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Phylogenic Analysis of Tetracycline-Resistant *Acinetobacter baumannii* Isolated from Different Clinical Samples

A Thesis

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By

Ruqaya Kareem Abass Ouda

B.Sc.in Microbiology\College of Science University of Babylon

Supervised by

Asst. Professor

Dr. Zaineb Adil Ghani Chabuck

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بسم الله الرحمن الرحيم

وَلَقَدْ ءَاتَيْنَا دَاوُۥدَ وَسُلَيْمَٰنَ عِلْمًاۖ وَقَالَا ٱلْحَمْدُ لِلَّهِ ٱلَّذِى فَضَّلَنَا عَلَىٰ كَثِيرٍ مِّنْ عِبَادِهِ ٱلْمُؤْمِنِينَ

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Asst. Professor

Dr. Zainab Adil Chabuck

University of Babylon

College of Medicine

/ / 2023

In view of the available recommendation, I put forward this thesis for debate by the examining committee

Professor

Dr. Hayam khalis Al-Masoudi

Head of Microbiology Department University of Babylon

College of Medicine

/ / 2023

Examination committee

We, the examiner committee, certify that we have read the thesis entitled (**Phylogenic Analysis of Tetracycline-Resistant *Acinetobacter baumannii* Isolated from Different Clinical Samples)** and have examined the student (**Ruqaya kareem abass Al-Ammery**) in its contents, and that in our opinion it is accepted as a thesis for degree in Master of Science in **Medical Microbiology** with excellent degree.

Signature
Professor
Dr. Maysa Salih Al-shukri
College of Medicine
University of Babylon
(Chairman)

Signature
Professor
Dr.Nada Khazal K. Hindi
College of Nursing
University of Babylon
(Member)

Signature
Professor
Dr. Jinan Mohammed Husein
College of Education for Girls
University of Kufa
(Member)

Signature
Assistant Professor
Dr. Zainab Adil Ghani Chabuck
College of Medicine
University of Babylon
(Member and supervisor)

Approved for the College Committee of Graduate Studies

Signature
Professor
Dr. Mohend Abbass Alshalah
Dean of College

Dedication

To those who carried my worries and occupied their thoughts with my thought to whom the heart beats echoing their name ….to the soul of my dear father and dear mother

To whom was my shadow when fatigue invaded me.. …. my faithful husband

To the seed of the heart and the hope of tomorrow……. my dear daughter

To my flower in life..…. my dear sisters

To my second family who supported me, helped me and surrounded me with love

To my beloved aunt and dear uncles who have never left me in the course of my life

To my friends and colleagues who have shared with me throughout my scientific career

To all my honorable teachers

To everyone who taught me a letter and supported me with a smile

I present to you my humble work

Ruqaya

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Finally, I extend my deep thanks to my family, praying to God Almighty to grant them health and wellness.

Ruqaya

Summary:

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen, causing severe infections difficult to treat, Tetracyclines' resistance mediated by efflux pump and other mechanism are problematic, and efflux pump inhibitors can be beneficial.

Thus this study aimed to Genotyping of Tetracycline resistant *Acinetobacter baumannii isolates.*

This study included one hundred and twenty five (125) clinical specimens were collected during the period extended from the beginning of October 2022 to the end of January 2023, from patients who were attended to Al-Hilla General Teaching Hospital; it was collected from different clinical sites suggested to have infection such as urinary and respiratory infections, burns and wounds infections. Then samples were cultivated on MacConkey and nutrient agar then incubated aerobically at 37 Cº for 24 hours, diagnosis was confirmed by cultivation, biochemical tests and VITEK test.

A. baumannii isolates were detected as 23/125 (18.4%) that distributed as 10/23 (25%) isolates from sputum, 5/23 (17.9%) from burns, while 4/23 (16%) and 4/23 (12.5%) isolates were from urine and wounds respectively.

All bacterial isolates were subjected to different members of Tetracyclines (Tetracycline, Minocycline, Doxycycline and Tigecycline) by disk-diffusion test and minimum inhibitory concentration, results showed that highest resistance against Minocycline 11/23 (47.8%) followed by resistance to Tetracycline and Doxycycline as 10/23 (43.5%) and 9/23 (39%) respectively, Tigecycline about 4/23 (17.4%).

Additionally, regarding Multiple Antibiotic Resistance Index; results revealed that 3/23 (13%) with MAR equal to 1.0 which mean resistance to all used

antibiotics, while 9/23 (39%) with MAR more than 0.2 and the remaining 11/23 (47.8%) with complete sensitivity to all members (MAR=0.0).

Regarding MIC, results showed that Tigecycline, being the most effective member (19/23), with MIC ≤ 1 μg/mL with a range (1 - 0.125 μg/mL) against these isolates, while the resistant isolates showed an MIC 8-16 μg/mL. Among the 23 *A. baumannii*, the 13 tetracycline-sensitive isolates, showing MIC values ranging from 2-8 μg/mL, while the resistant isolates with MIC range 16 - 256 μg/mL and mainly at 16 -32 μg/mL. Additionally, regarding Doxycycline and Minocycline both showed effective MIC range 1 – 8 μg/mL.

Concerning detection of *A. baumannii* efflux pump by ethidium bromide cartwheel assay, 8/10 (80%) isolates expressing an active efflux pump; 5/8 fluorescence at 2 mg/L and more, but the remaining 3/8 did not fluorescence even at 2.5 mg/L of EtBr similar to the positive control *A. baumannii* ATCC, while 2/10 (20%) isolates exhibited no activity. Later on water extract of chamomile was used as antimicrobial and efflux pump inhibitor, showed that it was an effective inhibitor especially at higher concentrations (40, 80 mg/ml) with inhibition to most of the tested isolates.

The last part of this study is the genetic section that included the detection of Tetracyclines' resistance genes (*tetA*, *tetB* and *tetM*) by the application of specific primers and by conventional PCR. Results of these amplifications showed that *tetA* gene appeared among only 7/23 (30.4%); while regarding *tetB* gene appeared among 18/23(78.3%), coexistence of *tetB* and *tetA* appeared as 4/23(17.4%). Also, 2/23(8.7%) appeared negative for both genes while ribosomal protection gene (*tetM*), all isolates were negative for this gene.

Repetitive extragenic palindromic PCR (REP-PCR) fingerprinting grouped *A. baumannii* isolates, among the (23) collected isolates of *A. baumannii* the 23 isolates were divided into two major clusters (A and B); in which cluster A contain

5 members, two of them showed no difference in the distance indicating that it is identical, and contain one Tetracycline-resistant isolate with MAR index 0.75.

While regarding Cluster B, it is subdivided into two subclusters (B1 and B2) and each of them again subdivided into two subgroups.

Subcluster B1 containing 4 isolates obtained from different sources and additionally contain the standard strain *A. baumannii* ATCC, 19606; also 3/4 of these isolates showing phenotypic Tetracycline-resistance 2 of them with MAR index 0.75 and one with MAR index 1.00 (complete Tetracycline resistance).

Subcluster B2 containing 14, that subgrouped into B3 and B4 subgroups, where B3 containing 8 isolates (most of them obtained from sputum), four of them showed no differences in the distance and indicating homology and relationship between these isolates with different Tetracycline resistance distribution.

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List of Abbreviations

Abbreviated form	Meaning
A. baumannii	*Acinetobacter baumannii*
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
Bp	Base pair
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
D.W	Distal Water
DDT	Disc diffusion test
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra acetic Acid
EPIs	Efflux Pump Inhibitor
EtBr	Ethidium bromide
GS	Gram Stain
H2O2	Hydrogen peroxide
HAIs	Hospital-acquired infections
HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
ICUs	Intensive care unites
MAR	Multiple antibiotic resistant
MATE	Multi-drug and toxic compound extrusion family
MDR	Multi-drug resistant
MFS	Major facilitator superfamily
Mg	Milligram
MgCL2	Magnesium Chloride
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
MIN	Minocycline
min	minute
mm	millimeter
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis

R	Resistant
REP-PCR	Repetitive extragenic palindromic PCR
RND	Resistant-nodulation-division
Rpm	Round per minute
S	Sensitive
SMR	Small multi-drug resistant superfamily
TBE	Tris-Borate-EDTA
TE	Tetracycline
TIG	Tigycycline
TSA	Trypticase Soy Agar
U	Unite
U.V	Ultra-Violate
UK	United Kingdom
USA	United States of America
UTI	Urinary Tract infection
WHO	World health organization

Chapter One

Introduction

and

Literatures review

1. Introduction and Literature Review

1.1. Introduction

Health problems have increased and the reason is due to emerging of multidrug resistant (MDR) among Gram-negative bacterial species, mainly *Acinetobacter baumannii*, about 25 different species of Acinetobacter, the most powerful *A. baumannii* is a multidrug-resistant bacterial pathogen that causes serious infections, especially in immunocompromised patients and is a major public health concern around the world (Al-Hilali, 2019)

Acinetobacter can be found in soil and water; in patients they are frequently cultured from the urine, Saliva, respiratory secretions, and open wound. The organism is also known to colonize intravenous fluids and other irrigation (Munier *et al*., 2019).

The increase in antimicrobial resistant to *A. baumannii* poses a threat to public health and therefore requires complex treatment. The *A. baumannii* is challenging to treat because it can easily innate resistant mechanisms and acquire a wide array of antimicrobial resistant genes. Resistant genes are either on the host plasmid or on the bacterial chromosome (Rahbar *et al*., 2019).

The pathogenic characteristics of *A. baumannii* are related to their ability to resist antibiotic and their tolerance to environment conditions, as well as their ability to survive for long periods in hospital. One of the resistance mechanisms that they poses in a change in the outer membraneproteins (OMP), which are considered one of the most dangerous types of resistance because they are general changing the number and dimeters of the channels in the membrane as well it's possession of efflux pump systems that work on the antibody outside the cell (Guilfoile *et al*., 2007).

The maximum worrying harms met through this data are the bacterium's capability to store varied machines of resistance then the rise of resistant strains to wholly commercially accessible antibiotics attached with the absence of novel antimicrobial mediators. This has caused in a partial best of antibiotics for handling of multidrug resistant of *A. baumannii*. The greatest vigorous agents alongside to multidrug resistant *A. baumannii* are the polymixinE (Colistine), polymixins-polymixinB and tigycycline (Lolans *et al*., 2006).

The basis of the different typing systems is based on phenotypic tests and molecular techniques. Biotyping, serotyping and bacterial type are examples of phenotypic typing classification methods that have now mostly been superseded by molecular classification systems such as plasmid profile analysis, ribotyping, pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intragenic consensus-polymerase chain reaction (ERIC-PCR) (Falah *et al*., 2019).

In *A. baumannii*, repetitive sequences of the gene which are called elements are often seen as the homologous non-coding protein of the DNA. As for the variable number and length of these repeat sequences, some primers have been designed. The length and number of bands obtained for each isolate are variable and the strains can be classified based on the diversity of the bands (Falah *et al*., 2019).

The most has been shown to have strong antibacterial potential against Gram-positive and Gram-negative bacteria is *Martricaria chamomilla* (Molnar *et al*., 2017).

The *Chamomilla* plant is known to have antibacterial , anti-flammatory, antiviral and antioxidant effects, due to the presence of a-bisabolol, luteolin, quercetin, and apigenin (Kolodziejczyk-czepas *et al*., 2015).

Aim of the study

Genotyping of Tetracycline resistant *Acinetobacter baumannii isolates*, and this was obtained by the following objectives:

1. Samples were taken from patients in different ages with different sites.

2. Bacterial isolation and identification of *Acinetobacter baumannii* will be done by culture media, microscopic morphology (Gram stain) and Biochemical tests, with confirmation by vitek2 system.

3. Studying Tetracycline susceptibility by the application of different Tetracycline generations by disk-diffusion test and MIC against bacterial isolates and Detection of the effect of plant extract as inhibitor against tetracycline resistance mechanism.

4. Extraction of DNA from bacteria by using specific kit for DNA extraction, Detection of some tetracycline resistance genes and Genotyping of *Acinetobacter baumannii* isolates by Rep-PCR.

5. Cartwheel methods.

1.2. Literature Review

1.2.1. Characterization of *Acinetobacter baumannii*

1.2.1.1. Background

Acinetobacter baumannii was considered a low-category pathogen in the past, but has now emerged as a leading cause of many hospital- and community-acquired infections (Pourhajibagher *et al*., 2016).

Acinetobacter calcoaceticus-baumannii complex is a group of aerobic, non-fermentative, Gram-negative coccobacillus that encompasses four different *Acinetobacteria*, comprising *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, and *Acinetobacter calcoaceticus*. The first three are implicated in infections, while the latter is rarely considered pathogenic (Bouvet and Grimont, 1987; Nemec *et al*., 2016).

A. baumannii is almost be considered new pathogen and as many researchers suggested the first appearance of this pathogen was in Iraq specifically in military Iraqi War and was called “Iraqibacter”, infections of this pathogen associated with military and injured soldiers in combat due to direct environmental contamination of wounds due to considering *A. baumannii* to become the potential and serious problem in military hospital (Dewachi, 2019).

1.2.1.2. General Criteria of *Acinetobacter baumannii*

The most common properties that characterize genus *Acinetobacter* are: according to Gram staining these bacteria Gram-negative appeared in short rod with 1.5µm in diameter and 1.5-2.5µm in length during the logarithmic phase and coccobacilli or coccoid during the stationary phase and according to cultural properties this genus considered strict aerobic with non-motile also no-fermenting

and fastidious colonies, additionally positive to catalase and negative to oxidase (Monem *et al*., 2020).

On culture media, most of the clinical isolates of genus *Acinetobacter* grow at 35-37°C or higher (especially species *baumannii*), while others grow only at lower temperatures, appearance of colonies on blood agar is creamy or white and mucoid or smooth colonies, on MacConkey agar colonies are faint pink color which indicates the non-lactose fermenter isolates (Biswas and Rather, 2019).

On CHROMagar *Acinetobacter* which is selective medium developed for the rapid identification of *A. baumanniii* with the appearance of colonies as convex and light purple with halo, with rounded colonies. This medium contains incorporates enzymatic substrates that enable color based preliminary identification of bacterial colonies recovered within 18 to 24 h of incubation (Perry, 2017).

1.2.2. Epidemiology of *A. baumannii*

Acinetobacter spp. are free-living saprophytic organisms and widely distributed in different environments including soil, water, wastewater, vegetables, and skin of animals and humans. They have been isolated from various body parts of healthy individuals, including the nose, ear, throat, forehead, trachea, conjunctiva, vagina and perineum, axillae, groin, hands, and toe webs; however, most strains isolated were other than *A. baumannii* (Maravić *et al*., 2016). In hospital environment, they reside on beds, curtains, walls, roofs, medical devices, and equipment, as well as on belongings of medical personnel, tap water sinks, telephones, door handles, hand sanitizers, dispensers, trolleys, bins, and even on computers (Al Atrouni *et al*., 2016).

Acinetobacter species. are found in abundance in the environment, as *A. baumannii* is responsible for approximately 90% of all *Acinetobacter* spp. clinical infections in humans. Its ability to withstand harsh conditions, such as desiccation and disinfection, contributes to its longevity and spread in hospital settings. Acinetobacter spp. have developed a variety of resistance strategies (Abdulzahra *et al*., 2018).

The *A. baumannii* has a propensity to cause outbreaks and then to become endemic. The Centers for Disease Control and Prevention (CDC) reports that close to 40% of *A. baumannii* isolates are impenem resistant, and more than 60% are multidrug resistant. One important feature of *A. baumanniii* is its tendency to cause outbreaks because of its resistance to antimicrobials and its ability to survive for prolonged periods on dry surfaces (Harris *et al*., 2019).

Outbreaks of multidrug resistant (MDR)-*A. baumannii* have been found to be mainly transmitted via the hands of healthcare workers, and contaminated equipment and healthcare environment. The potential of cross-transmission increases **first,** The patients is heavily colonized, **second,** The surfaces surrounding the patients are colonized or if the number of patients colonized in the unit at the same time is high; thus patients colonized in the unit at the same time is high with resistant clones are the predominant cause of outbreaks. Patients at high risk of MDR-*A. baumannii* infections are those with mechanical ventilation, particularly in case of prolonged duration, those with longer hospital or ICU stay, or those with greater degree of exposure to infected or colonized patients in the neighboring hospital environment (Escudero *et al*., 2017).

A. baumannii is resistant to dehydration, ultra-violet radiation in addition to common chemical sanitizers and detergents making it extremely difficult to eradicate and the increasing resistance of *A. baumannii* to antimicrobial drugs, with

persistence in hospital environments and propensity to cause outbreaks, as it can persist for a prolonged period in harsh environments (walls, surfaces, and medical devices) in the hospital settings (Amudhan *et al*., 2011).

1.2.3. Pathogenicity of *Acinetobacter baumannii*

The pathogenesis of *A. baumannii* infections are represented by a combination of multiple virulence factors leads to a variety of its disease; thus to establish a successful colonization and productive infection in the host, *A. baumannii* developed many specific and non-specific virulence factors including adherence, biofilm formation, invasion, serum resistance, *in vivo* survival, and killing of host cells. Biofilm formation is one of the important factors that enhance its adherence to biotic and abiotic surfaces, including those of host tissues and medical devices. Capsular polysaccharide prevents *A. baumannii* from phagocytosis by macrophages and facilitates its multiplication in fluid from human ascites and serum. Phospholipases C, and phospholipases D, are estimated as probable virulence factors in *A. baumannii* as they lead to apoptosis of host cells, increase survival as a response to heat shock and desiccation, and enhance survival in human serum and epithelial cells invasion (Runci *et al*., 2019; Gautam *et al*., 2022).

A. baumannii is considered as a low-virulence pathogen, unless it is isolated from patients having comorbidities such as neonates with low birth weights and elderly patients with chronic illnesses such as malignancy (Islahi *et al*., 2015).

In human specimens the *Acinetobacters* were the most commonly isolated non-fermenters after *Pseudomonas aeruginosa*. Although 1-3% of health care-associated infections are caused by *Acientobacter* spp. but these bacteria have a little risk to healthy people. However, people with weakened immune systems,

chronic lung disease and diabetes may be more susceptible to be infected with these bacteria (Almasaudi, 2018). The infection process is usually associated with various risk factors especially in ICUs these factors include the use of contaminated equipment such as ventricular or urinary catheters as well as the previously colonized health care staffs in addition to prolonged staying at intensive care units increased the risk of colonization and infection. Colonization is not necessarily symptomatic but it definitely increases the likelihood of causing subsequent infections which will proceed if the host natural barriers were weakened by trauma, surgery or other invasive procedures (Meschiari *et al*., 2021).

1.2.4. Infections of *Acinetobacter baumannii*

The nosocomial infections that mostly caused by *A. baumannii* isolates include ventilator-associated pneumonia, endocarditis, secondary meningitis, urinary tract infection (UTI) as well as blood stream infections. Also can cause septicemia, pneumonia, burns, wounds and soft tissue infections especially in the Intensive Care Unit (ICU) and burn unit. *A. baumannii* may colonize the skin, oropharynx, and gastrointestinal tract without causing infection. However, among immunocompromised hosts, particularly patients in ICU settings, *A. baumanniii* may cause serious infections including bacteraemia and wound infections (Snyman *et al*., 2019). The incidence of such infections has increased in the last decade, with an associated mortality rate of between 30% and 75% in many parts of the world

A. baumannii has ability to survive in the high stress environments as well as resistance to many classes of antibiotics which make them for surviving and spreading as a nosocomial bacterium specially in patients due to increase in morbidity and mortality. The intrinsic and acquired resistance of *A. baumannii* to

antibacterial agents and its propensity to cause outbreaks of hospital-acquired infections, especially in ICUs, is a major health concern (Monem *et al*., 2020).

Acinetobacter spp. especially *A. baumannii*, are emerged all-around the world, and its isolates have been rarely isolated from food products, especially meat, milk and vegetable (Gurung *et al*., 2013). However, data about the clonality of *A. baumannii* from food are lacking, which precludes any speculation about the eventual exchange of *A. baumanniii* clones between food and clinical settings (Askari *et al*., 2020).

The most medically important nosocomial infections of *A. baumannii* are blood stream, urinary tract and surgical site infections; where the blood stream and UTIs are the most frequently reported types (Mitchell *et al*., 2017).

1.2.4.1. Urinary Tract Infections

Urinary Tract Infections caused by *A. baumanniii* are usually hospital acquired and related to urinary tract catheterization or surgery. UTIs which involves the bladder, ureter and kidney are the commonest type (Mitchell *et al*., 2017).

A. baumannii isolates responsible for 1.6% of ICU-acquired urinary tract infection; the setting of indwelling urinary catheters usually causes the colonization of the urinary tract, leading to nosocomial urinary tract infections. It is uncommon for this bacteria to lead to complexed urinary tract infection in outside of hospital depending on results of study by (Di venanzio, 2019).

1.2.4.2. Respiratory Infections (Hospital- and Community-Acquired Pneumonia)

Acinetobacter pneumonia is observed predominantly in ICU patients who are under mechanical ventilation, and however, sometimes it is not easy to distinguish between airway colonization from true pneumonia. *A. baumannii* is the second commonest pathogen among Gram-negative bacteria causing hospital-associated pneumonia. The hospital-associated pneumonia caused by *A. baumannii* was around 3-5%, with a death rate of 30-75% being reported (Gautam *et al*., 2022).

While the community-associated *Acinetobacter pneumonia* shows sudden onset, which progresses rapidly, causing respiratory failure and hemodynamic instability, though the infection is rare. It has been reported in people who consume alcohol or in patients with chronic obstructive pulmonary disease from tropical areas of Asia and Australia during monsoon (Jaruratanasirikul *et al*., 2019).

1.2.4.3. Bloodstream Infections

A. baumanni bacteremia is an increasingly common and often fatal nosocomial infection, as the vascular catheters and respiratory tract are the commonest sources for it, and the origin remains unknown in about 21–70% cases, where about 1.5-2.4 % of the patients acquired infections nosocomially (Sun *et al*., 2020).

These infections are associated with various risk factors, including prolonged hospital and ICU stay, mechanical ventilation, surgery and other invasive procedures, wounds, burns, use of broad-spectrum antibiotics, and immunosuppression (Garnacho-Montero *et al*., 2015).

1.2.4.4. Meningitis

Meningitis followed by neurosurgery induced by multidrug resistant *A. baumannii* is a relevant issue. The certain risk factor associated with it includes surgery involving the brain and spinal cord, cerebrospinal fluid leakage, prior antibiotic treatment, and intracranial hemorrhage. Studies have shown that the mortality rate was about 20-30% and the survivors being left with severe neurologic deficits (Sari *et al*., 2021).

In 2019 large number of disease states of meningitis in post neurosurgical patients which caused by *A. baumannii* show that 21% of isolates were extensively drug-resistant isolates which have sensitivity to only colistin and tigecycline. On the other hand, the pediatric meningitis cases with high mortality rate specifically in China are caused by the same pathogens cultures of cerebrospinal fluid showed Pandrug-resistant *A. baumannii* after the neuro surgery (Xiao *et al*., 2019).

1.2.4.5. Skin, Soft Tissue, and Bone Infection

The soft tissue infection progressing to osteomyelitis caused by contaminated surgical and traumatic wounds is seen in the case of *A. baumannii* infections. It rarely causes other skin infections such as cellulitis, folliculitis, skin abscesses, and necrotising fasciitis. The wound and soft tissue infections caused by multidrug-resistant *A. baumannii* are mainly recognized after war injuries. Among different isolated organisms, *A. baumannii* accounted for 32% of the war victims of combat casualties in Iraq and Afghanistan war (Motbainor *et al*., 2020). Where nosocomial infections with MDR organisms are major global health issues. They are very difficult for treatment and main causes of poor clinical outcome, morbidity, mortality, prolonged hospitalization and high health care costs (Huang *et al*., 2018).

Burn and Wound infection the primary reason for skin graft failure and the mortality of burn and wound patients, particularly those in burn intensive care centers, as the most common burn and wound colonizers is multi-drug resistant *A. baumanniii* (Akers *et al*., 2019). Therefore *A. baumannii* pathogens infecting wounds and burns (especially in wars) represent more dangerous and threat life. So these pathogens should be prevented by treating the non-healing wounds which are possibly develop to biofilms with strong vital antimicrobial resistance medications (Dallo and Weitao, 2010).

1.2.5. Antibiotic Susceptibility of *A. baumannii*

The antimicrobial resistance of *A. baumannii* is of major concern. It is classified as an ESKAPE pathogen, an acronym referring to *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, A. baumannii, P. aeruginosa* and Enterobacter spp., all of which are recognized as nosocomial bacteria with high potential to exhibit MDR and virulence. Clinical isolates *A. baumannii* strains are frequently reported to demonstrate resistance to the most routinely prescribed antibiotics in varying degrees (Tacconelli *et al*., 2018).

A. baumannii has emerged as a significant cause of healthcare-associated infections, particularly in ICUs around the world. One of the most concerning features of this organism is its ability to rapidly develop resistance to multiple antibiotics, which has resulted in limited treatment options and increased morbidity and mortality rates. Understanding the antibacterial activity of *A. baumanniii* is critical for the development of effective treatment strategies and the prevention of the spread of this pathogen (Turton *et al*., 2010).

The *A. baumannii* resist antibiotic action through various mechanisms, including target alteration, drug inactivation, decreased permeability, and increased

efflux (Martins, 2013). As *A. baumannii* is known to have intrinsic resistance to several classes of antibiotics, including penicillins, first-generation cephalosporins, aminoglycosides and fluoroquinolones. This resistance is primarily due to the presence of efflux pumps, which actively pump out antibiotics from the bacterial cell before they can exert their antimicrobial effect. Additionally, *A. baumanniii* has a low permeability outer membrane, which restricts the entry of hydrophilic antibiotics, such as beta-lactams, and contributes to their reduced susceptibility (Asif *et al*., 2018).

In addition to intrinsic resistance, *A. baumanniii* has become increasingly adept at acquiring resistance to antibiotics through the acquisition of resistance genes via horizontal gene transfer. These genes can be carried on plasmids or transposons and can confer resistance to multiple classes of antibiotics, as the last-resort class of antibiotics for the treatment of multidrug-resistant infections. This ability to rapidly acquire and disseminate antibiotic resistance genes has made *A. baumanniii* a significant threat to public health, as the *A. baumanniii* has natural competence to incorporate exogenous DNA, and its genome incorporates foreign DNA at high frequencies, implying frequent horizontal gene transfer in this pathogen and it has amazing abilities to acquire drug resistance genes through these variety of mechanisms (Traglia *et al*., 2014).

1.2.5.1. Tetracyclines Resistance of *A. baumannii*

The Tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis, as it bind to the 30S subunit of the ribosome and preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Tetracyclines are bacteriostatic, broad-spectrum agents, exhibiting activity against a wide range of Gram-positive and Gram-negative

bacteria, atypical organisms such as *chlamydiae*, *mycoplasmas*, and *rickettsiae*, and protozoan parasites. The favorable antimicrobial properties of these agents and the absence of major adverse side effects has led to their extensive use in the therapy of human and animal infections (Beheshti *et al*., 2020). Tetracycline molecules comprise a linear fused tetracyclic nucleus (important unit for antibacterial activity) to which a variety of functional groups are attached that determine the different members and generations of Tetracyclines, as Tetracycline HCL is considered short-acting, and Doxycycline and Minocycline are long-acting, each having extended serum half-lives with more potent spectrums against some bacterial species (Pogue *et al*., 2011).

These alterations increase the molecule lipophilic properties facilitating tissue penetration and improving antibacterial activity. Minocycline is the most lipophilic of all Tetracyclines, and this compound has been recognized as the most potent agent in this class, followed by Doxycycline. Furthermore, Minocycline and Doxycycline have the capability to overcome many Tetracycline resistance mechanisms (Castanheira *et al*., 2014).

Tetracyclines are a class of antibiotics that have been used to treat *A. baumanniii* infections, but their efficacy has been limited by the emergence of resistant strains (Viehman *et al*., 2014). Streptomyces species were the source of

Tetracycline, which is considered as the first generation of Tetracyclines. Semisynthetic Tetracyclines, doxycycline and minocycline, as the second generation of this group have wider spectrum. Tigecycline, a glycylcycline is referred to the third generation (Liu *et al*. in 2021).

Tigecycline, being the first member of glycycline, is a novel drug approved by the US Food and Drug Administration in June 2005 for the treatment of complicated skin infections, community-acquired pneumonia, and intra-abdominal infections. It is also being used in the treatment of bacteremia and UTIs by multidrug-resistant (MDR) Gram-negative bacteria. It is active against a wide number of Gram-positive and Gram-negative bacteria including anaerobes. It has shown effectiveness against *A. baumannii* and other species of *Acinetobacter* in large number of studies (Pournaras *et al*., 2016).

In comparison with Tetracyclines, Tigecycline binds to corresponding ribosomal sites with greater affinity, and irrespective of the presence of mutations that confer resistance to Tetracyclines. Furthermore, Tigecycline evades Tetracycline efflux mechanisms (Babaei and Haeili, 2021).

The first case of Tigecycline resistance was reported by (Sader *et al.*, 2005) and in (Navon-Venezia *et al*., 2007) reported 66% Tigecycline resistance against *A. baumannii* in Israel. At times, varying percentages of resistance have been reported all over the world, with Turkey possessing the highest resistance rate (81%) (Dizbay *et al*., 2008). They also noted that some strains of *A. baumannii* were resistant to all Tetracyclines tested, including Tigecycline (Beceiro *et al*., 2011).

The most important mechanisms of resistance to Tetracyclines in *A. baumannii* isolates are efflux pumps followed by ribosomal protections and enzymatic inactivation (Viehman *et al*., 2014).

The mechanisms of Tetracycline resistance in *A. baumannii* that resistance was primarily mediated by the acquisition of genes encoding efflux pumps and ribosomal protection proteins (Twenty-nine different tetracycline resistance (*tet*) genes had been characterized.). These genes encode for proteins that belong to the

major facilitator superfamily. These proteins are located in the cytoplasmic membrane and decrease the tetracycline intracellular concentration by exchanging a proton for the tetracycline-cation complex (Castanheira *et al*., 2014).

Resistance to the first and second generations of Tetracyclines in *A. baumannii* isolates mainly resulted from the acquired major facilitator superfamily (MFS) efflux pumps, including *tetA*, *tetB*, *tetG*, *tetH*, *tetL*, and *tet39*, resistance nodulation division family (RND) efflux pumps nominated as *adeABC*, *adeIJK*, *adeFGH*, *adeM*, *adeDE*, and finally ribosomal protections *tetM* and enzymatic inactivation. *tetA* is responsible for the resistance to Tetracycline and Doxycycline whereas *tetB* has been found in the isolates that were also resistant to minocycline. It has also been found that Tet(A) pump acts synergistically with the resistance–nodulation–division (RND) superfamily of efflux pumps, such as AdeABC and AdeIJK, serving as an important resistance mechanism of resistance to Tigecycline in *A. baumannii* (Foong *et al*., 2020).

The genetic basis of these determinants was detected as a partially characterized Tn1721-like transposon containing the *tet(R)* and *tet(A)* genes, encoding, a regulatory protein and a resistance protein15, respectively, and *tet(B)* is carried by 5 to 9-kb plasmids in the multidrug resistant *A. baumannii* (Agers *et al*., 2007; Ardebili *et al*., 2012).

1.2.5.2. Efflux Pump

One of the major antimicrobial resistance mechanisms is the efflux pumps. They are membrane proteins that bind to substrates (often are antibiotics), and actively catalyze their translocation in an outward direction leading to a reduction in their intracellular concentrations and thus decrease the therapy efficacy.

Therefore, efflux systems can confer a multiple antimicrobial drugs resistance, causing serious public health concerns worldwide (Pu *et al*., 2017).

Additionally, efflux pump was required for the full virulence of *A. baumanniii* and it is also required for the biofilm formation of *A. baumanniii*, which is an important factor in the persistence and spread of *A. baumannii* and (Lin *et al*. in 2019). As the pumps are critical for bacterial resistance to exclude most of the unwanted entities until the cell gets required time for acquiring resistance (Venter *et al*., 2015).

The efflux systems are classified into two categories based on the mechanism by which they derive energy. The primary efflux pumps obtain energy by ATP hydrolysis, whereas the secondary efflux pumps derive energy from electrochemical potential difference created by pumping out Na+ and H+ outside the membrane (Dwivedi *et al*., 2017).

Secondary active transporters are highly substrate specific and their recognition sites are often antimicrobial drugs targets. Five major efflux pump families have been found in prokaryotes: ATP binding cassette (ABC) family, small multidrug resistance (SMR) family, multidrug and toxin extrusion (MATE) family, major facilitator superfamily (MFS) and resistance nodulation cell division (RND) family. The ABC family is primary active transporters, while, MFS, SMR, MATE and RND families are secondary active transporters. Bacterial efflux systems can be specific, extruding only one antibiotic class or MDR, extruding several antibiotic classes (Sharma *et al*., 2016).

Though efflux pumps from other families contribute to the antimicrobial resistance against certain antibiotics, RND pumps are the most potent drug efflux systems conferring resistance against clinically important antibiotics and biocides. Members of this family are known for their roles against a wide range of molecules

with dissimilar structures including antibiotics, biocides, organic solvents, antimicrobial peptides, detergents, dyes, and bile salts (Venter *et al*., 2015).

Furthermore, *A. baumannii* is known to harbor several horizontally acquired tet efflux pumps belonging to the MFS that confer tetracycline resistance, with an array of putative efflux pumps that may confer antibiotic resistance

Efflux pumps are a significant challenge in the management of *A. baumanniii* infections, and understanding their mechanisms and interactions with other resistance mechanisms is crucial for the development of effective treatment strategies. Research into new efflux pump inhibitors and combination therapies shows promise for overcoming multidrug resistance and improving outcomes for patients with *A. baumanniii* infections (Hirsch *et al*., 2016).

The genetic and biochemical mechanisms underlying the expression and function of the efflux pump was regulated by a regulatory system which responds to the presence of antibiotics in the environment; also efflux pump is capable of pumping out multiple classes of antibiotics, including tetracyclines, fluoroquinolones, and aminoglycosides. The efflux pump was also able to pump out dyes and detergents, indicating that it has a broad substrate specificity (Coyne *et al*. in 2010).

The poly-specificity of efflux pumps, their overexpression in response to drugs along with the phenomenon of heteroresistance seem key factors responsible for drug-resistance in a wide-range of bacterial species, especially in Gram-negative bacteria making them difficult to treat with conventional drug arsenal. The drug-efflux mediated bacterial drug resistance is an increasing threat to global healthcare, therefore they are gaining unprecedented attention not only from the perspectives of basic understandings that how they work and impart drug resistance but also as emerging targets for development of novel and potent adjunct

therapies for combating drug resistance in community and nosocomial infections. As a result, inhibition of drug efflux from bacterial cells via inhibiting or disrupting the pumps is an emerging approach for combating these threats (Shriram *et al*., 2018).

1.2.5.3. Effect of *Matricaria chamomilla* (chamomile بابونج) against Tetracyclines-Resistant *A. baumannii*

The most important aspect of the infection with *A. baumanniii* strains is their resistance to entirely known antibiotics, with rising antibiotic resistance and treatment difficulties suggesting the need for urgent action by the global health care community to find alternative drugs; many efforts to develop new antibiotics to combat *A. baumannii* have been largely unsuccessful, and alternative treatment strategies have focused on combination therapies, including the use of non-antibiotic agents such as efflux pump inhibitors or antibodies. Additionally, infection prevention and control measures, such as hand hygiene, environmental cleaning, and antimicrobial stewardship, are critical in reducing the spread of *A. baumannii* and limiting the development of antibiotic resistance (Intorasoot *et al*., 2017).

This continuous emergence of antibiotic-resistant bacteria urges the searching for alternative therapeutic options. A combination therapy of efflux pump inhibitors (EPIs) and antibiotics can be a promising option for the efflux-mediated bacterial resistance. For any compound to be used as an EPI, it must be selective, not target any eukaryotic efflux pumps, has ideal pharmacological properties, such as the non-toxicity and the high therapeutic effect. Many EPIs have been discovered, but, none of them was clinically approved because of their

low potency, narrow activity spectrum, inappropriate pharmacokinetics or high toxicity (Sjuts *et al*., 2016).

Combined actions of antibiotics and active components of plant extracts have been studied mostly as an alternative strategy. Many studies stated that the synergistic action of plant active components and the antibiotics could play a role to combat drug resistance and increase bacterial susceptibility, where many plant active compounds are being used worldwide as traditional remedies against several antibiotic-resistant bacteria, including *A. baumannii;* for example, flavones, tannins, and phenolic compounds are demonstrated to have inhibitory activity against *Acinetobacter* (Alanis-Garza *et al*., 2018).

Due to the safety of natural products derived from plant materials, they gained global attention as new antimicrobial compounds, particularly in traditional medicine. It is well-established that the secondary metabolites of plants possess antibacterial properties and the ability to modulate resistance. The adverse effects of conventional antibiotics can be minimized by the use of such compounds. Combination therapy has proven effective in the treatment of Gram-negative bacterial infections (Sinha *et al*., 2019).

The inhibition of active drug efflux by an inhibitor results into the elevated intracellular antimicrobial concentrations, and lowered or complete reversal of efflux-mediated bacterial drug resistance, prevention of microbial invasiveness by inhibiting the export of virulence factors and shortened adaptation time required for bacteria, prohibiting the emergence of mutant strains with high resistance (Shriram *et al*., 2018).

Major strategies developed for drug efflux inactivation are, **first**, alterations in regulatory mechanisms for activation/repression of pump gene expressions, **second**, deprivation of motive forces required for working of pumps by

diminishing the proton gradient, **third** structural modifications in existing antimicrobials to bypass the chemophore recognition by the pumps, **fourth** disrupting the pump-functionality by preventing assembly of pumps by targeting protein interfaces; interaction between protein motifs, thus obstructing the exit duct, and **fifth**, the trapping of pumps in the inactivated form by competitive binding of inhibitors and cytoplasmic membrane proteins (Abdali *et al.*, 2017).

Matricaria chamomilla also called *Matricaria recutita* from the *Asteraceae* family. In various cultures, the so-called chamomile is one of the prominent plant in traditional medicine as it is rich in active ingredients; it was reported to treat colic and diarrhoea as well as mouth, throat, and ear infections and infections in general; it like other medicinal herbs are a rich source of various biologically active compounds that are produced as secondary metabolites (Ginko *et al*., 2023).

For knowledge, Chamomile LD50 exceeded 5 g/kg. There is no standard dose of chamomile. Studies have used between 900 milligrams to 1200 milligrams daily in capsule form. The most common form is a tea, and some people drink one to four cups daily.

Chamomile is used both internally and externally to treat an extensive list of conditions. It is used externally for wounds, ulcers, eczema, gout, skin irritations mastitis and leg ulcers; as it is used topically by pads. Also used internally as a tea to treat many psycho- and neurological conditions in addition to treatment of wounds, sunburn and burns (Bown, 1995), Also Chamomile is widely used to treat inflammations of the skin and mucous membranes, and for various bacterial infections of the skin, oral cavity and gums, and respiratory tract, where drinking chamomile was associated with a significant increase in urinary levels of hippurate and glycine, which have been associated with increased antibacterial activity (Wang *et al*., 2005).

It shows antibacterial effects; thus it is widely applied as part of a cream for atopic eczema (Boroujeni et al., 2017). A mouthwash containing 1% chamomile extract was also successful in a clinical test in patients with gingivitis, as it reduces biofilm accumulation and bleeding (Goes et al., 2016).

Chamomile has exhibited bactericidal activity, with 120 chemical constituents have been identified as secondary metabolites, including 28 terpenoids, 36 flavonoids and 52 additional compounds with potential pharmacological activity, with anti-inflammatory activities have been attributed to some of chamomile's active ingredients, especially the flavonoids (anthemidin, apigenin, luteolin, among others), bitter glycosides, coumarins (herniarin and umbelliferone) and its volatile oils (containing alpha bisabolol and matricine, among others) (Dai *et al*., 2023).

EPIs are the substances that offer the most promising approach to blocking efflux pumps. They are the molecules that interfere with the process of removing toxic substances and antibiotics from the bacterial cell. Efflux pump inhibitors act as adjuvants to potentiate the activities of conventional medicines by inhibiting them either competitively or non-competitively. Several compounds have been discovered which are used as EPIs, with more techniques were required to design and quantify them and measure their kinetic parameters in the efflux pump components. These parameters were necessary for choosing between the general EPI that can stop the action of one transporter that expels various antibiotics in one species of bacteria or a specific EPI that stops the pumping of one antibiotic family in many bacteria (Rafiei *et al*., 2022; Wang *et al*., 2022).

Plant-derived EPIs are synergistic enhancers of drugs. Though they may not have any antimicrobial properties alone, when they are taken concurrently with standard drugs, they enhance the effect of that drug. Therefore, efflux pumps are

viable antibacterial targets, and the development of potent efflux pump inhibitors is a promising and valid strategy for restoring the susceptibility of resistant strains of bacteria to antibiotics that are substrates of efflux pumps (Cheesman *et al*., 2017; Reygaert, 2018).

1.2.5.4. Detection of Efflux Pump Activity

Multi-drug resistance can be the result of an over-expression of the bacterial efflux pump systems that expel unrelated antibiotics prior to their reaching the intended targets, thus there is a need to develop and implement improved methods for a real-time and quick identification of efflux mediated MDR phenotypes. Primarily, assessment of efflux activity has been conducted using a common substrate of efflux pumps, such as ethidium bromide (EtBr) that is widely used to monitor efflux in a given bacteria; for this reason to use Cartwheel assay which is a simplest, instrument-free, agar-based method that relies on the ability of bacteria to expel EtBr, which is a substrate for many efflux pumps. Efflux pump inhibitors (EPI) cause accumulation of EtBr within the cells and induce fluorescence at a much lower concentration of EtBr (Amaral *et al*., 2011; Bankan *et al*., 2021).

The principle of this fluorometric assay is the passage of EtBr across the cytoplasmic membrane and its subsequent intracellular accumulation inside the bacterial cell, it traverses the bacterial cell wall (in the case of Gram-negative bacteria *via* porin channels) and once inside, it can be concentrated to a point where it fluoresces when excited by ultraviolet light. Efflux pumps of MDR bacteria recognize this substrate and are able to extrude it to the medium. These efflux systems are temperature dependent and this process will continue if the concentration of EtBr in the culture medium does not overcome the capacity of the bacterial efflux pump itself. Therefore, loading of the bacteria with EtBr has to

take place at a concentration that is well below its minimum inhibitory concentration (MIC) (Blair and Piddock, 2016).

Where EtBr is a DNA-intercalating agent that fluoresces only when bound to DNA. Therefore, fluorescence is higher when intracellular accumulation than extracellular, and this is used to measure the amount of accumulation and pump activity (Martins *et al*., 2013).

When studying the efflux of antimicrobial substrates, it has been commonplace to use drug susceptibility measurements (such as the MIC) to reveal differences in drug efflux activity. The reason for using this method is that a bacterium with greater expression of an efflux pump will be less susceptible to various antimicrobials than its comparator with lower efflux pump expression. Thus EtBr- agar Cartwheel method is a good screening marker for efflux activity. It is easy to perform, less time-consuming and can be used to screen large numbers of bacterial strains, thereby facilitating the rapid identification of isolates displaying an MDR phenotype (Salumi and Abood, 2022).

1.2.6. Genotyping of *A. baumannii*:-

For bacterial typing to be useful, the development, validation and appropriate application of typing methods must follow many criteria. The ability to quickly and reliably differentiate among related bacterial isolates is essential for epidemiological surveillance systems. There are several typing methods used in laboratories today (Van Belkum *et al*., 2007).

The methods for bacterial phenotyping have a clear purpose in the confirmation and clarification of local and national healthcare-associated outbreaks due to bacterial strains these range from methods based on simple phenotypic features to DNA sequencing. Previously, the comparison of phenotypic characters,

such as colony morphology, color, odor, antibiogram-based typing, biotyping, serotyping and the ability to grow in the presence of specific substances, were used for differentiation. Today these methods are becoming old because they require strict standardization of experimental conditions since phenotypes are quite susceptible to the environmental conditions (Monem *et al*., 2020).

Microbial genotyping technologies are a significant tool for molecular epidemiology researches, especially in understanding pathogen transmission and population dynamics (Cinar *et al.*, 2020).

Molecular typing is becoming a paradigm for understanding the fundamental mechanisms of Acinetobacter infections in hospital settings to investigate the spreading, the clonality relationship among bacterial strains and its geographical spread. In infection control, molecular typing methods are currently an important tool to measure and identify the source of the original infection in hospitals outbreaks. Molecular typing methods have been used to investigate the nosocomial spread of *A. baumannii* outbreaks in hospital settings worldwide. Some examples of these techniques are plasmid typing, ribotyping, pulsed field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) based fingerprinting. As compared with other methods, PCR based fingerprinting methods are easier to perform and cost effective (Aljindan *et al.*, 2018).

There are dramatic increase in the use of different molecular methods for typing purposes, as they are more stable and less dependent on growth factors. Such methods are time effective and are used in finding the relationships between the microbial isolates and putting strains in specific groups; especially using ones evaluating the repetitive elements in bacterial genome due to the stability of such elements during evolution (Chan *et al*., 2020).

1.2.6.1. REP-PCR (Repetitive Extragenic Palindromic PCR) :-

Rep-PCR (repetitive extragenic palindromic PCR) is a genotyping technique that is widely used for strain typing and epidemiological investigations of bacteria. This technique targets the repetitive DNA sequences present in bacterial genomes, which are known as extragenic palindromic (REP) sequences. REP sequences are present in multiple copies throughout the genome, and their distribution and arrangement vary among different bacterial strains. The presence of these repetitive DNA sequences in genomes of *A. baumannii* was confirmed by several studies and by the application of different methods for the differentiation of *A. baumannii* strains and track the spread of antibiotic-resistant strains within healthcare settings (Villalón *et al.*, 2015).

Repetitive extragenic palindromic sequences (REPs) are a distinct class of abundant repeats important in regulation of certain bacterial functions. REPs are known to interact with several partners, by providing binding sites for proteins such as Integration Host Factor and DNA polymerase I, and providing the necessary cleavage sites for DNA gyrase to unwind DNA. REPs also increase mRNA stability and can cause transcription termination. It discovered nearly more than 30 years ago in enteric bacteria, REPs are 35-40 nucleotides long stem-loop structures often organized into larger units called bacterial interspersed mosaic elements (BIMEs). BIMEs comprise two REPs in inverse orientation separated by a linker sequence. One is called REP and the second inverted sequence is designated an iREP. REPs are found dispersed throughout the chromosome in many bacterial species, often in high copy number. They represent, for example, up to 1% of *E. coli* chromosomes. They are so frequent that their presence serves as the basis for ‘REP-PCR’ a method for the rapid identification of bacterial strains (Messing *et al*., 2012; Selim *et al*., 2022).

In Rep-PCR, a primer set is designed to amplify the REP sequences from bacterial genomic DNA. The resulting PCR products (diverse-sized DNA fragments) are then separated by electrophoresis and visualized using gel electrophoresis or other detection methods. The resulting banding patterns (Multiple amplicons of different sizes) are unique to each strain, and can be used to differentiate between different bacterial isolates (Meshkat *et al*., 2017).

Rep-PCR has been used extensively for genotyping and epidemiological studies of *A. baumanniii*. Studies have shown that Rep-PCR is a highly discriminatory method that can differentiate between *A. baumanniii* strains with high resolution. This technique has been used to identify outbreak strains, determine the spreading of antibiotic-resistant strains, and study the genetic diversity of *A. baumanniii* populations in different geographical regions (Ali *et al*., 2019).

Chapter Two

Materials and Methods

2. Materials and Methods:

2.1. Materials:

2.1.1. Equipment and Instruments:

Equipment and instruments used in the study were listed in **Table (2-1**).

Table (2-1): Equipment and Instruments Used in the study.

Equipment	**Manufacturing Company/ Origin**
Autoclave	Jeiotech/ South Korea
Centrifuge	Hettich / Germany
Deep-freezer	Hicool/ Denmark
Electrical balance	KERN/ Germany
Eppendorf tubes	Sterellin Ltd /UK
Gel electrophoresis apparatus	Cleaver/USA
Incubator, Shaker	Memmert/ Germany
Light microscope	Olympus /Japan
Microcentrifuge	Hettich/ Germany
Millipore filter (0.45 µm)	Schleicher and Schuel / USA
Nanodroop	Avans/UK
PCR Thermocycler	Clever/ England
Refrigerator	Crafft/ Saudiaarabia
Safety cabinet (HOOD)	Gallenkamp/ USA
UV- transilluminator	Cleaver/USA
Vitek 2 compact	Biomerieux/ France
Vortex	Fanem/ Brazil
Water bath	Memmert/ Germany
Water distiller	Paytekht Co.Ltd/ Iraq

2.1.2. Chemical and Biological Materials:

Chemical and biological materials used in this study were summarized in the **Table (2-2)**:

Table (2-2): Chemical and Biological Materials Used in this Study.

Chemical and biological material	Manufacturing Company/ Origin
Agarose	Promega/USA
Ethanol 70%, Absolute	BDH / UK
Ethidium bromide	Promega/USA
Glycerol	BDH / UK
Gram Stain kit	HiMedia/ india
Hydrogen peroxide (H_2O_2)	BDH / UK
Isopropanol 99%	BDH / UK
McFarland tube standard (0.5)	Mastgroup/England
N-N-N-Ntetramethyl-P-phenylene diamine dihydrochloride	Hi-Media/ India
Normal Saline	PSI/ Saudi Arabia
Nuclease Free Water	Promega/USA
Tris-Borate-EDTA (TBE) buffer	Promega/USA

2.1.3. Culture Media:

All the culture media were used in this study are listed in **Table (2-3**).

Table (2-3): Culture Media Used in this Study

Media	**Manufacturing Company/ Origin**
Agar-Agar	Himedia /India
Blood agar base	
Brain heart infusion broth & agar	
MacConkey agar	Oxoid / England
Mueller Hinton agar	
Nutrient broth & agar	
Trypticase Soy Agar	

2.1.4. Kits:

The diagnostic kits used in the current study were presented in Table 2-4.

Table (2-4): Diagnostic Kit Used in this Study.

Kit	**Manufacturing Company / Origin**
DNA extraction kit	Geneaid⁄USA
DNA ladder 100bp	Bioneer⁄Korea
Master mix Kit	Bioneer⁄Korea
Vitek 2 System	Vitek 2 System

2.1.5. Antibiotics:

2.1.5.1. Antibiotic Disks:

The antibiotic disks used in the current study for each isolate was determined and recommended by CLSI (2020), were presented in **Table (2-5).**

Table (2-5): Antibiotic Discs Used in this Study

Antibiotic	Code	Disk potency (µg/disc)	Manufacturing Company / Origin
Doxycycline	DOX	30	Bioanalyse/) (Turkey
Minocycline	MIN	30	
Tetracycline	TE	30	
Tigecycline	TIG	15	

2.1.5.2. Antibiotic powders:

The antibiotic powders used in the current study recommended by CLSI (2020), were presented in **Table (2-6).**

Table (2-6): Antibiotic powders used in the study

Antibiotics	concentration	Company	Origin
Doxycycline	100 mg/ Capsule	Medochemie	Europe
Minocycline	105 mg/ Tablet	Hikma	Iran
Tetracycline	250 mg/ Capsule	Ajanta	India
Tigecycline	50 mg/ Vial	LYKA lab	India

2.2. Methods

2.2.1. Reagents and Solutions

2.2.1.1. Reagents

A- Catalase Reagent:

It was prepared by adding 1ml of 30% Hydrogen peroxide to 9 ml of D.W., the final concentration of the solution was 3% and then it was kept in a dim bottle (Procop *et al*., 2017). This reagent was used for detecting capability of bacterial isolates to produce catalase enzyme.

B- Oxidase Reagent:

It was prepared by dissolving 1gm of N-N-N-N tetramethyl-P-phenylene diamine dihydrochloride in 100 ml of distilled water and kept in a dark bottle in refrigerator. This solution was used to detect the capability of bacterial isolates to produce oxidase C (Shields and Cathcart, 2010).

2.2.2. Preparation of Culture Media

2.2.2.1. Ready-Culture Media:

Ready-made culture media mentioned at **Table (2-3)** were prepared according to the manufacturing company instructions, while the other culture media were prepared in the laboratory according to the scientific references. All these media (with some exceptions) were autoclaved at 121°C for 15 minutes at 15 pound per square inch (Brown & Smith, 2014).

McConkey agar is used to isolate most Gram-negative bacteria and used to differentiate between lactose fermenter and non-fermenter bacteria.

Nutrient agar was used in general experiment such as cultivation and activation of bacterial isolates when it is necessary.

Trypticase Soy Agar used for detection of efflux pump activity by EtBr Cartwheel assay. Müller-Hinton Agar used for an antibiotic sensitivity test

2.2.2.2. Laboratory Prepared Culture Media:

A- Blood Agar:

According to the manufacturer company, Blood agar base was prepared. Then was autoclaved and cooled to (45-50)ºC, aseptically adding 5 ml of fresh human blood for each 95 ml of the medium mixing well to homogeneity, distributed into sterile Petri dishes. Finally, the prepared medium was cooled to 37ºC and was left to solidify at room temperature (25ºC) (Harley and Prescott, 1996).

B- Brain Heart Infusion (BHI) Broth-Glycerol Medium (Maintenance Medium):

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

C- Motility Semi-Solid medium:

This medium was prepared as nutrient broth with the addition of agar-agar (0.5%). The prepared medium was first dispensed in distilled water then pH was adjusted to 7-7.4, then 10 ml from this medium was dispensed in each sterile vial then autoclaved at 121ºC and 15 pound/inch2 for 15 minutes. Finally cooled to 37ºC and left to solidify at room temperature (25ºC) then used for identifying motile and non-motile bacteria (Forbes et al., 2007).

2.2.3. Ethical Approval:

The necessary ethical approval was taken from the ethical committee of College of Medicine/ University of Babylon and Al-Hilla General Teaching Hospital according to the document number 310 (at 26/10/2022), Moreover, agreement from the family and patients for sampling and carrying out this work was obtained.

2.2.4. Clinical Specimens' Collection:

One hundred and twenty five (125) clinical specimens were collected during the present study period extended from the beginning of October 2022 to the end of January 2023, from patients who were attended to Al-Hilla General Teaching Hospital; it was collected from different clinical sites suggested to have infection such as urinary and respiratory infections, burns and wounds infections.

Urine (mid-stream urine) was collected from patients suffering from symptoms of UTIs in a sterile screw-cap container. Swabs from the burns and wounds. Sputum were collected from patients before they take any antibiotics or cleaning. The samples were transferred immediately to the laboratory. Each sample was cultured on different media including MacConkey and nutrient agar then incubated aerobically at 37 C^{o} for 24 hours.

2.2.5. Isolation of Bacteria

In the laboratory and under aseptic conditions, the collected isolates were re-identified by culturing directly on blood agar and MacConkey agar, and all the isolates were incubated for 24 hours at 37^{o}C. The non- hemolytic opaque creamy colonies on blood agar and non-lactose fermenting on MacConkey agar.

2.2.6. Study design: Cross-sectional study.

The design of study was illustrated in figure 2-1.

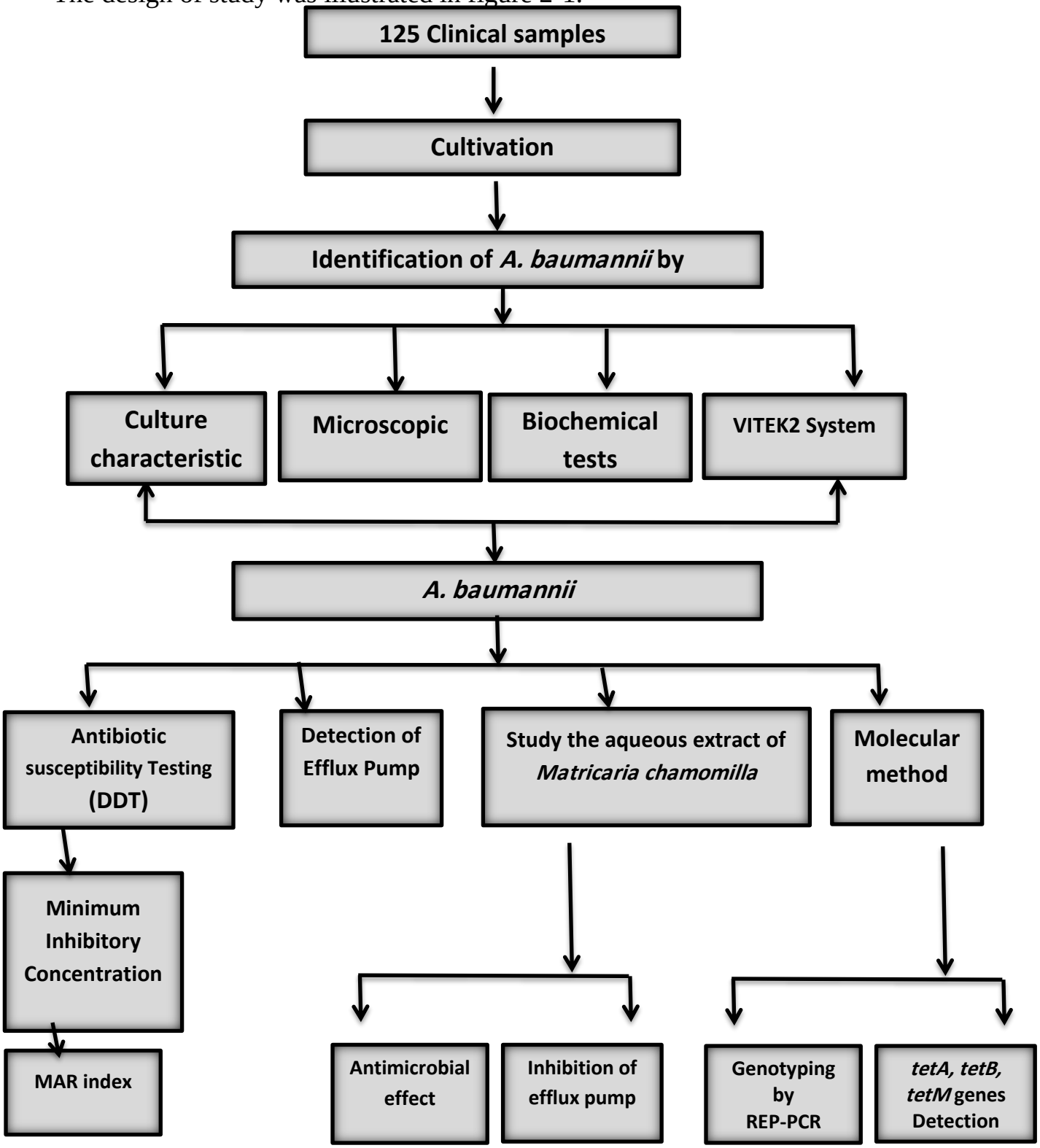

Figure (2-1): Scheme of the Study Design.

Then isolates are sub-cultured on another MacConkey agar plate and were incubated for another 24 hours at 37ºC to obtain pure well isolated colonies (Bailey and Scott, 1962; Forbes *et al*., 2007). The positive control of *A. baumannii* American type culture collection(ATCC, 19606) is a private, nonprofit, global biological resource center and standards organization that provides scientists with the biomaterials and resources they need to conduct critical life science research.The ATCC was cultured and the results compared with all isolates to give 100% consistency.

2.2.7. Bacterial Identification:

2.2.7.1. Microscopic Examination (Gram Stain):

One isolated colony was transferred to a microscopic slide; fixed then stained with Gram stain, cell shape and arrangement were recorded. The results were compared with (Brooks *et al.*, 2013).

2.2.7.2 Biochemical Tests

All the biochemical tests were carried out according to (Brown & Smith, 2014):

A- Catalase Test

On a clean microscope slide; this test was performed by using the end of a wooden swab, some cells from the bacterial culture were transferred to the surface of a clean microscope slide, two to three drops of 3% hydrogen peroxide **(2.2.1.1.A)** were added to the cells, then were mix with the wooden stick, and vigorous bubbles were observed indicating positive results.

B- Oxidase Test

A filter paper was saturated with little drops of 1%N-N-N-N-tetramethylpara- phenylenediamine dihydrochloride **(2.2.1.1.B)**. With a woody applicator, growth from an agar medium was smeared on the paper. Formation of purple color within 10 seconds indicates a positive results.

C- Motility Test

Motility of bacterial isolates was checked in semi-solid motility medium **(2.2.2.2.C)**. Cultures were inoculated with a sterile loop or needle into the center of the tube and incubated at 37°C for 24 hr. Movement away from the stab line or a cloudy appearance around the stabbing area indicated a motile organism.

D- Hemolysin Production

This test was used to detect hemolysin production on blood agar previously prepared at **(2.2.2.2.A.).** The plates were cultured with the tested bacteria by streaking method and incubated at 37˚C for 24 hours. A hemolysis around the colony should be observed for the positive result.

2.2.7.3. Vitek 2 System Diagnosis:

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

This system consists of:

1- A personal computer.

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

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

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

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

3- The standardized inoculums were placed into the cassette.

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

2.2.8. Preservation of Isolates:

2.2.8.1. Short Term Preservation:

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

2.2.8.2 Long Term Preservation:

Preservation media prepared at **(2.2.2.2.B)** was used for long term preservation (for months) as it should be stored at -20°C (Vandepitte *et al.*, 2003).

2.2.9. Tetracyclines Susceptibility Test:

2.2.9.1. Disk-Diffusion Test:

Kirby-Bauer method used to perform the antibiotic susceptibility test for four Tetracyclines' members; where bacterial suspension was prepared by picking 4-5 colonies of each bacterial isolate from pure culture and was suspended into a test tube containing 5 ml of normal saline, then turbidity was adjusted to obtain approximately 1.5 $x10^8$ CFU/ml (MacFarland 0.5 tube). By a sterile cotton swab a portion of bacterial suspension was transferred carefully and evenly spread on Mueller-Hinton agar medium, and then it was left for 10 min to dry. Then after the antimicrobial disks were placed on the agar surface with sterile forceps pressed firmly to ensure contact with the agar. Later the plates were inverted and incubated at 37 C° for 24 hr. Inhibition zones that developed around the disks were measured by millimeter (mm) unit by using a metric ruler. The isolate was interpreted as susceptible, intermediate, or resistant to particular antibiotic according to CLSI (2020).

2.2.9.2. Calculation of Multiple Antibiotic Resistance Index (MAR):

Multiple antibiotic resistance (MAR) index was determined for each isolate by using the formula:

MAR = a/b

where **a** represents the number of antibiotics to which the test isolate illustrated resistance and **b** represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility in the same study (Sandhu *et al*., 2016) and interpreted according to Krumperman (1983).

Where isolate showing index more than 0.2 can be classified as MAR, and those with index equal to one means that is resistant to all used antimicrobial types (Osundiya *et al*., 2013).

2.2.9.3. Determination of Minimum Inhibitory Concentration:

The two-fold agar-dilution susceptibility method was used for determination of MICs of Tetracyclines mentioned at **Table (2-6)** according to (CLSI, 2020 against all *A. baumannii* isolates. As **Table (2-7)** showed the acceptable break point (µg/ml) for quality control strain (*A. baumannii* (ATCC, 13304) (CLSI 2020), with the range of concentrations for each type of Tetracyclines' members.

Table (2-7): Break point (µg/ml) for quality control strain (*A. baumannii* (ATCC, 13304) and the range of concentrations for each type of Tetracyclines' members.

Tetracycline Type	Break point limits (µg/ml)		Ranges of Tetracyclines' Concentrations (µg/ml)
	R	S	
Tetracycline	**≥16**	**≤4**	**0.125-512**
Doxycycline	**≥16**	**≤4**	**0.125-512**
Minocycline	**≥16**	**≤4**	**0.125-512**
Tigecycline	**≥8**	**≤2**	**0.125-512**

Initially, stock solutions (100mg/1ml) were prepared from each antibiotic type, then after appropriate thirteen dilutions of each Tetracyclines' solutions were prepared according to the CLSI (2020).

The prepared dilutions of the all Tetracyclines solutions were added to the molten Müller-Hinton agar media separately that have been allowed to equilibrate in a water bath to 45-50 ºC.

The agar and antimicrobial solutions were mixed thoroughly and the mixture was poured into a sterile petri dish. The agar was allowed to solidify at room temperature.

A standardized inoculums prepared at (**2.2.9.1)**, 1µL aliquot of each inoculums was applied to the agar surface with standardized loop.

Antibiotic free media were used as negative controls and were inoculated; the inoculated plates were allowed to stand at room temperature (for no more than 30 min) until the moisture in the inoculums is absorbed by the agar. The plates were inverted and incubated at 35°C for 18 to 24 hrs.

To determine agar dilution break points, the plates were placed on a dark surface, and "the MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth". The MIC values were compared with the break points recommended by CLSI (2020).

2.2.10. Detection of Efflux Pump Activity of *A. baumannii:*

Efflux pump activity was evaluated using ethidium bromide-agar Cartwheel method as prescribed by Martins *et al*. (2013).

Plates of Trypticase Soy Agar (TSA) were prepared as **(2.2.2.1.)**, before it was solidify EtBr was added in different concentrations ranging from 0.0, 0.5, 1, 1.5, 2, 2.5 mg/L. The TSA plates should be prepared freshly on the same day of the experiment and kept protected from light.

Overnight cultures of the bacterial isolates to be tested are prepared in liquid media and in the following day their concentration adjusted to McFarland 0.5 standard (**2.2.9.1.**). The TSA plates are then divided into as many as eight sectors by radial lines, forming a cartwheel pattern. The adjusted bacterial cultures are then swabbed on the EtBr-TSA plates starting from the center of the plate to the margin. The TSA plates were then incubated at 37ºC overnight. After this period,

the TSA plates will be examined U.V. transilluminator for fluorescence, and the TSA plates were photographed.

Isolates without fluorescence indicated active efflux pump activity while those that fluoresced lacked efflux pump activity. Also, the minimum concentration of EtBr that produces fluorescence to be recorded (Salumi and Abood, 2022).

2.2.11. Study the Effect of *Matricaria chamomilla* (chamomile بابونج) against Tetracyclines-Resistant *A. baumannii*:

2.2.11.1. Preparation of Aqueous Extract of *Matricaria chamomilla*:

The dried flowers of chamomile were bought from the market and primed for the preparation of its aqueous extract, the stock solution was prepared as w/v suspension in a flask by adding hot boiled water. The flask was then placed on a shaker (200 rpm) for 4 h and the temperature was maintained at 37°C, containers were covered in order to keep all active elements. Later on solution was filtered by filter paper then sterilized by Millipore filter (0.45) and kept at 4°C until the time for further applications; where multiple dilutions were prepared as 5, 10, 20, 40 and 80 mg/ml (Srivastava and Gupta, 2009).

2.2.11.2. Inhibitory Antimicrobial Effect of *Matricaria chamomilla*:

The screening of antimicrobial activities of aqueous Chamomile extract on *A. baumannii* was determined on Muller-Hinton agar media, by the agar diffusion techniques using agar well diffusion method.

A standardized inoculums prepared at (**2.2.9.1)**, 1µL aliquot of each inoculums was applied to the agar surface with standardized loop or by a cotton swab and allow to dry. Later on, wells of 6 mm diameter and 5 mm depth were

made on the solid agar using a sterile glass borer; as 6 wells were made on each plate. Approximately 20µl of each concentration (5, 10, 20, 40, 80) of the extract were applied into each well, and the same amount of free normal saline or non-cultivated broth was applied to the sixth one as a negative control (three replicate plates were prepared for each isolate).

Then after plates were incubated at 37°C for 24 hrs., zones of inhibition were measured using a ruler in millimeters.

2.2.11.3. Efflux Pump-Inhibitory Effect of *Matricaria chamomilla*:

Ethidium bromide-agar Cartwheel method **(2.2.10.)** used to detect efflux pump activity was repeated again with the application of different concentrations of aqueous Chamomile extract **(2.2.11.1.)** in order to detect if there is an efflux pump-inhibitory effect of the Chamomile extract.

Ethidium bromide efficiently effluxes out and only accumulates in cells in the presence of an efflux pump inhibitor and emits strong fluorescence.

2.2.12 Genotyping assays of *A. baumanii*

2.2.12.1. DNA Extraction:

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid (UK). Chromosomal DNAs obtained were used as templates for all PCR experiments, and according to the following steps:

Step1: Cell Harvesting/pre-lysis

- One ml of bacterial suspension containing approximately up to 1.2×10^9 cell/ml (equal to 4.0 McFarland standard) was transferred to a 1.5ml

microcentrifuge tube, centrifugation for 1 minute at 14-16,000×g and discard the supernatant.

- A volume of 200 µl of GT buffer was added to the tube and resuspend the cell pellet by shaking vigorously or pipetting, then left at room temperature for 5 minutes.

Step 2: Lysis

- A volume of 200 µl of GB buffer was added to the sample and mix by shaking vigorously for 5 seconds, then incubated at 70ºC for 10minute
- or until the sample lysate is clear. During incubation, the tube was inverted every 3 minutes. At this time, the required Elution Buffer (200 µl per sample) incubated at 70ºC (for step 5 DNA elution).

Step 3: DNA Binding

- A volume of 200 µl of absolute ethanol was added to the sample lysate and immediately mixed by shaking vigorously. If precipitate appears, broke it up by pipetting.
- GD column was placed in a 2ml Collection Tube.
- All of the mixture (including any precipitate) was transferred to the GD Column and centrifuged at 14-16,000×g for 2 minutes.
- The 2 ml Collection Tube flow-through was discarded and placed the GD Column in s new 2 ml Collection Tube.

Step 4: Washing

- A volume of 400 µl of W1 Buffer was added to the GD Column.
- Then, centrifugation at 14-16000×g for 30 second

- The flow-through was discarded and placed the GD Column back in the 2 ml Collection Tube.
- A volume of 600 µl of Wash Buffer (ethanol added) was added to the GD Column.
- Centrifugation at 14-16,000×g for 30 seconds.
- The flow-through was discarded and placed the GD Column back in the 2 ml Collection Tube.

Step 5: DNA Elution

- The dried GD Column was transferred to a clean 1.5 micro centrifuge tube and 100 µl of preheated Elution Buffer was added to the center of the column matrix and let stand for 3-5 minutes or until the Elution Buffer is absorbed by the matrix.
- Centrifugation at14-16,000×g for 30 seconds to elute the purified DNA.

2.2.12.2. Detection of DNA concentration and purity by Nanodroop:

The extracted DNA was checked by using nanodrop spectrophotometer, which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260/280nm) as following steps:

1. After opening up Nanodrop software, chosen the appropriate application (Nucleic Acid, DNA).
2. A dry wipe was taken to clean instrument pedestals several times. then carefully pipette 2 µl of ddH_2O on to the surface of the lower measurement pedestals for blank system.
3. The sampling arm was lowered and clicked OK to initialized the nanodrop, then cleaning off the pedestals and 1 µl of extracted DNA carefully pipette onto the

surface of the lowered measurement pedestals, then concentration and purity of extracted DNA was checked (Wilfinger *et al.*, 1997).

2. 2.12.3. PCR amplification:

2.2.12.3.1. Oligonucleotides primer pairs:

All primer pairs used in this study table (2-9) were purchased from macrogen (Korea) in lyophilized form. Primarily, the stock solution was prepared by dissolving the lyophilized powder in nuclease free water buffer according to manufacturer's protocol.

2.2.12.3.2 PCR Master Mix:

A. Master Mix kit:

GoTaq Green Master Mix kit is a premixed ready-to-use solution for amplification of DNA templates by conventional PCR. This kit was purchased from Promega/USA. This master mix contains the following:

- *Taq* DNA polymerase: This enzyme responsible for the synthesis of new DNA strand during PCR reaction.
- Reaction buffer important for maintenance, pH about 8.5 also contains a compound that increases sample density.
- Deoxyribotriphosphates (dNTPs) 400 μM of the following: dATP, dGTP, dCTP and dTTP.
- Magnesium ions $MgCl_2$ (3mM), which is a co-factor for enzyme action.
- Loading dye: containing a mixture of yellow dye (xylene cyanol) and blue dye (bromophenol blue), important for direct PCR product loading for analyzed by agarose gel electrophoresis.

B. GoTaq Green Master Mix kit protocol:

The uniplex PCR amplification mixture used for detection of study genes were listed in T**ables (2-8)**.

Table (2-8): Contents of the Reaction Mixture of PCR

Contents of the reaction mixture	Volume
Green master mix	12.5µl
Upstream primer	2.5µl
Downstream primer	2.5µl
Nuclease free water	2.5µl
DNA template	5µl
Total volume	25µl

2.2.12.3.3 Primer Sequences and PCR conditions:

The primer sequences and PCR conditions that used are listed in T**able (2-9).**

Table (2-9): The primer sequences and PCR conditions with their amplicon size (Base pair (BP)).

Gene's Name	Primer Sequence (5´- 3´)	Size (BP)	Conditions	Reference
tetA	**F-** 5'GCGCGATCTGGTTCACTCG3' **R-** 5'AGTCGACAGYRGCGCCGGC3'	164	94°c 5min 1x 94°c 1min 57°c 1min 72°c 1min 30x 72°c 5min 1x	(Beheshti *et al.*, 2020)
tetB	**F-** 5'TACGTGAATTTATTGCTTCGG3' **R-** 5'ATACAGCATCCAAAGCGCAC3'	206	95°c 75s 1x 94°c 1min 57°c 1min 72°c 1min 30x 72°c 5min 1x	(Beheshti *et al.*, 2020)
tetM	**F-** '5TGGGCTTTTGAATGGAGGAA3' **R-** '5ATCTCCTCCTTTACACTTTA3'	1200	95°c 5min 1x 95°c 1min 57°c 1min 72°c 1min 35x 72°c 10min 1x	(Ribera *et al.*, 2003)
REP	**F-** 5'IIIGCGCCGICATCAGGC3' **R-** 5'ACGTCTTATCAGGCCTAC3	100-1500	95°c 5min 1x 95°c 30s 57°c 1min 72°c 1min 40x 72°c 7min 1x	(Selim *et al.*, 2022)

2.2.12.4. Detection of Amplified Products by Agarose Gel Electrophoresis:

PCR products were visualized on 1.5%. Agarose gel electrophoresis stained with Ethidium bromide according to (Lee *et al.*, 2012).

A- Preparation of Gel:

- The gel was prepared at a concentration of 1.5% by dissolving 1.5gram of agarose in 100ml of TBE working buffer (1X).
- Heating mixture until agarose was completely dissolved.
- The mixture was left to cool to about 50-60°C then 1 µl of ethidium bromide was added to the gel.

B-Preparation of Casting Horizontal Agarose Gel:

- The casting platform was placed with well former sideways in gel stand where the gel was poured.
- The gel was poured on an electrophoresis plate fixed on an even surface.
- The comb was placed and the gel was left to cool and solidify for 30 min at room temperature.
- After the gel was set, the combs were removed carefully and the tank was placed into electrophoresis system. Then added 1X TBE buffer until covered gel (approximately 1 - 2 mm above the gel surface).

C-Running of Products

- When the thermocycling was finished, the PCR tube was handled outside the thermocycler. 5 µl of each PCR product along with 100bp DNA ladder was loaded into the gel wells.
- The system cover put into place and then turned on. The gel was run for 90 min at 100 volts.

2.2.12.5. Visualization:

Following electrophoresis, visualization was conducted with a UV transilluminator and image was captured by the digital camera (Canon, USA).

2.2.12.6. REP-PCR Genotyping:

All the isolates were subjected to REP-PCR typing method for finding common REP-types among all of the study isolates. The primers used for REP-typing at **Table (2-9)** with its amplification condition and product size, in addition to the contents of the reaction mixture of PCR at **Table (2-8)**; amplification reaction was carried out by thermal cycler and the resulting multiple amplicons of different sizes can be fractioned by electrophoresis in a 1.5% agarose gel **(2.2.12.4.C.)** along with 100bp DNA ladder was loaded into the gel wells. Amplified products were detected by being stained with ethidium bromide and photographed and results were analyzed by PAST software for the construction of the dendogram.

2.2.13. Statistical analysis:

All study data were analyzed by the use of Chi-square through SPSS version 18.0. The 95% confidence interval of a proportion was used to calculate the significances. P-value that was obtained as less than the 0.05 the level of significance was considered statistically significant. Also, other data were represented by Number and frequency.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Frequency Distribution of *Acinetobacter baumanii* Isolates

During the period of this study, a total of (125) samples were obtained from different clinical specimens; after their cultivation and identification results revealed that *A. baumannii* isolates were detected as 23/125 (18.4%) that distributed as 10/23 (25%) isolates were obtained from sputum, 5/23(17.9%) isolates from burns, while 4/23 (16%) and 4/23 (12.5%) isolates were obtained from urine and wounds respectively, as shown in **Figure (3-1)**.

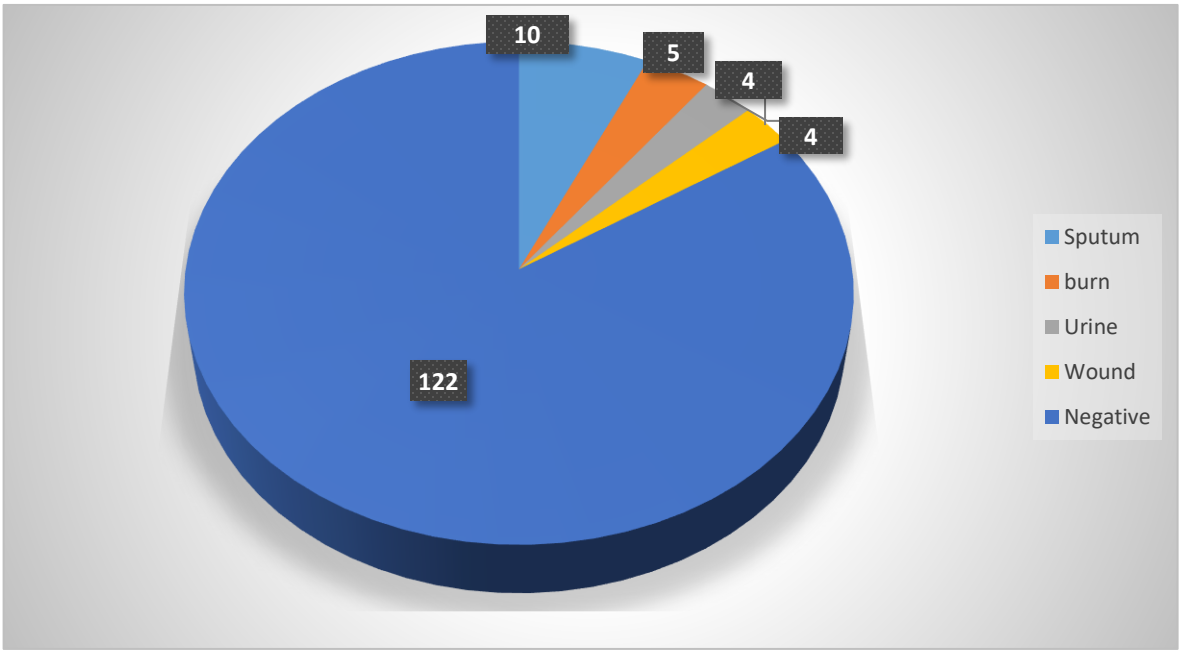

Figure (3-1): Distribution of *A. baumanii* According to Clinical Specimen.

These results suggested that *A. baumannii* isolation frequency was 18.4% with the highest number were obtained from respiratory tract infections followed by burns, while the lowest were obtained from both urine and wound.

The dissemination of *A. baumannii* in clinical samples could be due to its ability to cause different nosocomial infections and resistance to a wide range of antibiotics; where most cases in this study were collected from the Intensive Care Unit as their patients are usually on ventilators or other invasive supportive devices as the hospital environment itself is an increasingly important reservoir of *A. baumannii*

The *A. baumannii* are widely distributed in different environments, especially in hospital environment, they reside on medical devices, and equipment, with their capacity to survive for prolonged periods on inanimate objects. The factors that are responsible for their persistence in a hospital environment are resistance to key antimicrobial drugs and disinfectants and their ability to survive in desiccants (Evans *et al.*, 2013).

These abilities make this bacterium to infect not only hospitalized patients but also general population. In hospitals, it confers 26% mortality rate that goes up to 43% in ICUs, where *A. baumannii* is a principal agent of ventilator-associated pneumonia, which accounts nearly 15% of all hospital-acquired infections, with the highest morbidity and mortality in medical wards especially in the ICUs (Demirdal *et al*., 2016; Greene *et al*., 2016).

A. baumannii can be transmitted through the vicinity of affected patients, in addition to the contamination of respiratory support equipment, suction devices, and devices used for intravascular access is the key source of infection; thus major predisposing factors important in the acquisition of *A. baumannii* infection include prolonged hospital stay, mechanical ventilation, intravascular device, advanced age, immunosuppression, previous broad-spectrum antimicrobial therapy, previous sepsis, ICU stay, and enteral feedings (Islahi *et al*., 2015).

Since the coronavirus disease 2019 (COVID-19) pandemic, *A. baumannii* was recognized as a frequently implicated pathogen that contributed to outbreaks of multidrug resistant organisms in both ICU and non-ICU units (Polly *et al*., 2021). Numerous factors, such as the appropriate use of personal protective equipment, adherence to hand hygiene protocols, appropriate storage of personal protective equipment, and responsible antibiotic use, may all contribute to outbreaks of multi-drug-resistant organisms in hospital settings. However, these factors can potentially be modified to prevent the transmission of these organisms in healthcare facilities (Thoma *et al*., 2022).

Moreover, *A. baumannii* can survive desiccation better than other *Acinetobacter* spp. with its ability to form biofilm that involved in cell attachment on epithelial cell and smooth surfaces of medication instruments like urinary catheters and lung tubes. After attachment to the abiotic surface, *A. baumannii* survives in biofilm as viable or even dormant cells (Chapartegui-González *et al*., 2018). Forming of biofilms by *A. baumannii* can provide protection to bacteria from environmental damage such as host responses, antibiotics, cleansers, and disinfectants. The production of biofilms and their ability to withstand desiccation can heighten the probability of the establishment and persistence of *A. baumannii* in hospital environments, as well as increase the risk of acquiring antimicrobial resistance, and causing nosocomial infections and outbreaks (Ibrahim *et al*., 2021).

Different works concerning with the study of *A. baumannii* from various clinical sites and revealed varying frequencies; where study of Bayram and Al-Shukri (2022) at Babylon City/Iraq showed that *A. baumannii* was mostly isolated from respiratory tract infection with (27%) followed by

(14%) and (10%) from burns and urine respectively; while the lowest from wound (8%).

Also, another study was carried on at Babylon/ Iraq on the same year resulting in (20/100 (20%)) overall isolation divided into (7/20 (35%)) from burn swabs, (8/20 (40%) from wound swabs, while (5/20 (25%)) from urine (Kareem *et al*., 2022). In comparison to these two studies, a certain study identified only 20 isolates out of 600 specimens (3.33%) (Al-Hasnawy *et al*., 2021) and Mirazae *et al* (2020) got (9.51%); while Ribeiro and his colleges (2020) showed isolation frequency as (55.6%).

Additionally, Al-Sehlawi's group (2014) collected *A. baumannii* from urinary tract infection as (9.5%) and from wound (6.2%) while from lower respiratory tract infections as (5.1%).

3.2. Identification of *Acinetobacter baumanii* Isolates

All collected isolates subcultured into pure culture, in addition to other phenotypic tests for differentiation and identification of *A. baumannii*, where all the obtained *A. baumannii* gave similar results as cleared in the **Table (3-1)**, as these isolates were confirmed phenotypically beside the application of VITEK 2 system with a percentage of accuracy (99%) (appendix).

A. baumannii grows well on routine laboratory culture media such as blood agar, chocolate agar, and MacConkey agar. On blood agar, it forms colorless, non-hemolytic, shiny mucoid colonies, smooth in contexture with a diameter of 1–2 mm after 18–24 hours of incubation at 37°C. It produces colorless colonies on MacConkey agar which are shiny mucoid colonies, indicating its non-lactose fermenting ability (Almasaudi, 2018).

Table (3-1): Phenotypic Characterization of *A. baumannii*

Test	**Results**
Gram Stain	Gram-Negative
Morphology	Bacilli to cocobacilli
Growth on Blood agar	Pale, colorless colonies
Growth on MacConkey agar	Pale (Non-lactose fermenter)
Catalase	Positive
Oxidase	Negative
Motility	Non-motile
Haemolysis	Non-haemolytic

Acinetobacters have been identified as oxidase negative, nonmotile, nonfermentative, gram-negative coccobacilli that grow well only under aerobic conditions (Sehree *et al.*, 2021). Where, the isolates taken from MacConkey agar plate and stained by Gram stain appear under light microscope as a pink coccobacillus which referred Gram –ve bacteria. In biochemicals test, all *A. baumannii* isolates showed negative results for oxidase test and motility test, whereas a positive results for catalase (Kulkarni *et al*., 2017).

For confirmation of bacterial identification, VITEK 2 system was used for verification, as the accurate and rapid identification of *A. baumannii* was critical for the appropriate infection control in hospital settings. Up to date, the most common and widespread detection methods include characterization via a phenotypic system and commercial phenotypic methods (e.g., the Vitek-2 compact system, Biomerieux). It was used by many researches like (Jassim *et al*., 2016; Almaghrabi *et al*., 2018), where vitek used successfully to identify most *Acinetobacter* species.

3.3. Tetracycline Susceptibility Profile

3.3.1. Disk-Diffusion Test

All bacterial isolates (n = 23) in this study were subjected to different members of Tetracyclines (mentioned at **Table (2-5)**) by the Disk-Diffusion Test yielding results as in **Table (3-2)** and **Figure (3-2)**, showing

Table (3-2): Distribution of Tetracycline Resistance among *A. baumannii* isolates by Dick-Diffusion Test against Different Tetracycline Members, with their Multiple Antibiotic Resistance Index (MAR).

Isolate Number	Source	Tetracycline	Minocycline	Doxycycline	Tigecycline	MAR
1.	Sputum					0.0
2.	Sputum					0.75
3.	Burn					0.25
4.	Wound					0.75
5.	Burn					0.0
6.	Sputum					0.0
7.	Wound					0.0
8.	Burn					0.0
9.	Wound					0.0
10.	Sputum					0.75
11.	Urine					0.0
12.	Sputum					0.75
13.	Sputum					1.0
14.	Burn					0.75
15.	Sputum					1.0
16.	Burn					0.0
17.	Wound					0.0
18.	Sputum					0.25
19.	Urine					0.75
20.	Sputum					0.75
21.	Urine					0.0
22.	Urine					1.0
23.	Sputum					0.0

Red Square = Resistant, White Square = Sensitive

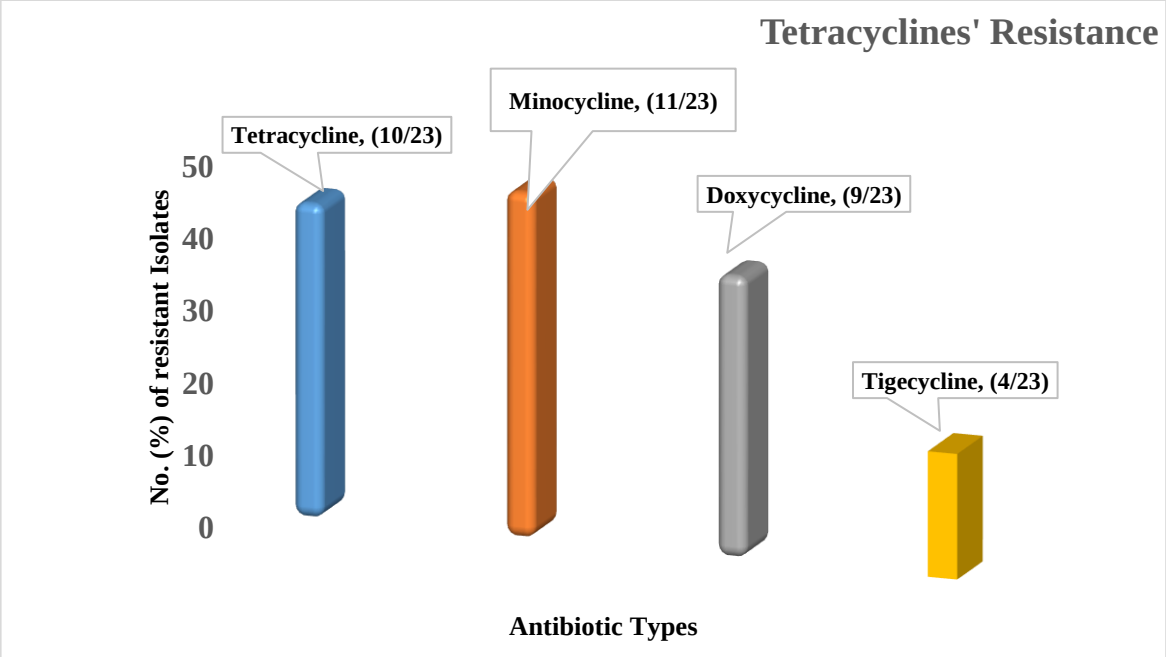

Figure (3-2): Overall Frequency Distribution of Tetracyclines Resistance among *A. baumannii* Clinical Isolates.

These results showed various distribution pattern of resistance against different members of the three generations of Tetracyclines, with the highest resistance against Minocycline 11/23 (47.8%) followed by resistance to Tetracycline and Doxycycline as 10/23 (43.5%) and 9/23 (39%) respectively. Moreover, with a shocking unexpected resistance to the third generation's member the Tigecycline about 4/23 (17.4%).

Additionally, regarding Multiple Antibiotic Resistance Index; results revealed that 3/23 (13%) with MAR equal to 1.0 which mean resistance to all used antibiotics, while 9/23 (39%) with MAR more than 0.2 and the remaining 11/23 (47.8%) with complete sensitivity to all members (MAR=0.0). Where most of isolates with MAR more than 0.2 were isolated from patients at ICU; as the isolates showing resistance to several antibiotics with MAR indices higher than 0.2, indicating that these are potentially hazardous to consumers.

A. baumannii has become a common cause of infections associated with high mortality and morbidity while bacteremia and pneumonia are most severe infections, and typically more prevalent in those that are immunocompromised, in ICU and from war wounds. Furthermore, it has become a multi-drug-resistant microorganism worldwide leading to a great concern in the medical community (Karakonstantis, 2020; Karakonstantis and Saridakis, 2020). The high resistance rates are likely to be associated with a wide range of empirical and therapeutic use of antibiotics at hospitals. The employed selective pressure by MDR strains emerging, which in turn may have led to the genes encoding resistance mechanisms (Mohammed *et al*., 2022).

The increasing rates of antibiotic resistance can be attributed to multiple causes. These are a direct relationship between the emergence of resistant strain and the magnitude of antibiotic consumption, resistance mechanisms can be transition from one bacterium to anther by either longitudinally, when inherited from horizontally by means of plasmids, and the latter may result in the transference of resistance among difference species. Also, the overuse of antibiotics in hospitals and constant rise in antibiotic resistance, to prevent new resistant strains appearance, evaluation of resistant isolates by susceptibility testing seems to be critical. Due to genetic alteration caused by unnecessarily prescribed antibiotics.

Also massive agricultural use of antibiotics, also the availability and low cost of antimicrobials has led to considerable reduction of investment into new alternative by the pharmaceutical industry (Vázquez-López *et al*., 2020).

Infections by antibiotic-resistant bacteria, especially multi-resistant bacteria, are challenging to treat, resulting in serious health issues and even

death due to extended hospital stays and unsuccessful treatment attempts (Lowe *et al*., 2018).

Thus, in spite of the importance of Tetracyclines as a part of *A. baumannii* treatment lines, but results showed resistance to all members with varying degrees even the member of the third generation. Regarding the first and second generations; Doxycycline appeared the effective one (susceptibility rate 61%) when compared to both Tetracycline and Minocycline, while Tigecycline was the most effective (susceptibility rate 82.6%) in comparison to other Tetracyclines' members.

A. baumannii is one the most clinically important nosocomial infections especially in debilitated patients, these infections chiefly with multidrug resistant strains, have been reported worldwide, so that only a few antibiotics have remained effective against MDR strains (Maleki *et al*., 2014); Tetracyclines are usually effective medications against different pathogenic bacteria, thus detection of bacterial susceptibilities to Tetracyclines is so valuable for supporting their therapeutic roles.

Resistance against Tetracyclines is mediated by various mechanisms, including active efflux of the antimicrobial mediated by resistance proteins in the bacterial cytoplasmic membrane and inhibition of ribosomal and tetracycline binding (Zhang *et al.*, 2021).

Study of Bayram and Al-Shukri (2022) at Hilla City, showed resistance rate to Tetracycline about 50% which is close to the present study result, also it is nearly identical to the results of Mahich *et al*. (2021) and Sehree *et al*. (2021) that was about (50-60%) and (31.7%) respectively.

Al-Tamimi *et al*. (2022) suggested that clinical *A. baumannii* isolates showed a low resistance rate 7.2% for Tigecycline and 23.8% for

Minocycline but higher for Tetracycline at 65.4%. Also, for Sepehr *et al*., (2022) who showed high resistances for Tetracycline and Tigecycline.

Beheshti with her colleagues (2020); their works showed that Doxycycline was the most active antibiotic tested, followed by minocycline and tetracycline, with susceptibility rates of 96.93% (95/98), 71.57% (68/98), and 43.87% (43/98), respectively.

In a study by Adibhesami *et al*. (2015) the number of minocycline and doxycycline-susceptible *A. baumannii* isolates was significantly higher than the number of tetracycline-susceptible ones. Maleki *et al*. (2014) have also found a resistance rate of 18% to doxycycline and 19% to minocycline against *A. baumannii* isolates, while 80% of isolates showed resistant to tetracycline. Also, Sandhu (2016) showed resistance to Doxycycline (31.2%), and most isolates with high MAR indices were isolated from ICU and surgery wards.

In an Iranian study, all of the *A. baumannii* isolates were sensitive to Tigecycline as it is a novel expanded broad-spectrum glycylcycline antibiotic that has a good activity against isolates which are either resistant and/or sensitive to tetracycline, minocycline and doxycycline. It seems that the activity of Tigecycline is not affected by the tetracycline resistance mechanisms (Maleki *et al*., 2014).

Although resistance to tigecycline is not commonly seen among *A. baumannii* isolates, it has been shown that resistance against these antibiotics may exist due to the over expression of AdeABC efflux pump. Both tigecycline highly resistant and sensitive *A. baumannii* have been reported from all over the world (Yin *et al*., 2016).

3.3.2. Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentrations (MICs) of the four used Tetracyclines' members against *A. baumannii* isolates were determined by the two-fold agar dilution susceptibility method, as in Table (3-3).

The MIC values were based on break point recommended by CLSI (2020) for estimation of the response. The break point represents the optimum concentration of the drug that can reach the serum and provide high level of therapy. The microorganism was considered sensitive if the estimated MICs were less than the break point.

Table (3-3): MIC values with Number (Percentages) of Tetracyclines against *Acinetobacter baumannii* isolates

Type of Tetracycline With break point µg/mL	Range of MIC Break Points µg/mL No. (%) of Isolates												
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Tetracycline (S≤4 - R≥16)*	0	0	1	0	3	5	4	3	4	1	1	1	0
Doxycycline (S≤4 - R≥16)*	1	0	0	3	4	2	4	2	4	1	0	1	1
Minocycline (S≤4 - R≥16)*	0	2	0	4	3	1	2	0	2	4	4	1	0
Tigecycline (S≤2 - R≥8)*	2	5	4	8	0	0	2	2	0	0	0	0	0

*Break points recommended by CLSI (2020); MIC=Minimum inhibitory concentrations
Green = Sensitive region, Blue = Resistant region

Results of the present study revealed that Tigecycline, being the most effective member against most tested *A. baumannii* isolates (19/23), with MIC ≤ 1 µg/mL with a range (1 - 0.125 µg/mL) against these isolates, while the resistant isolates showed an MIC 8-16 µg/mL.

Among the 23 *A. baumannii*, the 13 tetracycline-sensitive isolates, showing MIC values ranging from 2-8 µg/mL, while the resistant isolates

with MIC range 16 - 256 μg/mL and mainly at 16 -32 μg/mL. Additionally, regarding Doxycycline and Minocycline both showed effective MIC range 1 – 8 μg/mL.

One of the important causes behind failure of therapies is the drug selection pressure, especially when patients are ill, and drugs are chosen, administered in doses too small, which leads to the survival of a resistant bacterial population or induces antibiotic resistance mechanisms. Therefore, it is very important to use antibiotics in true bacterial infections and in effective doses so as to increase the likelihood of therapeutic effectiveness. Thus, determination of the MIC is important to determine the drug effectiveness and therapeutic selection, where the used antibiotic should be effective against probable pathogens and to stop the use of antibiotics already administered in case of the strain's resistance and to replace it with a drug to which the strain is susceptible.

For this awareness, determination of MIC of Tetracyclines' members are very valuable to decide their effectiveness and usage for treatment of hazardous *A. baumannii* different infections. As the *A. baumannii* isolates generally displayed elevated MIC values for most antimicrobial agents giving a picture of MDR in response to many antimicrobial agents, in addition to the spread of clones presenting with a higher prevalence of resistance determinants. In this study, varying values and ranges of MICs of Tetracyclines' members against *A. baumannii* were obtained even the not commonly used Tigecycline that could be due to harboring different resistance mechanisms and genes via horizontal gene transfer and development of novel resistance mechanisms.

Comparative studies, such as a study of Yang *et al*. (2022) revealed that Tigecycline MIC50/90 was 2/4 μg/mL against all the isolates, and

considered them as non-susceptible to Tigecycline; while Minocycline MIC50/90 was 4/16 μg/mL with susceptibility rate was 55%. Also, Beheshti *et al.* (2020) supposed that *A. baumannii*-Tetracyclines' Resistant isolates showed MIC values ranging from 128-256 μg/mL.

Moreover, group of Huband (2020) in a study for comparing Tetracycline, Minocycline and Doxycycline with another novel tetracyclines as Tigecycline and KBP-7072; supposed that MICs values for the 1st. three members as more than 8 with resistance frequencies as 55.6%, 19.4% and 30.7% respectively, while Tigecycline showed MIC about 4 with resistance rate 2.3% comparing to KBP-7072 sensitivity rate 99.2%.

Study of Maleki and his group (2014) showed that most isolates of *A. baumannii* with MIC50 = 32 μg/mL and MIC90 = 512 μg/mL in relation with Tetracyclin, thus classified as Tetracycline resistant. While regarding Tigecycline, showed MIC range from 2 – 0.125 μg/mL against all isolates revealing their sensitivity.

3.4. Detection of *A. baumannii* Efflux Pump by Cartwheel Assay

In this study, 10 isolates with antibiotic resistance to 2 and more out of four Tetracyclines were subjected to detection of efflux pump in comparison to positive control (*A. baumannii* ATCC, 19606) and Negative control and cultivated plates without EtBr.

As the results in **Figure (3-3)** and **Figure (3-4 A and B)** showed that 2/10 (20%) isolates exhibited fluorescence at all plates with different concentrations gradient of EtBr in comparison with controls, which mean that these two isolates had no phenotype of efflux pump, or even there may be a defect in their resistance genes whether mutation or even lost as their genes

are carried on plasmid, and their detected antibiotic resistance could be mediated by other resistance mechanisms.

While the other 8/10 (80%) isolates showed no fluorescence at lower concentrations gradients and started to fluorescence 5/8 at 2 mg/L and more, but the remaining 3/8 did not fluorescence even at 2.5 mg/L of EtBr similar to the positive control *A. baumannii* ATCC, which mean that these eight isolates expressing an active efflux pump that has ability to expel its substrate (EtBr) outside the bacterial cell, thus did not fluorescence on exposure to UV light.

Cartwheel assay is a simple method that relies on the ability of bacteria to expel Ethidium Bromide (EtBr), which is a substrate for many efflux pumps. Efflux pump inhibitors (EPI) cause accumulation of EtBr within the cells and induce fluorescence at a much lower concentration of EtBr (Abdi *et al*., 2019). Efflux pumps of MDR bacteria recognize this substrate and are able to extrude it to the medium, as this process will continue if the concentration of EtBr in the culture medium does not overcome the capacity of the bacterial efflux pump itself (Christena *et al*., 2015).

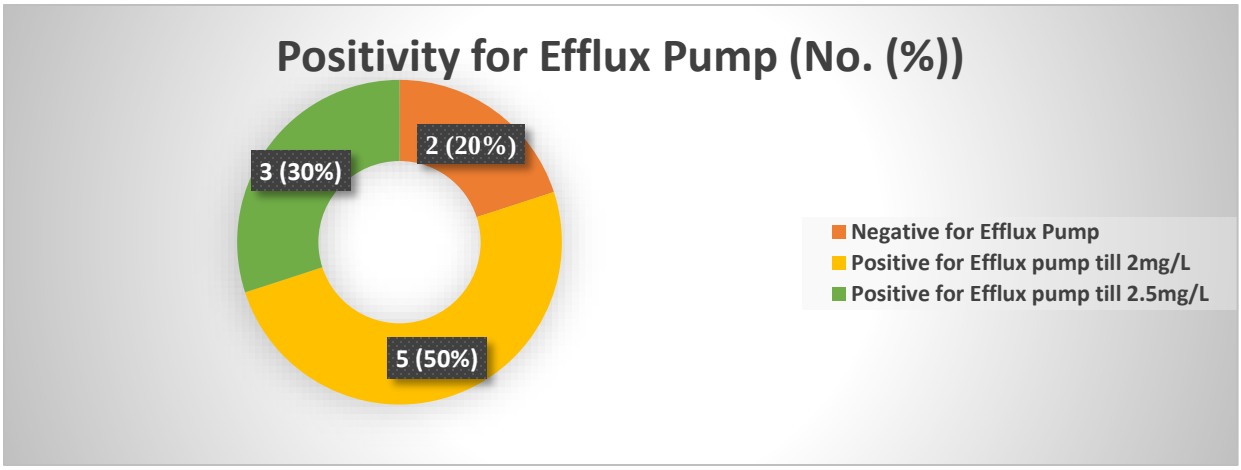

Figure (3-3): Positivity for Efflux Pump (No. (%)) Phenotype Production by *A. baumannii* Clinical Isolates by the application of Cartwheel assay.

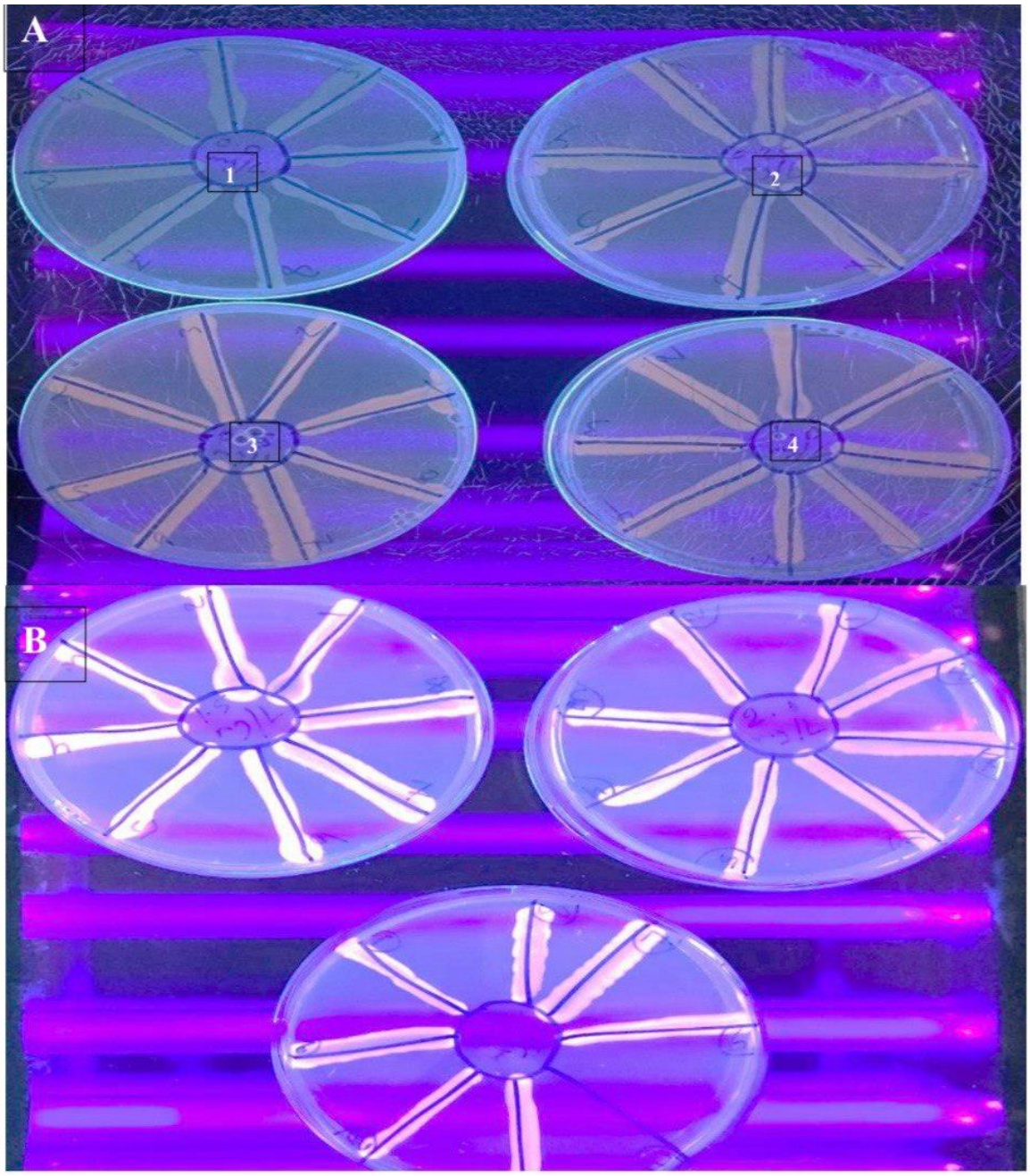

Figure (3-4 A and B): The EtBr-agar cartwheel Method for Detection of Efflux Pump Phenotype Production Applied to different *A. baumannii* Clinical Isolates. As A1 free of EtBr, A2,3,4 refer to Positive efflux pump with no fluorescence. B= Negative for efflux pump with fluorescence on UV light visualization.
A: 1= 0.0 mg/L, 2=0.25mg/L, 3=0.5mg/L, 4=1mg/L
B: 1.5, 2, 2.5 mg/L

Increasing multidrug resistance among bacterial isolates has become a significant challenge in health care settings for effective treatment. *A. baumannii* is a bacterium with a wide antibiotic resistance mechanism range. Among them, efflux pumps are considered one of the most important mechanisms for high antibiotic resistance (Khoshbayan *et al*., 2021). Mutation in amino acid sequence or amino acid replacement can increase the expression of efflux pumps and cause resistance to a wide range of drugs by reducing the intracellular concentration of antibiotics (Abdi *et al*., 2020).

This efflux pump is involved in resistance to Tetracyclines, Tigecycline and many other different classes of antibiotics, and unfortunately, the nosocomial infections caused by bacteria harboring these pumps and resistances have increased over the past decade, especially in patients admitted to intensive care units, burns, and surgery wards (Shi *et al*., 2020).

The basis for the expression of Tetracycline-resistance phenotype among clinical bacterial isolates has become a major concern since the therapy of the infections caused by these bacteria is problematic. The routine antimicrobial sensitivity tests fail to detect efflux pump mediated drug resistance. The high prevalence (80%) of an active efflux system in the present study in Tetracycline-resistance *A. baumannii* isolates indicated that efflux activity-based antibiotic resistance is prevalent among Tetracycline-resistance *A. baumannii* isolates. Hence, it is suggested that detection of active efflux pump must be included in the diagnostic regimen for providing a warning signal to the current therapy if continued as administered, may result in further acceleration of resistance possibly including additional classes of antibiotics and implementation of accurate therapy to the patients.

In targeting efflux, it is important to realize that efflux is only one of several available mechanisms of resistance in MDR bacteria to a given antimicrobial. Thus, its inhibition will have a significant therapeutic effect only for those antibiotics and in those organisms where efflux is the major contributor to resistance. The likelihood of false positives in such a screening cannot be ruled out as it has been reported that bacterial permeability to EB may also be highly decreased due to the down-regulation of porins (Martins, 2010).

Cartwheel- Ethidium bromide assay was used by many researchers for detection of efflux pump, where Sepehr *et al*., (2022) showed that the nine tested isolates with Tetracyclines' resistance resulting in the increased outflow of ethidium bromide from the bacteria, which decreased fluorescence compared to the control, also it was beneficial to detect the effect of efflux pump inhibitors.

3.5. Effect of Aqueous Extract *Matricaria chamomilla*

3.5.1. Antimicrobial Effect of Aqueous Extract of *Matricaria chamomilla* against *A. baumannii*

This study examined the effect of aqueous extract of *Matricaria chamomilla* in different concentrations (5, 10, 20, 40 and 80 mg/ml) for each one of *A. baumannii* isolates and their effect was expressed in percentage value and compare by chi square as p value; as revealed in **Figure (3-5)** and there were statically significant differences (P value = 0.032) between the inhibitory effects of different concentrations. The 1st. two lower concentrations showed little or no effect in comparison to other higher concentrations; also when compare them with effect of antibiotics.

These results showed that higher inhibition effect of Chamomile in (80 mg/ml) concentration; as the inhibition for *A. baumannii* growth was in a percentage (78.3%) and (69.6%) for (80 and 40 mg/ml) respectively; where as (20 mg/ml) concentration made (47.8%) inhibition effect of *A. baumannii* growth.

On the other hand , these results showed that the inhibitory activity of aqueous extract of *Matricaria chamomilla* is proportional linearly with it concentration; as it is increase when the concentration increase.

The treatment of bacterial infections is currently a great challenge for the medicine. Antibiotics that have been used for decades are not as effective due to MDR, and new ones rarely appear. Therefore, research focuses on adjuvant substances that can support the activity of antibiotics without the risk of developing secondary resistance. These adjuvants can directly inhibit the resistance mechanism in nontoxic concentrations or modulate virulence factors, and thus reduce the danger of bacterial strains. Plant extracts are a promising source because they contain a wide range of substances.

There are many biological activity studied on *M. chamomilla*. Among these studies, antimicrobial, antioxidant and antiinflammatory activity studies are the most common; where many antimicrobial activity studies on *M. chamomilla* mainly performed with its' extracts which obtained by using various solvents and extraction techniques or essential oil of the plant. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus* and *Salmonella typhi* bacterial strains and *Candida albicans, Aspergillus flavus* and *Aspergillus niger* fungal strains were the most common strains in these studies (Flemming *et al*., 2015; Cvetanović *et al*., 2017; Ozdemir *et al*., 2021).

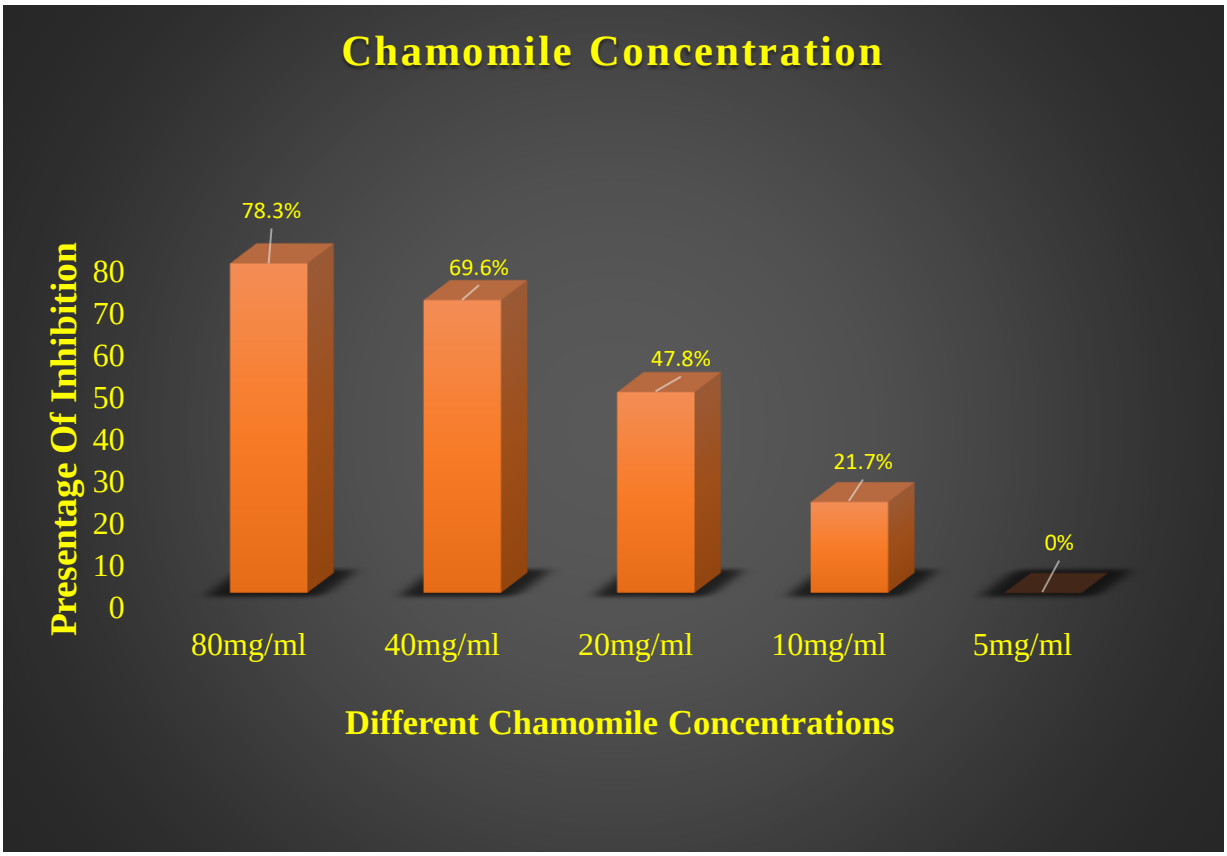

Figure (3-5): Inhibitory effect of Aqueous Extract of *Matricaria chamomilla* against *A. baumannii*

Several studies had been studied the antimicrobial activity of Chamomile and many other plants' extracts, and gave various MIC values that is mainly due to different extraction methods, type of extract and concentrations series that applied. Also, the presence of active compounds in the extract is also influenced by the conditions under which the plant grows (soil conditions, amount of rain, sunshine) and in which generation period the plant is at the time of collection (germination, full bloom). However, all extracts tend to be more active against Gram-positive than Gram-negative

bacteria at lower concentrations, which may be caused by poor permeation of active substances through the complicated membrane of Gram-negative bacteria (Milenkovi´c *et al*., 2015). Also, the use of different bacterial strains can also lead to different MIC values.

The sensitivity of bacteria to antibiotics is weakened not only by the expression of resistance genes but also by the formation of biofilms. The bacteria in biofilms can be up to 100 times resistant to antibiotics (Alav *et al*., 2018), thus extracts that have ability to destroy and overcome biofilm formation, also can potentiate and prevent bacterial growth and inhibiting antimicrobial resistance.

Ali and coworkers (2009) revealed that watery extract of Chamomile has an inhibitory effects on the microorganisms under investigation. Extract was containing azulene and apigenin compounds, after detection and quantitation using HPLC techniques; as the extract was used against both Gram-positive and negative isolates, it showed no inhibition in the control treatments, slight inhibition at 10 and 20 mg/ml while caused an inhibition zone with 15.2 mm at the concentration 40 mg/ml with more inhibition with concentrations increment.

Ismail and his group (2011) showed that the water extract of chamomile is more effective than the ethanolic one against different types of bacterial isolates; where higher concentrations gave better effects than the lower ones. *M. chamomilla* contains many active compounds, attributed to antimicrobial activity as terpene compounds, azulen and abisabolo. As the dried flowers of chamomile contain many terpenoids and flavonoids contributing to its medicinal properties.

Several chemical compounds from synthetic or natural sources enhance the activity of specific antibiotics and reverse the natural resistance

of specific bacteria to given antibiotics (Göger *et al*., 2018). Also, there has been an increase in the use of natural substances instead of synthetic chemicals.

Jafarzadeh *et al* (2022) explained the analysis of the chemical ingredients of *M. chamomilla* showed the presence of flavonoids, phenylpropanoids, polyyne compounds, coumarins, polysaccharides, and essential oils. *M. chamomilla* shows a wide range of biological activities, which are most often due to its phenolic compounds. It has been shown that the spasmolytic and anti-inflammatory activity of *M. chamomilla* is mainly related to its flavonoids.

An emerging approach to face the *A. baumannii* antibiotic resistance crises is to seek alternative strategies in order to identify effective sustainable treatment options. Natural products (NPs) represent a good repertoire for potential active moieties that can be effective antimicrobial agents. NPs have always been known as a valuable source for the discovery of antibacterial agents. Several studies have reported the effectiveness of NPs and their isolated compounds as antibacterial agents against Gram-negative resistant bacteria, including *A. baumannii*. Moreover, great advancements in the technologies employed to separate, purify, and identify biologically active ingredients have allowed for a more comprehensive understanding of these compounds (Vivas *et al*., 2019). Thus, synthetic drugs must be replaced with another antibacterial compounds have potent effect against *A. baumannii* multi-drug resistant pathogens and without side effects against individuals.

Aljanaby (2018) studied the antibacterial effect of watery extract of Chamomile against different pathogenic bacterial isolates and resulted in an excellent anti-bacterial activity against all bacterial isolates, the inhibition zone diameters of *E. coli*, *K. pneumoniae*, *A. baumannii*, *E. aerogenes*, *C.*

freundii, *P. mirabilis* and *S. saprophyticus* were 29.3 ± 0.2, 26.3 ± 0.4, 26.3 ± 0.2, 28.3 ± 0.3, 29.3 ± 0.1, 29.3 ± 0.5 and 28.3 ± 0.2, respectively. In spite of all these isolates appeared as MDR, but all of these isolates appeared as susceptible to the used watery extract with inhibition percentage 100%, especially with the hot water extract in comparison with cold water extract.

M. chamomilla is an important medicinal plant was used in treatment of many infections such as respiratory tract infections and gastrointestinal tract infections in many countries, also, it has been reported to have antibacterial, antipyretic, antifungal and anti-inflammatory as well as ulcer protective effect (Aljanaby, 2018)

M. chamomilla which have been associated with relieved hypertensive symptoms and decreases the systolic blood pressure, increasing urinary output and may be lead to decrease the numbers of pathogenic bacteria in urinary tract. Also, drinking *M. chamomilla* was associated with increase in urinary levels of hippurate and glucine, which have been associated with increased antibacterial activity. In the present study, boiling-water extract had a significant antibacterial activity as compare with cold-water extract; this may be due to that the boiling water has the ability to extracted more medical compounds from flowers more than cold water (Zeggwagh *et al*., 2009).

3.5.2. Detection of Inhibitory Effect of Aqueous Extract of *Matricaria chamomilla* against *A. baumannii* Efflux Pump by Cartwheel Assay

Later on after the detection of efflux pump phenotype among *A. baumannii* **(3.4.)** and detection of antimicrobial effect of aqueous extract of *Matricaria chamomilla* **(3.5.1.)**, this extract was applied as an efflux-pump inhibitor; as 6 isolates were used. Where the results to be detected by examining the fluorescence against UV transilluminator, appearance of fluorescence indicating failure of efflux pump

Results of **Figure (3-6), (3-7)** showed that the fluorescence against UV light appeared more frequently among isolates even with the lower concentrations of EtBr which is an indicator of loss of efflux pump function, as there was a decreased outflow of ethidium bromide from the bacteria, which increased fluorescence compared to the control. Where 5/6 (83.3%) isolates showed this inhibition of efflux pump especially with the highest concentration of the extract (80 mg/ml), as the inhibition started to some extent at (40 mg/ml) and appeared more frankly at the higher concentration.

Only one 1/6 (16.7%) of the isolates did not show any inhibition at both concentrations indicating pronounced efflux activity, this may be due to the inhibition can occur at higher concentrations of the extract, or even other factors including time, temperature, light or even change in the bacterial susceptibility.

The agar ethidium-bromide cartwheel method can be a suitable tool for the fast and accurate detection of efflux pumps and its inhibitors in MDR bacteria. Bacterial growth in a culture medium containing ethidium bromide causes the accumulation of this substance within the bacterium that detected

using ultraviolet (UV) light (Sepehr *et al*., 2022); as it been used to assess the intrinsic efflux activity in *E. coli, Staphylococcus aureus, Klebsiella pneumoniae,*and *Pseudomonas aeruginosa* (Rana *et al*., 2015).

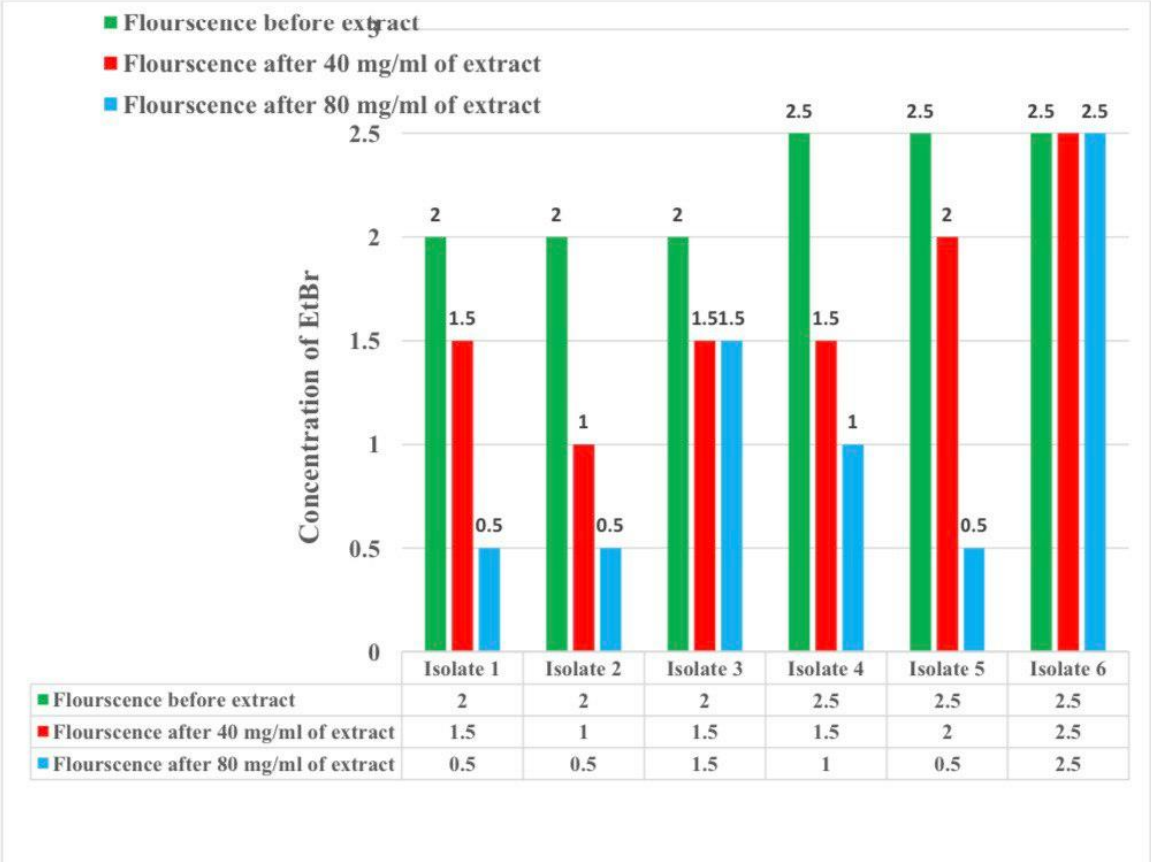

Figure (3-6): Efflux-Pump Inhibitory effect of Aqueous Extract of *Matricaria chamomilla* against *A. baumannii*

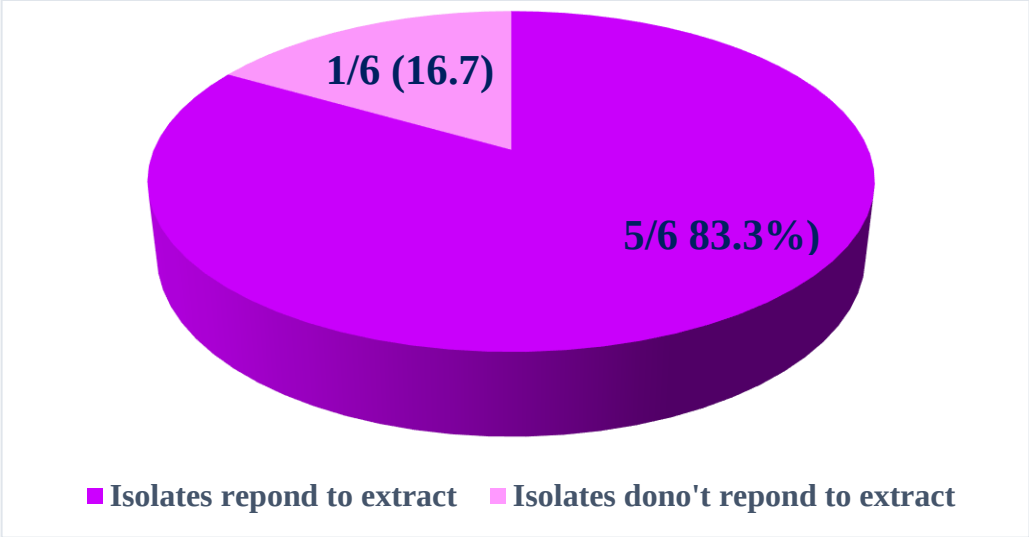

Figure (3-7): Number and Percentage of *A. baumannii* isolates responding to Efflux-Pump Inhibitory effect of Aqueous Extract of *Matricaria chamomilla.*

A combination therapy of efflux pump inhibitors (EPIs) and antibiotics can be a promising option for the efflux-mediated bacterial resistance; as EPI, must be selective, not target any eukaryotic efflux pumps, has ideal pharmacological properties, such as the non-toxicity and the high therapeutic effect.

The overexpression of bacterial efflux systems may be related to some of the measured activities. Inhibition of these systems can cause an increase in the concentration of antibiotics within the bacterial cell. Furthermore, it can complicate intercellular communication and thus reduce the production of virulence factors. Kˇríˇzkovsk´a *et al.* (2023) suggested that *M. chamomilla* at nontoxic concentrations can inhibit MDR-Gram negative efflux systems, and it is able to reduce the virulence of both Gram-positive and Gram-negative bacteria by inhibiting efflux pumps.

Even though drug efflux pumps play a critical role in the emergence of Tetracycline resistance and even MDR bacterial strains, there is no clinically approved EPI with which to inhibit these efflux pumps, Efforts are being made to develop a more effective EPI with fewer adverse effects and increased efficacy. Thus, suppression of efflux pump is a promising strategy for overcoming this problem.

Ismail *et al.* (2011) revealed that chamomile showed medicinal properties, where the flower is the main medicinal part of the herb; its products can be administrated in many ways as by oral, inhalation, and solution for bath and infusion. Also, it showed antitumor activity and contains several antibacterial, antifungal and antiseptic properties. It is used against different types of bacteria such as*: Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Micrococcus spp* and *pseudomonas aeruginosa*.

In comparison to these opinions, Ozdemir *et al*. (2021) presented that none of the used chamomile extracts showed antibacterial activity against *P. aeruginosa, K. pneumoniae* and *P. mirabilis* strains. Antifungal activity was observed only in extracts prepared from certain combination.

3.6. Molecular Study

3.6.1. Detection of Tetracycline Resistance Genes

Regarding detection of Tetracyclines' resistance genes (*tetA, tetB* and *tetM*) by the application of specific primers and by conventional PCR; as shown in **Figure (3-8)** and **Figure (3-9)**. Results of these amplifications showed that *tetA* gene appeared among only 7/23 (30.4%) as 3/23 appeared alone and 4/23 appeared in combination with *tetB*; while regarding *tetB* gene

appeared among 18/23 (78.3%); as 14/18 appeared alone and 4/23 appeared in combination with *tetA*. Thus coexistence of *tetB* and *tetA* appeared as 4/23(17.4%). Also, 2/23(8.7%) appeared negative for both genes; as shown in **Figure (3-10).**

Concerning detection of ribosomal protection gene *tetM*, all isolates were negative for this gene Due to the increase in the resistance to majority of antibiotics in *A. baumannii*, evaluating antimicrobial susceptibility of older agents that was not used in clinical practice is of interest to overcome the *Acinetobacter* infections, such as studying effectiveness of Tetracyclines against *A. baumannii in vitro.*

Six efflux pump-encoding genes have been reported in *Acinetobacter* species, including *tetA*, *tetB*, *tetG*, *tetH*, *tetL*, and *tet39*

Significantly, fact that there was neither *tetB* nor *tetA* in some isolates in this study indicates that additional genetic determinants other than *tet* genes may play a role in the expression of resistance to Tetracycline in some of these strains.

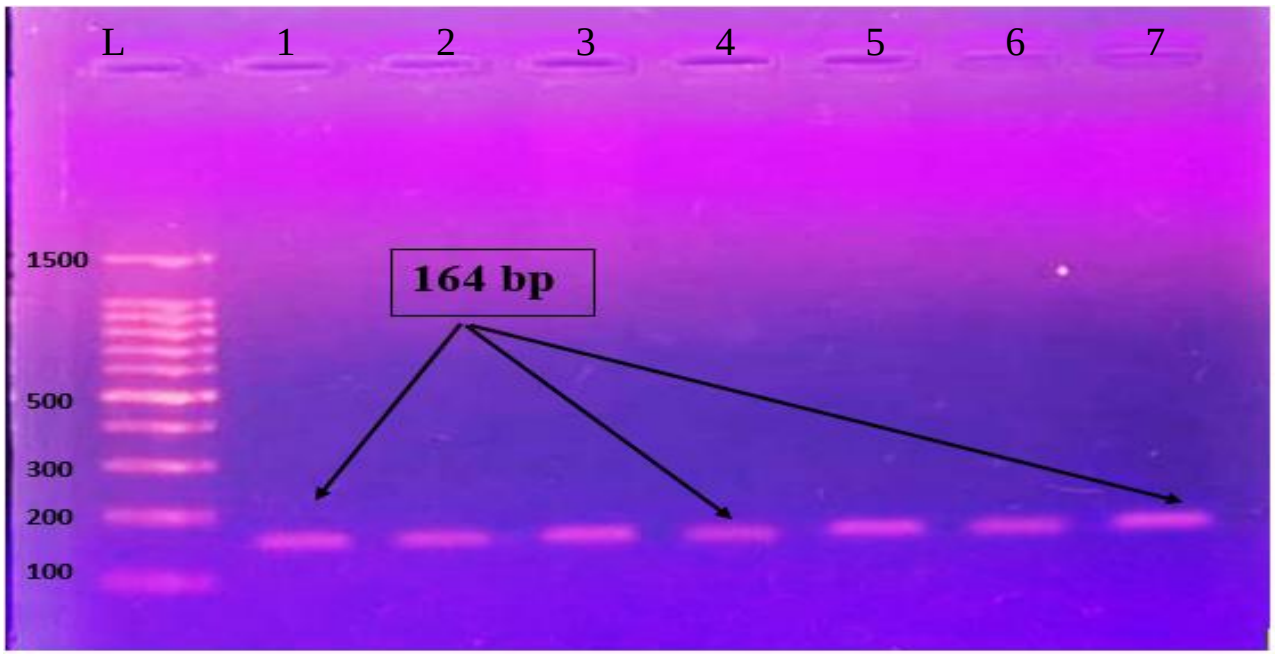

Figure (3-8): Agarose gel electrophoresis of *tetA* gene products visualized under U.V light after staining with ethidium bromide. L: 1500 bp ladder. The amplicons size is 164 bp.

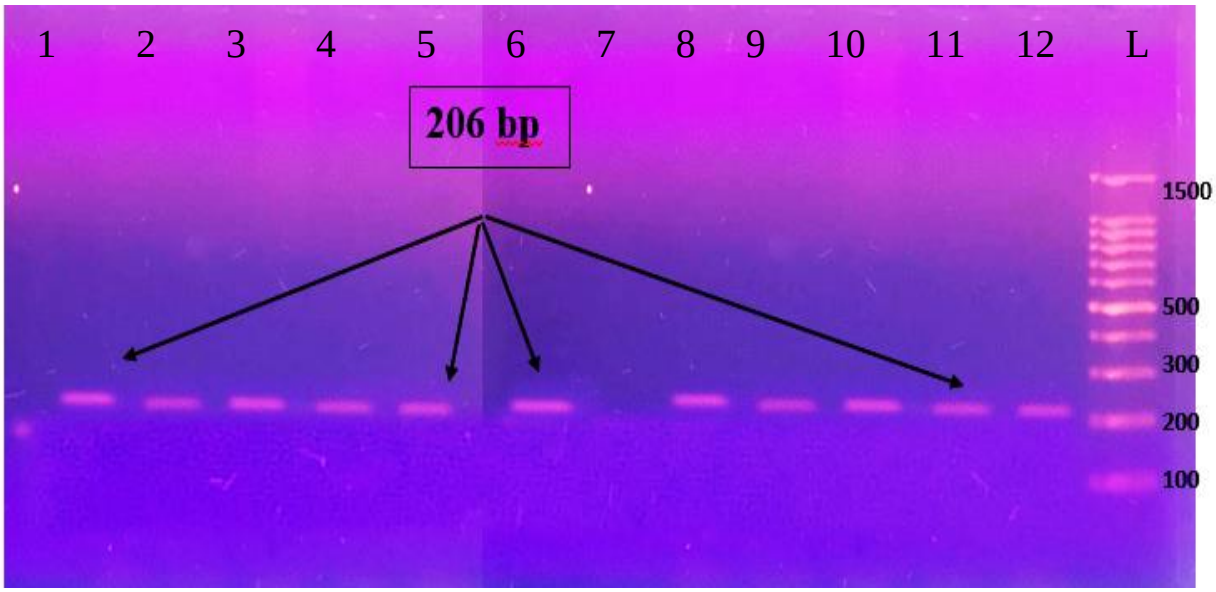

Figure (3-9): Agarose gel electrophoresis of *tetB* gene products visualized under U.V light after staining with ethidium bromide. L: 1500 bp ladder. The amplicons size is 206 bp.

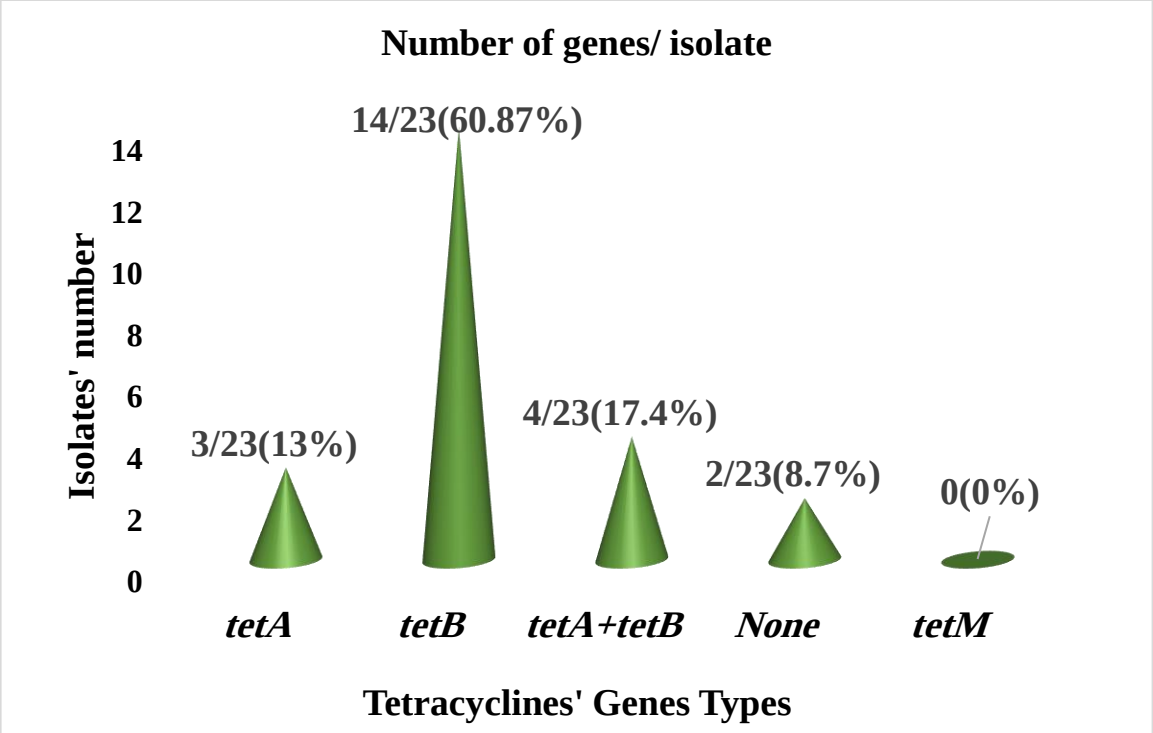

Figure (3-10): Distribution Tetracyclines' Genes among *A. baumannii* isolates.

tetA and *tetB* determinants conferring efflux phenotypes of resistance to Tetracycline have been known in *A. baumannii* isolates. The prevalence of *tetB* and *tetA* genes had been reported in at least 50% and 14%-46% of Tetracycline-resistant *A. baumannii* isolates (Grossman, 2016). Other study showed a high prevalence (61.7%) of *tetB* but not of *tetA* gene in tetracycline-resistant isolates. Similarly, Mosavat *et al*. (2018) detected the *tetB* determinant in a significant percentage of *A. baumannii* isolates about 95% but interestingly, *tetA* was not found in any of the isolates.

The prevalence of *tetB* in *A. baumannii* has increased over recent years, this phenomenon may be achieved by acquiring pre-existing resistance determinants via the mobile genetic elements, followed by amplification in response to selection, as is commonly observed in multi-drug resistant *A.*

baumannii, for example, insertion sequence is found to play a role in spreading of *tetB*, or genomic antibiotic resistance islands or transposons, by which that import *tetB* and other antibiotic resistance genes into the globally disseminated *A. baumannii* clones (Zhang *et al.*, 2012).

Maleki *et al.* (2014) revealed that nearly all tetracycline resistant isolates harbored at least one resistance gene. *tetB* was the most frequent encoding gene among study isolates (87.6%), and coexistences of *tet*A and *tetB* was seen among 1.1%.

The *tetA* and *tetB* determinants are common in Tetracyclines resistant *A. baumannii* isolates. A previous studies by Farsiani *et al*. in 2015 and Meshkat *et al.* (2017) reported that the *tetA* gene was not found in any of the strains whereas *tetB* was detected in 100% of the strains.

In contrast, Guardabassi *et al.* suggested a higher prevalence of the *tet*(A) gene among clinical isolates (6 out of 15 isolates, or 40%); who found that *tet*(A) and *tet*(B) are the genes responsible for tetracycline resistance that are most frequently encountered in clinical isolates of *A. baumannii*. They suggested that the *tet*(A) gene was identified in the strains resistant to tetracycline but not to minocycline, while the gene was not found in the strains resistant to both antibiotics. These strains may possess the Tet B determinant, which would confer resistance to both antibiotics.

Certain study suggested that *tetM* gene had been identified as having a Gram-positive origin, as this gene was studied in both Gram positive and negative bacterial isolates; and revealed that the frequency of the *tetM* gene in the Gram negative isolates (1 out of 15 isolates, or 6.6%) but with higher frequency among Gram positive isolates; this only detected gene was shown to be 100% homologous to the *tet*(M) gene of *S. aureus* suggests a horizontal

transference of genetic material between gram-positive and Gram-negative bacteria.

Although these genes possess a G + C content similar to that of Gram-positive organisms, they have been detected among both Gram-positive and negative genera (Roberts, 2020).

These genes encode for cytoplasmic proteins, which prevent tetracycline, doxycycline, and minocycline from binding to the ribosome, causing in vivo and *in vitro* resistance. These protection proteins interact with the protein within the ribosome, causing disruption of the primary tetracycline binding site. Consequently, the tetracycline molecule binding is reduced or released from the ribosome, which maintains or returns to a conformational state that allows protein synthesis (Castanheira *et al.* 2014).

3.6.2. Phylogenetic analysis by REP-PCR

REP-PCR fingerprinting grouped *A. baumannii* strains isolated from different specimens and from the same period of isolation and location. Among the (23) collected strains of *A. baumannii* the 23 strains were divided into two major lineages (clusters) denoted as genotype A and B on primary analysis; in which cluster A contain two small sub cluster with 5 members, where the 1st. with 2 members from the same source (wound) and the 2nd. contains 3 members from different sources and two of the showed no difference in the distance indicating that it is identical, but regarding phenotypic Tetracycline-resistance distribution this cluster include only one resistant isolate with MAR index 0.75.

Regarding Cluster B, it is subdivided into two subclusters (B1 and B2) and each of them again subdivided into two subgroups.

Subcluster B1 containing 4 isolates obtained from different sources and additionally contain the standard strain *A. baumannii* ATCC, 19606; also 3/4 of these isolates showing phenotypic Tetracycline-resistance 2 of them with MAR index 0.75 and one with MAR index 1.00 (complete Tetracycline resistance).

Subcluster B2 containing 14, that subgrouped into B3 and B4 subgroups, where B3 containing 8 isolates (most of them obtained from sputum), four of them showed no differences in the distance and indicating homology and relationship between these isolates with different Tetracycline resistance distribution; these four isolates were sourced from burn and sputum and noticeably depending on the isolates' history these four isolates were collected from two patients resides adjacent to each other in the same ward.

Finally, regarding subgroup B4 containing six isolates that collected from various sources, but the 1st. were collected from the same person, and this is the same about the 2nd. two isolates, and this is can be cleared by the absence of distance between them, all these data can be illustrated at **Figure (3-11**) and **Figure (3-12**).

Markedly, the Tetracycline-resistance phenotypes were grouped more commonly among members of cluster B especially subcluster B2. It is possible that the bacteria evolved as a result of the increasing use of antibiotics, which exerted selection pressure; also the standard strain *A. baumannii* ATCC, 19606 was located within the subcluster B1 and the nearest isolates to this strain were isolate number 12 (from sputum) and isolate number 22 (from urine).

There were also consistent REP-PCR DNA fingerprints among distinct antibiotype profiles within a genotype, which shows that resistance

acquisition may not have entailed gene insertion or deletion that affected the placement of the REP sequence elements. Acinetobacter spp. may have developed resistance to antibiotics because of other processes, such as gene mutation and gene silencing. REP-PCR amplification, on the other hand, may not have included any genomic area containing the acquired genes that contributed to antibiotic resistance (Selim *et al*., 2022).

Several authors have shown that repetitive extragenic palindromic PCR (REP-PCR) is a simple, rapid, and relatively low-cost method, useful in the characterization of nosocomial outbreaks of *A. baumannii* infections, being comparable to pulsed-field gel electrophoresis in regard to discriminatory power and reproducibility. Moreover, REP-PCR has a higher power of discrimination and reproducibility than do other PCR-based fingerprinting systems (Selim *et al*., 2022).

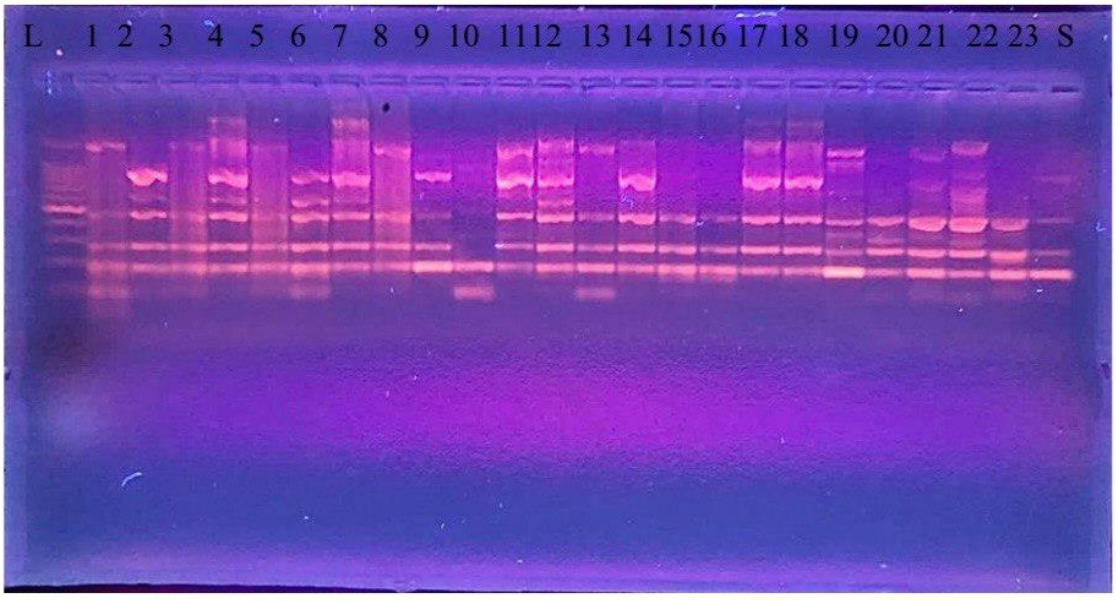

Figure (3-11): Agarose gel electrophoresis of REP-PCR products of 1-24 *A. baumannii* isolates, multiple bands products compared with 100 bp ladder.

Additionally, **Table (3-4)** showed the distribution of the different clones (A, B1, B3, B4) according to the sources of *A. baumannii*, where the five burn isolates were among clone B3 (3/5) and clone B4 (2/5); the four urine isolates were mainly from clone B1 (2/4) and 2 isolates from cloneA and B4, Where the four wound isolates were mainly from clone A (3/4). In contrast to them, the ten sputum isolates were distributed among the whole clones but mainly among clone B3 (4/10) and clone B4 (3/10).

Thus the nosocomial *A. baumannii* infections in this study were polyclonal, nineteen main different genotypes having been identified. On the contrary, epidemic outbreaks are mainly due to a single clone of *A. baumannii*. The distribution of clones throughout this study shows that the polyclonal character of *A. baumannii* explains why some patients with two *A. baumannii* isolates in different clinical samples had genotypically different *A. baumannii* isolates.

Many studies employed REP-PCR as a phylogenetic method especially during outbreaks or during dealing with antibiotic- resistant isolates indicating that REP-PCR is a useful and expeditious method with high discriminatory power for the epidemiological characterization of *A. baumannii* clinical isolates (Higgins *et al*., 2012; Pasanen *et al*., 2014; Selim *et al.*, 2022).

Meshkat and his co-workers (2017) applied REP-PCR as an analytic method, its results divided their isolates into ten distinctive REP-PCR clusters, in similar work to Ecker *et al.* (2006) by using molecular typing methods, The able to discriminates widespread clonal lineages of *A. baumannii* responsible for hospital clonal transition dynamics in outbreaks worldwide; and this was accomplished by the application of REP-PCR.

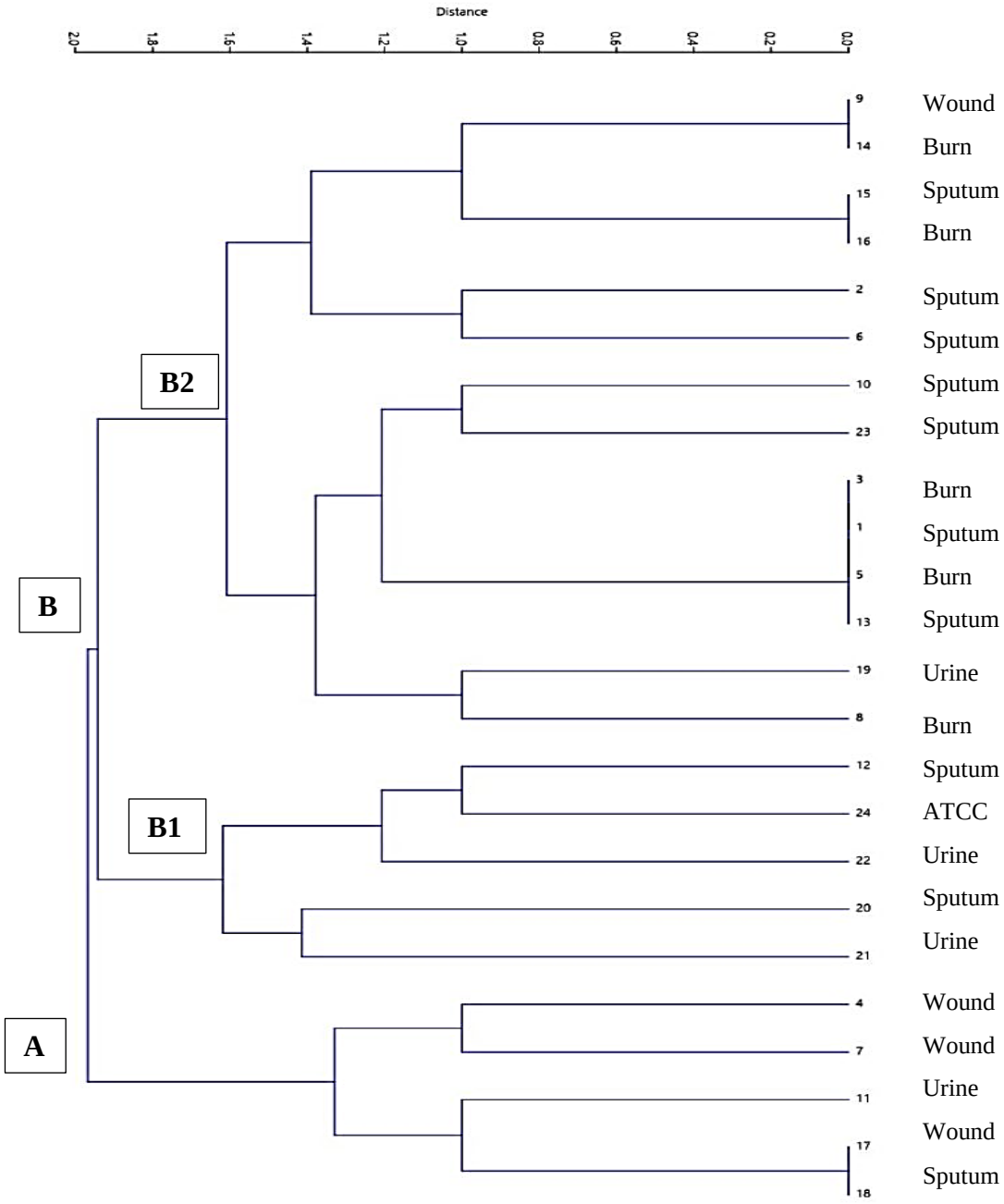

Figure (3-12): REP-PCR Phylogenetic Dendogram representing the relationship among 23 of *A. baumannii* in patients with different sample sources in comparison with (*A. baumannii* ATCC, 19606). Bar (above) represents the distance values. This cladogram was generated by PAST software system.

Conclusions

and

Recommendations

Conclusion

1- Bacterial resistance to antibiotics is a serious concern for the public health and the antibiogram along with MAR index serve an important epidemiological tool to monitor drug resistance.

2- High rates of antibiotic resistance were observed for most Tetracyclines, whereas the lowest resistance rates were observed for tigecycline, making them the most effective treatment options.

3- Ethidium bromide agar Cartwheel method can be a useful and economical tool in the detection of clinically relevant Tetracycline-resistance phenotypes of *A. baumannii* that utilize the efflux pump mechanism.

4- Plant-derived compounds can act as adjuvants to antibiotics to increase their ability to treat infectious diseases.

5- Rep-PCR was able to differentiate between *A. baumannii* strains with high discriminatory power, and identified several clusters of genetically related tetracycline-resistant isolates.

Recommendation

1- Application of strict antibiotic policy should be adopted in hospitals to estimate the impact of higher resistance in bacteria and to take steps for reducing this resistance.

2- The presence and diversity of tetracycline resistance genes in *A. baumannii* highlight the need for ongoing surveillance and infection control measures to prevent the spread of multidrug resistant *A. baumannii* strains in healthcare settings.

3- The inhibition of efflux pumps appears to be a promising strategy to restore antibacterial potency because active efflux of antibacterial agents plays a significant role in mediating drug resistance in bacteria and Evaluating only one efflux pump inhibitor (EPIs) is not valid for establishing a method. Therefore, further investigations using several EPIs for different bacteria are needed.

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Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed Oct 25, 2022 09:33 CDT

Patient Name: nawal, kadhim
Location:
Lab ID: 20221013

Patient ID: 20221013
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Acinetobacter baumannii

Source: Sputum

Collected: Oct 12, 2022

Comments:	

Identification Information	**Analysis Time:**	4.83 hours	**Status:** Final
Selected Organism	99% Probability **Bionumber:**	**Acinetobacter baumannii** 0241010301500210	
ID Analysis Messages			

Susceptibility Information	Analysis Time: 7.02 hours			Status: Final	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Ticarcillin	>= 128	R	Amikacin		
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	2	S
Piperacillin	>= 128	R	Tobramycin	<= 1	S
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Ceftazidime	>= 64	R	Pefloxacin		
Cefepime	>= 64	R	Minocycline	<= 1	S
Aztreonam			Colistin	<= 0.5	S
Imipenem	>= 16	R	Rifampicin		
Meropenem	>= 16	R	Trimethoprim/Sulfamethoxazole	<= 20	S

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

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	CARBAPENEMASE

bioMérieux Customer:

Microbiology Chart Report

Printed Oct 25, 2022 09:35 CDT

Patient Name: jabar, hassan ali
Location:
Lab ID: 2022109

Patient ID: 2022109
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Acinetobacter baumannii

Source: Urine

Collected:

Comments:	

Identification Information	**Analysis Time:**	5.82 hours	**Status:**	Final
Selected Organism	99% Probability **Bionumber:**	**Acinetobacter baumannii** 0201010103500210		
ID Analysis Messages				

Susceptibility Information	Analysis Time: 7.53 hours			Status: Final	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Ticarcillin	>= 128	R	Amikacin		
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	>= 16	R
Piperacillin	>= 128	R	Tobramycin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Ceftazidime	>= 64	R	Pefloxacin		
Cefepime	>= 64	R	Minocycline	4	S
Aztreonam			Colistin	<= 0.5	S
Imipenem	>= 16	R	Rifampicin		
Meropenem	>= 16	R	Trimethoprim/Sulfamethoxazole	>= 320	R

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

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	CARBAPENEMASE

الخلاصة

البكتريا البومانية هي أحد مسببات الأمراض الانتهازية سالبة الجرام ، وتسبب التهابات شديدة يصعب علاجها ، ومقاومة التتراسيكلين بوساطة مضخة التدفق وآلية أخرى تسبب مشاكل ، ويمكن أن تكون مثبطات مضخة التدفق مفيدة.

لذلك هدفت هذه الدراسة إلى الكشف المظهري والجيني لمقاومة البكتريا البومانية للتتراسيكلين، والكشف عن مثبطات مضخة التدفق وتوزيع العزلات المقاومة للتتراسيكلين.

تضمنت هذه الدراسة مائة وخمسة وعشرين (125) عينة سريرية تم جمعها خلال الفترة الممتدة من بداية تشرين الأول 2022 إلى نهاية كانون الثاني 2023 ، من المرضى الذين حضروا إلى مستشفى الحلة التعليمي العام. تم جمعها من مواقع سريرية مختلفة مثل التهابات المسالك البولية والجهاز التنفسي والحروق والتهابات الجروح. ثم زرعت العينات على اوساط زرعية مختلفة ثم حضنت هوائياً عند 37 درجة مئوية لمدة 24 ساعة ، وتم تأكيد التشخيص عن طريق الزراعة والاختبارات البيوكيميائية.

تم الكشف عن عزلات البكتريا البومانية على أنها 23/125 (18.4٪) موزعة على شكل 10/23 (25٪) عزلات من البلغم ، 5/23 (17.9٪) من الحروق ، بينما 4/23 (16٪) و 4/23 (12.5٪) عزلات كانت من البول والجروح على التوالي.

تم إخضاع جميع العزلات البكتيرية لانواع مختلفة من التتراسيكلين (التتراسيكلين ، المينوسكلين، الدوكسيسيكلين والتيجيسيكلين) باختبار الانتشار القرصي والتركيز المثبط الأدنى، وأظهرت النتائج أن أعلى مقاومة ضد المينوسكلين 11/23 (47.8٪) تليها مقاومة التتراسيكلين والدوكسيسيكلين 10/23 (43.5٪) و 9/23 (39٪) على التوالي، والتيجيسيكلين حوالي 4/23 (17.4٪).

بالإضافة إلى ذلك ، فيما يتعلق بمؤشر مقاومة المضادات الحيوية المتعددة ؛ أوضحت النتائج أن 3/23 (13٪) مع MAR يساوي 1.0 مما يعني مقاومة لجميع المضادات الحيوية المستخدمة، بينما 9/23 (39٪) مع MAR أكثر من 0.2 والباقي 11/23 (47.8٪) مع حساسية كاملة تجاه جميع المضادات الحيوية (MAR = 0.0).

فيما يتعلق بـ MIC ، أظهرت النتائج أن Tigecycline هو العضو الأكثر فاعلية (23/19) ، مع MIC 1 ميكروغرام / مل بنطاق (1 – 0.125 ميكروغرام / مل) ضد هذه العزلات ، بينما أظهرت العزلات المقاومة MIC 8. 16 ميكروجرام / مل. من بين 23 *A. baumannii*، 13 عزلة حساسة للتتراسيكلين ، تظهر قيم MIC تتراوح من 2–8 ميكروغرام / مل ، بينما العزلات المقاومة مع نطاق MIC 16–256 ميكروغرام / مل وبشكل رئيسي عند 16–32 ميكروغرام / مل. بالإضافة إلى ذلك ، فيما يتعلق بالدوكسيسيكلين والمينوسكلين أظهر كلاهما نطاق MIC الفعال 1–8 ميكروغرام / مل.

فيما يتعلق بالكشف عن مضخة تدفق *A. baumannii* بواسطة مقايسة عجلة بروميد الإيثيديوم ، 10/8 (80٪) عزلات تعبر عن مضخة تدفق نشطة ؛ 8/5 عند 2 مجم / لتر وأكثر ، ولكن لم تتألق البقية 8/3 حتى عند 2.5 مجم / لتر من EtBr مشابه للتحكم الإيجابي *A. baumannii* ATCC ، بينما لم تظهر 10/2 (20٪) عزلات نشاط. في وقت لاحق تم استخدام مستخلص البابونج المائي كمضاد للميكروبات ومثبط لمضخة التدفق ، وأظهر أنه مثبط فعال خاصة عند التراكيز الأعلى (40 ، 80 مجم / مل) مع تثبيط لمعظم العزلات المختبرة.

الجزء الأخير من هذه الدراسة هو القسم الجيني الذي تضمن اكتشاف جينات مقاومة التتراسيكلين (*tetA* و *tetB* و *tetM*) عن بأستخدام بادئات وعن طريق عملية البلمرة التقليدية. حيث أظهرت نتائج هذه التضخمات أن جين *tetA* ظهر بين 7/23 فقط (30.4%)؛ بينما فيما يتعلق بجين *tetB* ظهر بين 18/23 (78.3٪) مع ظهور الجينين معا في اربع عزلات فقط (17.4%) وكانت عزلتين سالبة لكلا الجينين (8.7%). بينما جين حماية الريبوسوم (*tetM*) كانت جميع العزلات سلبية لهذا الجين.

تم تجميع البصمات المتكررة PCR (REP–PCR) لسلالات البكتريا البومانية ، من بين (23) سلالة تم جمعها ، تم تقسيم السلالات الـ 23 إلى مجموعتين رئيسيتين (A و B) ؛ حيث تحتوي المجموعة A على 5 أعضاء ، لم يظهر اثنان منهم أي اختلاف في المسافة مما يشير إلى أنها متطابقة ، وتحتوي على عزلة واحدة مقاومة للتتراسيكلين بمؤشر MAR 0.75.

بينما فيما يتعلق بالمجموعة B ، يتم تقسيمها إلى مجموعتين فرعيتين (B1 و B2) وينقسم كل منهما مرة أخرى إلى مجموعتين فرعيتين.

المجموعة الفرعية B1 تحتوي على 4 عزلات تم الحصول عليها من مصادر مختلفة وتحتوي بالإضافة إلى ذلك على السلالة المعيارية ATCC *A. baumannii*، 19606؛ كذلك 3/4 من هذه العزلات أظهرت مقاومة التتراسيكلين المظهرية 2 منهم بمؤشر MAR 0.75 والآخر بمؤشر MAR 1.00 (مقاومة التتراسيكلين الكاملة).

المجموعة الفرعية B2 التي تحتوي على 14 ، والتي تم تجميعها في مجموعات فرعية B3 و B4 ، حيث تحتوي B3 على 8 عزلات (تم الحصول على معظمها من البلغم) ، لم تظهر أربعة منها فروقًا في المسافة وتشير إلى التماثل والعلاقة بين هذه العزلات بتوزيع مختلف لمقاومة التتراسيكلين.

أخيرًا، فيما يتعلق بالمجموعة الفرعية B4 التي تحتوي على ستة عزلات تم جمعها من مصادر مختلفة.

في الختام ، يمكن أن نقول ان REP–PCR يعتبر عملية مناسبة للتحليل الجيني للبكتريا البومانية المقاومة للتتراسيكلين.

وزارة التعليم العالي والبحث العلمي

جامعة بابل – كلية الطب

فرع الاحياء المجهرية الطبية

تحليل النشوء والتطور لل *Acinetobacter baumannii* المقاومة للتتراسايكلين والمعزولة من عينات سريرية مختلفة

رسالة

مقدمة الى مجلس كلية الطب – جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في العلوم/ الاحياء المجهرية الطبية

من قبل

رقـيــة كريـم عبـاس عوده

بكالوريوس علوم حياة–كلية العلوم–جامعة بابل

2015

باشراف

الاستاذ المساعد

د. زينب عادل غني چـــابـك

1445 2023