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Prevalence of type I secretion system (*Apr DEF*) and Alkaline protease (*AprA*) among *Pseudomonas aeruginosa* isolated from Burn infections.

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَمَنْ يَتَّقِ اللَّهَ يَجْعَل لَّهُ مَخْرَجًا * وَيَرْزُقْهُ مِنْ حَيْثُ لَا
يَحْتَسِبُ وَمَنْ يَتَوَكَّلْ عَلَى اللَّهِ فَهُوَ حَسْبُهُ إِنَّ اللَّهَ بَالِغُ
أَمْرِهِ قَدْ جَعَلَ اللَّهُ لِكُلِّ شَيْءٍ قَدْرًا *

صَدَقَ اللهُ الْعَلِيُّ الْعَظِيْمُ

سورة الطلاق

الآية ٢ -٣

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2023

Dedication

To the purest heart to my role model, and my ideal in life; He is the one who taught me how to live with dignity and loftiness… My dear father

To the heaven of God on earth, to the bridge that ascends me to heaven, to my ideal… My mother

To the eyes and heartbeat ...to … My brother & sisters

To my second half…My husband

To the endless love and the secret behind my existence… My children Hassan …Haider

Zahraa

Summary

Pseudomonas aeruginosa is an important Gram-negative opportunistic pathogen which causes many severe acute and chronic infections with high morbidity, and mortality rates. This study was designed as cross sectional study in burned patients with *Pseudomonas aeruginosa* infections. About 27 *Pseudomonas* isolates were isolated from 70 specimens of burned infections during (2021_2022) and 62 pure *P. aeruginosa* previously isolated from burned infections .These samples was collected from Al-Hilla teaching hospital, Imam Sadeq teaching hospital of Babylon Province during 2020_2021

The antibiotics susceptibility test the higher percentage of antibiotics resistant was (58.42 %) for amikacin (AK), intermediate resistant of antibiotics was (23.60 %) for piperacilline (PRL), and higher percentage of sensitivity of antibiotics was (47.19 %) for meropenem (MEM), in addition the frequency of antibiotic resistance of P. aeruginosa which appeared variable resistance to antibiotics and bout 23.6% of isolates were resist to five antibiotics and 21.34% of isolates were sensitive for all antibiotics.

The current study showed about 11 (12.36%) of these isolates appeared high-level of enzyme production (ratio =3-3.9), 39 (43.82%) of isolates produced Intermediate-level (ratio 2-2.9) and 29 (32.58%) isolates produced low-level (ratio 1-1.9). Whereas the rest of isolate had not any clear zone around their colonies (10: 11.244%) named non- extracellular protease producer.

The phenotypic and genotypic of selected *P. aeruginosa* isolates showed high-level protease producer for (*P. aeruginosa* 13- NR2) and multi-drug resistant for antibiotics ,while *P. aeruginosa* 18_GF showed intermediate level of extracellular protease and sensitive for antibiotics both isolates had complete *aprA* gene cluster which encodes to alkaline protease (*AprA*), alkaline protease inhibitor (*AprI*), and type I secretory system (TISS).

In PCR-DNA sequencing of *aprA* alkaline protease genes appeared both *P. aeruginosa* 13- NR2 and *P. aeruginosa* 61- NR1 had one a substitution mutation (C>T) in studied aprA region at the positions 60 and *P. aeruginosa* 18- GF had a

substitution mutation A>G at the positions 174, while for *aprI* gene the results appeared three substitution mutations in of *P. aeruginosa* 13- NR2 includes A>C, C>A and C>A at the positions 101, 123 and 141 respectively. In addition the Type I secretory system genes for *aprD & aprF for P. aeruginosa 18-GF*, 13-NR2 and 61-NR1 appeared identical sequences with sequences of those available in databanks (NCBI), the isolate P. aeruginosa 60-ZR had showed two mutations the first deletion (C) at position 30 and substitution mutation C>A in the position *139* , Finally *aprE* gene for local isolates P. aeruginosa 18-GF and 13-NR2 appeared one substitution mutation (G>C) at position 111 compared with sequences of those available in databanks (NCBI).

The molecular analysis of *toxA* gene showed amplified band at (150bp), where is about 95.5% of *P. aeruginosa* isolates harbor *toxA* gene, these isolates include 17 (89.47%) of sensitive to antibiotics, 34 (94.45%) of non-MDR isolates and all of the MDR isolates harbor *tox A* gene. Three *P. aeruginosa* isolates were randomly selected from three groups, MDR *P. aeruginosa 13-NR2*, Non-MDR *P. aeruginosa 61-NR1* and susceptible *P. aeruginosa 18-GF* isolate for further analysis using PCR-DNA sequencing technology. The results appeared *tox A* gene of the MDR isolate *P. aeruginosa 13-NR2* had one substitution mutation C > T at position 60.

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Abbreviations

Abbreviated Form	Meaning
ADCC	Antibody dependent cell cytotoxicity
AIDS	Acquired immunodeficiency
AprA	Alkaline protease
AprI	Alkaline Protease Inhibitor
CF	cystic fibrosis
CF	Cystic fibrosis
ENaC	Epithelial sodium channel
ESBLs	Extended-spectrum beta-lactamase
Fur	iron uptake
HCN	hydrogen cyanide
HCN	Hydrogen cyanide
ICU	Intensive care unit
LASB	Elastase B
LepA	Large exoprotease
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein
MDR	Multidrug-resistant
MIP-2	Macrophage-inflammatory protein-2
OMF	Outer membrane factor
PAAP	*P.aeruginosa* aminopeptidase
PAPS	*P.aeruginosa* small protease
PARs	Proteasome-activated receptors
PASP	*P.aeruginosa* small protease

PCR	Polymerase chain reaction
PE	Pseudomonas exotoxin
PIV	Type IV protease
PMN	Polymorph nuclear
RTX	Repeat-in-toxin
T2SS	Type II secretion system
TISS	Type I secretory system
TLR5	Toll-like receptor 5
TMDs	Transporters TM domains
Tox A	Toxin A
UTI	Urinary Tract Infection
VAP	Ventilation-related pneumonia
WHO	World Health Organization

1.1 Introduction

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium. It can be found as a single bacterium, in pairs, or in short chains and belong to the class γ-proteobacteria and family Pseudomonadaceae. It is a facultative aerobe that prefers to use oxygen as the final electron acceptor during aerobic respiration, although it is also capable of anaerobic respiration using other alternative electron acceptors such as nitrate (Wood *et al*., 2023). *P. aeruginosa* can also catabolize a wide-range of organic molecules for nutrients, making it one of the most biochemically versatile and ubiquitous bacterium found in many environments such as soil, water, vegetation, and even human skin and oral mucosa. It is motile via one or two polar flagella. (Alinaqvi *et al*.,2019; Sekhi, 2022).

Burn is described as a traumatic injury to the skin or other organic tissues, mainly caused by thermal or other acute exposures. Decreased immunity, hospital overstaying and breach in protective skin barrier are mostly responsible for the increase in hospital-acquired infections (HAIs) in these patients (Dou *et al*.,2017). Even with aggressive antibiotic therapy *P. aeruginosa* can carry out high morbidity and mortality in burn units. *P. aeruginosa* is one of the major pathogens causing hospital-acquired infections. It can easily develop antibiotic resistance through chromosomal mutations or by horizontal acquisition of resistant determinants.

P. aeruginosa encodes virulence factors such as LasA and LasB elastases, type IV protease (PIV), *P. aeruginosa* small protease (PASP), Large ExoProtease A (LepA), alkaline protease (AprA), *P. aeruginosa* aminopeptidase (PAAP), and MucD, these factors increase its fitness and

chances of survival within a human host (Mues *et al*., 2020), in addition they promoted bacterial growth and survival, maneuvering the host cellular machinery by causing devastating injuries, tissue necrosis, evasion, and immune system impairment (Li *et al*., 2019)

Alkaline protease, which is called aeruginolysin in *P. aeruginosa*, another metalloendopeptidase produced through T1SS (aprA gene encoded) which interferes with endothelial components (fibronectin and laminin) and degrades cytokines (IFN, TNF, and IL-6) and complement proteins (C1q, C2, and C3), allows phagocytic evasion (Tuon *et al*., 2022). It also cleaves free flagellin monomers, reducing the mucociliary clearance of bacteria through epithelial sodium channel activation and contributes to the production of pyocyanin (and other virulence factors) (Iiyama *et al*.,2017).

1.2 Aim of Study

Molecular detection for alkaline protease gene cluster and *tox A* gene as a major virulence factors among MDR and Non MDR *P. aeruginosa* isolates which infected the patients with burns.

Through achieving the following Objectives:

1.Isolation and identification of *P. aeruginosa* form burn infections

2. Investigation the antibiotic susceptibility and determine multidrug resistance isolates.

3. Molecular detection of extracellular alkaline protease gene cluster and exotoxin A gene using polymerase chain reaction (PCR) technique. Then further genetic analysis for these genes using PCR-DNA sequencing method.

2.1 *Pseudomonas aeruginosa*

The genus of Pseudomonas is Gram-negative bacilli cells, non-fermentative, oxidase-positive, catalase positive, aerobic, and motile by unipolar flagellum. Some members of *Pseudomonas* can produce pigments. The *Pseudomonas* genus comprises over 120 species belonging to the order Pseudomonadales, family Pseudomonadaceae includes the opportunistic human pathogen *P. aeruginosa*, animal and plant pathogenic bacteria, plant beneficial bacteria, universal soil bacteria with bioremediation abilities and other species that cause spoilage of milk and dairy products (Table 2-1),. and they are widespread in a moist environments such as water and soil ecosystems (Galdino *et al*., 2017; Silva *et al.*, 2019).

Table (2-1). The Classification of *Pseudomonas aeruginosa (Kim et al.*, 2012).

Domain	Bacteria
Phylum	Proteobacteria
Class	Gamma Proteobacteria
Order	Pseudomonadales
Family	Pseudomonadaceae
Genus	*Pseudomonas*
Species	*Pseudomonas aeruginosa*

Virtually all *P. aeruginosa* strains are Gram-negative rod- shape bacterium measuring 0.5–0.8 µm 1.5–3.0 µm, typically aerobic, but they also can grow in an anaerobic environment if nitrate, citrate, and arginine are available, ammonia producer, motile by a single polar flagellum that contributions the movement and colonization of a wide range of environmental places (Jayaseelan *et al.*, 2014). *P. aeruginosa* produced blue-green phenazine pigment that donates the green color to the bacterial

colony and also to the pus. This pigment and several others, such as pyoverdin (yellow, green, and fluorescent), pyomelanin (light-brown), pyorubin (red-brown), and pyochelin (purple-cyan), are secondary metabolites of *P. aeruginosa*, which play a vital role in bacterial nutrition, pathogenesis and iron acquisition (Jayaseelan *et al.*, 2014; Galdino *et al.*, 2017). This bacteria can grow in a wide range of temperatures (4 - 42 °C) in different habitats like soil and freshwater, salt and polluted, as well as on the surface of living hosts (plants, insects, animals, and humans) and nonliving environments, mainly in the hospital surroundings (disinfectants, distilled water, sinks, medical equipment and devices), become a main causative agent of nosocomial infections, particularly in intensive care units (ICUs) (Wiehlmann *et al*., 2007; Jayaseelan *et al.*, 2014; Galdino *et al.*, 2017). One of the impressive features of *P. aeruginosa* is its pan-genome, which benevolences a larger genetic repertoire than the human genome. This fascinating feature explains the broad metabolic abilities of *P. aeruginosa*, its adaptability and distribution in wide-ranging environments (Fariñas and Martínez-Martínez, 2013; Gellatly and Hancock, 2013, Ghodhbane et al., 2015). *P. aeruginosa* is most often associated with human infections. The bacterium is considered an opportunistic pathogen, causing mainly nosocomial infections in patients affected by an immune problem. Existing knowledge of *P. aeruginosa* pathogenesis is obtained mainly by studying clinical isolates, particularly those that cause chronic pulmonary infection in patients with cyst fibrosis (Silva *et al.*, 2019).

P. aeruginosa is high intrinsic antibiotic resistance, together with its rapid ability to gain new antimicrobial resistance. In general, resistance is usually caused by a combination of factors acting synergistically: (i) *P. aeruginosa* is intrinsically resistant to antimicrobial

agents due to the formation of its outer membrane or cell envelope that reduces the permeability of several drugs; and (ii) *P. aeruginosa* is a powerful repertoire of resistance mechanisms that can be developed through mutations in the genomic content regulating resistance genes, and also acquired from other organisms via plasmids, transposons, or phages (El Zowalaty *et al.*, 2015). This pathogen is an increasing problem for the pathology of infectious diseases, especially if the nosocomial originates, no medical trials exist to investigate the potential survival factors of hospitalized patients with *P. aeruginosa* urinary tract infections (UTIs), and the mortality of these patients except bacteremia is not understood (Horino *et al.*, 2012).

In critically diseased and weakened patients, particularly in ventilation-related pneumonia (VAP) and bloodstream infections, UTIs, intra-abdominal wounds, and skin soft tissue injury. *P. aeruginosa* is one of six ESKAPE pathogens that represent *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.*, capable of 'escaping' the bactericidal action of antibiotics and mutually representing new paradigms in pathogenesis, transmission, and resistance, that is the main cause of infectious nosocomial and is a global menace, as it becomes increasingly resistant to all antibiotics available (Pendleton *et al*., 2013; Lynch *et al*., 2017; Tümmler, 2019).

Nosocomial infections most often linked to *P. aeruginosa* include ventilator-related pneumonia, catheter-related UTI, serious burn patient wound infections, and multifactorial septicemia with pathogenesis. The bacterium is also able, via the type III secretions system, to produce many toxins, proteins, and enzymes such as elastase, phospholipase C, and siderophores (Streeter and Katouli, 2016).

P. aeruginosa demonstrates resistance to a broad range of antimicrobial drugs and expresses a variety of molecular mechanisms to tolerate different groups of antibiotic agents, such as β lactams, fluoroquinolones, tetracycline, and aminoglycosides. Although the external membrane is poor in permeability, its hydrophobicity and unspecified behavior to small molecular transport. The resistance of *P. aeruginosa* to different chemical agents is due to the bacterial chromosome carrying gene clusters encoding different mechanisms such as the inherited ability to form a biofilm which further improves the resistance under different environmental conditions (Mohanty *et al.*, 2021).

These bacteria are highly adapted to their environments, through the formation of biofilms at the site of infection which is facilitated by the flagella motility that leads to bacterial cell aggregation subsequently an increase in the bacterial tolerance to the surrounding conditions and improving antibiotic resistance and removal which documented in the context of lung infections, and otitis media, non-healing wounds, and soft tissue fillings, which do not comply with surface attachment (Demirdjian *et al*., 2019).

2.2 Antibiotic Susceptibility of *P. aeruginosa*

Several antibiotics have bactericidal or bacteriostatic effects against *P. aeruginosa*. These include antipseudomonal Penicillins that involve Carbenicillin and Ticarcillin, Mezlocillin, Azlocillin, and Piperacillin. The most commonly used antibiotic in this class is Piperacillin combined with Tazobactam. Another class is the antipseudomonal Cephalosporin which includes Ceftazidime, Cefepime, Ceftazidime/Avibactam, and Ceftolozane/Tazobactam. Furthermore, Aztreonam is the only available Monobactam that possesses

antipseudomonal activity (Giamarellou and Antoniadou, 2001). Carbapenems; Imipenem, Doripenem, and Meropenem, as well as some newer compounds such as Biapenem and Panipenem, have a wide spectrum of antimicrobial activity and can cover *P. aeruginosa* strains. Among the Fluoroquinolones available, Ciprofloxacin is the most active antibiotic and has a bactericidal effect against *P. aeruginosa*. Newer Quinolones such as Levofloxacin, Moxifloxacin, and Gatifloxacin have low to moderate activity against pseudomonas infections (less active than Ciprofloxacin). The last class is Aminoglycosides including Gentamicin, Tobramycin, and Amikacin, which have an important role in the therapy of serious *P. aeruginosa* infections (Bassetti *et al*., 2018).

Antimicrobial resistance is a serious public health problem worldwide ant it has become one of the most important problems that face clinicians. According to the World Health Organization (WHO), antimicrobial resistance has been declared a public health threat (Moremi *et al*., 2016). Misuse of antibiotics by humans, factories, and farms, as well as poor hygiene and untreated city and hospital sanitation, as well as ineffective infection prevention and control in healthcare settings and hospitals are important reasons for the emergence and distribution of antibiotic-resistant bacteria in the environment (Hocquet *et al.*, 2016; Lien *et al.*, 2017; Hashemi *et al*., 2018). Frequent and extensive use of antibiotics contributes to an increase in selective pressure in the bacterial community. This, in turn, leads to the development of multi-antibiotic resistance in bacteria and the gradual elimination of antibiotic-sensitive bacteria, leading to the spread of resistant strains. (Hashemi *et al*., 2018; Pazda *et al.*, 2019; Roulová *et al.*, 2022). Resistant bacteria can respond to selective pressure and adapt to new environmental conditions and are potential vectors for the spread of antibiotic resistance (Osiń ska *et al.*, 2017; Roulová *et al*., 2022). Moreover, selective pressure may also

induce native microorganisms to rapidly adapt to selective conditions by acquiring novel genetic traits (Santoro *et al.*, 2015; Proia *et al.*, 2018). In general, all environments such as human and animal intestinal flora, factory and hospital environments and wastewater in water treatment plants contain antibiotic-resistant bacteria, antibiotic-resistant genes, and environmental microbes that are constantly mixed with antibiotics, residues, and other substances with potential selective pressure that It can come from different sources (Rizzo *et al*., 2013; Santoro *et al.,* 2015; Manaia *et al.,* 2018). Which provide optimal conditions for the transfer of antibiotic resistance genes, the development of new antibiotic-resistant bacteria, and the creation of hotspots for the spread of bacteria and resistance genes in the environment (Nnadozie *et al.*, 2017; Osiń ska *et al.*, 2017).

The worst public health concern is the transfer of resistance genes between environmental bacteria and human pathogens (Moges *et al.*, 2014; Asfaw *et al.*, 2017). In addition, resistance properties accumulate after exposure to different antibiotics and cross-resistance between factors may lead to multidrug-resistant (MDR) bacteria (Valodon *et al.,* 2019; Krzyminski et al. 2019). Multidrug-resistant pathogens pose a significant threat to global public health and are a major clinical concern (McLain et al. 2016; Nnadozie *et al.,* 2017). The occurrence and prevalence of multidrug antibiotic resistance among bacterial pathogens may have severe consequences for human health (Pazda *et al.*, 2019). In the future, most of the antibiotics currently available in medical practice may be ineffective against resistant bacteria and infections caused by MDR pathogens will be completely untreatable (Asfaw *et al.*, 2017; Pazda *et al.*, 2019).

The opportunistic human pathogen *P. aeruginosa* is a universal microorganism, capable of surviving in many niches. It is a common

nosocomial pathogen responsible for severe hospital-acquired infections, especially in critically ill and immunocompromised patients. *P. aeruginosa* is the main pathogen of cystic fibrosis and is also involved in a variety of infections, including respiratory and urinary tract infections, wound and soft tissue infections, and infection of patients with thermal injuries (Fuentefria *et al*., 2011; Slekovec *et al*., 2012; Santoro *et al*., 2015; Rostami *et al*., 2018).

P. aeruginosa are essentially resistant to many antibiotics and are able to easily acquire antibiotic resistance determinants (Feng et al. 2017; Imanah *et al*., 2017). Moreover, P. aeruginosa has a high potential to develop multidrug-resistant phenotypes (Golle *et al*., 2017). The presence of diverse resistance mechanisms has an important clinical influence, as it limits therapeutic options for P. aeruginosa infection, impairs the effectiveness of antiapoptotic agents, and makes it very difficult to treat P. aeruginosa infection (Feng et al. 2017; Azam and Khan 2019; Rocha *et al*., 2019). P. aeruginosa strains have been described to be resistant to a wide range of presently available antimicrobial agents, such as fluoroquinolones, but also third-generation cephalosporins and carbapenems, which are chosen options in the treatment of serious infections caused by MDR strains (Imanah *et al*., 2017; Azam and Khan 2019). It is well known that infections caused by multidrug-resistant (MDR) Gram-negative bacteria, especially MDR *P. aeruginosa* have been associated with high morbidity, mortality, and costs (Paladino *et al*., 2002; Tuon *et al*., 2022).

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and the monobactam aztreonam. Infections with ESBL producing organisms have been associated with poor outcomes (Bryce *et al*., 2016; Bhoomika *et al*., 2016; Hashemi *et al*., 2018). One of the most

relevant mechanisms of resistance in *P. aeruginosa* that causes great clinical concern is the expression of extended-spectrum beta- lactamases (ESBLs), which confer resistance to penicillins, narrow and broad-spectrum cephalosporins (such as ceftazidime and cefotaxime), and monobactams (Canton *et al*., 2012). Many of the genes encoding ESBLs are hosted on plasmids that facilitate the transfer among bacterial species (Alonso *et al*., 2017; Dandachi *et al*., 2018). Multiple genetic mechanisms are implicated in the acquisition and dissemination of AMR. The *P. aeruginosa* strains have a variety of mobile and mobilizable genetic elements, including plasmids, transposons, insertion sequences, and different classes of integrin genes such as class I, class II and class III (Gillings, 2014).

Hence, the study of antibiotic resistance patterns of microorganisms is considered very crucial to guide clinicians in empirical therapy during life-threatening infections. Moreover, resistance patterns differ from one region to another and change continuously over time even in the same place which highlights the need for local annual resistance surveillance (El-Azizi *et al*., 2005; Moremi *et al*., 2016).

2.3 P. aeruginosa Pathogenicity

P. aeruginosa is one of the opportunistic human pathogens that preferentially infects patients with cancer or Acquired immunodeficiency syndrome (AIDS), immunocompromised patients by surgery, cytotoxic drugs or burn wounds, people with cystic fibrosis (CF), eye, ear and UTIs (Senturk *et al*., 2012).

The virulence factors facilitate adhesion and/or disrupt host cell signaling pathways while targeting the extracellular matrix. *P. aeruginosa* is capable of causing several diseases invading the organism and its

immune system leading to infections nearly impossible to eradicate (Skariyachan *et al.*, 2018). The expression of several virulence factors in *P. aeruginosa* depends on environmental conditions, including temperature, iron and oxygen levels, and osmolarity. The virulence of *P. aeruginosa* PAO1 is affected by the iron and oxygen-regulatory gene encoding the alternative sigma factor PvdS, which is regulated through the regulator of iron uptake (Fur). The iron regulator Fur contributes to the regulation of gene expression of several virulence factors by its downstream effect on the regulators of virulence factors through the production of the alternative factor sigma PvdS (Paula *et al*., 2001; Gaines *et al.*, 2007). PvdS belongs to the extracytoplasmic factor class of regulatory proteins. PvdS in turn regulates additional virulence genes such as ToxA (which encodes exotoxin A) and other regulatory genes, including *regA* and *ptxR* (Gaines *et al.*, 2007). Moreover, iron starvation factor sigma PvdS directs the transcription of pyoverdin siderophore (*pvdD*) genes. DNA protein binding analysis using recombinant PvdS showed that the PvdS-RNA polymerase holoenzyme complex specifically binds the *ToxA*, *regA*, and *ptxR* promoter regions. All three promoters contain a PvdS-binding site, the iron starvation box (Gaines *et al.*, 2007).

Among the pathogenicity caused by virulence factors, can be cited lipopolysaccharide (LPS), flagellum that participates in mobility, adherence, and internalization events, type IV pili that help bacterial adherence to the respiratory epithelial cells, type III secretion system, exotoxin A (inhibition of host protein synthesis), proteases, alginate, siderophores, quorum sensing, biofilm formation, type VI secretion systems, oxidant generation in the lung (Pier and Ramphal, 2005; Skariyachan *et al*., 2018). These are major virulence factors acting in different manners in the immune system. The LPS is a predominant

component of the outer membrane of *P. aeruginosa* (Figure 2-1) that induces cytokine production. Bacterial LPS typically consists of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating core oligosaccharide, and a distal polysaccharide (or O-antigen) (Pier and Ramphal, 2005).

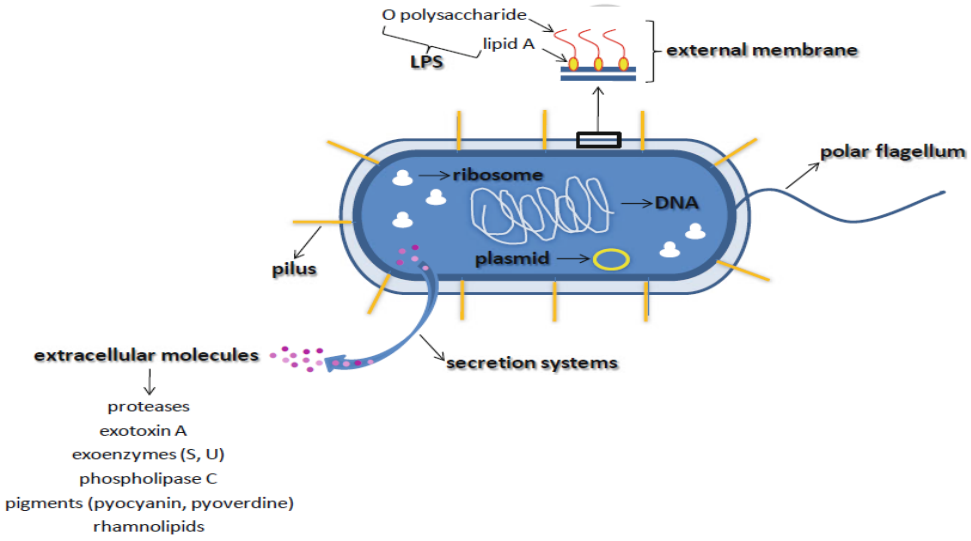


Figure (2-1). Virulence factors of *P. aeruginosa* (Galdino *et al.*, 2017).

Several extracellular proteases produced by *P. aeruginosa* such as metalloproteases (alkaline protease and elastases) destroyed host tissue; thus, it plays a significant role in both acute lung infections and burned wound infections (Bielecki *et al.*, 2005; Tuon *et al.*, 2022). As well as it produced exoenzyme S which induces a cytotoxic effect, exoenzyme U has an antiphagocytic effect, phospholipase C cleavage of membrane phospholipids, pigments that have many biological effects, like pyocyanin that induces free radicals in host cells, rhamnolipids act as detergent action, soluble lectins cause inhibition of beating of lung cells (Galdino *et al.*, 2017). Already the virulence factors alginate can produce a mucoid exopolysaccharide capsule, comprised of alginate, an acetylated random co-1-4 linked D-mannuronic acid (Poly-M) and L-guluronic acid (Pier *et al.*, 2001). The evidence of playing this role is believed due to the

overproduction of alginate in cell adherence within the CF in the lung and is thought to be involved in resistance to host defense by reducing susceptibility to phagocytosis, antifungal action, and host immune responses (Diggle *et al*., 2006).

Other mechanisms of virulence in *P. aeruginosa* such as antibiotic resistance (pump efflux and modifying enzymes) and cytotoxicity (hydrogen cyanide (HCN), exotoxin A, T3SS, and pyocyanin) (Tam *et al.*, 2010; Tuon *et al.*, 2022)

2.4. Proteolytic Enzymes of *P. aeruginosa*

A proteolytic enzyme also termed a protease, proteinase, or peptidase, is, a group of enzymes that break down polypeptide or protein molecules into shorter peptides and eventually into their components, amino acids. Proteolytic enzymes are found in bacteria, archaea, certain types of algae, some viruses, and plants; They are more abundant, however, in animals (Gupta *et al*. 2002; Verma *et al*. 2011; Masi *et al*. 2015).

There are different types of proteolytic enzymes, which are classified according to the active sites at which they catalyze the cleavage of proteins. The two main groups are exopeptidases, which target the terminal ends of proteins, and endopeptidases, which target sites within proteins. Endopeptidases use different catalytic mechanisms; Within this group are aspartate endopeptidases, cysteine endopeptidases, glutamic endopeptidases, metalloendopeptidases, serine endopeptidases, and threonine endopeptidases. The term oligopeptidase is for those enzymes that act specifically on peptides (Masi *et al*. 2015; Tuon *et al.*, 2022).

P. aeruginosa cells are capable of producing an enormous arsenal of virulence factors, particularly secreted molecules that act singly or in combination to ensure the establishment, maintenance, and persistence of a successful infection in susceptible hosts. In this context, the roles of *Pseudomonas* proteases are highlighted due to their ability to cleave key protein substrates of the host as well as modulate many biological processes, such as escape and modulation of bacterial host immune responses. At least eight proteases secreted by *P. aeruginosa*, including endopeptidases elastase A, elastase B (LasB), alkaline protease (AP), protease IV (PIV), *Pseudomonas* small protease (PASP), large protease A (LepA), MucD, and *P. aeruginosa* aminopeptidase (PAAP) that releases single amino acids from peptides (Galdino *et al*., 2017; O'Callaghan *et al*., 2019; Tuon *et al.,* 2022). These extracellular proteases are known to facilitate bacterial colonization by inducing damage to host tissue and actively subverting immune responses (Casilag *et al*., 2015; Tuon *et al.*, 2022).

2.4.1. Elastase A

An extracellular protease produced by *P. aeruginosa* is endopeptidases elastase A (LasA) encoded by *lasA*, also called staphylolysin or staphylolytic protease due to its ability to cleave the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus* (Barequet *et al.*, 2012). It is a metalloprotease belonging to subgroup A of the M23 family of staphylolytic or Beta-lytic zinc metalloendopeptidases. LasA has intracellularly synthesized as a pre-pro-elastase A protein with a molecular mass of 40 kDa (Ballok and O'Toole, 2013). Then it is secreted via the type II secretion system into the extracellular space, LasA is immediately converted to its mature and active form of 27 kDa due to the cleavage by other pseudomonal-secreted

endopeptidases, such as LasB, LysC, and protease IV (Wilderman *et al*., 2001; Kessler and Safrin, 2014).

2.4.2 Elastase B

One of the best proteases characterized in *Pseudomonas* is elastase B (LasB), also known as pseudolysin. This 33-kDa enzyme belongs to the M4 thermolysin-like family of neutral, Zn-dependent metalloendopeptidase. The first and the most studied substrate of elastase B is bovine and human elastin (Yang *et al*., 2015; Tuon *et al.*, 2022). This enzyme is encoded by *the lasB* gene as a pre-pro-protein, containing at the N-terminal region a signal peptide of 23 amino acids that transport the enzyme through the inner membrane to a periplasmic place by the bacterial secretory system (Hoge *et al*., 2010; Galdino *et al*., 2019).

LasB is able to cleave other host extracellular matrix proteins, such as elastin, collagen type III and IV, fibronectin, mucins, and components of the cellular junctions, such as vascular endothelial cadherin, inducing tissue injury, and bacterial dissemination (Flynn *et al*., 2017; Galdino *et al*., 2019). Elastase B can also interfere with the clearance of host bacteria by impairing several components of the innate and adaptive immune defense, including tumor necrosis factor A (TNF-a), interferon-c (IFN-c), and interleukin-2 (IL-2), monocyte chemoattractant protein 1 (MCP-1) and epithelial neutrophil-activating protein 78 (ENA-78) (Kuang *et al*., 2011; Nomura *et al*., 2014). In addition, LasB can inactivate key components of the complement system, such as fluid-phase and cell-bound C1 and C3 and fluid-phase C5, C8, and C9 (Bastaert *et al*., 2018; Tuon *et al.*, 2022), degrade other components of the immune defenses like interleukin 6 (IL-6) and interfere with bacterial killing by alveolar macrophages (Bastaert *et al*., 2018; Saint-Criq *et al.*, 2018). Furthermore, pseudomonal elastase can

interact with the host adaptive immune system by degrading Immunoglobulins, such as cleaving IgG molecules and its degradation products bound to IgG-receptors of human neutrophils, thereby inhibiting the opsonization of bacterial invaders (Lomholt and Kilian, 2008). Also, elastase B plays a key role in the differentiation of pseudomonal biofilms and it is essential for biofilm establishment (Yu *et al.*, 2014).

2.4.3 Protease IV

P. aeruginosa secretes a serine-type protease designated as protease IV (PIV) or lysyl endopeptidase (PrpL) or PvdS-regulated endoprotease, lysyl class. It encoded by *the piv* gene (PA4175) with molecular weight 26-kDa protease and related to the chymotrypsin family S1 that has been confirmed to be a significant virulence factor in the rabbit cornea but is originate in clinical isolates recovered from all the anatomical locations examined (Malloy *et al*., 2005; Hoge *et al*.,2010). The catalytic domain of PIV is formed by the triad His, Asp, and Ser. Moreover, it was verified that the residue Ser nearby to Ser is critical to the catalytic activity.

PIV contributes to tissue invasion/damage strategies and hemorrhagic activities due to the breakdown of fibrinogen. It is appropriately observed that fibrinogen is essential after vascular damage, however, the disruption of fibrinogen via PIV leads to hemorrhage during *P. aeruginosa* infection (Matsumoto, 2004). PIV is also vital to avoid host immune defenses due to the fact it is in a position to degrade plasminogen, immunoglobulin, C1q, and C3, and host antimicrobial peptide LL-37 (Ballok and O'Toole, 2013). Moreover, Malloy and co-workers (2005) found that PIV degrades the surfactant proteins, SP-A, SP-B, and SP-D, by way of a dose- and time-depended way in cell-free bronchoalveolar lavage fluid. Also, they indicated that

the destruction of the pulmonary surfactant proteins by protease IV reduced the connection between bacteria and alveolar macrophage. Remarkably, the incubation of pulmonary surfactant with pseudomonal protease IV decreased the potential of the surfactant to diminish the superficial tension inside the lung (Malloy *et al*., 2005). Protease IV has been exposed to be an iron-regulated protein, signifying that its expression is regulated regardless of the quorum sensing system, which is discrete from different pseudomonal proteases. Protease IV has additionally been correlated to ring abscess lesions existing in pseudomonal keratitis (Wilderman *et al*., 2001; Tuon *et al*., 2022).

2.4.4 *Pseudomonas* Small Protease

P. aeruginosa small protease (PASP) is described as an 18.5-kDa secreted zinc-dependent leucine aminopeptidase. *pasP* gene has been found in a large number of *P. aeruginosa* clinical strains, but its higher expression is found during ocular infection (Tuon *et al.*, 2022). According to Tang and co-workers (Tang *et al*., 2011), the sequence of the *pasP* gene appears to have a signal peptide consistent with that needed for the type II secretion system.

The *pasP* gene, like the gene for PIV, has been found in all *P. aeruginosa* isolates analyzed. Injection of purified recombinant PASP into the rabbit cornea causes severe ocular pathology, including epithelial and stromal erosions, edema, and neutrophil infiltration into the corneal stroma. PASP has also been shown to degrade host proteins important to the corneal structure, such as collagens, such as fibrinogen (but not fibrin), collagens, antimicrobial peptide LL-37and complement C3. Studies of PASP, coupled with those of PIV, powerfully provide the hypothesis that Pseudomonas proteases play a main role in keratitis (Tang *et al*., 2009, Tang *et al*., 2013; Tuon *et al.*, 2022).

2.4.5 Large Exoprotease A

P. aeruginosa produced another extracellular protease called large exoprotease A (LepA) with a molecular mass of about 100 kDa. LepA like thrombin and trypsin could cleave human protease-activated receptors (PARs) 1, 2, and 4 for activating the critical transcription factor NF-kB, which is associated with host inflammatory and immune responses (Kida *et al*., 2008; Kida *et al*., 2011).

LepA such as thrombin and trypsin can partition human proteasome-activated receptors (PARs) 1, 2, and 4 to activate the critical transcription factor NF-jB, which is associated with host immune and inflammatory responses (Tuon *et al.*, 2022).

2.4.6 Aminopeptidase

P aeruginosa aminopeptidase (PAAP) releases single amino acids from peptides and proteins, also known as *P. aeruginosa* leucine aminopeptidase (PA-LAP), or PepB which is identical to the name of the aminopeptidase coding gene, *pepB* (Galdino *et al*., 2019).

The *P. aeruginosa* aminopeptidase (PAAP) or leucine aminopeptidase has been speculated as a complementary enzyme to the activity of other endopeptidases. PAAP has an important function in bacterial physiology; it acts by releasing free amino acids/small peptides from protein fragments produced by the other *P. aeruginosa* endopeptidases, thereby providing low molecular mass nutrients that can be taken up by the bacterium, which in turn may promote bacterial growth and proliferation (Kessler and Safrin, 2014).

PaAP of *P. aeruginosa* is a heat-stable enzyme and a solvent-stable aminopeptidase. It is preferentially cleave N-terminal leucine

residues and lysine (Gaur *et al*., 2013; Wu *et al*., 2014), as well as an aminopeptidase that can cleave a synthetic insecticide called β-Cypermethrin (Tang *et al*., 2017; Galdino *et al*., 2019).

2.4.7 MucD

MucD was reported to be a serine endoprotease that is localized within the periplasmic space. Data suggest that MucD induced a significant reduction in the levels of IL-1b, neutrophil-chemoattractant chemokines KC, and macrophage-inflammatory protein-2 (MIP-2) in the early stages of bacterial infection as well as inhibited the recruitment of polymorph nuclear (PMN) cells into the cornea. Furthermore, a decrease in PMN cells recruited to the infection site favored the establishment of infection by *P. aeruginosa*. MucD may be secreted to the extracellular space, interfering with the biological functions of cytokines and chemokines, but further investigation is needed to understand the mechanisms underlying the role of MucD in keratitis (Mochizuki *et al.*, 2014; Tuon *et al.*, 2022).

2.4.8 Alkaline Protease

Alkaline protease (AprA) of *P. aeruginosa* (EC 3.4.24.40), also known as aeruginolysin, is a zinc-dependent metalloendopeptidase. Aeruginolysin is homologous to 50-kDa metalloproteinases secreted by *Serratia marcescens* and *Dickeya dadantii*. AprA is belonging to the metzincin superfamily of metalloendopeptidase and is a member of the serralysin family (Tuon *et al*., 2022). AprA is encoded by *aprA* gene which is the portion of *the apr* gene cluster located within a sequence of a 7.4-kb DNA fragment containing all five genes including *aprA, aprI,* and the type I secretory system encoding genes (*apr D, apr E, and apr F*) that is conserved among *Pseudomonas* species, *Serratia marcescens, and Erwinia chrysanthemi* (Galdino *et al*., 2019).

An analysis of *P. aeruginosa* aeruginolysin, *S. marcescens* metalloprotease, and protease C (PrtC), i.e. one of the four serralysins secreted by *D. dadantii*, had shown that these proteins have a C-terminal secretion signal located within the last 50 amino residues necessary to be translocated and secreted by AprD, AprE, and AprF membrane proteins, which form the bacterial type I secretory system (T1SS). TISS composed of a tripartite complex formed by an ABC-transporter, a membrane fusion protein, and a TolC-like outer membrane protein. T1SS is encoded by three genes located directly upstream of the *aprA* gene. In addition, there is another gene, *aprI*, located directly downstream of the *aprA* gene encodes a highly specific inhibitor of the alkaline protease known as alkaline protease inhibitor (AprI) as shown in figure (2-2) (Gellatly and Hancock, 2013; Galdino *et al*., 2017; Galdino *et al*., 2019)

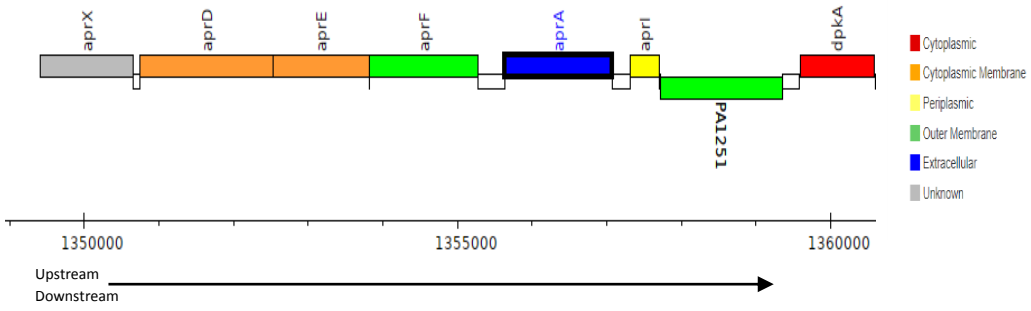


Figure (2-2): Alkaline protease gene cluster of *P. aeruginosa PAO1*
The *apr* gene cluster is composed of alkaline protease and alkaline protease inhibitor encoding genes (*aprA*, *aprI*), and the encoding genes of the type I secretory system include *aprF*, *aprE*, and a*prD*

The three-dimensional structure of the alkaline protease protein molecule exhibited two distinct structural domains. The enzyme is composed of an N-terminal catalytic domain of approximately 230 amino acid residues and a C-terminal beta-helix that is stabilized by binding calcium ions of about 240 amino acid residues. The N-terminal domain

is the proteolytic domain containing a zinc-binding active site motif HEXXHXUGUXH (X indicates arbitrary amino acid residues and U indicate bulky hydrophobic amino acid residues) and a conserved methionine located in a turn near the base of the metal binding pocket belongs to the astacin metalloprotease family. These domain homologs to the zinc-binding motif of a metalloprotease isolated from a European freshwater crayfish (Andrejko *et al*., 2019). The C-terminal calcium-binding domain consists of a 21-strand 3 sandwich known as the B sandwich domain (Fig. 2-3). Within this domain is a novel 'parallel (3 rolls' structure in which successive strands are wound in a right-handed spiral, and in which Ca^{2+} ions are bound within the turns between strands by a repetitive glycine-rich nanopeptide (which is a characteristic of repeat-in-toxin (RTX) proteins) and a secretion signal located within the last 70 residues. Pseudomonas aeruginosa alkaline protease possessing six of the RTX motifs with a consensus sequence X-(L/I/F)-X-G-G-X-G-(N/D)-D (where G indicates a glycine and X means any residue), revealed that the repeated sequences constitute a new type of calcium-binding binding structure (Fig. 2-3), and followed by a large hydrophobic residue. In this structure, the first six residues of each motif form a turn that binds calcium, and the remaining three residues build a short b-strand. This motif is found in a diverse group of proteins secreted by Gram-negative bacteria. (Baumann *et al.*, 1993; Miyatake , 1995; Andrejko *et al*., 2019).

There are structural changes around the active site when the substrate binds to the active site during the enzymatic reaction. In the uncoupling enzyme, Y216 acts as the fifth ligand for the active zinc ion site (Fig. 2-3). Upon binding to the substrate or inhibitor, Y216 may move to form a hydrogen bond with the carbon-oxygen of the P1 residue of the ligand peptide. D191 in the flexible loop, from Y190 to D196, above the active site cleft forms hydrogen bonds with the backbone atoms

of residues P1 and P2 from the bonding to close the entrance to the cleft. A water molecule that is the fourth ligand for the zinc ion is replaced by the carbonyl oxygen of the P1 residue (Miyatake *et al*., 1993).

Alkaline protease is important for phagocytic evasion and mainly interferes with fibronectin and laminin, two components of the endothelium (Galdino *et al*., 2017), and degrades complement proteins (C1q, C2, and C3) and cytokines (IFN-c, TNF-alpha and IL-6) allowing for phagocytic evasion (Laarman *et al*., 2012, Tuon *et al*., 2022). It contributes to the production of pyocyanin (and other virulence factors) (Butterworth *et al*., 2012; Tuon *et al*., 2022). It also cleaves free flagellin monomers and may reduce the mucociliary clearance of bacteria by activating the epithelial sodium channel (ENaC) (Bardoel *et al*., 2011; Butterworth *et al*., 2012)

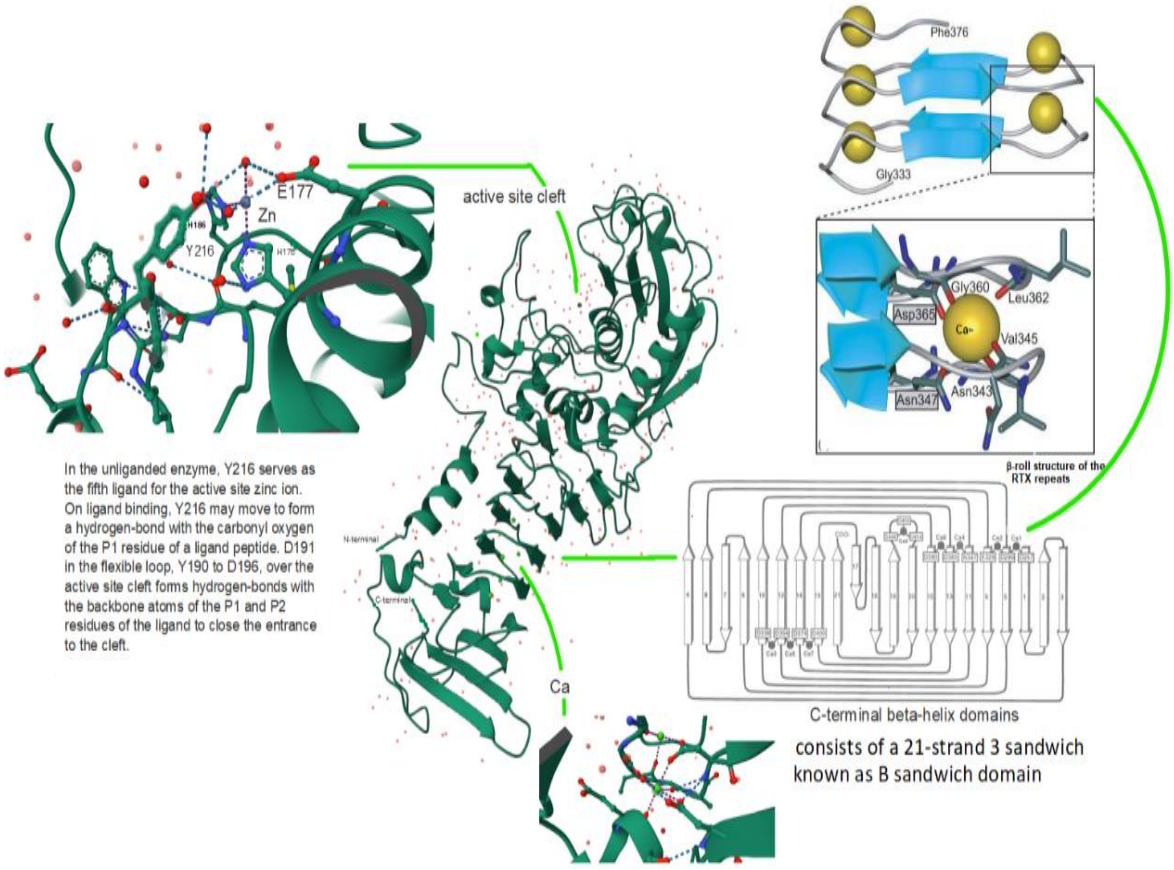


Figure (2-3). The tertiary structure of alkaline protease of *P.*

aeruginosa.

P. aeruginosa produces alkaline protease (AprA) as a virulence factor to improve its existence. AprA cleaves one of the key microbial awareness molecules, monomeric flagellin, and thereby diminishes Toll-like receptor 5 activation. The innate immune system plays an important role in the discovery and damage of microorganisms. Identification of very conserved bacterial molecules is essential to prevent bacterial invasion. The Toll-like receptor family identifies a wide-ranging variety of such molecules, and their activation triggers the creation of inflammatory cytokines and attracts cells of the immune system to the site of infection. The assembling block of all flagellated bacteria, flagellin, is recognized by Toll-like receptor 5 (TLR5) (Gellatly and Hancock, 2013; Galdino *et al*., 2017). Polymerized flagellin forms long filaments that are important for bacterial motility and virulence. TLR5 identifies a domain in flagellin that is required for filament assembly and motility. Recognition of the highly conserved flagellin by the innate immune system enables efficient clearance of a wide range of microorganisms (Galdino *et al*., 2019; Tuon *et al.*, 2022).

2.5 Alkaline Protease Inhibitor (AprI)

P. aeruginosa encodes a very strong inhibitor of alkaline protease (AprI) that is completely located in the periplasmic space, it is presumed to protect periplasmic proteins in opposition to secreted AprA. Structural and mutational studies reveal that the conserved N-terminal residues of AprI occupy the protease active site and are essential for inhibitory activity (Gellatly and Hancock, 2013; Galdino *et al*., 2017).

The exact biological function of this inhibitor remains unknown since alkaline protease is a secreted protein and Apr I is foretold to be a periplasmic protein. Putatively, Apr I is part of a safety mechanism that

protects self-proteins from degradation by Apr A before secretion (Galdino *et al*., 2019). Apr A and Apr I form a strong enzyme–inhibitor complex with a dissociation constant of 4 pM. In the crystal structure of this bimolecular complex, AprI contacts the Apr A through its N-terminus (residues 1–5) as shown in figure (2-4), the β-turn connecting β-strands s4 and s5, and β-strand s3. The N-terminal trunk of Apr I protrudes from the typical β-barrel structured protein and occupies the active-site cleft of Apr A as shown the figure (2-4) (Galdino *et al*., 2019; Tuon *et al.*, 2022). Additionally, the first AprI residue, a serine (S1), coordinates the catalytic zinc ion and, putatively, may contribute significantly to the high affinity of the inhibitor toward the protease (Fig. 2-4). The importance of the N-terminus is further illustrated by the decreased affinity of AprI lacking the first two to five N-terminal residues, whereas no complex is formed upon deletion of the sixth residue as well. The interactions between the β-barrel of the inhibitor and *AprA* may be crucial in the exact positioning of the extended N-terminal segment such that the zinc is chelated properly, and further insertion of the N-terminal segment and subsequent proteolysis is prevented. As a very strong inhibitor of the important virulence factor *AprA*, the protease inhibitor *AprI* may provide a basis for the development of molecules derived from this natural inhibitor that facilitate the treatment of *P. aeruginosa*-related diseases (Tuon *et al.*, 2022).

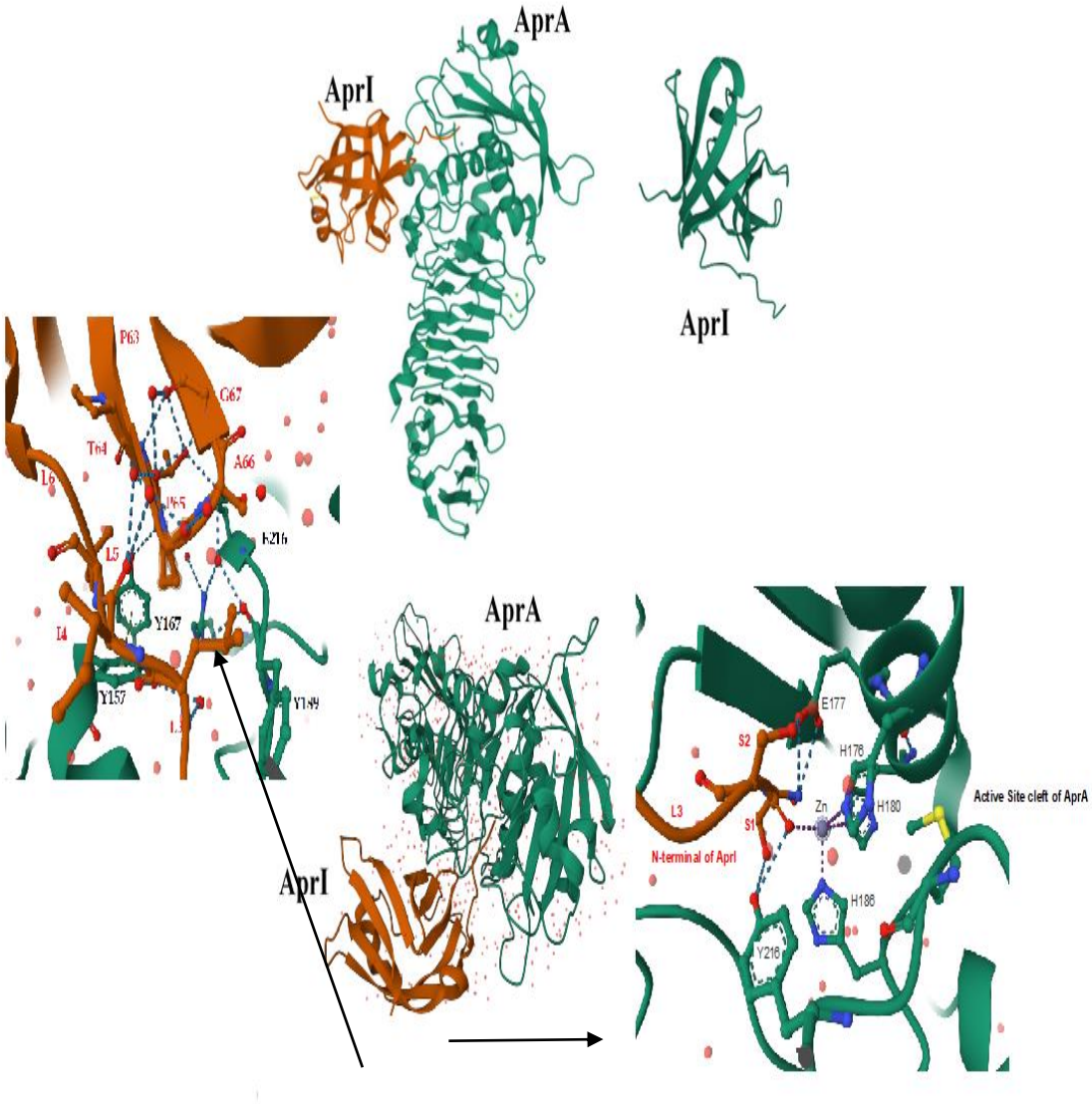


Figure (2-4). Interaction of Apr I with Apr A of *P. aeruginosa.*

2.6 Analysis of the Type I Secretion System of AprA *P. aeruginosa*

Nowadays, six different types have been identified which are recognizable by the characteristics of proteins/components forming the secretion machine. These are called type I to type VI secretion systems (T1SS–T6SS). The secretion systems could also be split into two

categories. Those that transport proteins at once across the bacterial cell envelope, thus directly from the cytoplasm to the cell surface, are known as a one-step secretion mechanism. Alternatively, the secreted proteins could transit through the periplasm before crossing the outer membrane. These systems are known as the two-step secretion mechanism (Durand *et al*., 2009; Filloux, 2011).

Type I secretion systems (T1SS) are widespread in Gram-negative bacteria, especially in pathogenic bacteria, and they secrete adhesions, iron-scavenger proteins, lipases, proteases, or pore-forming toxins in the unfolded state in one step across two membranes without any periplasmic intermediate into the extracellular space (Jacob *et al.*, 2017).

The exoprotease is secreted through a type I secretion system (TISS) consisting of a ternary complex consisting of an ABC transporter, a transmembrane fusion protein, and a TolC-like outer membrane protein. T1SS ABC transporters operate by a distinct mechanism with the unfolded polypeptide being processively moved through a pore formed by the transporter's TM domains (TMDs) (Thomas *et al.*, 2014; Kanonenberg *et al*., 2018). The three components of T1SS assemble into a complex spanning both membranes and providing a conduit for the translocation of unfolded polypeptides (Fig.2- 5). The ATP hydrolysis and assembly of the entire T1SS complex are necessary for protein secretion. T1SS substrates tend to be quite acidic and contain a calcium-binding "repeats-in-toxin" (RTX) domain, and both of these features are implicated in energizing secretion (Bumba *et al*., 2016; Locher, 2016; Kanonenberg *et al*., 2018).

T1SS substrates must be unfolded to be translocation competent (Bakkes *et al*., 2010), so the length of an unfolded T1SS substrate would far exceed the length of the transporter itself. These substrates vary

greatly in size and function and include proteases, toxins, and lipases. A conserved feature of substrate proteins is the presence of one or more RTX isoforms. The alkaline protease of *P. aeruginosa* contains six RTX isoforms and calcium regulates the multiple conformations of the enzyme. Calcium-induced RTX domain folding serves to chaperone the folding of the protease domain. Also, the studies found the disruption of the calcium-binding sites alters both the affinity and cooperativity of calcium-induced folding. The measuring of the RTX domain and in the full-length protease, and that the binding sites are not isoenergetic. Protein secretion was efficient when the passenger domain was maintained in an unfolded conformation and secreted into the medium with high calcium concentrations. Secretion efficiencies decreased with mutations in the RTX domain and with passenger domains that were stable and folded in the bacterial cytoplasm. Calcium may facilitate protease secretion via regulating protease conformation and may contribute to secretion efficiencies by maintaining specific protein conformations during translocation. The foundations for understanding the RTX proteins involved in calcium secretion from multiple bacterial pathogens will help devise ways to control bacterial virulence factors, inhibit their secretion of proteins that contribute to host tissue destruction, and reduce infection (Linhartova *et al*., 2010, Morgan *et al*., 2017).

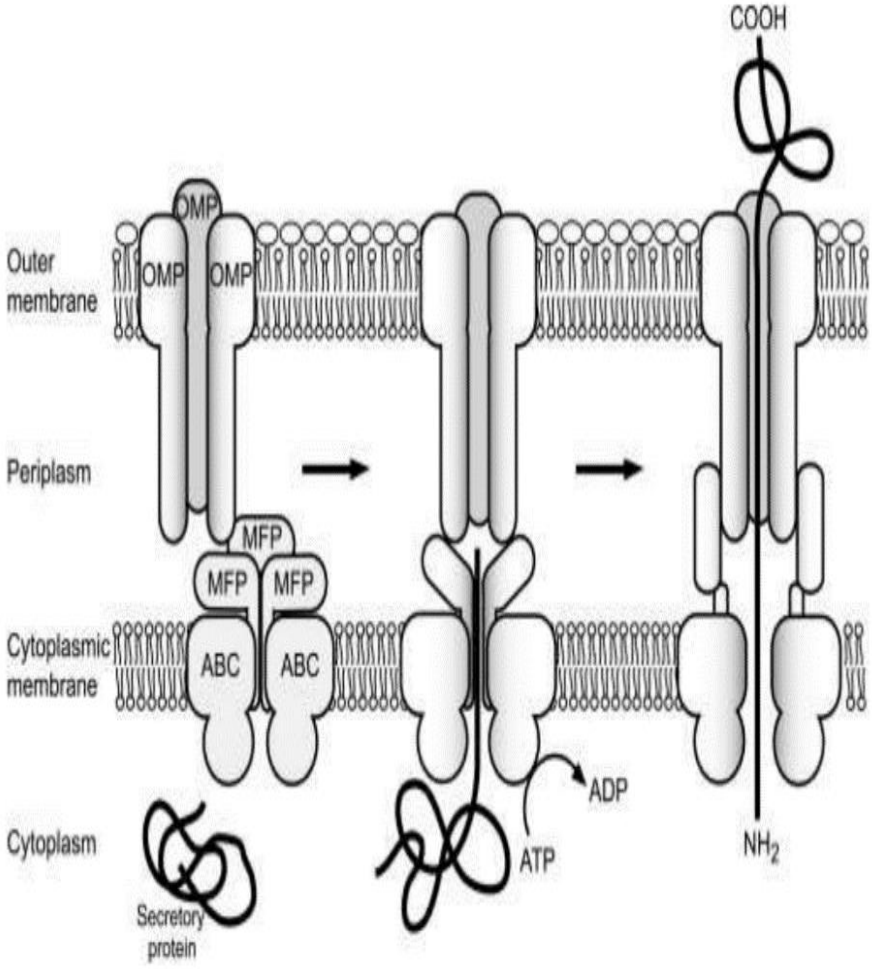


Figure (2-5). The schematic depiction of the TISS assembly operation.

Upon recognition of a C-terminal secretion signal on a given RTX protein translocation substrate, the inner membrane complex formed by an energy-providing ABC transporter and an MFP contacts the trimeric OMP. A sealed channel–tunnel assembly spanning across the entire Gram-negative bacterial cell envelope is formed, through which the RTX protein is exported in a single step from the bacterial cytoplasm directly to the external bacterial surface, without transiting through the periplasmic space. While concentrations of Ca^{2+}ions are typical of 100 nM in bacterial cytoplasm, allowing for the maintenance of an unfolded RTX domain, millimolar calcium concentrations are typically encountered in host extracellular space colonized by pathogenic bacteria. Loading of RTX repeats of the secreted protein by Ca^{2+}ions then promotes its folding and acquisition of biological activity (Linhartova *et al*., 2010, Morgan *et al*., 2017).

It was previously shown that the information encoded by the *aprA* structural gene is not sufficient to translocate AprA across both membranes of the cell envelope. A 6.6-kb DNA fragment, adjacent to *aprA*, appeared to encode several secretion functions. Three genes *apr D*,

E, and *F* are probably organized in a single operon. The stop codon of *aprD* overlaps with the start codon of *aprE.* The intergenic regions between *aprE and aprF, aprF and aprA, and aprA and aprl* are 3 bp, 357 bp, and 250 bp long, respectively. No obvious terminator structures were found in these regions. No inverted repeats could be identified between *aprF* and *aprA* (Galdino *et al*., 2019).

The first gene, *apr D,* codes for ABC transporter which is a protein of 593 aa with a deduced size of 63.7-kDa. The hydropathy profile of the amino acid sequence indicates that AprD lacks a signal peptide but possesses four hydrophobic domains corresponding to putative transmembrane segments, characteristic of inner membrane proteins (Higgins and Sharp, 1988). A homology search with the CLUSTAL4 program showed extensive homology (52% identity) of this protein with PrtD, one of the secretion proteins in *E. chryanthemi* (Lktoffi *et al.*, 1990). Some similarity was also found with HlyB of *E. coli* (Hess *et al.*, 1986). A consensus for the ATP-binding site (GXXGXGKS) is located in the C-terminal region of AprD, similar to PrtD and HlyB. These two proteins are members of both eukaryotes and prokaryotes subfamily of proteins involved in the export of various molecules. The MFP (membrane fusion protein) encodes by *apr E* and it contacts the trimeric OMP (outer membrane protein) encoded by *apr F*. Proteins of the MFP family function as auxiliary proteins or 'adaptors', connecting a primary porter in the cytoplasmic membrane of a Gram-negative bacterium with an outer membrane factor (OMF) protein that serves a porin or channel function in the outer membrane (Touzé *et al*., 2004). Thus, in conjunction with an MFP and an OMF, the primary porter in the cytoplasmic membrane pumps molecules out of the cytoplasm, across both membranes of the cell envelope into the external milieu without equilibration with solutes in the periplasm (Krishnamoorthy *et al.*, 2008).

Most MFPs are about 350-500 residues and probably either span the cytoplasmic membrane once at their N-termini or are anchored to the cytoplasmic membrane via a lipoyl moiety. These proteins cluster in the phylogenetic tree into subfamilies per the type of cytoplasmic membrane transport system [MFS; RND or ABC)], with which they interact (Yoneyama *et al.*, 2000).

2.7. Exotoxin A of *P. aeruginosa*

Pathogenic *P. aeruginosa* has virulence factors that increase infection severity such as Exotoxin A is the most prevalent and toxic virulence agent among pathogenic *P. aeruginosa* species that acquire ADP-ribosyltransferase activity (Douzi *et al*., 2012; Nader, 2019). This is the reason for the high mortality rates among experimentally infected animals in which a single 80 ng injection was sufficient to induce acute necrosis and swelling of the liver and hemorrhage in the lungs and kidneys within 48 hours of exposure (Wretlind and Pavlovskis 1981; Morgan *et al.*, 2021).

Pseudomonas exotoxin A (PE) has an enzymatic activity and belongs to the the mono-ADP-ribosyltransferase family (Liu, 1974). With regard to its function it is specified as NAD+-diphthamide-ADP-ribosyltransferase (EC 2.4.2.36) (Domenighini and Rappuoli, 1996). Later, it characterized as two-component AB toxin family, containing an A domain with enzymatic activity and a B domain as cell binding subunit (Odumosu et al., 2010), with respect to its function, has been identified as NAD^+-diphthamide-ADP-ribosyltransferase (Michalska and Wolf, 2015). Chromosomally *toxA* gene of *P. aeruginosa* expressed to *Pseudomonas* exotoxin A (PE) as a single pro-protein chain of 638 amino acids with 66 kDa molecular weight contains a highly hydrophobic leader sequence of 25 amino acids at its N-terminal and its removed during secretion. A mature toxin of 613 amino acids secreted into its

extracellular environment or the culture medium (Siegall *et al.*, 1989; Michalska and Wolf, 2015).

X-ray crystallography studies of functional or mature of *Pseudomonas* exotoxin A revealed that the toxin molecule consists of three domains. The N-terminal receptor-binding domain is the first domain that consists of two non-adjacent regions, Ia (1-252 aa) and Ib (365-404), which is composed of antiparallel ßsheets. The second domain is the membrane translocation domain (253-364 aa) with six consecutive α-helices, enables the toxin to translocate across cell membranes and the third domain is the ADP-ribosylation of elongation factor 2 (405-613 aa) at the C-terminus of the polypeptide (Figure 2-6). There are also four disulfide bridges, two located in domain Ia, one in domain Ib, and one in domain II (Siegall *et al*., 1989; Michalska and Wolf, 2015).

Pseudomonas Exotoxin A is secreted into the extracellular medium via the general secretory pathway, a two-step mechanism, which is highly conserved in Gram-negative bacteria (Voulhoux *et al.*, 2000; Gerard-Vincent *et al.*, 2002). After cytoplasmatic expression as an unfolded precursor protein, PE is initially transported to the periplasm using the Sec machinery (Douzi *et al.*, 2012). During translocation through the inner membrane, the N-terminal signal peptide is cleaved off and PE is released into the periplasmatic space. In the hydrophilic environment of the periplasm, PE is folded to a mature conformational protein in a manner that can be recognized by the type II secretion system (T2SS), specifically called Xcp in *P. aeruginosa*, for secretion into the extracellular space (Voulhoux *et al.*, 2000; Gerard-Vincent *et al*., 2002). Mutagenesis experiments gave evidence that two N-terminal glutamic acid residues at the +2 and +3 positions of domain Ia as well as domain II of PE are important for folding and extracellular secretion (Lu *et al.*, 1993). It is therefore speculated that the corresponding residues are part

of a still unknown conformational secretion signal of PE for recognition by T2SS or that they are important for the appropriate presentation of such a signal (Lu *et al.*, 1993; Voulhoux *et al.*, 2000). Once secreted, the terminal lysine (aa 613) of PE can be cleaved from the toxin in the extracellular environment, presumably by plasma carboxypeptidases of the host. This leads to a formation of a C-terminal motif from REDLK (aa 609–613) to REDL (aa 609–612), which enables the toxin to bind to KDEL receptors at the Golgi apparatus during subsequent intracellular trafficking (Hessler and Kreitman, 1997). On the host cell surface, PE specifically binds via domain Ia to CD91, which is also known as alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α2MR/LRP); Kounnas *et al.*, 1992; Yates and Merrill, 2004). Then, there are two pathways open for PE to reach the Endoplasmatic Reticulum: the KDELreceptor mediated pathway and the lipid-dependent sorting pathway. Upon endocytosis, PETA between Arg279 and Gly280 is cleaved by furin (a subtilisin like cell surface serine proteases widely distributed in the human body) in endosomes to generate a 37 kDa C-terminal enzymatic fragment, which is then transported into the cytosol (Zhaf and Schmidt, 2017). Once inside the cytosol, the enzymatically active C-terminal domain catalyzes the ADP-ribosylation and inactivation of EF2, which inhibits protein synthesis within the affected cell through its inability to lengthen polypeptide chains, and then irreversibly causes cell death (Jørgensen *et al.,* 2005; Dieffenbach and Pastan, 2020). This mechanism is similar to the action of diphtheria toxin but the difference is only limited to the respective receptors scattered on the cell surface to those of diphtheria toxin (Xing *et al*.,2010; Michalska and Wolf 2015).

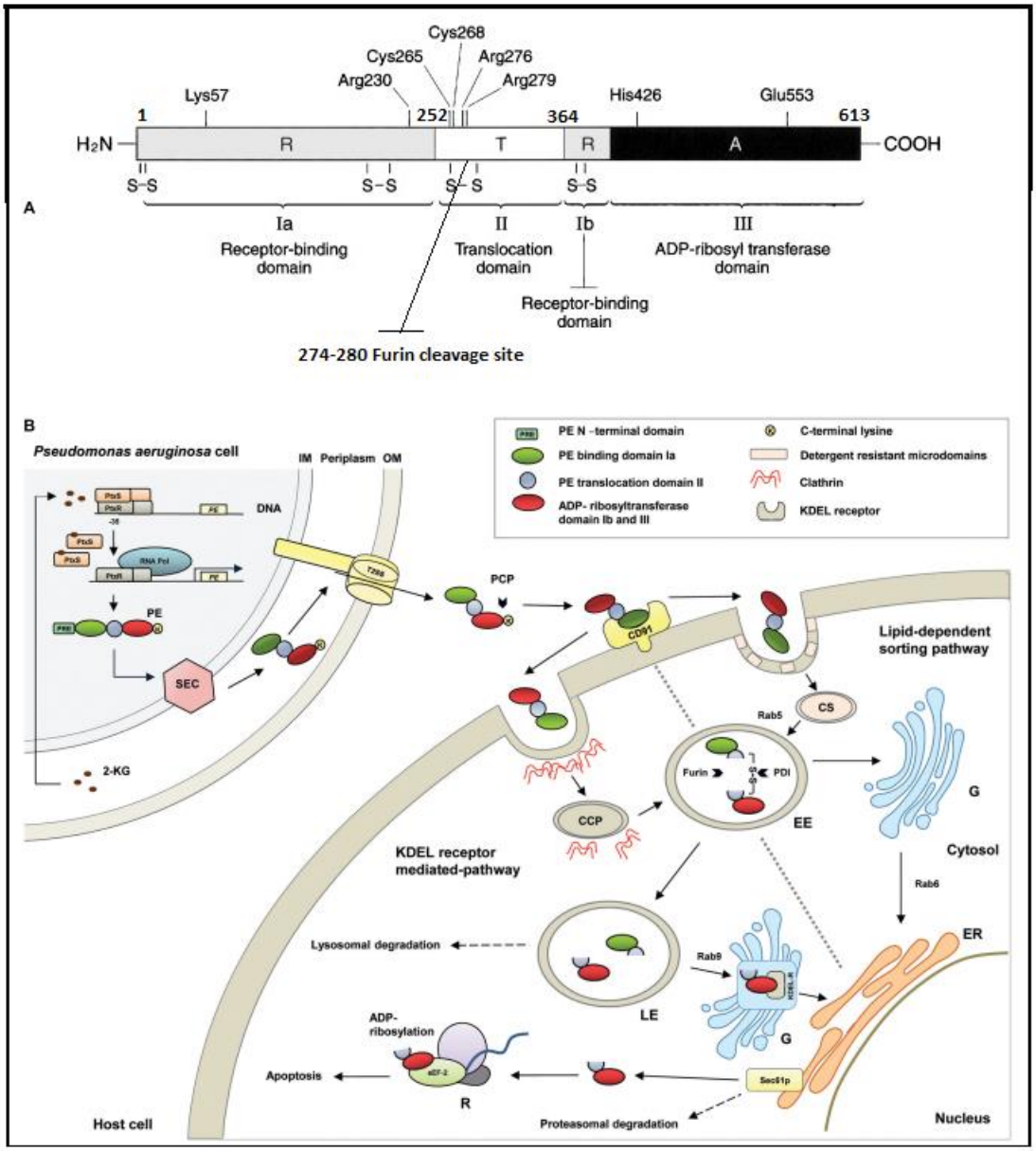


Figure (2-6). Structure and pathways of Exotoxin A of *P. aeruginosa*

(A) Schematic representation of the structural and functional domains of Pseudomonas Exotoxin A (PE). The enzymic domain (A) is shown in black, while the receptor-binding (R, Ia and Ib) and the translocation (T) domains are colored grey and white, respectively. The more relevant mutants affecting either cell binding or enzymic activity are reported in the upper portion of the figure. Disulfide bridges are also shown (Masignani *et al.*, 2006).

(B) Molecular pathways of PE. 2-KG, 2-ketogluconate; CCP, clathrin coated pit; CD91, CD91 receptor; CS, caveosome; EE, early endosome; eEF-2, eukaryotic elongation factor-2; ER, endoplasmatic reticulum; G, Golgi apparatus; KDEL-R, KDEL-receptor; PCP, plasma carboxypeptidases; PDI, protein disulfide isomerase; PtxR, PtxS, transcription regulators; R, ribosome; Rab, Rab-GTPase; RNA Pol, RNA polymerase; Sec61p, Sec61p translocon; T2SS, type II secretion system (Michalska and Wolf 2015).

Genetic studies, mainly based on expression of mutant forms of the gene encoding Pseudomonas exotoxin A in Escherichia coli, have shown that deletion of domain Ia results in non-toxic, enzymatically active

molecules that cannot bind to cells. A similar result can be obtained by mutating Lys57 into Glu (Jinno *et al.*, 1988). Alteration in domain II structure such as deletions in domain II lead to cell-binding molecules that are enzymatically active but not toxic. Similar result can be obtained by mutating Arg276, Arg279 and Arg230 (Jinno *et al.*, 1989) or by converting Cys265 and Cys268 to other amino acids (Figure 2-6). Mutation or deletions in the domain III lead to enzymatically inactive molecules (Siegall *et al.*, 1989). Whereas deletion of at least 2 or as many as 11 amino acids from the carboxylate end of PE does not affect ADP-ribosylation activity but produces non-cytotoxic molecules. The last five amino acids (Arg-Glu-Asp-Leu-Lys: REDLK) of PE are very important for its cytotoxic action and any deletions or substitutions between positions 602–611 of PE resulted in a complete loss of cytotoxicity. Mutagenesis analysis indicates that the essential amino acid (Arg-Glu-Asp-Leu) at 609, 610,611 and 612, respectively, is required for full cytotoxic activity. Lysine at 613 can be deleted or replaced by arginine but not by any other amino acids (Armstrong *et al.*, 2002). The REDLK segment is very similar to the KDEL motif which is a well-defined endoplasmic reticulum retention sequence that is also found on the C-terminus of other ADP-ribosyltransferases, such as cholera toxin and the thermostable enterotoxin of *Escherichia coli*. (Chaudhary *et al.*, 1990). It has been hypothesized that the REDLK sequence may be the recognition signal required for entry into the ADP-ribosylation domain of PE in the cytosol. PE can be described as a typical bacterial toxin with an A-B structure, and has a similar mechanism of action as diphtheria toxin. Both DT and PE induce apoptosis in human mast cells by a caspase-3 and -8-mediated mechanism (Jenkins *et al.*, 2004).

The catalytic site was identified and Glu553 was found to play a major role in the enzymatic activity of PE as it was the only NAD+

labeled amino acid (Carroll and Collier, 1987). Substitution of Glu553 with any amino acid, including Asp, reduced enzymatic activity by a factor of 1000 (Douglas and Collier, 1990), and deletion of Glu553 completely abolished PE toxicity. Similarly, iodination of Tyr481, which is also present in the catalytic site, has been shown to abolish enzymatic activity (Brandhuber *et al*., 1988). Other amino acids that, although not located in the catalytic site, have been shown to be essential for enzymatic activity are His 426 and residues 405–408. His 426 has been proposed to be essential for the interaction between PE and EF2 (Galloway *et al.*, 1989).

Understanding the structural and functional domains of toxins and improving molecular cloning techniques have revolutionized the development of recombinant information technology. The development of chimeric toxins by replacing the gene parts encoding for domain I with others encoding for cell-binding domains with different specificities. So far, nucleotides encoding domain I have been replaced by sequences encoding interleukin 2, interleukin 6, interleukin 4, T cell antigen CD4 or a tumor-targeting cytokine or an antibody fragment (scFv) and a growth factor. In all instances, expression of these genes in *E. coli* has given new toxins, which specifically kill the cells bearing the receptor recognized by the new domain I. Such molecules are promising candidates for the treatment of arthritis and allograft rejection (PE-IL2), AIDS (PE-CD4), hepatitis B and cancer and other diseases (Bachran *et al*., 2007; Havaei *et al*., 2021 ; Khoshnood *et al*., 2022).

3.Materials and Methods

3.1 Materials

3.1.1 Instrument and Equipment

Table (3-1): The equipment and Instrument were used in this study

Apparatus and Tools	Company	Manufacturer (Origin)
Autoclave	SHANDONG TECHNOLOGY CO., LTD.	China
Benson burner	Satorins	Germany
Calibrated Loop	Himedia	(India)
Centrifuge	Hermle/Labor Technik	Germany
Conical flask	HAD CO.	China
Disposable and Glassware	Cito CO.	China
Disposable gloves	TG Medical	Malaysia
Eppendorf Centrifuge	Hettich	Hettich Germany
Eppendrof tube	Sigma	UK
Gel Electrophoresis System	Cleaver Scientific	UK
Incubator	Memmert	Germany
Laboratory Distillation Unit	Technology laboratory equipment GFL	Germany
Laminar Flow Hood	Cryste	Korea
Latex Gloves	Broche	Broche /Malaysia
Micropipette tips (different size)		Germany
Micropipettes (0.5 – 10 µl, 5-50 µl, 100-1000 µl)	IVYX Scientific American company	USA
Para film	Afco-Dispo	Jordan
PCR Thermal Cycler	Techne	UK
PCR tube 1.5 ml and 0.2 ml	Biobasic	Canada
Petri dishes	-----	disposable
Plain tube 10 ml	Afco-Dispo	Jordan
Refrigerator	LG company	India
Sensitive Balance	Sartorius	Germany
Sterilized cotton	Afco-Dispo	Jordan
Sterilized needles (5,3 ml)	Shanchuan	China
Swab	Lab. Servic	Spain
Tips	Sterellin Ltd	UK
UV-Vis Spectrophotometer	Shimadzu	Japan
Volumetric cylinder	HAD CO.	China
Vortex	Griffin	England
Water bath	Memmert	Germany

3.1.2 Biological and Chemical Materials

The chemical materials, kits, media and antibiotic disks were used in this study are listed in the followed tables (3-2) and (3-3).

Table (3-2): Chemical Materials and molecular kits

Chemical Materials	Manufacturer company (Origin)
Agarose	Condalab /Spain
DNA Loading Buffer Blue	Eurx/Poland
Ethanol absolute	J.T. Baker /Netherland
Glycerol	Sigma/ USA
Gram stain 's	BHD /England
Hydrogen peroxide 3%	BHD /England
Normal Saline	Mehico /India
Nuclease Free Water	Bioneer /Korea
Simply Red Safe	Eurx /Poland
NaCl (Sodium chloride)	BHD /England
Tris-Borate-EDTA Buffer	Condalab /Spain
Culture Media	
Brain heart infusion broth	Biolife/ Italian
Cetrimide Agar Base	HiMedia/ India
MacConkey agar	Biolife/ Italian
MR–VP broth	Biolife/ Italian
Muller Hinton agar	Biolife/ Italian
Nutrient agar	Biolife/ Italian
Nutrient broth	Biolife/ Italian
Peptone water	Biolife/ Italian
Tryptic soy agar	Biolife/ Italian
Tryptic soy broth	Biolife/ Italian
Molecular Kits	
DNA extraction Kit	Favorgen /Taiwan
DNA ladder	IntronBio /Korea
Green master mix	USA/ Promega
Primers	Macrogen /Korea

Table (3-3): Antibiotic Disks, Symbol, and Potency.

Types of Antibiotics	Antibiotic disks	Symbol	Potency (µg/disk)	Manufacture (Origin)
Aminoglycosides	Gentamicin Amikacin	CN AK	10 30	Bioanalyse/ Turkey
Carbapenems	Meropenem Imipenem	MEM IPM	10 10	Bioanalyse/ Turkey
Fluoroquinolones	Ciprofloxacin	CIP	5	Himedia/ India
Pencillins	Piperacilline	PRL	100	Bioanalyse/ Turkey

3.1.3 Primer Pairs

The primer pairs used in this study are listed in the table (3-4)

Table (3-4): Primer Pairs, Sequences, Product Size and References

Gene	Nucleotide sequence (5'→3')	Product Size (bp)	T_m	Reference
aprI	F:TTTGCTTGCTCTGCGGTTT R:CCCAGGTCGTAGCCACTG	175	59.54 59.42	Designed
aprA	F:TGCATTGAAAGGTCGTAGCG R:TGACATACTTCCACGGCGTA	239	58.92 58.82	Designed
aprF	F:TGGTGTACAGCTACAACCGT R:GGTATAGGCTTCCAGGACCC	242	58.96 58.94	Designed
aprE	F:GCCTTCAACCAGAGCAAGAC R:ACAGGGGCTTGAACAGGTAA	229	59.12 58.85	Designed
aprD	F:AGTCACCCTGTTGATGCTGA R:CCTGGCCGGTGATGAACT	230	58.94 59.33	Designed
toxA	F:GGAGCGCAACTATCCCACT R:TGGTAGCCGACGAACACATA	150	60 60	Sabharwal *et al* ., 2014

3.2 Study Design

The study design was showed in figure (3-1).

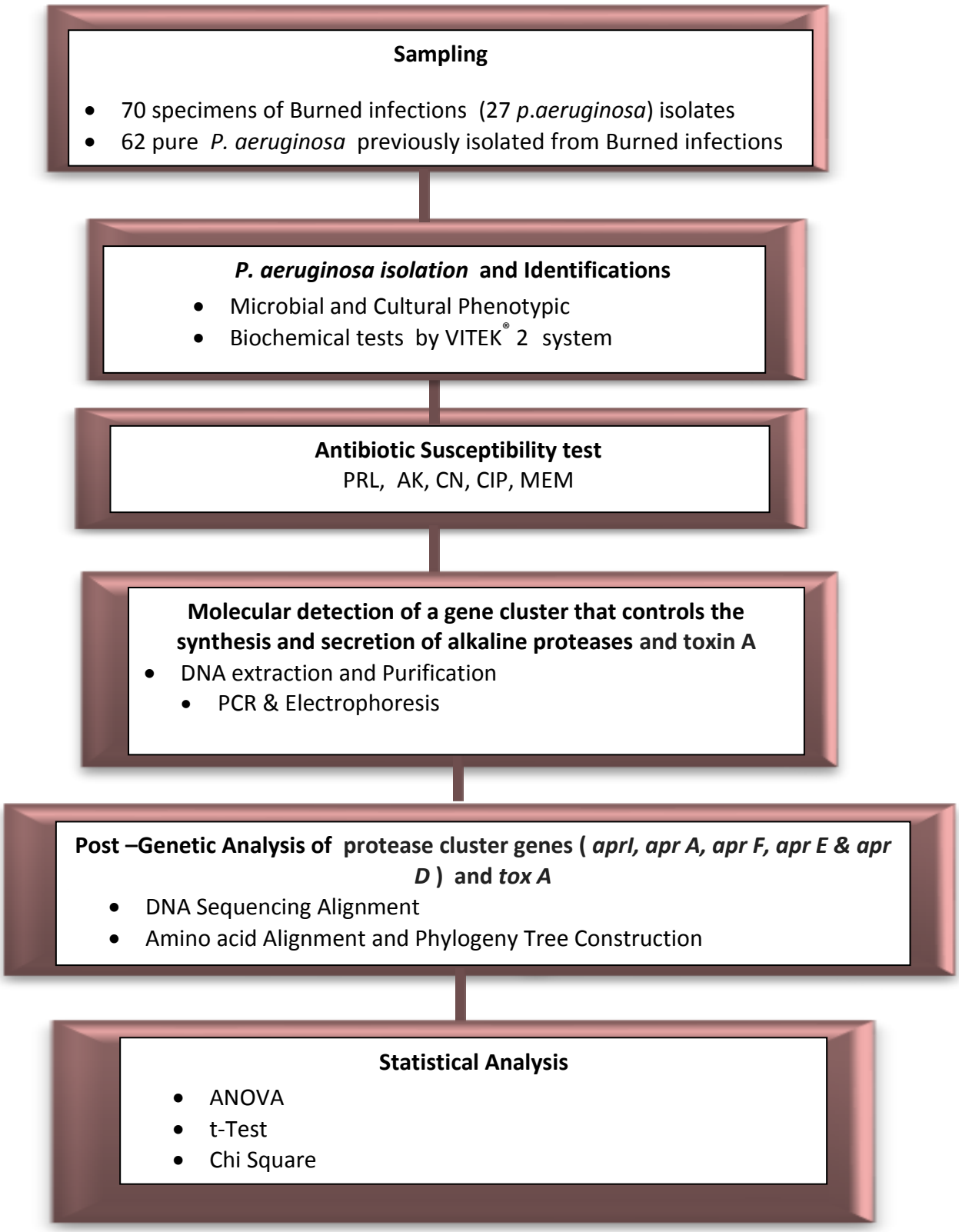


Figure (3-1): Flowchart of the study

3.3 Methods

3.3.1 Bacterial culture Media Preparations

Bacterial culture media used in the present study (Table 3-2) were prepared according to the Manufacturer's instructions and sterilized at 121°C (15 psi) for 15-20 min by autoclave .

3.3.2 Preparations of Solution and Buffers

The solutions and Buffers were sterilized at 121°C (15 psi) for 15-20 min by autoclave. or filtration (0.22 m Millipore filters). The base or acid solutions (1M NaOH or 1M HCl) were used to adjust the pH value of solutions before sterilization.

3.3.2.1 Turbidity Standard 0.5 McFarland's

The 0.5 McFarland standard tube was prepared by mixing 0.05 ml of 1.175% $BaCl_2.2H_2O$ with 9.95 ml of 1% H_2SO_4 to get barium sulfate precipitate. The bacterial suspension with 1.5×10^8 CFU/ml equal the turbidity of 0.5 McFarland's standard. The McFarland's standard tubes were tightly closed to prevent evaporation and stored in the dark at room temperature for 6 months. The accuracy of the prepared 0.5 McFarland's standard was verified using a spectrophotometer. The optical density (OD) should be between 0.08 and 0.1 when was measured at 625 nm (CLSI, 2019).

3.3.2.2 TBE Buffer

The Tris-Borate-EDTA (TBE) buffer was used in gel electrophoresis procedure for separating DNA molecules at the final concentration 1X TBE. Each 100ml of concentrated 10X TBE was diluted to 1X TBE by adding 900ml of sterile distal water (Green and Sambrook, 2012).

3.3.2.3 Loading Buffer

It was prepared by mixing Bromophenol blue (0.25%) and Sucrose (40%), then were kept at 4 °C (Sambrook and Rushell, 2001).

3.3.3 Specimens collection

The study included 70 swabs of burned infections collected from the hospitals of Babylon Province, during a period that extended from November 2021 to January 2022. These swabs were transferred in cooling box to Advanced Biotechnology Laboratory at Biology Department, College of Science, University of Babylon for bacterial isolation.

In addition to 62 pure *P. aeruginosa* isolates obtained from previous study were isolated from burned infections collected from hospitals in Babylon, Najaf and Karbala governorates during 2020-2021. These strains were previously identified using microbial, cultural and biochemical characteristic and were confirmed their diagnosis using VITEK® 2 system. After that these strains were preserved as glycerol stocks for long-term storage in freezing at -18°C in Advanced Biotechnology laboratory.

3.3.4 Bacterial isolation and identification

The isolating media such as blood agar and nutrient agar plats were inoculated with swab media of burned infections and incubated at 37°C for 24-48h. After that the single bacterial isolates were grown on MacConkey agar, Cetrimide agar and Chromoagar plates as a selective medium for isolation and identification of *P. aeruginosa* and incubated at 37 °C for 24 h. The pure pale- coloured (non-lactose fermented) colonies were then selected to subculture on the same medium for further purification.

On the other hand, the pure pseudomonad strains (62) were recovered from glycerol stocks by transferring a part of bacterial stock using a sterile loop, swabs and inoculated the Tryptic soy broth and incubated at 37°C for 24h. Subsequently, they were sub-cultured on Tryptic soy agar plats at 37°C for 24h. The pseudomonad strains were confirmed its purity by staining using Gram stain and re-cultured on selective media such as MacConkey agar, cetrimide agar and Chromoagar plats and incubated at 37°C for 24h.

All bacterial isolates were re-examined microscopically after staining by Gram stain to observe the color, size, shape, and arrangement of bacterial cells under a light microscope (Brooks *et al*., 2013; Tille, 2022). In addition to microbial and cultural features, the bacterial isolates were diagnosed according to the biochemical characteristics using VITEK® 2 system and they were preserved until used to complete the study.

3.3.5 Preservation of bacterial isolates

Bacterial isolates were preserved for a short period on nutrient agar plates and stored at 4 C° as daily -working stock and used for few days. The second stock as medium-term preservation up to four weeks was carried out by growing the bacteria on tryptic soy agar slant and stored at 4 C°. Also, Bacterial isolates were stored in a brain heart infusion broth containing 15% glycerol in deep freezing at -20C° for long term preservation (Tille, 2022).

3.3.6 Antibiotic susceptibility test

The Kirby-Bauer method according to CLSI (2020) were used to test different antibiotic susceptibility (Table 3-3) as follows:

A appropriate number of bacterial colonies from a pure and fresh

culture of *P. aeruginosa* were transferred to a sterile test tube containing 5 ml of normal saline and then it was compared with 0.5 McFarland standards (1.5×10^8 CFU/ml). A portion of bacterial suspension was transferred by a sterile cotton swab, a, carefully and consistently spread on Mueller-Hinton agar medium, then plates were left to dry. Antibiotic discs were positioned on the inoculated plate using a sterile forceps (duplicate was done for each antibiotic). Later the plates were incubated at 37ºC for 18-24 h . Depending on CLSI (2020), the inhibition zones around the disks were measured by millimeter (mm) using a metric ruler.

3.3.7 Detection of protease activity on skimmed milk agar plate

Bacterial isolate was grown on a skimmed milk agar plate (nutrient agar supplemented with 1% skimmed milk) for 48h and the clear area around colonies was measured in millimeters (mm) using a metric ruler. The ratio of area diameter / colony diameter was calculated.

3.3.8 Genomic DNA extraction

The bacterial genomic DNA was extracted using FavorPrep™ Genomic DNA Extraction Mini Kit according to the manufacturer's protocol. As follow:

1. The bacterial culture of each isolate was activated in LB broth for 18 h until growth reached a density of 10^9 CFU/ml. Approximately 1 ml of the broth was placed in 1.5 ml centrifuge tube and bacterial cells were precipitated at 14,000 rpm for 1–2 min by centrifuge. Pellets were resuspended by vortexing or pipettes in 1 mL of normal saline to wash bacterial cells and removing debris and then re-sedimented by centrifugation 14,000 rpm for 1–2 min.

2. Pellets were re-suspended in 200 µl of FATG buffer by vortexing and incubating for 5 at room temperature and then in 70°C for 10 min. During

incubation, the tube was inverted every 2- 3 min until the cell lysate became clear.

3. The elution buffer was pre-heated in water bath at 70°C which used in DNA elution step.

4. Up to 200 µl of 96-100% ethanol was added to the sample with vortexed for 10 sec, then the sample was mixed well by pipette to prevent any precipitate formed.

5. The FABG column was placed into the collection tube, then the sample mixture was carefully transferred to the FABG column, and centrifuged at 14,000 rpm for 5 min. The collection tube was discarded and the FABG column was placed in a new collection tube.

6. W1 buffer (400 µl) was added to the FABG column and centrifuged at 14,000 rpm for 30 sec. The flow-through was discarded and the FABG column returned to the collection tube.

7. The later step was repeated using 600 µl of ethanol-containing wash buffer instead of W1 buffer and centrifuged at 14,000 rpm for 30 sec. The flow-through was discarded and the FABG column returned to the collection tube.

8. The column was further centrifuged at 14,000 rpm for 3 min to discard any remaining liquid containing any inhibitors of subsequent enzymatic reactions.

9. The dry FABG column was transferred into a new 1.5 ml tube, and 100µl of preheated elution buffer or TE was loaded to the membrane center of FABG column to be completely absorbed.

10. The FAGB column was incubated at 37 °C for 10 min in an incubator . Then it was centrifuged for 1 min at a maximum speed of 14,000 rpm to elute the DNA . The DNA elution step was repeated for further DNA recovery to get 200 µl of DNA yield.

11. The concentration of genomic DNA (µg/ µl) and DNA purity (ABS_{260nm}/ABS_{280nm}) were measured by NanoDrop spectrophotometer, and then it was stored at −20 °C until use.

3.3.9 Molecular Detection for cluster genes of alkaline protease and other virulence genes by Polymerase Chain Reaction

3.3.9.1 Primer pairs preparation

Oligonucleotide primers were prepared by dissolving the lyophilized product by adding an appropriate amount of sterile ddH_2O depending on the instructions manufacturer to get 100 pmol/µl of the final concentration as a stock solution and stored in a deep freezer at -20 °C until use. The working primer solution was prepared by diluting the primer stock solution to 10 pmol /µl of the final concentration by mixing 10 µl of the stock solution with 90 µl of ddH_2O and kept in a deep freeze until use.

3.3.9.2 Reaction mixture of PCR

DNA amplification was accomplished with a final volume of 25 µl of reaction mixture as listed in the table (3-5).

Table (3-5): Reaction mixture components

Reaction mixture components	Volume (µl)
DNA template	4
Green master mix	12.5
10 pmol/µl of Downstream primer	2
10 pmol/µl of Upstream primer	2
Nuclease free water	4.5
Entire volume	25

3.3.9.3 Polymerase Chain Reaction (PCR)

Target DNA amplification was performed by conventional PCR using specific primer pairs for each studied gene previously mentioned in Table (3-3). The reaction was performed as three successive time steps including denaturation, annealing and extension as mentioned in Table (3-6). The amplified products were electrophoresed through an agarose gel at an appropriate time interval and were visualized and documented by the gel imaging system under UV light (254)nm

Table(3-6): PCR -Thermal Cycling conditions

Gene	Initial Denaturant. °C (sec)	Denaturant. °C (sec)	Anneal. °C (sec)	Extension °C (sec)	Final Extension °C (min)	Cycles
aprI	95 (2)	95 (30)	57.7 (30)	72(40)	72 (5)	35
aprA	95(2)	95(30)	57.7(30)	72(40)	72(5)	35
aprF	95(2)	95(30)	59.5 (30)	72(30)	72(5)	35
aprE	95(2)	95(30)	58.3(30)	72(30)	72(5)	30
aprD	95(2)	95(30)	57.8(30)	72(30)	72(5)	30
toxA	95(2)	95(30)	58.1(30)	72(20)	72(5)	30

3.3.10 Agarose Gel Electrophoresis

The integrity of the DNA molecules was verified by migration in agarose gel electrophorese. Agarose gel (1-2%) was prepared by dissolving agarose powder in 1X TBE buffer solution in the microwave for 1-3 min until all agarose particles were dissolved and the gel became clear. The agarose solution was cooled to about 50°C. An ethidium bromide dye or safe red dye was added to a final concentration of approximately 0.2-0.5 µg/mL (usually 1–2 µl of laboratory stock solution per 100 ml gel) and mixed well. The agarose gel was cast as a horizontal plate. Plastic combs were used to create wells into which DNA would be loaded. Agarose was gently poured into a gel tray with a fine comb in place. The gel is allowed to solidify and the gel tray is placed in a gel tank and filled with 1X TBE buffer. Prior to loading, 5 µl of the DNA sample was mixed with 1 µl of loading dye weighting the sample into solution, to prevent the DNA sample from leaving the well, and also includes a visible marker (Bromophenol blue dye) to track the progress of the run. Unknown DNA samples were often laden with running along with the DNA ladder. The electrodes are properly connected and an electric field is applied along the gel (4-5 V/cm) or at 80-100 V for 40-60 min (Green and Sambrook, 2012).

3.3.11 DNA sequencing of Amplified product

The amplified DNA was delivered to Macron sequencing corporation (Macrogen/Korea) according to Sanger method. The DNA sequence data of the studied genes were analyzed and aligned according to BioEdit and MEGA-X programs and compared with reference sequences available in the GenBank (NCBI) database for identification of polymorphisms and phylogenetic tree construction.

3.4. Statistical Analysis

Data for this study were statistically analyzed using SPSS version 19 by t-test, one-way ANOVA and Chi-Square test and the P value ($P \leq 0.05$) was statistically significant (Kirkpatrick and Brooke, 2015).

4.1 Bacterial Isolation and Identification

Of the 70 patients examined with second- and third-degree burns, 45 (64.3%) showed positive bacterial growth and 25 (35.7%) showed negative growth. Of the 45 positive cases, The dominant Gram-negative bacteria were *P. aeruginosa* 27 spesimens.

Although burn patients survive, complications of infection remain the leading cause of morbidity and mortality. Although invasive bacterial burn wound infection has been controlled, strict isolation techniques and infection control policies have significantly reduced the incidence of burn wound infection (Washington *et al*., 2006). The current study showed a high prevalence of bacterial infection among burn patients which is in agreement with the findings of other research (Al-Habib *et al*., 2011), but in contrast to another study (Amin and Kalantar, 2004). In the present study, a burn infection swab resulted in positive bacterial growth in 64.3% of the cases examined, which is similar to other researchers' observation (Askarian and Hossseini, 2004; Kehinde *et al*., 2004; Daher *et al*., 2007, Al-Habib *et al*., 2011). Single isolates were found in 56.7% of the studied cases which matched the result reported by Daher *et al*. (2007) who obtained pure isolates in 58.7% of their patients. Various types of Gram-positive and Gram-negative microorganisms were detected in the present study, including Gram-negative bacteria. It constituted (75.34%) Gram-positive (24.66%). This result is consistent with that of Gram-negative bacteria constituted (72%) and (83.8%) of their isolates respectively.

However, other researchers have reported lower incarceration rates ranging from 33–51.1% (Kehinde *et al*., 2004; Ekrami and Kalantar, 2007; Daher *et al*., 2007). Moreover, the frequency of hospital infections with Gram-negative bacilli especially *P. aeruginosa* has increased during the last decade. Other studies have described *P. aeruginosa* as the common cause of burn infection in hospital (Jefferson *et al*., 2003). In the current study, *P. aeruginosa* was proved to be the main cause in burn patients which accounted for 49.1% of Gram-negative isolates and 27 (36.99%) of the total isolates (73) and was found in 75% 64.3% of the positive growths. This result was in agreement with those of Song et al. (2001) and Al-Habib *et al.* (2011) who reported 50% of total isolates and 75% of the positive growths. However, other studies have reported a lower prevalence of P. aeruginosa in burn infections (Jefferson et al., 2003, Rastegar *et al*., 2005; Ekrami and Kalantar, 2007), On the other hand, Mansour and Klantar (2004) recorded a higher isolation rate (68.3%). The second most recovered organism in this work is *Klebsiella pneumoniae* (25.45%) which is in agreement with the result obtained by Kehinde *et al.* (2004) about 34.3%, and Al-Habib et al. (2011) about 26.7%, and in contrast to others (Gad *et al*., 2007) who report. In addition, E. coli recovered 18.18% of total Gram negative and 13.7 of the total cases and this rate was more than (10%) to what was discovered by Daher *et al.* (2007) and Al-Habib *et al.* (2011).

The difference in the prevalence of bacterial isolates can be attributed to the environmental conditions of a particular area and the contamination of the burning units. Due to the increasing resistance to various antibiotics and the spread of infection in the hospital environment, there is a clear change in the bacterial spectrum. A few

decades ago the dominant bacteria were *Streptococcus* which was later followed by *staphylococcus aureus* (Al-Habib *et a*l., 2011), but with frequent use of topical antibiotics, fungi and viruses become more prevalent. Also, due to the use of a wide range of antibiotics, Gram-negative and Gram-resistant bacteria are becoming more prevalent. This growing resistance to various antibiotics poses a challenge to burn care units as it reduces treatment efficacy and may increase morbidity and mortality.

Only *P. aeruginosa* isolates were selected to complete this study and others were excluded. Pseudomonad isolates were identified according to microbial and cultural characteristics on culture media such as MacConkey agar, Cetrimide agar and chromogenic agar. Bacterial cells resulting from slide smear film preparation were Gram-negative, rod-shaped, and occur singly, pairs, or short chains. The colony characteristics of *P. aeruginosa* isolates were studied using nutrient agar, MacConkey agar, Cetrimide agar and Chromogenic agar plates. They had the appearance of fried eggs, smooth with a flat edge and a raised appearance. All of these isolates produce pyocyanin (a bluish-green pigment). *P. aeruginosa* does not ferment lactose in MacConkey agar and is distinguished from lactose-fermenting bacteria (*Enterobacteriaceae*). Bacterial colonies can grow at 41°C but not at 4°C. These criteria are used to identify *P. aeruginosa* from other species; This is in agreement with (18), who found that *P. aeruginosa* had the ability to grow at 41 °C and produce pyocyanin pigment after growing on Cetrimide and chromogenic agar media. Bacterial colonies appeared blue-green on *Pseudomonas*™ chromogenic agar medium **(Appendices / Figure (4-1/A))** and other Gram-negative bacteria were inhibited. Biochemical chromogenic agar properties tested according to the VITEK® 2 system

confirming bacterial diagnosis **(Appendices / Figure (4-1/ B)).**

The total number of *P. aeruginosa* was 89 isolated from burn infections, including 27 isolates of this study isolated during four months (November 2021 to January 2022), in addition to 62 *P. aeruginosa* previously isolated during 2020-2021 obtained from hospitals in Babylon, Najaf and Karbala governorates which preserved as glycerol stock in the Biotechnology Lab. All of them examined phenotypically for antibiotic susceptibility (gentamycin, Amikacin, meropenem, ciprofloxacin and piperacilline), and extracellular alkaline protease production; and molecularly for detection of cluster of genes controlling synthesis and secretion of alkaline protease (*aprI, apr A, apr F, apr E & apr D*) and toxin A gene.

4.2 Antimicrobial Susceptibility of *P. aeruginosa*

The results showed that *P. aeruginosa* isolates had variable abilities to resist the studied antimicrobial drugs, including 6 antibiotics belonging to four different classes (Table 4- 1) that have different patterns or mechanisms in stopping the growth or killing of microorganisms.

Table (4-1) showed the antibacterial susceptibility test of 88 *P. aeruginosa* isolated from burn infections was identified using six antibiotics belonging to four classes. Where the higher percentage of antibiotics resistant was (58.42 %) for amikacin (AK), while the higher percentage of intermediate resistant of antibiotics was (23.60 %) for piperacilline (PRL), finally the higher percentage of sensitivity of antibiotics was (47.19 %) for meropenem (MEM) and most of isolates were sensitive (94.38) for Imipenem (IPM).

Table (4-1): Antibiotics susceptibility test of *P. aeruginosa* isolates

Drug	Breakpoints (mm)			Resistant No. (%)	Intermediate No.(%)	Sensitive No. (%)	Total No. (%)
	R ≤	I	S ≥				
AK	14	15-16	17	52 (58.42)	10 (11.24)	27 (30.34)	89 (100 %)
CN	12	13-14	15	34 (38.20)	19 (21.35)	36 (40.45)	
PRL	13	14-16	17	43 (48.31)	21 (23.60)	25 (28.09)	
CIP	21	22-25	26	46 (51.69)	10 (11.24)	33 (37.07)	
MEM	19	20-22	23	34 (38.20)	13 (14.61)	42 (47.19)	
IPM	19	20-22	23	0	5 (5.62)	84 (94.38)	

AK: Amikacin (30µg), PRL: Piperacilline (100µg), MEM: Meropenem(10µg), IPM : Imipenem (10µg), CIP: Ciprofloxacin (5µg) , and CN: Gentamicin(10µg) . R: Resistant, I: Intermediate and S: Sensitive.

The skin infections with pathogenic bacteria are very common, especially in burns and wounds patients, due to the contamination of the area with these microorganisms that called hospital acquired infection, so conducting antibiotic susceptibility testing for bacterial isolates and identifying the genes responsible for resistance is necessary to reduce the severity of infection (Gupta *et al*., 2015; Yanga *et al*., 2021). This is consistent with the current study, which was conducted on *P. aeruginosa* bacteria, which is considered one of the most pathogenic causes of skin diseases in burn patients, as it is classified as one of the most bacterial species that have antibiotic resistance genes.

In this study imipenem was the most effective antibiotic with 47.19% against bacteria in contrast to other studies have reported variable rates of imipenem resistance such as, 37% in Tunisia, 43% in Libya and 76% in Iran (Pang *et al*., 2019; Dégi *et al*., 2021). While another study

consistent with this study that showed the most effective antibiotic was meropenem with 37% resistance (Ullah *et al*.,2009). The rate of resistance against 6 types of antibiotic used in the present study ranged between 38.2-58.42% for burn isolates, in which 58.42% of the isolates were resistant to amikacin , 51.69% were resistant to ciprofloxacin , 48.31% were resistant to piperacilline and 38.2% were resistant to meropenem and gentamicin, while the results of some studies makes in some European countries showed the highest rates of resistance was to carbapenems and aminoglycosides (Ranjbar *et al*., 2011; Zoghlami *et al*., 2012).

In this study the effective agent against *P. aeruginosa* was imipenem which yielded the least resistance percentage (48.31%). This could be explained on the basis that piperacillin is not commonly prescribed against *Pseudomonas* infection in this locality. However, higher resistance to this drug (86.2%) was reported by other investigators (Strateva et al., 2007) where the use of this antibiotic is more frequent in their locality.

The sensitivity test of the six antibiotics used in this study against *P. aeruginosa* isolates was relatively low compared to the sensitivity pattern to the antibacterial drugs that were used in many other studies , This is due to the selective pressure exerted on bacteria for many reasons such as non-compliance with the hospital's antibiotic policy, and the excessive and indiscriminate use of extensive antibiotics , in addition the antibiotic sensitivity test against clinical isolates of *Pseudomonas* bacteria, especially those that have shown a high inhibition activity, imipenem and meropenem may help in the prevention and treatment of

multidrug-resistant pathogens in burn and wound infections (Rodloff *et al*., 2008).

The antibiotic study in the present work showed that most *P. aeruginosa* isolates were resistant to commonly used antibiotics such as gentamicin, ciprofloxacin and ceftriaxone, which were randomly prescribed as experimental treatment for a long time. This high resistance to the above-mentioned antibiotics has also been noted by other investigators (Strateva *et al*., 2007; Ekrami and Kalantar,2007; Al-Habib *et al*., 2011). In fact, MDR *P. aeruginosa* is currently a major problem. In the present study, it accounted for 44.94% of the total *P. aeruginosa* recovered, and this result was in agreement with Strateva et al. (49.8%) (Strateva *et al*., 2007) and Al-Habib *et a*l. (2011) about 44.4% although it was higher than that reported by other workers (Mansour and Klantar, 2004). The relationship between MDR *P. aeruginosaa* and antimicrobial consumption was also analyzed in this study. A statistically significant relationship was found to be with ceftriaxone, meropenem, ceftazidime and amikacin which was agreement with (Al-Habib *et al*., 2011) and in contrast to the study by Messadi et al, who found that the significant association was with ciprofloxacin use (Nudegusio *et al*., 2004). This discrepancy in results may be due to different antibiotic use in different settings.

The production of beta-lactamase enzymes is the mechanism by which *pseudomonas* are resistant to antibiotics. Moreover, the inducible β-lactamase producers were the MDR, and this result reflects the role of inducible β-lactamase in antibiotic resistance (Upathyay et al., 2010). This increased rate of MDR may be attributed to the inhibitory concentration of antibiotics in vivo due to administration of inappropriate dose of beta-lactam antibiotics, or regular administration of

aminoglycosides in combination with beta-lactam drugs that provide optimal conditions for persistence of MDR *P. aeruginosa* strains**.** These results highlighted the need for greater attention to disinfecting the non-living hospital environment and controlling contact between staff and patients in order to reduce *P. aeruginosa* transfer in burn units. Furthermore, the use of some antimicrobial agents should be restricted due to the presence of high resistance. Combined effective antibiotics are also recommended. In conclusion, bacteria isolated from burn unit patients are the best examples for studying pathogenic bacterial species, especially *P. aeruginosa*, other enteric bacilli and staphylococcus. The doctrine that is often responsible for human colonization. Also, *P. aeruginosa* and other Gram-negative bacilli are frequently associated with nosocomial burn infection. Moreover, most of the *P. aeruginosa* isolates from the flaring units are producers of β-lactamases and most of these isolates were MDR *P. aeruginosa*.

4.3 Detection of Extracellular Alkaline Protease

The virulence of *P. aeruginosa* is associated with the occurrence of several extracellular factors such as elastase and alkaline protease. These enzymes contribute to tissue destruction and aid bacterial invasion during infection. It, therefore, seems likely that identification of these virulence factors will be an important prognostic marker in the near future, for the initiation of antimicrobial agents that directly or indirectly inhibit microbial growth or virulence factor production. Perform a simple assay for the estimation of alkaline protease levels by the skimmed-milk agar plate method (Yagci *et al*., 2002). The results showed that the bacterial isolates displayed different abilities to produce extracellular proteases by showing the proteolytic zone around the bacterial colonies in the milk agar medium

due to the hydrolysis of casein (Fig.4-2). After calculation of the ratio between proteolytic zone diameter per colony diameter, these isolates showed three levels of extracellular protease production (Fig. 4-3); Including, 11 (12.36%) of these isolates appeared high-level of enzyme production (ratio =3-3.9), 39 (43.82%) of isolates produced Intermediate-level (ratio 2-2.9) and 29 (32.58%) isolates produced low-level (ratio 1-1.9). Whereas the rest of isolate had not any clear zone around their colonies (10: 11.244%) named non- extracellular protease producer due to defect or loosing coding gene (*aprA*) or they were not secreted extracellularly because they were trapped in the periplasmic space due to defect in controlling or protease secretory system which encoding by *aprF, apr E* and *apr D* genes respectively. These genes were detected using specific primer pairs by PCR in the next section.In the present study, although all of the pseudomonad isolates produced the pigment pyocyanin in chromogenic agar, only the isolates with the ability to produce high alkaline protease in skimmed milk agar revealed overproduction of pyocyanin (Fig. 4-4). This is because *aprA* has been considered to contribute to pyocyanin production in *P. aeruginosa* and bacteria regulate pyocyanin production by auto-inducible quorum sensing, which correlates with bacterial density (Latifi *et al*., 1995). *aprA* is one of the most important virulence factors in *P. aeruginosa*, and its overexpression led to the production of another virulence factor, pyocyanin, a phenomenon referred to as 'crosstalk between virulence factors'. Thus, overexpression of *aprA* in *P. aeruginosa* may exhibit hyper-virulence through a synergistic effect and pose a health threat. Various new strategies have been proposed to treat *P. aeruginosa* infection (Wagner *et al.*, 2016).

The quorum-sensing machinery and protein secretion system are involved in the production of multiple virulence factors; Therefore, these

systems are attractive therapeutic targets. *aprA* could also act as an effective target, since suppression of *aprA* may lead to reduction of both *aprA* and pyocyanin Thus, it is important to study the crosstalk between virulence factors in different pathogens in order to understand the pathogenesis and to develop new therapeutic strategies (Winsor *et al*., 2016; Wagner *et al*.,2016)

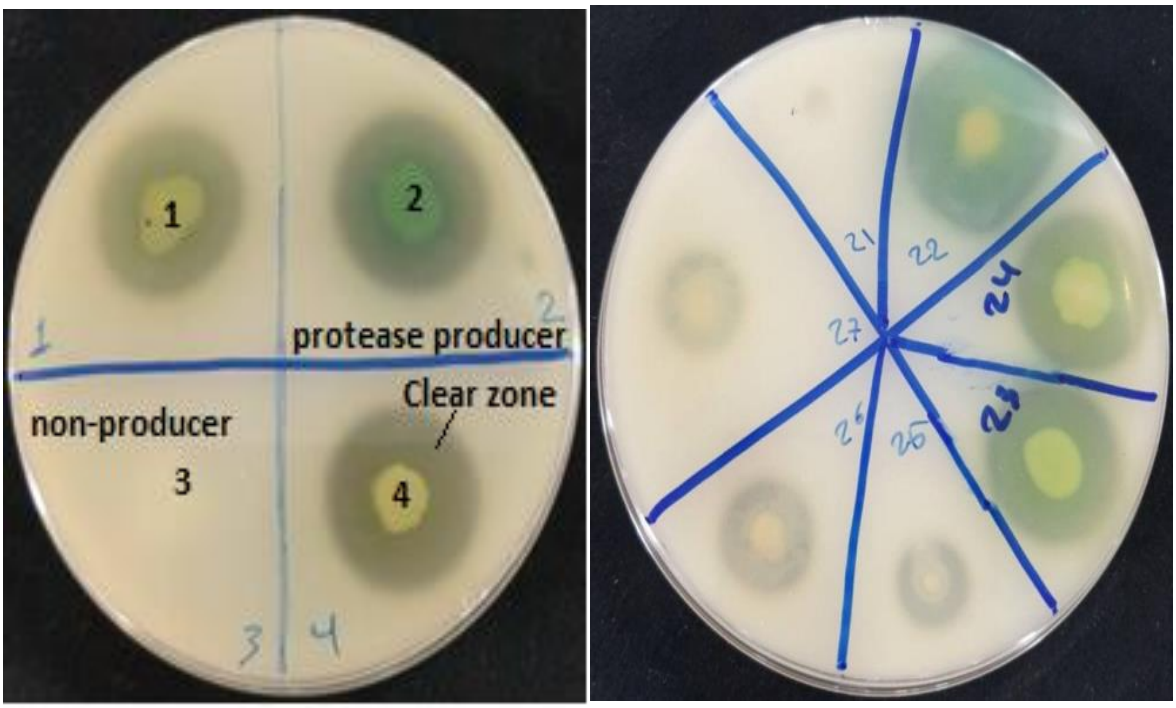


Figure (4-2): Detection of extracellular alkaline protease of *P. aeruginosa* in skimmed-milk agar medium

The alkaline protease producer isolates appeared clear zone around their colonies due to milk casein hydrolysis, while non-protease producer had not clear zone.

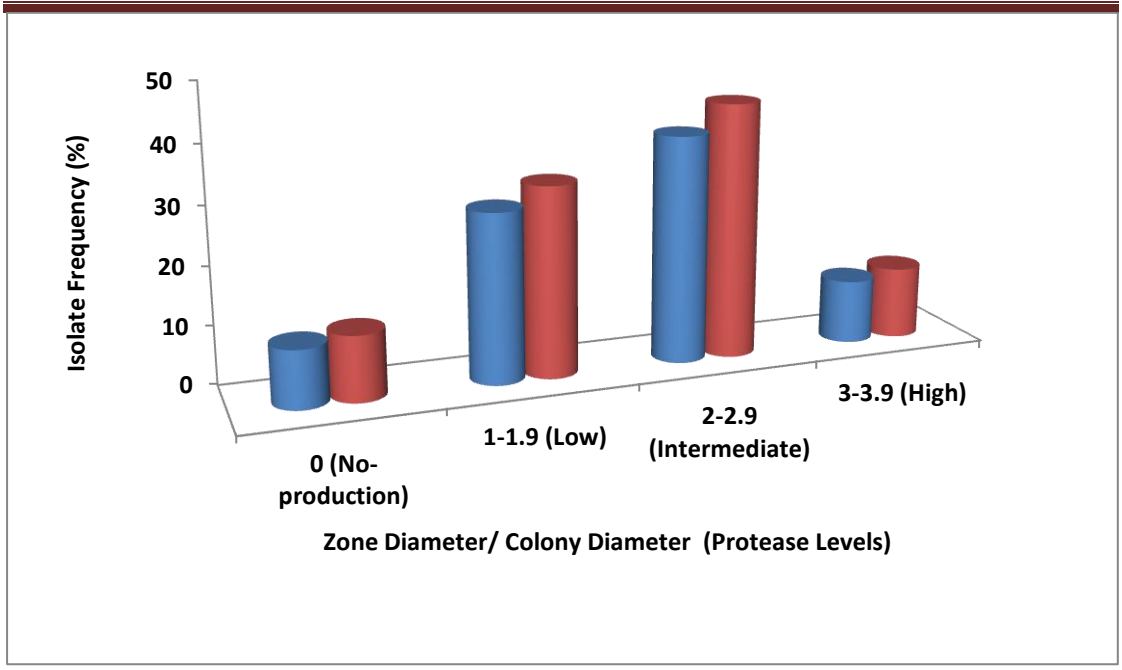


Figure (4-3): Extracellular alkaline protease levels and their percentage among *P. aeruginosa* isolates according to the (Senturk *et al*.,2012).

4.4 Molecular Detection of an Alkaline Protease Gene Cluster of *P. aeruginosa* by PCR

The results of molecular detection of an alkaline protease gene cluster (*aprI, apr A, apr F, apr E & apr D*) by PCR revealed that the presence of a single band in agarose gel electrophoresis for each gene was amplified. These bands include 175bp for the *aprI* gene, 239 bp for the *apr A* gene, 242 bp for the *apr F* gene, 229 bp for the *apr E* gene and 230 bp for the *apr D* gene, as shown in figure **(4-4/ A-E)**. The results indicate that the isolates contain complete the alkaline protease gene cluster. These results were consistent with previous studies, for which the genetic locus of alkaline protease (APR) synthesis and secretion in *P. aeruginosa* was first described (Lazdunski *et al*., 1990). The nucleotide sequence of the DNA fragment encoding these functions was determined and revealed the presence of five open reading frames: *aprA*, the structural gene encoding APR; *aprI*, which encodes a protease inhibitor; and *aprD, aprE, aprF*

whose products are involved in protease secretion. *P. aeruginosa* has a specialized secretory system that is very similar to secretory proteins AprD, AprE, AprF from *Erwinia chrysanthemi* proteases and is homologues to that of *Escherichia coli* alpha-haemolysin, and is widespread among Gram-negative bacteria (Guzzo *et al*., 1991; BardoelKok *et al.*, 2012).

P. aeruginosa secretes alkaline protease (AprA) to promote its survival, which interferes with the complement degradation of erythrocytes, and its actual role in bacterial complement damage is unknown. AprA effectively inhibited phagocytosis and killed *Pseudomonas* using the capacity of human neutrophils. Moreover, AprA inhibited the imprinting of microorganisms with C3b and formation of the C5a chemokine. AprA normally inhibits C3b deposition via the classical and lectin pathways, *P. aeruginosa* AprA interferes with the activation of classical complement and intermediate complement of the lectin pathway by C2 cleavage (Laarman *et al.*, 2012). Also, AprA cleaves one of the key microbial recognition molecules, monomeric flagellin, which reduces activation of the Toll-like receptor 5. Therefore, AprA activity plays an important role in suppressing the host's innate immune response and degrading host tissues such as complement proteins and cytokines. *P. aeruginosa* encodes a highly potent alkaline protease inhibitor (AprI) which is found only in the periplasm where it protects proteins surrounding the AprA-secreting protease. Therefore, enzyme-inhibitor interactions have been studied to provide a basis for future drug development with preliminary steps towards designing molecules derived from the natural inhibitor of the virulence factor AprA and their use in therapeutic applications in *Pseudomonas* and other Gram-negative infections (BardoelKok *et al.*, 2012).

The results of present study revealed the most of local *P. aeruginosa* isolates harbor alkaline protease cluster genes which encoding to the enzyme synthesis (*aprA*), controlling (*aprI*) and secretion system (*aprF*, *aprE* and *aprD*). About 94.38% of isolates harbor *aprA* gene, 91.01% for *aprI*, 94.38% for *aprF* and 88.76% for both *aprD* and *aprE* (Fig. 4-5). All of the protease producer isolates (100%) were carrying *aprA* gene (alkaline protease encoding gene), *aprI* (protease inhibitor encoding gene), *aprD* (ABC transporter) and *apr F* (outer membrane protein: OMP) and 83.15% harbor *aprE* (membrane fusion protein: MFP) which act as helper or 'adapter' proteins, delivering primary carriers into the cytoplasmic membrane. The MFP deficient leads to remain the enzyme in periplasmic space of bacteria that showed among 17.24% of low-protease producer isolates (Fig. 4-7). While 50% of the non-protease producer contains an incomplete protease gene cluster consisting of *aprA*, *aprE*, *aprF* and 20% for *aprI*, but they all lack the *aprD* gene that leads to the survival of the protease in the bacterial cytoplasm and its subsequent degradation. The rest of the non-protease producer isolates (50%) deficient for alkaline protease cluster, as shown in the figure (4-7). These results illustrate the diversity of protease production. If the bacteria possess complete genetic information required to synthesis alkaline protease and secreted into the growth medium. The variation of enzyme production levels depends on the gene expression of the enzyme and ability of type I secretory system. The isolates not produce alkaline protease due to harbor incomplete protease gene cluster or *aprA* cluster deficiency.

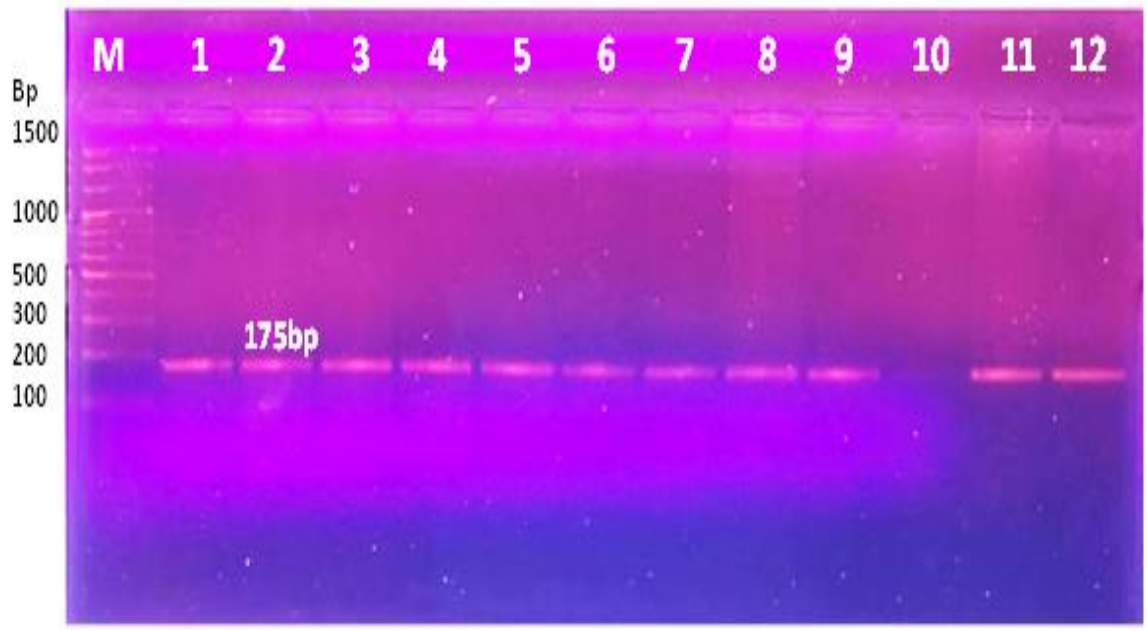


Figure (4-4/A): Gel electrophoresis of amplified products of an alkaline protease gene cluster to *aprI*: Alkaline protease inhibitor gene at (175bp), where the M= DNA markers (100-1550 bp). Line 1-12 = pcr bands of *aprI gene* , the Electrophoresis conditions: 1.5% agarose, 70 V, 60 min, Ethidium bromide staining)

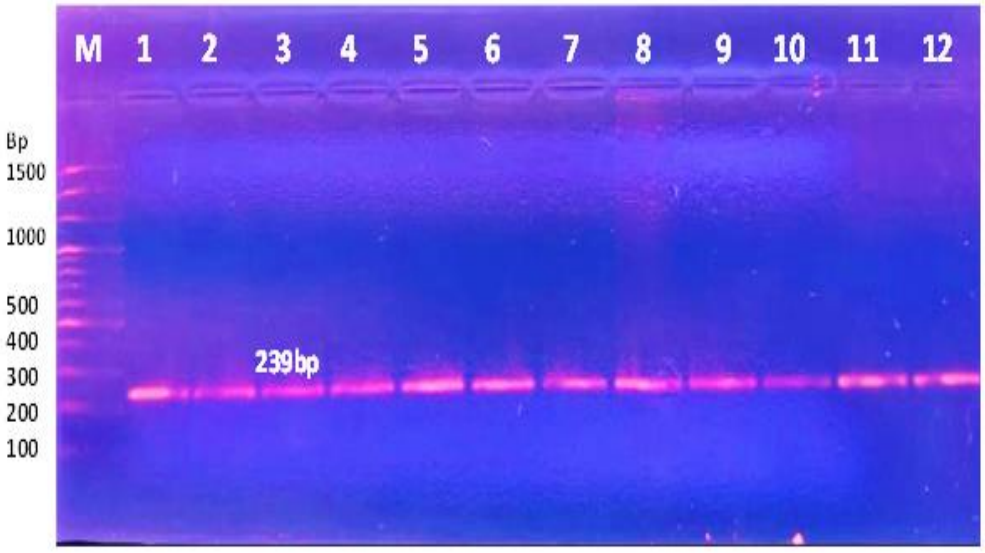


Figure (4-4/B): Gel electrophoresis of amplified products of an alkaline protease gene cluster to *aprA*: Alkaline protease inhibitor gene at (239 bp), where the M= DNA markers (100-1550 bp). Line 1-12 = pcr bands of *aprA gene* , the Electrophoresis conditions: 1.5% agarose, 70 V, 60 min, Ethidium bromide staining)

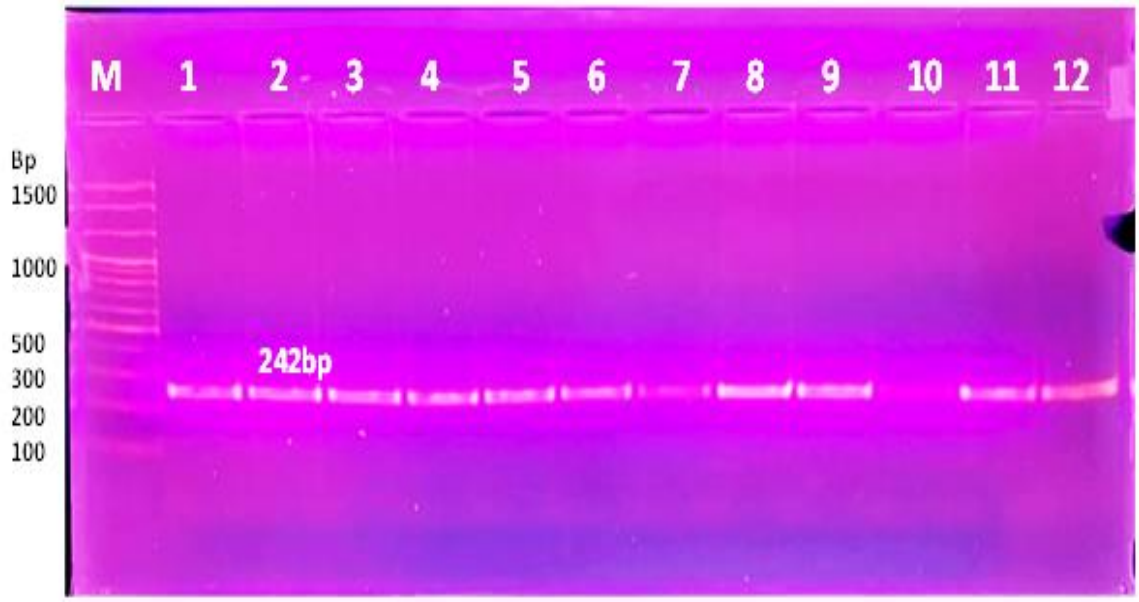


Figure (4-4/C): Gel electrophoresis of amplified products of an alkaline protease gene cluster to *aprF*: Alkaline protease inhibitor gene at (242 bp), where the M= DNA markers (100-1550 bp). Line 1-12 = pcr bands of *aprF gene* , the Electrophoresis conditions: 1.5% agarose, 70 V, 60 min, Ethidium bromide staining)

Figure (4-4/D): Gel electrophoresis of amplified products of an alkaline protease gene cluster to *aprE*: Alkaline protease inhibitor gene at (229 bp), where the M= DNA markers (100-1550 bp). Line 1-12 = pcr bands of *aprE gene* , the Electrophoresis conditions: 1.5% agarose, 70 V, 60 min, Ethidium bromide staining)

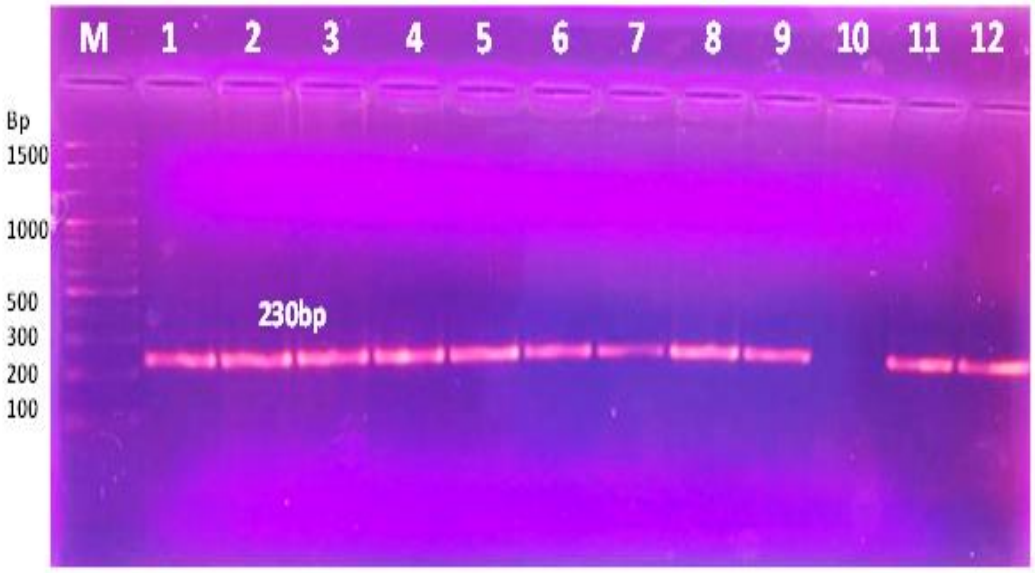


Figure (4-4/E): Gel electrophoresis of amplified products of an alkaline protease gene cluster to *aprD*: Alkaline protease inhibitor gene at (230 bp), where the M= DNA markers (100-1550 bp). Line 1-12 = pcr bands of *aprD gene* , the Electrophoresis conditions: 1.5% agarose, 70 V, 60 min, Ethidium bromide staining)

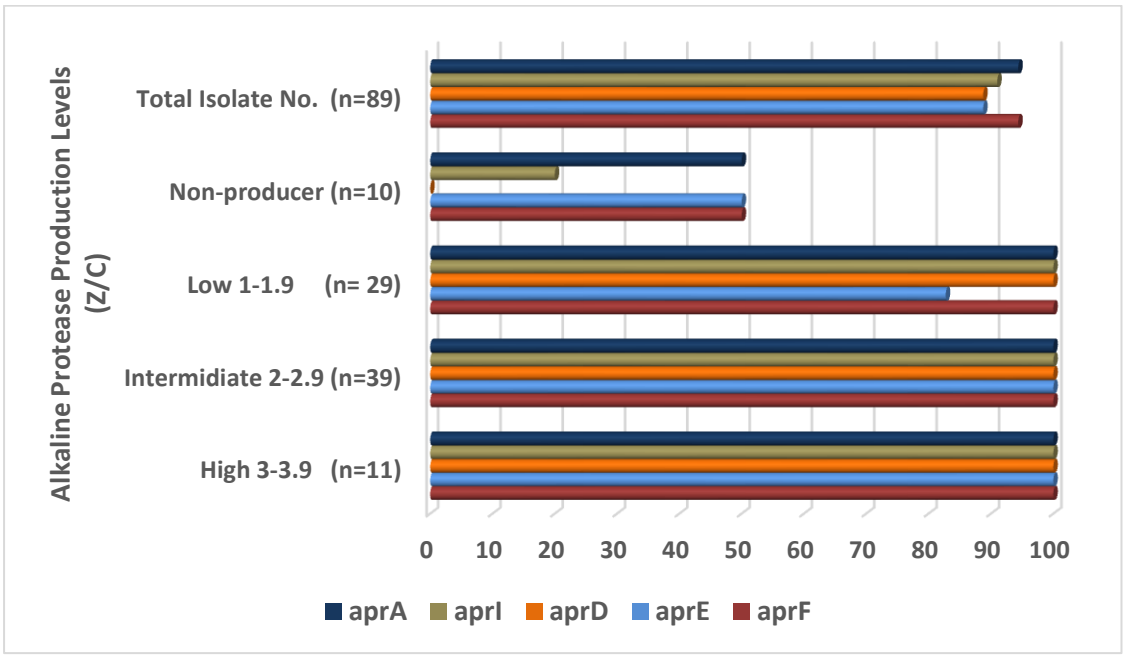


Figure (4-5): Distribution of an alkaline protease gene cluster among *P. aeruginosa* isolates according to extracellular alkaline protease production levels.

-An alkaline protease gene cluster composed of an alkaline protease encoding gene *apr A*, protease inhibiter gene *aprI and TISS secretory system includes apr F, apr E & apr D*).

- Z/C: refer to the ratio of clear zone diameter/ bacterial colony diameter.

Secretion of the alkaline protease is impaired by a deficiency of secretion genes that lead to intracellular protein survival and partial degradation. Each of the three *apr D, E, F* genes is necessary for alkaline protease secretion since any mutation in one of these genes abolished secretion (Guzzo *et al*.,1991; Galdino *et al.,* 2019). The present results consist with the previous finding that documented that in the absence of secretion functions, little APR was detected in *E. coli* (pJF2518) by Western blot analysis. Pulse-chase experiments showed that APR was very unstable inside the cells and was degraded with a half-life of approximately 160 s. Similar results were obtained with α-hemolysin and immunoglobulin A protease; when not secreted, these proteins were rapidly degraded (Klauser *et al*.,1990).

Table (4-2) showed all (100%) of the MDR *P. aeruginosa* isolates harbor complete *aprA* gene cluster, and more than 75% of Non-MDR isolates contain complete *aprA* gene. Whereas more than 73% of the sensitive isolates of *P. aeruginosa* harbor complete cluster. The presence of the *protease* gene cluster not associated with antibiotic resistance (*P*=0.18), but the MDR isolates became more virulent when they produce the alkaline protease.

Table (4-2): Distribution of an alkaline protease gene cluster among *P. aeruginosa* isolates according to antibiotic susceptibility

Gene	MDR (n=38)		Non-MDR (n=32)		Sensitive (n=19)		Total (n=89)	
	No.	%	No.	%	No.	%	No.	%
aprA	38	100	31	96.88	15	78.95	84	94.38
aprI	38	100	29	90.63	14	73.68	81	91.01
aprD	38	100	26	81.25	15	78.95	79	88.76
aprE	38	100	24	75.00	17	89.47	79	88.76
aprF	38	100	29	90.63	17	89.47	84	94.38

MDR: multidrug resistance; Non-MDR: non-multidrug resistance.

4.5 Genetic analysis of an alkaline protease gene cluster of *P. aeruginosa*

To understanding the relationship between the level of extracellular protease production and the presence of enzyme gene (*AprA*) cluster, four isolates were selected for further genetic analysis using PCR-DNA sequencing technique. Table (4-3) showed the phenotypic and genetic characteristics of the selected isolates. Both the high-level protease producer (*P. aeruginosa* 13- NR2) and intermediate level of extracellular protease (*P. aeruginosa* 18-GF) each had complete *aprA* gene cluster which encodes to alkaline protease (*AprA*), alkaline protease inhibitor (*AprI*), and type I secretory system (TISS). T1SS use an ATP binding cassette (ABC) transporter to translocate unfolded alkaline protease across the inner and outer bacterial membrane through a trans-envelope complex. Also, T1SS contain a periplasmic membrane-fusion protein (MFP) and outer-membrane porin (OMP). These components assemble in the presence of substrate, which contains a C-terminal secretion signal, to form a contiguous conduit across the periplasm (Morgan *et al*., 2017).

Table (4-3): Phenotypic and genotypic of selected *P. aeruginosa* isolates for further genetic analysis using PCR-DNA sequencing

Bacterial Isolates	Protease level (Z/C)	*apr I*	*apr A*	*apr F*	*apr E*	*apr D*	Antibiotic susceptibility
***P. aeruginosa* 13- NR2**	High (3.2)	+	+	+	+	+	MDR
***P. aeruginosa* 18 -GF**	Intermediate (2.5)	+	+	+	+	+	Sensitive
***P. aeruginosa* 61-NR1**	Low (1.2)	+	+	+	-	+	Non-MDR
***P. aeruginosa* 60 -ZR**	Non-producer (0)	-	+	+	-	-	MDR

(Z/C): Ratio of proteolytic zone diameter per colony diameter. MDR: multidrug

resistance; Non-MDR: non-multidrug resistance

T1SS substrates tend to be quite acidic and contain a calcium binding ‘‘repeats-in-toxin’’ domain, and both of these features are implicated in energizing secretion (Thomas *et al*., 2014; Bumba *et al*., 2016; Morgan *et al*., 2017).

The low-level protease producer (Non-MDR *P. aeruginosa* 61-NR1) had an enzyme gene (*apr A*) cluster with *apr E* deficiency which may be indicates the alkaline protease remain in the periplasm space because of lose the function of a MFP. While the non-extracellular protease producer (MDR *P. aeruginosa* 60 -ZR) lacks three genes that include *aprI, aprD and aprE* that encoded to *AprI* and two components of type I secretory system (TISS) includes ABC transporter and MFP. The deficiency of secretion genes lead to intracellular protein survival and partial degradation (Galdino *et al.*,2019).

4.5.1 PCR-DNA Sequencing of *aprA*

The DNA-sequencing of *AprA* results appeared both *P. aeruginosa* 13-NR2 and *P. aeruginosa* 61- NR1 had one a substitution mutation (C>T) in studied *aprA* region at the positions 60 and *P. aeruginosa* 18- GF had a substitution mutation A>G at the positions 174 (Fig. 4-6). These mutations not change amino acid (Fig. 4-7) and these isolate can produce native alkaline protease in comparison with standard strain. Whereas the isolate *P. aeruginosa* 60-ZR had one substitution mutation at position 42 (G>A) and deletion mutation at positions 44 and 45 (Fig. 4-8) that result in a change to the gene's reading frame of *AprA* protein that leads to change protein structure (Fig. 4-9). This indicates that the isolate cannot produce the alkaline protease and that the defective protein is degraded in the bacterial cells. This result illustrates there is no clear zone around non-producer isolates in the milk agar plate (Fig. 4-2).

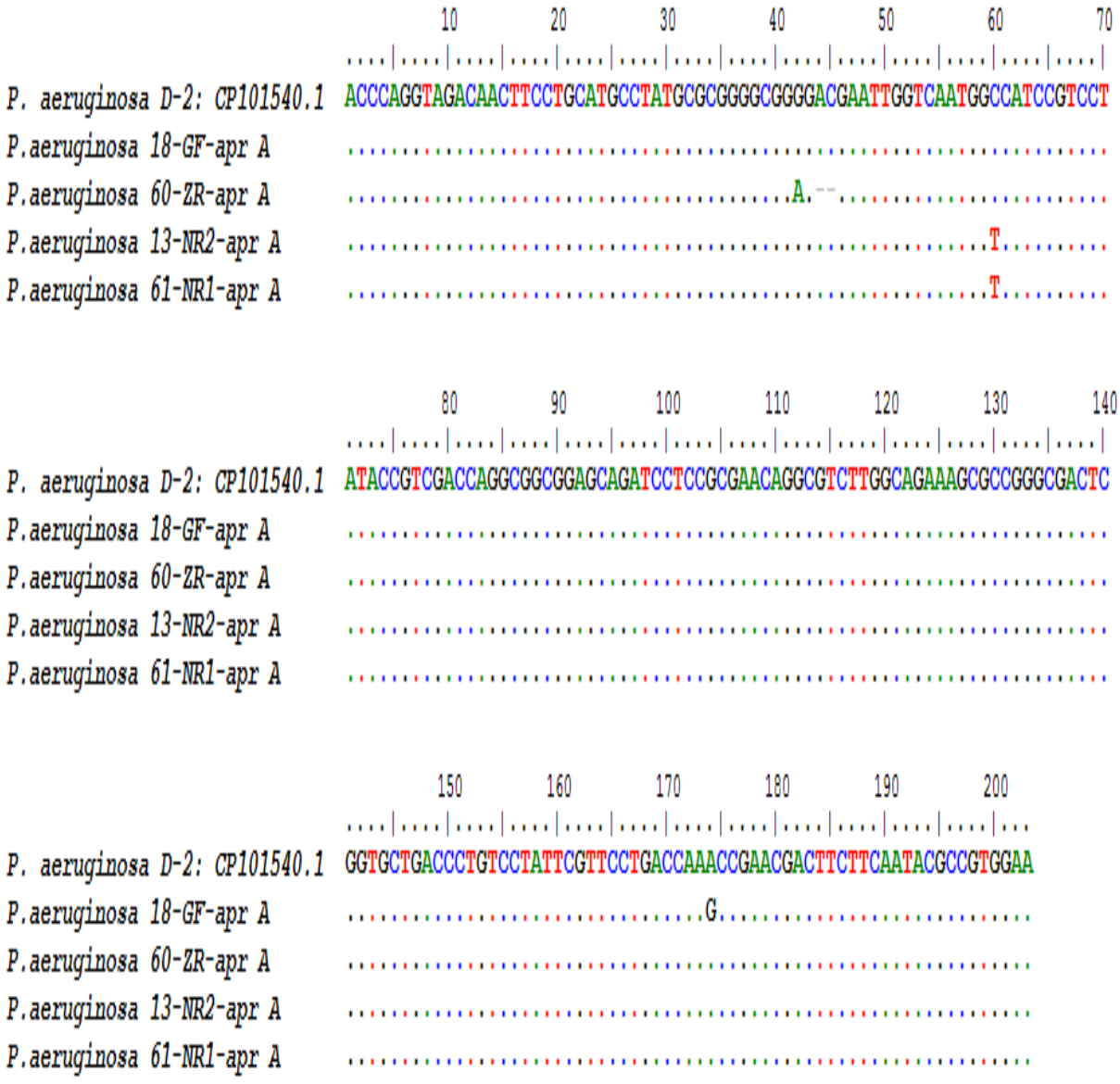


Figure (4-6). Nucleotides sequence alignment of the N-terminal of *aprA* gene among the local isolates and nucleotides sequence of those available in databanks.

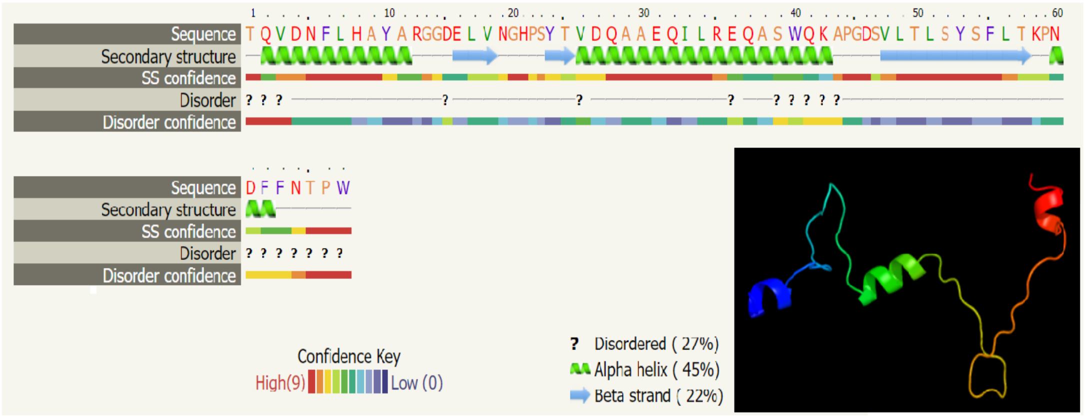
The local isolates including *P. aeruginosa 18-GF, 60-ZR, 13-NR*2 & *61-NR1* isolates. Data indicated that the position of nucleotides is different among isolates and identical data for all isolates are not shown by Bio Edit program version 7.2.5.

(1)

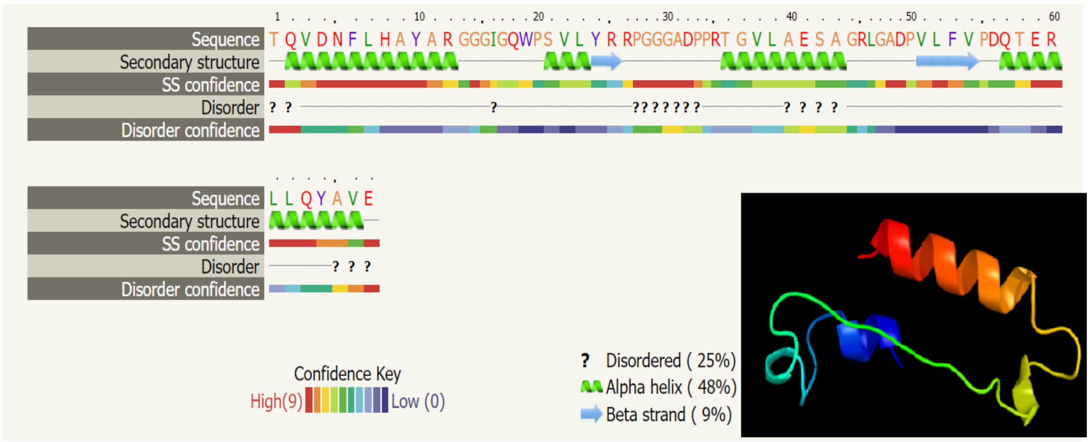
```
                                    10        20        30        40        50        60
                               ....|....|....|....|....|....|....|....|....|....|....|....|..
P. aeruginosa D-2: CP101540.1  TQVDNFLHAYARGGDELVNGHPSYTVDQAAEQILREQASWQKAPGDSVLTLSYSFLTKPNDFFNTPW
P.aeruginosa 18-GF-apr A       ..................................................................
P.aeruginosa 60-ZR-apr A       ..............GIGQWPSVL.RRPGGGADPP.TGVLAES.GRLGADPVLFVPDQTERLLQYAVE
P.aeruginosa 13-NR2-apr A      ..................................................................
P.aeruginosa 61-NR1-apr A      ..................................................................
```

(2)

Figure



A: Three-dimensional structure of Alkaline protease of *P.eruginosa* : A two-domain protein with Calcium binding parallel Beta-Roll motif in studied sequence of *P. eruginosa* D-2 standard strain and local isolates 18-GF , 13-NR2 and 61-NR1



B: Abnormal protease structure of *P. eruginosa* 60-ZR

(4-7). Alignment of predicted amino acid sequences and their three-dimensional structure of AprA protein.

(1): Amino acid alignment among the *P. aeruginosa 18-GF*, *60-ZR*, *13-NR*2 & *61-NR1* isolates and amino acid sequences of those available in databanks. draw based on the alignment of BioEdit program version 7.2.5.

(2): Three-dimensional structure of native AprA studied sequence of local *P. aeruginosa* isolates and standard strain (A) and abnormal protein of *P. aeruginosa* 60-ZR (B), http://www.sbg.bio.ic.ac.uk/phyre2/phyre2_output/93e1a320ca571186/summary.html .

4.5.2 PCR-DNA Sequencing of *aprI*

The DNA-sequencing of *AprI* results appeared there were three substitution mutations in studied fragment of *P. aeruginosa* 13- NR2 includes A>C, C>A and C>A at the positions 101, 123 and 141 respectively I (Fig. 4-10). Whereas the isolate *P. aeruginosa* 18 -GF had one substitution mutation at position 141 (C>A), that leads to change the amino acids of AprI protein of both isolates (Fig. 4-8/A). These results showed the transversion mutations among local isolates at the sequences were each mutation site of the active *AprI* at 20, 27 and 33 sequence number which converted aspartic acid > alanine, Histidine > glutamine and Serine > arginine respectively (Fig. 4-8/B).

Pro-*AprI* protein composed of 131 amino acids (aa) which activated after translocation into periplasmic space by cleaved the N-terminal transported sequence (25aa) by bacterial proteases and the active or functional *AprI* protein containing 106 aa .The studied fragment contains 57 aa of N-terminal at the sequence 12-68 including 14 aa of transported sequence and 43 aa of functional *AprI* protein. In the *P. aeruginosa* 13-NR2 the amino acids converted D>A at position 20, H>Q at 27 and S>R at 33 of N-terminal of functional AprI protein. Whereas the other isolates the amino acid change from serine to arginine (S>R) at 33 of N-terminal of active AprI protein as shown the figure (4-9).

The phylogenetic tree of *AprI* protein showed presence three types of *P. aeruginosa* namely 61-NR2 *AprI* ,18 –GF-aprI and 13NR2 *AprI* , as showed in Figure (4-10).

The biological function of this inhibitor (*AprI*) I is part of a safety mechanism that protects self-proteins in periplasmic space from degradation by Apr A before secretion. Apr I interact with the Apr A to form a strong

enzyme–inhibitor complex with a dissociation constant of 4 pM. *AprI* contacts the *AprA* through its N-terminus (residues 1–5) including serine 1, serine 2, leucine 3, isoleucine 4 and leucine 5 (as shown previously in the literature review, figure 2-4), and with the β-turn connecting β-strands to occupy the active-site cleft of *AprA* (Galdino *et al.*, 2019; Tuon *et al.*, 2022; https://www.rcsb.org/structure/1AKL). Also, the first AprI residue of active protein, a serine (S1), coordinates the catalytic zinc ion and, putatively, may contribute significantly to the high affinity of the inhibitor toward the protease. The importance of the N-terminus is further illustrated by the decreased affinity of *AprI* lacking the first two to five N-terminal residues, whereas no complex is formed upon deletion of the sixth residue as well. The interactions between the β-barrel of the inhibitor and *AprA* may be crucial in the exact positioning of the extended N-terminal segment such that the zinc is chelated properly, and further insertion of the N-terminal segment and subsequent proteolysis is prevented (Galdino *et al*., 2019; Tuon *et al.*, 2022). So the detected mutations in this study that located away from the binding sites of *AprI-AprA* complex may or slightly effects on function of AprI but may be effect on protein conformation as shown in the figure (4-12). The results revealed that the three transversion mutations (C>A *vs* A>C) in the Apr I of the isolate *P. aeruginosa* 18-GF is slightly effects on the protein secondary structure by change the of percentage of Beta-Strand 60% of the total studied protein sequence compared with the native of inhibitor in standard strain *P. aeruginosa* strain D-2 (ID: CP10154) which contains 63% beta-strand. While the enzyme inhibitor of the isolate *P. aeruginosa 13-NR2* is changed to form Beta-Strand 56% and alpha-helix 5% in comparison to Apr I of standard strain which has not alpha-helix structure as shown in the figure (4-10).

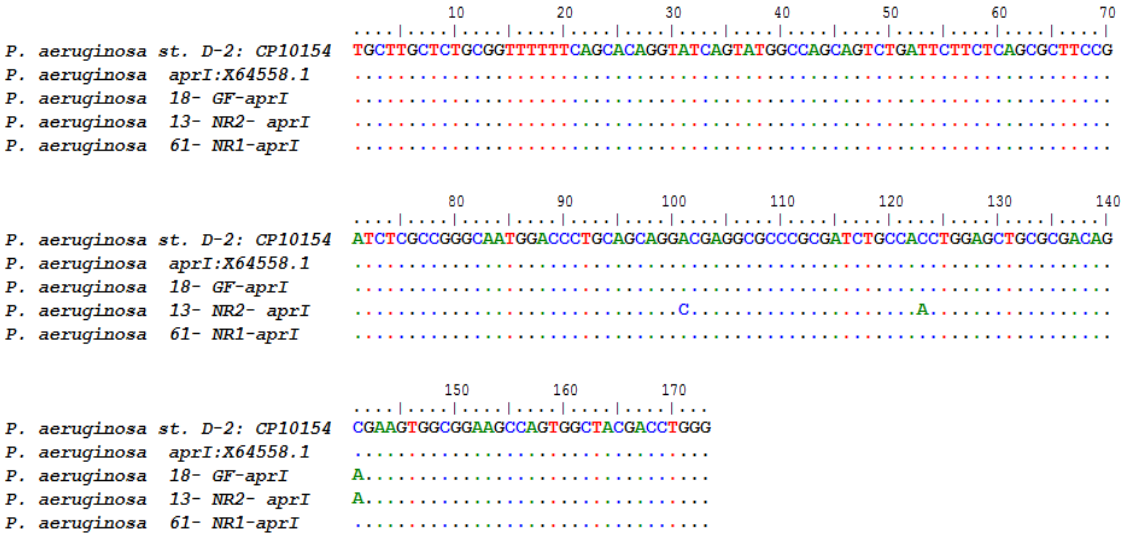


Figure (4-8) Nucleotides sequence alignment of the N-terminal of *AprI* gene among the local isolates and nucleotides sequence of those available in databanks.

The local isolates including *P. aeruginosa 18-GF, 13-NR2* & *61-NR1* isolates. Data indicated that the position of nucleotides is different among isolates and identical data for all isolates are not shown by Bio Edit program version 7.2.5.

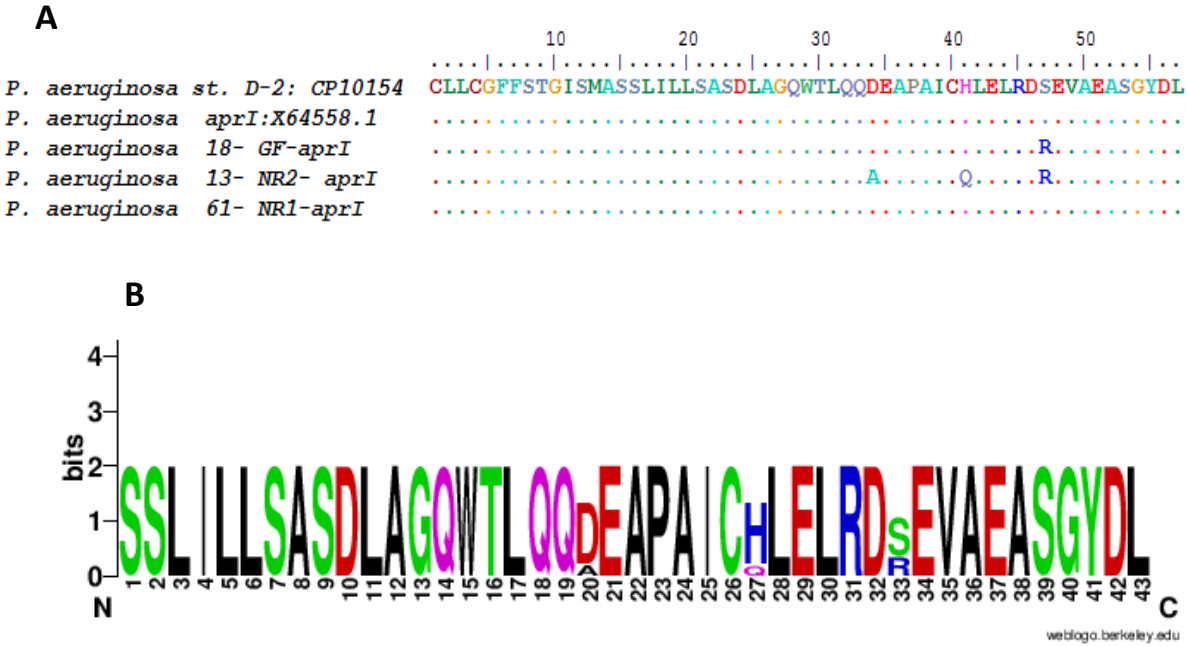


Figure (4-9) : Pair Fsequence alignment of the amino acid of the *P. aeruginosa AprI* protein.

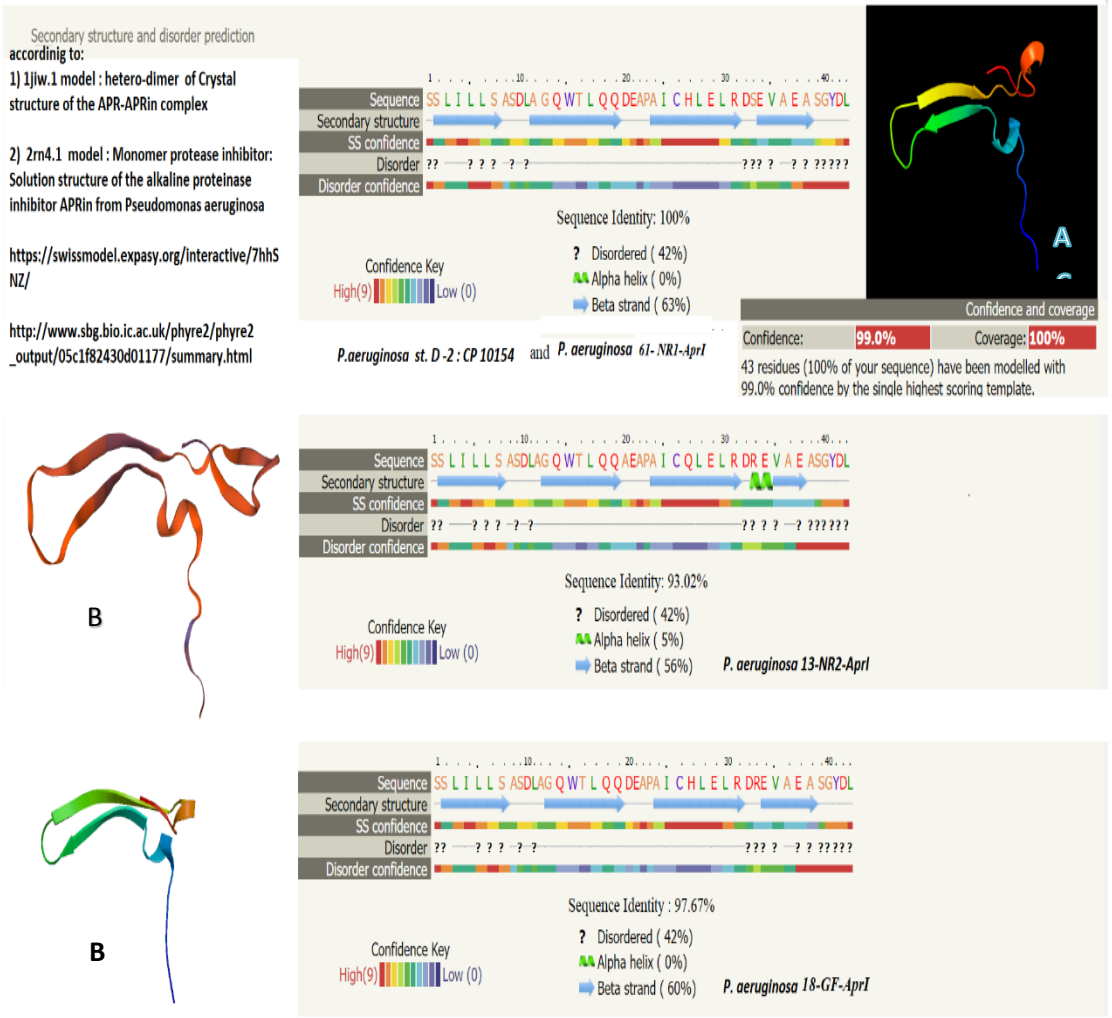


Figure (4-10). Secondary structure prediction of the active *AprI* protein of the standard and local strains of *P. aeruginosa.*

A: Amino acid alignment of AprI pro-protein among the *P. aeruginosa 18-GF*, *13-NR*2 & *61-NR1* isolates and amino acid sequences of those available in databanks. Draw based on the alignment of BioEdit program version 7.2.5

B: Weblogo of repeats of N-terminal of active AprI protein. The sequences were each mutation site of the *AprI* at 20, 27 and 33 sequence number which converted aspartic acid > alanine, Histidine > glutamine and Serine > arginine respectively.

the standard strain *P. aeruginosa* D-2 (ID: CP10154) and the local *P. aeruginosa 61-NR1***(A)**, *P. aeruginosa 13-NR*2 **(B)**, *P. aeruginosa 18-GF* **(C)** isolates which modeled with template 1jiw.1 of crystal hetero-dimer structure of *AprA-AprI* complex and the template 2m4.1 of monomer solution structure of alkaline protease inhibitor of *P. aeruginosa* according to http://swissmodel.expasy.org and http://www.sbg.bio.ic.ac.uk/phyre2 **.**

The figure (4-11) showed the phylogenic tree of *AprI* protein of studied isolates compared to amino acids sequences of those available in data bank (NCBI) which revealed the *AprI* amino acid sequence of *P. aeruginosa* 61_NR located at the same clade of reference strain (100% edintity).Whereas AprI of *P. aeruginosa* 18_GF located in other clade and similar to reference strain *P. aeruginosa* WP 134438153.Whil the inhibitor protein of *P. aeruginosa* 13_NR2 located in new clade (Fig 4-12).

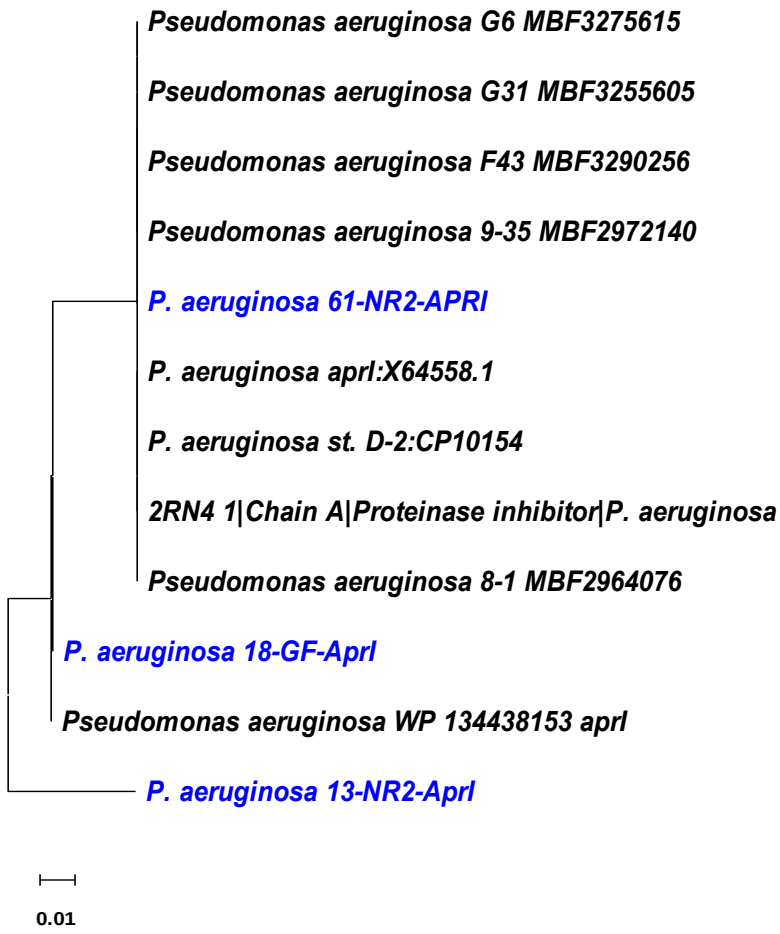


Figure (4-11) .phylogenic tree of *AprI* protein using the Neighbor-joining method Draw based on MEGAXI program.

4.5.3 PCR-DNA Sequencing of Type I Secretory System Genes

Type I secretory system (TISS) encoded by three genes including *aprD* encodes of ATP binding cassette (ABC) transporter to translocate unfolded alkaline protease across the inner and outer bacterial membrane through a trans-envelope complex. The MFP (membrane fusion protein) encodes by *aprE* and it contacts the trimeric OMP (outer membrane protein) encoded by *aprF* (Morgan *et al*., 2017).

The results of *aprD* DNA-sequence and predicated amino acid sequences appeared identical sequences with sequences of those available in databanks (NCBI) as shown in the figures (4-12) and (4-13).

The phylogenetic tree of *AprD* protein showed the *AprD* amino acid sequence located in two clade as showen in the figure (4-14)

Both *P. aeruginosa* 18_GF and 16_NRI located in one clade whereas *P. aeruginosa* 13_NR2 located in other clade.

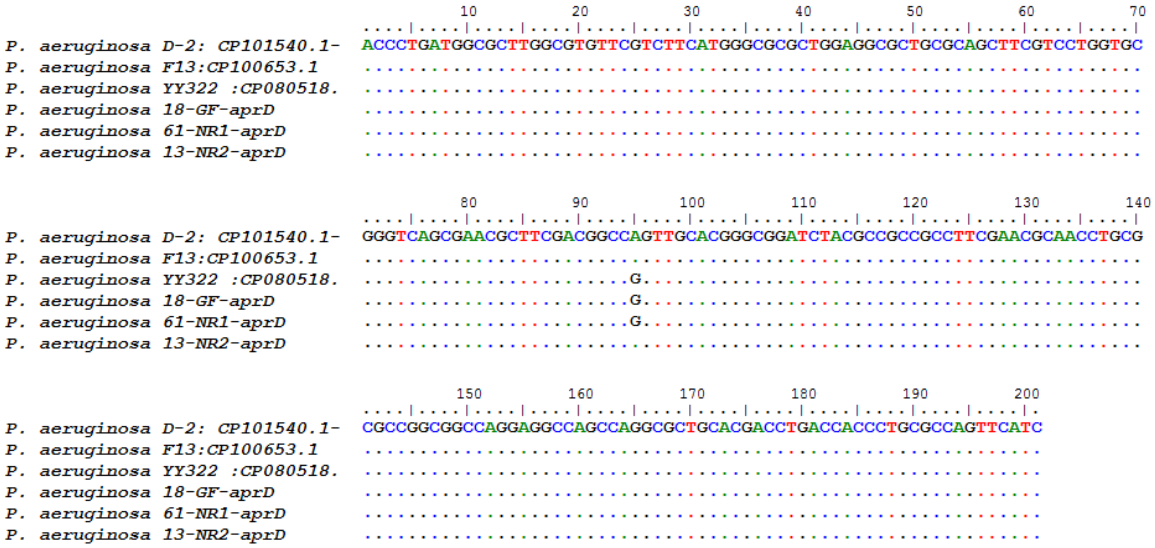


Figure (4-12) Nucleotides sequence alignment of the *aprD* gene among the local isolates and nucleotides sequence of those available in databanks.

The local isolates including *P. aeruginosa 18-GF, 13-NR2* & *61-NR1* isolates. Data analysis according to Bio Edit program version 7.2.5.

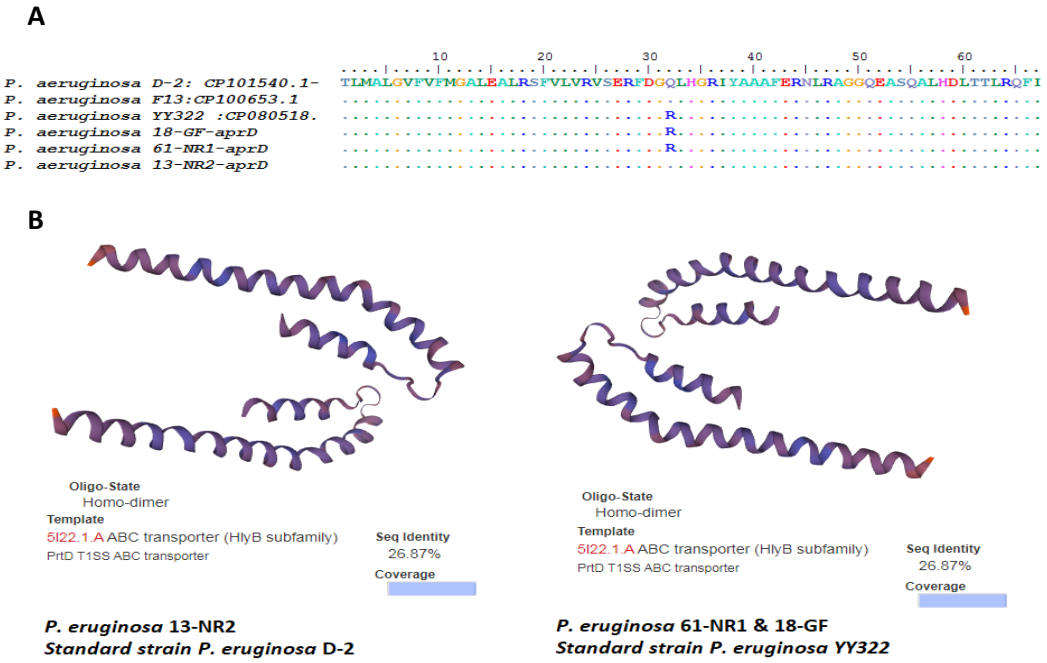


Figure (4-13) Pair sequence alignment of the amino acid of the *P. aeruginosa AprD* protein and their protein secondary structures. A: Amino acid alignment of *AprD* protein among the *P. aeruginosa 18-GF*, *13-NR*2 & *61-NR1* isolates and amino acid sequences of those available in databanks. draw based on the alignment of BioEdit program version 7.2.5 **B**: Protein secondary structures of local isolates and standard strains are identical which predicted according to https://swissmodel.expasy.org/interactive/LdHyaR/models/

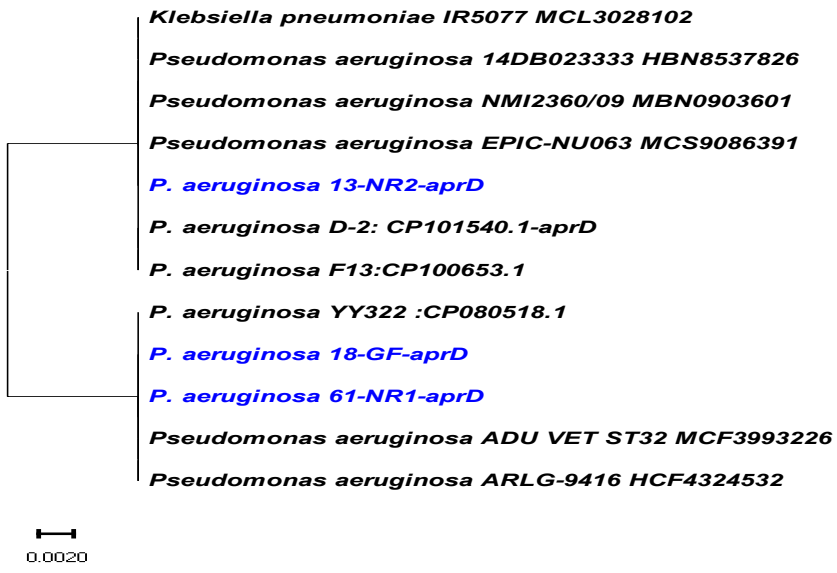


Figure (4-14). Phylogenic tree of *AprD* protein using the Neighbor-Joining method which draw according to MEGAXI program.

Figure (4-15) showed the results of *aprF* DNA-sequence which appeared the local isolates *P. aeruginosa 18-GF* and *13-NR*2 and *61-NR1* had identical sequences compared with sequences of those available in databanks (NCBI). Whereas the isolate *P. aeruginosa 60-ZR* had two mutations the first deletion (C) at position 30 and substitution mutation C>A in the position 139 of studied sequence . The deletion mutations lead to change the gene's reading frame of Apr F protein (OMP) (Fig. 4-16/A) that leads to change protein structure and then may be intracellularly degraded in comparison with native protein structure (Fig. 4-16/B).

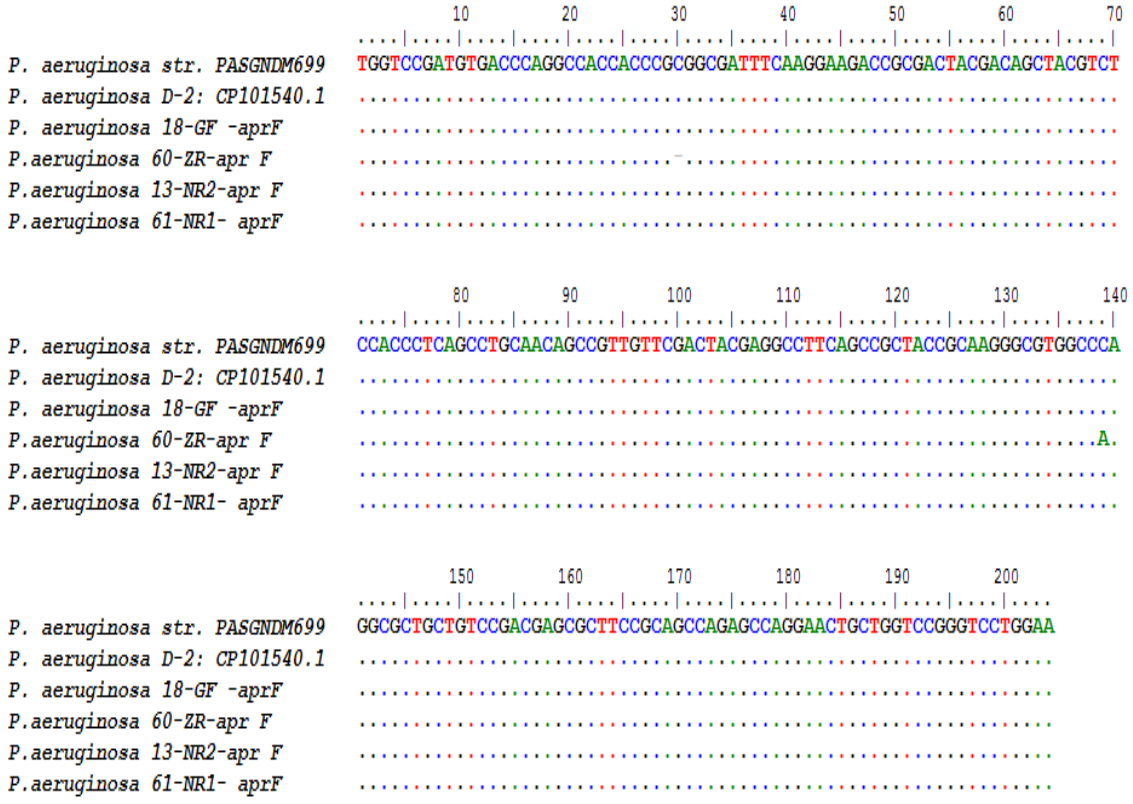


Figure (4-15) Nucleotides sequence alignment of the *aprF* gene among the local isolates and nucleotides sequence of those available in databanks.

The local isolates including *P. aeruginosa 18-GF, 13-NR*2, *61-NR1* & *60-ZR* isolates. Data analysis according to Bio Edit program version 7.2.5.

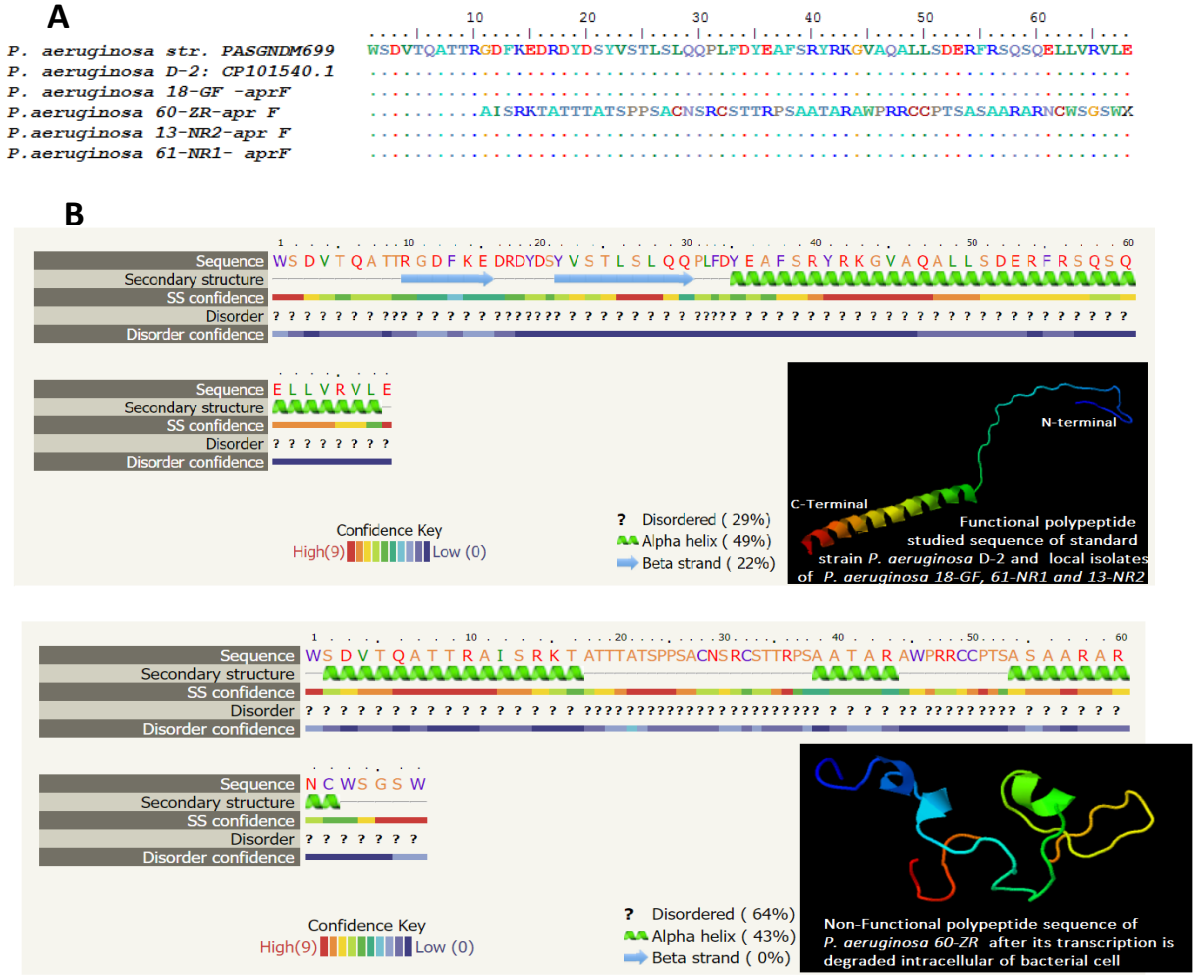


Figure (4-16) Pair sequence alignment of the amino acid of the *P. aeruginosa* AprF protein and their protein secondary structures.

A: Amino acid alignment of AprF protein among the *P. aeruginosa 18-GF*, *13-NR2*, *61-NR1* & *60-ZR* isolates and amino acid sequences of those available in databanks. draw based on the alignment of BioEdit program version 7.2.5

B: Polypeptide secondary structures of standard strains and the local *P. aeruginosa 18-GF*, *13-NR2*, *61-NR1* isolates are identical. While the polypeptide of *P. aeruginosa 60-ZR* is abnormal so it non-functional and then is intracellularly degraded. These structures were predicted according to http://www.sbg.bio.ic.ac.uk/phyre2/phyre2_output/928cd58e21209224/summary.html .

Figure (4-17) showed the results of *aprE* DNA-sequence which appeared the local isolates *P. aeruginosa 18-GF* and *13-NR2* had one substitution mutation (G>C) at position 111 of studied sequence compared with sequences of those available in databanks (NCBI). This

mutation leads to change amino acid from glutamine (Q) to histidine (H) (Fig. 4-18/A) which had slightly change or not on protein conformation as shown in the figure (4-18/B).

Figure (4-19) showed The phylogenetic tree of AprE protein of *P. aeruginosa*, 18-GF-aprI and 13-NR2 which located in the new clade compared with available amino acid sequences of data bank (NCBI)

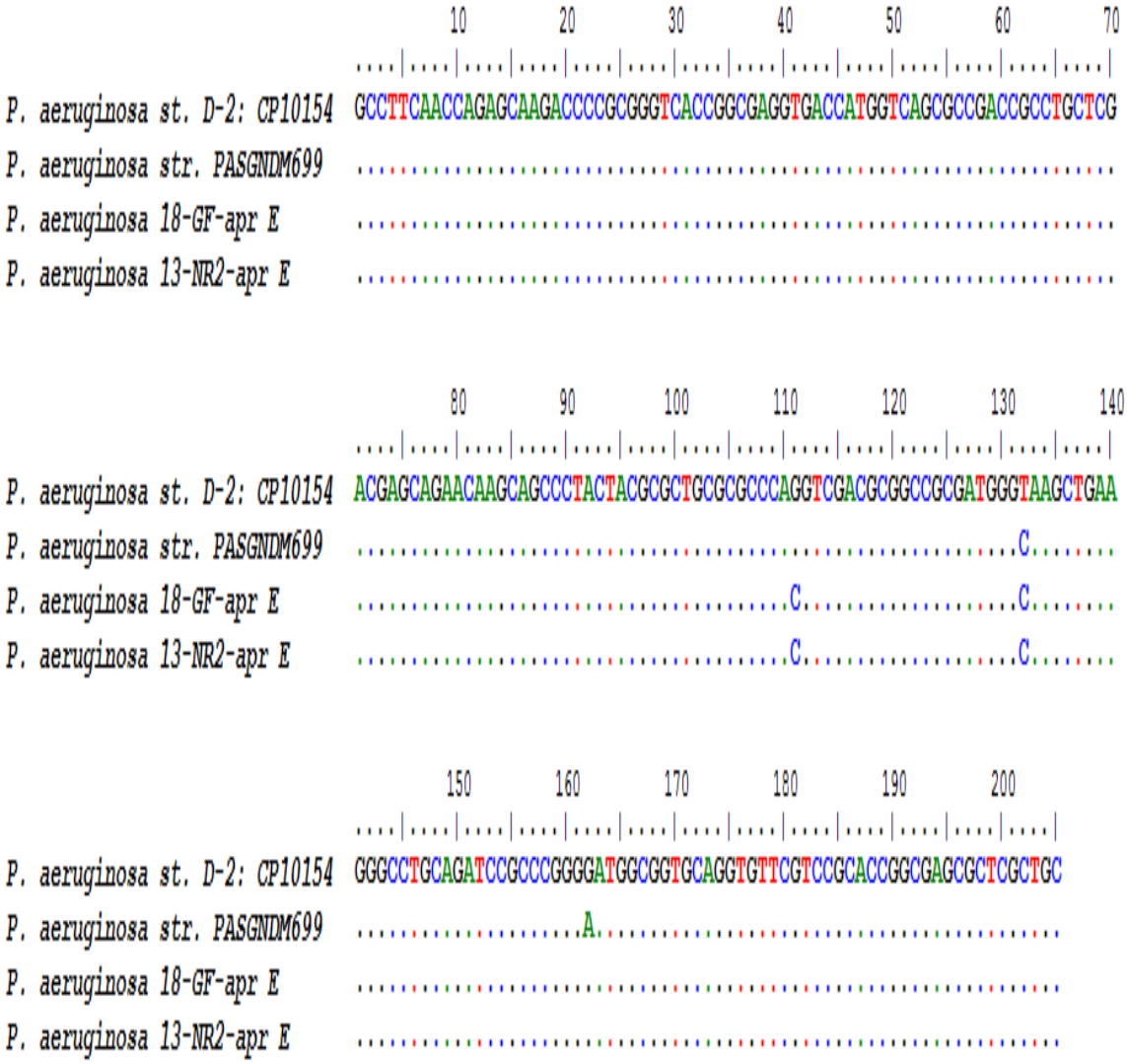


Figure (4-17) Nucleotides sequence alignment of the *aprE* gene among the local isolates and nucleotides sequence of those available in databanks.

The local isolates including *P. aeruginosa 18-GF and 13-NR*2 isolates. Data analysis according to Bio Edit program version 7.2.5.

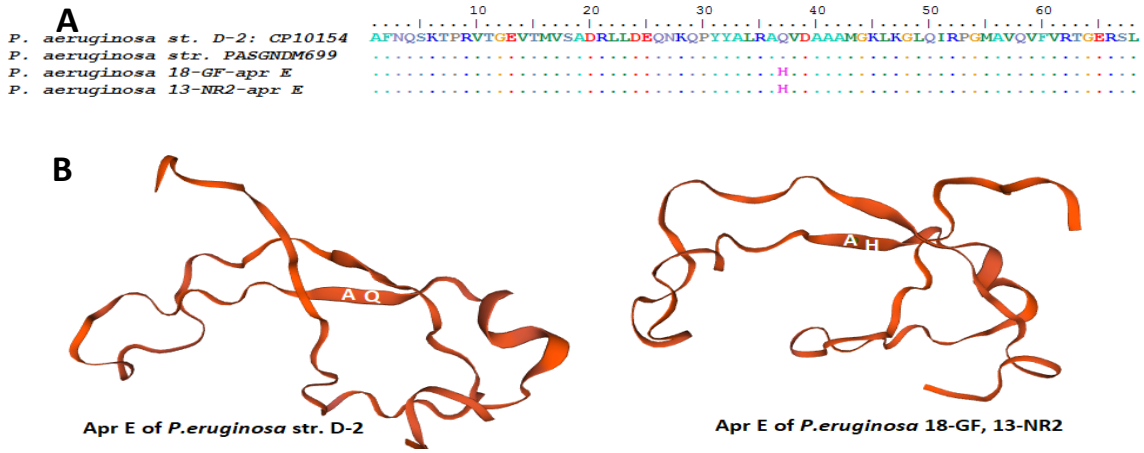


Figure (4-18). Pair sequence alignment of the amino acids and polypeptide secondary structures of the *P. aeruginosa AprE*.

A: Amino acid alignment of AprE protein among the *P. aeruginosa 18-GF & 13-NR*2 isolates and amino acid sequences of those available in databanks. draw based on the alignment of BioEdit program version 7.2.5

B: Protein secondary structures of local isolates and standard strains are identical according to https://swissmodel.expasy.org/interactive/3HXLwk/models/

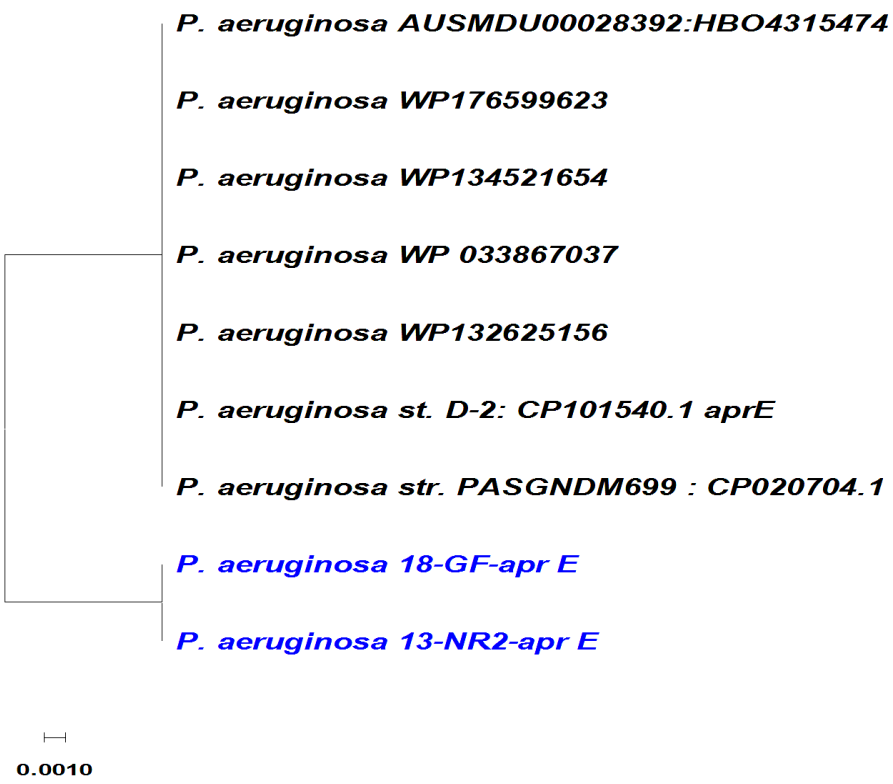


Figure (4-19). Phylogenetic tree of *AprE* amino acid sequence *P. aeruginosa* of using the Neighbor-Joining method which draw according to MEGAXI program

The overall of PCR-DNA sequencing results of alkaline protease gene cluster showed both the local *P. aeruginosa 18-GF and 13-NR*2 isolates had complete *apr A* gene cluster. But they were different in the extracellular alkaline protease levels (Table 4-2), *P. aeruginosa 13-NR*2 produce the enzyme more than *P. aeruginosa 18-GF* as indicated the levels (3.2 and 2.5 Z/C on milk agar medium respectively) that may be due to the gene expression of the enzyme, or the controlling protein inhibitor AprI of *P. aeruginosa 13-NR*2 which had three mutations compared with the inhibitor of isolate *18-GF* that may be effected. Also, the secretion system of *P. aeruginosa 13-NR*2 may be more efficient than the other isolate due to the ABC-transporter of this isolate contain a glutamine (Q) at position 32 of predicted amino acid sequence that homologues to standard strain *P. aeruginosa D-2* (Fig. 4-15). Whereas, the ABC-transporter of *P. aeruginosa 18-GF* contain an arginine (R) at position 32 of predicted amino acid sequence that homologues to standard strain *P. aeruginosa* YY322, may be the change of amino acid at this position had effect on alkaline protease transported or translocation from inner cytoplasmic membrane to outer membrane (into periplasmic space) via the recognition of a C-terminal secretion signal on a given RTX protein translocation substrate, the inner membrane complex formed by an energy-providing ABC transporter and an MFP contacts the trimeric OMP (Linhartova *et al*., 2010, Morgan *et al*., 2017). Finally, these isolates may produce other type of extracellular proteases such as alkaline protease VI or elastase B

The isolate *P. aeruginosa 61-NR1* harbor incomplete alkaline protease gene cluster lacking *apr E* gene which responsible for OMP expression that transport the enzyme from periplasmic space into environment or medium so

the enzyme survives in periplasmic space of bacteria. So the detected low-protease level may be due to releasing the enzyme after some bacterial cells lysis or the isolate produce other type of extracellular proteases (Galdino *et al.*, 2019 ; Tuon *et al.*, 2022). Isolate *P. aeruginosa 60- ZR* was unable to produce extracellular proteases on milk medium, despite harboring only two genes *aprA* and *aprF* depending on PCR detection. But these genes are non-functional due to the presence of frameshift mutations within the studied sequences according to the PCR-DND sequencing method. The isolates do not produce alkaline proteases due to an incomplete protease gene pool or lack of the *aprA* gene cluster. Secretion of the alkaline protease is impaired by a deficiency of secretion genes that lead to intracellular protein survival and partial degradation. Each of the three *apr* genes D, E, and F is required for the secretion of alkaline proteases because a mutation in one of these genes abolished the secretion (Guzzo *et al*., 1991; Galdino *et al.*, 2019).

4.6 Molecular Analysis of *toxA* Gene

Figure (4-20) showed the amplified band (150bp) of *toxA* gene on agarose gel electrophoresis. About 95.5% of *P. aeruginosa* isolates harbor *toxA* gene, these isolates include 17 (89.47%) of sensitive to antibiotics, 34 (94.45%) of non-MDR isolates and all of the MDR isolates harbor *tox A* gene (Fig.4-21). The presence of the *toxA* gene not associated with antibiotic resistance (P=0.45), but the MDR isolates became more virulent when they produce the exotoxin A.

Three *P. aeruginosa* isolates were randomly selected from three groups, MDR *P. aeruginosa 13-NR2*, Non-MDR *P. aeruginosa 61-NR1* and susceptible *P. aeruginosa 18-GF* isolate for further analysis using PCR-DNA sequencing technology. The results appeared *tox A* gene of the MDR isolate *P. aeruginosa 13-NR2* had one substitution mutation C > T at position 60 of studied sequence (Fig. 4-22?A). But this mutation is

Silent because of the change of a single DNA nucleotide within a protein-coding region of *tox A* gene does not affect the amino acid sequence (Serine: AGC> AGT) of the toxin A (Fig. 4-22/B) with identity about 100% compared with exotoxin A protein of standard strains (Fig.4-22).

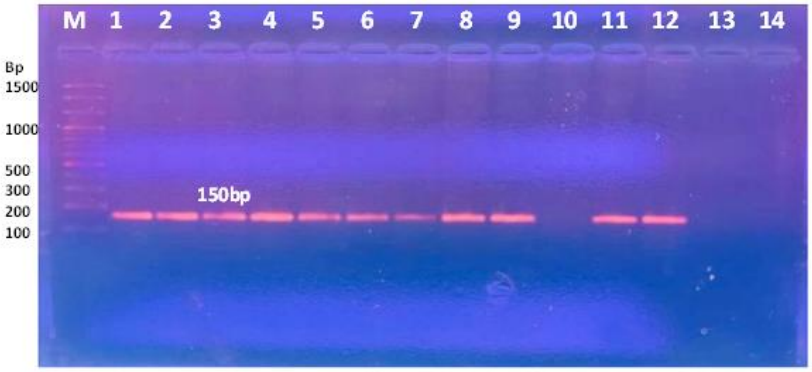


Figure (4-20). Agarose gel electrophoresis of *tox A* amplified product patterns of *P. aeruginosa* isolates

M: refers to DNA size marker (100-1500 bp); lanes 1 – 9 and 11-12 refer to positive results of PCR product (150 bp) of *tox A* and lanes 10, 13 and 14 refer to negative results for *tox A* of *P. aeruginosa* isolated from burn infections. Electrophoresis conditions: 2% agarose concentration; 100 V, 20 mA for 60 min. Staining method: precast Red Safe stain.

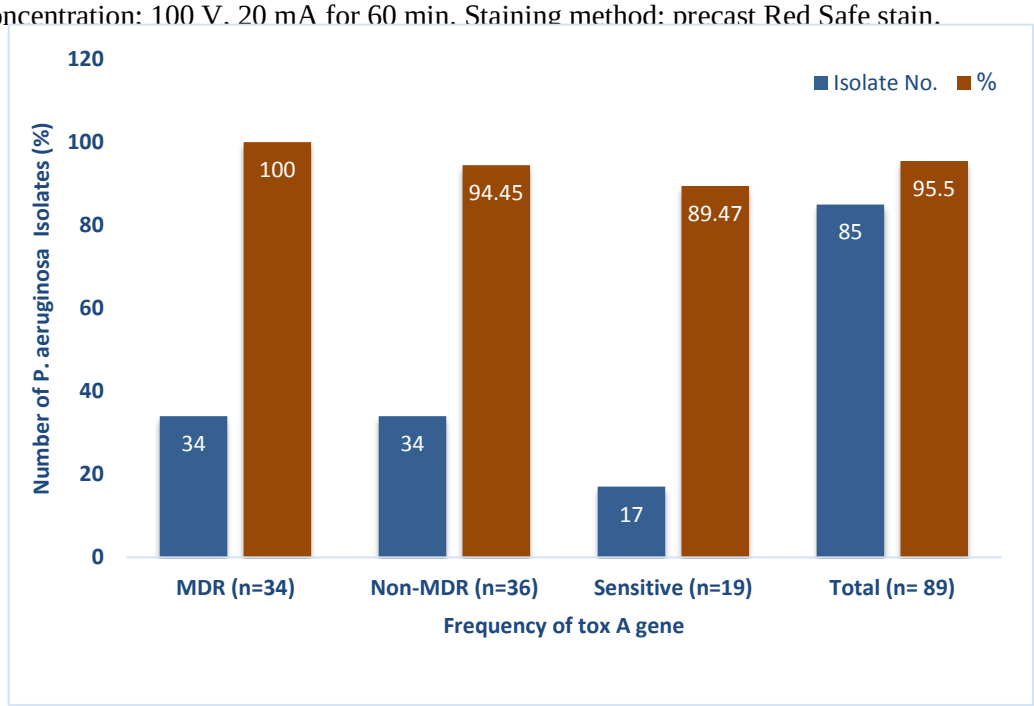


Figure (4-21). Frequency of the presence of *tox A* in *P. aeruginosa* isolated from burn infections

MDR: Multi-drug resistance *P. aeruginosa* which resist at least three class of antibiotics. Non-MDR *P. aeruginosa* which resist less than three class of antibiotics.

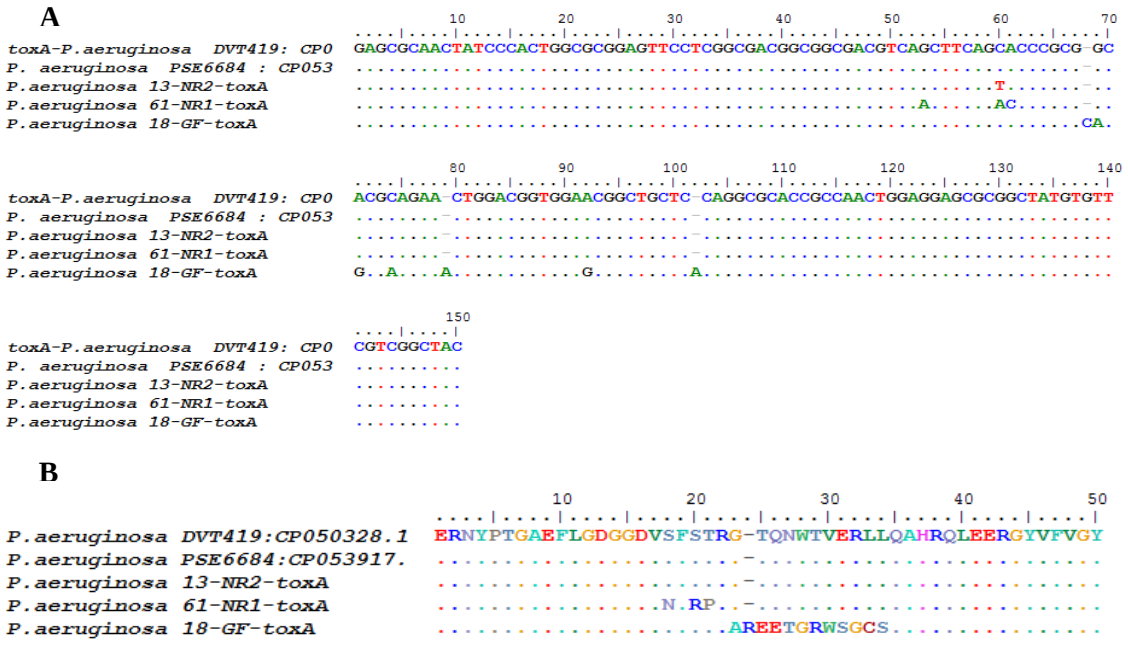


Figure (4-22). Nucleotides sequence and amino acid alignment of the *toxA* gene among the local *P. aeruginosa*

A: Nucleotides sequence alignment of the *toxA* gene among the local *P. aeruginosa* isolates and nucleotides sequence of those available in databanks. The local isolates including *P. aeruginosa* isolate 13-NR2, 61-NR1 and 18-GF. Data indicated that the position of nucleotides is different among isolates and identical data for all isolates are not shown by Bio Edit program version 7.2.5.

B: Pair sequence alignment of the amino acid of the *toxA* gene among the local *P. aeruginosa* isolate 13-NR2, 61-NR1 and 18-GF and amino acid sequences of those available in databanks. draw based on the alignment of Bio Edit program version 7.2.5

Whereas there are three substitution mutations in the *tox A* gene of Non-MDR *P. aeruginosa 61-NR1* includes G>A, C>A and A>C at positions 53, 60 and 61 of studied sequence respectively, that leads to change amino acids from serine to asparagine (S>N), from serine to arginine (S>R) and from tyrosine to proline (Y>P) respectively (Fig.4-21B). So this amino acid sequence had identity about 94% compared with exotoxin A protein of Genbank standard strains.

The studied DNA sequence of *toxA* gene in *P. aeruginosa 18-GF* revealed the presence several mutations within it includes three insertion

mutations at positions 68, 79 and 102 of studied sequence (and four substitution mutations at positions 69, 71,74 and 92 that leads to change 12 amino acid sequence located extended from 23-34 of the studied amino acid sequence and this region located in Domain III of exotoxin A from 413-424 of native protein sequence with identity about 84% compared with exotoxin A protein of standard strains of GenBank Data Base as shown in the figure (4-23/A). This amino acid change effect on protein conformation and may be on exotoxin function (Fig. 4-25B). which is participate to form exotoxin complexed with Nicotinamide and AMP. Whereas the mutations in other isolate (*P. aeruginosa 61-NR*1) also changed three amino acids as shown in the figure (4-23/B), which may be slightly effect on exotoxin conformation (domain III) and function.

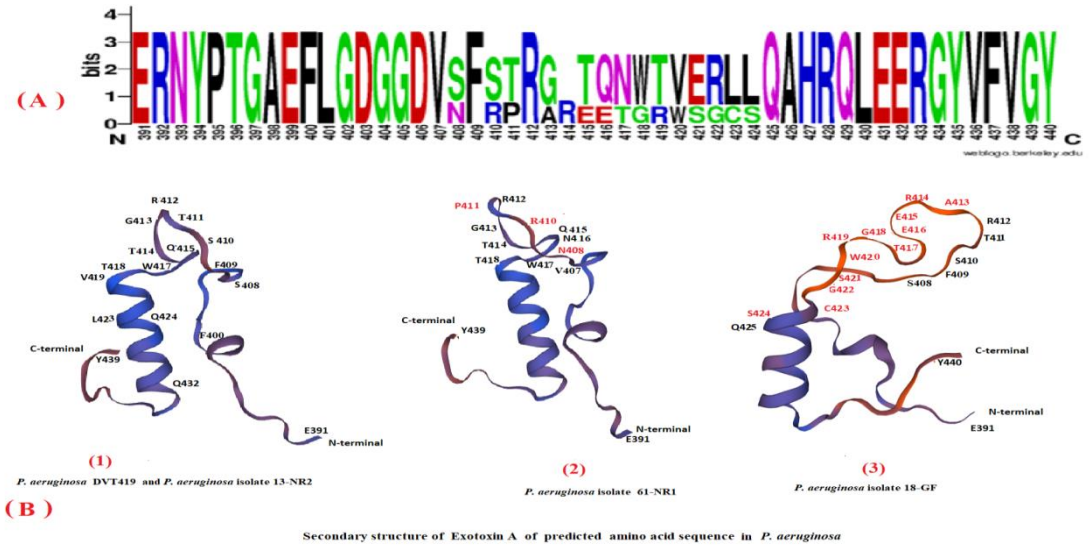


Figure (4-23). Secondary structure of *P. aeruginosa* Exotoxin A of predicted amino acid sequence.

A: The Weblogo of amino acids repeats of studied region (391-439) composed of the end sequence of binding domain Ib (365-404) and the beginning sequence of Domain III ADP-ribosylation of elongation factor 2 (405-613 aa) of exotoxin A.

B: Secondary structure of studied region of an Exotoxin A in *P. aeruginosa* isolates; 1: Standard strain *P. aeruginosa* DVT419 and *P. aeruginosa* 13-NR2; 2: *P. aeruginosa 61-NR1; 3: P. aeruginosa* 18-GF according to https://swissmodel.expasy.org/interactive/4nuJky/models/

The phylogenetic tree of *ToxA* protein showed the *P. aeruginosa* located in the new clade wherease the other isolates located in the same clade of data bank reference stains , as showed in Figure (4-24).

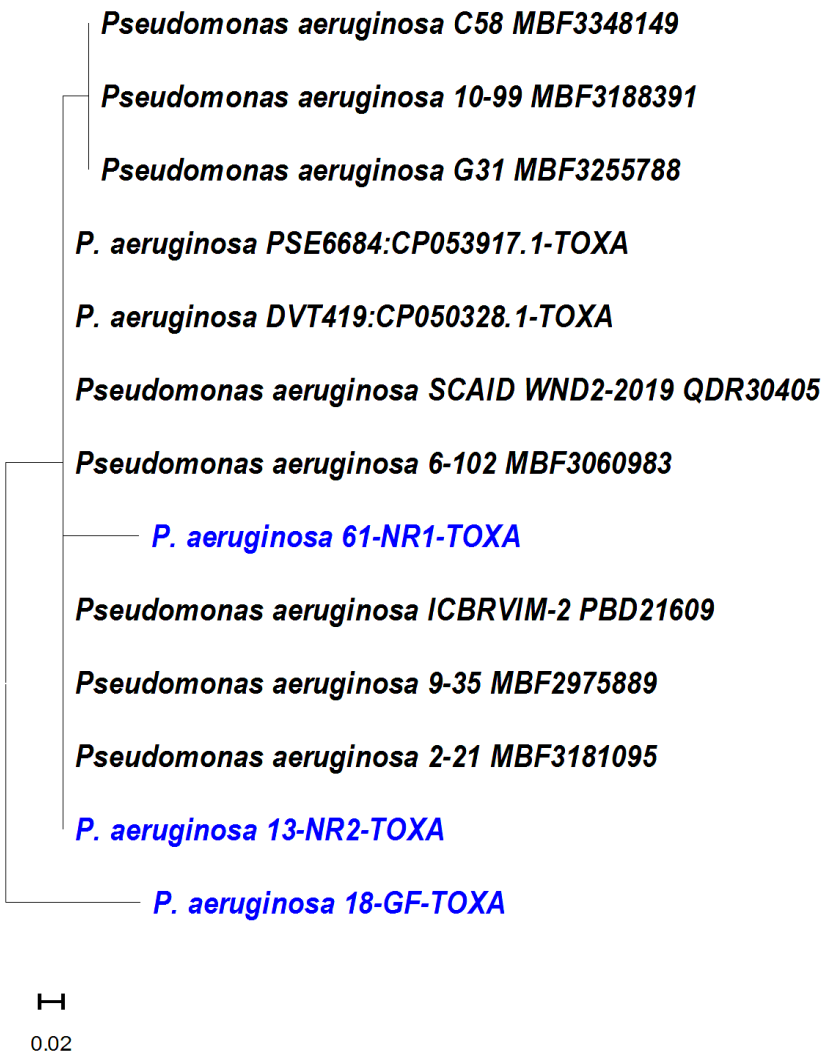


Figure (4-24). Phylogenic tree of ToxA amino acid sequence of *P. aeruginosa* using the Neighbor-Joining method which draw according MEGAXI program

Exotoxin A (ExoA) of *P. aeruginosa* is an important virulence factor belonging to the class of exotoxins secreted by pathogenic bacteria that cause human diseases such as cholera, diphtheria, pneumonia and whooping cough. The third domain of EoxA is involved in the formation

of a catalytic complex composed of ExoA with an elongation factor 2 (eEF2) and proper NAD (+), suggesting a direct role of two rings of the active site in ExoA during the catalytic cycle. Mutational studies of the two rings in the ExoA identify several important residues of catalytic activity, in particular Glu 546 and Arg 551, clearly supporting new complex structures that contribute to the formation of the transition state model of the toxin-catalytic reaction (Jørgensen *et al.*, 2008). Most of *P. aeruginosa* isolates which recovered from burn infections produce exotoxin A and generally resist to recently used antibiotics and some of them MDR isolates.

conclusions

1. Based on our results, the *Pseudomonas aeruginosa* isolates that possessed the gene clusters (*aprI, apr A, apr F, apr E & apr D*) for the Alkaline protease enzyme It may lead to making bacteria more virulent and resistant to antibiotic
2. Four substitution mutations at positions that leads to change 12 amino acid sequence located in Domain III of exotoxin A identity 84% compared with exotoxin A protein of standard strains of GenBank with This amino acid change effect on protein conformation and may be on exotoxin function By targeting new somatic cells or changing its effect inside the body
3. The DNA-sequencing of *AprA* P. aeruginosa 13- NR2 and P. aeruginosa 61- NR1 had one a substitution mutation that had slightly change of non-change in protein structure while *P. aeruginosa* 18- GF had two mutation and detection These mutations make the isolates can not produce alkaline protease.
4. There are three transversion mutations in the Apr I of the isolate P. aeruginosa 18-GF is slightly effects on the protein secondary structure .

5. The *aprE* DNA-sequence which appeared the local isolates *P. aeruginosa* 18-GF and 13-NR2 had one substitution mutation which leads to change amino acid from glutamine (Q) to histidine (H) that may leads to slightly change or not on protein conformation.

Recommendations

Study the gene expression of the studied gene and their ability on the production of enzyme.

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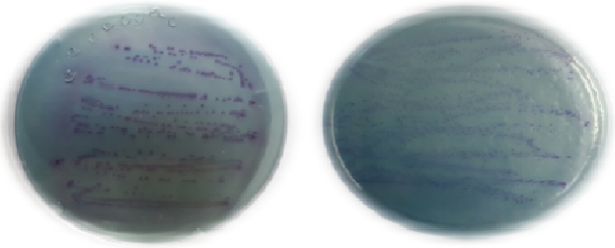
Appendix

A: Bacterial colonies appeared blue-green on *Pseudomonas*™ chromogenic agar



B: VITEK® 2 system for *P. aeruginosa* diagnosis

bioMérieux Customer: Microbiology Chart Report

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: Burn infections **Collected:**

Comments:	

Identification Information	**Analysis Time:**	5.80 hours	**Status:**	Final
Selected Organism	98% Probability	**Pseudomonas aeruginosa**		
	Bionumber:	0003451303500050		
ID Analysis Messages				

Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	+	62	ELLM	-	64	lLATa	-			

Figure (4-2). *P. aeruginosa* diagnosis by chromogenic agar and Biochemical tests using VITEK system.

A: Blue-*P. aeruginosa* colonies on chromogenic agar incubated at 37°C for 24h Due to produce pyocyanin pigment.

B: *P. aeruginosa* diagnosis by Biochemical tests using VITEK® 2 system.

الخلاصة

تعد بكتريا الزائفة الزنجارية السالبة لصبغة جرام من اهم مسببات الامراض الانتهازية حيث تسبب العديد من الالتهابات الحادة والمزمنة مع ارتفاع معدلات الاعتلال والوفيات. تم تصميم هذه الدراسة كدراسة مقطعية في المرضى الذين يعانون من عدوى *Pseudomonas aeruginosa*. تم عزل حوالي ٢٧ عزلة من *Pseudomonas aeruginosa* من ٧٠ عينة من مرضى الحروق خلال الفترة () و ٦٢ عزلة من النوع *P. aeruginosa* نقية معزولة سابقاً من مرضى الحروق، وتم جمع هذه العينات من مستشفى الحلة التعليمي ومستشفى الإمام صادق التعليمي في محافظة بابل خلال ٢٠٢٠_٢٠٢١

أظهر اختبار الحساسية للمضادات الحياتية ان النسبة الأعلى للمقاومة للمضادات كانت بنسبة (٥٨.٤٢٪) للمضاد الحيوي الأميكاسين (AK) وكانت متوسطة المقاومة للمضاد الحيوي للبيبراسيلين (PRL) بنسبة (٢٣.٦٠٪) ، بينما ظهرت أعلى نسبة حساسية للمضاد الحيوي الميروبينيم (MEM) بنسبة (٤٧.١٩٪) ، بالإضافة الى ان معظم عزلات بكتريا الزائفة الزنجارية كانت متعددة المقاومة للمضادات بنسبة ٢٣.٦٪ ، في حين ان نسبة ٢١.٣٤٪ من العزلات كانت حساسة لجميع المضادات الحيوية.

أظهرت الدراسة الحالية أن حوالي ١١ عزلة بنسبة (١٢.٣٦٪) اظهرت مستوى عالٍ من إنتاج انزيم البروتييز بمعدل (3-3.9) ،و ٣٩ عزلة اخرى بنسبة (٤٣.٨٢٪) كانت ذات معدل متوسط في انتاج الانزيم بمعدل (2-2.9) و ٢٩ عزلة بنسبة (٣٢.٥٨٪).) منخفضة الانتاج للانزيم بمعدل (1-1.9) ، في حين ١٠ عزلات اخرى بنسبة (11.244%) لم تظهر أي انتاج للانزيم

اعتمادا على التشخيص المظهري الوراثي لعزلات بكتريا *P. aeruginosa* ان عزلة البكتريا نمط (*P. aeruginosa* 13- NR2) لها القدرة على انتاج انزيم البروتييز القاعدي بمستوى عالي مما يجعلها مقاومه بشكل كبير للمضادات الحيوية ، بينما أظهر *P. aeruginosa* 18_GF انتاج متوسطً من انزيم البروتييز الخارج خلوي وحساسًا للمضادات الحيوية . حيث ان كلا النمطين كانا يملكان مجموعة جينية كاملة *aprA* والتي تشفر إلى البروتييز القاعدي (*AprA*) ومثبط البروتييز القاعدي (*AprI*) والنظام الإفرازي من النوع الأول (TISS).

أظهر اختبار التسلسل الجيني (sequence) للعينات التي اجري لها اختبار البلمرة المتسلسل لجينات البروتييز القاعدي من نوع *P. aeruginosa* 13- NR2 *aprA* و *P. aeruginosa* 61- كان لدى NR1 طفرة استبدال (C> T) في منطقة *aprA* المدروسة في الموضع ٦٠ و *P.*

aeruginosa 18- GF كان لديه طفرة استبدال (A> G) في المواضع ١٧٤ ، اما بالنسبة لجين *aprI* ، ظهرت ثلاث طفرات استبدال في *P. aeruginosa* 13- NR2 تشمل (A> C و C> A و C> A) في المواضع ١٠١ و ١٢٣ و ١٤١ على التوالي. بالإضافة إلى ذلك ظهرت جينات النظام الإفرازي من النوع الأول لـ *aprD* و *aprF* لـ *P. aeruginosa* 18-GF و NR2-١٣ و NR1-٦١ تسلسلات متطابقة مع متواليات من تلك المتوفرة في بنك الجينات (NCBI) ،في حين كان لعزل *p. aeruginosa* 60-ZR أظهر تحوران ، الحذف الأول (C) في الموضع ٣٠ وطفرة الاستبدال (C> A) في الموضع ١٣٩ ، أخيرًا ظهر جين *aprE* للعزلات المحلية *P. aeruginosa* 18-GF و NR2-١٣ طفرة استبدال واحدة (G> C) في الموضع ١١١ مقارنة بتسلسلات الجينات المتوفرة في بنك الجينات (NCBI).

أظهرت نتائج التحليل الجيني لجين *toxA* وجود تضخم عند الوزن الجزيئي (١٥٠ زوج قاعدة) ، حيث وجد هذا الجين في حوالي ٩٥.٥٪ من عزلات *P. aeruginosa* ، حيث كانت ١٧ (٨٩.٤٧٪) حساسة للمضادات الحيوية ، ٣٤ (٩٤.٤٥٪) متعددة المقاومة للمضادات الحيوية. تم اختيار ثلاث عزلات من *P. aeruginosa* عشوائياً من ثلاث مجموعات ، MDR *P. aeruginosa* 13-NR2 و Non-MDR *P. aeruginosa* 61-NR1 و *P. aeruginosa* 18-GF الحساسة لمزيد من التحليل باستخدام تقنية تسلسل PCR-DNA.

أظهرت النتائج أن الجين *toxA* لعزلة MDR *P. aeruginosa* 13-NR2 له طفرة إحلال واحدة C> T في الموضع ٦٠.

جمهـــــــــورية العـــــــــراق
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قســـــم علـــوم الحيـــــاة

انتشار نظام الافراز الاول (Apr DEF) وانزيم البروتييز القاعدي (AprA) في عزلات الزوائف الزنجارية المعزولة من اصابات الحروق .

رسالة مقدمة إلى

مجلس كلية العلوم – جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في العلوم / علوم ألحياة

من قبل

زهراء حمزة مرزة حمزه

بكالوريوس علوم حياة/ جامعة الكوفة (٢٠١١)

بإشراف

الاستاذ الدكتور

رباب عمران راضي الجيلاوي

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