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DETERMINATION OF THE GENETIC STRUCTURE OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM RAMADI POPULATION/IRAQ BY USING RAPD-PCR

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January 2023

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

DETERMINATION OF THE GENETIC STRUCTURE OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM RAMADI POPULATION/IRAQ BY USING RAPD-PCR

Omar Rajab Shahoodh SHAHOODH

Master of Science in Biology

Advisor: Asst. Prof. Dr. Emin BOZKURT

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January 2023

Totally 100 samples were taken from patients at two Ramadi Teaching Hospitals in Ramadi. These samples were collected from patients depending on the origins of their illnesses. The isolates were obtained from wounds, ear infections, and urine infections. The 75 samples of *P. aeruginosa* were characterized and identified by using enrichment, selective media, and biochemical testing. The genomic DNA of 12 *P. aeruginosa* isolates were extracted from these various sources and the concentration and purity of these genomic DNA samples were determined to be adequate for RAPD-PCR analysis. The amplification process with the two primers produced 27 bands and 24 of which were polymorphic and ranged in size from 250 to 1500 bp. The phylogenetic connection dendrogram among 12 *P. aeruginosa* isolates were constructed using 24 polymorphic RAPD markers, the genetic distance, and cluster analysis among different *P. aeruginosa* isolates were estimated by using a Jaccard matrix of similarity and analyzed by the UPGMA computer program to supply a phylogenetic tree. The results show the RAPD-PCR analysis is a good epidemiological screening method to detect genetic variation among *P. aeruginosa* isolates.

2023, 66 pages

Keywords: *P. aeruginosa*, RAPD-PCR, Genotype, Phylogenetic

ÖZET

RAMADİ POPÜLASYONUNDAN/IRAK'TAN İZOLE EDİLEN *PSEUDOMONAS AERUGINOSA*'NIN GENETİK YAPISININ RAPD-PCR İLE BELİRLENMESİ

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Ramadi'deki iki Ramadi Eğitim Hastanesindeki hastalardan toplam 100 örnek alınmıştır. Bu örnekler, hastalıklarının kökenlerine bağlı olarak hastalardan toplanmıştır. İzolatlar yaralardan, kulak enfeksiyonlarından ve idrar yolu enfeksiyonlarından elde edilmiştir. Zenginleştirme, seçici besiyeri ve biyokimyasal testler kullanılarak 75 *P. aeruginosa* karakterize edilmiş ve tanımlanmıştır. 12 *P. aeruginosa* izolatının genomik DNA'sı bu çeşitli kaynaklardan ekstrakte edilmiş ve bu genomik DNA örneklerinin konsantrasyonu ve saflığının RAPD-PCR analizi için yeterli olduğu belirlenmiştir. İki primer ile amplifikasyon işlemi 27 bant üretmiş ve bunların 24'ü polimorfiktir ve boyutları 250 ile 1500 bç arasında değişmektedir. 12 *P. aeruginosa* izolatı arasındaki filogenetik bağlantı dendrogramı 24 polimorfik RAPD markerı kullanılarak oluşturulmuştur, farklı *P. aeruginosa* izolatları arasındaki genetik mesafe ve küme analizi bir Jaccard benzerlik matrisi kullanılarak tahmin edilmiş ve filogenetik ağaç oluşturmak için UPGMA bilgisayar programı kullanılmıştır. RAPD-PCR bantlama modellerine dayanarak izolatlar iki birincil grup genotipinde kategorize elde edilmiştir. Sonuçlar, RAPD-PCR analizinin *P. aeruginosa* izolatları arasındaki genetik varyasyonu saptamak için iyi bir epidemiyolojik tarama yöntemi olduğunu göstermiştir.

2023, 66 sayfa

Anahtar Kelimeler: *P. aeruginosa,* RAPD-PCR, Genotip, Filogenetik

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Omar Rajab Shahoodh SHAHOODH

Çankırı-2023

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LIST OF SYMBOLS

%	Percentage
°C	Degree celsius
μm	Micrometer
bp	Base pairs
cm	Centimeter
DW	Distilled water
g	Gram
h	Hour
kDa	Kilo dalton
lbs	Bound
min	Minute
mL	Milliliter
mm	Millimeter
nm	Nanometer
pmol	Picomole
rpm	Round per minutes
V	Volt
α	Alpha
β	Beta
μL	Microliter

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AHL	Acyl homoserine lactone
BHI	Brain-heart infusion
CD	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic fibrosis fransmembrane conductance regulator
CRP	C-reactive proten
DNA	Deoxyribonucleic acid
EF	Elongation factor
ERIC	Enterobacterial repetitive intergenic consensus
ExoA	Exoenzyme A
ExoS	Exoenzyme S
ExoT	Exoenzyme T
IgG	Immunoglobulin G
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
LPS	Lipopolysaccharide
MHB	Mueller-hinton broth
MR	Methyl red
NTSYS	Numerical taxonomy system
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
QS	Quorum sensing
QSi	Quorum sensing inhibitory
RAPD	Random amplified polymorphic DNA
rRNA	Ribosomal ribonucleic acid
T3SS	Type III secretion system
TBE	Tris-borate-EDTA
TLR	Toll-like receptor
TMPD	Tetramethyl para phenylenediamine dihydrochloride
TSI	Triple sugar iron
UPGMA	Un-weighted pair-group method arithmetic
UTI	Urinary tract infections
UV	Ultra violate
VP	Voges-proskauer
WBC	White blood cell

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1. INTRODUCTION

Pseudomonas aeruginosa is a gram-negative, aerobic, non-sporulation organism that is found in soil, water, and living hosts. It moves by the use of polar flagella. An opportunistic pathogen, *P. aeruginosa* may infect practically every organ. Mucosal *P. aeruginosa* colonization of the pulmonary system that significant sickness and mortality causes among people with cystic fibrosis (CF) (Al-Wrafy *et al.* 2017). The majority of patients with nosocomial *P. aeruginosa* infection include those in critical care units, as well as those with catheters, burns, and chronic illnesses (Yetkin *et al.* 2006).

Conventional microbiological techniques for the detection of *P. aeruginosa* from clinical and environmental samples are reliable but time-consuming to complete. Rapid diagnosis of isolates that cause nosocomial infections is critical to patients' treatment decisions. By amplifying specific sequences of a given organism, PCR provides the ability to rapidly identify microbial species (Khan and Cerniglia 1994).

Two outer membrane proteins from *P. aeruginosa* called L and I lipoproteins are in charge of mycobacteria's resistance to antibiotics and disinfectants. These proteins can accurately identify *P. aeruginosa* in clinical specimens since they are present in *P. aeruginosa* (Nikaido and Vaara 1985).

Exotoxins A, exoenzyme S, elastase, and sialidase are virulence factors of *P. aeruginosa* that are all tightly regulated by cell-to-cell communication networks. Exotoxin A inhibits protein synthesis, and a type III partition system produces exoenzyme S which is a virulence factor. Las B, a zinc metalloprotease, has elastolytic activity in lung tissue. The sialidase enzyme, which aids in adherence to the respiratory tract, is produced by the *nan1* gene (Yahr *et al.* 1996).

In epidemiological studies conducted globally, the spread of illnesses that are resistant and exceedingly pathogenic has also been noted as a significant problem. The rRNA gene's chromosomal DNA restriction fragment length polymorphism (ribotyping) is a

useful technique for differentiating strains within and between species since rRNA sequences are highly conserved throughout eubacteria (Onteniente *et al.* 2003).

Aim of the study

The aim of the study investigates the presence of *P. aeruginosa* in wounds, UTIs, and otitis media from the patient's clinical appearance in Ramadi city. These samples were used and subjected to culture and RAPD-PCR methods. It also aims a comparison between *P. aeruginosa* isolates and identifies genetic diversity between *P. aeruginosa* detached using RAPD analysis through cluster analysis and the phylogenetic tree. It is demonstrated significant DNA variability between the genomics of several organisms belonging to the identical species. Also, it is identified the clonal nosocomial infection for determinnig the relationshap between the disease and the source of *P. aeruginosa* contamination.

2. LITERATURE REVIEW

2.1 *Pseudomonas* and Its Major Qualities

P. aeruginosa is a non-fermentative gram-negative type of bacteria that causes hospital-acquired infections and is impervious to several compounds. This bacterium has been isolated from a variety of facilities, most notably in intensive care units (Al-Awsi *et al.* 2019).

This bacterium pollutes water resources and generates epidemics, both in clinics and in terms of general hospital cleanliness (Sallman *et al.* 2018).

They facilitate bacterial motility and allow them to acquire nutrition more freely, and they allow them to dig deeper into tissues or cause greater injury to the tissues they inhabit by using enzymes (elastase, protease, and DNase) that can decompose various substances. However, biofilm seems to be the most infectious agent of bacteria (Mahmoud *et al.* 2022).

2.2 Epidemiology

P. aeruginosa is a sporeless, polarity flagella, negative bacteria with transposable elements that a typically an immediate release bacterium (Hematzadeh and Haghkhah 2021). *P. aeruginosa* does not digest sugars. It produces acid by oxidizing glucose in an oxidation-fermentation environment (Khalili *et al.* 2022).

Pseudomonas pseudomallei factor is mobile and usually coincides with malleus agents. It produces colonies varying in type from R to M. It creates a barrier on the broth's surface. It converts glucose, maltose, sucrose, and mannitol into acid influences gelatin. It contains oxidase. Certain strains can be destructive (Inglis and Sagripanti 2006).

While there is sufficient moisture, they can endure for an extended time on a tiny food supply. *P. aeruginosa* can be dangerous to the environment and mankind. Since it is inhabited by the mouth, mucous membrane, epidermis, as well as stools of healthy humans, it causes illness as an infectious agent in innate immunity patients. In a hospital setting, it is necessary to isolate patients from ventilators, baths, restrooms, toilets, beds, covers, bandages, bumpers, and flooring. It is among the key causes of care facility blood, urinary bladder, and respiratory problems. Patients undergoing burn treatment, oxygen therapy, chemotherapy, or surgery can become pathogens for patients who have received treatment. The bacterium's significance is increased by the fact that it generates nephrotic syndrome respiratory infection and is resistant to numerous antibiotics (Abdel-Rhman and Rizk 2021).

When cultivated in the medium, *P. aeruginosa* genotypes exhibit diverse hues. Among their other essential characteristics is that they produce dyes. The dyes pyocyanin blue, pyoverdine yellow-green, pyorubin red, and pyomelanin are involved in the creation of brown-black colo (Barati *et al.* 2021).

High in oxidase as well as catalase, *P. aeruginosa* isolates degrade carbohydrates by oxidation and produce acids. They respond to alkali and do not produce gas in three sugar-iron mediums. Several enzymes convert nitrates and nitrites into nitrogen gas (Ali *et al.* 2021a).

P. aeruginosa moves by using their polar flagella as well as measure 0.5-1.0 μm by 1.5-5.0 μm in size. Conditions between 30 and 37°C are suitable for reproduction. While it can breed successfully at 37°C, it can also grow at 42°C, setting *P. aeruginosa* apart from *Pseudomonas putida* and *Pseudomonas florescens* (Yang *et al.* 2020).

P. aeruginosa could manifest in a variety of colony shapes on medium. Alginate, a type of exopolysaccharide that is generated by some kinds, imparts a mucosal tissue quality to the bacterium. Isolated bacteria from immunocompromised patients and those with chronic lung disease commonly take the shape of mucosal tissue colonies (M colonies). Individuals separated out from milieu or other illnesses are mainly S-type colonies that

β-hemolyze or R-type colonies that produce little alginate. In blood agar, bacteria generate the characteristic green metallic sheen. They have a blue-green reproduction response on MacConkey Agar. In 24-48 hours of incubation in separation conditions. 2-aminoacetophenone of tryptophan in culture the unique grape odor produced by *P. aeruginosa* are detected the bacterium and recognized the odor when the petri dish is opened (Mombini *et al.* 2019).

2.3 Ailments Induced by *Pseudomonas*

Endocarditis caused by *P. aeruginosa* is especially prevalent in intravenous drug users. Most urinary infections caused by *P. aeruginosa* require hospitalization connected with catheter insertion for an extended period, surgery, or kidney transplantation. *P. aeruginosa* is the most prevalent bacterium responsible for disease in combustion injuries. The effects of hemodynamic changes are tissue necrosis and bacteremia. Common ecthyma gangrenosum tumors may form during *P. aeruginosa* septicemia (Vaiman *et al.* 2015).

Extensive infections, cellulitis, and bullae may develop. Common skin infections include folliculitis, caused by contact with moisture (swimming, heated water taps), and nail diseases that are caused by regular treatments. *P. aeruginosa* is frequently responsible for superficial otitis, also known as "swimmer's ear". Aggressive exterior otitis, noticed mainly in people with diabetes or elderly people, is a serious and contagious illness that could migrate towards the damaged tissue, causing deterioration of bone or injury to the cranial nerves. It can also result in a chronic middle ear infection (Blake Steele *et al.* 2020).

2.3.1 Infectious diseases caused by *P. aeruginosa*

2.3.1.1 Bacteremia

Among continuous patients with chronic kidney disease, the frequency of bacteremia ranges from 3.8% to 20%. Individuals with arteriovenous grafts who have blood albumin of less than 3.0 g/100 mL have a higher risk of developing bacteremia. In most cases, patients have a fever, chills, and a poisonous appearance. Nevertheless, there are situations when the symptoms are minimal. Gram-positive bacteria cause 50-74% of bacteremia, with *Staphylococcus aureus* being the most common. The entry points for bacteria include the vascular system, the digestive system, the urogenital system, and the skin. A blood sample has been necessary for diagnostic testing of bacteremia, so people with the disease suspected of having bacteremia should undergo blood testing. It may result in bacteremia, endocarditis, osteomyelitis, arthritis, metastatic abscess, and pulmonary embolism. If *S. aureus* is the possible cause of bacteremia, the average length of treatment for bacteremic patients is four weeks. It is desirable to examine culture and visual and interactive results when selecting antibiotics, but people who are infected with bacteremia should begin preventative treatment while having to wait for culture results. In most cases, successful pharmacological treatments for both gram-positive as well as gram-negative microorganisms are initiated promptly. Treatment delays increase death rates (Marei 2020).

2.3.1.2 Pneumonia

Inflammation of the lungs is popularly called pneumonia, a greater disease, and ranks fifth in terms of fatalities and first in terms of communicable diseases. Pneumonia is characterized by an acute and loud appearance (Asadpour 2018).

The first signs of normal pneumonia are a rising fever with chills, cough, yellow-green or soot phlegm, side aches when breathing, and difficulty breathing if the airway wall is affected. On a chest X-ray, lobar involvement is visible. Phagocytes, white blood cells

and C-Reactive protein (CRP) levels rise in the blood, and the formula shifts to the left. Typically, *Streptococcus pneumoniae, Hemophilus influenzae*, negative bacterial aerobes, and anaerobic bacilli are the causal culprits. The beginning is long and gradual. Early signs include joint and muscular discomfort, exhaustion, and decreased appetite. In classic pneumonia, signs include dry cough, wheezing, headache, and abdominal discomfort, in addition to the importance of extra-pulmonary systems (Hassuna *et al.* 2020).

Radiology reveals pericardial involvement and patchy infiltration. The leukocytes (WBC) count is normal or below average. Numerous microbes, including viruses, bacteria, and fungus, can cause pneumonia. The age of the person affects the etiologic agents of pneumonia. Group B *Streptococcus* and negative bacteria from the mother are common vertically transmitted causes in babies (0-3 months of age). The much more common bacterial source of pneumonia in kids elder than one week is *Streptococcus pneumonia*, which accounts for 14% to 35% of infections (Lynch III and Zhanel 2010).

Among kids between the ages of three months and five years, viral respiratory infections account for 50% to 60% of occurrences. Atypical organisms, such as *Mycoplasma pneumonia* and *Chlamydophila pneumonia* are more prevalent in school-aged children (>5 years) (Virkki *et al.* 2002).

2.4 Cystic Fibrosis with *P. aeruginosa* Infection

CF affects one in every 2500-3000 white individuals. It is unique among the most prevalent autosomal recessive illnesses even though a small number of research studies indicate that the prevalence of CF is 1 in 3000. It is evident that this rate was higher due to the prevalence of cousin marriage and the higher risk of death of children younger than 5 with diarrheal as well as respiratory problems without recognizing the actual issue. Since the first adult occurrence recorded in 1946, an increasing number of adult individuals eventually grown, despite the bacteria's previous reputation as a deadly childhood illness. With proper care and therapy, the average lifetime of diagnosed cases at a young age now, given the data gathered about the condition, is greater than it was

previously. Individuals with an unusual look and modest mutations that identified in adulthood could be detected as genetic information on CF expands (Emaneini *et al.* 2019).

The number of children who were unable to grow through nutritional inadequacies, who seemed to have a tense belly, pale complexion, and clear penalty diarrheal waste, perished in their second year owing to respiratory and pulmonary problems, as was initially documented as well as noticed. It was found that the infection mostly affected the respiratory system and that the lung tissue lumens were blocked by a greenish-gray, sticky inflammation (Nabilou *et al.* 2021).

This is one of the major complications encountered by CF sufferers. At the beginning of the 1950s, salt transport in the epithelial cells was the source of the disease. It was determined to be abnormal. It was demonstrated that the salt loss in the sweat of ill children was considerable (Oluborode *et al.* 2018).

Experts expressed their optimism that, because of their researches, the infectious activity of bacteria would be better comprehended, and, therefore, these recurrent diseases in CF individuals would be more effectively treated in the future (Goli *et al.* 2018).

Researches has revealed that even though existing anti-fibrotic medicines can halt the advancement of a deadly illness, they are unable to restore full lung function, which can only be accomplished through a liver transplant (Alag *et al.* 2020).

The views of nine idiopathic pulmonary fibrosis (IPF) patients (ages 57-83) and nine health professionals were gathered. All individuals remarked on the difficulties in identifying their complaints (Matsui *et al.* 1998). Medical experts talked about how important it is for expert staff to look closely at a patient's medical records (Xu *et al.* 2021).

The diagnostic method, all of the patients stated that they had been diagnosed by regional health facilities, even though some expressed dissatisfaction with the process.

Furthermore, two respondents emphasized the significance of how healthcare providers convey the prognosis. Individuals had unfavorable experiences if the prediction was prioritized over treatment. For example, a worker was fired after informing his boss that only had 3-5 years to live (Schauer *et al.* 2021).

2.5 Pathogen Therapies Influenced by *P. aeruginosa*

Samples of sputum, urine, pus, spinal fluid and swabs are required for the isolation of bacteria in *P. aeruginosa* cases based on the site of infection. In addition to its rod-shaped, gram-negative appearance, grape-like odor, colony type, and dye creation, this can be recognized by its oxidase positivity and non-fermentation in three sugar-ferrous media, as well as its ability to grow at 42°C. In addition to traditional and biochemical characterization, can provide rapid and accurate identification results (Khosravi *et al.* 2021).

With CF patients' increased life expectancy, 70-90% develops symptoms of microbial contamination with *P. aeruginosa*. In cystic fibrosis person, bacteria-caused respiratory viruses are attributed to the absence of the CF transmembrane conductance regulator in epithelial cells, according to studies (Majeed *et al.* 2021).

The oligosaccharide on the outer surface of *P. aeruginosa* is capable of binding to CF transmembrane conductance regulator (CFTR) gene in lung tissues. The epithelial cell connects the bacteria to itself with CFTR, which has become a transmitter for *P. aeruginosa*, endocytoses the bacteria as well as isolates it on the tissue surface. In CF sufferers, on the other hand, the faulty CFTR protein prevents the epithelial cell from ingesting the bacterium and triggering an inflammatory reaction. In this manner, germs might bypass the initial line of protection against infection, the immune response. Colonization of the respiratory system occurs when germs cannot be eliminated (Xia *et al.* 2022).

Primary colonization is typical with non-mucoid bacteria, and as invasion gets chronic, the bacterium transforms into a mucoid phenotype and dominates the lungs. The time-varying environment inside the airways of CF individuals increases the creation of alginate, which is essential for the transformation of the bacterium into a mucoid phenotype. Alginate additionally involves the production of biofilms. *P. aeruginosa'*s *mucA* gene encodes the anti-sigma factor. Under normal circumstances, the anti-sigma factor inhibits the *algT* gene (one of the key regulators of alginate synthesis), which is important for alginate production. Nevertheless, oxygen radicals produced through the inflammatory process in CF patients trigger mutations in the *mucA* gene and increase alginate concentration. The biofilm resulting from the production of alginate protects bacteria from medicines as well as the immune system (Stefańska *et al.* 2022).

2.6 *P. aeruginosa* Etiology Virulence Factors

2.6.1 Factors of virulence

Causative agents of *P. aeruginosa* are dependent on virulence genes that function at various levels throughout the invasion and enable *P. aeruginosa*'s viability in both the host organism and the environment. *P. aeruginosa'*s virulence factors are active both within and outside of the cell (Al-Azawi *et al.* 2021).

These virulence factors have specific roles and varied energy levels. In primary infection, infiltration, dissemination, colonizing, and tissue destruction predominate. On the other hand, *P. aeruginosa* could generate varying aspects of adaptability in persistent diseases and parasites, particularly in CF patients. In recurrent situations, *P. aeruginosa* is capable of isolating itself against the protective immune response by shedding immunogenic features including fimbria and flagellum, and by establishing a biofilm on the epithelial of the respiratory tract, therefore limiting immunological inhibition. Recurrent infections are characterized by prolonged inflammation and the release of external virulence agents. *Pseudomonas* virulence factors play a crucial role in acute illness (El-Badawy *et al.* 2021). Major virulence factors of *P. aeruginosa* were given in Figure 2.1.

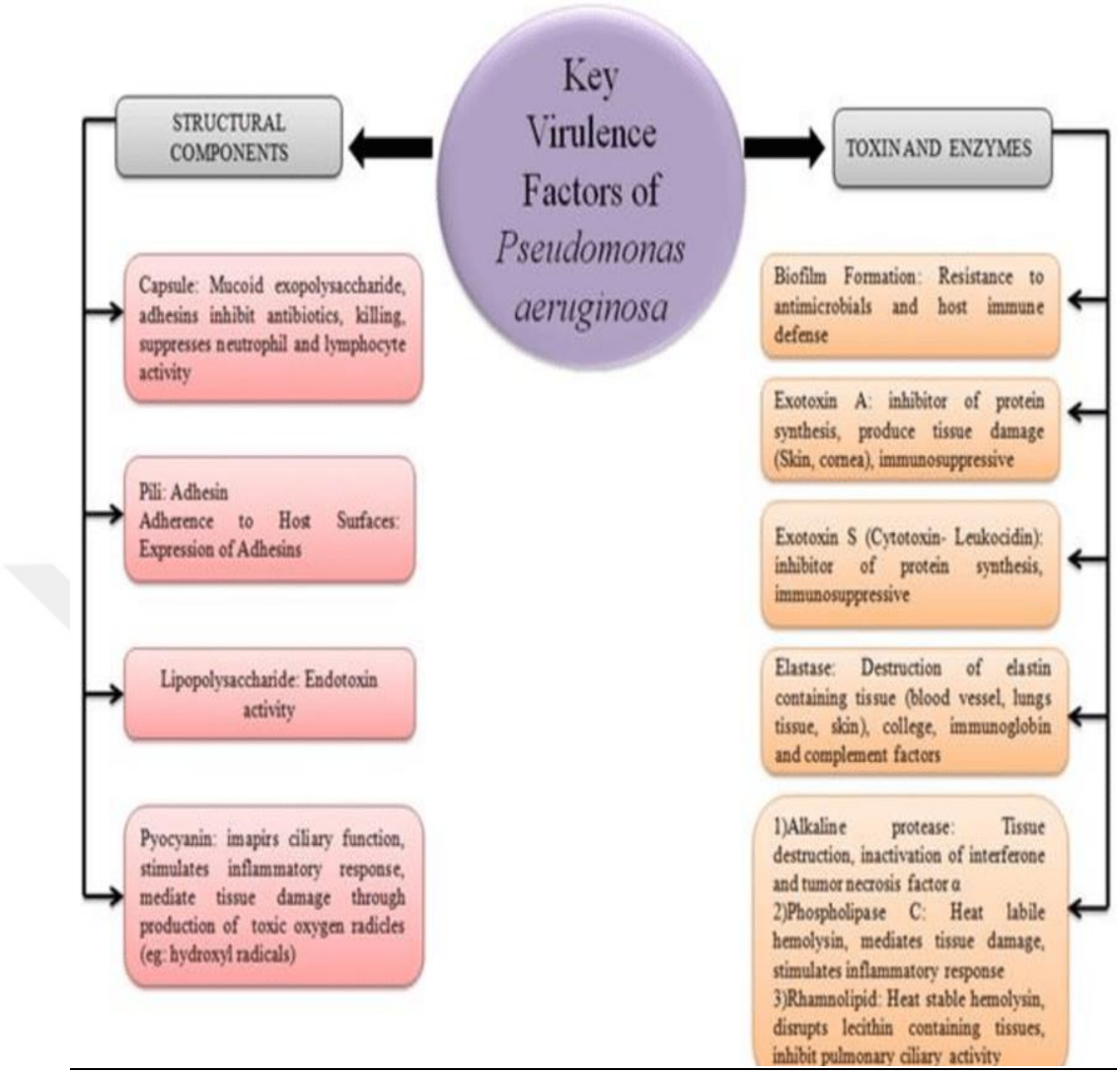

Figure 2.1 Major virulence factors of *P. aeruginosa*: mode of action and clinical outcomes (Bhardwaj *et al.* 2021)

2.6.2 Virulence factors affecting attachment

There are numerous adhesives responsible for exterior adherence. Immunogenically strong are the polar flagella, essential for things like the moving activity of the bacterium. By connecting to toll-like receptors (TLR), it promotes adhesion and promotes the immune response. Although polar flagella are occasionally detected in environmental and medical isolation, their presence is uncommon. This is related to the isolation of mutant strains. Although pili having short, fibrous surface areas are not responsible for stimulating the majority of bacteria, they do cause twitching. It contributes to the

production of pili biofilm that aids respiratory tract invasion. It identifies TLR4/CD14 or TLR4/MD-2 synapses and binds to CFTR, which is essential for virulence (Newman *et al.* 2022).

2.6.3 Flagellum bacteriophorum

It increases the NFib-dependent inflammatory reaction by engaging with cilia toll-like receptor sites (TLR5 and TLR2) and initiating interleukin-8 production via calcium-dependent kinase pathway activation. These are short, filamentous bacterial surface structures. Pili are not normally responsible for stimulating prokaryotic cells, but in *P. aeruginosa*, pili are necessary for twitching (Bogiel *et al.* 2021).

2.6.4 Four pilus varieties

Enterobacteriaceae species and *Pseudomonas* species were also present. It is a contrivance in which all the bacteria create a hole in the target tissue, construct a pilus-like connection between two cells, and then transfer activator protein to the eukaryotic cell cytoplasm across this bridge. This poison influences overall tissue injury and takes part in respiratory infections. Exoenzyme T (ExoT): ExoT, like ExoS, has a double effect, that plays a role in inducing rounded cell morphology and mediating the disruption of actin microfilaments in *Pseudomonas* internalization (Iglesias *et al.* 2008).

2.6.5 Lipopolysaccharide

Based on their antigenic characteristics, O-specific polysaccharide sequences are informative for recognizing *P. aeruginosa* serotypes. It has a considerable impact on pathogenicity because of its TLR4/CD14 or TLR4/MD-2 interface crediting characteristics and CFTR (King *et al.* 2009). The LPS is a major component of *P. aeruginosa'*s outer membrane; it is important in triggering the host's adaptive and innate immune responses, and, finally, it produces dysregulated inflammatory responses that lead to morbidity and death (Alhazmi 2015).

2.7 Efficient Infectivity Influences Conquest

By dividing groups according to their connection with quorum sensing (QS), it is feasible to analyze the pathogenicity components responsible for the successful colonization of *P. aeruginosa* in host cells. QS might be regarded as a novel discovery, and it is known as core detection, consensus detection, and quorum detection, based on the variety (Le Berre *et al.* 2008).

2.7.1 Infectivity variables associated with QS

The growth of multidrug-resistant isolates needs the creation of innovative infection-treatment strategies. The dominance of numerous virulence variables, including biofilm generation, in *P. aeruginosa* by QS has prompted the development of novel antibacterials targeting it. The lack of QS on bacterial growth and antibiotics' apparent resistance to QS suppression is significant. It is useful since it does not result in resistance to different strains (Wang *et al.* 2022).

In defining QS inhibitory (QSi), several substances with suppressive properties were identified by screening a large number of biological and man-made molecules. These substances exert their influence on three distinct levels. Researchers show that (i) Acyl homoserine lactone (AHL) production is stopped, (ii) the AHL signaling molecule is turned off, and (iii) the AHL receptor protein is turned off (LaSarre and Federle 2013).

The strategy that has received the least amount of research is signal generation blockage. Although it has been demonstrated that compounds such as adenosylhomocysteine, sinefungin, and butyryl-S-adenosylmethionine (butyryl-SAM) limit AHL formation, they are in vivo activity on bacteria will not be investigated. In addition, no one knows how well these chemicals, which are very important to how microbes work, affect other cellular activities (Abdelrahman and Ahmed 2021).

2.7.1.1 Exotoxin A (ExoA)

The majority of bacterial infections are derived from *P. aeruginosa,* which produces ExoA. It is an extracellular toxin Sort by type 2 excretion system. ExoA consumes a crucial part in the pathogenicity of *P. aeruginosa*. It exerts an impact comparable to that of diphtheria toxin by blocking elongated factor 2 (EF-2) and, by extension, protein production and by inducing cell decease via its ADP-ribosyl transferase property. It cause colonization and localized tissue destruction. It has also found to prevent the host's immune response. When scientists compared the mutant strain of *P. aeruginosa* with no ExoA formation to the wild-type strain, they found that the new strain is 20 times less dangerous (Li *et al.* 2007).

2.7.1.2 Elastases

Elastic is a protein that permits the lungs to expand and contract, so it accounts for around 30% of the proteins inside the lung. *P. aeruginosa*'s elastolytic action is attributed to LasA protease and LasB elastase. Two enzymes work together to disintegrate elastin. LasA is a serine metalloproteinase that breaks down proteins' pentaglycine peptidoglycan bonds in cell walls. LasA cannot degrade elastin; however, LasB increases elastase's efficiency. LasB is a zinc metalloproteinase for each molecular mass of 33 kDa that degrades elastin damaged by the LasA enzyme. LasB elastase has rising protein expression (Kalurazi *et al.* 2021).

Elastase, which is released by the external type 2 secretion system, has a significant impact on pathogenesis by inducing lung damage the initial stages of an infection as well as breaking down complementing elements and serum proteinase inhibitor. Elastin degradation leads to hemorrhages in a lot of elastin in the vascular structural frame. It degrades IgG, fibrin, collagen, and the surfactant components A and D. Furthermore, by disrupting the tight connections in the respiratory system epithelium, it enhances the porosity of the epithelium and produces a rise in the number of neutrophils in the infected spot. It promotes inflammation by enhancing the levels of elastase and IL-8 (Attiah *et al.* 2021).

2.7.1.3 Phospholipase C

Phospholipase C is classified into two kinds based on its hemolytic activity. Phosphatidylcholine and sphingomyelin are hydrolyzed by hemolytic phospholipase C. Phosphatidylcholine and phosphatidylserine are hydrolyzed by non-hemolytic phospholipase C. The type 2 secretion system is responsible for the removal of phospholipase C from the cell. It contributes to the pathophysiology of pulmonary disease by interacting with phospholipids that are part of eukaryotic cell membranes. It, like elastase, derives much of its pathogenicity from surfactant deactivation. Furthermore, it inhibits the host's neutrophil oxidative reaction (Al-Zubaidi 2021).

2.7.1.4 Rhamnolipids

It's hemolysin with hemolytic properties because of the glycolipid rhamnose in its structure, which has a biocatalytic action. It facilitates the action of phospholipase C via turning the pulmonary surfactant phospholipids hydrophilic. Moreover, it interferes with mucociliary transmission and ciliary activity (Allami and Dragh 2022).

2.7.1.5 Pyocyanin

It is *P. aeruginosa'*s blue pigment metabolite. The phzABCDEFG operon and the *phzH, phzM,* and *phzS* genes are associated with the formation of three-ringed pyocyanin from its precursor molecule, chorismic acid. Because *P. aeruginosa* are complex processes with multiple virulence genes, in vitro cellular research was conducted using isolated pyocyanin to determine the effects of pyocyanin alone on lung diseases. Research has revealed that pyocyanin slows cell respiration, hinders ciliary activities, halts epidermal growth, triggers the release of prostacyclin, and preserves calcium homeostasis. Additionally, pyocyanin deactivates the 1-protease inhibitor, upsetting the protease-antiprotease equilibrium and producing lung injury (Meletiadis *et al.* 2021).

2.7.1.6 Pyoverdin

It consists of a siderophore. It provides iron for *P. aeruginosa'*s metabolism by collecting iron in the surroundings. It makes the bacteria more dangerous by controlling how much of it is made and how much ExoA is made (Ugwuanyi *et al.* 2021).

2.7.1.7 Alkaline protease

It is an efficient fibrinolysis metalloprotease of *P. aeruginosa.* The *APR* gene encodes the 49 kDa enzyme, which is optimally alkaline. It is useful at various pH levels. Alkaline protease takes already been found to induce the development of infection by breaking the thick fibrin that forms in the alveoli during the early phase of acute lung damage. Even though the significance of alkaline protease in cell infiltration and bacteremia is not completely understood, it has been demonstrated that it shows a vital part trendy the pathogenesis in corneal infections (Faisal *et al.* 2021).

2.7.1.8 Protease IV

Protease IV was previously known for its function in the development of *P. aeruginosa* keratitis, but current research indicates that it can also be employed as a surfactant. It has been shown that this enzyme breaks down proteins A, D, and B, which can lead to lung diseases *P. aeruginosa* protease IV reduces the host defense and biophysical activities of pulmonary surfactant by degrading surfactant proteins (Caballero *et al.* 2004, Malloy *et al*. 2005).

2.7.2 Violent factors not connected to QS

2.7.2.1 Type III secretion system (T3SS)

The T3SS has received the most attention in pathogenesis, and numerous studies were written on the issue. With a few exceptions, plant-pathogenic gram-negative bacteria

invade their victims with T3SS.The system is utilized not just for virulence genes but also for plant mutualists and commensals. Injecting bacterial type 3 effectors straight through into a human host is the systems defining characteristic (Pakbin *et al.* 2021).

The T3SS is also known as the injectisome or the molecular needle because of its anatomical configuration shape and how it delivers proteins by touching the host cell. Having a sophisticated supramolecular construction, the T3SS traverses the internal membrane, periplasmic cosmos, external membrane, and extracellular planetary, besides recipient cell barriers trendy a single movement (across 5 steps). It was also reported that T3SS is physically and genetically similar to the flag system of bacteria. Without the T3SS phytopathogenic bacteria could not overcome the basic protections and effects on growth, the appearance of host sickness, or the hypersensitive reaction in the non-host. With the aid of T3SS phytopathogenic bacteria transfer pathogenicity effector proteins, which play crucial roles in pathophysiology, towards the cell cytoplasm of the growth medium. Numerous type 3 effector proteins were discovered from plant pathogens' bacteria (Hauser 2009).

2.8 Virulence Factors Impact Long-Term Infection

The majority of such microorganisms are typically harmless due to the host immune system's innate protection. The host's resistance is compromised in susceptible individuals, so when microorganisms from the flora enter, they commonly cause aggressive infectious illnesses. Nosocomial infections are viral infections that originate within a hospital. The typical flora includes *S. aureus* (found on this surface), *S. epidermidis*, and *Propionibacterium acnes*, as well as Bacteroides and Enterobacter (found in extremely small groups) (Anindyaswari *et al.* 2021).

2.8.1 Alginates and biofilm development

P. aeruginosa produced initiator in alginate bacterial growth plays a crucial function. A biofilm is a matrix composed of an extracellular matrix component that is implanted in

the bacterium that generates it, allowing microbes to cling to any surface or others. *P. aeruginosa* develops a biofilm on natural substrates, particularly in the lungs of people with CF. Since biofilm germs experience both phenotypic and metabolic alterations, they become refractory to treatment and can evade host response (Ramsey and Wozniak 2005).

Various signaling chemicals generated by Fungi's "QS System" (the majority sensing system) are hypothesized to play a function. Microbes can tell how many other bacteria are around them by how much chemical waste they make, and they respond to information by turning on many genes (Abed and Kareem 2021).

In gram-positive bacteria, the signal molecules of the system are tiny peptides, but in gram-negative bacteria, they are referred to as "auto-inducers". It is composed of certain chemicals. These autostimulators are AHL and LuxI and LuxR autostimulatory synthases. The transcription-activating protein produces this molecule. In situations with low cell density, the auto-stimulus is generated at a basal level, and its intensity declines because it extends into the environment (Mraidi and Lafta 2021).

Two QS systems can be defined in *P. aeruginosa*. These processes, which are related to the Las and Rhl systems, interact with one another. The Las structure controls the expression of the *LasB* gene and is necessary for the manufacture of the external pathogenicity components LasA elastase and ExoA. The Rhl QS system regulates the synthesis of rhamnolipids (Matsumoto *et al.* 2022).

2.9 RAPD-PCR Technique

While there are a number of methods for genetically analyzing bacterial isolates, the RAPD-DNA methodology is straightforward and doesn't require previous information on the nucleotide sequence of both the target cell. Additionally, a strain typing surrounded by 48 h of removing the colony first from agar media extremely quick and easy to carry out. It requires very little template DNA and is highly sensitive. But, little variations in

the precursor solution and temperature cycles can affect its sensitivity as well as reproducibility (Onasanya *et al.* 2003, Oldak and Trafny 2005).

It translates distinct structures and identifies unlike sorts of mutations in microbial DNA, RAPD-PCR produces diverse results. RAPD-PCR finds changes across the bacterial genome, not only in specific regions. As a result, this technique is useful in bacterial isolates over lengthy periods (Nanvazadeh *et al.* 2013).

3. MATERIALS AND METHODS

3.1 Laboratory Apparatus and Equipment

Table 3.1 and Table 3.2 list the instruments and tools utilized in this investigation.

Table 3.1 Devices and tools used in this study

Equipment and Apparatus	Company and Origin
Autoclave	Sakura (Japan)
Centrifuge	Hettich (Germany)
Cooling Centrifuge	Hermle (China)
Distilled Water	Elga (England)
Electric Balance	Elga (England)
Electric Oven	Olympos (Japan)
Electrophoresis System, Power Supply	Progress (Japan)
UV-Trans Illuminator	Bio Air (Italy)
Gel Document System	Apel (Japan)
Hood Cabinet	Fisher Hamilton (USA)
Hot Plate	Takashima (Japan)
Incubator	Yamato (Japan)
Micro Centrifuge	Beckman Coulter (USA)
Microtiter Plate (Polystyrene)	Coastline (USA)
Microwave	Panasonic (Japan)
Light Microscope	Olympos (Japan)
Refrigerator	National (Japan)
Shaking Incubation	Gallen Camp (England)
Spectrophotometer	Apel (Japan)
Thermal Cycler	TECHNE, TC-3000, Bibby Scientific Ltd, (USA)
Water Bath	TAFE SA Hannover (Germany)
VITEK-2	Biomerieux (France)
Vortex Mixer	Biocote (England)
Combi-Spin	Dig System (Germany)

Table 3.2 Other laboratory supplies

Materials	Company and Origin
Automatic Pipette (Different Size)	Brand (Germany)
Eppendorff Tube (Different Size)	Promega (USA)
Filter Papers Whatman (0.2 µm)	Schleicher and Schuel (USA)
Forceps	Olympos (Japan)
Plane Tubes (10 mL), Petri Dishes	Firm (Chinese)
Sterile Swab	Jlassco (India)

3.2 Chemicals

In Table 3.3, a list of the chemicals employed in the investigation is provided.

Table 3.3 Chemistries's list

Chemicals and Biologicals	Company and Origin
Absolute Ethanol (99%)	Scharlau (Spain)
Acetic Acid	BDH (England)
Alcohol	BDH (England)
Crystal Violet Powder	APCO (Jordan)
Deionized Sterile Distilled Water	Theba (Iraq)
Glycerol (99%)	Panreac (Spain)
Gram Staining	Apco (Jordan)
Hydrogen Peroxide 3% (Catalase Test)	Becton-Dickinson (USA)
McFarland Solution	Biomerieux (France)
Methanol	BDH (England)
Methyl Red	BDH (England)
Sodium Chloride (NaCl)	BDH (England)
Sodium Hydroxide (NaOH)	BDH (England)
Hydrochloric Acid (HCl)	BDH (England)
Iodine	Biomerieux (France)
Oil Immersion	Sigma Aldrich (Malaysia)
Para-dimethyl-amino benzaldehyde	BDH (England)
KOH	BDH (England)
Safranin	Biomerieux (France)

3.3 Methods

Clinical and practical researches were conducted in the Ramadi General Hospital in the city of Ramadi, Al-Anbar Province/Iraq.

3.3.1 Specimen collection

One hundred swabs were collected from patients with wounds, otitis media infections, and urinary tract infections (UTI). Before a bacteriological inspection, specimens were transferred by sterile delivery and stored at 4°C. The researcher discussed patients receiving inpatient care for infectious infections, and clinical. Samples were obtained from patients were recombened in Ramadi General Hospital and Al-Ramadi General

Hospital for Women and Pediatric. Those willing to participate in the study submitted notarized consent forms. With the agreement documents in hand, the hospital's ethics commission was contacted and authorization to perform the research was granted. Every patient provided a verbal and written report. All the samples were collected through the period since December 2021 toward February 2022.

Due to the Covid-19 pandemic, several limits and challenges were faced during the study, but the research was continued by taking the required safeguards.

Several factors were taken into account in selecting patients.

1) All patients are over the age of 18 years old.

2) The individuals have no additional conditions that could impair the experiment (assistance was received for the diagnosis and approval of the physician in charge).

3) Patients must be aware and possess sound decisions-making skills regarding the research operations.

4) Before taking the sample take the consent of patients to participate in the research.

Taking into account the gender distribution of the collected samples, wound infection samples were collected from 55 cases (23 males and 32 female patients), and UTI samples were collected from 15 cases (6 males and 9 female patients), and otitis media infection 30 cases (13 male and 17 female).

3.4 Culture Media

List of media used during this research were given as Table 3.4

Table 3.4 Media used during this research

Cultural Media	Company and Origin
Blood Agar Base	Micro Media (India)
Mannitol Salt Agar	Merk (Germany)
Methyl Red-Voges Proskauer Water (MRVP)	Oxoid (England)
Mueller-Hinton Broth (MHB)	Merk (Germany)
Müller-Hinton Agar	Merk (Germany)
Trypticase Soy Water	Oxoid (England)
Brain-Heart Infusion (BHI) Broth	HI media (India)
Cetrimide Agar	Macro media (Hungary)
DNase Agar	Oxoid (England)
Human Blood	RamadiTeaching Hospital Blood Bank
MacConkeyś Agar Medium	Micro media (Germany)
Nutrient Agar Media	Micro media (Germany)
Nutrient Broth Media	HI media(India)
Peptone Water Media	HI media(India)
Pseudomonas Agar Media	HI media (India)
Simmon Citrate Agar	Micro media (Germany)
Skimmed Milk Agar Media	Micro media (Hungary)

It is heated in a water bath to dissolve all of its ingredients and then autoclaved at 121℃ for 15 min. During 15 gram per square inch, poured in petri dishes, and stored at 4°C until use.

3.4.1 Blood agar

It was prepared by 1000 mL of distilled water and 40 g of blood agar base were combined, then the composition was sterilized by autoclaving at 121°C during 15 min to sanitize it. The media was added, thoroughly mixed, and then cooled to 45°C before being placed into the petri plate with 5% human blood (Niederstebruch *et al.* 2017).

3.4.2 Brain heart infusion

BHI was made by melting 37 g in 1000 mL of distilled water and bringing pH near 7.2. Before used, the medium was placed on sterile petri plates and autoclaved for sterilization. It was used to keep bacterial isolates safe (De Graaf *et al.* 2013).

3.4.3 Cetrimide agar

Cetrimide agar was made by melting 45.2 g of the substance in 1000 mL of distilled water that contained 10 mL of glycerol. The media was then autoclave sterilized. It was put into the petri dishes after cooling to a temperature of 45°C (Lilly and Lowbury 1972).

3.4.4 Skimmed milk agar

It was prepared by added 51.5 g in 1000 mL of distilled water and stir. Heat it to a boil, until the medium is well melted. At 15 pounds of pressure autoclave by pressurized steam for 15 min at 121°C. Put into sterile petri plates after thoroughly mixing (Brown and Foster 1970).

3.4.5 MacConkey agar

Gram-negative organisms can be selectively grown on MacConkey agar, which also distinguishes between lactose-fermenting and non-lactose-fermenting gram-negative rods. It is mostly used to find and isolate *Pseudomonas* species and members of the Enterobacteriaceae family. 51.5 g of this medium were melted in 1000 mL of distilled water, pH was corrected near 7.2, it was sanitized, chilled, then it was placed into sterile petri dishes (Humphries and Linscott 2015).

3.4.6 Nutrient agar

Using sterile petri dishes or sterile tubes, It was prepeared by created melting 25 g in 1000 mL, of distilled water, adjusting pH near 7.4, sanitizing, then cooling (slant). This medium was employed to grow and maintain the microorganisms (Al-Saffar and Jarallah 2019).

3.4.7 *Pseudomonas* agar

In 1 liter of deionized or distilled water, dissolve 52.4 g of powder. Glycerol supplement should be added in 10 mL. Stir until dissolved after bringing to a boil. Sterilization takes 15 min at 121°C. Cool to a temperature of 45 to 50°C.

3.5 Morphological Examination

On MacConkey agar, cultured isolates, cetrimide agar and blood agar were used to determine the main morphological features under light microscopy of bacterial growth, such as size, colonial shape, texture, edges, odor, colouration, and hemolysis (Sønderholm *et al.* 2017).

3.5.1 Discoloration of grams

Cell morphology, such as volume, structure, organization, as well as reactivity to the dye, is a crucial first step in the classification. All bacteria are inspected with a light microscope, and samples from the fresh culture are made and stained with gram staining.

To determine the size and organization of the microorganisms seen in gram staining, oil immersion microscopy was used (Beveridge *et al.* 2007).

It comprises all the subsequent steps.

- Crystal violet solution
- Lugo iodine solution
- Acetone alcohol mixture
- Saffron-based remedy

3.6 Growth at 4-42°C Test

Nutrient agar was used to cultivate bacterial cells, and each isolate of a single colony was seen individually on the medium before being incubated overnight at 42°C. The positive test results demonstrate that this species can develop at this temperature. These isolates were also incubated at 4°C for 18-24 h on nutrient agar. The expansion is viewed as a favorable sign.

3.7 Biochemical Tests

3.7.1 Catalase substance

It was made by 3% hydrogen peroxide from a 15% standard solution. It has been utilized to virus's capacity to manufacture the enzyme catalase. The substance was stored in a dark bottle.

3.7.2 Oxidase reagent

It was freshly prepared from 0.1g tetramethyl para phenylenediamine dihydrochloride (TMPD) in 10 mL distilled water in a dark bottle. This reagent was used for the detection of the cytochrome oxidase enzyme.

3.7.3 Voges-proskauer reagent

It was synthesized by combining 40% potassium hydroxide and 96% ethanol in 5% a-naphthol. It served as an indicator in the Voges-Proskauer test.

3.7.4 Kovac's reagent

It was prepared by carefully adding 5 mL of 1N HCl after 1 g of P-dimethyl amino benzaldehyde had dissolved in 15 mL of iso-amyl alcohol, and then setting the liquid aside at 4°C.

3.7.5 Indole test

An overnight examination of bacteria was followed by the addition of the bacteria to a tryptone broth medium, which was then put it incubater at 37°C for 24 h. Following that, 5 drops of Kovac's reagent 0.5 mL were immediately additional to the culture tube; a positive end result was well-thought-out to have been achieved if, following a gentle shake, a red ring developed at the top of the soup.

3.7.6 Methyle red reagent

It was prepared by melting 0.1 g of methyl-red coloring to 300 mL of 90% ethanol, which was then increased to 500 mL by adding distilled water. This substance served as the methyl red test indicator.

3.7.7 Citrate utilization test

Using a light inoculum selected from the center of a well isolated colony line forward and backward. For up to 24 h, incubate antennae at 35-37°C and note the color change from green to blue along the slope.

3.7.8 Gelatin liquefaction

When gelatin tubes were punctured in the center with an infected needle, they were incubated at 37°C for 24 h before being placed in the freezer at 4°C for 30 min.

3.7.9 Haemolysin test

The bacteria were plated on 5 % blood agar and cultured nightly at 37°C to identify β-hemolysis and the formation of a zone of total cell analysis surrounding the colony (Berk 1962).

3.7.10 Protease test

20 µL was isolated from bacterial culture and put in wells of petri plates using skimmed milk agar for 24 h at 37°C. The data were obtained by observing the well's observing zone.

3.8 Detection of *P. aeruginosa* via VITEK-2

The VITEK-2 is a completely automated system that identifies *P. aeruginosa* bacteria. Using a 64-well card barcoded with data on card type, expiration date, lot number, and unique card identification number, the VITEK-2 compact system combines a fluorogenic approach for organism identification and a turbidimetric method for susceptibility testing. Gram-positive cocci identification and gram-negative bacillus identification test kits are both offered (Joyanes *et al.* 2001).

3.9 Short-Term Storage

Bacteria were kept on nutrient agar plates for several weeks, covered in parafilm from the tilted surface and then incubated for 24 h at 37°C, and kept at 4°C.

3.10 Long-Term Storage in the Middle

Stabbing cultures have a finite shelf life for bacterial cultures. These cultures were grown in test tubes with lids using 5 mL of the nutrient agar plate and and they were kept at 40°C or room temperature.

3.11 Long-Term Storage

Bacterial isolates can be kept for a very long time at low temperatures in a medium containing 20% glycerol without experiencing any discernible loss of viability. 8 mL of BHI were combined with 2 mL of sterilized glycerol to achieve this. The tubes were kept in a deep freezer at -20°C for storage following 24 h exponential growth incubation at 37°C (Cody *et al.* 2008).

3.12 Preparation of Bacterial Inoculation

A colony was picked by a loop from the nutrient agar plate, according to the Clinical Laboratory Standards Institute (CLSI). The swab was spun after being dipped into the liquid medium. A sterile cap that was not airtight was loosely applied on top of the culture media. For 12 to 18 h, bacterial culture was cultured at 37°C in a shaking incubator. The subsequent standardization of the bacterial inoculation used to the McFarland tube (0.5) (Humphries *et al.* 2019).

3.13 Molecular Identification

3.13.1 Kits

Molecular kits used in the research were given in Table 3.5.

Table 3.5 Molecular kits used in this study and manufacturers

Kits	Manufacture (Country)
API Staph Kit	Biomerieux (France)
DNA Extraction Kit	Promega (USA)

3.14 Method of DNA Extraction

The DNA of bacteria was extracted using the Promega (USA) mini kit provided with the Wazard Genomic Kit for purifying DNA.

3.14.1 Cells pellet

The bacterial suspension was treated with 1 mL of BHI. The supernatant was removed after centrifuging the 1.5 mL eppendorf tube for two min at 13.000-16.000 rpm.

3.14.2 Cells lysis

It was prepared by using 600 µL of the nucleolysis solution. To resuspend the cell the cell was gradually compressed. For 5 min, the cells were heated to 80°C. Lys them, after which they were cooled to room temperature. Then to the cell solution 3 µL of RNase solution was added, and the tube was inverted two to five times to mix it. After being incubated at 37°C for 15-60 min, the material was cooled to room temperature.

3.14.3 Precipitation of protein

To an RNase-treated cell lysate, 200 µL of protein extraction solution was added. After being vortexed at high speed for 20 seconds to mix the protein extraction mixture with the cell lysate, the sample was incubated on ice for five min before being centrifugation at 13.000-16.000 rpm for three min.

3.14.4 Precipitation and hydration of DNA

- The DNA-containing supernatant was transferred into a clean 1.5 mL microcentrifuge tube with 600 µL room temperature isopropanol, gently mixed by inversion until the thread-like DNA strands formed a visible mass, then centrifuged at 13.000-16.000 rpm for 2 min.

- The supernatant was gently placed into the tube and emptied onto clean absorbing paper. 600 µL ambient temperature and 70% ethanol are added, and the tube is slowly inverted a few times to wash the DNA pellet. For two min, centrifuge at 13.000-16.000 rpm. Rehydrate the DNA by incubating the solution overnight at ambient temperature or 4°C and keeping the DNA at 2-8°C.
- Pour test tube in a clean filter papers after gently emptying the ethanol then leave the pellet dry about 15 min, then adding 100 µL of DNA rehydration solution to test tube then incubating for 60 min at 65°C, to rehydrate the DNA. Squeeze the tube gently repeatedly to mix the liquid. Incubate the solution overnight at 4°C to rehydrate the DNA. Finally, protect your DNA.

3.15 Methods of PCR

List of material used in PCR and electrophoresis were given in Table 3.6.

Table 3.6 List of PCR and electrophoresis materials

PCR and Electrophoresis Materials	Company and Origin
Agarose	Cleaver (England)
ddH_2O	NEB (England)
DNA Ladder Marker (1500 bp)	Promega (USA)
DNA Purification Kit	Promega (USA)
Hisenscripttm RH(-) RT Premix	Bio Laboratories (England)
Luna Universal qPCR Master Mix	Bio Laboratories (England)
Master Mix	Intron's premix (korea)
Primers/Integrated DNA Technologies/USA	Genewiz (USA)
Red Safety Paint	Bio Laboratories (England)
TBE Buffer 10 XT	Intron (Korea)

3.15.1 Primers

Primers were designed from an NCBI website according to the related study and were designed according to Integrated DNA Technologies company manufactured in USA. From Integrated DNA Technologies/USA, two random primers (OPX-13 and OPY-04) were gained. *P. aeruginosa* isolates tested on its own primers for the RAPD-PCR procedure (Table 3.7).

Table 3.7 Sequence and type of RAPD primers used in the PCR primer analysis

Primer	Sequence
OPX-13	5-ACGGGAGCAA-3
OPY-04	5-GGCTGCAATG-3

3.15.2 Primers solutions

By dissolving 10 µL in 90 µL of non-ionic nuclease-free distilled water (ddH_2O), a stock solution for each baseline was prepared, kept and mixed thoroughly, at -20°C. Mixed by vortex to homogeneity before use.

3.15.3 Maxime PCR premix kit

Intron's premix maxime PCR, kit contains not only different types of premixers according to the purpose of the experiment a 2X master mix remedy. The product that combines each ingredient i-Taq DNA, polymerase, dNTP mixture, reaction solution, and one tube for one rxn PCR is called the maxime PCR premix (i-Taq) kit (Table 3.8). With the greatest system, this product will produce the best results. The primer set, distilled water, and a DNA template are the only additional ingredients needed to do PCR for the first reason it already includes every component of a PCR. The gel loading may be performed without any preparation for the second reason: it comes with a gel loading buffer for electrophoresis. It is a rapid and easy manner of usage and is appropriate for diverse sample tests (Table 3.8).

Table 3.8 The maxime PCR premix design's ingredients (i-Taq)

Material	Volume
i-Taq DNA Polymerase	5 U/µL
dNTPs	2.5 mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

3.15.4 RAPD-PCR amplification

From 25 µL final product reactive containers with 0.5 µL, of genomic DNA, 2 µL of single primer, and 5 µL of Taq PCR mix, the reaction volume was rounded down to 25 µL, with the addition of 17.5 µL of nuclease-free water (Table 3.9). Amplification products (Table 3.10), were electrophoresed into 1.5% agarose gels visualized by staining with redsaft. Molecular standard markers were placed in each electrophoresis pathway. UV-illuminated gels were taken as photographs.

Table 3.9 Mixture of the specific interaction for PCR Mix

Components	**Concentration**
Taq PCR Premix	5 µL
Primer	10 p.mol/µL (2 µL)
DNA Template	0.5 µL
Distill Water	17.5 µL
Final Volume	25 µL

Table 3.10 The optimum condition of RAPD-PCR

Phase	**Tm (°C)**	**Time**	**No. of Cycle**
Initial Denaturation	94°C	5 min.	1 cycle
Denaturation	94°C	30 sec	40 cycle
Annealing	36°C	45 sec	
Extension	72°C	45 sec	
Final Extension	72°C	7 min.	1 cycle

One hundred samples were collected from patients who were admitted to the hospital. Three distinct locations wounds, ear infections, and urine infections, were used to collect the isolates. Biochemical besides enrichment tests, selective media, characterized and identified *P. aeruginosa*. 12 *P. aeruginosa* isolates using RAPD-PCR technology (Table 3.11).

Table 3.11 Sources of isolates subjected to RAPD-PCR analysis

No. of Isolates	Sources	No. of Isolates	Sources	No. of Isolates	Sources	No. of Isolates	Sources
1	Urine	4	Wound	7	Ear	10	Urine
2	Wound	5	Urine	8	Wound	11	Wound
3	Wound	6	Wound	9	Ear	12	Ear

3.16 Agarose Gel Electrophoresis

To identify DNA segments electrophoresis was performed after the extraction process or to detect the result of the RAPD-PCR, reaction while in the presence of a standard DNA, band size of marker for the PCR result on an agarose gel (Lee *et al.* 2012).

The agarose gel was prepared by melting 1.5 g of agarose in 100 mL of the previously created TBE solution. This resulted in a 1.5% condensation of the agarose. As soon as agarose reaches a boil, add 5 µL of redsafe TM nucleic acid staining solution (20.000x), to the agarose solution. Swirl the flask gently to mix the solution and avoid forming bubbles. It is allowed to cool at 45-50°C. Following the fixing of the comb to create holes that would contain the samples, the gel was poured into the pour plate in which the plate of agarose support had been created. To avoid creating air bubbles, the gel was carefully poured and given time to cool down. The comb was taken from the firm agarose. The plate has been fastened to its support in the horizontal electrophoresis unit, which is represented by the electrophoresis tank. TBE buffer is poured to the tank, completely enveloping the gel (Abdullah and Mezher 2021).

3.17 Preparation of Sample

The loading technique is now applied to the slots of the gel after combining 5 µL of DNA assay for electrophoresis (loading dye) and 3 µL of processor loading buffer (Intron/Korea). An electric current of 5 V/cm^2 was introduced for 90 min to allow the dye to cross the gel. The gel was immersed in a bath containing 500 mL of distilled water and

3 μL of a safe nucleic acid red staining solution and then analyzed under a UV lamp with a wavelength of 336 nm (Abdullah and Mezher 2021).

3.18 RAPD Information Recording

The PCR-based DNA marker RAPD, a straightforward technique for assessing the genetic diversity and fingerprinting of *P. aeruginosa* isolates acquired from distinct infection sites, was used. All of the information that was available was used to assess the RAPD findings. This information comprised the number of these polymorphic bands that could be seen longitudinally, the number of amplified DNA bands that were visible (1) or missing (0) through all *P. aeruginosa* isolates, and the overall number of amplified bands.

To acquire phylogenetic information and create a genetic distance tree, the Numerical Taxonomy System (NTSYS), 2.2 software was utilized, as well as the Jaccard coefficient of similarity (Meyer *et al.* 2007), arithmetic utilizing the unweighted pair group method (UPGMA) averages and cluster analysis (Micek *et al.* 2005). Each primer's efficacy and discriminatory ability were determined using two equations as described in (Nazik *et al.* 2007).

4. RESULTS AND DISCUSSION

4.1 Isolation and Identifecation of *P. aeruginosa*

4.1.1 Isolation

In the current investigation, 75 *P. aeruginosa* isolates were recovered from clinical sources from different isolates of *P. aeruginosa* were obtained 75% as shown in (Table 4.1).

Table 4.1 Shows the percentage of *P. aeruginosa* isolated from different sources

Specımen	**Number of samples(%)**	**Number of *P. aeruginosa* isolates%**	**Percentage within same group**
Wounds	55 (55)%	45 (60)%	81.8
Urine	15 (15)%	10 (13.3)%	66.7
Otitis media	30 (30)%	20 (26.7)%	66.7
Total	100 (100)	75 (100)	

4.1.2 Identification

4.1.2.1 Morphological features

Taking into account the design of bacterial cultures on both an enhanced and a selective and differential media, *P. aeruginosa* was identified. To assess the recognition, *P. aeruginosa* was used to identify the isolates. On blood agar medium develops into huge flat colonies with a grape-like scent, and the majority of isolates exhibit β-hemolysis after 24 h of incubation these results agree with the researches of (Saleem *et al.* 2021). Bacteria are distinguished by the creation of light yellow, tiny smooth spherical colonies that are characteristic of their growth on MacConkey agar indicating *P. aeruginosa* as the isolate as a result of their failure to ferment lactose. These result is compatible with the results of previous researches is consistent with the findings of earlier studies by (Forbes *et al.* 2002). On nutrient agar, the growth *P. aeruginosa* colonies was determined based on the

generation of smell and colours (grape-like odor), the colonies showing greenish coloration and this is compatible with previous researches by (DeBritto *et al.* 2020). Cetrimide agar, which differentiates between *Pseudomonas* and other infections and facilitates the induction of colors, because most of these colonies produce pyocyanin was used to culture these colonies. Colonies on cetrimide agar had a mucous appearance, flat edges, and a raised center. The results are consistent with (Laine *et al.* 2009). *Pseudomonas* agar was used to culture the isolates, so they were incubated at 42°C to confirm the identification and develop a key diagnostic property of *P. aeruginosa* other than the *Pseudomonas* genus species, and this is compatible with the results of (Carroll and Hobden 2019). Due to the infections' synthesis of pyocyanin, their development is distinguished by a glossy green color. Different growing mediums were employed to test the pathogenic isolates' pigment synthesis, and it was found that the creation of the pyoverdine pigments resulted in a colony that was somewhat yellowish-green in hue. *P. aeruginosa* bacteria that were still present most likely developed another pigment. Pyocyanin that's colonies appeared with blue-green colored colonies on different agar media king A, king B, citrimide and MH agar media (Ali *et al.* 2021b).

4.1.2.2 Microscopical and biochemical characterization

Microscopically, the isolates seemed to be *Pseudomonas spp*, with gram-negative pink color and rod-like bacteria that were first indicated *P. aeruginosa* in agreement with (Akanji *et al.* 2011). They created catalase, a substance that converts hydrogen peroxide (H_2O_2) into bubbles of both oxygen and water, and demonstrated positive findings for oxidase and catalase. *P. aeruginosa* colonies range in size from tiny to big mucus, and positive oxidase findings were linked to their capacity to manufacture cytochrome oxidase (tetramethylphenylenediamine oxidases). Isolates exhibit positive gelatin liquefaction due to their capacity to generate gelatin. Because citrate was the predominant carbon source, the results were substantial. The isolates further developed β-hemolysis on blood agar in contrast to other isolates, and some *P. aeruginosa* isolates were generated by a mutation under particular circumstances, including the presence of nutrients. As compared to other strains, virulence factors such as hemolysis were reduced 200-10.000 fold (Ostroff *et al.* 1989). Cetrimide agar is known to be poisonous and to hinder the

growth of other microbial flora. To culture *P. aeruginosa* was employed, and all *Pseudomonas* isolates could grow at 42°C. At 4°C, there was no growth. *P. aeruginosa* can endure the cold and creates a green pigment and this agrees with (Yilmaz 2017). The results of the IMViC test show that none of the isolates tested positive for indole, methyl red, or Voges-Proskauer. All findings support (Nathan 2011) (Table 4.2).

Table 4.2 *P. aeruginosa* clinical isolates

Name of Test	**Result**
Oxidase Test	Positive
Catalase Test	Positive
Indol Test	Negative
Methyle red	Negative
Voges-Proskauer	Negative
Citrate utilization	Positive
Hemolysis	β-hemolysis
Gelatine liquefaction	Positive
Growth at 42°C	Positive
Growth at 4°C	Negative

4.1.2.3 VITEK-2 system's diagnosis of *P. aeruginosa*

Overall, when identifying gram-negative bacteria, the VITEK-2 demonstrated compatibility with the industry-standard API strips from BioMerieux, correctly identifying 75 of 100 (75%) strains of *P. aeruginosa*. The accuracy was finally estimated to be 98%. Al-Shwaikh and Alornaaouti (2017) used VITEK-2 to determine isolates of the bacterium *P. aeruginosa* from patients' wounds and burns and other sources of infection that were constant in the current study 98%. Also compared with the results of (Nakasone *et al.* 2007), this shows the identifications obtained with the VITEK-2 gave 95.8% compatibility with the reference API strips (BioMerieux) in the gram-negative rods. Finally, the precision was determined. to be 98.3%. Also, >90% of identifications of gram-positive cocci and gram-negative rods were obtained within 7 h of incubations (APPENDIX 1).

Due to the fact that these infections are highly pathogenic and can bring disease to any flaw in the body's barriers, the results showed that *P. aeruginosa* is among the most prevalent species that creates wounds infections (Mulcahy *et al.* 2014).

Table 4.1 shows the percentage of isolates of bacteria from different sources of disease states, where the highest percentage of isolates of this bacteria from wounds was, where the number of isolates from *P. aeruginosa* isolated from wounds was 45 (55), with a percentage of same group 81.8% isolated, 10 (15) from UTI with a percentage of same group 66.7%, and 20 (30) from otitis media with an isolation rate of same group 66.7%. The results of the current research agree with the results of (Attiah *et al.* 2021). That study found a high percentage of bacteria isolated of *P. aeruginosa* were obtained from 16 (32%) burn swabs and 34 (68%) wound swabs. Mittal *et al.* (2009), also found that the highest rate of infection with *P. aeruginosa* that caused UTI infection was 25-30%. The isolation rate for ear infections of the current research agree with the results of (Mansoor *et al.* 2009) with 40%. *P. aeruginosa* and other bacteria, are found worldwide in soil and water. These bacteria prefer moist areas, such as washbasins, toilets, inadequately chlorinated swimming pools, hot tubs, and ineffective disinfectant solutions. These bacteria can be found in the armpits and genital areas of healthy people (Khalili *et al.* 2021). These bacteria can cause infections in the blood, skin, bones, ears, eyes, urinary tract, heart valves, and lungs, as well as in wounds (such as burns, injuries, or surgical incisions). *P. aeruginosa* catheter-associated UTIs are associated with a number of consequences. The genotyping of clinical isolates of *P. aeruginosa* is critical for understanding infection epidemiology. The bacterium is responsible for a significant number of virulence factors, yet it is very unstable phenotypically. This might aid in the development of immunotherapeutic strategies (Sabharwal *et al.* 2014). The causes for this large range in bacterial infection may be attributed to the patient's weakened immunity; also, various hospital-acquired bacterial infections may develop, and the patient becomes infected with *P. aeruginosa*. Because this bacteria is an opportunistic disease, the emergence of antibiotic resistance makes treating and eliminating it considerably more challenging. Other probable explanations include the source of the sample and the number of samples obtained from patients, geographical location, technique of sterilizing wounds and burns, and number of sterilization times. As a result, the level of health care must be

high from the patient's arrival at the hospital, as the patient's stay in the hospital for several days to receive treatment may lead to an increase in the rate of bacterial infection as a result of infection acquired from hospitals directly after contacting patients or indirectly through the use of contaminated surgical tools (Azam and Khan 2019, Hilliam *et al.* 2020) (Table 4.3).

Table 4.3 Shows the percentage of *P. aeruginosa* isolated depending on the gender

Gender	Number and percentage of positive cases with *P. aeruginosa*	Number and percentage of negative cases with *P. aeruginosa*	Total
Male	31 (73.8%)	11 (26.2%)	42
Female	44 (75.9%)	14 (24.1%)	58

Table 4.3 shows the percentage of isolates of *P. aeruginosa* bacteria depending on the gender of the patient. The highest percentage (75.9%) of isolates of this bacteria was from females, where the number of isolates while in the male it reached 73.8% of isolates. The findings of the present study are consistent with those of (Attiah *et al.* 2021) who discovered more positive results in females (54%) than males (46%) from burn and wound inflammation. Mohammed *et al.* (2014) discovered that women (64.91%) had higher rates of infection with bacteria than men (35.09%) that caused wound infections in Baghdad City. These types of infections may happen in deep puncture wounds (such as when stepping on a nail). In chronic suppurative otitis media, our results constant with the results of (Mansoor *et al.* 2009) that the rates of female (52%) higher than the male (48%).

As a result of RAPD-PCR, a total of 27 amplified DNA were observed for two primers (OPX-13 and OPY-04), 24 polymorphic amplified fragments ranging in size from 250 to 1500 bp. Usually noticed between the 12 *P. aeruginosa* isolates and for each primer gave unlike inherited shapes. The high sensitivity requires just a small quantity of template DNA. Primer OPX-13 showed the most RAPD patterns with 14 bands (Figure 4.1), whereas primer OPY-04 revealed the fewest with 13 bands (Figure 4.2). The massive number of bands might be explained by the original DNA of the isolates obtained having a significant quantity of primer annealing regions. Mahmmudi *et al.* (2015) found the

large number of bands was explained by the tested isolates' template DNA having a large number of primer annealing sites. A larger number of bands often increases the likelihood of identifying particular polymorphisms (Giroti 2006). Al-Assie *et al.* (2012) showed a high number of RAPD patterns (39 bands), while a lower number of RAPD patterns were 18 bands for *P. aeruginosa*. Onasanya *et al.* (2010) found that binding both molecular diagnostic and DNA fingerprinting have a potential for effective and reliable identification and differentiation of these bacterial pathogens.

The polymorphism level was shown using primers OPX-13 and OPY-04 (12 bands), polymorphisms in DNA can develop as a consequence of a variety of mutations, including single-base alterations in the genome's primer-annealing site that impede amplification by generating a mismatch at the 3'end of a DNA segment. Pethannan *et al.* (2018) found the primers OP-A11 and OP-D20 both produced distinct banding patterns between *P. aeruginosa* isolates. There were no monomorphic bands and all the bands were polymorphic. The acquired bands were in the 500-2000 bp. Results of (Pethannan *et al.* 2018) not agreement with our study (Figure 4.1) (Figure 4.2).

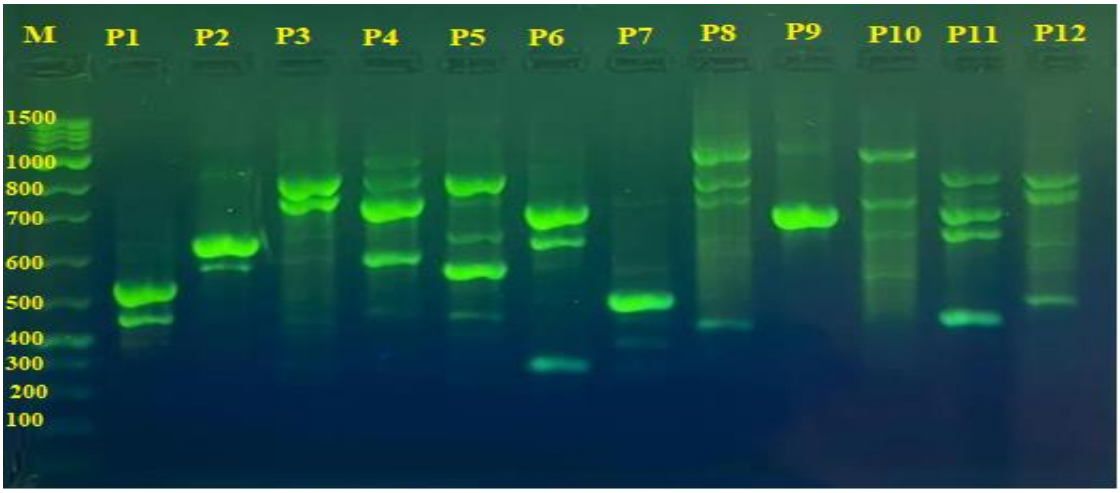

Figure 4.1 RAPD-PCR product of primer OPX-13. M: DNA ladder 1500 bp. The isolate number were given between P1 to P12

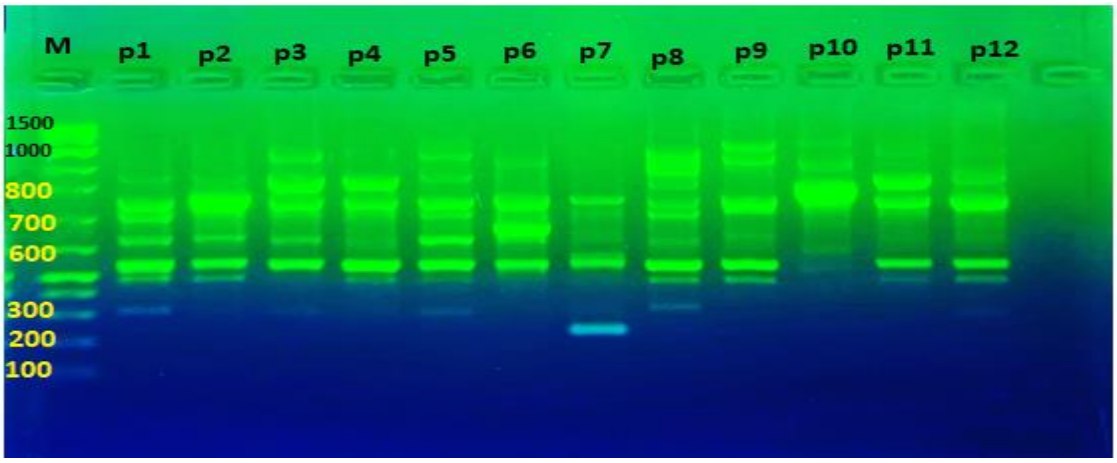

Figure 4.2 RAPD-PCR product of primer OPY-04. M: DNA ladder 1500 bp. The isolate number were given between P1 and P12

In addition, it was intended for this study to evaluate each primer's effectiveness (Table 4.4). Primers OPX-13 and OPY-04 both showed different estimated efficiencies, with OPX-13 having the greatest (51.8%) and OPY-04 having the lowest (48.14%). Al-Assie *et al.* (2012) reported primer efficiency ranged from 3.70% to 14.44%, which is not consistent with our data. The RAPD method, however, may be a useful tool for researching the molecular makeup and transmission of *P. aeruginosa* organisms.

Table 4.4 Details of RAPD's efficiency, discriminating power, and a number of bands as well as the number of polymorphic bands that the primer produced

Primer	No. of bands	Polymorphic Bands	Formal plurality of each prefix	Efficiency ratios	Discriminatory ability
OPX-13	14	12	85.71%	51.85%	50%
OPY-04	13	12	92.30%	48.14%	50%

The discriminating power of the different primers varies substantially. The discriminating strength of primers OPX-13 and OPY-04 was 50%, resulting in separate fingerprints (Table 4.4). This is explained by primers' ability to reflect differences in microbe genomes to the overall number of variants. A primer with strong discriminating power can yield a significantly more polymorphic bands than there are overall polymorphisms (Grundmann *et al.* 1995). The effectiveness and discriminating power of any primer must be determined because they utilized to limit how often the primer is blind-screened before

being used to examine the molecular underpinnings of additional individuals in the same species. This rules out the idea of monomorphic forms developing in individuals from various genetic backgrounds. It will also increase the likelihood of getting precise fingerprints of particular strains (Sandery *et al.* 1994). Because they needed specialized PCR reagents or heat flux for amplifications, or since every reaction parameter were identical for each primer, many primers failed to successfully amplify DNA (Sahu *et al.* 2018). This experiment proved the feasibility of molecular discrimination. Furthermore, it examines the whole genome, RAPD-PCR is discriminating. However, variations in the reaction mixture and temperature cycles can have an impact on its sensitivity and reproducibility. The usefulness of the method for fingerprinting is demonstrated by the presence of distinctive RAPD markers among the various *P. aeruginosa* genotypes. Numerous applications for RAPD fingerprinting exist, including cultivar purity evaluation, efficient management of genetic resource collections, and the detection of incorrectly tagged accessions. That the RAPD analysis would be the best choice for epidemiologic investigations since it is inexpensive and requires just a small quantity of DNA to produce high amplicons that can be easily evaluated. This agreement with (Abdel-Rhman and Rizk 2021). They demonstrated how the great level of discrimination was attained. When typing techniques were compared, RAPD-PCR produced the greatest mean percent of polymorphism per assay (76.85%) in comparison to the other studied techniques and strain categorization of *P. aeruginosa*. The RAPD-PCR analysis is a useful epidemiological scanning technique to identify genetic diversity among *P. aeruginosa* isolates, according to (Al-Awsi *et al.* 2019).

4.2 Phylogenetic Characterization of *P. aeuroginosa*

Phylogenetic trees were used to demonstrate the genetic link between the 12 *P. aeruginosa* isolates exposed to RAPD-PCR analysis.

The RAPD data produced by primer OPX-13 were used to create a dendrogram, and the UPGMA analysis of that dendrogram was completed and shown in the overall outcome of this analysis. It suggested that all 12 *P. aeruginosa* isolates were categorized into the primary genotype groups. The cluster analysis (Figure 4.3) of the twelve bacterial strain

genotypes was based on the similarity ratio results and used a method that allowed the studied genotypes to be placed under two main branches: the first branch included the strain P10, and the second branch contained the remaining strains. The two strains, P10 and P11, were the most branched. Divergent, while strains P1 and P5 were the most similar strains, as strained P3, P4, and P6. The genetic dimension values ranged from 0.5 to 1, with the lowest genetic dimension being between the structures P5 and P1, P6 and P3, and P6 and P4, which amounted to 1. P7 and P8, P1 and P2, P7 and P5, and P5 and P2, which amounted to 0.923, and this is the highest similarity between the two structures included in the study, and the largest genetic dimension (the least similarity) reached 0.5, between the structures P10 and P7, P10 and P2, and P9 (Table 4.5) (Figure 4.3).

Table 4.5 Similarity and distance indices for primer OPX-13

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**
P1	1	0.923	0.909	0.909	1	0.909	0.923	0.833	0.615	0.545	0.769	0.666
P2		1	0.833	0.833	0.923	0.833	0.854	0.769	0.714	0.5	0.615	0.615
P3			1	1	0.909	1	0.833	0.727	0.5	0.5	0.833	0.727
P4				1	0.909	1	0.833	0.727	0.5	0.6	0.833	0.727
P5					1	0.909	0.923	0.833	0.615	0.545	0.769	0.666
P6						1	0.833	0.727	0.5	0.6	0.833	0.727
P7							1	0.923	0.714	0.5	0.857	0.769
P8								1	0.769	0.545	0.876	0.833
P9									1	0.333	0.571	0.615
P10										1	0.666	0.545
P11											1	0.769
P12												1

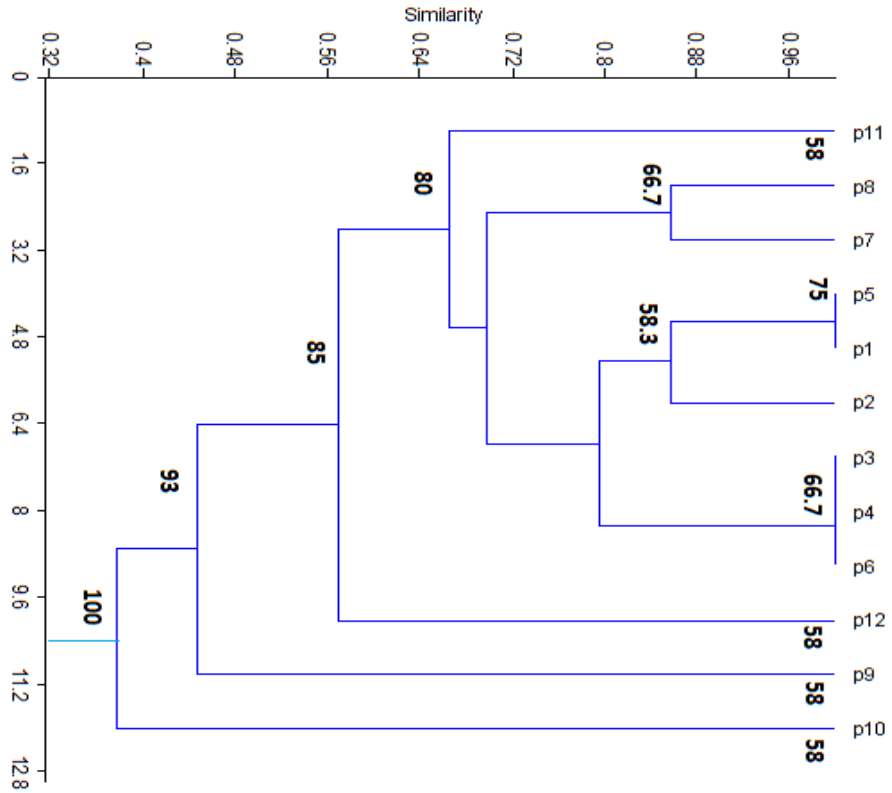

Figure 4.3 UPGMA tree of 12 *P. aeuroginosa* (P) isolates which are characterized by RAPD markers OPX-13

The UPGMA tree was built using the RAPD information recorded by primer OPY-04, and all 12 *P. aeruginosa* isolates were categorized into the primary genotype groups. Cluster analysis (Figure 4.4) developed for the twelve genotypes of bacterial strains based on the results of the similarity ratio and using a method where it was possible to place the studied genotypes under two main branches, the first branch included the strain P7 while the second branch contained the remaining strains, P7 and P12 strains were the most divergent, while the strains P4 and P2, and P6 and P10 were the most similar. The genetic dimension values ranged from 0.200-0.857, where the lowest genetic dimension was between the structures P2 and P4, P1 and P5, P3 and P8, P4 and P11, P4 and P12, as well as P6 and P10, which amounted to 0.857, P2 and P1, P5 and P4, P8 and P5, as well as P1 and P12, which amount to 0.800 and this is the highest similarity between the two structures included in the study, and the largest genetic dimension (the least similarity) reached 0.200, between P7 and P8, as P3 and P7 (Table 4.6) (Figure 4.4).

Table 4.6 Similarity and distance indices for primer OPY-04

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**
P1	1	0.8	0.615	0.666	0.857	0.222	0.444	0.615	0.333	0.4	0.6	0.8
P2		1	0.545	0.857	0.666	0.285	0.285	0.545	0.4	0.5	0.75	0.75
P3			1	0.4	0.8	0.6	0.2	0.857	0.615	0.727	0.545	0.545
P4				1	0.545	0.333	0.333	0.6	0.444	0.571	0.857	0.857
P5					1	0.545	0.363	0.8	0.571	0.666	0.666	0.666
P6						1	0.333	0.6	0.666	0.857	0.571	0.285
P7							1	0.2	0.222	0.285	0.285	0.285
P8								1	0.769	0.727	0.727	0.727
P9									1	0.6	0.6	0.4
P10										1	0.75	0.5
P11											1	0.75
P12												1

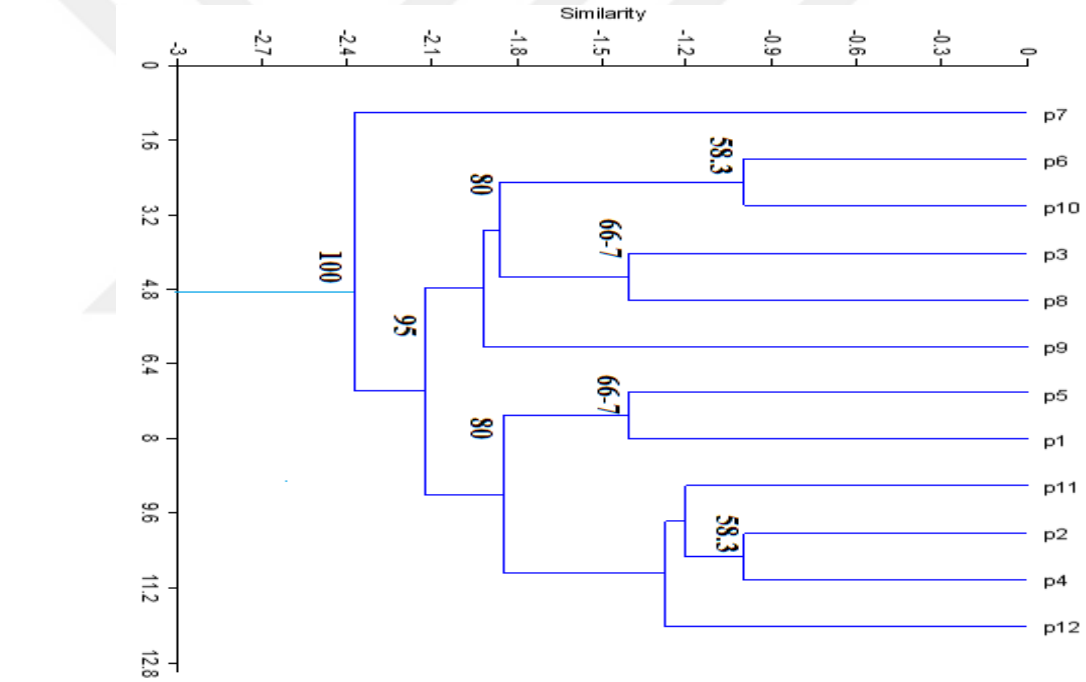

Figure 4.4 UPGMA tree of 12 *P. aeuroginosa* (P) isolates which are characterized by RAPD markers OPY-04

These results found the degree of genetic similarity or difference between the genotypes included in the study, this is due to two things. There is a high genetic similarity between the genotypes, which is contrary to the first thing that depends on the phenotypes. This is due to the similarity in the non-coding regions of genes. Strains can be inherited independently of the environment, and molecular markers may provide information about

the history and biology of the genotypes. This is consistent with many researchers who have used molecular markers, especially RAPD, in their studies. In addition, the rates of genetic recombination or mutation, resulted in the great amount of genetic diversity among the *P. aeruginosa* isolate population. Perron *et al.* (2008) found the effect of immigration may be analogous to that of an increased mutation rate in the genetic background in which mutations occur. *P. aeruginosa* has been found to survive in agricultural environments or water supplies, where it can trade resistance components or infect human and animal hosts. In agreement with other studies, (Al-Awsi *et al.* 2019) found substantial variety among the *P. aeruginosa* isolates. As shown previously a great amount of genotypes suggest that most *P. aeruginosa* isolates were derived from the patients themselves.

The regions of infection in the body of the isolates and their genetic diversity are related, and it turns out that isolates P2 and P6 were common in cases of wound infection and had a lot of genetic similarities. The most frequent UTI isolates were P1 and P5, whereas isolate P12 (ear) exhibited the highest genetic divergence from the novel strains. This suggests a possible connection between the genetic diversity of *P. aeruginosa* isolates and the types of environments in which they preferentially flourish. Genetic differences in *P. aeruginosa* strains may modify the location at which the organism is inclined to be virulent (Taheri *et al.* 2008). The second group, on the other hand, had a much higher proportion of UTI isolates of *P. aeruginosa*. The urinary system is a unique location with strong osmotic pressure, fluctuating pH levels, and liquid is the renal system. *P. aeruginosa* is frequently regarded as being particularly abundant in these features, which are identified in the illnesses and flora of this terrain as having strong ATPase wall transporters and adhesive properties. Parsa *et al.* (2020), Onasanya *et al.* (2010) showed the results of the RAPD-PCR method indicate that the isolated *P. aeruginosa* exhibits significant levels of polymorphism, most likely as a result of its rapid pace of genetic change. This considerable variation may be advantageous for a strain to adapt to its environment and could be helpful for domesticating and breeding wild strains. This situation lends credence to the idea that genetic variation can result through gene acquisition irrespective of environmental strains, cross-establishment, contamination with the same source, or gene acquisition. Although, the patient-to-patient transmission

rate is low but not zero. Each banding pattern in *P. aeruginosa* might reflect the dominant environmental strain in one location, and cross-contamination transmission confirmation is neither exact nor impossible given the various variables at play (Parsa *et al.* 2020).

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion

The identification of *P. aeruginosa* at certain ratios from the wounds, UTIs, and otitis media characterization and RAPD-PCR show there is clonal relatedness between clinical isolates and the environment, which could be the main reservoir for *P. aeruginosa* infection in the hospital. The rate of similarity in pattern between 12 *P. aeruginosa* strains refers to the carrier role of hospital equipment and personel. The efficiency of the RAPD-PCR method in detecting heterogeneity between isolates was high because each primer identified more than one genetic segment. Due to a high incidence of genetic variation, 12 *P. aeruginosa* specimens from patients at Ramadi Public Hospital are reportedly common, which affects the bacteria's favored environments for colonization and infection. We may thus say that the RAPD technique may be utilized to investigate the genetic traits as well as the epidemiology of *P. aeruginosa* pathogens.

5.2 Recommendation

- Search for phenotypic and genotypic heterogeneity generated in *P. aeruginosa* population during chronic infection and what impact this has on patient health.
- Study where the majority of bacterial strains that infect humans come from. Did they come from a transmissible epidemic strain or were they acquired from the environment?
- Furthering our understanding of *P. aeruginosa* infection by working with laboratory strains and understanding how accurately infection models capture the physiology of *P. aeruginosa* during human infection, which can help prevent infection among immunocompromised individuals.
- Studying what is the role of QS and biofilm during infection is social cheating between cells occurs during infection and what impact this has on population dynamics, virulence and antibiotic resistance.

- Identify whether the majority of *P. aeruginosa* strains that infect humans come from a transmissible epidemic strain or were they acquired from the environment.

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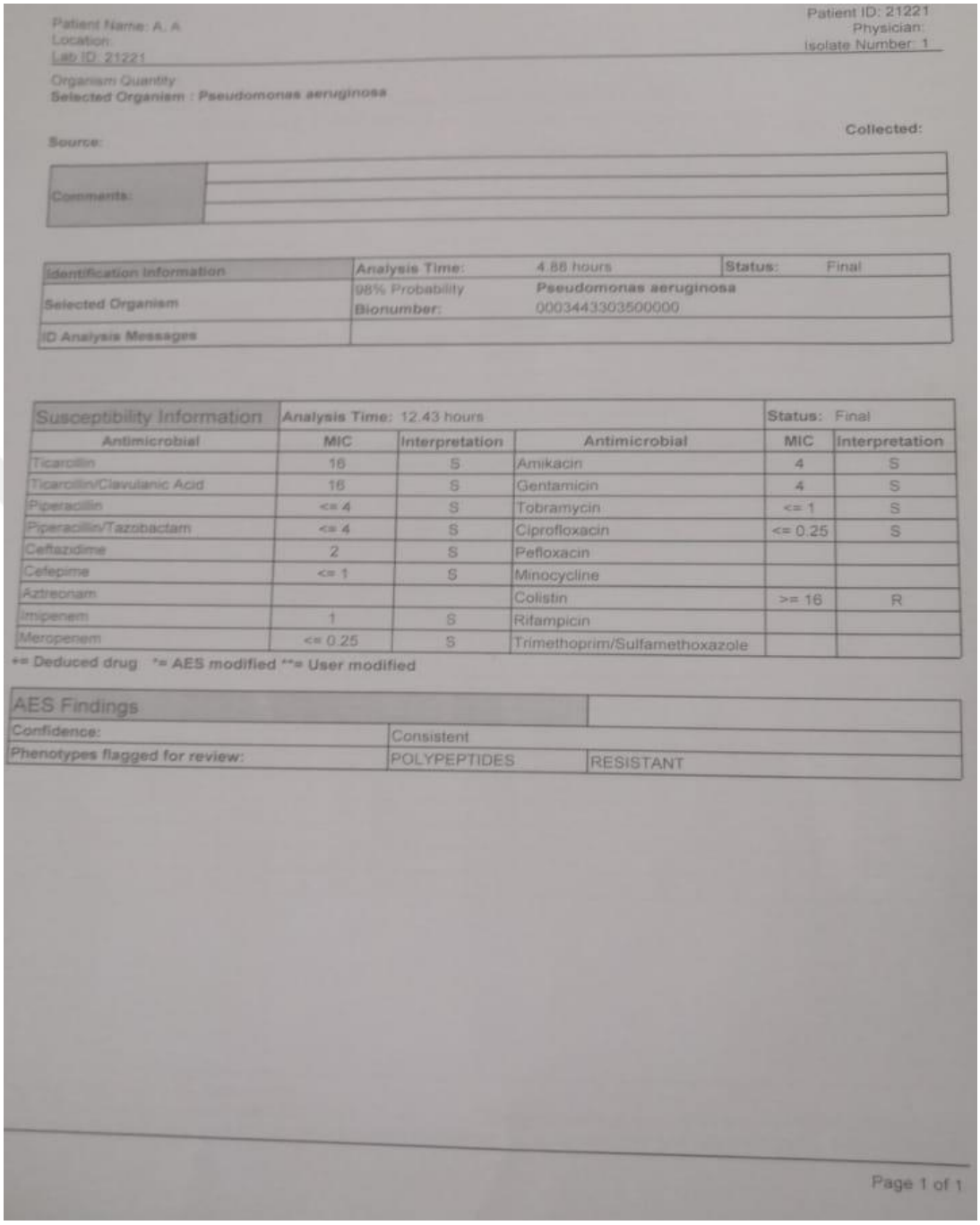
APPENDICES

APPENDIX 1. Confirmed test report *P. aeruginosa* by VITEK-2 system

APPENDIX 1. Confirmed test report *P. aeruginosa* by VITEK-2 system



Patient Name: A. A
Location:
Lab ID: 21221

Patient ID: 21221
Physician:
Isolate Number: 1

Organism Quantity
Selected Organism : Pseudomonas aeruginosa

Source:

Collected:

Comments:	

Identification Information	Analysis Time:	4.88 hours	Status:	Final
Selected Organism	98% Probability Bionumber:	Pseudomonas aeruginosa 0003443303500000		
ID Analysis Messages				

Susceptibility Information	Analysis Time: 12.43 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	16	S	Amikacin	4	S
Ticarcillin/Clavulanic Acid	16	S	Gentamicin	4	S
Piperacillin	<= 4	S	Tobramycin	<= 1	S
Piperacillin/Tazobactam	<= 4	S	Ciprofloxacin	<= 0.25	S
Ceftazidime	2	S	Pefloxacin		
Cefepime	<= 1	S	Minocycline		
Aztreonam			Colistin	>= 16	R
Imipenem	1	S	Rifampicin		
Meropenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole		

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

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	POLYPEPTIDES	RESISTANT

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CURRICULUM VITAE

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Education

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