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Molecular Detection of microRNAs and Interleukin-6 Gene Expression Profile in Recurrent Lower Urinary Tract Infection Caused by *Pseudomonas aeruginosa* Isolates

A Thesis

Submitted to the Council of College of Medicine-University of
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Degree of Doctorate of philosophy in Medical Microbiology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا
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صدق الله العليّ العظيم

{ سورة الإسراء / آية ٨٥}

Supervision certification

We certify that this thesis was prepared under our supervision at the department of Microbiology/College of Medicine/University of Babylon, as a partial requirement for the degree of philosophy in Medical Microbiology .

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Decision of Examination Committee

We, the examination committee, certify that we have read thethesis entitled (**Molecular Detection of microRNAs and Interleukin-6 Gene Expression Profile in Recurrent Lower Urinary Tract Infection Caused by *Pseudomonas aeruginosa* Isolates**) and have examined the student (**Bareq Abdullateef sebur Al-Ajealy**) in its contents, and that in our opinion it is accepted as a thesis for Degree of Doctorate of philosophy in Medical Microbiology with **Excellent** estimation .

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Bareq 2023

Dedication

To my father and Mother

To My Wife and My Son and daughters

To my supervisors

Prof. ***Dr. Maysa S. Al-Shukri***

And Prof. ***Dr. Mohammad R.Judi***

To my friends

With my love and respect

BAREQ

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ABBREVIATIONS

a.a	Amino acid
AFLP	Amplified fragment length polymorphism
BCDFs	B-cell differentiation factors
BSFs	B-cell stimulating factors
CAUTI	catheter-associated UTI
CDC	Centers for Disease Control
CF	cystic fbrosis
Clf A-B	Clumbing factors protein A-B
CLSI	Clinical and Laboratory Standards Institute
CRPA	carbapenem-resistant *P. aeruginosa*
cUTIs	Complicated urinary tract infections
D.W.	Distilled water
DC	Dendritic Cell
DD test	Disk Diffusion test
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Ebp	Elastin –binding protein
ECM	Extracellular matrix
ELISA	Enzyme linked immumossorpent assay
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EPS	Extracellular polymeric substance
ERIC	repetitive intergenic consensus

ERK-p38-NF-kB	extracellular signal-regulated kinase
ETs	Exofoliative toxin
ExoA	Exotoxin A
ExoS	Exotoxin S
ExoT	Exotoxin T
ExoU	Exotoxin U
ExoY	Exotoxin Y
FAME	Fatty acid modifying enzyme
Fnbp A-B	Fibronectin-binding proteins A-B
gm	Gram
HCN	hydrogen cyanide
HRP	Horseradish Peroxidase
IBCs	intracellular bacterial communities
IgG	Immunoglobulin G
IL	Interleukin
IL-6	Interleukin-6
IL6 -STP	IL6 -single transduction pathway
IL-8	Interleukin-8
IMFs	Intestinal Myo fibroblasts
IRAK4	Interleukin -1 Receptor –assisted kinases
IRF	Interferon Regulatory Factor
MAPK	Mitogen Activated Protein Kinases
MCP-1	Monocyte Chemo tactic protein
MDR	multidrug-resistant
MGEs	Mobile Genetic Elements

MHC II	Major Histocompablity class II
miRNAs	*MicroRNAs*
MLVA	multiple-locus variable-number tandem-repeat analysis
MRE	multiple response element
mRNA	messenger RNA
MYD88	Myeloid differentiation 88
NCBI	National Center for Biotechnology Information
NCCLS	National committee for clinical laboratory standards
NFκB	Nuclear Factor Kappa B cell
NK	Natural Killer
OD	Optical density
ORSA	Oxacillin resistant *staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
PAAP	*P. aeruginosa* aminopeptidase
PAMP	pathogen Associated Molecular pattern
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	pulsed-feld gel electrophoresis
pH	Power of hydrogen (H^+)
PI	Phosphatidylinsitol
PIA	Polysaccharide intercellular adhesion
PQS	*Pseudomonas* quorum sensing
QS	quorum sensing
RAPD	random amplifed polymorphic DNA

RUTI	Recurrent urinary tract infection
T-helper	T –lymphocyte helper cell
TIRF	Toll IFN regulatory factor
TLR	Toll-like receptor
UTI	urinary tract infection
VAP	ventilator-associated pneumonia
VNTR	variable-number tandem-repeat
WBCs	White blood corpuscles
WHO	World health organization
WT	wild type
β	Beta

Summary:

A total from(110) clinical specimens (Blood and Urine) were collected from patients with Recurrent Urinary tract Infection (RUTI) the result showed that (30) (27.3%) of isolate belonged to ***Pseudomonas aeruginosa*** and 80 (72.7 %) specimen give negative to ***P. aeruginosa*** result and no growth with a significant difference at P<0.0001.

A total of (30) positive cultured isolates belong to ***P. aeruginosa***, they were found that (18 of 30) (16.3%) of isolation obtained from males and (12 of 30) (10%) of isolated take from females .and result show a significant difference at p<0.05,while the age group of patients were distribution from (**20- <50**) years and the result show the highest percentage of *P . aeruginosa* belong to(40-50)age group while the lowest isolates belong to age (20-30)years , The present results showed the mean age of patients are 38.65 ± 10.97 years and 25± 5 years for control group (P= 0.739) .

The most common type of pigments produced by *P. aeruginosa* in this study is the pyocyanin (73.3%) followed by pyoverdine (26.7%).

The antibiotics susceptibility test of the *P. aeruginosa* isolates showed the highest resistance to antibiotics which including Piparacillin 26 (86.6%) , Ceftriaxone 24 (80%), Ceftazidiame25 (83.3%) Carbencillin 23 (76.6%), Meropenem 23 (76.6%) , Tobramycin 22 (73.3%, Amikacin 21(70%), Ciprofloxacin 20 (66.6%), Gentamicin which include 20(66.6%), while the isolates showed lower resistance to the Cefepime19 (63.3%), Norfloxacin 18 (60%) and Imipenem with percentages 16 (53.3%).

The present study found that the expression of Micro302b gene increased in the RUTI patients with *P. aeruginosa* when compared with

control group, as the expression gene is increased more than % 25 fold when compare with control group.

IL6 mRNA gene expression also studied by quantitative Real- time PCR (Relative gene expression) in patients with RUTI and the results show that the IL6 was increase in RUTI Patients with *P. aeruginosa* when compared with control groups. The expression of IL6 is increased more than (%30)fold when compare with control group ,also the result show highly significant increased to Secreted IL-6 levels at *P*. value 0.0001 when compared with control by Enzyme linked Immune Sorbent Assay (ELIZA) methods .

Multi locus Variable Number Tandem Repeat analysis (MLVA) were performed for all *p. aeuroginosa* isolates eight Variable Number Tandem Repeat (VNTR) loci (eight repetitive element loci) were selected for genetic typing of *p. aeuroginosa*. PCR were carried out, the products were electrophoresed on (1-1.5) % Agarose gel containing ethidium bromide and the allele profile were detected by MLVA . Analysis of MLVA profile showed that all *p. aeuroginosa* isolate were grouped into 16 distinct MLVA type with Tow cluster A and B.

The genetic diversity of (20) *P. aeruginosa* Isolates were assessed using ribotyping. The ribotyping assay were performed using two different marker Ribo_1 and Ribo_2 primers ,Ribo-1 primer distinguished the typable isolates though amplifying different bands (200pb to 1000pb), while Ribo-2 primer visible bands (300-900) pb.

Ribo-1 primer showed that the isolates where it classified in to 3 cluster, and 12 sub cluster and it found that isolates No 5,6 and 9 identical 100% No.11 and 12 100% identical No. 8 and 20 also identical 100%. No. 15, 18, 19 similar 100%. and finally ,No. 2 and 3 are similar 100%, So that primers showed highest similarity between isolates.

Ribo-2 primer showed that the isolates where it classified in to 2 cluster, and 12 sub cluster and it found that isolates No. 2, 3,5,6,8,9,12, identical 100%.No.10 and 13 identical 100%. 11 and 15 100% identical 16 and 17 also identical 100%. and finally 18,19 and 20 are similar 100%, So that primers showed highest similarity between isolates.

CHPTER

ONE

Introduction, Literatures Review

1.1. Introduction:

Recurrent urinary tract infection (RUTIs) are one of the most commonly diagnosed infections the pathobiology of RUTI is the disease can be modeled by an oscillating pattern of relapsed infection interspersed with periods of remission between infections (Jhang *et al.,* 2017). There are two main to explain the etiology of RUTI: repeated ascending infections from a reservoir outside the UT or reemergence from a persistent population residing within the UT (Forde *et al.*2019) recently showed the an elderly patient with RUTI and provided evidence of an intestinal reservoir for recurrence.

The significance of microRNAs has been established as one of the major regulators of gene expression that are able to target more than 60% of all human protein-coding genes as well as being involved in many diseases (Elbishbishy, 2019). Urinary miRNAs may be derived from the kidney and urinary tract cells, and could be passively filtered through the glomerulus or secreted by renal tubules, *microRNAs (miRNAs)* have been implicated in a spectrum of physiological and pathological conditions, including immune responses miR-302b has been implicated in cell differentiation. That miR-302b is induced by Toll-like receptor signalling upon Gram-negative bacterium *P. aeruginosa* infection (Zhou and Wu, (2018).

On the other hand the existence of persistent uropathogen populations within the bladder walls of human patients, a high incidence of same-strain RUTI with frequent RUTI , Identifying the mechanistic signals that trigger the reemergence of persistent bacterial populations within the UT is critical to understanding the pathogenesis of RUTIs (De Nisco *et al.*,2019).

Many bacterial virulence factors may contribute to the recurrence of UTI, such as flagella/pili, adhesins, extracellular polysaccharides, lipopolysaccharides, toxins, ureases, proteases and iron-scavenging siderophores. These factors allow uropathogens to survive during long periods in a nutrient limited habitat, helping them to adhere, colonize, damage and invade host cells, as well as to evade host defenses, ultimately increasing their persistence in the urinary tract has been reported in *P. aeruginosa* (Saini *et al.,* 2015).

The pathogenic *P. aeruginosa* encodes virulence factors that provide increased fitness and better chances of survival within the host. These virulence hallmarks promote bacterial growth and survival, thereby manoeuvring the host cellular machinery by causing devastating injuries, tissue necrosis, evasion and immune system impairment (Mues and Chu, 2020).

P. aeruginosa is one of the important causes of nosocomial infections. It represents around 10% of urinary tract infections and accounts for about 35% of mortality due to bacteremia.

This applied a multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) for an efficient genotyping of clinical *P. aeruginosa* strains isolated from patients and *P. aeruginosa* strains/variants were detected among the clinical isolates. (Jarych *et al*., 2021). Also to assess the performance of PCR- based typing techniques, ribotyping, tDNA, in determining the genetic diversity of *P. aeruginosa* urinary isolates (Ranjbar *et al*.,2018).

Interleukin-6 (IL-6) is produced in response to bacterial infections and are important mediators of inflammation , IL-6 is a multifunctional cytokine that regulates numerous body functions, such as the acute phase response, inflammation and organ development. The IL-6 receptor, to which IL-6 binds directly, is expressed by macrophages, neutrophils, T-helper cells, hepatocytes and podocytes. There is also a soluble IL-6 receptor, which, due to its ubiquitous expression, allows IL-6 to modulate a broad spectrum of target cells (Tramma *et al*.,2012) levels of IL- 6 and IL-8 are thought to be biomarkers of site of infection in the urinary tract(Su *et al.*, 2017).

This bacterium has low antibiotic susceptibility, which is attributable to low permeability of the bacterial cellular envelopes and action of multidrug efflux pumps. In addition to this intrinsic resistance, *P. aeruginosa* can get resistance by mutation either in chromosomally encoded genes or by horizontal acquisition of resistant determinants. Often these mechanisms exist simultaneously, thus conferring multiple resistances to many antibiotics (Eichenberger and Thaden, 2019).

Aim of the study:

This work investigates to analysis of miRNAs as abiomarker in urinary tract infection , and genotyping of clinical isolates of *P. aeruginosa* at Babylon hospitals.

Specific Objectives

- To identify and evaluate the frequency of *P. aeruginosa* clinical isolates.
- Using real-time PCR to confirm some of the unique miRNAs altered in positive patients and control .
- Detection the expression of Interleukin-6 genes expression by using real-time PCR (Relative gene expression) in patients and compared with control group .(healthy person free from any sign and symptoms).
- Determination of the concentration of Interleukin-6 by using ELISA in surum.
- To update the antipseudomonal susceptibility profiles of isolates in these organisms
- Assessment of different PCR-Based genotyping methods of *Pseudomonas aeruginosa* isolates by MLTS and Ribo typing .

1.2. Literatures review

1.2.1. Recurrent Urinary Tract Infection (RUTI):

1.2.2. General characterizations:

Recurrent Urinary Tract Infection (RUTI) are defined as two or more symptomatic episodes within 6 months or three or more symptomatic episodes in a timeframe of ≤12 months. From a pathophysiological point of view, a RUTI may present as a relapsing infection that corresponds to an incomplete clearance of the causative pathogen and occurs within 14 days of completion of treatment or as a re-infection presenting after 14 days of treatment completion. In the latter case, a new urine culture should be obtained. Overall, 90% of recurrences are classified as re-infections (Anger *et al*., 2019).

Recurrent Urinary tract infections RUTI are caused by multiple bacterial species and can be community-acquired or nosocomial. Among health care-associated infections, UTI are the most common after surgical site infections and pneumonia. Urinary tract infections UTIs are the infection of urethra (urethritis), bladder (cystitis), or kidney (pyelonephritis). It is among the most common infections worldwide, with substantial morbidity, mortality, and economic burden (Tullus and Shaikh, 2020).

Women are more vulnerable to UTIs due to the physiological and structural characteristics of female urethra. Over 60% of women will experience at least one UTI during their lifetime, and 20–30% of them will experience recurrent UTI within the next 6 months The epidemiology, species distribution, and susceptibility patterns of uropathogens varied greatly among different regions and population studied In addition, the prevalence of UTI increased with age, which could reach up to 20% among women older than 65 years mainly due to their weakened immune system and decreased estrogen level (Behzadi *et al.,* 2021).

1.2.3. Pathophysiology and Risk Factors:

Women are considered more vulnerable to UTIs than men, due to the urethra's proximity to the anus, which ensues in a distinct microbiome in the periurethral area and allows pathogens from the faecal reservoir, such as uropathogen an easier access to the bladder and renal pelvises. In addition, the female urethra is shorter than the male, further facilitating ascendance of pathogens into the bladder , Besides being female further host-related risk factors for RUTIs have been identified, including behavioural, genetic, and urological factors. Especially in young healthy women, sexual intercourse has been identified as a key risk factor. Particularly the frequency of intercourse, frequent partner changes, and the use of diaphragms or spermicides are linked to the occurrence of RUTIs In addition to sexual activity, further risk factors include early onset of UTI episodes and having a mother with a history of UTIs (Huang *et al.,* 2021).

The potential relevance of genetics is further underlined by the finding that certain blood groups have been linked to an increased susceptibility to RUTIs. The affiliation to Lewis-nonsecretor phenotypes has been associated with an increased predisposition to RUTIs, mediated by an increased adherence of uropathogens to the urothelial membrane by expression of unique globoseries-glycolipid-receptors (Zare *et al.,* 2022).

P. aeruginosa itself only represents about 5 to 10% of those reported UTI , Patients with a complicated UTI, outcomes are usually worst when infected with *P. aeruginosa* , Most *P. aeruginosa*-driven UTI are hospital-acquired through the use of urinary catheters, causing catheter-associated UTI (CAUTI). These infections are characterized by the formation of surface-associated biofilm inside indwelling catheters, leading to an entryway to the urinary tract. From there, *P. aeruginosa* can colonize the bladder and reach the kidneys. (Newman, *et al.,* 2017).

The role of *P. aeruginosa* virulence factors in UTI more extensively but slightly dated being the versatile pathogen that it is, *P. aeruginosa* uses multiple-virulence factors to facilitate its survival in the human host. In term of UTI, no specific virulence factor has been described as strictly required for these infections. However, most isolates obtained from UTI are notably proficient at forming biofilms, with producing exoproteases (LasB, AprA) and it causing hemolysis (Morin *et al.,* 2021).

Long-term survival of bacterial pathogens during persistent bacterial infections can be associated with antibiotic treatment failure and poses a serious public health problem. Infections caused by the Gram-negative pathogen *Pseudomonas aeruginosa*, which can cause both acute and chronic infections, are particularly challenging due to its high intrinsic resistance to antibiotics. The ineffectiveness of antibiotics is exacerbated when bacteria reside intracellularly within host cells where they can adopt a drug tolerant state. While the early steps of adherence and entry of *P. aeruginosa* into mammalian cells, the subsequent fate of internalized bacteria, as well as host and bacterial molecular pathways facilitating bacterial long-term survival, are not well defined. In particular, long-term survival within bladder epithelial cells has not been demonstrated and this may have important implications for the understanding and treatment of UTIs caused by *P. aeruginosa*. (Penaranda & Hung, (2021).

Demonstrate and characterize the intracellular survival of wild type (WT) *P. aeruginosa* inside bladder epithelial cells and a mutant with a disruption in the bacterial two-component regulator AlgR that is unable to survive intracellularly. Using simultaneous dual RNA-seq transcriptional profiling, define the transcriptional response of intracellular bacteria and their corresponding invaded host cells. The bacterial transcriptional response demonstrates that WT bacteria rapidly adapt to the stress encountered in the intracellular environment in contrast to ΔalgR bacteria.(Betin, *et al.,* 2019).

Analysis of the host transcriptional response to invasion suggests that the NF-κB signaling pathway, previously shown to be required for extracellular bacterial clearance, is paradoxically also required for intracellular bacterial survival. demonstrate that intracellular survival is important for pathogenesis of *P. aeruginosa* in vivo using a excremental models urinary tract infection. that the unappreciated ability of *P. aeruginosa* to survive intracellularly may play an important role in contributing to the chronicity and recurrence of *P. aeruginosa* in urinary tract infections. (Penaranda *et al.,* 2021).

Chronic persistent bacterial infections are a serious and growing public health problem worsened by the rise in antibiotic resistance, yet new approaches for treating these infections are lacking. These long-term infections can occur when bacteria invade and survive inside host cells where they can hide from the immune system and become less susceptible to killing by antibiotics (Gomila *et al.,* 2018).

Pseudomonas aeruginosa, a bacterium conventionally considered an extracellular pathogen, can cause chronic infections of many organ systems, including the urinary tract show that *P. aeruginosa* can in fact survive inside bladder epithelial cells and becomes tolerant to antibiotic treatment. Using gene expression analysis, show that bacteria quickly adapt to the intracellular environment while the corresponding host cells upregulate the NF-κB signaling pathway. that this response, which had previously been shown to be required for clearance of extracellular bacteria, is paradoxically also required for survival of intracellular bacteria. that the ability of *P.*

aeruginosa to survive intracellularly plays an important role in contributing to the chronicity and recurrence of *P. aeruginosa* infections and that targeting host pathways, such as NF-κB signaling, could transform this ability to manage chronic and/or recurrent infections(Yang *et al.,* 2022).

Through infection with *P. aeruginosa* can adhere to such surfaces as urinary catheters or foreign bodies inserted during urological surgery through production of adhesion factors and biofilm formation. This allows the microorganism to elude the immune system and the activity of antimicrobial agents, and persist in causing frequent relapses of infection. the indication for insertion of an indwelling urinary catheter and its duration should also be periodically evaluated, Complicated urinary tract infections (cUTIs) are among the most frequent healthcare-associated infections. In patients with cUTI, *Pseudomonas aeruginosa* deserves special attention, since it can affect patients with serious underlying conditions. insight into the risk factors and prognosis of *P. aeruginosa* cUTIs in a scenario of increasing multidrug resistance (MDR).Patients with *P. aeruginosa* cUTI had characteristically a serious baseline condition and manipulation of the urinary tract, although their mortality was not higher than that of patients with cUTI caused by other etiologies (Gomila *et al*., 2018).

1.2.4. The Mechanism of Recurrent UTI:

Recurrent urinary tract infection (RUTI) represents a massive burden for the economy, for healthcare systems and for the patients who suffer from them. Several hypotheses for the mechanism of RUTI (Thänert *et al.,* 2019).

One of the recurrence of UTI is that the gastrointestinal tract functions as a reservoir for uropathogens, which are repeatedly reintroduced into the urinary tract via contamination of the periurethral surface and subsequent retrograde ascension. Indeed, common uropathogens are abundant as gut commensals, and there are many papers supporting this mechanism (Jones-Freeman *et al.,* 2021).

Moreover, the now well-established observation that healthy urinary tracts are not sterile and that their microbiota is distinct from those of chronic UTI patients has highlighted the potential relationship between bladder dysbiosis and RUTI as an imbalance in the natural microbial community, may even affect several host protective mechanisms, such as those mediated by commensals.

Also, alteration of the urinary microbiota through the introduction of native microbes from either the gut or the vagina may potentiate the recurrence of UTI. and facilitated the exposure of intracellular reservoirs in the bladder (Gilbert *et al.,* 2017).*and* uropathogens canbecome the dominant vaginal species, which would facilitate their eventual transit to the urethra (Brannon *et al.,* 2020; Lewis and Gilbert, 2020).

Many strain-specific bacterial virulence factors may contribute to the recurrence of UTI, such as flagella/pili, adhesins, extracellular polysaccharides, toxins lipopolysaccharides, , ureases, proteases and iron-scavenging siderophores, This behavior has been reported in *Pseudomonas aeruginosa* and may even be facilitated by polymicrobial interactions during infection (Soga and Gaston, 2020)

Furthermore, a subpopulation of bacterial cells in biofilms, the so-called persistors, are known to reversibly reduce their metabolic activity, adopting a dormant state that can evade host defenses as well as treatments that target active metabolic pathways or activities such as cell division (Yelin, *et al.,* 2019).

Another bacterial mechanism implicated in RUTI is the ability of bacteria to invade host urothelial cells and establish intracellular bacterial communities (IBCs)., in which the bacteria subverted host defenses, invaded urothelial cells and formed IBCs that could later erupt and re-establish UTI (De Nisco *et al.,* 2019).

Bacterial resistance and resilience antibiotic resistance is a key issue in recurrent UTI, and is the most well-studied mode of treatment failure generally (Mattoo and Asmar, 2020).

One of the best studied bacterial resilient mechanisms involved in RUTI are biofilms,as in *Pseudomonas aeruginosa* which provide tolerance to external stresses, such as antibiotic treatments and host defenses ,Bacteria in polymicrobial UTI also have the ability to protect one other from clinically relevant antibiotics through the increase of tolerant/resilient phenotypes in the bacterial community (Murray *et al.*, 2021).

Host Factors that due to recurrence host urothelial cells can also change upon first infection, These changes influence host cell gene expression, shape, size, growth and proliferation, and depending upon what is changed, may make the urothelium more resilient, or more susceptible, to re-infection (O’Brien *et al.,* 2016). role in the chronicity of RUTI, as the recurrence of infection is frequently associated with a disturbed innate immune response and/or insufficient adaptive immunity (Lacerda Mariano and Ingersoll, 2020).

1.2.5. *Pseudomonas aeruginosa* pathogenesis:

1.2.5.1 Introduction to pathogenesis mechanisms:

Pseudomonas aeruginosa is associated with several human infections, mainly related to healthcare services. In the hospital, it is associated with resistance to several antibiotics, which poses a great challenge to therapy.

However, one of the biggest challenges in treating *P. aeruginosa* infections is that related to biofilms. The complex structure of the *P. aeruginosa* biofilm contributes an additional factor to the pathogenicity of this microorganism, leading to therapeutic failure, in addition to escape from the immune system, and generating chronic infections that are difficult to eradicate. *Pseudomonas aeruginosa* can be specified as one of the opportunistic bacteria related to healthcare infections, including ventilator-associated pneumonia (VAP), intensive care unit infections, central line-related blood stream infections, surgical site infections, urinary tract infections, burn wound infections, keratitis, and otitis media (Tuon *et al.,* 2022).

The problems of microbial infection and its clinical complications are increasing due to the increase in the resistance of microorganisms to antimicrobials and the development of this resistance to confront the new generations of antibiotics. *Pseudomonas aeruginosa* is a very common bacterium that is found in infections of wounds, burns, sputum, UTI , otitis and other. Also it is multidrug-resistant (MDR) It is found reside in different environments such as soil, water and moist environment. Approximately 100 species of Pseudomonas have been diagnosed (Tacconelli *et al.,* 2018).

The mortality rate by *P. aeruginosa* infections is ranging from 28% to 48% in intensive care unit due to the multiple drug resistance of the bacteria and the ineffectiveness of antimicrobial treatment (Anderson *et al.,* 2012). *P. aeruginosa* is classified as one of the important cause of chronic infections because its capability to form biofilms (Ciofu and Tolker, 2019).

Severe pathogenicity for *P. aeruginosa* due to have several virulence factors such as elastase enzyme, Biofilm formation, exotoxin A, proteases, alginate, lipopolysaccharide, flagellum, type IV pili and type III Secretion System (Rocha *et al.,* 2019).

The infectious diseases caused by *P. aeruginosa* are sometimes fatal for humans as it is a potential threat to people having less immunity like newborns, diseased persons and veterans. Notably, patients suffering from the diseases like cystic fibrosis, urinary tract infection, burning of the skin, leukaemia, HIV-AIDS, diabetes, patients having longer stay in hospital environments, and persons having organ transplantation are highly susceptible to *P. aeruginosa*, and releted to the disease,symptoms and its causes (Mohanty and Nayak, 2021).

1.2.5.2 . *Pseudomonas aeruginosa* virulence factors:

P. aeruginosa encodes virulence factors that provide increased fitness and better chances of survival within the host. These virulence hallmarks promote bacterial growth and survival, thereby manoeuvring the host cellular machinery by causing devastating injuries, tissue necrosis, evasion and immune system impairment (Mues and Chu, 2020).

P. aeruginosa possesses a large number of virulence factors of cell associated and extra-cellular this leading to increased pathogenicity and some of these factors help facilitate bacterial invasion whereas others support colonization (Rocha *et al.*, 2019).

Pseudomonas aeruginosa is an opportunistic pathogen and with mortality among immunocompromised patients in clinical setups. The hallmarks of virulence in *P. aeruginosa* encompass six biologically competent attributes that cumulatively drive disease progression in a multistep manner these multifaceted hallmarks lay the principal foundation for rationalizing the complexities of pseudomonal infections. They include factors for host colonization and bacterial motility, biofilm formation, production of destructive enzymes, toxic secondary metabolites, iron-chelating siderophores and toxins. (Pachori *et al.*, 2019). This arsenal of virulence hallmarks is fostered and stringently regulated by the bacterial signalling system called quorum sensing (QS), The central regulatory functions of QS in controlling the timely expression of these virulence hallmarks for adaptation and survival drive *the disease* outcome(Chadha *et al.,* 2022)

P. aeruginosa adheres to the cell surface and releases toxins that result in the recruitment of inflammatory cells leading to corneal scarring. These characteristics allow it to attach itself and survive on medical equipment and on other hospital surfaces, which favors the beginning of infections in immune-compromised patients (Khan *et al.* 2021).

1.2.6.3. Mechanisms of Virulence in *Pseudomonas aeruginosa* :

Pseudomonas aeruginosa initiates infection by virtue of cell adhesion molecules like adhesins, flagella and pili. These virulence-associated entities comprise the first hallmark and help govern motility and adherence of the bacterium to a defined substratum. Its characteristic polar flagellum allows *P. aeruginosa* to exhibit swimming and swarming motilities. Studies have indicated the involvement of an accessory appendage, type IV pilus, which imparts specialized twitching motility to this pathogen (Biedenbach, *et al.,* 2015).

P. aeruginosa possess the cytochrome oxidase enzyme, a phylogenetic taxonomic characteristic, and many members produce pigments (Ali *et al.,* 2022). can produce pigments, such as Pyocyanin, rhamnolipids and hydrogen cyanide: secondary metabolites on the siege. Secondary metabolites produced by *P. aeruginosa* play a crucial role in driving its virulence. Pyocyanin is a virtually beautiful blue pigment with redox activity involved in the invasion stage of *Pseudomonas* infection (Newman *et al.,* 2017).

As pyocyanine (green blue) and pyoverdin (yellow green) fluorescence . that all clinical isolates of *P. aeruginosa* recovered from urinary tract infections and catheter-associated urinary tract infections (CAUTIs) showed significant pyoverdine production. An investigation also suggested the critical role of pyoverdine in regulating the pathogenicity of *P. aeruginosa* strains isolated from cystic fibrosis patients (Kang *et al.*, 2019).

Biofilm formation in *P. aeruginosa* an invincible shield for emerging pathogenesis other factors like nutrient starvation, signaling by secondary messengers and sub-inhibitory concentration of antibiotics may also induce biofilm formation (Chadha and Khullar, 2021).

Clinical isolates of *P. aeruginosa* have pointed towards the implication of biofilm formation in chronic infections. 75% of the isolates from patients with ventilator-associated pneumonia exhibited strong biofilm formation, of which ~53% of isolates demonstrated multidrug resistance (Lima *et al.*, 2017).

Biofilms of *P. aeruginosa* are composed of embedded bacterial cells (~90%) with intense cell–cell interaction and channels for he EPS constitutes glycoprotein, glycolipids, polysaccharides and eDNA. The matrix exopolysaccharides include alginate in mucoid biofilms, glucose-rich pellicle (Pel) and mannose-rich polysaccharide synthesis locus (Psl) polymers in non-mucoid biofilms in a mucoid strain of *P. aeruginosa*. It has been shown to facilitate microcolony formation leading to biofilm development in chronic infections (Karygianni *et al.*, 2020).

Biofilm formation in *P. aeruginosa* is regulated by two interlinked mechanisms: QS systems and a key signalling molecule, cyclic-di-GMP. The cellular levels of this secondary messenger keep a check on the stringent command over the motility-sessility switch in this pathogen (Jenal *et al.*, 2017).

Quorum sensing instigator of virulence in *P. aeruginosa* has been shown to induce neutrophil histamine content and stimulate its secretion ,Histamine is not only a key modulator of the host immune system but also triggers chemoattraction and regulates virulence-related genes in *P. aeruginosa* (Matilla *et al.,* 2021).

These include a repertoire of genes associated with bacterial secretion systems, PQS and pyocyanin production, biofilm formation, siderophore production, secretion and iron uptake (Krell *et al.,* 2021). This affirms the role of histamine in regulating the virulence phenotypes of *P. aeruginosa* , In addition, chemotactic abilities and virulence in *P. aeruginosa* (Wang *et al.,* 2021).

Cytotoxicity by Exotoxin, , *P. aeruginosa* produces an array of deadly toxins, including the type III effector exotoxins (Exo): ExoS, ExoU, ExoT, ExoY and a type II toxin called exotoxin A. The type III secretion system that secretes ExoS, ExoU, ExoT and ExoY is composed of the needle complex and translocation apparatus encoded by the *psc* and *popBD-pcrV* genes (Javanmardi *et al.,* 2019).

The expression of type III secretion exotoxins has been detected in both acute and chronic infections of *P. aeruginosa* , exotoxins A and S are the prevalent ones that function as ADP-ribosyl transferases. ExoS is recognized to catalyse the transfer of ADP ribose moiety from NAD to essential cellular proteins like Ras, RalA, Rab, Rac1, Cdc-42, etc. (. Michalska *et al.,* 2015).

Anti phagocytic factor that effacement increased levels of ExoS have been reported across *P. aeruginosa* isolates recovered from the wound and urinary tract infections compared to tracheal infections with increased persistence (Tuon, *et al.,* 2022).

The *P. aeruginosa* T3SS, responsible for injecting toxic effectors into the host cell cytosol (injectisome), is composed of several proteins, including (i) a secretion apparatus (transmembrane transports effectors) and (ii) a translocation apparatus (moves the effectors through human cell membranes (Anantharajah *et al.,*2016).

P. aeruginosa extracellular proteases like LasA and LasB elastases, type IV protease (PIV), *P. aeruginosa* small protease (PASP), Large ExoProtease A (LepA), alkaline protease (AprA), *P. aeruginosa* aminopeptidase (PAAP), associated with critical invasion in acute infection caused by *P. aeruginosa* LasB and LasA elastases are secreted by T2SS regulated by QS systems degrading host elastin (Li and Lee, 2019) .

LasB elastase is a metalloprotease and the most abundant protease, the main extracellular virulence factor and is encoded by the lasB gene. Antibiotic resistance in clinical isolates of *P. aeruginosa* has been correlated with LasA expression. Alkaline protease, which is called aeruginolysin in *P. aeruginosa*, another metalloendopeptidase produced through T1SS (*aprA* gene encoded) which interferes with endothelial components (fibronectin and laminin) and degrades cytokines (IFN, TNF, and IL-6) and complement proteins (C1q, C2, and C3), allows phagocytic evasion. It also cleaves free flagellin monomers, reducing the mucociliary clearance of bacteria through epithelial sodium channel activation and contributes to the production of pyocyanin (and other virulence factors) (Iiyama *et al.*,2017).

Pseudomonas aeruginosa produces cell-associated virulence factors known to cause tissue damage. regulated by extracellular QS virulence factors include neuraminidase, pyocyanin, phospholipases, ATP ribosyltransferases, catalase, superoxide dismutase, (Kamal *et al.*, 2020).

1.2.6. Non-coding RNAs

1.2.6.1. MicroRNA:

MicroRNAs (miRNAs) are small single-stranded RNA molecules with 24 nucleotides in length that can bind to ¯3untranslated region (¯3-UTR) of target messenger RNA (mRNA) for enhancing or preventing translation (Zhou *et al.*, 2021).

Influence fundamental biological processes, including cell proliferation, differentiation, apoptosis, immune response, and metabolism ,The binding of miRNAs to target mRNAs changes the mRNA stability and translation efficiency , leading to degradation, suppression or up-regulation of the target mRNAs (Duval, *et al,*.2017). miRNAs are not translated into proteins and exert key roles in cellular and biological mechanisms (Witten & Slack 2020).

miRNA can be transcribed by RNA polymerase II and similar to messenger RNA (mRNA), miRNAs are polyadenylated and capped with mRNA. miRNAs can function as potential therapeutic, diagnostic and prognostic factors in cancer. As miRNAs can regulate apoptosis, differentiation, migration and angiogenesis under physiological conditions, dysregulation in miRNA expression results in the development of various pathological events, particularly cancer (Li *et al.*, 2020) .

It has also been found that miRNAs can selectively promote or inhibit proliferation and progression of cancer cells (Lee *et al*. 2020). Anti-tumor activity of miRNAs against cancer can be attributed to their effect on apoptosis stimulation and cell cycle arrest, (Lugano *et al.*, 2020).

1.2.6.2. Interaction between Host miRNAs and Bacterial Pathogens :

Evidence implies roles for miRNAs in bacterial infectious diseases by modulating inflammatory responses, cell penetration, tissue remodeling, and innate and adaptive immunity. ranging from the correlation between aberrant expression of miRNAs with bacterial infection progression to their profound impact on host immune responses. (Zhou and Wu, 2018).

miRNAs have also been recognized for their important role in the interactions between host and bacterial pathogens, either as an indispensable part of the host response to fight infection or as a molecular strategy utilized by bacterial pathogens to cause the dysregulation of host miRNA expression for their own benefit (Aguilar *et al.,* 2019). For example, miR-301b is involved in the augmentation of pro-inflammatory response during infection by *Pseudomonas aeruginosa* (Li *et al.,* 2016).

miRNAs hav emerged as important participants in the interactions between host and bacterial pathogens, which suggests an underlying mechanism that miRNAs are transmitted between host and bacteria (Gong *et al.* 2017) and Zhao *et.al.,* 2021).

The dysregulated miRNAs identified during different bacterial infections. the host signal transduction pathways utilized by bacterial effectors by which miRNA expression is dysregulated in mechanisms of modulation. the potential of miRNAs to serve as diagnosis biomarkers and treatment targets, (Zhou and Wu, 2018).

1.2.6.3. The role of microRNAs in **signaling pathways in inflammatory diseases:**

The host's immune response discriminates between microorganisms. One of the most extensively studied recognition mechanisms is through the toll-like receptor (TLR) family. The activation of the TLR signaling pathways is necessary to initiate the immune response to eliminate an infection; inappropriate activation, such as a persistent infection (i.e., bacteria, virus, or other microorganisms),(Xia and Zhu, 2021), can compromise immunological homeostasis, leading to pathologies such as autoimmune diseases, chronic inflammation, tumor development and even cancer. TLRs identify microbe-associated molecular patterns (MAMPs), which in turn triggers an intracellular signaling cascade involving adaptor proteins and the activation of transcription factors that prompt the production of cytokines (Moraga, 2020).

Once signaling has been initiated, transcription factors and message translation can be halted through post-transcriptional regulation of key proteins along the signaling cascade. This negative regulation can be achieved by the destabilization of encoding messenger RNA (mRNA) or by hampering the translation. One of the post-transcriptional regulatory mechanisms is through microRNAs (miRNAs), which are small non-coding RNAs, approximately 23 nucleotides that bind the seed region (2-7 nucleotides from the 5’ end) to the 3’ untranslated region (UTR) of the mRNA from target proteins (Chen,*et al*.,2018)

The sequences of miRNAs are conserved between species; they are not specific for a single protein. The importance of miRNAs as a regulatory mechanism for protein expression. Several of them imply diverse aspects of the immune system, particularly the inflammatory process. the role of some miRNAs in the regulation of TLRs and related signaling proteins, cytokines and their important roles in maintaining homeostasis, and the implications of this regulation in several diseases linked to the inflammatory response (Arenas & Mata ,2018).

MicroRNAs (miRNAs) can intervene in the initiation and modulation of the complex immunoregulatory networks via regulating the expression of TLRs and multiple components of TLR-signaling pathways including signaling proteins, transcription factors, and cytokines. (Banerjee *et al.*, 2021).

Moreover, the aberrant expression of TLRs can induce the expression of several miRNAs which in turn regulate the expression of TLR signaling components and TLR-induced cytokines, emerging roles of miRNA in the regulation of TLR signaling, the interaction between the miRNAs and TLRs, and their implication in inflammatory diseases closely related to the immune system response, such as cytokine and interleukin signaling, MAPK and ion channels routes, MyD88 pathways, NF-κβ and TLR7/8 pathways (Ureña *et al.,* 2020).

Regulation of Nuclear Factor-KappaB (NF-κB) signaling pathway by non-coding RNAs , NF-κB acts as a potential regulator of inflammation and is abnormal expressed in tumor cells, Noncoding RNAs can regulate NF-κB signaling by diverse mechanisms in cancer progression/inhibition, Long non-coding RNAs can regulate miRNA expression that may act as upstream mediators of NF-κB. That can bind to 3/-UTR of NF-κB to modulate its activation, and thereby affecting tumor genesis. MicroRNAs (miRNAs) can dually inhibit/induce NF-κB signaling Regulation of NF-κB by miRNAs may be mediated anti-tumor compounds can increase the expression of tumor-suppressor miRNAs in inhibiting NF-κB activation.(Mirzaei and Zarrabi *et al.,* 2021).

1.2.7. Expression profile of microRNAs :

The expression of microRNAs can be regulated on multiple levels. At the transcriptional level, expression of microRNA genes can change together with (intragenic miRNAs), or independently of (intergenic miRNAs), their host genes. Intergenic miRNAs have their own promoters, are expressed independently and can be regulated by separate transcription factors. In both cases, the expression of microRNA can change due to different mutations or can be regulated by methylation of the promoter Gulyaeva *et al.,* 2016).

On the post-transcriptional level the expression of microRNAs can be downregulated due to changes in the activity of key miRNA biogenesis enzymes, such as Dicer and Drosha. Activity of these enzymes can also be affected by mutations or epigenetic modifications, moreover, chemical compounds of endogenous origin (hormones, cytokines), and exogenous origin (xenobiotics), can alter microRNA expression. Activation of nuclear receptors by xenobiotics as their ligands can induce expression of both intergenic and intragenic microRNAs (Huang and Wu, 2020).

1.2.7.1. Expression profile of microRNAs 302b :

MicroRNAs (miRNAs) have been implicated in a spectrum of physiological and pathological conditions, including immune responses. miR-302b has been implicated in stem cell differentiation but its role in immunity remains unknown. Here that miR-302b is induced by Toll-like receptor 2 (TLR2) and TLR4 through ERK-p38-NF-kB signalling upon Gram-negative bacterium *Pseudomonas aeruginosa* infection. Suppression of inflammatory responses to bacterial infection is mediated by targeting IRAK4, a protein required for the activation and nuclear translocation of NF-kB. (Kirienko *et al.*, 2015).

Through negative feedback, enforced expression of miR-302b or IRAK4 siRNA silencing inhibits downstream NF-kB signaling and airway leukocyte infiltration, thereby alleviating lung injury and increasing survival in *P. aeruginosa* infection In contrast, miR-302b inhibitors exacerbate inflammatory responses and decrease survival in *P. aeruginosa*-infected reveal that miR-302b is a novel inflammatory regulator of NF-kB activation in respiratory bacterial infections by providing negative feedback to TLRs-mediated immunity (Zhou *et al.*, 2014).

The potential role of miR-302cluster in modulating mitophagy and bacterial clearance in macrophages. demonstrated that miR- 302cluster expression was significantly increased after *P. aeruginosa* infection. Overexpression of miR-302/367 cluster promoted mitophagy and enhanced the ability to scavenge ROS in macrophages, thus facilitating the elimination of intracellular *P. aeruginosa* and maintaining the cellular homeostasis. Importantly, identified NF-kB as a novel functional target of miR-302/367 cluster in eliminating intracellular *P. aeruginosa*. These findings shed new light on host defense mechanisms in *P. aeruginosa* infection.(Zhou and Li, 2018).

1.2.8. IL-6 inflammatory model and IL-6 gene expression:

Interleukin-6 (IL-6) was first identified as a factor derived from T-helper type 2 (Th2) lymphocytes biological abilities of IL-6 to stimulate B-cell differentiation, the interleukin was categorised among the B-cell stimulating factors (BSFs) and B-cell differentiation factors (BCDFs) .IL-6 controls the inflammatory response primarily through orchestration of pro-inflammatory and anti-inflammatory effects (Kistner *et al.*, 2022).

IL-6 gene expression is readily upregulated in cells in response to a wide variety of stimuli (viral and bacterial infections, inflammation and tissue injury, and other cytokines and growth factors). At the molecular level, the IL-6 promoter is activated through multiple signaling pathways, including activation of the protein kinase A, protein kinase C, and the NF-κB pathways through a super-enhancer region (“multiple response element,” MRE) of 100–200 nucleotides upstream of the RNA start site (Sehgal, 2022).

Secreted human IL-6 corresponds to differentially modified 184-amino acid proteins derived from a single gene located at 7p15–21 , The secreted IL-6 proteins are differentially O- and N-glycosylated, giving rise to multiple modified proteins in the size range of 21–30 kDa , This IL-6 heterogeneity is also reflected in the detection of multiple IL-6 species in human circulation and body fluids. IL-6 is a member of a family of IL-6 type cytokines which use the common gp130 β chain for signaling to the cell interior; this family includes interleukin-11, The IL-6 receptor on the surface of cells comprises the cytokine-binding 80 kDa α chain (the IL-6Rα) and the common signal transducer 130 kDa β chain (the gp130). While IL-6Rα has a restricted distribution in different cells, gp130 is present more ubiquitously “Classical” IL-6 signaling involves the binding of IL-6 to IL-6Rα associated with the plasma membrane and the binding of this binary complex to the membrane-bound gp130 (Kang *et al.,* 2020).

IL-6 in the circulation can also form complexes with other proteins such as C-reactive protein and albumin ,This complexity of modifications and protein interactions likely accounts for the difficulties encountered when evaluating IL-6 levels and biological activity in human circulation (Ene *et al.,* 2022).

IL-6 initiates signal transduction by first binding to either the membrane-bound form of the IL-6 receptor (IL6R), also known as glycoprotein 80 (GP80), or its soluble form (sIL6R; Proteolytic shedding of a 55-kD fragment in tissues expressing membrane IL6R results in circulating sIL6R bind the ubiquitously expressed membrane coreceptor IL-6 signal transducer, also known as glycoprotein 130 (GP130). (Kistner *et al.,* 2022).

The activity elicited by IL-6 and membrane IL6R is considered classical or cis signaling, while activity instigated by IL-6 with sIL6R is known as trans-signaling. Formation of either complex leads to trans-phosphorylation and activation of JAKs, which phosphorylate the transcription factor STAT3, promoting STAT3 dimerization and translocation to the nucleus (Rupert *et al.,* 2021).

IL-6 controls the inflammatory response primarily through orchestration of pro-inflammatory and anti-inflammatory effects, This is due to the activation of two different IL-6 pathways. The first is known as classic signaling, which operates in support of anti-inflammatory effects. Gp80 and gp130 are triggered through serum-derived free IL-6 in the cellular compartment (Trovato *et al.,* 2018).

This pathway is dependent on cellular expression of the IL-6R components and the concentration of free IL-6 in the serum , The second trans-signalling pathway, promotes pro-inflammatory activities via IL-6 ,in the serum compartment, free IL-6 recruits gp80 and the gp80/IL-6 complex activates cellular gp130 pathway, (Zanders *et al.*, 2022).

IL6-STP1 (single transduction pathway)transcripts have been shown to be involved in microbial defense processes Via activation of the IL-6 family cytokines, IL6-STP1 stimulates inflammatory cells to secrete acute-phase proteins (APP), including fibrinogen, antitrypsin and hepcidin , (Kuscuoglu *et al.*,2018)

Intriguingly, only IL-6 Trans-signaling is required for the pro-inflammatory properties of IL-6, while regenerative and anti-inflammatory functions are mediated via classic signaling. The sIL-6R is generated by different molecular mechanisms, including alternative mRNA splicing, proteolysis of the membrane-bound IL-6R and the release of extracellular vesicles. (Schumertl *et al.*, 2022).

There are some genes, pseudogenes and competitive endogenous RNA for molecular control of IL-6 signaling **,**In contrast to the parental genes composing IL-6R, a distinct pseudogene was demonstrated for IL-6Rα and the gp130 gene (https://www.genecards.org).These pseudogenes share much of their sequences with their corresponding parental genes , conversely, pseudogene transcripts are equivalent to non-coding RNA or to antisense RNA and therefore are unable to produce biologically active proteins (Millrine *et al.*, 2022) .

IL6-STP1 increased genetically proxied IL-6R signalling was associated with reduced levels of 10 circulating interleukins, chemokines and growth factors. The significant results include IL-10 (Rahman *et al.*, 2022).

The (miRNAs) and long non-coding RNAs However, by using the same code, the full set of ceRNAs competes with parental mRNA, ceRNAs absorb mRNA as a 'sponge' to dynamically balance mRNA levels for protein transcription efficiency as a consequence, the ceRNA matrix serves to regulate protein expression, (Trovato *et al.*, 2021).

Regulatory mechanisms that intervene in the initiation or modulation of inflammation include microRnas (miRnas), which have emerged as key post-transcriptional regulators of proteins involved in distinct cellular processes, such as regulation of the immune response. Data on the role of miRNA in the post-transcriptional regulation of IL-6 expression are gradually increasing in line with 15 miRNAs profiles have been recorded to have potential involvement with IL-6 expression (Yuan e*t al.* 2019).

Relapses of UTI are seen in a significant proportion of these adolescents and children, diagnosis of pediatric UTI is challenging, because of the wide ranging and often unspecific symptom presentation. Also, early diagnosis is of great value to avoid long term renal consequences and recurrence among these biomarkers, special attention has been paid to the urinary level of, IL-6 for instance, the levels of IL-6 and IL8 are significantly associated with the incidence of UTI and bacterial loading (Shaikh *et al.,* 2020).

clinically significant, current diagnosis of febrile UTI is comparably time consuming. However, the diagnostic ability of those IL6 in detecting febrile UTI is yet to be determined, since their optimal cut off.(Hosseini *et al.*, 2022).

1.9. Genotyping methods of *P. aeruginosa*

Several genotyping methods have been applied for the *P. aeruginosa* strains' discrimination. the most commonly used methods are amplifed fragment length polymorphism (AFLP), random amplifed polymorphic DNA (RAPD), ribotyping,, multilocus sequence typing (MLST),, multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) pulsed-feld gel electrophoresis (PFGE),Repetitive-element-based PCR, such as enterobacterial repetitive intergenic consensus (ERIC)-PCR and BOX-PCR target bacterial non-coding repetitive sequences and are highly conserved across species, A TRS-PCR is another method used for the efficient genotyping of clinical pathogenic strains, Tis method relies on the presence of microsatellites tri-nucleotide repeat sequences , (Jarych *et al*., 2021).

Due to the genetic flexibility among strains, on phenotypic and genomic diversity. To assess the genomic variability of *P. aeruginosa* strains, using different molecular techniques for tracking the epidemiological transmissions (Osawa *et al.*, 2019)

1.9.1. Multiple-locus variable-number tandem-repeat analysis (MLVA):

This applied a multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) for an efficient genotyping of clinical *P. aeruginosa* strains isolated from patients and compared results with a TRS-PCR typing, Based on the MLVA and TRS-PCR group assessment, *P. aeruginosa* strains/variants were detected among the clinical isolates. The study of *P. aeruginosa* isolates has revealed that during chronic bacterial infections, harbor different *P. aeruginosa* strains or variants within the same host over the years. *P. aeruginosa* genotypes diversity may result from infection with several strains and result from a microevolution process of an initially acquired strain. (Jarych *et al*., (2021).

The DNA typing tools such as MLVA combined with TRS-PCR may play an important role in routine epidemiological surveillance and the identification of the source of transmission of *P. aeruginosa* in patients. This suggests that both TRS-PCR and MLVA are suitable, inexpensive, fast, reproducible, and discriminatory DNA typing tools for effective epidemiological surveillance of potential transmissible *P. aeruginosa* isolates between patients. TRS-PCR provided results similar to those obtained by the MLVA typing. it seems that this method is, in some cases, more discriminatory compared to MLVA, and such genotyping methods combined with additional genetic, phenotypic, or other epidemiological data can play an essential role in making significant decisions on infection control issues(Camus and Moreau 2021).

Rapid diagnostic determination of isolate genotype and phenotype is essential to avoid the spread of dangerous super-resistant *P. aeruginosa* strains; however, epidemiological data should always be considered when deciding whether genetically related strains are also epidemiologically related. Examination of *P. aeruginosa* strains also revealed that a single bacterial colony cannot represent the entire MLVA/TRS-PCR type, as different genotypes of *P. aeruginosa* can simultaneously colonize patients, to analysis of few colonies from multiple sampling from the same period may be more representative of an entire genotype. To obtained results the differentiation of some strains with identical MLVA profiles (Cottalorda *et al.,* 2022).

To explore the diffusion of resistant strains and clonal relatedness of *P. aeruginosa* isolates, a large variety of genotyping methods has been developed such as Multilocus Sequence Typing (MLST). MLST has successfully identified epidemic high-risk clones resistant to antibiotics and responsible for healthcare-associated infections. On the other hand, within-host diversity of *P. aeruginosa* isolates has been identified in cystic fbrosis (CF) infections, which may complicate patient’s care few studies have explored the genetic diversity of *P. aeruginosa* urinary strains; they often included a limited number of strains and mainly focused on multi resistant ones, Thus, to known about both molecular epidemiology and within-sample diversity of *P. aeruginosa* urinary isolates (Wojkowska 2015)

Different study, in the MLVA typing technique with seven distinct VNTR loci was used to genetically characterize the isolated carbapenem-resistant *P. aeruginosa* (CRPA) strains, resulting in the identification of 34 types. Although the MLVA method is less accurate than techniques such as pulsed-feld gel electrophoresis (PFGE), because the strains are encoded based on the number of repeats in the VNTR loci, it is easy to examine the clonal correlation of a large number of isolates (Lashgarian *et al.*,2018).

The finding's isolates belonged to different cluster and mostly from one hospital but there was a lot of variety in sample type. Thus *P. aeruginosa* isolates from the same medical ward had remarkably high diversity. indicated that these organisms vary in genotype over time as well as within the same host (Jarych *et al.,* 2021).

Considering the high genetic diversity of carbapenem-resistant *P. aeruginosa* isolates, which are mainly found in ICU wards, it is difficult to properly implement infection control policies, the MLVA-7 showed good results in typing of *P. aeruginosa* isolates and all studied isolates were type able in a previous experiment from Canada, three not-tyable strains were detected using MLVA-7 as follows: (MS142, MS211, MS213, MS215, MS216, MS222, MS223). (Lalancette *et al.,* 2017)

1.9.2. Ribotyping of *P. aeruginosa* isolates:

Ribotyping describes a series of methods used to characterize the ribosomal DNA operon in bacteria and other more complex organisms. As all bacteria have rDNA, ribotyping is theoretically practicable for all bacterial species. Ribotyping has seen wide application as a molecular epidemiology tool in clinical bacteriology. Ribotyping, or the characterization of the ribosomal DNA operon, has become an established analytical tool for bacteria and other more complex organisms. this technique can be used for a wide variety of molecular epidemiology study (Rogers 2022).

based on the amplification of spacer regions or intervening sequences between 16S and 23S rDNA genes , Used for identifying *P. aeruginosa* using primers targeting 16S rRNA. PCR analysis of 16S-23S internal transcribed spacer primer targeting the interspaced regions between the 16 and 23S rRNA genes (PCR ribotyping) was evaluated for it s effectiveness to differentiate between the clinical isolates of *P. aeruginosa* belongs to ribotypes, which were show each isolate of *P. aeruginosa.* This method detects both the number of tRNA genes and the spacer length within the cluster, using primers complementary to the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene, which reveals length heterogeneity even within the same species, it mean it is used for studying genetic diversity.(Abdel-Rhman and Rizk, 2021).

The phylogenetic classification of prokaryotes with rRNA gene sequences is based on the assumption that the differences in sequences reflect the evolution of the organisms from which PCR ribotyping have extensively been used in the molecular epidemiology of different outbreaks and episodes of infections (Hassan *et al.*, 2014).

1.10. Antimicrobial Resistance of *P. aeruginosa*

Pseudomonas aeruginosa is a bacterial pathogen associated with a wide range of infections and utilizes several strategies to establish and maintain infection including biofilm production, multidrug resistance, and antibiotic tolerance. Multidrug resistance in *P. aeruginosa*, as well as in all other bacterial pathogens, is a growing concern. It is important to combat the rising number of recurrent and recalcitrant infections (Sindeldecker, and Stoodley, 2021).

Antimicrobial resistance is one of the great public health threats in 21st century(Feng *et al*., 2019). the World Health Organization (WHO) has listed carbapenem-resistant *P. aeruginosa* as one of three bacterial species in which there is a critical need for the development of new antibiotics to treat infections (Tacconelli *et al*., 2017).

P. aeruginosa displays resistance to a variety of antibiotics, including aminoglycosides, quinolones and β-lactams. Generally, The acquired resistance of *P. aeruginosa* can be realized by either mutational changes or horizontal transfer of resistance genes (Breidenstein *et al*., 2011). In *P. aeruginosa*, the best characterized mechanisms of adaptive resistance are the formation of biofilm and the generation of persister cells (Pang *et al.*, 2019).The frequency of XDR and MDR *P. aeruginosa* strains has increased during the last several years, with estimates ranging from 15% to 30% in Spain (Pea *et al*., 2015 ;Sader *et al*., 2018).

P. aeruginosa has been shown to possess a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell and production of antibiotic-inactivating enzymes such as β-lactamases (Breidenstein *et al*., 2011).

Most antibiotics used to treat *P. aeruginosa* infections must be able to penetrate the cell membrane to reach intracellular targets . To enter the bacterial cell, β- lactams and quinolones penetrate cell membranes through porin channels, whereas aminoglycosides and polymyxins promote their own uptake by interacting with bacterial lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria , such as *P. aeruginosa*, which acts as a selective barrier to prevent antibiotic penetration, (Klobucar and Brown, 2022).

Bacterial efflux pumps play an important role in expelling toxic compounds out of the cell, This compound has been shown to reduce virulence, diminish quorum sensing and increase antibiotic susceptibility of *P. aeruginosa* (Seupt *et al.*, 2020).

AmpC β-lactamase expression a wide-spectrum class C β-lactamase is also encoded by *P. aeruginosa*'s chromosomal drug-inducible gene, bla_{AmpC} , AmpC is one of the main causes of antibiotic resistance, particularly with respect to broad-spectrum cephalosporins (cephazolin, cefprozil), monobactams (aztreonam), and 3rd and 4th generation cephalosporins (ceftazidime, cefepime) *(*Berrazeg *et al.*, 2015 and El Shamy *et al.*, 2021).

Acquired Resistance Bacteria can gain antibiotic resistance through mutational changes or acquisition of resistance genes via horizontal gene transfer (Munita and Arias, 2016). Development of MDR/XDR strains, which increases the difficulty in eradicating this microorganism and leads to more cases of persistent infections (Henrichfreise *et al*., 2007).

Mutational Changes are able to cause reduced antibiotic uptake, modifications of antibiotic targets, and overexpression of efflux pumps and antibiotic-inactivating enzymes, thus, mutational modifications of the target sites in *P. aeruginosa* also contribute to its antibiotic resistance. Quinolone antibiotics inhibit bacterial DNA replication by targeting DNA gyrase and topoisomerase IV (Aldred *et al*., 2014)

Mutation causing overexpression of antibiotic-inactivating enzymes in *P. aeruginosa* is another well-characterized mechanism of acquired resistance (Munita and Arias, 2016). Some *P. aeruginosa* clinical isolates have overproduction of β-lactamases caused by mutations in a β-lactamase inducible gene *ampC,* which greatly increased the resistance to cephalosporins (Berrazeg *et al*., 2015).

Acquisition of Resistance genes antibiotic resistance genes can be carried on plasmids, transposons, integrons and prophages, plays a critical role in dissemination of antibiotic resistance among *P. aeruginosa* strains

(Khosravi *et al*., 2017). The main mechanisms of horizontal gene transfer involve transformation, transduction and conjugation , Acquisition of aminoglycoside and β-lactam resistance genes has been reported in *P. aeruginosa* (Hong *et al*., 2015).

In *P. aeruginosa*, the best characterized mechanisms of adaptive resistance are the formation of biofilm and the generation of persister cells, which result in persistent infection and poor prognosis in patients (Alford *et al*., 2021).

Biofilm-Mediated Resistance The survival of persister cells could possibly lead to a recurrent infection Clinically, biofilm related *P. aeruginosa* infections are commonly observed in chronic obstructive pulmonary disorder, cystic fibrosis, urinary tract infections, catheterization, intubation, and surgical site infections (Sindeldecker and Stoodley, 2021).

suggesting that the biofilm-mediated resistance is independent of genetic mutations, and it is an adaptive mechanism (Kahl and Dietrich, 2020).

Carbapenem Resistance in *P. aeruginosa* Carbapenem antibiotics (including imipenem, meropenem, ertapenem and doripenem. They are stable against most chromosomal broad spectrum β-lactamases and cephalosporinases found in Gram-negative bacteria (Li *et al*., 2020). Carbapenems are one of the latest β-lactam with one of the broadest spectrum Their stability against hydrolysis by most β-lactamases makes them major weapons in the treatment of severe nosocomial infections (Patrier and Timsit, 2020).

Aminoglycosides resistance in *P. aeruginosa* are potent, broad-spectrum antibiotics that act through inhibition of protein synthesis. The aminoglycosides that have been, or are still, important in medical practice are amikacin, gentamicin, isepamicin, kanamycin, neomycin, netilmicin, paromomycin, sisomicin, streptomycin and dihydrostreptomycin, and tobramycin (Studies and Information, 2016).

16SrRNA methylation this mechanism involved methylation step occurs via a methylase enzyme at specific nucleotides in 16S rRNA, a site causing restriction in binding between antibiotics to its target due to loss the affinity of binding, thus producing elevated resistance to the clinically important aminoglycosides such as amikacin, tobramycin and gentamicin (Moura *et al.*, 2017; Braun *et al.*, 2018).

CHPTER

TWO

Materials and methods

2.1 Materials and Methods

2.1 Materials

2.1.1 Laboratory apparatus and materials

The following Equipment and Instrument used in this study are listed in table (2-1) below:

Table (2-1): Laboratory Instruments.

Instruments	Company	Origin
Autoclave	Daikyo	Japan
Biosafety cabinet class II	ESCO	Singapore
Burner	Amal	Turkey
Centrifuge	Fisher Scientific	USA
Compound light microscope	Olympus	Japan
Cooling Centrifuge	Eppendorf	Germany
Deep Freeze -80	Binder	Germany
Distillator	Kottermann	Germany
Electronic balance	Kern	Germany
Electrophoresis system	Cleaver	UK
Gel Documentation	Cleaver	UK
Hot plate with magnetic stirrer	Gallenkamp	UK
Incubater	Memmert	Germany
Laminar air flow	Gallenkamp	UK
Micropipette set (1-1000ml)	Eppendorf	Germany
Microplate reader	Organon Teknika	Austria
Digetal camera	Compact	France
Oven	Memmert	Germany
PCR Thermocycler	ThermoFisher	USA
Ph meter	Radiometer	Denmark

Instruments	Company	Origin
Nanodrop spectrophotometer	philips	Holland
Refrigerator	LG	Japan
Shaker incubator	GFL	Germany
ThermoMixer	Bioer	Japan
Vortex	Fanem	Brazil
Water bath	GallenKamp	UK
Vitek 2 System	BioMerieux	France

2.1.2 Disposable Materials

The following disposable materials used in this study are listed in table (2-2) below:

Table (2-2): Disposable Materials

Disposable Materials	Company	Origin
Loop	JRZ	Canada
Eppendrof tubes	Eppendorf	Germany
Filter paper	Sartorius	Germany
Latex Gloves	Broche	Malaysia
Petri dish	Treumpo	Japan
Plan tubes (different volume)	Afco-Dispo	USA
Sterilized cotton swabs	ABBDOS	India
Tippes	Eppendorf	Germany
Universal tube	Difco	USA

2.1.3 Chemicals Materials

Chemicals materials used throughout this study are summarized in table (2-3).

Table (2-3): Chemicals Materials utilized in this Study.

Chemical and Biological Materials	Manufacturing Company	Origin
N, N, N, N – tetramethyl-p-phenylene- Diamine dihydrochloride	BioMerieux	France
Absolute ethyl alcohol (99.9%)	Diamound	France
catlase	BDH	England
Crystal violet	Himedia	India
Ethanol 95%	ROMIL pure chemistry	UK
Glucose	Hi-media	India
Glycerol	GCC	India
Hydrogen Peroxide (H_2O_2)	BDH	UK
McFarland tube No.(0.5)	BioMerieux	France
Normal saline	Pioneer	Iraq
Nuclease free water	Promega	USA

2.1.4. Molecular Materials:

Genetics materials used throughout this study are listed in table (2-4).

Table (2-4): molecular Materials utilized in this Study.

Chemical and Biological Materials	Manufacturing Company	Origin
Agarose	Cleaver	England
T. E 10 × buffer	Geneaid	Korea
Phosphate buffer saline (PBS)	BioUltra	UK
DNA ladder 100 bp,5000	Promega	USA
DNA loading dye	Promega	USA
Ethidium Bromide	Promega	USA
Loading buffer (6X)	Promega	USA
Nuclease free water	Promega	USA
Proteinase k	Promega	USA
RNAlater	Thermo Scientific	USA
Tris-Borate-EDTA (TBE) buffer	Promega	USA
TRIzol Reagent	Thermo Scientific	USA

2.1.6 . The Culture Media

The Media are used in illustrated in table (2 -5).

Table (2–5): Ready and prepared Media utilized in this Study.

Media	Manufacturing Company	Origin
Blood agar base	Himedia	India
Brain Heart Infusion broth	Himedia	India
Cetrimide agar medium	Himedia	India
MacConkey agar	Himedia	India
Muller Hinton agar	Himedia	India
Nutrient agar	Himedia	India
Nutrient broth	Himedia	India

2.1.5. Antibiotic discs

Antibiotic discs that were tested according to CLSI (2021) in table (2–6).

Table (2–6): Antibiotic discs that used and Diameter interpretive standards of inhibition zones based on"CLSI 2021"

No.	Antibiotics	Code	Inhibition Zones Diameter (mm)		
			Susceptible	Inter mediate	Resistant
2	Amikacin	AK	≥ 17	15 – 16	≤ 14
6	Carbencillin	CAR	≥ 28	24– 27	≤ 23
5	Ceftazidiame	CAZ	≥ 18	15– 17	≤ 14
7	Ceftriaxone	CRO	≥ 21	14 – 20	≤ 13
12	Ciprofloxacin	Ctx	≥23	15 – 22	≤ 14
9	Cefepime	FEP	≥ 18	15 – 17	≤ 14
3	Gentamicin	GN	≥ 15	13 – 14	≤ 12
10	Imipenem	IMP	≥ 19	16– 18	≤ 15
11	Meropenem	MEM	≥ 22	19– 21	≤ 18
8	Norfloxacin	NOR	≥ 17	13 – 16	≤ 12
1	Piparacillin	PRL	≥ 21	15– 20	≤ 14
4	Tobramycin	TOB	≥ 15	13 – 14	≤ 12

2.1.7 Kits

The following kits used in this study are listed in table (2-7).

Table (2-7): Kits for identification, extraction and purification

Kit	Manufacturing Company	Origin
DNA extraction kit	Geneaid	USA
RNA extraction kit	Geneaid	USA
DNA ladder 100bp,5000bp	Bioneer	Korea
Green master mix 2X Kit	Promega	USA
Vitek 2 System	BioMerieux	France
ELAS IL6	RayBio®	USA
Gram stain kit	Crescent	KSA

2.1.7.1. DNA extraction kit content solution for genomic bacterial and genotyping analysis study:

Materials and solution are listed in table (2-8).

Table (2-8): DNA extraction kit content solution:

Materials and solution
1- Cell lysis: -GB Buffer
2- DNA binding alcohols:- - Absolute ethanol alcohol
3- Wash solution: - W1Buffer - Wash buffer
4- DNA Elution: - Preheated Elution Buffer

The molecular assay and main diagnostic kits used in this study are listed in table (2-9) as follows

Table (2-9) Content DNA extraction kit :

Ethanol alcohol (96-100%), Proteinase K solution for precipitation proteins, Wash buffer, W1 buffer solution, FABG buffer, ddH$_2$O, Elution buffer, RNase solution.

2.1.7.3. Master Mix Used in PCR:

Table (2-10): contents of master mix

NO.	Materials
1	DNA polymerase enzyme (Taq)
2	dNTPs (400µm dATP, 400µm d GTP, 400µm dCTP, 400µm
3	MgCl$_2$ (3mM)
4	Reaction buffer (pH 8.3)

2.1.7.3 DNA ladder

Table (2-11): composition of ladder and loading dye

NO.	Materials
1	Ladder consists of 11 double-stranded DNA with size 100-1500bp and (100-5000)bp
2	Loading Dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA)

Table (2-12): Contents of Reaction Mixture

Contents of reaction mixture	Volume
Master Mix	12.5 µl
Template DNA	5 µl
Forward primer (10 pmol/µl)	1.5 µl
Reverse primer (10 pmol/µl)	1.5 µl
Nuclease free water	4.5µl
Total volume	25 µl

2.2 . Methods

2.2.1 Media preparation and sterilization technique:

Nutrient agar, MacConkey agar, Brain Heart Infusion broth, Nutrient broth and Muller Hinton agar, were prepared according to the manufacturing company instructions. They were brought to boil in a water bath to dissolve all constituents completely, and sterilized by autoclaving at 121ºC for 15 min at 15 pounds/inch^2, then the media were incubated at 37ºC for 24hr. to ensure sterility.

٢.2.2 Laboratory prepared culture media

٢.2.2.1 Blood agar

These media were prepared according to manufacturing company instructions sterilized by autoclaving and cooled to 45°C then 5% of sterile fresh human blood was added. These media were used for cultivation most of pathogenic bacteria and examined their ability to hemolysis blood and showed type of hemolysis (Musafer, 2013).

٢.2.2.2 Cetrimide agar

Amount of 45.3 gm of this medium is added in 800ml distilled water, and is completely dissolved and separately 10 ml glycerol dissolved in 100ml D.W too. Then, they were mixed well with media and completed volume to 1000ml with D.W thereafter autoclaved later cooled. Finally, This media is selective for *P. aeruginosa* growth and identification (Rijal, 2015).

2.2.2.3. Nutrient Agar Medium:

Nutrient agar medium was prepared according to the method suggested by the manufacturing company by dissolved 28gm in 1000mlD.W. It was used for the cultivation of the bacterial isolates when necessary.

2.2.2.4. Müller-Hinton Agar

This medium was prepared according to the instruction of supplier company by dissolving 38gm from the medium in 1L of distilled water and sterilization by autoclave at 121 Cº for 15 min., this medium used for an antibiotic sensitivity test.

2.2.2.5. Brain Heart Infusion (BHI) Broth -Glycerol Medium(Maintenance Medium)

This medium used to preserve the bacterial isolated as the standard for a long time. This medium was prepared by adding 5ml of glycerol with 95 ml of BHI broth and sterilization by autoclave at 121 Cº for 15min. (Forbes *et al.*, 2007).

2.2.2.6. MacConkey Agar Medium: Key components of the MacConkey medium included crystal violet dye, bile salts, lactose, and neutral red (pH indicator). Crystal violet dye and bile salts halt the growth of gram-positive bacteria. This allowed only gram-negative species to form colonies on MAC agar(Lagier *et al*. 2015). It was used for the primary isolation of most Gram-negative bacteria and to differentiate lactose fermenters from non-lactose fermenters (Forbes, Sahm, and Weissfeld 2007). A Weight (51.5gm.) was produced by dissolving in (1 L. of Distillated water). Heat until the sugar had dissolved completely. It was decontaminated for 15 minutes in an autoclave at 121°C, then cooled to 50°C before pouring onto petri dishes.

2.2.3 Preservation of Isolates :

2.2.3.1 Short Term Preservation

Bacterial isolates were maintained for few weeks on nutrient agar plates which were wrapped tightly with parafilm and then stored at 4ºC(Harley & Prescott, 1996) .

2.2.3.2 Long Term Preservation

Brain heart infusion broth addition to 5% glycerol was distributed into plain tube, autoclave and inoculated with 24 hr. ancient culture of bacteria isolates as well as incubated at 37°C for 24 hr. then stored at -20°C (Vandepitte *et al.*, 2003).

٢.2.3 Solutions and reagents used in bacterial identification:

٢.2.3.1 Gram stain solutions

The ready kit consists of four containers: crystal violet solution, iodine, decolorization solvent and 0.5% standard safranine as counter-stain. This stain serves as differential between two type's gram bacteria and used to study bacterial cells morphology and their arrangement (Forbes *et al.*, 2007).

٢.2.3.2 Catalase reagent

A drop of catalase agent which is a ready commercial 3% hydrogen peroxide (H2O2) reagent was placed on a clean microscopic glass slide. This test used for investigating the ability of bacteria to produce catalase enzyme which causative degradation H**2**O**2** releasing free O**2**(Acharya, 2013).

٢.2.3.3 Oxidase reagent

It has been prepared by dissolving 1mg of N, N, N, N-tetramethyl-ρ-phenylenediaminedihydro chloride to 100 ml of distilled water, then it was stored in a dark bottle and used immediately. This test used for detecting the ability of bacteria to produce oxidase enzyme, (O’Hara,*et a*l.,2014).

2.2. 4. Preparation of molecular materials

Gel electrophoresis solutions of extracted DNA consists of;1X TBE buffer, loading dye, DNA ladder marker, ethidium bromide (10mg/ml). The solutions were prepared as described by (Sambrook and Russell2001), as follows:

2.2.4.1 Tris-Borate-EDTA Buffer (TBE)

Working buffer (1X) was prepared by adding 100 ml from TBE buffer (10 X) to 900 ml of D.W.

2.2.4.2 Loading dye

It was used by following the manufacturer instruction. This dye is used for loading DNA samples into gel electrophoresis wells and tracking migration during electrophoresis. Only 1-2µl of the loading dye was mixed with DNA sample. It was used in genomic DNA profile.

2.2.4.3 Ethidium bromide

Ethidium bromide was prepared in a concentration of 10 mg/ml by dissolving 1g of the dye in 100 ml of sterile D.W and stored at 2-8°C in a dark bottle until use.

2.2.4.4 Agarose gel

Agarose gel was prepared by dissolving agarose powder in 1X TBE buffer. An amount of agarose can be dissolved by TBE buffer depending upon the purpose for which agarose gel is used. 1% agarose gel was used for visualization DNA after the processing extraction. It was then run at75V for 30 min for better resolution. On the other hand, 1-1.5% of agarose gel was visualization using PCR product was used for running at 75V for90 min. All gels were stained in an ethidium bromide stock solution, and the concentration was 10mg/ml. Only 3µl of ethidium bromide stock solution was added to100 ml of the melting agarose gel to get a final concentration of 0.3 µg/ml and was visualized under ultraviolet transilluminator.

2.3. Isolation and Identification of *Pseudomonas aeruginosa*:

2.3.1 Identification by conventional and biochemical tests

2.3.2.1. Gram's stain

The first step in the identification of bacteria was Gram stain. The isolates were stained by Gram staining technique and smear which examined under light microscope. Gram’s reaction was observed for each bacterium whether it was gram positive bacteria which appeared as purple or if it was gram’s negative stained pink. Also notice the shape and arrangementof bacteria was cocci, bacilli, strep or cluster shape (Christopher and Bruno, 2003).

2.3.2.2. Catalase test (slide test) : This test detects the ability of bacteria to produce the catalase enzyme. A drop of catalase reagent of ready commercial (3% H2O2) hydrogen peroxide was placed on a glass slide. A colony of bacteria was tested by mixing with the reagent on the surface of a clean dry glass slide, presence of gaseous bubbles indicates a positive result, while bacteria did not give reaction indicates a negative result. Catalase enzyme which broke down H2O2 into oxygen and water (Forbes *et al.*, 2007).

2.3.2.3 Oxidase test

A single colony of bacteria isolates was transferred to a piece of filter paper by wooden applicator sticks, and then 2-3 drops of 1% oxidase reagent were added to the filter paper. Then, the color was fast changed within 5-10 sec. An appearance of dark purple color indicates positive result and the absence of change in color indicated the negative results. This test was used to identify the ability of bacteria to produce the enzyme oxidase (O’Hara *et al*., 2014).

2.3.2.4. Lactose fermentation test

MacConkey agar plates were inoculated by streaking and incubated at 37°C for 24hrs. The positive result indicated by the red colonies associated with lactose fermentation, while the pale yellow colonies gave a negative result to non-lactose fermentation (MacFaddin, 2000).

2.3.2.5. Hemolysin production test

Bacterial culture was streaked with Blood agar. Then incubated for24hrs at 37°C. A positive result for *P.aeruginosa*in blood agar is typically display beta hemolysis (Gao *et al*, 2019).

2.3.2.6. Pigment production test

One colony of fresh culture was streaked on cetrimide medium plates and incubated at 37°C for 24hrs. Cetrimide agar is used for the selective isolation of *P.aeruginosa* inhibits the growth of many microorganisms while allowing *P.aeruginosa* to develop typical luxuriant colonies (Quinn *et al*., 2011).

2.3.2.7. Vitek 2 system diagnosis

The Vitek 2 System was used to confirm the result of the manual biochemical test, this system used to identify microorganisms . This system was performed according to the manufacturer's instructions (Biomerieux-France).

This system consists of :

1- A personal computer.

2- Reader/incubator that consisting of multiple internal components including: card cassette, card filler mechanism, cassette loading processing mechanism, card sealer , bar code reader, cassette carousel and incubator.

3- The system also contains: transmittance optics , waste processing, instruments control electronics and firm ware. This system was performed according to the manufacturer's Instructions (Biomerieux-France):

1- Three ml of normal saline were placed in plane test tube and inoculated with a loopfull of single colony of overnight culture.

2- The test tube was inserted into a dens check machine for standardization of colony to McFarland's standard solution (1.5×10^8 CFU/ml).

3- The standardized inoculums were placed into the cassette.

4- Then a sample identification number was entered into the computer software via barcode . Thus the vitek 2 card was connected to the sample ID number. The cassette was placed in the filler module, when the cards were filled, transferred the cassette to the reader/incubator module.

2.4. Antibiotic Susceptibility Test:

Kirby -Bauer method used to perform carry out the antibiotic susceptibility test for 12 different antimicrobial agent Bacterial suspension was prepared by picked 4-5 colonies of each bacterial isolate from original culture and was suspended into a test tube containing 5 ml of normal saline, then turbidity was adjusted to obtain approximately 1.5 $x10^8$ CFU/ml (MacFarland tube). By a sterile cotton swab a portion of bacterial suspension was transferred carefully and evenly spread on Mueller - Hinton agar medium, and then it was left for 10 min. Then after the antimicrobial discs were placed on the
agar with sterile forceps pressed firmly to ensure contact with the agar. Later the plates were inverted and incubated at 37 C° for 24 hr. Inhibition zones that developed around the discs were measured by millimeter (mm) unit by using a metric ruler. The isolate was interpreted as susceptible, intermediate, or resistant to particular antibiotic according to CLSI 2021(Clinical & M100, 2021).

2. 5 . Patients Specimens :

A case-control study was conducted in, Hilla Hospital from several hospitals in Babylon governorate. (Teaching Hospital ,Merjan General Hospital) and out patients from both gender with age ranged from 20years to 80 years, between February 2021 to the March 2022. Individuals admitted these centers with a suspected of Recurrent Urinary tract infection according to physician undergone physical examinations, and patients history with recuurence.

Total of 110 Patients with confirmed RUTI by patient history and pathological reports according to urologist were recruited in the study includes age range (20-80) years. urine samples were obtained from Recurrent Urinary tract infection (RUTI) patients ,and All urine sample cultured on selective and differential media for bacteriological identification study, and genotyping analysis. DNA extraction were done to all bacterial samples in order to be for molecular study.

Two ml of blood were obtained from all patients after they were confirmed to have positive culturing for *pseudomonas aeruginosa* and when they have history with recurrence by puncture of the vein and placing it in Ethylene di amine tetra acetic acid (EDTA) tubes. RNA was extracted from EDTA tubes for molecular study, and gene expression study.

One 1 ml of blood put in gel tube used for serological measurement of IL6 by ELAS method .

2.5.1. Control group :

Twenty individuals with the clinical diagnosis that are free from any sign and symptoms and free from recurrent infection RUTI and who were agreed to participate in our study. Blood specimen was taken from all those twenty control for serology and the RNA extraction from these sample were done in order to be used for molecular study , and urine negative culturing result.

2.5.2. Inclusion criteria:

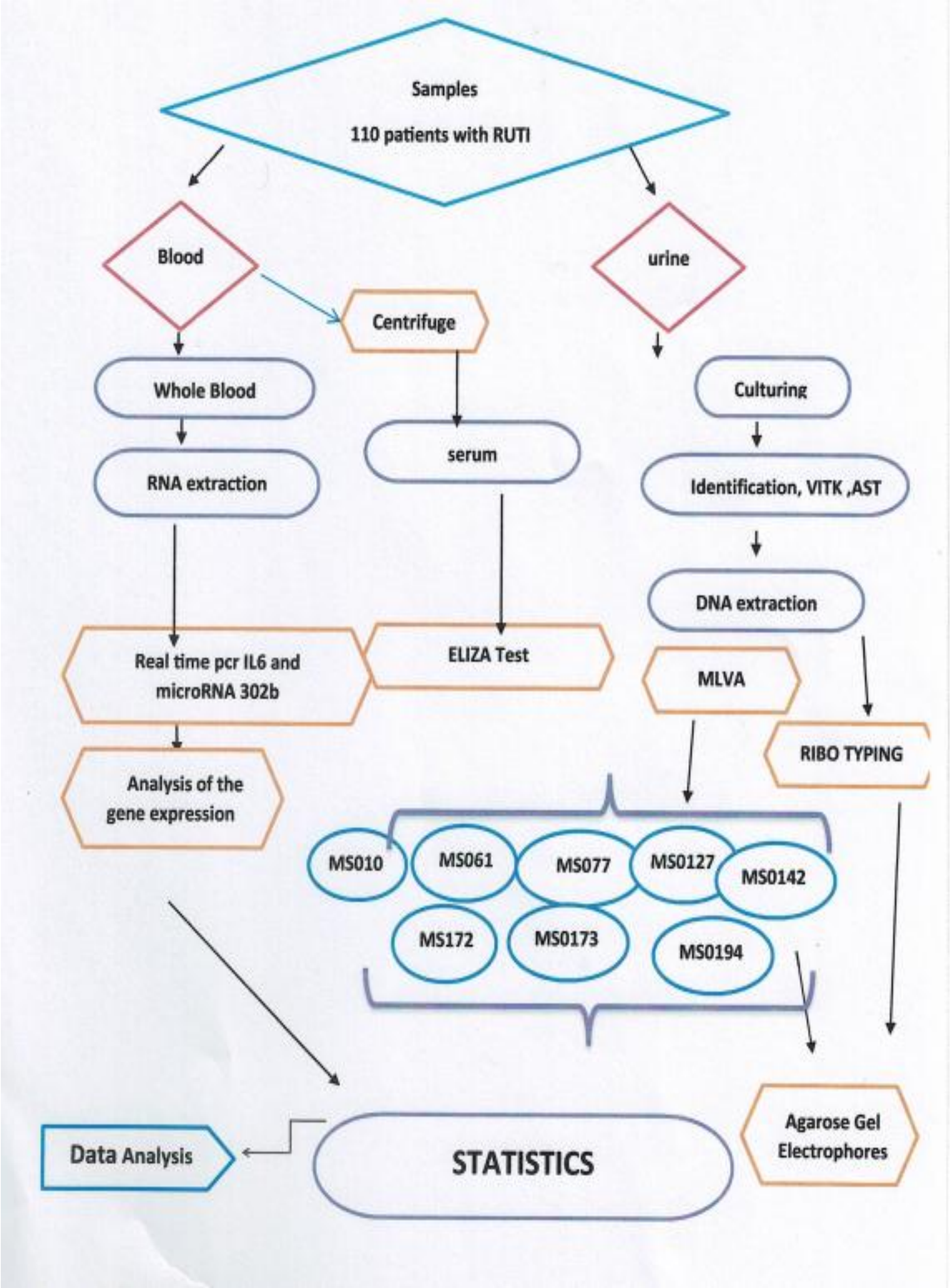
Patients with Recurrent Urinary tract infection (RUTI) who meet the diagnostic criteria and were diagnosed as recurrence for *P. aeruginosa* positive culture examination.

2.5.3. Exclusion criteria:

- Patients who received adjuvant chemoradiotherapy before sampling.
- Those who suffer from chronic diseases such as Patients with any infection and COVID. And Endocrine disorder, and diabetes.
- Patient who received antibiotic treatment .
- Those who refuse to participate in our study.

Study Design :

Everyone was acquaint of the investigation's goals and approved to follow protocol, and they signed the consent to participate in scientific research, as Show up in (appendix 1). A questionnaire was filled to enclose name, age, gender,



2.6. Ethical Approval :

The necessary ethical approval was obtained by verbal consent from patients. This study was approved by the committee of publication ethics at College of Medicine, Babylon Province, Iraq, under the reference No. BMS /0203/ 016.

2.7. Molecular Analysis :

2.7. 1. Genomic RNA extraction fom human blood:

The RNA extraction from fresh whole blood specimens were carried out according to the manual of manufacturer of Geneaid company **:**

The Genomic RNA extracted kit for blood Component show in Table (2- 13).

Table (2-13) RNA extraction kit for blood contents

Component	Volume Final
RBC Lysis Buffer	10 ml
RB Buffer	2ml
DNase I Reaction Buffer	200 µl
W1 Buffer	2 ml
Wash Buffer (Added Ethanol)	1.5 ml (6 ml)
RNase-free Water	1 ml
RB Column	4
2 ml Collection Tube	8

2.7.2. 1. Protocols of RNA extraction :

1. A volume of 300 µl of whole human blood was added to a sterile 1.5 ml microcentrifuge tube. Mixed by inversion.

2. A volume of 1 ml of RBC Lysis Buffer was added to the tube and incubated on ice for 10 minutes (briefly vortex twice during incubation).
3. Centrifuge at 3,000 x g for 5 minutes then remove the supernatant completely.
4. A volume of 400 µl of RB Buffer was added, and the tube was incubated at room temperature for 5 minutes.
5. A volume of 400 µl of Wash Buffer was added to the RB Column. Ethanol was confirmed to be added to the RB Column and centrifuge at 14000 x g for 30 seconds. Flow- through was discarded and the RB Column was placed in the 2 ml collection tube.
6. DNase 1 solution was prepared in a 1.5 ml microcentrifuge tube (RNase-free) as follow: **Table (2-14)**

Table (2-14) The DNase I prepared contents

Content	Volume
D Nase 1	5 µl (2 U/µl)
DNase 1 Reaction Buffer	45 µl
Total Volume	50 µl

7. By pipette DNase 1 solution was mixed carefully.
8. A volume of 50 µl DNase 1 solution was added into the RB column and Incubated for 15 minutes at room temperature (20-30ºC).
9. A volume of 400 µl of W1 Buffer was added into the RB Column, centrifuge at 14000 x g for 30 seconds. Flow-through was discarded then the RB Column was placed in the 2 ml collection Tube.

10. A volume of 600 µl of Wash Buffer was added into the RB Column. Centrifuge at 14000 x g for 30 seconds .
11. A volum of 600 µl of Wash Buffer was added into the RB Column, Centrifuge at 14000 x g for 30 seconds . The RB Column was placed back in the 2 ml collection tube and centrifuge at 14000 x g for 3 min to dry the column.
12. The dried RB Column was place in a clean 1.5 ml microcentrifuge tube.
13. A volume of 50 µl of RNase-free Water was added into the column. It was left for at least 1 minute to ensure that the RNase-free water was absorbed. Centrifuge at 14,000 x g for 1 minute to elute the purified RNA.
14. DNA digestion in Solution the DNase 1 reaction in a 1.5 ml microcentrifuge tube (RNase-free)was prepare as follows : **Table (2-15)**

Table (2-15) The DNase I reaction contents

Content	Volume
RNA in RNase-free Water	1-40 µl
DNase I	0.5 µl/µg RNA
DNase I Reaction Buffer	5 µl
RNase-free Water	Added to final
Total Volume	50 µl

15. By pipette DNase 1 solution was mixed carefully.
16. The microcentrifuge tube was incubated at 37ºC for 15-30 minutes.
17. The tube containing RNA was kept at -20 °C.

2.7.2. B . Measuring RNA with Nano Drop :

Measuring the amount and purity of purified RNA is crucial for determining the amount of each sample to use in q RT-PCR. NanoDrop Spectrophotometers (NDS), such as the one below, are very convenient instruments for assessing RNA quantity and quality.

Procedure:

1. Samples of RNA as well as water used to separate them were prepared on ice to a spectrophotometer.
2. The sample reader was blank with molecular grade water and dried with Kim wipe
3. Following the program instructions, 2 µl of water was loaded to initialize the system.
4. The computer setting was changed to RNA.
5. Two µl of sample was loaded and the Measure button was clicked.
6. After the reading was completed, the A260/A280 and A260/A230 ratios plus the amount of RNA recovered (ng/µl) were recorded.
7. The sample reader was wiped by Kimwipe clean and dry between samples and the steps were repeated.

2.7.2. 2. Interpreting the Results:

A lower than expected concentration of RNA indicates low cell numbers in the sample, poor homogenization of samples, or too much volume of water used in the elution step of RNA purification.

1. Very pure RNA will have an **A260/A280** ratio of ~2.1. Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential DNA or protein contamination. A low A260/A280

ratio is likely due to mixing phases when removing the upper aqueous phase or is also more common in samples with a very low yield of RNA.

2. The **A260/A230** ratio should also be above 2.0. A low A260/230 ratio indicates contamination with the wash solutions, chaotropic salts, phenols or protein. A low A260/A230 ratio is most likely due to contamination of the samples with washing buffers during the Mini spin washes.

2.8. Bacterial Genomic DNA extraction

2.8.1. A. Genomic DNA Mini Kit for bacteria:

The DNA extraction from fresh bacterial culture specimens were carried out according to the manual of manufacturer of Geneaid company **:**

The Genomic DNA extracted kit for bacteria contents show in Table (2-9).

2.8.1.2. DNA Extraction:

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (UK).Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a Thermal Cycler. Before PCR assay, DNA profile was performed by using bacterial DNA and loading buffer without thermal cycling conditions, and according to the following steps:

- One ml of bacterial suspension containing approximately up to1.2×10^9 cell/ml (equal to 4.0 McFarland standard) was transferred to a 1.5ml microcentrifuge tube, centrifugation for 1 minute at 14-16,000×g and discard the supernatant.

-A volume of 200µl of Gram Buffer in the 1.5 ml microcentrifuge tube ,incubated at 37^oC for 30 minutes. During incubation the tube was inverted every 10 minutes.

- A volume of 20 µl of proteinase K was added then mixed by vortex, incubated at 60^{o}C for at least 10 minutes. During incubation the tube was inverted every 3 minutes.

-A volume of 200 µl of GB Buffer was added to the sample and mix by vortex for 10 minutes.

-The sample lysate was incubated at 70ºC for at least 10 minutes. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 µl per sample) was pre-heated to70ºC (for step 5 DNA Elution).

-Following 70ºC incubation, 5 µl of RNase A (10mg ml) was added to the clear lysate and mixed by shaking vigorously.

-The lysate was incubated at room temperature for 5 minutes.

-A volume of 200 µl of absolute ethanol was added to the clear lysate and immediately mixed by shaking vigorously, the precipitate was broken up by pipetting.

-A GD Column was placed in a 2ml collection tube.

-All of the mixture was transferred (including any precipitate) to the GD column, centrifuged at 14000-16000 xg for 2 minutes.

-The 2 ml collection tube was discarded containing the flow-through and the GD column was placed in a new 2 ml collection tube.

-A volume of 400 µl of W1 buffer was added to the GD Column, Centrifuged at 14000-16000 g for 30 seconds.

-The flow-through was discarded and placed the GD column back in the 2ml collection tube.

-A volume of 600 µl of wash buffer (ethanol added) was added to the GD column, centrifuged at 14000-16000 xg for 30 seconds.

-The flow-through was discarded and placed the GD column back in the 2ml collection tube, Centrifuged again for 3 minutes at 14000-16000 xg to dry the column matrix.

-The dried GD column was transferred to a clean 1.5 ml centrifuge tube.

- Avolume of100 µl of preheated elution buffer or TE was added to the center of the matrix, centrifuged at 14000-16000 xg for 30 seconds to elute the purified DNA.

-Then detection of DNA by horizontal gel electrophoresis, and concentration was measured by Nanodroop DNA.

2.8.1.3.Estimation of the DNA concentration and purity:

The DNA concentration is detected by using the Nanodrop. The Nanodrop uses to measure the optical density (O.D) at wavelength of 260 nm and 280 nm by adding (1 micro liter) of the extracted DNA (Stephenson, 2003). The DNA purity ratio estimates according to this formula: DNA purity ratio = O.D 260 nm / O.D 280 nm

2 .9. PCR amplification:

2.9.1 . Real-time PCR (RT-PCR)

2.9.2. A. Detection of Gene expression of (miRNA302b and IL6) by PCR technique :

The real-time qPCR reactions were performed by using specific primers targeting reference gene GAPDH and the target genes microRNA 302b and IL6 gene expression (Table :2-16). Conversion the total RNA to cDNA and amplification of DNA was done according to instructions provided by GoTaq® 1-Step RT-qPCR System (Promega) using BRYT Green® dye, where RT-qPCR mixture and conditions were summarized in tables (2-17) and (2-18), where the final volume of RT-qPCR reaction was 20 µl. Relative expression fold was calculated by delta delta method (2^-(ΔΔCt) according to Livak and Schmittgen, 2001.

Table (2-16): Primers sequence and condition used in qRT-PCR mi302b and IL6 gene expression :

Primer name	*Sequence 5'-3'*	*step*	*Temp /Time/ cycles*
miR-302b-F	*GCGTAAGTGCTTCCATGTT*	-Revers transcription *-Reverse transcriptase inactivation and GOTaq DNA polymerase activation* *-Denaturation* *Annealing and data collection* *-Extension*	*37c° for15min.* *95c° for10min* *1 cycles* *10 c° for 30sec.* *37 c° for 30sec.* *40 cycles* *72c c° for 30sec.*
miR-302b-R	*TCCAGGGACCGAGGA* **(Ge *et al*.,2014)**		
GAPDH-F	*GTCTCCTCTGACTTCAACAGCG*		
GAPDH-R	*ACCACCCTGTTGCTGTAGCCAA*		
IL-6 Sense	GACAACTCATCTCATTCTGC G		
IL-6 AntiSense	TAACAACAACAATCTGAGGTG **(Allahbakhshian *et al*.,2020)**		

Table (2-17): RT-qPCR Mixture content

Component	Volume
GoTaq® qPCR Master Mix, 2X	10 µl
GoScript™ RT Mix for 1-Step RT-qPCR (50X)	0.5 µl
Forward primer (20X)	1µl
Reverse primer (20X)	1µl
RNA Template	5 µl
Nuclease-Free Water	2.5 µl
Total	20 µl

Table (2-18): RT-qPCR conditions

Step	Cycles	Temperature	Time
Reverse Transcreption	1	37 °C	15 minutes
Initial denaturation	1	95°C	10 minutes
Denaturation		10°C	30 seconds
Annealing	40	37°C	30 seconds
Extension		72°C	

2.10. Determination of IL-6 by Enzyme Linked Immunosorbent Assays (ELISA IL-6):

2.10. 1.Estimation of Human Interleukin - 6

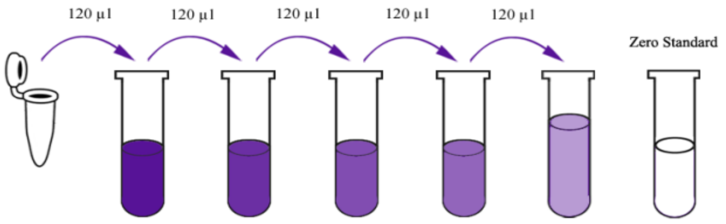
2.10. 2. A- Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human IL-6 antibody. IL-6 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human IL-6 Antibody is added and binds to IL-6 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-6 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human IL-6. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.10. 3. B- Reagent Preparation

1. All reagents should be brought to room temperature before use.
2. Standard Reconstitute the 120µl of the standard (640ng/L) with 120µl of standard diluent to generate a 320ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (320ng/L) 1:2 with standard diluent to produce160ng/L, 80ng/L, 40ng/L and20ng/L solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

320ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
160ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
80ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
40ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
20ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
640ng/L	320ng/L	160ng/L	80ng/L	40ng/L	20ng/L

Figure (2- 1) The Diluting of Stock Solution of IL – 6 .

3. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

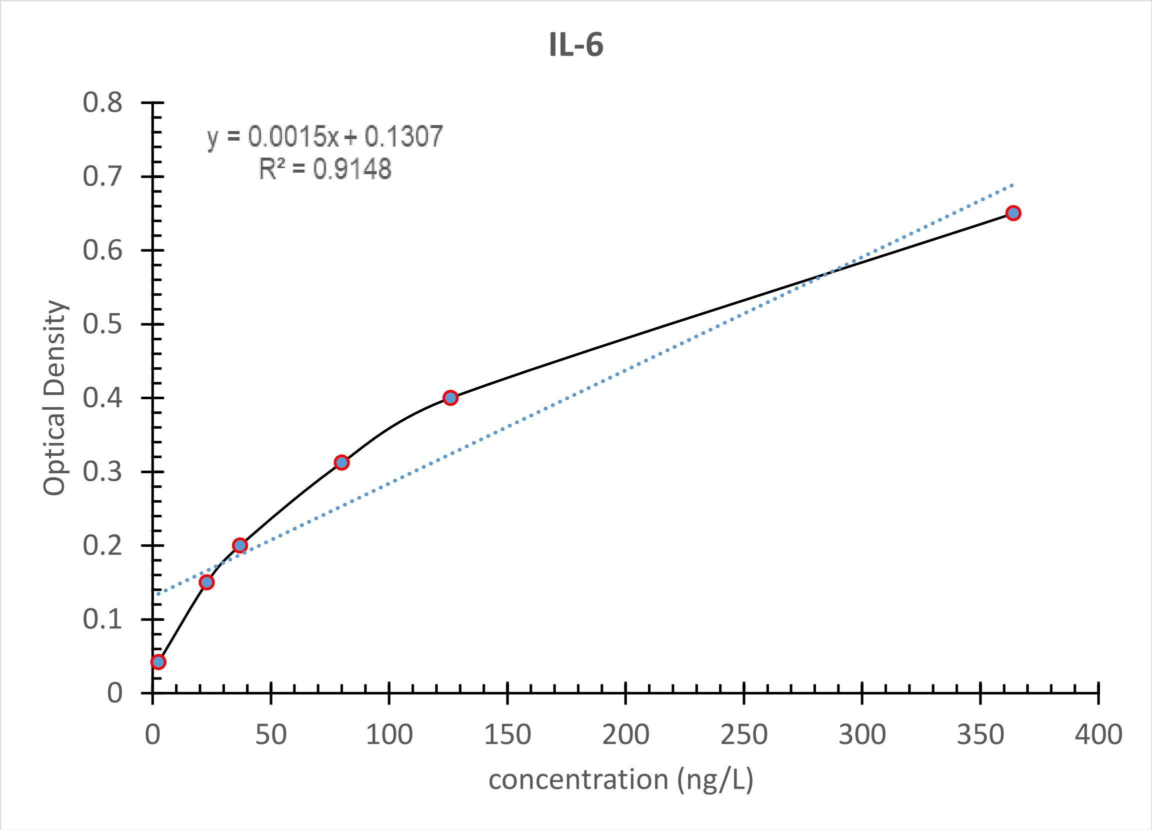
2.10. 3.C- Assay Procedure

1. Prepared all reagents, standard solutions and samples as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.
2. Determined the number of strips required for the assay. Inserted the strips in the frames for use. The unused strips were stored at 2-8°C.
3. Added 50μl standard to standard well. Note: Didn't add antibody to standard well because the standard solution contained biotinylated antibody.
4. Added 40μl sample to sample wells and then added 10μl anti-IL-6 antibody to sample wells, then added 50μl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mixed well. Covered the plate with a sealer. Incubated 60 minutes at 37°C.
5. Removed the sealer and washed the plate 5 times with wash buffer. Soaked wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated or decanted each well and washed 5 times with wash buffer. Blotted the plate onto paper towels or other absorbent material.
6. Added 50μl substrate solution A to each well and then added 50μl substrate solution B to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Added 50μl Stop Solution to each well, the blue color will changed into yellow immediately.
8. Determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.10. 4. D- Standard Curve

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best fit curve was drawn through the points on the graph as shown in figure (2. 2).



$y = 0.0015x + 0.13$

$R^2 = 0.9148$

Figure (2-2): Standard curve for Serum Human Interleukin 6 concentration

2.11. Molecular typing *P. aeruginosa* isolates.

2.11.1. MLVA of clinical *P. aeruginosa* isolates.

In this study, 30 isolates were DNA extraction was conducted for genotyping analysis by MLVA method. Subsequently, VNTR sequences located in several genes of bacterial genomes such as (MS-077, MS-127, MS-142, MS-172, MS-010, MS-061 and MS-173) were amplified by specific primers using PCR technique as in table (2-12) .

2.11.2. Minisatellite PCR amplification and genotyping conditions :

The PCR mixtures contained 1 ng of DNA, 1 *Taq* Reaction Buffer , 1 U of *Taq* DNA polymerase, 200 M each deoxynucleoside triphosphate, and 0.3 M each flanking primer .

Initial denaturation at 96°C for 5 min was followed by 30 cycles of denaturation at 96°C for 20 s, annealing at 60°C for 30 s, and elongation at 65°C for 90 s. The final extension step was 5 min at 65°C. Different annealing temperatures and/or elongation times were used for ms173-5186_243bp and ms194-5915_12bp (for ms173, the annealing temperature was 64°C and the extension time was 5 min at 70°C; for ms194, the annealing temperature was 65°C and the extension time was min at 70°C). Five microliters of each of the PCR products was run on standard 1% (ms173), 2% (ms142, ms172, ms194) or 3% (ms010, ms061, ms077, ms127) agarose gels in 0.5 TBE (Tris-borate-EDTA) buffer at a voltage of 10 V/cm. Gel runs (bromophenol blue position) of 20 cm (ms127, ms142), 30 cm (ms010, ms061, ms077, ms172), or 40 cm (ms173) were used according to the PCR product size and motif length. Gels were stained with ethidium bromide, visualized under UV light, and photographed.

After confirming the PCR amplification using electrophoresis and visualization of their bonds on agarose gel, relationship evolutionary graph for the different strains was constructed based on MLVA technique.

After the electrophoresis of PCR products and determination of VNTR copy-numbers, 30 strains were classified as types and genetic evolutionary tree was also constructed based on VNTRs Data. According to the MST algorithm, 20 clinical strains divided into clonal complexes which these criteria is interpreted as genetic distance based on the difference of VNTR copy numbers for each group.

Table (2-19) Primers used in of MLVA of clinical *P. aeruginosa* isolates according to (Onteniente *et al.*, 2003).

Locus Name		**Open reading frame**	Nucleotide Sequence	Size pb
1	ms077-2263_39bp	*pcoA*	*L: GCGTCATGGTCTGCATGTC* *R: TATACCCTCTTCGCCCAGTC*	**349-520**
2	ms127-3496_15bp	PA3115	*L: CTCGGAGTCTCTGCCAACTC* *R: GGCAGGACAGGATCTCGAC*	***210-225***
3	ms142-3873_115bp	A3463	*L: AGCAGTGCCAGTTGATGTTG* *R: GTGGGGCGAAGGAGTGAG*	***200-775***
4	ms172-5083_54bp	PA4541	*L: GGATTCTCTCGCACGAGGT* *R: TACGTGACCTGACGTTGGT*	***573-843***
5	ms194-5915_12bp	*algP*	*L: CCTTAGGAGGCGCTGGTC* *R: AGCTGCTGGCAAGGCTCT*	***600-700***
٦	ms010-0098_6bp	PA00816	*L: GCAGGAACGCTTGCAGCAGGT* *R: CTTCGCCGACCCAGGGATCA*	***143-233***
٧	ms061-1844_6bp	*pscP* 6 12	*L: CTTGCCGTGCTACCGATCC* *R: CCCCCATGCCAGTTGC*	***85-139***
8	ms173-5186_243bp	PA4625	*L: CTGCAGTTCGCGCAAGTC* *R: ATTTCAGCCAGCGTTACCAA*	***1,073-4,718***

2.11.3. Drawing an evolutionary link diagram and determine the strain type:

To draw the evolution chart, the data is entered into the software available at http: //mlva.upsud. fr/mlvav4/genotyping, and the resulting dendrogram for typing the isolates based on the stratified coefficient and the UPGMA algorithm. For this purpose, strains with 80% or more than 80% of the same repeat count (based on the difference in 2 loci VNTR (DLV)) were placed in one strain and other strains were placed in different strains.

2.12. Ribotyping *P. aeruginosa* isolates.

PCR-based methods have been proposed for identifying this cluster. Intergenic Transcribed Spacer PCR ribotyping (PCR-ribotyping) uses specific primers as show in table (2-13) below that amplifies sequences between the 16S and 23S gene and has been applied for molecular identification of bacteria at the species level and the discrimination of bacterial strains .

Primer Name		Nucleotide Sequence (5`to 3`)
1	Ribo-1	*F (5´-TTGTACACACCGCCCGTCA-3´)* *R (5´-GGTACCTTAGATGTTTCAGTTC-3´)*
2	Ribo-2	*F (5´-GAAGTCGTAACAAGG-3´)* *R (5´-CAAGGCATCCACCGT-3´)*

Table (2-20) Primer sequence used in Ribotyping methods of *P. aeruginosa* isolates.

2.12.1. PCR ribotyping - PCR ribotyping conditions:

PCR ribotyping - PCR ribotyping was done by ampliication of 16S-23S rRNA Intergenic Spacer Region (ISR) using universal primers forward and reverse ,The PCR assay was standardized with 1.5mM MgCl2 and 1U of *Taq* DNA polymerase , The temperature profile used was 60 sec at 95°C, 120 sec at 52°C, and 120 sec at 73°C. The amplification was done for 35 cycles in a thermal cycler PCR System , The product was electrophoresed in 2 percent agarose gel with the DNA molecular weight marker (100bp DNA ladder) and visualized after staining with (0.5**µ**g/ml) ethidium bromide in a gel documentation system .

2.12.2. Draw an evolutionary link diagram and determine the strain type:

To draw the evolution chart, the data is entered into the software available and the resulting dendrogram for typing the isolates based on the stratified coefficient and the UPGMA algorithm. were placed in one strain and other strains were placed in different strains.

2. 13. Agarose Gel Electrophoresis:

Agarose gel was prepared by dissolving 1.5% of agarose powder in 100ml of TBE buffer (pH:8) in boiling water bath, allowed to cool to 50°C and ethidium bromide at the concentration of 0.5mg/ml was added.

The comb was fixed at one end of the tray for making wells used for loading DNA sample.

The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber which was filled with TBE buffer covered the surface of the gel, 5µl of DNA sample was transferred in to the wells in agarose gel, and in one well, we put the 5µl DNA ladder mixed with 1µl of loading buffer.

The electric current was allowed at 60 volt for 80min. UV transilluminater in 280 nm was used for the observation of DNA bands, and gel was photographed using a digital camera (Deuk *et al* ., 2019) .

2. 13. Statistical Analysis:

Genetic analysis was performed by using Chi-square(x2) test. P values less than (0.05) is considered. Statistical analysis was performed by using SPSS 19 version. Data were expressed as (mean ± SD). The normality of the distribution of all variables was assessed by the student's ANOVA test and Pearson correlation analyses that have been used to determine the significant difference between the groups. Finally, a method T-test was used to find out the variables and calculate the results to some groups in the study.

2.14. 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. (Bergen, and Shelhamer, 1996) .

CHPTER

THREE

Result and Discussion

3. Results and Discussion:

3.1. Isolation and Identification of *P. aeruginosa* isolated from RUTI patients:

A total from (110) clinical specimen were collected from patients with recurrent Urinary tract Infection (RUTI), the result show that (30) (27.3%) of isolate belonged to ***pseudomonas aeruginosa*** and 50 (45.4%) specimen give other bacteria growth result and 30 of 110 (27.3) No growth, with a significant difference at P<0.0001 as shown in **Table (3-1)** .

Table (3-1) Distribution and percentage of *P. aeruginosa* strains isolated from patients with RUTI diseases.

Results of culturing		*No.*	*Percentage %*	*P value*
***P. aeruginosa* Positiv**		**30**	**27.3%**	**<0.0001***
Other bacterial growth	**G+V**	**22**	**24.2%**	
	G-Ve	**28**	**30.8**	
No growth		**30**	**27.3%**	
Total		**110**	**100%**	

*** represents a significant difference at P<0.05**

According to the results of the present, study 30 (27%) *P. aeruginosa* were recovered and identified from urine clinical samples in Babylon hospitals, Results in this study may be totally agree with previous studies whose found that dominance of *P. aeruginosa* among UTIs, (Inci *et al.*, 2016).

A study by Kamali *et al.*, (2020) who found that the percentage of *P. aeruginosa* isolate which isolated from different clinical specimens indicated that, the high percentage of isolating detect in urine (32.5%). Study by (Karami *et al.*, 2019) found that the percentage of *P. aeruginosa* isolates in Urine samples was (41.37%).

New study found that the frequency of *P. aeruginosa* in urinary tract infections were (8.3%) which disagreed with present study (Motbainor *et al.*, 2020).

A slightly higher percentage was recently reported by Al-Janahi (2020), found that a total from 4904 tested clinical samples where 3.1% *P. aeruginosa* isolates were detected .

In addition, the present finding is higher than the prior national research works, where isolation rate (26.1%, 18.8%, and 5.2%) were recorded by Belal (2010), Al-Shara (2013), and Rasool (2015), respectively.

When compared to previous studies carried out in Iraq, higher frequency rates were recorded in other similar studies in Hilla (Saleh, 2012), Al-Diwaniya (Al-Janabi, 2011) and Al-Nasiriya (Abdul-Wahid, 2014), in which 13.2%, 28.2%, and 18.3% of *P. aeruginosa* percentage rates were reported, respectively. This difference in prevalence rate among above studies could be attributed due to differences in sample number, hygienic strategies and geographical location.

A higher isolation rate (41.5%) was detected by Sedighi *et al*. (2015) in (Iran), where 106 *P. aeruginosa* isolates were identified from 255 collected from clinical samples including urine specimen.

Also Gomila, *et al.*,(2018) show that (9.6%) were belong to *P*. aeruginosa that can recurrently in cause RUTI and this difference in rate of isolation duo to number of sample collected.

Urinary tract infection is one of the leading causes of infection worldwide. In present studies, the frequency of *P. aeruginosa* are dissimilar to previous studies (Rasool, 2015; Al-Janahi, 2020). The lower frequency of *P. aeruginosa* was recovered from urine samples (2.6%).

Chronic persistent bacterial infections are a serious and growing public health problem worsened by the rise in antibiotic resistance, yet new approaches for treating these infections are lacking. These long-term infections can occur when bacteria invade and survive inside host cells where they can hide from the immune system and become less susceptible to killing by antibiotics. *P. aeruginosa*, a bacterium conventionally considered an extracellular pathogen, can cause chronic infections of many organ systems, including the urinary tract.(yang *et al.,* 2022).

Interestingly, present study found that *P. aeruginosa* was predominantly isolated from patients visited to hospitals (outpatients) as compared to inpatients, which was Actually, the dissemination of MDR *P. aeruginosa* from hospitals to the natural environment may increase the occurrence of community-acquired infections (Elshafiee *et al.,* 2019). Similarly, in previous study Al-Janahi (2020) showed a high prevalence of *P. aeruginosa* infection among outpatients to be 56% as compared with inpatients, 44%. In contrast, several studies reported the isolation of *P. aeruginosa* to be more common in hospitalized patients (Lila *et al.,* 2018). This may be explained by the failure of sterilization and inadequate application of standard precautions for infection control.

The result show that 50 (45.4%)of specimen give negative result to *P. aeruginosa* this may be due to other bacterial species and 30(27.3) show no bacterial growth ,the no–growth culture is either a viral infection or an acute urethral syndrome, which is a condition in which there are complaints suggesting UTI, but when cultured, organism is below the number that could be effectively cultured (Hamdan *et al*., 2011).

Moreover, symptomatic patients whose urine culture didn’t show appreciable growth might be due to other less frequent UTI causing microorganisms, such as parasites, fungi and viruses (Fürnkranz and Walochnik, 2021).

The present study included recurrent urinary tract infections. A Genetic factors are considered to be an important risk factor for susceptibility to UTI.(Godaly and Svanborg 2015). There is a genetic predisposition to recurrent RUTI and renal scarring (Fürnkranz and Walochnik, 2021) .

Penaranda *et al.*,(2021) showed that *P. aeruginosa* can in fact survive inside bladder epithelial cells and becomes tolerant to antibiotic treatment and the bacteria quickly adapt to the intracellular environment while the corresponding host cells upregulate the NF-κB signaling pathway according to gene expression analysis study.

One of the best studied bacterial resilient mechanisms involved in RUTI are biofilms,as in *P. aeruginosa* which provide tolerance to external stresses, such as antibiotic treatments and host defenses (Olsen, 2015). Bacteria in polymicrobial UTI also have the ability to protect one other from clinically relevant antibiotics through the increase of tolerant/resilient phenotypes in the bacterial community and causing recurrence (de Vos *et al.*, 2017).

Many strain-specific bacterial virulence factors may contribute to the recurrence of UTI, such as flagella/pili, adhesins, extracellular polysaccharides, toxins lipopolysaccharides, , ureases, proteases and iron-scavenging siderophores, This behavior has been reported in *Pseudomonas aeruginosa* (Ivanova *et al.*, 2015; Saini *et al.*, 2015), and may even be facilitated by polymicrobial interactions during infection (Gaston *et al.*, 2020).

These genetic factors such as mutation and polymorphism in some of genes are capable of interfering with any step during the process of bacteria invasion and regulate the inflammatory response before, during, and after UTI. These factors are different gene products such as cytokines, receptors, and adhesion molecules (Hussein *et al*. 2018).

The present study included *Pseudomonas aeruginosa* is one of the major pathogens as a cause recurrent urinary tract infection and this agreed with (Lambert *et al.*, 2011). This organism creates a serious public health disaster resulting in an enormous burden of morbidity, mortality in both developing and developed countries (Morales *et al.*, 2012). One of the reasons for the high pathogenicity of *P. aeruginosa* is the intrinsic high resistance to several antibiotics, as well as the development of multiple drug resistance (Langendonk *et al*., 2021). *P. aeruginosa* has been reporte as the most commonly isolated organisms in hospitals and the study is to evaluate the occurrence of clinical *P. aeruginosa* isolates and to find out whether *P. aeruginosa* has shown up in clinical samples the findings could be useful for detection an effective strategy to eliminate infections.

Pseudomonas aeruginosa is one of the common pathogens of urinary tract infection, especially after surgical interference. The presence of these bacteria is referred to the urinary tract through limited factors such as urinary catheterization in urinary tract patients, especially since most of these patients are elderly or suffer from immune-suppression for any reason, as these bacteria are characterized by their ability to colonize the roofs of urinary catheters and the formation of biofilms and, therefore, when transferred to the patient's urinary system causing urinary tract infections and becoming resistance to many antibiotics (Cole *et al*., 2018).

The difference in the isolation rates of *P.aeruginosa* isolates and their types in local and international studies may be due to several reasons, including different seasons of collection of samples, times of isolation and isolation source, different geographical location of isolation and number of samples, and other factors affecting, as well as "the most important factor is antibiotics and their misuse, whether the patient is using drugs that prevent the growth of bacteria or those resisted by bacteria grow, which contributed significantly to their spread (Iskandar *et al.*,2021) .

3.1.2. Frequency distribution of culture positivity of RTUI patient according to gender:

A total of (30) positive culturing isolate belong to ***P. aeruginosa***, it was found that (18 of 30) (16.3%) of total isolation obtained from males and (12 of 30)

(10%) of total isolation from females .and the result show a significant difference at p<0.05 as show in **Table (3- 2):**

Table (3-2): Frequency of culture positivity according to growth culture and gender in RUTI .

Positive growth	**Percentage an (NO.)**	**Gander (% &NO.)**	
		male	**Female**
P. aeruginosa	**30 (27.3) %**	**18(16.3)%**	**12(10.9)%**
Other bacteria	**50 (45.4)%**	**20(18.1)%**	**30(27.3)%**
No growth	**30 (27.3) %**	**10(9.00%**	**20(18.1)%**
Total	**110 (100)%**	**48(43.6)%**	**62(56.3)%**
P value	**p<0.05**		

The higher rate of infection with *P. aeruginosa* found in male than females, this due to virtue of male occupations, which exposed to different injuries making them more susceptible to these bacteria. On the other hand females which being high in UTI is due to female's urogenital tract being shorter than of males making then more susceptible to urinary tract infections(Anjum, & Mir 2010 and Pai *et al.*,2022)**.**

Recurrence of UTI were global statistics state that around 20-30% of women may suffer from re-infection within 3–4 months of the first occurrence. Several studies have reported the evolution of drug-resistant UTIs in the female population, especially among pregnant women. Factors such as age, structural changes, altered immune response, familial history, comorbid conditions, prior or current catheterization, lack of personal hygiene, and pregnancy could be some of the major factors attributing to the higher prevalence of UTI among females (Paudel *et al.*,2018 Murray*et al.*,2021).

In addition, history of UTI incidence, catheterization, poor socio-economic status, educational status, age between 25– 34 years, frequent sexual intercourse in the pregnancy period, recent contraceptive use, gestational age, and multiparity exhibit significant association with infection (Derese,*et al*.,2016), also The gestational stage in women is crucial due to several predisposing factors such as physiological and hormonal alterations during pregnancy, infection route, and urinary instrumentation that could induce the incidence of infection (Belete *et al.*,2020)

3.1.3.Prevalence of *P. aeruginosa* isolates according to age group:

A total of (30) isolate belong to ***P. aeruginosa*** were distribution according to age range from (**20- <50**) years , and the result show the highest percentage of *p . aeruginosa* isolates belong to(40-50)age group while the lowest isolate belong to age (20-30)years as show in **Table (3- 3):**

Table (3-3): Frequency of *P. aeruginosa* isolates according to age groups:

age groups (year	***NO. and rate of P. aeruginosa* isolates**
20-30	**4(13.3%)**
30-40	**5 (16.7%)**
40-50	**11(36.7%)**
50<	**10(33.3%)**
Total	**30(100%)**

The highest percentage of isolation of *p . aeruginosa* were (36.7%) in 40-50 year this may be due to they have weak immunity when Compare with lower age group. (Khan *et al.*, 2016).

The Men older than 50 years tend to get more infection than in younger .also the men get recurrent infection when they do not properly empty their bladder because of an enlarged prostate .also man may not empty their bladder if they have nerve damage from stork, uncontrolled diabetes or injury in the spine (Kallenbach, 2020).).

This study found that the high percentage of isolation detect in (40-50) years and(< 50)years age group, this may be due to this age are low immunity and which make them difficult to treated for UTI so the RUTI occur**.**

Gomila *et al.,* (2018). Found that factors associated with *P. aeruginosa* were age group with different factors such as male sex, steroid therapy, low functional capacity, having had antibiotics and manipulation of the cUTI, either insertion of urinary catheter or surgical procedures that anatomically modify the Urinary Tract Of these, anatomical UT modification was a risk factor for MDR *P. aeruginosa* cUTI and RUTI .

Chronicity and recurrence of *P. aeruginosa* infections and that targeting host urinary tract pathways in young age interestingly increased the infection with ability of *P. aeruginosa* to survive plays an important role in contributing to the chronicity and recurrence and that targeting host urinary tract pathways (Yang*et al.,*2022).

The present results showed the mean age of patients are 38.65 ± 10.97 years and 25± 5 years for control group (P= 0.739), these results agree with results of(I E Ali *et al.* 2018), which showed the mean age of the patients at diagnosis time was 26.49±4.623 years (range 19-43 years). Regarding to the age groups, this study showed that the affected age groups were different according to the sex as shown in **table (3-4).**

The affected ages in males were mostly distributed in old-ages , thus, 11 (36.7 %) of the cases were 40-50 years old. While, in females the distribution of cases were; 12(40 %) in 20->50 years old age groups**.**

So that affected females were distributed in the age between 20->50 years old. The relationship between sexual activity and UTI was well establish in younger women. During intercourse, vaginal bacteria gained access to the urinary tract by colonizing the periurethral mucosa and ascending to the bladder through the urethra (Hooton 2012)**.**

Table (3-4): Demographic Characteristics of patients with recurrent urinary tract infection associated with *P. aeruginosa* and control subjects.

Characteristic	**Patients** ***N0* = 30**	**Control** ***N0* =20**	***P***
Age (years)			
Mean ±SD	38.65 ± 10.97	25 ± 5.0	**0.739 †** **NS**
Range	20-<50	20 -<50	
20-30, n (%)	4 (13.3 %)	5 (25.0 %)	**0.062 ¥** **NS**
30-40, n (%)	5 (16.7 %)	5 (25.0 %)	
40-50, n (%)	11 (36.7 %)	5 (25.0 %)	
≥ 50, n (%)	10 (33.3 %)	5 (25.0 %)	
Gender			
Male, n (%)	18 (60.0 %)	12 (60.0%)	**0.001** **S**
Female, n (%)	12 (40.0 %)	8 (40.0%)	
Male: Female	3: 2		

***N0*: number of cases; SD: standard deviation; †: independent samples t-test; ¥: Chi-square test; S: significant at $P > 0.05$.**

Generally RUTI in males are more prevalent in older age groups than other age groups, it can be due to some factors such as prostate enlargement, or an age-related decrease of antibacterial activity in prostate secretions that lead to urinary tract infection in males at old-age or urological operations or diabetes and diabetic cystopathy (Agbugui and Osaigbovo 2016).Also this might be explained with immunosenescence and/or degenerative changes in the prostate and decreased defense mechanisms of the urethral mucosa(Stangl-Kremser *et al.* 2019) .

Generally, The present findings were in consistent with the results of (Akgül and Karakan, 2018). That show the females are more susceptible to UTI than males, because their urethra is shorter and closer to anus than in males, so that the intestinal flora which ascend to urinary tract and cause UTI , For physiological and anatomical reasons, women are more prone to bacteriuria than men, because structurally the female urethra is less effective in preventing the bacterial entry for colonization (Themphachana *et al.* 2015).

(Biggel *et al.* 2019) showed that females are more susceptible to asymptomatic UTI than males. The present findings were inconsistent with the results of(Bazaid *et al*. 2021), which showed the majority of the patients with UTIs were young female**.**

Males patients with urinary tract infection were more affected in age group of more than 50 years old. Inconsistence with current findings,

The result of this study agree with (Chu *et al.,* 2018),show *p. aeruginosa* isolated from RUTI varied across different age groups to up recurrent uncomplicated UTI, culture and sensitivity analysis should be performed at least once while the patient is symptomatic.

That according to gender, higher percentage detected with males (18) than female (12),result indicating that males show greater vulnerability for infections. however, the results in the current study revealed that, there is significant association between *P. aeruginosa* infections and age. It is noteworthy to mention that result was disagreed with several studies who were reported the high rate of *P. aeruginosa* infection among age group more than 50 years (Srinivas *et al.,* 2012; Ahmed *et al.,* 2019). In light of this study advancing age might be one of the predisposing factors.

This finding be consistent with DoToka (2021) who found that dominance of *P. aeruginosa* among UTIs and the number of isolates obtained from males was 12 (60%) and 8 isolates from females (40%),. This result may be consolidated in all similar studies conducted in different regions in the world (Ahmadi *et al.,* 2016; Amankwa, 2017).

Durmaz, and Celik (2018), showed in postmenopausal women, vaginal PH drops due to the lack of estrogen, the colonization of lactobacilli decreases. Thus, it facilitates colonization of the vagina with the possible uropathogens originating from the gastrointestinal tract. This can be explained by the high prevalence of UTI in females in these age group. Also the present findings agree with Mohan *et al*.,(2017), Who found that the disease effects all age group but the females are affected mostly between 50-60 years old. also revealed an increase in UTI incidence among older females (>50 years old) (Simmering *et al.,* 2017), and this variation in age groups can be due to differences in the study cohort.

3.2. Identification of *P. aeruginosa* from Recurrent Urinary Tract Infections.

All the *P. aeruginosa* isolate were phenotypically identified on (Blood agar, MacConkey agar, Cetrimide agar, and Nutrient agar) revealed that the colonies on the blood agar appear as white to gray color, sticky textures bacteria, and their ability to hemolyze blood and display form of hemolysis were examined. while On MacConkey agar, the colony appear of *P. aeruginosa* non-lactose fermenter .

Furthermore, *P.aeruginosa* can grow on Nutrient agar at 42°C but not at 4°C. *P.aeruginosa* is distinguished from other Pseudomonas species by its ability to grow at high temperatures(Rouhi & Ramazanzadeh, 2020).

The isolates were then examined microscopically by using Gram stain, and they were mostly tinted with pink color referred to gram negative, and appeared straight or slightly rods shapes. Biochemical tests verified all *P. aeruginosa* isolates as Gram (-ve) with typical bacterial characteristics. The oxidase test (positive result) with violet color, and bubble formation (positive result) indicates catalase's ability to degrade H_2O_2 (Gillespie and Hawkey,2006) suggested using these findings for diagnosis *P. aeruginosa*. The results of the phenotypic characterization based on physiological, morphological, and biochemical tests as shown in **table (3-5).**

A specimen was considered positive for RUTI if a single organism was cultured at a concentration of $\geq 10^5$ cfu/ml. Bacterial identification was made using biochemical tests, namely , oxidase, decarboxylase, lactose fermentation , catalase, coagulase, (Bonadio *et al*. 2001). and urine nitrite positive indicates the presence of nitrate-reducing bacteria(Demilie *et al*. 2014).

Table (3-5): Biochemical test for identification of *Pseudomonas aeruginosa* from different clinical sample.

Biochemical test	Result
Catalase test	**+ Ve**
Oxidase test	**+ Ve**
Gram stain	**- Ve**
Hemolysis production	**+ Ve**
Lactose fermentation	**- Ve**
Growth at 42°c	**+ Ve**
Growth on Cetrimide agar and Pigments Production	**+ Ve**

(+) Positive test, (-) Negative test

The gold standard for diagnosing recurrent RUTIs is still the urine culture, followed by antimicrobial susceptibility testing (AST). using the clean-catch midstream urine samples (Sathiananthamoorthy *et al.* 2019). urine culture and antibiotic susceptibility testing are recommended because diverse species can cause infections under these situations (Bonkat *et al*. 2019).

The collected urine samples were processed and using a calibrated loop (0.001 ml) urine specimens were inoculated in to blood agar and MacConkey agar plates and citrimaid agar. After overnight incubation at 37 °C for 24–48 h' colonies were counts to check significant growth. Colony counts of bacterial growth of $> 10^5$/ml of urine were significant(Tadesse *et al.* 2018).

To confirm a diagnosis of recurrent RUTIs, there should be documentation of positive urine cultures associated with appropriate urinary symptoms. (Anger *et al*. 2019).

Further the identification for *P. aeruginosa* VITEK 2 system (bioMe´rieux, Marcy l'Etoile, France) has been shown to be a reliable fully automated instrument for the identification of microorganisms , Automated methods are the quickest, and most accurate of the bacterial identification methods The Vitek2 system consists of a plastic reagent cards consist of microliter quantitative of several biochemical test media in 30 wells supply a biochemical profile that is used for organism diagnosis(Maina and Kagotho 2014) . Using VITEK 2 system used the identification card for Gram negative strains (ID-GNB). The obtained result shows an interesting percentage of accuracy (99%) (Appendix).

3.2.1. Pigment Production on Cetrimide Agar

The result of this study show that all isolate of *P. aeruginosa* are produce to pigment which varies between pyocyanin and pyoverdine, The development of blue greenish color pigments and a fruity odor on selective medium cetrimide agar was particularly associated with *P. aeruginosa*, and the production of certain pigments on Nutrient or Muller-Hington agar was especially associated with *P. aeruginosa*(AL-Shamaa *et al*., 2016).

On the selective medium (Cetrimide agar), colonies of *P. aeruginosa* were circular, mucoid and smooth. The most common types of pigments produced by *P. aeruginosa* in this study were the pyocyanin were(73.3%) followed by pyoverdine were (26.7%). Cetrimide enhances the production of *Pseudomonas* pigments such as pyocyanin and pyoverdine, which show a characteristic green in (15)(50%), and yellow-green color in(7)(23.3), blue-green in(5)(16.7%), and yellow –brown in(3)(10%) respectively The types of pigments produced by *P. aeruginosa* isolates are shown in **table (3-6).**

Table (3-6): Pigments production by *P. aeruginosa* on Cetrimide agar.

No.	Types of Pigments	No. of Isolates	Percentage of *P. aeruginosa* isolates
1	Green (pyocyanin)	15	50%
2	Yellow- Green (pyocyan	7	23.3%
3	Blue-Green (pyoverdin	5	16.7%
4	Yellow – Brown (pyoverdine)	3	10%
5	No pigment	0	0%
Total		30	100%

The results of current study revealed the ability of (73.3%) *P. aeruginosa* isolates to produce pyocyanin making them more virulent and have great harmful consequences due to implication of pyocyanin in tissue damage, interfering with immune response and triggering proinflamattory responses (Hall *et al*., 2016).

Pseudomonas and *Streptomyces* produce natural phenazines, which are versatile secondary metabolites. Two precursors give rise to all phenazine-type metabolites: phenazine-1-carboxylic acid (PCA) in *Pseudomonas* Pyocyanin biosynthesis was investigated via detection of Phenazine gene (*phzA*) which encode for phenazine-1-carboxylic acid (PCA) who subsequently converted to pyocyanin by two enzyme: Phenazine-1-carboxylate N-methyltransferase and Flavin-containing monooxygenase respectively. (Guo *et al*., 2017). Pyocyanin has shown to interfere with extracellular DNA and affect physicochemical interactions and cell surface properties to facilitate cell-to-cell interactions between *P. aeruginosa* cells. Pyocyanin has also been related to the development of biofilms by encouraging the production of extracellular DNA (Das and Manefield, 2012).

Recurrence and reinfection associated with UTI, Pyocyanin production strain is involving it can facilitate biofilm formation via an iron-independent pathway. (Das and Manefield, 2012; Saranya and Thayumanavan, 2013; Das *et al*., 2013).

The predicted cell cytotoxicity is in accordance with the reported sites of infections by *P.aerugenosa* . As it causes chronic urinary tract infections, and also induces apoptosis in neutrophils via PCN. PCN has the ability to modulate expression profiles in various tissue types, as well as already established immunomodulatory role (Cole *et al.*, 2018).

higher pathogenicity and resistant isolate from RUTI agree with Sakhtah *et al*.(2016) showed that a human population infected with PCN-producing *P. aeruginosa* this on the urolological and the vascular system, with mechanisms other than oxidative stress implicated in a number of circumstances with PCN-producing *P. aeruginosa* have a diverse range of toxic effects have been observed, physiologically predominantly mediated by free radical and pro-inflammatory cytokine production. (Hall *et al*., 2016).

P. aeruginosa possess a phylogenetic taxonomic characteristic, and many members produce and can produce pigments, such as pyocyanin (green blue) and pyoverdin (yellow green) fluorescence pigments (Durán,*et al*., 2022).

Pyocyanin is an important virulence factor, which is one of the numerous pigmented phenazines produced by *P.aeruginosa*. Pyocyanin is produced by this bacteria during biofilm formation and play a role in acute and chronic infections. In addition, it serves as a redoxactive antimicrobial compound and enables anaerobic survival (Hotterbeekx *et al*., 2017).

By its inter-and intracellular signaling, pyocyanin enables *P. aeruginosa* to display successful competition with other bacteria and even fungi (Tashiro *et al*.,2013).Furthermore, the presence of G+ve organisms, in urinary tract including some Staphylococcus spp., can stimulate the PQS system due to induce pyocyanin production in *P.aeruginosa* (Whiley *et a*l., 2014).

Non-fluorescent bluish pigment pyocyanin is often produced by *P.aeruginosa,* which diffuses into the agar. Other *Pseudomonas* species do not produce pyocyanin. Many strains of *P.aeruginosa* also produce the fluorescent pigment pyoverdin, which gives a greenish color to the agar, some strains produce the dark red pigment pyorubin, or the black pigment pyomelanin (Brooks *et al.*, 2013). *P.aeruginosa* strains that do not produce pyocyanin have a low pathogenicity and higher susceptibility to the immune response (Nowroozi *et al.*, 2012). This suggest highly virulent isolate in current study from recurrent urinary tract infection .

Production of virulence factors in *P.aeruginosa* is a strategy for survival to evade the immune defense of host (Feng *et al.*, 2016). they are mainly under the regulatory control of the quorum sensing systems (García- Contreras, 2016). this plays a critical role during pathogenesis for survival and colonization by coordinating progress of acute to chronic phenotype regulating, (Barr *et al.*, 2015).

3.3 Antimicrobial susceptibility test

Thirty of *P. aeruginosa* were subjected to an antibiogram examination according to the Clinical and Laboratory Standards Institute to determine possible resistance to 13 antibiotics . The results were interpreted according to the recommendation of CLSI (2021) which listed in appendix (2).

P. aeruginosa clinical isolates showed a wide range of resistance against 13 antimicrobial agents: aminoglycosides class (amikacin, tobramycin and gentamicin), cephalosporins class (Cefepime, ceftazidime and ceftriaxone), carbepenems class(imipenem and meropenem), Fluoroquinolones class (norfloxaccin, Ciprofloxacin), β-Lactamase inhibitors class(piperacillin, (carbencillin). and the result show in figure (3-1)and table (3-7).

The susceptibility test of the *P. aeruginosa* in the current study showed the highest resistance to the following antibiotics which including

Piparacillin 26 (86.6%) , Ceftriaxone 24 (80%), Ceftazidiame25 (83.3%) Carbencillin 23 (76.6%), Meropenem 23 (76.6%) , tobramycin 22 (73.3%, Amikacin 21(70%), Ciprofloxacin 20 (66.6%), Gentamicin which include

20(66.6%), while the isolates showed lower resistance to the antibiotics Cefepime19 (63.3%), Norfloxacin 18 (60%) and Imipenem with percentages 16 (53.3%).

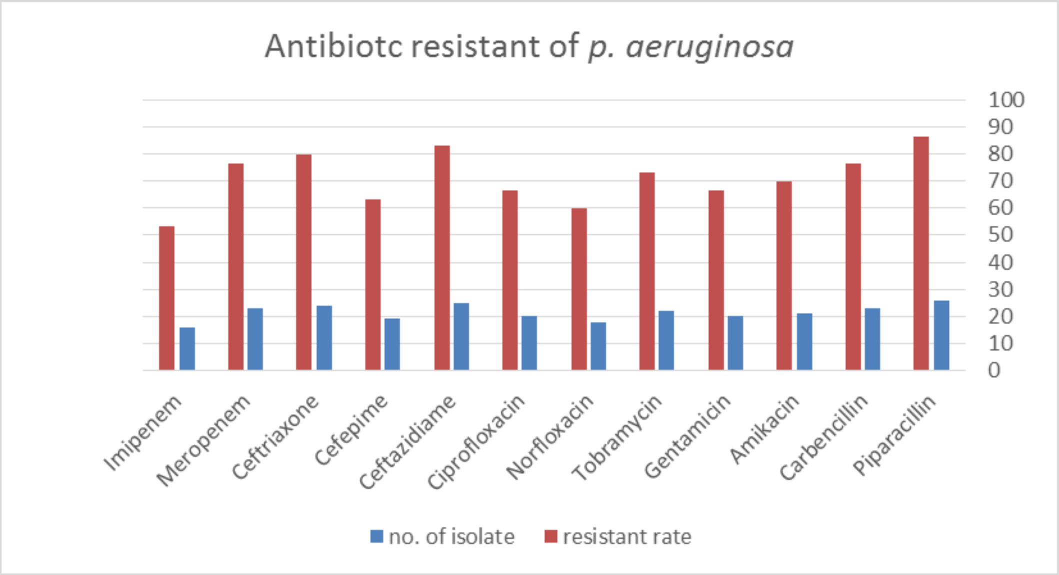


Figure (3-1) percentage of resistance result of the *P. aeruginosa* isolated from RUTI to different antibiotics.

Pseudomonas aeruginosa is intrinsically resistant to a wide range of antimicrobials as well as able to get acquired resistance to effective antibiotics which lead to problematic conditions (Pang *et al.*, 2019).

The diversity of antibiotic resistance mechanisms contributes to the development of multiple antibiotic resistant isolates, and makes conventional antibiotics ineffective for the treatment of *P. aeruginosa* infections (Peterson and Kaur, 2018).

The periodic surveillance, testing and monitoring of antibiotic resistance of *P. aeruginosa* is very important in order to provide updated information about the current activity of commonly used antipseudomonal antibiotics in hospitals and would enable the physicians to detect the trends in the resistance pattern to the commonly prescribed antibiotics in a given organism. (Hasan *et al.*, 2019; Al-Khikani, 2020).

Table (3-7): Antibiotic susceptibility results (N0 = 30).

N	Type of antibiotics	Resistanc isolates No.	%	Intermedia isolates No.	%	ensitive isolate No.	%	P-value
1	Piparacillin	26	86.6	0	0	4	13.4	0.0001 **
2	Carbencillin	23	76.6	0	0	7	23.4	0.0001 **
3	Amikacin	21	70	1	3.3	8	26.6	0.0001 **
4	Gentamicin	20	66.6	0	0	10	33.4	0.0001 **
5	Tobramycin	22	73.3	2	6.6	6	20	0.0001 **
6	Norfloxacin	18	60	1	3.3	11	36.6	0.0001 **
7	Ciprofloxacin	20	66.6	2	6.6	8	26.6	0.0001 **
8	Ceftazidiame	25	83.3	1	3.3	4	13.3	0.0001 **
9	Cefepime	19	63.3	3	10	8	26.6	0.0001 **
10	Ceftriaxone	24	80	1	3.3	5	16.6	0.0001 **
11	Meropenem	23	76.6	1	3.3	6	20	0.0001 **
12	Imipenem	16	53.3	2	6.6	12	40	0.0001 **
P-value		---	0.0002 **	---	0.041	-	0.0014 *	---
*** (P≤0.05), ** (P≤0.01).**								

It was found that the vast majority of *P. aeruginosa* isolates showed high levels of resistance to most commonly-used antibiotics and there was no antibiotic which inhibits all tested *P. aeruginosa* isolates (Table 3-7). This result is in accordance with those of other studies conducted by Al-Shara (2013) and AlKhudhairy and Al-Shammari, (2020) .

In this study, piperacillin, the extended spectrum penicillin, was tested for the susceptibility of *P. aeruginosa* isolates. Current finding indicated that 86.6% of isolates were found to be resistant to piperacillin, while previous resistance rate for this antibiotic was reported 60.5%, 59.6% and 77.7% by Al-Shara (2013), Rasool (2021), and Al-Janahi (2020), respectively. The high resistant rate of this antibiotic in *P. aeruginosa* may be due to the frequent use of this antibiotic in both of community and clinical settings in the country.

P.aeruginosa can produce β-lactamase enzymes which is the most common and important mechanism of resistance in Gram-negative bacteria. β-lactamase diffuses through or directly traverse porin channels in the outer membrane of Gram-negative bacterial cell walls. *P.aeruginosa* either decreased porin production or increased efflux and reduced permeability (Idowu, *et al*., 2019).

Third generation cephalosporin group are regarded as antipseudomonal drugs, particularly ceftriaxone and ceftazidime, which were tested against *P. aeruginosa* isolates. The data of the present study showed high presentage of resistance were found 80% and 83.3% respectively. This finding is consistence with other findings (Rasool, 2021; Al-Janahi, 2020). In addition, these high values of resistance which were observed were considerably comparable to those of the reports, with resistance values of 76.9%, and 50.8% against ceftriaxone, and ceftazidime, respectively (Abdul-Wahid, 2014).

The reduced of susceptibility to third generation cephalosporin may be related to the more extensive use of these antibiotics in hospital. On the other hand, additional resistance mechanisms especially production of ESBLs and other enzymes may contribute to cephalosporins resistance (Ahmad *et al.*, 2016).

Cefepime, a fourth-generation cephalosporin, is one of the few agents that remaining has reliable activity against *P. aeruginosa* isolates. The increased frequency of resistance to cefepime among *P. aeruginosa* has been reported globally (Akhabue *et al.*, 2011).The data of the present study showed high level of resistance to cefepime (63.3%), which is quite worrisome and may reflect the increased use of cefepime in recent years in Babylon hospitals. Prior treatment with cefepime can lead to emergence of cefepime-resistant *P. aeruginosa* (Akhabue *et al.*, 2011). In addition, resistance may arise by the acquisition of plasmids encoding β-lactamases (Bennett, 2008). However, present results are similar to the findings of local studies done by Al-Muhannak (2010) and Al-

Janahi (2020) in which resistance rate of cefepime against clinical isolates of *P. aeruginosa* was 93.2% and 80.4%, respectively.

Due to the stability against ESBLs, carbapenems (imipenem and meropenem) are considered as the most potent agents for multiple antibiotic resistant *P. aeruginosa* (Çiçek *et al*., 2021). however, due to the inappropriate drug use and improper dosage of carbapenem, development of drug resistance can occur in clinical settings and increasing worldwide (Xu *et al.,* 2020).

Also current study found that 53.3% isolates were resistant to imipenem and 76.66% were resistant to meropenem. This rate of resistance reflects a risk restricting of therapeutic options. It is reasonable to explain that this may be correlated with the extent use of carbapenems in recent years as well as a selective pressure from increased prescriptions of carbapenem. The WHO has listed carbapenem resistant *P. aeruginosa* as one of three bacterial species in which there is a critical need for the development of new antimicrobial agents to treat infections (Tacconelli *et al.,* 2017).

Aminoglycosides are broad-spectrum antibiotics of high potency that have been traditionally used for the treatment of serious infections caused by Gram negative pathogens, such as *P. aeruginosa* infections (Gholami *et al*., 2017). In the current study, aminoglycosides appear to be one of the effective classes of antibiotics against the examined *P. aeruginosa* isolates. This study found that 70%, 66.6% and 73.3% of the isolates were resistant to the aminoglycosides amikacin gentamicin, and tobramycin, respectively. However, moderate rates of resistance to aminoglycosides antibiotics, was also reported by Al-Janahi (2020) in Najaf. Additionally, rates of resistance of *P. aeruginosa* isolates to tobramycin and amikacin were somewhat dissimilar to previously reported by Rasool (2015) in (32.7% and 23%, respectively) and Abdul-Wahid (2014) in (32.3% and 18.5%, respectively). Among the several studies, aminoglycosides, especially amikacin, showed a significant high susceptibility rate in some countries. In Saudi Arabia and Indonesia amikacin had the lowest resistance rate 6% (Al-Tawfiq *et al*., 2020) and 10.5% (Rustini *et al*., 2017).

The high frequencies of *P. aeruginosa* isolates resistant to aminoglycosides have also been reported in other health institutions (Murphy-belcaster *et al*., 2021). These varying of resistance rates might be due to the difference in selective pressure exerted by antibiotic usage or the quality of infection control practice.

Fluoroquinolones like ciprofloxacin and levofloxacin are some of the most commonly prescribed effective antimicrobials against *P. aeruginosa* infections (Gholami *et al*., 2017). However, *P. aeruginosa* rapidly becomes resistant to these drugs during antibiotic therapy. The principle mechanism of fluoroquinolones resistance in *P. aeruginosa* involves mutations in the genes of DNA gyrase and topoisomerase IV(Yang *et al.*, 2015) Interestingly, in this study 66.6%, 25.2%, and 60% resistance of *P. aeruginosa* isolates to ciprofloxacin, levofloxacin, and norfloxacin is agree with in Germany, France, Spain, Italy, who found the resistance rates to fluoroquinolones were 85.8%, 84.9%, 79.9%, 74.9%, respectively (Litwin *et al.,* 2021). dissimilarly to present findings, in the study done by Al-Janahi *et al*. (2020) founds 32%, 32.7%, and 35.3% of isolates exhibited resistance to ciprofloxacin, levofloxacin, and norfloxacin, respectively. Unfortunately, overuse of fluoroquinolones in Iraq has promoted bacterial resistance to fluoroquinolones in recent years, which has caused a huge challenge in the anti-infective therapy of *P. aeruginosa* (Hadi, 2015). Therefore, the monitoring of antimicrobial susceptibility is crucial for selecting effective antimicrobial agents in the treatment of the disease.

The study showed that the multi drug resistance (MDR) isolated which resistant to three classes of antimicrobials, increased amongst these organisms making it difficult to choose appropriate suitable antimicrobial therapy and the increased resistance of *P. aeruginosa* to numerous antibiotics, as a result of excessive antibiotic administration, is now leading to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of MDR forms of *P. aeruginosa* (Yayan *et al*., 2015) .

The MDR strain of *P. aeruginosa* may be found in RUTI patient due to frequently exposed to antimicrobial agents throughout their hospitalization, increasing the likelihood of colonization or infection with drug-resistant organisms (Nader *et al*., 2017) .

According to the infections caused by potentially harmful pathogen in the hospital environment and health center act as possible reservoirs for *P.aeruginosa* (Doughari *et al*., 2018).

3.4. Gene expression of micro302b by RT-PCR quantitation:

A Total from 30 blood patient sample, RNA was extracted to detect the gene expressing of microRNA302b by RT-qPCR(Relative gene expression[2ΔCt] methods ,in this method the expression level of micro302b gene in test sample as well as in control sample normalized with house-keeping gene GAPDH as show in figure(3-٢) .

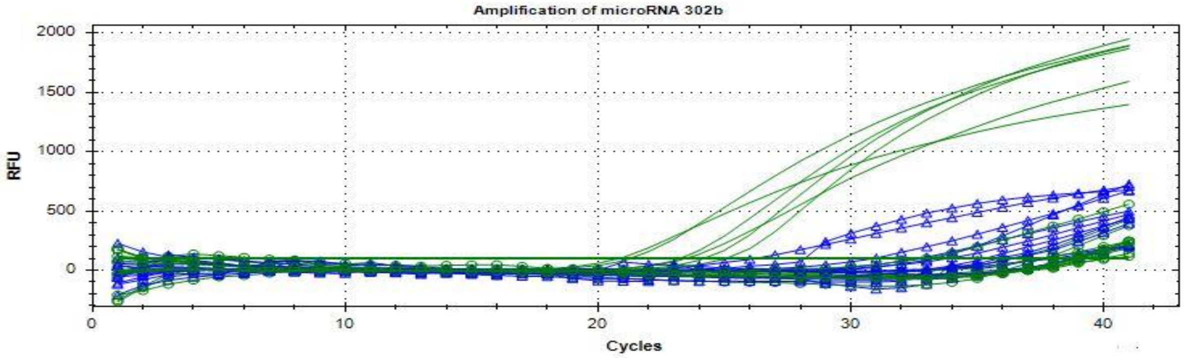


Figure (3-٢): microRNA 302b gene expression level. This is the first run for 15 samples, ⁄ represents amplification of Reference gene (GAPDH), △ represents amplification of samples RUTI Patients, ○ represents amplification of control samples

The results of this work show the microRNA unregulated during infection with recurrent urinary infection (RUTI) with *p. aeruginosa* that microRNA unregulated during infection with recurrent urinary infection,

as (Elbishbishy, 2019) show that microRNA-mediated regulation in signaling networks is particularly important in addition, the evolutionary analysis shows that humans microRNA-mediated regulation of receptors have diverged significantly increase the chances of microRNA regulation. One of the major regulators of cell signalling are microRNAs that regulate gene expression post-transcriptionally in many diseases.

Also Huang *et al.*,(2020) found that mi302b expression was significantly elevated with *P. aeruginosa* .at 3h and 6h,these finding suggest that both mi302a and mi302b may be involved in the process of host defense during *P. aeruginosa* infection, As all other non-coding sequences, the significance of microRNAs has only been established, as one of the major regulators of gene expression that are able to target more than 60% of all human protein-coding genes as well as being involved in many diseases.

The present study found that the expression of Mi302b gene expression increased in the RUTI patient with *P. aeruginosa* when compared with control group, so the expression gene is increased more than (% 25) fold when compare with control group,as show in figure (3-3) .

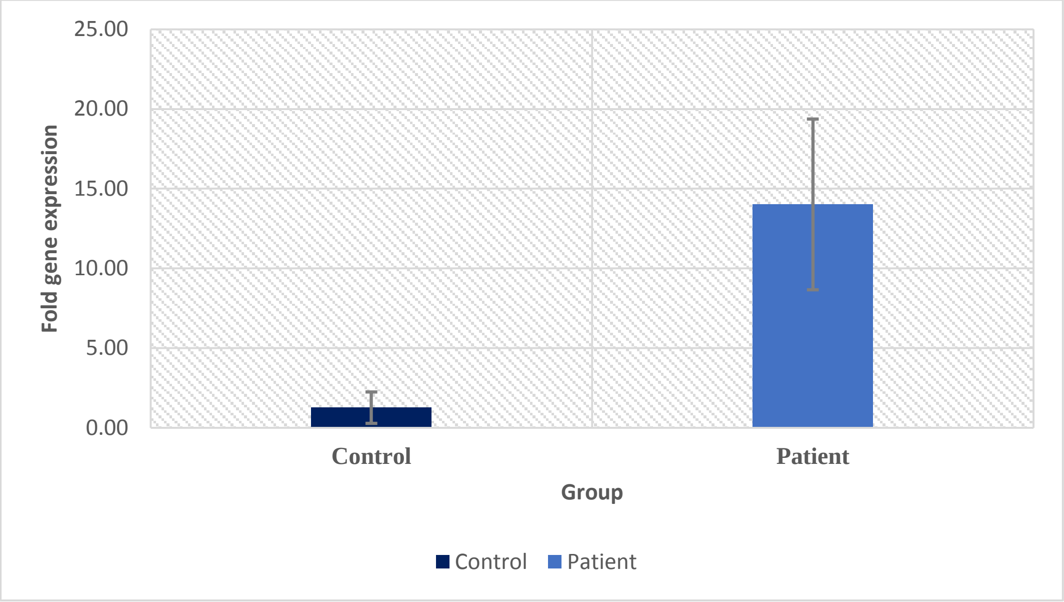


Figure (3-3): microRNA 302b fold Gene Expression among Control and RUTI Patients versus the reference gene (GAPDH).

Godlee, (2012). Showed the alterations in the microRNA profile observed following a variety of inflammatory insults, innate immune miRNA and negative regulation pathways which determine susceptibility to secondary bacterial complication that susceptibility to bacterial complication extends infection leads to Toll-like receptor desensitization, an increase in the expression of innate negative regulators, and a dramatic change in the microRNA profile of these cells. (Godlee , 2012).

A Study was investigated whether miRNAs are involved in the regulation of inflammatory responses upon Gram-negative bacterial infection.The analysis of the expression patterns of 302b miRNAs, and found that miR-302b is among the most highly induced in the early stage of Gram-negative bacterial infection and the miR-302b expression is up regulated bacterial infection, to play important roles in the regulation of diverse biological processes. Whether the expression of miRNAs might be regulated by *P. aeruginosa* infection, (Bushati and Cohen, 2007).

Relative expression levels of target genes were determined by normalizing reaction cycle thresholds (CTs) to the housekeeper genes, GAPDH. ΔCT values were used in the Equation [ΔΔCt] for calculation of the relative mRNA expression levels of target genes, as previously described using PCR efficiencies and mean crossing point deviations between samples and controls.

The results described microRNA 302b fold using real time PCR efficiencies and mean crossing point deviations between samples and controls, represent a significant difference at $p \leq 0.0001$as show in Table (3-8)and Table (3-9)show the microRNA 302b (CT) Expression in RUTI patient associated with *P. aeruginosa* infection .

Table (3-8): microRNA 302b fold gene expression in control and patients versus the reference gene (*GAPDH*).

Groups	**No.**	**Expression levels (2^- (ΔΔCt))**		
		Mean	**SD**	**SE**
Control	20	1.26	0.99	0.22
Patient	30	14.01	5.36	0.98
P value		<0.0001*		

* represent a significant difference at $p \leq 0.05$.

Table (3-9): microRNA 302b (CT) expression in control and patients versus the reference gene (*GAPDH*).

Groups	**N0.**	**Ct**		
		Mean	**SD**	**SE**
Control	20	39.72	0.9028	0.2018
Patient	30	36.10	0.9029	0.1648
P value		<0.0001*		

* represent a significant difference at $p \leq 0.05$.

The identification of miR-302b is a crucial regulator of inflammatory response in host defense against *P. aeruginosa* invasion and the result agreement with (Zhou *et al.*,2014)That show this type of microRNA correlated with in host defense against *P. aeruginosa* may involved in host defense against *P. aeruginosa* invasion.

MiRNA molecules have been observed to complete the intercellular communication and preventing endogenous degradation, miRNAs dysregulation observed in tissues and serum associated with disease activity which considered as a potential biomarker for clinical diagnosis (Hossian *et al.,* 2019; Chen *et al.,* 2020),

The result of this study demonstrated that miRNA302 expression was significantly increased after *P. aeruginosa* infection, overexpression of miR-302 which agree with Huang, *et al.*, (2020) demonstrated that miR- 302 expression was significantly increased after *P. aeruginosa* infection. Overexpression of miR-302 promoted mitophagy and enhanced the ability to scavenge ROS in macrophages, thus facilitating the elimination of intracellular *P. aeruginosa* and maintaining the cellular homeostasis. Importantly, that identified NF-kB as a novel functional target of miR-302 cluster in eliminating intracellular *P. aeruginosa*. These findings shed new light on host defense mechanisms in *P. aeruginosa* infection.

Zhou*et al.,* (2014). showed a novel negative regulatory network for TLR/NF-kB signalling at the level of miRNAs. in Gram-negative bacterial infection could induce upregulation of miR-302b expression in epithelial cells through TLR/NF-kB- ependent pathways. Then, have proved that miR-302b negatively regulates bacteria-triggered proinflammatory cytokine production, thus indicating a new mechanism to counteract the bacterial evasion of innate immune. that IRAK4 is a potential target for miR-302b. Mechanistically, inhibition of miR-302b target, IRAK4, could suppress NF-kB-dependent cytokine production.

Therefore, that bacterial infection is first sensed by TLR and in turn TLR initiates the production of proinflammatory cytokines against bacterial infection; however, at the same time, bacterial infection can also upregulate the expression of miR-302b which in turn inhibits innate antibacterial immune response by blocking TLR signalling and TLR triggered proinflammatory cytokines production, thus providing a balanced immune response. As one of the most essential cell types in the antibacterial immunity, macrophages function as the predominant cell for making response to *P. aeruginosa* infection (Li *et al*., (2013).

Molecular mechanisms of communication: specific host molecule effect on bacteria, many classes of bioactive molecules operate within an organism in order to maintain its physiological milieu. The involvement of host molecules such as host microRNA (miRNA), hormones, cytokines, metabolites, and related inflammatory molecules in eukaryotic to prokaryotic communication (White,*et al.,* (2020).

Effects of endogenous factors in host microRNA expression the expression of microRNAs can be regulated on multiple levels at the transcriptional level, expression of microRNA genes can change together with (intragenic miRNAs), or independently of (intergenic miRNAs), their host genes. Intergenic miRNAs have their own promoters, are expressed independently and can be regulated by separate transcription factors. In both cases, the expression of microRNA can change due to different mutations or can be regulated by methylation of the promoter. On the post-transcriptional level the expression of microRNAs can be downregulated due to changes in the activity of key miRNA biogenesis enzymes, such as Dicer and Drosha. Activity of these enzymes can also be affected by mutations or epigenetic modifications (Gulyaeva and Kushlinskiy, 2016).

Moreover, chemical compounds of endogenous origin (hormones, cytokines), and exogenous origin (xenobiotics), can alter microRNA expression. Activation of nuclear receptors by xenobiotics as their ligands can induce expression of both intergenic and intragenic microRNAs,_Various physiological and pathological stimuli, such as steroid hormones or stress, can affect miRNA expression. Several reports claimed that estrogens can affect miRNA expression (Katchy and Williams, 2016).

Also the Estrogen-induced miRNAs are important for adrenomodulin balance the key regulator of cardiac activity among women (Wetzel *et al.,* 2016). MiRNA expression can also be affected by corticosterone, closely related genes involved in the development, inflammation and depression (Dwivedi *et al.*,2015)

The discovered miRNAs possess binding sites on the glucocorticoid receptor (GR), which suggests a common regulatory mechanism with the genes controlled by GR. believe that this is a novel mechanism of GR action on the expression of miRNAs, which can aid in understanding of the development of depression. Interferon (IFN) can induce miRNA expression (Yang*et al*.,2015) and this serves as an important component of the host immune response during the development of malignant tumors or viral infections. Also The concluded that STAT3 and the p65 subunit of NFκB can bind to the miRNA promoter and alter its expression(Jain*et al.,* 2012).

Changes in intragenic miRNA expression can occur due to changes in the expression of host genes where the miRNA is encoded. Several studies have suggested links between miRNA expression and transcription factors, host genes and targets of mRNAs in various malignant tumors were study network of microRNAs, transcription factors, target genes and host genes in human renal cell carcinoma (Song *et al.,* 2015)

Li and co-authors 2013, analyzed miRNA expression, transcription factors and their gene targets. Authors utilized several databases of TF-miRNA feed forward loops (FFLs) and chose FFLs typical of cancer and associated with transcription factors E2F1 and RB1 as an important family in the regulatory network of interaction between miRNA and transcription factors. Another study demonstrated dependence of miRNA expression on a host gene (Elton *et al.*,2013).

3.5. Gene expression of IL6 by quantitative Real-time RT-PCR:

A total 30 blood patient sample, RNA was extract to revision the gene expressing of IL6 by means of RT-PCR([ΔΔCt] methods in this method the level of expression to IL6 gene in the patients sample as well as in control sample normalized by house-keeping gene *GAPDH* as show in figure(3-4), and the results show that the IL6 was increase in RUTI Patients with *P. aeruginosa* when compared with control groups . The patient expression of IL6 is increased more than (%30) fold when compare with control group .

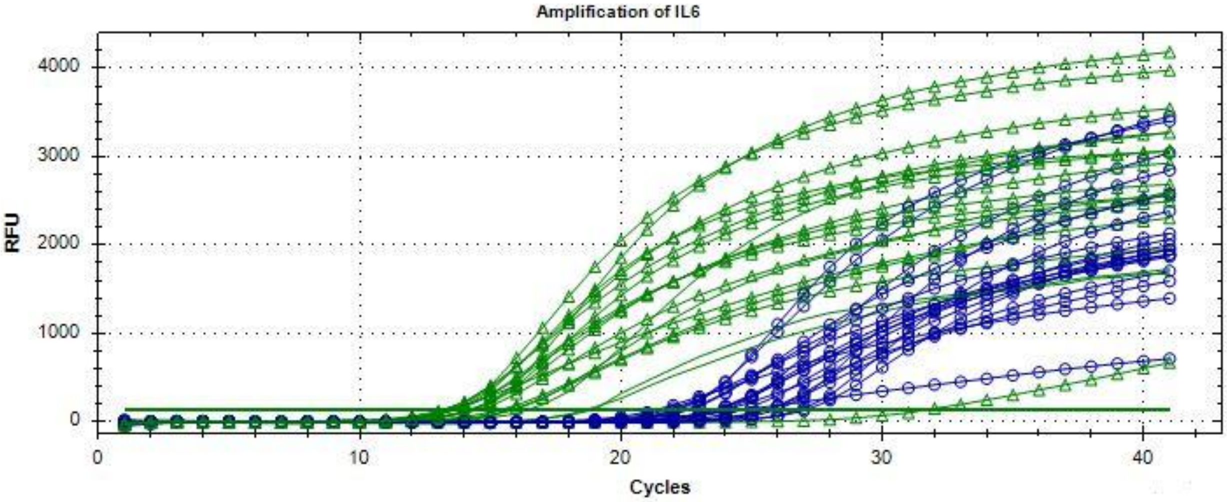


Figure (3-4): The level IL6 expression. This is the first run for 15 samples, ═ represents amplification of Reference gene (GAPDH), △ represents amplification of samples RUTI Patients, —○— represents amplification of control samples.

Gene expressing (IL-6) can be secondhand as a biomarker for initial UTI detection because it is unique of the greatest significant components of the inflammatory reaction and released by urothelial cells in comeback to exposure to UTI-causing substances (Brauner *et al.*,2001).

The detection of cytokines in the serum and gene expressing have been employ in this work to aid in the diagnosis and follow-up of numerous urological disorders. However, when compared to the controls group in the current investigation, the IL-6 mean levels were substantial (P = 0.001)as show in Table (3-10)and (3-11).

This result were agree with other Clinical and test findings, were performed by the measurement of IL-6 in the blood and it was found to be useful in separating upper from lower involvement and aiding in the diagnosis of UTIs(Otukesh *et al.*,2010).

a wide range of stimuli (bacterial infections, inflammation, tissue injury response, and other cytokines and growth factors) IL-6 gene expression is easily elevated in cells. The promoter of IL-6 is turned on at the molecular level via a number of signaling mechanisms (Darogha,*et al.*, 2012).

The present study show the IL-6 gene expression is easily elevated in bacterial urinary tract infection and agree with other study that suggest (IL-6) as a marker of bacterial urinary tract infection as a indication for inflammation (Aziziaram,*et al.*, 2021).

Table (3-10): gene expression fold of IL 6 among control and RUTI patients versus the reference gene (*GAPDH*).

Groups	**No.**	**Expression levels (2^-(ΔΔCt))**		
		Mean	**SD**	**SE**
Controls	**20**	**1.91**	**2.51**	**0.56**
Patients	**30**	**142.99**	**29.93**	**5.47**
P. value		**<0.0001***		

*** represent a significant difference at p≤ 0.05.**

The result showed increase the Expression of Ct IL 6 gene among Control and RUTI Patients versus the reference gene (*GAPDH*). Expression levels in *p. aerugenosa* associated infection represent a significant difference at p≤ 0.0001. as shown in Table (3-11).

Table (3-11): gene expression fold (ct) for IL6 among control and RUTI patients versus the reference gene (*GAPDH*).

Groups	N	Ct		
		Mean	SD	SE
Controls	20	24.6480	1.13072	.25284
Patients	30	17.5207	1.13191	.20666
P. value		<0.0001*		

*** represent a significant difference at p≤ 0.05.**

Due to The host immune response to pathogens has a big impact on the pathogenesis of UTI. and play essential roles in the host defense against pathogens, they also cause harm when present in excess or are dysregulated. for example, in acute conditions, uroepithelial cells and inflammatory cells, in response to uropathogen stimulation, produce a number of pro-inflammatory mediators (e.g., IL-6, TNF-a, and IL 8), which (if present in excess) cause epithelial inflammation/ damage, allowing bacteria to enter the underlying tissue (Godaly and Svanborg 2015).

In the course of acute inflammatory reactions in urinary tract infections, cellular and molecular processes and interactions successfully reduce the risk of approaching harm or infection brought on by microorganisms invading (Masajtis *et al.*, 2017).

Inflammatory reaction by urothelial cells in comeback to exposure to UTI-causing substances as bacterial infection and release of immune mediator a pro inflammatory cytokine (IL-6) as a unique of the greatest significant components of the inflammatory reaction biomarker for initial UTI detection (Catalano *et al.*,2023).

The expression of IL6 is increased more than %30 fold when compare with control group as shown in figure (3-5) , and this increase of production interleukin as a reason, enhancing systemic immunity and mucosal immunity should be important to prevent and control urinary tract infection, especially the recurrent urinary tract infection . When UTI affects patients with low immune function, antibiotics might be used much longer and more frequent. in this state, infection, drug resistance, and dysbacteriosis associate and bring many difficulties for the clinical treatment of urinary tract infection, and recurrent UTI induces pyelonephritis (Gupta and Trautner, 2017) .

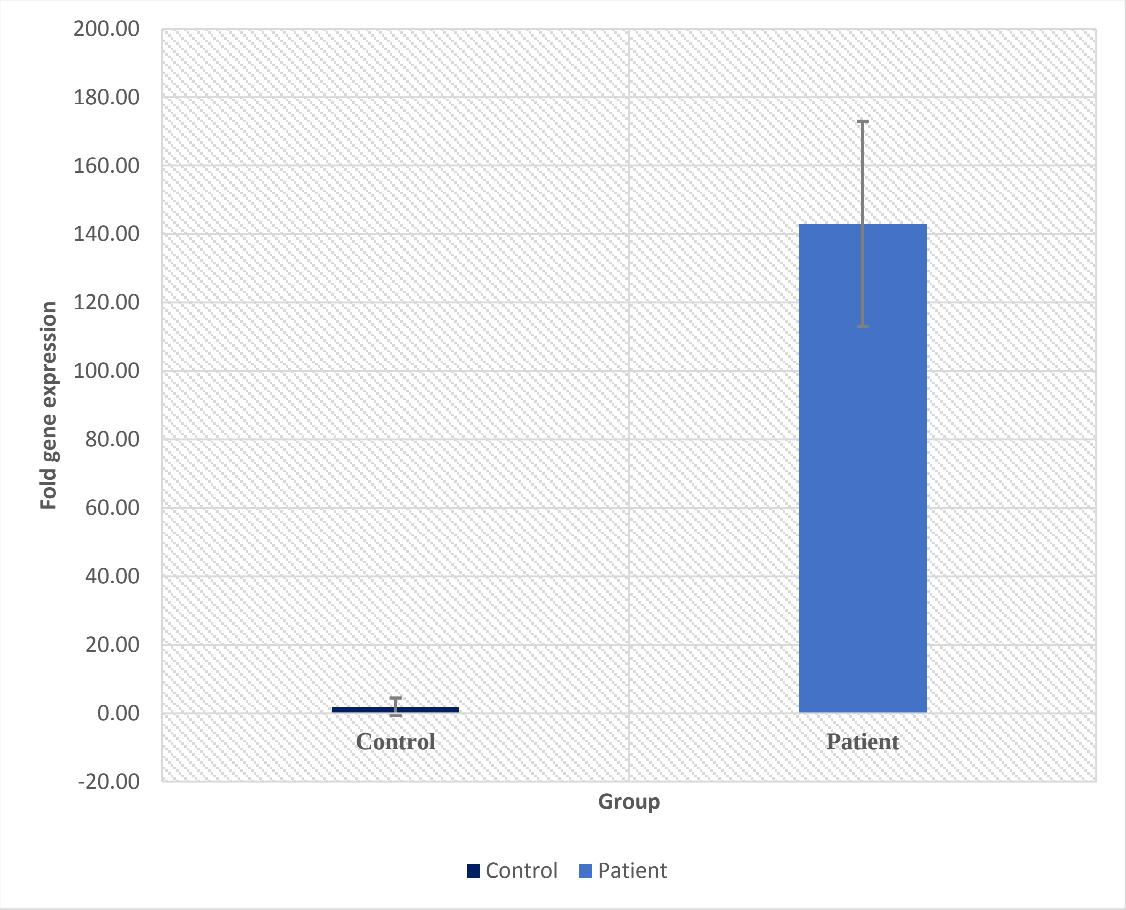


Figure. (3-5): IL 6 gene expression fold among control and RUTI patients versus the reference gene (*GAPDH*).

3.6. Enzyme Linked Immunosorbent Assay (ELISA) for measuring IL6:

The result show increasing secreted IL-6 levels produced according to the table (3-12) and the figure (3-6) which indicate highly significant at *P*. value 0.0001 when compared with control and gender association .

Table (3-12) Secreted IL-6 ELISA for Measuring Levels Produced According to Patient and Control.

	Case	No.	Mean	Std. Deviatio	Std. Error Me	*P*. value
IL6	Contr	**20**	**186.0145**	**14.450275**	**3.231180**	**٠.001**
	Patien	**30**	**286.6514**	**81.470098**	**14.874337**	

The detection of cytokines in the serum has been employed in this work to aid in the diagnosis and follow-up of numerous urological disorders. but when compared to the controls group in the current investigation, the IL-6 mean levels were substantial (*P* = 0.001). this result were agreed with other Clinical and test findings, were performed by the measurement of IL-6 in the blood and was found in studies to be useful in separating upper from lower involvement and aiding in the diagnosis of UTIs (Otukesh*et al*., 2010)

Also a result of Olsyzna *et al.*, (2001)found that urinary IL-6 were measured and increased in the UTI group,

The results were disagree with the study of (Naghshidaian *et al*., 2017) they showed that IL-6 was not a good biomarker for urinary tract infection and differentiation the upper tract involvement from the lower tract.

Transurethral inoculation of uropathogenic bacteria induced cystitis in a TL4-dependent manner by causing IL-6 release, urothelial Stat3 phosphorylation, and activation of antimicrobial peptide production in urothelial cells, IL-6 induced Stat3 phosphorylation, and universal IL-6 injection increased urothelial Stat3 phosphorylation and antimicrobial peptide production (Alubaidi*et al.*,2022).

Chronic IL-6 reduction also resulted in severe pyelonephritis and an increase in the amount of bacteria in the kidneys. Thus, infected urothelium expresses antimicrobial genes through a transcriptional pathway driven by IL-6/Stat3 signaling, which is crucial for preventing epithelial invasion and spreading infection. through significant roles in regulating epithelial attack and ascending infection (Butler *et al.*,2022).

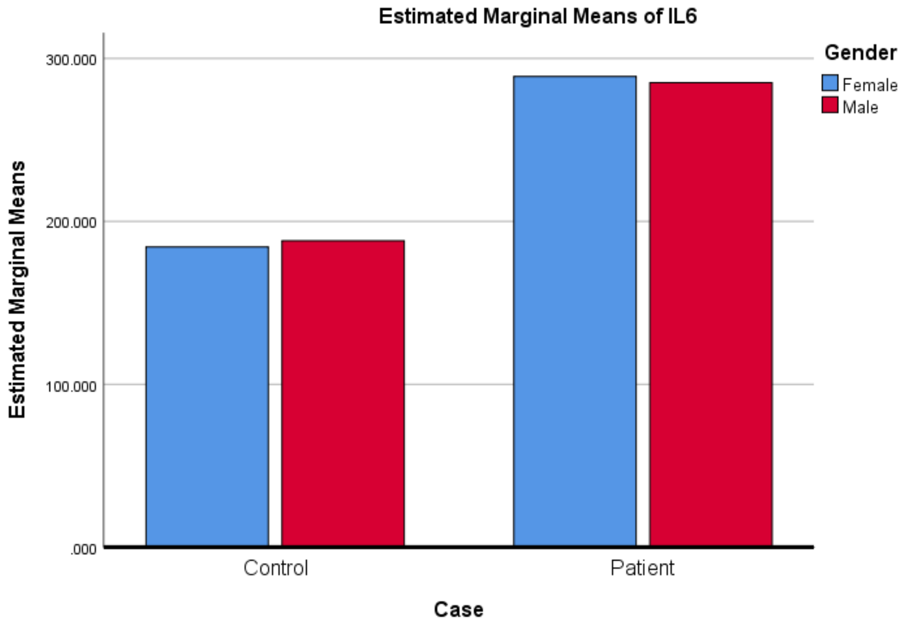


Figure (3-6) Establish mean of Secreted IL-6 ELISA for measuring levels produced according to patient and control and gender male and female association.

Both Wawrysiuk *et al*, (2019), and Sundvall *et al*., 2014)who demonstrated that cytokines are essential elements of the indigenous host-cell comeback and that they are talented biomarkers for distinguishing upper from lower UTIs. Additionally, they may be helpful in assessing the degree of local immune reaction during simple lower UTIs, which might aid in choosing a non-antibiotic course of treatment .

Production of interleukin in lower UTI consists of one main mechanisms of innate immune responses and urothelium barrier function (Abraham and Miao 2015). The innate immune system is composed of (1) pattern recognition receptors like toll-like receptors (TLR); (2) plasma proteins, chemokines, and cytokines; (3) cellular components like epithelial cells, bone marrow-derived phagocytes, dendritic cells, and natural killer cells; (4) toxic molecules such as reactive oxygen and reactive nitrogen intermediates; and (5) antimicrobial peptides (AMPs). Additionally, normally present local microbiota in the urogenital system and intestinal tract serve as another source of innate immunity, altering the pH of the local environment and producing their own antimicrobial products to help control UTI, as well as simply acting as competitive inhibitors of more virulent bacterial strains (Ching, *et al.* 2020).

A persistent and dysregulated inflammatory phase, an enhanced production and release of pro-inflammatory are marked by cytokines such as (IL-6) in RUTI in male and female at p value (P=0.55) as shown in table (3-13).

Table (3-13) Secreted IL-6 ELISA for measuring levels produced according to patient and control and gender association.

	Gender	No.	Mean	Std. Deviation	Std. Error Mean	*P*. value
IL6	Female	23	238.91843	80.997864	16.889222	0.55
	Male	27	252.76700	81.161829	15.619601	

Increased duration of cytokine response, increased pro-inflammatory cytokine and impaired local cytokine production leads to a dysregulated cytokine response in uncontrolled immune suppress person further increasing susceptibility to severe infections (Nagendra *et al*., 2022).

Macrophages, dendritic cells, NK cells, and granulocytes are part of the innate immune system. The cells of the innate immune system control and clear invasion via various mechanisms, such as release of cytotoxic molecules, engagement of more immune cells, complement pathway activation, or activation of the phagocytosis process and interleukin production process (Shihab *et al*., 2020).

In antibody responses, B cells are activated to secrete immunoglobulin which bind to the invading microbial antigens and block their binding to receptors on host cells (Nagendra *et al*., 2022).

T cells which produce interleukin and also are activated by certain cytokines and antigen presenting cells (APC), react directly against a foreign antigen that is presented to them on the surface of a host cell or themselves secrete cytokines that activate macrophages to destroy the invading microbes after phagocytosis. dysregulation between anti-inflammatory and pro-inflammatory cytokines and defects at the level of antigen presenting cells leads to dysfunction of T cells (Nagendra *et al*., 2022).

RUTI are marked by a persistent and dysregulated inflammatory phase, an enhanced production and release of pro-inflammatory cytokines such as (IL-1β, IL-6 and TNF-α) causes disturbance the balance between pro-inflammatory and anti-inflammatory cytokines (Raheem *et al*., 2019).

The increase of pro-inflammatory cytokines but also from a decrease of anti-inflammatory and healing-associated cytokines like TGF-β and IL-10. Because these antagonistic cytokines usually inhibit each other's action, the distinction between the cause and the effect In fact, the fine balance between inflammation and pathogen tolerance is mainly regulated by T-cells. These cells also control the adaptive response through cell–cell interactions or through cytokines (Moura *et al*., 2017).

3.7. Genotype of *P. aeruginosa* isolate by Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA):

MLVA ware performed for all *p. aeuroginosa* isolate eight VNTR loci (eight repetitive element loci) were selected for genetic typing of *p. aeuroginosa*. PCR were carried out the PCR products were electrophoresed on (1-1.5) %

Agarose gel containing ethidium bromide and the allele profile were detected by MLVA .

Analysis of MLVA profile showed that all *p. aeuroginosa* isolate were grouped into (16) distinct MLVA type with (2) cluster (A) and (B).

The amplicon size distribution in (20) *p. aeuroginosa* isolate for each VNTR which were 140- 250 bp in MS010, 80-150bp in MS061,350-530bp inMS077,200-250bp in MS127, 200-800bp in MS142 ,600-850bp in MS172 and 1050-5000bp in MS173 and 600-700bp in MS 194, as shown in figures **(3-7),(3-8), (3-9),(3-10),(3-11), (3-12),(3-13),(3-14).**

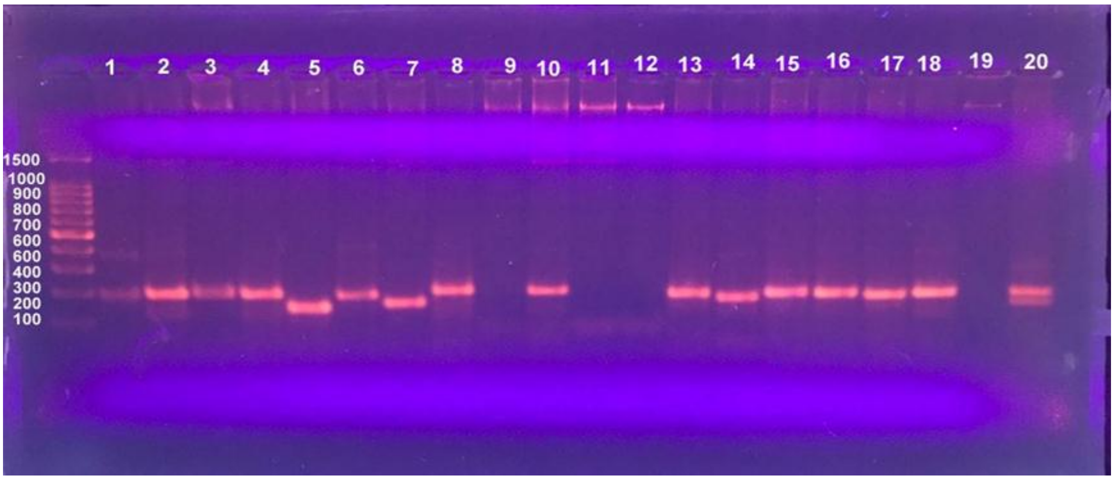


Figure (3-7) Agarose gel electrophoresis at 70 volt for 50 min. for MS010 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive(9,11,12,19 were positive after re electrophoresis) , for this gene with amplicon size 140-250 bp .

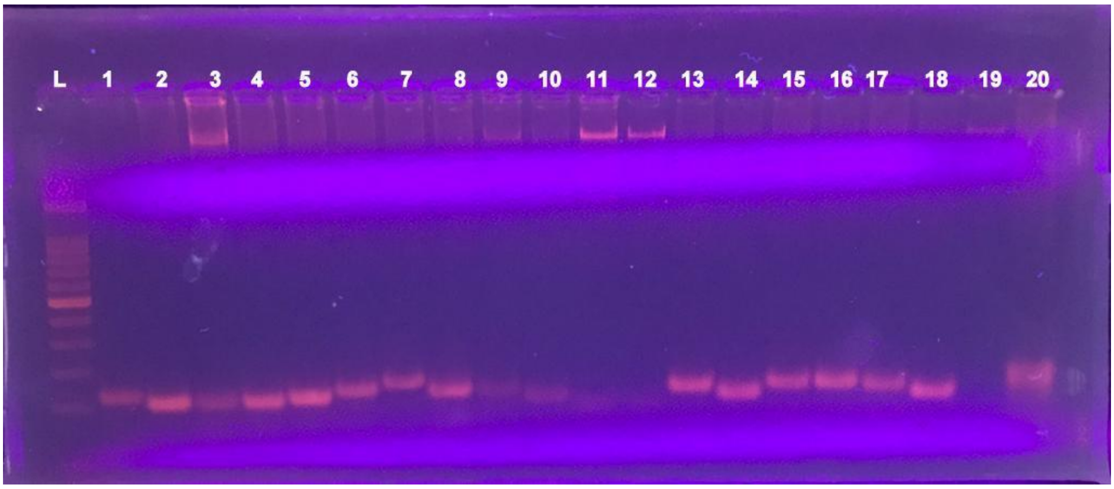


Figure (3-8) Agarose gel electrophoresis at 70 volt for 50 min. for MS061 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive,(No19) were positive after re electrophoresis for this gene with amplicon size 80-150bp

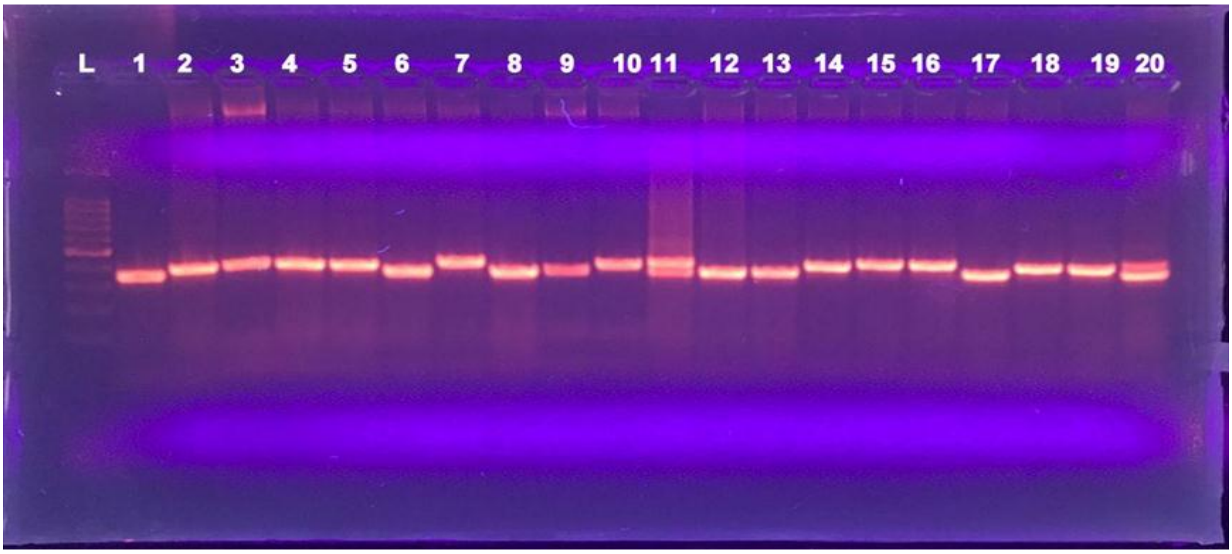


Figure (3-9) Agarose gel electrophoresis at 70 volt for 50 min. for MS077 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive, for this gene with amplicon size 350-530bp

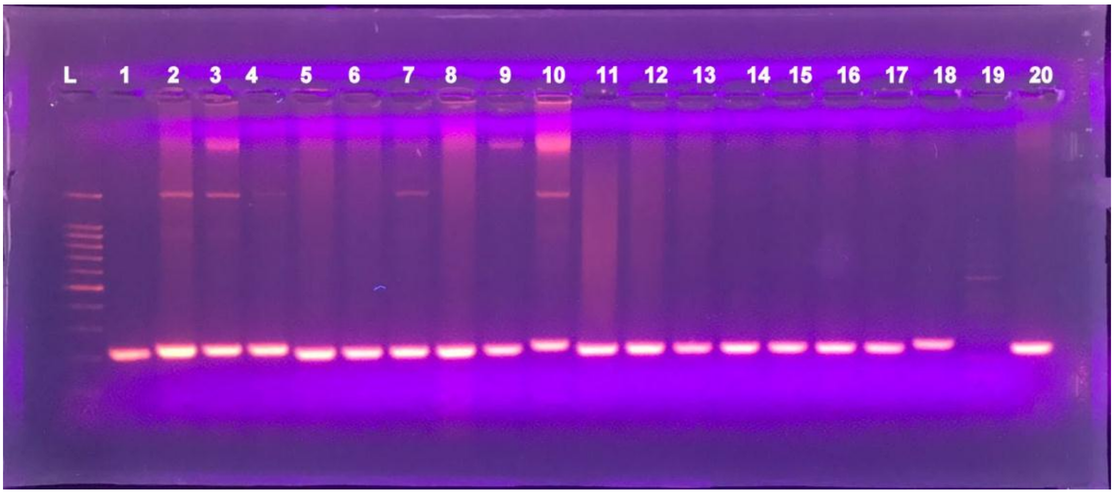


Figure (3-10) Agarose gel electrophoresis at 70 volt for 50 min. for MS127 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive, (No19) were positive after re electrophoresis for this gene with amplicon size 200-250bp.

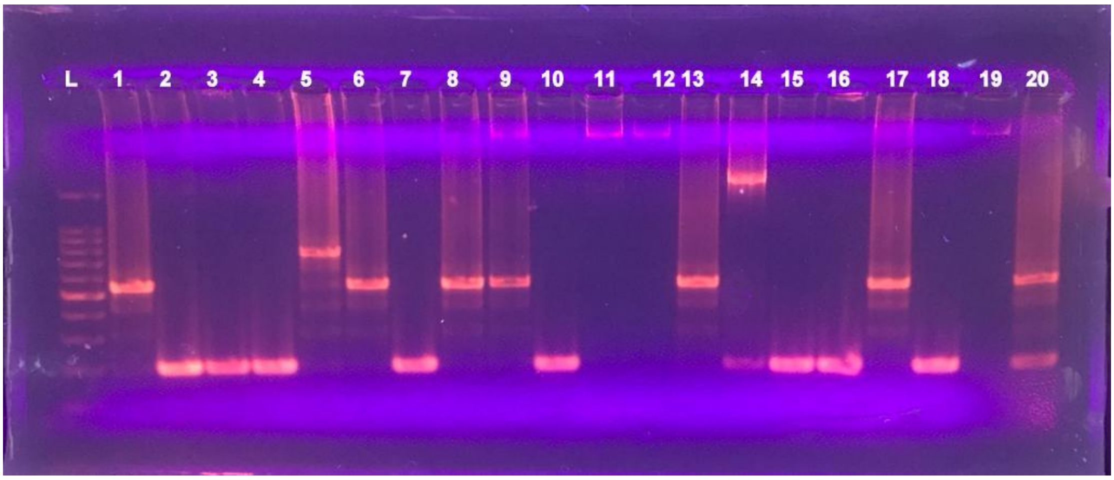


Figure (3-11) Agarose gel electrophoresis at 70 volt for 50 min. for MS142 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive, (No 11,12,19) were positive after re electrophoresis for this gene with amplicon size 200-800bp

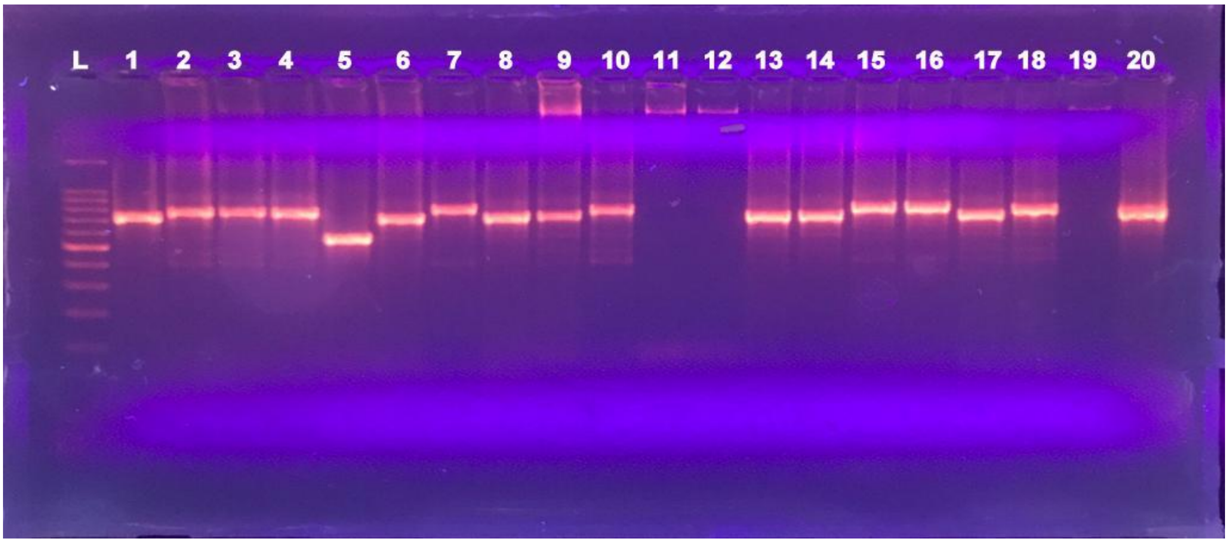


Figure (3-12) Agarose gel electrophoresis at 70 volt for 50 min. for MS172 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive,(No. 11,12,19 were positive after re electrophoresis for this gene with amplicon size 600-850bp

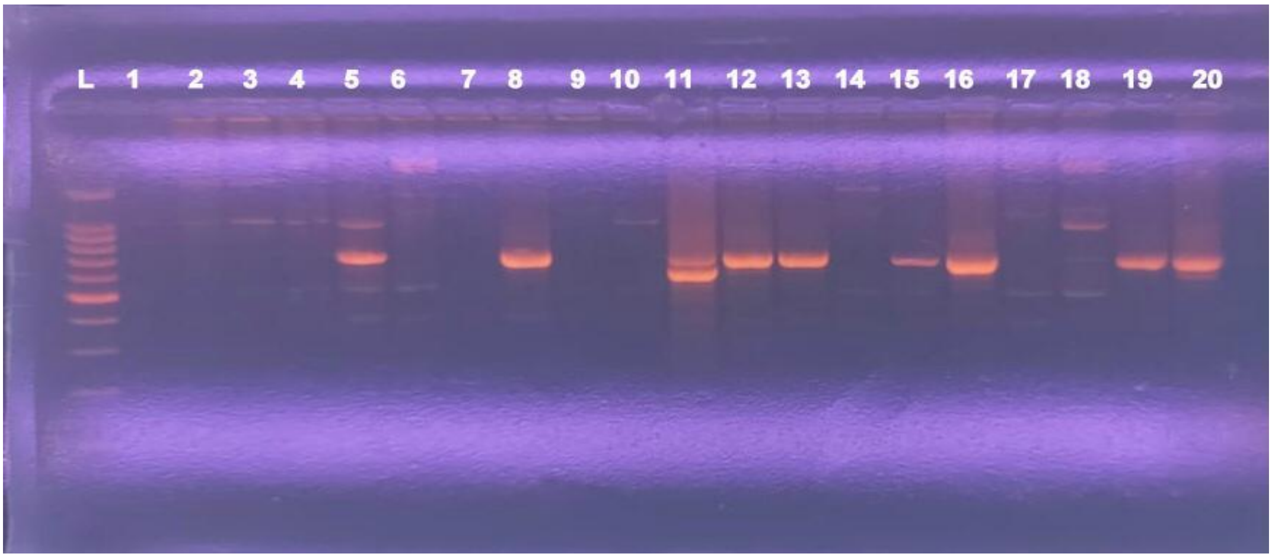


Figure (3-13) Agarose gel electrophoresis at 70 volt for 50 min. for MS194 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive, for this gene with amplicon size 600-700bp

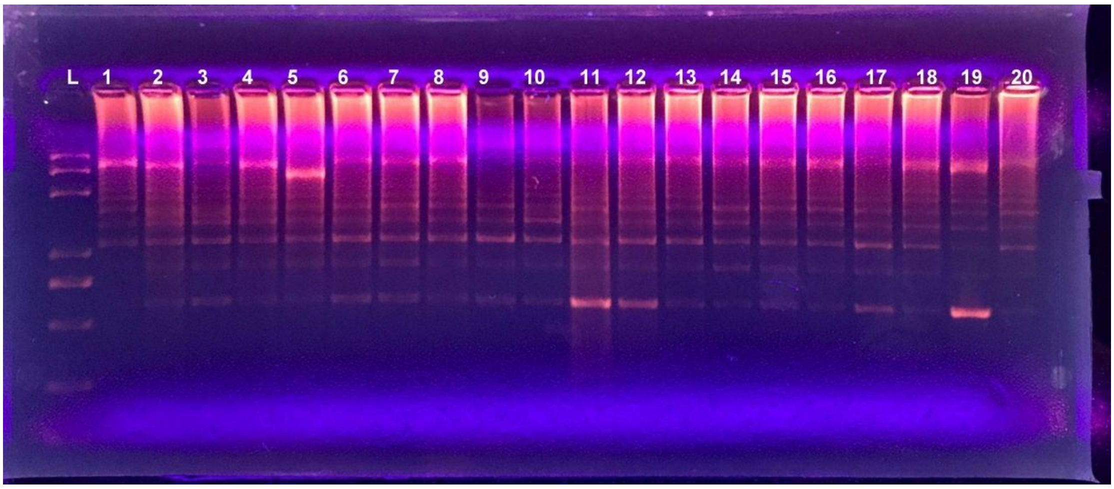


Figure (3-14) Agarose gel electrophoresis at 70 volt for 50 min. for MS173 locus in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 5000 bp. 1-20 were positive, for this gene with amplicon size 1050-5000bp

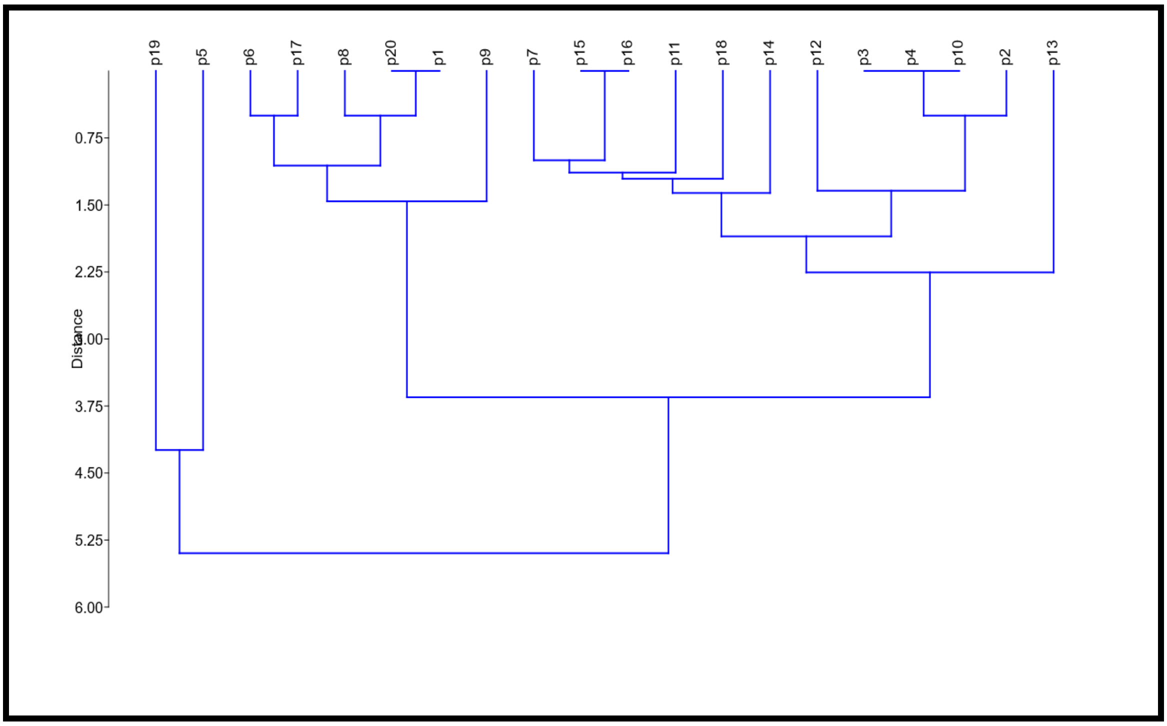


Figure (3-15) phylogenetic tree for MLVA eight VNTR locus in *p. aeruginosa,* the cluster (A) contain (two isolate) while cluster (B) contain (18) isolate designed by mega 6 data base .

The result show the cluster (A) contain (two isolate) while cluster (B) contain (18) isolate as show in figure (3-15).

In addition, the result show that the cluster (B) were divided in two sub-cluster, and sub-cluster (NO.1) contain (6) isolate were sub-cluster (NO.1and NO. 2) are identical 100% and have the same distance, while sub-cluster (NO.2) contain (12) isolate were isolate are (100%) similarity and isolate NO.3,4,10 has the same distance so the similarity 100%.

The result in this study obtained data to accurate the diversity of (8) TR loci .and detect the different alleles.

The high level of diversity was related to MS172 and MS127 this due to it was the most polymorphic locus while the lowest level of diversity detected in MS77.

MLAV method provide data in the form code that can be saved in the database transferable between different isolate ,also this method allowed to establish association between genotypes and parameter such as virulence factor(Farajzadeh *et al*.,2020).

The data which was obtain in this result, indicate the phenotypic and genomic diversity in *P. aeruginosa* in RUTI patient , which confirm the existence of strain diversity during RUTI by *P. aeruginosa* which may serve as a marker of disease progression and be used in novel therapies .

Jarych *et al*.,(2021) distinguished 63 genotype of *P. aeruginosa* isolate perform by MLVA ,the allelic profile for each strain can be described by the a code corresponding to the number of repeats at the selected VNTR based on the PCR product size .

Also lashgarian *et al*., (2018) found that from 70 isolate of *P. aeuginosa* isolated from UTI,39 types were obtained ,the length of each branch different in the number of repetition in different branches .

To develop an MLVA scheme , one needs to identify polymorphic mini satellite loci which must then be individually checked for variation of repeat number ,the genome of *P. aeuginosa* strain is relatively rich in tandem repeats. So evaluation of this polymorphic loci allows a high degree of discrimination among strain with high degree of reproducibility (ontenient *et al.,* 2003).

shiralizadeh *et al.,* (2023) showed that among 15 clinical isolate of *P. aeuginosa* isolate were evaluated by the MLVA method regarding the amplification of ten different VNTR region ,there were 11 different MLVA genotypes of *P. aeuginosa* strains .

The finding's isolates belonged to different cluster and mostly from one hospital but there was a lot of variety in sample type. Thus, *P. aeruginosa* isolates from the same medical ward had remarkably high diversity. indicated that these organisms vary in genotype over time as well as within the same host (Jarych *et al.,* 2021).

Considering the high genetic diversity of resistant *P. aeruginosa* isolates, which are mainly found the MLVA-7 showed good results in typing of *P. aeruginosa* isolates and all studied isolates were type able, three not-type able strains were detected using MLVA-7 as VNTR in study of (Lalancette *et al.,* 2017)

In another study by Johansson *et al.,*2015), an agreement of 91.0% was observed between MLVA in clone identification of *P. aeruginosa* isolates from patients. Johansson, Seven VNTR markers used were the same as study and indicated different genotypes among *P. aeruginosa* isolates which offered a moderate discriminatory power by these VNTR markers.

3.8.Genotyping of *P. aeruginosa* by Ribotyping PCR methods:

In present study the genetic diversity of (20) ***p. aeruginosa*** Isolates was assessed using ribotyping. The ribotyping assay were performed using two different marker Ribo_1 and Ribo_2 primers

Ribo-1 primer distinguished the typable isolates though amplifying different bands (200bp to 1000bp), while Ribo-2 primer visible bands (300-900) bp as shown in **figure (3-16) and) (3-17)**.

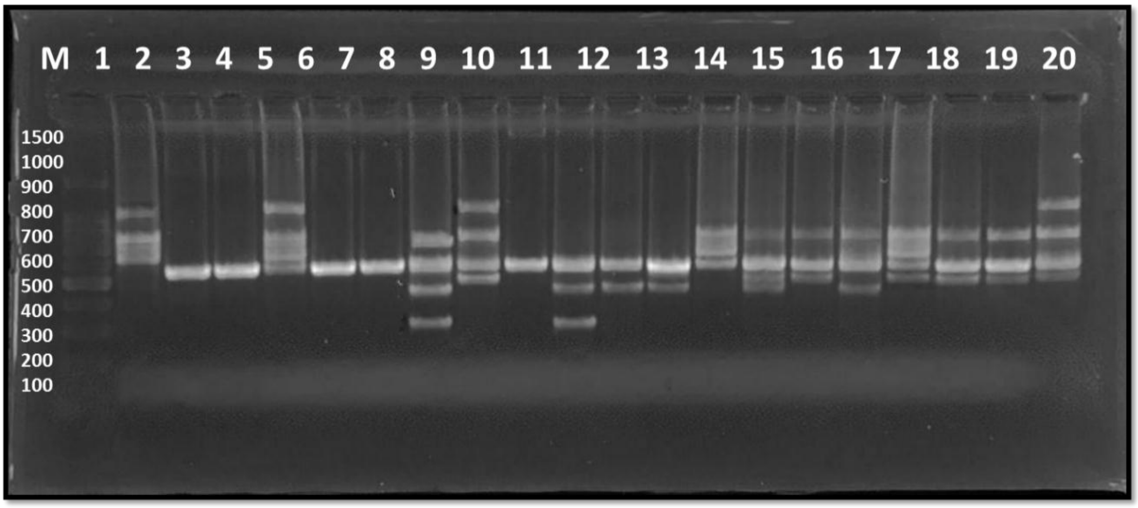


Figure (3-16) Agarose gel electrophoresis at 70 volt for 50 min. for Ribo-1 in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive, for this gene with amplicon size 200-1000 bp.

Ribo-1 primer showed that the isolates classified in to 3 cluster, 12 sub cluster and it found that isolates No 5,6 and 9 identical 100% NO.11 and 12 100% identical ,NO. 8 and 20 also identical 100%. NO. 15, 18, 19 similar 100%. and finally,NO. 2 and 3 are similar 100%, So that primers showed highest similarity between isolates as show in figure (3-17).

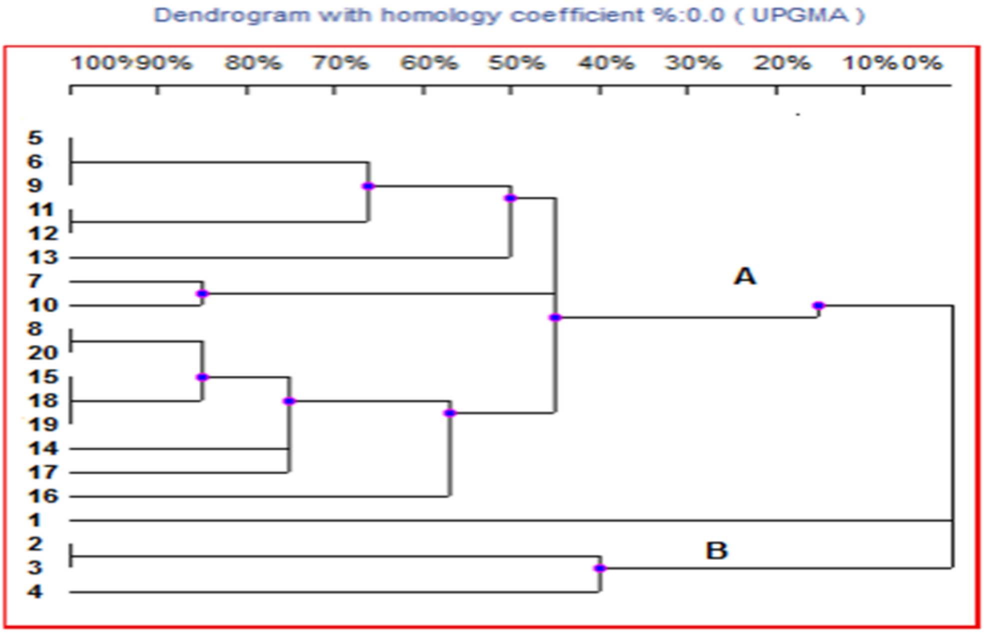


Figure (3-17): The RIBO-1-PCR-derived cladogram representing the relationship among 20 of *p. aerugenosa* strains. Bar represents the distance values. This cladogram was engendered by ''Unweighted Pair Group Method with Arithmetic mean (UPGMA)"

Ribo-2 primer showed that the isolates where it classified in to 2 cluster, and 12 sub cluster and it found that isolates No. 2, 3,5,6,8,9,12, identical 100%.NO.10 and 13 identical 100%. 11 and 15 100% identical 16 and 17 also identical 100%. and finally 18,19 and 20 are similar 100%, So that primers showed highest similarity between isolates as show in figure (3-18)and (3-19).

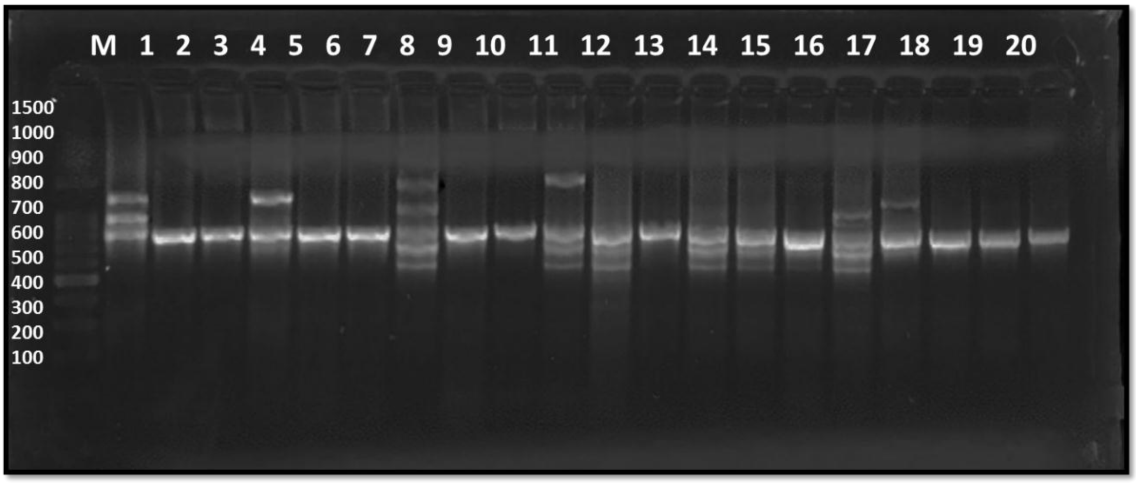


Figure (3-18) Agarose gel electrophoresis at 70 volt for 50 min. for Ribo-2 in *p. aeruginosa* PCR product visualized under U.V light at 320nm. After staining with ethidium bromide. L: Ladder with 1500 bp. 1-20 were positive, for this gene with amplicon size 200-900 bp.

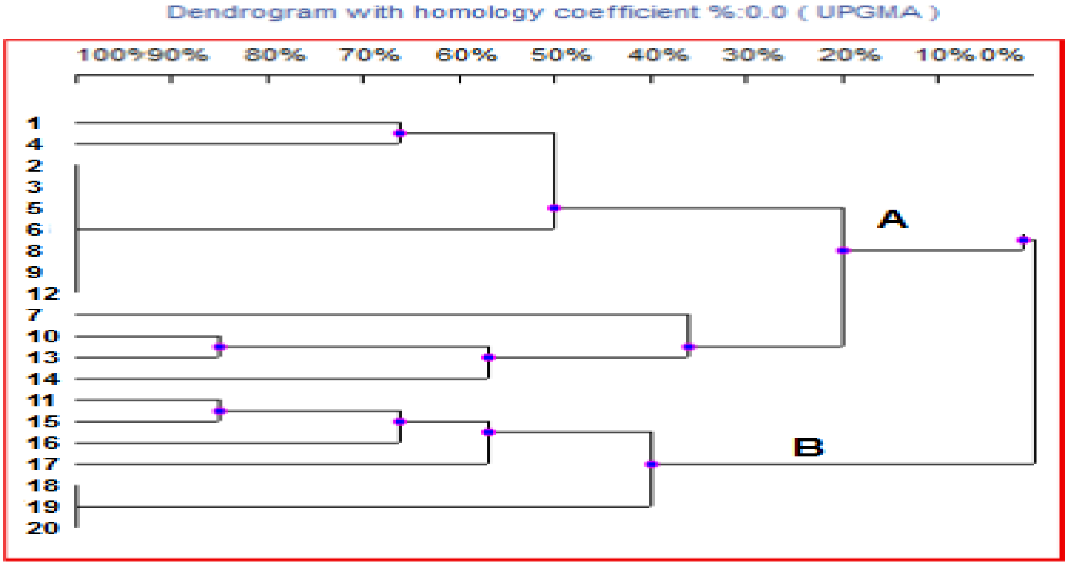


Fig. (3-19): The RIBO-1-PCR-derived cladogram representing the relationship among 20 of *p. aerugenosa* strains. Bar represents the distance values. This cladogram was engendered by "Unweighted Pair Group Method with Arithmetic mean (UPGMA)"

The present investigation the results of PCR ribotyping (date which concentrate on the analysis of the segments of the ribosomal genes) in the whole bacterial genome) for clinical *P. aeruginosa* isolates.

So the Ribotyping by (Ribio-1 and 2) revealed (16) type from (20) isolates, in which divided in 2cluster A and B ,and cluster A contain 9 isolates .

Where isolates No.p18 and p19 are identical 100%. While in cluster B contain 11 isolate where isolates No. P5 and p6 and p9 are identical 100%. Which indicates that these isolates with the same similarity may originate from the same source as show in Figure (3-20)

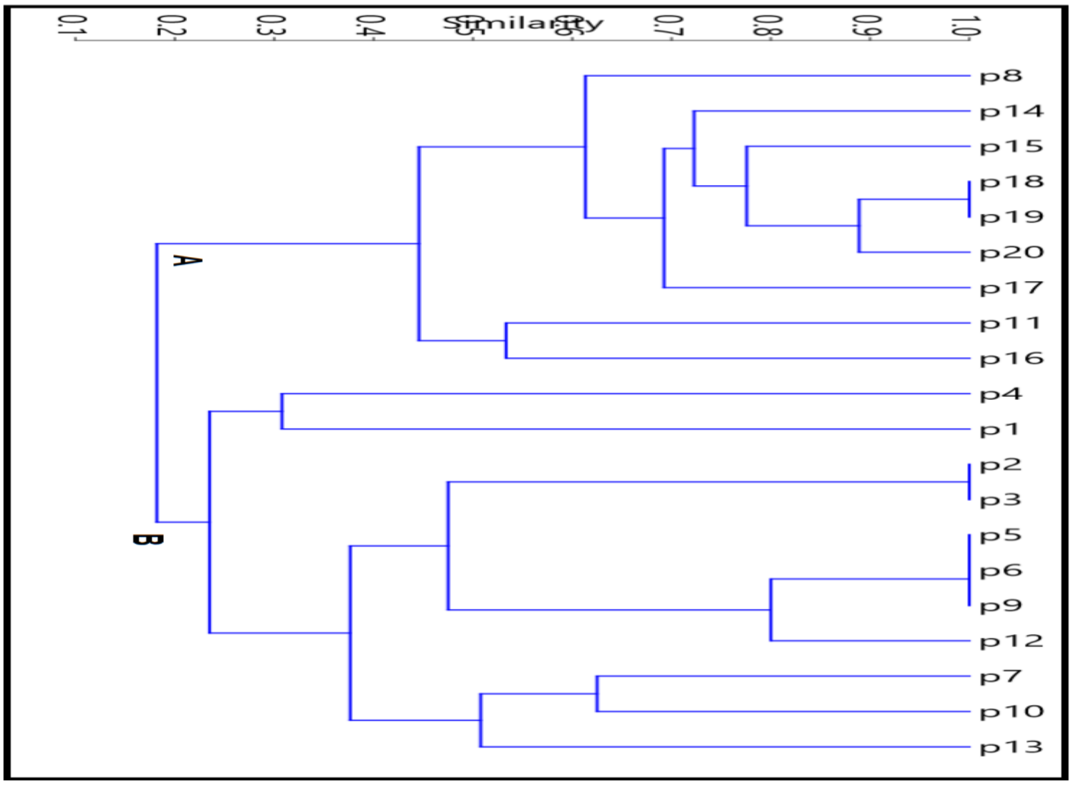


Figure (3-20): The RIBO-1-2 similarity PCR-derived cladogram representing the relationship among 20 of *p. aerugenosa* strains. Bar represents the distance values. This cladogram was engendered by "Unweighted Pair Group Method with Arithmetic mean (UPGMA)"

The rRNA genetic loci of prokaryotes have three types of gene (16s,23s,5s) that are separated by spacer regions with diverse lengths and sequence in the genus and species, the spacer region between 16sand 23s can be used for identification of bacteria by detection of the polymorphism of Pcr product of the region, of many bacteria has multiple copies (alleles)of rRNA operons (Abel - Rahman and Rizk, 2021)

The study of (Wolska and szweda ,2008) found that *P. aeruginosa* belong to one of 3 ribotypes which were showed in one to 3 bands , bands varied between 220_900 bp in size .

Also Pacov *et al.*,(2006) refer that PCR-Ribotyping is at unique used to detect polymorphism in intragenic spacer region of rRNA or tRNA Intragenic spacers region (ISRs) are expected to inferior evolution pressure and thus demonstrated broader genetic variations .

That Azimirad *et al.*(2019) showed that's PCR-Ribotyping gave highly discrimination of isolates as PFGE. The copy numbers , and nucleotide sequence and size in this three rRNA genes are highly conserved within bacterial species, so it used in identification and taxonomic classification (pal *et al.*,2014).

PCR-based methods have been proposed for identifying the gene cluster,PCR ribotyping (PCR-ribotyping) uses specific primers that amplifies sequences between the 16S and 23S gene and has been applied for molecular identification of bacteria at the species level and the discrimination of bacterial strains and also study the variant between different isolate with distance PCR-derived ribotyping as show in Figure (3-21) :

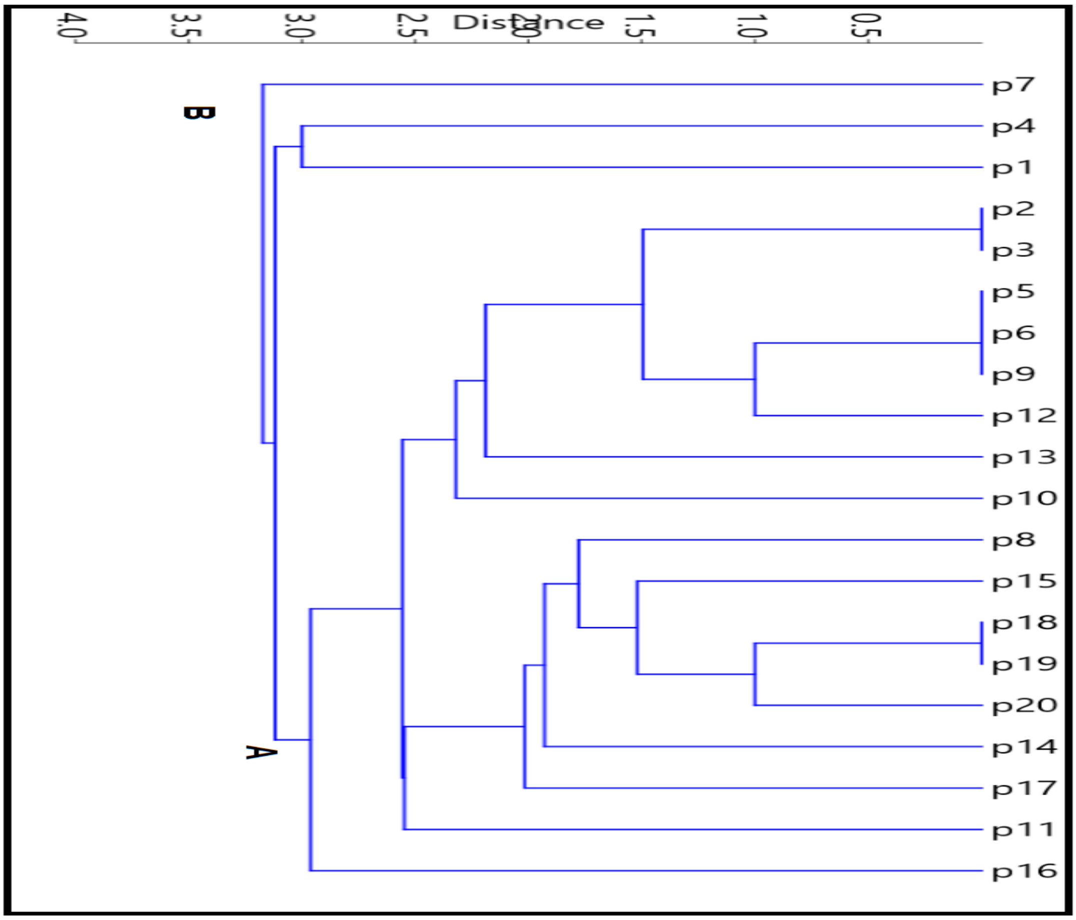


Figure (3-21): The RIBO-1- 2 distance PCR-derived cladogram representing the relationship among 20 of *p. aerugenosa* strains. Bar represents the distance values. This cladogram was engendered by ''Unweighted Pair Group Method with Arithmetic mean (UPGMA)"

This method detects the spacer length within the cluster , another highly promising method for bacterial identification is based on the PCR length polymorphisms of the intergenic spacers spread along the bacterial genome. The tRNA genes are highly conserved among eubacteria and occur in multiple copies throughout the bacterial genome, within and outside rDNA gene clusters. These genes are generally clustered and are separated by spacers whose length and sequences are subjected to a higher degree of variations . This method is regarded as producing species-specific banding patterns and has been applied to the differentiation of various bacterial species (Clementino *et al.*, 2001).

Ribotyping methods were evaluated by (Dawson and Dancer, 2002) utilizing 62 clinical strains of *P, aeruginosa*, to assess their useful as tools to study the bacterial diversity within this genetic diversity was determined by PCR ribotyping by these methods, **36** genotypes

According to our results, *P. aeruginosa* isolates from the same medical case had remarkably high diversity. these findings indicated that these organisms vary in genotype over time as well as within the same host , Considering the high genetic diversity of clinical isolates, which are mainly it is difficult to properly implement infection control policies(Jarych *et al.*, 2021) .

The investigations of nosocomial infections caused by *P. aeruginosa* are hampered by the inadequate discriminatory capacity of phenotypic markers, but with the advent of DNA-based techniques, stable and discriminatory epidemiological markers have become available (Lewis,*et al.*, 2005).

Conclusions
and
Recommendation

Conclusion:

1. Recurrent urinary tract infections were more frequent in old males than young females with significant statistical difference.
2. *P. aeruginosa* was the most frequent bacterial isolations in RUTI.
3. The majority of *P. aeruginosa* bacterial isolations harbored the virulence factor *pyocyanin* pigment production in(73.3%) .
4. The susceptibility test of *P. aeruginosa* in the current study showed the highest resistance to the many antibiotics .
5. It was found that the expression of miR-302b was significantly increased in patients with recurrent urinary tract infection associated with *pseudomonas aeruginosa* when compared with control group. as biomarker for recurrent urinary tract infections.
6. The role of IL-6 in intermediating pro- inflammatory according to gene expression and phenotypes was study in recurrent Urinary tract infection ,These findings provide pre-clinical justification for clinical investigations of IL-6 in recurrent urinary tract infection.
7. Analysis of MLVA profile showed that all *P.. aeuroginosa* isolates were grouped into (16) distinct MLVA type with (2) main cluster.
8. PCR ribotyping for the clinical *P. aeruginosa* isolates were done and the Ribotyping results by Ribio-1 and 2 revealed (16) type from (20) isolates, in which divided in 2cluster.
9. MLVA profile PCR ribotyping for the clinical *P. aeruginosa* isolates are considered as highly promising typing method for phylogenetic analysis.

Recommendations:

1. New technology has hardly been used in the laboratory, like other miRNAs and artificial anti-miRNAs may have a promising future in human medicine. which could be targeted for developing new antimicrobial therapies
2. More studies exploring the impact of circulating miRNAs on altered transcriptional programs in recipient cells.
3. The application of new typing methods like PFGE type or combination typing methods may result in a better discrimination of isolates
4. HLA analysis to verify the associated target alleles in patients with recurrent urinary tract infections is recommended.
5. Study the role of others' specific pro and anti-inflammatory cytokines levels and their gene polymorphism, which may have impact on the disease management.
6. Assessment of the possible alterations in the cell mediated immune response in patients with urinary tract infections is recommended which might have an impact on the disease severity, prognosis and the possible planned management.

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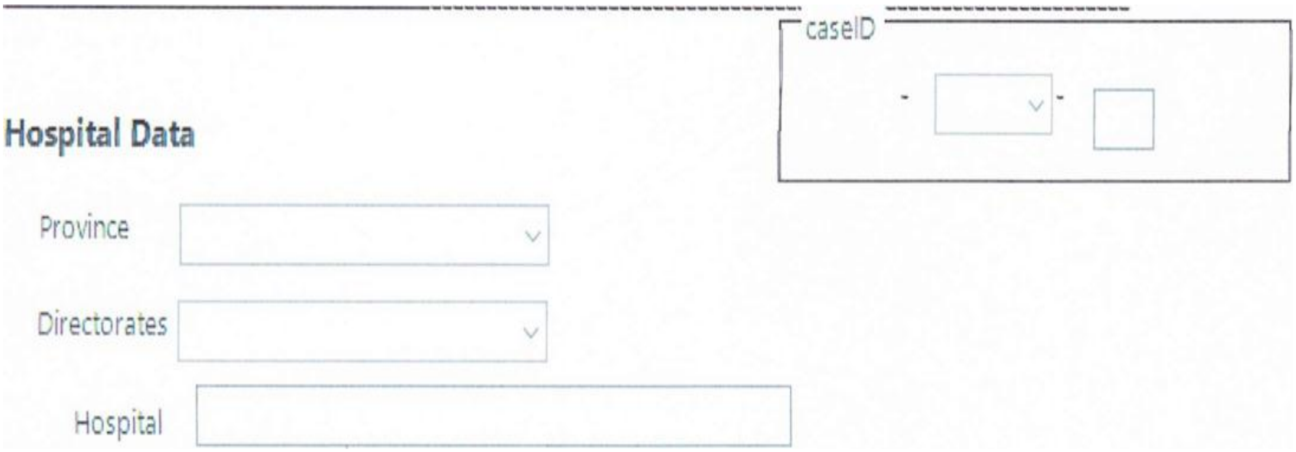
Appendix

Appendices:

Appendice-1

Recurrent urinary tract infections Case Investigation form (Questioner)



caseID

Hospital Data

Province

Directorates

Hospital

Patient Data

Patient and Father Name Mother Name

Age in Years Age in Months Age in Days Sex

Patient's Governorate

Patient's District

Patient's Village

Patient's telephone Number

Appendice-2

Clinical Data / Signs and Symptoms Frequent urination Urine that is cloudy A strong urge urination Urine dark or red Urine with strong, Unpleasant odor	Pain or pressure in pelvic area A feeling of pain or burning during urination Pain during sex Fever Vomiting

Past History

Is the patient a contact of someone with similar illness

If yes mention the relation with the patient

if other Specify

Has the patient been treated with antibiotics in the week prior to collection urine sample

Risk factor for developing recurrent urinary tract infection

UTI Information

Is Urine culture Done Date culture

Appearance a urine

Laboratory Data

Urine analysis

Urine Appearance

Glucose ,Protein

WBCs count(Pus cell) RBCs Cell count

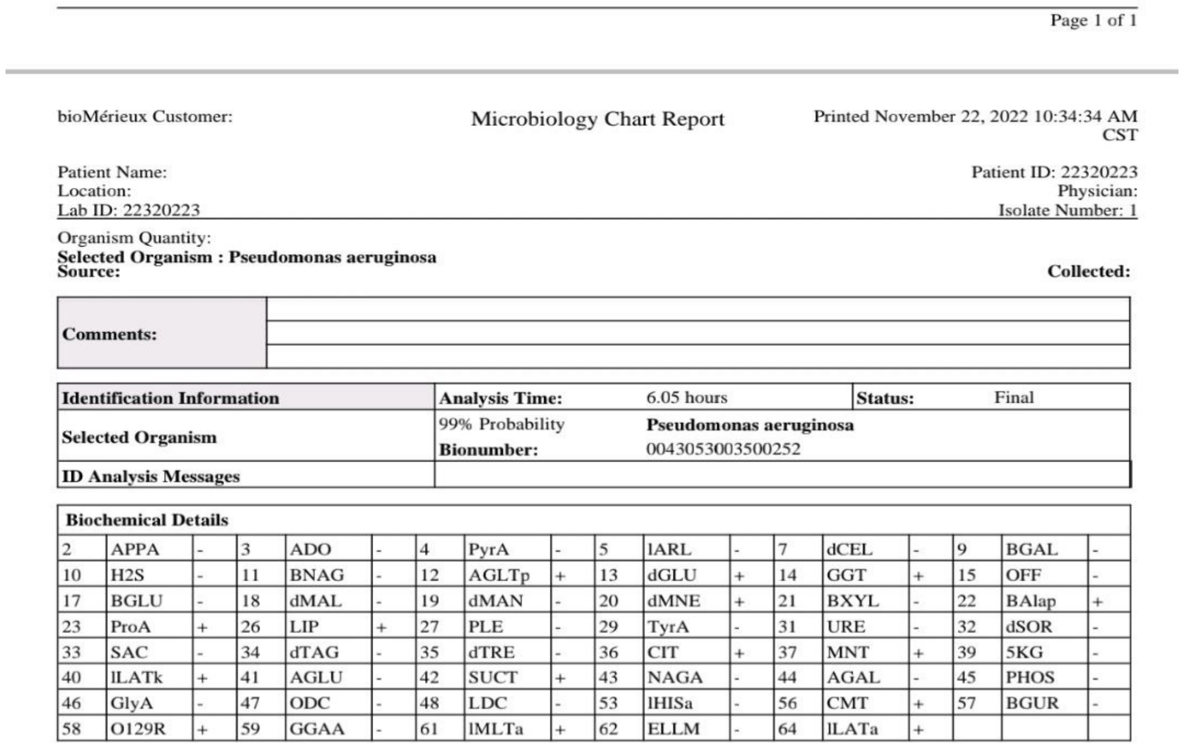
Cast Crystals Epithelial cell Bacteria

Gram Stain Done

Gram Stain Result under microscopic

Urine culture Done Urine culture Result

Appendice-3: VITK report:



Page 1 of 1

bioMérieux Customer:

Microbiology Chart Report

Printed November 22, 2022 10:34:34 AM CST

Patient Name:
Location:
Lab ID: 22320223

Patient ID: 22320223
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa
Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	6.05 hours	**Status:**	Final
Selected Organism	99% Probability **Bionumber:**	**Pseudomonas aeruginosa** 0043053003500252		
ID Analysis Messages				

Biochemical Details

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

Appendices 4: data of real time PCR of micro RNA302b and IL6:

	IL6					MicroRNA 302b			
Sample	Reference	Reference	IL6 (1)	IL6 (2)		Reference	Reference	MicroRNA (	MicroRNA (2)
Control 1	18.8	18.3	24.7	25.2		18.8	18.3	38.2	37.1
Control 2	18.9	19.5	24.0	27.0		18.9	19.5	37.9	38.1
Control 3	21.9	22.5	26.9	25.9		21.9	22.5	41.0	40.0
Control 4	20.0	19.3	23.6	25.3		20.0	19.3	40.0	39.2
Control 5	20.7	20.9	23.6	25.0		20.7	20.9	39.4	40.5
Control 6	20.1	21.0	24.0	26.0		20.1	21.0	39.9	41.0
Control 7	21.0	20.0	22.1	23.5		21.0	20.0	38.9	40.3
Control 8	22.2	22.9	27.1	26.3		22.2	22.9	41.0	40.0
Control 9	22.4	20.0	23.7	24.3		22.4	20.0	40.2	40.9
Control 10	20.7	21.0	25.6	24.6		20.7	21.0	40.9	40.3
Control 11	22.4	20.9	21.8	23.5		22.4	20.9	41.0	40.2
Control 12	20.7	21.2	22.2	23.5		20.7	21.2	38.2	39.3
Control 13	20.1	20.2	23.5	24.5		20.1	20.2	38.6	39.7
Control 14	19.0	20.0	25.5	24.2		19.0	20.0	39.4	39.0
Control 15	18.6	18.3	23.5	25.5		18.6	18.3	39.3	40.2
Control 16	20.1	19.5	25.5	26.5		20.1	19.5	39.4	40.2
Control 17	19.7	20.2	25.5	24.3		19.7	20.2	39.6	40.0
Control 18	20.7	21.3	26.0	24.6		20.7	21.3	38.5	39.6
Control 19	22.1	22.9	22.6	24.6		22.1	22.9	39.5	39.6
Control 20	21.5	20.4	26.0	24.6		21.5	20.4	41.0	41.5
	Reference	Reference	IL6 (1)	IL6 (2)		Reference	Reference	MicroRNA (	MicroRNA (2)
Treated 1	21.0	18.3	16.0	17.0		21.0	18.3	34.0	35.0
Treated 2	18.9	19.5	18.0	14.1		18.9	19.5	37.0	36.9
Treated 3	21.9	22.5	19.0	18.9		21.9	22.5	36.0	38.0
Treated 4	20.0	19.3	17.2	16.3		20.0	19.3	37.3	38.0
Treated 5	20.7	20.9	18.0	17.9		20.7	20.9	36.0	35.5
Treated 6	20.1	21.0	16.0	18.3		20.1	21.0	35.0	38.0
Treated 7	21.0	20.0	19.0	16.4		21.0	20.0	36.0	35.6
Treated 8	22.2	22.9	18.9	19.3		22.2	22.9	38.0	37.0
Treated 9	22.4	20.0	18.9	17.9		22.4	20.0	37.0	36.0
Treated 10	20.7	21.0	17.9	18.3		20.7	21.0	37.0	35.0
Treated 11	22.4	20.9	18.0	18.3		22.4	20.9	35.8	37.0
Treated 12	20.7	21.2	18.3	17.5		20.7	21.2	35.0	37.0
Treated 13	20.1	20.2	16.5	18.3		20.1	20.2	35.8	34.9
Treated 14	19.0	20.0	17.3	16.0		19.0	20.0	34.9	35.0
Treated 15	18.6	18.3	14.2	16.0		18.6	18.3	36.8	35.9
Treated 16	20.1	19.5	14.6	18.0		20.1	19.5	36.0	35.0
Treated 17	19.7	20.2	18.3	16.0		19.7	20.2	36.0	34.1
Treated 18	20.7	21.3	17.0	18.3		20.7	21.3	36.0	37.0
Treated 19	22.1	22.9	19.3	19.3		22.1	22.9	37.0	38.0
Treated 20	21.5	20.4	16.0	19.3		21.5	20.4	37.0	35.0
Treated 21	19.0	20.0	15.4	18.3		19.0	20.0	35.5	34.0
Treated 22	18.6	18.3	13.0	17.0		18.6	18.3	36.0	35.0
Treated 23	20.1	19.5	18.3	16.0		20.1	19.5	34.3	36.0
Treated 24	19.7	20.2	17.5	17.3		19.7	20.2	34.0	36.0
Treated 25	20.7	21.3	17.3	18.9		20.7	21.3	36.0	37.0
Treated 26	22.1	22.9	19.3	19.3		22.1	22.9	37.0	37.8
Treated 27	20.1	19.5	15.4	18.3		20.1	19.5	35.7	35.0
Treated 28	19.7	20.2	18.3	16.1		19.7	20.2	35.5	35.9
Treated 29	20.7	21.3	17.2	19.2		20.7	21.3	36.0	37.0
Treated 30	22.1	22.9	19.3	19.3		22.1	22.9	37.0	38.0

الخلاصة:

بينت النتائج ان من مجموع ١١٠ عينه سريريه(دم وادرار) من مرضى التهاب المجاري البولية المتكرر ان ٣٠ ٢٧.٣% عزلة تعود الى بكتريا الزوائف الزنجاريه. وتكون البكتريا الأخرى بنسبه ٨٠ % مع نسبه البكتريا الأخرى وعديمة النمو البكتيري للزرع مع فرق ذو دلاله معنويه عند نسبه ٠.٠٠١ .

من مجموع ٣٠عزله تعود الى بكتريا الزائفة ال نجاريه وكانت موزعه على مديات عمريه للمرضى من ٢٠-٥٠ سنه وكانت بنسبة ٣٦.٧% والنتيجة بينت ان اعلى نسبع عزل لبكتريا الزائفة الز نجاريه تعود الى عمر ٤٠-٥٠ سنه والدراسة الحالية بينت ان معدل عمر المرضى يكون ٢٨.٦٦+-١٠.٩٧ و ٢٥+-٥ لمجموعه السيطرة عند p=0.739.

الصبغة المنتجة من بكتريا الزائف الز نجاريه في هذه الدراسة الأكثر شيوعا كانت البايوسانين بنسبه ٧٣.٣% متبوعة ب البايوفردين كانت بنسبه ٢٦.٧%.

أظهرت النتائج اختبار الحساسية الدوائية لعزلات بكتريا الزائف الز نجاريه المعزولة مستويات عاليه من المقاومة للمضادات التي شملت الببارسلين ٢٦(٨٦%) ،والسفترياكزون بنسبه ٢٤(٨٠%)،والسفتازديم ٢٥(٨٣%)،والكاربنسلين ٢٣(٧٦.٦%)،والميروبنيم ٢٣(٧٦.٦%)والتبومايسين ٢٢(٧٣.٣%)،ووالاميكاسين ١٢(٧٠%) و السبروفلوكساسين ٢٠(٦٦.٦%) ،والجنتامايسين ٢٠(٦٦.٦%) بينما بينت النتائج مستوى اقل من المقاومة للسيفابيم ١٩ (٦٣.٣) ،والنوروفلوكساسين١٨(٦٠%) والامبينيم بنسبه ١٦(٥٣.٣%).

الدراسة الحالية وجدت ان التعبير الجيني رنا المتناهيه الصغر(٣٠٢ب) قد ازداد في مرضى الالتهاب المجاري البولية المتكرر المرتبط مع بكتريا الزائفة الزنجارية مقارنه بالأصحاء وبزياده تعبير جيني بأكثر من ٢٥%ضغف مقارنه بالأصحاء.

كذلك دراسة التعبير الجيني اللانترلوكين -٦ بواسطه البلمرة الكمي اللحظي(التعبير الجيني النسبي) في مرضى الالتهاب المجاري البولية المتكرر والنتيجة كانت ارتفاع في مستوى التعبير الجيني لدى المرضى مقارنه بالأصحاء والتعبير الجيني كان بزياده ٣٠ %ضعف مع المقارنة بالأصحاء وكذلك زياده معنويه للانترلوكين -٦ المفرز عند قيمه ودلاله إحصائية عند ٠.٠٠٠١ المقاس بواسطه الايلايزا مقارنه بالأصحاء.

قد جرى تحليل تعدد المواقع المتباين الاعداد المتكررة قد انجز لكل العزلالت البكتيرية للزوائف الزنجاريه ول ٨ مواقع قد اختيرت للتنميط الوراثي لبكتريا الزائف الزنجاريه بواسطه تفاعل البلمرة المتسلسل والترحيل الكهربائي بسبه ١-٥.١% نسبه الاكاروز الملون بصبغه بروميد الاثيديوم و ملف الاليلات قد حدد باستخدام تحليل تعدد المواقع المتباين المتكرر ،وكانت نتيجة هذا التحليل كل العزلات البكتيرية وضعت في ١٦ مجموعه مختلفة لهذ التحليل مع عنقودين رئيسين ا وب .

في هذه الدراسة التنوع الجيني ل٢٠ عزله من الزوائف الزنجاريه كذلك تم تقييمها بواسطه التنميط الرايبي .التنميط الرايبي قد انجز باستخدام نوعين من هذا التنميط الأول والثاني باستخدام بادئين الأول والثاني والتميز بين هذه العزلات من خلال التضخيم الجيني للحزم للأول من ٢٠٠-١٠٠٠ زوج قاعدي والبادي الثاني لحزم بحجم ٣٠٠-٩٠٠ زوج قاعدي.

التنويع الرايبي الأول قد بين ان العزلات صنفت الى ٣ عناقيد رئيسه و١٢ عناقيد فرعيه وكانت العزلات رقم ٥و٦و٩ متشابهة تماما ١٠٠%و١١و١٢ أيضا متماثله ١٠٠% و٨و٢٠كذا أيضا متماثله بنسبه ١٠٠% و٢و٣ أيضا متماثله لذلك هذا البادي يبين تشابه العالي بين العزلات.

التنويع الرايبي الثاني بين ان العزلات صنفت الى عنقودين رئيسين و١٢ عناقيد فرعيه وقد وجد ان العزلات برقم ٢و٣و٥و٦و٨و٩و١٢و متشابهة تماما بنسبه ١٠٠% ورقم ١٠و١٣ومتشابهه والعزلات رقم ١١و١٥متشابيه تماما و١٦و١٧ايضا متماثله وأخيرا ١٨و١٩و٢٠ كانت متشابهة بنسبه ١٠٠%والبادي بين مستوى كبير من التشابه بين العزلات.

وزارة التعليم العالي والبحث العلمي

جامعة بابل -كلية الطب

الكشف الجزيئي عن التعبير ال ار ان اي المتناهية الصغر والانترلوكين ٦ في عدوى المسالك البولية السفلية المتكرر التي تسببها الزائفة الزنجارية

أطروحة

مقدمة الى مجلس كليه الطب /جامعة بابل

وهي جزء من متطلبات نيل درجة الدكتوراه فلسفه في علم الأحياء المجهرية الطبية

من قبل

بارق عبد اللطيف صبر برير العجيلي

بكالوريوس في الأحياء المجهرية ٢٠١٢/ كلية العلوم -ماجستير احياء مجهرية طبيه /كليه الطب ٢٠١٧-جامعة بابل

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