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Integration of Phenotypic, Molecular and Computational Approaches for the Study of Antibiotic Resistance in *Pseudomonas aeruginosa* Clinical Isolates

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{وَإِن مِّن شَيْءٍ إِلَّا عِندَنَا خَزَائِنُهُ
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صَدَقَ الله العَلي العَظيم

سورة الحجر/ الآية ٢١

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Was prepared under our supervision in the College of Medicine/ University of Karbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology.

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Dedicated to….

My dear mother, the soul of my father, the Support of

my brothers & my lovely wife

To my soul & and my life my sons with lots of love

Alaa

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Summary:

Pseudomonas aeruginosa is a complicated pathogen that causes infections with high mortality rates. Because of its ability acquired resistance to many first-line antibiotics, this study was designed to explore the distribution and association of *carbapenemase , ESBL* and *PMQR* genotype pattern *(VIM, NDM, OXA, CTX, QNR-A, and QNR-B*) with antibiotic resistance in local clinical *Pseudomonas aeruginosa* isolates.

A total of 240 samples (100 urines, 100 burn swabs, and 40 wound swabs) were included in this study. Among these Only 43 isolates of *Pseudomonas aeruginosa* were collected from different specimens between November 2024 to May 2025. The isolates included 29 from burns, 5 from wounds, and 9 from urinary tract infections (UTIs). Identification of the isolates was carried out using microscopically and cultural characterization using MacConkey agar, blood agar and cetrimide agar. identification was carried out by the VITEK_2_Compact system.

To assess the frequency of antibiotic resistance in *P. aeruginosa* (MDR and XDR), the antibiotic susceptibility test was done by the VITEK_2_Compact system. The test included two groups of antibiotics, totaling seven agents. The antibiotic susceptibility of *Pseudomonas aeruginosa* were analyzed according to the type of infection, whether UTIs, burns, or wounds. For Impenem, the highest percentage of isolates resistant to this antibiotic was those isolated from burns, at 93.1.

Regarding Meropenem, the highest percentage of resistant isolated from burns, with 86.2%.

As for Levofloxacin, Ciprofloxacin, Ofloxacin, and Norfloxacin, the highest percentage of isolates resistant to this antibiotic was those isolated from burns, at 62.1%, according to Nalidixic acid, all *Pseudomonas aeruginosa* Isolates Were Resistant to Nalidixic acid.

Gene Detection analysis was perform for six genes (*OXA,VIM,CTX,NDM,QNR-A* and *QNR-B*) across different infection type.

(A) UTI Cases :The *OXA*, *QNR_A*, *QNR_B*, and *VIM* genes were 100% detected in UTI cases; the *CTX* gene was detected at 55% in those cases, while the *NDM* gene was detected only in 11.2% of UTI cases.

(B) BURN cases, all genes of *P. aeruginosa* showed high gene detection in burn cases, except the *NDM* gene, which was not detected in 90% of those samples (only 10% were positively detected), The highest percentage of genes that detected was *QNR-B* (97.7%).

(C) WOUND cases: The *oxa*, *qnr_a*, *qnr_b*, and *vim* genes were 100% detected in wound cases, the *CTX* and *NDM* genes were detected at 80% and 60%, respectively, in those cases.

Genetic sequence analysis revealed the spread of several mutations, among *Pseudomonas aeruginosa* Isolates. The *CTX* gene exhibited highest number of mutations followed by the *OXA*, VIM, and *QNR-A* genes in succession, while the *NDM* gene was the least prevalent in terms of the number of mutations.

Also, when studying mutations through a bioinformatics study, there were many missense and silent mutations, across the examined genes. The effect of these mutations on the resulting protein was studied through molecular docking.

In conclusion, the results of this study revealed a considerable prevalence of *carbapenemase, ESBL* and *PMQR* genes in *Pseudomonas aeruginosa* isolated from Al-Diwanyih hospitals. In addition, it was shown that the vast majority of carbapenemase *Pseudomonas aeruginosa* isolates were classified as XDR. Therefore, carbapenemase screening should be implemented for routine laboratory practice in Iraqi hospitals. In addition, infection control measures are needed to prevent further spreading of these organisms.

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List of abbreviations

Abbreviation	Key
bla gene	**β-lactamases gene**
CFU	**Colony forming units**
CLSI	**Clinical and Laboratory Standards Institute**
CRPA	**Carbapenem resistant *Pseudomonas aeruginosa***
CTX-M	**Cefotaximase, β-lactamase active on cefotaxime**
DNA	**Deoxyribonucleic acid**
EDTA	**Ethylene diamine tetra acetic acid**
ESBL	**Extended-spectrum β – lactamase**
I	**Intermediate**
MBL	**Metallo- β-lactamase**
MDR	**Multi drug resistance**
MHA	**Mueller-Hinton agar**
MIC	**Minimum inhibitory concentration**
NDM	**New Delhi Imipenemase**

OXA	**Oxacillinases, β-lactamase active on oxacillin**
PCR	**Polymerase chain reaction**
PDR	**Pan-drug resistance**
PW	**Peptone Water**
R	**Resistance**
S	**Sensitive**
Spp.	**Species**
UTI	**Urinary tract infection**
VIM	**Verona integron-encoded metallo-β-lactamases**
WHO	**World health organization**
XDR	**Extensive-drug resistance**
VAP	**ventilator-associated pneumonia**
ETA	**exotoxin A**
CAUTI	**catheter-associated urinary tract infections**
PBPs	**penicillin-binding proteins**
PA	***Pseudomonas aeruginosa***
TBE Buffer	**Tris-Borate-EDTA**
PMQR	**plasmid-mediated quinolone resistance**
ESBL	**Extended-Spectrum Beta-Lactamases**
COPD	**chronic obstructive pulmonary disease**
SNPs	**single nucleotide polymorphisms**
NGS	**Next-Generation Sequencing**
IMP	**Imipenem**
MER	**Meropenem**

CIP	**Ciprofloxacin**
NOR	**Norfloxacin**
LEV	**Levofloxacin**
NA	**Nalidixic acid**
OFX	**Ofloxacin**
PDB	**Protein Data Bank**
SIFT	**Sorting Intolerant From Tolerant**
PolyPhen-2	**Polymorphism Phenotyping**
PhD-SNP	**Predictor of human Deleterious Single Nucleotide Polymorphisms**
Meta-SNP	**metadata" for single nucleotide polymorphisms**
mSCM	**Mutation cutoff scanning matrix**
SDM	**Site directed mutator**

Chapter One

Introduction &

Review of Literature

1.1 Introduction:

Pseudomonas aeruginosa (*P. aeruginosa*), constitute a great challenge in the microbiology specialty and therapy of infectious diseases. *P. aeruginosa* is an opportunistic pathogen known for its adaptability and high resistance to a wide range of antibiotics, which makes healing these bacteria a complex matter.

P. aeruginosa is usually found in various environments, such as soil, water, and hospitals, where it can cause opportunistic infections, especially in those who have weak immune systems (Lister *et al.*,2009). The clinical outcomes of pseudomonal infections are significant and emphasize the requirement for an inclusive comprehension of their pathogenic mechanisms and resistance aspects, as well as the therapeutic approaches available to combat these challenging pathogens. Since the global health landscape is promoting the escalating prevalence of antibiotic-resistant strains of *P. aeruginosa*, there is a critical necessity for effective surveillance, investigation, and development of novel antimicrobial therapies (Abdelrahim S. *et al.*,2024).

P. aeruginosa is an important opportunistic pathogen characterized by its ability to induce a range of infections, especially in immunocompromised people, the pathogenic potential of *Pseudomonas spp.*, particularly *P. aeruginosa*, is attributed to a variety of virulence factors that enable their survival and propagation within host tissues (Xiong *et al*., 2021).

Biofilms formation, is a principal aspect of pathogenicity of *P. aeruginosa* characterized by organized aggregations of microorganisms embedded in a self-produced polymeric matrix, this matrix enables the adherence of *P. aeruginosa* to both living and non-living surfaces. This feature presents a considerable challenge in clinical settings, as biofilms are associated with persistent infections, such as those in cystic fibrosis cases and infections linked with medical devices. The protective features of biofilms not only enhance resistance to antimicrobial agent but also offer a protection

against host immune defenses (Saki, *et al*.,2022). Beyond of biofilms formation, *P. aeruginosa* utilizes a wide spectrum of secreted toxins and enzymes that damage ordinary cellular functions. The most important of which are exotoxin S and exotoxin U (*ExoS* and *ExoU)*, which can trigger the apoptosis for host cells or intervene with cellular signaling process. Additionally, *P. aeruginosa* produces proteolytic enzymes and phospholipases that disrupt host tissues, expediting the spread of infection. Collectively, these virulence factors enable the establishment of infections and evading immunological detection (Yamba *et al*., 2023).

In addition to biofilm formation, intrinsic resistance mechanisms to several antibiotics are significantly contribute to the pathogenicity of *P. aeruginosa* include the presence of efflux pumps, that emit antimicrobial agents from bacterial cell, and the capacity to develop resistance genes via horizontal gene transfer, both of which aggravate the challenge of treating the Pseudomonal infections ,the rinsing prevalence of antibiotic-resistant strains further complicates the clinical management, causing treatment failure along with redoubled morbidity rates (Saki *et al*., 2024).

The rising antibiotics resistance among *Pseudomonas* spp. has great implications for public health, especially in immunocompromised individuals and those with chronic conditions (Tesalona *et al*., 2024).

The resistance capacity of *P. aeruginosa* is attributed to multiple factors, such as the presence of a highly impermeable outer membrane and having the expression of efflux pumps that effectively detach antimicrobial substances. Furthermore, particular genetic modifications may permit *Pseudomonas spp*. to acquire resistance against multiple classes of antibiotic, thus complicating the medication strategies. The excessive and inappropriate usage of antibiotic has accelerated the increase of resistant isolates, leading to a modern "post-antibiotic era" characterized by restricted medications alternatives (Aguilar-Salazar *et al*., 2023).

The clinical consequences of this resistance are significant. Infections caused by multidrug-resistant *P. aeruginosa* strains are associated with escalating morbidity and mortality, prolonged hospitalizations, and heightened healthcare costs. Standard treatment frequently involves combination therapies; however, these methods are becoming ineffective as resistance mechanisms continue to develop. Therefore, ongoing research into therapeutic options. is essential, including the development of new antibiotics and alternative treatment strategies (Cheng *et al*., 2024).

The resistance of *P. aeruginosa* is multifaceted, involving a combination of intrinsic factors such as a unique outer membrane structure and efflux pump systems, alongside acquired resistance through horizontal gene transfer. Prominent among these are the genes producing β-lactamases, like metallo-β-lactamases (MBLs) as *bla_VIM* and *bla_IMP*, which confer resistance to carbapenems antibiotics often considered the last- treatment for severe infections (Kashefieh *et al*., 2022).

It is very important for healthcare professionals to be aware of these resistance mechanisms. Considering the genetic foundation of resistance in *P. aeruginosa* is decisive for developing successful treatment strategies and combating the escalation of antibiotic resistance. Ongoing investigation efforts aimed at elucidating these mechanisms will assuredly play a crucial role to address this public health threat (Phetburom *et al*., 2023).

1.2 Aim and Objectives of the Study

This study aimed to identify the occurrence of carbapenem resistant *P. aeruginosa* isolates carrying carbapenemase, ESBL and PMQR genes in clinical samples.

P. aeruginosa had become a notable pathogen, particularly in healthcare settings, as it usually reveals resistance to antibiotics. The main aim of the current investigation is to conduct genotyping of *P. aeruginosa* strains obtained from different clinical samples. This research will include estimating the genetic diversity among these strains and identification of their putative virulence factors.

1- A crucial part of the current investigation is the molecular analysis of virulence genes related with resistance mechanisms, especially, the production of carbapenemases, ESBL, and Plasmid-Mediated Quinolone Resistance (PMQR). By reconnoitering these resistance mechanisms, the current study aims to identify the genetic factors that enhance the pathogenicity and drugs resistance of *P. aeruginosa*.

2- In addition, the current investigation aims to establish an association between the existence of specific virulence genes and their associated clinical consequences, contributing to a deeper comprehension of the epidemiology and clinical relevance of these resistant strains. By genotypic and phenotypic description, the outcomes may inform therapeutics strategies and guide public health measures directed to control infections caused by this exceptionally pathogen. Overall, the current study prospects to improve the understanding of *P. aeruginosa* with in clinical settings, addressing a critical need in the field of infectious disease researches and administration.

3- The study of the genetic sequencing of certain genes (*OXA, NDM,VIM,CTX,QNR-A* and *QNR-B*) related to antibiotic resistance to interpret the mutations and variations that have occurred by bioinformatics study and their impact on the bacterial resistance to these variations.

1.3. Literature Review

1.3.1. *P. aerugino*sa: General characteristic:

P. aeruginosa is a ubiquitous and adaptable pathogen that has garnered remarkable attention in both microbiology and clinical fields for its distinct features and impact on human health, this Gram-negative, rod-shape organism belongs to *Pseudomonadaceae* family and is recognized for its metabolic variety, pathogenicity, along with environmental adaptability. understanding the general features of *P. aeruginosa* is pivotal to understand its role in diverse ecological surroundings, its clinical implications, and its resistances mechanization (Zhao *et al*., 2024).

This bacterium possesses a remarkable metabolic variety and an outstanding capacity to grow in various environments, ranging from soil and water to the surfaces of plants and animals. This ability to adaptation is assigned to its respiratory resilience and the ability to use a wide range of organic and inorganic compounds as energy sources. *P. aeruginosa* can metabolize simple sugars, fatty acids, amino acids, as well as aromatic hydrocarbons, enabling it to exploit different ecological surroundings. This ability is bolstered with a complex set of enzymes and transporter systems, that expedite the uptake and employment of various substrates (Conceição *et al*., 2024).

In addition to its metabolic adaptation, *P. aeruginosa* is known for a peculiar morphology and staining features. As any Gram-negative bacterium, it has a thin peptidoglycan layer enclosed by an outer membrane, which contains lipopolysaccharides, possessing such a structural feature is essential for their pathogenicity, as polysaccharides facilitate evasion of host immune defenses. Under the microscope, *P. aeruginosa* manifests as straight or lightly curved rods. Typically, its diameter ranges from 0.5-0.8 micrometers, while its length is about 1-3 micrometers, the ability of these bacteria to move is due to its possession of polar flagella, which empower it to move over aquatic environments and began infections in host cells (Mudaliar *et al*., 2024).

P. aeruginosa can be detected through biochemical tests, including pigment production. It is able to form several pigments, such as pyocyanin (blue green color) and pyoverdine (yellow green fluorescent color), Pyocyanin has significance due to its distinct role, as it works as a virulence factor, it also function as a mediator in oxidation-reduction reactions, disrupting the host tissues and modifying the immunological responses , the pigments not only enhance the bacterium's adaptability across diverse environments, but as well serve as main discriminatory indicators in clinical microbiology (Hall *et al*., 2016).

This organism distinguished by several phenotypic traits; including its positivity for oxidase and catalase tests, in addition to its ability to produce pyocyanin pigment, which imparts a characteristic coloration in cultures. It's rod-shaped and motility enables it to grow in various environments including the human's cells, preserving its survival even in its natural nutrient-scarce environment and supporting its dual life strategy (as saprophyte in environmental habitat and pathogen in human host). Noticeably, *P. aeruginosa* has a strong biofilm-forming ability, which is responsible for its persistence in natural and clinical environments (Rasamiravaka *et al*., 2015).

1.3.2. *P. aeruginosa* : Taxonomy

Comprehensive knowledge of its taxonomy is not just an academic exercise; it is instrumental in combating the threats posed by this complecated pathogen. As microbial research, progresses the necessity of understanding *P. aeruginosa's* taxonomy will undoubtedly play a critical role in the development of effective public health policies and therapeutic strategies, ultimately improving outcomes for affected patients (George, *et al.*, 2024)

Pseudomonas aeruginosa is classified as follows:

- Domain: Bacteria
- Phylum: Proteobacteria
- Class: Gammaproteobacteria
- Order: Pseudomonadales

- Family: Pseudomonadaceae
- Genus: Pseudomonas
- Species: *Pseudomonas aeruginosa*

1.3.3. *P. aeruginosa*: Historical Context

The genus Pseudomonas was first described in the late 19th century, while *P. aeruginosa* was recognized as a significant pathogen place in the early 20th century. Initially categorized along with other pseudomonads based on phenotypic characteristics and environmental isolation, advancements in molecular biology and genetic sequencing have prompted a more refined understanding of its taxonomy. Recent studies have allowed for the identification of numerous genomic sp. within the *Pseudomonas* genus, including *P. aeruginosa*, thus enabling taxonomists to draw clearer phylogenetic relationships (Mudaliar *et al*., 2024).

1.3.4. *P. aeruginosa*: Pathogenicity

The pathogenic capacity of *P. aeruginosa* is a vital focus of interest, especially in the context of hospital infections. It affects individuals with weak immune system, such as patients with cystic fibrosis, burns, or individuals undergoing invasive procedure. A key factor that exacerbates its pathogenicity is its ability to produce biofilms. these biofilms on surfaces like catheters, ventilator, and other medicinal devices complicates the therapeutic interventions and contributing to persistent infections. Besides that the bacterial species that form biofilms are exhibit heightened resistance to antibiotics and immunological responses, making infections intractable to exterminate (George *et al*., 2024).

The physiological effect of *P. aeruginosa* infections is deep, often leading to chronic inflammation, tissue damage, and, in severe cases, systemic infection. In patients with cystic fibrosis or those with chronic obstructive pulmonary disease (COPD), persistent colonization of the lungs by *P. aeruginosa* can lead to progressive lung damage and eventually respiratory failure. The complex interactions between the bacterium and the host immune

system can also contribute to the formation of chronic wounds and harmful biofilm-associated infections in burn patients (Ábrahám *et al*., 2024).

Various risk factors predispose individuals to *P. aeruginosa* infections, with the most significant category being compromised immune function. Patients with conditions like cystic fibrosis, chronic obstructive pulmonary disease (COPD), cancer, or individuals receiving immunosuppressive therapy are particularly weak. Patients with Cystic fibrosis being at high risk due to the presence of thick mucus in the lungs, forming an environment consistent to bacterial colonization, in addition to underlying clinical conditions, other risk factors include extended hospitalization, invasive procedures, and the use of broad-spectrum antibiotics, which damage the normal flora, thereby creating favorable conditions for opportunistic pathogens can like *P. aeruginosa* to thrive. The misuse and overuse of antibiotics have not only augmented the prevalence of infection but also contributed to the emergence of multidrug-resistant (MDR) strains, posing a considerable challenge to public health (Mudaliar *et al*., 2024).

1.3.4.1. Mechanisms of Pathogenicity

P. aeruginosa pathogenesis can be attributed to a complex interaction between virulence factors and host interaction, which can be categorized into the following principle mechanisms:

A- Adhesion and Biofilms Formation: One of the initial steps in *P. aeruginosa* infection is its adherece to host surfaces. *P. aeruginosa* utilizes a number of adhesion agents, such as pili (fimbriae) and surface polysaccharides, which expedite the attachment to epithelial cells and medical tools. Once adhered, *P. aeruginosa* began to form the biofilms, structured cluster of bacteria surrounded with a protective extracellular matrix, the process of Biofilm formation is important since it boosts the pathogen's resistance to host's immunological response and to antibiotics treatment, thereby tangling the eradication of infections (Goltermann *et al*., 2024).

B- Secretion System: *P. aeruginosa* has a number of specialized secretion systems that deliver effector proteins into host cells. The Type III secretion system (T3SS) is of particular interest. It delivers a group of virulent effectors directly into eukaryotic cells, which may modulate host cellular pathways, induce apoptosis, and enhance inflammations. Moreover, the bacteria utilizes the Type II secretion system (T2SS) to secrete of proteases and toxins that promote the degradation of host plant tissues and prevent immunological responses (Swart *et al*., 2024).

C- Toxins Producing: *P. aeruginosa* produces various toxins, such as, exotoxin A, which inhibits protein synthesis in the host cells, and several hemolysins and phospholipases causing damage to the cellular membranes. Production of these toxins is a key factor of its virulence by which this bacterium is able to harm tissues and circumvent the host immune response (Swart *et al*., 2024).

D- Resistance to antibiotics: The intrinsic ability of resistance toward a wide range of antibiotics is a critical factor in its pathogenicity. This bacterium has effective efflux pumps that expel several antimicrobial factors, reducing their activities. Also, it has an outer membrane being less permeable to many medications due to the existence of specific porins, further complicating treatments. Besides, *P. aeruginosa* could acquire resistance genes via horizontal gene transfer, especially in hospital environments where antibiotics use is widespread (Elfadadny *et al*., 2024).

E-Immune Evasion: *P. aeruginosa* has developed several strategies to escape the host immunological response. The bacterium can alter the expression of its surface structures, making it less recognizable to the immune system. Additionally, enzymes produced by the bacterium can degrade immune mediators, such as antibodies and complement proteins, inhibit the host's ability to mount an effective response to infection. (Conceição *et al.*, 2024).

1.3.4.2. Pathogenesis of *P. aeruginosa* in Wound Infections

The initial colonization of a wound by *P. aeruginosa* is often facilitated by skin breakdown due to trauma, surgical incisions, or pre-existing skin conditions. Upon entering the wound, *P. aeruginosa* employs numerous virulence factors that enable it to adhere to host tissues, evade the immune response, and cause tissue damage (Kim, *et al*. 2015).

A significant virulence factor is the production of biofilms, structured communities of bacteria encased in a protective extracellular matrix. Biofilm formation enable the pathogenic bacteria to counter the phagocytosis and the activity of antimicrobial agents, thus establishing persistent infections that are difficult to eradicate (Khan, 2024).

In addition to biofilm formation, *P. aeruginosa* possesses several virulence determinants, including exoenzymes, exotoxins, and quorum sense molecules. Regarding to Exoenzymes, such as elastase and alkaline protease, which degrade host tissues and facilitate bacterial invasion, Exotoxins including cytotoxin, and endotoxin that damage host tissues and contribute to inflammations. Quorum-sensing molecules control gene expression and coordinate group behaviors, such as biofilms formation, thereby enhancing the pathogenicity of bacteria (Liao, *et al*. 2022).

The existence of *P. aeruginosa* in wound infections forms considerable clinical challenges. Infected wounds often undergone delayed recovery, augmented pains, and exudates production, leading to patient discomfort and conceren. Furthermore, the persistence of *P. aeruginosa* could cause in severe complications like sepsis, which may entail extended hospitalization and more invasive treatment interventions. The antibiotics resistance of *P. aeruginosa* further complicates treatment regimens, necessitating multidisciplinary approaches that may involve combination therapies and emerging approaches such as therapy with bacteriophages (Serra *et al*., 2015).

1.3.4.3. Pathogenesis of *P. aeruginosa* in Urinary Tract Infections

There are several risk factors predisposing subjects to urinary tract infections (UTIs) caused by *P. aeruginosa*. The most consequential of these include the existence of indwelling urinary catheters, which erect a direct pathway for bacterial infections. Such catheters not only serve as a substrate for biofilm formation but also compromise the host's anatomical defenses. Hospitalized and immunocompromised patients, such as those suffering from diabetes mellitus, malignancies, or undergoing immunosuppressive therapy, are at heightened risk due to their potentially weakened immune systems and the presence of invasive procedures that may disrupt normal urinary tract function (Goltermann *et al.*, 2024).

Other contributing factors include structural abnormalities of the urinary tract, such as congenital malformations, urinary retention, and the presence of calculi. Additionally, the use of broad-spectrum antibiotics can inadvertently promote the growth of multidrug-resistant *P. aeruginosa* strains, as these treatments can disrupt the normal bacterial flora of the urinary tract, creating an ecological niche for opportunistic pathogens (Mittal *et al*., 2009).

The clinical presentation of UTIs caused by *P. aeruginosa* often resembles that of other common uropathogens, often leading to dysuria, frequency, urgency, and suprapubic pain. However, patients with P. aeruginosa infections may also exhibit atypical symptoms, such as more severe systemic manifestations, including fever and chills, particularly in cases of pyelonephritis or bacteremia (Khan, 2024).

In some instances, the characteristic "grape-like" odor associated with *Pseudomonas* infections may be noted in the urine, a distinctive feature that can provide clinical clues for diagnosis. Moreover, the presence of blood or pus in the urine (hematuria or pyuria) may indicate more severe infection or the presence of simultaneous bacterurea, necessitating prompt medical evaluation and management, the diagnosis of UTIs caused by *P. aeruginosa*

typically involves a combination of clinical assessment and laboratory testing. Urinalysis and urine culture remain essential tools for confirming the presence of the organism and establishing its susceptibility to antibiotics. Given the organism's notorious ability to develop resistance, susceptibility testing becomes paramount in guiding effective treatment strategies (Swart *et al*., 2024).

Recent advances in molecular diagnostic techniques, such as polymerase chain reaction (PCR), provide rapid identification of pathogens and, in some cases, specific resistance genes, allowing for improved management of complicated infections. Nonetheless, traditional culture methods remain the gold standard, particularly in clinical settings where timely diagnosis and appropriate intervention are critical (Wei *et al*., 2024).

Urinary tract infections caused by *P. aeruginosa* represent a complex challenge in modern medicine, particularly among at-risk populations. The opportunistic nature of this organism, coupled with its multifaceted virulence strategies and increasing antimicrobial resistance, necessitates a comprehensive understanding of its epidemiology, pathophysiology, and management. Continuous research aims to elucidate the complexities of *P. aeruginosa* infections, thereby informing more effective prevention and treatment strategies that can ultimately improve patient outcomes and public health (Zhao *et al*., 2024).

1.3.5. The Virulence Factors of *P. aeruginosa*

P. aeruginosa has evolved a multitude of virulence factors that facilitate its survival and pathogenicity in a host. This various virulence factors associated with *P. aeruginosa*, focusing on their mechanisms of action and their contributions to the bacterium's overall pathogenic potential (Qin *et al*., 2022).

One of the primary virulence factors of *P. aeruginosa* is its ability to form biofilms. Biofilm formations occur when bacterial cells adhere to a certain surface and secreting polysaccharides matrix that envelops them,

making a protective environment. The biofilm construction not only protects the bacteria from host immunological responses but also boosts their resistance to antibiotics. In clinical surroundings, biofilms are a noteworthy concern; especially in patients with cystic fibrosis and those treated by indwelling medical devices. The resilience of biofilm is ascribed to its complex community architecture, which assists nutrient exchange and genetic diversity among bacterial cells, thus enhancing their survival under challenging conditions (Swart et al., 2024).

The secretion of exotoxins represents a major pathogenic strategy because they play a pivotal role in the pathogenesis of *P. aeruginosa*. Among these exotoxins A (ETA) is the most well-recognized, it prohibits proteins synthesis in host cells by stimulating ADP-ribosylation of elongation factor-2, thus leading to cells death. In addition to causing localized tissues damage, ETA causes the host immune response, thereby enabling *P. aeruginosa* to evade the recognition and clearance. Overwhelmingly, ETA is associated with critical clinical aspects, like pneumonia and septicemia, underscoring its importance as a virulence factor (George *et al*., 2024).

Additionally, to exotoxins, *P. aeruginosa* produce the proteases, including elastase and alkaline protease, which degrade tissues and modulate the immunological responses of host. Elastase could break down elastin, a main component of connective tissues, enhancing tissues damaging and inflammations. Moreover, these proteases can cleave immunoglobulins and complement elements, lessening the host's capacity to mount an effective immune response against infections. This strategical interference with immunological defenses makes the host more susceptible to the impacts of *P. aeruginosa* and accelerates its proliferation (Mudaliar *et al*., 2024).

Another notable feature of *P. aeruginosa* is the presence of adhesions, which enable it to promptly attach to host tissues. These adhesins like fimbriae and pili are crucial for colonization and the early stages of infection. These structures facilitate the bacterial interaction with host extracellular matrix

components, strengthening their capacity to counter clearance by mucociliary activity and phagocytosis. Adherence of bacteria to surfaces is a key stage in the establishment of chronic infections, as in the case of lung diseases in patients with lung functions abnormalities including infected cystic fibrosis (Khan *et al.*, 2024).

The acquisition of mobile genetic elements, such as plasmids and transposons, can rapidly change genetic profile of the pathogens allowing the dissemination to their virulence traits and enhancing survival even in presence of host immune response and therapeutic interventions (Conceição *et al*., 2024).

1.3.6. Challenges in Treatment and Prevention

The treatment of *P. aeruginosa* infections presents considerable challenges due to its inherent resistance mechanisms and the ability to form biofilms. Multidrug-resistant strains have emerged as a result of selective pressure from antibiotics, making infections increasingly difficult to treat. The management of *P. aeruginosa* infections often requires the use of combination therapies, designed to specific susceptibility profiles, and necessitates continuing research into novel antibiotics and adjuvant therapies that can enhance the efficacy of existing treatments (Zhao *et al.*, 2024).

Prevention strategies focus on stringent infection control measures in healthcare settings, including thorough disinfection protocols and careful monitoring of patients at high risk of infection. The development of vaccines targeting *P. aeruginosa* remains an area of active research, with the goal of providing an additional layer of protection for weak populations (Khan *et al*., 2024).

P. aeruginosa example the complexity of microbial pathogenicity, exhibiting a range of mechanisms that enable it to thrive in various environments and evade host defenses. Its ability to cause significant morbidity and mortality, particularly within healthcare settings, underscores the importance of understanding its pathogenic mechanisms. As antibiotic

resistance continues to form a complicated challenge, ongoing research and creativity in treatment and prevention strategies will be essential to combat the threat formed by this opportunistic pathogen. Through a combined effort of scientific investigation, clinical vigilance, and public health initiatives, the load of *P. aeruginosa* infections can potentially be relaxed, improving outcomes for affected individuals (Tuon *et al*., 2022).

1.3.7. *P. aeruginosa*: epidemiology and Clinical Relevance

P. aeruginosa is a multilateral, opportunistic pathogen that has got considerable concern in the field of the microbiology and infectious disease epidemiology, its important role in both community-acquired and Hospital-acquired infections. It is a member of *Pseudomonadaceae* family, *Pseudomonas* was first characterized in 1882 and has since appeared as a considerable contributor to morbidity and mortality, especially among immunocompromised individuals and patients with chronic lung diseases (Swart *et al*., 2024).

Recent scientific studies emphasize that *P. aeruginosa* is a major multidrug-resistant pathogen widely implicated in hospital-acquired infections globally, particularly pneumonia, bloodstream infections, and surgical site infections. It is a member of the ESKAPE group and poses a critical threat in healthcare and community settings due to its environmental reservoirs and ability to infect immunocompromised hosts. Genetic diversity with high-risk clones and varied virulence profiles significantly impact chronic infections, especially in cystic fibrosis patients. Hospital reservoirs include water sources and medical devices, and prevalence has been rising alongside intensive care advances and antibiotic use. Multidrug resistance rates are notably increasing, emphasizing the need for continuous surveillance and infection control efforts (Gouveia *et al*., 2025& Allan J. *et al.,* 1984).

The presence of *P. aeruginosa* is omnipresent of nature, especially in soil, water, and others environments contributing to its global environmental resilience Its presence in varieties of habitats spanning from hospital setting

to community settings underscores the versatility and resilience of *P. aeruginosa*. Epidemiological studies suggest that *P. aeruginosa* can be transmitted through skin-to-surface contact and medical instruments, aerosolize droplets and environment source such as water and respiratory equipment. its involvement with biofilms, particularly in damp conditions, further complicates infection control protocols—biofilms provide a protective barrier against antimicrobials and immune responses (George *et al*., 2024).

Epidemiological surveillance researches have demonstrated that the prevalence of MDR strains of *P. aeruginosa* has expanded significantly in last few decades. The World Health Organization (WHO) had classified *P. aeruginosa* as a "critical priority" pathogen, underscoring the urgency to address this growing public health threats via strengthened infection prevention approaches, antimicrobial administration programs, and continued toward novel therapeutic agents (Ábrahám *et al*., 2024).

In hospitals, mostly in intensive care units (ICUs) and surgical wards, *P. aeruginosa* is one of the critical causes of infections. It mainly influences severally affected patients, including those with ventilator-associated pneumonia (VAP), catheter-associated urinary tract infections (CAUTI), as well as bloodstream infections. This pathogen is known for its capacity to colonize medical instruments, such as endotracheal tubes and catheters, leading to escalated rates of hospital-acquired infections (Swart *et al*., 2024).

1.3.7. *P. aeruginosa*: Clinical Manifestations

P. aeruginosa is well-known for its capacity to cause a wide range of infections, consistent with its classification as an opportunistic pathogen. The clinical appearances of infections could range from mild to severe, based on the host's immunological status and infection site. In healthy individuals, this bacterium is mostly logical; nevertheless, in immunocompromised

individuals, it may lead to serious consequences such as pneumonia, sepsis, skin infection, and mucous colonization (Goltermann *et a*l., 2024).

1.3.8. *P. aeruginosa*: Antimicrobial Resistance Patterns

The intrinsic and acquired antibiotic resistance are probably one of the most alarming points in the epidemiology of *P. aeruginosa*. The bacterium has multiple mechanisms to evade the impacts of antibiotic, including biofilms, efflux pumps, and production of beta-lactamases. The emergence of carbapenem-resistant *P. aeruginosa* (CRPA) has become a global issue, making therapeutic options severely restricted (Ábrahám *et al*., 2024).

Resistance to antimicrobial agents is an important concern related with *P. aeruginosa*, for its intrinsic properties along with acquired resistance mechanism. Its outer membrane serves as a barrier to various antibiotics, whereas efflux pumps effectively expel toxic compounds out of cell. Besides, the mutations at target sites, enzymatic degradation of antibiotic, and acquiring of resistance genes further contribute to its MDR phenotype. The mounting prevalence of MDR isolates form a considerable challenge to public health and requires the innovation of new curative strategies (Elfadadny et al., 2024).

Recently, several initiatives have been stated against the rising rate of antibiotics resistance, involving surveillance programs for understanding epidemiological patterns of *P. aeruginosa*. These initiatives focus on encouraging the responsible antibiotic usage, improving infection control measures in hospitals and clinics, and supporting research into new therapeutic approaches, including bacteriophage therapy and vaccine development (Zhao *et al.*, 2024 & Swart *et al*., 2024).

1.3.8.1. Emerging Strategies for Combatting Resistance

Given the challenges posed by *P. aeruginosa* resistance, it is indispensable to explore and apply novel strategies to eradicate this pathogen. One hopeful approach involves the development of novel antimicrobial agents that pick out unique bacterial pathways. Studies are investigating several

compounds, including those that suppress biofilms formation. Disrupting biofilms formation or enhancing biofilms dispersal may boost the efficacy of the current antibiotics (Kunz Coyne *et al*., 2022).

Furthermore, utilization bacteriophages that able to infect the bacteria seem to be another exciting approach to combat P. aeruginosa infections. Bacteriophage therapy has demonstrated effectiveness against antibiotic-resistant strains and could target certain bacterial phenotypes, offering a promising treatment strategy. However, challenges remain related with regulatory approval, and more investigations are requiring to determine the optimal conditions for clinical application (Khan *et al*., 2024).

Moreover, the significance of antibiotics administration cannot be exaggerated in the combating of *P. aeruginosa*. Implementing hard protocols to guide the proper usage of antibiotic in clinical practices may alleviate the emergence of resistance. Educating healthcare professionals and individuals about the outcomes of unsuitable antibiotic utilization is necessary to preserve the efficacy of existing medications (Mensa *et al*., 2018).

The resistance of *P. aeruginosa* is a urgent issue that needs multifaceted responses from the medical communities, researchers, and public health administrators alike. The bacterium's intrinsic abilities in avoiding antibiotics action, in addition to its exceptional adaptability, form considerable challenges in clinical administration of infections. Addressing *P. aeruginosa* resistance demands planned efforts in research, monitoring, and supervise, to evolve new treatment strategies while optimizing the usage of existing antibiotics. As we proceed in our understanding of this complex pathogen, cooperating endeavors will be vital to limit the effects of this pathogen and protect the public health in the coming years (Mudaliar *et al*., 2024).

1.3.8.2. Carbapenems : Mode of Action and Resistance Strategy

Carbapenems are a subclass of β-lactam antibiotics exhibiting broad-spectrum activity against many gram-positive and gram-negative bacteria. Drugs within this class, such as imipenem, meropenem, doripenem, and

ertapenem, are characterized by their structured β-lactam ring that interferes with bacterial cell wall synthesis. The primary mode of action of carbapenems, like other β-lactams, is the inhibition of penicillin-binding proteins (PBPs), which are crucial for the integrity of the bacterial cell wall (George *et al*., 2024).

Upon entering the bacterial cell, carbapenems bind to PBPs, particularly PBP-1 and PBP-2 figure (1-1), leading to the prevention of cross-linking in peptidoglycan, a vital component for maintaining the rigidity and stability of the bacterial cell wall This inhibition results in osmotic lysis of the bacterium, ultimately causing cell death. The effectiveness of carbapenems is further enhanced by their ability to penetrate the outer membrane of gram-negative bacteria, making them valuable for treating infections caused by resistant strains (Zhao *et al*., 2024).

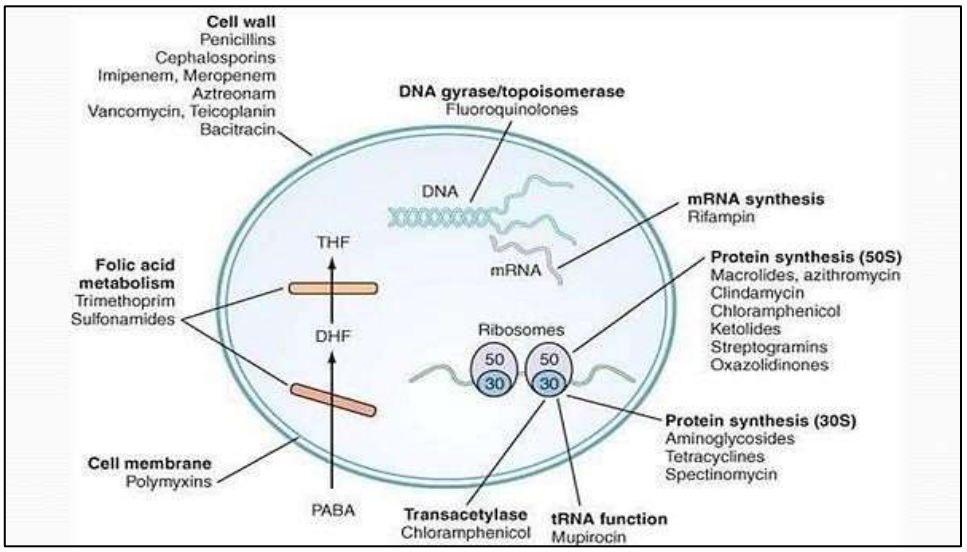


Figure 1-1: Mechanisms of antibiotics action.(Gigante *et al*.,2024)

Despite the efficacy of carbapenems, *P. aeruginosa* has developed number resistance mechanisms that undermine the therapeutic potential of these antibiotics. Understanding these mechanisms is crucial for managing infections and guiding treatment strategies.

A-Decreased Permeability: The outer membrane of *P. aeruginosa* represents an effectual barrier to the entrance of several antibiotics. Mutations or loss of functions in porin proteins, particularly OprD, considerably lessen

carbapenems's uptake. OprD acts as a channel for the uptake of small, hydrophilic molecules, together with carbapenems. When expression of porin is reduced, the drug's capacity to penetrate the cell wall is notably compromised (Conceição *et al*., 2024).

B-Efflux Pumps: *P. aeruginosa* has several efflux systems prone to expel many antimicrobial agents. The MexAB-OprM and MexXY-OprM efflux pumps are particularly important to mediate the resistance to carbapenems. They actively transfer carbapenems out of cell, minimizing their intracellular levels and effectiveness (Swart *et al*., 2024).

C- Production of β-lactamase: Producing of β-lactamases, particularly MBLs and ESBLs, poses a notable threat to the efficacy of carbapenems. MBLs, like New Delhi metallo-β-lactamase (*NDM*), have acquired importance in *P. aeruginosa*, hydrolyzing carbapenems and making them ineffective. The ability of *P. aeruginosa* to gain plasmids encodes these enzymes facilitating fast dispersal of resistance among bacterial populations (Swart *et al*., 2024).

D- Chromosomal Mutation: Mutations in genes encoding PBPs or other principal proteins involved in cell wall synthesis can also give resistance. Changes in PBP-3 may affect binding affinity of carbapenems, leading to decreasing susceptibility (López-Causapé *et al*., 2018).

E-Biofilm Formation: The ability of *P. aeruginosa* to produce biofilms adds another layer of complexity to carbapenems resistance. Biofilms acts as protective matrices that hinder antibiotics penetration and boost bacterial persistence, leading to chronic infections that are usually resistant to treatment with carbapenems (Zhao *et al*., 2024).

1.3.8.3. The Mechanism of Resistance: Ambler Class B β-Lactamases

Ambler class B β-lactamases (also called metallo-β-lactamases or *MBLs*) are a subgroup of β-lactamases that need zinc ions for their enzymatic actions, distinguishing them from other β-lactamases, which either do not need metal ions or employ another mechanisms of action. BiaIMP and

BiaVIM from this class and are capable to hydrolyze may β-lactam antibiotics, such as penicillin, cephalosporins, and, carbapenems, which are usually resistant to wide range kinds of β-lactamases. The existence of these enzymes in bacterial pathogens widely complicates curative options and forms a big challenge to infection's management (Lin *et al*., 2022).

The enzymatic action of Ambler class B enzymes hinges on their ability to bind zinc ions in their active site. Upon binding, the enzyme undergoes a conformational change that facilitates the hydrolysis of the β-lactam ring, rendering the antibiotic ineffective. While the precise mechanism can vary among different variants, the core functionality remains consistent across the Ambler class B family. Specifically, BiaIMP and BiaVIM utilize their metal-binding properties to exhibit an extremely broad substrate profile, allowing them to inactivate carbapenems and other β-lactam agents effectively (Khan *et al*., 2024).

1.3.8.4. Quinolone: Mode of Action and Resistance Strategy

Quinolones represent a significant class of synthetic antibacterial agents, widely utilized in the treatment of various infectious diseases. Their into clinical medicine has marked a pivotal advance in antimicrobial therapy, particularly against a broad spectrum of gram-negative and some gram-positive pathogens. The structural characteristics of quinolones, along with their pharmacological properties, and most importantly, their mode of action, distinguished them from other antibiotic classes (Swart *et al*., 2024).

Quinolones first gained prominence in the 1960s, with the synthesis of nalidixic acid, the first compound in this class, which was initially intended for treating urinary tract infections. However, the structural of quinolones quickly paved the way for the development of a second generation of fluoroquinolones, such as ciprofloxacin and ofloxacin, which exhibited enhanced antimicrobial activity and a broader spectrum of efficacy (George *et al*., 2024). The typical structure of quinolones comprises a bicyclic ring system that includes a pyridine, which is essential for their biochemical

activity. The substitutions on this core structure allow for the distinction between various quinolone derivatives, which can significantly influence their pharmacokinetics, spectrum of activity, and resistance profiles (George *et al*., 2024).

The quintessential mode of action of quinolones is predicated upon their ability to inhibit the activity of DNA gyrase and topoisomerase IV. These enzymes are pivotal in maintaining the integrity of bacterial DNA, and thus, the inhibition results in the disruption of DNA replication and transcription processes.

1. Inhibition of DNA Gyrase: The quinolone molecule interacts with the DNA gyrase-DNA complex. Upon binding to the enzyme, quinolones stabilize a cleavable complex formed between the enzyme and DNA, inhibiting the re-ligation of the DNA strands. This stabilization inhibits the activity of both DNA gyrase and topoisomerase IV. As a direct consequence, replication forks cannot proceed, leading to the accumulation of double-strand breaks in bacterial DNA. Consequently, this results in bacterial cell death, particularly evident in rapidly growing bacteria (Khan *et al.*, 2024).

2. Inhibition of Topoisomerase IV: The inhibition of topoisomerase IV further exacerbates the issue, as the separation of duplicated chromosomes during bacterial cell division becomes compromised. This dual mechanism promotes the bactericidal effect of quinolones, as the bacteria are unable to replicate or repair their vital genetic material (Zhao et al., 2024).

Resistance Mechanisms: Despite their efficacy, the emergence of quinolone resistance, particularly in pathogenic strains, poses a significant clinical challenge. Resistance can arise from mutations in the genes encoding DNA gyrase and topoisomerase IV, which lead to amino acid substitutions that reduce the binding affinity of quinolones. Additionally, the acquisition of efflux pumps can expel the antibiotic molecules from bacterial cells, further diminishing their intracellular concentrations. Understanding these resistance

mechanisms is crucial for developing strategies to counteract the problem and for guiding empirical therapy (Conceição *et al*., 2024).

The quinolone class of antibiotics, with its distinctive mechanism of action centered on the inhibition of DNA gyrase and topoisomerase IV, has been integral to contemporary antimicrobial therapy. Their ability to halt bacterial growth and finally induce cell death promote their role as invaluable agents against an infection. Continuous research efforts are warranted to address the growing threat of antibiotic resistance and to develop derivatives that can over one existing resistance mechanisms. As the understanding of the modes of action of quinolones progresses, it hold promise to enhance future applications of these antibiotics in clinical settings and protection their role in the arsenal against infectious diseases , the accuracy interplay between their efficacy, pharmacokinetic properties, and emerging resistance remains a focal point of ongoing scientific inquiry, underscoring the necessity for management of antibiotic supervise , *P. aeruginosa* often employs various genetic mechanisms to counteract the effects of antibiotics. Among these mechanisms, plasmid-mediated quinolone resistance (*PMQR*) has garnered significant attention due to its role in enhancing bacterial resistance to quinolones, a class of antibiotics widely utilized in treating infections caused by *P. aeruginosa* (Ábrahám *et al*., 2024).

The development of resistance to quinolones is multifactorial and can be attributed to several genetic and biochemical changes within bacterial cells.

1. Target Modification:

One of the principal mechanisms of quinolone resistance entails mutations in target enzymes, DNA gyrase and topoisomerase IV. Certain mutations within genes encoding these enzymes can reduce the binding affinity of quinolones, therby making these antibiotics less successful. It is reported that mutations in gyrA and parC genes encoding subunits of DNA gyrase and topoisomerase IV, respectively, are often noted in resistant strains **(**Swart et al., 2024).

2. Efflux Pumps:

Bacteria can also develop resistance via the over expression of efflux pumps (figure 1-2) which act to expel antibiotics away from the cell, thus decreasing intracellular drug levels. Notably, the AcrAB-TolC efflux pump system in *Escherichia coli* is often linked to reduced susceptibility to quinolones. The activation of such efflux systems can significantly diminish the effectiveness of quinolone therapy (Mudaliar *et al*., 2024).

3. Porin Channel Alterations:

In Gram-negative bacteria, the outer membrane contains porin channels that allow the diffusion of small molecules, including antibiotics. Alterations or loss of these porin channels can impede quinolone entry into the bacterial cell, further contributing to resistance (figure 1-2). Mutations or deletions in genes encoding porins such as OmpF can lead to reduced accumulation of quinolones, thereby enhancing resistance (Swart *et al*., 2024).

4. Plasmid-Mediated Resistance:

Another avenue of resistance is the horizontal gene transfer of resistance determinants via plasmids. Certain plasmids carrying genes that encode for modified targets or efflux systems can disseminate resistance traits among bacterial populations, leading to an increase in quinolone-resistant strains. (George *et al*., 2024).

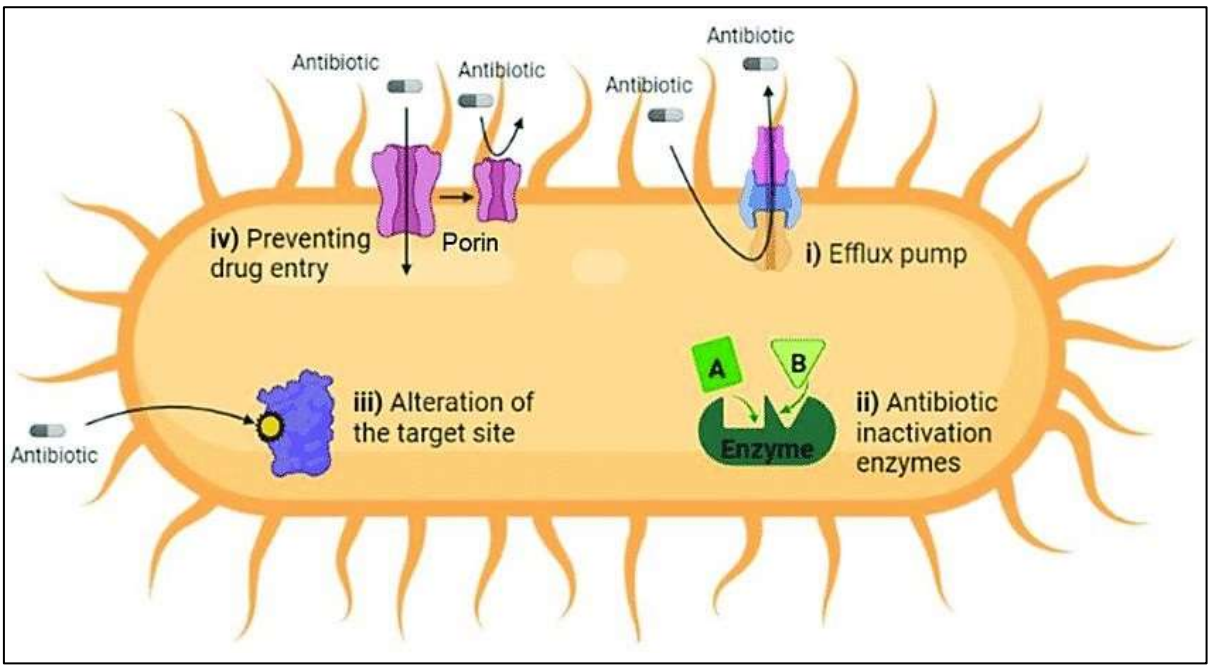


Figure 1-2: Main antimicrobial resistance mechanisms in pathogenic bacteria (Rice L. B. ,2012)

1.4. Bacterial genotyping by DNA Sequencing

The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of fluorescence-based sequencing methods with a DNA sequences. DNA sequencing has become easier and orders of magnitude faster (Behjati *et al*., 2013).

Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as medical diagnosis, biotechnology, forensic biology, virology and biological systematics. The rapid speed of sequencing carried out with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species. Several new methods for DNA sequencing were developed in the mid to late 1990s and were implemented in commercial DNA sequencers by the year 2000 (Celesti *et al.*, 2017) .

Nowadays, knowing the nucleotide sequence of the genome of an organism is extremely important in different biotechnology research fields. DNA sequencing is a molecular biology process able to determine the right nucleotide sequence of a DNA molecule, which is constituted by the alternation of the four nucleotides: adenine, thymine, guanine and cytosine. The information obtained from sequencing process are used for basic biological research and in other specific fields such as comparative genomic, metagenomics, biological systematic, medical diagnosis, early detection of cancer, single nucleotide polymorphisms (SNPs) research, the regulation of gene expression, forensic biology and many others. In recent years, modern high-performance sequencing methods have been developed. In such a context, Next-Generation Sequencing (NGS) indicates a number of different modern DNA sequencing techniques. NGS solutions allow to speed up DNA sequencing tasks. Even though it is possible to analysis short fragments of nucleic acids in a more efficient fashion, the possibility to carry out a large number of parallel sequencing tasks causes a huge amount of genomics data that need to be processed in a short time. Therefore, the huge amount of genomics data brought by NGS techniques is an example of the well-known problem of "big data". These techniques are applied for genome sequencing, genome resequencing, transcriptome profiling (RNA-Seq), DNA-protein interactions (ChIP-sequencing), and epigenome characterization. NGS allows researchers to perform numerous parallel sequencing processes to obtain a large number of sequences in a short time and at low cost, compared to traditional chain-termination method (Sanger sequencing)(Celesti *et al.*, 2017) .

1.5. *P. aeruginosa*: Genetic and Molecular Insights

With the advent of advanced molecular techniques, insights into the genetic architecture of *P. aeruginosa* have evolved significantly. *P. aeruginosa* has relatively large genome, which consists of nearly 6.3 megabase pairs and encompassing about 5500 genes. Notably, the genetic

diversity found within *P. aeruginosa* populations, leads to a wide ray of strains that vary in virulence and antibiotic resistance profile. This genetic variability is plays a pivotal role in its adaptability and persistence in hostile environments, including evasion of the human the immunological response of human (Swart *et al*., 2024).

Moreover, horizontal gene transfer is a notable factor of *P. aeruginosa* evolution, facilitating the gaining of novel features, like antibiotics resistance mechanisms, these mechanisms involve β-lactamases production, efflux pump systems, and modulation of target sites, Collectively, all these features complicate treatment interventions (Conceição *et al*., 2024).

1.5.1. ESBL genes families

ESBL gene families in *P. aeruginosa* include blaTEM, blaSHV, blaCTX-M, blaVEB, blaPER, blaGES, and blaOXA types. Their presence is linked to high-level resistance to beta-lactam antibiotics, complicating treatment of infections caused by this pathogen.

1.5.1.1. *CTX-M*

CTX-M-type β-lactamase enzymes were initially reported in the late 1980s, emerging concomitantly in several locations. The nomenclature *CTX*-M (cefotaximase from Munich) was initially used in a report from Germany (Bauernfeind *et al.*, 1990). *CTX-M* variants have been reported among several members of the order Enterobacterales and in *P. aeruginosa* and *Acinetobacter* spp. (Picão *et al*., 2009).

Isolates carrying *CTX-M*-encoding genes have been detected in nosocomial and community settings as well as in companion animals, the environment, food products and livestock (Liu *et al*., 2018).

The early *CTX-M* variants efficiently hydrolysed cefotaxime and ceftriaxone, hence the name cefotaximase (Bonnet et *al*., 2004). Contrary to the *TEM* and *SHV*-type *ESBLs* reported to that point, the early *CTX-M* enzymes had limited activity against ceftazidime, however *CTX-M* variants with enhanced ceftazidime hydrolytic activity were later described. Important

examples of *CTX-M* enzymes displaying ceftazidime hydrolysis are *CTX-M*-15 and *CTX-M*-27, which are from the *CTX-M*-1 and *CTX-M*-9 groups, respectively (Bonnet *et al*., 2004).

1.5.1.2. *OXA*-type β-lactamases

The *OXA*-type β-lactamases hydrolyse oxacillin and are grouped as Ambler class D and Bush-Jacoby-Medeiros functional group 2d enzymes (Bush *et al*., 1995). In general, *OXA*-type enzymes are a broad group that displays variability in substrate profiles and amino acid sequences. However, several *OXA*-type variants have been noted to hydrolyse cephalosporins, cephems, and/or monobactams. These *OXA* enzymes with an ESBL phenotype are categorized in Bush functional subgroup 2de (Bush *et al*., 2010). Whether or not these oxacillinases with activity against expanded-spectrum cephalosporins are defined as *ESBLs* is debatable (Livermore ,2008).

According to a recent review, there are 27 oxacillinase enzymes described as extended spectrum. These enzymes' substrates include third- and/or fourth-generation cephalosporins in addition to penicillins and early cephalosporins (Yoon *et al*., 2020). Most extended-spectrum oxacillinases derive from OXA-10 (also named PSE-2) and OXA-2. The OXA-10 derivatives include OXA-11, OXA-13, OXA-14, OXA-16, OXA-17, OXA-19 and OXA-28 (Evans BA. et al., 2014). In addition, OXA-16 has only a partial sequence submitted as its first description (GenBank #AF043100). Among the OXA-2 derivatives, OXA-15, OXA-32, OXA-34, OXA-36 (partial sequence), OXA-53, OXA-141, OXA-161, OXA-210 and OXA-226 have been described (Yoon *et al*., 2020). Many OXA-2 and OXA-10 derivatives are detected in isolates of *P. aeruginosa*.

1.5.2. *MBL* genes families

Major MBL gene families in *P. aeruginosa* include *blaVIM, blaIMP*, and *blaNDM*, among others, encoded mostly on transferable genetic elements that enable rapid spread of carbapenem resistance, these enzymes play a

crucial role in the pathogen's multidrug resistance profile and complicate clinical treatment.

1.5.2.1. *VIM*

VIM-1 (Veronese imipenemase) was first to be identified in *P. aeruginosa* in Italy (Gupta, 2008). The $bla_{\text{VIM-1}}$ gene was also integrated as a gene cassette into a class 1 integron (Lauretti *et al*., 1999). *VIM*-1 has also been detected in *P. putida* isolates in Italy (Queenan and Bush, 2007). This *MBL* has also been reported in *E.coli* from Greece, and *K. pneumoniae* from France (Scoulica *et al*., 2004). VIM-2 was identified for the first time in *P. aeruginosa* in 1996 from southern France (Poirel *et al*.,2010). *VIM*-2 is closely related to *VIM*-1 and is encoded by a gene cassette. The gene was located on a 45 kb non conjugative plasmid.*VIM*-2 producing *P. aeruginosa* has also been reported from other countries throughout the world showing its global distribution (Gupta, 2008). Some of the enterobacterial species also show the presence of *VIM* type *MBLs* but predominantly they are seen in *P. aeruginosa* strains (Queenan and Bush, 2007).

1.5.2.2. *NDM*

The *NDM*-1 (for New Delhi MBL) a new *MBL* emerged out of India and Pakistan, it was first reported in 2009 from a *K. pneumonia* isolate obtained from a Swedish patient of Indian origin, who had received medical treatment in New Delhi-India in 2007 (Yong *et al*., 2009).

It has now been identified mostly in *E. coli* and *K. pneumoniae* and, to a lesser extent, in other *Enterobacteriaceae* species (Yong *et al*., 2009; Kumarasamy *et al*., 2010; Nordmann *et al*., 2011). Located on a 180 kb plasmid, it expressed high-level resistance to all penicillins, cephalosporins, aztreonam, cefoxitin, carbapenems and ciprofloxacin. It was susceptible only to colistin (Grundmann *et al*, 2010). Furthermore, the $bla_{\text{NDM-1}}$ gene has been carry resistance to macrolides, aminoglycosides, rifampicin, sulfamethoxazole and aztreonam, contributing to their MDR patterns. The highly resistant phenotype, coupled with the increased prevalence of these

enzymes among clinical isolates makes them a major concern for public health (Walsh, 2005; Grundmann *et al*., 2010; Nordmann *et al*., 2011).

1.5.3. *QNR-A* and *QNR-B*

The *qnrA* and *qnrB* genes in *Pseudomonas* refer to plasmid-mediated quinolone resistance (*PMQR*) genes that encode Qnr proteins. These proteins protect bacterial DNA gyrase and topoisomerase IV from inhibition by quinolone antibiotics, thereby conferring resistance to these drugs. The *qnr* genes are a significant mechanism of quinolone resistance in Gram-negative bacteria, including *P. aeruginosa* (Venkataramana *et al*., 2022)

The *qnrA* gene was among the earliest identified plasmid-mediated quinolone resistance genes and has been detected in clinical *P. aeruginosa* isolates. The *qnrA* gene blocks the action of ciprofloxacin on bacterial enzymes DNA gyrase and topoisomerase IV, the *qnrB* gene is also a plasmid-mediated quinolone resistance determinant found among *Pseudomonas* and other Gram-negative bacteria. Its prevalence in *P. aeruginosa* clinical isolates in regional studies varies. These genes encode pent peptide repeat proteins that protect the bacterial target enzymes from quinolones. (Saki *et al*., 2022).

Chapter Two

Materials and Methods

2. Materials and methods

2.1. Materials

2.1.1. Laboratory Equipment and Apparatus.

The apparatuses and equipment in the study with manufacturing companies and origin of countries are recorded in Table (2-1).

Table (2-1): Instruments and Apparatus Used in the Study**.**

Kind of equipment	Company of manufacture (origin)
Autoclave	Hirayama (Japan)
Centrifuge	Eppendrof (Germany)
Deep freezer	GFL (Germany)
Different volume micropipette	Eppendrof (Germany)
Distillator of water	GFL (Germany)
Cold centrifuge high speed	Hettich (Germany)
Documentation system for gel	Biometra (Germany)
Electrophoresis unit	Labner (Taiwan)
Electric balance	Memmert (Germany)
PCR cabinet	USA
Incubator	Faithful (Germany)
Light microscope	Novel (Japan)
Loop	Himedia (India)
PCR gradient	Biometra (Germany)
PCR system	Gene Amp. (Singapore)
Petri dish	China
PH-meter	APEL (Japan)
Refrigerator	Ishtar (Iraq)
Thermo mixer comfort	Eppendrof (Germany)
VITEK2 system	Biomerieux (France)
Vortex	cyan (France)
Water bath	Memmert Western (Germany)
McFarland chek.	Biomerieux (France)

2.1.2 Chemical Substances

The biochemical and chemical substances used for the study are shown in Table (2-2).

Table (2-2): Biochemical and chemical substances used in the study

Materials	company (Origin)
Agarose	Promega (USA)
DNA loading buffer	Promega (USA)
DNA extraction kit	Geneaid Genomic DNA Purification Kit (Turkey)
Ethylene diamine tetra-acetic acid (EDTA)	AppliChem (Germany)
Ethanol (96%)	BDH (USA)
Nuclease Free water	Promega (USA)
Glycerol	Fluka (Switzerland)
Gram stain	Himedia (India)
$HgCl_2$	Sigma (USA)
HCl	BDH (USA)
H_2O_2 (3%)	Himedia (India)
α-Naphthol ($C_{10}H_8O$)	BDH (USA)
N.N.N.N Tetramethyl p-phenyline diamine dihydrochloride	BDH (USA)
Phenol red	BDH (USA)
Ethidium bromide	Intron (USA)
Sodium chloride (NaCl)	Promega (USA)
(TE) Tris- EDTA buffer molecular grad	Promega (USA)
(TBE) Tris-borate-EDTA buffer	Promega (USA)

2.1.3 Culture Media

The culture media used in this study are shown in Table (2-3).

Table (2-3): The powder for culture media used in the study from Accumix media (India) and Lioflichem (Italy)

No	Type of medium	Purpose of use
1	Brain heart infusion broth	Bacterial activation
2	Blood agar	Detection of hemolysin production
3	MacConkey agar	Isolation and identification of *pseudomonas* sp.
4	Nutrient broth	General purpose medium
5	Nutrient agar	General purpose medium
6	Cetrimide agar	Selective media for identification *pseudomonas. sp.*

2.1.4 Diagnostic kits

The types of kits that are used in this study are listed in Table (2-4)

Table (2-4): **Types of kits that are used for the study**

Type of kit	Origin
GN ID(VITEK2)	Biomerieux (France)
GN AST N222(VITEK2)	Biomerieux (France)
DNA Extraction	Geneaid (Turkey)

2.1.5 Polymerase chain reaction (PCR) materials

2.1.5.1 Master Mix

The master mix used during the study is shown in Table (2-5).

Table (2-5): Master mix Tag green used in the study

Type	Description	Origin
Master mix Tag Green	2X Green Taq Reaction buffer pH 8.5, 400µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3mM $MgCl_2$.	Promega (USA)

2.1.5.2. Ladder of molecular DNA

The ladder of molecular DNA used for the study is presented in Table (2-6).

Table (2-6): Marker of DNA Molecular Weight Used in the Study

DNA Marker	Description	Origin
100 bp Ladder with Loading dye	DNA Ladder 100-3000 base pairs. The DNA ladder consists of 12 double strand DNA fragments ranging in sizes from 100 to 1,000 bp increments, and additional fragments of 1,500 and 3,000 bp. The 500, and 1500bp bands are two to three times brighter for easy identification	Promega (USA)

2.1.5.3 Solution Preparation

Table (2-7): Solutions used in this study with their purpose of use.

No.	Solutions name	Preparation Source	Purpose
1	1X TBE buffer	(Green and Sambrook, 2012)	Used to dissolve agarose and in electrophoresis process.
2	Normal saline	Ready to use	preparation of culture suspension.

2.1.5.4 The Primers

All primers used for the study are shown in Table (2-8).

Table (2-8): The primers used for the study

Gene type		Primer	Gene name	Oligo sequence (5'-3')	reference	Product size (bp)
ESBL		**CTX-M**	***bla*CTX-M**	**F**: CGCTTTGCGATGTGCAG	Ashraf *et al*., 2004	550
				R: ACCGCGATATCGTTGGT	Ashraf *et al*., 2004	
		OXA	**OXA-10**	**F:** TATCGCGTGTCTTTCGAGTA	Gurung *et al*., 2020	760
				R: TTAGCCACCAATGATGCC	Gurung *et al*., 2020	
Carbapenemases	**Class B**	**NDM**	***bla*NDM**	**F:** CAGTCGCTTCCAACGGTTTG	Xiao Wang *et al*., 2021	740
				R: ATCACGATCATGCTGGCCTT	Xiao Wang *et al*., 2021	
		VIM	***bla*VIM-2**	**F**: AAAGTTATGCCGCACTCACC	Yan J *et al*., 2001	815
				R: TGCAACTTCATGTTATGCCG	Yan J *et al*., 2001	
PMQR		**QNR-A**		**F**: ATTTCTCACGCCAGGATTTG	Izadi *et al*., 2017	516
				R:GATCGGCAAAGGTTAGGTCA	Izadi *et al*., 2017	
		QNR-B		**F**:GATCGTGAAAGCCAGAAAGG	Yang *et al*., 2025	469
				R:ATGAGCAACGATGCCTGGTA	Yang *et al*., 2025	

2.2 Methods

2.2.1 Preparation of Reagents and Buffers

The reagents were prepared as labeled in the procedures.

2.2.1.1 Oxidase Reagent

The reagent was ready to use. It was used to distinguish the capability of bacteria for produce oxidase enzyme.

2.2.2 Culture Media Preparation

2.2.2.1 Ready Prepared Culture Media

The culture media used in this study are shown in Table (2-3). Each medium uses prepared according to the instruction of the manufacturer**,** followed by autoclaving at 121 C for 15 minutes to ensure sterilization. Adding 5% of blood supplement to blood agar medium after sterilization and waiting to cooling at 45 C before distribution in to the petri dishes (Carroll *et al*., 2015).

2.2.2.2 Preparation of Laboratory Media

2.2.2.2.1 Luria Bertoni Medium

This medium was prepared according to (Sam brook and Russell 2001) by mixing the following: (Yeast extract, 5gm, Trypton,10gm NaCl,10gm 1000 ml D.W. The pH has been regulated to 7.5 and autoclaved for 15 minutes at 121°C. The medium was used to support the growth of bacterial isolates for DNA extraction.

2.2.2.2.2 Conservation Medium

The medium was formed as the base medium of the nutrient broth, complemented by 15% glycerol. and it was autoclaved and left to cool to 56˚C and 5 ml was distributing to test tubes, then stored at 4°C before being used. this medium has been used for the long-term preservation of bacterial isolates under freezing condition at -70C (Mahon *et al.,* 2007).

2.2.3 Study Design, Samples, and Identification of Bacteria

A Cross-sectional study was performed at two major hospitals (Afak Hospital and the specialized center for burns in Diwaniyah) in al- Diwaniyah province between November 2024 to May 2025. This study aimed to identify the occurrence of carbapenem resistant *P. aeruginosa* isolates carrying carbapenemase , ESBL and PMQR genes in clinical samples attained. Ethical approval was obtained from the University of Kerbala / College of Medicine, Ministry of Health, in addition to, the research ethics committees at each hospital. Different clinical samples were collected from infected patients, including burn exudate, wound exudate, and urine. The samples were labeled and transported to the Afak Hospital Microbiology Laboratory for processing as describe by (Collee *et al., (*1996).

Midstream urines were collected into sterile containers, while Burn and Wound samples were taken by sterile swab. All clinical samples were transported to the laboratory, cultured on MacConkey , blood and cetrimid agar media, by using the standard loop method, streaking samples onto media. The plate was incubated aerobically at 37 °C for 24 hours. Urine culture was considered positive when the bacterial count was $\geq 10^5$ (CFU)/ml. After 24 hours of incubation, the culture media were examined for the presence of *P. aeruginosa* growth. All isolates were initially identified by macroscopic feature on media and positive for oxidase test confirmed with VITEK-2 automated system using the GN ID and AST N222 cards.

Molecular study involves detection the following genes (VIM, NDM, CTX, OXA, QNR-A and QNR-B) by conventional PCR technology. Sequencing was also conducted to determine the variations occurred in the genes through an insilico study and the effect of these variations on the structure of the resulting protein through Molecular Docking.

2.2.3. study design

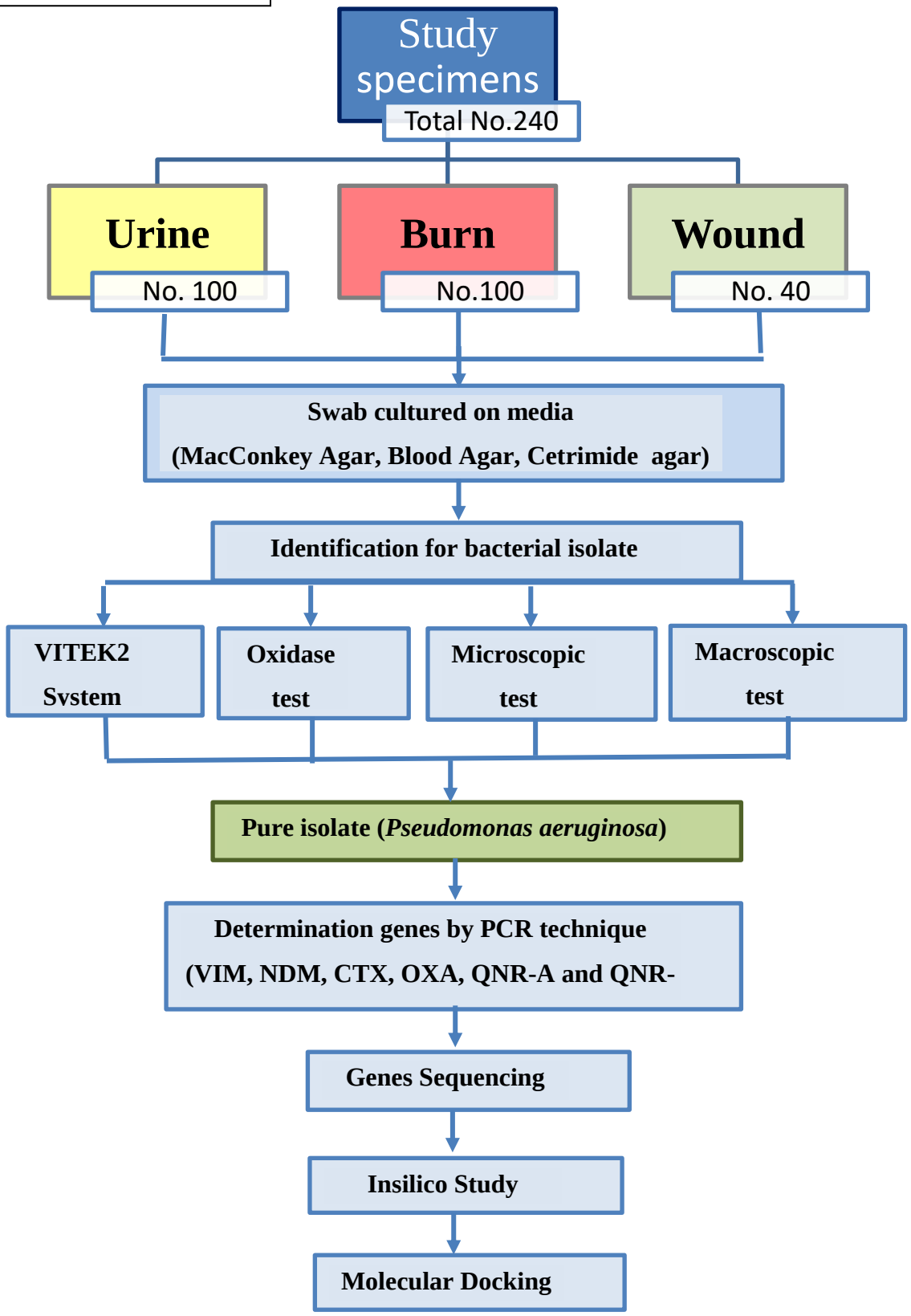


Figure (2-1) study design

2.2.4 Biochemical Tests

2.2.4.1 Oxidase Test

On the surface filter paper was taken and saturated with few drop of 1% oxidase reagent, then a pure colony was taken using a sterile wooden stick and applied over the filter paper, the positive result appeared as purple color (MacFaddin, 2000).

2.2.4.2 Growth on Cetrimide Agar

Cetrimide agar serves as a selective medium for *P. aeruginosa* isolate. These isolates were cultured on this medium and incubated at 37 c for 24 hr.
A blue-green pigment indicates the production of pyocyanin.

2.2.5 Preservation of bacterial samples

The conservation of bacterial isolates for short time by culturing on slant of the nutrient agar to 4°C. Isolates were sub-cultured monthly by growing on new culture media. For long term preservation, transferred fresh colon by loop into nutrient broth with 15% glycerol and storing at –70°C (Mahon *et al*., 2007).

2.2.6 Subculture of Preservative Isolates

Preserved bacterial isolates were sub cultured from frozen isolates on blood and MacConkey agar plates. Incubated aerobically for 24 hours at 37°C (Mahon *et al*., 2007).

2.2.7 Identification by VITCK 2 compact kits.

The biomirux Vitek 2 system was used for confirmation of suspected isolates of P. aeruginosa and for AST (by bioMérieux origin France).

2.2.7.1 Principles

The VITEK 2 is an automated microbiology system utilizing growth –based technology. the system is available in three formats (VITEK2compact, VITEK2, and VITEK2XL) that differ in increasing levels of capacity and automation. All three systems accommodate the same colorimetric reagent cards that are incubated and interpreted automatically.

2.2.7.2 Reagent Cards

There are 64 wells in the reagent cards that can each hold an individual test substratum. In the presence of inhibitory compounds, substrates calculate different metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and development. An optically transparent film on both sides of the card allows oxygen delivery levels to be sufficient while retaining a sealed vessel that avoids interaction with the admixtures of the organism-substrate. Each card has a transfer tube used for inoculation that is pre-inserted.

Cards have barcodes that contain product type information, lot number, expiration date, and a special product type information identifier that can be linked to the sample either before or after loading the card onto the system.

2.2.7.3 Culture Requirements

The on-line product information contains a culture requirements table that lists parameters for appropriate culture and inoculum preparation. These parameters include acceptable culture media, culture age, incubation conditions, and inoculum turbidity.

2.2.7.4 Suspension Preparation

A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted accordingly and measured using a turbidity meter called the Densi Chek . Mcfarland turbidity range for Gram negative bacteria is between (0.50 - 0.63) .[fig.2-1]

Figure (2-2) GN card used in AST of VITEK2

2.2.7.5 Inoculation

Identification cards using an incorporated vacuum apparatus are inoculated with microorganism suspensions. In a separate rack (cassette), a test tube holding the microorganism suspension is placed and the identity card is placed in the adjacent slot while the transfer tube is inserted into the corresponding suspension tube. Up to 10 tests will handle the cassette (VITEK 2 Compact; [Fig. 2-1] Or up to 15 (VITEK 2 and VITEK 2 XL) tests. The loaded cassette is put in the vacuum chamber station either manually (VITEK 2 compact) or automatically transported (VITEK 2 and VITEK 2 XL). After the vacuum is applied and air is reintroduced into the station, the suspension of the organism is pushed through micro-channels that fill all the test wells via the transfer tube.

Card Sealing and Incubation

Inoculated cards are moved through a process that, before loading into the carousel incubator, cuts off the transfer tube and seals the card. Up to 30 or up to 60 cards will handle the carousel incubator. All types of cards are Incubated at (35.5 + 1.0) oC online. Each card is withdrawn once every 15 minutes from the carousel incubator, transferred for reaction readings to the optical device and then returned to the incubator before the next reading time. Data is obtained at intervals of 15 minutes during the entire incubation process.

2.2.8 Extraction of DNA

The DNA extraction, was done according to manufacturing origin company protocol (Geneaid Genomic DNA Purification Kit (Turkey)).

2.2.8.1 Extraction of Genomic DNA from Bacterial Culture

Genomic DNA was extracted from *P. aeruginosa* isolates using the Geneaid Genomic DNA Purification Kit (Turkey) according to the manufacturer's instructions. the bacterial culture was seeded in 10 ml nutrient broth medium and cultured overnight at 37 C in a shacking incubator. Transferred to 1 x 109 bacterial cells to a 1.5 ml micro centrifuge tube and centrifuged for 1 minute at 14-16,000 x g, then discard the supernatant.

1. A total of 180 µl of GT Buffer were added, then the cell pellet was re-suspended using a vortex or pipette, then 2 µl of Proteinase K was added (make sure ddH2Owas added). The tube was then reversed every 3 minutes while incubating at 60C for at least 10 minutes.
2. Two hundred µl of GB Buffer were added, vortex mixed for 1 second, and incubated for at least 10 minutes at 70 °C. To confirm that the examined sample was free of contamination, the tube was inverted every 3 minutes during the incubation period. At this point, the needed elution buffer (200 l per sample) was warmed to 7°C in preparation for the following step (DNA elution).
3. Two hundred µl of 100% ethanol were added and vigorously blended right away. If a precipitate was formed, the shaking was stopped as much as possible using a pipette, and the mixture (including any insoluble precipitate) was transferred to the GD column in a 2 ml collection tube and centrifuged at 14-16,000 x g for 2 minutes. The flow-through 2 ml collection tube was discarded, and the GD column was put into a fresh 2 ml collection tube.
4. Four hundred µl W1 Buffer was added to the GD Column and centrifuged for 30 seconds at 14-16000 x g; the flow was discarded by placing the GD column back into a 2 ml collection tube.

5. Six hundred µl of washing buffer (with ethanol) was added to the GD column, which was then centrifuged for 30 seconds at 14-16000 x g, the flow was discarded, and the GD column was inserted back into the 2 ml collection tube and spun for 3 minutes at 14-16000 x g to dry the column matrix.
6. The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube, and 1 µl of pre-heated elution solution was added to the middle of the column matrix and allowed to incubate for at least 3 minutes before centrifuging at 14-16,000 x g for 30 seconds to extract the pure DNA. After that, the DNA was kept at 2-8 °C.

2.2.8.2 Estimation of DNA Concentration

The purity of the extracted genomic DNA was determined using a Nano-drop spectrophotometer, which measures DNA concentration (ng/ µl) and reads the absorbance at (260/280 nm). The following is how the device was used:

1- The DNA measuring program was selected after powering on the Nanodrop instrument.

2- The scale substrate was filtered twice by putting 2 microliters of ionic distilled water over the surface of the scale substrate using a sterile micropipette.

3- One microliter of each extracted DNA sample was placed on the device scale substrate, and then OK was pushed to begin the DNA concentration measurement process. The device scale was then cleaned before measuring the next sample.

4- The purity of the extracted DNA samples was also tested using a Nanodrop Spectrophotometer and measuring the absorbance at two wavelengths (280/260nm), with the extracted DNA being declared pure when the absorbance ratio was equal to (1.8).

2.2.9 PCR Reaction Mixture Volumes Protocol

The protocol of volumes mixture of PCR reaction are showed in Table (2-9).

Table (2-9): Mixture for PCR reaction used in the study.

Mixed reaction of the PCR	Protocol to promega (final volume 50µl)
Master mix 2X Forward primer (10µM) Reverse primer (10µM) DNA template PCR grade water (free nuclease)	25 µl 3 µl 3 µl 3 µl 16 µl

2.2.10 Programs for PCR Thermocycling Conditions for Gene Detection

The thermo cycling condition programs for detection genes fragment are showed in Table (2-10).

Table (2-10): Thermo cycling conditions for gene fragment detection in the study

Gene name	Temperature (°C)/Time					Cycle number
	First denaturation	Condition of cycling			Last extension	
		Denaturation	Annealing	Extension		
*bla*NDM	95/5 min.	95/30 sec.	56/ 30 sec.	72/ 45 sec.	72/ 7 min	35
bla_{VIM}	**95/5 min.**	**95/30 sec**	**51/30 sec**	**72/45 sec**	**72/7 min**	**35**
bla_{CTX-M}	95/5 min	95/30 sec	53/30 sec	72/45 sec	72/7 min	**35**
bla_{OXA}	95/5 min	95/30 sec	52/30 sec	72/45 sec	72/7 min	**35**
Qnr A	95/5 min	95/30 sec	54/30 sec	72/45 sec	72/7 Min	**35**
Qnr B	**95/5 min**	**95/30 sec**	**53/30 sec**	**72/45 sec**	**72/7 Min**	**35**

2.2.11 Agarose Gel Electrophoresis of Extracted DNA

By dissolving agarose powder in 1X TBE buffer, an agarose gel was created. The amount of agarose that may be dissolved depends on the application of the agarose sheet. The DNA after extraction was visualized using a 0.7 percent agarose gel, whereas the PCR product was seen using a 1.5 percent -2 percent agarose sheet with Ethidium bromide dye concentration of 10 mg/ml. In 100ml of melting agarose gel, just 5 drop of Ethidium bromide dye was applied (Green and Sambrook, 2012).

2.2.12 Preparation of Primer Pairs

The primers used in this study were prepared according to the manufacturer's instructions by adding deionized distill water (ddH2O) to lyophilized primers based on the volume fixated on the tube containing primers, mixing thoroughly with a vortex mixer to obtain stock solutions 100X (Pico moles microliter), and then storing at -20°C. To achieve a 10x concentration of primer, ten micro liters of stock solution were transferred to an RNase – DNase free tube, and 90 micro liters of ddH2O were added, well mixed by vortex mixer, and stored at -20°C.

2.2.13 Reaction Mixture of PCR

The reaction mixture was made using a kit applied from Biological Materials Industries (ABM), following the manufacturer's instructions:

•The polymerase chain reaction mixture was created in PCR tubes fitted with the reaction components kit, and the other components were added to the reaction mixture according to the manufacturer's instructions, as shown in the table below (2-9).

•After completing the reaction mixture preparation, the tubes were closed and gently stirred for 10 seconds with the Vortex device.

• To finish the amplification of the target gene, the tubes were transferred to the PCR Thermocycler.

2.2.14. Polymerase Chain Reaction (PCR)

Using specified primer pairs, conventional PCR was utilized to amplify the target genes (table 3.10). the process consists of three stages performed for a set number of cycles to produce a PCR result (amplicon) that can then be observed using an agarose gel electrophoresis

2.2.15. Gel Electrophoresis

All resistance genes' PCR products were analyzed in 1.5 percent Agarose as follows (Sambrook and Rusell, 2001):

1- An agarose gel was made by dissolving 1.5 g of agarose in 100 ml of 1X TBE and allowing it to cool to 45-50°C.

2- Then, into the agarose gel solution, 5 drops of Ethidium bromide stain were added.

3- Agarose gel solution was poured into tray, and a comb was placed in appropriate position, and it was permitted to harden at room temperature for 30 minutes. The comb was then carefully removed from the tray.

4- In the electrophoresis chamber, the gel tray was fixed and filled with 1XTBE buffer.

5- In each comb well, ten ml of PCR product were added, as well as ten ul of (100 bp Ladder) in one well.

6- After that, an electric current of 80 volts was applied, for 45 minutes.

7- According to the amplicon size in the table, PCR products were visualized using a gel documentary system .

2.2.16. Biosafety and Hazard Material Disposing

Biosafety aspects were followed during the work which includes disposing of all swabs, petridishes, and all contaminated supplies by autoclaving and then incineration. All benches cleaned with alcohol (70%) before and after the work. Simply safe were used instead of ethidium bromide to reduce biohazard (Bergen, and Shelhamer, 1996).

2.2.17. Methods of In Silico Analysis

A- Detection of Nucleic Acid Variations

The resolved PCR amplicons were commercially sequenced from the forward direction, following to instruction manuals of the sequencing company (Macrogen, South Korea). By comparing the observed nucleic acid sequences of the local bacterial samples with the retrieved nucleic acid sequences, the virtual positions and other details of the retrieved PCR fragments were identified. The sequencing results of the PCR products of the targeted sample were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, USA). The observed variations in the sequenced sample were numbered in PCR amplicons as well as in their corresponding position within the referring genome. The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome.

B- Detection of Amino Acid Variations

The amino acid sequences of the targeted bacterial proteins were retrieved online from the NCBI server (http://www.ncbi.nlm.nih.gov). The observed nucleic acid variants in the coding portions were translated into a reading frame corresponding to the referring amino acid residues in the encoded protein using the Expasy online software (http://web.expasy.org/translate/). Multiple amino acid sequence alignment was conducted between the reference amino acid sequences and our amplified sample using the "align" script of the BioEdit server. The amplified regions of each used protein were highlighted, and the identified amino acid substitutions were positioned in their corresponding locations in the entire amino acid sequences of the proteins.

C-Annotation of the Detected Variations

The allelic variation of the detected variants was described, and the sample IDs containing these variants were also shown. The position of each identified

variant was indicated in both the PCR amplicon and the genomic sequences from which it was retrieved. Furthermore, the effect of each variant, whether silent (synonymous) or missense (non-synonymous), was also presented.

D-Three-Dimensional Proteins Modelling

Due to the absence of crystallized structures for the investigated proteins in the Protein Data Bank (PDB) server (https://www.rcsb.org/), the three-dimensional structures of each protein were generated using the SWISS-MODEL tool (https://swissmodel.expasy.org/) (Waterhouse *et al*., 2018). By using the modeling script of the SWISS-MODEL suite, the most suitable 3D models for each of the analyzed proteins were obtained based on homology modeling. The generated models were then validated using the Ramachandran plot tool, which is also integrated within the same suite. The Ramachandran plot was utilized to assess the stereochemical quality of the protein models by evaluating the backbone dihedral angles (ϕ and ψ) of amino acid residues, ensuring that they occupy energetically favorable regions. Based on the generated and verified 3D structures of the investigated OXA, CTX-1, blaVIM-1, and qnrA genes, the amino acid substitutions were created using the Mutagenesis Wizard of the PyMol software (The PyMOL Molecular Graphics System, Version X.Y, Schrödinger, LLC). Using this tool, the effects of the identified missense SNPs on the 3D structures were assessed using various in silico prediction tools.

E-Prediction of Protein Level Effects of the Identified Variants

To assess whether the identified variants exhibit neutral or deleterious effects on their corresponding proteins, a series of bioinformatics tools were used. These tools were grouped into two categories: one set focused on evaluating the effects of the variants on protein structure and function, and another set focused on assessing their impact on protein stability. Four in silico tools were utilized in the first group, including SIFT, PolyPhen-2, PhD-SNP, and Meta-SNP. The SIFT tool is used to predict the impact of amino acid substitution on protein function based on sequence homology and the physical properties of amino acids (Ng and

Henikoff, 2003). Whereas PolyPhen-2 was employed to assess the potential impact of an amino acid change on the structure and function of a human protein using physical and comparative considerations (Adzhubei *et al*., 2013).

Meanwhile, PhD-SNP was utilized to evaluate whether a missense single-nucleotide polymorphism (SNP) is likely to be disease-associated or neutral (Capriotti *et al*., 2006). In turn, the meta-SNP tool was used to combine predictions from multiple individual tools to provide a consensus prediction on the functional impact of the variant (https://snps.biofold.org/meta-snp/). After assessing the potential structural and functional effects of each detected SNP on the protein of interest, the impact of these SNPs on protein stability was also investigated. Three in silico tools were used to perform this analysis: mCSM, SDM, and DUET. All these tools rely on the modeled 3D structures of the proteins to generate their predictions. The mCSM tool was used to assess changes in protein stability upon mutation by analyzing the impact of missense SNPs on interatomic interactions through graph-based signatures (Pires *et al*., 2012). In contrast, SDM was utilized to evaluate the effects of missense SNPs on protein stability using environment-specific substitution tables (Worth *et al*., 2012). Whereas the DUET tool uses a combined approach that integrates mCSM and SDM predictions to improve accuracy (Pires *et al*., 2014).

2.2.18. Molecular Docking

After assessing the possible consequences of each detected SNP on the structure, function, and stability of the corresponding proteins, another bio-computational approach was conducted to evaluate the potential effect of these SNPs on the binding affinity of the proteins with their substrates or receptors. The most effective way to perform this analysis was through molecular docking of the wild-type proteins and their mutant counterparts. For proteins that typically bind to small molecules (ligands), protein–ligand molecular docking was performed. In these types of dockings, the SDF formats of the most suitable ligands for these proteins were retrieved from PubChem sever

(https://pubchem.ncbi.nlm.nih.gov/). For proteins that do not bind with small molecules, protein – protein interaction dockings was performed. In these types of dockings, the closest proteins to which these proteins bind were selected from the uniprotKB server (https://www.uniprot.org/). For protein–ligand interactions, the Cavity-detection guided Blind Docking (or CB-Dock) tool was used to assess the binding affinity and interaction patterns between each protein and its corresponding ligand. This tool first automatically detects the binding cavity on the protein and calculates sizes and positions of dock box size before performing docking (Liu *et al*., 2020). Further annotations for the protein – ligand interactions were visualized by Discovery Studio software (Jejurikar and Rohane, 2021). For proteins that do not bind to ligands, the HawkDock server was used to evaluate the possible effects of the detected SNPs on the binding affinity of the protein to its receptor or partner protein (Zhang *et al*., 2025). Further annotations for the protein – protein interactions were visualized by PDBSum generate tool (Laskowski *et al*., 2018). In both cases, the docking results of the wild-type proteins were compared with those of the mutant forms to determine the extent to which these SNPs affected their binding capacity with substrates or receptors.

2.2.19. Statistical Analysis

The statistical analysis for the present investigation was done using the Statistical Package for the Social Sciences software, version 26 (IBM, SPSS, Chicago, Illinois, USA). Descriptive statistics were performed on the participants' data of each group. Data was presented as percentages; Chi-Square analysis was employed to compare between percentages. The Results of all hypothesis tests with p-values <0.05 (two-side) were considered to be statistically significant. In addition, SPSS program and Microsoft Excel 2010 program were employed to draw chart figures (Duncan *et al*., 1983; Basher, 2003).

2.2.20. Ethical approval

The study protocol was submitted to the relevant ethical committee at the health directorate in Al-Diwaniyah [Ethical No. 41 on 25-11-2024].

Chapter Three

Results

3.Results

3.1. Characteristics Data for Patients

Table (3-1) illustrates the Characteristics data for patients, including distribution of patients according to age, sex and Site of infection. Patients were classified into four age categories: 1-16 y, 17-32 y, 33-48 y, and 49->64y, The results of statistical analysis revealed significantly higher proporation of patients (42.1%) within the age category 17-32 y, while the lowest propartion was (16.3%) within age category 49->64 y. Sex distribution ,with (44.6%) males and (55.4%) females.

Table (3-1): Characteristics Data for Patients

Age group (year)				
1-16 y	**17-32 y**	**33-48 y**	**49->64 y**	**Total**
49 (20.4%)	101(42.1%)	51(21.3%)	39 (16.3%)	240 (100%)
Sex				
Male	**Female**	**Total**		
107 (44.6%)	133 (55.4%)	240 (100%)		
Antibiotic up take				
No	**Yes**	**Total**		
104 (43.4%)	136 (56.6%)	240 (100%)		

3.2. Identification of *P. aeruginosa:*

3.2.1 Morphological Features:

To assess the identification, the isolates were initially diagnosed as *P. aeruginosa*. Bacterial isolates were cultivated on Nutrient agar, Blood agar, MacConkey agar, and Cetrimide agar (with nalidixic acid). On MacConkey agar, *P. aeruginosa* colonies appeared pale yellow, small smooth and round .On Blood agar, large flat colonies with a grape-like smell, and also most isolates showed β-hemolysis after 24 hours of incubation. Isolates that showed growth in both media means, were identified as Gram-negative bacteria . These colonies were cultivated on Cetrimide agar that distinguishes between *Pseudomonas* and other pathogens and enhances the induction of pigments. Colonies grown on Cetrimide agar

appeared to be mucous and smooth, with flat edges, an elevated center, creamy in color, and fruity in odor .

The growth of the pathogens is characterized by a shiny green pigment due to the production of pyocyanin. After this, all *P. aeruginosa* isolates were characterized using the Vitek 2 system (Bio-Mérieux (French) and indicated by biochemical testing.

3.2.2 Microscopically and Biochemical Characterization

Microscopically, the study showed that these isolates likely belong to *Pseudomonas* spp. which appeared Gram-negative , rod-shaped cells, that are non-spore forming which indicative of *P. aeruginosa* .And positive results for oxidase. *P. aeruginosa* colonies differ by medium and source of infection, ranging from small to large mucoid, and the positive results of oxidase were related to their ability to produce cytochrome oxidase (tetramethylphenylenediamine oxidases). 40 isolates cause β-hemolysis in the blood agar but no hemolysis in 3 isolates of *P. aeruginosa* are caused by a mutation but under certain conditions. Cetrimide agar is recognized to be toxic and inhibiting other microbial flora. It was used to cultivated *P. aeruginosa*, and all *Pseudomosa* isolates were able to grow at 42^{o} C. in contrast, no growth was noticed at 4^{o}C . *P. aeruginosa* can tolerate low temperatures and produce green pigment.

3.3 Bacterial Culture

The percentage of bacterial growth in each type of infection is shown in Figure (3-1), The majority of patients with UTIs (86%), burn (73%), and wounds (67.5%) had bacterial growth; while only (14%), (27%), and (32.5%), respectively of those patients had no growth of bacteria.

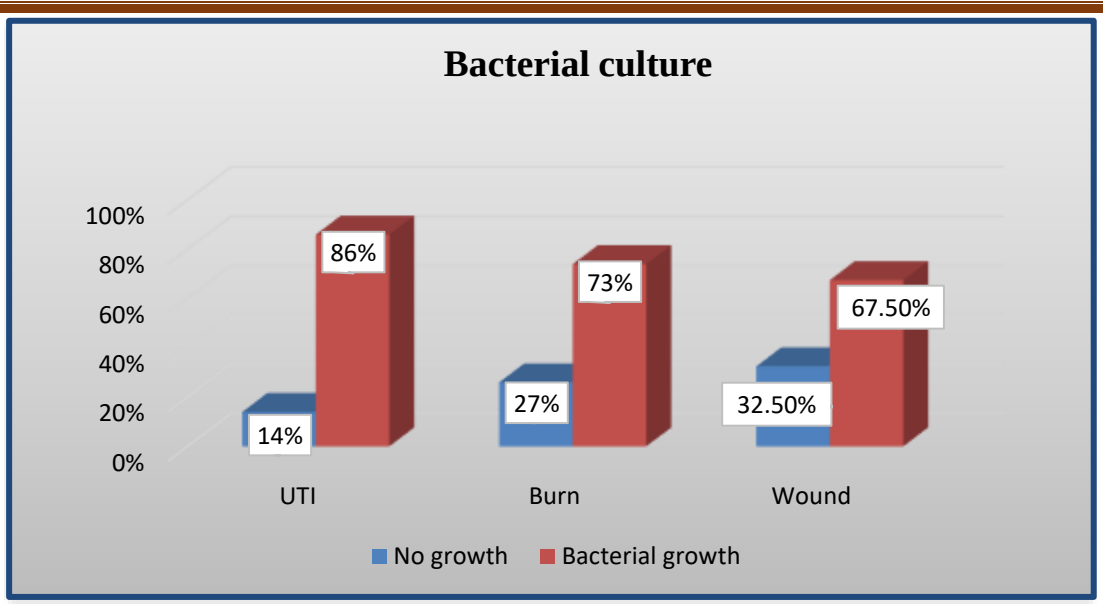


Fig. (3-1): Bacterial culture

3.4 Analysis of *P. aeruginosa* Infection in Patients:

3.4.1 Distribution of *P. aeruginosa* Isolation Based on Age and Sex:

Table (3-2) Distribution of *P. aeruginosa* Isolation Based on Age and Sex. Among males, the highest (48.1%) prevalence of *P. aeruginosa* was in 17-32 y age groups, followed by identical (18.5% each) frequencies in both 1-16 y and 33-48y age groups, while the lowest prevelance (14.8%) in those aged 49->64y. Overall, males accounted for 62.8% of the isolates, showed a significant association (P=0.0001).

Among females, isolate prevalence was overwhelmingly concentrated in the 17-32 y age groups with (87.5%), Much lower frequencies were recorded in 1-16 y and 33-48 y age groups (6.25% each), whereas no isolates observed in the 49->64y age group. Female patients accounted for 37.2% of the isolates, with a significant association (P=0.0001) as well.

Overall, the 17-32y age group had the highest occurrence of *P. aeruginosa* isolates (62.8%), followed by same distributions in 1-16 y and 33-48 y age groups (13.9%), and the lowest prevalence was in 49->64y age group (9.3%).

Table (3-2): Distribution of *P. aeruginosa* Isolation Based on Age and Sex:

***Pseudomonas aeruginosa* appearance No. (%)**						
Sex	Age group				**Total**	***P value* (P ≤ 0.05)**
	1-16 y	**17-32 y**	**33-48 y**	**49->64 y**		
Male	5 (18.5%)	13 (48.1%)	5 (18.5%)	4 (14.8%)	27(62.8%)	0.0001*
Female	1(6.25%)	14 (87.5%)	1(6.25%)	0 (0%)	16 (37.2%)	0.0001*
Total	6 (13.9%)	27 (62.8%)	6 (13.9%)	4 (9.3%)	43 (100%)	
*Significant difference at the 0.05 level by chi-square test						

3.4.2 Contribution of Infection Sources to the Total Number of *P. aeruginosa* isolates :

Figure (3-2) displays the distribution of *P. aeruginosa* isolates according to infection types. This figure shows that the highest (67.44%) prevalence of *P. aeruginosa* was in burns specimens, while the lowest (11.63%) prevalence was in wound specimens.

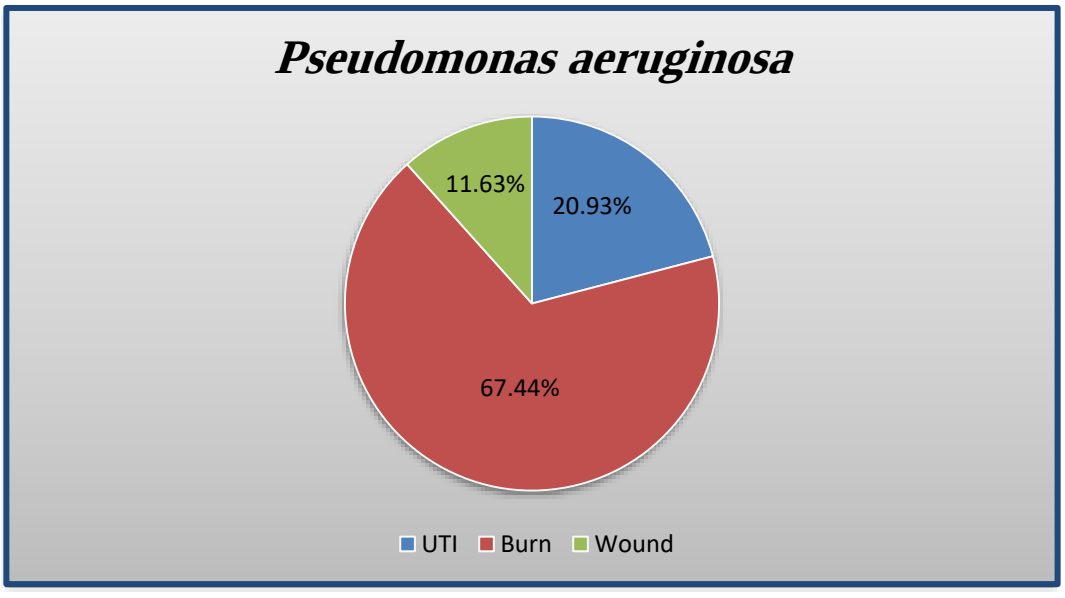


Figure. (3-2): sources of *P. aeruginosa* According to Infection Types

3.5 Antibiotics Resistance:

3.5.1 Antibiotic Resistance State of *P. aeruginosa* in Patients:

Figure (3-3) shows the antibiotic resistance type of *P. aeruginosa* in patients. In UTIs cases, 66.7% of *P. aeruginosa* have MDR resistance pattren, whereas only 33.3% have XDR. In burn cases, 72.4% of *P. aeruginosa* isolates have XDR resistance and 27.6 were MDR. In wound cases, 60% of isolates have XDR and 40% have MDR resistance. With notably highly significant (p=0.000) differences in each of them.

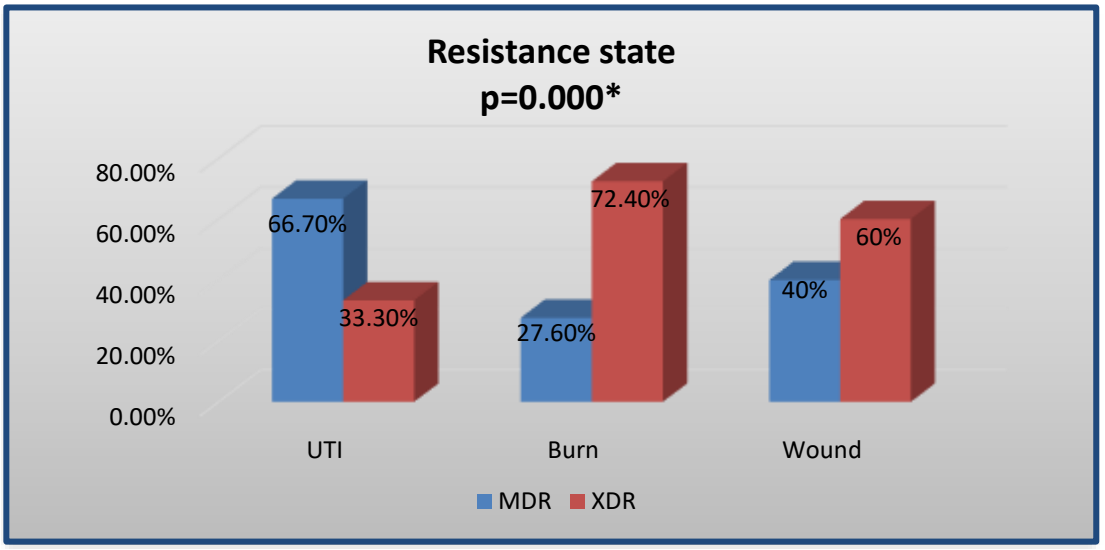


Figure. (3-3): Distribution of Antibiotic Resistance Pattern According to the Source of the Isolates

3.5.2 Antibiotics Resistance Patterns of *P. aeruginosa* Across Infection Types and Age Groups

Table (3-3) illustrates the distribution of multi-drug resistant (MDR) and extensively- drug resistant (XDR) *P. aeruginosa* isolates of across different infection types specimens (UTIs, burn, and wound) and age categories, where all findings showed statistically significant differences (P=0.0001).

In UTIs patients, MDR *P. aeruginosa* isolates were significantly most prevalent in 17-32 y age category with percentage of (66.6%), followed by equal percentages in 1-16y and 33-48y age groups (16.6% each), and absent

in those aged 49->64 years; XDR isolates were concentrated in the younger age categories, with the highest (66.7%) in the 1-16y age group and the remaining in 17-32 age group (33.3%), with none found in patients older than 32 years.

For burn specimens, MDR isolates were primarily observed in the 17-32 age group (62.5%), while the smaller percentage in 1-16 (12.5%) age group. No MDR *P. aeruginosa* isolates were detected in the 33-48y age group. XDR isolates showed significantly highest (47.6%) prevalence in 17-32y age group, with smaller yet significant distributions in 33-48y (23.8%) and 49->64y (23.8%) groups, while minimal prevalence (4.8%) was found in 1-16 group.

In wound specimens, MDR *P. aeruginosa* isolates were exclusively observed in 17-32 age group (100%), with no MDR *P. aeruginosa* in other age groups; XDR isolates were evenly distributed across the 1-16y, 17-32y, and 33-48y age groups (33.3% each), with no isolates observed in patients aged 49->64 years.

Table (3-3): Antibiotic Resistance for *P. aeruginosa* in Patients According to Age Groups

Infection types	Antibiotic resistance	Age category No. (%)				Total	P value
		1-16 y	17-32 y	33-48 y	49->64 y		
UTI (n=9)	MDR	1 (16.6%)	4 (66.6%)	1 (16.6%)	0 (0%)	6	0.0001*
	XDR	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	3	0.0001*
Burn (n=29)	MDR	1 (12.5%)	5 (62.5%)	0 (0%)	2 (25%)	8	0.0001*
	XDR	1 (4.8%)	10 (47.6%)	5 (23.8%)	5 (23.8%)	21	0.0001*
Wound (n=5)	MDR	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2	0.0001*
	XDR	1 (33.3%)	1 (33.3%)	1 (33.3%)	0 (0%)	3	0.0001*
*Significant difference at the 0.05 level by chi-square test							

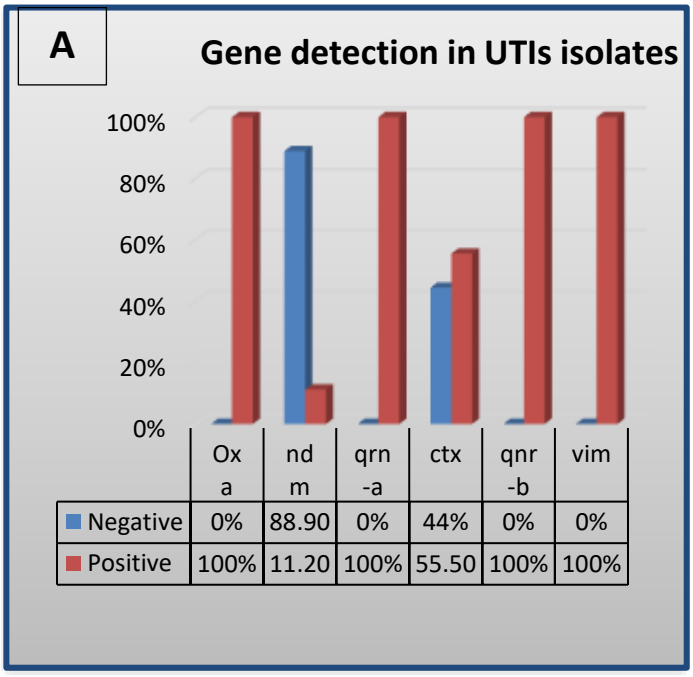
3.6 Gene Detection

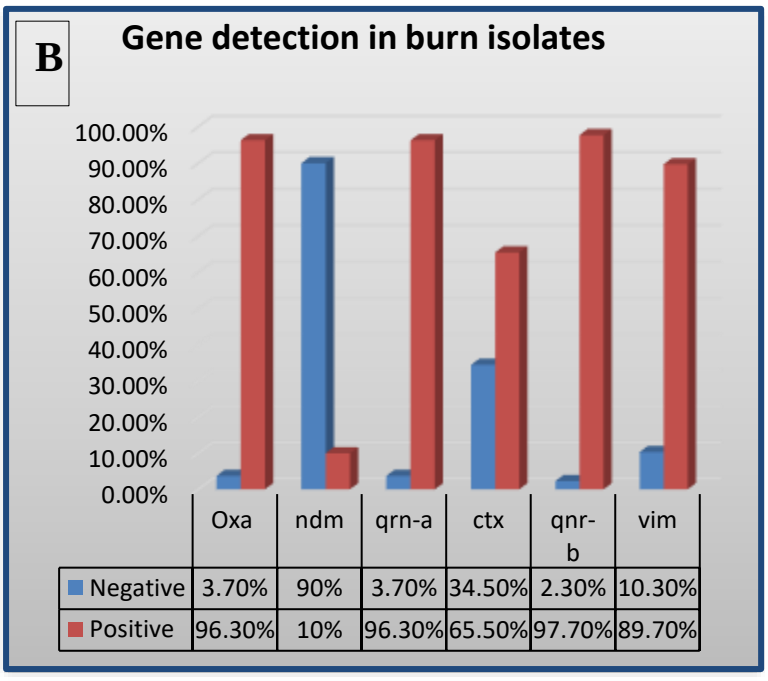
3.6.1 Gene Detection of *P. aeruginosa* in UTIs, Burns, and Wound Infections

Figure (3-4) displays the gene detection of *P. aeruginosa* in: A) UTIs cases, B) burn cases, C) wound cases. The *OXA*, *QNR_A*, *QNR_B*, and *VIM* genes showed 100% detection in UTIs cases; *CTX* gene was detected at 55% in those cases, on the other hand, *NDM* gene detected only in 11.2% of UTIs cases (figure 3.4a).

In burn cases, all genes of *P. aeruginosa* showed high gene detection in burn cases, except for the *NDM* gene, which was not detected in 90% of those samples (only 10% was positively detected) (figure 3.4b).

As for wound cases, the *OXA*, *QNR_A*, *QNR_B*, and *VIM* genes were 100%, the *CTX* and *NDM* genes were detected at 80% and 60% respectively





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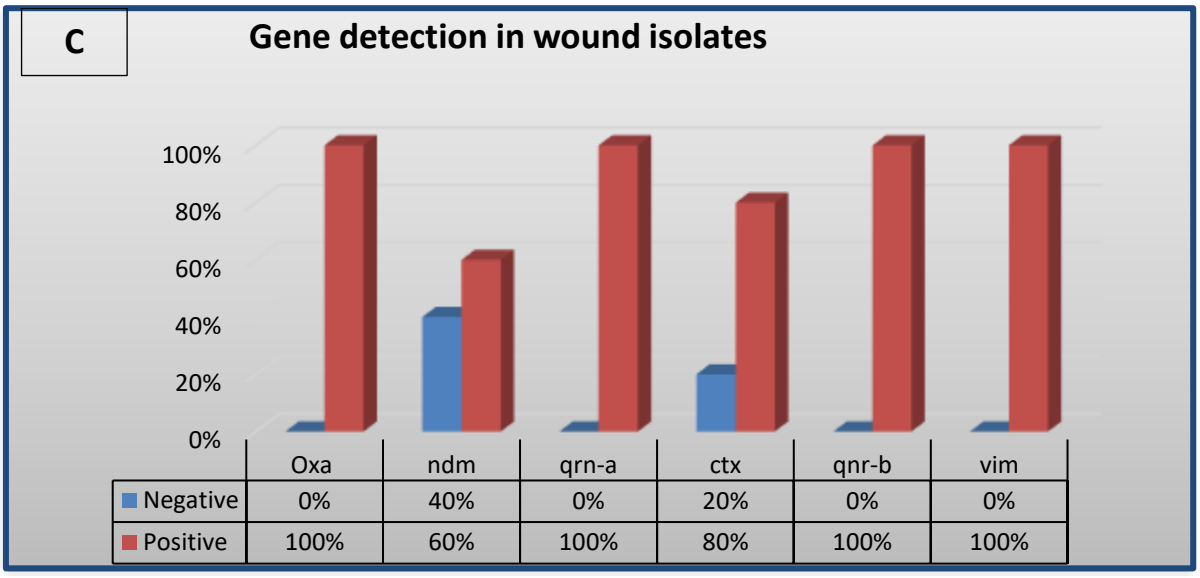


Figure. (3-4): Gene detection of *P. aeruginosa* in: A) UTIs isolates, B) burn isolates, C) wound isolates

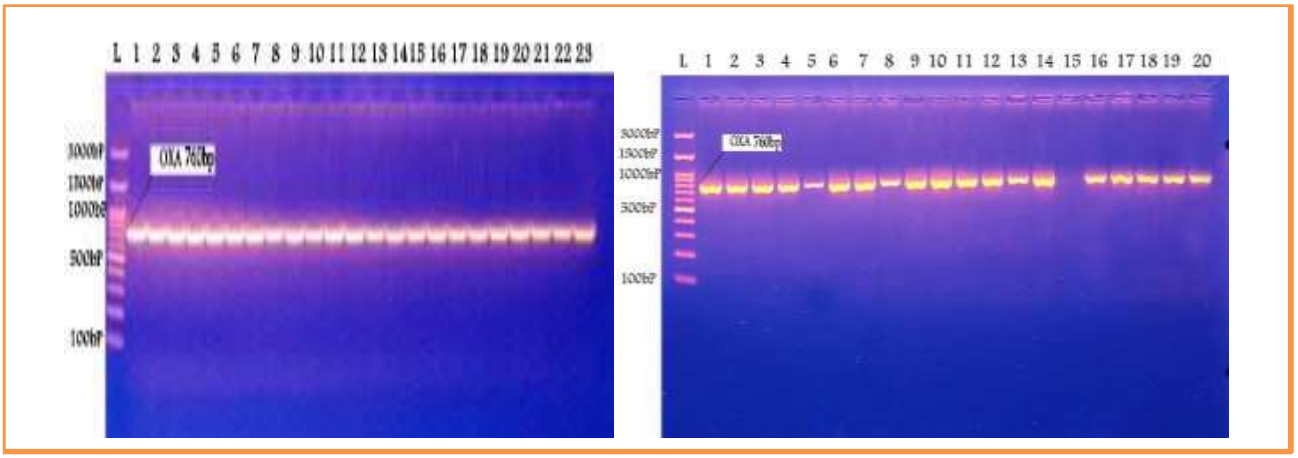


Figure (3-5): Agarose gel with red safe stained with mono-plex PCR amplified product from extract DNA of *P. aeruginosa* samples with *bla*$_{OXA}$ gene primer. The electrophoresis performed at 80 volt for 120 minutes. Lane (L) is molecular size of DNA marker (3000-bp ladder). Lanes (15) showed Negative results while the other showed positive result by *bla*$_{OXA}$ gene (760 bp).

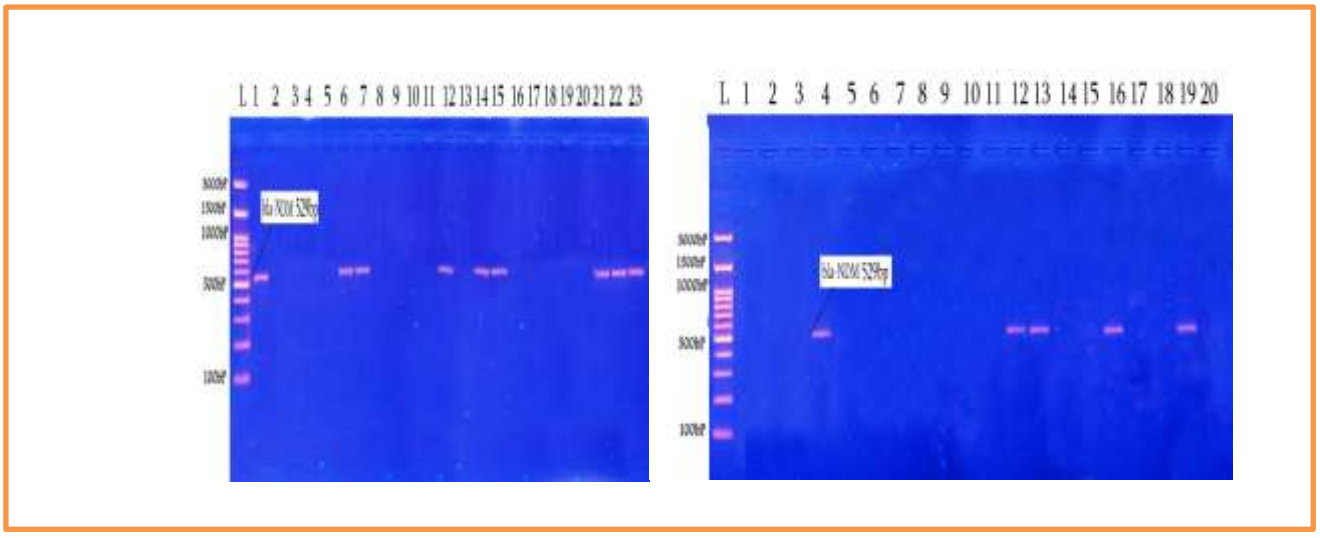


Figure (3-6): Agarose gel with red safe stained with mono-plex PCR amplified product from extract DNA of *P. aeruginosa* samples with *bla*$_{NDM}$ gene primer. The electrophoresis performed at 80 volt for 120 minutes. Lane (L) is molecular size of DNA marker (3000-bp ladder). Lanes 3,12,13,16,19,20,26,27,32,34,3541,42,43 showed positive result by *bla*$_{NDM}$ gene (529 bp).

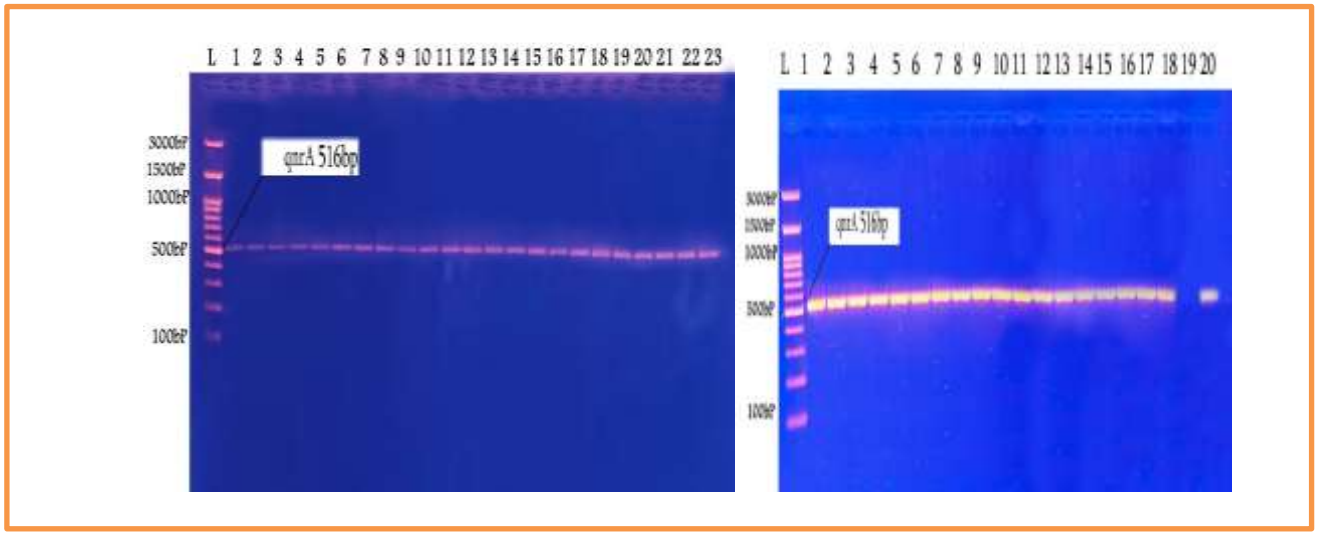


Figure (3-7): Agarose gel with red safe stained with mono-plex PCR amplified product from extract DNA of *P. aeruginosa* samples with *qnr-a* gene primer. The electrophoresis performed at 80 volt for 120 minutes. Lane (L) is molecular size of DNA marker (3000-bp ladder). Lanes (19) showed Negative results while the other shoed positive result by *qnr-a* gene (516 bp).

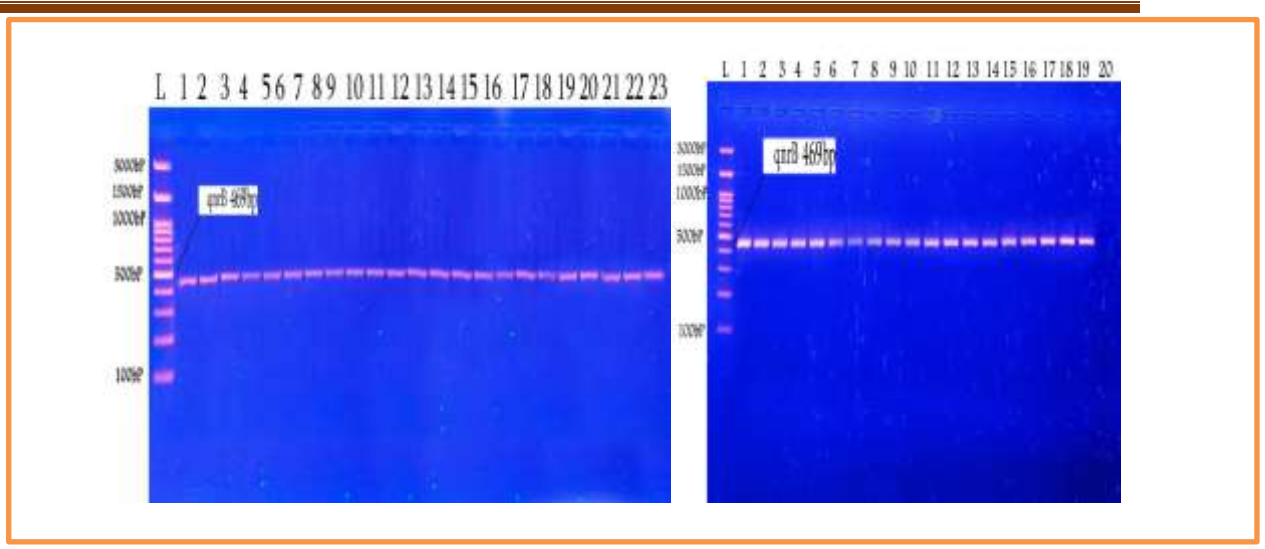


Figure (3-8): Agarose gel with red safe stained with mono-plex PCR amplified product from extract DNA of *P. aeruginosa* samples with qnr-b gene primer. The electrophoresis performed at 80 volt for 120 minutes. Lane (L) is molecular size of DNA marker (3000-bp ladder). Lanes showed positive results in all samples by *qnr-b* gene (469 bp).

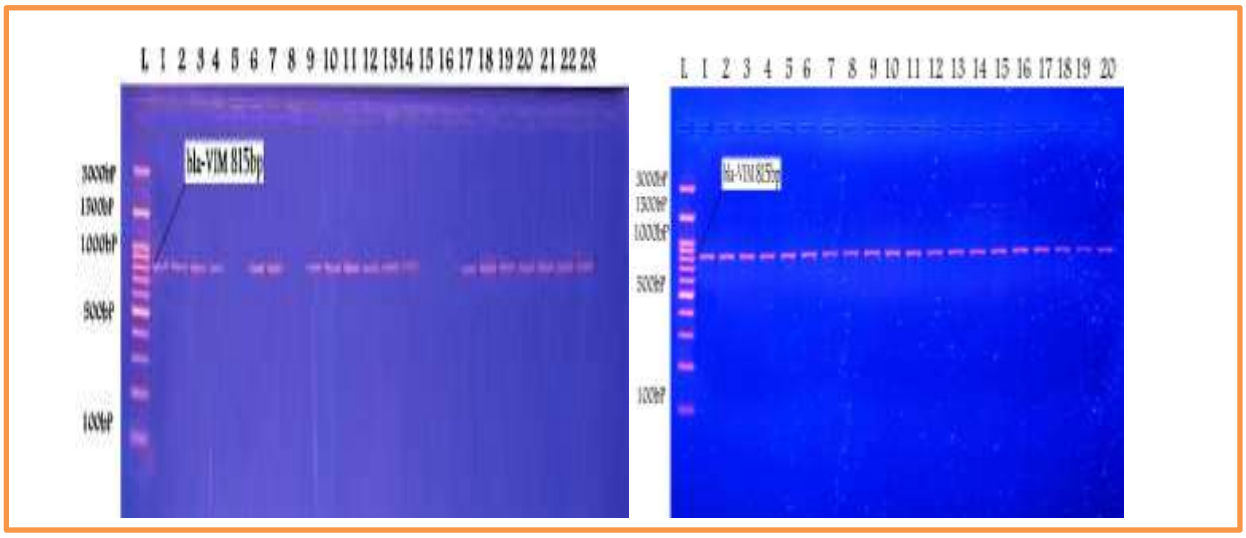


Figure (3-9): Agarose gel with red safe stained with mono-plex PCR amplified product from extract DNA of *P. aeruginosa* samples with *bla*$_{VIM}$ gene primer. The electrophoresis performed at 80 volt for 120 minutes. Lane (L) is molecular size of DNA marker (3000-bp ladder). Lanes (25,28,35,36) showed Negative results while the other showed positive result by *bla*$_{VIM}$ gene (815 bp).

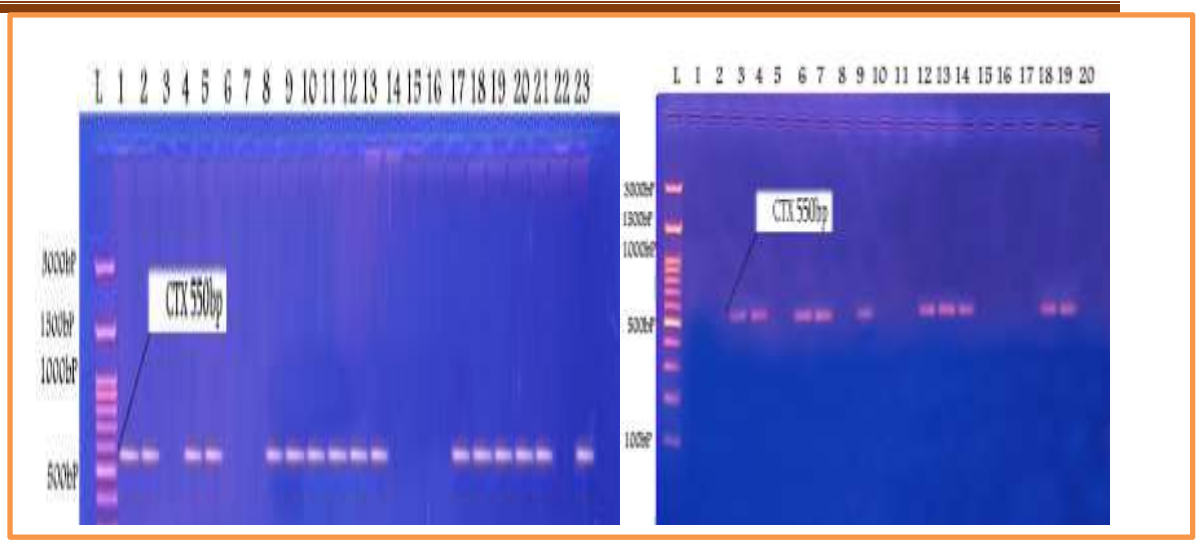


Figure (3-10): Agarose gel with red safe stained with mono-plex PCR amplified product from extract DNA of *P. aeruginosa* samples with *bla*$_{CTX}$ gene primer. The electrophoresis performed at 80 volt for 120 minutes. Lane (L) is molecular size of DNA marker (3000-bp ladder). Lanes (1,2,5,8,10,11,15,16,17,20,23,26,27,28,34,35,36,42) showed Negative results while the other showed positive result by *bla*$_{CTX}$ gene (550 bp)

3.6.2 Association Between Gene Detection and Patterns of Antibiotic Resistance for *P. aeruginosa* in UTIs, Burns, and Wounds Infections

The study analyzes the association between genes detection and antibiotics resistance in infections across UTIs, burn, and wound cases as explained in Table (3-4).

The *OXA* gene showed a significant association between its positive detection and escalated antibiotics resistance (both MDR and XDR) over all infection types, with P-values of 0.0001 in all these comparisons.

The *NDM* gene exhibited varying patterns, where its negative detection is generally associated with increased XDR and MDR resistance in UTIs cases, while its negative detection showed associated with significantly increase in only XDR resistance, especially in burns cases, whereas its positive detection associated with significantly increase in only XDR resistance in wounds cases.

Regarding to *QNR_A and QNR_B* genes, their positive detection is dominant over all infection cases and resistance patterns, while negative detection was mostly absent, and these findings are supported by significant p-values of 0.0001.

The positive detection of the *CTX* gene was more commonly linked to significantly increase in only MDR antibiotic resistance in UTIs cases, while in burn and wound cases their positive detection was associated with significant increase in both MDR and XDR resistances. Finally, vim gene demonstrated a strong link between its positive detection and significantly (p=0.0001) in both MDR and XDR resistances across all cases, with negative detection being exceedingly rare.

Table (3-4): Association between Gene detection and Patterns of Antibiotic Resistance for *P. aeruginosa* in UTIs, Burns, and Wounds Infections.

Gene type	Gene detection	Type of infection and Antibiotic resistance					
		UTI(n=9)		Burn (n=29)		Wound (n=5)	
		MDR	XDR	MDR	XDR	MDR	XDR
Oxa	- ve	0 (0%)	0 (0%)	0 (0%)	1(4.7%)	0 (0%)	0 (0%)
	+ve	6(100%)	3(100%)	8(100%)	20(95.2%)	2(100%)	3(100%)
Total		6	3	8	21	2	3
P value		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Ndm	- ve	5(83.3%)	3(100%)	4(50%)	15(71.4%)	1(50%)	1(33.3%)
	+ve	1(16.7%)	0 (0%)	4(50%)	6(28.6%)	1(50%)	2(66.7%)
Total		6	3	8	21	2	3
P value		0.0001*	0.0001*	1.000^{NS}	0.0001*	1.000^{NS}	0.0001*
qnr_a	- ve	0 (0%)	0 (0%)	1(14.3%)	0 (0%)	0 (0%)	0 (0%)
	+ve	6(100%)	3(100%)	7(87.5%)	21(100%)	2(100%)	3(100%)
Total		6	3	8	21	2	3
P value		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Ctx	- ve	2(33.3%)	2(66.7%)	3(37.5%)	7(33.3%)	0 (0%)	1(33.3%)
	+ve	4(66.7%)	1(33.3%)	5(62.5%)	14(66.7%)	2(100%)	2(66.7%)

Total		6	3	8	21	2	3
P value		0.0001*	0.0001*	0.0120*	0.0001*	0.0001*	0.0001*
qnr_b	**- ve**	0 (0%)	0 (0%)	1(11.1%)	0(0%)	0 (0%)	0 (0%)
	+ve	6(100%)	3(100%)	8(88.9%)	20(100%)	2(100%)	3(100%)
Total		6	3	9	20	2	3
P value		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
Vim	**- ve**	0 (0%)	0 (0%)	0 (0%)	3(14.2%)	0 (0%)	0 (0%)
	+ve	6(100%)	3(100%)	8(100%)	18(85.7%)	2(100%)	3(100%)
Total		6	3	8	21	2	3
P value		0.0001*	0.0001*	0.0001*	0.0001*	0.0001*	0.0001*
*Significant difference at the 0.05 level by chi-square test NS: Non-significant difference							

3.6.3 Antibiotic Susceptibility and Gene Detection for *P. aeruginosa* Isolates.

Table (3-5) elucidates the dataset of antibiotics susceptibility in relation to gene detection (*OXA, NDM, QNR_A, CTX, QNR_B,* and *VIM*) over various antibiotics, including: Imipenem, Meropenem, Levofloxacin, Ciprofloxacin, Ofloxacin, Nalidixic Acid, and Norfloxacin. Where the resistant isolates called (R), intermediate resistance (I), and sensitive isolates (S).

According to the results of this table, the resistant isolates are predominantly linked to positive gene detection for all genes, with consistently high rates, especially for Imipenem and Meropenem. Also it was found that all isolates showed resistance to Nalidixic acid with increased gene detection of all genes.

Intermediate resistant isolate was less frequent, *P. aeruginosa* isolates showed this type of resistance to Meropenem only, and the highest percentage was in isolates with positive gene detection.

As for the sensitive isolates, *P. aeruginosa* showed varying degrees of sensitivity to all antibiotics, except nalidixic acid as shown in this table. The

percentages were close in some antibiotics between the isolates detection and non-detection the studied genes.

Overall, the present findings emphasized the dominant role of positive gene detection in driving resistance for antibiotics, highlighting the studied genes as potential markers for multidrug-resistant isolates.

Table (3-5): Antibiotic Susceptibility and Gene Detection for *P. aeruginosa* Isolates

Antibiotic	R/I/S	Gene detection No. (%)											
		oxa		ndm		qnr_a		ctx		qnr_b		vim	
		-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
IMPEN-EM	R	1 (100)	35 (83.3)	23 (79.3)	10 (76.5)	1 (100)	35 (83.3)	11 (73.3)	25 (89.3)	0 (0)	35 (83.3)	3 (100)	33 (82.5)
	S	0 (0)	7 (16.6)	6 (20.6)	4 (23.5)	0 (0)	7 (16.6)	4 (26.6)	3 (10.7)	1 (100)	7 (16.6)	0 (0)	7 (17.5)
Total		1	42	29	14	1	42	15	28	1	42	3	40
MEROPENEM	R	1 (100)	31 (73.8)	21 (72.4)	11 (78.5)	1 (100)	31 (73.8)	10 (66.7)	22 (78.5)	0 (0)	31 (73.8)	3 (100)	29 (72.5)
	I	0 (0)	4(9.5)	2 (6.9)	2 (14.3)	0 (0)	4(9.5)	1 (6.6)	3 (10.7)	0 (0)	4 (9.5)	0 (0)	4 (10)
	S	0 (0)	7 (16.6)	6 (20.6)	1 (7.1)	0 (0)	7 (16.6)	4 (26.6)	3 (10.7)	1 (100)	7 (16.6)	0 (0)	7 (17.5)
Total		1	42	29	14	1	42	15	28	1	42	3	40
LEVOFLOXACIN	R	0 (0)	23 (54.7)	15 (51.7)	8 (57.1)	0 (0)	23 (54.7)	8 (53.3)	15	0 (0)	22 (52.3)	2 (66.7)	21 (52.5)
	S	1 (100)	19 (45.2)	14 (48.3)	6 (42.9)	1 (100)	19 (45.2)	7 (46.7)	13	1 (100)	20 (47.6)	1 (33.3)	19 (47.5)
Total		1	42	29	14	1	42	15	28	1	42	3	40
CIPROFLOXACIN	R	0 (0)	23 (54.7)	15 (51.7)	8 (57.1)	0 (0)	23 (54.7)	8 (53.3)	15 (53.3)	0 (0)	22 (52.3)	2 (66.7)	21 (52.5)
	S	1 (100)	19 (45.2)	14 (48.3)	6 (42.9)	1 (100)	19 (45.2)	7 (46.7)	13 (46.4)	1 (100)	20 (47.6)	1 (33.3)	19 (47.5)
Total		1	42	29	14	1	42	15	28	1	42	3	40
OFLOXACIN	R	0 (0)	23 (54.7)	15 (51.7)	8 (57.1)	0 (0)	23 (54.7)	8 (53.3)	15 (53.3)	0 (0)	22 (52.3)	2 (66.7)	21 (52.5)
	S	1 (100)	19 (45.2)	14 (48.3)	6 (42.9)	1 (100)	19 (45.2)	7 (46.7)	13 (46.4)	1 (100)	20 (47.6)	1 (33.3)	19 (47.5)
Total		1	42	29	14	1	42	15	28	1	42	3	40
NALIDIC (n=43)	R	1	42	29	14	1	42	15	28	1	42	3	40
NORFLOXACIN	R	0 (0)	23 (54.7)	15 (51.7)	8 (57.1)	0 (0)	23 (54.7)	8 (53.3)	15 (53.3)	0 (0)	22 (52.3)	2 (66.7)	21 (52.5)
	S	1 (100)	19 (45.2)	14 (48.3)	6 (42.9)	1 (100)	19 (45.2)	7 (46.7)	13 (46.4)	1 (100)	20 (47.6)	1 (33.3)	19 (47.5)
Total		1	42	29	14	1	42	15	28	1	42	3	40

3.6.4 Antibiotic Susceptibility for *P. aeruginosa* in Patient According to Type of Infection

Table (3-6) shows the antibiotic susceptibility for *P. aeruginosa* according to the type of infection, whether it is UTIs, burns or wounds. For Impenem, the highest percentage of isolates resistant to this antibiotic was those isolated from burns, at 93.1%, while the highest percentage of isolates sensitive to this antibiotic was those isolated from UTIs, at 44.9%.

Regarding to Meropenem, as we mentioned above, there were three types of resistance isolates: resistant, intermediate, and sensitive. The highest percentage of resistant isolates was isolated from burns, at 86.2%. The isolates that showed intermediate resistance, which appeared only to this type of antibiotic, were the lowest percentage of those isolated from UTIs, as well the highest percentage of isolates sensitive to this antibiotic was those isolated from UTIs, at 44.4%

As for Levofloxacin, Ciprofloxacin, Ofloxacin, and Norfloxacin, the highest percentage of isolates resistant to this antibiotic was those isolated from burns, , while the highest percentage of isolates sensitive to this antibiotic was those isolated from UTIs,

According to Nalidixic acid, all *P. aeruginosa* isolates were resistant to Nalidixic acid, and the highest percentage was that isolated from burns, at 67.4%.

All these associations showed statistically highly significant differences, ($p<0.05$).

Table (3-6): Antibiotic susceptibility of *P. aeruginosa* in Patient According to Type of Infection

Antibiotic	R/I/S	Site infection No. (%)			Total	P value (p ≤ 0.05)
		UTIs	Burn	Wound		
IMPENEM	R	5 (55.5%)	27(93.1%)*	4 (80%)	36 (83.7%)	0.0074*
	S	4 (44.9%)*	2 (6.9%)	1(20%)	7 (16.3%)	0.0001*
Total		9	29	5	43	
MEROPENEM	R	3 (33.3%)	25 (86.2%)*	4 (80%)	32 (74.4%)	0.0001*
	I	2 (22.2%)*	2 (6.8%)	0 (0%)	4 (9.3%)	0.0001*
	S	4 (44.4%)*	2 (6.8%)	1 (20%)	7 (16.3%)	0.0001*
Total		9	29	5	43	
LEVOFLOXACIN	R	2 (22.2%)	18 (62.1%)*	3 (60%)	23 (53.4%)	0.0001*
	S	7 (77.8%)*	11 (37.9%)	2 (40%)	20 (46.5%)	0.0001*
Total		9	29	5	43	
CIPROFLOXACIN	R	2 (22.2%)	18 (62.1%)*	3 (60%)	23 (53.4%)	0.0001*
	S	7 (77.8%)	11 (37.9%)	2 (40%)	20 (46.5%)	0.0001*
Total		9	29	5	43	
OFLOXACIN	R	2 (22.2%)	18 (62.1%)*	3 (60%)	23 (53.4%)	0.0001*
	S	7 (77.8%)*	11 (37.9%)	2 (40%)	20 (46.5%)	0.0001*
Total		9	29	5	43	
NALIDIXIC ACID	R	9 (20.9%)	29 (67.4%)*	5 (11.6%)	43 (100%)	0.0001*
NORFLOXACIN	R	2 (22.2%)	18 (62.1%)	3 (60%)	23 (53.4%)	0.0001*
	S	7 (77.8%)	11 (37.9%)	2 (40%)	20 (46.5%)	0.0001*
Total		9	29	5	43	

*Significant difference at the 0.05 level by chi-square test

3.7. IN SILICO ANALYSIS

3.7.1. Detection of Nucleic Acid Variations

For *OXA* locus, two isolates were selected in the present study, namely AMKMM1 and AMKMM2. These isolates were screened to partially amplify the OXA gene sequences within the *P. aeruginosa* sequences to assess their polymorphisms. Using the NCBI Blastn tool, the sequencing reactions indicated the exact identity of the analyzed locus after pairwise alignment with the closest reference sequences in the NCBI server.

Concerning the AMKMM1 and AMKMM2 samples, the NCBI BLASTn engine showed about 99% sequence similarities between the sequenced samples' genomic sequences of OXA gene belonging to P. aeruginosa (GenBank acc. CP156941.1). The alignment results of the AMKMM1 and AMKMM2 isolates revealed the presence of four nucleic acid variations compared with the most similar reference nucleic acid sequences of the bacterial OXA gene sequences. These nucleic acid variations, as determined in the PCR amplicons, are 45A>C and 417T>C in AMKMM1 sample, and 404A>C, 417T>C, and 443G>A in AMKMM2 sample (Fig. 3-11a).

Concerning the AMKMM3 sample, the NCBI BLASTn engine showed an entire sequence homology between the sequenced samples' genomic sequences of NDM gene of *P. aeruginosa* (GenBank acc. CP107257.1). The alignment results of the AMKMM3 sample revealed the presence of no nucleic acid variation compared with the most similar reference nucleic acid sequences of the bacterial NDM gene sequences (Fig. 3-11b). This entire similarity with the bacterial cataloged sequences highlights the presence of highly conserved sequences in the studied amplicons.

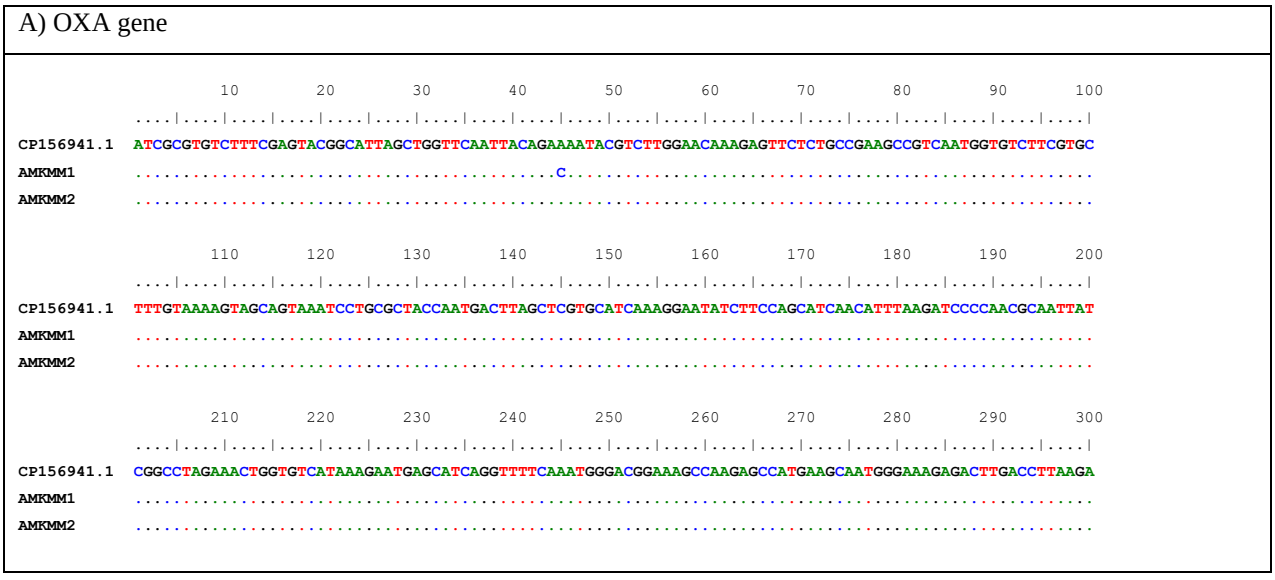
Concerning the AMKMM4 and AMKMM5 samples, the NCBI BLASTn engine showed about 99% sequence similarities between the sequenced samples' genomic sequences of CTX-1 gene belonging to P. aeruginosa (GenBank acc. NG_056171.1). The alignment results of the AMKMM4 and AMKMM5 samples

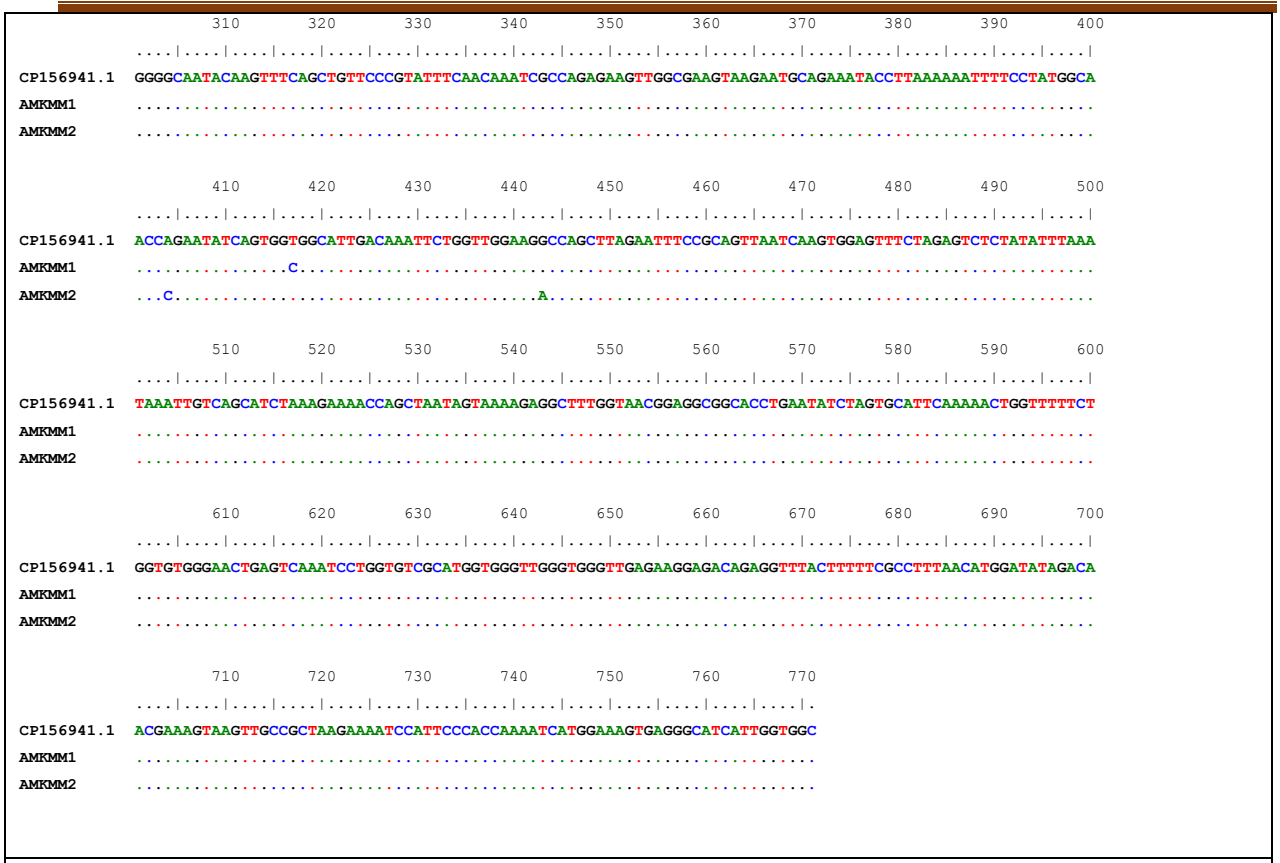
revealed the presence of nine nucleic acid variations compared with the most similar reference nucleic acid sequences of the bacterial CTX-1 gene sequences. These nucleic acid variations as determined in the PCR amplicons are 281A>G in AMKMM4 sample, and 38C>T, 74G>A, 112C>T, 148A>G, 226G>T, 381G>T, 408C>T, and 477A>T in AMKMM5 sample (Fig. 3-11c).

Concerning the AMKMM7 and AMKMM8 samples, the NCBI BLASTn engine showed that the AMKMM7 sample exhibited 100% sequence similarity between the sequenced samples' genomic sequences of blaVIM-1 gene belonging to P. aeruginosa (GenBank acc. OP329418.1). Whereas the alignment results of the AMKMM8 sample with the same GenBank sequences revealed the presence of two nucleic acid variations. These nucleic acid variations, as determined in the PCR amplicons, are 164A>G and 428A>G (Fig. 3-11d).

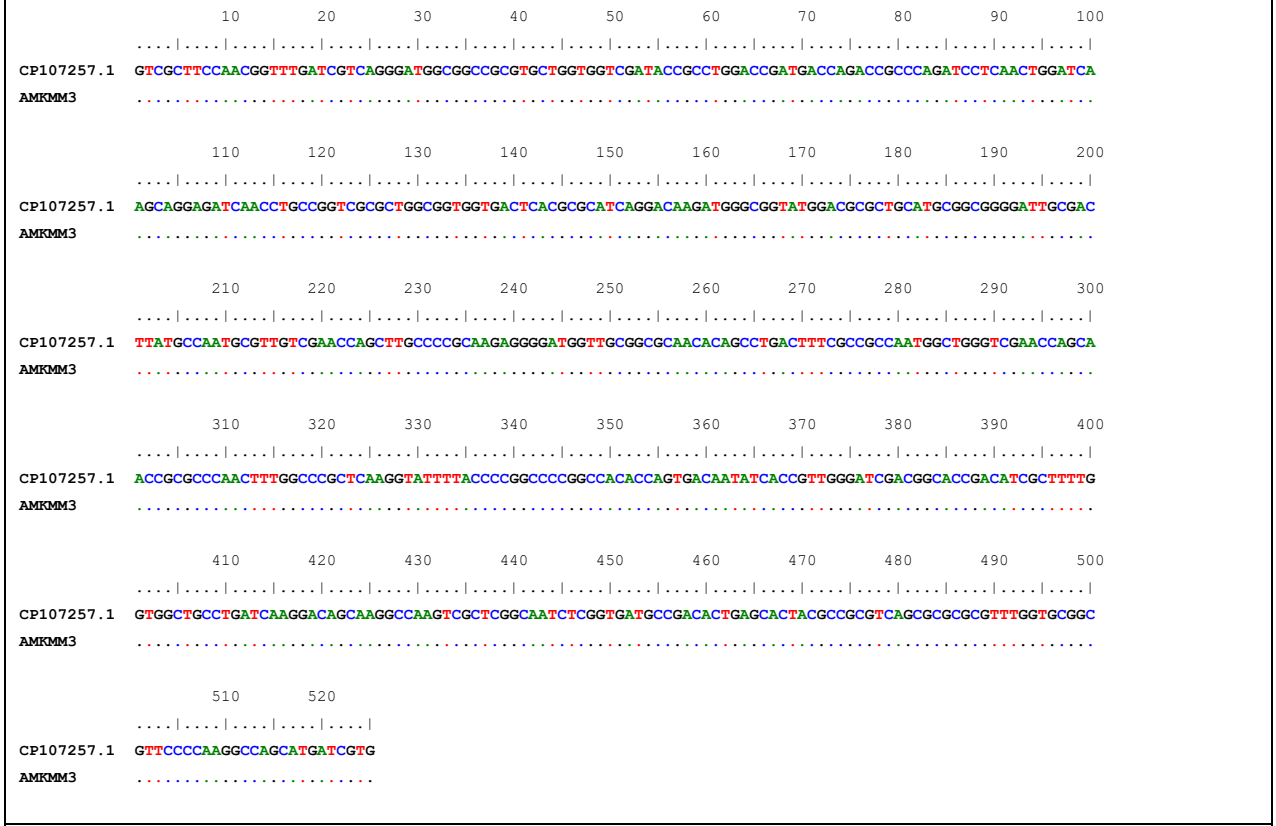
With regard to the AMKMM11 and AMKMM12 samples, the NCBI BLASTn engine showed about 99% sequence similarities between the sequenced samples' genomic sequences of qnrA gene belonging to P. aeruginosa (GenBank acc. CP109684.1). The alignment results of the AMKMM11 sample revealed the presence of only one nucleic acid variation compared with the most similar reference nucleic acid sequences of the bacterial qnrA gene sequences. This nucleic acid variation as determined in the PCR amplicons, is 373G>A. The same nucleic acid variant is also detected in the AMKMM12 with an additional variant, namely 444T>A (Fig. 3-11 e).

A) OXA gene

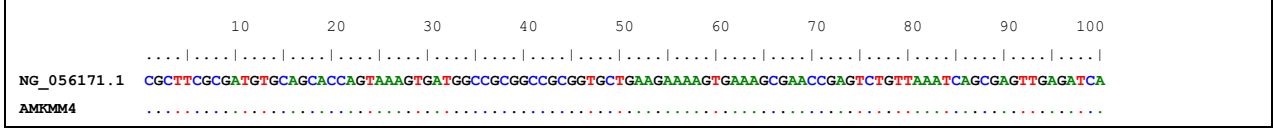


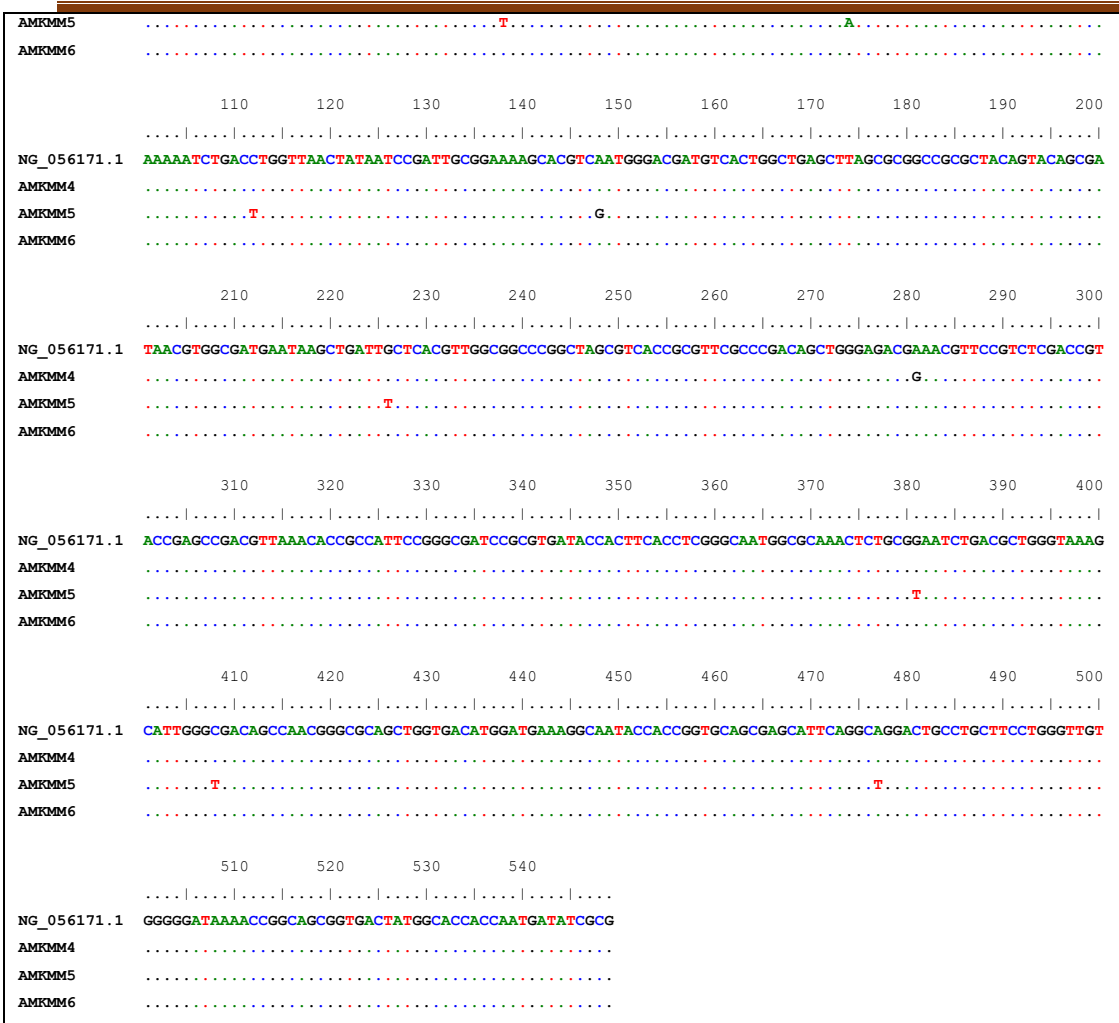


B) NDM gene

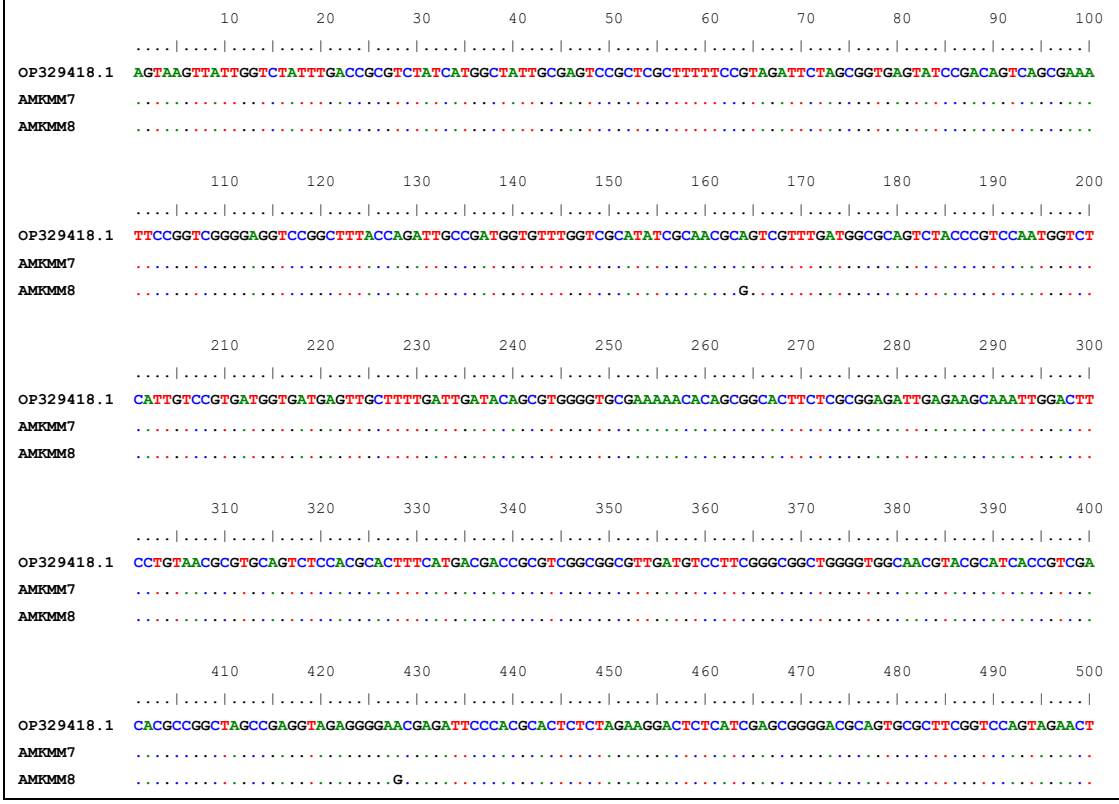


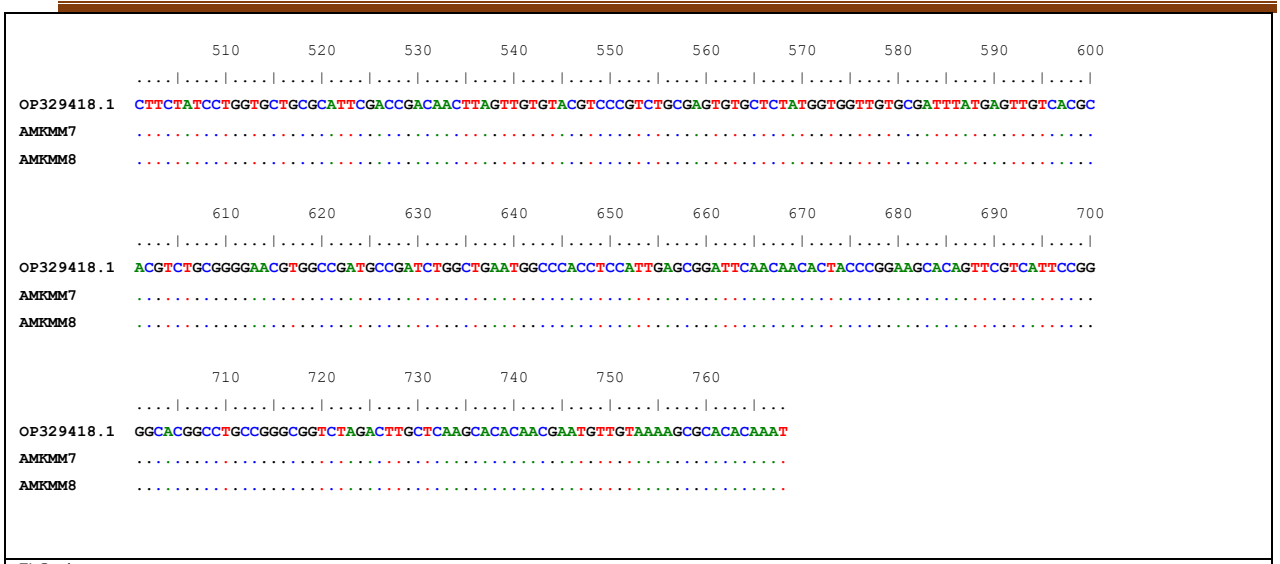
C) CTX-1 gene





D) blaVIM-1 gene




510 520 530 540 550 560 570 580 590 600
OP329418.1 CTTCTATCCTGGTGCTGCGCATTCGACCGACAACTTAGTTGTGTACGTCCCGTCTGCGAGTGTGCTCTATGGTGGTTGTGCGATTTATGAGTTGTCACGC
AMKMM7
AMKMM8
610 620 630 640 650 660 670 680 690 700
OP329418.1 ACGTCTGCGGGGAACGTGGCCGATGCCGATCTGGCTGAATGGCCCACCTCCATTGAGCGGATTCAACAACACTACCCGGAAGCACAGTTCGTCATTCCGG
AMKMM7
AMKMM8
710 720 730 740 750 760
OP329418.1 GGCACGGCCTGCCGGGCGGTCTAGACTTGCTCAAGCACACAACGAATGTTGTAAAAGCGCACACAAAT
AMKMM7
AMKMM8

E) QnrA gene


10 20 30 40 50 60 70 80 90 100
CP109684.1 ATGGATATTATTGATAAAGTTTTTCAGCAAGAGGATTTCTCACGCCAGGATTTGAGTGACAGCCGTTTTCGCCGCTGCCGCTTTTATCAGTGTGACTTCA
AMKMM11
AMKMM12
110 120 130 140 150 160 170 180 190 200
CP109684.1 GCCACTGTCAGCTGCAGGATGCCAGTTTCGAGGATTGCAGTTTCATTGAAAGCGGCGCCGTTGAAGGGTGTCACTTCAGCTATGCCGATCTGCGCGATGC
AMKMM11
AMKMM12
210 220 230 240 250 260 270 280 290 300
CP109684.1 CAGTTTCAAGGCCTGCCGTCTGTCTTTGGCCAACTTCAGCGGTGCCAACTGCTTTGGCATAGAGTTCAGGGAGTGCGATCTCAAGGGCGCCAACTTTCC
AMKMM11
AMKMM12
310 320 330 340 350 360 370 380 390 400
CP109684.1 CGGGCCCGCTTCTACAATCAAGTCAGCCATAAGATGTACTTCTGCTCGGCTTATATCTCAGGTTGCAACCTGGCCTATACCAACTTGAGTGGCCAATGCC
AMKMM11 .....A.....
AMKMM12 .....A.....
410 420 430 440 450 460 470 480 490 500
CP109684.1 TGGAAAAATGCGAGCTGTTTGAAAACAACTGGAGCAATGCCAATCTCAGCGGCGCTTCCTTGATGGGCTCAGATCTCAGCCGCGGCACCTTCTCCCGCGA
AMKMM11
AMKMM12 .....A.....
510 520 530 540 550 560 570 580 590 600
CP109684.1 CTGTTGGCAACAGGTCAATCTGCGGGGCTGTGACCTAACCTTTGCCGATCTGGATGGGCTCGACCCCAGACGGGTCAACCTCGAAGGAGTCAAGATCTGT
AMKMM11
AMKMM12
610 620 630 640 650
CP109684.1 GCCTGGCAACAGGAGCAACTGCTGGAACCCTTGGGAGTAATAGTGCTGCCGGATTA
AMKMM11
AMKMM12

Figure. (3-11) Multiple nucleic acid alignments of the investigated bacterial samples alongside the most homologous GenBank reference sequences. Branches A–E refer to the analyzed OXA, NDM, CTX-1, blaVIM-1, and qnrA genes.

3.7.2. Detection of Amino Acid Variations

Given that all identified SNPs are located within the coding regions of the investigated genetic loci, it is necessary to assess whether these SNPs exert silent (synonymous) or missense (non-synonymous) effects on the encoded proteins. Additionally , the translation of nucleic acids into their corresponding amino acids will describe how much region amplified from the targeted gene. To this end, the nucleic acid sequences detected in all analyzed genetic loci were translated into their corresponding amino acid sequences using the Expasy translate tool.

Concerning the OXA genetic sequence, translation of the OXA nucleic acid sequences into their corresponding amino acid sequences within the oxacillin-hydrolyzing class D beta-lactamase OXA-10 indicates that the currently amplified region of the OXA gene covers the majority of the coding portion of this protein, which consists of 257 amino acid residues. These coding portions extend from the tenth amino acid residue to the last amino acid in the OXA-encoded protein. After translating the identified nucleic acid variations into their corresponding amino acid sequences, three missense SNPs and one silent SNP were identified. The identified missense SNPs are p.E24D, p.Q114P, and p.G157D, which result from the 45A>C, 404A>C, and 443G>A substitutions, respectively. In contrast, the 417T>C variant exerts a silent effect on the protein, namely p.G148= (Fig. 3-12a).

Concerning the NDM genetic sequence, translation of the NDM nucleic acid sequences into their corresponding amino acid sequences within the metallo-beta-lactamase NDM-1 indicates that the currently amplified region of the NDM gene covers about 64.8% of the coding portion of this protein, which consists of 270 amino acid residues. These coding portions extend from the 73^{th} amino acid residue to the 247^{th} amino acid in the NDM-encoded protein. Due to the entire homology of the investigated AMKMM3 sample with the reference sequences of

the NDM gene, no genetic variation has been shown in the translated nucleic acid sequences (Fig. 3-12b).

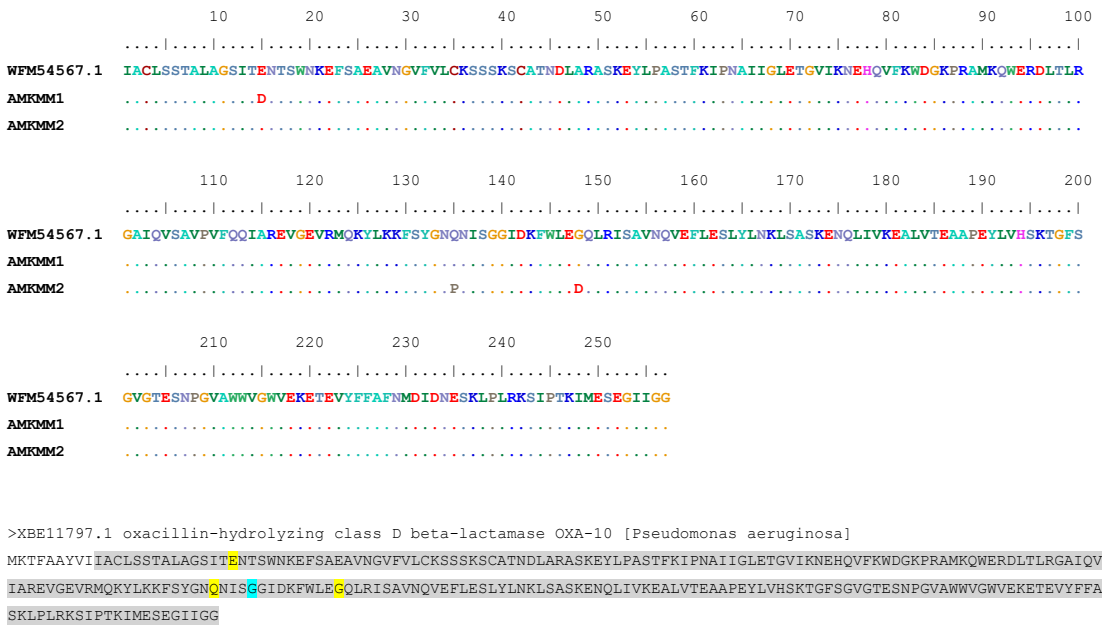
Concerning the CTX genetic sequence, translation of the CTX nucleic acid sequences into their corresponding amino acid sequences within the beta-lactamase CTX indicates that the currently amplified region of the CTX gene covers approximately 62.9% of the coding portion of this protein, which consists of 291 amino acid residues. These coding portions extend from the 68th amino acid residue to the 250th amino acid in the CTX-encoded protein. After translating the identified nucleic acid variations into their corresponding amino acid sequences, five missense SNPs and four silent SNPs were identified. The identified missense SNPs are p.80A>V, p.92N>S, p.117D>N, p.143S>A, and p.161E>G, which result from the 38T>C, 74A>G, 148G>A, 226T>G, and 281A>G substitutions, respectively. In contrast, the 112T>C, 381T>G, 408T>C, and 477T>A variant exerted the silent effects of p.105L=, p.194R=, p.203G=, and p.226A=, respectively (Fig. 3-12c). While AMKMM6 sample did not exhibit any variation, eight variations detected in the CTX gene attributed to the AMKMM5 sample. Whereas the AMKMM4 sample showed only one missense SNP of p.161E>G.

The translation of the blaVIM-1 nucleic acid sequences into their corresponding amino acid sequences within the VIM family beta-lactamase indicates that the currently amplified region of the blaVIM-1 gene covers 82.3% of the coding portion of this protein, which consists of 311 amino acid residues. These coding portions extend from the 51th amino acid residue to the 306th amino acid in the blaVIM-1-encoded protein. After translating the identified nucleic acid variations into their corresponding amino acid sequences, two missense SNPs were identified. The identified missense SNPs are p.105Q>R and p.193N>S, which result from the 164A>G and 428A>G nucleic acid substitutions, respectively (Fig. 3-12d).

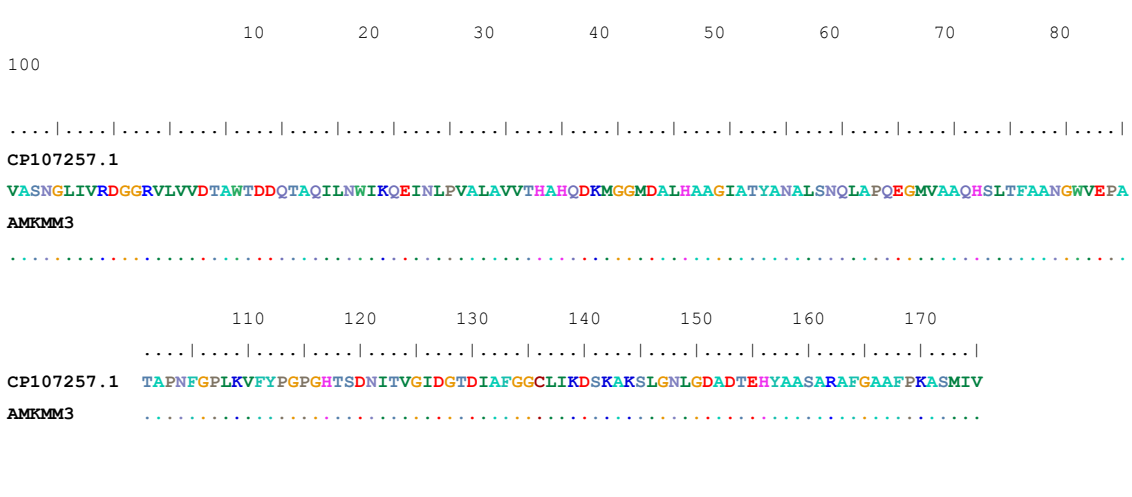
The translation of the *qnrA* nucleic acid sequences into their corresponding amino acid sequences within the quinolone resistance pentapeptide repeat protein

QnrA1 indicates that the currently amplified region of the qnrA gene covers all the coding portion of this protein, which consists of 218 amino acid residues. After translating the identified nucleic acid variations into their corresponding amino acid sequences, two missense SNPs were identified. The identified missense SNPs are p.125A>T and p.148N>K, which result from the 373G>A and 444T>A nucleic acid substitutions, respectively (Fig. 3-12e). While AMKMM11 sample exhibited both missense variations, the AMKMM11 sample showed only one missense SNP of p.125A>T.

A) OXA gene



B) NDM gene



>UYM59573.1 subclass B1 metallo-beta-lactamase NDM-1 [Pseudomonas aeruginosa]

MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQHTSYLDMPGFGAVASNGLIVR
DGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLT
FAANGWVEPATAPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAFPKA
SMIVMSHSAPDSRAAITHTARMADKLR

C) CTX-1 gene

```
                    10        20        30        40        50        60        70        80        90       100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
WP_102607455.1  RFAMCSTSKVMAAAAVLKKSESEPSLLNQRVEIKKSDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDR
AMKMM4          ..........................................................................................G.....
AMKMM5          ............V..........N.....................D.....................S.....................
AMKMM6          ..........................................................................................

                    110       120       130       140       150       160       170       180
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
WP_102607455.1  TEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGSGDYGTTNDIA
AMKMM4          ..............................................................................
AMKMM5          ..............................................................................
AMKMM6          ..............................................................................
```

>WP_102607455.1 MULTISPECIES: extended-spectrum class A beta-lactamase CTX-M-206 [Gammaproteobacteria]

MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQILYRADERFAMCSTSKVMAAA
AVLKKSESEPSLLNQRVEIKKSDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDET
FRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGSGDY
GTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL

D) blaVIM-1 gene

```
                    10        20        30        40        50        60        70        80        90       100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OP329418.1      SKLLVYLTASIMAIASPLAFSVDSSGEYPTVSEIPVGEVRLYQIADGVWSHIATQSFDGAVYPSNGLIVRDGDELLLIDTAWGAKNTAALLAEIEKQIGL
AMKMM7          ..........................................................................................
AMKMM8          ..........................................R...............................................

                    110       120       130       140       150       160       170       180       190       200
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
OP329418.1      PVTRAVSTHFHDDRVGGVDVLRAAGVATYASPSTRRLAEVEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGAAHSTDNLVVYVPSASVLYGGCAIYELSR
AMKMM7          ..........................................................................................
AMKMM8          ..................................S.......................................................

                    210       220       230       240       250
               ....|....|....|....|....|....|....|....|....|....|.
OP329418.1      TSAGNVADADLAEWPTSIERIQQHYPEAQFVIPGHGLPGGLDLLKHTTNVVKAHTN
AMKMM7          ........................................................
AMKMM8          ........................................................
```

>WCS41564.1 VIM family beta-lactamase [Pseudomonas aeruginosa]

MPRASKQQARYAVGRCLMLWSSNDVTQQGSRPKTKLCRTHPHGVLMFKLLSKLLVYLTASIMAIASPLAFSVDSSGEYPTVSEIPVGEVRLYQIADGVWSHIATQSFDGAVYPSNGLIVRDG
DELLLIDTAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHDDRVGGVDVLRAAGVATYASPSTRRLAEVEGNEIPTHSLEGLSSSGDAVRFGPVELFYPGAAHSTDNLVVYVPSASVLYGGCA
IYELSRTSAGNVADADLAEWPTSIERIQQHYPEAQFVIPGHGLPGGLDLLKHTTNVVKAHTNRSVVE

E) QnrA gene

```
                    10        20        30        40        50        60        70        80        90       100
```

Figure. (3-12): Multiple amino acid alignments of the investigated bacterial samples alongside the most homologous GenBank reference sequences. Branches A–E refer to the analyzed OXA, NDM, CTX-1, blaVIM-1, and qnrA proteins. Grey color refers to the covered amplified coding regions. Yellow and blue colors refer to the missense and silent SNPs, respectively.

3.7.3. Annotation of the Detected Variations

After describing the detected SNPs in the selected isolates , it is mandatory to summarize the overall SNPs in these samples. Accordingly, detailed information on the SNPs identified in the investigated bacterial samples was conducted to assess the number, affected samples, positioning, and effects of these SNPs in the OXA**,** CTX-1**,** blaVIM**,** qnrA, and NDM genes, using specific GenBank reference sequences. Four SNPs were detected in the OXA gene. Three of these are missense variants, resulting in amino acid substitutions: p.E24D, p.Q144P, and p.G157D. One silent SNP of p.G148= was found in one sample. These variations are located within the region encoding oxacillin-hydrolyzing class D beta-lactamase, potentially affecting resistance profiles. CTX-1 gene represents the most polymorphic gene since nine SNPs were identified in this genetic locus. Six were missense mutations, leading to the following substitutions: p.A80V, p.N92S, p.D117N, p.S143A, p.E161G, and one isolates carried multiple SNPs simultaneously. Three silent mutations were also found, which are p.L105=,

p.R194=, p.G203=, and p.A226=. The presence of these silent SNPs indicates potential genetic variability without altering the amino acid sequence in these positions. Two missense SNPs were detected in blaVIM, resulting in p.Q105R and p.N193S substitutions, possibly affecting the enzyme's activity and antibiotic resistance. Likewise, two missense SNPs were found in qnrA: p.A125T and p.N148K. These variations may influence quinolone resistance. Noteworthy, no SNPs were detected in the NDM gene across all analyzed samples, indicating a conserved sequence in this set.

Table (3-7): Details of the identified SNPs in the study. GenBank accession numbers used for OXA, CTX-1, blaVIM, and qnrA in the verified variations are CP156941.1, NG_056171.1, CP109684.1, and OP329418.1, respectively. No variations have been detected in the analyzed NDM gene (GenBank CP107257.1).

Gene	position in amplicon	position in the genome	native	allele	isolates	position in protein	effect
OXA	45	1666522	A	C	AMKMM1	24E	missense (p.24E>D)
OXA	404	1666205	A	C	AMKMM2	144Q	missense (p.144Q>P)
OXA	417	1666166	T	C	AMKMM1	148G	silent (p.148G=)
OXA	443	1666166	G	A	AMKMM2	157G	missense (p.157G>D)
NDM	-	-	-	-	AMKMM3	-	-
CTX-1	38	239	T	C	AMKMM5	80V	missense (p.80A>V)
CTX-1	74	275	A	G	AMKMM5	92N	missense (p.92N>S)
CTX-1	112	313	T	C	AMKMM5	105L	silent (p.105L=)
CTX-1	148	349	G	A	AMKMM5	117D	missense (p.117D>N)
CTX-1	226	427	T	G	AMKMM5	143S	missense (p.143S>A)
CTX-1	281	482	A	G	AMKMM4	161E	missense (p.161E>G)
CTX-1	381	582	T	G	AMKMM5	194R	silent (p.194R=)
CTX-1	408	609	T	C	AMKMM5	203G	silent (p.203G=)
CTX-1	477	678	T	A	AMKMM5	226A	silent (p.226A=)
blaVIM	164	15971	A	G	AMKMM8	105Q	missense (p.105Q>R)
blaVIM	428	16235	A	G	AMKMM8	193N	missense (p.193N>S)
qnrA	373	275098	G	A	AMKMM11&12	125A	missense (p.125A>T)
qnrA	444	275169	T	A	AMKMM12	148N	missense (p.148N>K)

3.7.4. Three-Dimensional Proteins Modelling

To evaluate whether the identified missense SNPs cause neutral or deleterious impacts on the structure, function, and stability of their proteins, several in silico tools were used. These tools were categorized into two main groups: those concerned with predicting the effects of these SNPs on structure and function, and those that predict their effects on protein stability.

The conducted homology modeling of SWISS-MODEL for a beta-lactamase protein OXA-10 resulted in the generation of a model using the template 6skp.1.A, which corresponds to a beta-lactamase OXA-10_IPM. This template was chosen because it has 100% sequence identity to the intended target, providing a very reliable basis for modeling. In the 3D visualization, the protein is shown in cartoon representation embedded within a translucent surface (Fig. 3-13a).

The conducted homology modeling for a CTX beta-lactamase protein resulted in the generation of a model using the template 7u48.1.A, which corresponds to a CTX beta-lactamase protein. This template was chosen because it has 98.63% sequence identity to the intended target, providing a very reliable basis for modeling. In the 3D visualization, the protein is shown in cartoon representation embedded within a translucent surface (Fig. 3-13b).

The conducted homology modeling for a blaVIM metallo-beta-lactamase resulted in the generation of a model using the template 6bm9.2.A, which corresponds to a metallo-beta-lactamase. This template was chosen because it has 93.75% sequence identity to the intended target, providing a very reliable basis for modeling. In the 3D visualization, the protein is shown in cartoon representation embedded within a translucent surface (Fig. 3-13c).

The conducted homology modeling for a qnrA protein or quinolone resistance pentapeptide repeat protein resulted in the building of a model using the template M4W996.1.A, which corresponds to a quinolone resistance pentapeptide repeat protein. This template was chosen because it has 100% sequence identity to the intended target, providing a very reliable basis for

modeling. As in the case of the other generated 3D models, the generated 3D structure of the qnrA protein is shown in cartoon representation embedded within a translucent surface (Fig. 3-13d). To verify the accuracy of the generated proteins and to assess their suitability for in silico analysis, the structures of the four generated proteins were evaluated by Ramachandran plot. This method analysis is widely performed for homology-modeled proteins to evaluate the stereochemical quality and reliability of the predicted three-dimensional structures. This analysis examines the backbone dihedral angles (phi and psi) of amino acid residues, identifying whether they adopt conformations that are energetically and sterically allowed based on empirical data from high-resolution crystal structures.

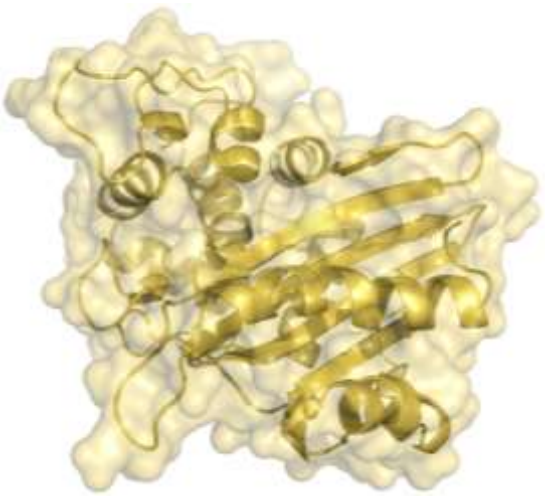
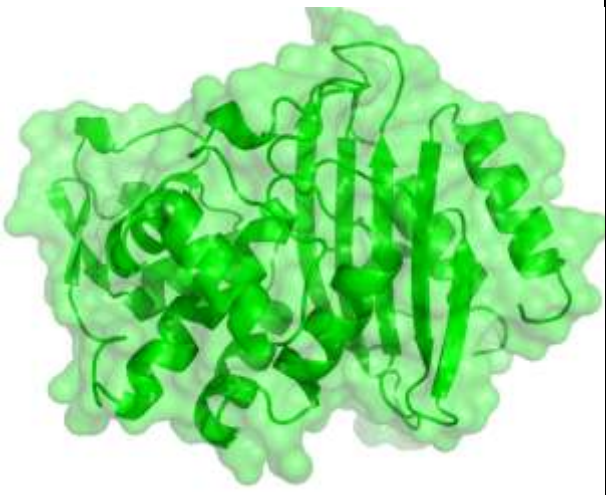
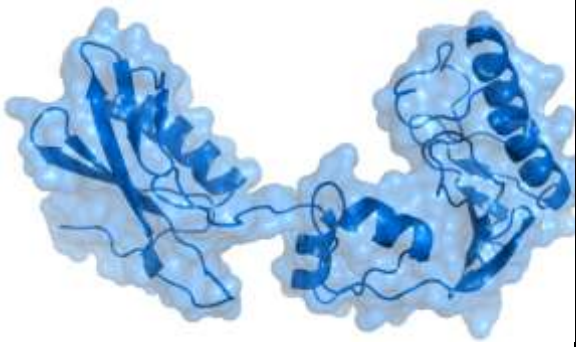
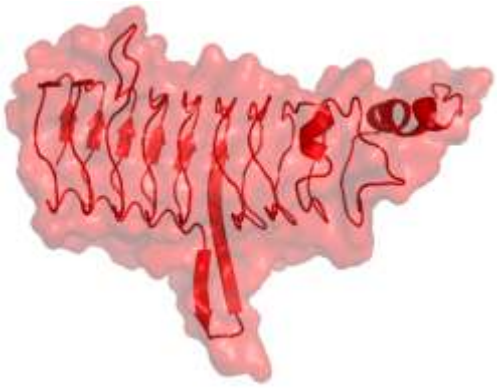
A) OXA protein	B) CTX protein
	
C) blaVIM-1 protein	D) QnrA protein
	

Figure. (3-13): Three-dimensional modeling of OXA (yellow), CTX (green), blaVIM-1 (blue), and qnrA (red) proteins as shown in branches A, B, C, and D, respectively.

By quantifying the percentage of residues in favored, allowed, and outlier regions, the Ramachandran plot provides a rigorous assessment of the model's geometric plausibility. A high proportion of amino acid residues in favored regions suggests that the model has realistic local conformations consistent with known protein structures. The presence of 90% or more than 90% of the generated protein indicates confidence in its use for in silico prediction and molecular docking reactions. Conversely, the presence of significant outliers may indicate errors in model model-building process that require correction or refinement. The Ramachandran Plot for the OXA protein, the model shows 97.12% of residues in the favored regions and no outliers, indicating excellent stereochemical quality (Fig. 3-14a).similarly The CTX protein has 95.94% in favored regions with 1.53% outliers, still suggesting good overall geometry but with minor regions needing attention (Fig. 3-14b). The blaVIM-1 protein has 93.83% favored residues and 0.88% outliers, reflecting acceptable structural quality with minimal stereochemical strain (Fig. 3-14c). Likewise, the qnrA protein showed 97.69% favored residues and no outliers, reflecting excellent stereochemical quality (Fig. 3-14d).

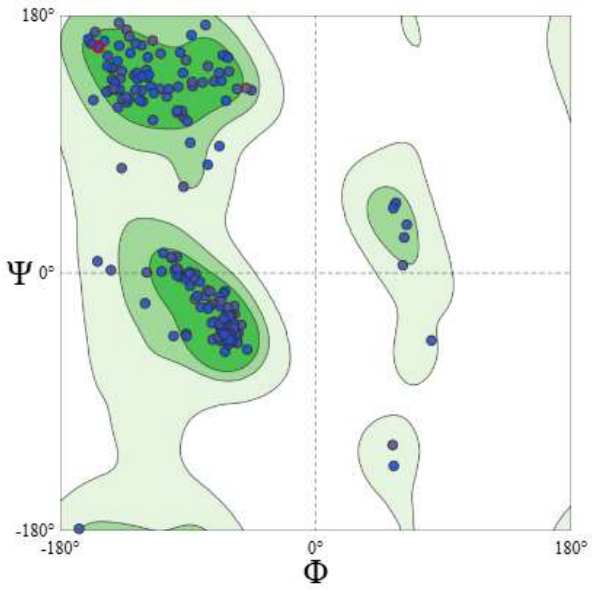
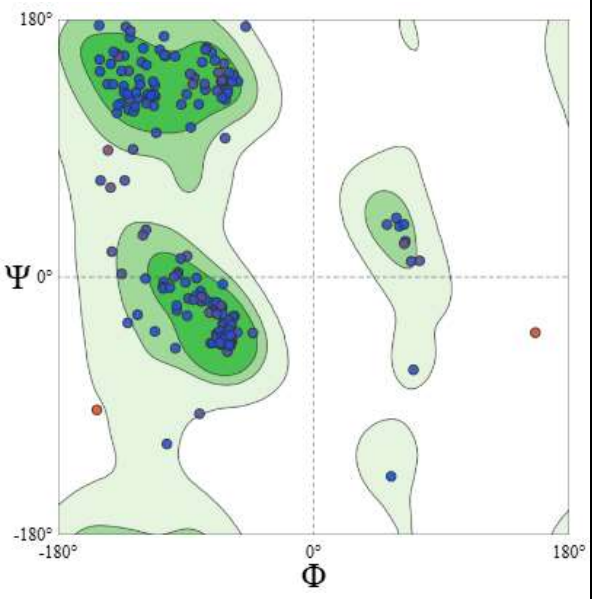
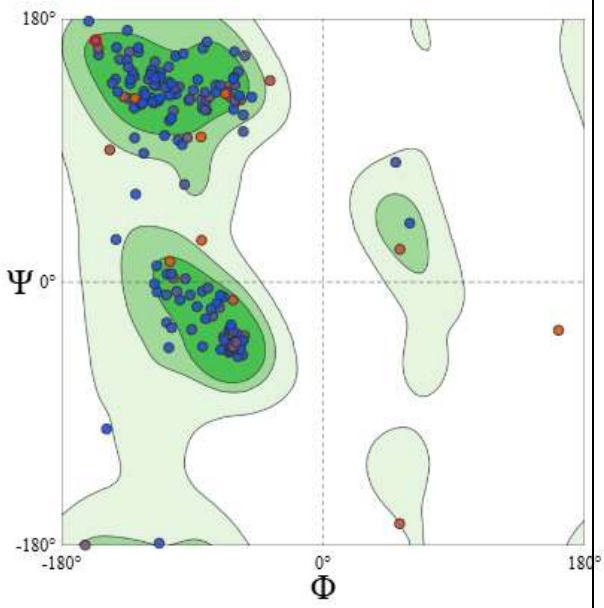
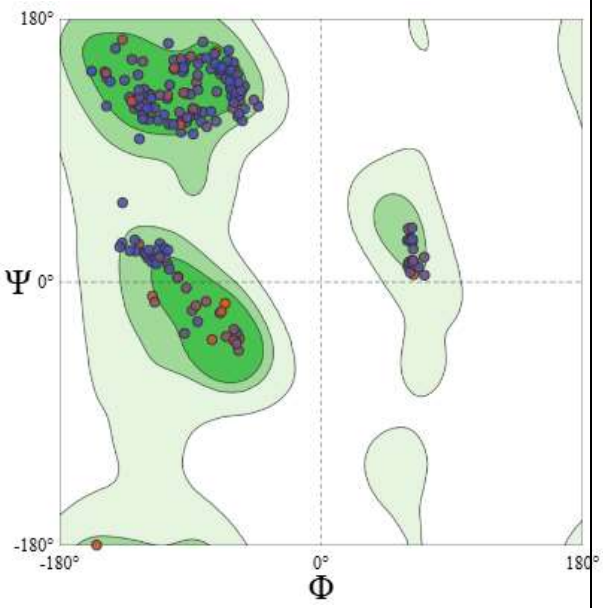
A) OXA protein	B) CTX protein
	
Ramachandran favoured 97.12% Ramachandran outliers 00.0%	Ramachandran favoured 95.94% Ramachandran outliers 1.53%
C) blaVIM-1 protein	D) QnrA protein
	
Ramachandran favoured 93.83% Ramachandran outliers 0.88%	Ramachandran favoured 97.69% Ramachandran outliers 0.00%

Figure. (3-14): Verification of the generated 3D models of OXA, CTX, blaVIM-1, and qnrA proteins as shown in branches A, B, C, and D, respectively.

3.7.5. Predicting the Effects of the Identified Variants on Proteins

The wild-type and altered structures of the OXA protein were compared to assess the impacts of the three variants Glu24Asp, Gln144Pro, and Gly157Asp. The conversion of the Glu-24, Gln-144, and Gly-157 residues in their native conformations into Asp-24, Pro-144, and Asp-157 in the altered model showed variable structural and functional deviations at each site (Fig. 3-15 a and b).

The Glu24Asp, substitution involves replacing glutamate with aspartate. In silico predictions classified this variant as having moderate impact. This is due to the neutral prediction from SIFT, a moderate impact of the SNP by PolyPhen-2, the neutral effects by PhD SNP and meta-SNP, the destabilizing effects by mCSM, SDM, and DUET tools. This suggests a modest but potentially significant impact on local charge interactions or hydrogen bonding that could slightly reduce stability or alter local electrostatics without grossly destabilizing the entire protein.

The Gln144Pro, the substitution of glutamine with proline has severe predicted effects. The majority of tools classify it as deleterious, including PolyPhen-2, PhD SNP, meta-SNP, and SDM. Proline introduces a rigid cyclic structure that can disrupt backbone conformations and local secondary structures such as helices or loops. This likely introduces kinks or destabilizes folding in the effect region, which severely compromises the structural integrity and potentially impairs protein function.

Concerning Gly157Asp, in which glycine is replaced with aspartate, the utilized computational methods have consistently shown the deleterious predictions of this SNP across all tools. Glycine’s minimal side chain permits flexibility and tight turns in the polypeptide backbone. Substituting it with the bulkier and negatively charged aspartate introduces steric clashes and charge repulsion. This sort of alteration can significantly distort local conformation, compromise proper folding, and reduce stability.

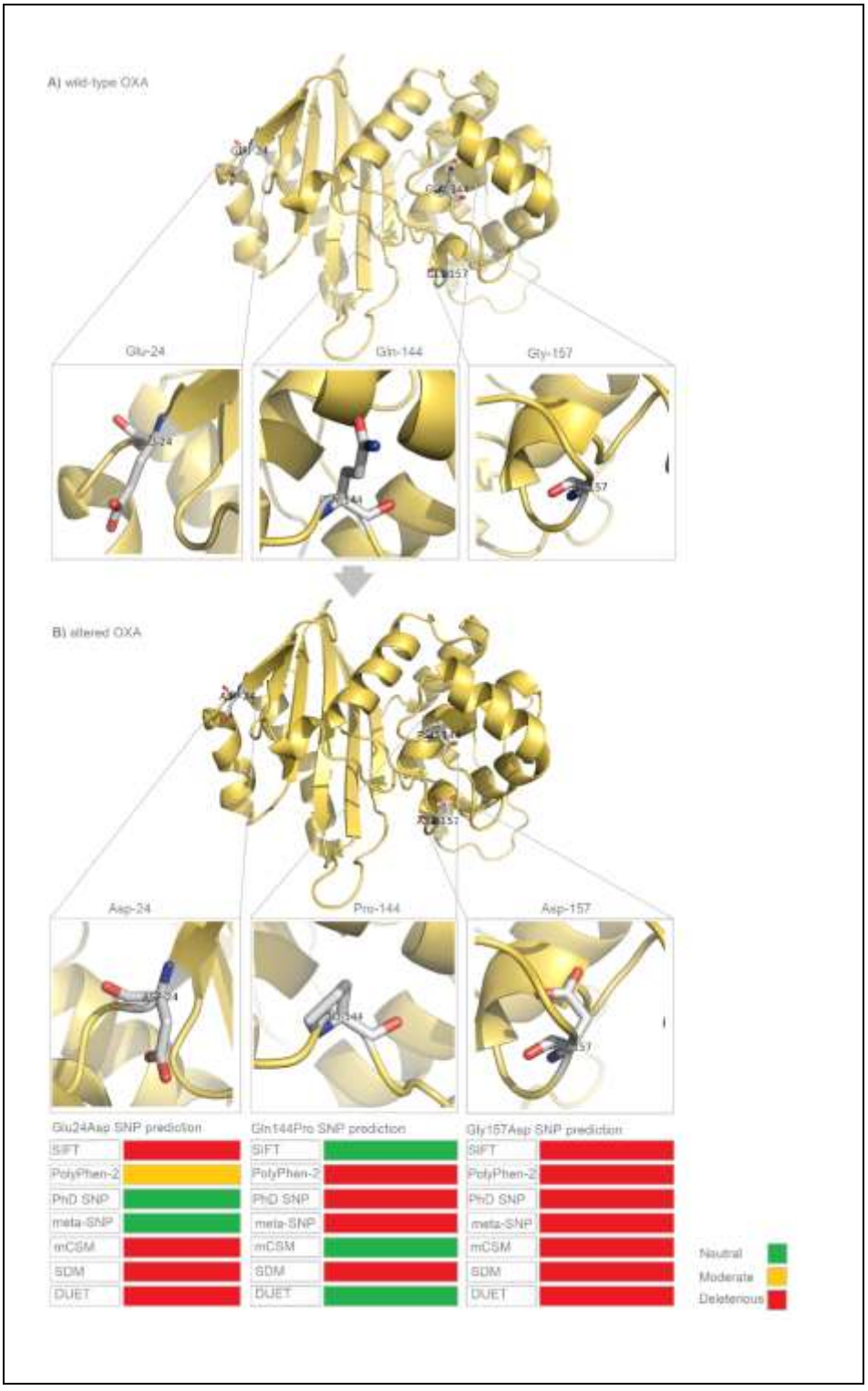


Figure. (3-15): In silico prediction of the effects of the detected missense variants on the OXA protein structure, function, and stability between the wild-type (branch A) and its altered form (branch B) as inferred by seven computational tools.

A structural and computational analysis of the impact of five detected SNPs on the CTX protein is shown by comparing the 3D structure of the wild-type CTX protein and its altered form (Fig. 3-16 a and b). In the wild-type CTX protein, the five key residues of Ala-80, Ser-92, Asp-117, Ala-143, and Glu-161 were highlighted. Each of these residues is shown in close-up to depict their native positions and side-chain orientations within the folded protein structure. In the altered CTX structure, five specific missense SNPs, Ala80Val, Ser92Asn, Asn117Asp, Ala143Ser, and Glu161Gly, were shown instead. The highlights of these mutations correspond to SNPs detected in this comparison and result in amino acid substitutions that can potentially affect CTX protein function and stability. Structural views of the mutated residues reveal changes in side-chain chemistry and positioning compared to the wild-type, which suggest possible structural perturbations. The in silico predictions of these mutations were conducted using seven computational tools: SIFT, PolyPhen-2, PhD-SNP, meta-SNP, mCSM, SDM, and DUET. These computational tools assess whether a SNP is likely to be neutral or stable, moderately impactful, or deleterious or destabilizing based on sequence conservation, structural modeling, and energetic effects.

For the Ala80Val SNP, the structure-function prediction tools of SIFT, PolyPhen-2, PhD-SNP, and meta-SNP predict a neutral effect of this amino acid substitution. Whereas the stability prediction tools of mCSM, SDM, and DUET showed a destabilizing impact of this SNP on the protein.

The Asn117Asp and Ala143Ser variants are largely predicted to be neutral and stabilizing across the majority of tools. The only exception to their neutral prediction is mCSM tool, which suggested a destabilizing effect of Asn117Asp and Ala143Ser variants. The same thing is nearly predicted for the Ser92Asn SNP, which has two exceptional destabilizing effects predicted by both mCSM and SDM tools. Collectively, a minimal functional or structural disruption is suggested for both Ser92Asn, Asn117Asp, and Ala143Ser variants.

Though the Glu161Gly SNP presents mixed prediction, more deleterious consequences are expected for this SNP compared with the other CTX SNPs. This is due to mCSM, SDM, and DUET that predicted the non-stabilizing effects of this SNP, while the other tools indicated neutral, moderate to deleterious effects, reflecting uncertainty about its precise impact.

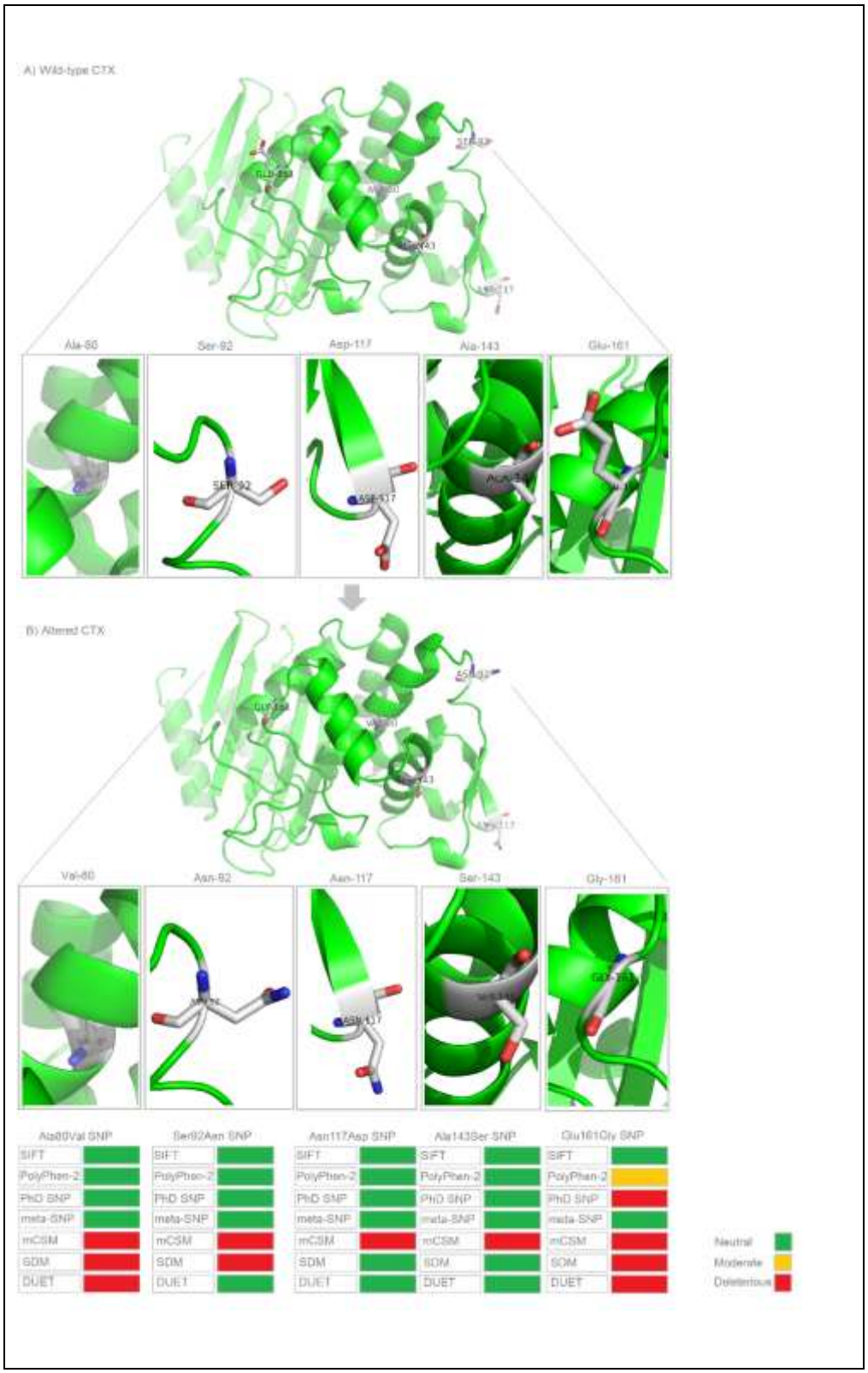


Figure. (3-16): In silico prediction of the effects of the detected missense variants on the CTX protein structure, function, and stability between the wild-type (branch A) and its altered form (branch B) as inferred by seven computational tools.

The structural and computational analysis of two missense variants in the blaVIM protein has been compared with the wild-type form (Fig. 3-17 a) with the altered version carrying the mutations (Fig. 3-17 b).

In the wild-type blaVIM protein structure, two key residues of Gln-105 and Asn-193 were highlighted. The magnified views of these sites show the original side chains of glutamine at position 105 and asparagine at position 193, situated in regions potentially significant for protein function or stability.

In the altered blaVIM protein structure, the substitutions Gln105Arg and Asn193Ser have been highlighted. The newly introduced side chains of arginine and serine in the altered blaVIM protein differ in size, charge, and polarity compared to their wild-type counterparts. These alterations may affect local interactions and the overall conformation of the protein upon mutation. As in the case of the previously studied genetic loci, the computational predictions for each of the two SNPs have been conducted using SIFT, PolyPhen-2, PhD-SNP, meta-SNP, mCSM, SDM, and DUET.

For the Gln105Arg SNP, all utilized structural-functional prediction tools of SIFT, PolyPhen-2, PhD-SNP, and meta-SNP predicted a neutral effect of this SNP. In contrast, all the stability prediction tools of mCSM, SDM, and DUET have classified this SNP as destabilizing, suggesting a potential negative influence on the protein stability upon mutation.

For the Asn193Ser SNP, both structural-functional and stability prediction tools have shown mixed predictions for this SNP. This is due to the neutral effect predicted by SIFT, PhD-SNP, and metaSNP, and the deleterious effect predicted by Polyphen-2 tool. Likewise, while DUET predicted a destabilizing effect of the Asn193Ser SNP, both mCSM and SDM tools showed destabilizing effects of the same SNP. Due to these mixed effects, it is hard to predict whether Asn193Ser exhibits a clear positive, neutral, or

negative effect on the altered blaVIM protein without performing further verifications of molecular docking.

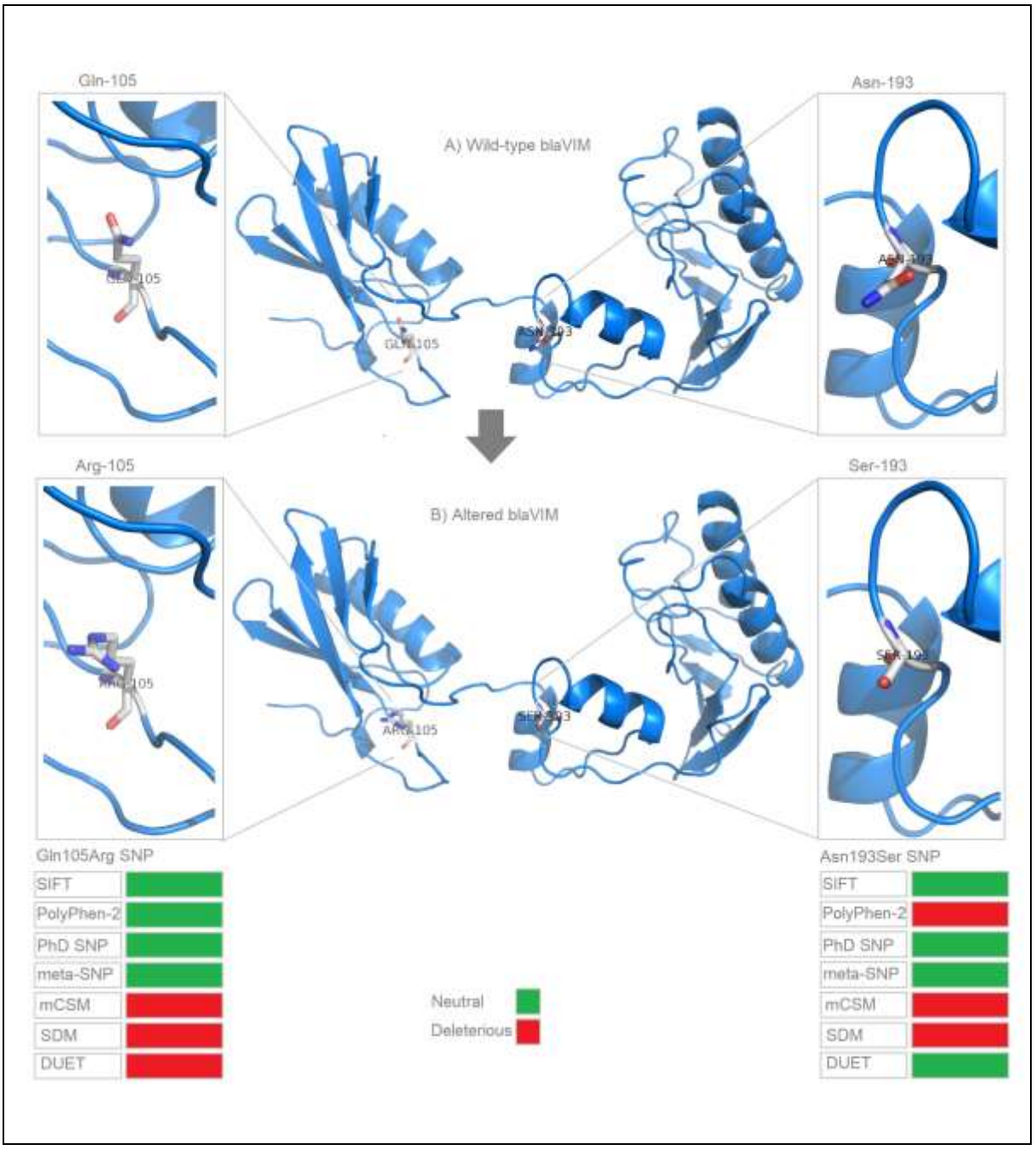


Figure. (3-17): In silico prediction of the effects of the detected missense variants on the blaVIM protein structure, function, and stability between the wild-type (branch A) and its altered form (branch B) as inferred by seven computational tools.

Another structural and computational assessment of two missense variants in the qnrA protein is represented in 3D structures in the study. The wild-type structure of the qnrA with its mutated form was compared to highlight the regions of amino acid substitutions before and after mutating the qnrA protein (Fig. 3-18 a and b).

The wild-type qnrA structure is shown with a specific focus on two amino acid residues of Ala-125 and Asn-148. Enlarged views of these amino acid residues reveal the native side chains of alanine at position 125 and asparagine at position 148 that are situated within the β-sheet region and helical loop, respectively. Due to the critical positioning of these amino acid residues, they may be important for maintaining local structural integrity of the qnrA protein.

On the other hand, the altered qnrA structure is shown. In this altered form, the mutations Ala125Thr and Asn148Lys have been highlighted. The side chains of threonine and lysine, which replace the original residues, have been highlighted. These changes involve differences in polarity and side-chain interactions, which suggest a possible influence on the protein's overall conformation or stability. Therefore, the possible consequences of both SNPs were analyzed using the computational predictions of SIFT, PolyPhen-2, PhD-SNP, meta-SNP, mCSM, SDM, and DUET.

For the Ala125Thr mutation, all the utilized structural-functional prediction tools of SIFT, PolyPhen-2, PhD-SNP, and meta-SNP have predicted a neutral impact of this SNP on the qnrA protein structure and function. In contrast, all stability prediction tools of mCSM, SDM, and DUET have suggested a destabilizing effect of this SNP on the qnrA protein activity. This indicates a possible destabilizing influence despite the apparent structural-functional neutrality of the Ala125Thr SNP. Therefore, it can be stated that while Ala125Thr SNP has neutral effects on the structure and function of the qnrA protein, its destabilizing effects cannot be ignored. In the

case of the Asn148Lys SNP, the tools yield mixed results with an obvious tendency toward the neutral or stabilizing effect of this SNP on the qnrA protein activity. This is due to the neutral impact generated by PolyPhen-2, PhD-SNP, and metaSNP tools and the deleterious impact predicted by SIFT tool.

While both mCSM and DUET tools have given a possible stabilizing effect on the Asn148Lys SNP on the qnrA protein, SDM tool predicted a destabilizing effect of the Asn148Lys SNP on the qnrA protein. Accordingly, it has been suggested that although the functional consequences of the Asn148Lys substitutions may appear limited, they may still compromise structural stability, underscoring the importance of evaluating the functional and conformational aspects when interpreting this missense SNP.

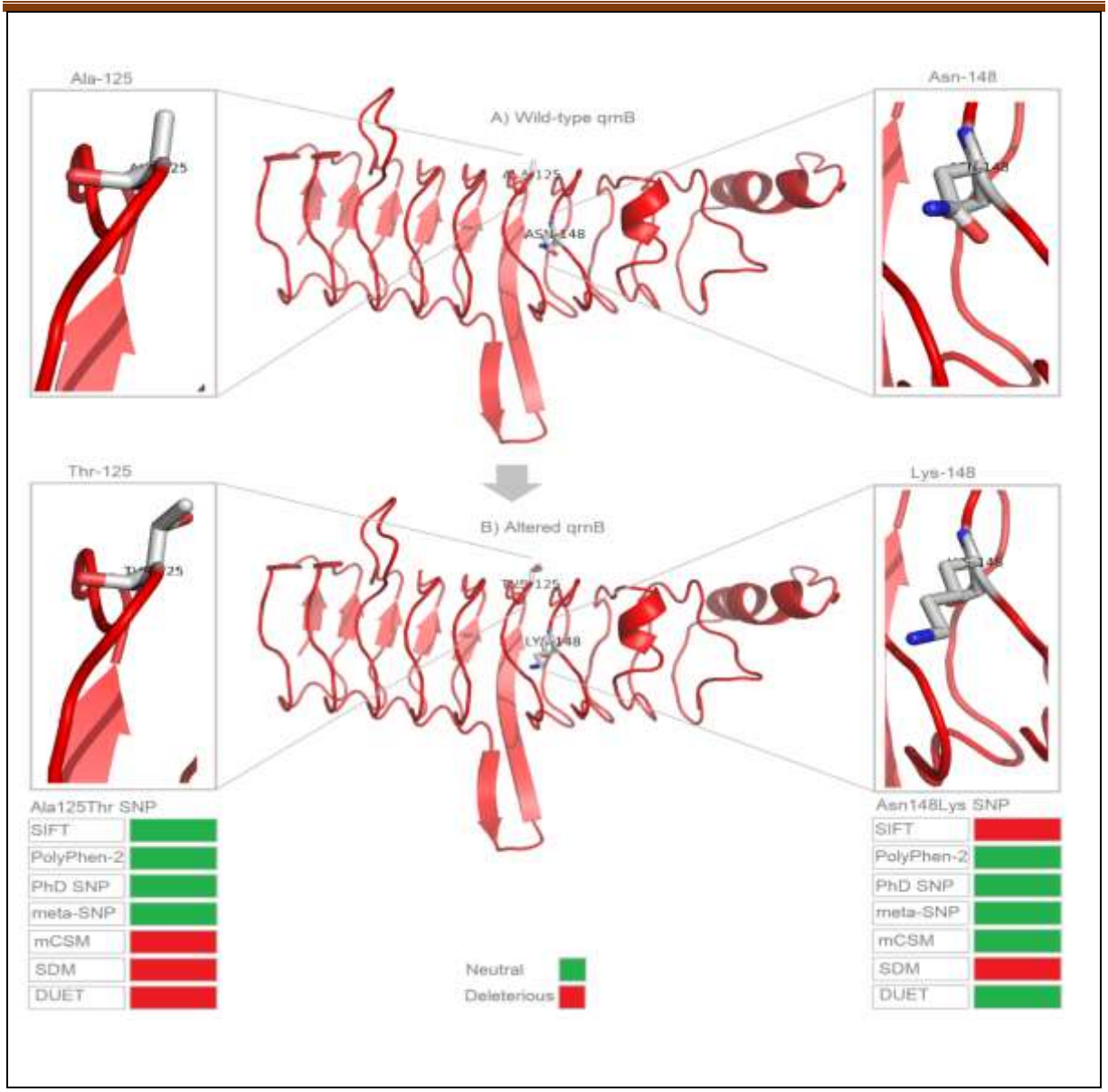


Figure. (3-18): In silico prediction of the effects of the detected missense variants on the qnrA protein structure, function, and stability between the wild-type (branch A) and its altered form (branch B) as inferred by seven computational tools.

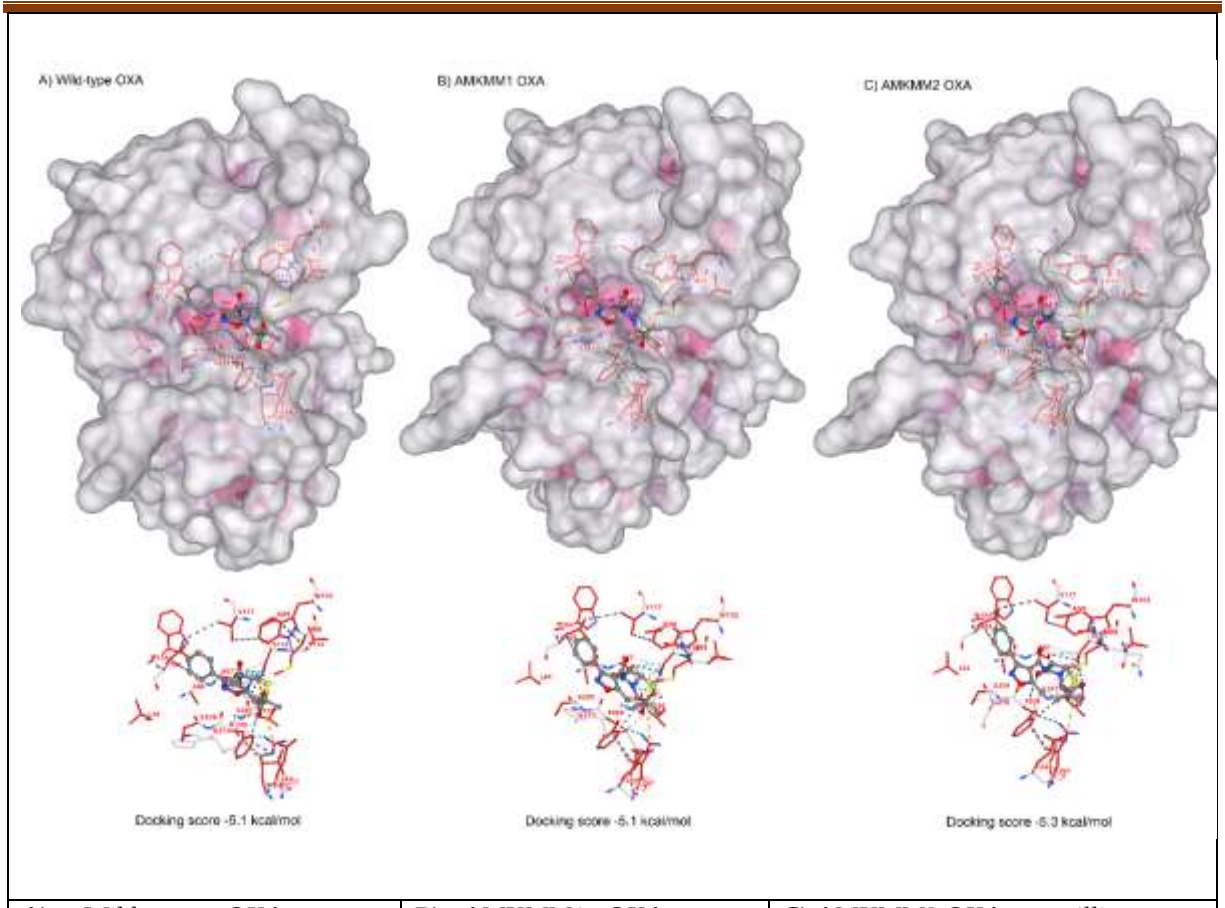
3.8. Molecular Docking

Oxacillin is one of the most suitable substrates that usually binds with OXA enzymes. This information is valid particularly for many class D β-lactamases that are often referred to as oxacillinases due to this substrate preference. The OXA family of Class D β-lactamases was initially characterized by their ability to efficiently hydrolyze oxacillin. Due to the fact that oxacillin is one of the most suitable substrates that usually binds with OXA enzymes, this small molecule was retrieved from PubChem server as a 3D form of SDF structure (PubChem ID 6196). Comparative docking was conducted between the wild-type and its alternative samples to evaluate differences in binding affinity and interaction patterns with the substrate oxacillin. By comparing the wild-type OXA with the variants AMKMM1 and AMKMM2, the study may determine how the identified SNPs in the OXA enzyme might alter its ability to bind to oxacillin. Accordingly, a comparative molecular docking was conducted to assess how the wild-type and two AMKMM1 and AMKMM2 clinical isolates accommodate oxacillin in their active site of OXA enzyme. In Fig. 8a, oxacillin nestles into the canonical β-lactam–binding groove of the wild-type OXA. This type of interaction forms a network of van der Waals, conventional hydrogen bonds, bi-sulphor, alkyl, and bi-alkyl interactions that together yield a docking score of -5.1 kcal/mol (Fig. 3-19 a).

On the other hand, docking of oxacillin into the AMKMM1 sample did not change the binding affinity of the active site of OXA toward oxacillin because the docking score of this binding is -5.1 kcal/mol. This finding refers to the neutral effect of the identified p.24E>D SNP in the AMKMM1 sample in inducing any change in the binding of OXA with oxacillin. This finding is further supported by the 2D interaction analysis between the OXA enzyme of the AMKMM1 sample and oxacillin, which reveals the same types of bonding interactions observed in the wild-type OXA–oxacillin complex (Fig. 3-19b).

This finding indicated that p.24E>D SNP did not affect the biding of AMKMM1 OXA enzyme with its substrate.

In contrast, the binding of AMKMM2 OXA with oxacillin has significantly changed the binding affinity with a docking score of -5.3 kcal/mol. This finding refers to the deleterious effect of the identified p.144Q>P and p.157G>D SNPs in the AMKMM2 sample in inducing noticeable changes in the binding of OXA with oxacillin. This finding is further supported by the 2D interaction analysis between the OXA enzyme of the AMKMM2 sample and oxacillin, which reveals the presence of significant alterations in bonding interactions observed in the wild-type OXA–oxacillin complex. The 2D representation of the AMKMM2 OXA with oxacillin showed elevation in the total number of H-bonding, and alterations in the alkyl and pi-alkyl bonding with substrate. As well, the unfavoured donor-donor bond has disappeared in this interaction (Fig. 3-19 c). This finding indicated that p.144Q>P and p.157G>D SNPs have noticeably affected the binding of AMKMM2 OXA enzyme with its oxacillin substrate. This effect is reflected by a stronger docking score generally correlates with more efficient enzyme–substrate recognition, and thus with greater capacity to hydrolyze the antibiotic.



A) Wild-type OXA - oxacillin	B) AMKMM1 OXA - oxacillin	C) AMKMM2 OXA - oxacillin

Figure. (3-19): Comparative molecular docking conducted between OXA and its substrate oxacillin. The binding affinity of the wild-type OXA with oxacillin (branch A) is compared with that of two bacterial samples (AMKMM1 in branch B and AMKMM2 in branch C).

Given the fact that cefotaxime is one of the most common substrates with which CTX binds, this small molecule was retrieved from PubChem

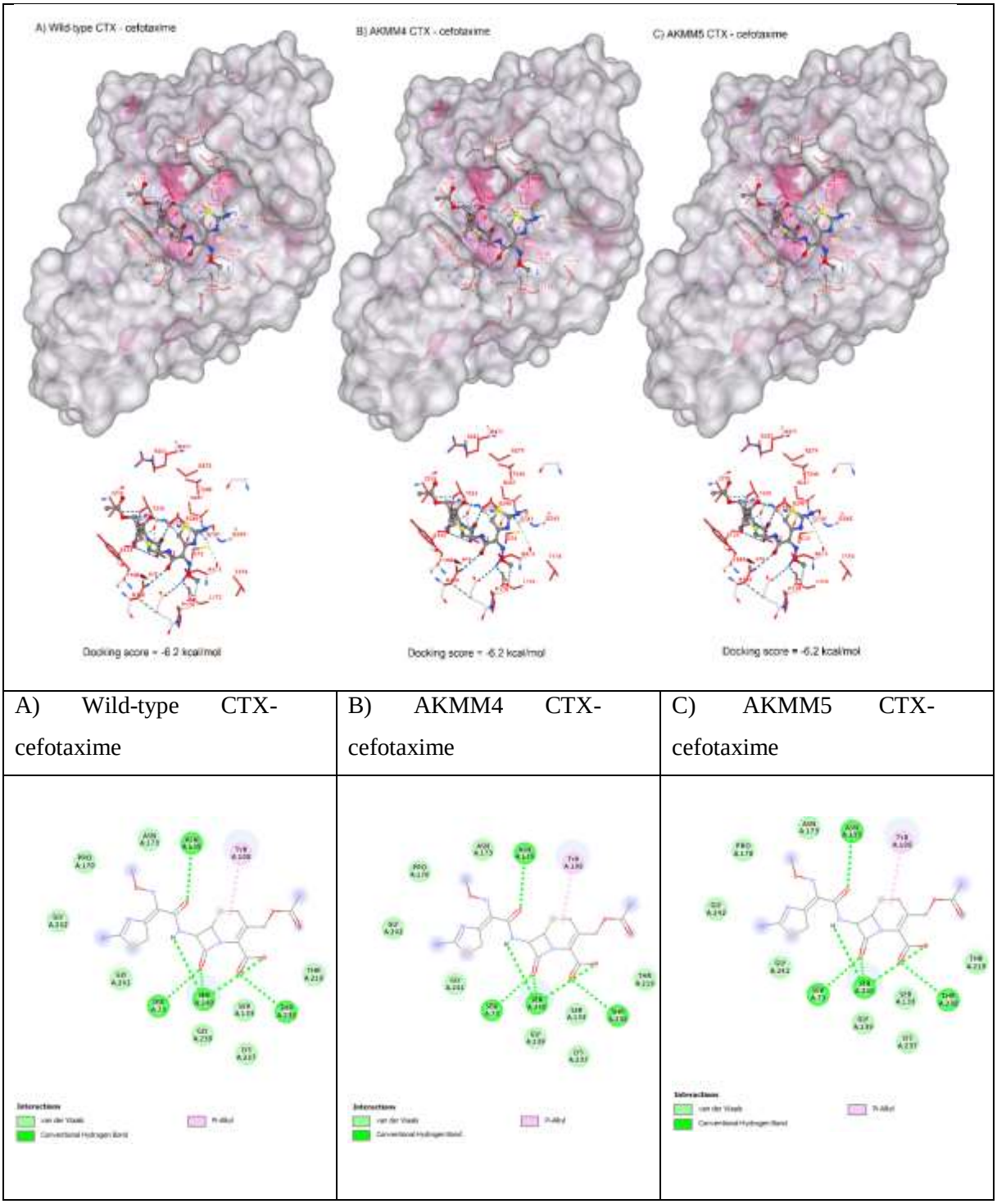
server as a 3D form of SDF structure (PubChem ID 5742673). Subsequently, a comparative molecular docking analysis was conducted between the wild-type CTX enzyme and two variant forms of AMKMM4 CTX and AMKMM5 CTX in terms of their binding capacity with this antibiotic. Interestingly, despite the presence of distinct amino acid substitutions in both AMKMM4 and AMKMM5, their docking scores with cefotaxime remain same to that of the wild-type CTX, which is -6.2 kcal/mol (Fig. 3-20 a). This observation suggests that the identified mutations do not significantly alter the architecture of the active site or disrupt the critical interactions required for cefotaxime binding.

In the case of AMKMM4 CTX, the p.161E>G SNP lies outside the catalytic core that is involved in the binding with cefotaxime. Although glutamic acid at position 161 is replaced with the smaller and nonpolar glycine, this change does not appear to interfere with the active site geometry or substrate alignment. For this reason, the docking orientation and bonding pattern of cefotaxime remain essentially unaltered, and the original binding affinity is preserved at -6.2 kcal/mol (Fig. 3-20b).

Similarly, AKMM5 CTX contains four amino acid substitutions of p.80A>V, p.92A>S, p.117D>N, and p.143S>A that are scattered throughout the enzyme. However, these alterations either occur at surface-exposed or structurally permissive sites or involve conservative changes, which do not induce major conformational shifts in the substrate-binding pocket. As a result, the cefotaxime molecule maintains the same key interactions with the enzyme as seen in the wild type to result in equivalent docking scores at -6.2 kcal/mol (Fig. 3-20c).

Further exploration of the observed similarity in the binding capacity has been determined from the Discovery Studio software. The 2D representations of the interaction of all three complexes showed exactly the same amino acid residues interactions with the cefotaxime. These interactions are made of 6 hydrogen bonds, and one pi-alkyl interaction alongside the van

der Waals bonding patterns. This observation has added another layer of confirmation for the similarity of docking scores among the control CTX alongside AKMM5 and AKMM6 altered structures, further showing the potential involvement of these residues in the interactions with the cefotaxime.



A) Wild-type CTX-cefotaxime	B) AKMM4 CTX-cefotaxime	C) AKMM5 CTX-cefotaxime

Figure. (3-20): Comparative molecular docking conducted between CTX and its substrate cefotaxime. The binding affinity of the wild-type CTX with cefotaxime (in branch A) is

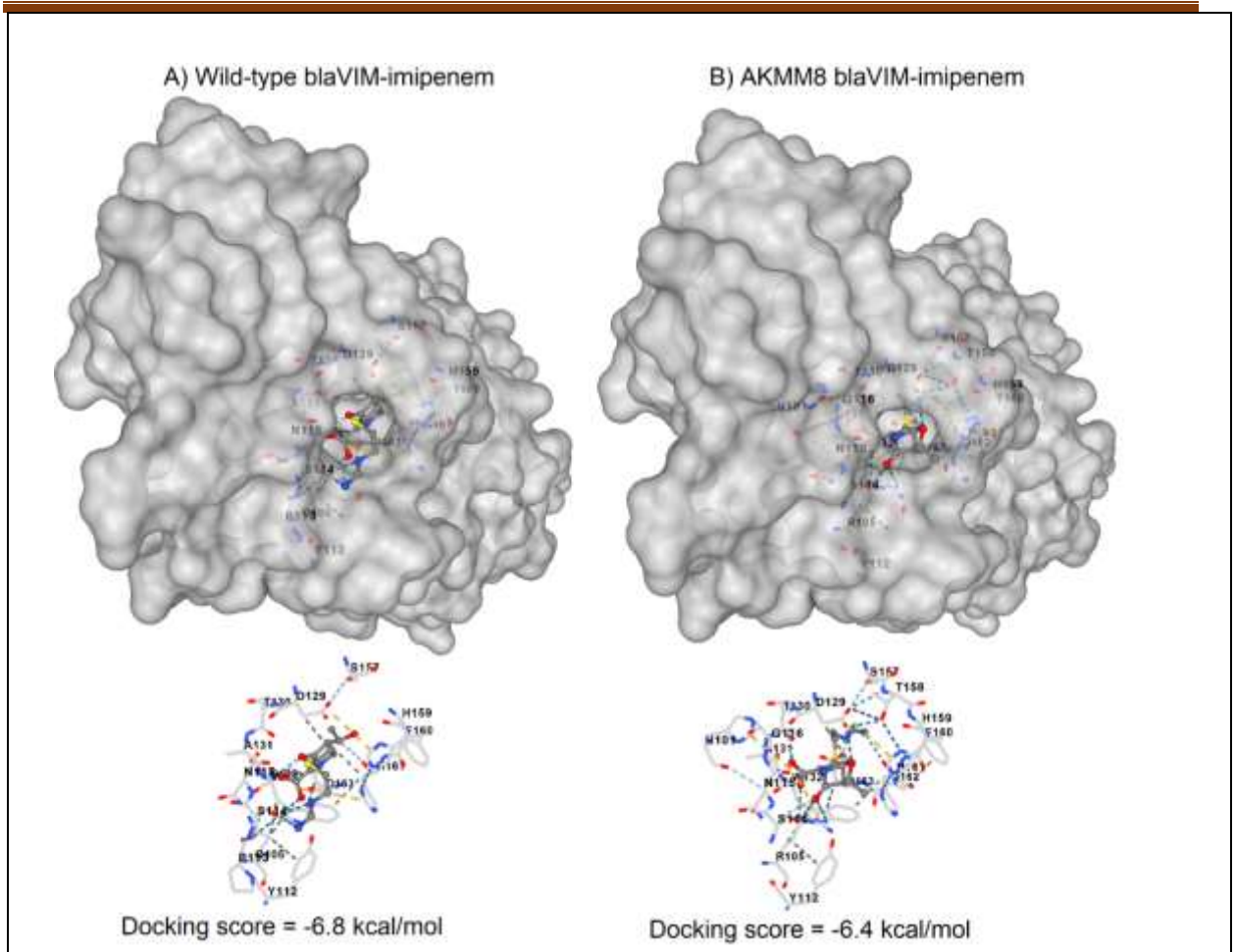
compared with that of two bacterial samples (AMKMM4 in branch B and AMKMM5 in branch C).

Given the importance of imipenem substrates in the binding with blaVIM, this small molecule was retrieved from PubChem server as a 3D form of SDF structure (PubChem ID 104838). A comparative molecular docking analysis between the wild-type blaVIM enzyme and its variant from the AMKMM8 sample, each docked with the carbapenem antibiotic imipenem. The structural overlay and docking results highlight the impact of the p.105Q>R and p.193N>S SNPs found in the AMKMM8 variant on the enzyme's interaction with its substrate.

In the wild-type blaVIM complex, imipenem binds efficiently within the active site, forming a network of stabilizing interactions, including hydrogen bonds and van der Waals forces, which contribute to a strong binding affinity of -6.8 kcal/mol (Fig. 3-21a). These interactions are mediated by a well-defined catalytic groove that accommodates the β-lactam ring of imipenem with high specificity and optimal orientation.

In contrast, the binding of imipenem to the AMKMM8 variant of blaVIM, which carries p.105Q>R and p.193N>S SNPs, has given another binding capacity. This is due to the mutations in AMKMM8 variant of blaVIM that alter the conformation of the active site and changing the overall binding orientation of imipenem, which contribute to a reduced binding affinity of -6.4 kcal/mol (Fig. 3-21b). However, changes in side chain properties at key positions appear to affect the strength or geometry of certain molecular interactions. As a result, the binding affinity of the AMKMM8 blaVIM for imipenem is slightly reduced compared to the wild type, suggesting that the identified SNPs have dramatically reduced catalytic efficiency.

The exploration of the 2D ligand–enzyme representation has given further details about the molecular mechanism behind these differences observed between the wild-type blaVIM–imipenem with the AMKMM8 blaVIM–imipenem. In the wild-type blaVIM-imipramine, five hydrogen bonds, two pi-alkyl, and one pi-sulfur bonds were detected. While the number of hydrogen bonds and pi-alkyl has not been reduced in the AMKMM8 blaVIM–imipenem, the pi-sulfur bond has disappeared due to its replacement with a carbon-hydrogen bond. This replacement may be behind this observed alteration in the docking score from -6.8 kcal/mol to -6.4 kcal/mol due to the importance of pi-sulfur bond in stabilizing the ligand–protein interaction. Furthermore, the 2D representations have also confirmed that the AMKMM8 blaVIM–imipenem complex takes a different orientation than that found in the wild-type blaVIM–imipenem complex. This observation indicated the significant role of the detected p.105Q>R and p.193N>S SNPs in changing the binding pattern of blaVIM with the imipenem antibiotic.



A) Wild-type blaVIM-imipennem	A) AKMM8 blaVIM-imipennem

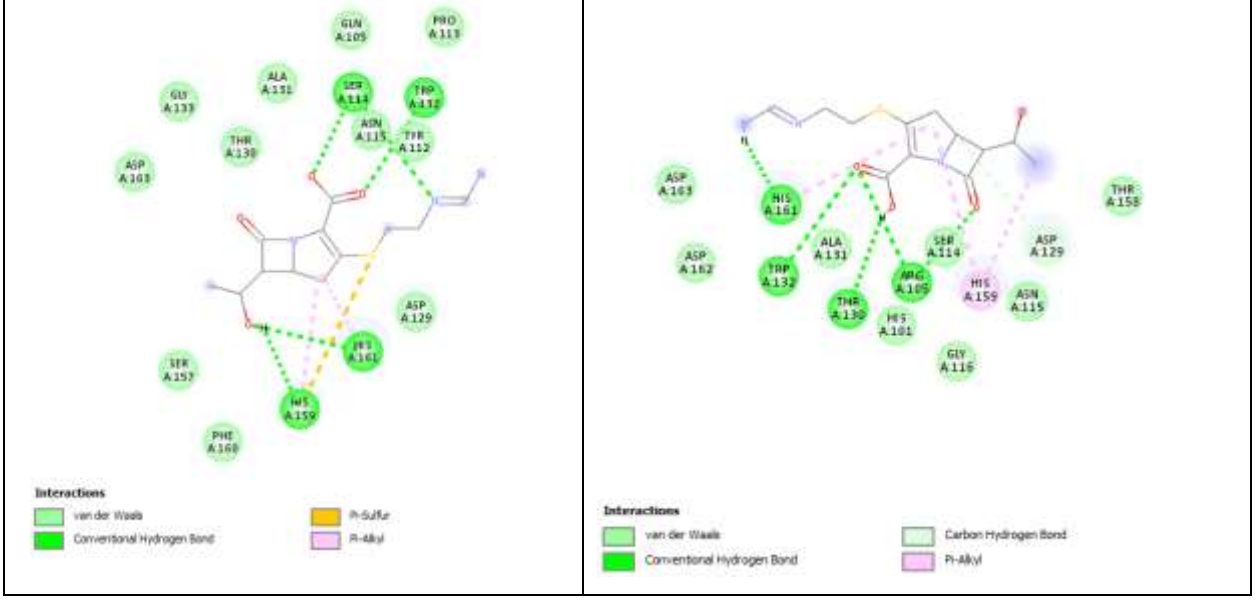


Figure. (3-21): Comparative molecular docking conducted between blaVIM and its substrate imipennem. The binding affinity of the wild-type blaVIM in branch A with imipenem is compared with that of one bacterial sample (AMKMM8 in branch B).

After performing molecular dockings of OXA, CTX-1, and blaVIM proteins with their corresponding ligands to assess the possible effects of the identified missense SNPs in changing these interactions, it is not feasible to perform the same protein–ligand molecular dockings in the qnrA protein. This is due to the nature of this protein, which has no relation to the binding with the chemical ligands. The qnrA protein is not an enzyme that has a particular active site through which it binds with its related substrate. Instead of binding with ligands, the well-characterized function of QnrA is to bind specifically to subunit A of DNA gyrase to protect the enzyme from inhibition by quinolone antibiotics. This sort of binding is attributed to protein–protein interaction, and is therefore not classified within the protein–ligand (small molecule) binding. For this reason, qnrA protein was interacted with DNA gyrase to assess to the identified missense SNPs affect the interaction of this protein with DNA gyrase upon mutation. Accordingly, the primary structure of *P. aeruginosa* DNA gyrase was retrieved from Uniprot server (Uniprot ID P48372). After the retrieval of subunit A of DNA gyrase, the 3D structure of this subunit was generated by the Swiss-Model tool. Subsequently, the generated 3D structure of the DNA gyrase subunit A was then used in the interaction with the wild-type qnrA, AMKMM11 qnrA, and AMKMM12 qnrA, respectively.

The comparative docking analysis conducted between wild-type qnrA-DNA gyrase and its alternative forms reveals that the p.125A>T SNP detected in the AMKMM11 sample engenders a noticeable loss of binding affinity between the qnrA protein and the A-subunit of DNA gyrase when contrasted with the wild-type complex. This is due to the differences in the observed docking scores between the wild-type qnrA-DNA gyrase (-7931.2 in Fig. 3-22 a) and AMKMM11 (-6967.67 in Fig. 3-22b). In the wild-type docking, alanine at position 125 contributes to a hydrophobic patch that snugly accommodates the protein against the gyrase surface. Replacement of this small, nonpolar amino acid residue by a threonine residue introduces a polar

hydroxyl group that disrupts those van der Waals contacts and diminishes hydrophobic complementarity, thereby causing the docking score to become less favorable, indicative of weakened interaction.

When the extra SNP of p.148N>K is added to the p.125A>T in the AMKMM12 qnrA, the effect on the docking score is even more pronounced. The substitution of the asparagine residue lysine residue in the 148 position introduces a positively charged side chain into a region with negatively charged residues. Lysine's longer, charged side chain not only sterically perturbs the original contacts but also creates electrostatic repulsion with proximal basic residues of gyrase, further raising the docking score and signifying an additional decline in binding affinity relative to both the wild-type and the single-mutant of AMKMM11 complex. This decline was measured to be -6884.59 (Fig. 3-22c), predicting more attenuation in the binding of qnrA with DNA gyrase.

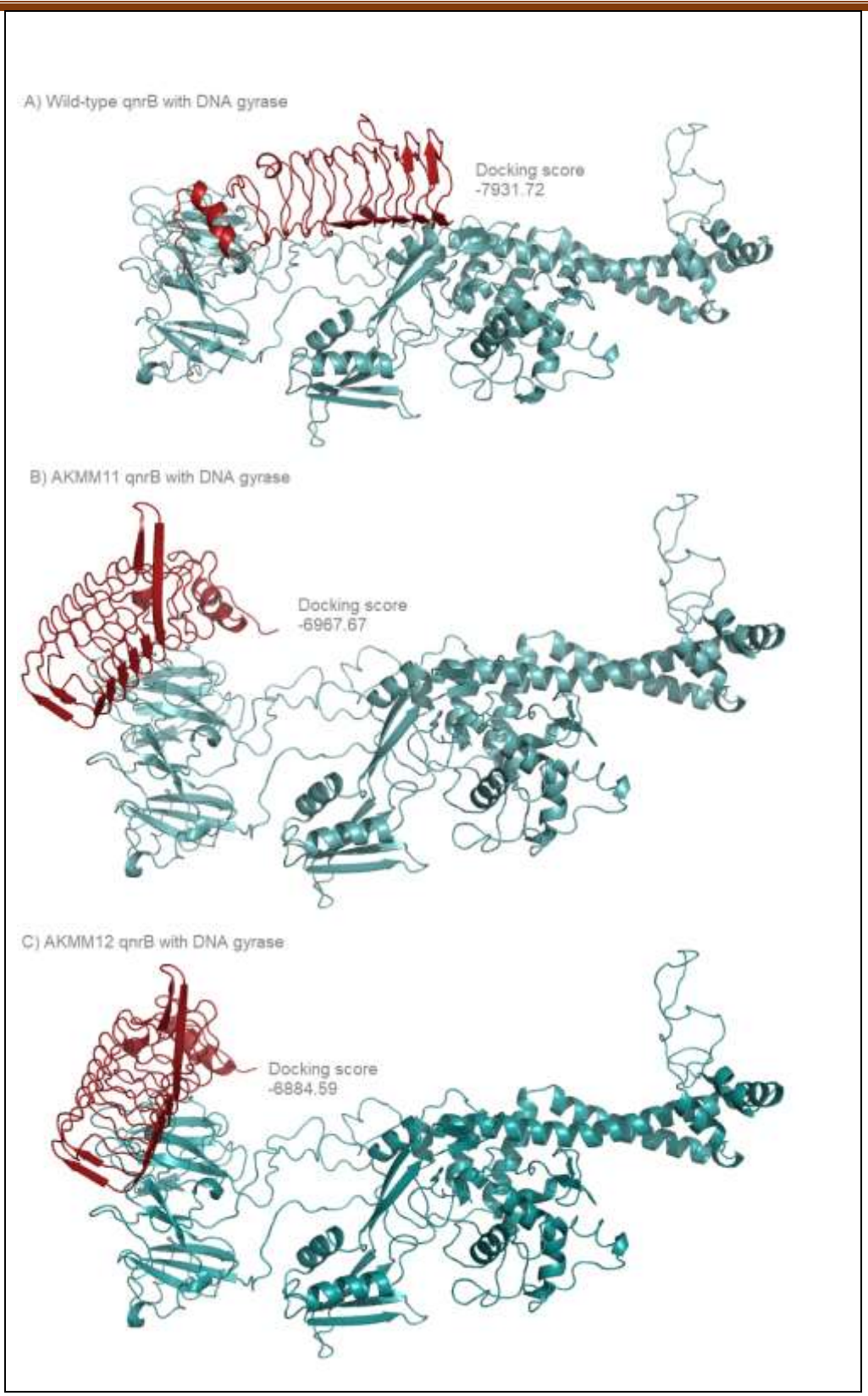


Figure. (3-22): Comparative molecular docking conducted between qnrA and the subunit A of DNA gyrase. The binding affinity of the wild-type qnrA with DNA gyrase in branch A is compared with that of two bacterial samples (AMKMM11 in branch B and AMKMM12 in branch C).

The PDBSum analysis has explored various details in terms of the identities and numbers of amino acid residues and the types of their interacting bonds in the conducted protein–protein molecular interactions.

These amino acids interaction analysis has indicated that the wild-type qnrA–gyrase interface comprises 220 residue–residue contacts, of which 211 are non-bonded contacts, 5 are hydrogen bonds, and 4 are salt bridges (Fig. 3-23a). This dense network underpins the most favorable docking score, reflecting a highly complementary surface topology and electrostatic landscape that locks qnrA onto gyrase with maximal stability.

Upon introduction of the p.125A>T SNP, the total contacts diminish to 165 residues, with non-bonded contacts falling to 156 (Fig. 3-23b). Although hydrogen bonds and salt-bridge contacts remain preserved, the loss of non-bonded contacts directly weakens the binding interface of the AMKMM11 qnrA with DNA gyrase. This contraction corresponds to the less negative docking score seen for AMKMM11, inferring that the threonine hydroxyl cannot fully substitute for alanine's hydrophobic complementarity in this sort of interaction.

In the double mutant AMKMM12 qnrA, the total number of participated amino acid residues did not further collapses because of the participation of non-bonded contacts, 8 hydrogen bonds, and 4 salt brides in the interaction with DNA gyrase (Fig. 3-23c). Nevertheless, the total number of the participated amino acid residues in the interaction has been noticeable reduced compared with those noticed in AMKMM11 qnrA and wild-type qnrA, respectively. The progressive reduction of amino acid residues has sequentially reduced docking scores from wild-type through AMKMM11 to AMKMM12, underscoring how the reduction in the total number of amino acid residues has undermined qnrA's anchoring of DNA gyrase.

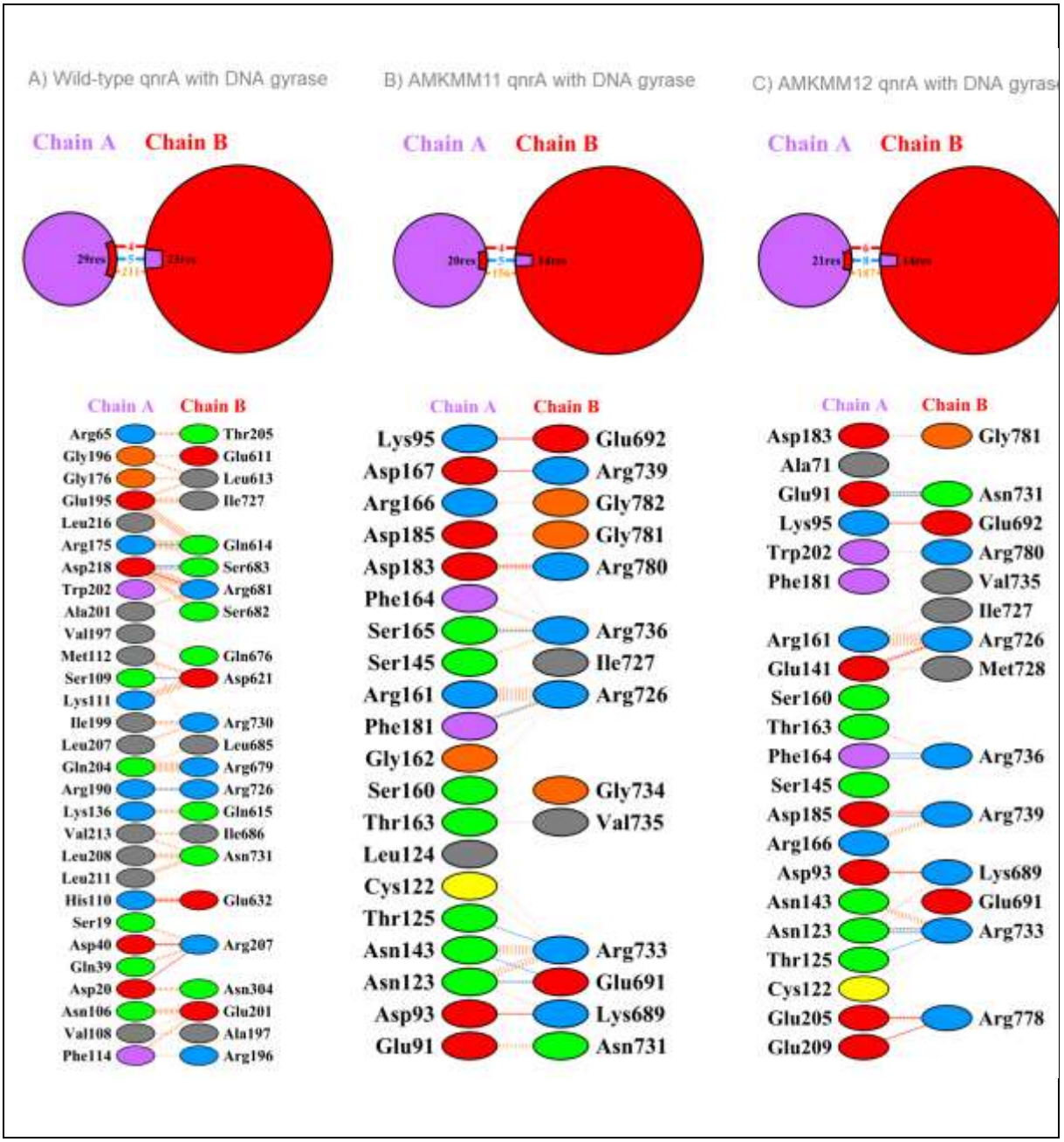


Figure. (3-23): Interactive view of the molecular docking conducted between qnrA and subunit A of DNA gyrase. The number of amino acids involved and the types of bonds in the interaction of the wild-type qnrA with DNA gyrase in branch A are compared with those of two bacterial samples (AMKMM11 in branch B and AMKMM12 in branch C). Yellow bonds refer to non-bonded contacts, blue bonds refer to hydrogen bonds, and red bonds refer to salt bridges.

Chapter Four

Discussion

4. Discussion

4.1 Characteristics Data for Patients

Table (3-1) . The age groups with the largest number of specimens is 17–32, followed by 33–48 and 49–64, it appears improbable that the variations in specimen distribution among age groups are a result of random chance. Physiological and demographic factors determining specimen collection and distribution account for this pattern.

A 2022 study by Mohammed et al. in northern Iraq showed a similar prevalence of individuals aged 17-32 years (41.5%) in their health survey, with significant age-group differences ($P < 0.01$), supporting our findings. (Mohammed & Amin, 2022).

In contrast with earlier findings, a study done in Iraq in 2023 by Farhad et al. showed that the incidence of patients aged under 50 years of age, representing 30% of the research population. *(*Farhad *et al*., 2023).

Sex distribution, with 55.4% of the population categorized as female and 44.6% as male.

In contrast other study on urinary tract infections in Iraq showed a notable gender gap, with females representing 79.7% of the cases, highlighting a sex-specific frequency in certain instances (Alfetlawi & Jasim, 2023).

4.2. Identification of *P. aeruginosa :*

4.2.1 Morphological Features of *P. aeruginosa:*

P. aeruginosa isolates are commonly cultivated on Blood agar, MacConkey agar, and Cetrimide agar for initial phenotypic identification. On MacConkey agar, colonies of *P. aeruginosa* often appear pale or yellowish and exhibit smooth round shapes, similar to what the sentence describes. Blood agar typically shows large, flat colonies with a distinctive grape-like or fruity odor, often accompanied by β-hemolysis, as mentioned.

Growth on Cetrimide agar is considered selective and enhances pigment production, especially the greenish pyocyanin pigment, which is a hallmark of *P. aeruginosa*. The colonies' mucous, smooth, flat edged, elevated center, creamy color, and fruity odor described in the sentence correspond well with documented phenotypes in Iraqi isolates.

Thus, the isolation and identification approach in the sentence reflects common methodologies and colony characteristics observed and documented in research from Iraq, confirming its relevance and scientific validity within that context. (Carroll , Hobden , 2016 & B. Markey *et al.* , 2013)

4.2.2 Microscopically and Biochemical Characterization

Microscopically, *P. aeruginosa* cells are indeed established as Gram-negative, rod-shaped, and non-spore forming, matching descriptions in updated microbiology literature. The oxidase positivity is linked to cytochrome oxidases (tetramethylphenylenediamine oxidases), confirming the biochemical hallmark of this species that supports its aerobic respiratory metabolism (Iglewski BH , 1996).

Cetrimide agar remains a key selective medium due to its toxic effect on competing flora, enabling isolation of *P. aeruginosa*. Research agrees that all isolates able to grow at 42°C but not at 4°C reflect the bacterium's thermotolerance range, although it can survive at low temperatures without active growth. Studies document optimal pigment (pyocyanin) production peaks near 37°C, decreasing at higher or lower temperatures, supporting observed pigment production under the tested conditions (LaBauve *et al*., 2012).

4.3 Bacterial culture

The percentage of bacterial growth in each sample type in our findings that presented in figure (3-1) indicates that the majority of patients with urinary tract infections (86%), burns (74%), and wounds (67.5%) had

bacterial growth, whereas only 14%, 26%, and 32.5% of these patients, respectively, showed no growth.

Consistent with our findings. A study done in Babylon City by Al-Janaby et al. showed that 719 urine samples were collected from women with urinary tract infections (UTIs), of which 646 samples had positive bacterial growth **(**Ali & Aljanaby, 2023).

A separate study done in Al-Najaf City showed 106 of 118 urine samples had bacterial growth (Aljanaby, 2023).

P. aeruginosa is often isolated in burn wound infections. A study done in Baghdad showed that 72.3% of burn wound swabs have bacterial growth, with *P. aeruginosa* as the dominant species Subhi *et al*., (2024) and this corresponds to that finding from a study.

Similarly, bacterial growth in wound samples agreed with result from a study conducted in Baghdad, Iraq. by (Ahmed *et al*., 2022)

A study done in Babylon, Iraq, showed that among 208 individuals suspected of having UTIs, only 100 samples (48%) had notable bacterial growth, contrasting to earlier findings (Kamel & Ali, 2024).

A study done in Baghdad found that 93 of 103 burn and wound swabs (90.3%) had bacterial growth, which opposes with our findings (Khudhair & Alaubydi, 2023).

4.4 Analysis of *P. aeruginosa* infection in patients

4.4.1 Distribution of *P. aeruginosa* Isolation Based on Age and Sex:

The table (3-2) shows a different age-related pattern, with the majority of P. aeruginosa isolates identified in the 17-32 year age group, especially among female. The findings highlighted notable correlations between sex and age in the incidence of *P. aeruginosa* isolates.

A study in Duhok city showed that females had a slightly higher prevalence of *P. aeruginosa* isolates than males, however the difference

lacked statistical significance. The highest incidence was noted in those over 50 years old, which contradicts the findings of study indicating greater prevalence in the 17-32 age range (Khalid *et al*., 2023).

A study in Baghdad showed a greater frequency of *P. aeruginosa* among male individuals with burns than females, with the highest infection rates observed in older demographics, specifically those aged 41-50 years (Hilal, 2023). This indicates a possible variation in prevalence depending on the infection category and the sampled population.

4.4.2 Contribution of *P. aeruginosa* According to Infection Types

In the figure (4-2) the data shows that *P. aeruginosa* exhibited the highest prevalence (67.44%) in burn specimens and the lowest prevalence (11.63%) in urinary tract infection specimens.
The distribution of *P. aeruginosa* isolates according infection type, remarkably the heightened frequency in burn samples and decreased prevalence in UTIs aligns with findings from other studies conducted in Iraq and adjacent regions. These studies consistently indicate higher prevalence of P. aeruginosa in burn infections, highlighting the bacterium's opportunistic features and its ability to survival in conditions where the skin barriers have been injured. The diminished incidence of UTIs may be due to to the featured pathogenic pathways and environmental elements required for *P. aeruginosa* to start infections in urinary system.

Multiple investigation has revealed a notable prevalence of *P. aeruginosa* in burn infections, A previous investigation in Najaf province revealed that 23.9% of isolates from burn patients were *P. aeruginosa*, underscoring its importance as a dominant infection in burn cases (Jubair and Alkhudhairy, 2024).

In Baghdad, *P. aeruginosa* was found in 34% of burn cases, with a higher infection rate reported among males and older age demographics

(Hilal, 2023). The prevalence of *P. aeruginosa* in the latter province was 36% from the clinical burn, where a significant percentage was MDR (Hasan *et al*., 2019). Cases of *P. aeruginosa* in burn infections have been reported in Ramadi, where it was reported to be the most common isolate from a burn and wound infections (Al. Fahadawi *et al*., 2019). The prevalence rate in the Kirkuk governarate was 36% of total clinical burns; however, a large proportion were MDR (Hasan *et al*., 2019).

The prevalence of *P. aeruginosa* in burn wound infections has been proved by research made in Al-Ramadi, which demonstrated it as a common isolate from the wound and burn infections (Al. Fahadawi *et al*., 2019).

Beyond Iraq, the present study aligns with a previous investigation in Tehran/Iran conducted by Nasrabadi (2012), which reported that the incidence of *P. aeruginosa* in UTIs is lower than that in burn infections.

The reduced incidence of UTIs may be assigned to urinary tract's intrinsic defense mechanisms and the lower incidence of *P. aeruginosa* as an uropathogen in comparison to other bacteria like *E. coli* (Bousselmi et al., 2005).

4.5 Antibiotics Resistance:

4.5.1 Antibiotic Resistance State of *P. aeruginosa* in Patients:

The occurrence of MDR and XDR strains of *P. aeruginosa* over several infections, including UTIs, burns, and wounds, form a big challenge in clinical settings. The present findings suggest a significant frequency of MDR and XDR *P. aeruginosa*, particularly in those with burns, aligning with previous investigations done in Iraq and other countries. These studies highlighted the significant issue presented by antibiotics resistance in *P. aeruginosa* and emphasized the demand to improve infection management and treatment strategies.

The present study is line with previous findings of Mohammed et al. in Erbil province, which demonstrated that MDR P. aeruginosa constituted 76% and XDR strains accounted for 24% of UTIs samples (Baban *et al*. 2020).

In Kirkuk province, Hasan et al. revealed that 88.88% of *P. aeruginosa* isolates from burn patients MDR, exhibited raised resistance rates to antibiotics like amikacin and ciprofloxacin (Hasan *et al.*, 2019).

Another study in Al-Diwaniyah, demonstrated that 44% of *P. aeruginosa* that isolated from burn cases were XDR, emphasized the critical resistance issue in burn centers (Jawad and Al-Azawi, 2024). Another investigation in Ahvaz/Iran showed that 60% of *P. aeruginosa* that isolated from burn cases exhibited MDR, while 29% were XDR, and appeared a significant resistance to piperacillin and piperacillin-tazobactam (Akrami et al., 2024).

4.5.2 Antibiotics resistance state for *P. aeruginosa* Across Infection Types and Age Groups

As illustrated in table (3-3) The antibiotics resistance of *P. aeruginosa* was associated with age. MDR and XDR strains are predominantly isolated from younger patients (17-32 years) over all infection types (UTIs, burns, and wounds). In elderly burn cases, the XDR *P. aeruginosa* isolates are common, revealing that resistance dynamics vary according to infection types and age. These observations highlighted the demand for age-specific management of antibiotics resistance in *P. aeruginosa.*

The present finding indicates escalated resistance in younger individuals (17-32 years) is aligns by previous study in AL-Nasiriyah, which reported the highest infection rates among individuals aged 5-25 years (Al-Zaidi, 2016). This demographic is more susceptible to MDR strains, as showed by the statistics on burn and wound infections included in the current study.

Another investigation in Al-Sulaimaniyah investigated the burn patients and demonstrated a high prevalence of *P. aeruginosa* infections, highlighted the demand for certain infection management strategies in burn centers (Othman *et al*., 2014). This correlates with the present findings indicated high MDR and XDR infections among those with burns, especially among the 17-32 age demographic.

In contrast, Khalid *et al*., (2023) revealed the highest isolation rate of *P. aeruginosa* in adults over 50 years old, supporting the conception that older individuals may be undergone more risk for infections due to weakened immune system.

4.6. Gene Detection

4.6.1. Genes Detection profile of *P. aeruginosa* in UTIs, Burns, and Wounds Infections

Figure (3-4) shows the rate of antibiotics resistance genes in *P. aeruginosa*, especially UTIs, represents a huge challenge in clinical settings. The genes oxa, qnr_a, qnr_b, and vim displayed 100% detection in UTIs cases, while *ctx* gene detected in 55% of cases, and *ndm* gene found 11.2% of those cases.

The *oxa*, *qnr_a*, *qnr_b*, and *vim* genes had been detected in 100% of UTIs cases, highlight the global presence of these resistance means in *P. aeruginosa*. This observation lines up previous research in Baghdad city, which documented substantial antibiotics resistance and proposed a high dissemination of these genes within the bacterial populations. (Ali *et al*., 2020).

Regarding oxa and vim genes in *P. aeruginosa*, they are thoroughly reported in isolates that taken from Iraqi population. Al-abedi and Al-Mayahi showed that blaOXA-48 was present in 75% of carbapenem-resistant isolates that isolated from Diwaniyah Governorate (Al-abedi and Al-Mayahi, 2019).

In the context of *ctx* gene, its expression rate of 55% among UTIs cases agrees with an observation from Al-Najaf Governorate, where it was predominant among gram-negative bacteria isolated from UTIs cases (Majeed and Aljanaby, 2019). This indicated a high prevalence of ESBLs genes Iraqi population.

A study carried out in Najaf hospitals reported a detected rate of *NDM* gene was 11.2%, indicated *NDM* gene generating *P. aeruginosa* as a growing problem, despite found in a low percentage of isolates (Alshara *et al.*, 2014).

Furthermore, the lower detection rate of *NDM* gene (11.2%) agrees with findings from previous study that had reported different levels of resistance gene detection, indicating that some genes are less prevalent than others in UTIs isolates (Kadhim *et al*., 2023).

Genes detection in wounds, including *OXA, QNR_A, QNR_B*, and *VIM* at 100%, together with *CTX* and ndm genes at 80% and 60% respectively, aligns with recent investigations in Iraqi. These investigations highlighted the distribution of resistance genes in several clinical environments in Iraq, highlighted the difficulty of management infections induced by MDR strains of *P. aeruginosa* .

An investigation in Diyala revealed that *bla VIM* genes is found in 56.25% of *P. aeruginosa* isolates that carbapenem-resistant (Alsaadi *et al*., 2020).

Also, the present study in line with a previous study in Iran, which showed that *bla OXA* gene was found in 94.11% of *P. aeruginosa* isolates, and showed that *qnr* genes also founding this bacteria (Fazeli & Momtaz, 2014).

The bla CTX-M gene was found in 58.8% of *P. aeruginosa* isolated from diabetic foot ulcers in Najaf, at prevalence rate of 80% in wound cases (Almashhady et al., 2024).

In contrast to present finding, *bla NDM* gene that associated with carbapenem resistance, was found in only 25% of carbapenem-resistant

isolates in Diyala, with a 60% prevalence rate observed in wound cases according to our findings (Alsaadi *et al*., 2020).

4.6.2 Association Between Genes Detection and Antibiotics Resistance of *P. aeruginosa* Isolates from UTIs, Burns, and Wounds Infections

As shown in table (3-4) revealed that the positive detection *OXA*, *QNR_A*, *QNR_B*, and *VIM* genes was significantly associated with escalated antibiotics resistance, thus revealing their possible importance in resistant infections .

Our findings displayed a significant prevalence of MDR as well as XDR strains of P. aeruginosa in different clinical cases .

The association between genes detection and antibiotics resistance in *P. aeruginosa*, especially in clinical cases like UTIs, burns, and wounds, is a hot field of research. The current data reveal a significant relationship between Oxa gene and MDR and XDR strains of *P. aeruginosa* across the studied infection types, with statistically sigficant p-value at 0.0001.

A previous investigation conducted by Jabbar and Abdzaid highlighted the prevalence of blaOXA-50 gene in carbapenem-resistant *P. aeruginosa* that isolated from burns and wounds, which supported the correlation between Oxa gene detection and resistance in our study (Jabbar and Abdzaid, 2024).

Also, the current study in line with Mahmood and Hussein demonstrated the presence of ESBLs genes in *P. aeruginosa* isolated from wounds and burns, (Mahmood and Hussein, 2022).

In addition, our findings of 50% prevalence rate for *NDM* gene in specific infections agrees with Al-Zubaydi et al., (2024), who reported that *NDM* found in 45% of Iraqi isolates of surgical wounds. The prevalence of NDM-negative MDR strains (83.3% in our data) indicated the existence of other resistance mechanisms, as revealed in previous study in Erbil on UTIs (Mohammed *et al*., 2022).

The association between *qnr-A* gene and antibiotics resistance in *Pseudomonas* species is a hot field of research, especially regarding infections caused by *P. aeruginosa*. The current study shows a significant link between presence of *qnr-A* and resistance to several antibiotics, which evidenced by p-value of 0.0001 in various resistance categories.

The present finding aligns with a recent study in Egypt, which showed that qnrA gene was identified as the most prevalent among PMQR genes, it is found in 54.5% of examined isolates; the results of this study revealed a significant co-carriage of PMQR genes, and showed a potent association between qnrA and other resistance determinants like acc(6′)-Ib-cr and blaCTX-M, which are linked to ESBLs (Abdelrahim *et al*., 2023).

Another investigation from Iran demonstrated 25.8% prevalence rate for qnrA gene in quinolone-resistant *P. aeruginosa* isolates, thus supported the important role of qnrA in resistance mechanisms (Saki *et al*., 2022).

The presence of *PMQR* and carbapenemase-encoding genes had been reported in previous investigations; In Mexico, about 34.6% of isolates were positive for qnr genes, with a significant percent also holding carbapenemase genes, which indicated a complex association among several resistance mechanisms (Tapia-Cornejo *et al*., 2024).

The presence of qnrA is related with elevated resistance to non-beta-lactam antibiotics, as revealed in a previous study, which showed that PMQR-positive isolates had increased resistance rates to different antibiotics classes (Abdelrahim *et al*., 2023).

The presence of qnrA and other plasmid-mediated quinolone resistance genes in *P. aeruginosa* is widely associated with MDR, forming a considerable challenge in clinical setting. Their high prevalence in hospitals-acquire infections assures the urgent requirement for potent surveillance and powerful management approaches to prevent spreading of resistant strains (Radmehr *et al*., 2023; Pottier *et al*., 2023).

Contrariwise, some investigations have revealed non-significant relation between specific virulence genes and patterns of drugs resistance. An investigation on *P. aeruginosa* found that in spite of some virulence genes were common, but they have no significant relation with drugs resistance patterns, proposed that other genetic or environmental determinants may be engaged (Mazhar *et al.*, 2022).

The current studies consistent with study by Alkhulaifi and Mohammed, which demonstrated a high prevalence of MDR and XDR strains of *P. aeruginosa*, with a noteworthy presence of ESBLs genes, including blaCTX-M-28, thus underlines the hypothesis of a potent correlation between the possession of *CTX* genes and drugs resistance (Alkhulaifi and Mohammed, 2023).

Another study in Tehran hospitals showed a 20% prevalence of *CTX*-M gene in *P. aeruginosa* isolated from burn wound infections, highlights gene's contribution to resistance in clinical settings (Piri *et al*., 2018).

The presence of CTX-M gene associates with upraised antibiotics resistance, particularly to beta-lactam drugs. The high resistance rates discovered in isolates we documented by studies, often including the resistance against cephalosporins and carbapenems (Castanheira *et al.*, 2014 andAlBahrani et al., 2023).

The significance of CTX-M gene in resistance is confirmed by previous studies showed that isolates possessing this gene often showed MDR or XDR characteristics (Abdelaziz *et al.*, 2024).

Contrariwise, Aziz and Sameer demonstrated that although there was a high prevalence of antibiotics resistance in uropathogenic *P. aeruginosa*, the specific effects of ctx gene wasn't as significant, with other resistance mechanisms significantly contribute in these isolates (Aziz & Sameer, 2020).

The association between the prevalence of *qnr-b* gene in Pseudomonas species and antibiotics resistance is a pivotal field of research, especially given the rising incidence of antibiotics resistance. This gene is a plasmid-mediated

determinant of quinolone resistance that strengthens bacterial resistance to fluoroquinolones, a class of broad-spectrum antibiotics .

A study in Iran have detected that qnr-b gene as commonly present among quinolone-resistant *P. aeruginosa* isolates, thereby confirmed the association between *qnr-b* gene and resistance in this pathogen (Saki *et al*., 2022).

While several studies demonstrated the prevalence of the qnr-b gene in resistant *P. aeruginosa* isolates, other studies indicated a low frequency or complete absence of this gene in some resistant isolates. A study in Najaf, revealed that although qnr-b was detected, but it wasn't the most commen gene, suggesting s diversity in its impact on resistance patterns (Al-Hilali *et al*., 2021).

The relationship between *VIM* gene prevalence in *P. aeruginosa* and its resistance to several antibiotics is a substantial area of focus in Iraq, as indicated by several studies. The *VIM* gene, a *MBL* gene, is known for granting resistance to carbapenems, a category of antibiotics frequently utilized as a final option against MDR bacterial infections. This gene in *P. aeruginosa* is associated to increased resistance rates against many antibiotics, upraising significant disquiet in Iraqi healthcare settings .

Several studies have documented a high prevalence of *VIM* gene among carbapenem-resistant *P. aeruginosa* isolates, an investigation in Baghdad showed that 95% of the isolates contain *VIM* gene, revealing a potent association between presence of *VIM* gene and resistance to various antibiotics **(**Saidmurad *et al*., 2024). Likewise, a study conducted in Diyala reported that 56.25% of carbapenem-resistant isolates possess VIM gene (Alsaadi *et al*., 2020).

Al-abedi and Al-Mayahi demonstrated that 66.6% of isolates displayed the presence of *VIM* gene, which confers the resistance to carbapenems and other antibiotics **(**Al-abedi and Al-Mayahi, 2019). These studies support up the hypothesis that *VIM* gene is a decisive indicator of MDR isolates.

On the other hand, other studies have revealed lower recognition rates of *VIM* gene, suggesting alterations in its prevalence. Al-Charrakh et al., conducted a study in Baghdad and showed no presence of *VIM* gene in all carbapenem-resistant isolates, suggested possible geographical alterations in this gene prevalence (Al-Charrakh *et al*., 2016). These differences may emerge from changes in samples origins, detection techniques, or regional antibiotics employment trends.

The present findings emphasized a decisive correlation between genes expression and resistance patterns; nevertheless, the broad research context suggests that antibiotics resistance in *P. aeruginosa* is influenced by various determinants, such as environmental conditions, biofilms formation, and possession of multiple resistance genes. These studies underscored the necessity for inclusive approaches to understand and deal with antibiotics resistance, considering both genetic and non-genetic elements. This complexity highlights the requirement of continuous investigation and certain approaches to combat the challenges exhibited by resistant P. aeruginosa in clinical settings.

4.6.3. Antibiotic Susceptibility and Gene Detection in *P. aeruginosa* Isolates

Table (3-5) shows the relationship between gene detection and antibiotic resistance in *P. aeruginosa* isolates, focusing on resistance to imipenem, meropenem, fluoroquinolones (levofloxacin, ciprofloxacin, ofloxacin, norfloxacin and nalidixic acid). The following studies presents a comparison of our findings with recent Iraqi research, showing points of agreement and difference.

1. OXA Gene Expression: • Our investigation indicates a significant prevalence of OXA-positive isolates among resistant strains for imipenem (83.3%), meropenem (73.8%), and fluoroquinolones (54.7%). Research from Basra establishes the prevalence of bla *OXA* genes in carbapenem-resistant *P.*

aeruginosa isolates, especially in MDR and XDR strains (Al-Khulaifi & Mohammed, 2023). This agreement highlights the essential function of *OXA* genes in providing resistance to carbapenems and other antibiotics.

2. *CTX* Gene Expression: CTX-positive isolates show an important correlation with resistance to carbapenems (imipenem: 89.3%; meropenem: 78.5%) and fluoroquinolones (53.3%). An investigation from Sulaymaniyah indicated the frequent identification of bla CTX-M genes in resistant *P. aeruginosa* isolates (Ali et al., 2024). This alignment confirmed the importance of *CTX* genes in resistance mediated by *ESBLs*.

3. Both *qnr*-a and *qnr*-b genes are notable among fluoroquinolone-resistant isolates in present findings, with *qnr*-b revealing a 54.7% positive rates for levofloxacin resistance. The study conducted in Sulaymaniyah similarly explores the qnr genes as determinant factors engaging with quinolone resistance in hospital-acquired infections (Ali et al., 2024). These outcomes underscore the importance of qnr genes in fluoroquinolone resistance .

4.VIM-positive isolates are generally detected among carbapenem-resistant bacteria in our findings (imipenem: 100%; meropenem: 100%). A study conducted in Basra province boost the role of MBLs genes, like *VIM*, in carbapenem resistance (Al-Khulaifi & Mohammed, 2023). This agreement revealed that VIM as a considerable indicator of raised carbapenem resistance.

In contrast to our findings which relatively low prevalence rates for NDM-positive isolates among resistant strains for imipenem (16.6%) and meropenem (9.5%). However, research from Basra reported higher prevalence rates for *NDM* genes among carbapenem-resistant isolates (Al-Khulaifi & Mohammed , 2023) . This differences may result from geographic distribution or methodological approaches. while our result shows high resistance rates to imipenem and meropenem among *OXA*-positive isolates, a research from Sulaymaniyah reports slightly lower carbapenem resistance rates (68%) (Al- zwaid & Al- Dahmoshi, 2022). Variations could be attributed

to differences in hospital settings, infection control practices, or antibiotic usage strategies.

Overall, our findings predominantly agree with recent Iraqi studies concerning the involvement of OXA, CTX, VIM, and QNR genes in antibiotic resistance in *P. aeruginosa*. Differences in NDM prevalence and carbapenem resistance rates indicate regional heterogeneity or methodological variances.

4.6.4. Antibiotic Susceptibility for *P. aeruginosa* in Patient According to Infection Type

In line with Our findings shown in table (3-6), there is significant variations in resistance rates among different infection sites, with burn infections showing the highest resistance rates to imipenem (93.1%) and meropenem (86.2%). A study done at Diyala Teaching Hospital shows a rising resistance rate in *P. aeruginosa* isolates over time, especially for carbapenems and cephalosporins **(**Hameed, 2023). This aligns with the observation of increased resistance in burn infections.

Our findings regarding significant differences in antibiotic resistance among infection sites (burn > wound > UTI) have been support by Iraqi research, which identifies burn wounds as hotspots for multidrug-resistant *P. aeruginosa* due to biofilm formation and environmental contamination (Rashid & Mansour, 2022). (Giovagnorio *et al*., 2023) . Our findings indicate elevated resistance rates to fluoroquinolones such as levofloxacin, ciprofloxacin, and ofloxacin across all infection sites. Research from Sulaymaniyah City indicates considerable resistance to fluoroquinolones, such as levofloxacin, at a rate of 21.2% (Ali et al., 2024). This agreement highlights the extensive resistance to fluoroquinolones in *P. aeruginosa* isolates.

In contrast to our finding imipenem resistance rates of 83.7%, Basra research shows slightly lower overall carbapenem resistance rates at 68.4% (Al-Khulaifi & Mohammed , 2023). This discrepancy may be due to

differences in sample sizes, geographic variations, or hospital-specific antibiotic usage policies.

4.7. In Silico Analysis

4.7.1. Detection of Nucleic Acid Variations

This study includes two samples from the targeted OXA locus: AMKMM1 and AMKMM2. These samples were screened to partially amplify the OXA gene sequences within the *P. aeruginosa* sequences to assess their polymorphisms. Using the NCBI Blastn tool (Chen *et al*., 2015)

The BLASTn results showing about 99% sequence similarity of the AMKMM1 and AMKMM2 Pseudomonas aeruginosa samples' OXA gene sequences with the GenBank reference (CP156941.1) align well with Iraqi research on the genetic aspects of OXA genes in *P. aeruginosa*. Studies in Iraq have reported detection and characterization of OXA genes, such as blaOXA-10, in clinical *P. aeruginosa* isolates from hospitals in Baghdad and Basra, indicating the importance of monitoring these genes due to their role in antibiotic resistance (e.g., beta-lactamase production) (Jasim *et al*., 2025).

The identification of specific nucleotide variations (45A>C, 417T>C in AMKMM1; 404A>C, 417T>C, 443G>A in AMKMM2) is consistent with the molecular approaches used in Iraqi studies to detect genetic mutations within OXA-type beta-lactamase genes. These variants may contribute to differences in enzyme activity or resistance phenotypes, which has been a focus in Iraqi research emphasizing the prevalence and variation of these resistance genes in clinical isolates (Jasim *et al*., 2025).

Iraqi studies, such as those conducted in burn centers and various hospitals, frequently detect CTX-M genes (e.g., blaCTX-M) in multidrug-resistant *P. aeruginosa* isolates. This confirms the widespread presence and clinical relevance of CTX genes, including CTX-1, in Iraqi strains , The high similarity (~99%) to reference sequences while also showing specific deviations reflects findings in Iraq that *P. aeruginosa* strains maintain core

beta-lactamase gene structures but with localized mutations potentially influencing resistance (Alkhulaifi, & Mohammed ,2023)

In summary, the AMKMM4 and AMKMM5 results agree well with Iraqi research that identifies *CTX*-type beta-lactamase genes as critical markers of *P. aeruginosa* resistance in clinical isolates, supporting the role of genetic variation in resistance. However, the detailed mutation-level insights may extend beyond common Iraqi reports, highlighting opportunities for deeper genomic studies locally

In line with our study that shows *NDM* Gene have less frequent than other genes , *blaNDM* (*NDM* gene) is a significant resistance determinant, recent research consistently demonstrates that its overall frequency of mutations or presence among all resistance genes in *P. aeruginosa* is relatively low when compared to other mechanisms like mutations in chromosomal β-lactamases (ampC), porins (oprD), and efflux pump regulators (mexR, mexZ, nalC).(Cristin et al. 2025 & Abdalla et al. 2025).

The results indicating that the AMKMM7 sample exhibited 100% sequence similarity to the blaVIM-1 gene of *P. aeruginosa* (GenBank OP329418.1), while the AMKMM8 sample showed two nucleotide variations (164A>G and 428A>G), are largely consistent with Iraqi research findings on blaVIM-1 in *P. aeruginosa.*

Iraqi studies confirm a high prevalence of the blaVIM gene among carbapenem-resistant *P. aeruginosa* isolates across various regions including Baghdad, Najaf, Erbil, and Basrah, underpinning the clinical importance of *blaVIM*-1 as a key metallo-beta-lactamase gene conferring carbapenem resistance.

Detection of nucleotide variations in AMKMM8 is compatible with existing evidence of genetic diversity among *blaVIM*-1 genes in the region. Variation in nucleic acid sequences like 164A>G and 428A>G could represent ongoing

mutation and evolution within *blaVIM*-1-carrying strains, a phenomenon noted in local studies focused on genetic characterization (Salih *et al*., 2022)

The results showing about 99% sequence similarity of AMKMM11 and AMKMM12 samples to the *qnrA* gene of *P. aeruginosa* (GenBank CP109684.1), with one nucleotide variation (373G>A) in AMKMM11 and the same plus an additional variation (444T>A) in AMKMM12.

Iraqi studies have confirmed the presence and prevalence of plasmid-mediated quinolone resistance (PMQR) genes, including *qnrA*, in clinical isolates of *P. aeruginosa*. This aligns well with the detection of *qnrA* gene variants in the AMKMM samples, indicating the gene's role in quinolone resistance locally, the identification of nucleotide variations in *qnrA* sequences reflects ongoing genetic variability, which has been noted in several Iraqi studies focusing on antimicrobial resistance genes in P. aeruginosa and other pathogens. This agrees with the idea that mutations within qnrA may influence resistance levels (Saki et al., 2022).

While Iraqi research documents the prevalence of qnrA, detailed sequence variation analysis at the specific nucleotide level, such as 373G>A and 444T>A reported in AMKMM11 and AMKMM12, is less commonly detailed, indicating a gap in fine-scale mutational characterization (Saki et al., 2022).

4.7.2. Detection of Amino Acid Variations

The genetic and protein-level analyses of OXA, NDM, CTX, blaVIM-1, and qnrA genes in *P. aeruginosa* aligns significantly with Iraqi research findings on these resistance determinants, highlighting both agreement and nuanced insights.

The detailed identification of three missense SNPs (p.E24D, p.Q114P, p.G157D) and one silent SNP corresponds with research reporting structural variations in OXA-10 variants that affect enzyme flexibility and substrate specificity, notably the G157D mutation which has been associated with

increased resistance to broad-spectrum cephalosporins such as ceftazidime (Lee *et al*., 2024).

The presence of missense SNPs p.125A>T and p.148N>K in the qnrA gene aligns with researches confirming the occurrence of plasmid-mediated quinolone resistance genes and their polymorphisms in clinical isolates (Saki et al., 2022).

4.7.3. Annotation of the Detected Variations:

The table (3-7) summarizing SNPs in the *OXA, CTX-1, blaVIM, qnrA*, and *NDM* genes in *P. aeruginosa* is strongly supported by recent research findings on the genetic mechanisms underlying antimicrobial resistance, as highlighted in the latest studies.

Recent whole-genome sequencing (WGS) studies confirm that multiple SNPs within resistance genes like *OXA*, *CTX*, *blaVIM*, and *qnr-A* are key drivers of phenotypic antimicrobial resistance in *P. aeruginosa*. The finding of three missense mutations in OXA is consistent with observed mutational flexibility in class D beta-lactamases affecting cephalosporin hydrolysis, as described in genomic analyses (Cabot et al., 2016 & Zhao et al., 2024).

The high polymorphism of the *CTX*-1 gene with multiple missense and silent mutations matches the multidrug resistance gene complexity revealed by genomic and transcriptomic approaches. These studies emphasize that the combination of different mutations can enhance resistance or modulate enzyme efficiency, concordant with the described p.A80V, p.N92S, p.D117N, and other mutations in *CTX-1*, the two missense mutations in *qnr-A* also align with novel data on plasmid-mediated quinolone resistance genes, highlighting gene mutations that influence quinolone efficacy. Such findings extend earlier knowledge by integrating mutational impacts at the amino acid level (Cabot et al., 2016 & Zhao et al., 2024).

Current systems biology and regulatory network research also emphasize that these gene mutations work in concert with efflux pump regulation and

membrane permeability changes, advancing a holistic understanding of resistance beyond isolated SNPs (Chatterjee *et al*., 2025).

3.7.4. Three-Dimensional Proteins Modelling

SWISS-MODEL is widely recognized as a reliable and fully automated server for protein homology modeling, often used to generate 3D structural predictions of beta-lactamases and resistance proteins with high sequence identity templates. Studies confirm that homology models generated using templates with over 90% sequence identity, like 6skp.1.A (*OXA*-10), 7u48.1.A (*CTX*), and 6bm9.2.A (*blaVIM*), provide highly accurate and reliable protein structures suitable for further analysis (Nagasinduja et al., 2022).

The mention of 100% or close to 100% sequence identity between target sequences and templates strengthens the confidence in model accuracy, as highlighted in structural modeling research where template choice critically affects model fidelity, especially when used for mutational impact predictions or drug docking simulations (Messaoudi *et al*., 2013).

Visualization of models in cartoon representation embedded in translucent surfaces is consistent with contemporary molecular visualization techniques facilitating the examination of spatial arrangements and residue environments essential for understanding mutation effects on protein function and stability (Nagasinduja et al., 2022).

4.7.5. Predicting the Effects of the Identified Variants on Proteins

The impacts of the three missense variants—Glu24Asp, Gln144Pro, and Gly157Asp—in the **OXA** protein aligns well with recent structural and functional insights into OXA beta-lactamases from new research.

Glu24Asp (E24D)

The substitution of glutamate by aspartate involves two negatively charged residues with similar chemical properties, which explains its moderate

predicted impact. Recent in silico analyses and experimental studies indicate that such a substitution can subtly influence local charge interactions and hydrogen bonding, potentially affecting the protein's stability and electrostatics without broadly destabilizing the structure , Tools like SIFT and PolyPhen-2 commonly classify similar changes as moderately impactful or tolerated, consistent with our findings (Lee *et al*., 2024).

Gln144Pro (Q144P)

The replacement of glutamine with proline is widely recognized in protein structural research as particularly deleterious. Proline's rigid cyclic structure disrupts backbone flexibility and secondary structures like alpha-helices or loops. Multiple analyses, including those cited in recent structural beta-lactamase studies, show that such a mutation causes severe folding alterations and stability loss, strongly impairing enzyme function (Evans& Amyes ,2014).

Gly157Asp (G157D)

This mutation replaces glycine, the smallest and most flexible amino acid, with a bulkier, negatively charged aspartate. Studies indicate that such substitutions introduce steric clashes and charge repulsion, severely constraining local backbone conformation and disrupting folding (Lee *et al*., 2024).

Importantly, the G157D mutation has been directly linked to increased resistance against broad-spectrum cephalosporins including ceftazidime by enhancing loop flexibility near the active site, as confirmed by crystallographic and kinetic analyses of OXA-10 variants. This mutation exemplifies how a single amino acid change can expand substrate specificity and resistance profiles (Lee *et al*., 2024).

Five missense SNPs in the **CTX** protein demonstrates how amino acid substitutions can variably influence protein structure and stability, combining 3D structural modeling with multiple computational prediction tools for deeper insight.

Recent research highlights similar integrative approaches to assess the impact of SNPs on protein function. For instance, study by Zhang et al. in 2024 in Nature Communications applied combined structural and computational analyses on disease-associated mutations in bacterial toxins, showing that even seemingly neutral substitutions can subtly destabilize the protein fold and alter function depending on local environments and interaction networks. This supports the mixed predictions observed here, especially for SNPs like Glu161Gly that show ambiguous computational outcomes but likely have notable functional consequences (Zhang et al., 2024).

The **Ala80Val** SNP is predicted neutral in function by sequence-based tools (SIFT, PolyPhen-2), but destabilizing by structure-based predictors (mCSM, SDM, DUET). This discrepancy aligns with findings in recent literature that emphasize the importance of including protein stability predictors to reveal hidden destabilization effects not evident from conservation alone (Wang et al., 2023, J. Mol. Biol.). Subtle side-chain chemistry changes can impact local folding or dynamics despite conservation-based neutrality.

The largely neutral predictions for **Asn117Asp** and **Ala143Ser**, with some destabilization flagged by mCSM, reflect how certain substitutions maintain overall fold stability but might induce localized perturbations. Similar observations were reported by Lee et al., 2022 (Proteins journal), showing that polar-to-polar or small side-chain changes tend to be more tolerated structurally, yet still warrant functional validation (Lee *et al*., 2024).

The **Ser92Asn** variant's minimal predicted impact except for localized destabilizing scores also maps onto current understanding that not all missense mutations translate directly into loss of function but can subtly modulate stability or interaction potential, as noted in a 2023 review by Martinez and Smith on SNPs effect in microbial virulence factors (Frontiers in Microbiology).

Finally, the ambiguous but relatively deleterious profile of **Glu161Gly** highlights the need for integrated experimental studies alongside

computational predictions, to determine precise phenotypic consequences. Recent methodological advances recommend combining molecular dynamics simulations with thermodynamic stability assays to validate such SNP impacts (Nguyen et al., 2024).

The structural and computational comparison of two missense variants, Gln105Arg and Asn193Ser, in the **blaVIM** protein with the wild-type illustrates the nuanced impact of mutations on protein stability and potential function.

Recent research on blaVIM metallo-β-lactamase variants supports these observations. Molecular studies on carbapenem-resistant Pseudomonas aeruginosa confirm that blaVIM alleles, including mutations near key residues such as Gln105 and Asn193, may influence resistance phenotypes through altered enzyme stability and activity (e.g., the study from Italy on blaVIM-2 variants). While some mutations show neutral predictions for functionality, stability-focused computational tools frequently detect destabilizing tendencies, paralleling the destabilization seen here for Gln105Arg (Punda , 2000).

Mixed computational predictions for Asn193Ser, including deleterious and neutral categorizations, reflect the complexity of predicting mutation effects in beta-lactamase enzymes. This is consistent with broader literature, where amino acid substitutions in active or binding regions often present ambiguous impacts without biochemical or docking validations (e.g., molecular docking and kinetic assays are recommended in recent reviews on resistance enzyme mutations).

The structural and computational analysis of Ala125Thr and Asn148Lys variants in the **qnrA** protein highlights the complex balance between functional neutrality and protein stability.

Recent research into qnrA mutations aligns with these findings. Nawaz et al. (2015) characterized mutations involved in quinolone resistance, emphasizing that while some amino acid substitutions may appear neutral in function, they

can affect protein stability or interaction with DNA gyrase and topoisomerase IV, which qnr-A protects. This supports the observed destabilizing prediction for Ala125Thr despite its neutral functional predictions (Nawaz et al., 2015)

3.8. Molecular Docking :

The molecular docking results showing oxacillin binding with wild-type and variant OXA enzymes are consistent with recent research elucidating the binding mechanisms and substrate specificity of class D β-lactamases (OXA enzymes).

Studies such as June et al. (2014) have detailed the crystal structures of oxacillinase OXA enzymes, showing that oxacillin binds into a canonical β-lactam-binding groove involving hydrogen bonds, van der Waals forces, and hydrophobic interactions with specific residues in the active site. (June *et al*., 2014).

The finding that the AMKMM1 variant with the p.24E>D SNP displays an unchanged docking score and similar interaction pattern supports the notion that some mutations distant from critical active site residues may have neutral effects on substrate binding. This aligns with observations from Avci et al. (2022), demonstrating how certain mutations do not disrupt the active site's structural integrity or enzyme activity with oxacillin (Avci *et al*.,2022).

On the other hand, the AMKMM2 variant carrying p.144Q>P and p.157G>D SNPs shows increased binding affinity (-5.3 kcal/mol) with alterations in hydrogen bonding and alkyl interactions. This corresponds with literature reporting how mutations near or in the active site can modify substrate interactions and potentially enhance hydrolysis efficiency, as high docking affinity usually correlates with efficient antibiotic hydrolysis and resistance. Zhang et al. (2025) showed a similar correlation with OXA variants exhibiting stronger binding leading to greater antibiotic degradation (Zhang et al., 2024).

The molecular docking results between cefotaxime and CTX enzyme variants (AMKMM4 and AMKMM5) align well with recent structural and mechanistic studies on CTX-M β-lactamases. The finding that mutations in

these variants do not change the binding affinity or interaction pattern with cefotaxime (docking score -6.2 kcal/mol, identical to wild type) suggests that these SNPs do not perturb the active site architecture or key substrate interactions.

Research by Adamski et al. (2015) detailed the catalytic specificity of CTX-M enzymes for cefotaxime, showing that critical active site residues maintain direct hydrogen bonding, pi-alkyl, and van der Waals interactions essential for binding and hydrolysis. Variants with surface-exposed or conservative substitutions often retain these interactions and thus maintain substrate binding affinity, corroborating the docking similarity observed here (Adamski *et al*., 2015).

Additionally, a 2023 study by Judge et al. further mapped substrate specificity determinants, emphasizing that active site geometry and key residues like Ser237 and Arg276 are pivotal for cefotaxime recognition. Mutations outside these critical areas, like the p.161E>G in AMKMM4 and scattered substitutions in AMKMM5, are often tolerated without functional disruption (Judge et al. 2023).

The molecular docking results on the interaction between the carbapenem antibiotic imipenem and wild-type versus AMKMM8 variant blaVIM enzyme are consistent with recent research on metallo-β-lactamase (MBL) resistance mechanisms.

Studies such as those by Mirbagheri et al. (2015) and Sedighi et al. (2015) describe the prevalent role of blaVIM genes in conferring imipenem resistance in *P. aeruginosa* through β-lactamase activity, which hydrolyzes carbapenem antibiotics. The wild-type blaVIM enzyme's strong binding affinity (-6.8 kcal/mol) for imipenem correlates with its efficient hydrolysis, supported by a well-defined catalytic groove enabling multiple stabilizing interactions, including hydrogen bonds and pi-sulfur bonds critical for substrate stabilization (Mirbagheri et al.,2015)

The observed slight reduction of binding affinity (-6.4 kcal/mol) in the AMKMM8 variant carrying p.105Q>R and p.193N>S SNPs corresponds to findings in structural studies that mutations at or near the active site can alter the enzyme's conformation, affecting substrate orientation and interactions (e.g., loss of pi-sulfur bonding replaced by a weaker carbon-hydrogen bond). Such changes often translate to decreased catalytic efficiency but may still preserve partial resistance phenotypes, as demonstrated in research on blaVIM variants with altered substrate profiles (Lauretti *et al*., 1999).

The molecular docking results showing the impact of p.125A>T and p.148N>K SNPs in *qnr-A* on its binding to the DNA gyrase A subunit align well with recent mechanistic studies on plasmid-mediated quinolone resistance.

The wild-type *qnr-A* protein forms a hydrophobic patch at Ala-125 that promotes tight binding to DNA gyrase, consistent with classical models of quinolone resistance protein function where qnr proteins protect the gyrase by binding near the DNA-binding groove and preventing quinolone inhibition. Introducing the threonine mutation with its polar hydroxyl disrupts these hydrophobic contacts by allele substitution, weakening van der Waals interactions and thereby reducing binding affinity, as reflected in the less favorable docking score. This mechanistic insight parallels the importance of hydrophobic interactions in stabilizing protein-protein complex interfaces in such resistance pathways (Tran *et al*., 2005).

Addition of the p.148N>K SNP further diminishes affinity by introducing a positively charged lysine residue into a negatively charged region on DNA gyrase. This charge perturbation likely causes electrostatic repulsion, steric clashes, and conformational disruption, further attenuating *QNR-A* binding. This observation is consistent with structural and biochemical studies highlighting that charged residue substitutions in protein-protein interfaces can severely affect binding specificity and stability, thereby modulating the protective effect of *QNR* proteins against quinolones (Tran *et al*., 2005).

Conclusions and Recommendations

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Conclusions

Considering all the above results**,** the following conclusions can be drawn:

1. This study confirms a occurrence, prevalence and incidence of MDR and XDR phenotypes among the *P. aeruginosa* isolates in Al-Diwanyah City.
2. The occurrence of carbapenem resistant *P. aeruginosa* isolates in hospitals was high.
3. The majority of the carbapenem resistant *P. aeruginosa* isolates carried carbapenemase, ESBL and PMQR genes either alone or in combination.
4. The high rate of mutations in the isolates taken in the study makes these bacteria highly resistant to antibiotics.

Recommendations:

1. The detection of MDR/XDR *P. aeruginosa* isolates co-harboring multiple carbapenemase genes is worrying in Al-Diwanyah hospitals. Underscores the need to perform routine screening of carbapenemases production for the purpose of infection control and trace the source.
2. Attention should be focused on the possible rapid spread of carbapenemases producing *P. aeruginosa* isolates in Iraqi hospitals, for as this species may become a reservoir for transmissible resistance mechanisms.
3. sustained antibiotic surveillance, infection control precautions, and promotion of antibiotic stewardship programs should be emphasized to limit the emergence and transmission of carbapenem resistant isolates in Iraqi hospitals.
4. Continued research is urgently needed to determine the most appropriate treatment for serious carbapenem resistant *P. aeruginosa* infections.
5. Antibiotic resistance research is ongoing due to the evolution of bacterial resistance to effective antibiotics, so we recommended study the prevalence of MDR *P. aeruginosa* variation according to geographical areas.
6. Additional studies – with the inclusion of other isolates are needed to provide clarity on this subject.
7. Additional studies – to the prevalence of this bacteria in a larger number of wound samples and measuring the extent of resistance genes in them are needed.
8. Additional studies –to link molecular testing with computational assessments.

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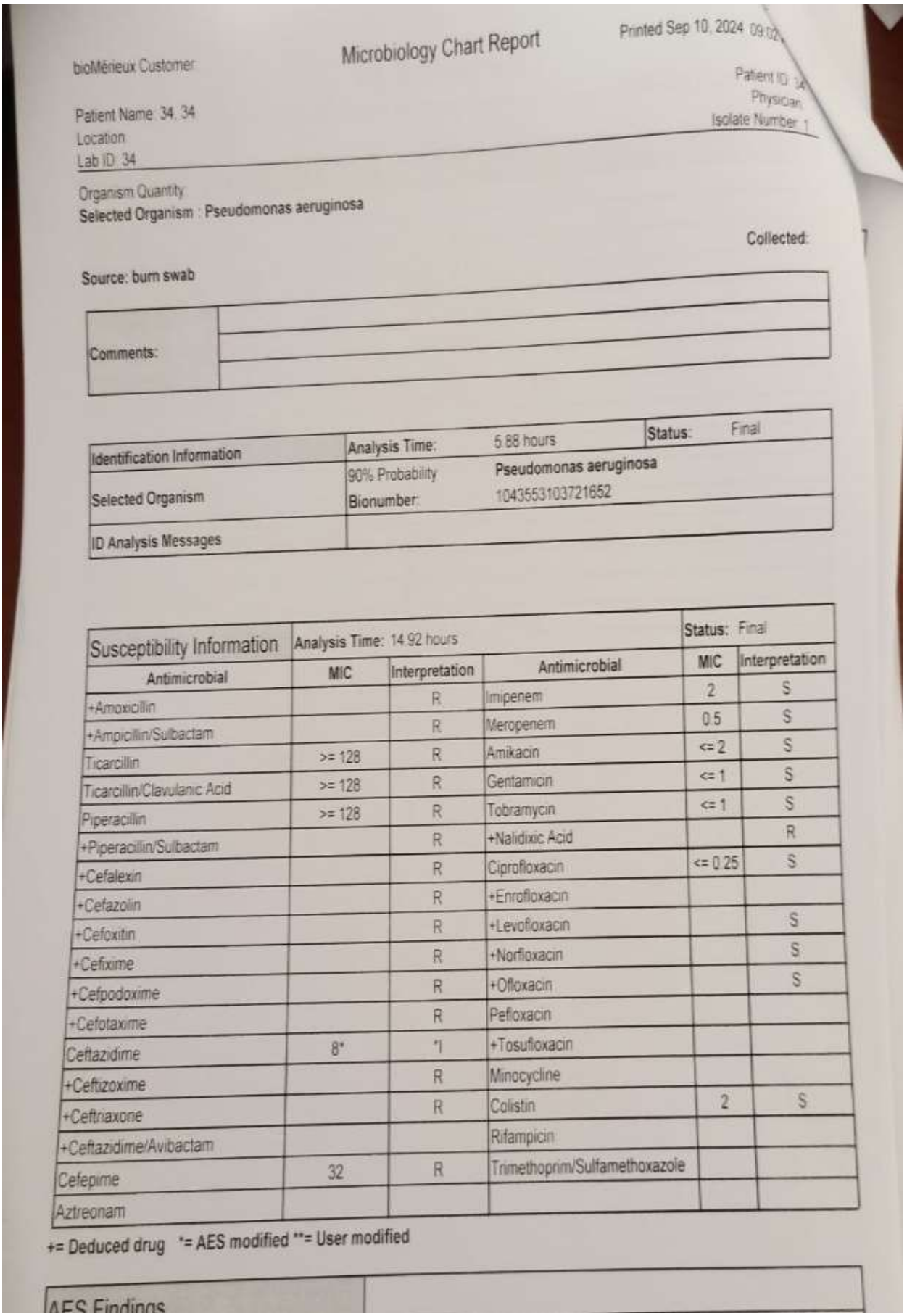
- **Appendix 1: Confirmed test report *Pseudomonas aeruginosa* *by* Vitek 2 system**



Microbiology Chart Report

Printed Sep 10, 2024 09:02

bioMérieux Customer

Patient ID: 34
Physician
Isolate Number: 1

Patient Name: 34, 34
Location
Lab ID: 34

Organism Quantity
Selected Organism : Pseudomonas aeruginosa

Collected:

Source: burn swab

Comments:	

Identification Information	Analysis Time:	5.88 hours	Status:	Final
Selected Organism	90% Probability	Pseudomonas aeruginosa		
	Bionumber:	1043553103721652		
ID Analysis Messages				

Susceptibility Information	Analysis Time: 14.92 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Imipenem	2	S
+Ampicillin/Sulbactam		R	Meropenem	0.5	S
Ticarcillin	>= 128	R	Amikacin	<= 2	S
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	<= 1	S
Piperacillin	>= 128	R	Tobramycin	<= 1	S
+Piperacillin/Sulbactam		R	+Nalidixic Acid		R
+Cefalexin		R	Ciprofloxacin	<= 0.25	S
+Cefazolin		R	+Enrofloxacin		
+Cefoxitin		R	+Levofloxacin		S
+Cefixime		R	+Norfloxacin		S
+Cefpodoxime		R	+Ofloxacin		S
+Cefotaxime		R	Pefloxacin		
Ceftazidime	8*	*I	+Tosufloxacin		
+Ceftizoxime		R	Minocycline		
+Ceftriaxone		R	Colistin	2	S
+Ceftazidime/Avibactam			Rifampicin		
Cefepime	32	R	Trimethoprim/Sulfamethoxazole		
Aztreonam					

+= Deduced drug *= AES modified **= User modified

AES Findings	

Appendix 2: ***Pseudomonas aeruginosa on MacConky agar and blood agar***

Appendix 3**:** ***culture media***

Appendix 4: some of instruments

الخلاصة :

الزائفة الزنجارية هي ممرض معقد يسبب التهابات بمعدلات وفيات مرتفعة. بسبب قدرته على مقاومة العديد من المضادات الحيوية من الدرجة الأولى، تم تصميم هذه الدراسة لاستكشاف توزيع وترابط نمط الجين المتعلق بـ Carbapenemase، وESBL، و PMQR
(VIM، NDM، OXA، CTX، QNR-A، و QNR-B)مع مقاومة المضادات الحيوية في عزلات الزائفة الزنجارية السريرية المحلية. شملت هذه الدراسة ما مجموعه 240 عينة (100 ادرار، 100 مسحة حروق، و40 مسحة جروح). من بين هذه العينات، تم جمع 43 عزلة فقط من الزائفة الزنجارية من عينات مختلفة بين تشرين الثاني 2024 مارس 2025. تضمنت العزلات 29 من الحروق، و5 من الجروح، و9 من التهابات المسالك البولية .(UTIs) تم تحديد العزلات باستخدام الفحص المجهري والتوصيف المظهري باستخدام أجار ماكونكي، وأجار الدم، وأجار السيتريميد. وتمت عملية التشخيص بواسطة نظام.VITEK_2_Compact
لتقييم تكرار مقاومة المضادات الحيوية في بكتيريا الزائفة الزنجارية (MDRوXDR)، تم إجراء اختبار حساسية المضادات الحيوية بواسطة نظام .VITEK_2_Compact شمل الاختبار مجموعتين من المضادات الحيوية، بإجمالي سبعة مضادات. تم تحليل حساسية المضادات الحيوية لبكتيريا الزائفة الزنجارية وفقًا لنوع العدوى، سواء كانت التهابات المسالك البولية أو الحروق أو الجروح. بالنسبة للـ Impenem، كانت أعلى نسبة من العزلات المقاومة لهذه المضاد الحيوي هي تلك المعزولة من الحروق، بنسبة 93.1%. فيما يتعلق بالـMeropenem ، كانت أعلى نسبة من العزلات المقاومة هي المعزولة من الحروق، بنسبة 86.2.%
بالنسبة لمجموعة الكينولونات (Levofloxacin، Ciprofloxacin، Ofloxacin، Norfloxacin) فإن أعلى نسبة من العزلات المقاومة لهذه المضادات الحيوية كانت تلك المعزولة من الحروق، بنسبة 62.1%. اما nalidixic acid، كانت جميع العزلات مقاومة لحمض nalidixic.
تم إجراء تحليل للكشف عن الجينات لستة جينات OXA ، VIM، CTX، NDM، QNR-Aو QNR-Bعبر أنواع مختلفة من العدوى. (أ) حالات عدوى المسالك البولية: تم الكشف عن جينات OXA، QNR_A، QNR_B، و VIM بنسبة 100% في حالات عدوى المسالك البولية؛ بينما تم الكشف عن جين CTX بنسبة 55% في تلك الحالات، بينما تم الكشف عن جين NDM فقط في 11.2% من حالات عدوى المسالك البولية.
(ب) في حالات الحروق، أظهرت جميع جينات الزائفة الزنجارية ظهورا عاليًا في حالات الحروق، باستثناء جينNDM ، الذي لم يتم اكتشافه في 90% من تلك العينات (فقط 10% تم اكتشافها إيجابيًا)، وكانت أعلى نسبة من الجينات التي تم اكتشافها هي.(97.7%) QNR-B

(ج) حالات الجروح: تم اكتشاف جينات OXA و QNR_A وQNR_B وVIMبنسبة 100% ، بينما تم اكتشاف جينات CTX و NDMبنسبة 80% و60%، على التوالي، في تلك الحالات.
كشفت تحليل تسلسل الجينات عن انتشار عدة طفرات بين عزلات الزائفة الزنجارية .أظهر جين CTX أكبر عدد من الطفرات يتبعه جين OXA ثم VIM ثم جينQNR-A ، بينما كان جين NDM هو الأقل انتشارًا من حيث عدد الطفرات. أيضًا، عند دراسة الطفرات من خلال دراسة insilico study، كانت هناك العديد من الطفرات غير المدروسة وصامتة عبر الجينات التي تم فحصها. تمت دراسة تأثير هذه الطفرات على البروتين الناتج من خلال molecular docking

في الختام، كشفت نتائج هذه الدراسة عن انتشار كبير لجينات ESBL و PMQR في بكتيريا الزائفة الزنجارية المعزولة من مستشفيات الديوانية. بالإضافة إلى ذلك، أظهرت النتائج أن الغالبية العظمى من عزلات الزائفة الزنجارية الحاملة لجينات Carbapenemase تم تصنيفها على أنها مقاومة للأدوية بشكل موسع .(XDR) لذلك، يجب تنفيذ فحوصات Carbapenemase كجزء من الممارسة المخبرية الروتينية في المستشفيات العراقية. بالإضافة إلى ذلك، هناك حاجة إلى تدابير لمكافحة العدوى لمنع المزيد من انتشار هذه الكائنات.

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة كربلاء/ كلية الطب
فرع الاحياء المجهرية

تكامل الأنماط الظاهرية والجزيئية والحاسوبية لدراسة مقاومة المضادات الحيوية في العزلات السريرية لبكتيريا الزائفة الزنجارية

رسالة

مقدمة الى مجلس كلية الطب - جامعة كربلاء وهي جزء من متطلبات نيل درجة الماجستير في علم الاحياء المجهرية الطبية

من قبل

علاء حسن جبار غريب البديري

بكالوريوس علوم حياة / جامعة القادسية

2018

بإشراف

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1447هجري **2025** ميلادي