoresis of mexP gene amplicon (528 bp). M represent 100bp DNA ladder, lane 34-60 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

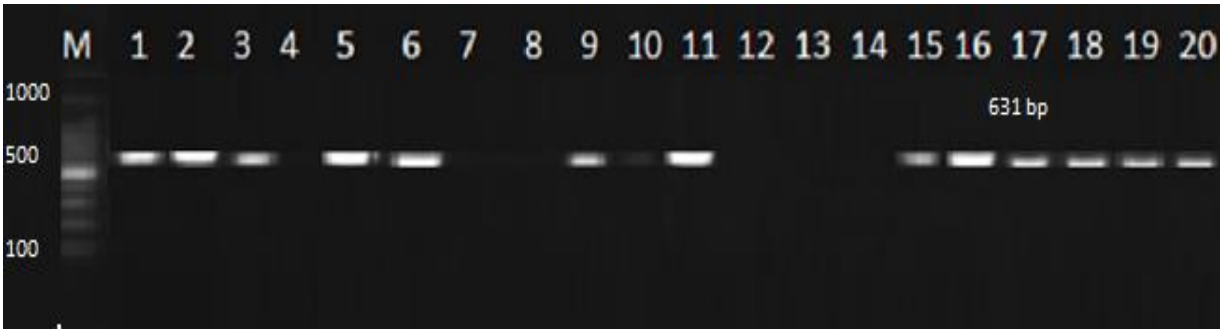


Figure (3-12): 1.5% agarose gel electrophoresis of *mexQ* gene amplicon (631 bp). M represent 100bp DNA ladder, lane 1-28 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

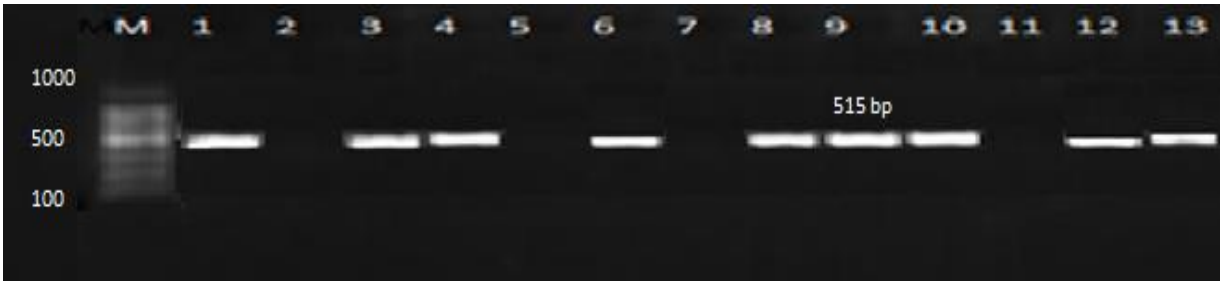


Figure (3-13):1.5% agarose gel electrophoresis of *opmE* gene amplicon (515bp). M represent 100bp DNA ladder, lane 1-13 represent the isolates, TBE 1x, at Voltage 110 volt for 50 min.

Figures from (3-14 to 3-17). PCR assay for *muxABC-opmB* genes with 59.49% (47 isolates) for muxA followed by 37.97% (30 isolates) for muxB, muxC 48.1% (38 isolates) and 62.02% (49 isolates) for opmB gene.

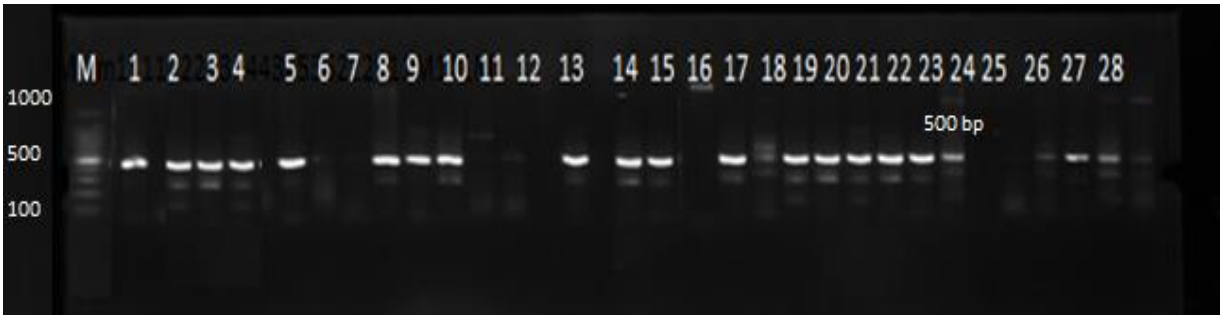


Figure (3-14): 1.5% agarose gel electrophoresis of *muxA* amplicon (500bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-28 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

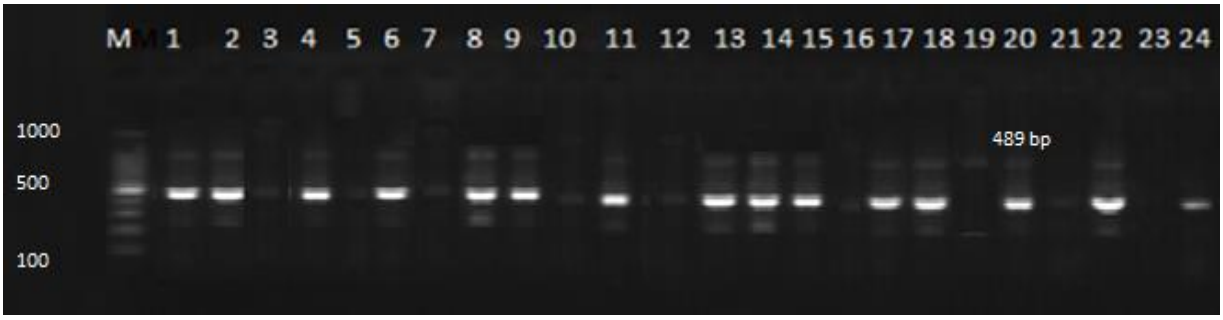


Figure (3-15): 1.5% agarose gel electrophoresis of *muxB* amplicon (489 bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-24 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

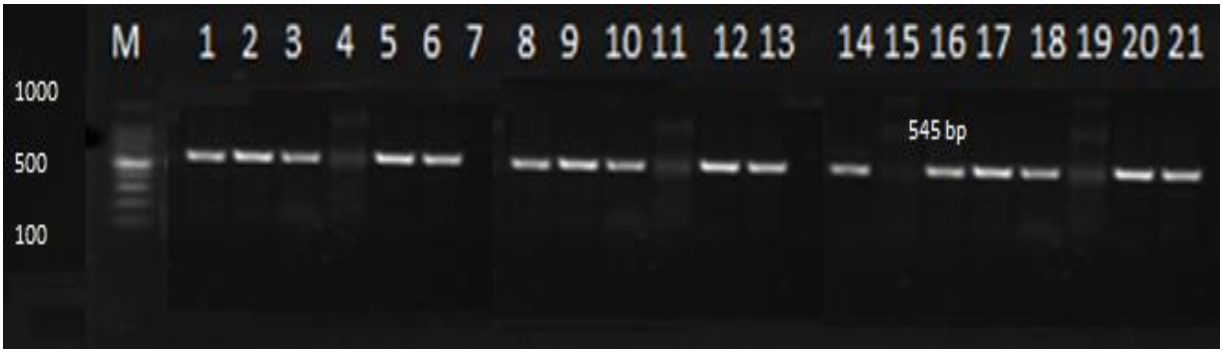


Figure (3-16): 1.5% agarose gel electrophoresis of *muxC* amplicon (545bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-79 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

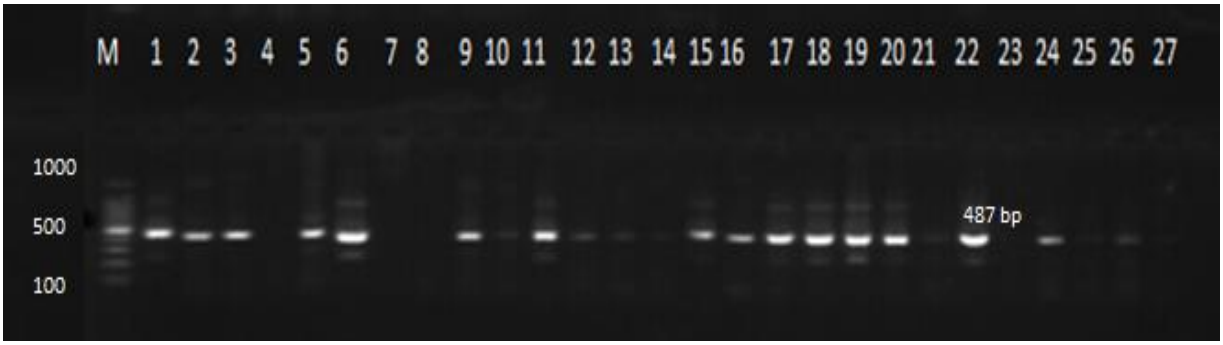


Figure (3-17): 1.5% agarose gel electrophoresis of *opmB* amplicon (487bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-27 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

The molecular detection of *mexCD-oprJ* efflux revealed *mexC* 41.77% (33 isolates), *mexD* 48.1% (38 isolates) and *oprJ* 81.01% (64 isolates) (Figure 3-18 to 3-20).

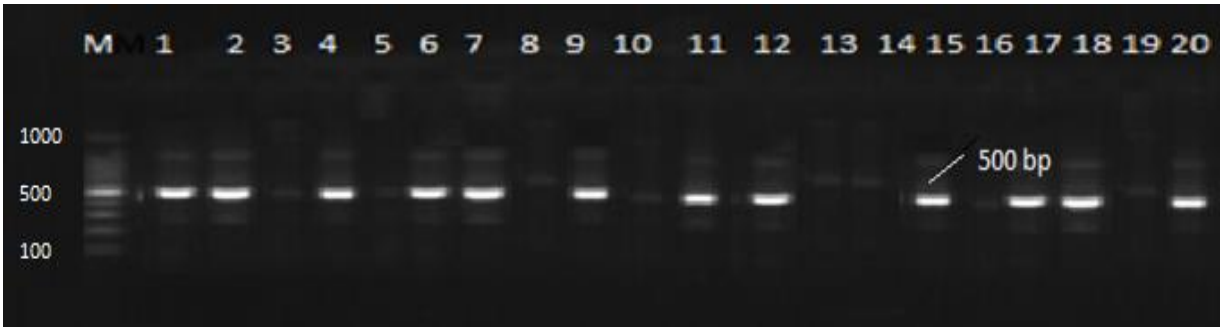


Figure (3-18): 1.5% agarose gel electrophoresis of *mexC* amplicon (500bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

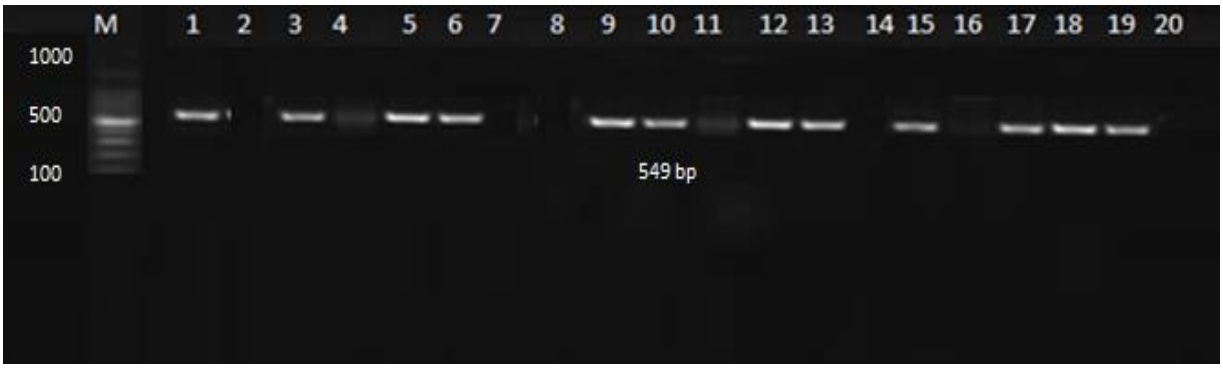


Figure (3-19): 1.5% agarose gel electrophoresis of *mexD* amplicon (549bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-79 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

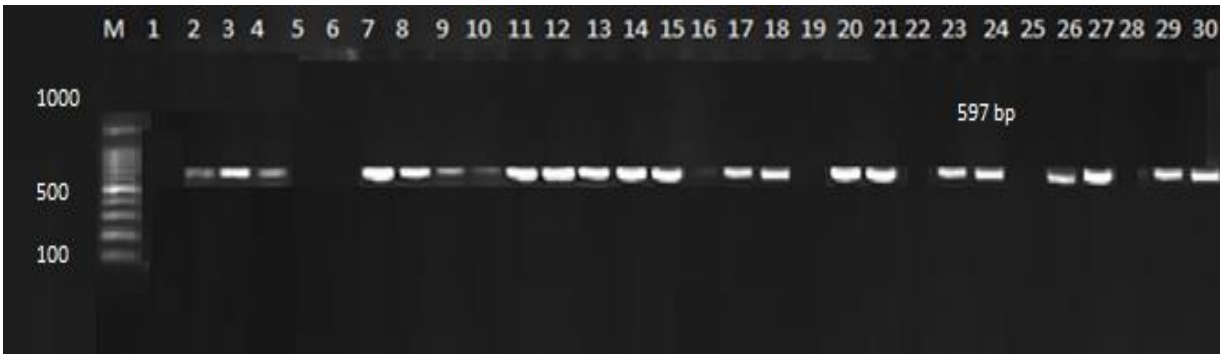


Figure (3-20): 1.5% agarose gel electrophoresis of *oprJ* amplicon (597bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-30 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

PCR assay for *mexGHI-opmD* genes with 68.35% (54 isolates) for mexG followed by 49.36% (39 isolates) for mexH, 37.97% (30 isolates) for mexI and 20.25% (16 isolates) for the opmD gene (Figure 3-21 to 3-24).

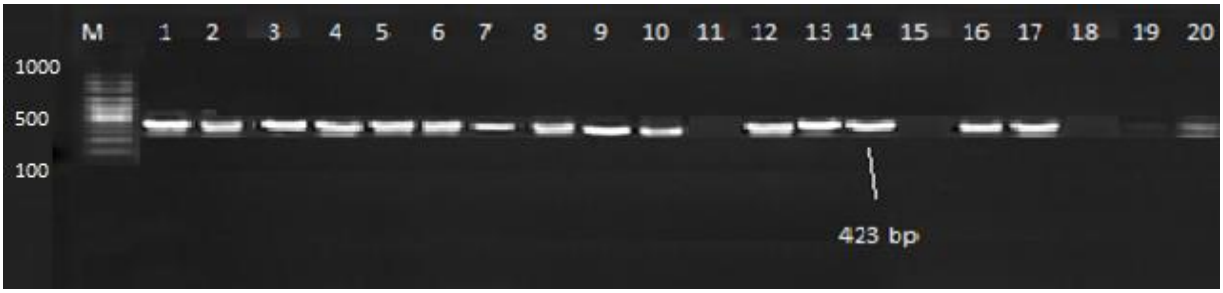


Figure (3-21): 1.5% agarose gel electrophoresis of *mexG* amplicon (423bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

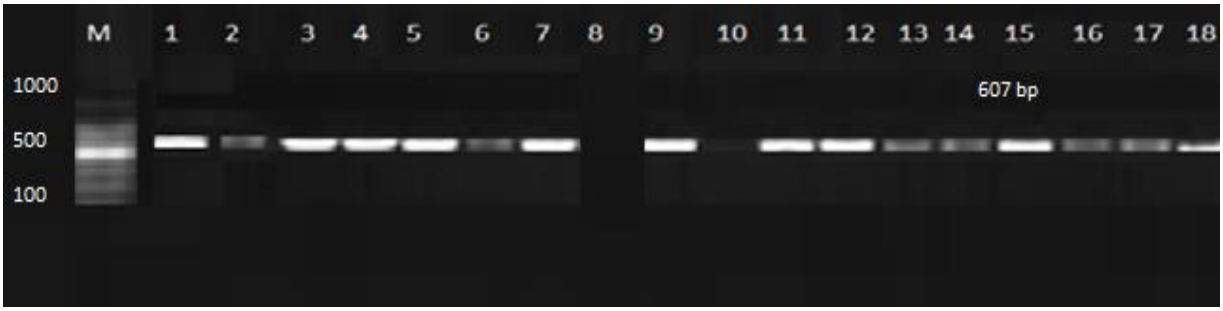


Figure (3-22): 1.5% agarose gel electrophoresis of *mexH* amplicon (607bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

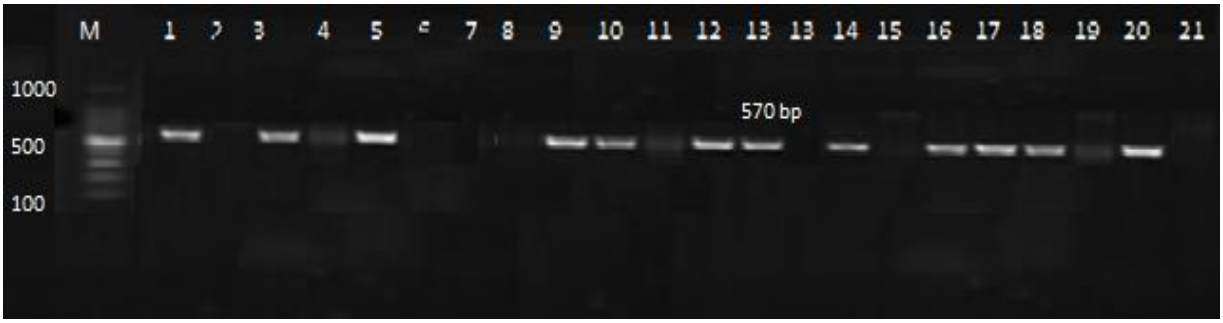


Figure (3-23): 1.5% agarose gel electrophoresis of *mexI* amplicon (570bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-21 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

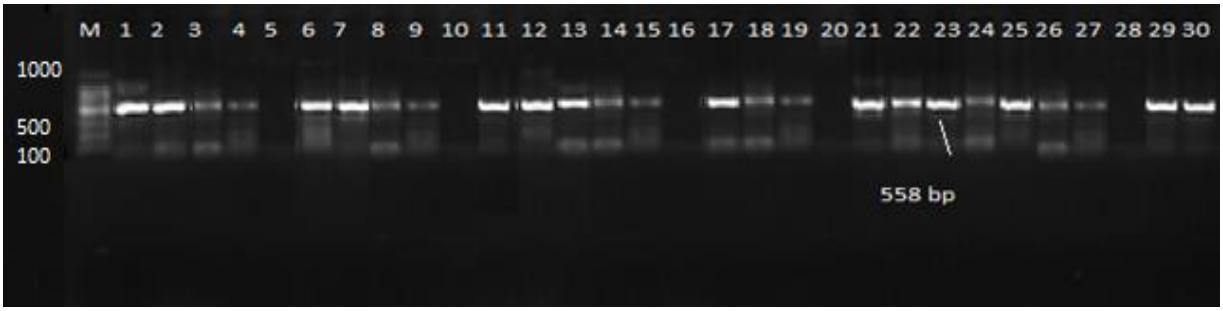


Figure (3-24): 1.5% agarose gel electrophoresis of *opmD* amplicon (558bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-30 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

The molecular detection of *mexJK-oprM* efflux revealed *mexJ* 37.97% (30 isolates) *mexK* 34.17% (27 isolates) and *oprM* 48.1(38 isolates) (Figure 3-25 and 3-26).

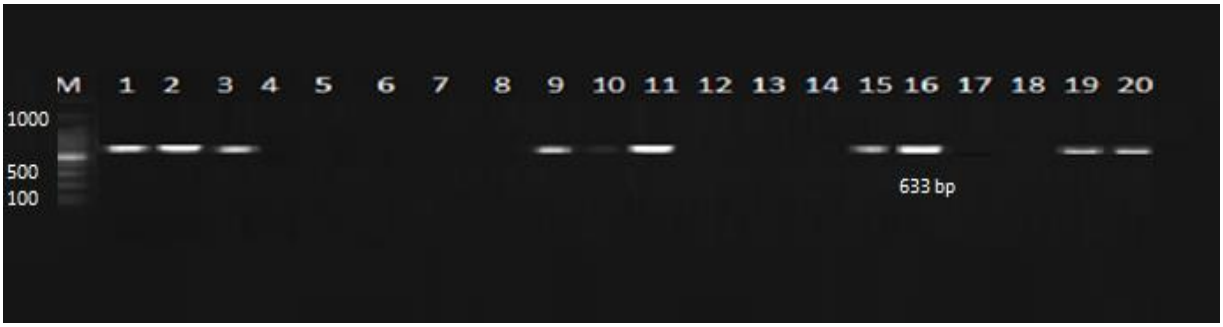


Figure (3-25): 1.5% agarose gel electrophoresis of *mexJ* amplicon (633bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

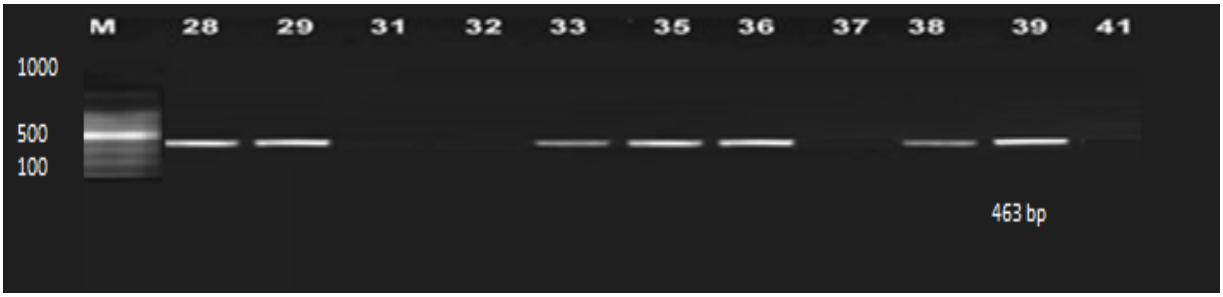


Figure (3-26): 1.5% agarose gel electrophoresis of *mexK* amplicon (463bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 28-41 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

The molecular detection of *mexMN-oprM* efflux revealed *mexM* 69.62% (55 isolates), *mexN* 50.63% (40 isolates) and *oprM* 48.1% (38 isolates) (Figure 3-27 and 3-28).

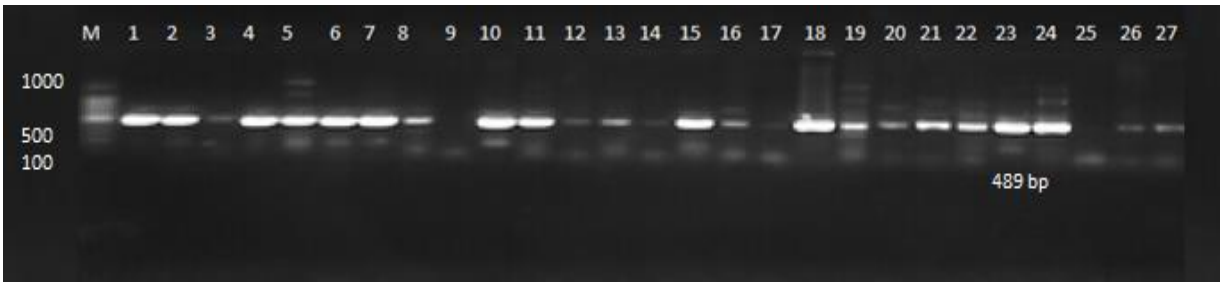


Figure (3-27): 1.5% agarose gel electrophoresis of *mexM* amplicon (489bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-27 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

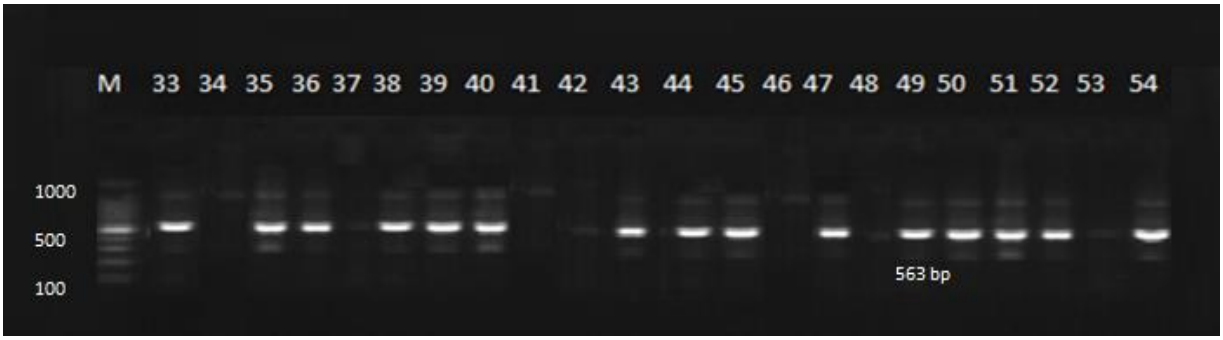


Figure (3-28): 1.5% agarose gel electrophoresis of *mexN* amplicon (563bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 33-50 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

The molecular detection of *mexVW-oprM* efflux revealed *mexV* 92.40% (73 isolates), *mexW* 91.13% (72 isolates) and *oprM* 48.1% (38 isolates) (Figure 3-29 and 3-30).

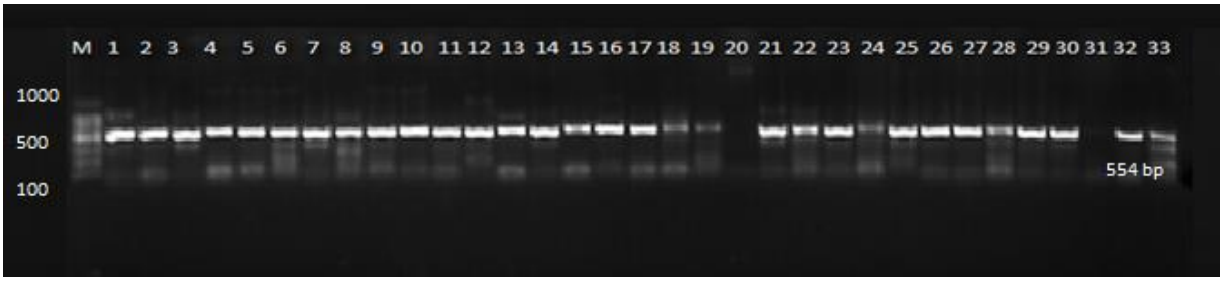


Figure (3-29): 1.5% agarose gel electrophoresis of *mexV* amplicon (554bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-33 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

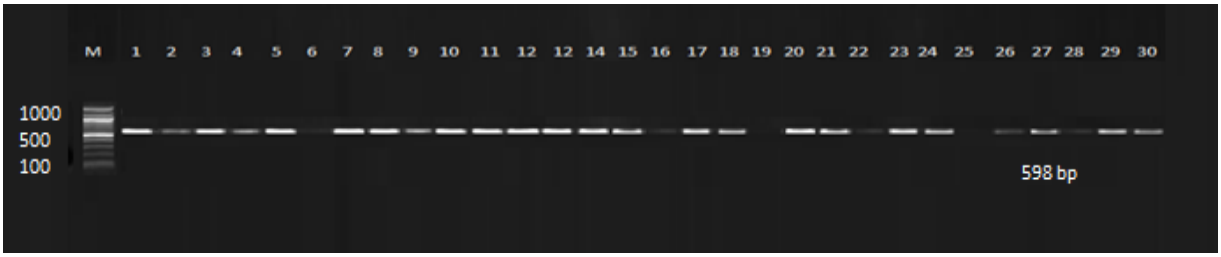


Figure (3-30): 1.5% agarose gel electrophoresis of *mexW* amplicon (598bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-79 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

PCR assay for triABC-opmH genes with 55.69% (44 isolates) for triA followed by 16.45% (13 isolates) for triB, 50.63% (40 isolates) for triC and 49.36% (39 isolates) for the opmH gene (Figure 3-31 to 3-34).

Figure (3-31): 1.5% agarose gel electrophoresis of *triA* amplicon (532bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-79 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

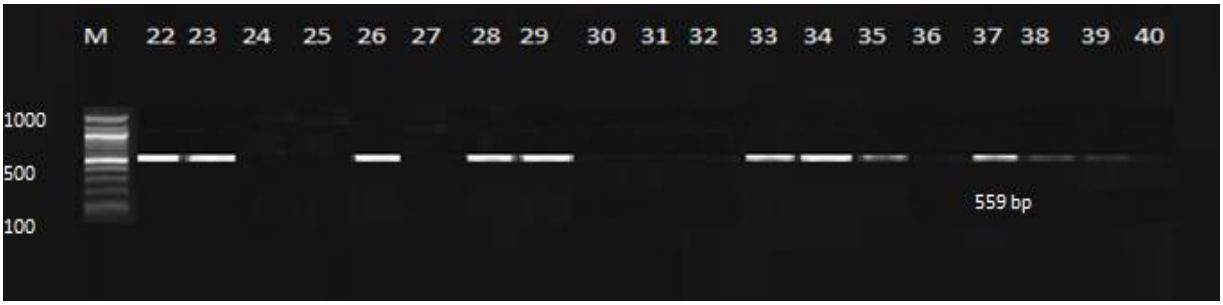


Figure (3-32): 1.5% agarose gel electrophoresis of *triB* amplicon (559bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 22-40 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

Figure (3-33): 1.5% agarose gel electrophoresis of *triC* amplicon (597bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-24represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

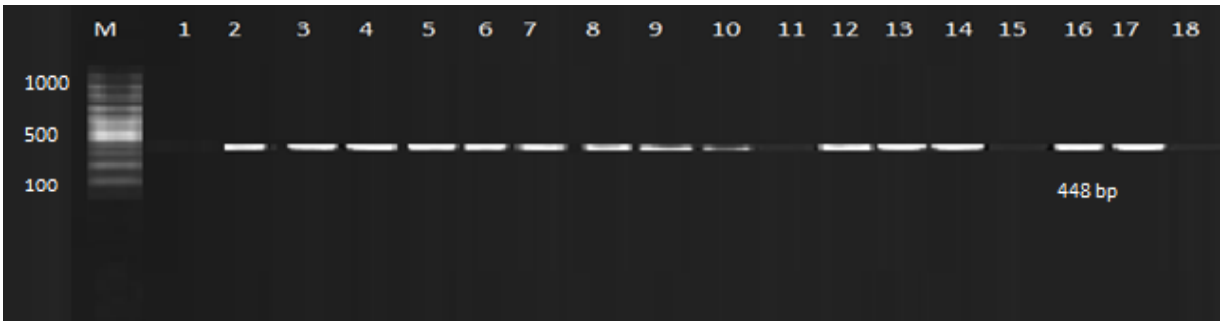


Figure (3-34): 1.5% agarose gel electrophoresis of *opmH* amplicon (448bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-18 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

PCR assay for mexEF-oprN genes with 41.77% (33 isolates) for mexE followed by 39.24% (31 isolates) for mexF (Figure 3-35 and 3-36).

Figure (3-35): 1.5% agarose gel electrophoresis of *mexE* amplicon (555bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-79 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

Figure (3-36): 1.5% agarose gel electrophoresis of *mexF* amplicon (562bp) among *P. aeruginosa* isolates. M represent 100bp DNA ladder, lane 1-18 represent the isolates, TBE 1x at Voltage 110 volt for 50 min.

3.6. Evaluating the Efflux Pump Inhibitor's Effect on Selected MDR Isolates:

In order to confirm the effective role of the efflux pump in our isolates, the MICs of antibiotics (levofloxacin and papracillin) were compared with and without the inhibitor PaβN. Among the MDR *P. aeruginosa* isolates, all MDR isolates had MICs ranging from (8-32) µg/mL in the absence of PaβN for Levofloxacin. However, the Levofloxacin MIC was reduced by one-fold, ranging from (4-16) µg/mL, except in the cases of samples 18 and 35, which stayed the

same, and doubled to 64 μg/mL in the case of sample number 38. On the other hand, pipracilin had an MIC ranging from ≥1024 to 1024 μg/mL for all isolates and when treated with PaβN, the number fell by 8-fold in the case of sample 3, 4-fold in sample 13, and 2-fold in sample 9, 11. Other samples stayed the same; PaβN was less effective at reducing the MIC in these samples (Table 3-3 and 3-4).

Table (3-3): Minimum inhibitory concentration (MIC, μg/mL) of piperacillin among MDR *P. aeruginosa* isolates before and after addition of the inhibitor (PAbN).

Sample	Efflux pumps genotypes	Without inhibitor (PABN) piperacillin MIC	With inhibitor (PABN) piperacillin MIC
3	mexA, mexB, oprM, mexV, mexW, mexX, mexY,mexC, mexD, oprJ, mexJ, mexK, mexG, opmE, triA, triC, opmH, mexM	1024	128
9	mexA, mexB, oprM, mexV, mexW, triC, opmH, mexJ, muxA, muxC, opmB,	>1024	1024
11	mexA, mexB, oprM, mexV, mexW, mexC, mexD, oprJ, mexJ, mexK, mexG, opmE, muxA, triC, opmH	>1024	512
12	mexA,oprJ, mexV, mexW, mexX, mexD, mexJ, mexK, mexG, opmE, triC, opmH	1024	1024
13	mexA, oprM, mexV, mexX, mexY,mexC, mexD, oprJ, mexJ, mexK, mexG, opmE, triB, triC, opmH, mexN	1024	256
18	mexB, mexV, mexW, mexX, mexC, oprJ, mexK, mexG, opmE, triA, mexM, mexE	>1024	>1024
24	mexA, mexV, mexW, mexY, mexC, mexJ, mexK, mexG, triC, triB, muxA	>1024	>1024
30	mexA, mexW, mexY, mexC, mexJ, mexG, triC, triA, mexF,	>1024	>1024
35	mexI, mexA, oprJ, mexC, opmH, triB, triC, mexV, mexW	>1024	>1024
38	mexA, mexW, mexY, mexD, mexJ, mexK, mexH, triA, triB, opmE,	>1024	>1024

Table (3-4): Minimum inhibitory concentration (MIC, µg/mL) of levofloxacin among MDR *P. aeruginosa* isolates before and after addition of the inhibitor (PAbN).

Sample	Efflux pumps genotypes	Without inhibitor (PABN) Levofloxacin MIC	With inhibitor (PABN) Levofloxacin MIC
3	mexA, mexB, oprM, mexV, mexW, mexX, mexY,mexC, mexD, oprJ, mexJ, mexK, mexG, opmE, triA, triC, opmH, mexM	32	16
9	mexA, mexB, oprM, mexV, mexW, triC, opmH, mexJ, muxA, muxC, opmB,	8	4
11	mexA, mexB, oprM, mexV, mexW, mexC, mexD, oprJ, mexJ, mexK, mexG, opmE, muxA, triC, opmH	32	16
12	mexA,oprJ, mexV, mexW, mexX, mexD, mexJ, mexK, mexG, opmE, triC, opmH	32	16
13	mexA, oprM, mexV, mexX, mexY,mexC, mexD, oprJ, mexJ, mexK, mexG, opmE, triB, triC, opmH, mexN	32	16
18	mexB, mexV, mexW, mexX, mexC, oprJ, mexK, mexG, opmE, triA, mexM, mexE	16	16
24	mexA, mexV, mexW, mexY, mexC, mexJ, mexK, mexG, triC, triB, muxA	32	16
30	mexA, mexW, mexY, mexC, mexJ, mexG, triC, triA, mexF,	32	16
35	mexI, mexA, oprJ, mexC, opmH, triB, triC, mexV, mexW	32	32
38	mexA, mexW, mexY, mexD, mexJ, mexK, mexH, triA, triB, opmE,	32	64

In general, our findings from utilizing the inhibitor helped to clarify the important function that pumps conduct and how resistance develops in resistant isolates. The isolates that showed a decrease in MIC are consistent with the studies by (El-Said *et al.*, 2012; Iman *et al.*, 2018), that measured MICs for different MDR *P. aeruginosa* isolates in the presence and absence of the efflux pump inhibitor PaβN, among isolates that were resistant, most of them showed a reduction in

MIC. On the other hand, the isolates that remained unchanged with the MIC despite the use of the inhibitor, this prompts us to think of other reasons for resistance, including the occurrence of mutations in the genes encoded for pumps may reduce their efficiency, and they usually accompany resistant isolates that are more susceptible to it than sensitive isolates as agreed with the study by (Sanz-García *et al*., 2022) which investigates multidrug-resistant mutants that overproduce efflux pumps, as well as another study that investigates the role of efflux pumps in multidrug resistance rather than susceptibility and the role of mutation (Nabilou *et al*., 2022).

Another study agreed, which examined the correlation between efflux pumps and bacterial antibiotic resistance and using PaβN for efflux activity among burn isolates that were non susceptible to levofloxacin, some isolates showed a ≥three-fold reduction in MIC with PaβN which 87.5% and 100% of them exhibited an overexpression of the efflux genes (Goli *et al.,* 2018). Other studies indicated that the use of inhibitors like PaβN improved the susceptibility of several clinical isolates of *P. aeruginosa* with a wide range of resistance phenotypes to imipenem and piperacillin (β-lactams), gentamicin (aminoglycosides), ciprofloxacin, and levofloxacin (fluoroquinolones), where the efflux systems demonstrated the simultaneous multidrug extrusion (Goli *et al.,* 2016; Talebi *et al.,* 2016 and Rahbar *et al.,* 2021).

3.7. Use Quantitative Real Time PCR to estimate the expression of the efflux pump gene (*oprM*):

The gene expression patterns were evaluated in MDR and non-MDR bacterial *oprM* gene. This gene was selected as the outer membrane of the pump(mexAB-oprM) of medical importance, which has been dealt with by previous research and

studies, also it shares five pumps (mexAB-oprM, mexJK-oprM, mexMN-oprM, mexXY-oprM, and mexVW-oprM) within the family RND. By normalization with the expressed housekeeping gene *rpoS* (a Pseudomonas internal control gene). All that analysis by qRT-PCR was considered a standard analysis technique to assess gene expression of potential gene (*oprM*), as shown in the amplification and melting curve (Figure 3-37 and 3-38).

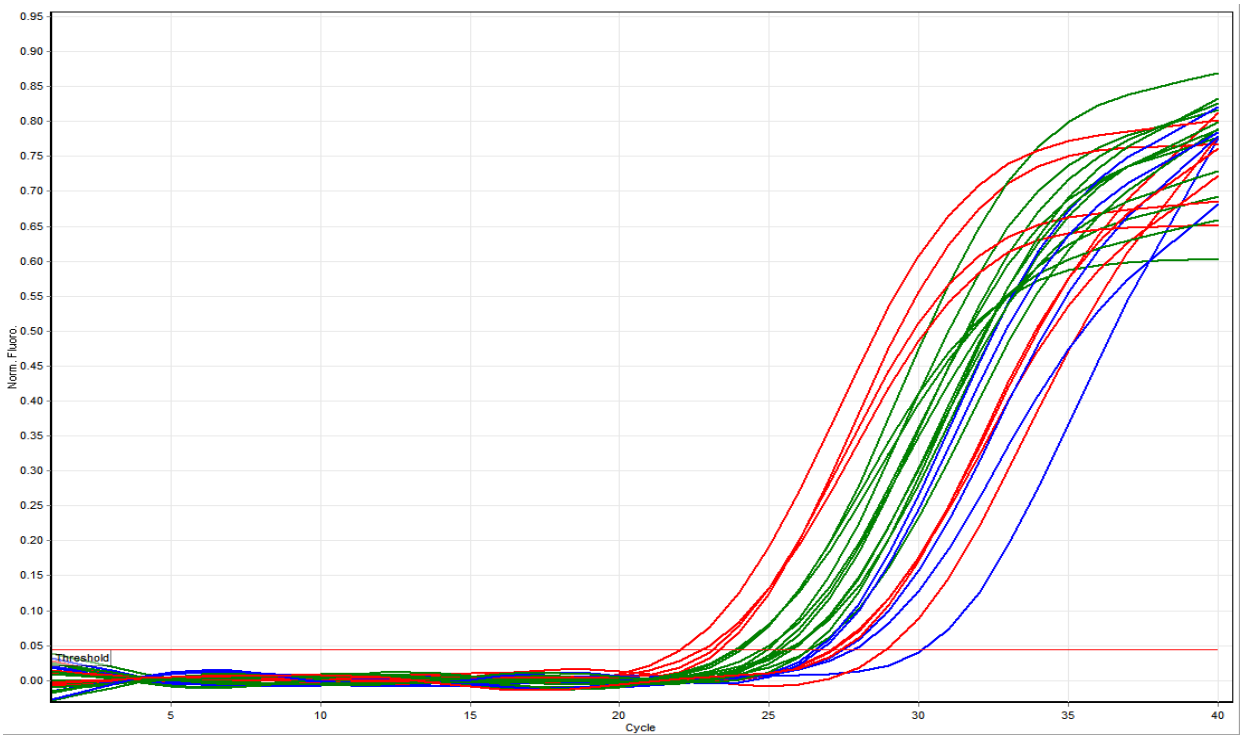


Figure (3-37): Amplification of qRT-PCR of the *oprM* gene in resistance and sensitive for MDR and non MDR *P. aeruginosa* isolates, red target gene in resistance samples, blue target gene in sensitive samples, green housekeeping gene.

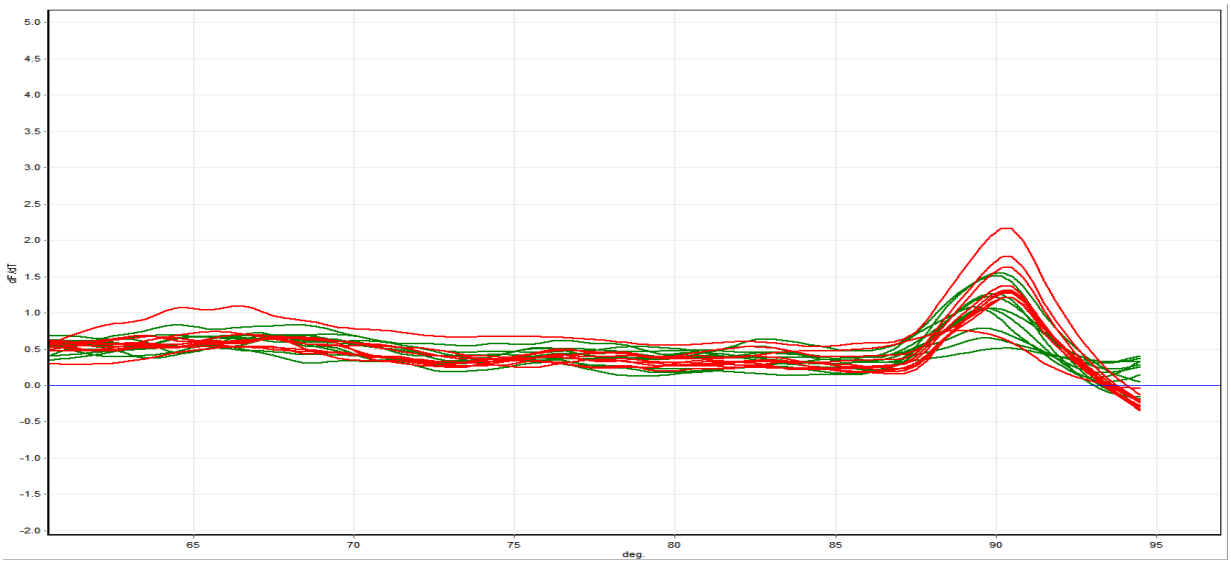


Figure (3-38): Melting curve (red trace for gene, green trace for housekeeping gene) in resistance and sensitive for MDR and non-MDR *P. aeruginosa* isolates.

Quantification of gene expression contributes to the study of *P. aeruginosa* responses to changes in circumstances and provides an understanding of resistance mechanisms. To obtain reliable gene expression results using real-time qRT-PCR, stable internal control genes should be selected and used for the accurate normalization of target gene expression, which is in agreement with the study (Li and Plesiat, 2016).

According to a previous study, which studied the expression stability of 13 housekeeping genes (*rpoS, proC, recA, rpsL, rho, oprL, anr, tipA, nadB, fabD, ampC, algD, and gyrA*) during a carbon shortage of *P. aeruginosa*, it *was* discovered that *rpoS* was the only gene that remained stable under carbon shortage in *P. aeruginosa* (Alqarni *et al.*, 2016).

3.7. 1. Estemate *oprM* Expression in selected MDR and non MDR *P. aeruginosa*

The current study included nine candidate MDR isolates (1–9) that showed resistance to all of the antibiotics used in our experiment and were isolated from UTI,RTI, burns, and wound samples, as well as five sensitive isolates (10–14) to assess *oprM* gene expression. The results of the study revealed that *P. aeruginosa* isolates showed an increase in the fold change of the gene expression levels of the efflux pump gene *oprM* in all MDR isolates compared with non-MDR isolates. The highest difference in the value of gene expression between MDR and non-MDR isolates was in the isolate number (8, 9), and the lowest value was in the amount of difference between the gene expression of MDR and non-MDR isolates (2, 7).

This investigation was completed by employing the formula (2^-ΔΔCT), which was used to standardize the *oprM* gene level in samples of appropriate housekeeping quality (*rpoS*), that was used to standardize the *oprM* gene quality levels in *P. aeruginosa* isolates.

Table (3-5): Estimated fold change for the expression of *oprM* in MDR and non MDR *P. aeruginosa* isolates by relative quantity investigation.

Isolate	Ct *oprM*	Ct *rpoS*	ΔCt-MDR	ΔCt-non-MDR	ΔΔC	relative Quantity (RQ)=2^-ΔΔCt
MDR1	19.7	22.71	-3.01	**2.95**	-5.96	62.25
MDR2	24.8	25.51	-0.71	**2.95**	-3.66	12.64
MDR3	26.93	29.19	-2.26	**2.95**	-5.21	37.01
MDR4	24.51	26.56	-2.05	**2.95**	-5	32
MDR5	20.98	22.6	-1.62	**2.95**	-4.57	23.75
MDR6	25.18	26.44	-1.26	**2.95**	-4.21	18.5
MDR7	22.14	23.2	-1.06	**2.95**	-4.01	16.11
MDR8	21.53	26	-4.47	**2.95**	-7.42	171.25
MDR9	20	24.25	-4.25	**2.95**	-7.2	147.03
Isolate		**Ct *oprM***		**Ct *rpoS***		**ΔCt-non-MDR**
non-MDR1(control1)		25.27		25.4		-0.13
non-MDR2 (control2)		29.1		21.73		7.37
non-MDR3 (control3)		29.4		24.06		5.34
non-MDR4 (control4)		25.61		27.26		-1.65
non-MDR5 (control5)		28.22		24.4		3.82
Mean ΔCt = 2.95						

From the current study, the results showed that the samples of UTIs (35.4%), RTIs (29.1%), wounds and burn infection (18.9%) are the most clinical samples of *P. aeruginosa* isolates that showed resistance to the antibiotics used in our experiments, such as ceftazidime (CAZ), cefepime (FEP), and piperacillin (PRL)were recorded at 93.6%, 77.2%, and 68%, respectively, according to the results of the table (1-3) and figures (2-2, 2-3, 2-4), and this is in agreement with studies that exhibit a greater resistance to ceftazidime and cefepime as well as piperacillin antibiotics (Sallman *et al*., 2018; Chand *et al*., 2021; Saeed *et al*., 2022; Al-Thabhawee and Al-Dahmoshi, 2022).

The isolates that showed resistance to the antibiotics used had been chosen and tested for the cause of their resistance by using the efflux pump inhibitor PaβN according to the tables (3-3, 3-4), which showed that the MIC of antibiotics (LVE and PRL) was reduced in most cases and gave proof that the most common cause of resistance was the presence of efflux pump action that supported our results that showed the presence of efflux pump coded genes according to the table (3-2). On the other hand, the correlation between EPI PaβN and the reduction of antibiotic resistance in *P. aeruginosa* has been shown in previous studies (Ferrer *et al.*, 2019; Elsheredy *et al.*, 2021; Sanz *et al.*, 2022).

After that, these resistant samples were selected and compared with sensitive ones. Both of them contain the same coded gene *oprM. It* was found that the gene expression of this gene increased in resistant isolates by an amount compared to sensitive subjects, who showed lower gene expression, which proves the importance of the work of these pumps and their impact on the high rate of resistance in our clinical isolates. This is consistent with the study's assumption that some of the multidrug resistance observed in *P. aeruginosa* isolates could be attributed to overexpression of multidrug efflux pumps because of their broad substrate specificity. Every efflux pump expels several antibiotic classes, such as β-lactams and quinolones (Talebi *et al*., 2016).

Another study by Llanes *et al. (*2004) showed the overexpression of efflux pumps in *P. aeruginosa* clinical strains isolated in France. In Brazil, Xavier (Xavier *et al.*, 2020) detected overexpression in efflux genes. The overexpression of *oprM* had a significant correlation with resistance to tested antibiotics as indicators for phenotypic detection of efflux pump overexpression, where piperacillin and gentamicin are the specific substrates of MexAB-OprM and MexXY-OprM. The obtained results are comparable to another study (Shigemura

et al., 2016), which demonstrated a significant relationship between the increased expression of efflux pump genes and complicated urinary tract infection (UTI). In addition, they observed a significant association between the increased expression of these genes and resistance to levofloxacin. The overexpression exhibited a significant correlation with the MDR phenotype in our *P. aeruginosa* clinical isolates and the overexpression of the *oprM* gene.

Conclusions & Recommendations

Conclusions:

The present study concludes the following

1. Prevalence of RND efflux pumps in *P. aeruginosa* local isolates is high
2. The most prevalent efflux pumps among MDR and non MDR isolates are MexVW-OprM and MexAB-OprM
3. The most prevalent out membrane protein among MDR and non-MDR isolates is the *oprJ* gene
4. The efflux pump inhibitor (PaβN) has a positive effect and improves the sensitivity of MDR isolates to piperacillin and levofloxacin.
5. Gene expression of efflux pump *oprM* gene increased in MDR *P. aeruginosa* isolates

Recommendations:

The present study recommends the following:

1. Further investigation of inhibitory effect of PaβN on all component of efflux pumps gene expression and on all outer membrane protein gene expression
2. Studying the cytotoxicity and selectivity of PaβN
3. Studying the inhibitory effect of natural products on efflux pumps gene expression
4. Study the inhibitory effect of PaβN on susceptibility of other antibiotics
5. Study gene expression of other efflux pump genes that have a correlation with antibiotic resistance.

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وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية العلوم

قسم علوم الحياة

التنميط الجيني لمضخات لفظ المضادات الحياتية المسؤولة عن المقاومة في العزلات السريرية للزوائف الزنجاريه

اطروحة مقدمة إلى

مجلس كلية العلوم / جامعة بابل وهي جزء من متطلبات نيل شهادة الدكتوراه فلسفة في العلوم /علوم الحياة

من قبل

افراح جواد عبد زويد

(بكالوريوس /علوم حياة / احياء مجهرية / 2004)

(ماجستير /علوم حياة / احياء مجهرية / 2016)

اشراف

ا.د. حسين عليوي مطلب الدهموشي

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الخلاصة

الزائفة الزنجارية هي سبب رئيسي للعدوى المكتسبة من المستشفيات. تمثل هذه العدوى تحديًا علاجيًا صعبًا للغاية وترتبط بالمراضة والوفيات.

يُعد الإفراط في التعبير عن مضخات تدفق الأدوية المتعددة (EPs) أحد أهم الآليات الأساسية. يمكن لمضخات التدفق هذه بثق المضادات الحيوية من السيتوبلازم إلى البيئة خارج الخلية وبالتالي فقدان فعالية الدواء. الهدف من هذه الدراسة هو التحقق من انتشار خصائص مضخات التدفق بين العزلات السريرية من الزائفة الزنجارية ومساهمتها في النمط الظاهري MDR. الهدف الآخر هو قياس تأثير استخدام مثبط مضخة التدفق (EPI) ؛ فينيل ألانين-أرجينين-بيتا-نافثيلاميد (PAβN) على الحد الأدنى من التركيز المثبط (MIC) لبعض المضادات الحيوية ودراسة التعبير الجيني للجين المشفر للغشاء الخارجي لمضخة التدفق (*oprM*) في MDR والعزلات الحساسة.

أظهرت نتائج الدراسة الحالية نسبة عالية من عزلات الزائفة الزنجارية 79 موزعة على مرضى التهابات المسالك البولية 35.4٪ ، مرضى عدوى الجهاز التنفسي السفلي 29.1٪ ، إصابات الجروح والحروق 18.9٪ ، 8.8٪ لمرضى التهابات المسالك البولية. التهاب الأذن الوسطى 2.5٪ تجرثم الدم 3.7٪ التهاب المهبل الجرثومي و 1.2٪ التهاب السحايا.

بالنسبة لاختبار الحساسية للمضادات الحيوية ، سجلت قيم مقاومة عزلات الزائفة الزنجارية تجاه السيفتازيديم (CAZ) والسيفيبيم (FEP) عند 93.6٪ و 77.2٪ على التوالي ، بينما يمثل 65٪ البيبراسيلين (PRL) ، ويمثل 40.5٪ الجنتامايسين (CN).). تم اكتشاف سيبروفلوكساسين (CIP) في 37.9٪ فقط من العزلات. من ناحية أخرى ، تم تسجيل 41.7٪ توبراميسين ((TOB، 39.2٪ أزتريونام ((ATM، 45.5٪ أميكاسين ((AK، 43٪ أوفلوكساسين ((OFX، 37.9٪ نيتيلميسين ، 13.9٪ إيميبينيم (IPM)) ، و 25.3٪ للميروبينيم (MEM). أظهرت دراسة أنماط مقاومة المضادات الحيوية أن 59٪ (47 عزلة) من العزلات مقاومة للأدوية المتعددة و 41٪ (32 عزلة) غير مقاومة للأدوية المتعددة.

تم إجراء اختبار PCR للجينات المشفرة بواسطة PCR التقليدي لجميع العزلات (79 عزلة). تم اكتشاف المضخة mexAB-oprM مع جينات *MexA* و *mexB* و *oprM* في 82.2٪ (65 سلالة) و 63.29٪ (50 سلالة) و 48.1٪ (38 سلالة) من العزلات على التوالي. كشفت المضخة

mexXY-oprM أن مكس 41.7٪ (33 سلالة) ، مكسي 50.6٪ (40 سلالة) ، 48.1 *oprM*٪ (38 سلالة). كشفت مضخة mexPQ-opmE أن 36.70 *mexP*٪29) سلالة) ، *mexQ* (37٪46.83 سلالة) و 50.6 *opmE*٪40) سلالة) لجينات muxABC-opmB، 59.4٪47) سلالة) لـ *muxA* ، تليها 37.9٪ (37 سلالة). 30 سلالة) لـ *muxB* و 48.1 *muxC*٪38) سلالة) و 62.02٪ (49 سلالة) لجين *opmB*. كشف الكشف عن تدفق mexCD-oprJ أن *mexC* (33٪41.77 سلالة) ، 48.1 *mexD*٪38) سلالة) و 81.1 *oprJ*٪64) سلالة) ، لجينات mexGHI-opmD بنسبة 68.35٪ (54 سلالة) لـ *mexG* تليها 49.36٪ (39 سلالة) لـ *mexH* ، 37.9٪30) سلالة) لـ *mexI* و 20.2٪ (16 سلالة) لجين *opmD*.

كشف الكشف عن تدفق mexJK-oprM أن 37.9 *mexJ*٪30) سلالة) ، 34.17 *mexK*٪27) سلالة) و 38) 48.1 *oprM* سلالة). كشف الكشف عن تدفق mexMN-oprM أن *mexM* (55٪69.62 سلالة) و 50.63 *mexN*٪40) سلالة) و 48.1 *oprM*٪38) سلالة). كشف الكشف الجزيئي عن تدفق mexVW-oprM أن 92.40 *mexV*٪73) سلالة) ، *mexW* (72٪91.13 سلالة) و 48.1 *oprM*٪38) سلالة) لجينات triABC-opmH ، مع 55.69٪ (44 سلالة) لـ *triA* ، تليها 16.4٪ (13 سلالة) لـ *triB*، 50.63٪40) سلالة) لـ *triC* و 49.36٪ (39 سلالة) لجين *opmH*. فحص PCR لجينات mexEF-oprN بنسبة 41.77٪ (33 سلالة) لـ *mexE* تليها 39.24٪ (31 سلالة) لـ *mexF* ،

كان الإفراط في التعبير عن جين *oprM* أعلى بشكل ملحوظ في عزلات MDR من العزلات التي لا تحتوي على MDR. أدت إضافة EPI، PAβN ، إلى انخفاض كبير في MIC في تهوية العادم المحلي و PIP. كانت المقاومة بوساطة مضخة التدفق آلية مهمة تساهم في مقاومة الأدوية المتعددة في العزلات السريرية لـ الزائفة الزنجارية ومع ذلك ، ينبغي النظر في آليات مقاومة الأدوية الأخرى. على الرغم من إضافة برنامج التحصين الموسع ؛ أدى PAβN إلى خفض MIC في سلالات MDR. لذلك ، من المهم الاستمرار في استكشاف برامج التحصين الموسعة الأكثر فعالية ، ويوصى بدراسات المراقبة واسعة النطاق للكشف عن انتشار الإفراط في التعبير عن مضخة التدفق بالإضافة إلى الآليات المحتملة الأخرى بين عزلات الزائفة الزنجارية وارتباطها بمقاومة الأدوية المتعددة.