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Molecular Detection and Gene Expression for *hcp* and bla_{OXA-51} Genes in *Acinetobacter baumannii* Isolated from Different Clinical Sources

A thesis

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By

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Dedication

To the great creator … The God

To those who have given… their lives to our Iraq

To the strong wedge and the towering mountain ….. My dear father and to the soul of my deceased mother

To my partner and support …My Husband Yasser

To my three angels My lovey daughters… Rawan, Rahaf, Welddan

To the pure hearts and loyal hands who assisted me in life …My Brother and Sisters … and to all who supported me

Shaymaa 2020

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Summary

Acinetobacter baumannii one of the important multidrug-resistant (MDR) opportunistic nosocomial pathogens, in part due to its high capacity of acquiring resistance to diverse antibiotic groups. Twenty isolates (9.7%) of *Acinetobacter baumannii* were obtained from (207) clinical specimens including infections of wounds ,burns ,sputum and blood from both male and female, different ages, diverse local regions .One hundred and sixty-two specimens had been given positive growth while Forty-five specimens showed no growth. The isolates were collected during the study period from the initial September /2019 till at the end of January /2020. The samples were collected from visitors and hospitalized patients in governmental hospitals in Baquba / Diyala. The visitor's percentage 57.97%, but the hospitalized patients' percentage was 42.03%.

A. baumannii initial diagnosis was made by culture media (MacConky and blood agare), and depending on the features of the cultures, biochemical tests, and VITEK 2 Compact system GN,final confirmation of diagnosis and identification was done using bla_{OXA51} gene.The positive numbere of *A.baumannii* isolates were high percentage in burns 8(40%), then blood 7(35%), wound 4 (20%) and the later was sputum only 1(5%) isolate.

The antimicrobial susceptibility of *A.baumannii* isolates was determined by disk diffusion method. The results showed that all the isolates 20(100%) were resistance Pipercillin-tazobactam,Cefotaxime, Ticarcillin–clavulanic acid,Amikacin, Gentamicin, Levofloxacin, (95%)for Imipenem,Meropenem(, (90%)for Ceftrixone, Ceftazidime, (65%)for Tetracycline, (55%)for Ampicillin-sulbactam, and(20%)for Doxycycline

The twenty isolates of *A.baumannii* were differentiation for two patterns according to antibiotic resistance, 15(75%) MDR isolates were resisted to (5-

9)antibiotics, while 5 (25%) of XDR isolates were resisted(12 -13) antibiotics.

The results for all 20 of MIC showed that the values for Imipenem ranged from(8-≥1024) µg/ml more than the MIC values for(≤4-128) µg/ml for Meropenem.

The β-Lactmases had been detected for three classes were (ESBLs, AmpC, MBLs). The results of ESBLs showed that all the isolates were with high resistance (100%) for Cefotaxime and Ceftazidim. The isolates also showed high resistance to piperacillin (PRL) and Augmentin (AMC) (100%).However, there was no synergy between the antibiotic discs.

Cefoxitin resistant test was ok to detect AmpC production. The results showed that 6 (30%) isolates were positive, three from burns, one was from blood, one isolate was from sputum, and one isolate was from the wound.

The results of MBLs phenotypically were positive for *A. baumannii* isolates that formed MBLs enzyme was 18 (90 %) isolates, but 2(10 %) isolates were negative. The results of molecular detection by conventional PCR were showed that 14 (70%) isolates of *A. baumannii* were possessed bla_{VIM} gene, but 6 (30%)isolates did not possess it, and all the 20 isolates did not possess $bla_{\text{NDM-1}}$, bla_{IMP} genes.

Detection of virulence factor was done to determine the aggressiveness of *A. baumannii*. Biofilm formation was detected by two methods, qualitative assay by the tube method, this is the first step of the ability of bacteria to form a biofilm, The results showed that all isolates showed purple ring after staining by crystal violate, and quantitative assay by Micro-titer plate method. The results were 7 (35%) of the isolates were strongly biofilm-forming while 12 (60%) for moderately biofilm-forming and 1(5%) weak biofilm formation.

The colorimetric method was used to detect quorum sensing (Q.S). The isolates were producer to Q.S signals in various percentages, 7(35%) of *A. baumannii* isolate were a high producer of Acyl-Homoserine-Lactones molecules, while 11(55%) isolates that exhibited moderate, and 2(10%) isolates were no formation activity of AHL.

conventional PCR technique was used to detect *hcp* gene as a molecular marker of a functional T6SS. The results showed that the prevalence of this gene 95%, it presented in 19 isolates, but one isolate did not have it.

Real-Time qRT PCR Technique (Syber green) was used to study gene expression after treatment by (128)µg/ml (sub MIC) concentration of Imipenem for two genes, bla_{OXA51} gene for Carbapenems resistance and *hcp*(T6SS). The bla_{OXA51} gene gave a high expression of different degrees in 3 3isolates from wounds, burns, and blood. The level of folding with the average of 1.45 was more than the expression of control, but The *hcp* gene gave a low expression compared with the control in different degrees in these 3 isolates with average folding 0.88.

Sequencing was performed for one(Ab.13) isolate of *A. baumannii*. Concerning 16S rRNA amplicons, the results indicated that the amplified 16S rRNA fragment exhibited five nucleic acid variations, g.422C>T, g.431A>C, g.433C>T, g.806T>C, and g.918-919G ins compared with the referring ribosomal sequences. Concerning the *hcp* amplicon, the results showed that the amplified *hcp* locus exhibited one silent mutation (g.279978C>T) compared with the referring region sequences. The generated *hcp*-based phylogenetic tree indicated that the investigated sequences were accurately positioned in the *A. baumannii* sequences. The utilization of a comprehensive tree for the phylogenetic positioning of the currently investigated sequences using the *hcp* gene had given confirmed identity with respect to the

phylogenetic relationships of the investigated sample as well as to the accurate phylogenetic positioning within the sequences of *A. baumannii*. This notion, in turn, indicates the feasibility of utilizing the currently targeted *hcp* gene fragment in the precise determination of the investigated identity of *A. baumannii.*

One strain of *A. baumannii* was isolated from a human source in Baquba city /Diyala and the sequences have a symbol code (SHRRWY.80) with the accession (MT551041), and variation (MT551041.1) for 16s rRNA, and with the accession (LC553000)and variation (LC553000.1) for *hcp* gene.

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List of Abbreviation

Abbreviation	Key
ACB complex	(*Acinetobacter haemolyticus* , *Acinetobacter junii* , *Acinetobacter radioresistans*)
AHLs	Acyl-Homoserine-Lactones
AmpC	Ambler class C beta-lactamase
BP	Base Pair
CDC	Centers for Disease Control
CEDT	Combined EDTA disc test
CLSI	Clinical & Laboratory Standards Institute
CRAB	Carbapenems resistant *Acinetobacter baumannii*
DDST	Double Disk Synergy Test
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay

EP	Efflux Pump
ESBL	Extended Spectrum Beta lactamase
ESKAPE	pathogens are *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *A.baumannii* (A), *Pseudomonas aeruginosa* (P), and *Enterobacter* species
Eth.Br	Ethidium bromide
Hcp	Hemolysin coregulated protein
ICU	Intensive Care Unite
ISAba1	Insertion Sequences A.baumannii
ISs	Insertion Sequences
MBLs	Metallo-β-lactamases
MDR	Multidrug resistant
MDRAB	Multidrug resistant *Acinetobacter baumannii*
MIC	Minimum Inhibitory Concentration
Nm	Nanometer
OD	Optical density
OMPs	outer membrane proteins
PCR	Polymerase chain reaction
PH	Hydrogen Ion
Pmole	Picomoles
qRT PCR	Quantitative Reverse Transcriptase Real-Time
QS	Quorum Sensing
T6SS	Type six secretion system
UV	Ultraviolet
WHO	World Health Organisation
XDR	Extensively Drug Resistance

CHAPTER ONE
INTRODUCTION

1. 1 Introduction

Acinetobacter baumannii is a gram-negative coccobacillus initially considered to be an opportunistic pathogen, which plays a vital role as a major cause of healthcare-associated infections. In recent years, *A.baumannii* has become resistant to most effective antimicrobial agents and causing a high incidence rate of morbidity and mortality especially in the intensive care unit in many countries (Moulana *et al*.,2020). *Acinetobacter baumannii* strains have the ability to colonize several ecological niches including soil, water, and animals, including humans. They also survive under extremely harsh environmental conditions thriving on rare and recalcitrant carbon compounds (Yakkala *et al*., 2019). *Acinetobacter baumannii* is frequently described as the etiologic agent for ventilator-associated pneumonia, urinary infections, wound infections, and bloodstream infections (Yadav *et al*., 2020).

Acinetobacter baumannii has become a major cause for concern in conflict zones, and has gained particular notoriety in the resent desert conflicts in Iraq, earning it the moniker "Iraqibacter." In particular, high incidences of MDR bacteremia (bloodstream infections) have been noted among US Army members (Howard *et al*.,2012).

Acinetobacter baumannii one of the important multidrug-resistant (MDR) opportunistic nosocomial pathogens, in part due to its high capacity of acquiring resistance to diverse antibiotics groups (Da Silva *et al*., 2016). Different mechanisms play a role in the acquisition of multidrug resistance (MDR) phenotype among *Acinetobacter baumannii* strains. This is because they possess a wide range of genes that they are encoded to antibiotic resistance, both intrinsic and acquired (Fallah *et al*., 2014). The ability to produce Carbapenemase enzymes such as oxacillinases (Ambler class D OXA-type) and Metallo-β-lactamases (Ambler class B) are the most frequent

resistance mechanisms in *A.baumannii* (Shoja *et al* .,2016), accordingly, the name of *A.baumannii* is associated with an acronym (CRAB) that mean Carbapenem-resistant *A. baumannii* (Bardossy *et al*., 2020). The genes coding for these enzymes can be passed from cell to cell via (chromosomal and plasmid-borne, integrons, and transposons), allowing this bacterium to survive the challenge by many classes of antibiotics (Nasiri *et al* ., 2020).

The *A.baumannii* whole-genome repertoire(the 'pan-genome') is impressively large of coding sequences (over 8800 CDSs for 12 strains) and increases exponentially as new genomes become available (an open pan-genome), which high lights the importance of gene acquisition and loss events in the evolution and adaptation of this human pathogen (Antunes *et al*.,2014).

The combinatorial approaches involving genomic, phenotypic, and infection model analyses have helped in the identification of virulence factors important for *A. baumannii* pathogenicity (Wong *et al.*,2017). Quorum sensing (Acyl homoserine lactone molecules) is a regulatory mechanism among Gram-negative bacteria and plays important role in expression of virulence genes, antibiotic resistance, biofilm formation, and T6SS in *A. baumannii* (Bhargava *et al*.,2010; Morris *et al*.,2019).

The Type VI secretion system (T6SS) is a protein nanomachine deployed by many Gram-negative bacteria. The primary function of the T6SS is as a device for inter-bacterial competition (Coulthurst *et al*.,2019)Expression of the T6SS varies among different strains of *A. baumannii*. several multidrug-resistant strains of *A. baumannii* harbor a large, self-transmissible resistance plasmid that carries the negative regulators for T6SS (Weber *et al*.,2016).

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. Bacterial gene expression depends not only on specific regulatory mechanisms but also on bacterial growth

because important global parameters such as the abundance of RNA polymerases and ribosomes are all growth-rate dependent. The changes in gene expression often reflect changes in the environment. Understanding these global effects is necessary for a quantitative understanding of gene regulation and important physiological functions such as antibiotic resistance and tolerance (persistence) (Klumpp *et al* .,2009).

The aims of the study

The current study aimed to a phenotypic and molecular investigation of multidrug-resistant (MDR) *Acinetabacter baumannii*, especial that should be resistant to Carbapenems (Imipenem, Meropenem), and measure the expression of the of $bla_{OXA51,}$and *hcp* genes and compare the gene expression in the presences of Antibiotic, and in the absence of it. in order to show the role of this gene in the resistance of *Acinetabacter baumannii*. For this aim, the following steps were performed:

1- Isolation and identification of *Acinetobacter baumannii* from different clinical infections.

2-Investigations of the occurrence of multi-drug resistant and antibiotic susceptibility profile in *Acinetobacter baumannii* isolates, as well as the minimum inhibitory concentration (MIC) for certain antibiotics.

3- Phenotypic detection of the Extended-spectrum β-lactamase (ESBLs) AmpC, and Metallo beta-lactamase (MBLs) enzymes

4- Detection of some virulence factor phenotypic, Quorum sensing, biofilm formation, and T6SS.

5- Molecular investigation of some genes coded for resistance toβ-lactams, using PCR technique *(*bla_{IMP}, bla_{VIM}, bla_{NDM-1},bla_{OXA-51}) and molecular detection to *hcp* gene that is a hallmark of present T6SS.

6- Studying gene expression of *bla*$_{\text{OXA-51}}$ and *hcp* genes using quantitative RT-PCR technique.

CHAPTER TWO

LITERATURES REVIEW

2. Literatures Review

2.1.History of *Acinetobacter baumanii*

The history of the genus Acinetobacter dates back to the early 20th century, in 1911, when Beijerinck, a Dutch microbiologist, isolated a microorganism from the soil by enrichment in minimal medium contains calcium acetate (Dijkshoorn, 2008). A major breakthrough in the long and complicated history of the genus was achieved in 1986 by Bouvet and Grimont, who—based on DNA-DNA hybridization studies—distinguished 12 DNA (hybridization) groups or genospecies, some of which were given formal species names, including *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, and *A. lwoffii*. In 1971, the sub-committee on the Taxonomy of Moraxella and Allied Bacteria officially acknowledged the genus *Acinetobacter* based on the results of Baumann's 1968 publication (Howard *et.al,*2012).

Acinetobacter baumannii is one of the most strains that during the past three decades has emerged from an organism of questionable pathogenicity to an infectious agent of importance to hospitals worldwide. Clinically the most important of 25 *Acinetobacter* genospecies, increased from 9% in 1995 to 40% in 2004 (Munoz-Price *et al*.,2008). Bacteriological studies on the antibiotic resistance in *A. baumannii* in Iraq are relatively new; the first published paper appeared in 2001. Since then, more data have been made available and the real situation of antibiotic resistance in hospitals is alarming (AL-Marjani *et al*,2016).

2.2. Taxonomy

This Bacteria was classified as members of the genus *Acinetobacter* have a long history of taxonomic changes, moving from the family

Neisseriaceae to the family Moraxellaceae. Within the genus Acinetobacter, studies based on DNA/DNA hybridization have resulted in the description of 25 “genomic species” that fulfilled the criteria to be considered distinct species(Fournier(a) *et al*.,2006).

The *Acinetobacter* genus currently comprises 34 species, with the *Acinetobacter* calcoaceticus-Acinetobacter baumannii (ACB) complex including four phenotypically related species: *A. baumannii*, *A. calcoaceticus*, *A. pittii*. Advances in molecular genotyping and whole genome sequencing have made possible delineation of individual Acinetobacter species within clinical isolates of the ACB complex (Fitzpatrick *et al*.,2015).

The species’ names have endured substantial taxonomic changes over the years due to the advanced understanding of molecular methods of the genetic make-up of this group of microorganisms. Recent classifications that seem to have gained wide acceptance among bacterial taxonomists have accepted this group of bacteria as gamma Proteobacteria categorized in the order Pseudomonadales and the family Moraxellaceae. Thus the taxonomical classification is given as; Domain: Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Pseudomonadales, Family: Moraxellaceae, Genus: *Acinetobacter*. The species *A. baumannii*, *Acinetobacter haemolyticus* and *A. calcoaceticus* are of clinical significance (Almasaudie,2018).

2.3. General characteristics:

The Maine characters on the most study of *A. baumannii* is a Gram-negative bacillus that is aerobic, pleomorphic, non-fermenting, non-fastidious,non-motile, catalase-positive, oxidase-negative, and the bacteria with a DNA G + C content of 39% to 47% (Howard, *et.al,*2012). Even though they are non-motile, some "twitching" or "gliding" on semisolid media has occasionally been reported; This may be due to the activity of type IV

pili, also, they do not form spores and flagella are absent (Juni,2015). Morphologically *A.baumannii* is short, plump, typically (1.0–1.5) μm by (1.5–2.5)μm in size as measured during the rapid phase of their growth but often develop into more coccoid in the stationary phase, usually present in pairs or long chains of different in length .*A.baumannii* is metabolically versatile and can be grown easily on simple microbiological media, forming domed, smooth colonies of ˜2 mm diameter, convex, with some species being pigmented pale yellow or grey(Visca, *et al*.,2011). It was able to survive at a low pH(3.37), desiccation, and a high temperature of 40°C (Lal *et al*,2019).

2.4.Epidemiology

Acinetobacter. baumannii is primarily a healthcare-associated pathogen and many reports indicated it as the causative of outbreaks and nosocomial infections including septicemia, bacteremia, ventilator-associated pneumonia, wound sepsis, endocarditis, meningitis, and urinary tract infections (Almasaudi,2018; Sen *et al*.,2016). The World Health Organization (WHO) has recently identified antimicrobial resistance as one of the three most important problems facing human health. The most common and serious MDR pathogens have been encompassed within the acronym "ESKAPE," standing for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp* (Howard *et.al*,2012).

Acinetobacter baumannii became one of the most prevalent pathogens during wars in Lebanon, Afghanistan, and Iraq, causing multiple outbreaks of MDR infections among combat casualties(Bazzi et al.,2020). A key risk group for *A. baumannii* infection is members of the armed forces who have been deployed to conflict zones, particularly Iraq, earning *A.baumannii* the notorious moniker of 'Iraqibacter.' The dry, sandy conditions associated with these desert campaigns provide an ideal environment for the physiologically

robust *A. baumannii*, making it the main source of infection among injured soldiers(Camp *et al* .,2010).

2.4.1.Habitat:

The natural habitats of the bacteria are soil, water, animals, and humans. It is normal inhabitants of human skin and is frequently isolated from the throat and respiratory tract of hospitalized patients (Fournier(a) *et al*.,2006). However, over the last decade, there have been reports on the existence of *A. baumannii* in environments influenced by humane waste. Urban wastewater represents one of the largest proportions of human waste and it consists of different types of wastewaters that are generated in cities such as domestic, industrial, hospital, and storm wastewaters. Of these, hospital wastewaters are recognized as the source of *A. baumannii* of clinical significance (Higgins *et al*.,2018).

2.4.2. Reasons for outbreaks and methods of transmission

One of the important feature of *A. baumannii* ; its tendency to cause outbreaks due to its resistant antimicrobial agents and its ability to overcome desiccation (Fournier(a) *et al*., 2006). In the past decade strains of *A. baumannii* often have emerged as a major cause of healthcare-associated infections (HAIs)and the organism shows a formidable capacity to complex coexistence of epidemic and endemic infections (Gordon *et al*.,2010). It has the ability to survive on dry surfaces under nutrient limiting conditions facilitates their persistence and transmission in the natural and medical environment. Furthermore, colonized medical devices and equipment could serve as reservoirs in prolonged hospital outbreaks, it is more frequently found on inanimate objects and hands of staff in the (Intensive care unit)ICU than *Staphylococcus aureus* and *Pseudomonas spp*(Almasaudi,2018; Townsend *et al* .,2015).

2.5 Pathogenicity

Acinetobacter baumannii has a wide distribution in most environments and it was considered the second pathogen after *Pseudomonas aeruginosa,* which spread in hospitals (Vishnu Preya *et al*., 2019). The attributable mortalities in patients with *A. baumannii* healthcare-associated infections, of which ventilator-associated pneumonia and bloodstream infections are the most common, can range from 5% in general hospital wards to 54% in the intensive care unit (ICU)(Ayoub Moubareck *et al*.,2020). On the other hand, several various infections are related to this microorganism such as Pneumonia, ventilator-associated pneumonia (VAP) is the most related infection with *A. baumannii* especially in intensive care units (Dexter *et al*.,2015)and bacteremia, It has become a leading cause of bloodstream infections in health care settings with intravenous catheters or the respiratory tract representing a frequent source of infection (Oliveira *et al*.,2015).

Acinetobacter baumannii has been repeatedly isolated from skin and soft tissue in patients with severe burns, wounds, or trauma, for instance, soldiers injured during military operations or victims of natural disasters (Akrami *et al*.,2019; Lerner *et al*.,2020) and it is one of the causes urinary tract infections (UTIs), especially with indwelling urinary catheters. Moreover, it is unusual for this organism to cause uncomplicated UTI in healthy outpatients (Di Venanzio(a) *et al*.,2019). Nosocomial meningitis due to *A. baumannii* remains an increasing threat in intensive care neurosurgery units, with mortality approaching 70%, especially in patients on indwelling ventriculostomy tubes or cerebrospinal fistulae and receiving post-surgical antimicrobial therapy (Xiao *et al*.,2019). However, *A. baumannii* is also reported from osteomyelitis, infections, dental plaques, chronic and aggressive periodontitis, and polymicrobial bacterial infections (Akrami *et al*.2019).

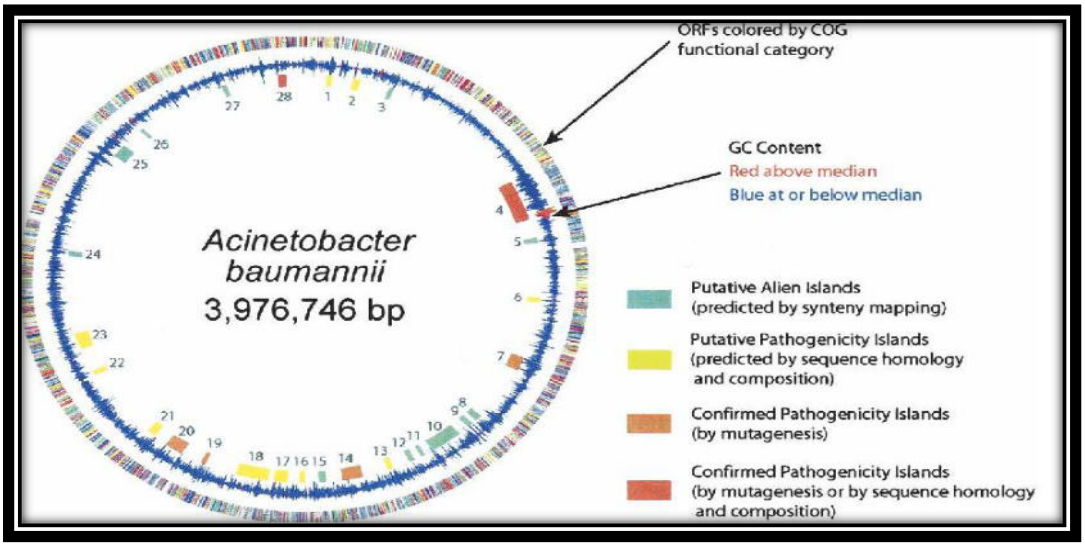
2.6 Genetic Content of *Acinetobacter baumannii*

The chromosome of this bacteria is described as a single chromosome containing 3,976,747 base pairs, 3,454 bp of which are specialized for protein synthesis, and with a DNA G + C content of 39% to 47%, and it has 3830 open reading frame (ORFs). A significant fraction of (ORFs) (17.2%) is located in 28 putative alien islands, indicating that the genome has acquired a large amount of foreign DN (Smith *et al*., 2007). "A total of 3539 *A*. *baumannii* genome sequences are available in the NCBI database. All these genomes display resistance traits acquired through horizontal gene transfer (HGT) as part of the accessory genome"(Yakkala *et al*.,2019). It has much strain such as SD002, SDF, and others, but the famous strain is AYE that it as an XDR strain of *A. baumannii* complex.AYE has 86 Kb resistance island (RI) was identified carrying 45 resistance genes (Yakkala *et al*.,2019), and the strain AYE also contains two plasmids, pACICU1 and pACICU2 their size 28.2 and 64.3 base pairs, respectively. The pACICU1 plasmid contains no antibiotic-resistance genes, while the pACICU2 plasmid carries two copies of the bla_{OXA58} gene encoded for Carbapenem resistance. Also, it contains an area of about 20 kbp comprehensive genes involved in transport genes, indicating that this plasmid may be conjugated (Imperi *et al*., 2011). Most of the *A. baumannii* strains contain on an average 30–40 Genomic islands per genome, indicating a high degree of horizontal mobility of DNA among the strains of the *A. baumannii* complex (Yakkala *et al*.,2019).

The bacteria possesses conserved regions of seven housekeeping gene, and 305- to 513-bp internal fragments of seven such genes—*gltA*, *gyrB, gdhB, recA, cpn60, gpi*, and *rpoD*—were sequenced for all strains. This housekeeping genes are encoding for this respective products: citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*),

glucose-6-phosphate isomerase (*gpi*) and RNA polymerase 70 factors (*rpoD*) (Bartual *et al*.,2005).

The bacteria carries ISAba 125 gene and ISAba1is one of important insertion element present upstream gene, and that is significant requires to act as a strong transcriptional promoter for $bla_{\text{OXA-51}}$ like gene, the last responsible for hydrolyzing penicillins (benzylpenicillin, ampicillin, ticarcillin, and piperacillin) and carbapenems (imipenem and meropenem), and it has been suggested its use as a marker for identification of the species (Gordon, *et al*.,2010; Turton(a) *et al*.,2006). The role of ISAba1 and other insertion sequences in modulating the expression of *A. baumannii* resistance genes has also been established for genes involved in cephalosporin resistance (*amp*C) (Turton(a) *et al*.,2006) . *A. baumannii* strains harbor a plethora of plasmids that play key roles in the dissemination of antimicrobial resistance. However, the basic biology of Acinetobacter plasmids is poorly understood. Multiple globally distributed *A. baumannii* strains carry large conjugative plasmids (LCPs) ranging in size from 150 to 200 kb and are characterized by three conserved regions: a locus encoding the type IV secretion system (T4SS) conjugative machinery; a region encoding two TetR transcriptional regulators; and a transposon rich resistance island containing antibiotic resistance genes and regulation the T6SS activity (Di Venanzio(b) *et al*.,2019). Figure 1 showing the Circular map of *A. baumannii*.



Figure(2- 1). Circular map of *the A. baumannii* genome (Smith et al., 2007).

2.7Antibiotic

Antibiotics are secondary metabolites produced by some organisms active against bacteria and is the most important type of antibacterial agent for fighting bacterial infections. Antibiotic medications are widely used in the treatment and prevention of such infections. They may either kill or inhibit the growth of bacteria (Kang and Park, 2015). While some antibiotics can completely kill other bacteria, These are called bactericidal, and some of them are able to inhibit bacterial growth, those are called bacteriostatic (Holten and Onusko, 2000). The excessive use of antibiotics has led to the development of some resistant bacterial species, as it must be used according to medical instructions or the need to take it in order to avoid and reduce the emergence of antimicrobial resistance (Le Page *et al*., 2019).

2.7.1 Classification of Antibiotic

There are several ways to classify antibiotics; however, the most common classification schemes are based on their molecular structures, mode of action, and spectrum of activity (Calderon and Sabundayo, 2007), others include the route of administration (injectable, oral, and topical). Antibiotics

within the same structural class will generally show a similar pattern of effectiveness, toxicity, and allergic-potential side effects. Some common classes of antibiotics are based on chemical or molecular structures such as Beta-lactams, Quinolones, Aminoglycosides (Van Hoek *et al.,* 2011; Adzitey, 2015).

2.7.1.1 β-Lactam Antibiotics

Beta-lactam antibiotics are one of the most commonly prescribed drug classes with numerous clinical indications. From a biochemical point of view, these drugs have a common feature, which is the beta-lactam ring. The beta-lactam ring is an integral part of the chemical structure of several antibiotic families of beta-lactams. It is a heterocyclic ring, formed by the cycling of an amide group, and therefore, composed of three atoms of carbon and one of nitrogen (Gomez *et al*., 2015; Qin *et al* .,2014). The class includes Penicillins, Cephalosporins, Carbapenems, Monobactams, and Beta-lactamase inhibitors. This is a broad category of drugs, so the drugs get categorized functionally, which makes it easier to relate and prescribe. The basis of grouping is the activity spectrum, therapeutic, and susceptibility testing (Balsalobre *et al*.,2019). Beta-lactam antibiotics have a bactericidal mechanism of action, include inhibit the growth of rapidly multiplying bacteria by inhibiting their cell wall synthesis; this occurs through inhibition of the enzymes responsible for cell wall biosynthesis located in the cell membrane, the most common enzyme being penicillin-binding proteins (PBPs) (Drawz and Bonomo, 2010). This binding preventing the final transpeptidation of the peptidoglycan in the cell wall, ultimately resulting in the cell breaking down its cell wall, causing cell death (Balsalobre *et al*.,2019).

2.7.1.1.1: Penicillins

Members of Penicillin class include Penicillin G, Penicillin V, Oxacillin (dicloxacillin), Methicillin, Nafcillin, Ampicillin, Amoxicillin, Carbenicillin, Piperacillin, Mezlocillin and Ticarcillin which inhibit cell wall synthesis in bacteria (Boundless, 2016).

2.7.1.1.2: Cephalosporins

Cephalosporins are similar to the penicillins group regarding their structure and mode of action: (1) binding to specific PBPs that serve as drug receptors on bacteria, (2) inhibit cell wall synthesis by blocking the transpeptidation of peptidoglycan, and (3) activate autolytic enzymes in the cell wall that can produce lesions resulting in bacterial death. Cephalosporins are subdivided into generations (1st-5th) according to their microbial target but later generations increasingly become more effective against Gram-negative bacteria. (Brooks *et al*, 2010). The generations include(first generation Cephalosporins(Cefactor), Second-generation Cephalosporins (Cefoxitin), Third-generation cephalosporins(Ceftazidime), Fourth-generation Cephalosporins (Cefepime), Fifth-generation cephalosporins(Ceftobiprole)) (Vallerand and Sanoski, 2014).

2.7.1.1.3: Carbapenem

Carbapenems possess the broadest spectrum of activity and high potency against Gram-positive and Gram-negative bacteria. (Torres *et al*., 2007). Imipenem is a carbapenem and has the same activity as β-lactams. It inactivates the penicillin-binding proteins and causes lysis of the cell wall. It is has a wide spectrum of activity and is used when there are limited treatment options for infection treatment (Papich, 2015). Carbapenems are consist of

Imipenem, Meropenem, and Faropenem. In general, this group is the main drug used to treat *A .baumannii* (Qin *et al* .,2014).

2.7.1.1.4: Monobactams

Aztreonam which resembles aminoglycosides in activity. Monobactams are active against gram-negative rods primarily through binding to PBP3 but not against gram-positive bacteria or anaerobes (Brook, 2010). Piperacillin/Tazobactam is a Beta-lactam/ penicillanic acid sulfone combination made up of Piperacillin and Tazobactam. Tazobactam inhibits β-lactamases produced by the microorganism whilst Piperacillin inhibits cell wall synthesis (Berger, 2014).

2.7.1.2: Quinolones

Quinolones group are consists of Ciprofloxacin Norfloxacin, Levofloxacin, and moxifloxacin. Ciprofloxacin is a Fluoroquinolone antibiotic that is used to treat diverse infections. It is mainly used in the treatment of infections caused by gram-negative organisms. Quinolones (Ciprofloxacin and Norfloxacin) inhibit DNA gyrase and topoisomerase IV therefore DNA replication in the bacteria is blocked by inhibition of these enzymes (Francis *et al.,* 2015).

2.7.1.3: Aminoglycosides

Aminoglycosides include Gentamicin, Amikacin, Tobramycin, Netilmicin, and streptomycin. A typical example of an aminoglycoside is gentamicin (Larson, 2015). Gentamicin is produced by the species Micromonospora and it is active against both gram-negative and positive bacteria. It has effects on the bacterial protein synthesis by misreading of mRNA (Larson, 2015).

2.8.β –lactamases enzymes

Bacterial β-lactamases are members of an enzyme family (EC 3.5.2.6) that provide multi-resistance to β-lactam antibiotics such as Penicillins, Cephalosporins, Cephamycins, and Carbapenems (ertapenem), although Carbapenems are relatively resistant to beta-lactamase, and these enzymes deactivate β-lactam antibiotics by splitting the amide bond of the β-lactam ring. they have considered as the main way of acquiring drug resistance in MDR of *A.baumanii* (Spencer *et al*., 2001). β-Lactamases are extracellular, membrane-associated enzymes in Gram-positive bacteria, and periplasmic proteins in Gram-negative bacteria (AL-Marjani *et al*.,2016).

2.8.1 Mechanisms of Resistant for β-lactams Antibiotic in *Acinetobacter baumannii*

Resistance to β-lactams is an alarming and growing phenomenon. Most information about healthcare-associated *A. baumannii* infections is based on outbreak investigations (Ayoub Moubareck *et al*.,2020). It is further divided into three categories, in turn, due to its resistance to antigens, those are “multidrug resistance” (MDR) that is resistant to three or more classes of drugs that would otherwise serve as treatments for Acinetobacter infections, and extremely drug-resistant strains (XDR), which are non-susceptible (or nearly so) to all classes of antimicrobials (Munoz-Price *et al*.,2008). The last term “pan resistance”(pan- drug) has been used to describe strains of Acinetobacter that are resistant to all standard antimicrobial agents tested (Qin *et al* .,2014). Figure (2-2) showed multiple resistant methods.

2.8.1.1 *Acinetobacter baumannii* non-enzymatic β-lactam Resistance Mechanisms

2.8.1. 1.1Penicillin-binding proteins.

Alterations to penicillin-binding proteins (PBPs) such that β-lactam antibiotics can no longer bind to them is a mechanism of resistance that is observed in many bacterial species (Zapun *et al*., 2008). Alteration of PBPs in *A. baumannii* is poorly studied, and it is likely that alterations in PBPs make a small but important contribution towards carbapenem resistance, particularly in *A. baumannii* isolates that lack an acquired carbapenemase (Džidić *et al* .,2008).

2.8.1.1.2 Outer membrane proteins

Outer membrane protein A (OmpA), a major component of outer membrane proteins (OMPs) in Gram-negative bacteria is a key virulence factor which mediates bacterial biofilm formation, eukaryotic cell infection, antibiotic resistance and immunomodulation. The resistance mechanism that is found in many bacteria that can confer reduced susceptibilities to many antibiotic classes is the loss of outer membrane proteins (OMPs) that facilitate the transport of the antibiotic molecules across the cell membrane (Nie *et al.*, 2020).

2.8.1. 1.3 efflux pumps

Efflux systems that actively pump antibiotics out of the cell are able to confer resistance to a wide range of antibiotic classes and are common amongst bacteria. There are many different families of related efflux pumps, but in *A. baumannii* the best studied of these belong to the resistance nodulation division (RND) family, in particular the AdeABC pump, and these

have been found to be able to transport β-lactams out of the bacterium(He *et al* .,2015).

2.8.1.2 *Acinetobacter baumannii* enzymatic β-lactam resistance mechanisms

2.8.1.2 .1Classification of β-lactamases

Two major classification schemes exist for categorizing β-lactamase enzymes: Bush-Jacoby-Medeiros, based on substrate and inhibitor profile, and Ambler classes A through D, based on amino acid sequence homology (Drawz and Bonomo, 2010).

2.8.1.2 .1.1Functional Classification(Bush and Jacoby GA., (2010))

Group 1: Cephalosporinases which are not inhibited by clavulanic acid, belonging to the molecular class C.

Group 2: Penicillinases, Cephalosporinases, or both inhibited by clavulanic acid, corresponding to the molecular classes A and D reflecting the original TEM (Temoneira; patient from which the first isolate was identified) and SHV (sulfhydryl Reagent Variable) genes. However, because of the increasing number of TEM- and SHV-derived β-lactamases, they were divided into two subclasses.

Group 3: Zinc-based or Metallo β-lactamases, corresponding to the molecular class B (not inhibited by clavulanic acid), which are the only enzymes acting by the metal ion zinc. Metallo β-lactamases are able to hydrolyze penicillins, cephalosporins, and carbapenems. Thus, carbapenems are inhibited by both groups (a serine-based mechanism) and (a zinc-based mechanism).

Group 4: penicillinases that are not inhibited by clavulanic acid, and they do not yet have a corresponding molecular class.

2.8.1.2 .1.2 Molecular Classification (Ambler, 1980)

The molecular classification of β-lactamases is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognized (A-D). Molecular classes A, C, and D contain β-lactamases with serine in their active site, while group B contains Metallo β-lactamases with zinc in their active sites (Kurokawa *et al*.,2003).

2.8.1.2 .1.2 .1 Class A serine β-lactamases

The properties of these enzymes involve the presence of an active site serine at location 70 and the presence of a disulfide bond between Cys69 and Cys238 (changes the overall shape of the active site). All the enzymes have the ability to hydrolyze(Penicillins), early and extend spectrum (Cephalosporins), (Aztreonam) as wells as(Carbapenems) (Bush and Bradford, 2016). This enzyme group includes the majority of the extended-spectrum β-lactamases (ESBLs) as it encompasses the TEM, SHV, and CTX-M enzyme families. Class A β-lactamases appear to be reasonably widespread in *A. baumannii* (Kim *et al*., 2008).

2.8.1.2 .1.2.2 Class B β-lactamases

Class B β-lactamases (often called the Metallo-β-lactamases) differ from the class A, C, and D enzymes in that they have one or two metal ions, usually zinc, in their active site rather than a serine residue((B1 and B3 enzymes contain two Zn2+ ions, while B2 enzymes contain only one Zn2+ ion)) (Nakano *et al*., 2015). Ambler class B β-lactamases which are termed as Metallo β-Lactamases consisting of NDM, VIM, IMP, SPM, GIM, SIM, KHM, AIM, DIM, SMB, TMB and FIM (Abdu *et al* .,2019). Enzymes of the

IMP, NDM, and VIM families, in particular, are becoming more common in *A. baumannii* especially in areas such as the Far East, South America, and Greece (Tsakris *et al*., 2008). Plasmid-mediated IMP-type carbapenemases (IMP stands for active-on-imipenem), 19 varieties of which are currently known, became established in Japan in the 1990s both in enteric Gram-negative organisms and in *Pseudomonas* and *Acinetobacter* species (Abdu *et al* .,2019). (Verona integron-encoded Metallo-*β*-lactamases) was first described in a multidrug-resistant *P. aeruginosa* strain in Italy during the 1990s and has since been reported worldwide. More than 33 different VIM allotypes are described (Fallah *et al* .,2014). NDM-1 (New Delhi Metallo-β-lactamase) originally described from New Delhi in 2009, this gene is now widespread in *Escherichia coli* and *Klebsiella pneumoniae* from India and Pakistan. As of mid-2010, NDM-1 carrying bacteria have been introduced to other countries (including the United States and the UK), most probably due to the large number of tourists traveling the globe, who may have picked up the strain from the environment, as strains containing the NDM-1 gene have been found in environmental samples in India (Fallah *et al* .,2014).

2.8.1.2 .1.2.3 Class C serine β-lactamases

The genes for Ambler Class C β-lactamases, also referred to as AmpC enzymes, are commonly found on the chromosome in Gram-negative organisms. As well as these intrinsic enzymes, there are several families of plasmid-borne ampC genes that are spreading particularly within the Enterobacteriaceae (Jacoby, 2009). In Acinetobacter species the class C enzymes are referred to as ADC enzymes, for Acinetobacter-derived Cephalosporinases (Hujer *et al*., 2005).

2.8.1.2 .1.2.4 Class D serine β-lactamases

The OXA -lactamases were among the earliest -lactamases detected; however, these molecular class D -lactamases were originally relatively rare and always plasmid-mediated. They had a substrate profile limited to the penicillins, but some became able to confer resistance to cephalosporins. From the 1980s onwards, isolates of *A. baumannii* that were resistant to the carbapenems emerged, manifested by plasmid-encoded -lactamases (OXA-23, OXA-40, and OXA-58) categorized as OXA enzymes because of their sequence similarity to earlier OXA –lactamases (Evans *et al*.,2014).

Additionally, it seems that the majority of *A. baumannii* strains produce oxacillinases represented by OXA-51/69 variants (Al-Haideri *et al*.,2019). These enzymes show weak hydrolytic activity towards carbapenems. However, isolates bearing the ISAba1-$bla_{\text{OXA-51}}$ gene, show higher rates of resistance to imipenem and meropenem (Da Silva *et al*.,2016). The $bla_{\text{OXA-51}}$-type is usually located in the chromosome. It has been suggested its use as a marker for identification of the species (Turton(b) *et al*.,2006), but it has also been shown that other species may eventually carry this oxacillinase in plasmids (Vijayakumar *et al*.,2019). Detection of target site duplications surrounding transposon Tn6080, which carry the $bla_{\text{OXA-51}}$ gene in *A. baumannii* plasmids, suggests that this genetic element has moved by transposition from the chromosome of this species (Chen *et al*.,2010). Transfer of plasmid-carrying $bla_{\text{OXA-51}}$ by HGT, probably conjugation, to non-baumannii species has probably occurred. Conjugation of plasmids carrying the $bla_{\text{OXA-51}}$ gene from *Acinetobacter nosocomialis* to *A. baumannii* was observed (Lee *et al* .,2012). The most common mechanism of carbapenem resistance in *A. baumannii* is the production of Carbapenem-hydrolyzing class D β-lactamases (CHDLs) (carbapenemases) Four major groups of CHDLs

have been identified in *A. baumannii* represented by bla_{OXA-23},bla_{OXA-24}, bla_{OXA-51} and bla_{OXA-58} enzymes (Frère *et al*.,2020).

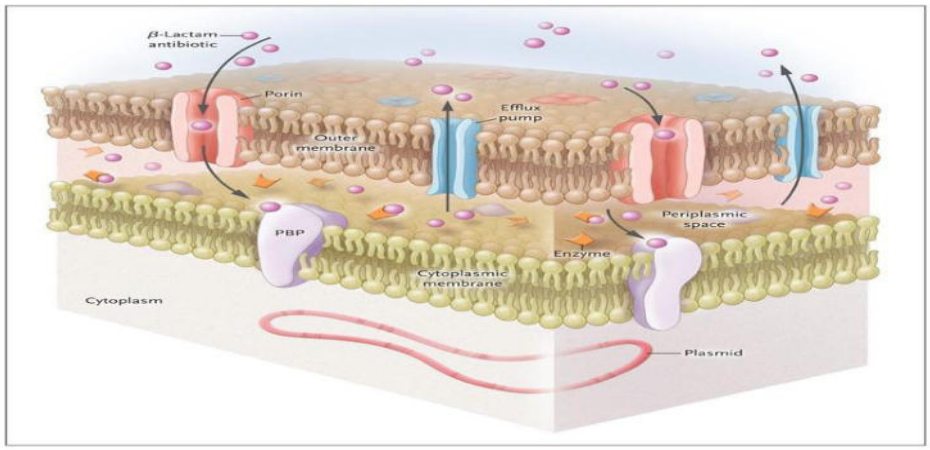


Figure2 -2. Potential Mechanisms of Antimicrobial Resistance in *Acinetobacter baumanii* (Munoz-Price *et al*.,2008).

2.8.1.3The role of mobile elements in resistance

2.8.1.3.1 Insertion sequences (ISAba)

Insertion sequences (IS) are the smallest and the most abundant transposable elements (2.5 kb) capable of independent transposition in microbial genomes. They cause insertion mutations and genome rearrangements and enhance the spread of resistance and virulence determinants within species (Mugnier *et al*.,2009). ISAba1 has been identified in *A. baumannii* isolates from 2001, where it was found upstream of genes encoding the chromosomal class C βlactamases, the ADC enzymes (Corvec *et al*., 2003; Böhm *et al* .,2020). ISAba1 has been identified in association with several antibiotic resistance genes in *A. baumannii* (Joshi(a) *et al*.,2017). A significant contribution to β-lactam resistance by OXA-51, and OXA-23 enzymes, therefore, requires the presence of an insertion element ISAba1 upstream of the gene, able to act as a strong transcriptional promoter (Gordon *et al*., 2010).

2.8.1.3.2 Integrons

Integrons are genetic elements that are able to capture and express multiple genes. They contain an integrase gene, intI, and an attI recombination site. Genes that are associated with an attC recombination site, called gene cassettes, that is recognized by the integrase can then be inserted into the integron. This can occur successively such that an integron carries multiple gene cassettes. Integrons are sorted into classes based upon the sequence of their integrase gene (Engelstädter *et al*.,2016). In *A. baumannii*, integrons are often identified with the majority being of the class 1 type. They commonly carry genes conferring resistance to the aminoglycosides, sulphonamides, chloramphenicol, trimethoprim and β-lactams While genes conferring resistance to the carbapenems of the bla_{IMP} and bla_{VIM} types have been found in *A. baumannii*, they are not generally widespread as yet, except in the Far East (Evans, 2010)

2.8.1.3.3. Resistance Islands

The term resistance island (RI) in bacteria refers to a region in a genome containing a high concentration of genes that encode resistance to antimicrobials that have been inserted into the bacterial chromosome. The first RI to be discovered in *A. baumannii* was published in 2006 and contained 45 resistance genes located within a ~96 kb region inserted into an ATPase gene (Fournier(b) *et al*., 2006) AbaR-type genomic islands (AbaRs) are important elements responsible for antimicrobial resistance in *A. baumannii* (Hamidian *et al* .,2018)

2.9 Virulence Factors

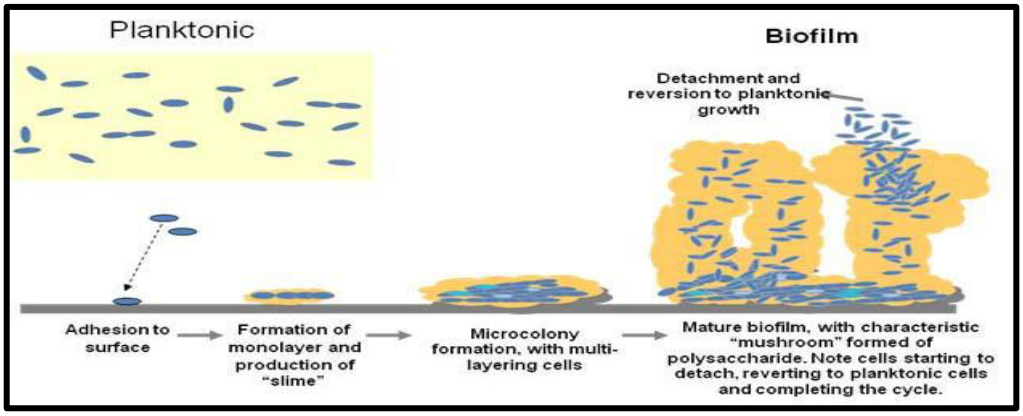
Some microbes including *A. baumannii* possess a number of properties that permit them to be more effective as pathogens. These characteristics may

be virulence factors such as toxins or toxin transfer systems that straight affect the host cell (Ellis and Kuehn, 2010), the ability to form biofilm or motility in a different mechanism, and it is possessing genes that encode to produce anti-resistant enzymes and types secretion systems. These properties are impartial some of the identified factors which make *A.baumannii* active as a pathogen (Cho and Blaser, 2012).

2.9 .1 *Acinetobacter baumannii* biofilm formation.

Biofilms are a bacterial lifestyle, constituting dynamic community environments, comprised of a heterogeneous protein matrix, nucleic acids, polysaccharides, and bacterial microcolonies, dispersed with water channels (Morris *et al*.,2019).*A. baumannii*, an important emerging pathogen of nosocomial infections, is known for its ability to form biofilms, and it forms biofilms on both biotic and abiotic surfaces, promoting survival on indwelling medical devices, hospital surfaces, or in otherwise unfavorable conditions (Lin *et al* .,2020). Especially for *A. baumannii* which is one of the most common bacterial causes of biofilm-related contamination of medical devices (Qi *et al*.,2016). The mechanisms to explain the increased drug resistance of bacteria related to biofilms are various and at least included delayed penetration of the antimicrobial agents into the biofilm and reduced growth rate of the microorganisms within the biofilm (Donlan, 2000). A positive correlation between biofilm formation and antimicrobial resistance in *A. baumannii* has been confirmed (Badave *et al*.,2015), although one study suggested an inverse relationship between biofilm production and meropenem resistance in nosocomial *A. baumannii* isolates (Perez, 2015). The ability to form a biofilm may affect antibiotic susceptibility and clinical failure, even when the dose administered is in the susceptible range (Kim *et al*., 2015). Biofilm formation is a multistage process, commencing with the initial attachment, proceeding to strong adhesion and aggregation of cells into

microcolonies, followed by biofilm growth and maturation, before cell dispersal into the environment (Hall-Stoodley *et al*., 2004). The first stage is attachment to the bacteria in a planktonic phase, contact with a surface, either of human matrix or foreign body material, and attempt to adhere to it (Kostakioti *et al.,* 2013) as shown in figure (2-3).



Figure(2-3): Various stages of biofilm formation and development (Vasudevan,2014).

In the second stage, collective cells bond on the surface then split into daughter cells, multiply outward and upward from the point of bond to form cell clusters, the separating cells produce exopolysaccharides (EPS) and quorum sensing molecules, so accumulating cells in Micro colonies and biofilms stick to surface on which it is made (Colquhoun *et al*.,2020) , aggregate number of organisms cause microcolonies which come to be bigger and rise of quantity of EPS produced likewise increasing of signaling molecules (Malic *et al*., 2009). The completely mature biofilm structure consists of the polymer matrix, bacterial cells and interstitial water channels that simplify the exchange of nutrients and wastes in and out of the biofilm into the periphery environment (Li, 2017).

2.9.2 Quorum Sensing

Quorum sensing (QS) is a bacterial cell-cell communication process that involves the production, detection, and response to extracellular signaling

molecules called autoinducers (AIs). Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Rutherford *et al*.,2012)**.** *Acinetobacter baumannii* has one quorum sensing (QS) system, which plays an integral role in regulating virulence factors, T6SS, biofilm formation, and surface motility (Colquhoun *et al*.,2020).

Four common features are found in nearly all known Gram-negative quorum-sensing systems. First, the autoinducers in such systems are acyl-homoserine lactones (AHLs) or other molecules that are synthesized from *S*-adenosylmethionine (SAM), and they are able to diffuse freely through the bacterial membrane. Second, autoinducers are bound by specific receptors that reside either in the inner membrane or in the cytoplasm. Third, quorum sensing typically alters dozens to hundreds of genes that underpin various biological processes. Fourth, in a process called autoinduction, autoinducer-driven activation of quorum sensing stimulates the increased synthesis of the autoinducer, which establishes a feed-forward loop that is proposed to promote synchronous gene expression in the population. (Papenfort *et al*., 2016). In the LuxRI family of proteins, LuxI synthesizes the acyl-homoserine lactone, which interacts with the LuxR protein mediating the transcriptional response (Bhargava *et al*., 2010). In *A. baumannii*, abaI is responsible for the synthesis of N-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C12-HSL), functioning in conjugation with the abaR LuxR homolog, to regulate biofilm maturation (Niu *et al*., 2008; Guo *et al*.,2017). Furthermore, the importance of this system in virulence has been demonstrated by the significant increase in murine survival rates during infection with abaI mutants(Bhuiyan *et al*., 2016).

2.9.3 Type six secretion system (T6SS)

The type VI secretion system (T6SS) is a molecular machine , an important virulence system that exists in many bacterial pathogens and used by a wide range of Gram-negative bacterial species .T6SS is responsible for the secretion of many toxic effector molecules that can kill both prokaryotic and eukaryotic prey cells(Wang *et al*.,2018). The function of T6SS is similar to the tail of bacterial phage and consists of a floor structure corresponding to the cytosol dynamic organelles connected to the cytomembrane (Shneider *et al*.,2013).

The T6SS was officially described in 2006, with two publications detailing the requirement of a conserved gene cluster in *Vibrio cholerae* and *Pseudomonas aeruginosa* for the secretion of a protein called Hcp (Pukatzki *et al*., 2006). Similarly, Hcp was secreted in a T6SS-dependent manner in *P. aeruginosa* and could be detected in the sputum from chronically infected cystic fibrosis patients (Mougous *et al*., 2006). Both studies strongly implicated the T6SS as a virulence factor in these organisms, and bioinformatic analyses suggested the T6SS was widespread among Gram-negative bacteria (Boyer et al., 2009).

2.9.3.1 Core structural components

Through bioinformatics and mutagenesis studies, the T6SS is now known to possess six mechanisms for effector secretion elucidated in gram-negative bacteria, particularly notable for its significant structural homology with T4 bacteriophage. The basic structure consists of thirteen proteins necessary for a function which are (Hcp, VgrG, TssB/TssC, TssE, TagL/TssL, TssJ, TssM, TssA, TssF, TssF, TssK),Table 1explain structural information available on T6SS core (Silverman *et al*.,2012; Coulthurst and S.,2019;

Cascales *et al*.,2012; Douzi *et al*.,2018) and the (figure 2-4) shows the details of the core structure.

Table 1. Summary of the structural information available on T6SS core components and their function (Silverman *et al*.,2012; Coulthurst and S.,2019; Cascales *et al*.,2012; Douzi *et al*.,2018).

Categories	T6SS subunit	Localization	Function to apparatus
The trans-membrane complex spans between the cytoplasm and outer membrane	TssL	inner membrane	it is located at the distal end of the tail where it acts as a stopper
	TssM	inner membrane	It is for the assembly of the membrane-bound components
	(TssJ) or lipoproteins SciN	outer membrane lipoprotein	required for T6S activity
The tail, a highly dynamic and energetic system	Hcp/TssD	soluble, forms hexameric rings, putative pilus	Hcp (Hemolysin Coregulated Protein)VgrG (Valine-Glycine Repeat Protein G)The PAAR protein that has a position at the tip of VgrG allows to sharpen the puncturing device and to dock effectors The tip of VgrG is too narrow to allow the passage of protein substrates, and therefore VgrG probably dissociates upon entry to the recipient cell, exposing the hollow Hcp tubule
	VgrG /TssI&pAAR	cell puncturing device, forms trimers	
	TssB	soluble protein, OM associated	
	TssC	soluble protein, OM associated	
	TssE	putative soluble protein	it is necessary for the assembly of the T6SS
	TssH/CLp	cytosolic protein, AAAþ ATPase	The AAA+ protein ClpV utilizes ATP hydrolysis to dissemble another T6SS tubular structure composed of interacting TssB/TssC proteins
The baseplate secrets	TssA	putative cytosolic protein	The proteins provide the drastic conformational changes between extended and contracted tail sheath, its role might be also to accommodate these changes to keep the system Strongly assembled
	TssF	putative soluble protein	
	TssG	putative soluble protein	
	TssK	putative cytosolic protein	

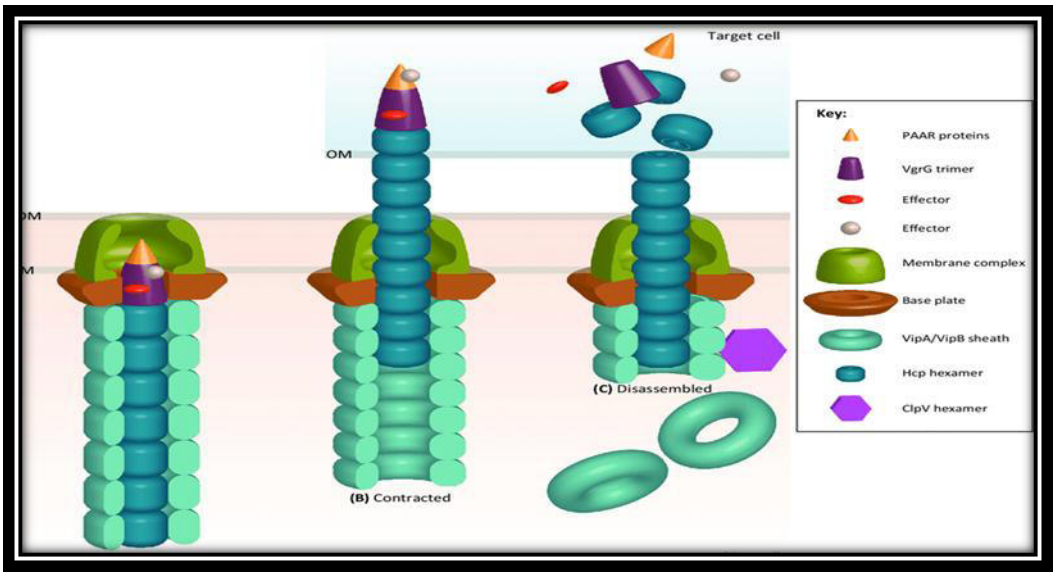


Figure (2-4) details of the core structure to T6SS(Joshi(b) *et al* .,2017).

2.9.3.2 Inputs that regulation T6SS expression and activity

Recent findings have demonstrated various classes of regulators sensitive to environmental cues specifically modulate the activity of the T6SS. In most bacteria, the expression of the T6SS is tightly controlled by numerous different mechanisms (Silverman *et al*., 2012). Several regulatory proteins and environmental signals regulate T6SS in other bacteria, including iron, zinc, osmolarity, quorum sensing, and temperature (Brunet *et al*., 2011; Silverman *et al*., 2012). Also, regulation of T6SS often varies between different strains of the same species and may reflect the particular niche those strains inhabit (Bernardy *et al*., 2016). The dynamic nature of the T6SS requires a large input of energy, and therefore a constitutive expression of the system is likely a costly process (Basler, 2015).

Any bacterial cell possessing an antibacterial T6SS must possess a means to prevent self-intoxication by its effectors (cytoplasmic-acting effectors, before secretion) and intoxication by effectors delivered into it by its neighboring sibling cells (all effectors, incoming)(Coulthurst,2019) The multicomponent T6SS apparatus facilitates a dynamic contact-dependent injection of toxic

effector proteins into prey cells, and expression of cognate immunity proteins prevents self-inflicted intoxication. (Schwarz *et al*., 2010). Figure (2-5). Schematic representation of the diverse regulatory systems that modulate T6S expression and activation in assorted bacteria (Silverman *et al*., 2012).

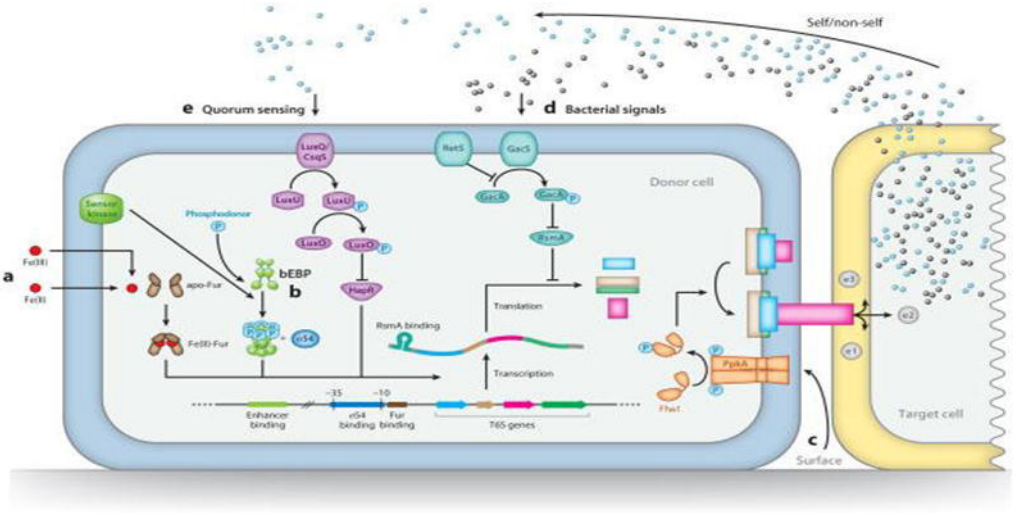


Figure 2-5. Schematic representation of the diverse regulatory systems that modulate T6S expression and activation in assorted bacteria (Silverman *et al*., 2012).

2.9.3.3 A multidrug resistance (MDR) plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*

Multidrug resistance (MDR) *A. baumannii* is emerging as a frequent cause of difficult-to-treat nosocomial infections, and some isolates are resistant to all clinically relevant antibiotics (Di Venanzio(b) *et al*.,2019). As mentioned earlier, this system is composed of several conserved proteins involved in the formation of the secretory apparatus, One of these components, hemolysin-coregulated protein (Hcp), that are robustly secreted to the culture supernatants in bacteria with an active T6SS, allowing it to be used as a molecular marker for T6SS activity(Kim *et al*., 2017). T6SS is

silenced in most strains and only activated under specific conditions perturbations (Basler ,2015).

Kim *et al*.,(2017); Weber *et al*.,(2017)and Di Venanzio *et al* .,(2019) reports have proved and shown that multiple globally distributed *A.baumannii* strains carry large conjugative plasmids (LCPs)(pAB3) ranging in size from 150 to 200 kb and are characterized by three conserved regions and its functions to repress the T6SS by encoding negative regulators of its activity. This plasmid is a link between MDR and T6SS, on one hand, it encodes to antibiotic resistance cassettes against tetracyclines, aminoglycosides, and Carbapenems, and on another hand, it encoded TetR transcriptional regulators completely represses the T6SS of their *A.baumannii* host resulting in loss of Hcp secretion. The expression and secretion of Hcp protein varied among the clinical isolates and its concretion varies from time to time on the same isolation, depending on condition. The (figure2 -6) explain the role of the plasmid to regulator the T6SS.

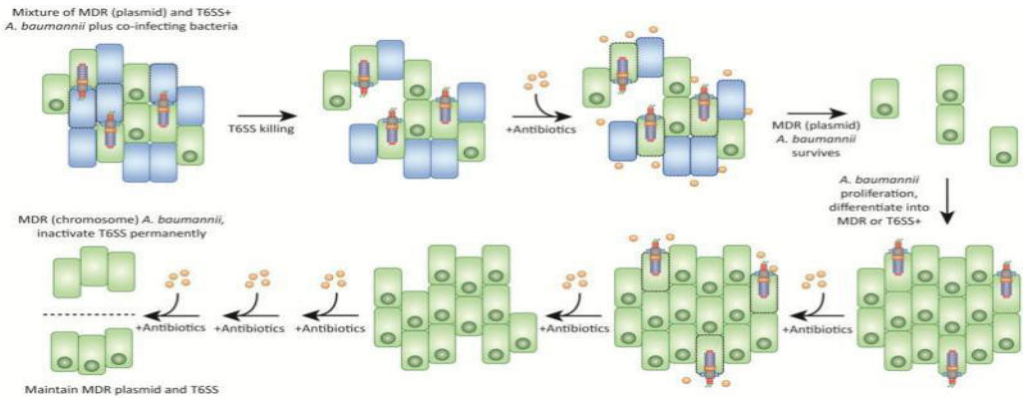


Figure 2-6. Hypothetical Model for T6SS Deletion and MDR Maintenance in Clinical Isolates of *A. baumannii* The genome sequences of several MDR *A. baumannii* clinical isolates have shown they lack a full T6SS cluster. This model proposes that pressure to maintain drug resistance by antibiotic treatment may select for strains that either repress their T6SS or degrade the T6SS cluster, permanently inactivating the secretion system in favor of antibiotic resistance (Weber *et al* 2017) .

2.9.3.4 Mode of action T6SS, function and effectors

Type VI secretion systems (T6SS) are a class of macromolecular secretion machines, and play a major role in the intracellular lifecycle of these organisms (Weber *et al*.,2013), which can facilitate the injection of effector proteins into target cells. Injection of effectors via T6SS into eukaryotic cells has been linked to phenotypes such as inhibition of phagocytosis and induction of apoptosis, which could potentially explain the link between T6SS in certain bacteria and virulence(Carruthers *et al* .,2013). It also has a role in the competition between bacterial species through the contact-dependent injection of effectors, which may have bacteriostatic or bactericidal activities, in target bacteria because it delivered toxins to kill competing bacteria (Lopez *et al*.,2020).

More recently, T6SS-mediated killing has also been shown to lead to DNA release and enhanced horizontal gene transfer events which may contribute to the spread of antibiotic resistance(Repizo *et al*.,2015).

In *V. cholerae*, the VgrG1 protein contains a C-terminal actin cross-linking domain and was shown to be secreted into phagocytic cells, thereby blocking bacterial uptake and causing host-cell death (Ma *et al*., 2010). VgrG3 from *V. cholerae* was found to encode a C-terminal extension with peptidoglycan degrading activity (Brooks *et al*., 2013)

The bacterial-specific peptidoglycan layer is a common target for several T6SS effectors, which cleave linkages in both the peptide and glycan moieties (Durand *et al*., 2014) *Pseudomonas aeruginosa* activates a T6SS during infection of cystic fibrosis patients(Weber *et al*.,2013). It has recently emerged that the T6SS can also be used as a powerful weapon against fungal competitors, and the first fungal-specific T6SS effector proteins, Tfe1 and Tfe2, have been identified. These effectors act via distinct mechanisms

against a variety of fungal species to cause cell death. Tfe1 intoxication triggers plasma membrane depolarization, whilst Tfe2 disrupts nutrient uptake and induces autophagy (Trunk *et al*.,2019).

CHAPTER THREE

MATERIAL AND METHOD

Material and Method

3.1 Materials

3.1.1 Equipment and Apparatus

The equipment and apparatuses were used in this study are listed in Table (3-1).

Table (3-1): Equipment and apparatuses

NO	Equipment and apparatus	Company	Origin
1	Autoclave	GEMMY	Taiwan
2	Centrifuge	Fisher Scientific	USA
3	Distillatar	Gallenkamp	England
4	Incubator	Memmert	Germany
5	Eppendorf tubes	Sterellin Ltd	UK
6	Gel Imaging System	Major Science	Taiwan
7	Inoculating loop	John Bolton	UK
8	Laminner Flow Hood	Lab Tech	Korea
9	Light Microscope	Olympus	Japan
10	Mic qPCR Cycler	Bio Molecular system	Australia
11	Mic Tupe	Bio Molecular System	Australia
13	Micropipettes different sizes	Slamed	Germany
14	Micro spin Centrifuge	My Fugene	China
15	Microcentrifuge	Eppndrof	Germany
16	Microwave Oven	GOSONIC	China
17	Millipore Filters (0.22µ)	Millipore Corp.	Germany
18	OWLElectrophoresis System	Thermo	USA
19	pH meter	Hana	Italy
20	Quantus Florometer	Promega	USA
21	Refrigerator	TEKA	Spani
22	Sensitive balance	OHAUS-PioNEER	USA
23	Sterilized cotton swabs	Sterellin Ltd.	UK
24	Swab with media	Sterellin Ltd.	UK
25	Tips	Sterellin Ltd.	UK

26	Thermo Cycler	BioRad	USA
27	VITEK-2 system	BioMerieux	France
28	Vortex	Quality Lab system	England
29	Water bath	Memmert	Germany
30	Shaker incubator	Sartorius	Germany
31	ELISA reader	HS-Human Reader	Germany
32	UV-transilluminator	Scope – 21	Japan
33	Well flat bottom plate	Coastar	USA
34	Cooling Centrifuge	Hettich	Germany
35	mini-column eppendorf tubes	Sterellin Ltd	UK
36	1.5ml, 0.5ml and 0.2ml Tube	JET BIOFIL	Singapore

3.1.2 Chemical and Biological Materials

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

Table(3-2): Chemicals and biological materials

NO	Chemical and biological material	Company	origin
1	Absolute Ethanol and Isopropanol	Romil pure chemistry	USA
2	Ethylene diamine tetra-acetic acid (EDTA)	Fluka	Switzerland
3	Chloroform	LiChrosolv	Germany
4	Crystal violet	BDH	England
5	Catalase Reagent	Analar	England
7	Ethyl acetate	BDH	England
8	Ferric chloride	BDH	England
9	Gram Stain Kit	Syrbio	Syria
10	Glycerol	Fluka	Switzerland
11	Human blood	Diyala blood bank	
12	Hydroxyl Amin (NH2OH)	BDH	England
13	Hydrochloric acid HCl ,Sodium chloride NaCl, Sodium hydroxide NaOH	BDH	England
14	Hydrogen peroxide (H_2O_2)	BDH	England
15	Methyl red staining,Kovac's Indol Reagent Vogaes-prskour Reagen	BDH	England
16	Methanol solution	BDH	England

17	**Normal saline**	**PSI**	**(SaudiArabia)**
18	**Oxidase Reagent :Oxidase indicator [N,N,N,Ntetramethyl-p-phenylenediamine dihydr chloride%1]**	**Fluka**	**Switzerland**
19	**Standard MacFarland's solution(0.5)**	**Bio Mérieux**	**France**
20	**Urease**	**CHD**	**India**
21	**Glucose**	**CHD**	**India**

3.1.3 Culture Media

Culture media in the table (3-3) are prepared according to instructions of manufacturing company**.**

Table (3-3): Culture media

No.	Culture Media	Company\ Origin	utilization	references
1	**Brain heart infusion broth**	**Himedia India**	Use for activation the isolates after preservation, used as a conveying medium, and Use to detect the biofilm formed by bacteria	**(Macfaddin *et al* .,2000 ; Atlas &Snyder,2006)**
2	**Brain heart infusion agar**	**Himedia India**	Use for activation the isolates and for longtime preservation	**(Macfaddin *et al* .,2000)**
3	**Blood agar**	**Himedia India**	Use it to test the ability of bacteria to blood hemolysis and to detect the type hemolysis	**(Forbes *et al*., 2007).**
4	**MacConkey agar**	**Himedia India**	Used for the purpose of preliminary diagnosis, and to detect its ability to lactose fermentation	**(Forbes *et al*., 2007).**
5	**Muller –Hinton agar**	**Oxoide England**	Use this medium to test for antibiotics susceptibility and detection of β-lactamase enzymes	**(Macfaddin *et al* .,2000)**
6	**Nutrient agar**	**Himedia India**	Use for activation the isolates and for preservation	**(Macfaddin *et al* .,2000)**
7	**Nutrient broth**	**Himedia India**	Use for activation the isolates and for preservation, apical assay,	**(Macfaddin *et al* .,2000)**
8	**Muller –Hinton broth**	**Oxoide England**	Use this medium to test for antibiotics susceptibility and MIC	**(Macfaddin *et al* .,2000)**
9	**Trypticase soy broth**	**Oxoide England**	Use to detect the biofilm formed by bacteria	**(Snyder,2006)**

3.1.4 Kits and Materials Used in Molecular Study

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

Table (3-4): Kits and materials

No.	Kits	Components of kit	Company/origin
1	ABIOpureTM Total DNA	• CLBuffer, • Proteneinase K solution • BL Buffer • BW Buffer, • TW Buffer andAE Buffer • Ethanol (95%)	ABIOpure, USA
2	Master mix	• Go Taq ® Green Master Mix 2x,(pH = 8.5) • Taq DNA polymeerase • 400mM of dATP, dGTP, dCTP, and dTtp • 3mM of MgCl2 • Deionized Nuclease –Free water	Promega/USA
3	Electrophoresis migration material	• Agarose , • Ethidium Bromide Solution (10mg/ml), • 1X TAE-electrophoresis • Ladder (100bp DNA Marker)	Promega/USA
4	TRIzol Reagent Protocol(RNA Exraction)	• TRIzol Reagent • Chloroform • Isopropanol • 70%Ethanol • Nuclease free water	Thermo Scientific, USA
5	Real Time PCR kit (Syper green) GoTaq® 1-Step RT-qPCR System	• qPCR Master Mix • RT mix • MgCL2 • dATP, dGTP, dCTP, and dTTP • Taq DNA polymeerase • Nuclease Free Water	Promega/USA
6	Quantitation of DNA	• TAE 40X • Quantiflor dsDNA System	Promega/USA

3.1.5 Antibiotic powder

Table (3-5): Antibiotics powder

Meropenem \100mg	Astrazeneca\UK
Imipenem \ 500 mg	KOCAK FARAM\Turkey

3.1.6 Antibiotics

Antibiotic disks were used are listed in table (3-6).

Table (3-6): Antibiotics disks

Class of antibiotics	Antibiotic discs	code	MG/ Concentration Disc	Zone Diameter interpretive Criteria ≥S , I , ≤R mm	Company (origin)
Beta Lactam	Meropenem	MEM	10	18, 15-17 ,14	Mast Group Ltd.,\ UK
	Imipenem	IMP	10	22, 19-21 ,18	
	Ampicillin-sulpuctam	AMS	10\10	15, 12-14 ,11	
	Piperacillin-tazobactam	PRL	100\10	22, 18-20 ,17	
	Ticarcillin-clavulante	TCC	75\10	15, 12-14 ,11	
	Augmentin	AMC	30	15, 12-14 ,11	
	Ceftazidime	CAZ	30	18, 15-17 ,14	
	Cefotaxime	CTX	30	23, 15-22 ,14	
	Cefixime	CFX	5	21, 14-20 ,13	
	Ceftriaxone	CRO	30	21, 14-20 ,13	
	Cefoxitin	FOX	30		
Aminoglycosid	Gentamicin	GM	10	15 , 13-14 ,12	
	Amikacin	AK	30	17, 15-16 ,14	
Tetracycline	Tetracycline	TE	30	15, 12-14 ,11	
	Doxycyclin	DO	30	13 , 10-12 ,9	
Fluro quinolone	Levofloxacin	LEV	5	17, 14-16 ,13	

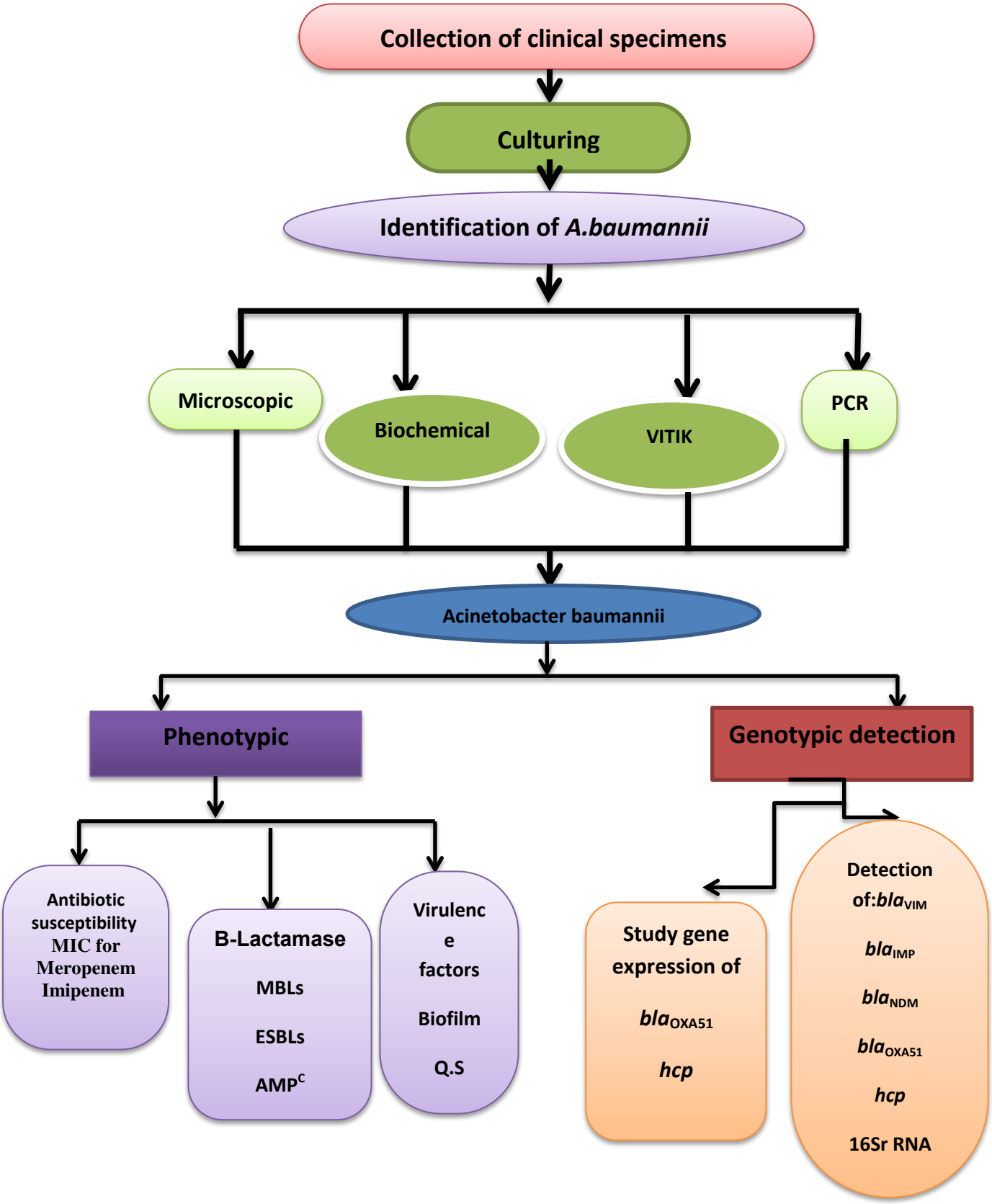
3.1.7 Primers

Table (3-7): The primers used in the current study for gene detection (from Macrogen, Korea).

Primer Name	Sequenc	Annealing Temp. (oC)	Product Size bp	Reference
bla $_{OXA51}$F	5`-TAATGCTTTGATCGGCCTTG-3`	52	353	(Joshi *et al* .,2017)
bla $_{OXA51}$R	5`-TGGATTGCACTTCATCTTGG-3`			
bla$_{NDM-1}$F	5`-GGTTTGGCGATCTGGTTTTC-3`	52	624	
bla$_{NDM-1}$R	5`-CGGAATGGCTCATCACGATC-3`			
bla$_{VIM}$ F	5`-GATGGTGTTTGGTCGCATA-3`	55	390	
bla$_{VIM}$ R	5`-CGAATGCGCAGCACCAG-3`			
bla$_{IMP}$ F	5` CTACCGCAGCAGAGTCTTTG -3`	54	578	
bla$_{IMP}$R	5`- AACCAGTTTTGCCTTACCAT -3`			
hcp F	5`-TGCTGAGCGTGTTGAACATT-3`	55	142	(Kim *et al* .,2017)
hcp R	5`-ACGTTTATCGCCATTTGCAC-3`			
16S RNA F	5`-CAGCTCGTGTCGTGAGATGT-3`	55	150	(Higgins *et al*., 2004)
16S RNA R	5`-CGTAAGGGCCATGATGACTT-3`			
27F	5`-AGAGTTTGATCCTGGCTCAG-3`	60	1500	(Mukherjee *et al* .,2013)
1492 R	5`-TACGGTTACCTTGTTACGACTT-3`			

3.2 Methods

*Study Design



3.2.1 Laboratory Prepared Culture Media

All media offered in the table (3-5) except blood agar, peptone water, and Urea Agar were prepared to render to the manufacturing company directions; the ingredients were dissolved in distilled water, pH was adjusted to 7.2 ± 0.2, then heated in the water bath to dissolve all ingredients entirely. The media was sterilized by autoclaving at 121˚C for 15 min at 15 pounds/inch^2, subsequently dispersed into sterile Petri dishes; otherwise, the media were incubated at 37 ºC for 24 hours to confirm sterility (Harley and Prescott, 2002).

3.2.1.1 Blood Agar Medium

This medium was prepared according to the manufacturing company Instructions (40gm/L) sterilized by autoclaving and cooled to 45-50°C, Then 5-10% of expired sterilized fresh human blood was added. The medium was used for isolation; cultivating most of the pathogenic bacteria and examining their ability to hemolysis blood and showed a type of hemolysis (Atlas and Snyder, 2006).

3.2.1.2: Peptone Water Medium

This medium was prepared by dissolving 10g of Peptone and 5g of NaCl in 1 liter of D.W. pH was adjusted to 7.4, distributed into sterile tubes then sterilized by autoclaving at 121°C for 15 min. It was used for detecting the bacterial ability to produce indole from tryptophan (Atlas and Snyder, 2006).

3.2.1.3 Urea Agar Medium

Urea Agar Medium was Prepared by dissolving 2.4 g of Urea Base agar in 95 mL of distilling water, then sterilize the medium, it was left to cool at 45 ° C, then 5ml of Urea Supplement at 40% was added, then it was poured into sterile tubes, left diagonally hardened, use the medium to detect the ability of bacteria to produce urease (Forbes *et al.,*2007).

3.2.2 Reagents and Solutions

3.2.2.1 Reagents

3.2.2.1.1 Catalase reagent

As stated by (Forbes *et al*., 2007), Hydrogen peroxide H_2O_2 (3%) was prepared from the stock solution (15%) and then it was kept in a dark tube.

3.2.2.1.2 Oxidase reagent

As stated by(Shields and Cathcart 2010) it was prepared via dissolving 1g of (N-N-N-N-tetramethyl para phenylene diamine dihydrochloride) in 100 ml of distal water. It was used for noticing the isolate's capability to yield oxidase enzyme.

3.2.2.2 Solutions

3.2.2.2.1 Ferric chloride ($Fecl_3$) (10%) in 4M HCl solution

First HCL was prepared by adding 3.33ml of HCL to 6.66ml of D.W. to get 10ml of HCl. Then ferric chloride ($Fecl_3$) was prepared, by dissolving 1gm $Fecl_3$ in a little volume of the HCl and then, volume completed to 10ml, then kept until been use in phenotypic detection of AHL of Q.S (Baldiris *et al*., 2016).

3.2.2.2.2 Sodium Hydroxide Solution (NaOH)

It is prepared by dissolving 1.4gm NaOH in a little volume of distilled water and completed 10ml, then kept until been used in the detection of phenotypic detection of AHLs molecules of Q.S (Baldiris *et al*., 2016).

3.2.2.2.3 Hydroxylamine 2M Solution (NH_2OH)

It is prepared by dissolving 1.3gm NH_2OH powder in a little volume of distal water and completed 20ml, then kept until been used in the detection of phenotypic detection of AHLs molecules of Q.S (Baldiris *et al*., 2016).

3.2.2.2.4: EDTA solution

Ethylene-diamine-tetra-acetic acid disodium salt (18.6 g) was dissolved in 50 ml deionized water and the pH was adjusted to 8 using NaOH pellets. The volume was completed to 100 ml with distilled water and autoclaved (Bhalerao *et al*., 2010).

3.2.2.2.5: Antibiotic stock Solutions

The antibiotic stock solutions were prepared with a final concentration of 100000 µg/ml as primarily stock, for both Meropenem and Imipenem. 1gm of antibiotic was dissolved in 10ml completing the volume to 100ml then sterilized by filters 0.22 µm pores and stores in a refrigerator, then prepare the stock 2 (2084 µg/ml) by (C_1*V_1= C_2*V_2) from100000 µg/ml as primarily stock, this according to CLSI (Weinstein *et al*.,2020)

3.2.3: Samples collection

The total of 207 clinical samples (wounds, burns, blood, and sputum) were collected from Baquba Teaching Hospital and Consultative Clinic in Diyala from hospital inpatients and hospital visiting patients with different ages ranging from 2 to 75 years, the collection beginning from September 2019 to January 2020. Patient-related information was recorded in the hospital, age, gender, and sample source. As in the form shown in Appendix 10, the samples were cultured immediately after sampling for diagnostic purposes. The samples of wounds and burns had been collected by using cotton swab ,but the samples of sputum had been collected and then added normal saline to sample before cultured by using cotton swab. As for blood samples, they were collected and cultured by the following steps:

1)A tourniquet was applied and location of vein was determined by touch, then the tourniquet was released while the skin was being prepared for venipuncture, and a bactericidal disinfectant (2%tincture of iodine, and 70% alcohol) was used at the venipuncture site and the skin was cleaned in

concentric circles and increasing diameter. The disinfectant was allowed to evaporate on the skin surface before blood was withdrawn for at least 30 seconds. The skin was not touched after preparation.

2) The tourniquet was reapplied, venipuncture was performed and 10 ml of blood was withdrawn, then 5ml of the blood was transferred into blood culture bottles.

3) blood culture had been by used BACT\Alert 3D system was used. This system is closed for continuous monitoring of the blood culture and employed to detect bacteremia and fungemea. This is possible because the blood stream is usually a sterile environment. Inoculated bottles were placed in the instrument where they were incubated and continuously monitored for the presence of microorganisms' growth. When there was a bacterial growth, the device emitted a signal, the device was pointing when there was no microorganism growth, and the result was regarded negative and the bottle came out of the device (depending on the manufactures instructions).

3.2.4 Isolation of *Acinetobacter baumannii* isolates

Blood Agar of and MacConkey agar were used to collected specimens by directly streaked, incubated for 24 hours at 37ºC. The non-lactose fermenting colonies on MacConkey agar and non-hemolytic vague creamy colonies on blood agar were sub-cultured on an additional MacConkey agar plate and incubated for 24 hours at 37ºC. Non-lactose fermenting colonies were selected and cultured on additional MacConkey agar plates to attain pure isolated colonies. More identification tests involved the morphological appearances and biochemical tests were carried out according to Forbes et al., (2007).

3.2.5 Identification of *Acinetobacter baumannii*

3.2.5.1 Gram's Stain Examination

According to Mahon *et al*., (2018) all the bacterial isolates were examined by Gram's stain, shape, and color of the cells were observed via a light microscope by taking one bacterial colony and transported to the microscopic slide, fixed then stained by using Gram's stain. Cell arrangement and shape were observed with oil immersion.

3.2.5.2 Morphological Examination

Morphological examination based on morphological characteristics including colony color, shape, edges, and texture (as primary diagnostic tests) of isolates which grown on brain heart infusion agar, and MacConkey agar which was incubated at 37°C overnight (Biswas and Rather, 2019).

3.2.5.3 Biochemical tests

As stated by Biswas and Rather, (2019) all biochemical tests were carried out as follow:

3.2.5.3.1 Oxidase Test

It is performed to detect bacterial capability to release the oxidase enzyme. It was done via saturating a filter paper with oxidase reagent (1% -N-N-N-N-tetramethyl para-phenylenediamine dihydrochloride. A colony from tested organisms was transported to the filter paper and rubbed onto the reagent with an applicator stick. A purple color would develop in (20-30) seconds was a positive reaction; whereas the color of the colony rests the same was a negative reaction.

3.2.5.3.2 Catalase Test

For the detection of catalase enzyme production, slide method was used. Pure isolated colonies were transferred via a wood stick to a glass slide of the microscope, using a dropper, 1 drop of (3 %) H_2O_2 was added to the colony.

This test was used to identify the capability of the tested bacteria to yield the catalase enzyme. The presence of bubbles was indicated as catalase positive.

3.2.5.3.3 Kligler Iron Agar (KIA)

This test was used to identify the capability of bacteria to ferment sugars; glucose and lactose. By using the streaking and stabbing method, bacterial isolates grown on MacConkey agar were cultured on the Kligler iron agar, incubated for 24 hours at 37ºC. The positive consequence was detected via the change of phenol indicator from red to yellow color as a consequence of sugar fermentation and acid formation. Cracks and bubbles might seem in the medium at a stabbing place concerning gas creation from sugar aerobic fermentation. Black precipitate would develop at the bottom of the tube as a consequence for the reaction between H_2S yielded from anaerobic fermentation with the ferrous sulfate in the medium, the negative consequence was detected via keeping phenol indicator its red color, bubbles are not made and black precipitate would not develop.

3.2.5.3.4 Lactose Fermentation Test

This test was used to distinguish between gram-negative bacteria grown on MacConkey agar medium for lactose fermentation. MacConkey agar was inoculated with tested bacteria via streaking on the medium by a sterile loop and incubated for 24 hours at 37ºC. The neutral red which is the indicator in the medium must turn into yellow in alkaline pH and pink in acidic pH as a consequence for acid formation from lactose fermentation. Positive consequence revealed pink colonies as a consequence for lactose fermentation; whereas pale colonies indicate to the non-lactose fermenters.

3.2.5.3.5 Growth at 44ºC

All isolates were streaked on nutrient agar medium and were incubated for 24hours at 44ºC. The results revealed that the ability of the tested bacteria to grow at 44 ºC. This test was done to notable *A. baumannii* which can grow at

44 ºC from other *Acinetobacter* species which incapable to grow at 44 ºC (MacFaddin, 2000).

3.2.5.3.6. IMViC tests (McFadden, 2000)

*** Indol test**

Peptone water that was previously prepared was inoculated with a loopful of bacterial culture**.** After incubation, 5 drops of Kovac's reagent was added. The formation of a red surface ring indicated a positive result.

*** Methyl Red Test**

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

*** Voges-Proskauer Test**

MR-VP medium was inoculated with bacterial culture and incubated, then 0.6ml of VP1 (α-naphthol) and 0.2ml of VP2 (KOH) were added; the formation of red color after 15min indicated a positive result.

*** Citrate Utilization test**

Simmon citrate slants that previously prepared have been inoculated with *Acinetobacter baumannii* isolates then incubated at 37°C for 24 hrs. The transformation of medium color from green to blue due to sodium citrate utilization as a result of PH change indicates a positive result.

3.2.5.3.7 Identification of *Acinetobacter baumannii* via VITEK2 Compact System

The VITEK2 system was used for the identification of bacterial isolate. The bacterial isolates were subcultured on MacConkey agar plates. Bacterial suspensions identically to MacFarland 0.5 standard in 0.45% sterilized NaCl solution was used. The bacterial suspensions turbidity was adjusted by a

densitometer. Cards of the VITEK 2 ID-GN, AST-No. 12 cards and bacterial suspension were manually loaded into the VITEK 2 compact system. Each test card was mechanically filled with a bacterial suspension, sealed, and incubated for 6 h. through this period the cards were recited via kinetic fluorescence measurement every 15 min. The VITEK 2 compact system software first analyzed the data and then reported the consequences mechanically (Aslanzadeh,2006).

3.2.6 Preservation of Isolates

As stated by Vandepitte *et al*.,(2003) Preservation of isolates were done as follow:

3.2.6.1 Preservation for Short Time

Acinetobacter baumannii was conserved after being the assurances of its purification via transported a single pure isolated colony to nutrient agar slant in a screw-capped tube or plan tube, incubated for 24 hours at 37ºC, thence stored in the refrigerator at 4°C for short time (relatively one weak) preservation.

3.2.6.2 Preservation for Long Time

A. baumannii was conserved after being the assurances of its purification via transported single pure isolated colony to glycerol brain-heart broth (the conservation medium was prepared by adding 15 ml from glycerol to 85 ml Brain Heart Infusion Broth (BHIB). Then 10 mL from this medium was allotted in each sterile and well-capped screwed test tube and then sterilized by autoclaving.

3.2.7 Antibiotic Susceptibility *Acinetobacter baumannii*

3.2.7.1 Preparation Suspension of bacteria

Transfer numbers of colonies from a pure culture by sterile swab and suspend in 3 ml of sterile saline in a clear test tube. The turbidity was

compared with the MacFarland tube (equivalent to 1.5×10^8 CFU/ml) (Wiegand *et al*.,2008).

3.2.7.2 Standard Disc Diffusion Technique

As stated by Magiorakos *et al.*, (2012), Kirby-Bauer method was subordinated to carry out the susceptibility test of 13 antibiotics on table 3-5. The suspension of bacteria was prepared via picked 4-5 isolated colonies of *A. baumannii* from the indigenous culture and presented into a test tube having 4 ml of normal saline to yield a bacterial suspension of modest turbidity likened with the standard turbidity solution. By using a sterile cotton swab a part of bacterial suspension was transported cautiously and regularly spread on Mueller- Hinton agar medium previously prepared, then it was left for 10 min. Subsequently, the antimicrobial discs were sited on the agar with a sterile forceps pushed firmly to confirm contact with the agar. After that, the plates were reversed and incubated for (18-24) hours at 37ºC. The zones of inhibition were measured by a ruler and compared with the standard diameters determined by CLSI (Wayne, 2019) specific to *A.baumannii*.

3.2.7.3 Minimum inhibitory concentration:

The MIC for all isolates have been determined according to (Hancock, 1999) as follows:-

1. The isolates were grown on MacConky agar for 24 hr at 37°C.

2. About 3-4 colonies were transported to normal saline until the turbidity equal to 0.5 McFarland.

3. The antibiotic solution of Imipenem and Meropenem , that it was used in the current study prepared and diluted in Müeller Hinton Broth to (stock 2). the top concentration in the test 10^5 µg /mL (stock 1), that is prepared in

advance according to paragraph(3.2.2.2.5) had been diluted to 2048µg/mL (stock 2).

4. 100µL of Müeller Hinton Broth was added into all wells of the microtitre plate, using a sterile micropipette and it's a tip.

5. A micropipette was used to move 100µL of (stock 2) antibiotic solution into the wells of column 1, this for getting 1024µL\ml.

6. A micropipette was used to mixed the antibiotics by sucking up and down 5-8 times.

7. A handdred µL was withdrawn from column 1 and add to column 2. Mix by sucking and transfer to column 3. Repeat the procedure to column 9,this for has been get 9 dilution (4, 8, 16, 32, 64, 128, 256, 512, 1024).

8. A handdred µL was Discard from column 9.

9. A fiveµL had been pour of bacteria into wells in columns 1 to 9 and column 12 that contained only broth and it has been prepared as a positive control. Added 100µL from stock 2to wells of column11 as control negative and hadn't been added anything to the column 10 (only broth), this it was been controlled negative too.

10. the plates was been Incubated at 37°C for 24 hours.

11. The reading of results had been made manually using a black card and electronically with an ELISA reader on 630 nm wavelengths.

3.2.8 Phenotypic Detection of β-Lactamase production

3.2.8.1: Detection of Metallo-beta-lactamase production by combined EDTA disc test (CEDT)

Suspensions of *A.baumannii* isolates were prepared (after 24hr of incubation) by transporting a single colony to 5 ml of normal saline until

equivalent to 0.5 McFarland. About 0.1ml of the bacterial suspension was then spread by sterile cotton swab to Muller Hinton agar plates and left to dry at room temperature. Two discs of Imipenem (10µg) was placed 20 mm apart on the Mueller Hinton agar plate then 5 µl of EDTA (that was prepared on paragraph 3.2.2.2.3) was added to one of the Imipenem discs and was incubated overnight at 37° C. An enhanced zone of inhibition around Imipenem + EDTA disc for about 7mm or above compared to the disc without EDTA suggested a positive result for MβLs production (Anoar *et al*., 2016).

3.2.8.2: Production of ESβLs detection:

ESBL production in *A.baumannii* was identified by the double-disk synergy test (DDST) as described by Jarlier *et al*.,(1988).

Mueller–Hinton agar plates were streaked with inoculum (equivalent to 0.5 McFarland) using a sterile cotton swab. An Augmentin (20 µg amoxicillin and 10 µg of clavulanic acid) disk placed in the center of the plate and, Cefotaxime (30 µg), Cefixime (30 µg) Piperacillin (30 µg) were placed 20 mm apart center to center on the plates with a sterile forceps (Al Salam, 2012). The plates were then incubated for 18-24 hr at 35-37°C. An enhanced zone of inhibition from 5 mm or more in the presence of Augmentin is suggested as a positive result for the production of ESBL enzyme (Felicita, 2010).

3.2.8.3: Production of AmpC detection:

A suspension of *A.baumannii* isolates was prepared (after 24hr of incubation) by transporting a single colony to 5 ml of normal saline until equivalent to 0.5 McFarland. About 0.1ml of the bacterial suspension was then spread by sterile cotton swab to Muller Hinton agar plates and left to dry at room temperature .One disc of Cifocxitin on (30 µg) placed on the center of the plate. The plates were then incubated for 4 days at 28 °C. The inhibition zone around the disc was observed after the end of the incubation period and

it was determined whether the isolate was positive or negative According to CLSI 2019 measurements of this test (El-Taie M. I. and El-Hasso M. Z.,2009).

3.2.9: Detection of some virulence factors in *Acinetobacter baumannii*

3.2.9.1: Detection of biofilm formation

3.2.9.1.1 Detection of biofilm using the tube method (TM)

About 5ml of Trypticase soy broth with 1% glucose was inoculated with isolates from an overnight culture broth and incubated at 37ºC for 24hrs. The cultures then decanted and the tubes were washed with normal saline. The tubes were left to dry and then stained with 0.1% crystal violet. The excess stain was washed with distilled water and the tubes dried in an inverted position. A visible stained film was seen lining the wall and the bottom of the tubes indicated a positive result. The result was read as absent (-)(Mathur *et al.,* 2006).

3.2.9.1.2: BiofilmFormation (Quantitative Biofilm Production Assay)

The biofilm formation test was done according to the method described by Ghellai *et al.* (2014) included:

1. Overnight bacterial culture,20 µl was used to inoculate wells of flat-bottom microtiter, containing 180µl of Brain Heart infusion broth with 2% sucrose. Control wells contained only 200µl of Brain heart infusion broth with the 2% sucrose (0.5 gm sucrose for each 25ml of brain-heart broth)
2. The covered microtiter plate was sealed with Parafilm during incubation at 37 °C for 24h.
3. Unattached bacterial cells were removed by washing the wells three times with normal saline pH= 7.2.

4. Drying at the room temperature for 15 min, then 200µl of crystal violet solution (0.1%) was added to the wells for 15 min.

5. After removing a crystal violet solution, all wells were washed three times by distal water to remove the unbounded dye, allowed to dry at room temperature.

6. Extracted with 200µl of ethanol 95%.

7. The absorbance of each well was measured at 630 nm using an ELISA reader. The OD value for the control well was deducted from all the test OD values.

8. Results were classified according to absorbance into 3 categories: strong, moderate, and weak, this classification is demonstrated in the table below, (Bose *et al*.,2009).

(3-8) classification of *A.baumannii* as biofilm formation

"OD" ≤ "ODc"	**non-biofilm**
"ODc < OD ≤ 2 x ODc"	**Moderately biofilm producer**
"2 x ODc < OD"	**strong biofilm producer**

*"OD" Mean optical density reader average of Isolate.
*"ODc" Mean optical density reader average of control.

3. 2.9.2 Detection of AHLs by Colorimetric Method

Quantification of the AHL molecules activity as described by Baldiris *et* al.,(2016) as follows: One pure overnight bacterial colony of *A.baumannii* was cultured in 5ml of nutrient broth at 37°C for 24h. From the previous culture, 1.5ml were centrifuged for 15 min at 10000 rpm. transferred the supernatant and this step was repeated twice. Extraction the liquid by ethyl acetate for 10m and removed the organic phase on the top. The next step dried the samples from remained ethyl acetate at 42°C for (1-2)h in the incubator, transport 40µL to a microtiter plate and about 50µl was added of two

solutions included, 1:1 solution of $Fecl_3$10%(3.2.2.2.1) in 4 M of HCL: 95% of Ethanol and 1:1 solution of 2 M hydroxylamine

(3.2.2.2.3): NaOH 3.5 M as prepared previously at (3.2.2.2.2).

Finally, the OD was measured by ELISA reader at 630 nm.

The isolates will be considered negative or weak AHLs produce if the OD is below 0.98 while controls include the positive control (+) with AHLs signal molecules and negative control (-) without AHLs signal molecules.

3.2.9.3 Detection possession of clinical isolates *Acinetobacter baumannii* to T6SS

According to the method followed by Kim *et al*., (2017) to discover this system, by detecting the presence of a gene *hcp*. hemolysin coregulated protein (Hcp) serves as molecular markers of a functional T6SS. The primer for this gene mentioned in the table (3-7) was used and the gene was detected by a PCR program as mentioned in the molecular study in paragraph (3-3).

3.3: Genotyping study of *Acinetobacter baumannii*

3.3.1 DNA Extraction

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure Extraction as the following steps:

1. pellet cells obtained as 1ml of overnight culture for 2min at 13000 rpm. The supernatant was then discarded.
2. The pellet cell was re-suspended completely in 200µl of Buffer CL.
3. For protein digestion and cell lysis, 20µl of Proteinase K solution (20 mg/ml) was added to 200µl of Buffer CL and cell pellet, then the tube mixed vigorously using vortex and incubated at 56˚C for 30 min in water bath, for farther lysis incubated 30min at 70˚C.
4. After incubation, 200µl of BL Buffer was added to the sample then the tube mixed vigorously using vortex and Incubated at 70˚C for 30 min in water bath.

5. From absolute ethanol 200µl was added to the sample, pulse-vortex to mix the sample thoroughly.

6. All the mixtures were transferred to the mini-column carefully, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replace with a new one.

7. From Buffer BW 600µl was Added to the mini-column, then centrifuge for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replaced with a new one.

8. From Buffer TW 700µl was applied. Centrifuge for 1 min at 6,000 x g above (>8,000 rpm). The pass-through was discarded and the mini-column was reinserted back into the collection tube.

9. The mini-column was Centrifuge at full speed (>13,000 x g) for 1 min to remove the residual wash buffer, then the mini-column was placed into a fresh 1.5 ml tube.

10. From Buffer AE 100µl was added and incubated for 1 min at room temperature, then centrifuge at 5,000 rpm for 5min.

3.3.2 Quantitation of DNA

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

3.3.3 Primer preparation

All primers under study were supplied by the "Macrogen Company" in a lyophilized form .All Lyophilized primers in table (3-9) were dissolved in (300 µl) nuclease-free water to give a final concentration of 100 pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease-free water to obtain a working primer solution of 10 pmol/µl.

3.3.4 Preparation PCR mixture

All of DNA that extracted from *Acinetobacter baumannii* isolates in the current study went through a PCR procedure in order to target the different genes that are under study. Each PCR reaction had a final volume of 20 µl. In the table (3-7) illustrated primers that are used in this study and their appropriate volume for the PCR mixture. Promega master mix was used. The mixture was composed of, PCR Master Mix (2x) solution which was melted at room temperature and mixed by vortex for homogenizing before being used. Primer's solutions were mixed well by vortex before being used

Table (3-9) Protocol of PCR reaction mixture volumes used in the current study. (Monoplex)

Master mix componenents	Stock	Unit	Final	Unit	Volume	
					1 Sample	20.5 sample
Master Mix	2	X	1	X	10	200.5
Forward primer	10	µM	1	µM	1	20.1
Reverse primer	10	µM	1	µM	1	20.1
Nuclease free water					5	100.3
DNA		ng/µl		ng/µl	3	
Total volume					20	
Aliquot per single rxn	17µl of Master mix per tube and add 3µl of Template					

3.3.5 Gradient PCR amplification procedure

A gradient PCR is often done to optimize PCR protocol and to figure out what annealing temperatures work best .PCR conditions were optimized by repeated changing annealing temperatures (from 48Cº to 60Cº according to primers) and the number of cycles (30) according to the current study till being fixed at the conditions listed in the table (3-11a). This method offers significant time-savings and minimizes reagent use, relative to a standard PCR optimization protocol.

3.3.6Thermal cycling conditions

All components of each PCR mixture were mixed together in the Eppendorf tube by vortex before settings into thermocycler. The reaction was performed in a PCR thermal cycler apparatus, and after several trials, and according to the manufacture's guide. The following program in the table (3-10a-3-10b) was adopted. Usually, the process started with the initial denaturation step (95ºC for 5 min) followed by repeated cycles which consist from the denaturation step (ranged from 95ºC to 96ºC), the annealing step (depends on the primers) then the extension step (mostly at 72 ºC) followed by final extension step (usually at 72°C).

Table (3-10a) Programs of PCR thermocycling conditions for detection of genes(all the primers)

PCR Program			
Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	Depend on primer	00:30	
Extension	72	01:30	
Final extension	72	07:00	1
Hold	10	10:00	

Table (3-10b) Programs of PCR thermocycling conditions for annealing temperature and number of cycle of each primer genes

Genes Monoprix	Annealing	No of Cycle
bla_{OXA51}	52°C/30sec	30
bla_{VIM}	55°C/30sec	30
bla_{NDM}	52°C/30sec	30
bla_{IMP}	55°C/30sec	30
hcp	55°C/30sec	30
16sRNA	55°C/30sec	30
univesel16sRNA	60°C/30sec	30

3.3.7 Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

3.3.7.1 Solutions of agarose Gel Electrophoresis

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

3.3.7.2 Preparation of agarose

1. One hundred of 1X TAE was taken in a beaker.
2. One and a half (1.5)gm (for 1.5%) agarose was added to the buffer.
3. The solution was heated to boiling (using Microwave) until all the gel particles were dissolved.
4. Oneμl of Ethidium Bromide (10mg/ml) was added to the agarose.
5. The agarose was stirred in order to get mixed and to avoid bubbles.
6. The solution was allowed to cool down at 50-60C°.

3.3.7.3 Casting of the horizontal agarose gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

3.3.7.4 DNA loading

For the PCR product, 10μl was directly loaded to well. Electrical power was turned on at 100v/mAmp for 90min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system.

3.3.8Molecular study of *Acenitobacter baumannii* RNA

3.3.8.1 RNA Purification

RNA was isolated from the sample according to the protocol of TRIzol™ Reagent as the following steps:

A-Sample lysis

Cells were grown in suspension :

For pellet calls, 1.4 ml of cell culture was precipitated by centrifugation for 2 min at 13000 rpm, supernatant then discarded and 0.6 mL of TRIzol™ Reagent was added to the pellet.

The lysate was homogenized by pipetting up and down several times.

B-For three phase's separation

• For each tube, 0.2 mL of chloroform was added to the lysate, then the tube cap secured.

• All mixes were Incubate for 2–3 minutes then centrifuge for 10 minutes at 12,000 rpm, the mixture was separated into a lower organic phase, interphase, and a colorless upper aqueous phase.

C-For RNA precipitation

• 0.5 mL of isopropanol was added to the aqueous phase and incubated for 10 minutes then centrifuge for 10 minutes at 12,000 rpm.

• Total RNA was precipitate formed a white gel-like pellet at the bottom of the tube .

• Supernatant then discarded.

D-For RNA washing

• For each tube, 0.5mL of 70% ethanol was added and vortex briefly then centrifuge for 5 minutes at 10000 rpm.

• Ethanol then aspirated and air-dried the pellet.

E-For RNA solubility

• Pellet was rehydrated in 20-50 µl of Nuclease Free Water then incubated in a water bath or heat block set at 55–60°C for 10–15 minutes.

3.3.8.2 Determine RNA concentration (Fluorescence Method)

Quantus Fluorometer was used to detect the concentration of extracted RNA in order to detect the goodness of samples for downstream applications. For 1 µl of RNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature in a dark place, RNA concentration values were detected.

3.3.8.3 Primers preparation for gene expression

The primers that were used to study gene expression in the current study were *bla*$_{OX51}$, *hcp* genes, and as a reference gene had been used the16S rRNA, this primers preparation as in paragraph (3.3.3).

3.3.8.4 Confirm phenotypic Drug Resistance by quantitative RT-PCR of *bla*$_{OXA51}$, and *hcp* gene expression

To confirm and select the highest and lowest drug resistance isolates through of gene expression which is determined and based on comparing the different values of mean CT of *bla*$_{OXA51}$ and *hcp* genes and confirmed and normalized them by 2-mean ΔCT, where the ΔCT; is the difference in CT threshold cycle between the target and reference gene obtained from quantitative real time-PCR(qRT-PCR) for bla$_{OXA51}$ and hcp (target genes) and 16S rRNA (reference gene) each isolate, so ΔCT= CT gene - CT reference gene, where CT; is threshold cycle value for the amplified gene (Schmittgen and Livak, 2008). The measurement of gene expression of the two genes (*bla*$_{OXA51}$ and *hcp*) in the bacterial isolates was done before the treatment with the Imipenem on 128 µg/ml concentration as sub MIC, and after the treatment, it was studied by RT-PCR. The (XDR) isolates were selected for gene expression befor and after treatment for the two genes were from three

sources (wounds, burns, and blood). The standard protocol was applied on extracted RNA with TRIzol™ GoTaq®(Syber green) Master Mix (1-Step RT-qPCR) System used according to manufacturer's instructions and applies to a single reaction where only template, primers. All reagents should be thawed on ice, gently mixed, and briefly centrifuged before use. The following table showes the recommended component volumes:

Table(3-11): Ingredients and their volumes for the qRT-PCR technique of the(*bla*$_{OXA51}$, *hcp)* genes and 16S rRNA *(* housekeeping) genes.

Master mix components	Stock	Unit	Final	Unit	Volume	
					1 Sample	6.1 sample
qPCR Master Mix	2	X	1	X	5	30.3
RT mix	50	x	1	x	0.25	1.5
MgCl2					0.25	1.5
Forward primer	10	µM	1	µM	0.5	3.0
Reverse primer	10	µM	1	µM	0.5	3.0
Nuclease Free Water					2.5	15.1
RNA		ng/µl	ng/µl	1		
Total volume				10		
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template					

-The reactions are mixed thoroughly by pipetting or gentle vortexing followed by a brief spin in a microcentrifuge.

-Transfer tubes into a Real-time PCR instrument and the program runs as the following table(3-12).

Table(3-12):qRT-PCR programmed detection of the (*bla*$_{OXA51}$, *hcp)* genes and 16srRNA *(* housekeeping) genes.

Steps	°C	m: s	Cycle
RT. Enzyme Activation	37	15:00	1
Initial Denaturation	95	10:00	
Denaturation	95	00:15	40
Annealing	52 OR 55 depending on the primer	00:30 acquiring on Green	
Extension	72	00:30	
Melt on Green\ Melt from 72°C to 95°C at 0.3°C/s			

Quantitative real-time PCR and gene expression was performed using the Mic qPCR Cycler -RT-PCR Detection System (BioMolecular System, Australia). Based on the result of mean ΔC_T and $2^{-\Delta CT}$ with the least and highest values respectively.

"ΔCt = Ct target gene **-** Ct reference gene"

Then the difference between the "ΔCt" of the unknown and the "ΔCt" of the calibrator is calculated, giving the "ΔΔCt" value, as shown in the following equation: "ΔΔCt" = "(Ct target **-** Ct reference)" sample – " (Ct target**-** Ct reference)" calibrator. The normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$ (Dumas *et al*.,2006).

Folding =$2^{-\Delta\Delta CT}$

ΔΔCT =ΔCT $_{Treated}$ - ΔCT $_{Control}$

ΔCT =CT $_{gene}$ - CT $_{House\ Keeping\ gene}$

3.3.9 Sequencing and Blasting

One isolate(A.b13)(XDR) was selected for sequencing ,PCR product were sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software as in the follwoing**.**

3.3.9. 1 PCR to Universal 16SrRNA and *hcp gen*

Two PCR fragments were selected for amplification, which was supposed to partially cover two different genetic loci in *Acinetobacter baumannii*, 16S rRNA, and *hcp* amplicons sequences (Table 3-13). PCR reaction was performed as a paragraph (**3.3.4**) and (**3.3.5**)**.**

Table (3-13). The specific primers' pairs were selected to amplify 16S rRNA and *hcp*-gene within the *Acinetobacter baumannii* sequences.

Locus	Primer Name	Specific primer sequences	Annealing Temp. (°C)	Product size (bp)	Ref.
16S rRNA	27F	5′ -AGAGTTTGATCCTGGCTCAG-3′	60	1500	(Kim *et al* .,2017)
	1492R	5′ -TACGGTTACCTTGTTACGACTT-3′			
hcp gene	F	5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′	55	142	(Mukherjee et al .,2013)
	R	5′-GAGMGGKATRTACCCATGAGRTCGGC-3′			

3.3.9.2 DNA Sequencing of PCR amplicons 16S rRNA and *hcp*

Sequencing was performed for 16SrRNA and hcp gene to 1(Ab.13) isolate. Uniplex PCR products of the genes sample of *A. baumannii* isolate were stored at -20C°, then the nucleotides sequence of the gene carried out by sending the samples and primer to(Macrogen Inc. Geumchen, Seoul, South Korea). After the results were received, the resolved PCR amplicons were commercially sequenced from termini, forward, and reverse, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystems) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences of the bacterial database, the virtual positions, and other details of the retrieved PCR fragments were identified.

3.3.9.3 Interpretation of sequencing data

The sequencing results of the PCR products were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were

numbered in PCR amplicons as well as in its corresponding position within the referring genome.

3.3.9.4 Translation of nucleic acid variations into amino acid residues

The amino acid sequences of the targeted protein of *hcp gene* were retrieved online from the protein data bank (http://www.ncbi.nlm.nih.gov). The observed variants in the coding portions were translated into a reading frame corresponds to the referring amino acid residues using the Expasy online program (http://web.expasy.org/translate/). Multiple amino acid sequences alignment was conducted between the referring amino acid sequences and its observed mutated counterpart using the "align" script of the BioEdit server.

3.3.9.5 Comprehensive phylogenetic tree construction

A specific comprehensive tree was constructed in this study according to the neighbor-joining protocol described by Sarhan *et al.* (2020). The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (Zhang *et al*. 2000). Then, a full inclusive tree, including the observed variant, was built by the neighbor-joining method and visualized as a cladogram tree using iTOL suit (Letunic and Bork, 2019). The sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

3.4: Statistical analysis

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

CHAPTER FOUR

RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Isolation of *Acinetobacter baumannii*

The results in the current study revealed that a total number of 20 (9.7%) isolates of *Acinetobacter baumannii* were obtained from (207) clinical specimens including wounds ,burns ,sputum and blood of infections from both male and female, different ages, diverse local regions, 162 specimens had been given positive growth while 45 specimens showed no growth as appeared in figure(1-4). The isolates were collected during the study period from the initial September /2019 till the end of January /2020. The collection was from visitors and hospitalized patients in governmental hospitals in Baquba / Diyala.

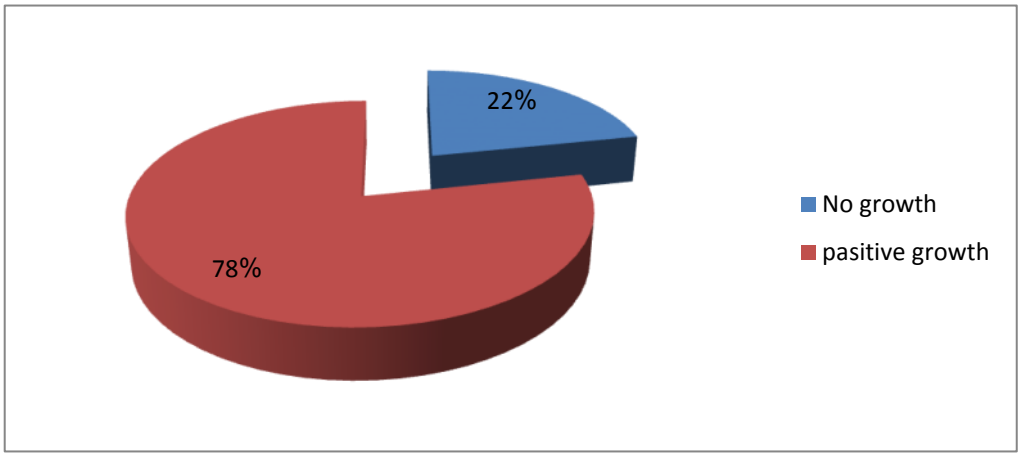


Figure (4-1): The percentages of clinical specimens

4.1.1 Distribution of the specimens according to visitors and hospitalized patients

The Samples were collected randomly from hospitalized patients or visitors to the consulting clinic, and on this basis, their percentages were as shown in the table (1-4).

Table (4-1): The percentages of clinical specimens according to visitors and hospitalized patients.

patients	number of specimens	percentages%	number of target bacteria	percentages %
visitors	120	57.97	5	2.42
hospitalized patients	87	42.03	15	7.25
total	207	100	20	9.67

4.1. 2 Distribution of the specimens according to sources

The results showed in table (4-2), *A.baumannii* isolates isolated in high percentage in burn 8(40%), then blood 7(35%), wound 4 (20%)and the later was septum only 1(5%) isolate.

table (4-2) Distribution of the specimens according to sources

sources	Total number of specimens	NO. of positive A.b Isolates	percentages % A.b Isolates
Burns	50	8	16%
blood	17	7	41.2%
wound	90	4	4.4%
septum	5	1	20%
Total	162	20	12.35%

4.2 Identification of *Acinetobacter baumannii*

The results showed that from the total 162 clinical specimens which cultured on blood agar and maconky agar only 20(9.7%)isolates were primary identification as *Acinetobacter baumannii*.

The conventional methods of bacterial identification relying on the phenotypic identification of the causative organism using bacteriological methods, including culturing on selective media, Grams staining, colonial morphology, and microscopically characteristics.

4.2.1 Microscopic Examination (Gram stain)

All of 20 *A.baumannii* isolates were examined by Gram stain. The results showed that all isolates were Gram-negative coccobacilli, occurring in singly, or in short chains and occasionally arranged in diplococcic .

4.2.2 Identification the morphological on media growth

In the present study, all 20 isolates were cultured on the MacConky agar and blood agar. Growth MacConkey agar revealed a pale pinkish tint, due to the non-lactose fermenting colonies, no pigmentation with small size and regular edges. The colonies of *A.baumannii* isolates on blood agar appeared white to cream-colored, smooth and circular with entire edges. Most species were non-hemolysis. Colonies became more mucoidal upon further incubations (Macfaddin *et al*.,2000). This appears on the figure (4-2)

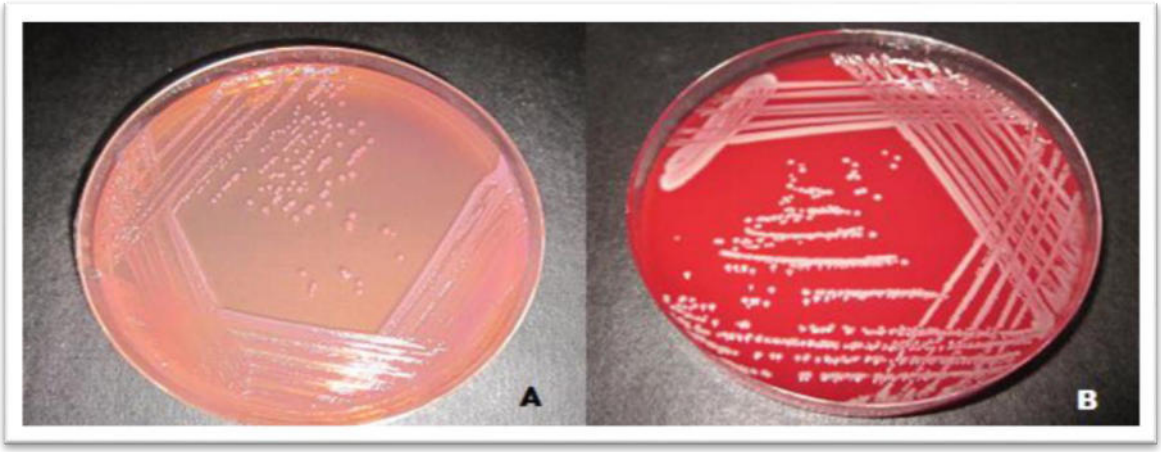


Figure 4-2: *Acinetobacter baumannii* colonies on MacConkey agar (A) and Blood agar (B), after 24 hrs of incubation at 37ºC.

4.2.3 Biochemical Identification and Heat tolerance

A series of biochemical tests were performed to identify the bacteria listed in Table (4-3). These tests allowed quickly to identify the unknown isolate based on the color changes that occur in the various tests. *A.baumannii* isolates gave in biochemical tests after 24 hrs of being incubated at 37°C. In the case of the catalase test, all the suspected isolates showed positive results for this test by the formation of gas bubbles after adding a hydrogen peroxides

reagent to colonies. However, they gave negative results to the oxidase test. In the Kliglar iron agar test, they gave alkaline/acid type of growth slant and did not change the bottom and H2S negative without gas production because they were strictly aerobic. Appendix (1) show this result.

Biochemical tests showed that all isolates no indole production, vogus proskauer test, and red methyl test gave negative results. The positive results for the test appeared in Simmons citrate, while the urease test gave variable results. In the present study, these conventional biochemical tests were carried out, and the results were compared with the standard result documented by (Macfaddin, 2000). *Acinetobacter* is easily isolated in typical cultures but is relatively nonreactive in many biochemical tests that are usually used to distinguish among gram-negative bacilli (Munoz-Price, 2008).

All isolates of *A.baumannii* had the ability to grow at 44°C were positive on nutrient agar media. They can produce heavy growth after 24 hrs of incubation. This test was used to differentiate *A.baumannii* from other *Acinetobacter* species reading the ability to grow at this temperature degree. This is considered the best method to identify *A.baumannii* from other bacteria (Sohrabi *et al*., 2012).

Table (4-3): Identification of *Acinetobacter baumannii* by biochemical test.

NO	Biochemical tests	results
1	Gram stain	-
2	Microscopic shape	Coccobacilli
3	Growth at 44°C	+
4	Lactose fermentation	-
5	Hemolysin production	- γ hemolysis
6	Oxidase test	-
7	Catalase production test	+
8	Methyl red	+

9	**Citrate utilization**	+
10	**Voges- Proskauer**	-
11	**Indol production**	-
12	**Urease production**	**Variable**
13	**Kliglar iron agar**	**Alkaline slant /No change bottom, No gas, No H2S**

+ = Positive result, - = Negative result

4.2.4 Identification of *Acinetobacter baumannii* by VITEK 2 Compact system GN

The confirmatory diagnosis of the 20 isolates of *A. Baumannii* screened in the current study was made by using the GN ID Card of the VITEK 2. The using of a Gram-negative bacterial card gives accuracy through "47 tests" within 8-5 hours. Permitting the diagnosis of isolates during the typical time without any mutations in the isolates with high resolution (about 99% accuracy) and by very little error (Pincus, 2011). The table (4-4) shows the result of "64 tests" (VITEK 2).

After the diagnosis, it was as certained that 100% of bacterial isolates included in the current study were of the same type *A. baumannii*, according to the biochemical tests shown in Table 4-4, Appendix (2).

Table (4-4): Results of the biochemical test to *Acinetobacter baumannii* by using the VITEK-2 system.

Test type	Result	Test type	Result	Test type	Result	Test type	Result	Test type	Result	Test type	Result
APPA	-	ADO	-	PyrA	-	IARL	-	Dcel	+	BGAL	-
H2S	-	BNAG	-	AGLTp	-	dGLU	+	GGT	-	OFF	-
BGLU	-	dMAL	-	dMAN	-	dMNE	+	BXYL	-	BAlap	-
ProA	-	LIP	-	PLE	-	TyrA	+	URE	-	dSOR	-
SAC	-	dTAG	-	dTRE	-	CIT	+	MNT	-	5KG	-

ILATk	+	AGLU	-	SUCT	+	NAGA	-	AGAL	-	PHOS	-
GlyA	-	ODC	-	LDC	-	IHISa	+	CMT	+	BGUR	-
O129R	+	GGAA	-	IMLTA	-	ELLM	-	ILATa	-		

***+ = Positive result,* - = Negative result**

4.2.5 Molecular identification of *Acinetobacter baumannii*

4.2.5.1 Genomic DNA Extraction

Extraction results were good and the DNA quantification (concentration) was directly performed by Quantus Fluorometer. DNA concentration extracted from all the isolates ranged between (13 to26) ng/µl, and this show on the table of (appendix 3). All of the PCR product under study was confirmed and analyzed by the horizontal gel electrophoresis in 1.5% agarose for 90min and turned on at 100v/m Amp , and then was exposed to U.V light where the DNA appears as compact bands.

4.2.5.2 Molecular Identification of *A.baumannii* by the Detection $bla_{\text{OXA-51}}$ Gene

After the initial diagnosis by biochemical tests to the isolates, a genotypic method was carried out for the genomic identification of *A.baumannii*. Conventional Polymerase Chain Reaction (PCR) with a specific primer was used for the detection of the presence of $bla_{\text{OXA-51}}$ gene the identified species level for each DNA extracted sample has been used. The results showed thatthe PCR products were confirmed by comparing their molecular weight with 100 bp DNA Ladder by the analysis of the bands on gel electrophoresis, to $bla_{\text{OXA-51}}$ gene presented in all 20(100%) of DNA *A.baumannii* isolates with a PCR product size single amplicon 353 bp as shown in Figure(4-3)and(4-4).

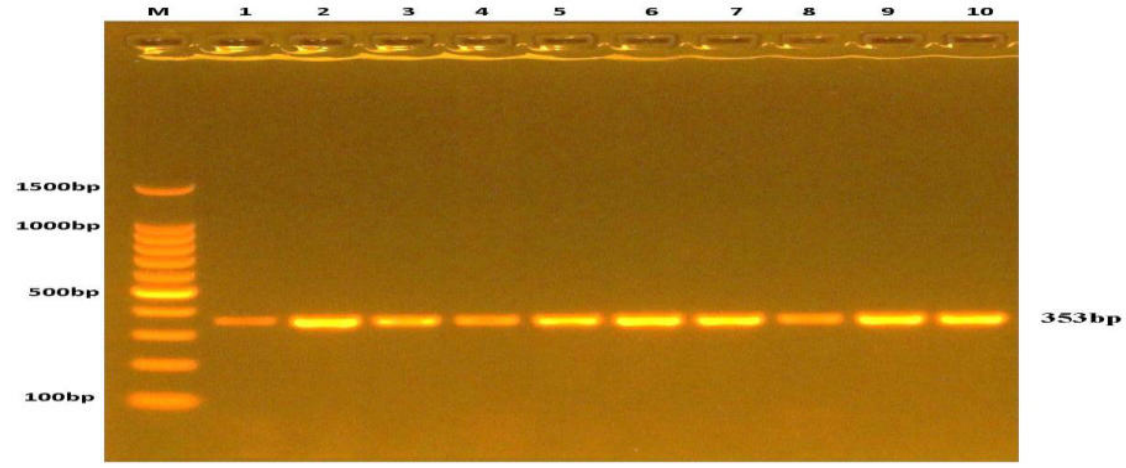


Figure :(4-3) The amplification of *bla*$_{OXA-51}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel(90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-10 resemble 353bp PCR products.

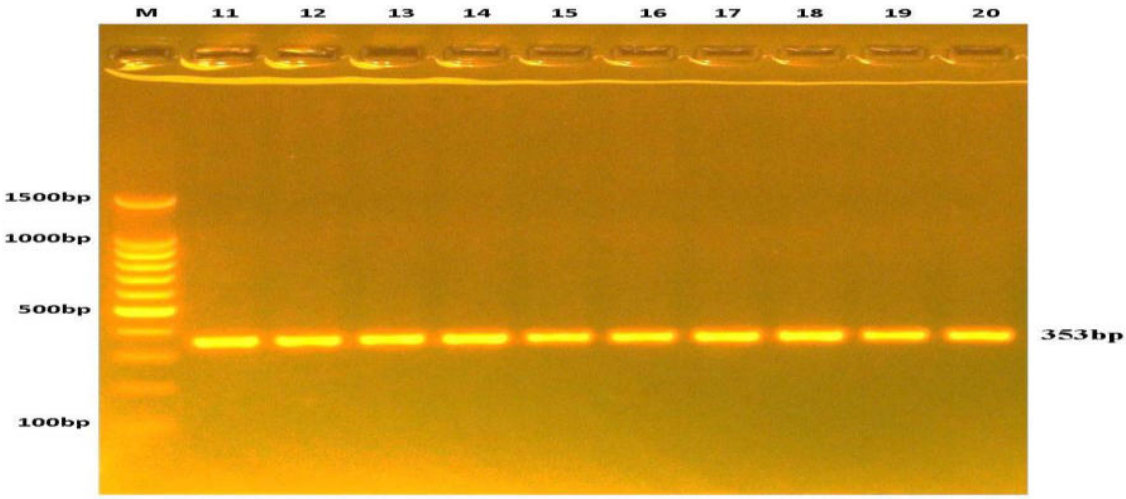


Figure (4-4) The amplification of *bla*$_{OXA-51}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel (90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 11-20 resemble 353bp PCR products.

The results of the amplification of *bla*$_{OXA-51}$ gene (detection gene) indicated that all 20 clinical isolates were identified as *A.baumannii.*

The current results were parallel with most of the quondam studies; a matter the proves the occurrence of *bla*$_{OXA-51}$ gene in all clinical isolates of *A.baumannii*. (Ghaima, 2016; Kazim, 2019). The confirmation by using *bla*$_{OXA-51}$ primers and observing ~ 353 bp band in the agarose gel it was in agreement with most preceding studies, (Al-Agamy (a)*et al*. 2014) in Egypt, (Nowak *et al*. 2012) in Korea and others, which demonstrated that the

presence of this gene in all clinical isolates of *A.baumannii* was positive for the amplification of the *bla*$_{OXA-51}$ gene. Therefore, the *bla*$_{OXA-51}$ gene was normally considered to be species-specific to *A.baumannii* (Khalilzadegan *et al*., 2016).

The detection of the *bla*$_{OXA-51}$ gene has been considered a simple, rapid, and reliable method for *A.baumannii* genomic species identification as these are naturally occurring carbapenemases genes to *A.baumannii*, that have enhanced the understanding of the clinical importance and epidemiology of *A.baumannii* (Khalilzadegan *et al*., 2016). The high rate of *bla*$_{OXA-51}$ gene is back to the information that this gene is a ubiquitous, an intrinsic, and chromosomally located. It explains its role in the specific identification of *A.baumannii* (Bonnin *et al*., 2012).

4.3 Phenotypic Detection of Antimicrobial agents

4.3.1 Antibiotic Susceptibility Test

The antimicrobial susceptibility of *A.baumannii* isolates was determined by the disk diffusion method (Kirby-Bauer) in recommendation with the clinical and laboratory standards institute guidelines CLSI (2019) depending on the diameter of inhibition zone (mm), listed in the materials and method Table (3-5) and paragraph (3.2.7.2.) This test was conducted to all 20 *A.baumannii* isolates against 13thirteen antimicrobial agents: β-Lactamase inhibitors class (Ticarcillin–clavulanic acid, Ampicillin-sulbactam, Piperacillin-tazobactam), cephalosporins class (Cefotaxime, Ceftazidime, Ceftriaxone), Carbapenems class (Imipenem, Meropenem), quinolones class (Levofloxacin), aminoglycosides class (Amikacin and Gentamicin), and Tetracyclin class (Doxycycline, Tetracycline).

The results revealed that all *A.baumannii* clinical isolates have a very high level of resistance to the tested antimicrobial agents under test as presented in

Figures (4-5) for isolate A.b13, the table of the appendix (4) showed the different susceptibility of isolates towards these antibiotics.

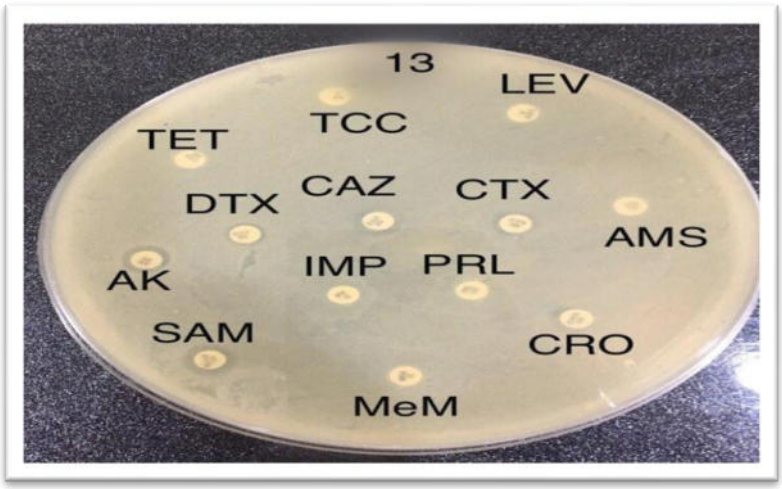


Figure (4-5): Antibiotic Susceptibility Test for *A.baumannii* Using 13 Antibiotics The isolate (Ab 13) is Resistance to 13 Antibiotics. (PRL= Pipercillin-tazobactam,CTX= Cefotaxime , TCC= Ticarcillin–clavulanic acid ,AK= Amikacin,GT= Gentamicin , LEV= Levofloxacin , IMP= Imipenem, MEM= Meropenem, CRO= Ceftrixone ,CAZ=Ceftazidime,TE= Tetracycline, AMS= Ampicillin-sulbactam,DO= Doxycline)

The results of the current study demonstrated that the highest resistance to almost all β-lactam antibiotic classes understudy was as follows cephalosporins (Piperacillin-tazobactam, Cefotaxime, and Ticarcillin–clavulanic acid) showed complete resistance with a percentage rate of 20(100%), a high resistance to (carbapenems) imipenem and meropenem with a percentage of (95%) and(Ceftrixonem, Ceftazidime) with a percentage of (90%). These results were similar and quite an agreement to the previous studies in Iraq by AL-Kadmy *et al.* (2018); Fallah *et al*. (2017); Maryam *et al*. 2015) reported that their *A.baumannii* clinical strains were resistant to those antibiotic with relative percentages. The percentages of bacterial susceptibility to antibiotic resistance are listed as shown in the figure(4-6)

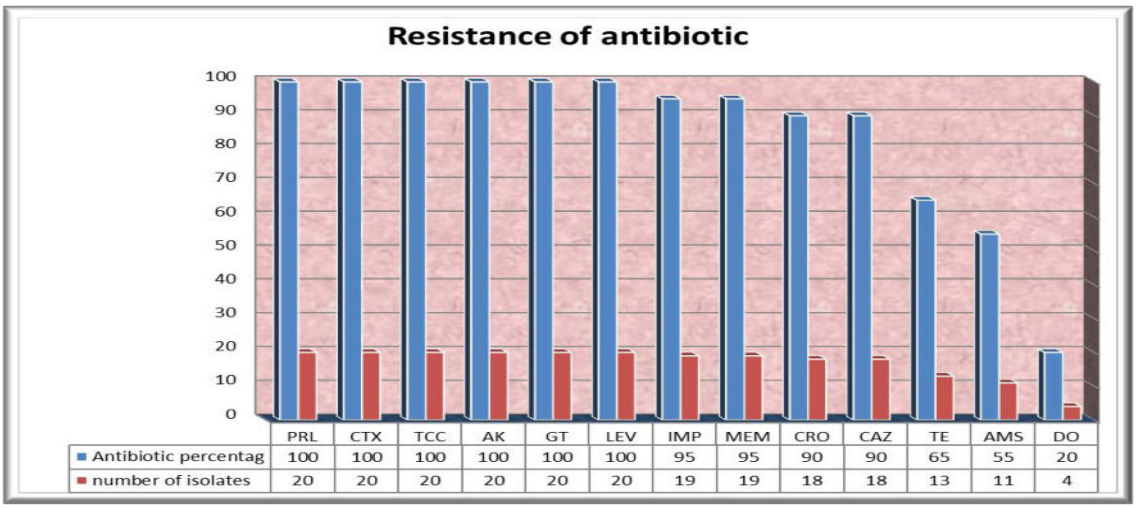


Figure (4-6): The Percentage of Susceptibility for *A.baumannii* Isolates against Antibiotics(PRL= Piperacillin-tazobactam,CTX= Cefotaxime , TCC= Ticarcillin–clavulanic acid ,AK= Amikacin,GT= Gentamicin , LEV= Levofloxacin , IMP= Imipenem, MEM= Meropenem, CRO= Ceftrixone ,CAZ=Ceftazidime,TE= Tetracycline, AMS= Ampicillin-sulbactam,DO= Doxycycline)

The results of (Imipenem and meropenem) whic they considered as drug choice of treatment with(90% percentage), the resulting agreement to study of the neighboring countries, such as Turkey (98%) and Pakistan (100%), and Iran(90%)and Saudi Arabia 90% (Al-Agamy(b) *et al*., 2014; Begum *et al*., 2013; Güven et al., 2014; Rahbarnia *et al*.,2020), but disagree with a study in Iraq that the resistant to Imipenem and meropenem was(75%) (Kadom *et al*.,2020).

Imipenem and Meropenem are from the Carbapenem antibiotic group. The reason for the emergence of resistance by bacteria to the antibiotic of this group is the ability of the bacteria to produce two types of β-lactamase enzymes, those are Metallo β-lactamase enzymes and Carbapenem hydrolyzing class D of β-lactamase (oxacillinases), that hydrolysis and break down carbapenems antibiotics, these enzymes have been detected both phenotypically and genetically for the isolates understudy, and that any

change occurs in proteins associated with the outer membrane OMPs leads to bacterial resistance to carbapenems antibiotic(Gallego,2015).

The isolates under study showed high resistance to antibiotics, those are (Piperacillin-tazobactam100%, Cefotaxime100%, Ticarcillin – clavulanic 100%, Ceftriaxone 90%, Ceftazidime 90%), these were agree with a local study of AL-Marjani *et al*.,(2016), and Iran study reported high percentages resistance of this antibiotics PEYMAnI *et al*.,(2012), and this study also agreed with a result of Ampicillin-sulbactam where it was (65%).

The isolates showed high resistance to some Aminoglycosides antibiotics, such as(Gentamicin and Amikacin) with a ratio of resistance (100%) these were agreed with a local study of AL-Marjani *et al*.,(2016), but does disagree with the Iranian study which lower than the percentage Fallah *et al*.,(2014) as the ratio(44%). The main cause of resistance to Aminoglycosides is due to the presence of modified Aminoglycosidase (AMEs). The high resistance levels against these antibiotics are often associated with the 16S rRNA methylase enzyme as well as the efflux mechanism and reduced the outer membrane permeability.

As for the Tetracycline (Doxycycline, Tetracycline) group showed the lowest percentage of resistance which was (20%,65%) respectively. These results were near the study of (Huband *et al*.,2020), but did not agree with India's study (Subramanian *et al.,*2020). The mod of action of the Tetracycline group inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (Subramanian *et al.,*2020), but resistance mechanisms are by efflux and ribosomal protection that cause resistance in older-generation Tetracycline (Huband *et al*.,2020).

The Results in current study were in agreement with (Genteluci *et al*. ,2016) who reported that *A.baumannii* isolates developed resistance to different

antibiotic classes, including Fluoroquinolones in high resistance rates(100%). The resistance to Fluoroquinolones, including Levofloxacin, was due to mutations in some of these bacteria, resulting in a change in membrane permeability (Lahiri *et al*., 2004). Concerning fluoroquinolone resistance of *A. baumannii,* it is often caused by modifications in the structure of DNA gyrase or topoisomerase IV secondary to mutations in the quinolone resistance-determining regions. It is the main cause of the resistance of *A.baumannii* to quinolones. These changes result in a lower affinity for the binding of the quinolone to the enzyme-DNA complex. The second mechanism of resistance to the quinolones is mediated by efflux systems that decrease the intracellular drug accumulation (Potron *et al*., 2015).

4.3.2 Distribution of the isolates according to multidrug-resistant categories

According to the Centers for Disease Control (CDC), the recently devised National Strategy for Combating Antibiotic-Resistant Bacteria, *A.baumannii* is a (serious) threat level pathogen (MAJ Ford *et al*., 2017). The prevalence and Severity of *A.baumannii* infections have earned the bacterium recognition as part of a group of pathogenic bacteria causing the largest share of hospital-acquired (MDR) infections: the ESKAPE pathogens. The ESKAPE pathogens are *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *A.baumannii* (A), *Pseudomonas aeruginosa* (P), and *Enterobacter* species (E) (Boucher *et al*., 2009). According to antibiotic Susceptibility; the isolates divided into the current study into three categories as shown on the table (4-5).

Table (4-5): Distribution of isolates according to multidrug-resistant categories

categories	NO.of isolates	No. and percentage %
MDR	A.b1,A.b2,A.b4,A.b5,A.b6,A.b7,A.b8,A.b9, A.b10,A.b11,A.b12,A.b14,A.b15,A.b16,A.b17	n=15(75%)
XDR	A.b3,A.b19	n=2(10%)
	A.b20,A.b18,A.b13(resistant all the antibiotic under study)	n=3(15%)

4.3.3Minimum Inhibitory Concentration (MIC) Imipenem and Meropenem against Resistant *Acenetobacter baumannii*

All the 20 isolates were subjected to the Minimal inhibitory concentration test for Imipenem and Meropenem. MIC had been a determined by the microtitre broth dilution standard method by on Müeller Hinton Broth. After 24 hours incubation for the microtiter plate, the results appeared as shown in the figure (4-9) and they were read by the ELISA reader under a wavelength of 6300nm and the readings were as shown in (Appendix4).

This microtitre broth dilution is the best method to determine the MIC of antibiotics, due to its ease to use, saving time and cost, in addition to reading results accurately with an ELISA reader.

The results of these isolates were identified as sensitive or resistant based on the CLSI 0f (Weinstein *et al.,* 2020) breakpoint as the basis for the response calculation and are defined as the optimal concentration that the antibiotic in the serum can provide the highest treatment limit. The bacteria is Susceptible when the dose MIC is calculated less than the breakpoint.

As in the figure (4-7), the MIC well is clear and the following well is highly turbid. The results were read and compared with positive and negative control.

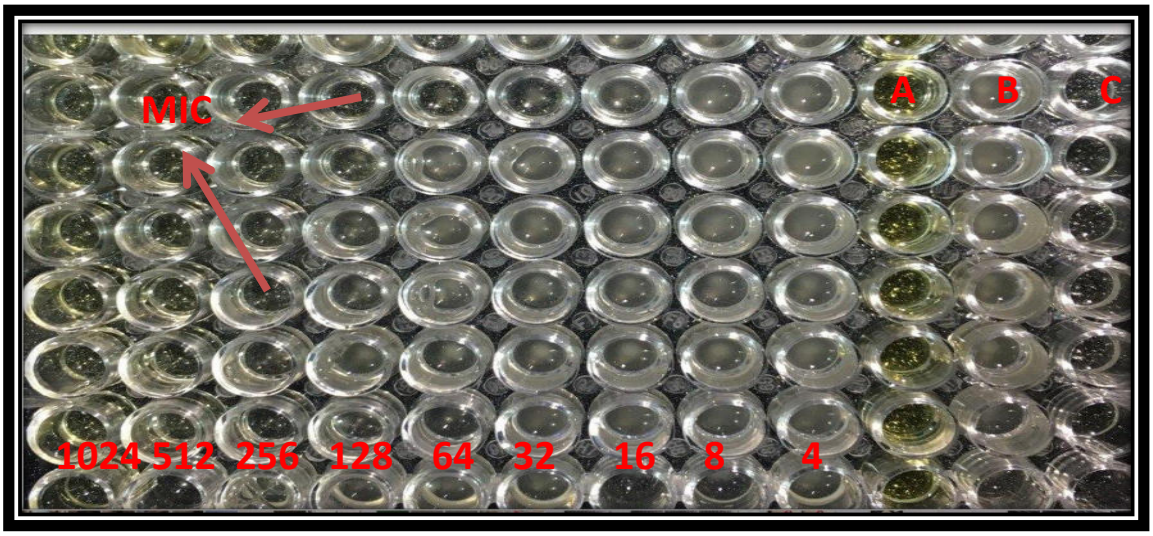


figure (4-7) MIC Result on microtiter plate ,A: control-ve(broth +Antibiotic),B=control +ve (bacterial growth),C=control –ve(only broth).

The MIC was determined for 20 isolates to the antibiotics that used under the current study as shown in the table (4-6), that showed MIC values for IMP ranged from(8-≥1024) µg/ml and the MIC for MEM values for(≤4-128) µg/ml, this results disagree with Iraqi study on Dhi Qar Governorate where was the range from 1-256 µg/ml(Kadom et al.,2020), but the results were similar to a study conducted in Baghdad governorate (Al-Saleem,2013).

The results of an previous study (Fallah *et al.*, 2014) were almost similar to the results of the current study in terms of high resistance to Carbapenems.

From the MICs, values can notice that the isolates had a high level of resistance to imipenem and meropenem which reached 1024µg/ml. These results reflect the physiological of bacteria in their possessing of resistant methods, whether mechanical or enzymatic, and this will be explained later in phenotypic and molecular detection of β- lactamase enzyme.

Table (4-6): MIC values for imipenem and meropenem

Antibiotics NO. of isolates	Imipnem	Meropenem
breakpoint	≤2(S) \≥8(R)	≤2(S) \≥8(R)
A.b1	256	16
A.b2	128	16
A.b3	256	32
A.b4	1024	32
A.b5	1024	32
A.b6	8	32
A.b7	1024	32
A.b8	256	8
A.b9	1024	16
A.b10	512	≤4
A.b11	256	16
A.b12	512	8
A.b13	256	16
A.b14	512	128
A.b15	256	16
A.b16	1024	64
A.b17	16	32
A.b18	512	32
A.b19	64	64
A.b20	≥1024	64

4.4 Detection of β-Lactmases production

4.4.1 Detection of extended-spectrum β-Lactamase production

All 20 isolates were subject to the double-disk synergy test (DDST) as described by Jarlier *et al*., (1988) to the detection the extended-spectrum β-Lactamase production. All 20 isolates of *A. baumannii* were tested, they were showing the high resistance (100%) to cefotaxime and ceftazidime, these results were indicated to the producing of ESBLs. The isolate also showed high resistance to piperacillin (PRL) and Augmentin (AMC) (100%) as shown in figure(4-8). So that there was no synergy between the disk of antibiotics. This does not mean the absence of the ESBLs enzymes, since the resistance is present, perhaps the isolates possess beta-lactamase enzymes from other types such as AmpC enzymes or they use other mechanisms in the resistance such as efflux pump or PBPs.

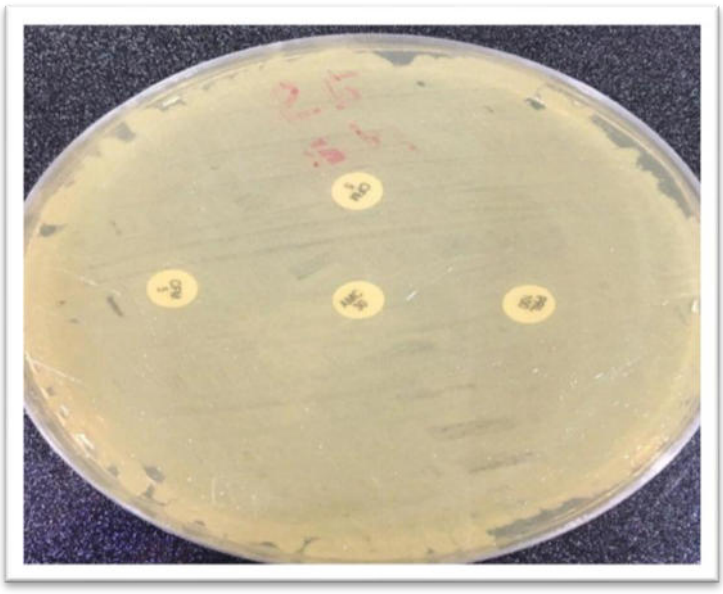


Figure (4-8) : (DDST) test for phenotyp detecation ESBLs

The results were disagree to an Iranian study, where the results of this study for phenotype detection of ESBLs were shown by (21%) (Fallah *et al*.,2014),

While the results agreed with a Jordanian studyBatchoun *et al* .,(2009), where *A.baumannii* isolates of this study showed(0%) as a percentage of production ESBLs as phenotype detection.

Research undertaken by the World Health Organisation (WHO) suggests the highest levels of antimicrobial resistance and infection by .β-Lactamase producers (above 50%) in densely populated countries like India and China. This was mainly attributed to the poor quality of antibiotics and its unsupervised use in most of the regions in these countries (Vasant *et al* .,2020).

4.4.2Phenotyp Detcation Ambler class C beta-lactamase (AmpC)

The current study had been followed AmpC disk cefoxitin resistant test to detect AmpC production, AmpC beta-lactamases are known to bestow resistance to cephalosporins in the oxyimino group and are not affected by available β-lactamase inhibitors (Thomson ,2001). The results as in the table(4-7) showed that from total 20 isolates of *A.baumannii* 6(30%) had the ability to produse AmpC which due to resistance to Cefoxitin after a four-day incubation period at 28 ° C as cleared in the figure (4-9) as positive results. Pervious study in an Indian done by Hans *et al* ., (2015), they detected (56% rate) from total 50 isolates of *A.baumannii* were production AmpC,in other study in Nepal was carried out byYadav *et al*., (2020) their results approval with the results of current study which recorded 38.5 %.

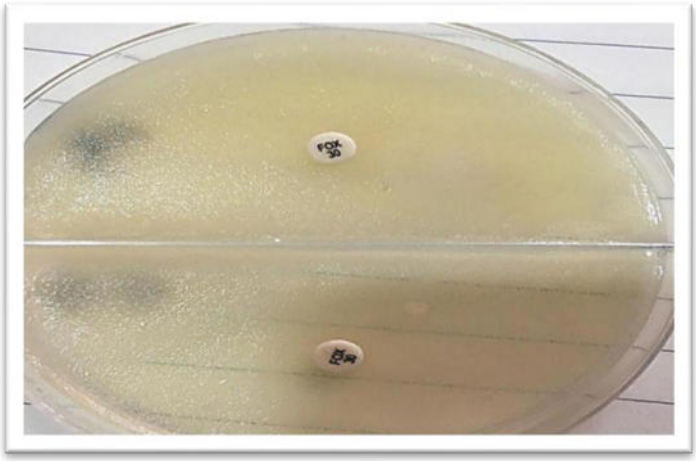


Figure (4-9) : Test of AmpC production

Table(4- 7) : of AmpC beta-lactamases production by the *A.baumannii*

No.	The source	AmpC results	No.	The source	AmpC results
A.b1	Wound	-	A.b11	Wound	-
A.b2	Burn	+	A.b12	Burn	+
A.b3	Burn	-	A.b13	Blood	+
A.b4	Burn	-	A.b14	Blood	-
A.b5	Blood	-	A.b15	Burn	-
A.b6	Blood	-	A.b16	Burn	+
A.b7	Burn	-	A.b17	Sputum	+
A.b8	Burn	-	A.b18	Blood	-
A.b9	Blood	-	A.b19	Blood	-
A.b10	Wound	-	A.b20	Wound	+

+ = Positive result, - = Negative result

4.4.3 Detection of Metallo-beta-lactamase production

4.4.3.1 Phenotypic Detection of Metallo-beta-lactamase production by combined EDTA disc test (CEDT)

The CEDT test was used for phenotypic detection MBLs of *Acinetobacter baumannii,* figure (4-10). Isolates showing ≥7 mm increase in the inhibition zone size of Imipenem-EDTA disc than the Imipenem disc alone were considered as MBL producers.

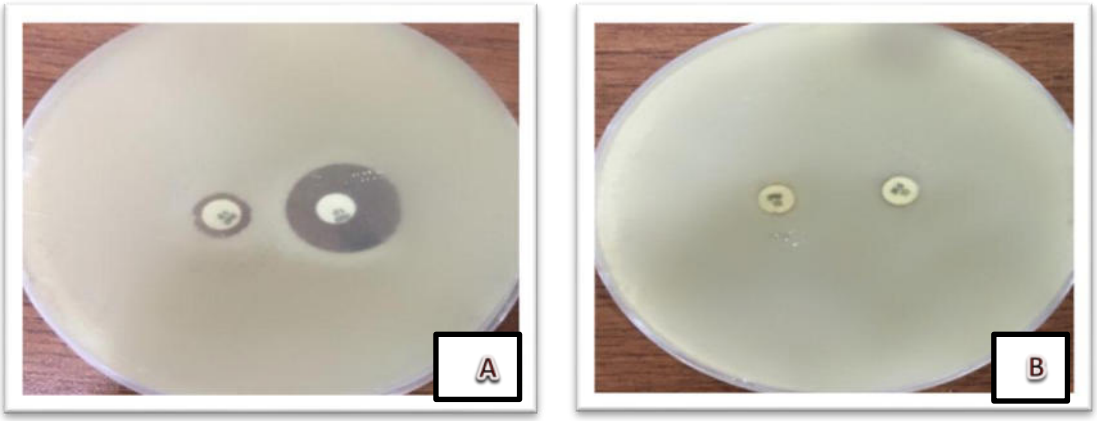


Figure (4-10) : Imipenem EDTA Combined Disc Test (ECDT) for MBL Screening; Imipenem and EDTA Zone Size > 7mm than IMP Zone Size ,A=Positive result ,B=negative result.

The total number of positive *A. baumannii* isolates that formed MBLs enzyme was 18 (90 %) and 2(10 %) isolates were negative, as showed in table 4-8. This enzyme is responsible for the antimicrobial resistance to beta-lactam groups such as Imipenem and Meropenem. The findings were similar to the Test for Antibiotic Susceptibility, in which all 19 isolates were resistance to Imipenem and Meropenem, while (A.b 8) it was sensitive to them, but the A.b 17 is resistance to them while it did not possess MBL enzyme as this detection, perhaps it resistance by another method.

Table (4- 8): Phenotype detection for the MBLs

No.	The sourse	MBL results & zone diameter (mm)EDTA	No.	The sourse	MBL results & zone dimeter(mm) EDTA
A.b1	Wound	+18	A.b11	Wound	+12
A.b2	Burn	+14	A.b12	Burn	+15
A.b3	Burn	+15	A.b13	Blood	+10
A.b4	Burn	+10	A.b14	Blood	+11
A.b5	Blood	+14	A.b15	Burn	+20
A.b6	Blood	+18	A.b16	Burn	+20
A.b7	Burn	+19	A.b17	Septum	- 5
A.b8	Burn	-4	A.b18	Blood	+14
A.b9	Blood	+20	A.b19	Blood	+15
A.b10	Wound	+ 21	A.b20	Wound	+15

+ = Positive result, - = Negative result

The current results inconsistent with an Iranian study conducted by Moulana *et al* ., (2020) they detected form 50 isolates 30% were produced MBLs. On the other Iraqi study done byAbed AL-Gayle,(2019) disagree with the current study, which he reported that 30% of the isolates produced MBLs , while Ranjbar *et al* ., (2019) were recorded phenotype detection of MBLs showed 90.1 %(n=147) of isolates were MBL producing isolate.

4.4.3.2 Molecular detection of MBLs genes by Polymerase Chain Reaction (PCR)

4.4.3.2.1 Detection of *bla*$_{VIM}$ gene

The conventional PCR was performed to all of 20 *A. baumannii* isolates in this study, by using a specific primer to *bla*$_{VIM}$ gene, Results showed that 14 isolates (70%) of *A. baumannii* were possessed *bal*$_{VIM}$ gene. Figure (4-11 A,B) showed the bands (390 bp) of DNA. This result disagree with the local study of Abed AL-Gayle,(2019) that recorded the frequency of *bal*$_{VIM}$ was (30-40%) and it also disagree with another Iraqi study (Ridha *et al* .,2019) that recorded the frequency 12.10% of the same gene in *A.baumannii* isolates, while the current study results agreed with Kadom *et al*., (2020) they recorded that 65% of the isolates were possessed the *bla*$_{VIM}$ gene.

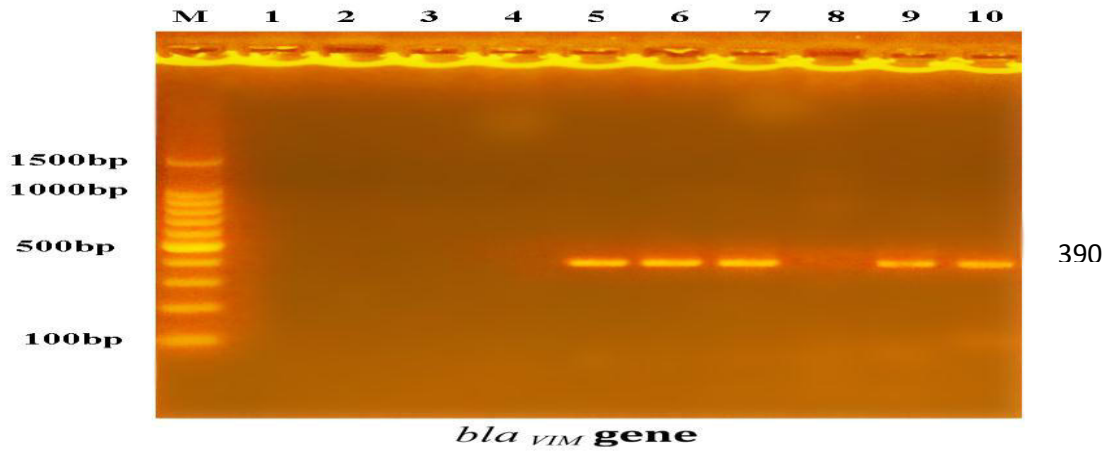
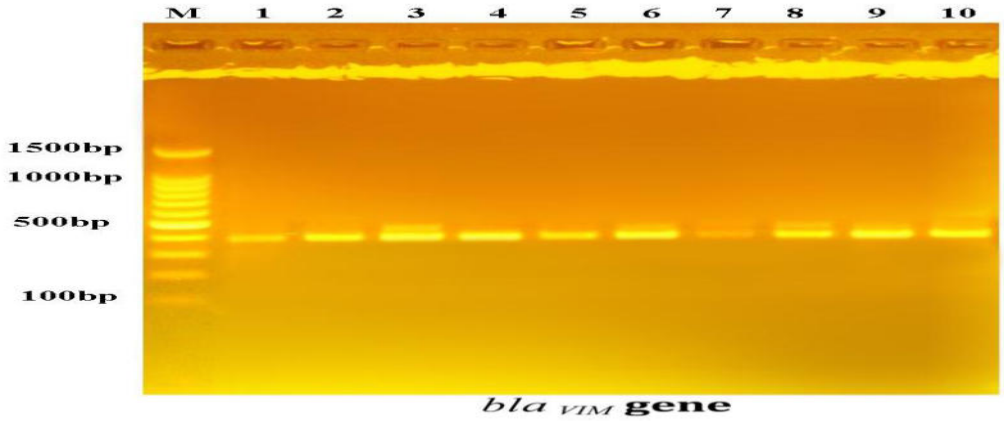


Figure (4-11 A): The amplification of *bla*$_{VIM}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel(90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-10 resemble 390bp PCR products.



390

Figure (4-11 B) : The amplification of *bla*$_{VIM}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel (90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 11-20 resemble 390bp PCR products.

4.4.3.2.2 Detection of *bla*$_{IMP}$ gene

The conventional PCR was performed to all of 20 *A. baumannii* isolates in this study, by using a specific primer to *bla*$_{IMP}$ gene, Results showed that all the 20 isolates (0%) of *A. baumannii* did not possess *bal*$_{IMP}$ geneas shown in Figure (4-12A,B) this result agreed with results of Iranian study done by Moulana *et al* ., (2020), but it disagree with local study done Ridha *et al* ., (2019) with 79% at the same gene.

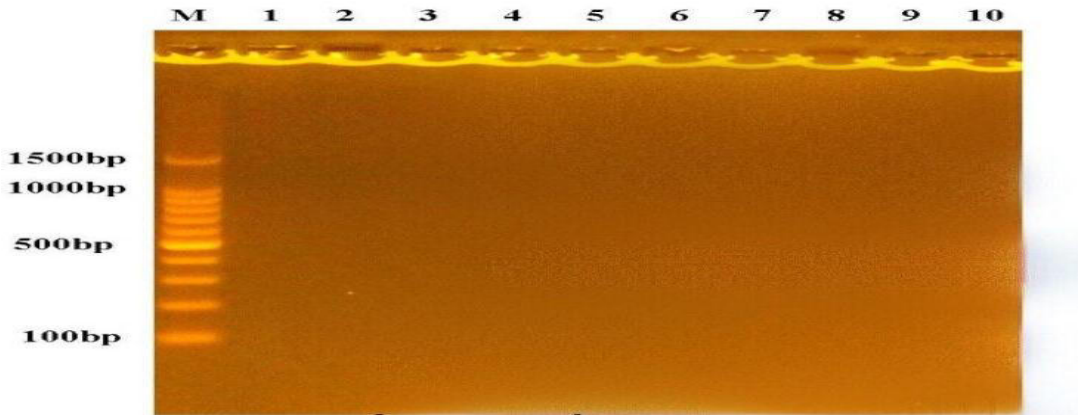


Figure (4-12) A: The amplification of *bla*$_{IMP}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel(90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-20 resemble.

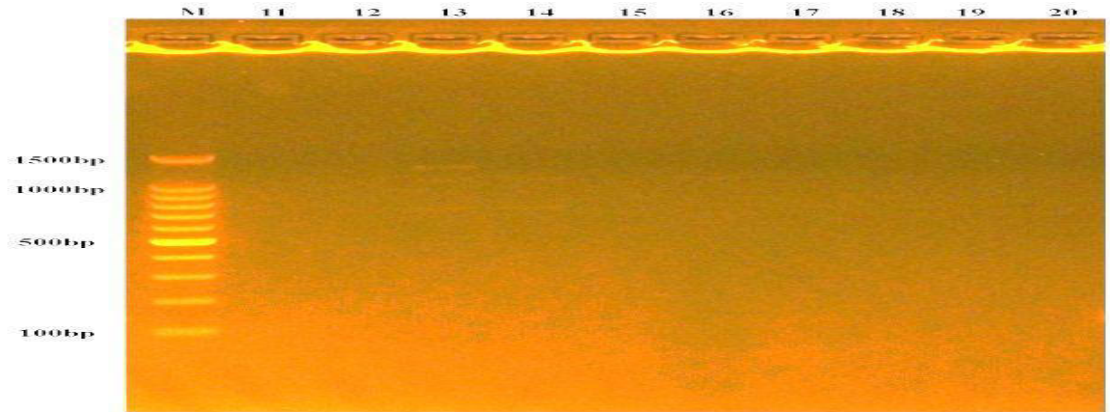


Figure (4-12)B: The amplification of *bla*$_{IMP}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel(90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-20 resemble.

4.4.3.2.2 Detection of *bla*$_{NDM\ -1}$ gene

The results showed as appeared in figure(4-13 A, B) that all 20 isolates of *A. baumannii* did not possessed gene *bla*$_{NDM\ -1}$(0 %) . These results agree with the study of Fallah *et al* .,(2014), were recorded the prevalence of *bla*$_{NDM\ -1}$ was (0%) on clinical *A. baumannii* isolates,but it disagree with the local study (Ridha *et al* .,2019) which recorded 14.50% for the same gene.

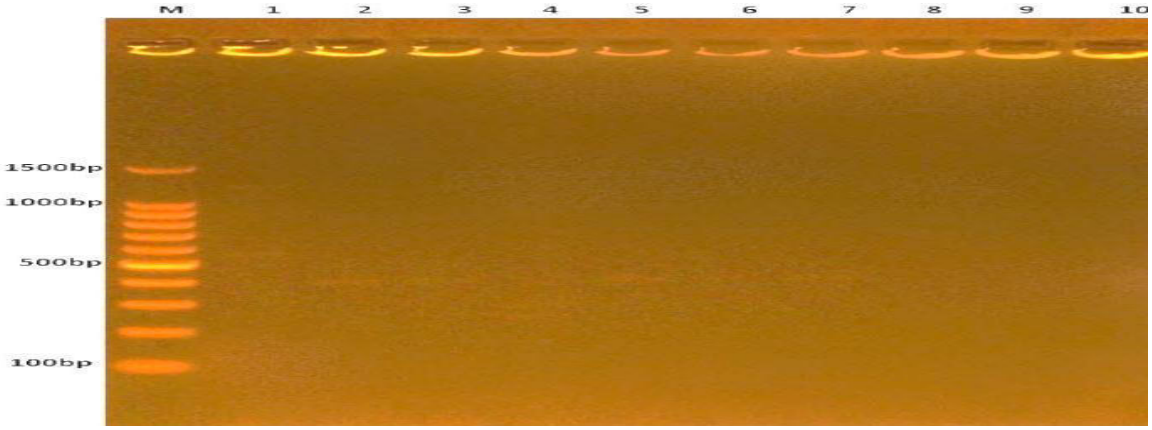


Figure (4-13)A: The amplification of *bla*$_{NDM-1}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel(90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100 bp ladder marker. Lanes 1-20 resemble.

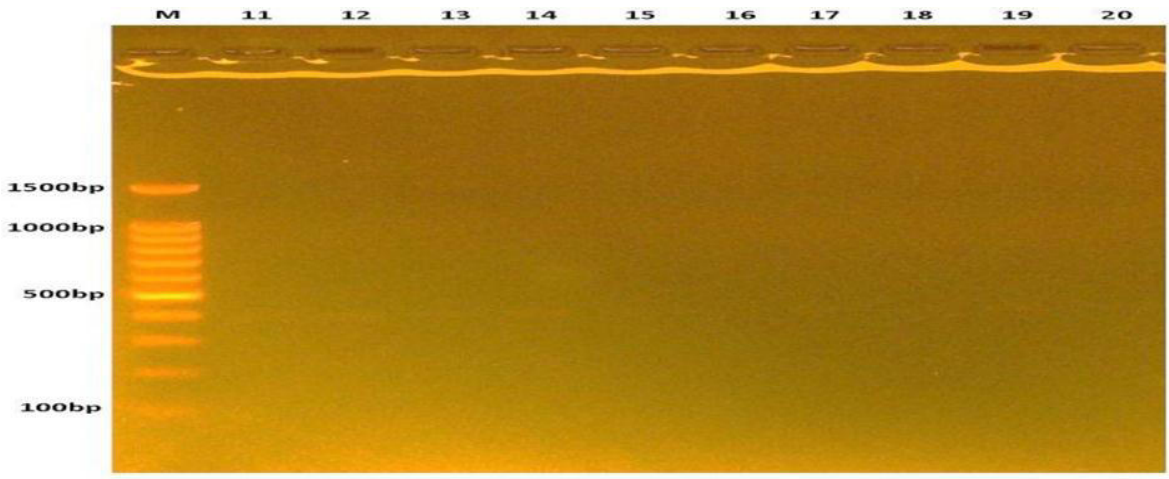


Figure (4-13)B: The amplification of *bla*$_{NDM-1}$ gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose(90 min at 100 volts) gel electrophoresis stained with Eth.Br. M: 100 bp ladder marker. Lanes 1-20 resemble

The results of current study as agarose gel electrophoresis were showed the prevalence of *bla*$_{VIM}$, *bla*$_{IMP,}$ and *bla*$_{NDM-1}$ genes were 70%,0%,0% respectively. The (A.b8, A.b17) isolates did not have any of that three gene of MBL, this result corresponds to the phenotype detection of MBLs, while the (A.b 1,2,3,4) isolates gave a positive result for MBL yet they did not harbor MBL genes under study, which may be due to the presence of putative proteins belonging to the MBL other than IMP, VIM, and NDM type predominant MBLs or other groups MBL genes (Lee *et al* .,2008).

4.5 Biofilm formation in *Acinetobacter baumannii*

The clinical importance of *Acinetobacter baumannii* is partly due to its natural ability to survive in the hospital environment. This persistence may be explained by its capacity to form biofilms and interestingly, *A.baumannii* can form pellicles at the air-liquid interface more readily than other less pathogenic *Acinetobacter* species(Chabane *et al*.,2014).

4.5.1 Detection of biofilm using the tube method

The method is considered a Qualitative assay for detecting the biofilm formation ability based on the thickness and intensity of the biofilm-associated with the inner wall of the test tube. The results showed that all isolates had the ability to adherent and forming pellicle at the surface of the broth after 24ohr incubation, it showed as a purple ring as in figure (4-14) after staining by crystal violate. This was the indicate to the ability of the bacteria to form a biofilm(Chabane *et al*.,2014).

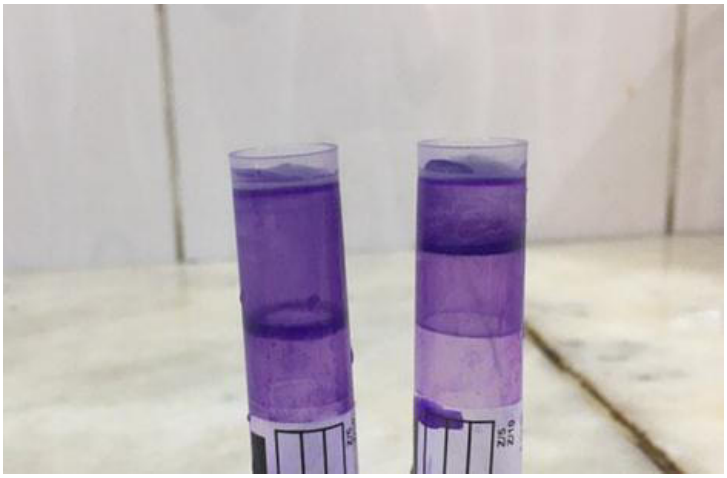


Fig (4-14): Tube method for biofilm production *A.baumannii*

4.5.2 quantitative of biofilm formation by Micro-titer plate method

The test is a quantitative assay to detect the development of biofilms. It uses the ELISA reader to calculate the amount of biofilms produced by adhesion to the surfaces of the calibration plates to provide a numerical absorbance value over a wavelength of 630 nm as figure (4-15).

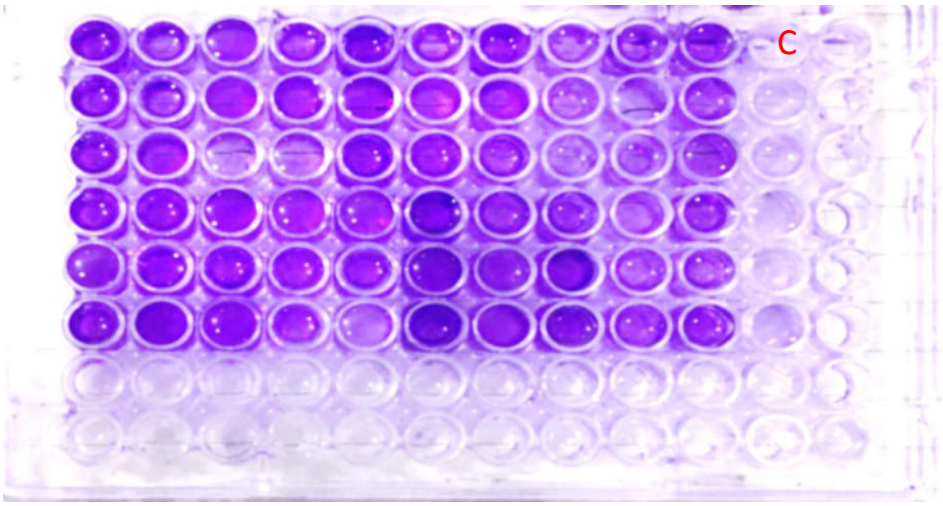


Fig (4-15): Micro-titer plate for biofilm determination

The results were shown in Table (4-9), all 20 isolates included in the study had the properties of biofilms formation and the absorbency value was ranged from (0.182 – 0.060), this method described by Tang *et al*., (2011) in the detection of the production of the biofilms. 7 (35%) of isolates were strongly biofilm-forming while 12 (60%) for moderately biofilm-forming and 1(5%) weak biofilm formation, the result nearly to result of the local study AL-Mosusawi, (2018), but disagree with Ridha *et al*., (2019) as their results showed a rate of 94% for high-yield biofilm isolates, and the moderate was 10%. In a Taiwanese study conducted on clinical isolates of *A.baumannii*, the Results showed that among the 154 tested isolates, 15.6% of the clinical isolates were weak biofilm producers, while 32.5% and 45.4% of them possessed moderate and strong biofilm formation ability, respectively Yang *et al* ., (2019).

Table 4-9: Absorbency values and biofilm pattern by MTP method.

Sample	Absorbency at 630 nm	Biofilm level compared to (ODc=0.058)(2*ODc=0.116)	Sample	Absorbency at 630 nm	Biofilm level compared to (ODc=0.058)(2*ODc=0.116)
A.b1	0.128	strong	A.b11	0.157	strong
A.b2	0.083	moderate	A.b12	0.108	moderate
A.b3	0.148	strong	A.b13	0.094	moderate
A.b4	0.159	strong	A.b14	0.121	strong
A.b5	0.128	strong	A.b15	0.065	moderate
A.b6	0.07	moderate	A.b16	0.089	moderate
A.b7	0.078	moderate	A.b17	0.064	moderate
A.b8	0.105	moderate	A.b18	0.131	strong
A.b9	0.096	moderate	A.b19	0.060	weak
A.b10	0.067	moderate	A.b20	0.079	moderate

The discrepancy among the findings of the current research and the previous studies may be due to the different components used in the process, such as the using TSA or N.B media, microbial concentration or incubation period (24 hours), as the density of the cells in the biofilm increases as the incubation period increases, and the dye concentration affects the results as the concentration of 0.5 percent gives better results when compared to 1% (Wagner, 2016).

A microtiter plate is an important tool for studying the early stages of biofilm formation. This approach has the advantage of economical quantitative technique for identifying serious factors and standard conditions of culture for

in *vitro* forming biofilms. It is a 100 percent higher sensitivity in positive strain exposure (O'Toole, 2011).

4.6 Colorimetric method to detect quorum sensing

The results showed 18 isolates under study were producer to quorum sensing Q.S but in vary percentage ranged from (strong to moderate) according to the value of absorbance that was measured by ELISA reader, the table (4-10) showed 7(35%) of *A. baumannii* isolate were a high producer of AHL molecules with absorbance value (1.551-2.69), while11(55%) isolates that exhibited moderate activity of AHL with absorbance value(1.145-1.550) and 2(10%) isolates were no formation activity of AHL that is because the isolates will be considered negative or weak AHLs produce if below a threshold level (OD≥0.985). The optical density (OD) was measured by ELISA reader at 630 nm (Modarresi *et al*.,2015).

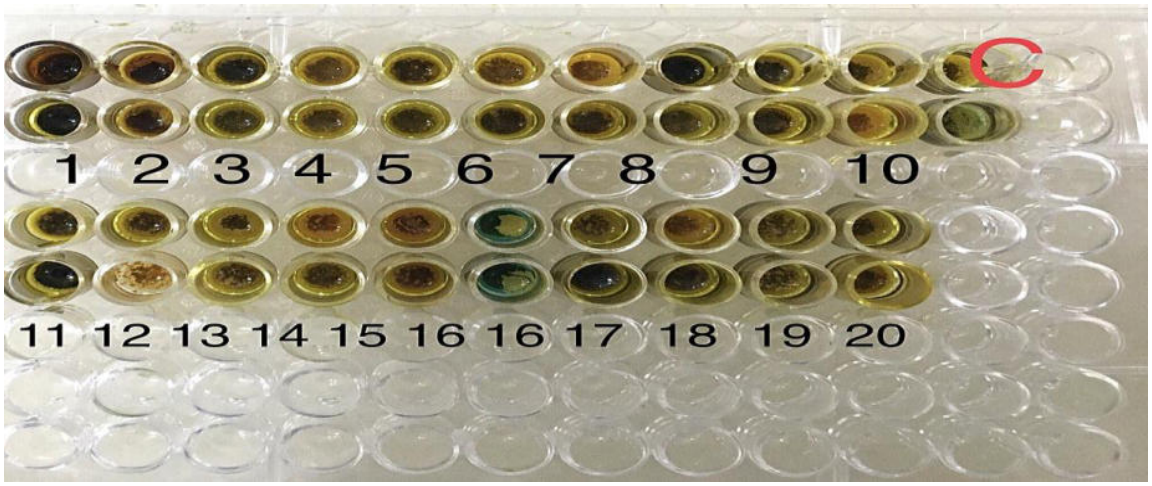


Figure (4-16) Quantification of AHLs measured in ELISA reader at 630 nm

Figure (4-16) showed a dark brown color is produced in the samples containing AHL molecules, but in some cases, it may change to yellow color depending on the concentrations of the compounds.

Table 4-10: Quantification of AHLs optical density measured in ELISA reader at 630 n

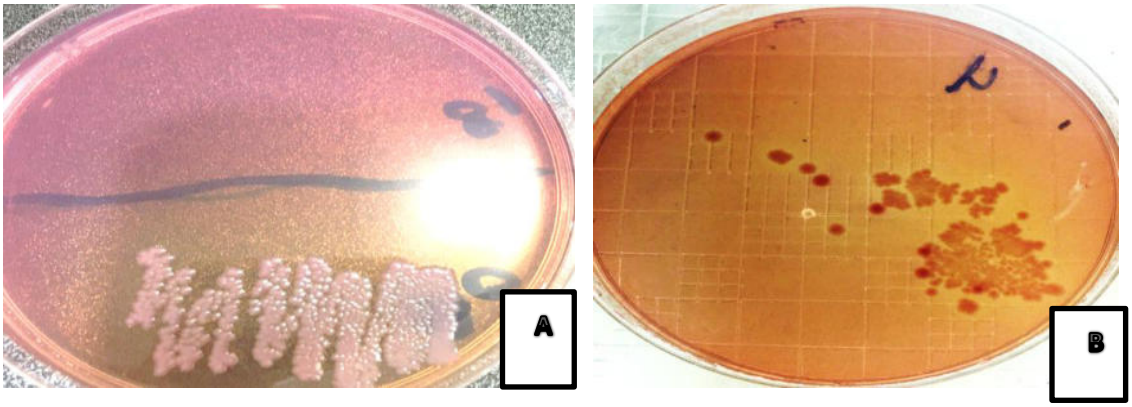
Sample	Absorbency at 630 nm	Q.S level compared to (ODc=0.931)(0.981)	Sample	Absorbency at 630 nm	Q.S level compared to (ODc=0.931)(0.981)
A.b1	2.569	strong	A.b11	1.829	strong
A.b2	1.968	strong	A.b12	0.993	no formation
A.b3	2.115	strong	A.b13	1.372	moderate
A.b4	1.487	moderat	A.b14	1.145	moderate
A.b5	1.551	strong	A.b15	1.26	moderate
A.b6	1.256	moderate	A.b16	1.801	strong
A.b7	1.299	moderate	A.b17	1.229	moderate
A.b8	1.393	moderate	A.b18	1.332	moderate
A.b9	1.269	moderate	A.b19	1.405	moderate
A.b10	0.995	non formation	A.b20	1.559	strong

Modarresi *et al*. ,(2015) stated AHLs formed by various bacteria differ in the length of the side-chain of the R-group, and both the production of AHL and the formation of biofilms were dose-dependent regulation of iron concentration. These findings show that iron limitation plays a significant regulatory role in AHL and siderophore growth, resulting in strong or weak biofilm, thus helping to ensure that the organism persists in less available nutrients climates.

4.7 Detection of the *hcp* gene in clinical *Acinetobacter baumannii* isolates .

The presence of T6SS was genetically evaluted by detecting the *hcp* gene using PCR technique, Considering hemolysin coregulated protein (Hcp) that is one of the external protein of T6SS, Since Hcp is shed into the extracellular milieu by activation of the system, they serve as molecular markers of a functional T6SS as mentioned by Kim *et al*., (2017).

During the first stages of this study, specifically in the stage of collection and diagnosis, some of wound swabs ,when streaked on surfaces of culture media ;appeared pure colony of *A. baumannii* as shown in figure (4-17 A),while the culture of burn sample(isolate Ab4) appeared mixed with fewer E.coli colonies compare with *A. baumannii* . This was a clear indication of the ability of bacteria to compete within the microbial pool, whether in wounds or burns, or other sources, and this increases its ferocity, and this was what has been recorded in previous studies on the importance of this system in virulence (Repizo *et al* .,2015; Wang *et al*.,2018).



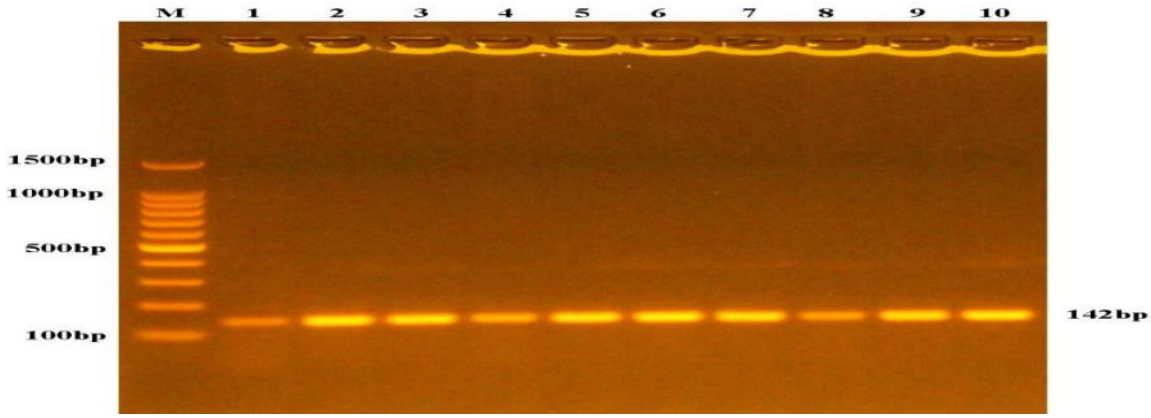
Figure(4-17): The growth of *A. baumannii* on agar

(A- wound sample) (B -burn sample)

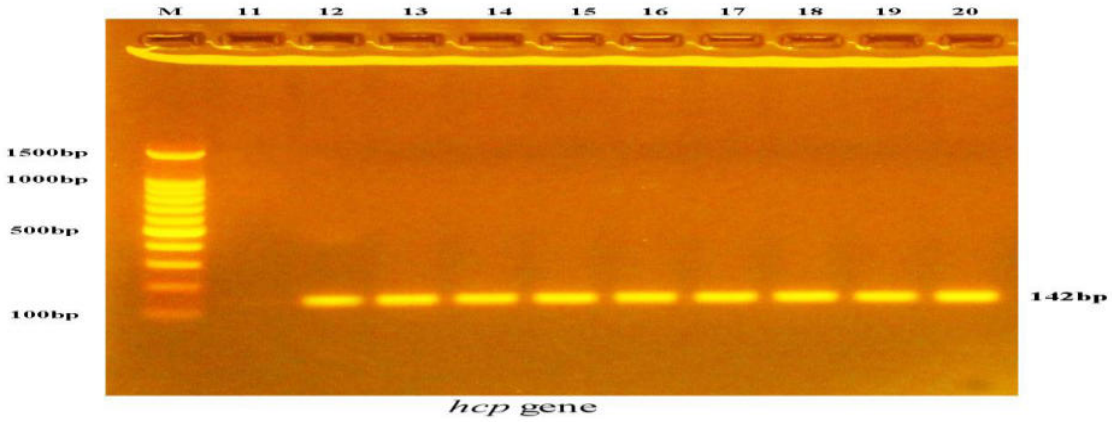
All 20 genomic DNA extracted of isolated understudy subjected for conventional PCR by used specific primer of *hcp* gene .the results showed

according to the agarose electrophoresis, as in figure (4-18 A,B), where it was found the prevalence of the present this gene 95%, it presented in 19 isolates, except the(A.b11) which did not have it. This study for this system, it was the first study in Iraq. The T6SS system is one of the first lines of defense for pathogenic bacteria, especially MDR. Therefore, it was highlighted and its gene expression studied also after treatment with antibiotics, this is what will be explained in the study of gene expression in the next paragraph. The result did not agree with the study on South Korea were mentioned ((Among the 162 *A. baumannii* clinical isolates, 51 isolates(31.5%) contained an *hcp* gene from MDR and XDR isolates)) (Kim et al., 2017).

Di Venanzio**(b)** *et al* .,(2019) recorded Most *A.baumannii* strains harbor a constitutively active type VI secretion system (T6SS) that mediates indiscriminate, the contact-dependent killing of neighboring nonsister bacterial competitors.



Figur (4-18 A): The amplification of *hcp* gene of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel (90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-10 resemble 142bp PCR products.



Figur (4-18 B): The amplification of *hcp gene* of *Acinetobacter baumannii* samples were fractionated on 1.5% agarose gel (90 min at 100 volts) electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 11-20 resemble 142bp PCR products.

4.8 Relationship between MDR of *A.baumannii* and virulent factors

Bacteria are perpetually at war against multiple competitors and thus require weapons to conquer new territory or persist in an ecological niche. Type VI Secretion Systems (T6SSs), Quorum Sensing (QS), and social behavior in biofilms, all these interconnected mechanisms are important for bacterial survival; T6SSs allow bacteria to battle other cells and attack the host target cell, QS is devoted to the perception of bacterial cell density, and biofilm formation is essentially controlled by QS.Table(4-11) show the summary the relationship between MDR isolates and virulent factors.

As shown in the table the (A.b(2,13,16,20) 20% isolates had all virulent factors under study, in addition they were high resistant to antibiotic, but the rest of the isolates 80% were varying in possession virulent factors.

Table(4-11): The summary the relationship between MDR isolates and virulent factors.

no.isols	MDR	Biofilm	Q.S	T6SS	MβLs	AmpC	no.isols	MDR	Biofilm	Q.S	T6SS	MβLs	AmpC
A.b1	MDR	strong	strong	+	+	-	A.b11	MDR	strong	strong	-	+	-
A.b2	MDR	moderate	strong	+	+	+	A.b12	MDR	moderate	no formation	+	+	+
A.b3	XDR	moderate	strong	+	+	-	A.b13	XDR	moderate	moderate	+	+	+
A.b4	MDR	strong	moderat	+	+	-	A.b14	MDR	strong	moderate	+	+	-
A.b5	MDR	moderate	strong	+	+	-	A.b15	MDR	moderate	moderate	+	+	-
A.b6	MDR	moderate	moderate	+	+	-	A.b16	XDR	moderate	strong	+	+	+
A.b7	MDR	moderate	moderate	+	+	-	A.b17	MDR	moderate	moderate	+	-	+
A.b8	MDR	strong	moderate	+	-	-	A.b18	XDR	strong	strong	+	+	-
A.b9	MDR	weak	moderate	+	+	-	A.b19	MDR	weak	no formation	+	+	-
A.b10	MDR	moderate	no formation	+	+	-	A.b20	XDR	moderate	moderate	+	+	+

All the isolates were able to form biofilm formation, but in varying proportions , and 90% of them also produced the quorum. Cepas *et al.,* (2019) mentioned that the Gentamicin and Ceftazidime resistance was related to biofilm formation, this explains the cause of high resistance that was shown by isolates in this study to two of these antibiotic , and Gallique *et al* ., (2017) mentioned Quorum Sensing (QS) is crucial for collective adaptive responses (similar to a social behavior) and regulates both bacterial virulence and biofilm formation and several relationships between the ability to form biofilm and antimicrobial resistance, being different for each species,and the Q.S one of regulater of T6SS according to infection and enviromental.

All these mechanisms of resistance that used the bacteria , which is also a mechanism of virulence; provide the bacteria's first line of defense in order to survive in harsh conditions, thus enhancing their multiple resistance to antibiotics.

4.9 Molecular study of *Acinetobacter baumannii* RNA

4.9.1 Extraction of total RNA

The total genomic RNA extracted from target three isolates from different clinical sources(wound A.b20, burn A.b16, blood A.b13) to measure the $bla_{OXA51}$and *hcp* genes expression level before and after treatment with an antibiotic using of sub-MIC. In highly precise conditions and avoid any contamination especially RNase and the protection came from using TRIzoL (guanidinthiocynate) with the ready kit. The quantity measured by the Quantus Fluorometer device which gave a range of values(20-96.8 ng/µl) as shown in (Appendix6).

4.9.2 Gene Expression Analysis by Using Quantitative Reverse Transcriptase Real-Time qRT PCR Technique (Syber green)

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is distinguished from other methods for gene expression due to the accuracy, sensitivity, and fast results. This technology represents the golden standard for analyzing gene expression. It is important to note that the studies are generally involved in comparing the degree of expression of a particular gene between different samples in a relative quantification analysis (Derveaux *et al*., 2010).

4.9.2.1 Real-time PCR Quantification of *bla*$_{OXA51}$ Gene Expression

The experiment of the quantitative RT-PCR reaction was completed by using three (3) selected out of (20) of *A.baumannii* isolates. The sources of these isolates were (wound, burn, blood). The *bla*$_{OXA51}$ gene gave a high expression of different degrees in these 3 isolates. This variation refers to the source of isolates. The Ct value of *bla*$_{OXA51}$ gene in the present study was shown in Table (4-12) and Appendix (7) that show the pattern of the amplification of the gene and TM temperature of DNA.

Table (4-12): Ct values and fold of gene expression of *bla*$_{OXA51}$ gene of *A.baumannii* that was treated with Imepinime

sours	Samples	CT H.K 16SrRNA	CT *bla*$_{OXA51}$	Δ Ct	Δ Δ Ct	Folding= $2^{-\Delta\Delta ct}$	Average of folding
blood	13C	16.08	22.24	6.16	0.00	1.00	1
burn	16C	14.01	20.94	6.93	0.00	1.00	
wound	20C	15.79	20.14	4.35	0.00	1.00	
After treated with antibiotic							
blood	13	15.61	21.08	5.47	-0.69	1.61	1.45
burn	16	15.66	21.89	6.23	-0.71	1.63	
wound	20	16.50	20.68	4.18	-0.17	1.12	

According to the qRT-PCR results of gene *bla*$_{OXA51}$, it is noticed there is a change in the Ct of the gene after the treated, and this leads to overexpression in *bla*$_{OXA51}$ as formula ($2^{-(\Delta\Delta CT)}$), so that it showed height level of folding with the average of 1.45 after treated with Imepinim at the concentration of128µg/ml. The overexpression of *bla*$_{OXA51}$ showed in all 3 isolates (A.b13,

A.b16, and A.b20), that were with higher MIC of Imepinim (512-1024-226 µg/ml).

An Iraqi study of Al-Haideri *et al*. (2019) to investigate the gene expression of the *bla*$_{OXA51}$ gene of clinical isolates of *Acinetobacter Baumannii* after treatment with Imepinim, they were found that the gene expression did not increase compared to the control, while another American study for Figueiredo *et al.,*(2009), where mention resistance to carbapenems in *Acinetobacter baumannii* increasingly reported and is mostly associated with expression of carbapenemases, this is because the bacteria have genes of β-lactamase class D, which they include *bla*$_{OXA51}$. The study proved that the existence of a novel insertion sequence named (ISAba1) immediately upstream of the promoter of the *bla*$_{OXA-51}$ gene is the reason for overexpression after treated with Imepinim and Meropinem in isolates that possess (ISAba1) compared with isolates that did not have it, which was treated with same antibiotics did not affect to *bla*$_{OXA-51}$ gene expression for them.

The results agree with an Iranian study of Mohammadi *et al*.,(2017) conducted on clinical *Acinetobacter baumannii* isolates, and they were recorded that *bla*$_{OXA-51}$ encoded genes naturally occur in *A. baumannii* isolates, and they have also been identified worldwide and also they were mention to important of an ISAba1 element was detected in all the *A.baumannii* strains. This co-existence has been shown to confer high levels of carbapenem resistance and increase the level of gene expression.

4.9.2.1 Real-time PCR Quantification of *hcp* Gene Expression

The experiment of the quantitative RT-PCR reaction was completed by using three(3) elected out of (20) of *A.baumannii* isolates. The different sources of these isolates were distributed as follows (wound, burn, blood).

The *hcp* gene gave a low expression compared with control in different degrees in these 3 isolates. This variation refers to the source of isolates. The Ct value of *hcp* gene in the present study is shown in Table (4-13) and Appendix (7) that show the pattern of the amplification of the gene and TM temperature of DNA.

Table (4-13) : Ct values and fold of gene expression of *hcp* gene of *A.baumannii* that was treated with Imepinime

sours	Samples	CT H.K 16SrRNA	CT *hcp*	Δ Ct	Δ Δ Ct	Folding= $2^{-\Delta\Delta ct}$	Average folding
blood	13C	16.08	20.45	4.36	0.00	1.00	1
burn	16C	14.01	18.08	4.07	0.00	1.00	
wound	20C	15.79	17.98	2.19	0.00	1.00	
After treated with antibiotic							
blood	13	15.61	20.05	4.44	0.07	0.95	0.88
burn	16	15.66	19.87	4.21	0.14	0.91	
wound	20	16.50	19.05	2.55	0.35	0.78	

The CT range for *hcp* gene before the treatment was (17.98-20.45) while the range of CT after treated was (19.05-20.05), this result according to formula ($2^{-(\Delta\Delta CT)}$) of RT-PCR, refer to low of folding in all three isolates. The highest gene expression after treatment was in blood isolation (0.95), then in burn isolation (0.91), then in wound isolation (0.78). The variation in gene expression follows the bacterial need for this system and is associated with the infection status(Hu *et al* .,2018).

one the results of a study in South Korea for Kim *et al* ., (2017) which were the study investigate the role of the T6SS in *A. baumannii* both in and around the human host, so the study on MDR *A. baumannii* isolates, those were highly resistant to Carbapenemse. The results showed that *A. baumannii* clinical isolates with high *hcp* expression have an active T6SS (T6ss +), whereas isolates with low *hcp* expression have an inactive T6SS (T6SS–) under the same conditions.

The reason behind the difference in gene expression of *hcp* after treatment with an antibiotic, that *A. baumannii* harbors an MDR plasmid that encodes repressors of T6SS. In the absence of antibiotics, this plasmid is lost in a subset of the population and results in T6SS activation. The activation of the T6SS prepares *A. baumannii* for competition and imparts the ability to kill other bacteria that may try to enter the same environment. Upon (re)introduction of antibiotics, plasmid-less *A. baumannii* will die, and the rest of the *A. baumannii* cells will be resistant and ensure the survival of the population, Weber *et al* ., (2016*)* proved when describing T6SS in a different strain of *A. baumannii* .

4.10 Sequencing and phylogeny analysis of *A. baumannii* (A.b13)

4.10.1 Sequencing and phylogeny analysis of 16S rRNA

The sequencing reactions indicated the exact positions after performing NCBI blast. This engine showed about 99% sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine indicated the presence of remarkable homology with the expected target that covered a portion of the 16S rRNA within the *Acinetobacter baumannii* sequences. This genetic fragment was the primary target for the detection of this bacterium as

it was characterized by highly conserved sequences for this organism. By comparing the observed DNA sequences of this local sample with the retrieved DNA sequences (GenBank acc. HQ631951.1), the exact positions and other details of the retrieved PCR fragment were identified (Figure-19).

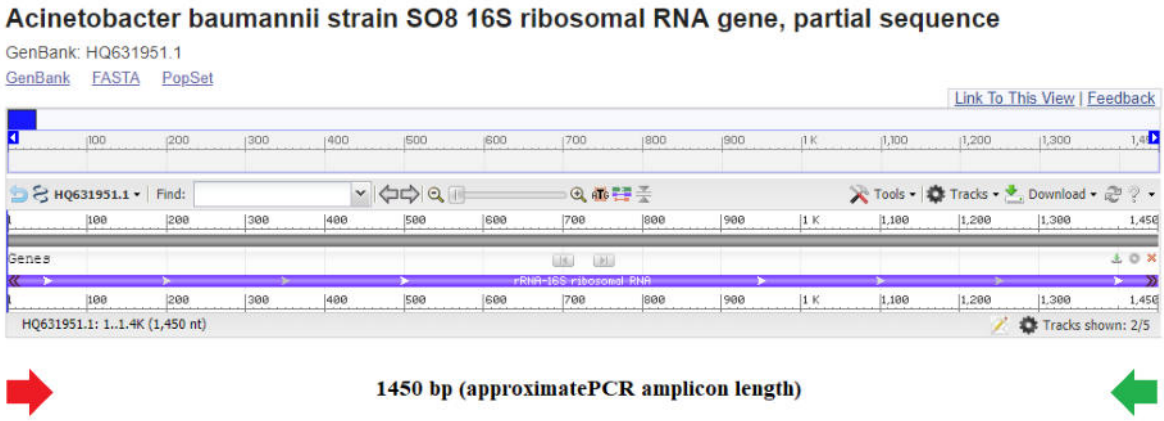


Figure (4-19): The exact position of the retrieved 1450 bp amplicon that covered a portion of 16S rRNA locus within the *Acinetobacter baumannii* genomic sequences (acc. no. HQ631951.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 1450 bp amplicons' sequences within the 16S rRNA of *Acinetobacter baumannii* sequences, the details of these sequences were highlighted, starting from the position of the forward primer to the position of the reverse primer within the same sequences (Table 4-14).

Table 4-14. The position and length of the 1450 bp PCR amplicons were used to amplify a portion of the 16S rRNA within the *Acinetobacter baumannii* genomic sequences. The amplified sequences were extended from 1 to 1450 nucleotides within the NCBI reference DNA sequence (GenBank acc. no. HQ631951.1).

Amplicon	Reference locus sequences (5′ - 3′)	length
16SrRNA sequences	GAATCGGAGGGGCCGGGCTTACACATGCAGTCGAGCGGGGGAAGGTAGCTTGCT ACCGGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGG GGGACAACATCTCGAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCA GGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGG TGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCC GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA ATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG CCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTCTAGTTAATACCTA GGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC GCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGC GTAGGCGGCTTATTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCA TTCGATACTGGTGAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCG GTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCC TAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTG GTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGT GGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAA ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG ATGCAACGCGAAGAACCTTACCTGGCCTTGACATACTAGAAACTTTCCAGAGATG GATTGGTGCCTTCGGGAATCTAGATACAGGTGCTGCATGGCTGTCGTCAGCTCGT GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGC CAGCATTTCGGATGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGC GGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACA ATGGTCGGTACAAAGGGTTGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCG ATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGT AATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC CGTCACACCATGGGAGTTTGTTGCACCAGAAGTAGCTAGCCTAACTGCAAAGAGG GCGGTACCTGTAGATTTTCAGAAGGCCG	1450 bp

The alignment results of the 1450 bp samples revealed the detection of five mutations in comparison with the referring sequences of the GenBank acc. HQ631951.1 (4-20). These five mutations were represented by four nucleic

acid substitutions and one insertion mutation detected in the investigated sample.

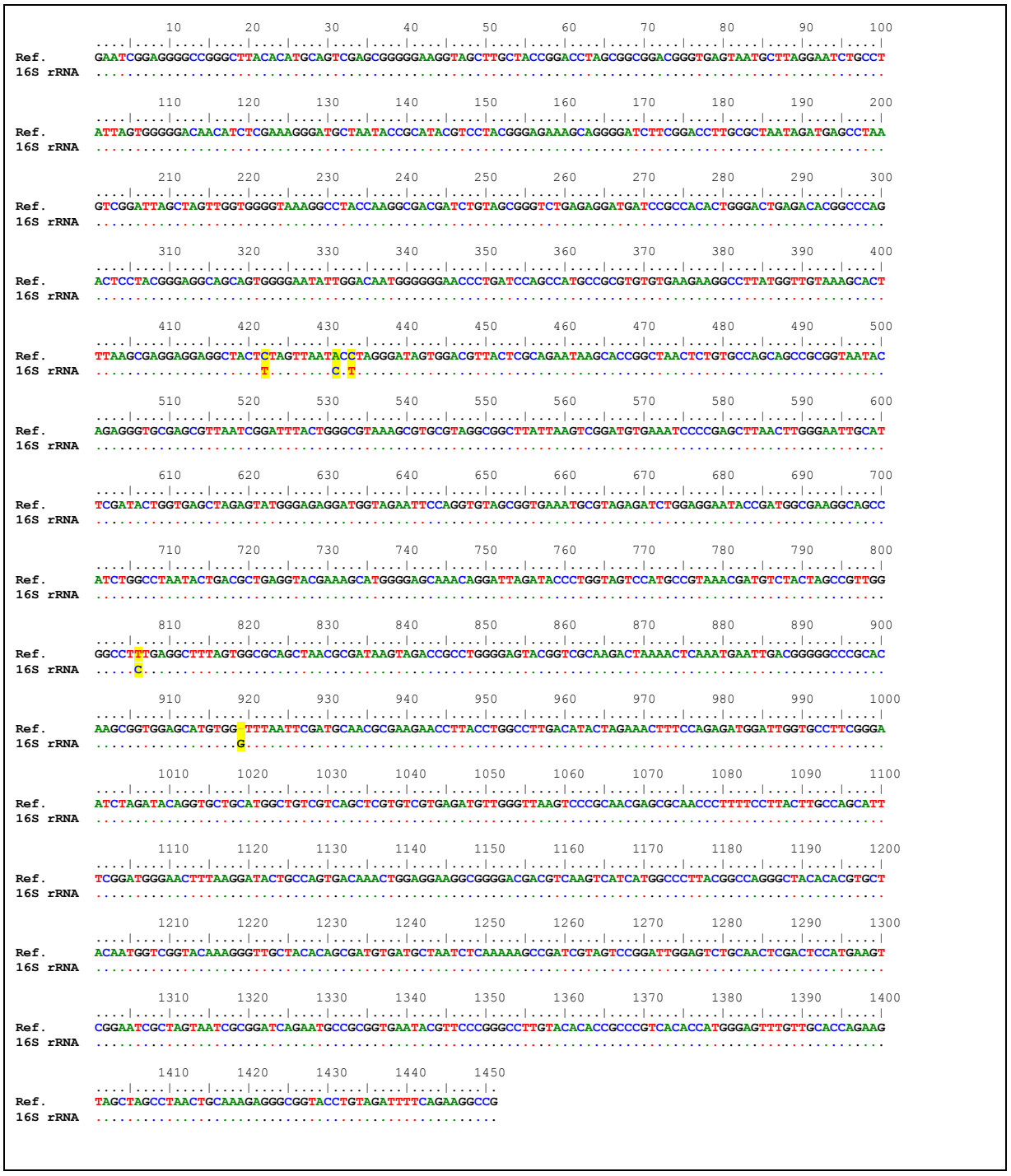


Fig. (4-20). DNA sequences alignment of one local sample with their corresponding reference sequences of the 16S rRNA within the *Acinetobacter baumannii* genomic sequences. The symbol "ref" refers to the NCBI reference sequences, while "16S rRNA" refers to sample code.

The sequencing chromatogram of DNA sequences as well as its detailed annotations was documented and the pattern of these variants within the amplified sequences was shown (Fig. 4-21).

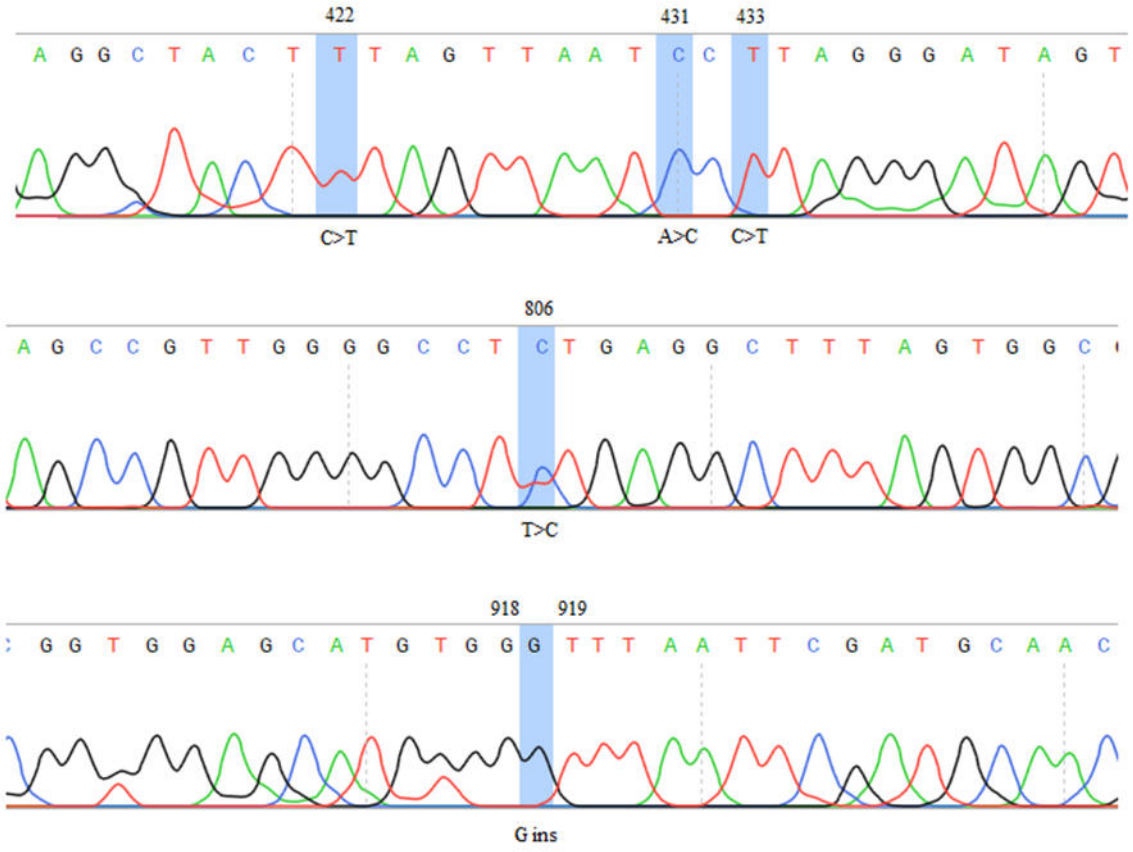


Figure4-21. The chromatogram profile of the observed genetic variants of the 16S rRNA within the *Acinetobacter baumannii* local isolate. Each substitution mutation is highlighted according to their position in the PCR amplicon. The symbol ">" refers to "substitution" mutation, while the symbol "ins" refers to "insertion mutation".

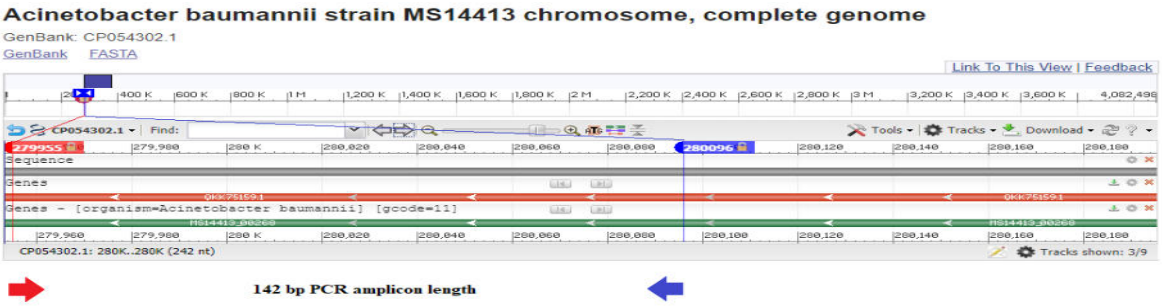
To summarize all the results obtained from the sequenced 1450 bp fragments, the exact positions of the observed mutations were described in (Table 4-15).

Table 4- 15 The pattern of the observed mutation in the 1450 bp of the 16S rRNA amplicon in comparison with the NCBI referring sequences (GenBank acc. no. HQ631951.1).

Native	Allele	Position in the PCR fragment	Position in the reference genome	Type of mutation	Variant summary
C	T	422	422	Nucleic acid substitution	HQ631951.1;g.422C>T
A	C	431	431	Nucleic acid substitution	HQ631951.1;g.431A>C
C	T	433	433	Nucleic acid substitution	HQ631951.1;g.433C>T
T	C	806	806	Nucleic acid substitution	HQ631951.1;g.806T>C
-	G	918-919	918-919	Nucleic acid insertion	HQ631951.1;g. 918-919G ins

4.10.2 Sequencing of The *hcp* gene of *Acinetobacter baumannii*

The sequencing reactions indicated the exact positions after performing NCBI blastn for this PCR amplicon. NCBI BLASTn engine shown about 99% sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine indicated the presence of remarkable homology with the expected target that covered a portion of the *hcp* gene within the genomic DNA sequences of the *Acinetobacter baumannii*. By comparing the observed DNA sequences of this local sample with the retrieved DNA sequences (GenBank acc. CP054302.1), the exact positions and other details of the retrieved PCR fragment were identified (Figure. 4-22).



Figure(4 -22). The exact position of the retrieved 142 bp amplicon covered a portion of the *hcp* region within the *Acinetobacter baumannii* sequences (acc. no. CP054302.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 142 bp amplicons' sequences within the *hcp* region of the *Acinetobacter baumannii* sequences, the details of these sequences were highlighted, starting from the position of the forward primer to the position of the reverse primer within these sequences (Table 4-16).

Table(4 -16). The position and length of the 142 bp PCR amplicons used to amplify a portion of the *hcp* region within the *Acinetobacter baumannii* genomic sequences. The amplified sequences were extended from 279955 to 280096 of the NCBI reference DNA sequence (GenBank acc. no. CP054302.1). The grey color refers to both forward and reverse primers sequences.

Amplicon	Reference locus sequences (5′ - 3′)	length
hcp gene sequences	AACGTGTTGAACATTCTGACATGGTTTTCGTGAAAGACTTAGACGCA ACTAGCCCTAAATTATGGGAAGCTTGTTCAGCTGGTTATACATTTGA TGAAGTACAAATCGACTTCTATCG**C**GCAAATGGCGATAAACGTATCA A	142 bp

The alignment results of the 142 bp bacterial sample revealed the presence of only one mutation in comparison with the referring core region of the

bacterial genetic sequences (Figure. 4-22). This mutation was represented by one nucleic acid substitution detected in the investigated *hcp* sample.

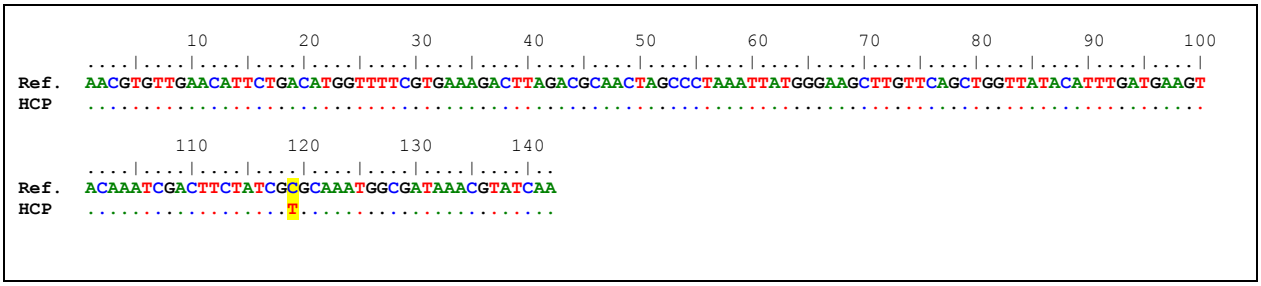


Figure (4-23). DNA sequences alignment of one local sample with their corresponding reference sequences of the *hcp* locus within the *Acinetobacter baumannii* genomic sequences. The symbol "ref" refers to the NCBI reference sequences, while "*hcp*" refers to sample number.

The sequencing chromatogram of DNA sequences as well as its detailed annotations were documented and the pattern of this variant within the amplified sequences was shown (Figure. 4-24). At the position 119th of the PCR amplicon, one nucleic acid substitutions, C>T 119, was observed.

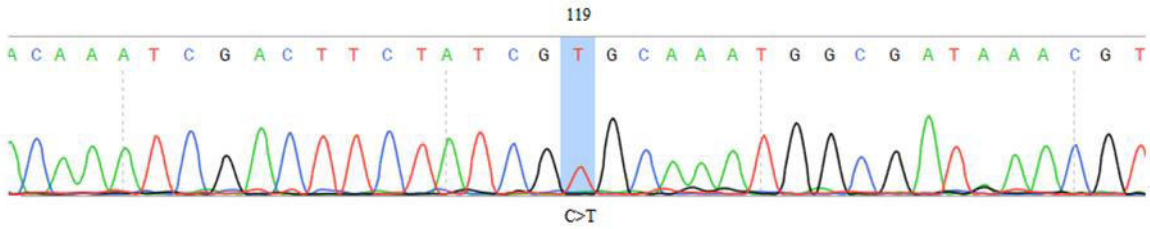


Figure (4- 24) The chromatogram profile of the observed genetic variant of the *hcp* gene within the *Acinetobacter baumannii* local isolates. The detected substitution mutation is highlighted according to its position in the PCR amplicon. The symbol ">" refers to "substitution" mutation.

The observed mutation was further analyzed to identify whether such nucleic acid substitution induces a possible alteration in its corresponding position in the encoded amino acids from the *hcp* region. All nucleic acid sequences were translated to their corresponding amino acid sequences using the Expasy translate suite. It was found that the detected nucleic acid substitution was

found in the amino acid Arg in the position 39th within the amplified *hcp* locus. No amino acid substitution was observed and this mutation has a silent effect on the resulting protein (Figure 4-25).

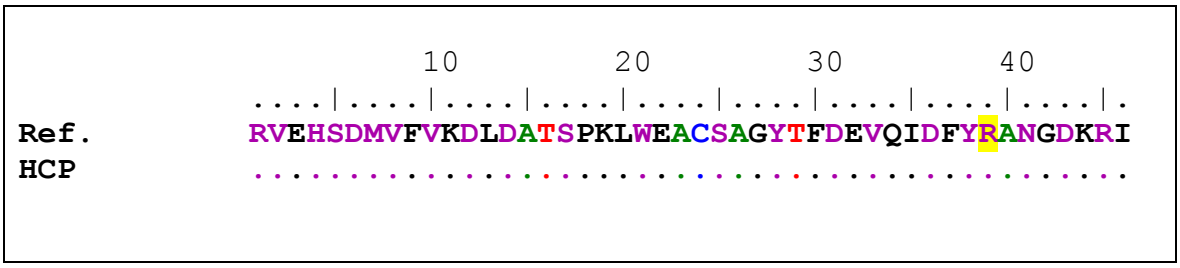


Figure (4- 25) Amino acid residues alignment of the detected variations within the investigated sample of *Acinetobacter baumannii* sequences. The detected silent mutations were localized according to their positions within the encoded amino acid sequences of the *hcp* amplicon. The yellow highlighted colors refer to the detected amino acid at which the nucleic acid substitution was detected.

To summarize all the results obtained from the sequenced 142 bp fragments, the exact position of the observed mutation was described in (Table 4-17).

Table (4-17). The pattern of the observed mutation in the 142 bp of the *hcp* amplicon in comparison with the NCBI referring sequences (GenBank acc. no. CP054302.1).

Native	Allele	Position in the PCR fragment	Position in the reference genome	Variant type	Variant summary
C	T	119	279978	Nucleic acid substitution (Silent variant)	CP054302.1;g.279978C>T

A comprehensive phylogenetic tree was generated in the present study, which was based on the observed nucleic acid variation detected in the investigated sample. This phylogenetic tree was contained this *hcp* sample, alongside with other relative reference *hcp* sequences. A total number of the aligned nucleic acid sequences in this comprehensive tree was 95. This generated comprehensive tree indicated the presence of only one type of organism, *Acinetobacter baumannii*, which represents the majority of the incorporated

organisms within the tree. In addition to *Acinetobacter baumannii* sequences, other sequences of *Acinetobacter pittii* and *Acinetobacter indicus* were also incorporated as out-groups.

The currently generated comprehensive tree showed extremely close genetic distances among all the incorporated organisms. These close genetic distances were attributed to a small genetic scale (0.1) inferred from these sequences. Based on the *hcp* gene sequences, the currently investigated sample was clustered within the same clade of the deposited *Acinetobacter baumannii* sequences (Figure 4-26). Though on nucleic acid substitutions were observed in this locus, CP054302.1;g.279978C>T, there was no deviation from *Acinetobacter baumannii* as this detected mutation was only minor variations within the bacterial sequences of this species. Within the incorporated *hcp* sequences, it was found that the currently investigated (*hcp*) sample was clustered beside the GenBank acc. no. CP050388.1, which was belonged to the Indian isolate of *Acinetobacter baumannii* sequences of the strain VB473. For this reason, it was clearly shown that the investigated sample sequences may exhibit high similarity to the Indian origins of the same species.

This *hcp* – based observation indicated another distinct role of the generated phylogenetic tree in the accurate detection and origination of the *hcp* sample. This comprehensive tree has also provided an extremely inclusive tool about the high ability of such genetic sequences to efficiently identify the *hcp* sample using this genetic fragment. This, in turn, gives an additional indication of the power of the currently utilized *hcp*-specific primers to identify the currently investigated isolate of *A. baumannii*.

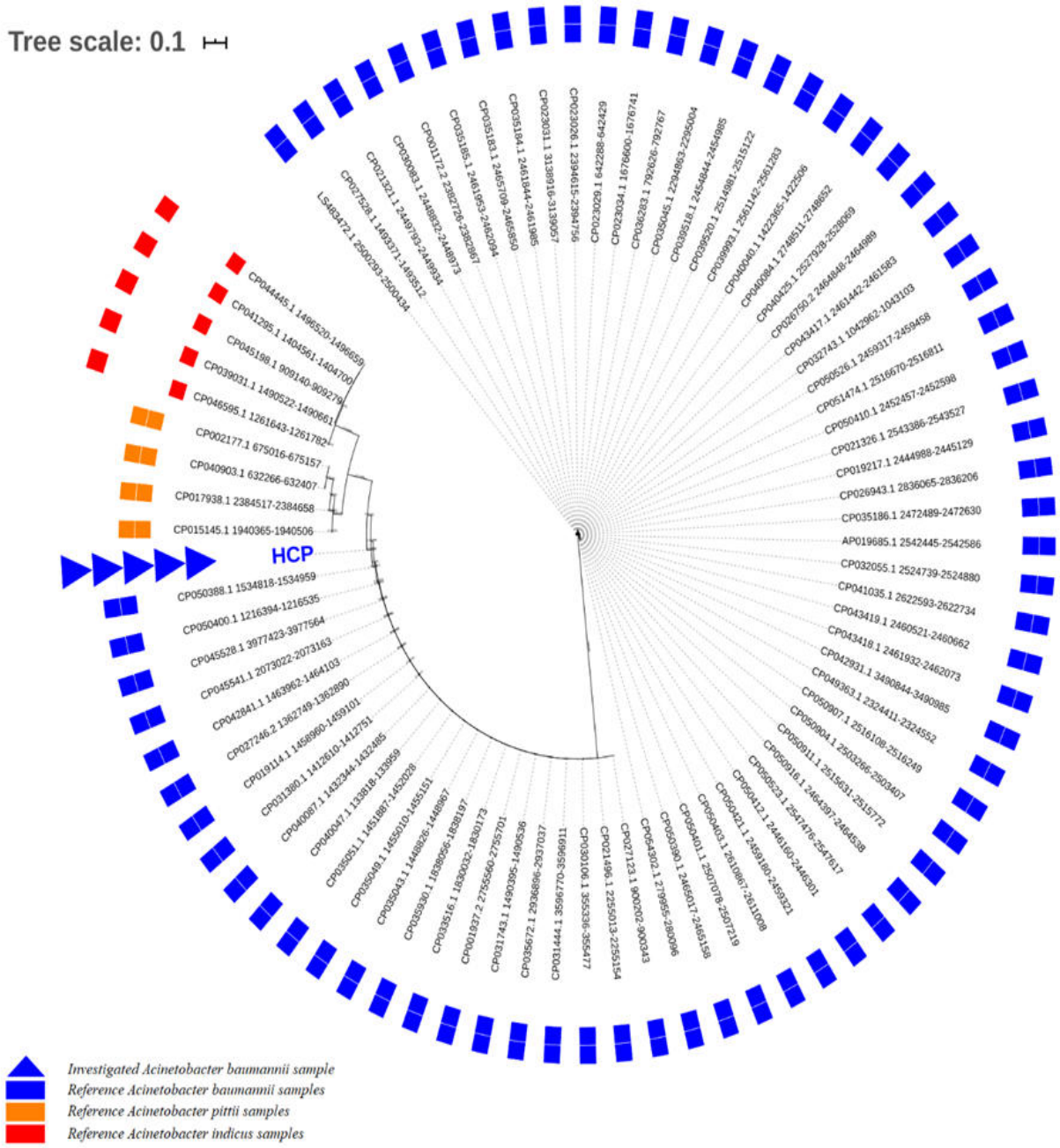


Figure. 4-26 The comprehensive phylogenetic tree of genetic variants of the *hcp* gene within the *Acinetobacter baumannii* local isolate. The dark blue color refers to the analyzed variants, while the orange and red color refers to the related referring Genbank acc. no NCBI deposited sequences of the *Acinetobacter pitti* and *Acinetobacter indicus*, respectively. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The symbols *hcp* refers to the code of the investigated sample.

4.10.3 Recording Iraqi *Acinetobacter baumannii* isolate in gene bank-NCBI

Qne strains of *A. baumannii* was isolated from human source in Baqubacity /Diyala and the sequences has a symbol code (SHRRWY.80). 16s rRNA and *hcp* gene sequences had been submitted to Gen Bank, the results of these sequences were analyzed and examined by professional staff in gene bank. This strain was published in the national center for biotechnology information (NCBI) and the database of the strain was recorded in the DNA Data Bank of Japan (DDBJ) and Gene Bank for DNA sequences with accession (MT551041),and variation (MT551041.1)for 16s rRNA , and with accession (LC553000)and variation (LC553000.1) for *hcp* gen . The sequence accepted in gene bank .The (Appendix 8) showed the report of it.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

According to the results of the present study, the following conclusions could be exposed :

- *Acinetobacter baumannii* had demonstrated widespread resistance to many modern antibiotics.
- *Acinetobacter baumannii* isolates were possessed a high percentage of MBLs and chromosomal AmpC enzymes.
- Microtiter broth standard method to identification MIC of antibiotic is a high-quality method because it reduces the time and cost of materials in addition to its accurate readings by ELISA reader.
- One of the reasons for the high resistance of *A. baumannii* to Carbapenems is that they have bla_{VIM} and bla_{OXA51} genes that encode the production Carbapenemase enzyme.
- Doxycycline is the best drug choice for *A.baumannii* isolates according to the susceptibility test.
- *Acinetobacter baumannii* isolates have many virulent factors such as biofilm, quorum sensing, which they considered as a resistance agents against many antibiotics.
- When the bacteria were treated at a concentration sub their MIC, the gene expression of the resistance bla_{OXA51}gene increased, but the gene expression of *hcp*(T6SS) gene decreased.
- One strains of A. baumannii was isolated from human source in Baqubacity /Diyala and the sequences has a symbol code (SHRRWY.80). 16s rRNA and hcp gene sequences had been submitted to Gen Bank.

Recommendations

- Molecular Study of local *A. baumannii* isolates that resistance to polymyxins and determine the mutations that occurred, utilizing sequencing technology, to compare them with international isolates.
- Studying the gene expression of virulence factors for *A.baumannii* by RT-PCR after treatment with nanoparticle materials.
- Molecular detection of the presence ISAb1 insertion sequence element in MDR isolates is necessary.
- A molecular and descriptive study of T6SS system and detection multidrug-resistant plasmids repress chromosomally encoded T6SS to enable their dissemination.

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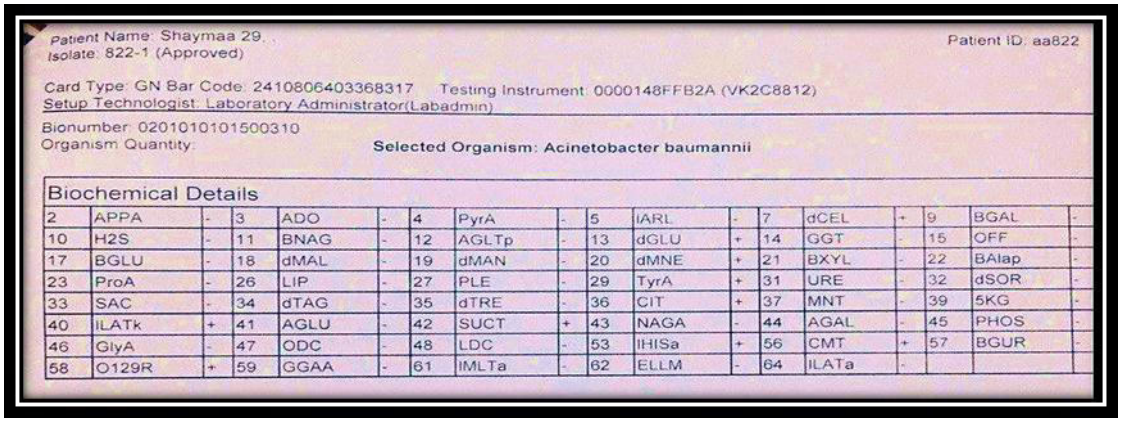
APPENDIXES

Appendix (1)

Results of biochemical tests

Appendixe(2)



Patient Name: Shaymaa 29
Isolate: 822-1 (Approved)
Patient ID: aa822

Card Type: GN Bar Code: 2410806403368317 Testing Instrument: 0000148FFB2A (VK2C8812)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0201010101500310
Organism Quantity:
Selected Organism: Acinetobacter baumannii

Biochemical Details

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

Results of the biochemical test to *Acinetobacter baumannii* by using VITEK-2 system .

Appendixe(3)

DNA Concentration (ng/µl)

Sample	Conc.	Sample	Conc.
1	15	11	13
2	13	12	18
3	23	13	20
4	14	14	26
5	15	15	24
6	21	16	24
7	22	17	22
8	21	18	15
9	20	19	15
10	17	20	19

Appendixe(4)

Source of infection and result of susceptibility test of different antibiotics(R= Resistant, I=Intermediate, S= Sensitive).

No. of Isolate	source of infection	Antibiotic resistant patterns												
		AMS	PRL	TCC	CAZ	CTX	CRO	IMP	MEM	GM	AK	DO	TE	LEV
A.b1	Wound	R	R	R	R	R	R	R	R	R	R	I	R	R
A.b2	Burn	R	R	R	R	R	R	R	R	R	R	S	I	R
A.b3	Burn	R	R	R	R	R	R	R	R	R	R	S	R	R
A.b4	Burn	S	R	R	R	R	R	R	R	R	R	S	I	R
A.b5	Blood	I	R	R	R	R	R	R	R	R	R	S	R	R
A.b6	Blood	R	R	R	R	R	R	R	R	R	R	S	I	R
A.b7	Burn	S	R	R	R	R	R	R	R	R	R	S	R	R
A.b8	Burn	R	R	R	R	R	R	I	I	R	R	R	R	R
A.b9	Blood	I	R	R	S	I	I	R	R	R	R	S	R	R
A.b10	Wound	I	R	R	R	R	R	R	R	R	R	S	I	R
A.b11	Wound	S	R	R	R	R	R	R	R	R	R	S	R	R
A.b12	Burn	I	R	R	R	R	R	R	R	R	R	S	R	R
A.b13	Blood	R	R	R	R	R	R	R	R	R	R	R	R	R
A.b14	Blood	R	R	R	R	R	R	R	R	R	R	S	I	R
A.b15	Burn	R	R	R	R	R	R	R	R	R	R	S	I	R
A.b16	Burn	R	R	R	R	R	R	R	R	R	R	S	R	R
A.b17	Septum	R	R	R	R	R	R	R	R	R	R	S	S	R
A.b18	Blood	R	R	R	R	R	R	R	R	R	R	R	R	R
A.b19	Blood	I	R	R	S	R	S	R	R	R	R	S	R	R
A.b20	Wound	R	R	R	R	R	R	R	R	R	R	R	R	R

Appendix (5)

ELISA reader reads for MIC results

Isolates	OD OF ELISA reader					
	Imipenem		Meropenem		controls	
	MIC well	After MIC well	MIC well	After MIC well	Con.+ve	Con.-ve
A.b1	0.073	0.569	0.044	0.662	0.688	0.051
A.b2	0.066	0.606	0.036	0.278	0.546	0.056
A.b3	0.071	0.819	0.041	0.152	0.677	0.050
A.b4	0.061	0.140	0.040	0.494	0.545	0.050
A.b5	0.041	0.153	0.049	0.117	0.573	0.057
A.b6	0.060	0.097	0.042	0.160	0.664	0.043
A.b7	0.74	0.110	0.045	0.236	0.481	0.055
A.b8	0.074	0.361	0.038	0.309	0.451	0.058
A.b9	0.053	0.397	0.037	0.235	0.499	0.047
A.b10	0.081	0.196	0.054 all wells no growth		0.107	0.053
A.b11	0.053	0.121	0.035	0.130	0.701	0.046
A.b12	0.054	0.108	0.038	0.565	0738	0.045
A.b13	0.076	0.486	0.049	0.360	0.405	0.038
A.b14	0.062	0.252	0.039	0.95	0.775	0.042
A.b15	0.052	0.401	0.055	0.100	0.441	0.034
A.b16	0.083	0.461	0.044	0.479	0.701	0.039
A.b17	0.035	0.766	0.034	0.174	0.539	0.055
A.b18	0.075	0.202	0.034	0.90	0.617	0.045
A.b19	0.050	0.818	0.034	0.749	0.816	0.050
A.b20	0.190 growth on all wells		0.037	0.442	0.615	0.056

Appendixe(6)

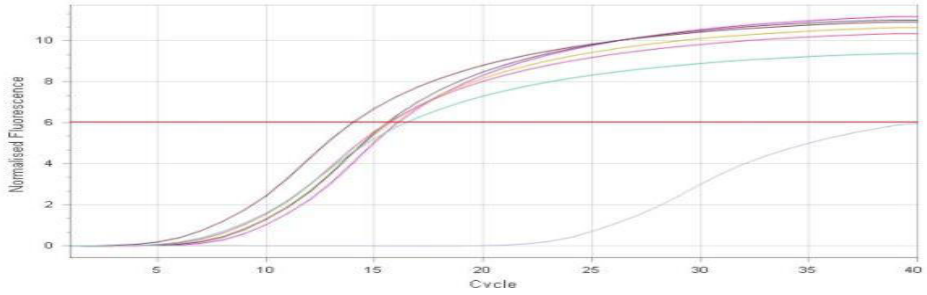
RNA Concentration (ng/µl)

Sample	Conc.
13C	39.4
16C	81.1
20C	85.8
13	20
16	96.8
20	32.4

Appendixe(7)

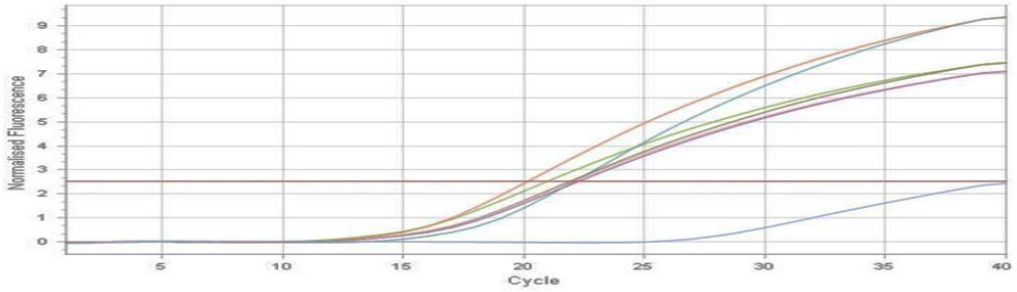
Cycling: 16SrRNA

Target	16SrRNA → 16SrRNA
Normalisation	Dynamic
Exclusion	Extensive with fluorescence cutoff of 5%
Threshold	6.030 starting at cycle 1

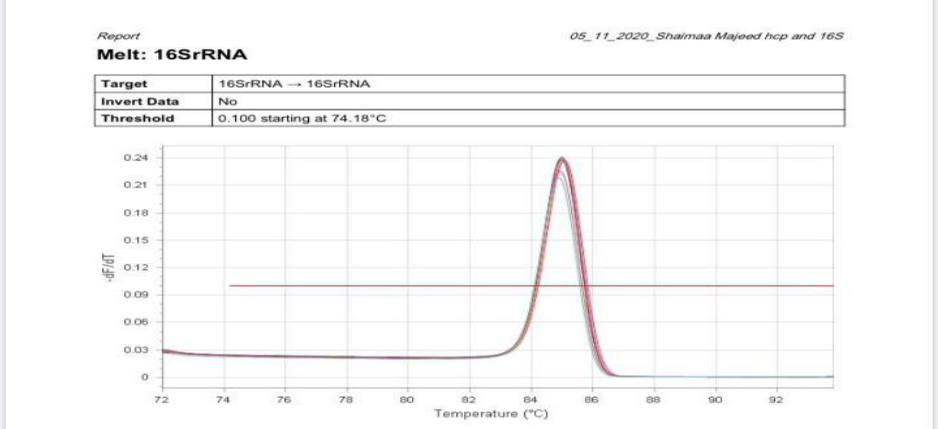


The pattern of the amplification of 16SrRNA

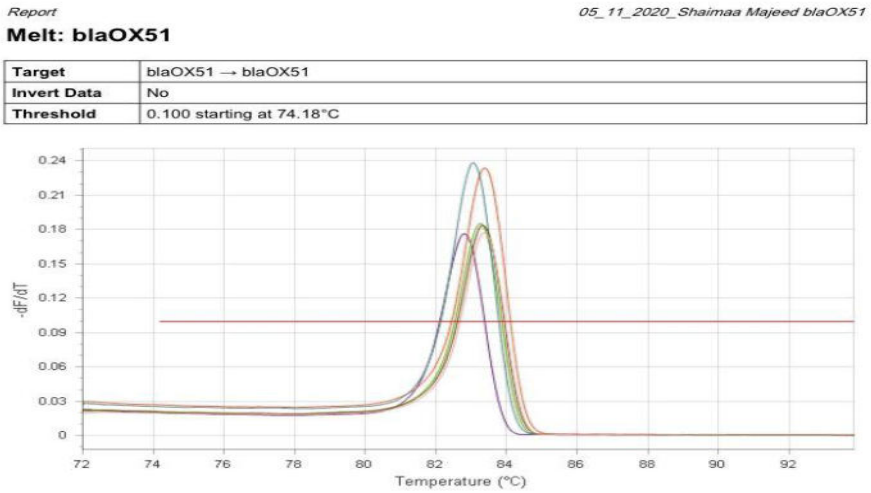
Target	blaOX51 → blaOX51
Normalisation	Dynamic
Exclusion	Extensive with fluorescence cutoff of 5%
Threshold	2.518 starting at cycle 1



The pattern of the amplification of *bla*$_{OXA51}$

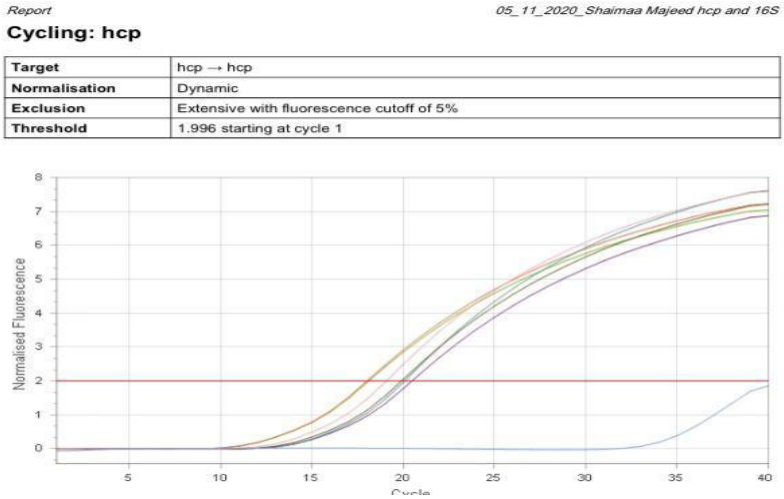


The melting temperature pattern of the amplification of 16SrRNA

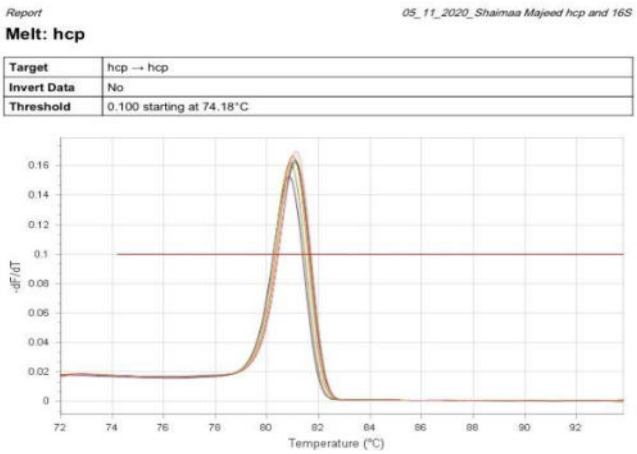


The melting temperature pattern of the amplification of *bla*$_{\mathbf{OXA51}}$

Appendix (8)



- **The pattern of the amplification of *hcp* gene**



The melting temperature pattern of the amplification of *hcp* gene

Appendix(9)

Acinetobacter baumannii strain SHRRWY.80 16S ribosomal RNA gene, partial sequence

GenBank: MT551041.1
FASTA Graphics

Go to:

```
LOCUS       MT551041                1402 bp    DNA     linear   BCT 08-JUN-2020
DEFINITION  Acinetobacter baumannii strain SHRRWY.80 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   MT551041
VERSION     MT551041.1
KEYWORDS    .
SOURCE      Acinetobacter baumannii
  ORGANISM  Acinetobacter baumannii
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter; Acinetobacter calcoaceticus/baumannii
            complex.
REFERENCE   1  (bases 1 to 1402)
  AUTHORS   AL-Dahlaki,S.M., AL-Taai,H.R. and AL-Qertani,Y.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2020) Biology Department, Diyala University,
            College of Science, Diyala, Diyala, Iraq 32001, Iraq
COMMENT     Sequences were screened for chimeras by the submitter using
            geneious 11.

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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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```

Report of Acinitobacter baumannii strain (SHRRWY.80) from GenBank

Appendix 10: Patients information form

Sample NO.:

Patient name:

Patient age:

Gender:

Address:

Sample type:

Isolation place:

Collection date:

الخلاصة

الراكدة البومانية واحدة من مسببات الأمراض الانتهازية ذات المقاومة المتعددة للأدوية (MDR) ، ويرجع ذلك خصوصا إلى قدرتها العالية على اكتساب المقاومة لمجاميع المضادات الحيوية المتنوعة. تم الحصول على عشرين عزلة (9.7%) من *Acinetobacter baumannii* من (207) عينة سريرية. تضمنت جروح ، حروق ، بلغم ، الدم من الإصابات من كلا الجنسين على حد سواء ، وأعمار مختلفة ، ومناطق محلية متنوعة ، وقد أعطت (162)عينة نمو إيجابي بينما (45) عينة لم يظهر فيها نمو. تم جمع العينات خلال فترة الزمنية من سبتمبر / 2019 وانتهى في نهاية يناير / 2020، من مجموعة من المراجعين والمرضى الراقدين في المستشفيات الحكومية في بعقوبة / ديالى. حيث بلغت نسبة المراجعين (57.97%) بينما بلغت نسبة المرضى الراقدين (42.03%).

التشخيص الأولي لـ *A. baumannii* تم باستخدام الاوساط الزرعية اكار الدم والماكونكي ، واعتمادًا على الميزات على الأوساط الزرعية والاختبارات البيوكيميائية ونظام VITEK 2.وقد تم التأكيد النهائي باستخدام طريقة التشخيص الجزيئي باستخدام تقنية تفاعل البلمرة المتسلسل بواسطة جين bla_{OXA51} للكشف عن نوع A. baumannii. كان العدد الموجب لعزلات *A. baumannii* مرتفعًا في الحروق 8 (40%) ، ثم الدم 7 (35%) ، الجروح 4 (20%) والاخيرة كانت البلغم 1 (5%) عزلة.

تم تحديد الحساسية لمضادات الميكروبات لعزلات *A. baumannii* بطريقة الانتشار القرصي ،لـ 13 مضاد حيوي ، وكانت نتائج مقاومة العزلات كما يلي: (Piperacillin-tazobactam 100%)،(Cefotaxime 100%)، (Ticarcillin-clavulanic acid 100%) ،(أميكاسين 100%) ، (جنتاميسين 100%)،(ليفوفلوكساسين 100%) ،(إيميبينيم 95%)، (ميروبينيم 95%) ، (سيفتريكسون 90%) ،(سيفتازيديم 90%) ،(تتراسيكلين 65%) ،(أمبيسلين - سولباكتام 55%) ، (دوكسيسيكلين 20%).

تمايزت العشرين عزلة من *A. baumannii* قيد الدراسة لنمطين حسب مقاومة المضادات الحيوية في اختبار الحساسية ، جميع العزلات كانت متعددة المقاومة ، 15 (75%) عزلة MDR قاومت (5-9) مضاد حيوي ، 2 (10%) من العزلات XDR قاومت (12) مضاد حيوي ، وثلاث عزلات (15%) من عزلات XDR قاومت كل المضادات الحيوية التي تم استخدامها في الدراسة.

تم تحديد MIC (التركيز المثبط الأدنى) لجميع العزلات العشرين لمضادين حيوين (Imipenem، Meropenem). وباستخدام الطريقة المعيارية بواسطة لوحة العيار الدقيقة عن طريق التخفيف التسلسلي لـ Müeller Hinton Broth .أظهرت النتائج أن قيم MIC للـ Imipenem تراوحت من (8-≤1024) ميكروغرام / مل و قيم MIC لـ Meropenem تراوحت (≤4-128) ميكروغرام / مل.

اجري فحص الكشف عن اصناف لـβ-Lactmases وهي (ESBLs، AmpC، MBLs). اضهرت النتائج للكشف عن ESBLs أن جميع العزلات ذات مقاومة عالية لـ (100%) للسيفوتاكسيم والسيفتازيديم . كما أظهرت العزلات مقاومة عالية للبيبراسيلين (PRL) وأوجمنتين (AMC) (100%)) ولكن لم يكن هناك أي تآزر بين أقراص المضادات الحيوية .

تم استخدام اختبار مقاومة سيفوكسيتين للكشف عن إنتاج AmpC ، وكانت النتائج الموجبة لـ 6عزلات (30%) قد انتجت الانزيم ، ثلاثة عزلات كانت من الحروق)، عزلة واحدة من الدم، وعزلة من البلغم، عزلة من الجروح.

تم الكشف عن النمط الظاهري لـ MBLs وكانت النتائج كانت18 (90%) منتجة لإنزيم MBLs بينما 2 (10%) عزلة سلبية .وكانت نتائج الكشف الجزيئي بواسطة تقنية الـ PCR التقليدية أن 14 عزلة (70%) من *A. baumannii* كانت تمتلك جين bla_{VIM} و 6 (30%) عزلات لا تمتلكها ، وجميع العزلات العشرين لا تمتلك جينات bla_{NDM-1}، bla_{IMP}.

تم إجراء عملية الكشف عن عوامل الضراوة من أجل تحديد مدى امراضية *A. baumannii*.حيث تم الكشف عن تكوين الغشاء الحيوي بطريقتين ، الفحص النوعي بطريقة الأنبوب ، وهذا أول مؤشر على قدرة البكتيريا على تكوين غشاء حيوي ، وأظهرت النتائج أن جميع العزلات كونت حلقة أرجوانية بعد تصبيغها بصبغة الكرستال البنفسجية ، و طريقة المعايرة الكمية بواسطة لوحة العيار الدقيقة ، كانت النتائج 7 (35%)من العزلات كونت غشاءً حيويًا قويًا بينما 12 (60%) كونت غشاء حيوي معتدل و 1 (5%) كونت غشاء حيوي ضعيف.

تم استخدام طريقة القياس اللوني للكشف عن استشعار النصاب الحسي. كانت العزلات منتجة لإشارات النصاب الحسي بنسب مختلفة ، 7 (35%)عزلات من *A. baumannii* كانت عالية الإنتاج لجزيئات (Acyl-Homoserine-Lactones) ، بينما 11 (55%) عزلة كانت معتدلة الانتاج ، و 2 (10%) عزلة لم يكن لها نشاط تكوين AHL.

تم استخدام تقنية تفاعل البوليميراز المتسلسل (PCR) التقليدية للكشف عن جين *hcp* الذي يعد علامة جزيئية وظيفية لنظام الـ T6SS ، وأظهرت النتائج أن هذا الجين تواجد في 19(95%) عزلة ، ولكن عزلة واحدة لم تمتلك الجين .

تم استخدام تقنية qRT PCR في الوقت الحقيقي باستخدام (Syber green) لدراسة التعبير الجيني بعد المعاملة بتركيز (128) ميكروغرام / مل من (Imipenem) لجينين ،جين bla_{OXA51} احد جينات مقاومة Carbapenems وجين الـ *hcp* (T6SS) . أعطى الجين bla_{OXA51} تعبيرًا عاليًا بدرجات مختلفة في 3 عزلات من ثلاثة مصادر (جروح ، حروق ، دم) ،حيث كان مستوى التعبير الجيني بمتوسط (1.45) أكثر من التعبيرالجيني لعزلات السيطرة ، لكن جين *hcp* أعطى مستوى تعبير جيني منخفض مقارنة مع التعبير الجيني لعزلات السيطرة بدرجات مختلفة حيث كان متوسط مستوى التعبير الجيني0.88.

تم إجراء التسلسل لجيني (16S rRNA) وجين *hcp* ضمن العزلة (13 *A.baumannii*). وقد بينت نتائج الدراسة إلى أن الجزء 16S rRNA المُضخم أظهر خمسة اختلافات في الأحماض النووية ، g.422C> T، g.431A> C، g.433C> T، g.806T> C ، وطفرة ادخال g.918-919G مقارنة مع متواليات الريبوسوم. فيما يتعلق بجين *hcp* ،بينت النتائج أن موضع *hcp* المتضخم أظهر طفرة صامتة واحدة (g.279978C> T) مقارنة بتسلسلات المنطقة المرجعية. أشارت شجرة النشوء والتطور التي تم إنشاؤها على أساس *hcp* إلى أن التسلسلات التي تم فحصها تم وضعها بدقة في تسلسل *A.baumannii*. إن استخدام شجرة شاملة لتحديد المواقع التطورية للتسلسلات التي تم فحصها حاليًا باستخدام جين *hcp* قد أعطى هوية مؤكدة فيما يتعلق بالعلاقات التطورية للعينة التي تم فحصها بالإضافة إلى تحديد الموقع الدقيق للتطور داخل تسلسلات *A.baumannii* . تشير هذه الفكرة ، بدورها ، إلى جدوى استخدام جزء الجين *hcp* المستهدف حاليًا في التحديد الدقيق للهوية التي تم فحصها *A.baumannii*.وتم تسجيل سلالة واحدة من *A. baumannii* من مصدر بشري في بعقوبة / ديالى ولها رمز (SHRRWY.80) وكان رمز التسلسل (MT551041) ورمز التغاير (MT551041.1) لتسلسل الجينات 16S rRNA وكان رمز التسلسل (LC553000) ورمز التغاير(LC553000.1) لجين *hcp* .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ديالى

كلية العلوم

قسم علوم الحياة

التحري الجزيئي والتعبير الجيني لجني *hcp*,*bla*$_{OXA51}$ لبكتريا *Acinetobacter baumannii* المعزولة من عينات سريرية مختلفة

رسالة مقدمة الى

مجلس كلية العلوم – جامعة ديالى

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل الطالبة

شيماء مجيد محمد الدهلكي

بكالوريوس علوم حياة / جامعة بغداد (2002)

بإشراف

أ. د هادي رحمن رشيد الطائي

1442هـ **2020 م**