Republic of Iraq
Ministry of Higher Education
and Scientific Research
University of Diyala
College of Science
Department of Biology

Molecular identification of *Pseudomonas aeruginosa* using multiple locus variable number of tandem repeat units

A Thesis
Submitted to the Council of College of Science/University of Diyala
in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

by

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July, 2021 Muharram ,1443

بسم الله الرحمن الرحيم

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Acknowledgements

First, in the name of Almighty “ALLAH”, the glorious creator of the universe, praise and peace be upon Mohammed His prophet and upon his family.

I extend my sincere thanks to the supervisors professor Dr. Hadi Rahman Rasheed and Dr. Esam Hamid Hameed for their guidance and constant supervision as well as for providing necessary information regarding the work and for their support in completing this work.

Also, I would like to thank the deanship, the head of the department and all the faculty of the Department of Biology at the College of Science at Diyala University for being helpful with me. Special thanks and gratitude to my dearest Friends Dr. Lena, Dr. Ebtehal, Shaemaa, Neyaf and Ali for support me to complete this work.

Then my sincere thanks and appreciation also go to all my fellow graduate students thank you. Finally, I have taken efforts in this work however, it would not have been possible without the kind support and help of many individuals, I would like to extend my sincere thank you to all of them.

Ansam Esaa, 2021

Dedication

To the great creator ... The God

To those who sacrifice their lives to our Iraq

To those who are my heaven in this world and the afterlife,

To who guided and support me … My father

To who filled me love and kindness …My Mother

To My partner and My Husband… Mousa

To the …. My lovey kids and resone of my life

Ahmed .. Mariam … Yousif

To the pure hearts and loyal hands who assisted me in life …

My Brothers Hussam..Ali…Bassam and Sisters Angham ..Safa …Esraa

To all who supported me

everywhere Dedicate this humble effort.

Ansam Esaa, 2021

Summery

Pseudomonas aeruginosa is Gram negative, an opportunistic, aerobic bacterium. It is one of the important multidrug-resistant (MDR), nosocomial pathogens cause severe infections in local communities in Iraq. This study aimed to evaluate the prevalence and diversity of local different clinical isolates of *P. aeruginosa* by determine Variable Number Tandem Repeats (VNTRs) polymorphism. Two hundred eighty clinical samples were collected from different sources (burns, wounds and urine) from governmental hospitals in Baqubah City/Diyala Province between October 2020 and January 2021.

The results showed that fourty bacterial isolates were identified as *P. aeruginosa* by conventional microbiological methods, biochemical tests, and confirmed by VITEK 2 automated system and 16S rRNA gene assay. 16S rRNA test for only 9 isolates showed that 9(22.5%) isolates were positive. The antibiotic susceptibility profile of 40 isolates were tested using 12 different antibiotics against each isolate by Kirby-Bauer method.

The results showed that a higher resistance percentage 42.5% was obtained against Ticarcillin/Clavulanic acid and the Netilmicin. The lower percentage 15% of the resistance was against Piperacillin/Tazobactam. The percentage resistance for Amikacin, Aztreonam, Cefepime and Ciprofloxacin was 37.5%. With Meropenem and Ceftazidime the resistance was 35% and 32.5% with Imipenem. The isolates were resist Gentamicin and Levofloxacin with 30%.

The antibiotics sensitivity test of the total isolates was showed differentiation into two patterns according to the resistance, MDR and XDR type. MDR profile showed that 5(12.5%) were subdivided to 2(5%) resiste to 3 classes and 3(7.5%) isolates were resiste to 4 classes of antibiotics. XDR type showed 13(32.5%) isolates were subdivided to 3(7.5%) were resiste to 5 classes

and 10(25%) were resist to 6 classes of antibiotics. The MIC values for the forty isolates with Ceftazidime were ranged from (16-1024) μg/ml. The β-lactamases was detected for three enzymes (ESBLs, AmpC and MBLs). 12 (30%) of the isolates were ESBLs producer with resistant to Cefotaxime and Ceftazidime. Phenotypically, 10(25%) of the isolates were MBLs producer and 30(75%) were non-producers. AmpC production was found in 18(45%) isolates. According to the antibiotics resistance, nine isolates were subjected to Multiplex PCR. Four loci (MS-213, MS-214, MS-215 and MS-142) were selected to analyze *P. aeruginosa* isolates based on VNTR feature using the Multilocus variable number of tandem repeats (MLVA).

Capillary electrophoresis data showed variation in amplicon sizes. MS-213 exhibited 3 different amplicons, 350pb (4 isolate), 370pb (3 isolate) and 290pb (2 isolate). MS-214 exhibited 4 different amplicons, 730pb (3 isolate), 640pb (1 isolate), 540pb (1 isolate) and 350pb (4 isolate). In case of MS-142 locus, 650pb (3 isolate), 720pb (1 isolate), 530pb (2 isolate) and 310pb (3 isolate) were obtained. Finally, one type of amplicon was identified in MS-215.

In order to get a broader view for phylogentical analysis, the amplicon sizes were converted to repeat numbers by using flanking regions. The number of repeats obtained for three loci was used to generate allelic profile. All the 9 *P. aeruginosa* isolates clustered into the 10 MLVA type or genotypes without applying the cut off. The loci were considered 100% similarity in the repeats. Most of the genotypes are generated from the genotype 9 which represented by the isolates No. 9.

The use of MLVA resulted in high rapidity and accuracy providing a phylogenetic information by which the source of genotype relationships and evolutionary histories can follow. It also reflects the relationship between the source of infection and the type isolates.

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List of abbreviations

AIDS	Acquired Immunodeficiency Syndrome
Amp^c	Ambler class C beta-lactamase
bp	Base pair
CDC	Centers for Disease Control and Prevention
CE	Capillary Electrophoresis
CF	Cystic fibrosis
CFC	Cephalothin, Fucidin, Cetrimide
CLSI	Clinical and Laboratory Standards Institute
D.W	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended-Spectrum β –Lactamase
Eth.Br	Ethidium bromide
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infections
MLVA	Multilocus variable Number of Tandem Repeats Analysis
MB	Megabyte
MBL	Metallo Beta-Lactamase
MDR	Multidrug-Resistant or Resistance
MIC	Minimum Inhibitor Concentration
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
pH	Potential Hydrogen
QS	Quorum Sensing
UTI	Urinary Tract Infection
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization
XDR	Extensively-drug resistant
µl	Microliter

CHAPTER ONE

INTROCUTION

1. Introduction

Pseudomonas aeruginosa is one of the most clinically and epidemiologically important bacteria (Rocha *et al.*, 2019). *P. aeruginosa* is Gram-negative bacterium classified as one of the six highly antibiotic resistant bacteria. These aggressive bacteria included *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. figure (1-1) which designated as ESKAPE (De Oliveira *et al.*, 2020). Recently, World Health Organization (WHO), is also listed *P. aeruginosa* as a critical priority pathogen for which new antibiotics are urgently needed (WHO, 2017; Bonneau *et al*., 2020).

P. aeruginosa is the major cause of opportunistic and nosocomial infections. It causes various inflammation and infectious diseases such as burn infections, wounds, urinary tract infection, meningitis, otitis media, eye injuries, respiratory infections pneumonia, especially in people with a cystic fibrosis disease, bone and joints, gastrointestinal and soft skin infections (Levinson, 2016; Ullah *et al.*, 2017; Pedersen *et al*., 2018).

This, pathogen is responsible for morbidity and mortality in immunocompromised patients (McCutcheon *et al.*, 2018 and Rocha *et al*., 2019). Burn infection, *P. aeruginosa* is very strong relation with it associated sepsis due to more than 50% of fatalities in seriously burned patients (Shehab *et al*., 2020). Due to ability to resist a wide range of antibiotics, the treatment of infections by *P. aeruginosa* represents a serious challenge to the physicians.

The fact that *P. aeruginosa* have different mechanisms to resist a wide range of antibiotics such as production of antibiotic-hydrolysis enzymes, Moreover, the emergence and proliferation of Gram-negative resistant bacteria can contribute to poor availability of effective antibiotics to treat these organisms. This can be explaining the needs to find effective strategies to

control their expansion and aggressiveness (Hawken and Snitkin, 2019). Most of *P. aeruginosa* isolates are multi-drug resistant (MDR) with more than 50 % producing β-Lactamase. Almost 90% of the isolates produced Extended Spectrum β-Lactamases (Ahmed *et al*., 2020). In addition, it has multiple virulence factors such as Biofilm Formation (Jurado-Martín *et al*., 2021). *P. aeruginosa* strains especially MDR cause serious problems in many countries, including Iraq (Hawken and Snitkin, 2019).

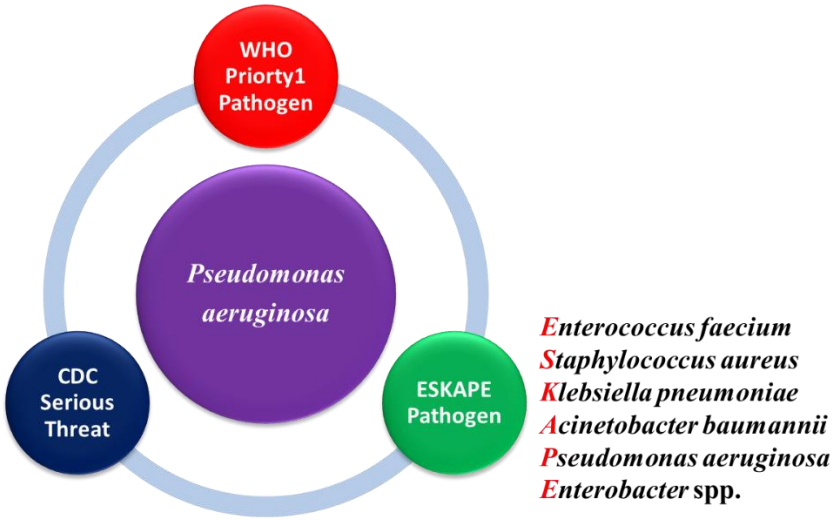


Figure (1-1) The importance of *Pseudomonas aeruginosa* as a pathogenic bacterium according to different categories (Botelho *et al*., 2019).

P. aeruginosa is characterized with high genetic variation which is related to several infectious diseases lead to differences in their pathogenicity. Therefore, *P. aeruginosa* are isolated from several hospital setting determines their genotypes (WHO, 2017; De Sales *et al*., 2020). Molecular typing is a technique that apply to identify specific strains of microbes such as bacteria, fungi, and viruses through analysis their genetic material. This technique is helpful in outbreak identification and determination of the most virulent strains that cause severe diseases (Magalhães 2020).

Molecular typing included several methods such Pulsed Field Gel electrophoresis (PFGE), Multilocus Sequence Typing (MLST) and Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) (Yang *et al*., 2020). MLVA is a molecular subtyping method based on amplification and fragment size analysis of tandem sequence repeats (VNTR) that are found in microbial genome of most bacterial species. MLVA utilizes the naturally occurring variation in the number of tandem repeat DNA sequences. MLVA is rapid and highly discriminatory assay and their output can be interpreting and standardize among laboratories as codes easily (Chen *et al*., 2018; Farahani *et al*., 2020).

The aims of the study

This study aims to find out the variances and track evolutionary change between *P. aeruginosa* clinical isolates from different infections using MLVA molecular typing technique, so that useful in investigating epidemiology of *P. aeruginosa.* This study could be the first one in Iraq that uses MLVA technique for molecular typing of *P. aeruginosa* as Gram-negative bacteria for detection of localized outbreaks.

1) Isolation and identification of several isolates of *P. aeruginosa* from clinical samples (wounds, burns, urine).
2) Study the antibiotic resistance profile of the isolates and categorized into multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria.
3) Detection of beta-lactamase and Metallo-β-lactamases enzyme activity.
4) Molecular detection of the isolates by 16S rRNA gene.
5) Carry out the molecular profiling for a number of multi-drug resistance isolates by Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) technique of some selected genes (MS-213, MS-214, MS-215, and MS-142).
6) Draw the phylogenetic tree to find the relationship between *P. aeruginosa* isolates and their sources.

CHAPTER TWO

LITERATURES REVIEW

2. Literatures Review

2.1 General characters of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is Gram negative, obligatory aerobic, rod shape bacteria with dimension of (0.5-0.8) μm x (1.5-3) μm. *P. aeruginosa* grow in a single or short chains, motile by one polar flagellum or several flagella, non-spore forming. It gets their energy from the carbohydrates by the oxidation process but not by fermentation and so called non-fermenter bacteria of glucose (Diggle and Whiteley, 2020). The optimum temperature of their growth is 37 °C with maximum temperature up to 42 °C and the optimum pH is 7±0.5 (Brooks *et al.*, 2016).

P. aeruginosa shows positive test for oxidase, catalase, urease and citrate (Anthony *et al*., 2014). It has the ability to grow and survive even with simple growth factors and that help the bacteria to survive for a long time on the surfaces of the medical equipment causing hospital infections. *P. aeruginosa* produces two dyes when diagnosed. Pyocyanin is one of these substances which can stain pus in injured wounds and burns with a greenish-blue color. The second dye is Pyoverdine (Fluorescein) with a yellowish-green color that shines under ultraviolet light. Pyoverdine has a remarkable ability to resist the disinfectants and therefore play a role in hospital acquired infections (Sudhakar *et al.*, 2015).

The pigments that produce by *P. aeruginosa* can inhibits the growth of other kinds of bacteria. Pyocyanin, water soluble blue pigments, and Pyoverdine (also known as Pseudobactin) are toxic pigments to the host cells, and dark pyorubin, and dark black pyomelanin, (Orlandi *et al.*, 2015; Cohen *et al*., 2017). *P. aeruginosa* can be initiative identified by its distinct odor in-vitro and also by the color of the colonies which is mostly bluish green (Brooks *et al.*, 2016).

2.2 Classification of *P. aeruginosa*

P. aeruginosa is the type species of the genus *Pseudomonas*. *P. aeruginosa* belongs to the family Pseudomonadaceae (Riedel *et al*., 2019). *P. aeruginosa* is one of the subtypes a group of 12 members (Rocha *et al*., 2019). This bacterium includes various species, both non-pathogenic and pathogenic bacteria (Moore and Flaws, 2011). The scientific classification of *P. aeruginosa* is depending on 16S rRNA as the following (Slonczewski *et al*., 2014):

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma proteobacteria

Order: Pseudomonadales

Super Family: Ribosomal RNA I

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *aeruginosa*

2.3 Pathogenicity of *P. aeruginosa*

P. aeruginosa is one of the nastiest bacteria that causes severe diseases for humans and animals (Patricia, 2014). It is a very well documented as important and frequent in nosocomial and opportunistic microorganisms (Merza *et al*., 2018). This microorganism continues to create an important complication in burns and wounds infections associated with morbidity and mortality over all the world (Heister *et al.,* 2017). As opportunistic bacterium, *P. aeruginosa* is consider as a pathogen that cause a wide range of acute and chronic injures and diseases in humans (Kordes *et al*., 2019). Therefore, it can easily spread and causes some nosocomial infections (Olivares *et al.,* 2020). The pathogenicity of *P. aeruginosa* is intervene by its capability to produce a wide range of virulence factors which is hardened the bacterial intrinsic resistance to the environmental stresses and synthetic chemical agents such as antibiotics, heavy metals and disinfectants materials (Singer *et al.,* 2016). The infection by *P. aeruginosa*

begins by the adhesion stage through the cilia, flagella and the cytotoxic exo-enzyme S, which works on tissue necrosis, then the infections started by other virulence factors (Neamah, 2017).

P. aeruginosa causes a serious and life-threatening infections such as sepsis, urinary tract infections and soft tissue infections (Laudy *et al*., 2017). It also causes diabetic foot and respiratory infections such as pneumonia especially cystic fibrosis disease (Pedersen *et al*., 2018). *P. aeruginosa* habitat the skin surface facilitates to penetrate and causing many inflammations such as otitis media, bone and joints infections (Falkinham *et al*., 2015).

Multi-drug resistance (MDR) Gram-negative microorganisms result in high mortality, extend durations in hospital admission and an elevated in healthcare costs when compared with conditions accompanying susceptible strains. This pathogenic bacterium is also experiences extensive rearrangements of its chromosomal DNA which permit phenotypic conversion (Merza *et al*., 2018).

2.4 Epidemiology of *P. aeruginosa*

P. aeruginosa is widespread microorganism in natural habitats. It can be isolated from different environmental niches such as water, soil, plants, animals and humans (Kordes *et al*., 2019). However, it is rarely isolated from sea water (except the water outfalls from sewage and polluted river estuaries) (Schroth *et al*., 2018).

It has the ability to be alive and adapt to different environments such as municipal wastewater and hospital (Lee and Zhang, 2015). *P. aeruginosa* can also survive within lower nutritional requirements and tolerate a diversity of the physical conditions allowing the bacteria to exist in both hospital settings and community (De Sales *et al*., 2020). Due to their high metabolic ability, *P. aeruginosa* can metabolite a high concentrations of salts, dyes and antibiotics

(Gillespie and Hawkey, 2006). *P. aeruginosa* is the most common type in genus of *Pseudomonas* that causes of hospital infections, accounting for approximately 10% of total infections (Abbasi *et al.,* 2017). *P. aeruginosa* represent about 36.2% of the common Gram negative organisms isolated from patients with lower respiratory tract infections (LRTIs) acquired during period stay in the Intensive Care Unit (ICU) (Claeys *et al.*, 2018). It also considers the second most common pathogen associated with ventilator-associated pneumonia (Bhatta *et al.*,2019).

Many Iraq's studies have shown that the frequency of *P. aeruginosa* in burn infections was high, as it ranged between 32-66% in provinces of Karbala, Kirkuk, Hilla and Baghdad, with the highest percentage recorded in Baghdad, Karbala, Hilla and Kirkuk (Al-Saadi, 2009; Al-khafaji, 2016; Zein and Khorshid, 2017). Also, *P. aeruginosa* isolated from wound infections recorded as highest percentage (23.5%) among the other bacterial genera isolated in Baghdad Province (Ali *et al*., 2009). In Tikrit city, *P. aeruginosa* was recorded the highest rate in middle ear infections reached up to 42% (Kamal *et al.*, 2015).

The direct and indirect contact between patients, and using the contaminated surgical instruments facilitates the spreading of these organism**.** Whereas, the bacterium possesses the ability to survive in disinfectants, treatments and liquid medications, such as eye drops and anesthesia masks (Al-Daraghi and Al-Badrwi, 2020). In 2017, the Centers for Disease Control and Prevention (CDC) estimated 2.700 deaths out of 32.600 cases in hospitalized patients (CDC, 2019).

2.5 Virulence factors of *P. aeruginosa*

Most of *P. aeruginosa* strains involved in infections are both invasive and toxigenic, as consequence of the production of surface virulence factors (allowing bacterial attachment, colonisation and invasion) and virulence factors secreted when injure tissues and trigger the production of cytokines, respectively. The pathogenesis of *P. aeruginosa* is due to its production of many virulence factors depending on a numbers of cellular and extracellular factors that have a role in bacterial pathogenesis and their invasion of tissues (Pang *et al*., 2019). The cellular and extracellular factors can be categorized into two groups:

(A) Group I (the acute infection)

These factors are either on the bacterial surface or secreted into the medium included: (Jurado-Martín *et al.*, 2021).

- The cilia, they are associated with the epithelial cell of the host.
- The external enzyme S which performs tissue necrosis and inhibits the process of phagocytosis.
- Generating exotoxin A.
- Haemolysin.
- Production of lecithinase, lipase and leukocidin which is A type of cytotoxin.
- Protease production four types of proteolytic enzymes such as the Alkaline protease enzyme, catalase, and Elastase, Ureases production.
- Pigments production.

(B) Group II (the chronic infection)

- **Siderophore** (pyoverdin, pyochelin) pyochelin is a salicylate-based siderophore with a lower affinity for iron, pyoverdine (PVD) has a peptide nature and is considered the major siderophore (Jurado-Martín *et al*., 2021).

- **Alginate layer** protects the bacteria from the phagocytosis process (Pedersen *et al*., 2018).

- **Quorum sensing** system this bacterium contains a system controls of the production of virulence factors in these bacteria and has an important role in the process of regulation and expression of genes responsible for these factors (Jurado-Martín *et al*., 2021).

- **Biofilm** is a community of microorganisms that adhere to a biotic or biotic surface surrounded by a matrix of extracellular polymeric substances (EPS). Biofilm is main virulence factor in *P. aeruginosa* which collect the monocytes and forming a multicellular structure (Soares *et al*., 2020).

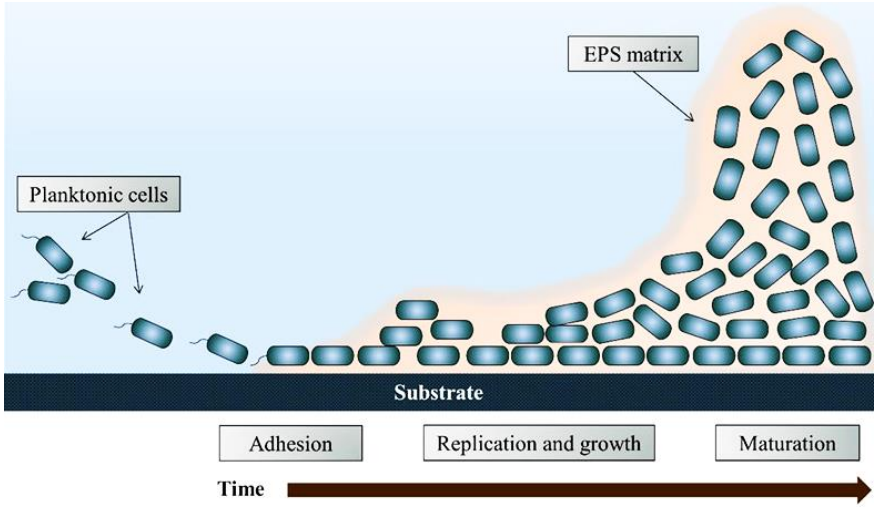


Figure (2-1): Stages of bacterial biofilm formation (adapted from: Gora *et al*., 2019).

The function of EPS is to guarantee the protection of the microorganisms existing in its interior from the attack of the other microorganisms in the external or internal environment. Thus, when *P. aeruginosa* is under stressed conditions, the formation of biofilms is often associated with higher antimicrobial resistance when compared to the planktonic form and assists in the

evasion of the host immune response (Skariyachan *et al*., 2018). The initiation of biofilm formation and non-opsonic phagocytosis is mediated by phagocyte receptors that recognize corresponding adhesions on the microbial surfaces (Rocha *et al*., 2019). The adhesion to the surface of the target cell is by the pili type 4, flagellum, exoenzyme S, fimbria and polysaccharide (Kose and Yapar, 2017).

In *P. aeruginosa*, the biofilm has many functions including antibiotics resistance, that is responsible for the initial inflammatory stages and resistance to phagocytosis by neutrophils, escape from defense mechanisms and expulsion of free radicals released by macrophages and neutrophils, causing the establishment of a chronic infection (Wagner *et al*., 2016). When *P. aeruginosa* exposed to an environmental pressure and a changes occur in its growth mode, it will trigger generation the biofilm forming process (Rocha *et al*., 2019).

The formation of biofilm in *P. aeruginosa* is responsible on chronic and intractable injuries. These diseases such as wounds, burns, cardiac arrhythmia, cystic fibrosis and middle ear inflammation, injuries are related to the contaminated of medical instruments, catheters and inflammation of gum and dental decay. genetic expression of the biofilm are specific genetic mechanisms, selection of resistant mutants, or survival of tolerant cells (Olivares *et al*., 2020).

2.6 Antibiotics

Antibiotics are therapeutic agents that used in modern healthcare due to its powerful activity in fighting of life-threatening infections. Antibiotics are defined as natural organic substances with the ability to inhibit/kill the other competitive organisms. It is produced by some particular microorganisms at low concentrations **(**CDC, 2019). A long time ago, human tried to find treatments of microbial infections. Dyes, molds, and even heavy metals were thought to hold promise for healing (Gould, 2016). The practice of antimicrobial stewardship

revolves around the concept of optimizing antimicrobial therapy and reducing adverse events through economically responsible methods (Cunha *et al*., 2018).

2.6.1 Classification of antibiotics

Antibiotics are classified to(Chaudhary *et al*., 2019).

(1) β-lactam antibiotic class

- Penicillins
- Cephems (Cephalosporins)
- Carbapenem
- Monobactams

(2) Aminoglycoside antibiotic class

(3) Fluoroquinolone antibiotic class

(4) Lipopeptides antibiotic class

2.7 Antibiotics resistance of *P. aeruginosa*

Antibiotic resistance can be defined as the ability of microbes to defeat the antimicrobial agents intended to kill them. Recently, antibiotics resistance is the greatest global public health challenges. In 2017, the World Health Organization (WHO) published a list of 12 genera and/or families, prioritized for the development of alternative antimicrobials. They categorized them into critical, high, and medium. Carbapenems-resistant genera were classified as one of the main priority 1:critical group of antimicrobial resistance of clinical, environmental, and control strains of *P. aeruginosa* (Shrivastava *et al.*, 2018). Unfortunately, nowadays, many people around the world are dying due to the untreatable infections caused by the emergence and spread of antibiotic resistance (CDC, 2019). In addition, the antibiotics resistance causes significant economic loss to the health care centers and hospitals of the high cost of treatment and the long duration of the patient's stay in hospital (Yusuf *et al*., 2017; Ullah *et al*., 2017). The inherent and acquired antibiotic resistance of *P. aeruginosa* due to only limited number of antibiotics are remain unclear in

treatment of infections caused by this bacterium. The excessive use of antibiotics during treatment accelerates the development of multidrug-resistant in *P. aeruginosa* strains. This lead to an ineffectiveness of the use empirical antibiotic therapy against this microorganism (Esmaeili *et al*., 2019).

In 2013, the Center for Disease Control (CDC) was published a list of antibiotic resistance threats and then updated the list in 2019. The list was organized in a levels of “urgent threats”, “serious threats” and "concerning threats". The serious threats are those antibiotics that need monitoring as they have the ability to become imminent threats to the public health. Those Gram-negative bacteria included multi-drug resistant of *P. aeruginosa* (CDC, 2019). The high percentage in morbidity and mortality in affected patients is because the elevate in antibiotic resistance in immunocompromised patients and healthcare-associated with this pathogen (Rocha *et al.,* 2019; McCutcheon *et al*., 2018). Based on the drug resistance patterns of the organism, *P. aeruginosa* is divided into different phenotypes. These are included multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) (Gill *et al*., 2016).

1) Multidrug-resistant (MDR) as being acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Hawkey *et al.*, 2018). The MDR of *P. aeruginosa* strains is associated with increase morbidity and mortality and prolonging hospital stay.

2) Extensive drug resistance (XDR) is defined as non-susceptible bacteria to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) (Magiorakos *et al.*, 2012).
3) Pan drug-resistant (PDR) is defined as non-susceptibility to all agents in all antimicrobial categories. To ensure the correct application of these

definitions, bacterial isolates should be tested against all or almost all of the antimicrobial agents within the antimicrobial categories (Magiorakos *et al.*, 2012). The pan-drug resistance, the lack of effective antibiotics against MDR *P. aeruginosa* strains and continues the dissemination of such strain pose serious challenges in the infection control management (Murugan *et al*., 2018).

2.8 The mechanisms of resistance in *P. aeruginosa*

The mechanisms resistance are defense strategies developed by microbes to help them survive and avoid the effects of antibiotics (CDC, 2019). *P. aeruginosa* has become an important and frequent opportunistic nosocomial pathogen. This organism is characterized by an innate resistance to multiple classes of antimicrobials causing difficult-to-treat infections lead to high morbidity and mortality (Hassuna *et al*., 2020). As well known, *P. aeruginosa* is difficult to control by agents like antibiotics and antiseptics.

The extensive use of antibiotics to treat the infections caused by this bacterium has led to an emerge of strains with multiple antibiotic resistance increased over time, making it one of the most common health problems, specifically for those patients remaining in hospitals (Dantas and Sommer, 2014). *P. aeruginosa* associated resistance is frequently categorized into intrinsic/innate resistance and acquired or mutational resistance (Blair *et al.*, 2015). Figure (2-2) summarize the types of mechanisms of resistance in *P. aeruginosa.*

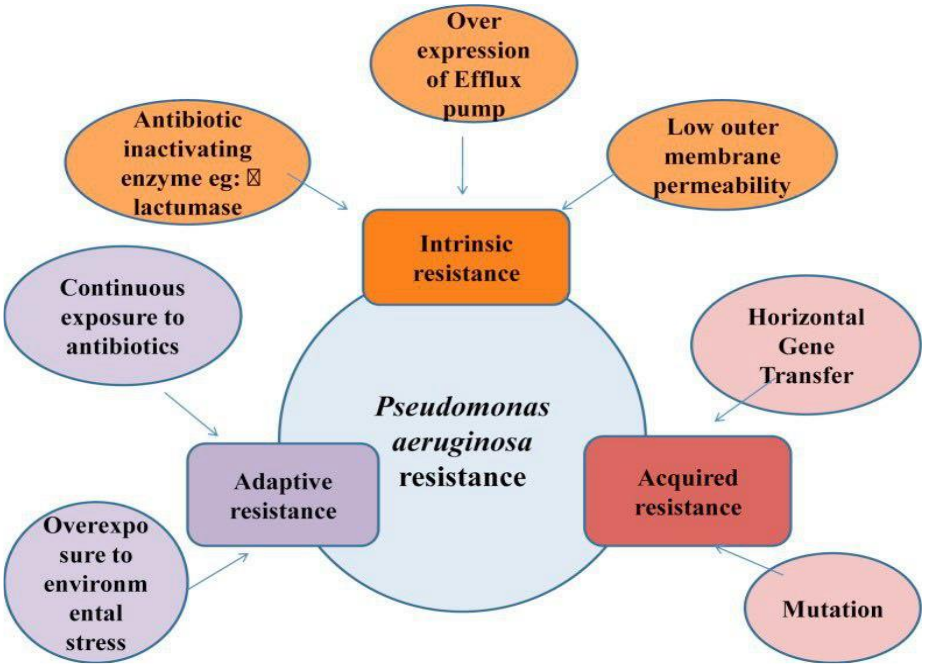


Figure (2-2) *P. aeruginosa* have different mechanisms to resist a wide range of antibiotics (Pachori *et al*., 2019).

2.8.1 Intrinsic antibiotic resistance

P. aeruginosa strains are naturally resistant (less susceptible) to several antibacterial drugs. Intrinsic mechanisms of *P. aeruginosa*, are heritable and stable resistance determinants (Yaeger *et al*., 2021). *P. aeruginosa* was possess a high level of intrinsic resistance to most antibiotics includes restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell and production of antibiotic inactivating enzymes such as β-lactamases AmpC **(**Pappa *et al.*, 2020)

2.8.1.1 Efflux system

Efflux pumps is one of the bacterial mechanisms that have developed to resist drugs (Puzari and Chetia, 2017). Beside the bacterial efflux systems capable of ejecting antimicrobials, they are mostly encoded by chromosomal genes (Avrain *et al*., 2013). The efflux pumps mechanisms were divided into five groups based on the similarity of the amino acid sequence and the source of

energy required for export any foreign. The major facilitator superfamily (MFS) (Blanco *et al*.,2016):

1. The ATP-binding cassette family (ABC).
2. The small multi-drug resistance family (SMR).
3. The multi-drug and toxic compound extrusion family (MATE).
4. The resistance-nodulation-division family (RND).

2.8.1.2 Outer membrane permeability

Gram-negative bacteria, including *Pseudomonas*, have an outer membrane (OM) with less permeability to the hydrophobic agents due to presence of lipopolysaccharide (LPS). The outer membrane in Gram-negative bacteria is slowed the influx and uptake the antibiotics (Zgurskaya *et al.,* 2015). Also, the outer membrane acts as a selective barrier to prevent penetration of the antibiotics. It is an asymmetric bilayer of phospholipid and LPS embedded with porins that form β-barrel protein channels. The LPS-containing bilayers become more rigid than the normal bilayers slowing passive diffusion of hydrophobic compounds. While the narrow pores are limited by size the penetration of hydrophilic drugs (Jurado-Martín *et al*., 2021). *P. aeruginosa* is set up to lack outer membrane porin D (OprD) proteins. OprD are pores that allow the entrance of Carbapenems. When these pores are lost, carbapenems have to face the challenges of resistance (Dantas *et al*., 2017)**.** In any changes in the locations of these pores can leads to antibiotic resistance (Cox *et al*., 2015).

2.8.1.3 Target site alteration

In this type of intrinsic resistance, PBPs are represent the site of binding of beta-lactam antibiotic. The genetic mutation in genes responsible for PBPs are carried on plasmids. These mutations lead to change in PBPs site and thus failure of the antibiotic to recognize PBPs sites in the bacterial cell wall. The

failure of the antibiotic to react with PBPs will change the profile of the bacteria from sensitive to resistant (Sun *et al*., 201 4).

2.8.1.4 Inactivating enzymes mechanism

Inactivating enzymes is one of the major mechanisms in bacterial intrinsic resistance. This mechanism included production of antibiotic-inactivating enzymes that break down or modify the applied antibiotics. Many antibiotics have chemical bonds such as (amides and esters) that are susceptible to hydrolysis (Pang *et al*., 2019). These enzymes are commonly produced by *P. aeruginosa*, such as β-lactamases and aminoglycoside-modifying enzymes (Wolter and Lister, 2013). Gram-negative bacteria are secreted antibiotic-inactivating enzymes in the periplasmic region. The genes that encoding of enzyme beta-lactamase are transmitted to the plasmid, chromosomal or on a transposon (Boussoualim *et al*., 2014).

2.8.2 Acquired antibiotic resistance

This type of antibiotic resistance appears after the exposure to a particular antibiotic, caused by the presence of plasmid (RP1), transposon, or integrons, or chromosomal mutations (Ekizogl *et al.*, 2016). Therefore, bacteria can gain and passes this type of resistance by horizontal gene transfer (Munita and Arias, 2016). The resistance to MDR can develop via successive mutations through the dissemination of multi resistant plasmids genes (e.g. transposons), or through a combination of both processes. Acquired antibiotic resistance can narrow the antibiotic choices for definitive clinical therapy (Hawkey *et al.*, 2018).

2.8.3 Adaptive antibiotic resistance

Adaptive resistance refers to a resistance that occurs due to environmental conditions, such as transcriptional changes in genes that determine resistance/susceptibility, and increase the ability of a bacterium to survive an

antibiotic attack due to transient changes in gene and/or protein expression in response to an environmental stimulus and is reversible when environmental conditions (for example, complex adaptive growth states such as swarming, biofilm formation, or exposure to stresses, including antibiotics) are reversed or can be reversed when the stimulus is removed (Schroeder *et al*., 2017; Pang *et al*., 2019).

2.9 Beta lactamase enzyme

Many species of bacteria are able to produce enzymes that break down antibiotics. Beta lactamase enzymes are one of the most important resistant mechanisms in Gram-negative bacteria against β- lactam antibiotics group. Indeed, occurrence of point mutations in the sequence of the primary β-lactamase gene results in production of different types of enzymes (Ali and Ahmed, 2021). Beta-lactamase enzymes are classified within hydrolase group enzymes.

It belongs to serine family that contains serine in its hydroxyl group in the structure of the active site. The hydrolysis process of β-lactam antibiotics by the β-lactamase activity occurs though formation of an ester bond between the serine in the active site (or with zinc ions in the case of the metallo-β-lactamases) of the β-lactamase enzyme and the β-lactam ring of the antimicrobial agent. Based on Ambler classification system, β-lactamase are categorized into four groups according to their primary proteinous structure called A, B, C and D (Bush and Bradford, 2020).

Originally, Ambler identified two main classes of β-lactamases based on the molecular hydrolysis mechanism of the β-lactamase ring: (i) class A, ser-β-lactamases, which is operate through the action of a Ser nucleophile active site and (ii) class B, metallolactamase (MBLs) activate water through Zn^{+2} center (Yoon and Jeong, 2021). There was a new class of serine β-lactamases discovered later, that bore little similar sequence to class A enzyme. Class

designated C, its known as the b-lactamases (AmpC) (Minhas and Sharma, 2015).

This enzyme has the ability to hydrolysis Penicillins, Cefotaxime, Ceftazidime or Aztreonam and inhibits by Clavulanic acid, Sulbactam, and Tazobactam (Keshri *et al*., 2018). These enzymes are complex, diverse, rapidly evolving and challenge many of the available antibiotics (Shaikh *et al*., 2015). Carbapenems among antibiotics, are considered as the strongest β-lactam against MDR isolates owing to the high affinity for penicillin-binding protein, stability against β-lactamases (ESBLs), and the permeability of bacterial outer membrane (Wi *et al.*, 2018; Yin *et al.*, 2018).

2.9.1 Types of *P. aeruginosa* β-Lactamases

2.9.1.1 Extended spectrum β-lactamases (ESBLs)

In Gram-negative bacilli bacteria, there are more than 200 type of ESBLs have identified. Among them, 32 type are present in *P. aeruginosa*. These types are subdivided into 8 subgroups included TEM, SHV, CTX-M, PER, VEB, GES, BEL, and OXA. OXA and GES Which they are commonly observed in *P. aeruginosa* (Horcajada, 2019).

These major genetic subgroups of ESBLs are TEM, SHV, PER and CTX-M Extended-spectrum β-lactamase are plasmid-encoded enzymes which mediate resistance to a wide range of antibiotic generations (Mendes *et al.*, 2019). The enzymes have the ability to hydrolysis Penicillins, Cefotaxime, Ceftazidime or Aztreonam. Nevertheless, the β-lactamase inhibitors (clavulanic acid with ticarcillin and tazobactam with piperacillin) provides a protection against some of bacterial plasmid-mediated enzymes (Keshri *et al.*, 2018).

2.9.1.2 Metallo beta-lactamases (MBLs)

MBLs are mineral beta-lactamase enzymes which are important as clinical problem of concern in all societies in the world because it acts as inhibitors most of beta-lactam antibiotics (Ali and Ahmed, 2021). Production of these enzymes makes *P. aeruginosa* strains more resistant (Ferreira *et al*., 2017). The mechanisms of resistance in Metallo B-lactamase in *P. aeruginosa* are multiple resistance type. They produced at high level. The encoding genes of these enzymes are located on integrons, transposon, plasmids or chromosome and incorporated as gene cassettes (Ghasemian *et al*., 2018).

They play a key role in bacterial resistance because it can pass from one cell to another due to the intensive and wrong uses of antibiotics (Mohamudha *et al*., 2012). The genes of MBL families are various in types as shown below and have been identified in *P. aeruginosa* including (Azimi *et al*., 2018):

- Imipenemase (IMP)
- Verona integron-encoded metallo-β-lactamase (VIM)
- São Paulo metallo-β-lactamase (SPM)
- Germany imipenemase (GIM)
- Seoul imipenemase (SIM)
- New Delhi metallo-β-lactamase (NDM) types

2.9.1.3 Beta-lactamase AmpC

AmpC is one of cephalosporinases enzymes belonging to the class C of ß-lactamase. According to the molecular classification of Ambler A 1980. It is the first group in functional classification based on Bush and others 1995 (Bush and Jacoby, 2010). All *P. aeruginosa* strains possess the AmpC gene for the inducible chromosomal β-lactamase. The β-lactams induce synthesis of a new penicillin-binding protein, PBP2a, which does not bind any β-lactam (Brooks *et al*., 2016). They are differentiated from other ESBLs by their ability to

hydrolyze Cephalosporins, as well as other extended spectrum Cephalosporins (Torrens *et al*., 2019).

2.10 The genome of *P. aeruginosa*

P. aeruginosa has one of the largest genome among human pathogenic bacteria with an average size of (~5 MB to ~7 MB) (Jurado-Martín *et al*., 2021). The genome is complex with 66.6% of nitrogenic bases, cytosine and guanine (C+G). It consists of chromosomal and extra-chromosomal component called plasmid (Bachta *et al.,* 2018; NCBI, 2020). The structure of *P. aeruginosa* genome is a mosaic resulting from multiple acquisitions from different donors during its evolution and the horizontal gene transfer (Bachta *et al*., 2018).

Basically, the genome of *P. aeruginosa* consists of core genome (90%) and accessory genome (10%). A pangenome is the whole set of genes (including core and accessory) found in a phylogenetic group (Whelan *et al*., 2021). The accessory genome consists of integrative and conjugative elements (ICEs), harbored by some strains of *P. aeruginosa*, which may be considered part of the accessory genome (Botelho *et al*., 2019). In addition, the occurrence of large number of deletions and rearrangements genes in *P. aeruginosa* (about 5.567 genes) make their genome more capacity than other prokaryotes (Alayande *et al.,* 2018).

The ICEs are defining as modular mobile genetic elements that can integrate into a host genome and be vertically propagated through the cell replication or transfer horizontally following excision from the chromosome (Botelho and Schulenburg, 2021). The genome of *P. aeruginosa* encodes an unusually high attribution of proteins that involved in regulation, transport and virulence functions.

This may explain the high fluctuate and adaptive capacity of this organism. There is 0.3% of the total genes encode for proteins complicated in antimicrobial resistance (Gimenez *et al*., 2018). Genomic islands found in *P.*

aeruginosa possess encoding genes factors that are represented by genes in genetic mobility and in various virulence traits such as iron uptake functions, antibiotic resistance, type III secretion systems, biofilm synthesis, toxins and adhesions that support their ability to survive in diverse hosts and cause of disease (Yoon and Jeong, 2021).

2.11 Elements of mobile genetics

Horizontal gene transfer (HGT) are playing a key role in bacterial evolution and it is a major source of genome expansion (Botelho and Schulenburg, 2021). HGT is defined as a transfer of the genetic elements from one bacterial cell to another rather than parental inheritance, also, HGT is responsible on the spreading of antibiotic resistance among bacterial strains. The mutational events that causes antibiotic resistance comes from genes harbored on mobile genetic elements such as transposons, integrons or plasmids. These factors can be transferred readily between others members belong to the same species or to different genera (Odoi, 2017; Wheatley and MacLean, 2021). Passing of horizontal gene involve main mechanisms: transformation, transduction and conjugation. They can transfer from one microbe to another and from genome to genome (Pang *et al*., 2019). HGT can be divided into the following elements:

- **Plasmids** are an extra-chromosomal pieces of DNA that exists in most of Gram-negative and Gram-positive bacteria. Plasmids are circles in shape and self-replicating that carry genes and spread by transfer between the bacteria by different ways. Their function is regulatory role in cellular metabolic processes and virulence characteristic of bacteria (Odoi, 2017; Cepas and Soto, 2020).

- **Transposons** are small pieces of DNA that can insert into the cell and change the overall DNA of the cell. Transposons can move from a

chromosome (which carry the genes essential for germ survive) to a plasmids and vice versa (Botelho *et al*., 2019, CDC 2019).

- **Phages** are a groups of viruses that attack microbes and can carry a piece of DNA from one germ to another (Botelho *et al*., 2019; CDC 2019).

- **Integrons** are genetic structures that capable of expression and acquisition of gene cassettes exchange. They consist of promoter and attachment site (attI). Integrons may be located on transposons, plasmids or chromosomal DNA (Gillings, 2014). There are five different classes of mobile integrons have been described based on the homology of the integrase gene but only three of them have been involved in multidrug-resistance of antibiotics (Pang *et al*., 2019)**.**

- **Pathogenicity islands (PAIs)** Pathogenicity islands are well identified and characterized elements as members of mobile ICEs group (Flannery *et al*., 2009). PAIs are gene clusters incorporated into the genome of *P. aeruginosa* chromosomally or extra-chromosomally region distinct and large. They are usually absent from those nonpathogenic organisms of the same or closely related species, also, PAIs range from 10-200 kb length and encode genes that are contribute to the virulence of the respective pathogen. The GC bases content and codon usage of pathogenicity islands often differs from that of the rest genome. Potentially, the detection of PAI can help in DNA sequencing, unless the donor and recipient of the PAI have similar GC content (Cepas and Soto, 2020).

2.12 Typing of bacteria

Typing is a term that mean identification of different types of organisms such as bacteria within a specific species, or it is differentiation of the isolates or strains to subspecies (Ruppitsch, 2016). Previously, many traditional methods

were used in bacterial typing based on a set of traits included serotype, biotype, phage-type or antibiogram which they called phenotype method. Another way emerged for bacterial typing is the molecular typing which differentiate the bacterial isolates and their relatedness at the molecular level (Sabat *et al*., 2013). Different methods of molecular typing can be classified into several categories (Nutman and Marchaim, 2019):

- Amplification-based, variable-number tandem repeat typing.
- Fragment-based, Pulsed-field gel electrophoresis.
- Sequence-based, Multi-Locus Sequence Typing.
- Genomics-based methods.

The choice of which technique suitable for an appropriate molecular typing is significantly based on the problem or case to solve and the epidemiological context in this method. It based on the time and geographical scale of their uses. The human pathogens are comprising of very diverse organisms. Hence, typing techniques should have an excellent ability to be able to type the isolates under study (Nutman and Marchaim, 2019).

Typing of a pathogen is very important in detection the source of the microbial outbreaks. It also provides an implement effective way to control and prevent the pathogens from spreading (Maccannell, 2013). Typing of bacteria has greatly contributed to increase the effectiveness of surveillance systems and providing an important clue to the public health control strategies.

However, the main role of bacterial typing is to determine the clonal relatedness between a microbial strain, that it is essential in determination the routes and source of infections. Typing of bacteria is essential to determine the relationships between microbial isolates comes from different sources, the outbreaks in rule or confirm, trace cross-transmission of healthcare associated pathogens, recognize virulent strains, assess the effectiveness of control measures (Perez-Losada *et al*., 2013). *P. aeruginosa* bacteria has a very variable and complicate genome which make it the cause of a wide range of infections.

This character makes *P. aeruginosa* able to discard or acquire genomic segments via recombination or horizontal gene transfer (Al-Kubaisy, 2018). A brief an overview of some typing techniques which they used in study of bacterial epidemiology and active surveillance:

2.12.1 Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis is a popular method used for large-scale epidemiological investigations due to its discriminatory powers (DIs) (Tang *et al.,* 2017). In this technique, the whole bacterial genome is restricted and analyzed in a pattern without use a probes. PFGE has been successfully as a short-term for many bacterial pathogens, proving a very accurate and reproducible method (Martak *et al*., 2019). However, for *P. aeruginosa* this method is time consuming, expensive, and needs specialized equipment and expert operators (De Sales *et al*., 2020). In compare with Whole Gene Sequence -based typing, Martak and co-worker (2020). Found that the PFGE can be used with a confidence in the outbreak investigation by this bacterium.

2.12.2 Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR)

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR method is based on using oligonucleotides for targeting a short repetitive sequences sparse throughout different bacterial genomes, like Enterobacteriaceae indifferent directions. This will allow to enable the differentiation at the level of genus and serovars depending on their electrophoretic amplification products (Qin *et al*., 2016).

2.12.3 Random Amplified Polymorphic DNA (RAPD)-PCR

Random Amplified Polymorphic DNA assay used to avoid the drawbacks of PFGE. Moreover, it is rapid, inexpensive, simple, and easy to use due to its widely used for typing of the bacterial isolates in cases of the outbreaks. based

on, the use of short arbitrary sequences, as primers 20-25 base pairs (bps) for targeting several unspecified genomic sequences. RAPD amplicon, by depending on the labeling of primers with a suitable fluorescent dyes the products can be analyzed by either DNA sequencing or agarose gel electrophoresis (Sharma and Park, 2020).

2.12.4 Multi-Locus Sequence Typing (MLST)

(MLST) Multilocus sequencing typing, analysis is an electronically portable, universal, and definitive bacterial typing method that focuses solely on conserved housekeeping genes and the combination of each allele (Wendt and Heo, 2016). MLST is a genetic method with a high power resolution; based on fragments sequencing of (7 genes of 450 to 500) bp, (with a high variability degree). The analysis detects variations, in the different loci and permits the identification of identical microorganisms the (clones) or of highly related ones (clonal lines or genotypes). Which, they are markers that have remained stable along evolution and they used for the comparison of strains in large time scales or from different geographical regions (Tümmler, 2020).

2.12.5 Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA)

There are specific sequences with distinct sizes in bacterial genome that repeated in a copy numbers in tandem way. These loci called Variable-Number Tandem Repeats (VNTRs) which consider as characteristic fingerprint the bacterial DNA for each strain. Appearance polymorphism in the mini-satellite loci between strains, as VNTRs that are based on PCR (Babenko *et al*., 2017).
MLVA is a simple, rapid, affordable, and reliable approach used in characterization of microorganisms. MLVA is a promising tool that used in determination of the outbreak of pathogenic strains of *P. aeruginosa* (Shokoohizadeh *et al*., 2020). MLVA analysis was developed for the first time

in typing of *P. aeruginosa* by Onteniente and co-workers in 2003. Subsequently, this method was improved by adding new epidemiologically information markers (De Filipps and McKee, 2012).

MLVA is a potential assay because of their sensitivity specificity as detection test in clinical laboratory to target the pathogenic strains of *P. aeruginosa* (Basu *et al.,* 2015). The discriminatory ability of MLVA in bacterial strains is much higher than MLST and that power is not less than PFGE compared to the other methods (Farahani *et al.*, 2020). The early detection of *P. aeruginosa* treated with aggressive antibiotic is important in order to prevent or postpone the chronic colonization in patients (Douraghi *et al*., 2014).

For example, burn infections caused by *P. aeruginosa* could lead to severe health consequences such as pneumonia, sepsis, and necrosis (De Sales *et al.,* 2020). Recently, MLVA-based typing, in those aggressive isolates are evaluated by a number of replicates in several genetic regions in their genome. MLVA technique also developed to type many bacteria such as *Salmonella*, *Escherichia coli, Bacillus anthracis* (Shokoohizadeh *et al*., 2020). Due to the high variation in the pathogenic strains that carry the genes of antibiotic resistance, the importance of MLVA techniques become more common physicians. In high numbers of clinical specimens, MLVA could be a potential way to differentiate between those close related clinical strains (Hertz *et al*., 2016).

Considering, pathogenic strains of the hospital that typing can provide very useful information for designing an effective therapeutic approach to physicians and medicine staff. The main limitation of MLVA assay is that the studies comes from different laboratories cannot be compared precisely. The reason behind that is the amplicons products are observed as banding profile by using horizontal agarose gel electrophoresis. In this case, the recognize the band in the pattern will be more complicate regarding the target in PCR reaction.

Also, this approach is a high assay-specific for the different organisms and lacks standardization for the most of published assays (Chen *et al*., 2018).

2.12.5.1 The process of MLVA technique

MLVA are Multiplex PCR-based method that based on the analysis of variable copy numbers of tandem repeats (VNTR). It utilizes the naturally occurring variation in the number of tandem repeated DNA sequences in multiple loci of the genome which then detected by PCR using flanking primers. The process of MLVA assay started from selecting the desired locus, designing the primer, extracting the DNA of a desired strain, PCR proliferation of the sequences that containing the number of VNTR loci (Lashgarian *et al.*, 2018) figure (2-3) and figure (2-4).

The products (number of VNTR) loci obtained from the Multiplex PCR reaction, after amplification was subjected to capillary electrophoresis together with reference DNA fragments (known as DNA size markers). That resulted as peaks to be measured the size of each locus. From this size and this steps the number of the replicates is calculated, depending on the amplicons sizes and flanking regions which define the flank that does not contain any repeats). These sequential repetitive sequences called number of tandem repeat units (TRs) are in different sizes and variable from one isolates to another.

The number of TRs for each locus is collected in a code (numeric digit code) of the isolates, that called the MLVA profile of the analyzed organism and could be used later for clustering of *P. aeruginosa* isolates to evaluate genetic variability and relatedness. By comparison of bands, the genetic distance was calculated using (UPGMA) method (Bachelerie *et al*., 2016; Babenko *et al*., 2017).

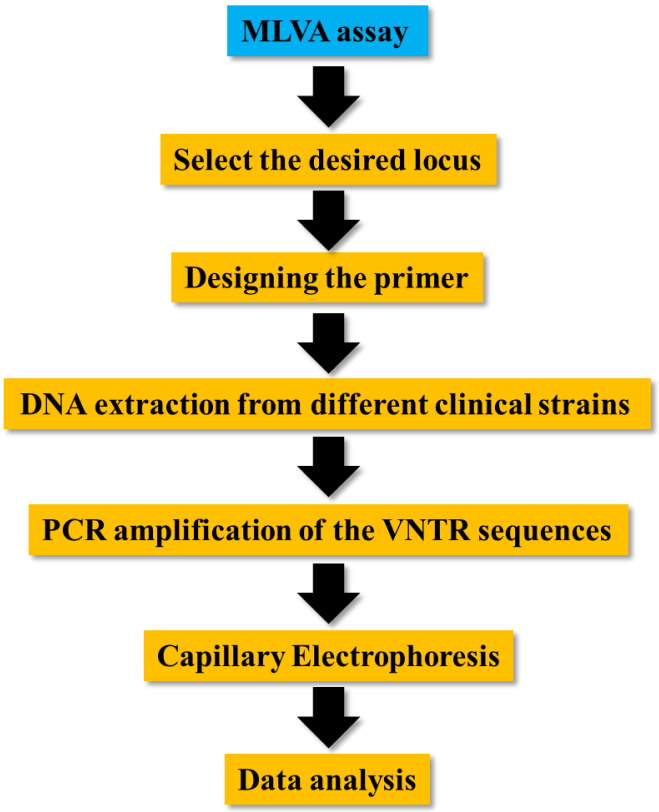


Figure (2-3): Diagram showing the steps of the MLVA.

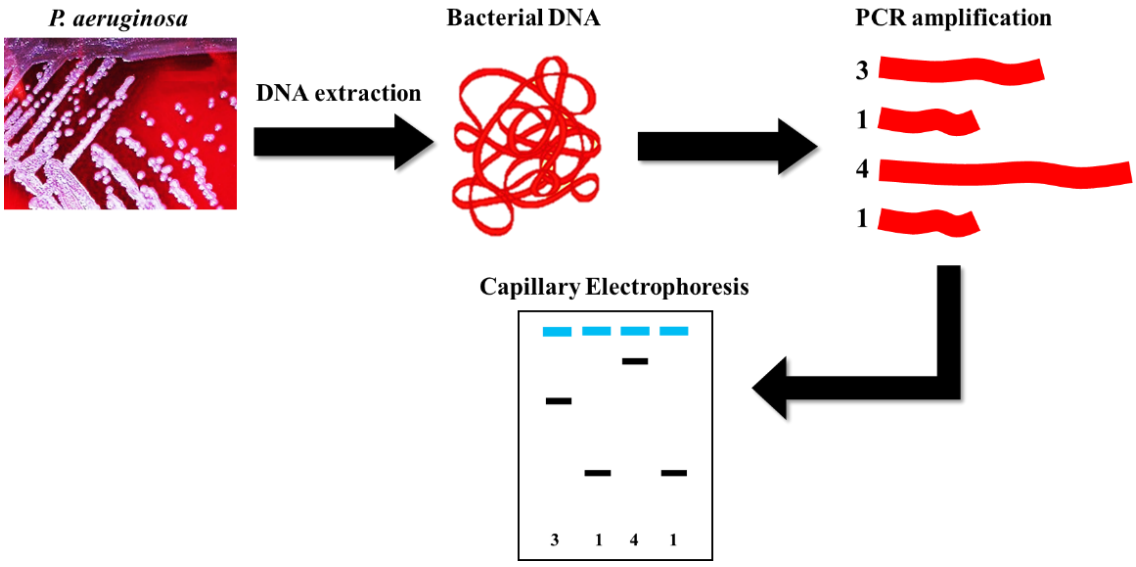


Figure (2-4): Steps of MLVA.

The final information of analysis can be compared with databases (reference) easily. Once the assay has been standardized and compatible. MLVA has become a major first line typing tool in a number of hospital pathogens (Nadon *et al.*, 2013). In Pseudomonas genus, the repeated sequences have been

identified based on different loci included MS-213, MS-214, MS-215 and MS-142 (Lashgarian *et al.*, 2018).

These loci selected in this study, to determine the variant and evolution of local *P. aeruginosa* isolates. Accordingly, *P. aeruginosa* from different patients or different locations can be categorize into different subspecies. Each categorized strain is described by a code corresponding to the number of the repeats at a specific VNTR. Hence, it will be easily to compare these codes between the laboratories based on the web databases (available at http://bacterial-genotyping.igmors.u-psud.fr) (Le Fle`che *et al*., 2002)**.**

The Variable number of tandem repeats (VNTR) is the discovery of unique DNA sequences that replicate throughout the genome, and are one of a kind in terms of (length and copy number). These repeats are organized in a particular way and have a specific copy number inside an organism's genome. of the relatedness degree among a population's individuals can be determined, As a result to the difference in the number of repeats. Since these repeats are found in the genome, they can be used to track evolutionary change and as DNA fingerprinting (Naseer *et al*., 2012; Nadon *et al*., 2013)

CHAPTER THREE

MATERIALS AND METHOD

Materials and Methods

3.1 Materials

3.1.1 Equipment and apparatus

The Equipment and apparatus that were used in this study are shown in table (3-1).

Table (3-1): Equipment and apparatus.

Equipment and apparatus	Company	Origin
Autoclave	GEMMY	Taiwan
Centrifuge	Thermo Scientific™	USA
Distillator	Gallenkamp	England
Gel Imaging System	Major Science	Taiwan
Horizontal Gel Electrophoresis System	Thermo Scientific™	USA
Incubator	Memmert	Germany
Laminar air flow	LabTech	Korea
Light Microscope	Olympus	Japan
Microcentrifuge	Eppendorf	Germany
Micropipettes of different sizes	Human	Germany
Microplate reader	Human	Germany
Microspin Centrifuge	My Fugene	China
Microwave Oven	GOSONIC	China
Millipore Filters (0.22µ)	Millipore Corp.	Germany
pH meter	Hanna	Italy
Quantus Fluorometer	Promega	USA
Refrigerator	TEKA	Spain
Sensitive balance	OHAUS- PioNEER	USA
Shaker incubator	Sartorius	Germany
Sterilized cotton swabs	Sterellin Ltd.	UK
Thermal Cycler (Veriti)	Thermo Fisher Scientific	USA
VITEK-2 system	BioMerieux	France
Vortex	Quality Lab system	England
Water bath	Memmert	Germany
Well flat bottom microplate	Coastar	USA

3.1.2 Chemical and biological materials

Chemical and biological materials that were used in current study are listed in table (3-2).

Table (3-2): Chemical and biological materials

Chemical and biological material	Company and origin
Absolute ethanol	Romil/France
Catalase solution: Hydrogen peroxide 3% (H_2O_2)	Analar/England
Glycerol	Fluka/Switzerland
Oxidase reagent: Oxidase indicator [N,N,N, N-tetramethyl-p-phenylenediamine dihydrochloride%1]	Himedia/India
Ethylene diamine tetra-acetic acid (EDTA)	Fluka/Switzerland
Kovac‘s Indol Reagent: p-dimethylamino benzaldehyde, isomylalcohol	BDH/England
Methyl red Reagent	BDH/England
Vogaes-prskour Reagent: alpha-naphthol, Potassium hydroxide(KOH)	BDH/England
Hydrochloric acid (HCl), Sodium chloride (NaCl), Sodium hydroxide (NaOH)	BDH/England
Standard McFarland’s solution (0.5)	Bio Mérieux/Bio Mérieux

3.1.3 Culture media

Culture media that used in current study listed in the table (3-3) are prepared according to instructions of manufacturing company. Briefly, the powder was dissolved in distilled water and heated by hot plate with continue stirring to dissolve all the ingredients entirely then sterilized by autoclave. The sterilized media were poured in sterile Petri dishes. The plates were incubated for 24 h at 37 °C to ensure their sterility after being prepared. They kept at 4°C till they are used.

Table (3-3): The culture media.

Media	**Company and origin**
Brain-Heart Infusion agar (BHIA)	MAST group- UK
Brain heart infusion broth (BHIB)	
Muller Hinton agar (MHA)	
Muller Hinton broth (MHB)	
Nutrient agar (NA)	
Nutrient broth (NB)	
MacConkey agar	
Pseudomonas Agar Base with CFC supplement	
Simmons Citrate agar	
Peptone water broth	
Methyl red, Voges-Proskauer broth	

3.1.4 Kits and materials used in molecular study

Kits and materials that were used listed in table (3-4).

Table (3-4): Kits and chemicals.

Kit	Company and origin
Presto™ Mini gDNA Kit	Geneaid/Taiwan
▪ Agarose ▪ Ethidium Bromide Solution (10mg/ml), ▪ GoTag Green Master Mix, Nuclease free water ▪ TAE 40X ▪ Quantifluor dsDNA System	Promega/USA

3.1.5 Primers

Primers that used in this study were described by Spilker and co-workers (2004) and Lashgarian *et al*., (2018) as shown in table (3-5).

Table (3-5): The primers used in the current study for gene detection (supplied by Macrogen, Korea).

Primer Name	Oligo sequence (5'-3')	Annealing (°C)	Dye
16S rRNA-F	5` GGG GGA TCT TCG GACCTCA3`	54	-
16S rRNA-R	5` TCC TTA GAG TGCCCA CCCG3`		
MS-213-F	5`-TGGCGTACTCCGAGCTGATG-3`	60	FAM*
MS-213-R	5`-CTGGGCAAGTGTTGGTGGATC-3`		
MS-214-F	5`-CCATCATCCTCCTACTGGGTT-3`		VIC**
MS-214-R	5`-AAACGCTGTTCGCCAACCTCTA-3`		
MS-215-F	5`-CTGTACAACGCCGAGCCGTA-3`		NED
MS-215-R	5`-GACGAAACCCGTCGCGAACA-3`		
MS-142-F	5`-GTGGGGCGAAGGAGTGAG-3`		SAV
MS-142-R	5`-AGCAGTGCCAGTTGATGTTG-3`		

*6-FAM= Fluorescein amidites (6-carboxyfluorescein)

**VIC= 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein.

3.1.6 Antibiotics

3.1.6.1 Antibiotics powder

Ceftazidime (1 g) was used in this study which supplied in powder from LDP/Barcelona/Spain.

3.1.6.2 Antibiotic disks

Antibiotic disks (Mast group Ltd./UK) were used are listed in table (3-6) according to (CLSI, 2020).

Table (3-6): Antibiotics disks.

Antibiotic class	Antibiotic type	Antibiotic symbol	Concentration mg/disc
ß-lactam combination agent	Ticarcillin/clavulanic acid	TIM	75/10
	Piperacillin/Tazobactam	PTZ	100/10
Cephalosporins	Ceftazidime	CAZ	30
	Cefepime	CPM	30
Fluoroquinolones	Ciprofloxacin	CIP	5
	Levofloxacin	LEV	5
Aminoglycosides	Gentamicin	GN	10
	Amikacin	AK	30
	Netilmicin	NET	30
Monobactams	Aztreonam	ATM	30
Carbapenems	Imipenem	IMP	10
	Meropenem	MEM	10

3.2 Methods

This study was carried out according to the following diagram Figure (3-1):

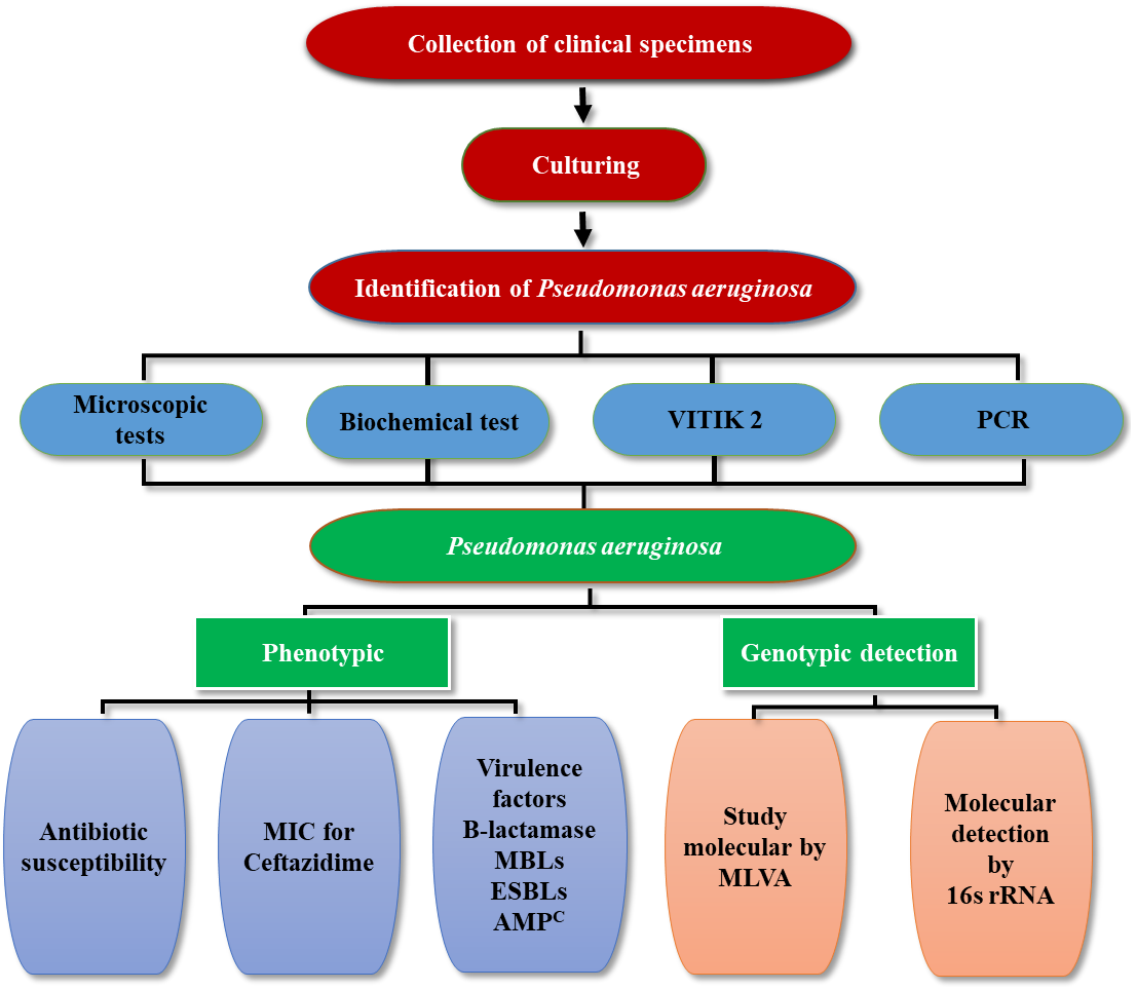


Figure (3-1): Illustration of the study plan.

3.2.1 Methods of sterilization

- Sterilization by autoclave: The media were sterilized by the moist heat sterilization method by autoclave for 15 minutes at 121°C and pressure 15bar/in^2.
- Sterilization by hot air oven: All the glassware was sterilized and dried by thermal oven at 180 °C for one hour.
- Sterilization by Millipore filter: The solutions that affected by high temperature were sterilized with Millipore filter 0.22 µm.

3.2.2 Preparation of reagents and solutions

3.2.2.1 Reagents and solution were prepared according to MacFaddin (2000) as follow:-

3.2.2.1.1 Catalase reagent

A commercial hydrogen peroxide 3% (H_2O_2) was prepared from 15% stock solution and kept in dark tube

3.2.2.1.2 Oxidase reagent

Oxidase reagent was prepared by dissolving 1g of N-N-N-N–tetramethyl-P-phenylene diamine hydrochloride in 100 ml of distilled water and stored in dark container in the refrigerator and used within a week.

3.2.2.1.3 Methyl red reagent

Prepared by dissolving a 0.1 g of methyl red in 300 ml of methyl alcohol at 95% concentration and the volume was completed up to 500 ml by adding 200 ml of distilled water.

3.2.2.1.4 Vogas-Proskaur reagent

This reagent consists of:

- **Alpha nephthol 5%**: prepared by dissolving 5 g of alpha nephthol in 100 ml of 95% ethyl alcohol.
- **Potassium hydroxide KOH 40%:** 40 g of potassium hydroxide dissolved in 100 ml of distilled water.

3.2.2.1.5 Kovac's reagent

Five grams of para-dimethyl aminobenzaldehyde dissolved in 75 ml isopropyl alcohol using water bath at 50 °C for 15 min and completed up to 100

ml with HCl. The color of the solution was pale yellow. The reagent was stored in dark bottle in the refrigerator.

3.2.2.2 Solutions

3.2.2.2.1 Antibiotic stock solutions

The antibiotic stock solutions were prepared with a final concentration of 100000 µg/ml as primarily stock, 1 g of antibiotic was dissolved in 10 ml completing the volume to 100 ml then sterilized by filters 0.22 µm pores and stores in a refrigerator, then prepare the stock 2 (2048 µg/ml) by the formula: $C_1V_1 = C_2V_2$, from100000 µg/ml as primarily stock, this according to CLSI (Weinstein and Lewis, 2020).

3.2.3 Samples collection and bacterial isolation

A total of 280 clinical specimens were collected beginning from October 2020 to January 2021. Patients with different ages ranging from 4-70 years from both genders were choosing from different hospitals in Baqubah city/Diyala Province, Iraq included Baqubah Teaching Hospital, Al-Batool Teaching Hospital and the Public Health Laboratories. The clinical specimens were collected from wounds and burns using cotton disposable swabs and transported into the sterile medium in plastic bottles. All the swabs and urine containers of 280 specimens were taken under sterile conditions and transferred, inoculated immediately on MacConkey agar and incubated at 37 °C for 18 or 24 h. After that, pure growth colonies were cultured on Pseudomonas agar. The biochemical examination were carried out for identification diagnosis and characterization according to the standard routine techniques. The isolates that attributes to *P. aeruginosa* were confirmed by using Vitik2 System. These tests are summaries below:

3.2.4 Phenotypic identification

3.2.4.1 Morphological examination

The initially diagnostic tests were based on the morphological characteristic of the bacterial growth on Pseudomonas agar and MacConkey agar. These characters were including colony shape, colony texture, color and edges, odor and the color of the produced pigment (Baron *et al*., 2007).

3.2.4.2 Microscopic examination

A pure, single colony of *P. aeruginosa* developed on nutrient agar for 18-24 h at 37 °C was tested under microscope according to (Procop *et al*., 2017).

3.2.4.3 Biochemical tests

As stated by Cappuccino and Welsh, (2020), all the biochemical tests were carried out as the follow:

3.2.4.3.1 Catalase test (slide test)

The catalase test was done by mixing a picked up single colony of the isolate with a drop of 3% hydrogen peroxide (H_2O_2) on the clean glass slide. The appearance of bubbles immediately (gas liberation O_2) pointing to a positive test, whereas the bacteria with no reaction which is a catalase negative test.

3.2.4.3.2 Oxidase test

A single colony of an isolate was transferred to a piece of filter paper by wooden stick and 2-3 drops of oxidase reagent were added. The change in color to purple would develop within 20-30 sec indicates a positive test, whereas bacteria with negative oxidase will not produce such color.

3.2.4.3.3 IMViC tests (McFadden, 2000)

(A) Indole test

A sterile peptone water was inoculated with a loopful of bacterial growth. After incubation, 5 drops of Kovac's reagent was added. The positive result indicated by formation a red surface ring.

(B) Methyl Red Test

MR-VP medium was inoculated with a bacterial growth and incubated for 24 h at 37 ºC. A few drops of methyl red solution were added to the broth culture. The change in color of the medium from yellow to red indicates a positive result.

(C) Voges-Proskauer (VP) test

MR-VP medium was inoculated with a colony of the tested bacteria and incubated for 48 h at 37 °C, anaerobically. A 60 µl of α–naphthol reagent and 200 µl of Potassium hydroxide **(**KOH) reagent was applied. The formation of red color after 15 min is indicative of the existence of acetoin (acetyl methyl carbinol) as a positive result.

(D) Citrate utilization test (Simmons Citrate Agar)

Citrate medium was used to assess the bacteria's ability to use citrate as the sole source of carbon. A slants of Simmons Citrate Agar was inoculated with *P. aeruginosa* isolates and incubated at 37 °C for 24 h. on the base of the ability of the isolate to use citrate as sole of carbon source indicates positive result with change in medium color from green to blue.

3.2.4.3.4 Growth on Pseudomonas agar

Pseudomonas agar was used as selective media to isolate *P. aeruginosa* from clinical specimens by inhibiting the growth of other microorganisms allowing *P. aeruginosa* to develop.

3.2.4.3.5 Diagnosis of *P. aeruginosa* via VITEK 2 Compact System

This device equipped by bioMérieux/France was used for bacterial diagnosis with a high degree of accuracy (HernándezDurán *et al*., 2017). This device includes 64 biochemical tests and an antibiotic sensitivity tests as well. VITEK 2 Compact system is an automated microbiological system utilizing growth-based technology. VITEK 2 is used to confirm the identification of the both bacteria Gram negative and Gram-positive. It was characterized by accommodating the colorimetric reagent cards that are incubated and interpreted (see Appendix 1). The steps of VITEK 2 Compact System as below:

1) *P. aeruginosa* isolate was grown on MacConkey agar and incubated for 24 h at a temperature of 37 °C.
2) VITEK2 card checking perform a Gram stain proper.
3) A single pure colony of the bacteria was picked and diluted with a special holder in 3 ml of saline solution contained in a sterile tube brought.
4) The turbidity of the solution was measured (0.5-0.36) using VITEK2 DESNICHEK.
5) The suspension containing in a tubes were placed in the VITEK2 cassette and inside the tubes a negative bacterial card was placed.
6) VITEK2 Cassette transferred to the device for the diagnosis of bacteria though 64 well holes. Each well contains a dried medium and a color guide in which the biochemical tests are performed. The system works to record the color changes that occurred as a result of bacteria growth (see Appendix 1) and the result recovered after 24 h.

3.2.4.4 Molecular Identification

Recently, PCR based methods were developed as an alternative way for accurate identification (Crone *et al*., 2020). Sequences amplification of 16S rRNA gene is the most commonly used method for identification and classification of the bacterial isolates such as *P. aeruginosa*. After extraction of DNA firstly, Primers were used to perform PCR screening 16S rRNA gene which is responsible for diagnosis of *P. aeruginosa*.

3.2.5 Maintenance of the bacterial isolates

3.2.5.1 Short period maintaining

A slants of nutrient agar were prepared and inoculated with a single pure colony of the isolates and incubated at 37 ^{o}C for 24 h. After that, the slants were stored at 4 ^{o}C in the refrigerator for one month (Fugelsang and Edwards, 2007).

3.2.5.2 Long period maintaining

For long term storage, the isolates were inoculated in Brain Heart Infusion broth and incubated for 24 h at 37 ^{o}C. The broth culture was preserved by adding a glycerol to the final concentration of 20% and stored at -20 ^{o}C for 1-2 years.

3.2.6 Antibiotic susceptibility test

The antimicrobial susceptibility was tested for all the isolates according to CLSI (2020). The steps of standard disc diffusion technique as following:

3.2.6.1 Preparation of bacterial inoculum

The bacterial inoculum was prepared for using in the bioassays by adjust the cell concentration to 0.5 McFarland turbidity (1.5×10^{8} cfu/ml). A few colonies were picked up from overnight agar culture and mixed in 3 ml of sterile

normal saline by vortex and used immediately in the bioassay (Wiegand *et al*., 2008).

3.2.6.2 Standard disc diffusion technique

As Kirby-Bauer method stated by Magiorakos and co-workers (2012):

- Muller-Hinton agar plates prepared in advanced and streaked with the bacterial suspension by a sterile cotton swab. The plates were left to dry for a while.
- Different antimicrobial discs (table 2-6) were used with a maximum six discs placed on the surface of the media. The plates were incubated for 24 h at 37 °C (a duplicate was done for each test).
- The diameter of the clear zone was measured of each antibiotic disc and the results were recorded. The results were recorded as Resistant (R) and Sensitive (S) or Intermediate (I) according to the (CLSI, 2020).

3.2.7 The Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of an antibiotic that can inhibits the visible growth of bacteria. The MIC values for all of the isolates were determined according to Hancock (1999) as the following:

1) The isolates were grown on Pseudomonas agar at 37 °C for 24 h. Bacteria suspension was prepared according to (3.2.6.1).
2) The antibiotic solution of Ceftazidime, that it was used in the current study prepared and diluted in Mueller Hinton Broth (1x) to (stock 2).
3) The top concentration in the test 100 mg/ml (stock 1), that is prepared in advanced according to paragraph (3.2.2.2.2) and diluted to 2048 µg/ml (stock 2).
4) 100 µl of Mueller Hinton Broth (1x) was added into all wells. A 100 µl of (stock 2) antibiotic solution transferred into the wells of column 1.

5) A hundred µl was withdrawn from column 1 and add to column 2. Mix by sucking and transfer to column 3 and so on until column 9 to get 9 dilutions with a concentration of (4, 8, 16, 32, 64, 128, 256, 512, 1024 µg/ml). A hundred µl was discard from column 9.
6) A five µl of bacteria suspension was added into each well in columns 1 to 9 and column 12 (positive control).
7) Added A 100 µl from stock 2 and sterile broth to the wells in columns 10 and 11 as negative controls, respectively.
8) The plates were incubated at 37 °C for 24 h.
9) A micropipette was used to mixed the antibiotics by sucking up and down 5-8 times.
10) The reading of results had been made manually using a black card and electronically with an ELISA reader on 630 nm wavelength.

3.2.8 Phenotyping detection of some virulence factors

3.2.8.1 Detection of Extended Spectrum β-lactamase (ESBL)

The method of combined disk or double-disk diffusion test was used to detect of the ability of the isolates to produce ESBLs according to (Patel *et al*., 2017). The procedure is summarized as below:

1) A bacterial suspension was prepared according (3.2.6.1) and spreaded on Mueller Hinton agar and left for 10 min to dry.
2) The antibiotic disk contains combination of Amoxicillin/Clavulanic acid (30 µg/disk) placed in the center of the inoculated plate.
3) Then surrounded by the antibiotic disk of Aztreonam and third generation of cephalosporin Cefotaxime and Ceftazidime in a distance of 3 cm from of the disk in the center.
4) The plates were incubated for 24 h at 37 °C.
5) Inhibition of zone from 5 mm or more in the presence of Augmentin is suggested as a positive result for the production of ESBL enzyme.

3.2.8.2 Detection of Metallo-beta lactamase

The antibiotic consortium method was used to detect bacterial production of the mineral beta-lactamase enzymes by combined EDTA disc test (CEDT) as follows:

1) A bacterial suspension was prepared as mention in (3.2.6.1).
2) A bacterial suspension of 0.1 ml was spreaded by sterile swab on the surface of solid Muller-Hinton medium and allowed to dry at room temperature for 5 min.
3) Two antibiotic disks of Imipenem with a concentration of 10 micrograms were placed on the surface of the medium with 3 cm distance between them.
4) Five µl of EDTA solution was added to Imipenem disk.
5) Plates were incubated at 37 ° C for 24 h.
6) The inhibition zone was observed which was more than 7 mm around the Imipenem with EDTA disk compared with Imipenem disk alone. The result is considering positive and the bacteria are known as the mineral **β**-lactamase enzyme producers (Bashir *et al.*, 2011).

3.2.8.3 Detection of AmpC-beta lactamase

The confirmatory test for detection of AmpC enzymes by disk antagonism test by the following steps:

1) A bacterial suspension was prepared as mentioned in (3.2.6.1) and spreaded on the Muller–Hinton agar plates.
2) Antibiotics Cefotaxime (30 mg/ml) and Cefoxitin (30 mg/ml) disks were placed in 2 cm apart from each center.
3) After incubation overnight, the isolates showing blunting of Cefotaxime of inhibition zone adjacent to the Cefoxitin disk or reduced susceptibility to each other were showed as a positive result for AmpC-beta lactamase production (Smatha and Parveen, 2011).

3.2.8.4 Detection of pigments production

A pure isolates of *P. aeruginosa* were cultured on nutrient agar and incubated for 24 h at 37 °C to visual analysis colony morphology and pigments production of Pyoverdine and Pyocyanin (Winn *et al*., 2008).

3.3 Genotyping study of *P. aeruginosa* isolates

3.3.1 DNA extraction

The genomic DNA of the isolates were extracted from bacterial growth according to the protocol of Geneaid extraction kit as the following steps:

(A) Suspension and protein digestion

1) For the pellet cells, one milliliter of the overnight culture was centrifuged for 3 min at 12000 rpm. The supernatant was discarded.
2) 180 µl of GT buffer was added to the pellet and mixed by vortexing for prepared the suspension.
3) For protein digestion, 20 µl of Proteinase K was added to the previous suspension.
4) All the mixtures were incubated at 60 ºC for at least 10 min.

(B) Cell lysis

1) For cell lysis, 200 µl of GB buffer was added to the sample and mixed by vortex for 10 sec.
2) The mixture was incubated at 70 ºC for at least 10 min to ensure the sample lysate is clear.
3) After incubation, the samples were spanned in a centrifuge for 10 sec to remove the bubbles.

(C) Binding

1) For DNA binding, 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously. Next, the mixture (including any insoluble precipitate) was transferred to the GD column and centrifuged at 12000 rpm for 1 min.
2) The GD collection tube containing the flow-though was discarded and the GD column was placed into a new GD collection tube.

(D) Washing

1) For washing, 400 µl of W1 buffer was added to the GD column and centrifuged at 12000 rpm for 30 seconds then the flow-though was discarded.
2) The GD column placed back in 2 ml collection tube and 600 µl of wash buffer was added to the GD column (make sure ethanol was added).
3) Centrifugation at 12000 rpm for 3 min was performed, then the flow-though was discarded and the GD column was placed back in the 2 ml collection tube.
4) The empty column matrix was centrifuged at 12000 rpm for 3 min to dry the column matrix.
5) The dried GD column was transferred into a clean 1.5 ml microcentrifuge tube.

(E) Elution

1) The dried GD column was transferred into a clean 1.5 ml microcentrifuge tube.
2) Aliquot of 100 µl pre-heated elution buffer1 was added into the center of the column matrix. After waiting at least 3 min (to allow Elution Buffer to be completely absorbed).
3) Centrifugation at 9000 rpm for 3 min was performed to elute the purified DNA.

3.3.2 Quantitation of the extracted DNA

A Quantus™ Fluorometer was used to detect the concentration of the extracted DNA in order to determine their quality for the subsequent applications. For One microliter of DNA, 199 µl of diluted Quanty Fluor Dye was mixed. The DNA concentration was measured after 5 min of incubation at room temperature. DNA concentration values were detected ranged from (12 to 22) ng/µl, (see Appendix 5).

3.3.3 Primers preparation

As a stock solution, the lyophilized primers were dissolved in a nuclease-free water to a final concentration of 100 pmol/µl. A working primer solution of these primers was made by combining 10 µl of primer stock solution (stored at -20 ^{o}C) with 90 µl of nuclease-free water to yield a working primer solution of 10pmol/µl.

3.3.4 Preparation PCR mixture

3.3.4.1 PCR master mix for 16S rRNA gene detection

The DNA of nine *P. aeruginosa* isolates that have been extracted in this study, went through PCR procedure in order to target 16S rRNA gene. Each PCR reaction had a final volume of 20 µl. Table (3-7) illustrates the primers that were used in this study and their appropriate volume for PCR mixture.

Table (3-7): PCR master mix components and their size of 16S rRNA.

Master mix components	Stock	Unit	Final	Unit	Volume	
					1Sample	9.05 Samples
Master Mix	2	X	1	X	10 µl	90.5
Forward primer	10	µM	1	µM	1	9.1
Reverse primer	10	µM	1	µM	1	9.1
Nuclease Free Water					6	54.3
DNA		ng/µl		ng/µl	2	
Total volume					20	
Aliquot per single rxn	18µl of Master mix per tube and add 2µl of Template					

All the components of each PCR mixture were mixed together in 200 µl Eppendorf tube by vortex before setting into Thermal cycler. The program is shown in table (3-8).

Table (3-8): Conditions of PCR reaction program for 16S rRNA gene.

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	54	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

3.3.4.2 Multiplex PCR for detection of typing gene

All the DNA samples that extracted from *P. aeruginosa* isolates after being diagnosed by 16s rRNA, were passed through a multiplex PCR procedure in order to target different genes for typing in this study. Each multiplex PCR reaction had final volume of 25 µl. Table (3-9) illustrates the primers that were used and their appropriate volume for the Multiplex PCR mixture. Promega master mix was used. The mixture was composed of PCR Master Mix (2x) solution which was melted at room temperature and mixed by vortex for

homogenizing before being used Primer's solutions were mixed well by vortex before being used.

Table (3-9): Multiplex PCR master mix components and their size of genes.

Master mix components	Stock	Unit	Final	Unit	Volume (µl)	
					1 Sample	9.05 Samples
Master Mix	2	X	1	X	10	90.500
MS-213-F	10	µM	1	µM	1	9.05
MS-213-R	10	µM	1	µM	1	9.05
MS-214-F	10	µM	1	µM	1	9.05
MS-214-R	10	µM	1	µM	1	9.05
MS-215-F	10	µM	1	µM	1	9.05
MS-215-R	10	µM	1	µM	1	9.05
MS-142-F	10	µM	1	µM	1	9.05
MS-142-R	10	µM	1	µM	1	9.05
DNA		ng/µl		ng/µl	2	
Total volume					20	
Aliquot per single rxn	18 µl of Master mix per tube and add 2 µl of Template					

To confirm the presence of amplification, PCR amplification was followed by agarose gel electrophoresis. The extracted DNA criteria were completely dependable on PCR, table (3-10).

Table (3-10): Multiplex PCR program Condition.

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

3.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was adopted to confirm the amplification after PCR run. PCR was completely dependable on the extracted DNA criteria.

3.3.5.1 Solutions

1X TAE buffer, loading dye, DNA ladder marker, Ethidium bromide (10 mg/ml).

3.3.5.2 Agarose Preparation of agarose

1) agarose 1.5 g was added to 100 ml of 1x TAE buffer in a flask.
2) The solution was heated to boiling by microwave until the gel particles had been dissolved.
3) One µl of ethidium bromide (10mg/ml) was added to the gel.
4) The gel was stirred in order to get mixed and to avoid bubbles.
5) The solution was left to cool down at 50-60 ^{o}C.

3.3.5.3 Agarose casting for horizontal agarose gel

After sealing both edges with cellophane tapes, the agarose gel was poured into the tray and allowed to solidify at room temperature for 30 min. After carefully removing the comb, the gel was placed in the tank. The tank was filled with 1x TAE-electrophoresis buffer until reached 3-5 mm above the gel's surface.

3.3.5.4 DNA loading

PCR products were loaded directly. DNA loading buffer was add to Five microliters and directly loaded to the well. An electrical power was turned on at 100v/mAmp for 60 min. The DNA moves from cathode to the anode poles. The ethidium bromide-stained bands in gel were visualized using gel imaging system.

3.3.6 Capillary electrophoresis

Capillary electrophoresis was performed to all the samples using a 3130xl Genetic Analyzer (Macrogen Corporation/Korea). Using GeneMapper Software, each variable number tandem repeat (VNTR) locus was identified by color and size. The differences in fragment size were used to identify allele variation.

3.3.7 The MLVA procedure includes:

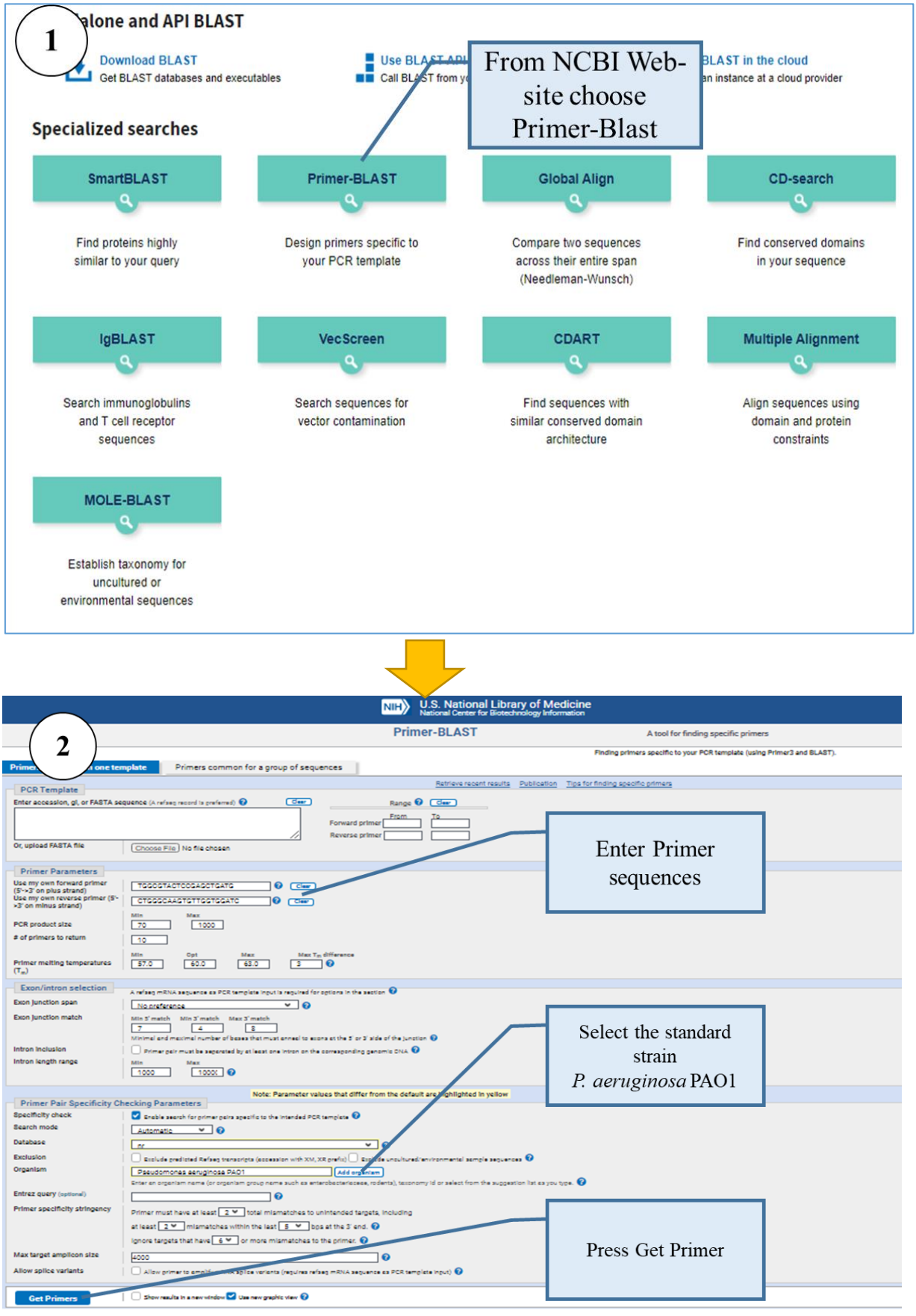
MLVA measures the specific regions of the genome, variable number of tandem repeat units (VNTRs) in bacteria. This protocol describes the MLVA procedure using 96-well PCR plates and the described dyes are suitable for ABI:

- Amplification of the VNTRs with fluorescent labelled primers; (primer MS-213:FAM-Blue, primer MS-214: VIC-Green, primer MS-215: NED and MS-142: SAV- Red.
- Detection of fragment length by capillary electrophoresis; and
- Calculation of the actual number of repeat units (Nielsen, 2011).

In less than 4 days could be genotyped almost 100 isolates (Chen *et al*.,2018).

3.3.8 Variable Number Tandem Repeat Calculation and Multilocus Variable Number Tandem Repeat Analysis

The Tandem Repeats Finder Program 4.03 (http://tandem.bu.edu/trf.html) was used to calculate tandem repeat counts for all the loci from sequenced strains *Pseudomonas aeruginosa* PAO1, also known as normal strains received from NCBI (Benson, 1999). Using PCR results from these strains as a guideline, the number of repetitions in the study isolates was estimated. The following formula was used to measure the repeat numbers: number of repeats (bp) = fragment size (bp)-flanking regions (bp)/ repeat size (bp) (Zaid, 2021) (figure 3-2): (see Appendix 3).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Download BLAST
Get BLAST databases and executables
Use BLAST API
Call BLAST from yo
BLAST in the cloud
an instance at a cloud provider
From NCBI Web-site choose Primer-Blast
Specialized searches
SmartBLAST
Find proteins highly similar to your query
Primer-BLAST
Design primers specific to your PCR template
Global Align
Compare two sequences across their entire span (Needleman-Wunsch)
CD-search
Find conserved domains in your sequence
IgBLAST
Search immunoglobulins and T cell receptor sequences
VecScreen
Search sequences for vector contamination
CDART
Find sequences with similar conserved domain architecture
Multiple Alignment
Align sequences using domain and protein constraints
MOLE-BLAST
Establish taxonomy for uncultured or environmental sequences
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U.S. National Library of Medicine
National Center for Biotechnology Information
Primer-BLAST
A tool for finding specific primers
Finding primers specific to your PCR template (using Primer3 and BLAST).
Primers common for a group of sequences
Retrieve recent results
Publication
Tips for finding specific primers
PCR Template
Enter accession, gi, or FASTA sequence
Range
From
To
Forward primer
Reverse primer
Or, upload FASTA file
Choose File
No file chosen
Primer Parameters
Use my own forward primer (5'->3' on plus strand)
Use my own reverse primer (5'->3' on minus strand)
PCR product size
# of primers to return
Primer melting temperatures
Enter Primer sequences
Exon/intron selection
Exon junction span
No preference
Exon junction match
Intron inclusion
Intron length range
Select the standard strain P. aeruginosa PAO1
Primer Pair Specificity Checking Parameters
Specificity check
Search mode
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Primer specificity stringency
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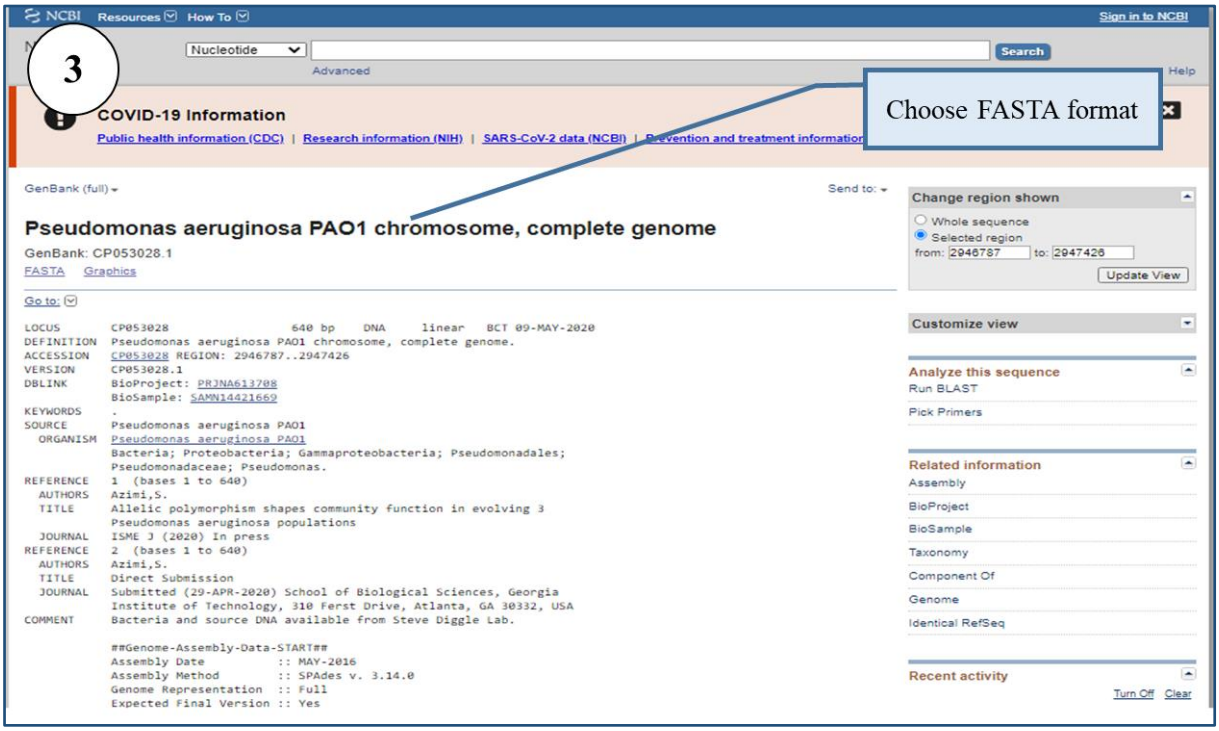
Allow splice variants
Get Primers
Press Get Primer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BioSample
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Genome
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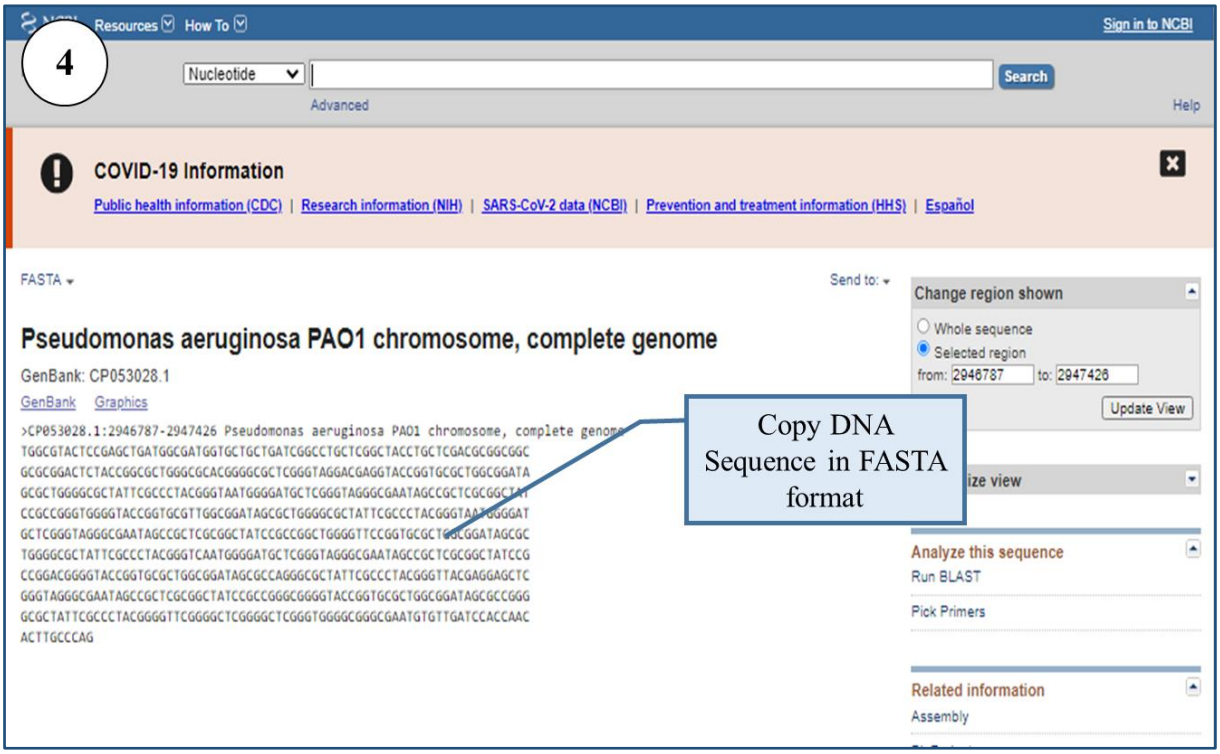
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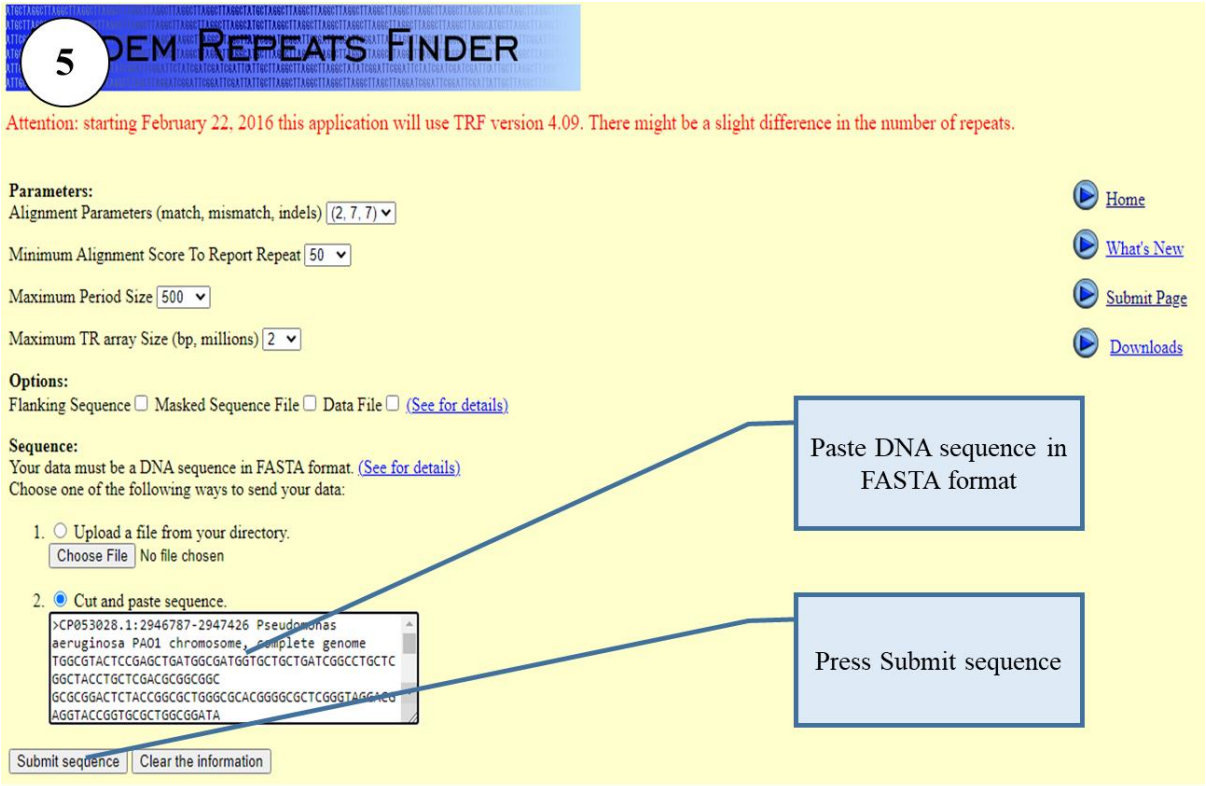
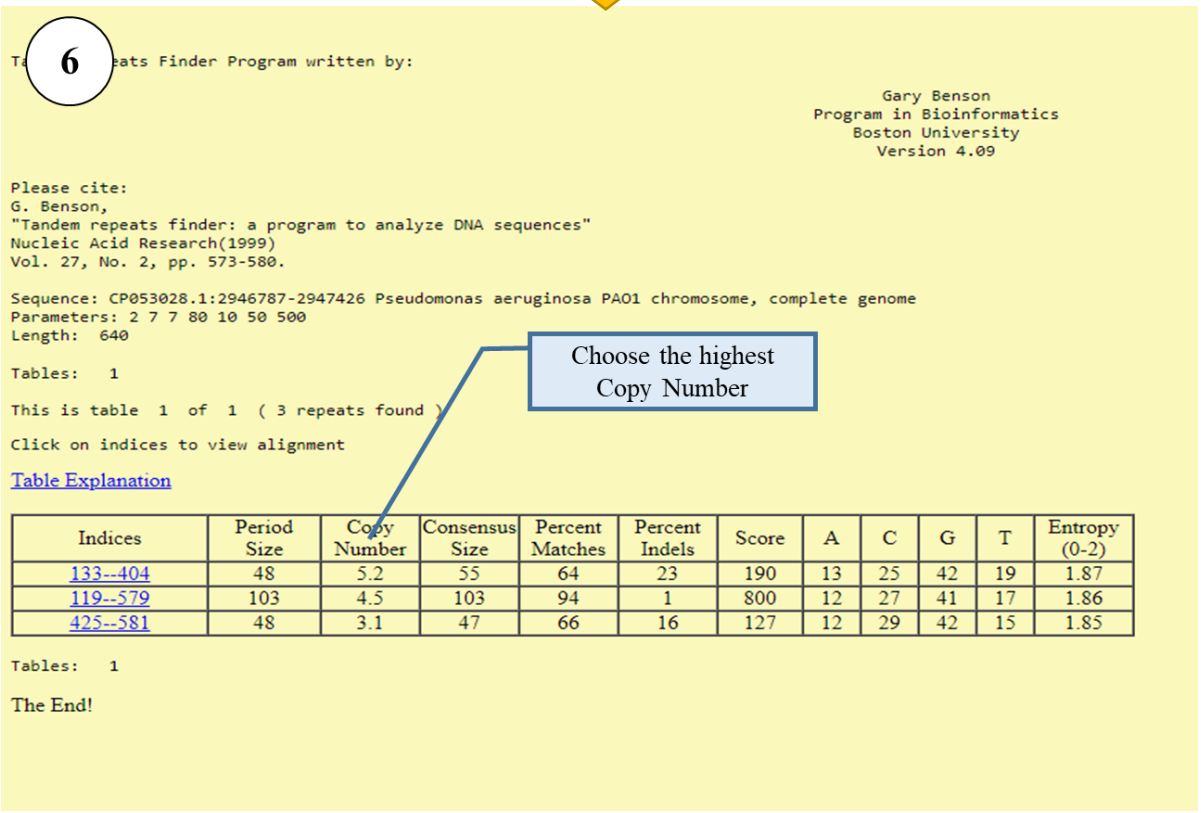
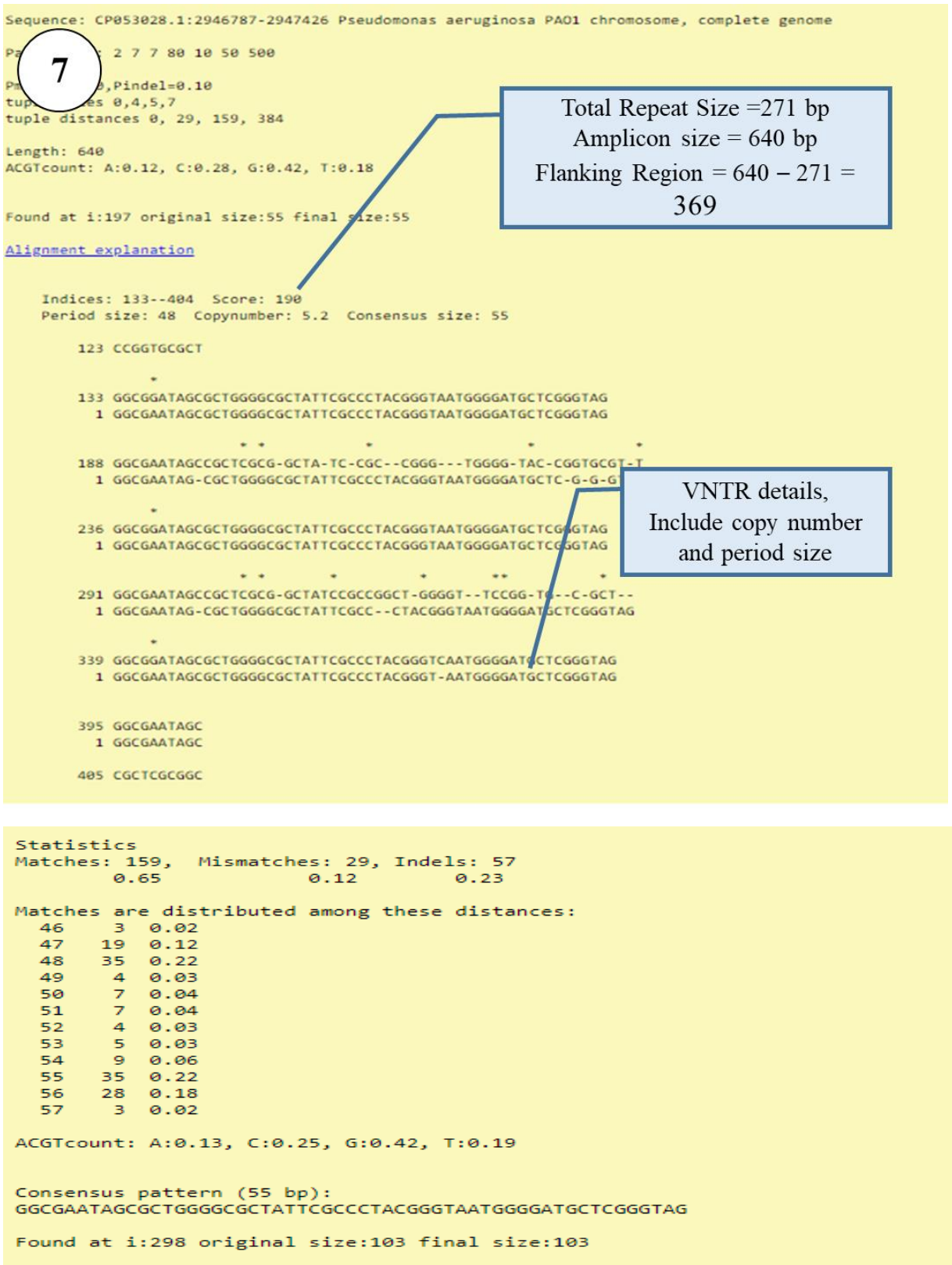
Update View
Analyze this sequence
Run BLAST
Pick Primers
Related information
Assembly





Indices	Period Size	Copy Number	Consensus Size	Percent Matches	Percent Indels	Score	A	C	G	T	Entropy (0-2)
133--404	48	5.2	55	64	23	190	13	25	42	19	1.87
119--579	103	4.5	103	94	1	800	12	27	41	17	1.86
425--581	48	3.1	47	66	16	127	12	29	42	15	1.85




```
Statistics
Matches: 159,  Mismatches: 29, Indels: 57
         0.65              0.12         0.23

Matches are distributed among these distances:
  46     3  0.02
  47    19  0.12
  48    35  0.22
  49     4  0.03
  50     7  0.04
  51     7  0.04
  52     4  0.03
  53     5  0.03
  54     9  0.06
  55    35  0.22
  56    28  0.18
  57     3  0.02

ACGTcount: A:0.13, C:0.25, G:0.42, T:0.19


Consensus pattern (55 bp):
GGCGAATAGCGCTGGGGCGCTATTCGCCCTACGGGTAATGGGGATGCTCGGGTAG

Found at i:298 original size:103 final size:103
```

Figure (3-2): The instructions of using of Tandem repeats finder software program by using (MS-213) gene as an example.

3.3.8 Analysis of Phylogenetic Tree

The acquired tandem repeats numbers for all sites and each lineage were entered into the bioinformatics software program, as well as in the MLVA plugin for bioNumerics (Applied Maths NV.) to generate the phylogenetic tree. The circles in this tree indicates the isolates (the genotype), and the color of the circles indicates the origins and locations of isolation. The variance in the number of iterations is shown by the lines connecting the circles. Different isolates at a single locus are connected with short bold lines, whereas those that differ in many loci are linked with long bold lines.

3.4 Statistical analysis

The statistical analysis system (SAS) was used to calculate the influence of different factors in the study parameters. The results were presented in numbers and percentages (Allison, 2012).

CHAPTER FOUR

RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Isolation of *Pseudomonas aeruginosa*

In this study, a total number of 280 clinical specimens were isolated from different sources of infections included swabs of wounds, burns and urine samples, from male and female. Patient-related information was recorded in the hospital included age, gender, and sample source as shown in Appendix (4). The swabs of clinical samples were obtained from governmental hospitals in Baqubah City/Diyala Province: Baqubah Teaching Hospital, Al-Batool Teaching Hospital and Public Health Laboratory. The isolates were collected during the study period from October, 2020 to January, 2021. The positive growth was 217(77.5%) samples while the negative growth (no growth) gave 63(22.5%) samples (figure 4-1). 40(18.4%) *P. aeruginosa* isolates were obtained from 217(77.5%) positive growth samples detected by morphological and biochemical test and confirmed by using VITEK® 2 Compact Automated System.

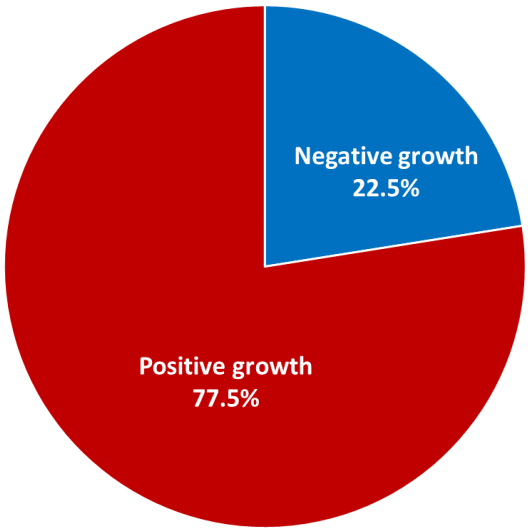


Figure (4-1): The percentage of samples of *P. aeruginosa* growing on (MacConkey agar) for 24 h at 37 ^{o}C.

4.1.1 Distribution of the specimens according to source of infection

The results showed in table (4-1) *P. aeruginosa* isolated in high percentage in burns 19(17.6%), wounds 10(20.8%), and urines 11(18%) isolate.

Table (4-1): Distribution of the specimens according to source.

Source of collection	Total number of the specimens	No. of positive *P. aeruginosa* Isolates	percentages of *P. aeruginosa* Isolates
Burns infections (Swab)	108	19	17.6%
Wounds infections (Swab)	48	10	20.8%
Urinary tract infections (Urines)	61	11	18%
Total	217	40	18.4%

4.2 Identification of *P. aeruginosa*

The results showed that 217 clinical samples were positively grew on MacConkey agar and Pseudomonas agar. These samples were taken from three different infectious sources (burns, wounds, urines). From these positive samples, 40(18.4%) isolates were primary identified as *P. aeruginosa*. The conventional methods of bacterial identification relying on the phenotypic identification of the causative organism using bacteriological methods including culturing on selective media, colonial morphology, and microscopically characteristic.

4.2.1 Microscopic examination

All of 40 *P. aeruginosa* isolates were examined by Gram staining technique. The results showed that all the isolates were Gram-negative, mostly had been appeared straight or slightly rods shapes.

4.2.2 Identification the morphology on culture media

In the present study, all fourty isolates were cultured on the MacConkey agar media as a selective medium (inhibit most Gram positive bacteria and allow Gram negative bacteria to grow). This medium also differentiates lactose ferment Gram negative and non-lactose ferment Gram negative such as *P. aeruginosa* which grew with a pale yellowish color. The growth of *P. aeruginosa* on Pseudomonas agar (selective media) gives blue greenish color colonies due to pyocyanin production after 24 h incubation. This help to distinguish *P. aeruginosa* from other species of *Pseudomonas*. The production of some pigments on both nutrient agar and Muller-Hinton agar was especially associated with *P. aeruginosa*. The result was in table (4-2). the emergence of glamorous green colonies with distinctive odor proof that the identified isolates were *P. aeruginosa*. These results agreed with AL-Shamaa *et al*. (2016); who identifying *P. aeruginosa* and other bacteria isolated from burns and wounds samples from Iraqi patients figure (4-2):

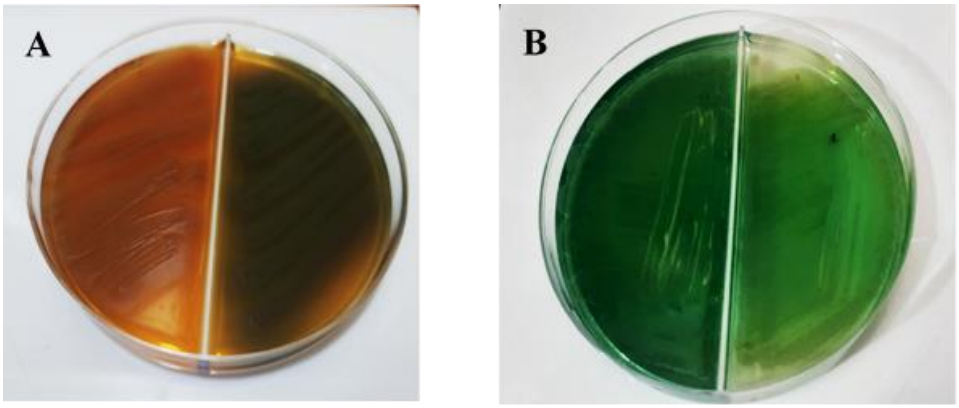


Figure (4-2): *P. aeruginosa* cultured at 37 °C for 24 h on (A) MacConkey agar and (B) Pseudomonas agar.

The pigments produced by *P. aeruginosa*, in addition to their significance in pathogenicity as virulence factors, play a clear role in pigmentation, which remains a prominent feature among the diagnostic features in the *Pseudomonas* species (Novik *et al.*, 2015; Hotterbeekx *et al.*, 2017).

Table (4-2): pigments production of *P. aeruginosa* isolates on nutrient agar.

Colorless	Pale yellowish color	Light green color	Blue-greenish color
8	19	6	7

4.2.3 Biochemical test

All 40 isolates subjected to a series of biochemical tests were performed to identify them included oxidase test, and catalase test. These tests gave positive results and allowed quickly to identify the unknown isolate. Oxidase test based on the color changes by *P. aeruginosa* isolates after 24 hrs incubation at 37 °C. In case of catalase test, all the suspected isolates showed positive results by formation a gaseous bubble after adding a hydrogen peroxides reagent to the colonies. Simmons citrate test was positive in all the bacterial isolates. The biochemical tests that gave negative results in all the isolates were Voges-Proskauer (VP), methyl red (MR) and Indole production (Todar, 2004). Table (4-3).

Table (4-3): The results of the biochemical tests of *P. aeruginosa*.

Biochemical tests					
Oxidase	Catalase	Citrate	VP	MR	Indole
+	+	+	-	-	-

4.2.4 Identification of *P. aeruginosa* by VITEK 2 Compact system

The confirmatory diagnosis of the 40 isolates of *P. aeruginosa* screened in the current study was made by using the (GN ID) Card of VITEK 2 automated system. This system has unconventional properties not found in other diagnostic equipment such as analytical profile index (API), permitting the diagnosis of isolates during typical time with high accuracy about 99% (Pincus, 2011). The results of current study were similar with the study of AL-Mayyahi (2018) in Wasit Province, Iraq. 100% of bacterial isolates included in this study were the same type of *P. aeruginosa*, according to the biochemical tests (see Appendix 1).

4.2.5 Molecular identification of *P. aeruginosa* 16S rRNA

The extracted DNA from 9 isolates was quantified by Quantus Fluorometer method. The concentration of the extracted DNA ranged from (12 to 22) ng/µl, as seen in (Appendix 4). The Genotypic identification results of nine isolates revealed that a 100% were *P. aeruginosa* which showed the amplicons size of 956bp for housekeeping gene 16S rRNA. Figure (4-3) illustrates a shine bands of positive isolates compared with 1500bp DNA ladder. The genotypic analysis by PCR reaction with specific 16S rRNA ended the confusion in diagnosis confirmation process. Several studies were used this technique because it is more rapid and reliable method. The results of the current study agreed with the results of local study in 2020 by ALsaadi in Diyala Province who found that all *P. aeruginosa* isolates were a 100% positive for 16S rRNA gene.

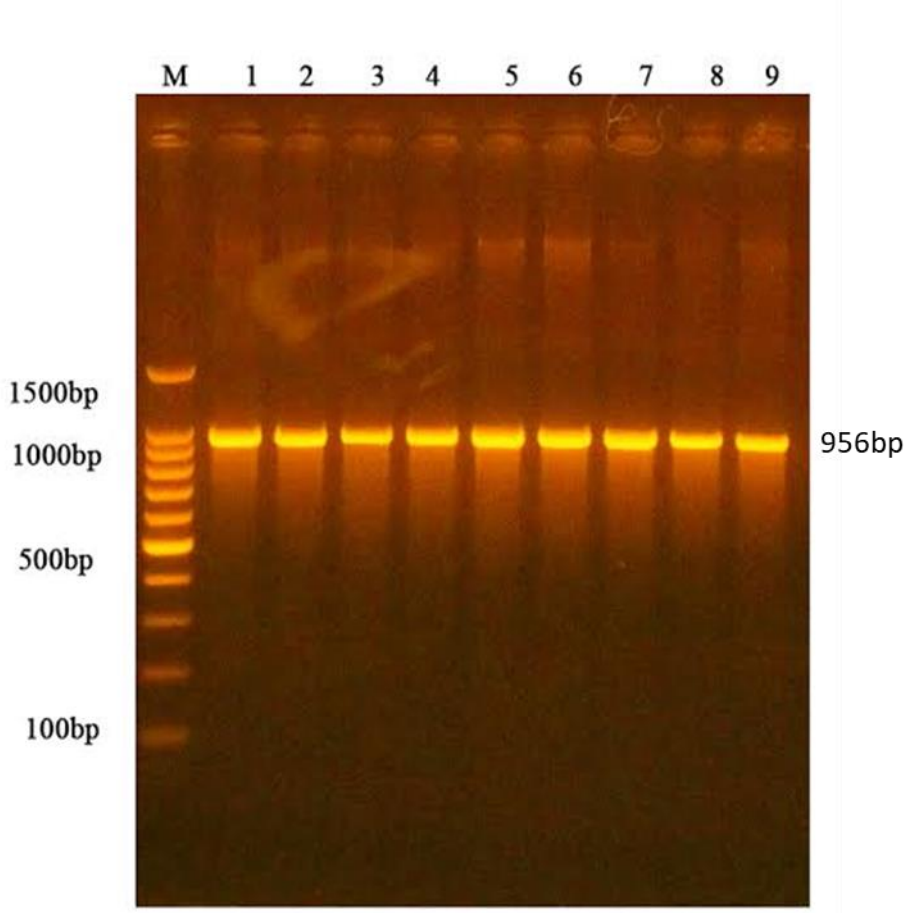


Figure (4-3): The amplification of 16S rRNA gene of *P. aeruginosa* samples fractionated on 1.5% agarose gel at 100v/mAmp for 60 min. stained with ethidium bromide. Lanes 1-9 *P. aeruginosa* isolates ; M: 100bp DNA ladder used as marker; resemble 956bp PCR products.

4.3 Phenotypic detection of antimicrobial agents

4.3.1 Antibiotic susceptibility test

Antibiotic susceptibility tests were applied on 40 isolates of *P. aeruginosa* isolated from different clinical sources using disc diffusion method (Kirby-Bauer method) against twelve classes of antibiotic. The results were compared according to CLSI (CLSI, 2020). It was found that lower percentage (15%) of resistance was seen against Piperacillin/Tazobactam and this result was close to what Shokoohizadeh *et al*. (2020) found 22.2% but disagree with the results of Elhariri *et al*. (2017) which was 76.2%. The percentage of resistance for Levofloxacin and Gentamicin was 30% which is agree with Aziz *et al*. (2019),

ALageedi, (2021), and Hosu *et al*. (2021) who reported Levofloxacin resistance with 36.2%, 34.6% and 30.6%, respectively. However, the percentage found in this study disagrees with Ahmad *et al*. (2020) which was 16.7 %.

The percentage of Gentamicin resistance agreed with Hasan *et al*. (2020) and Sameet *et al*. (2020) who reported Gentamicin resistance which was 30% and 29.09%, respectively. This result disagreed with Al-Obaidi and Al-Dahmoshi (2020) which was 50%. For Imipenem, the percentage resistance was 32% which is close to the findings of Macin and Akyon (2017); Shidiki *et al*. (2019) and Mohamed, (2021) with 28%, 27.6% and 30.23%, respectively, and differ from Osman *et al*. (2018) which was higher (75%).

The resistance to Ceftazidime and Meropenem was 35% which is agreed with study carried out by Najnin *et al*. (2018) ; ALageedi, (2021) who reported a resistance of 35% and 34.6%, respectively. In contrast, this result disagreed with Pungcharoenkijkul *et al*. (2021) which was 11.11%. The resistance percentage to Meropenem agrees with Namuq *et al*. (2019) and ALageedi, (2021) who demonstrated 41.7% and 38.4% while differ from 9.41% found by Emaneini *et al*. (2019).

The resistance rate of Cefepime, Aztreonam, Ciprofloxacin and Amikacin was 37.5%. Aziz and co-workers (2019) and Ferman, (2019) recorded approximate rate for Cefepime was 35% and 38%, respectively. The resistance rate to Aztreonam agreed with Aziz *et al*. (2019) and Khadim and AL Marjani (2019) which was 38.7% and 39.6%, respectively in contrast with 11.7% found by Bhuiya *et al*. (2018). Regarding the percentage of resistance of Ciprofloxacin, it agrees with Yang *et al*. (2020); Mohamed, (2021) and ALageedi, (2021) who reported a resistance of 34.2%, 34.6% and 34.88%, respectively. Also, these figures were lower than Qayoom *et al*. (2019) with 72.2%. In regard to Amikacin, the resistance rate agrees with Macin *et al*.

(2017) and ALageedi, (2021) who reported 29.5% and 30.7%, respectively, and disagreed with Saleh *et al*. (2020) which was 60%.

Finally, the resistance percentage of Ticarcillin/Clavulanic acid and Netilmicin was the highest in current study (40%, 42.5%), respectivly compared with other tested antibiotics. Netilmicin resistance rate agreed with Holbrook and Garneau-Tsodikova (2018) and local study by ALageedi, (2021) who reported Netilmicin resistance was 33.6% and 38.46%, respectively. And compared with Jebur (2018) which was higher 94.3%. The resistance percentage of Ticarcillin/Clavulanic acid was showed a variance with Alsaadi, (2020) who reported Ticarcillin/Clavulanic resistance which was 71.6%. The figure (4-4).

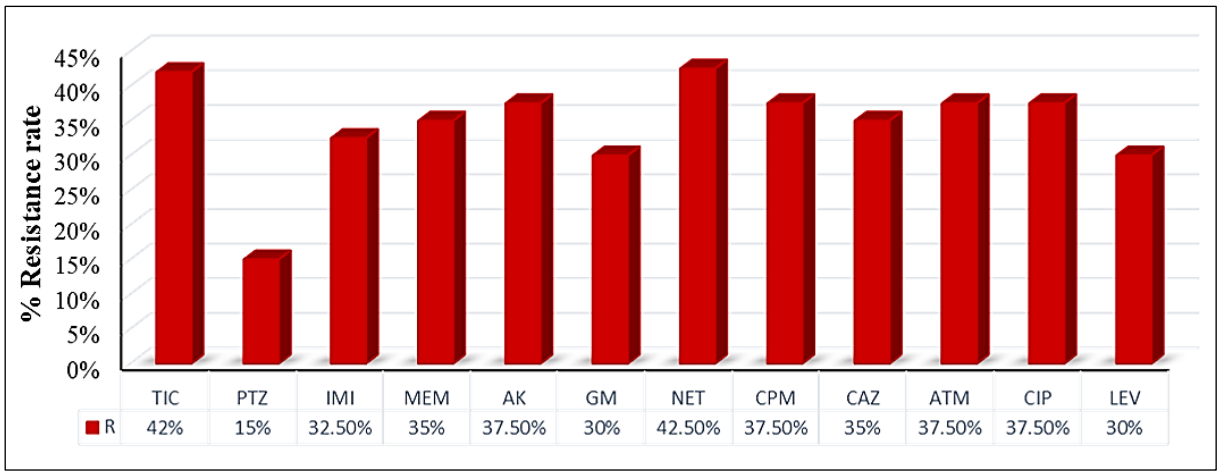


Figure (4-4): The percentage of antibiotic resistance for *P. aeruginosa* isolates.

TIM = Ticarcillin/clavulanic acid, MEM = Meropenem, NET = Netilmicin, CPM = Cefepime, ATM = Aztreonam, CIP=ciprofloxacin, LEV= Levofloxacin, CAZ = Ceftazidime, AK= Amikacin, GM = Gentamycin, IMI = Imipenem, PTZ = Piperacillin/Tazobactam.

In the current study, a lower prevalence of antibiotic resistance was observed. In contrast to other local studies that showed a high prevalence of antibiotic resistance see (Appendix 2). This could occur due to the differences in *P. aeruginosa* isolates obtained and may be related to differences in

antibiotic use in different environments and selective pressure and this result may be due to the difference in sample size for these bacterial isolates in the current study Mwinyikombo, (2018). In general, the resistance of different types of antibiotics is attributed to many reasons, including altered cell membrane permeability, alterations in target site structures Sheikh *et al*. (2015). Table (4-4):

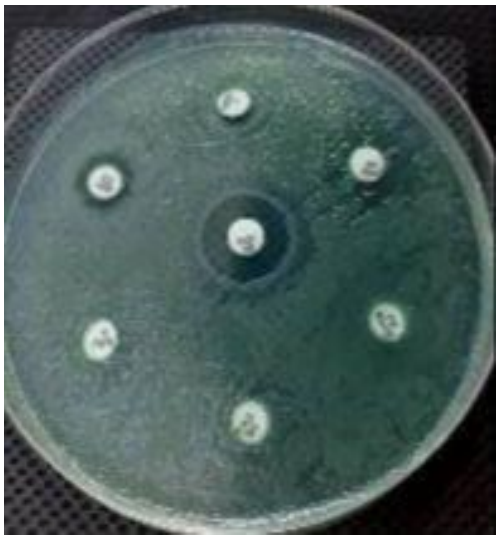
Table (4-4): The percentage of susceptibility for *P. aeruginosa* isolates against antibiotics tested by Kirby-Bauer method.

Antibiotic	Resistance		Intermediate		Sensitive		Mode of action
	Isolates No.	%	Isolates No.	%	Isolates No.	%	
Ticarcillin/ clavulanic acid	P17	42.5	P10	25	P13	32.5	Inhibit cell wall synthesis
Piperacillin/ Tazobactam	P6	15	P9	22.5	P25	62.5	
Ceftazidime	P14	35	-	-	P26	65	
Cefepime	P15	37. 55	P1	2.5	P24	60	
Aztreonam	P15	37.5	P5	12.5	P20	50	
Imipenem	P13	32.5	P1	2.5	P26	65	
Meropenem	P14	35	P1	2.5	P25	62.5	
Gentamicin	P12	30	P3	7.5	P25	62.5	Inhibit protein synthesis
Amikacin	P15	37.5	-	-	P25	62.5	
Netilmicin	P17	42.5	-	-	P23	57.5	
Ciprofloxacin	P15	37.5	P1	2.5	P24	60	Inhibit DNA synthesis
Levofloxacin	P12	30	P3	7.5	P25	62.5	

4.3.2 Distribution of the isolates according to multidrug resistant categories

All 40 clinical isolates of *P. aeruginosa* that were tested in this study were classified into a groups based on the antibiotic resistant. Table (4-5) showed several isolates of Multi-Drug Resistance (MDR). MDR is defining as the resistance of a bacterial isolate towards antimicrobial drugs from at least three of the six-antipseudomonal classes of the drugs used in this study (β-lactamase inhibitor combination, aminoglycosides, monobactams, carbapenems, cephalosporins, and quinolones). Extensive drug resistance (XDR), in contrast, means the resistant of an isolate to all but except one or two classes of antibiotics (Magiorakos, 2012).

The increasing in resistance rate of *P. aeruginosa* to numerous antibiotics can be due to excessive antibiotics administration figure (4-5). This will lead to the accumulation the resistance and cross-resistance between the antibiotics and the appearance of multidrug-resistant (MDR) formed in *P. aeruginosa* (Bahador *et al*., 2019).



(Figure 4-5): Disk diffusion test for *P. aeruginosa* isolate resistant, extensive drug resistance (XDR).

Table (4-5): Distribution pattern of the isolates according to Multidrug resistance (MDR), extensive drug resistance (XDR) and Multidrug sensitive (MDS).

Categories	Number of class resistance	Number of antibiotic resistance	Percentage of isolates (%)
MDR	MDR (3)	2/5	12.5
	MDR (4)	3/5	
XDR	MDR (5	3/13	32.5
	MDR (6)	10/13	
MDS	-	22	55

Based on the definition of MDR, 5(12.5%) of the isolates were confirmed as MDR table (4-5). A 2/5 (40%) of them resist three classes of antibiotics and 3/5(60%) MDR isolates resist four tested classes of antimicrobials. Among these isolates, 13(32.5%) were resisted 5 or 6 classes of antibiotics meeting the criteria for XDR organisms. The majority of XDR isolates (3 of 13) were resistant to 5 classes of antibiotics representing 23 % of all the isolates. Ten out of thirteen of XDR isolates (76.9%) were resistant to 6 classes of antibiotics which agreed with ALsaadi (2020). While 13(32.5%) of the isolates showed a pattern of XDR including carbapenem, cephalosporins and monobactams, ticarcillin.

4.3.3 Minimum Inhibitory Concentration (MIC) of ceftazidime against *P. aeruginosa*

Ceftazidime was selected as antibiotic for the MIC measurement in all 40 isolates. The MIC values of each isolate were determined by choosing the lowest concentrations in which no growth occurs. The MIC was calculated by using broth microdilution standard method. After 24 h incubation, the MIC results of 40 isolates appeared as shown in the table (4-6). This method is considering as the best method to determine the MIC of the antibiotics because

of easy to use, saving cost and time, more accurate since the plate can be read by measuring the absorbance in microplate reader. The MICs values of ceftazidime were ranged between 16-1024 µg/ml which result was agreement with recent local study of Alageedi (2021) but differ from another Iraqi study by Alsaadi (2020) and Najeeb (2020) with MIC values ranged from 8-512 µg/ml and 64 - 512 µg/ml, respectively.

Table (4-6): The MIC values (µg/ml) for Ceftazidime.

No. of the isolates	Ceftazidime (µg/ml)	No. of the isolates	Ceftazidime (µg/ml)	No. of the isolates	Ceftazidime (µg/ml)
P1	64	P15	32	P29	1024
P2	64	P16	32	P30	128
P3	16	P17	16	P31	32
P4	32	P18	256	P32	512
P5	128	P19	64	P33	128
P6	256	P20	128	P34	128
P7	64	P21	256	P35	512
P8	256	P22	128	P36	512
P9	128	P23	32	P37	128
P10	128	P24	64	P38	64
P11	64	P25	32	P39	64
P12	128	P26	128	P40	128
P13	32	P27	128	-	-
P14	32	P28	128	-	-

4.4 Detection of some virulence factors

4.4.1 Detection of β-Lactamase enzymes production

4.4.1.1 Phenotypic screening for Extended-Spectrum β-Lactamase (ESBL) production

All 40 isolates were subjected to the double-disk synergy test (DDST) as described by Jarlier *et al*. (1988) to detect the production of extended-spectrum β- Lactamase (ESBLs). ESBLs are plasmid mediated enzymes that produced by a number of Gram negative bacteria (Collins *et al.*, 2004). The results showed 12(30%) isolates produce ESBLs enzyme figure (4-6), while 28(70%) of the isolate are non ESBLs enzyme producer. These findings are consistent with the local study of Aziz (2020) which showed 34.8% of isolates were ESBLs enzyme producer, but differ with Alwan (2020) 62.59% isolates. According to WHO research, the highly populated nations such as India and China have a greatest level of antibiotic resistance and infections by β-Lactamase producers (over 50%). This was connected mostly to the poor quality of antibiotics and their uncontrolled usage in majority of these regions (Vasant *et al*., 2020).

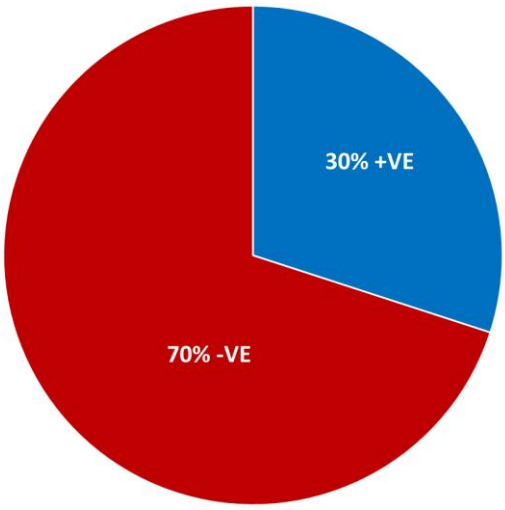


Figure (4-6): Phenotypic screening for ESβLs production (30% +VE) and non-producing of ESβLs (70% -VE).

4.4.1.2 Phenotypic screening for Metallo β-Lactamase MBLs in *P. aeruginosa*

P. aeruginosa isolates that were investigated for the presence of chromosomal or plasmid-mediated MβLs genes by combined EDTA disc test (CEDT) or imipenem-EDTA synergy test . MβLs is responsible for resistance to beta-lactam antimicrobial agents like Imipenem and Meropenem. Among 40 isolates of *P. aeruginosa* 10(25%) of them were MBL producers. The remaining isolates 30(75%) were non-MBL producers. The inhibition zone with Imipenem-EDTA is more than 7 mm than the imipenem disc alone figure (4-7).

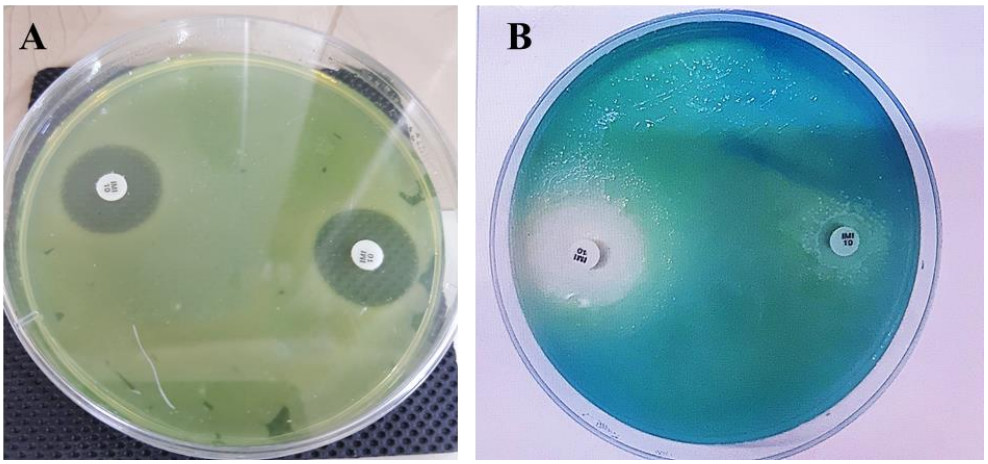


Figure (4-7): Combined disc diffusion test MBL. (A) Negative test, (B) Positive test.

In the current investigation, Metallo-lactamase was found in 10(25%) of *P. aeruginosa* isolates, which was consistent with Hyford's findings (2016) who found that 31% of carbapenem resistant *P. aeruginosa* isolates from patients have MBL enzyme. However, Najeeb (2020) showed that all isolates were enzyme nonproducing (100%) and Kazeminejad *et al*. (2017) who demonstrate 73% of carbapenem resistant isolates have MBL enzyme. Early detection of these MBL producing isolate in a routine laboratory could help to avoid treatment failure, as often the isolates producing this enzyme show a susceptible phenotype in routine susceptibility testing.

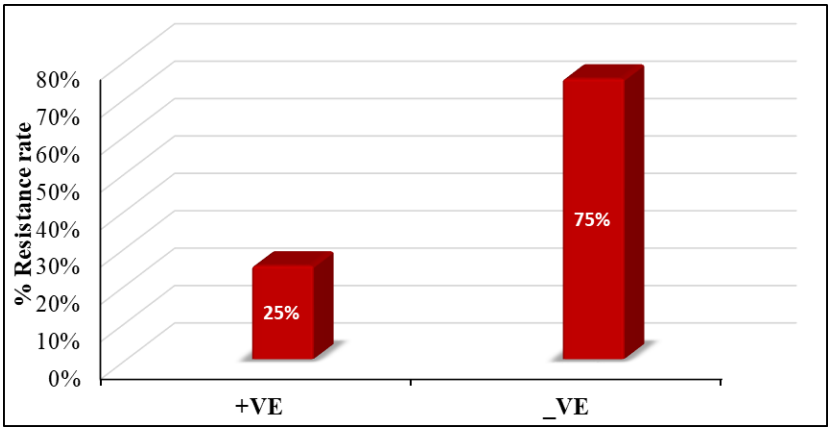


Figure (4-8): Phenotypic screening of *P. aeruginosa* for producing and non-producing of MBL.

4.4.1.3 Phenotypic Detection of Ambler class C beta-lactamase (AmpC)

Disk antagonism test was performed to detect AmpC enzymes for all the 40 isolates. The isolates showed blunting of the inhibition zone of cefotaxime adjacent to the cefoxitin disk or reduced susceptibility to each of them were showed a positive for AmpC B-lactamase production (Samatha and Parveen, 2011). The results showed that 18(45%) of the isolates had the ability to produce AmpC enzymes while lower than 7(38.8%) found by the local study conducted by (AL-Jubori *et al*., 2014).

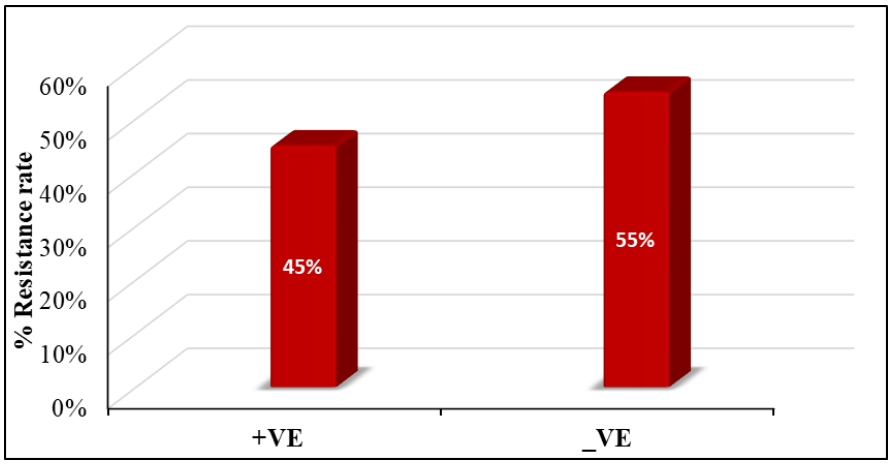


Figure (4-9): Phenotypic screening of *P. aeruginosa* for producing and non-producing of AmpC.

4.5 Molecular study

4.5.1 Multilocus Variable Number of Tandem Repeats Analysis (MLVA) of *P. aeruginosa* isolates

In order to find different variants of *P. aeruginosa* strains from different clinical samples, a PCR based on MLVA were designed to amplify different variable number tandem repeat (VNTR) in the genome of *P. aeruginosa*. These regions have been reported to cover highly polymorph regions which allow the identification of various bacterial species. These regions of VNTR in *P. aeruginosa* including MS-213, MS-214, MS-215, MS-217, MS-222, MS-223, MS-142, and MS-173. From these regions, four loci were selected in the current study which were MS-213, MS-214, MS-215 and MS-142**.**

4.5.1.1 Multiplex polymerase chain reaction for detection of repetitive loci MS-213, MS-214, MS-215 and MS-142

Multiplex polymerase chain reaction figure (4-10), was carried out in order to study four loci in *P. aeruginosa* isolates based on MLVA. Nine isolates were chosen based on the antibiotic susceptibility profile. The isolates that developed the maximum degree of antibiotic resistance. The amplified product was also tested on an agarose gel stained with ethidium bromide before capillary electrophoresis to ensure that the amplification was effective.

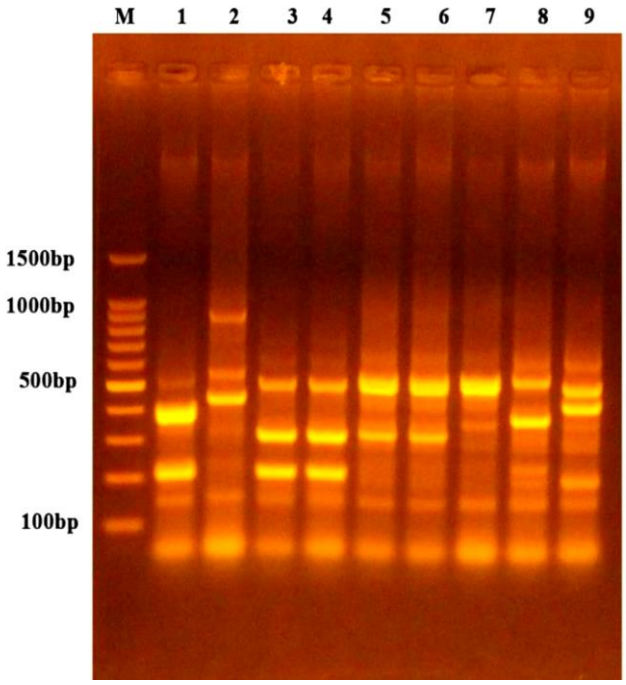


Figure (4-10): Agarose gel electrophoresis of MS-213, MS-214, MS-215 and MS-142. The DNA samples were fractionated on 1.5% agarose gel at 100v/mAmp for 60 min stained with Eth.Br., M: 100bp ladder marker.

4.5.1.2 Capillary electrophoresis analysis

The outcomes for the current study by comparing the amplicon sizes on the gel to the DNA ladder, it was demonstrated how difficult it was to compute the exact amplicon sizes. Because the amplicon from two loci might occasionally overlap or the difference between them is so little that it is difficult to distinguish them, as shown in figure (4-10), additionally, DNA fragments marked with various fluorescent dyes might generate electrophoresis mobility discrepancies, which can lead to mistakes in DNA fragment size estimations) (Tu *et al*., 1998; Bachelerie *et al*., 2016).

This explains why the present study's findings for some genes differ from what was expected by agarose gel electrophoresis. The ABI genetic analyzer was used to do capillary system electrophoresis on the PCR results produced from one multiplex PCRs. The Applied Bio system Gene Mapper^{TM} Software 5 was

used to analyze the data collected from capillary electrophoresis in the form of peaks in order to determine the exact PCR product scale shown in figure (4-11), and figure (4-12).

Traditional multilocus variable number tandem repeat fingerprinting (MLVF) depends on agarose gel electrophoresis, which reduces the specificity and reproducibility of the method. Furthermore, some analytical strategies that rely on comparing several banding patterns on agarose gels are difficult to compare between laboratories, preventing the development of international databases. MLVA uses a different VNTR-dependent typing process, Capillary Gel Electrophoresis (CGE) rather than traditional agarose gel electrophoresis, this resulted in data that is more precise and detailed than conventional MLVA, and it encourages the generation of genotypes in the form of a code that can be stored in a database and easily compared and exchanged among laboratories. Still no unanimity on which sets of markers should be utilized or which one is the best scheme (Babenko e*t al*.,2017).

The results of the current study showed that, the gene scan analysis revealed different kinds of amplicons in 9 isolates, for MS-213 loci, 3 different types which were 350pb (4 isolate), 370pb (3 isolate) 290pb (2 isolate). These differences between MS-213 bands due to the numbers of tandem repeats that different in each amplicon of different *P. aeruginosa* isolate table (4-7).

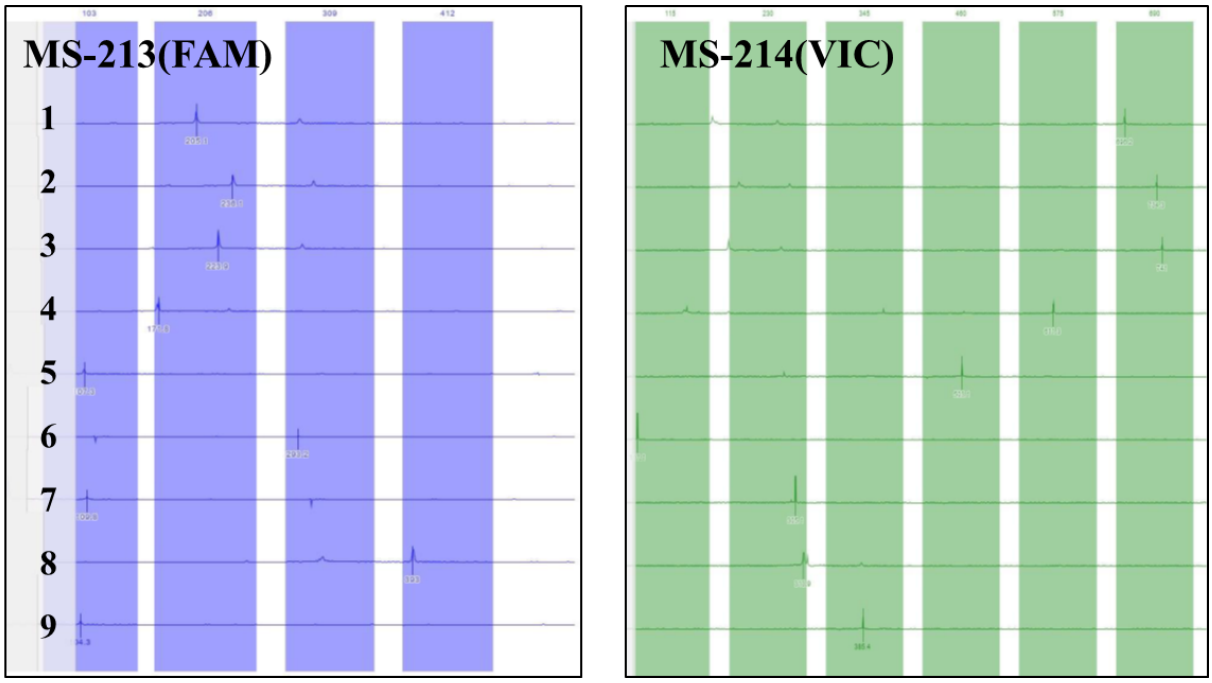


Figure (4-11): Capillary electrophoresis results of (VIC) and (FAM) using ABI- genetic analyzer and analyzed by the Applied Bio system Gene Mapper™ Software 5 (see Appendix 6,7).

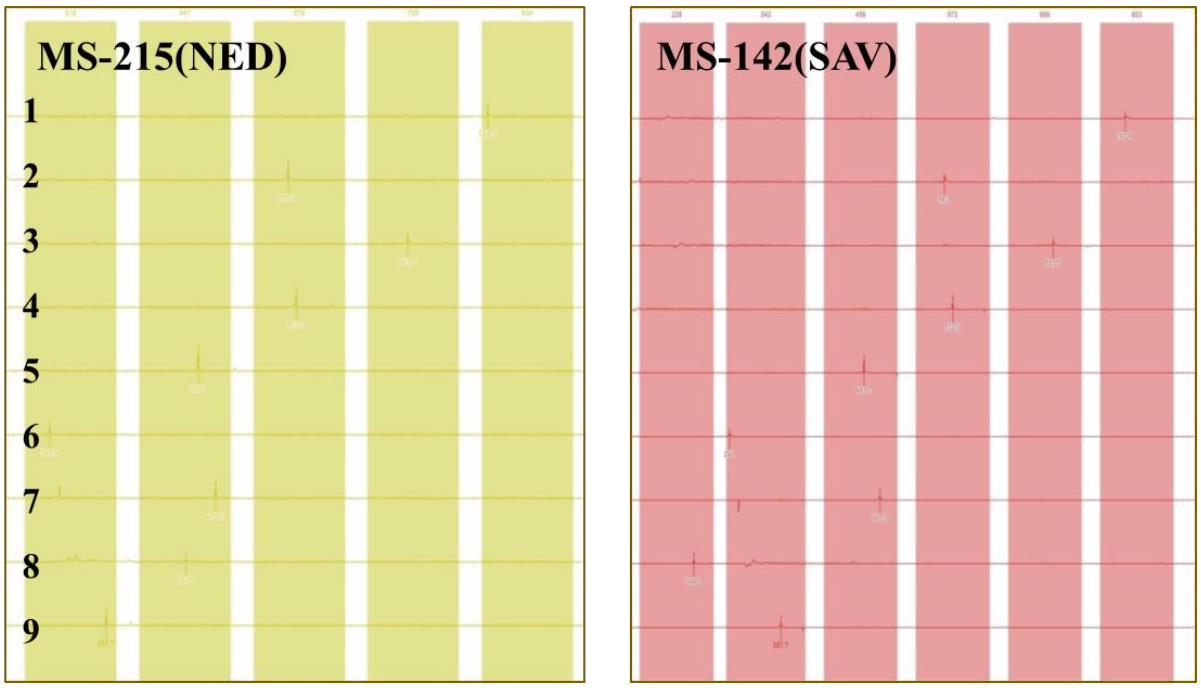


Figure (4-12): Capillary electrophoresis results of (NED) and (SAV) using ABI- genetic analyzer and analyzed by the Applied Bio system Gene Mapper™ Software 5 (see Appendix 8,9).

In case of MS-214 loci, 4 different types of amplicons were obtained with *P. aeruginosa* isolates. These amplicon sizes were 730pb (3 isolate), 640pb (1 isolate), 540pb (1 isolate) and 350pb (4 isolate) these differences due to variations of tandem repeats in different amplicons of *P. aeruginosa* isolates. Finally, MS-142 loci, 4 different kinds of amplicons with all of 9 isolates of *P. aeruginosa* were detected. These amplicon sizes as follows: 650pb (3 isolate), 720pb (1 isolate), 530pb (2 isolate) and 310pb (3 isolate) these variations among amplicon sizes due to variations of tandem repeats in these amplicons of different *P. aeruginosa* isolates.

4.5.1.3 The MLVA allelic profile using bioinformatics analysis

As indicated MLVA typing is a combination of different loci is used to group epidemiologically homogeneous isolates. So a combination of four loci was used for converting the amplicon sizes into the repeat numbers in order to get a broader view for phylogenetic analysis (Pourcel *et al*., 2011). The sizes of fragment obtained for each locus were converted into the repeats number. For this purpose, the sequence data for the standard strains *P. aeruginosa* PAO1(taxid:208964) that was used as a gene references, genomic structure of *P. aeruginosa* strain PAO1 has relatively a large number of tandem repeats.

That were imported into the Tandem Repeats Finder, a program in bioinformatics written by Gary Benson, Boston Version 4.0 (Benson, 1999; http://tandem.bu.edu/trf.html). The software identified the numbers of repeats present in the DNA sequences of the loci. The sizes of the flanking regions were assessed by subtracting the repeat containing region from the total size of the amplicon. In this way it was obtained the length of flaking region, length of VNTR region, and size of repeat unit from the Tandem repeat finder version 4.0. Then this approximately was used to calculate the number of repeats found at PCR products acquired from other *P. aeruginosa* isolates. Total number of

repeats was calculated by using the formula: the size of the product of PCR minus flanking region size divided repeat unit size. So according to the above the outcomes of the present study revealed the number of VNTR for all the loci which are given in the table (4-7).

Table (4-7): Nine *P. aeruginosa* isolates and strains obtained after Gene Scan (Positive control) *P. aeruginosa* PAO1 for the repetitive loci (MS-213, MS-214 and MS-142).

Sample	MS-213			MS-214			MS-142		
	Band (bp)	Repeat size	Repeat No.	Band (bp)	Repeat size	Repeat No.	Band (bp)	Repeat size	Repeat No.
1	350	170	3	730	557	5	650	495	4
2	370	190	4	730	557	5	650	495	4
3	370	190	4	730	557	5	720	565	5
4	350	170	3	640	467	4	650	495	4
5	290	110	2	540	367	3	530	375	3
6	290	110	2	350	177	2	310	155	2
7	350	170	3	350	177	2	530	375	3
8	370	190	4	350	177	2	310	155	2
9	350	170	3	350	177	2	310	155	2
P. aeruginosa PAO1	536	356	3	426	253	2	928	773	6

The data in the table (4-7) shows and represent the IDs of isolates (numeric digit code), then the amplicon sizes obtained by gene scan, length of VNTR region present in these amplicons and total number of repeats as calculated by using the formula as mentioned previously in material and methods. Three different amplicon sizes were detected in MS-213 loci, hence this means there are three different types of repeat numbers were presented in these amplicons which are (2, 3 and 4) depending on the amplicon sizes and VNTR lengths. For MS-214, four different types of amplicon sizes were identified, this means there were four different types of repeat numbers (2, 3, 4, and 5) with respect to amplicon sizes and length of VNTRs. For MS-142, four different types of amplicon sizes were identified, this means there were four

different types of repeat numbers (2, 3, 4 and 5) with respect to amplicon sizes and length of VNTRs. In this way the results obtained for the number of repeats to the three loci (MS-213, MS-214 and MS-142), that's used to generate allelic profile. But, In case of MS-215 locus, there are no repeat numbers were detected, so this locus were excluded from generating allelic profile. This explained probably to presence unique DNA sequences of a *p. aeruginosa* genome.

4.5.2 Phylogenetic tree (minimum spanning tree)

In order to draw the minimum spanning tree (Phylogenetic tree), the repeats obtained with three loci (MS-213, MS-214 and MS-142) for each isolate were feed into the BioNumerics program. The outcomes of MST of nine *P. aeruginosa* isolates obtained from VNTR loci of (MS-213, MS-214 and MS-142) loci utilizing BioNumerics software was shown in figure (4-13 A) and figure (4-13 B). The findings as showed in these figure (A), each circle reflects one isolate, while the colors represent the isolation sources for each isolate such as the green color represents the isolates taken from wounds, the red color represents the isolates taken from the urine and the blue color represents the isolates taken from the burn. While the figure (4-13 B) showed colors representing places for each isolate. The different isolates were linked with repetition at VNTR with bold lines, and the numbers within the bold line between isolates represent the number of different locations between isolates and the standard strain was also included in this tree. All 9 isolates were grouped into 10 genotypes MLVA before applying cut of i.e. the similarity between the replicates in four sites was considered 100%. In figure (4-13 A) and figure (4-13 B), it is clear that these isolates are closely related to each other, as the majority of MLVA species are linked by bold lines originating from the genotype 9 represented by isolation No. 9 and isolated from wound in Baqubah Teaching Hospital.

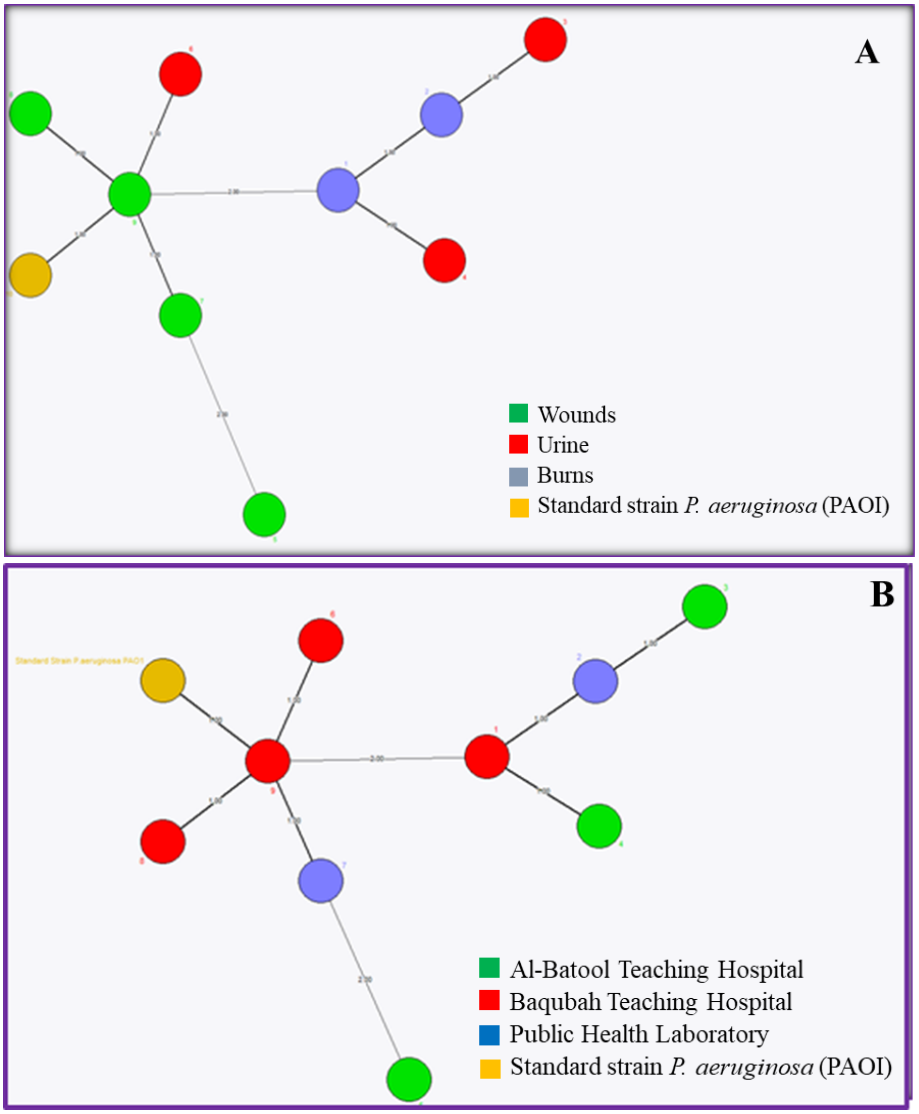


Figure (4-13): Minimum spanning tree of 9 *P. aeruginosa* isolates derived from VNTRs for MS-213, MS-214 and MS-142 loci using BioNumerics software. A: the colors represent the sources of each isolate and B: represent the places of isolation.

The dendrogram was drawn by using the allelic profile. The dendrogram obtained by the genotypes (MLVA types) of the 9 *P. aeruginosa* isolates in addition to the standard strains is shown in figure (4-14). The dendrogram building method was unweighted pair group method with arithmetic mean (UPGMA) with categorical distance coefficient and a cluster cutoff of 50%. Isolates names, source of isolate and place of isolation are also indicated in the figure. All the 9 *P. aeruginosa* isolates were clustered into 10 genotypes at 100% similarity (without cut off) this means the isolates that clustered together

are dissimilar in amplicon sizes for all loci, Consequently, they have asymmetric numbers of VNTRs, that is, they do not duplicate in any position from each other as shown on the dendrogram.

The following genotypes were obtained after clustering: Each of Genotype (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) contained one isolate which are (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) respectively. (Vu-Thien, 2007) performed an MLVA on a number of isolates the results were analyzed revealed varying in repeat number that was obtained between the locus. The repeat number values for MS-142 was (7) compared with standard isolate PAO1 and this the same standard isolate used in this study, MS-213 was (5), and MS-214 was (3), this result is similar to the result of the current study.

Also, Shokoohizadeh *et al*., (2020) showed high results in repeat number that was obtained between the locus. As follow MS-213, MS-215, MS-214 The repeat number values were (9) and MS-214 was (8) an MLVA. For example, from the figure (4-14) at 50% cut off, the genotypes 1 and 2 clustered together at 60% cut off and they clustered together again with 3 to form a group (1) in cut off 50%. Similarly genotypes 6, 8, and 9 clustered together at cut off 60%, and they clustered together again with 7 to form another group (2) in cut off 50%.

The genotypes 4 and 5 did not cluster into any group after 50% cut off because the isolates in these genotypes are varied by a repeat at two or more loci from each other. In the Pseudomonas genus, the repeated sequences that have been identified, which in this study several of them were MS-213, MS-214, MS-215, MS-142 that can be used to typing strains. These sequences, which have different replications in different strains of *P. aeruginosa*, are an appropriate means for categorizing *P. aeruginosa* subspecies from different sources or from different locations. Considering that the typing of pathogenic strains of the hospital can provide very useful information for designing an effective

therapeutic approach to physicians and medical staff, this study was conducted with the aim of reaching a clear and reliable pattern.

Therefore, in this study, the *P. aeruginosa* bacterial strains isolated from different sources of infection burn, wound, and urine specimens, based on sequential tandem repeats were investigated by MLVA technique. MLVA stands for discriminating power, which is defined as the ability to distinguish between unrelated bacterial isolates. It also elucidates the relationships between organisms obtained from the same sources (Bachelerie *et al*., 2016; Liu *et al*., 2016).

In comparison to those of randomly amplified polymorphic DNA PFGE, the concurrent analysis related to number of repeats unit in 7 specific genes gives MLVA with reproducible discriminatory power. This method was known to be low-cost, simple, sensitive, and simple to understand, and it had to be accurate to compare data from different laboratories. Furthermore, MLVA type may be easily predicted using multiplex PCR primer sequences labeled with various fluorescent pigments. This structure may eliminate the inherent challenge of comparing results from different laboratories using procedures that rely on standard agarose gels to separate DNA fragments. (Shokoohizadeh *et al*., 2020).

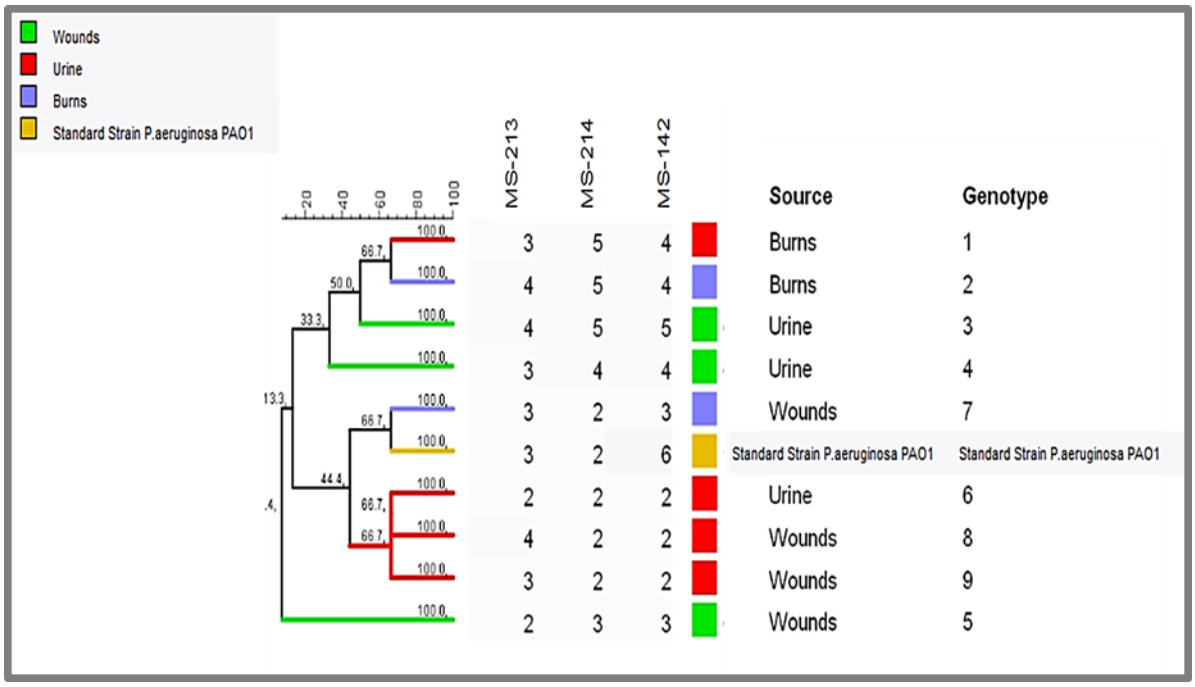


Figure (4-14): Dendrogram base on clustering with respect to MLVA types of *P. aeruginosa* isolates with similarity calculated by Dice coefficient and represented by UPGMA. 10 haplotypes are shown at 100% similarity, which clustered into 5 groups when 50% cut off technique was used According to sources.

CONCLUSIONS

AND

RECOMMENDATIONS

Conclusions

The results of the current study concluded the following points:

1. MLVA is a genetic tool for inter-laboratory surveillance that is able to distinguish the bacteria with different genotypes into different groups despite their phenotypic similarity.
2. Gene scan for Capillary electrophoresis revealed all three repetitive loci showed different types of amplicons except loci MS-215, which were unique DNA repeat.
3. Capillary Electrophoresis is a high resolution technique to detect the presence of resistance genes and repetitive loci (fingerprinting DNA) more than the conventional PCR.
4. The early detection of the pathogenic bacteria by MLVA analysis could control their prevalence in the community.

Recommendations

1. Conducting an epidemiological study to detect prevalence of *P. aeruginosa* focusing on different governorates and sources.
2. Using more *P. aeruginosa* isolates to differentiate more of genotypes and screen them using other VNTR loci in this bacteria.
3. Conducting epidemiological studies for other types of Gram-positive or Gram-negative bacteria by MLVA analysis and the VNTR feature.
4. The importance of spreading health consciousness by warning not to use antibiotics excessively and indiscriminately and without prescription.
5. VNTRs can be applied as a routine typing analysis for early warning of outbreaks and initiating epidemiological investigations for clinical isolates.
6. The necessity of performing a laboratory analysis (culture) before prescribing any antibiotic to the patient.

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Appendixes

Appendix (1) VITEK2 compact system report

bioMerieux Customer:
System #:

Laboratory Report

Printed Oct 3, 2020 08:22 GMT-06:00
Printed by: LabAdmin

Patient Name: nor aly kadem(pus)
Isolate Group: 9158-1

Patient ID: 948006

Card Type: GN Testing Instrument: 0000148FF4B8 (10140)

Bionumber: 4043453303500270
Organism Quantity:

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2411143203	**Expires:**	Jan 12, 2021 12:00 GMT-06:00
	Completed:	Oct 1, 2020 15:16 GMT-06:00	**Status:**	Final	**Analysis Time:**	5.00 hours
Selected Organism	90% Probability		**Pseudomonas aeruginosa**			
	Bionumber:	4043453303500270			**Confidence:**	Good identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Pseudomonas aeruginosa GGAA(1),PyrA(3),URE(16),						

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	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	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	+	61	IMLTa	+	62	ELLM	-	64	ILATa	-			

Installed VITEK 2 Systems Version: 07.01
MIC Interpretation Guideline:
AES Parameter Set Name:

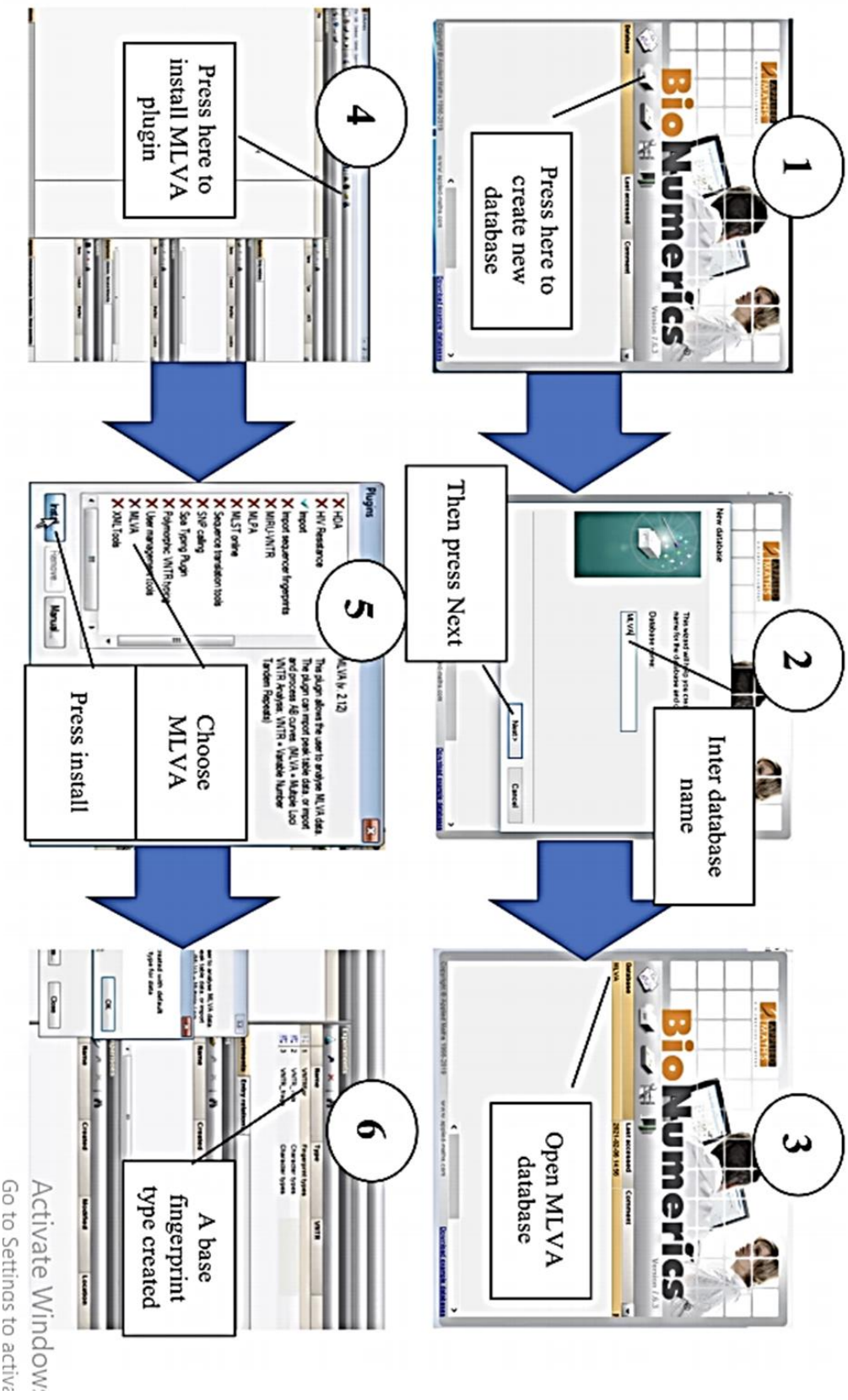
Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

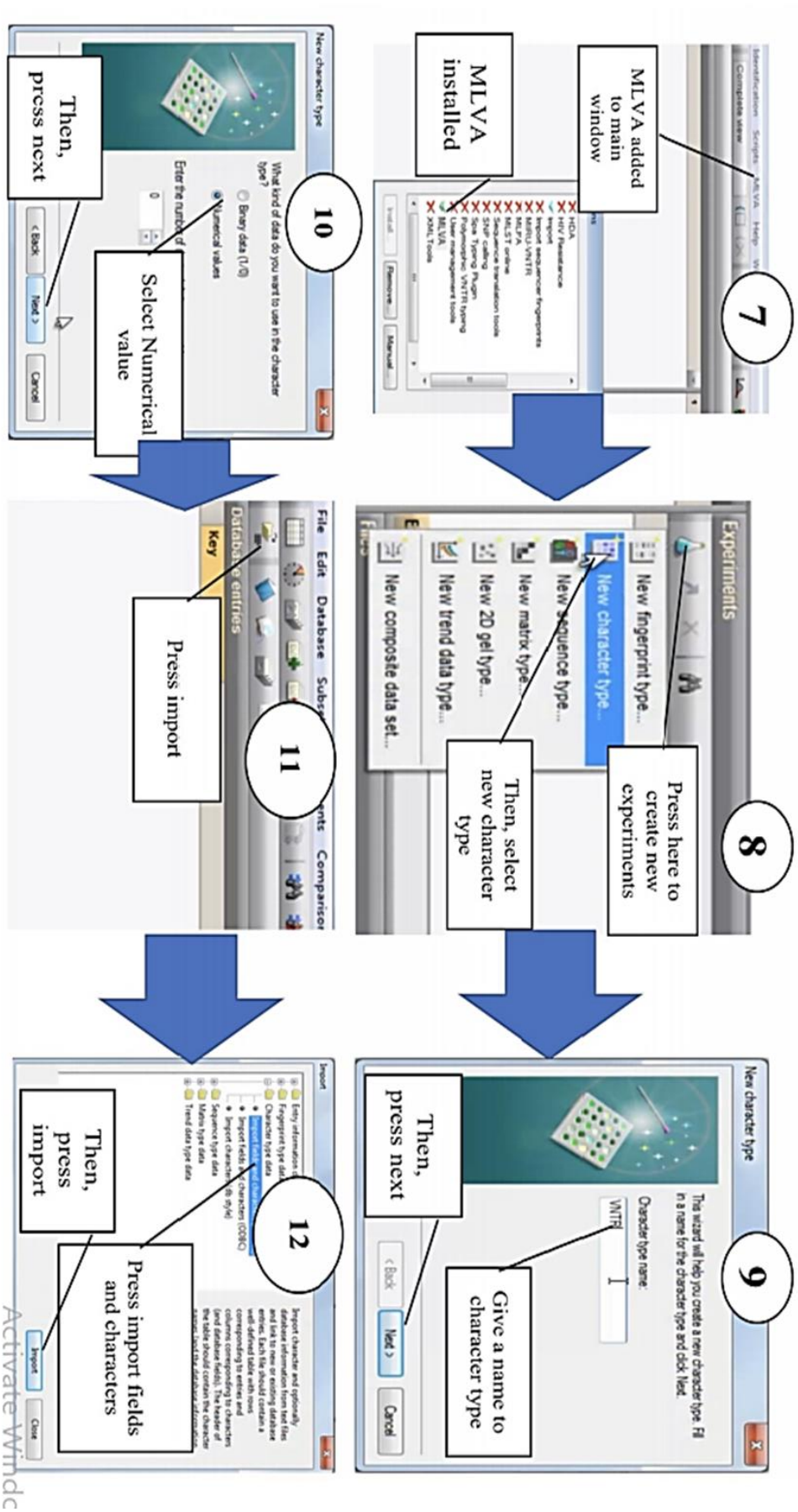
Page 1 of 1

Appendix (2) Antibiotic susceptibility table

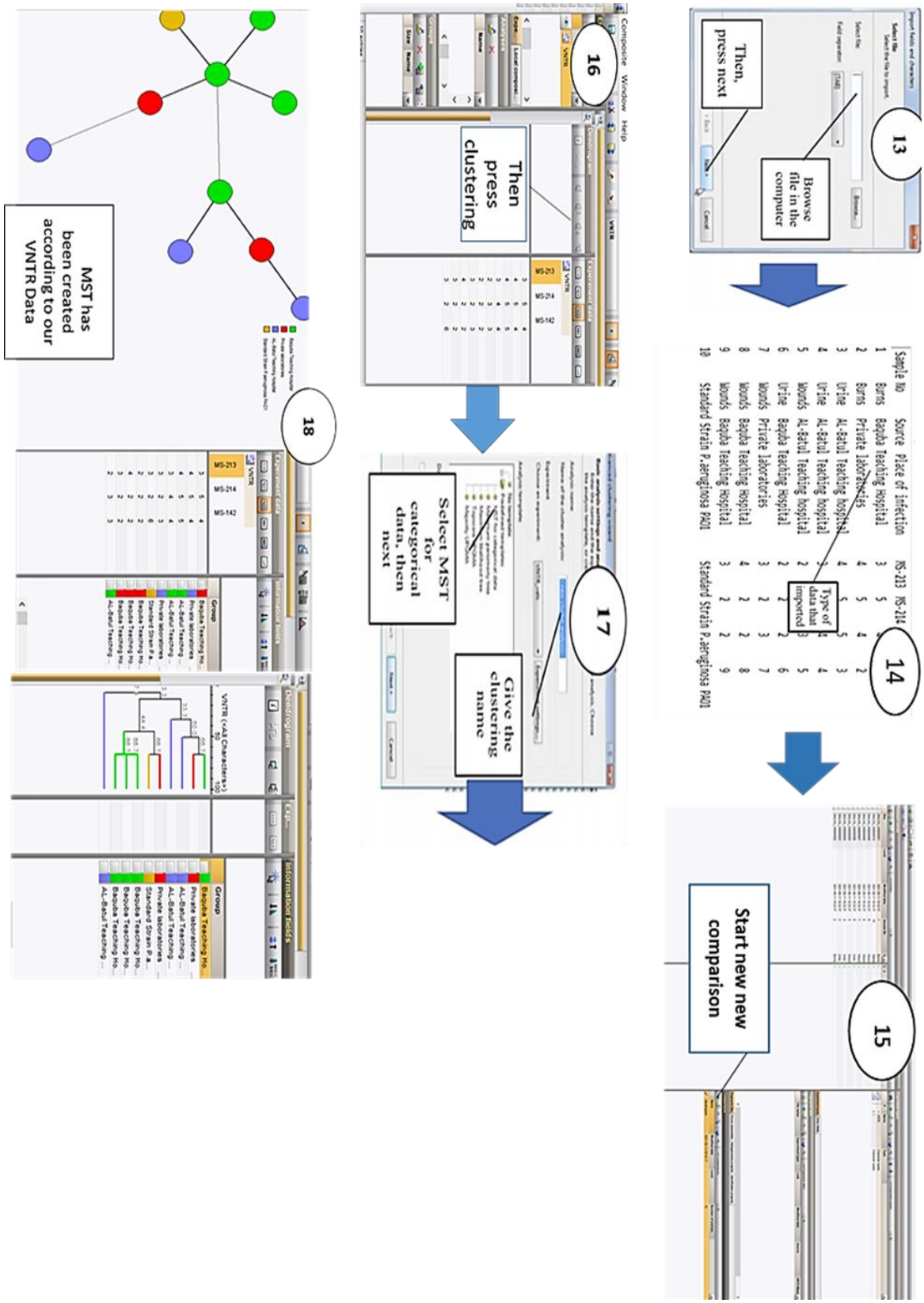
Isolate No.	IMI	MEM	ATM	CPM	CAZ	LEV	TIM	AK	CIP	NET	PTZ	GM
P1	S	S	S	S	S	S	I	S	S	S	S	S
P2	S	S	S	S	S	S	S	S	S	S	S	S
P3	S	S	S	S	S	S	I	S	S	S	S	S
P4	S	S	S	S	S	S	I	S	I	S	S	S
P5	S	S	S	S	S	S	I	S	S	S	S	S
P6	S	S	I	S	S	S	S	S	S	S	S	S
P7	S	S	I	S	S	S	I	S	S	S	S	S
P8	R	S	S	I	S	I	R	S	R	R	R	S
P9	S	S	I	S	S	S	I	S	S	S	S	S
P10	S	S	S	S	S	S	I	S	S	S	S	I
P11	S	S	R	R	S	S	R	R	S	R	I	R
P12	S	S	S	S	S	S	I	S	S	S	S	S
P13	R	R	R	R	S	S	R	R	S	R	I	R
P14	R	R	R	R	R	S	R	R	S	R	R	R
P15	S	S	S	S	S	S	S	S	S	S	S	S
P16	S	S	S	S	S	S	S	S	S	S	S	S
P17	S	R	S	S	S	S	S	S	S	S	S	S
P18	R	R	R	R	R	R	R	R	R	R	I	R
P19	I	R	R	R	S	R	R	R	R	R	I	R
P20	S	S	I	S	S	S	I	S	R	S	R	S
P21	S	S	S	S	S	S	S	S	S	S	S	S
P22	R	R	R	R	R	R	R	R	R	R	I	R
P23	R	R	R	R	R	R	R	R	R	R	S	S
P24	R	R	R	R	R	R	R	R	R	R	S	R
P25	R	R	R	R	R	R	R	R	R	R	I	I
P26	S	S	S	S	S	S	R	S	S	S	S	S
P27	S	S	S	S	S	S	S	S	S	S	S	S
P28	S	S	S	S	S	S	I	S	S	S	S	S
P29	R	R	R	R	R	R	R	S	R	R	I	R
P30	R	R	R	R	R	R	R	R	R	R	I	I
P31	R	R	R	R	R	R	R	R	R	R	I	R
P32	S	R	R	R	S	R	R	R	R	R	I	R
P33	S	S	S	S	S	R	R	S	R	R	R	S
P34	S	R	S	S	R	I	S	S	S	S	S	S
P35	S	S	I	S	R	R	S	R	R	R	S	R
P36	R	S	R	R	R	S	S	R	S	S	I	S
P37	R	S	R	R	R	S	R	R	S	R	S	R
P38	S	S	S	S	S	S	S	S	S	S	S	S
P39	S	S	S	S	S	S	S	S	S	S	S	S
P40	S	R	S	S	S	I	S	S	R	S	S	S

Appendix (3) MLVA steps:




7
MLVA added to main window
MLVA installed
8
Press here to create new experiments
Then, select new character type
9
Then, press next
Give a name to character type
10
Then, press next
Select Numerical value
11
Press import
12
Then, press import
Press import fields and characters

Appendixes


13
Browse file in the computer
Then, press next
14
Type of data that imported
15
Start new new comparison
16
Then press clustering
17
Give the clustering name
Select MST for categorical data, then next
18
MST has been created according to our VNTR Data

Appendix (4) Patients information form

Sample NO.:

Patient age:

Gender:

Sample type:

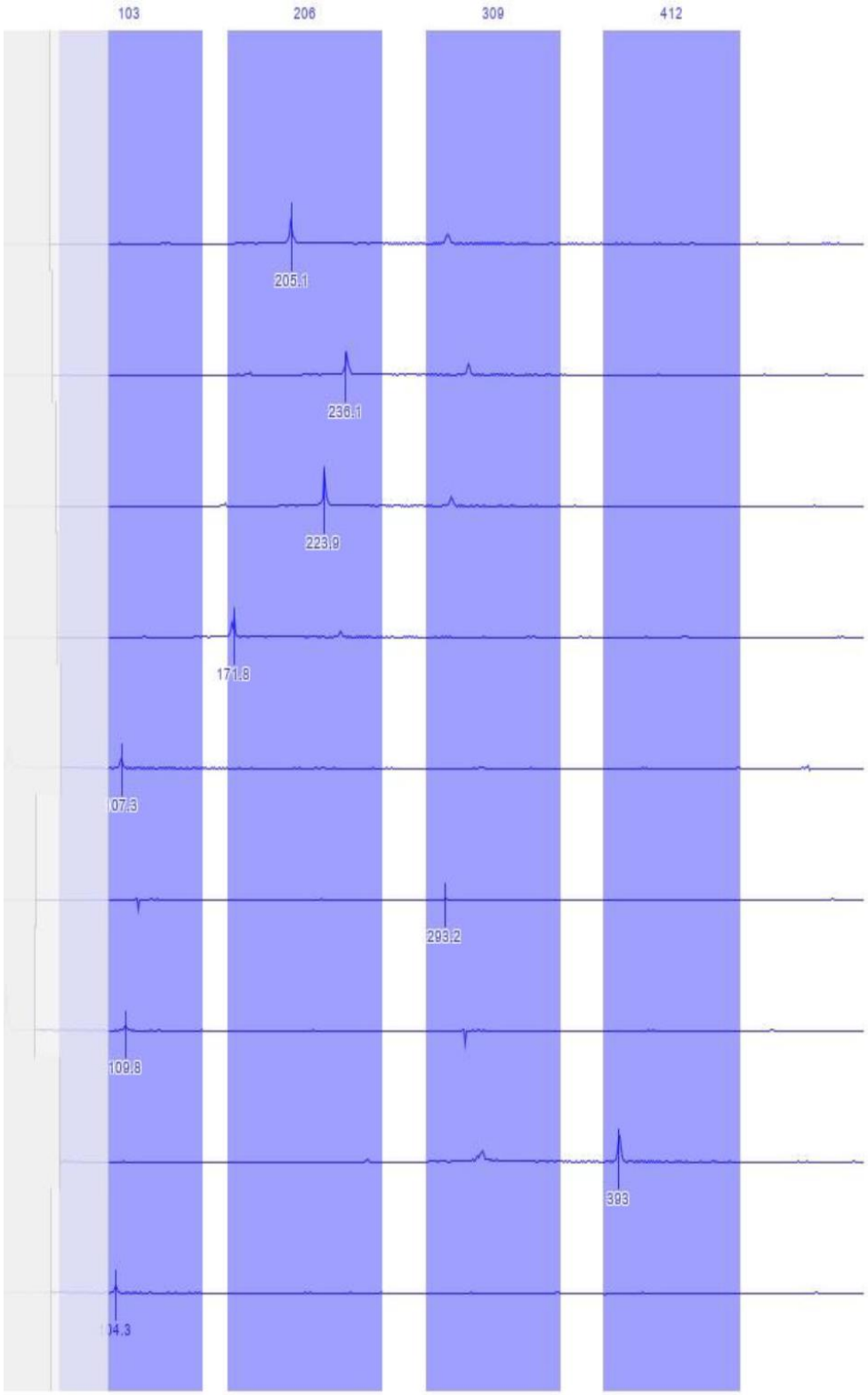
Collection place:

Collection date:

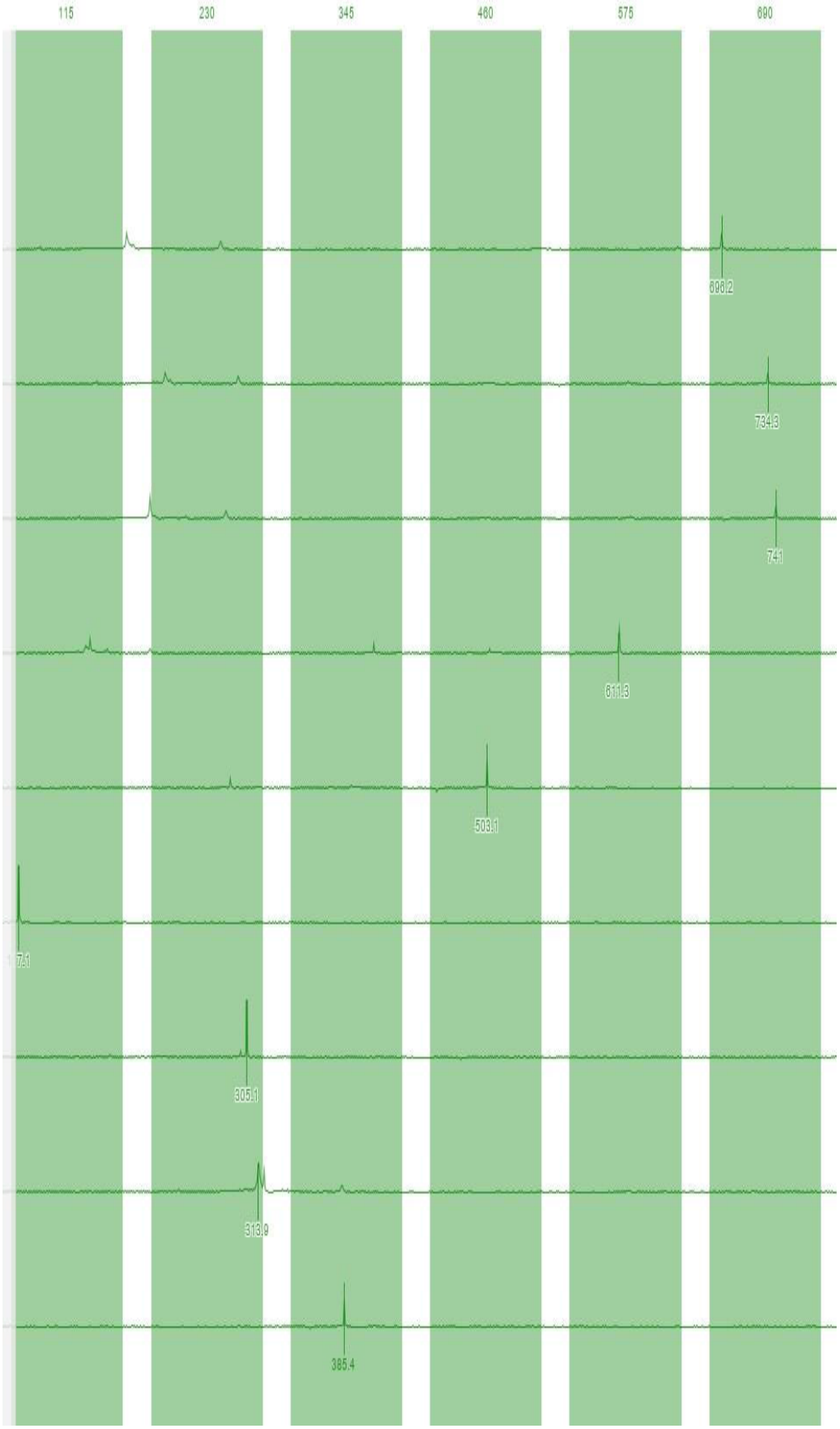
Appendix (5) The concentration of DNA samples in ng/µl.

Sample	Concentration (ng/µl)
1	19
2	20
3	15
4	12
5	14
6	13
7	15
8	22
9	18

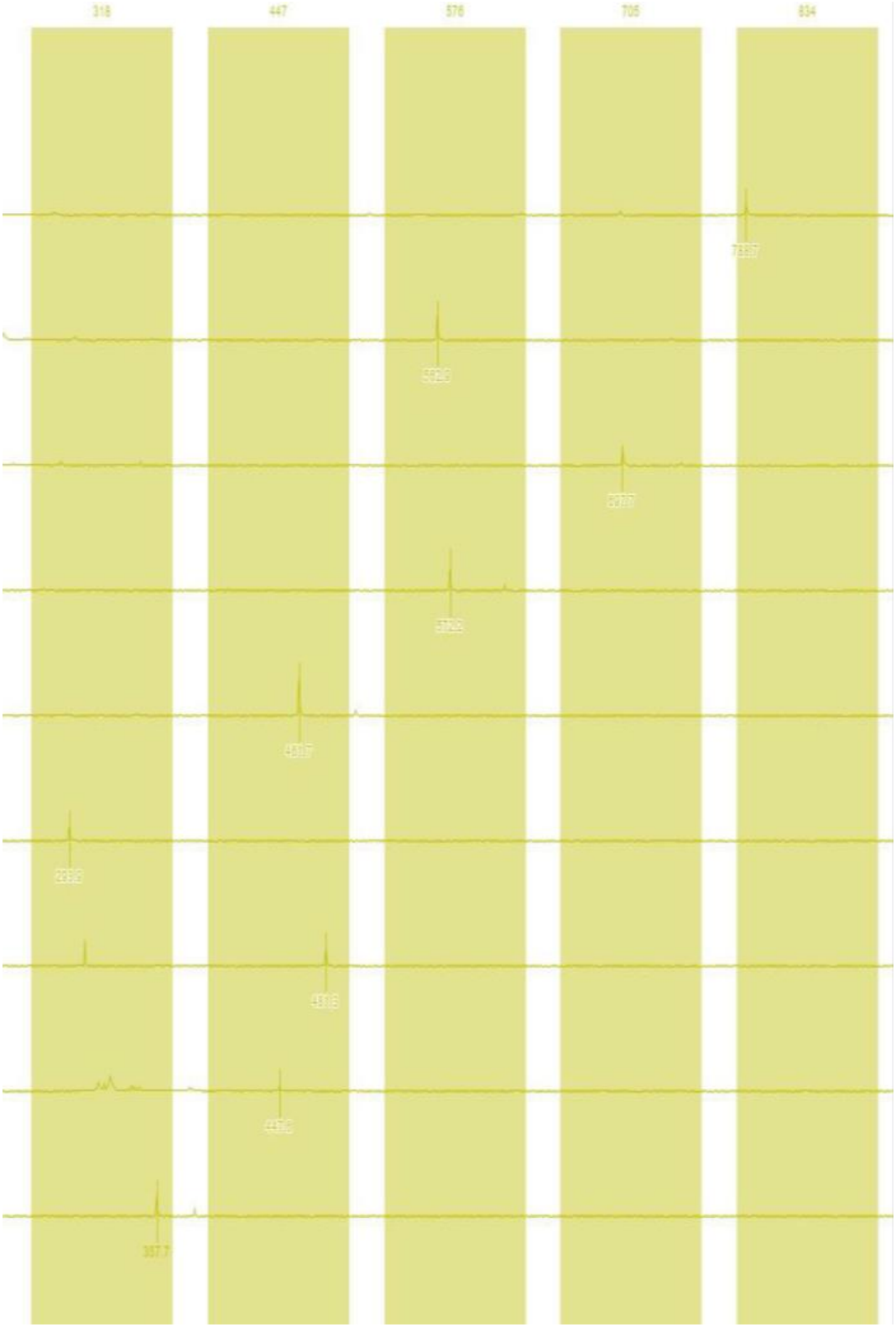
Appendix (6) Data Analysis of Locus MS-213



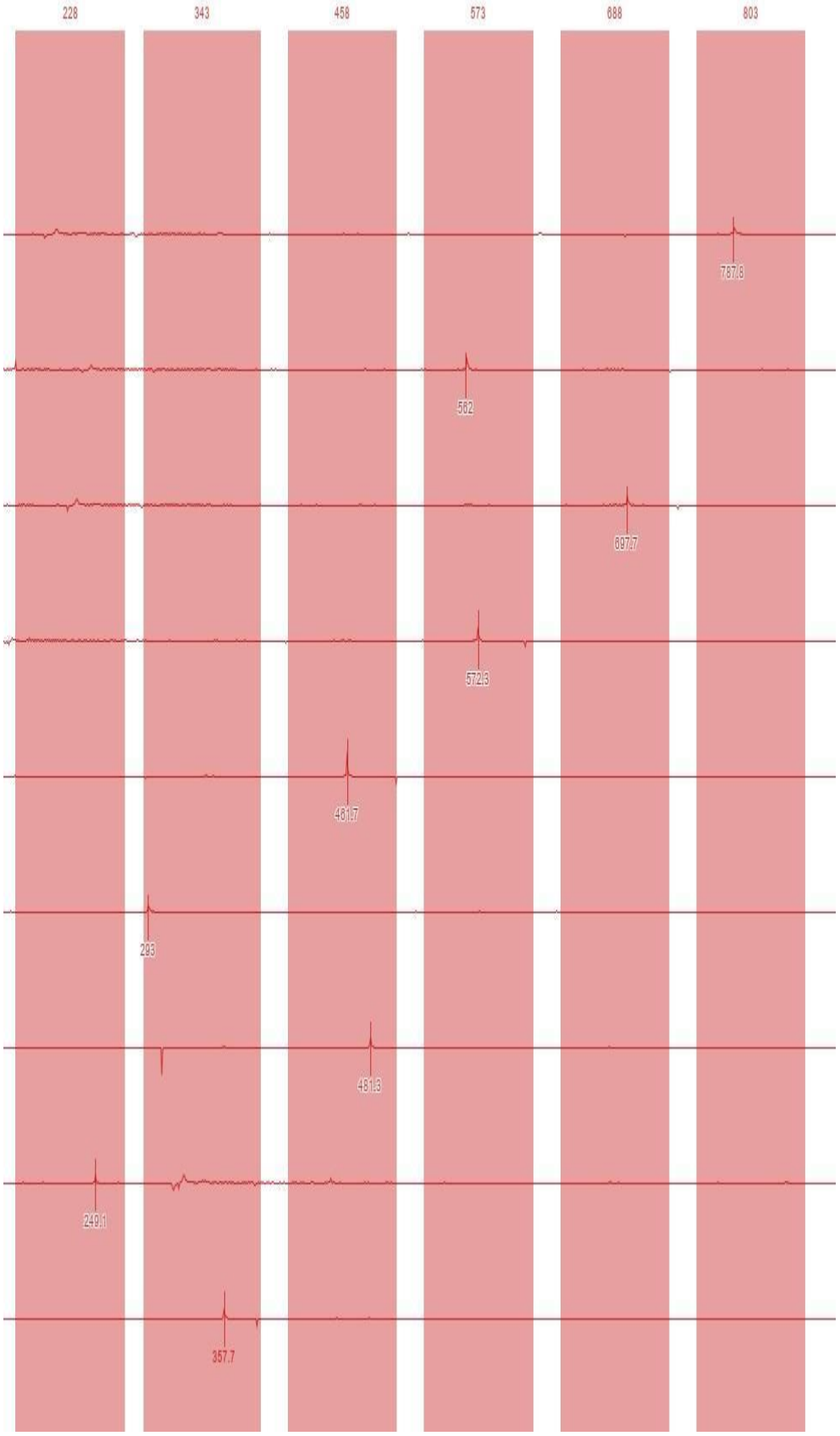
Appendix (7) Data Analysis of Locus MS-214



Appendix (8) Data Analysis of Locus MS-215



Appendix (9) Data Analysis of Locus MS-142.



الخلاصة

الزائفة الزنجارية هي بكتيريا سالبة لصبغة كرام، انتهازية هوائية. وهي واحدة من اهم مسببات العدوى المكتسبة في المستشفيات. تقاوم البكتريا العديد من الأدوية وتسبب التهابات شديدة في المجتمعات المحلية في العراق. تهدف هذه الدراسة إلى تقييم مدى انتشار وتنوع العزلات السريرية المحلية من الزائفة الزنجارية من خلال تقنية تحليل التكرارات الترادفية للأرقام المتغيرة (VNTRs). جمعت مائتان وثمانون عينة سريرية من مصادر مختلفة (حروق وجروح وادرار) من المستشفيات الحكومية في مدينة بعقوبة/محافظة ديالى بين تشرين الأول ٢٠٢٠ وكانون الثاني ٢٠٢١. أظهرت النتائج تحديد ٤٠ عزلة بكتيرية كونها زائفه زنجارية بالطرق الميكروبيولوجية التقليدية، اختبارات كيموحيوية وتم تأكيدها باستخدام تقنية VITEK 2 واختبار 16S rRNA. أظهر أختبار 16S rRNA أن ٩ عزلات (٢٢.٥%) كانت موجبة. تم أختبار حساسية المضادات الحيوية لأربعين عزلة باستخدام ١٢ صنف مختلف من المضادات الحيوية تجاه كل عزلة بطريقة (Kirby Bauer Disc Diffusion). أظهرت النتائج أن أعلى نسبة مقاومة (٤٢.٥%) تم الحصول عليها ضد Ticarcillin/Clavulanic acid و Netilmicin. وكانت النسبة الأقل (١٥%) من المقاومة ضد Piperacillin/Tazobactam. كانت نسبة المقاومة (٣٧.٥%) لكل من Amikacin و Aztreonam و Cefepime و Ciprofloxacin. بالنسبة للمضادين Meropenem و Ceftazidime كانت المقاومة (٣٥%) و (٣٢.٥%) مع Imipenem. كانت عزلات المقاومة Gentamicin و Levofloxacin بنسبة (٣٠%). أظهر اختبار الحساسية للمضادات الحيوية لمجموع العزلات نمطين حسب طبيعة المقاومة، نوع MDR ونوع XDR. أظهر النوع MDR أن ٥ (١٢.٥%) قسمت إلى ٢ (٥%) مقاوم إلى ٣ فئات و ٣ (٧.٥%) قاومت ٤ أصناف من المضادات الحيوية. أظهر النوع XDR أن ١٣ عزلة (٣٢.٥%) قسمت إلى ٣ عزلات (٧.٥%) قاومت ٥ فئات و ١٠ (٢٥%) قاومت ٦ أصناف من المضادات الحيوية.

تراوحت قيم التركيز المثبط الأدنى (MIC) للعزلات الأربعين مع Ceftazidime من (١٦-١٠٢٤) ميكروغرام/ملليتر. جرى الكشف عن β-lactamases لثلاثة إنزيمات شملت ESBLs وAmpC و MBLs. كانت ١٢(٣٠%) عزلات منتجة لأنزيمات ESBLs ومقاومة للمضادين Cefotaxime و Ceftazidime. كانت ١٠(٢٥٪) عزلات منتجة لأنزيمات MBLs و ٣٠ (٧٥%) غير منتجة. تم الحصول على عزلات منتجة لأنزيم AmpC في ١٨ (٤٥%). وفقا لمقاومة المضادات الحيوية، تم إخضاع تسعة عزلات لتقنية Multiplex PCR. تم اختيار أربعة مواقع شملت MS-213 و MS-214 و MS-215 و MS-142، لتحليل عزلات الزائفة الزنجارية استناداً إلى خاصية VNTR باستخدام طريقة عدد متغير متعدد التركيز من التكرارات الترادفية (MLVA).

أظهرت بيانات الترحيل الكهربائي الشعيري تبايناً في أحجام Amplicon. أظهر الموقع MS-213 ثلاثة Amplicons مختلفة هي ٣٥٠(٤ عزلات)، ٣٧٠(٣ عزلات) و ٢٩٠(٢ عزلة). أظهر الموقع MS-214 أربعة Amplicons مختلفة هي ٧٣٠(٣ عزلات)، ٦٤٠ (عزلة واحد)، ٥٤٠ (عزلة واحد) و ٣٥٠ (٤ عزلات). أما الموقع MS-142 فقد أظهر أربعة Amplicons شملت ٦٥٠ (٣ عزلات)، ٧٢٠ (١ واحدة)، ٥٣٠(٢ عزلة) و ٣١٠(٣ عزلات). أما الموقع MS-215 فقد انتج Amplicon واحد فقط. من أجل الحصول على عرض أوسع للتحليل التطوري، تم تحويل أحجام Amplicon إلى أرقام متكررة باستخدام flanking region. تم الاستعانة بعدد التكرارات التي تم الحصول عليها لثلاثة مواضع من أجل إنشاء ملف تعريف أليلي. تجمعت جميع عزلات الزائفه الزنجاريه التسعة في نوع أو الطرز الوراثية من نوع MLVA 10 دون تطبيق القطع. تم اعتبار المواضع تشابهًا بنسبة ١٠٠% في التكرارات. يتم تكوين معظم الطرز الجينية من النمط الجيني رقم ٩ الممثل للعزلة رقم ٩. أدى استخدام MLVA إلى السرعة والدقة العالية في توفير معلومات النشوء والتطور التي يمكن أن تتبع مصدر علاقات التركيب الجيني والتاريخ التطوري. كما يعكس العلاقة بين مصدر الإصابة وأنواع العزلات.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة ديالى/كلية العلوم
قسم علوم الحياة

التشخيص الجزيئي لبكتريا الزائفة الزنجارية *Pseudomonas aeruginosa* بأستخدام طريقة multiple locus variable number of tandem repeat units

رسالة مقدمة إلى مجلس كلية العلوم/جامعة ديالى
وهي جزء من متطلبات نيل شهادة الماجستير في علوم الحياة

من قبل

أنسام عيسى علي الحيالي

بكالوريوس علوم حياة/كلية العلوم/جامعة ديالى ٢٠٠٥

إشراف

أ. د. هادي رحمن رشيد **أ.م.د. عصام حامد حميد**

١٤٤٣هـ ، ٢٠٢١م