جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية الطب

دراسة جزيئية لبعض البروتينات في مقاومة البكتريا البومانية المعزولة من عينات سريرية مختلفة

رسالة

مقدمة الى مجلس كلية الطب/ جامعة بابل كجزء من متطلبات الحصول على درجة الماجستير في العلوم/ الاحياء المجهرية الطبية

من قبل

انتظار نعيم كريم كاظم

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اشراف

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الخلاصة :

Acinetobacter baumannii هي واحدة من مسببات الأمراض ESKAPE التي تؤدي إلى التسبب في التهابات المستشفيات في جميع أنحاء العالم. معظمهم من العزلات المقاومة للأدوية المتعددة ، والتي تعد واحدة من أكبر التحديات في الممارسة السريرية.

هدفت الدراسة الحالية إلى توصيف دور بروتينات الغشاء الخارجي (*car*O و *opr*D) في عزلات *A.baumannii* المقاومة المأخوذة من عينات سريرية مختلفة في مستشفى الديوانية التعليمي. خلال الفترة من سبتمبر 2021 إلى ديسمبر 2022 ، تم جمع 100 عينة في هذه الفترة. تضمنت العينات مسحات الحروق (37) ومسحات الجروح (38) والبول (25) لفحص *baumannii* Acinetobacter أو النمو البكتيري.

باستخدام وسط التشخيص كروم أجار تم أخذ 100 عينة مختلفة منهم 20 عزلة (20%) *baumannii* Acinetobacter من أصل 100 و 69 عزلة (69٪) تم تحديدها على أنها أنواع بكتيرية أخرى ، بينما لم يتم العثور على نمو في 11 عينة (11٪).

تم استخدام التقنية الجزيئية بواسطة تفاعل البلمرة المتسلسل لتأكيد عزلات *A.baumannii* باستخدام جين 16sRNAكعلامة تشخيصية ، وقد وجد أنه من بين 20 عزلة تم الكشف عنها بواسطة كروم اكار الأولي ، تم تأكيد 19 عزلة (95٪) على أنها *A.baumannii*. تم إجراء اختبارات الحساسية للمضادات الحيوية على 19 عزلة.

تـم إخضـاع هـذه العـزلات لملـف حساسـية المضـادات الحيويـة عـن طريـق اختبـار انتشـار القـرص الصـلب ، وأظهـرت معـدلات مقاومـة لجميـع المضـادات الحيويـة المسـتخدمة فـي الدراسـة الحاليـة باسـتثناء الإميبينـيم أظهـرت مقاومـة فـي 15 عزلـة (79٪) ، مـن ناحيـة أخـرى ، أظهـرت جميـع العـزلات مقاومـة بمعـدل (100٪) إلـى (/ Piperacillin Tazobactam، Tetracycline، Ciprofloxacin، Cefotaxime، amikacin، Cefepime and Colistin) عـلاوة علـى ذلـك ، تـم اسـتخدام طريقـة الحـد الأدنـى مـن التراكيـز المثبطـة باسـتخدام تخفيـف أجـار فـي الدراسـة الحاليـة لتأكيـد قابليـة إلاميبينـيم فـي الخمـس عـزلات التـي تـم اكتشـافها بواسـطة اختبـار انتشـار القـرص الصـلب سـابقاً ، أظهـرت 5 عـزلات مقاومـة للاميبينـيم (100٪) بـثلاث تركيـزات مـن الإميبينـيم (64-128-256 مجـم / مـل) ، بينمـا أظهـرت عـزلات 2 فقـط مقاومـة (20٪) بتركيـز (64 مجم / مل).

تـم اسـتخدام جـين إنـزيم carbapenemase الجزيئـي (مثـل *bla*OXA-51) فـي الدراسـة الحاليـة لتقيـيم مقاومـة الكاربـابينيم الجوهريـة جزيئيًـا ، ووجـد أن 17 مـن أصـل 19 عزلة تم اكتشافها بواسطة 16sRNA ، كانت تحمل جينًا شبيهًا بالبلاوكسا -51.

تـم اكتشـاف جينـات بروتينـات الغشـاء الخـارجي (*car*O و *opr*D) فـي العـزلات المقاومـة للإميبينـيم فقـط التـي تحمـل جـين carbapenemase (مثـل *bla*OXA-51) ، وكانـت النتـائج إيجابيـة لجـين *opr*D فـي 8 عـزلات مـن أصـل 17 (47٪) و 9 عـزلات خـارج مـن 17 (52٪) لجـين *car*O. عـلاوة علـى ذلـك ، فـي الدراسـة الحاليـة للكشـف الجزيئـي عـن جينـات الغشـاء الخـارجي *car*O و *opr*D ، لتحليـل التسلسـل النـاتج لتفاعـل البلمـرة لكـل مـن العـزلات المقاومـة للإميبينـيم والعـزلات الحساسـة للاميبينـيم للكشـف عـن الطفـرات

المختلفة في البنية الأولية للبروتين في بروتينات الغشاء الخارجي (*carO* و *oprD*) ، وجد أنه تم العثور على العديد من الطفرات في العزلات المقاومة والحساسة التي قد تؤثر على التعبير الظاهري لمقاومة الإميبينيم في *A.baumannii* في هذه الدراسة.

Ministry of Higher Education and
Scientific Research University of
Babylon College of Medicine

Molecular study of some Proteins in Resistance *Acinetobacter baumannii* Isolated from Different Clinical Samples

A Thesis

Submitted to the Council of the College of Medicine- University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master in science/ Medical Microbiology

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1444 A.H. **2022 A.D**

بسم الله الرحمن الرحيم

(رب أوزعني أن أشكر نعمتك التي أنعمت علي وعلى
والدي وأن أعمل صالحا ترضاه وأدخلني برحمتك في
عبادك الصالحين)

صدق الله العلي العظيم

(النمل الاية. 19)

Dedication

To the soul who taught me the meaning of loss, as the pain is not only in the first days of loss, but when the happy moments come, we find that whoever the soul needs to share those moments sincerely is gone
My beloved father, I give you this little

To whom taught me patience and diligence in all aspects of life, my beloved mother
To my dear husband, the supportive assistant, the beloved, the life partner, and the firm trunk who was the best support in my research and scientific journey
To those who made an effort to help me, my sisters and brothers were the best support

To my sons and daughters who enjoyed ripping papers and scribbling on books, the most beautiful moments are the fruits of you.
Asadullah, Rama, Ali Redha, Dora

To the professor Dr Huda Hadi Al-Hasnawy who helped me with scientific information in my specialty
To everyone who taught me letters of gold and words from pearls and phrases from my name and the most beautiful phrases in science to those who have come to me who taught them letters and from their thoughts is a lighthouse

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***Intidhar* 2022**

Supervisors Certification

We certify that this thesis entitled (*Molecular study of Some Proteins in Resistance Acinetobacter baumannii Isolateted from Different Clinical Samples*) was made by (*Intidhar Naeem Kareem*) and prepared under my supervision at the college of Medicine,Department of Microbiology University of Babylon as partial requirement for the degree of Master in Medical Microbiology.

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

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Date: / / 2022

Committee Certification

We certify that we have read this thesis entitled " **Molecular study of some proteins in resistance *Acinetobacter baumannii* isolated from different clinical samples** " and as an examining committee, examined the student (**Intidhar Naeem Kareem**) in its content and in our opinion, it meets standard of thesis for degree of master in medical microbiology with (Excellent) estimation.

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Summary

Acinetobacter baumannii is one of the ESKAPE(*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species)* pathogens which are leading to cause nosocomial infections throughout the world. Most of them are multidrug resistant isolates, which is one of the greatest challenges in clinical practice. The present study was aimed to characterize the role of outer membrane protiens (*car*O and *opr*D) in resistant *A.baumannii* isolates recovered from different clinical specimens in Al-Diwaniya Teaching hospital. During the period between September 2021 to December 2021, 100 specimens were collected in this period. The specimens included burns swabs(37),wound swabs(38) and urine(25), in order to investigate *Acinetobacter baumannii* or other bacterial growth, The specimens were grown on Macconkey agar. The suscpected *Acinetobacter baumannii* were diagnosed using biochemical test in addition to the usage of diagnostic medium chromagar. A total of 100 different specimens were taken of them 20 isolates of *Acinetobacter baumannii* were recovered (20%). 69 isolates out of 100 (69%) were identified as other bacterial species, While no growth was found in 11 specimens (11%). Molecular technique by PCR was used to confirm *A.baumannii* isolates using 16SrRNA gene as adiagnostic marker, it was found that out of 20 isolates detected by chromagar initialy, 19 isolates(95%) was confirmed as *A.baumannii*. Antibiotics susceptibility tests were performed on These 19 *A. baumannii* isolates. These isolates were subjected to antibiotic susceptibility profile by Disk diffusion test (DDT), showed rates of resistance to nearly all antibiotics used in present study except imipenem showed resistance rate in 15 isolates (78.9%), on the other hand, all isolates exhibited resistance rate (100%), to (Piperacillin/Tazobactam, Tetracycline, Ciprofloxacin, Cefotaxime, amikacin, Cefepime and Colistin). Furthermore, Minimum Inhibitory Concentrations (MIC) method using agar dilution was used in current study to confirm imipemem susceptibility

detected by DDT previously, the sensitive 5 isolates showed imipenem resistance (100%) at three concentrations of imipenem (64,128,256 mg/ml),While only 2 isolates showed resistance (40%) at a concentration (64mg/ml).

Moleculary, carbapenemase enzyme gene (*bla*OXA-51 like), was used in the current study to assess the intrinsic carbapenem resistant molecularly, it was found that 17 out of 19 isolates detected by 16sRNA, were carrying *bla*OXA -51 like gene. Outer membrane proteins genes (*car*O and *opr*D) ,were detected in imipenem resistant isolates only that carry the carbapenemase gene (*bla*OXA-51 like) , the results were positive for *opr*D gene in 8 isolates out of 17 (47%) and 9 isolates out of 17(52%) for *car*O gene.

Furthermore, in current study for molecular detection of outer membrane protiens *car*O and *opr*D genes, Sequence analysis for PCR product for both imipenem resistant *A.baumannii* (IRAB) and imipenem susciptible *A.baumannii* (ISAB) for detection different mutations in protein primary structure in outer membrane protiens (*car*O and *opr*D), it was found that many muatations were found in IRAB and ISAB isolates that may affect the phenotypic expression of imipenem resistance in *A.baumannii* in the present study.

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List of Abbreviations

Abbreviated Form	Meaning
AK	**Amikacin**
AAC	**aminoglycoside acetyltransferases**
ANT	**aminoglycoside nucleotidyltransferases**
APH	**aminoglycoside phosphotransferases**
ARG	**antimicrobial resistance genes**
AME	**aminoglycosides modifying enzymes**
AST	**Antibiotic Susceptibility Test**
BHI	**Brain Heart Infusion**
C3H8O3	**Glycerol**
CDC	**Center diseases Control**
FEP	**Cefepime**
CRAB	**carbapenem- resistant A. baumannii**
CHDLs	**carbapenem-hydrolysing class-D –lactamases**
CIPs	**Complement inhibitory proteins**
CIP	**Ciprofloxacin**
CLSI	**Clinical Laboratory Standards Institute**
CV	**Crystal violate**
CT	**Colistin**
Dab	**Diaminobutyric acid**
DDT	**Disk diffusion Test**
D.W	**Distilled water**
DNA	**Deoxyribonucleic Acid**
EDTA	**Ethylene Diamine Tetra Acetic Acid**
GRY	**Gyrase**
HCL	**Hydrochrolic Acid**
HAP	**hospital-acquired pneumonia**
H2O2	**Hydrogen Peroxide**
H2s	**hydrogen sulfide**
HCAI	**Healthcare Associated Infections**
ICU	**Intensive care unite**
ISAB	**Imipenem susceptible A.baumannii**
IRAB	**imipenem-resistant A.cinetobacter**
IMP	**Imipenem**
ISAbal	**Insertion sequence**
LAMP	**loop-mediated amplification**

KOH	**Potassium hydroxide**
LPS	**Lipopolysaccharied**
BSI	**bloodstream infections**
MGE	**mobile genetic elements**
LRTI	**Lower respiratory tract infection**
MBLs	**metallo-β-lactamases**
MATE	**multidrug and toxic compound extrusion**
MFS	**major facilitator superfamily**
MDR	**Multidrug Resistance**
Mg	**Milligram**
MIC	**Minimal Inhibitory Concentration**
MR-VP	**Methyl Red / Voges-Proskauer**
NCBI	**National Center ForBiotechnology Information**

CHAPTER ONE INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Acinetobacter baumannii is one of the ESKAPE pathogens which causes respiratory tract infection, pneumonia and urinary tract infections. It is an aerobic, pleomorphic and non-motile bacillus that can be classified as Gram-negative, catalase-positive, oxidase-negative, non- fermenting coccobacilli. Organisms of the *Acinetobacter* genus are often known to be widespread in nature as they can be collected from virtually all samples of soil and surface water. As a pathogen, *A. baumannii* specially targets moist tissues such as mucous membranes or exposed areas of the skin, either by accident or injury. According to Shirin, (2018), this species is often produced from the sputum or respiratory secretions, wounds, and urine of hospitalized patients.

Acinetobacter commonly colonizes irrigation solutions and intravenous solutions in a hospital setting. The prevalence of this pathogen increases gradually in the clinical setup where it can grow on artificial surfaces like contaminated medical instruments including ventilators, catheters, respirometers, pillows, bed mattresses etc, Tiwari *et al*., (2015).

Its pathogenic potential includes the ability to adhere to surfaces, form biofilms, display antimicrobial resistance and acquire genetic material from unrelated genera, making it a versatile and difficult adversary to control and eliminate. Sequence similarity and phylogenetic analyses confirmed that most of the resistance genes found in the *Acinetobacter* strain had been acquired from bacteria of the genera Pseudomonas, Salmonella or Escherichia Howard *et al*., (2012). Antibiotics have played a major role in the treatment of *A. baumannii* infections. However, the misuse or overuse of antibiotics emerged evolvement of bacterial strains harboring antibiotic resistance gene.

The Centers for Disease Control and Prevention (CDC) recognizes multidrug-resistant (MDR) *A.baumannii* as a source of global outbreaks and epidemics especially due to its effectiveness in colonizing hospital environments and due to its increase resistance to commercially available

antibiotics, including *β*-lactams, Fluoroquinolones, Tetracyclines, and Aminoglycosides Cai *et al*.,)2012).The increasing antimicrobial resistance rates of this bacterium pose a serious threat to public health and lead to complicated treatment.Multidrug resistant (MDR) *A. baumannii* isolates. is challenging to treat because it can easily upregulate innate resistance mechanisms and acquire a wide array of antimicrobial resistance genes. Resistance genes are either on the plasmid or on the bacterial chromosome Rahbar *et al*., (2019) . One such weapon in the arsenal of *A. baumannii* is the outer membrane protein (OMP) gathering. OMPs in *A. baumannii* play distinctive roles in facilitating the bacterial adaptation to antibiotic- and host- induced stresses. OMPs are major immunogenic proteins in bacteria conferring bacteria host-fitness advantages including immune evasion, stress tolerance, and resistance to antibiotics and antibacterials Uppalapati *at el* ., (2020). Polymerase chain reaction (PCR) was used for molecular detection of *A.baumannii* isolates based on *bla*-OXA-51 like gene and 16SrRNA gene.

16sRNA gene conserved ,reliable and more accurate for detection and can we used alone. The relationship between *car*O or *opr*D mutation and imipenem resistance has been the focus of controversy. It is reported in several studies that *car*O and *opr*D participate in the resistance of imipenem with nonspecific and specific monomeric channel in *A baumannii*, re- spectively.(Dupton ,2005 ; Catel-ferreira,2012 and Zahn,2015). Three-dimensional structural modeling showed that significant modifications such as deletion, insertion, or polarity reversal of *car*O amino acids mostly occurred at the position of β folds. Conformational changes in porin *car*O caused by *car*O gene mutations eventually reduce the permeability of outer membrane and lead to drug resistance. This result is consistent with the reports of Benmahmod *et at* .,(2019), but different from that of Moran- Barrio J *et al*., (2017). It may be due to different epidemic types of the bacteria and multiple Drugs resistance mechanisms involved in different regions

Aim of study

The aim of the study is to characterize the imipenem resistance and study the possible role of outer membrane protiens in *A. baumannii* isolates recovered from patients with clinical infections from hospitals, this is achieved by the following **objectives:**

1- Isolation and identification of bacterial isolates from different clinical specimens.

2- Dtermination of Antibiotic susceptibility Profile in *A.baumannii* isolates.

3- Detection of imipenem resistance in these isolates by disc diffusion test and minimum inhibitory concentrations.

4- Molecular characterization of outer membrane protiens by PCR.

5-Sequencing analysis of outer membrane protiens.

1.2 Literature Review

1.2.1. *Acinetobacter.*

Genus *Acinetobacter* includes 50 species of nonmotile Gram-negative rods that are strictly aerobic, adapted to a wide range of temperatures, and able to survive on abiotic surfaces. Many species belonging to the *Acinetobacter* genus are able to cause infections, favored by the presence of indwelling devices, in immune- compromised human hosts Nocera *et al*., (2021). The lethality of *Acinetobacter* infections is elevated in more than 50% of cases . Among the *Acinetobacter* spp. *A. baumannii* is the most prevalent, responsible for 95% of infections and outbreaks in hospitals, followed by *A. nosocomialis* and *A. pittii*.

The *Acinetobacter* genus was first identified in 1911,while Beijernick named it *Micrococcus calcoaceticus* when he described soil species. In 1957, all the non-motile species belonging to *Achromobacter* were included in the genus *Acinetobacter*. Finally, the genus comes with a broad comparative biochemical analysis. The genus *Acinetobacter calcoaceticus* was identify and named. The genus *Acinetobacter* have a long history of taxonomic changes, moving from the family Neisseriaceae to the family Moraxellaceae Within the genus *Acinetobacter* Carvalheira *et al*., (2021). *Acinetobacter* was proposed as a separate genus in 1954,but then several comprehensive revisions have been carried out. The genus has long been considered a member of the Neisseriaceae family, but it has now been assigned to the Moraxellaceae family Gordon and Wareham, (2010), together with Moraxella and Psychrobacter as follows: Kingdom= bacteria, Phylum = Proteobacteria, Class=Gamma Proteobacteria, Order =Pseudomonadales , Family=Moraxellaceae, Genus=*Acinetobacter,* Species=*Baumannii.*

Several global epidemics have occurred, sustained by a few strains belonging to successful lineages, namely, clonal complex I-III, as characterized by multilocus sequence typing . Recently, another lineage with the potential for global diffusion, delineated as sequence type (ST) 25, has emerged preventing the introduction of *A. baumannii* into hospital settings could contribute to preventing the further spread of multidrug-resistant isolates Kornelsen *et al*., (2021). Although its reservoir remains unknown, this organism has been found in soil, water, and food, including fish, milk, raw vegetables, and meat, which has earned it the definition of "ubiquitous." The presence in retail meat samples of *A. baumannii* isolates belonging to a clonal complex commonly associated with multidrug-resistant clones invites the speculation that food may carry organisms into hospital settings Choi *et al*., (2021). The genus *Acinetobacter* is a large and diverse group of biochemically, physiologically, and naturally multi-skilled bacteria. *Acinetobacter* spp, a ubiquitous coccobacillus genus, is characterized as glucose non-fermentative, non- motile, catalase-positive, oxidase-negative, and non-fastidious Gram-negative bacteria . The taxonomy of this genus is complicated due to the numerous and closely related species, which are often impossible to distinguish from each other by phenotypic and chemotaxonomic methods. Since the first description by Beijerinck in 1911 ,this bacterial taxonomy has been reclassified under various names. The *Acinetobacter* genus today contains 65 species with validly published names.

Currently, six species, namely, *A. calcoaceticus*, *A. baumannii*, *A. pittii*, *A. nosocomialis*, *A. seifertii*, and *A. lactucae* (a later heterotypic synonym of *A. dijkshoorniae*) belonging to the A.b.c. complex (*Acinetobacter calcoaceticus–Acinetobacter baumannii* complex) have been associated with human diseases. Even though these species differ in their pathogenicity, antimicrobial resistance, and epidemiology. Highly found in the environment, bacteria of this genus can be recovered from different habitats, such as soil, surface water, foods, vegetables, and arthropods . *Acinetobacter* can be retrieved as commensals of skin,wound

and respiratory and gastrointestinal tracts (Rangel *et al*., 2021).

In the hospital environment, *Acinetobacter* is also easily isolated, especially the *A. baumannii* species. When recovered from clinical samples, most species may have some significance as human pathogens . All of the *Acinetobacter* species, *A. baumannii* is the most critical pathogenic member (Wang *et al*., 2019).

1.2 .2 Characteristics of *Acinetobacter baumannii*

These bacteria are gram-negative bacteria that can be found in many places, such as on soil, water, human skin surfaces, equipment and hospital floor. *A.baumannii* is also an opportunistic bacterium whose pathogenicity is low, but over the time, these bacteria have high pathogenic capability because they have become resistant bacteria not only on one type of antibiotics but also on several types of antibiotics (Multi Drugs Resistance/ MDR) and has been reported to have both Pan resistance and Extremely Drug Resistance *A.baumannii* (XDRAB) (Lupo *et al*., 2018).

Acinetobacter baumannii is non-motile, Gram-negative coccobacilli, opportunistic extracellular human pathogen. It is emerging as an important nosocomial pathogen causing a variety of infections. It is increasingly related to serious infections among patients on these life support systems. *A. baumannii* have become resistant to almost all currently available antibacterial agents (Abdul- Hussein *et al.,* 2019).

Acinetobacter baumannii is a cause of increasing patient mortality and morbidity as well as length of hospitalization, the increase in patient mortality due to *A. baumannii* is 17-46%. Some of the diseases often caused by *A.baumannii* are ventilator associated peumonia, urinary tract infections and surgical wounds, meningitis and endocarditis. The existence of infection due to MDR A. *baumannii* makes the therapy choice arrower, increases mortality and morbidity, as well as increases cost and length of hospitalization, and makes it

one of the important public health issues to be solved immediately (Lei *et al*., 2016).

Healthcare Associated Infections (HCAI) is an infection acquired in hospitalized patients over 48 hours with a history of previous hospital care (within 90 days) or receiving treatment (open wound, intravenous injection or intravenous therapy) by health workers or their families at home (within 30 days) or routinely for intravenous therapy such as hemodialysis or chemotherapy (within 30 days). *A. baumannii* is one of the causes of HCAI and usually these bacteria have become MDR bacteria (Taylor *et al*.,2016).

1.3 Transmission:

Acinetobacter baumannii is mainly transmitted by direct contact with infected persons or indirect contact with contaminated environments. However, airborne route also plays an important role in transmission of *A. baumannii* infections in hospitals. Although, airborne transmission was considered as a route for acquisition of *A. baumannii* infections Al-Taliby *et al*., (2019). Hospital acquired infections are most commonly seen in critically ill patients specific risk factors for developing an *Acinetobacter baumannii* infection includes prolonged hospital stays, immune suppression, advanced age, presence of comorbid disease Morris *et al., (* 2019).The cell surface structures are crucial for bacterial pathogens in sensing the environment and interacting with the host Skerniškyte *et al*., (2019).

In order to persist in clinical settings *A. baumannii* must be equipped with a set of cell surface features enabling it to adhere to the abiotic surfaces such metal and plastic found in

medical devices and hospital equipment as well as to survive under desiccation stress (Chiang *et al*., 2017).

1.4 Epidemiology

Overall, *A. baumannii* is accountable for more than 12% of the cases of hospital-acquired bloodstream infections (BSI) in ICU, with wide geographic variations, it is frequent in Southern Europe, median Eastern countries, Asia, and South America, whereas it is rare in Northern European countries and Australia. *A. baumannii* is a common cause of ICU- acquired pneumonia, particularly late onset pneumonia. In countries were *A. baumannii* is spreading, it is the predominant pathogen isolated from patients with hospital-acquired pneumonia (HAP) Volpicell *et al*., (2021).

A. baumannii might be accountable of more than 36% of HAP cases in Asia. Nevertheless, it only represents 1–2% of nosocomial pneumonia episodes in some countries. In addition, *A. baumannii* might be involved in out-of hospital healthcare- associated infections.

Thus, a comparison of two large multicenter cohort studies found an increase in out-of-hospital cases from 1.2% in 2000 to 14.2% in 2010. Conversely, the incidence of *A. baumannii* in the ICU seems to have diminished in the last years Herwanto *et al*., (2018). One important feature of *A. baumannii* is its tendency to cause outbreaks because of its resistance to antimicrobials and its ability to survive for prolonged periods on dry surfaces. Outbreaks of multidrug resistant (MDR), *A.baumannii* have been found to be mainly transmitted via the hands of healthcare workers, and contaminated equipment and healthcare environment (Garnacho-Montero& Timsit, 2019).

The potential of crosstransmission increases if the patients is heavily colonized, if the surfaces surrounding the patients are colonized or if the number of patients colonized in the unit at the same time is high. Specific resistant clones are the predominant cause of outbreaks Woon *et al*., (2021). Three European clones (designated as I, II, and III) have disseminated in geographically distinct areas, and in specific institutional outbreaks, the majority of MDR *A. baumannii* isolates usually belongs to a single clone. Whole genome sequencing techniques may help in differentiating outbreak from nonoutbreak strains. Although limited to *A. baumannii* endemic areas, MDR, *A. baumannii* risk is highly variable according to the countries. Patients at high risk of MDR, *A. baumannii* infections are those with mechanical ventilation, particularly in case of prolonged duration, those with longer hospital or ICU stay, or those with greater degree of exposure to infected or colonized patients in the neighboring hospital environment (Huang *et al*., 2018).

1.5 Virulence Factor and pathogensis of *Acinetobacterbaumannii*

Acinetobacter baumannii has developed a number of specific and non-specific virulence factors for successful colonization and infection in the host. *A. baumannii* virulence factors play critical roles in serum/complement resistance, bacterial replication, cell adhesion and cytotoxicity, biofilm formation, immune evasion, and interaction with other bacteria during a polymicrobial infection. However, unlike many other bacterial pathogens, no distinct toxins with virulence potential were identified from *A. baumannii*. The pathogenicity and toxicity of *A. baumannii* are still unclear Lee *et al*., (2017). A type V secretion system, is a cell surface protein of Gram negative bacteria that plays an important role in bacterial adhesion to host cells and extracellular matrix proteins. TAAs (type Vc secretion system) are obligate homotrimeric structures with a common N-terminal head-stalk- membrane anchor-C-terminal

architecture Koiwai *et al*., (2016). Type Vc systems are considered probably like the most complex autotransporter systems and only their translocation pore is an oligomeric structure among subtypes of type V secretion systems (Fan *et al*., 2016). *Acinetobacter baumannii* is an aerobic pathogen responsible for health care- associated infections of the respiratory tract, skin, bacteremia, urinary tract, and other soft tissues. This pathogen can survive for long periods in hospital environments and can easily acquire different antibiotic resistance mechanisms (Navarrete& Terrón, 2020). The increment in the number of infections and the appearance of multidrug-resistant strains has prompted studies on the infection mechanism with the aim of identifying new virulence factors that could be targets for developing novel treatments (Pacios *et al*., 2020).

During the process of infection, bacteria adapt to different environments modifying their gene expression. It is known that tissue hypoxia occurs in the course of infection, due to a higher rate of oxygen consumption by immune cells and pathogens together with a reduction in the perfusion caused by vascular dysfunction. This means that oxygen levels in the foci of infection are much lower (<1%) than in healthy tissues. Moreover, there are different common medical conditions that produce hypoxemia and peripheral tissue hypoxia and they are often associated with infection and inflammation (Gil-Marqués *et al*., 2020). *A. baumannii* has also developed redundant and complex iron utilization and regulatory mechanisms (Parquet *et al*., 2019; Runci *et al*.,2019).

Iron availability or lack is critical for bacterial growth and host defense (nutrition immunity), respectively; iron may also play an important role in blood dissemination during *A. baumannii*-associated pneumonia (Parquet *et al*., 2019). *A. baumannii* also possessed a *mumR*-mediated transcriptional mechanism for survivingMn starvation and oxidative stress (Green *et al*., 2020).

Acinetobacter baumannii is one of the most challenging bacterial pathogens because of its unique antibiotic resistance characteristic. *A. baumannii* is clinically very significant because it is involved with nosocomial infections and is intrinsically resistant to wider classes of antimicrobials with a high propensity to developing resistance. The unique ability of *Acinetobacter* to survive desiccation renders its viability in inanimate objects for months and thus facilitates its spread in the hospital. Multidrug-resistant *Acinetobacter* spp. has been associated with prolonged hospital admissions, morbidity, and deaths (Raut *et al* ., 2020).

1.6 Antimicrobial resistance mechanisms

Acinetobacter baumannii is characterized by its great persistence in the environment enabling it to spread rapidly and the extraordinary capability to develop resistance to antibiotics. Although, *A. baumannii* has innate resistance mechanisms against multiple antimicrobials on its core genome, the strains can easily acquire new resistance determinants via various mobile genetic elements. Almost all mechanisms of antimicrobial resistance have been described including enzymatic inactivation, alteration of bacterial targets, permeability barriers to uptake of antimicrobials, or active efflux pumps. In many isolates, the genes that confer resistance to antimicrobials are clustered together which accumulates in specific genetic regions of the large accessory genome (Garnacho-Montero *et al.,* 2019).

Acinetobacter baumannii has become one of the most successful pathogens in modern healthcare because of its amazing ability to acquire antimicrobial resistance. Several strains of *A. baumannii* are highly resistant to most clinically available antibiotics (Lee, 2017). *A. baumannii* has a number of resistance mechanisms, including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects and modifications of

target sites. The accumulation of several resistance mechanisms in *A. baumannii* has gradually decreased the number of antibiotic classes available to treat *A. baumannii* infections in clinical practice (Ayoub *et al*., 2020).

1.6.1 Intrinsic Resistance of *Acinetobacter baumannii*

Acinetobacter baumannii exhibits an intrinsic reduced susceptibility to several antibiotic classes, including beta-lactams, macrolides, trimethoprim, and fosfomycin. The mechanisms underlying such intrinsic resistances consist of natural membrane impermeability, basal efflux activity, and the presence of two chromosomally encoded beta-lactamases, an ADC cephalosporinase and an OXA-51 oxacillinase D'Souza *et al*., (2019).

Three efflux systems belonging to the resistance-nodulation-division family have been characterized in *A. baumannii*, encoded by the *ade*ABC, *ade*FGH, and *ade*IJK operons. Homologs of these operons have been recovered in other *Acinetobacter* spp. such as *A. calcoaceticus*, *A. nosocomialis*, and *A. pittii*, among others (Mathlouthi *et al*., 2017). The *Ade*IJK efflux system is constitutively expressed and contributes to resistance to beta-lactams, tetracyclines, macrolides and lincosamides, phenicols, fusidic acid, and fluoroquinolones (Pokludová, 2020).

1.6.2 Acquired Resistance of *Acinetobacter baumannii*

The development of acquired resistance can occur by two processes: mutation in chromosomal structures and the acquisition of exogenous genes by horizontal gene transfer. Mutations in the two-component regulatory system *Ade*RS in the regulators AdeL and AdeN have been shown to lead to the overproduction of the efflux pumps AdeABC, AdeFGH, and AdeIJK, respectively, and consequently to an increase in resistance. In particular, over production of AdeABC contributes to an increase of resistance to beta- lactams, aminoglycosides, fluoroquinolones, tetracyclines and tigecycline,

macrolides and lincosamides, and chloramphenicol, whereas overproduction of AdeFGH contributes to resistance to quinolones, antifolates, and chloramphenicol (Gerson, 2020). The molecular mechanism of drug resistance includes enzymatic hydrolysis such as carbapenemase, the overexpression of active efflux pump, and reduced permeability as a result of outer membrane proteins OMPs loss or modification. Carbapenemase was considered the main cause of imipenem resistance (Zhu *et* al., 2019).

1.6.3 Resistance to carbapenems in *Acinetobacter baumannii*

Carbapenems, including imipenem, meropenem, and doripenem, are the antibiotics of choice for the treatment of serious infections due to *A. baumannii*. The prevalence of *A. baumannii* isolates that are resistant to carbapenems, carbapenem-resistant *A. baumannii*(CRAB) is increasing(Sheng *et al*., 2010). Carbapenem- resistant *Acinetobacter baumannii* (CRAB) is one of the most frequent multi-drug resistant opportunistic pathogens causing infections.

The Centers for Disease Control and Prevention (CDC, 2019) lists it as one of the urgent threat pathogens. CRAB has emerged in the last few decades, and the spread of clonal lineages is well known to be associated with specific resistance determinants Gaiarsa *et al*., (2019).Predominance of carbapenemases OXA-23-, OXA-24/40- and OXA-143- like enzymes is well documented. Whole Genome Seguence WGS has clarified the understanding of antimicrobial resistance genes (ARG) spread among *Acinetobacter* isolates in the hospital environment, elucidating the role of mobile genetic elements (MGE) involved in the acquisition of these resistance determinants. Carbapenem, aminoglycosides, tetracycline, and even polymyxin resistances, can result from acquisition of ARG in MGE, namely, OXA-type carbapenemases, aminoglycosides modifying enzymes (AME), *tet* genes and *mcr*-like genes, respectively (Camargo *et al*., 2020).

carbapenem including imipenem, meropenem and dorepenem have generaly been consider the agents to treat *A. baumannii* infections, due to their effective activity against this organism and their favorable safety (Doi *et al.*,2015). However, the decreased susceptibility of *A. baumannii* to carbapenems has forced clinicians and researchers to explore alternative therapeutic approaches Doi *et al*., (2015).

Because carbapenem-resistant *A. baumannii* strains are often resistant to all other commonly used antibiotics as well, these strains remain susceptible to only limited antibiotics, such as minocycline/tigecycline and polymyxins (colistin and polymyxin B) (Doi et al., 2015) (Rahi et al., 2021) . However, the recent increase of tigecycline- or colistin-resistant *A. baumannii* increasingly poses a serious threat to public health worldwide (Cai et al., 2016)(Rahi et al., 2021). The increasing emergence of imipenem resistant *Acinetobacter baumannii* worldwide has created severe challenges to therapeutic strategies. It is significant to explore the resistance mechanism of *A. baumannii* to imipenem.

The molecular mechanism of drug resistance includes enzymatic hydrolysis such as carbapenemase, the overexpression of active efflux pump, and reduced permeability as a result of outer membrane proteins OMPs loss or modification. Carbapenemase was considered the main cause of imipenem resistance Zhu *et al*., (2019). These enzymes encoding by multiple genes of resistance, which is associated with different mobile genetic determinant, thus conferring resistance to various classes of antimicrobials.

Sulbactam is a β-lactamase inhibitor and also has affinity for penicillin- binding proteins of *A. baumannii*. Combined therapy of ampicillin with sulbactam is effective for treating bloodstream infections due to MDR *A. baumannii* Ampicillin/sulbactam/carbapenem combination therapy is also effective for treating MDR *A. baumannii* bacteremia, skin and soft tissue infection of carbapenem- resistant *A. baumannii* but not in ventilator-associated pneumonia (Sakoulas *et al.*,2019)

1.6.4 Expression of OMPS in Antibiotic Resistant *A.baumannii*

The diversity of antibiotic resistance and virulence determinants and *A. baumannii* specific regulatory networks lead to the spread of bacteria in different environments, one group of bacterial proteins, termed outer membrane proteins (OMPs) due to their localization, have been studied with most interest due to their distribution, functional relevance and stipulated role in both antibiotic resistance and virulence (Uppalapati *et al*., 2020).

Outer membrane proteins in general are beta barrel-shaped monomeric or trimeric porins that allow diffusion of small molecules into and out of periplasmic space of Gram-negative bacteria. *A. baumannii* outer membrane holds scores of OMPs including *omp*A, *car*O, *opr*D- like OMPs, OMP 33-36 kDa, AbuO, TolB, DcaP, Oma87/BamA, NmRmpM, CadF, OprF, etc. (Bhamidimarri *et al*., 2019; Rasooli *et al*., 2020). OMPs participate in a wide range of functions that assist the bacterium in enduring the harsh environmental conditions, in combating the threat posed by antimicrobial compounds (Srinivasan *et al*., 2015; Wang *et al*., 2015),). Immunization with *A. baumannii* OMPs ensued significant rise in protective immune parameters Bazmara *et al*., (2019) and antibodies against OMPs passively protected experimental animals (Goel and Kapil, 2001). Clinical studies frequently identify differential expression of OMPs in antibiotic resistant *A. baumannii* strains, establishing their role in conferring resistance (Mostachio*et al*., 2012).The outer membrane of Gram-negative bacteria primarily consists of phospholipids, lipopolysaccharide and outer membrane proteins (OMPs) , OMPs play an important role in bacterial pathogenicity (Lin *et al*., 2002). *A.baumannii omp*A mediates serum resistance as well as bacterial adherence and invasion of epithelial cells and macrophages(Sukumaran *et al*., 2003). This suggests that

A.baumannii ompA contributes to overcoming the host defence and proliferation in blood cells or host cells to maintain a high level of bacteraemia, leading to the onset of infection. *A.baumannii* also induces inflammatory lung responses in pneumonia by adhering to epithelial cells (Choi *et al*., 2008) and subsequently inducing cell death through caspase-3 activation Smani *et al*., (2011). The studies indicate that *ompA* is vital to the pathogenicity of *A. baumannii* infection. *carO* is associated with resistance to carbapenem antibiotics (imipenem, doripenem and meropenem). In addition, *A. baumannii* Omp33-36 induces apoptosis kda and modulates autophagy in human epithelial cells and plays an important role in the pathogenicity of *A. baumannii* infections. Although, the role of *A. baumannii carO* remains unknown, a multidrug-resistant clinical isolate of *A. baumannii* with decreased *carO* expression was shown to exhibit reduced cytotoxicity to lung epithelial cells (Fernández-Cuenca *et al*., 2011), suggesting that *carO* is also associated with virulence (Sato *et al*., 2017).

The currently identified virulence factors, outer membrane protein A (*ompA*) is one of the best characterized. *ompA* is highly conserved, with molecular similarities among different clinical isolates of *A. baumannii*, and plays an important role in the pathogenesis of other Gram-negative bacterial pathogens. *ompA* interacts with host cells to induce host inflammatory and innate immune responses, although the potential of *ompA* as a target for vaccines and immunotherapeutics remains inconclusive (Chen, 2020). One group of bacterial proteins, termed outer membrane proteins (OMPs) due totheir localization, have been studied with utmost interest due to their distribution, functional relevance and stipulated role in both antibiotic resistance and virulence (Nie *et al*., 2020).

carO protein, generally considered as an eight-stranded β-barrel protein of 29 KDa, plays a key role in the influx of imipenem (but not meropenem) into *A baumannii*. It could be divided into two groups, *carOa* and

*carOb, car*Ob was showed to be twice more specific for imipenem from *car*Oa Zahn *et al*., (2015). Mutations in the *car*O gene would alter the structure, decrease or delete the expression of these porins, resulting in reduction of antibiotics entry into the bacteria.

*opr*D, a 43 KDa porin, is the main and specific porin for uptake of carbapenems into *A baumannii*. *A baumannii* also possesses an *opr*D homologue, which is normally considered to be involved in carbapenems resistance. However, a few studies suggested that *opr*D homologue in *A baumannii* does not really associate with resistance to carbapenem(Vahhabi *et al* ., 2021).

The concern of carbapenemase- producing Gram- negative bacteria that emerged currently is due to it is often related to the occurrence of multiple drug resistant isolates for which few choice of antimicrobials stay available. Carbapenem are B- lactam antibiotics that used most frequently as last resource antibiotic for treating of multidrug -resistant Gram negative bacilli -causing infections, they have the wide spectrum of bactericidal action and their stability aginst most of the B- lactamase. The most common mechanisms of resistance is the production of carbapenem -hydrolysing enzymes, that hydrolyse most B-lactams, (Tarashis *et al*., 2016).

1.6.5 Role of carO proteins in *A.baumannii*

The OMPs have been a hot research topic in recent years. The major OMPs associated with imipenem resistance include *car*O and *opr*D. *car*O protein, generally considered as an eight-stranded B-barrel protein of 29 KDa, plays a key role in the influx of imipenem (but not meropenem) into *A baumannii.* It could be divided into two groups, *car*Oa and *car*Ob, in which *car*Ob was showed to be twice more specific for imipenem than *car*Oa . Mutations in the *car*O gene would alter the structure, decrease or delete the expression of the porin, resulting in reduction of antibiotics entry into the bacteria Zahn *et al*., (2015). Carbapenem susceptibility

porin or *car*O was first reported in imipenem sensitive *A. baumannii*(ISAB) isolates that acquired resistance upon the loss of a *car*O (29) kDa protein Limansky *et al*., (2002). *car*O is an 8-stranded beta barrel-shaped outer membrane channel protein that does not have a continuous channel (Zahn *et al*., 2015) but mediates influx of beta lactams (selectively imipenem) into *A. baumannii* Mussi *et al*., (2005). However, contradicting these observations, liposome model system embedded with *car*O revealed its ability to transport small amino acids such as glycine and ornithine, but not carbapenem antibiotics Zahn *et al*., (2016). Despite this lonesome tangential observation, the excessive evidence from diverse research groups denotes the role of *car*O in antibiotic resistance (Uppalapati *et al*., 2020).

*car*O is classified into two sub-groups; *car*Oa and *car*Ob; of which *car*Ob exhibits a two-fold greater specificity for IMP (Catel-Ferreira *et al*., 2011). However, there has been call to rethink the *car*O classification system based on phylogenetic analysis Novovic *et al*., (2015). So far, at least six polymorphic variants of *car*O have been reported to co-exist in *A. baumannii* populations with varied specificities to imipenem, highlighting the importance of the protein.

The alterations in *car*O gene are posited to be a result of rapid adaptation of *A. baumannii* to diverse habitats and hosts. Besides gene alterations, conformational changes in primary structure, intra-genic insertion sequences , posttranscriptional and transcriptional regulation dramatically affect *car*O function (Lee *et al*., 2011), (Cardoso *et al*., 2016), (Kuo *et al*., 2017) .

The identification *car*O is significantly up-regulated in an Hfq deletion mutant strain of *A. baumannii* indicate that it is kept under post-transcriptional control by the bacterium to regulate its expression in response to the changing environment Kuo *et al*., (2017). The occasional isolation of antibiotic resistant strains with a loss of *car*O gene signifies the diversity of resistance mechanisms

in *A. baumannii* Li *et al*., (2015). The studies linking carbapenem resistance to the loss of *car*O, there are a few reports of the presence of *car*O porin on the OM of carbapenem resistant clinical isolates of *A. baumannii*. However, this can possibly be explained by the "porin-localized toxin inactivation" model, where carbapenemases like Oxa-23 interact with the periplasmic region of OMPs like *car*O or *omp*A to act as an efficient selective filter to inactivate incoming antibacterial compounds Royer *et al*., (2018).The clinical relevance of *car*O has also been ascertained by many hospital epidemiological studies.

These revealed that there is a prevalence of *car*O deficiency amongst carbapenem resistant isolates expressing bla_{oxa} Abbasi *et al*., (2020) and TEM-1 (Nan *et al*., 2018) genes among the hospital isolates of *A. baumannii*. carbapenem resistance is hydrolysis of carbapenems by carbapenemase enzymes, which are encoded mainly on plasmids and are highly transmissible .The Ambler classification system categorizes β-lactamase enzymes into 4 groups (A, B, C, D) based on their central catalytic domain and substrate preference. Of these, classes A, B, and D include carbapenemases, whereas class C enzymes hydrolyze primarily cephalosporins . Enzymes in classes A, C, and D have serine in the active catalytic site, whereas class B enzymes are metallo-β-lactamases (MBLs) with zinc in the active site.

Among the newer agents, avibactam inhibits class A, class C (eg, ampicillin chromosomal cephalosporinase AmpC, and only some class D (eg, oxacillin carbapenemase/oxacillinase OXA, serine-β-lactamases, but does not significantly inhibit the activity of class B MBLs (eg, imipenemase metallo-β-lactamase [IMP], Verona integron-encoded metallo-β-lactamase [VIM], (Nordmann *et al*.,2019). Various carbapenem resistant clinical isolates demonstrated a disruption in the *car*O gene by insertion sequences like ISAba1, ISAba125, ISAba825, ISAba10, ISAba15, and ISAba36 (Mirshekar *et al*., 2018). When exposed to a high concentration of monovalent cations ex:Nacl, *A. baumannii* release a variety of OMPs including *car*O into the surrounding

media and becomes more tolerant to IMP stress, the release of outer membrane

proteins in high NaCl, including porins Caro whose loss or inactivation, is associated with antibiotic resistance(Hood *et al*., 2010).

1.6.6 Role of *opr*D Proteins in *A.baumannii*

Outer Membrane *opr*D Proteins was first identified during outer membrane investigations of carbapenem resistant *A. baumannii* isolates (Dupont *et al*., 2005). It is an orthologous protein to a porin involved in the basic amino acid and imipenem transport in *A.baumannii* (Hancock and Brinkman, 2002). *opr*D, a 43 KDa porin, is the main and specific porin for uptake of carbapenems into *A baumannii. A baumannii* also possesses an *opr*D homologue, which is normally considered to be involved in carbapenems resistance. However, a few studies suggested that *opr*D homologue in *A baumannii* does not really associate with resistance to carbapenem Vahhabi *et al* ., (2021).The studies of a conserved *A.baumannii opr*D revealed a monomeric 18-stranded β-barrel structure characterized by a very narrow pore constriction (Biswas *et al*., 2007). The amino acid conservation at structural domains between *A. baumannii* and *P. aeruginosa opr*D porins indicate its putative function in *A. baumannii*. However, sequence and homology analysis of *A. baumannii opr*D showed that it belongs to *P. aeruginosa opr*D, a protein involved in resisting low-iron or magnesium and low oxygen stresses Catel-Ferreira *et al*., (2012).

Recombinant *A. baumannii opr*D did not conduct antibiotics but partially bound to Fe^{2+} andMg^{2+} cations. An isogenic deletion mutant of *A. baumannii opr*D did not affect MICs of β-lactams (Smani and Pachón, 2013), but in *A. baylyi* spp, a significant reduction in MIC of imipenem and meropenem is observed (Morán- Barrio *et al*., 2017). Despite these two heralding reports on lack of relationship between *opr*D and antibiotic resistance in *A. baumannii*, single nucleotide polymorphisms and insertional elements in *opr*D have been frequently identified in MDR *A. baumannii* signifying its role in resistance. *A.*

baumannii

clinical strains Lai *et al*., (2018). Insertion of mobile element ISAba1 upstream to the gene was also associated with increased carbapenem MICs of *A. baumannii* sequence Costa *et al*., (2019). *opr*D was renamed to OccAB1 by(Zahn *et al*., 2016), while solving its crystal structure. In their work,(Zahn *et al*., 2016) resolved structures of four carboxylate channels OccAB1, 2, 3, and 4 and showed that OccAB1 has the largest channel size with corresponding high rates of small-molecule shuttle, including amino acids, sugars, and antibiotics, contrary to previous observations. The particularly large pore size of OccAB1 facilitates the objective translocation of both positive and negative substrates at low energy cost (Benkerrou and Ceccarelli, 2018).

1.6.7 β-Lactamases

The B- Lactamases form a large family of enzymes that can hydrolyze the B-Lactam ring of antimicrobial agents containing this feature (Bush, 1989). Lactamases are the primary cause of bacterial resistance to β-lactam antibiotics Inactivation of β-lactams by β-lactamases is a major antibiotic resistance mechanism in *A. baumannii*. Based on sequence homology, β-lactamases are grouped into molecular classes, A, B, C, and D Jeon *et al*., (2015). All four classes of β-lactamases were identified in *A. baumannii*. Recent studies have shown that *A. baumannii* has natural competence to incorporate exogenous DNA and its genome has foreign DNA at high frequencies, implying frequent horizontal gene transfer in this pathogen Traglia *et al*., (2020). Additionally, albumin, a main protein in blood, enhances natural competence of *A. baumannii* . Therefore, natural competence of *A. baumannii* may contribute to identification of a large number of β-lactamases in this threatening human pathogen (Traglia *et al*., 2019).

Class A β-lactamases inhibited by clavulanate hydrolyze penicillins and cephalosporins more efficiently than carbapenems, except for some KPC type enzymes Jeon *et al*., (2015). A number of class A β-lactamases, including TEM, SHV, GES, CTX-M, SCO, PER, VEB, KPC, and CARB, have been identified in *A. baumannii*. Some of these enzymes, such as TEM-1, CARB-4, and SCO-1, are narrow-spectrum β-lactamases, whereas other enzymes (e.g., PER-1, TEM-92, CARB-10, SHV-5, PER-2, CTX-M-2, CTX-M-15, VEB-1, GES-14, and PER-7) are ESBLs. Some carbapenemases, such as GES-14 and KPC-2, have been detected in *A. baumannii* Bogaerts *et al*., (2010). Unlike the serine-dependent β-lactamases (classes A, C, and D), class B β-lactamases are metallo-β-lactamases (MBLs) that require zinc or another heavy metal for catalysis Jeon *et al*., (2015). Due to a broad substrate spectrum, MBLs catalyze the hydrolysis of virtually all β-lactam antibiotics including carbapenems, but not

monobactams Jeon *et al*., (2015). *A* variety of class B β-lactamases have been identified in *A. baumannii*. Class C β- lactamases pose therapeutic problems because they can confer resistance to cephamycins (cefoxitin and cefotetan), penicillins, cephalosporins, and β-lactamase inhibitor combinations, but are not significantly inhibited by clinically used β- lactamase inhibitors, such as clavulanic acid (Jeon *et al*., 2019).

Acinetobacter baumannii has an intrinsic AmpC cephalosporinase (Gordon and Wareham, 2010). Several clinical isolates of *A. baumannii* have the ampC gene transcribed from a strong promoter contained within a putative insertion sequence element (ISAba1-like sequence), which results in high resistance to ceftazidime Segal *et al*., (2004). This sequence has been identified in ceftazidime-resistant *A. baumannii* isolates, but is absent in ceftazidime-susceptible *A. baumannii* isolates Heritier *et al*.,(2006).

Class D β-lactamases are called OXAs (oxacillinases), because they commonly hydrolyze isoxazolylpenicillin oxacillin much faster than benzylpenicillin (Jeon *et al*., 2015). More than OXA-type enzymes have been identified and many variants actually possess carbapenemase activity. The presence of carbapenem- hydrolyzing class D β-lactamases or MBLs is one of the major carbapenem resistance mechanisms in *A. baumannii* (Lin and Lan, 2014).

The subgroups of carbapenem-hydrolyzing OXAs, such as the OXA-23, OXA-24, OXA-51, and OXA-58 subgroups, are prevalent in *A. baumannii*. The OXA-23 enzyme was first identified in an *A. baumannii* isolate in the United Kingdom in 1985 Perez *et al*., (2007). The blaOXA-23 gene has been disseminated worldwide, and the frequency of OXA-23-producing *A. baumannii* strains is significantly high Al-Agamy *et al*., (2016) *A. baumannii* isolates are resistant to carbapenems, and OXA-23 β-lactamases have been found in isolates Al-Atrouni *et al., (*2016). Insertion of ISAba1 in the *blaOXA-23* promoter sequence has been reported to be associated with overexpression of *blaOXA-23*,

blaOXA-51, or *blaOXA-58* in *A. baumannii* (Vrancianu *et al*., 2020).

The *blaOXA-51* like gene is intrinsic to *A..baumannii* and originally was confine on the chromosomes of this species. The *bla OXA-51* like gene for species level marker for species identification Ghaith *et al* ., (2017). The *blaOXA-51*-like genes are present in at least the vast majority of isolates of *A. baumannii*, there has been some debate as to whether they are present in all isolates of this species. If they are consistently found and are also unique to this species, then their detection could provide a simple and convenient method of identifying *A. baumannii* which could more easily be carried out than current definitive methods. Therefor, their detection could provide simple and convenient of *A.baumanii* identification Nowak *et al*., (2012). resistance genes due to their uniqueness in *A. baumannii*, particularly the bla_{OXA-51}-like β-lactamases, the genetic determinant of which is inherent in *A. baumannii* chromosome and can be readily overexpressed as a result of promoter activation by insertion sequences such as IS*Aba1* (Wong *et al* .,2019).

Some genes were also found in all *A. baumannii* strains but were not consistently associated with ISAba*1*, which is believed to provide the promoter required for expression of linked antibiotic resistance genes (Hamed *et al*., 2019).

1.6.8 Efflux Pumps

Efflux pumps lead to release of antibiotics from the cell, which reduces drug accumulation. Each efflux pump contains three components: the outer membrane channel, the periplasmic lipoprotein, and the inner membrane transporter. Four classes of efflux pumps including major facilitator superfamily (MFS), the resistance-nodulation cell division (RND) family, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion (MATE) family are associated with *A. baumannii* antimicrobial resistance.

These several pumps, the MFS and RND families of transporters have been studied in detail. AdeABC and RND-type efflux pump are not only related to aminoglycoside resistance but also involved in the resistance to many other antibiotics such as tigecycline lactams, chloramphenicol, erythromycin, and tetracycline as well. From the five superfamilies of pumps, resistance-nodulation- division (RND) systems are the most important ones in multiple resistant *A. baumannii*. This system generally demonstrates an extensive substrate variety as well as antibiotics, dyes, biocides, detergents, and antiseptics.

Efflux pumps can be specific for one substrate or can transport a range of structurally dissimilar compounds as well as antibiotics of different chemical classes. Such pumps that transport numerous compounds can be related to multidrug resistance (MDR). Several efflux pumps have clinical significance since they can reduce the fatality of bacterial infection by the agent(s) of choice. These pumps exist in nearly all bacterial species. Encoding genes of this class of proteins can be located on chromosomes or plasmids(Abdi *et al* ., 2020).

1.6.9 Permeability Defects

Permeation of compounds across the OM is a key factor that defines their accumulation in bacteria. This process is not instantaneous even when specific pores exist in the cellular membrane that allow compounds a seemingly unfettered access to the periplasm. The rate of transmembrane transport is not only finite but also limited, and the magnitude of this limit can have dramatic effects on the overall performance of efflux transporters. An excellent analysis of transmembrane diffusion can be found in prior literature (Acosta-Gutierrez *et al.,* 2018). Transmembrane diffusion is the central event during drug permeation. The rate of the diffusion can be expressed as a flux across the outer membrane. Whereas the permeability of the membrane is defined by the highest region on the energy diagram, where the compound concentration is at its lowest, the apparent affinity of the membrane to compounds is decided in its

most populated region, at the trenches in the energy profile (Zgurskaya &Rybenkov, 2020).

1.6.10 Aminoglycoside-Modifying Enzymes

The aminoglycoside antibiotic class is commonly used in the treatment of healthcare–associated infection(HAIs) from gram-negative bacilli, including *A. baumannii* strains. The bactericidal activity of aminoglycosides depends on their concentration rather than on the exposure duration to inhibitory concentrations of them. Recently, different resistance mechanisms had been developed against these antimicrobial drugs(Kishk *et al* ., 2021).

Development of resistance to newer semisynthetic aminoglycosides such as tobramycin, isepamicin, amikacin and sisomicin are being described in many countries worldwide. The resistance mechanisms of *A. baumannii* to aminoglycoside agents are different and typically include aminoglycoside-modifying enzymes (AME) production, which might be classified into aminoglycoside phosphotransferases (APH) (3′)-Via (aphA6), aminoglycoside acetyltransferases (AAC) (3)-Ia (aacC1), besides aminoglycoside nucleotidyltransferases (ANT) (2′’)-Ia (aadB) and ANT(3”) -Ia (aadA1) (Upadhyay *et al* ., 2018).

The main mechanism of aminoglycoside resistance is enzymatic alteration of amino-or hydroxyl-groups of aminoglycosides. Aminoglycoside enzymatic modification results in decreased binding to the ribosome of the aminoglycoside molecule. Previous studies suggested several mechanisms of aminoglycoside resistance in *Acinetobacter* spp. Enzymatic inactivation by AAC, ANT, and APH is the most prevalent resistance mechanism. Although aminoglycosides present nephrotoxicity risks and other side effects, they are considered to be important antimicrobial agents and are used to treat HAIs (Parte *et al* ., 2018). The high rates of aminoglycoside resistance could cause a serious issue for combination therapy of aminoglycoside with broad-spectrumβ-

lactams including cephalosporins and carbapenems against *A. baumannii* infections. AME and efflux pumps are the most important sources of aminoglycoside resistance among *A. baumannii* isolates where their genes encoding these aminoglycoside resistance mechanisms can be distributed mobile elements (Rashvand *et al*., 2021).

1.6.11 Alteration of Target Sites

A better understanding of what makes *A. baumannii* so difficult to treat is critical for improved strategies that attack the pathogen. The evolution of drug resistance in *A. baumannii* in large part is due to acquisition of inactivating enzymes or drug target mutations blocking antibiotic lethal action (Butler *et al*., 2019). These acquired alterations, which vary across isolates, act in concert with conserved mechanisms tightly linked to reduced drug penetration, including a low-permeability cell envelope and upregulation of efflux pumps. Insight into the intrinsic envelope- level defenses has the potential to inform ways to enhance antibiotic killing across diverse isolates (Geisinger *et al*.,2020).

1.6.12 Molecular identification of *Acinetobacter baumannii*

Clinical diagnostic assays targeted to nucleic acid (NA) markers are becoming an increasingly important part of the clinician's toolbox. Many disease states are difficult to diagnose due to the lack of specific and well-characterized biomarkers in an accessible specimen. These generalizations apply in particular to infectious disease diagnostics. The clinical signs of infection are often nonspecific and may originate from many possible sources, yet the treatments are more often specific and require an accurate diagnosis to be effective. There are many infectious diseases endemic where the lack of simple, instrument-free, NA diagnostic tests is a critical barrier to effective(LaBarre *et al*., 2011).Treatment, in part because of co-morbidities that confounddifferential

diagnosis. Millions of lives are lost and a huge morbidity burden is incurred through inadequate diagnosis and treatment of these diseases. In many cases, the need for rapid diagnostics is appropriate that mediocre performance tests are preferred to less accessible but better performing NA tests. Clearly, any technology that can increase the practicality and availability of NA assays in could have a significant impact on global public health. Nucleic acid detection, to date, has mainly been confined to wealthy, developed countries or to the large centralized facilities in the developing world that can marshal the resources required to perform these techniques. Like many molecular diagnostic assays, nucleic acid amplification techniques (NAATs) typically require a significant investment in equipment, training, and infrastructure(Huang *et al*., 2020).

Economic and infrastructural realities dictate that diagnostics for the developing world need to be foremost inexpensive; but also, accurate, reliable, rugged, and suited to the contexts of these low-resource settings. Recent guidelines published by the World Health Organization recommend that diagnostic devices for developing countries should be ASSURED: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipmentfree, and Deliverable to end users. In some diagnostic contexts, rapid diagnostic tests, albeit with limited sensitivity and specificity (Silva *et al*., 2020).

Nucleic acid assays that use polymerase chain reaction (PCR) amplification are capable of providing excellent sensitivity and specificity but generally fail to meet the ASSURED guidelines for affordability, rapidity and robustness, equipment-free operation, and deliverability. Appropriate, low-cost, equipment-free, pathogen-specific NA marker assays that characterize medical care in much of the developing world remain unavailable(Ahmed *et al*., 2020).

PCR is inherently ,reliable electrical power, complex equipment, training, reagent storage, quality programs and clean water, are intermittent or absent. There have been significant developments in a class of NAATs. A

comprehensive review of these techniques, and their application has recently been published. These isothermal amplification techniques vary in amplification temperature andduration, as well as complexity of reagents required—and many are proprietary—but all have the potential to be simpler and require less complex equipment than PCR-based assays Hsieh *et al*., (2021).

These methods use a variety of reaction principles to specifically amplify NA targets through isothermal melting, exponential amplification and intermediate target generation; and which, in several cases, can be detected directly without the need for an instrument. Nevertheless, almost all investigators and manufacturers currently use some type of electrically powered equipment to achieve and maintain the temperature required for amplification, although this equipment can be much simpler than the typical PCR thermocycler. This inherent simplicity makes isothermal amplification more appropriate for diagnostics. We are currently developing a non-instrumented nucleic acid platform that requires no detection instrument, no electrical power, no batteries, and no external reagents. We believe this can be achieved by combining isothermal amplification with a novel method for generating the required temperature profile without electrical power in a simple disposable that contains the lyophilized assay reagents (Hsieh *et al*., 2021).

Our first prototype of this platform uses loop-mediated amplification (LAMP) as the model for an isothermal amplification technique and malaria as a model diagnostic target. The amplification protocol requires incubating the reaction mixture at 65uC for at least 60 minutes (Van *et al*., 2021)

This temperature requirement is sufficiently flexible that small excursions (+/21.5uC) around this target are tolerable. LAMP (and several other isothermal techniques) have been shown to be far less sensitive to inhibitors than PCR, to the point where direct assay of whole blood and other unpurified specimens is feasible.

In those cases, no power or instruments, as is the case with PCR. In addition, recent advances in protein stabilization make it likely that the reagents can be dried-down in the reaction tubes with sufficient stability to avoid the need for a cold chain during delivery and storage. Thus, another power consuming ‘‘instrument’’ iseliminated(Walker& Hsieh *et al*., 2019).

We have not yet attempted to package all of these features and advances into a single prototype device; however, the successful demonstration of electricity-free temperature-controlled heating in a disposable format reported here is an important first step toward the long-term goal significant temperature variation within incubation time, and a lack of run-to-run repeatability was observed. To the performance goals implied in the guidelines, an optimized heating unit should be engineered to eliminate or minimize all sources of variation. When combined with the temperature- moderating characteristics of engineered phase change materials(Zhong& Ji *et al*., 2021).

The accurate and rapid identification of *A. baumannii* is critical for appropriate infection control in hospital settings. To date, the most common and widespread detection methods include DNA- based testing such as PCR (16sRNA gene amplification, *blaOXA- 51*-like gene),which have been used to successfully identify most *Acinetobacter* species. However, there are some limitations in these methods (Álvarez- Buylla *et al*., 2012). identification utilizing genus and species-specific primers to detect the 16sRNA and $bla_{\text{OXA-51}}$ was also applied.Molecular techniques have been successfully applied with high specificity using 16sRNA and *blaOXA- 51*-like gene as a simple and reliable method to differentiate *A. baumannii* strains Ghajavand *et* al., (2015). Molecular identification was considered more accurate in identification of bacteria and eliminates the variable phenotypic problem. The using of 16 sRNA gene in bacterial species classification have been well established because 16sRNA genes are highly conserved among species and all organisms possess various unique species-specific region that

allow for bacterial identifications . The 16sRNA gene is a suitable marker gene for the quantification of microbiota on taxonomic and phylogenetic levels. PCR with 16sRNA gene-based specific primers has been utilized as a sensitive and rapid method to quantifyintestinal microbiota Yang *et al*., (2015).16sRNA gene sequencing is one of the most commonly used for bacterial identification (Kim and Jang, 2012). The 16sRNA gene sequencing can be used in hospitals to identify bacterial species that are difficult to identify using ordinary methods. However, 16sRNA gene sequencing is not sufficiently polymorphic to distinguish all *Acinetobacte*r species Lee *et al*., (2014). The use of 16sRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons.

These reasons include

(i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16sRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16sRNA gene (1,500 bp) is large enough for informatics purposes (Petti, 2007). In 1980 in the Approved Lists, 1,791 valid names were recognized at the rank of species. Today, this number has ballooned to 8,168 species, a 456% increase. The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16sRNA gene sequencing studies as opposed to the more cumbersome manipulations (Janda *et al*.,2007).

1.6.13 The *bla*OXA-*51*-like gene

Carbapenem resistance in *A. baumannii* is commonly conferred by carbapenem-hydrolysing class-D -lactamases (CHDLs) also known as oxacillinases (OXA). There are 5 OXA subgroups associated with *A. baumannii*, an intrinsic OXA-51 with over 70 variants, and 4 of which are acquired; OXA-23, OXA-40, OXA-58, and OXA-143(Woodford *et al*., 2006). *Acinetobacte*r spp. possesses several

mechanisms that mediate antibiotic

resistance. One of the most potential ways is the antimicrobial inactivating enzymes, which includes a wide spectrum of β-lactamases that degrade and confer resistance to penicillins, cephalosporins and carbapenems. According to amino acid sequence consensus, these β-lactamases are classified into four major molecular classes; A, B, C and D, where A, C and D are inactivate the β-lactam ring via active catalytically serine residue, amongst these enzymes, OXA-51 was found to be involved in carabapenem resistance, when it was characterized form two isolates of *A. baumannii* clones in Argentina (5). OXA- 23 was firstly mentioned in 1995 Al-Haideri *et al*., (2019).

The *bla*OXA-*51*-like genes were used for strain characterization. The *bla*OXA-*51*-like gene is intrinsic to *Acinetobacter baumannii* and originally was confined on the chromosome of this species Dijkshoorn et al., (2007). Therefore, its detection has been used as a method of *A. baumannii* identification (Turton, 2006). However, the genetic structzure *bla*OXA- *51*-like has integrated into plasmids, probably via a transposition event . The plasmids carrying *bla*OXA-51-like had disseminated into *A. baumannii* isolates (Chen, 2010). Although it is clear that $bla_{\text{OXA-51-like}}$ genes are present in at least the vast majority of isolates of *A. baumannii*, there has been some debate as to whether they are present in all isolates of this species .

If they are consistently found and are also unique to this species, then their detection could provide a simple and convenient method of identifying *A. baumannii* which could more easily be carried out than current definitive methods.Here we describe the results of testing large numbers of well-characterized clinical *Acinetobacter* isolates for $bla_{\text{OXA-51-like}}$ genes by PCR with group-specific primers. OxA-51-like B- lactamases are present in all isolates of *A. baumannii* and carbapenem resistance has been associated with this gene, where it may provide a promoter sequence enhancing the expression of *bla*OxA- *51*-like genes Alsultan *et al*., (2009).The first report of this gene described $bla_{\text{OXA-51}}$ (Brown *et al,* 2005), but since then a large number of closely related variants have

been found with OXA numbers 64, 65, 66, 67, 68,

69, 70, 71, 75, 76, 77, 83, 84, 86, 87, 88, 89, 91, 92, 94, and 95) (Brown *et al*., 2005 and Turton *et al*., 2006)**,** and we have referred to them collectively as "*bla*$_{\text{OXA-51-like}}$" genes. An additional unexpected development has been the discovery worldwide of molecular class D β-lactamases in clinical isolates that have acquired the ability to hydrolyse carbapenems. These OXA-type enzymes are normally found in bacteria such as *Acinetobacter baumannii*, and are not usually associated with carbapenem resistance. So far, two subgroups of OXA-type carbapenemases have been identified in *A. baumannii*.

CHAPTER TWO

MATERIALS

And METHODS

2- Materials and Methods:

2.1 Materials:

2.1.1 Laboratory Equipment and Instrument:

The laboratory Equipment and Instruments used in this study were listed below (Table 2-1).

Table (2-1): Equipment and Instrument used in the present study.

Equipment/Instrument	Company	Country of Origin
Autoclave	Tripod	Turkey
Burner	Amal	Turkey
Centrifuge	Hittich	Germany
Conical flask	HAD	China
Cooling centrifuge	Eppendorf	Germany
Digital camera	Sony	Japan
Distillator	Memmert	Germany
DNA extraction tubes 100 µl.	Eppendorf	Germany
Eppendorf centrifuge	Hettich	Germany
Eppendrof tubes	Eppendrof	Germany
Filter paper	Memmert	Germany
Gel documentation system	Sony	Japan
Gloves	Broche	Malaysia
Gradient Thermal Cycler	BioRad	Singapore
Horizontal electrophoresis system	Mupid	Japan

Incubator	Selecta	Spain
Lap Safty Cabinet	Bio LAB	South Korea
Light microscope	Olympus	Japan
Microcentrifuge	Hettich	Germany
Micropipettes size(5-50 µl, 100- 1000 µl , 0.5 – 10 µl)	Eppendorf	Germany
Millipor filters	Sartorius	Germany
Nano drop	Avans Biotechnology	Taiwan
Oven	Memmert	Germany
Parafilm	BDH	England
PCR tubes	Eppendorf	Germany
Petridishes	Sterilin	England
Plain tubes	DMD-DISPO	Syria
Refrigerator	Kiriazi	Egypt
Sensitive electron balance	Sauter	Switezeland
Sterilized cotton swabs	Afco-Dispo	Jordan
Sterilized needles	Afco-Dispo	Jordan
Swab media	Difco	China
Tips	Sterellin Ltd	England
Turbidity meter	Biomerieux	U.S.A
UV-transilluminator	UtraViolete	U.S.A
Volumetric cylinder	HAD	China
Vortex mixer	DAIHAN	Korea
Water Bath	DAIHAN LabTech	Korea

2.1.2 Chemical and Biological Materials:

Table (2-2), below listed the biological and chemical materials used in this study.

Table (2-2): Chemical and biological materials used in study.

Materials	Manufactuer (origin)
Agar	Biolife (Italy)
Agarose	Promega (USA)
Deionized sterile water	Bioneer (Korea)
DNA Loading dye	Promega (USA)
Ethanol 96%	BDH (England)
Ethidium bromide	Promega /USA
Ethylene diamine tetra-acetic acid (EDTA)	Bioneer (korea)
Glucose	BDH(England)
Glycerol ($C_3H_8O_3$)	GCC (England)
Gram stain Kit	Himedia (India)
Hydrochrolic Acid(HCl)	BDH (England)
Hydrogen peroxide (H_2O_2)	BDH (England)
Iodine	GCC (England)
Methyl red	BDH (England)
Peptone	Difco (China)
Phosphate buffer	BDH (England)
Potassium hydroxide (KOH)	BDH(England)
Tris-Borate-EDTA buffer (TBE)	Bioneer (korea)

2.1.3 Culture Media

In the present study, cultural media were illustrated in table (2-3).

Table (2-3): Culture media used in this study.

Type of media	Manufacturing company	Origin
Blood agar base	Himedia	India
Brain heart infusion Broth	Himedia	
Chrom agar	Biomeriux	France
MacConkey agar	Himedia	India
MR–VP broth	Himedia	
Muller Hinton agar	Himedia	
Nutrient agar	Himedia	
Nutrient broth	Himedia	
Peptone water	Himedia	
Simmons' citrate agar	Himedia	
Urea agar base	Diffco-Michigan	USA

2.1.4 Antibiotic disks:

The antibiotic discs presented in Table (2-4) were used for detecting the susceptibility of *A. baumannii* isolates according to the standard guidelines recommended by National Committee for Clinical laboratory Standard (CLSI, 2021).

Table (2-4): The Antibiotic disks used in this study.

No.	Antibiotics	Disk symbols	Concentration (µg)	Manufacturing Company
1-	Amikacin	AK	30 µg	Bioanalyse/U.K
2-	PiperacillinTazobactam	PTZ	10 µg	
3-	Cefepime	FEP	30 µg	
4-	Cefotaxime	CTX	30 µg	
5-	Ciprofloxacin	CIP	5 µg	
6-	Colistin	CT	25 µg	
7-	Imipenem	IPM	10 µg	
8-	Tetracycline	TE	30 µg	

2.1.5 DNA Amplifications Materials:

2.1.5.1 DNA Extraction Kit:

Whole bacterial DNA was extracted using DNA extraction kit/G- spin^{TM} Genomic DNA Extractions Kit is used in this study and its contents were listed below table (2-5).

Table (2-5): DNA extraction kit/ G-spin^{TM} Genomic DNA Extractions Kit.

Lable	Description	Contain	Origin
G-Buffer	For Gram Negative Bacteria	20 ml	Korea
Binding Buffer	DNA binding Solution	15ml	
Washing Buffer A	With absolute EtOH	9ml	
Washing Buffer B	With absolute EtOH	10ml	
RNaseA(Lyophilized powder)	Dissolve in DW	3mg	
ProteinaseK(Lyophilized Powder)	Dissolve in DW	1.76 g	

2.1.5.2 Master Mix:

Table (2-6) The contents of Master mix used in PCR.

PCR Master Mix	Company	origin
Taq DNA polymerase	Bioneer	Korea
dNTPs (dATP, dCTP, dGTP, dTTP)		
Tris-HCl pH 9.0		
KCL		
MgCl2		

2.1.6 PCR Materials:

The content of PCR materials were listed in Table (2-7).

Table (2-7) The PCR materials.

PCR Material	Manufacturing company	Origin
Master mix	Bioneer	Korea
Free nuclease water	Promega	USA
100 – 1500 bp DNA ladder	Bioneer	Korea
TBE (Tris-Borate EDTA) buffer	Bioneer	Korea
DNA loading dye	Promega	USA

2.1.7 Primers:

The following primers were used in this study to identify the target genes in *A. baumannii* isolates as listed in Table (2-8).

Table (2-8): Primers used in this study.

Primer Name	Sequence (5'_3')	Product size (bp)	References
16SrRNA	F5'-CAGCTCGTGCGTGAGATGT -3'	150	(Ghaima, 2016)
	R5'-CGTAAGGGCATGATGACTT -3'		
blaOXA51	F5'-TAATGCTTTGATCGGCCTTG -3'	353	
	R5'-TGGATGCACTTCATCTTGG -3'		
carO	F5'-ATGAAAGTATTACGTGTTTTAGTGACAAC 3'	**370**	(Zhu, 2019)
	R5'-TTACCAGTAGAATTCTACACCAACT-3'		
oprD	F5'-ATGCTAAAAGCACAAAAACTTACATTAG C A-3'	587	
	R5'-TTAGAATAATTTCACAGGAATATCTAAGA A -3'		

2.2 Methods:

2.2.1 Study Design:

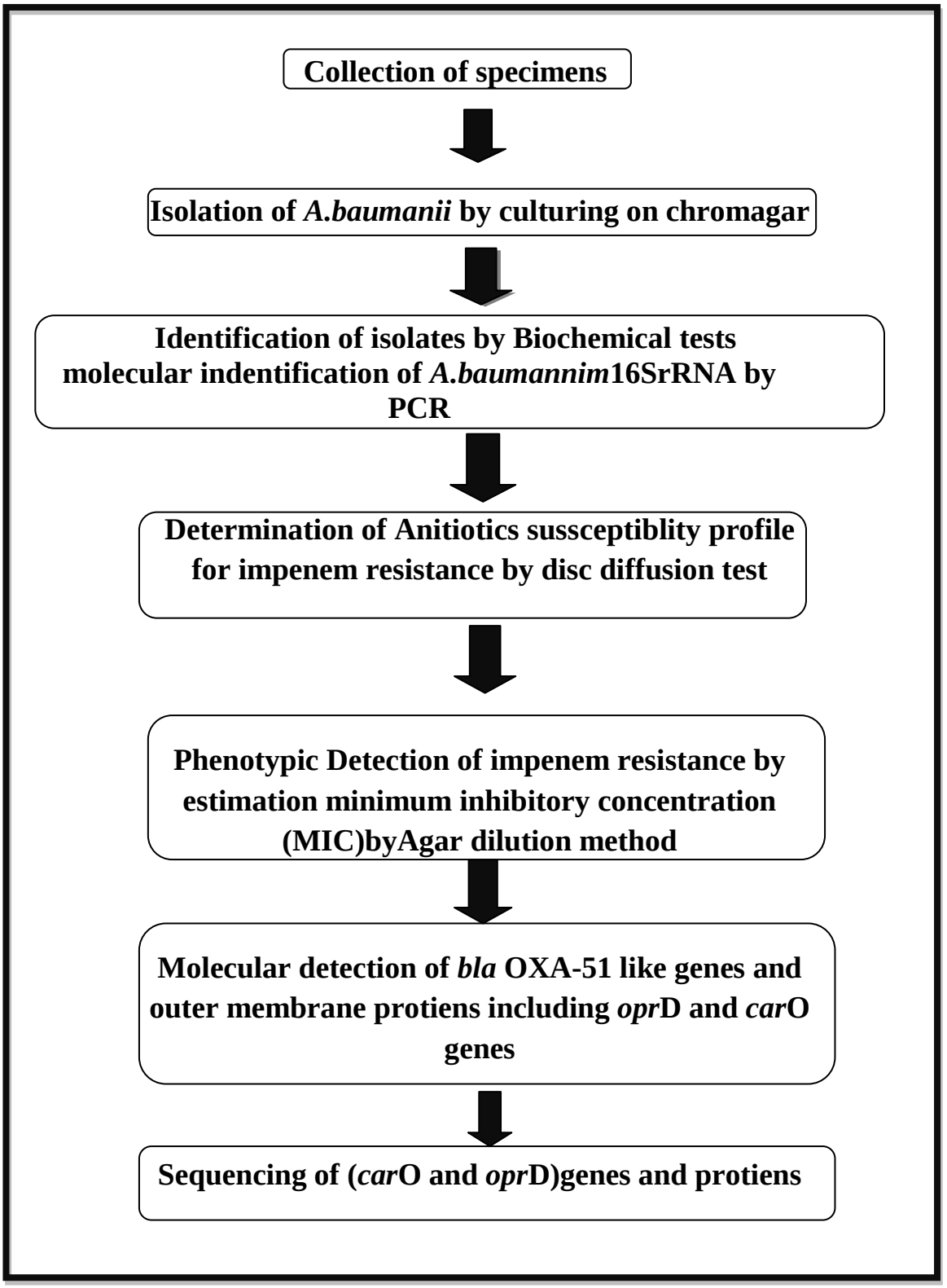


Figure (2-1): Scheme of the Study Design.

2.2.2 Specimens collection

100 specimens were collected from Al-Diwaniya Teaching Hospital, the period from September 2021 to December2021. The clinical specimens included burns, wound, and urine,Burn and wond swab were taken from the burn or wound depth. For urinary tract infection,the specimens were usually collected from the mid-stream for standardized sterile plastic containers, then centrifuged for 15 min at 5000 rpm,specimens were immediately transported to the laboratory.

2.2.3 Specimens Culturing

20 Specimens were streaked on MacConkey agar, and Chrom agar and then incubated for 24 hours at 37°C under aerobic conditions. Reports of the culture have been described as non fermenting lactose and nonfermenting bacteria. Non-lactose fermenting isolates were sub-cultured, incubated for additional overnights. Colonies morphology was examined for all isolates,then microscopically stained by Gram stain. Suspected isolates of *A. baumannii* were described using the following biochemical tests .The reports were dependent on the quality of the specimen and the time between the collection and processing. Apprporiate methodology needs to be followed for collection, amount, type, labeling, transportation, and processing of the specimens especially for *Acinetobacter* species Lal *et al*., (2019).The identification of *Acinetobacter baumannii* depends on the ability of organisms to produce certain enzymes or to utilize certain compound to be identified by biochemical tests (MacFaddin, 2000) .

2.2.4 Isolation and Identification of *Acinetobacter baumannii*

Isolates were identified depending on the morphological properties and biochemical tests . Atotal of 100 specimens were collected from urine, and full depth burns and wounds from patients at different hospitals in Diwaniya city, to isolate *A. baumannii*. Clinical relevance were determined by the clinical microbiologists and attending physicians. The specimens were inoculated

initially on MacConkey agar and chromagar then incubated for 24hr at 37 °c .

2.2.5 Culture Characteristics

All bacterial isolate were identified according to general cultural characteristics (color, shape and size) of colony and their effects on media such as lactose fermentation and the color of the colony on chrom agar, (Mahon *et al*., 2015).

2.2.6 Microscopically Examination

Gram stain procedure entails fixing clinical material to the surface of the microscope slide by heating. The slides were air-dried before staining. After fixation the primary stain, crystal violet (CV) was applied. Then A mordant, Gram's iodine (I), was applied. The decolonization step by alcohol (acetone) was done to distinguishes gram positive from gram negative cell and the secondary stain or counter stain safranin was added to stain the colorless gram-negative bacteria pink (Tille, 2016).

2.2.7 Preparation of Culture Media:

2.2.7.1 Nutrient Agar Medium:

Nutrient agar medium was prepared according to manufacturing company suggested by dissolving 28 gm in1000 ml D.w. Then the medium was sterilized by autoclave at a temperature of 121ºC and a pressure of at least 15 p.s.i. for15 min, and then cool down to 45 ºC. It was used for the cultivation of the bacterial isolates when necessary (MacFaddin, 2000).

2.2.7.2 MacConkey Agar Medium:

MacConkey agar medium was prepared according to the manufacturing firm's preferred process. It was used to isolate (G-ve) bacteria primarily, in addition to distinguish between lactose and non-lactose fermentation (Winn, 2006).

2.2.7.3 Muller-Hinton Agar:

Muller-Hinton agar medium was prepared according to the instruction of the manufacturing company by dissolving 38 gm in 1000 ml D.W. Then the

medium was sterilized by autoclave at a temperature of 121ºC and a pressure of at least 15 p.s.i. for 15 min, and then cool down to 45 ºC. It was used to test the sensitivity of *A.baumannii* isolates to antibiotics (Tille, 2016).

2.2.7.4 Brain Heart Infusion:

This medium was prepared according to the instruction of the manufacturing company by dissolving 37 gm in 1000 ml D.W. Then the medium was sterilized by autoclave at 121ºC and pressures at least 15 p.s.i. for 15 min, cold to 45Cº. This medium used for the cultivation of *A.baumannii* (MacFaddin, 2000).

2.2.7.5 Blood Agar Medium:

Blood agar medium was prepared according to manufacturer by dissolving 40 gm blood agar in 1000 ml D.W. The medium was sterilized by autoclave at a temperature of 121ºC and a pressure of at least 15 p.s.i. for 15 min, and then cool down to 45 ºC and it was supplemented with 5% of fresh human blood. This medium was used to determine hemolytic reactions (Forbes *et al*., 2007).

2.2.7.6 Urea Agar Medium:

This medium was ready by inserting (10ml) solution of urea (20%) then sterilized by millipore filter paper in the volume of autoclaved urea agar base and finishing up to (100 ml) of distilled water and cooling up to 50 °C, changing the pH to (7.1).Then was dispersed into test tubes that were sterilized and allowable to solidify in a slant pattern. This was used to check bacteria capacity to yield enzyme of urease (MacFaddin, 2000).

2.2.7.7 Peptone Water Medium:

This medium was ready by melting (8gm) of peptone into 1000 ml of distilled water, formerly spread and autoclaved into test tubes. It was used to determine the bacterial capacity to decay the amino acid tryptophan to indole (MacFaddin, 2000).

2.2.7.8 MR-VP Medium:

The medium was ready according to the manufacturing company and used

to distinguish partial and whole hydrolysis of glucose (MacFaddin, 2000).

2.2.7.9 Brain Heart Infusion Broth with (15%glycerol):

The medium consisted of brain heart infusion broth as a basal medium, supplemented with 15% glycerol, after the medium was sterilized by autoclave at 121ºC and pressures at least 15 p.s.i. for 15 min, cold to 45Cº. It was distributed in 5 ml sterile test tube. This medium was used to preserve the bacterial isolates at - 20 °C for long term storage (Collee *et al*.,1996).

2.2.7.10 Simmons' citrate Medium:

The Simmons' citrate medium was used to conclude bacteria capacity to use citrate as their sole source of carbon (MacFaddin, 2000).

2.2.7.11 Kligler Iron Agar:

This medium was used as a main step in the identity of (G-ve) bacteria for the determination of glucose and lactose fermentation and possible hydrogen sulfide (H2S) production (McFaddin, 2000).

2.2.7.12 Chromagar Medium:

This type media used as selective and differential media for *A.baumannii*. It give red color colony according to (Mahon *et al*.,2015).

2.2.8 Antibiotic Susceptibility Test(AST):

2.2.8.1 Bacterial Suspension Preparation

A loop full of 24 hours of bacterial growth on a nutrient agar was transported separately to (5 ml) of nutrient broth (pH=7) and was incubated at 37 ºC for (2- 3hr).The turbidity has been calibrated by turbidity meter equal to No.0.5 fit for the density of cell(1.5×10^8 cells /ml bacteria) (McFaddin,2000).

2.2.8.2 Disc diffusion test (DDT):

It was performed by using a pure culture of previously identified bacterial organism. The inoculum to be used in this test was prepared by adding growth from colonies grown on nutrient agar plates to 5 ml of nutrient broth, this culture was then incubated for 2-3 hours to produce a bacterial suspension of

moderate turbidity .A sterile swab was used to obtain an inoculum from the standardized culture, this inoculum was then swabbed on Muller–Hinton agarplate.

1. The antibiotic discs were placed on the surface of the medium at evenly spared intervals with flamed forceps, then incubated at 37ºC for a full 18 hours before reading the results.
2. Antibiotics inhibition zones were measured using a transparence ruler. Zone size was compared to standard zones from the (CLSI, 2021), to determine the susceptibility of organism to eachantibiotics.

2.2.8.3 Minimum Inhibitory Concentration(MIC):

2.2.8.3.1 Agar dilution:

The ranges of appropriate dilution of imipenem for MIC determinations was used as described by Mshachal *et al*., (2017),from (0.125-256) µg/ml. Two-fold agar dilution susceptibility method was used for determination of MIC of imipenem.Label petridishes with the imipenem concentration to be poured.

Add 2ml of the imipenem solution with 18mlof melted Muller –Hinton agar at 55°C and mix thoroughly.

Pour the mixture on to the petri dish with appropriate lable and allow to set. Dry the plates at 37°C With their lids tipped for 20-30 min in an incubator. A standardized inoculum for agar dilution method was prepared by growing bacteria to the turbidityof 0.5 measured by turbidity meter.

Inoculate with wire loop or Droppers(Plastic Pasteur Pipette) and spread over a5mm to 8mm spot on the Muller-Hinton agar containing imipenem agents for each concentration The inoculated plates were allowed to stand at room temperature(for no more than 30 min)until the moisture in the inoculum spots was absorbed by the agar.The plates were inverted and incubated at 33 °C for 16-20 hrs.

To determine agar dilution break points, the plates were placed on adark surface, and the MIC was recorded as the lowest concentration ofthe imipenem that

completely inhibits growth (disregarding asingle colony or afainthaze caused by the inoculum)or that concentration at which no more than two colonies were detected.The MIC values were compared with the breakpoints recommended by CLSI(2021). The following test describe the concentration and dilution and its contents were listed in Table (2-10).

Table (2-10): The Concentration and dilutions for agar dilution test (MIC test).

Steps.	Conc.	Source	Diluent (D.W)	Intermediate conc.(µg/ml)	Final conc. Dilution in Agar (µg/ml)
1	2560 µg/mL	Stock	-	2560	256
2	2560	Stock	3ml	1280	128
3	2560	Stock	7ml	640	64
4	640	Step 2	2ml	320	32
5	640	Step 2	3ml	160	16
6	640	Step2	7ml	80	8
7	80	Step 5	2 ml	40	4
8	80	Step 5	3ml	20	2
9	80	Step 5	7ml	10	1
10	10	Step 8	2 ml	5	0.5
11	10	Step8	3ml	2.5	0.25

2.2.9 Preparation of Solutions and Reagents

2.2.9.1 Ready Prepared Reagents:

six reagents were used in the present study, catalase, ,oxidase , methyl red, , Kovac´s, Urea , Ethidium Bromide and buffer,Tris-Borate-EDTA (TBE) Buffer, Tris-EDTA Buffer (TE).

2.2.9.1.1 Catalase Reagent:

It was designed to be held in a dark bottle by adding (3%) of hydrogen peroxide to (100 ml) of D.W.It used to determine the bacterial capacity for catalase enzyme production (Forbes *et al*., 2007).

2.2.9.1.2 Oxidase Reagent:

It was ready by dissolving (0.1gm) of (tetra methyl-ρ-paraphenylene diamine dihydrochloride) in (10ml) of D.W: then placed in a freshly prepared, (dark) container (Forbes *et al*., 2007).

2.2.9.1.3 Methyl Red Reagent:

The reagent was ready by dissolving 0.1 g of 99 percent ethanol methyl red in 300 ml, and then the distilled water raised the volume to 500 ml. This reagent assists in understanding whole hydrolysis of glucose (MacFaddin, 2000).

2.2.9.1.4 Kovac´s Reagent:

It was prepared in (75ml) of amyl alcohol by dissolving 5 g of (P-dim ethyl aminebenzaldehyde) and then adding (25ml) of concerted Hcl. This was used for detection indole production (MacFaddin, 2000).

2.2.9.1.5 Urea Solution:

It has been prepared to dissolve 20 g of urea for small volume of distilled water, completed up to 100 ml D.W., and. Then, sterilized with a millipore by filter .This was used to detect urease positive bacteria (MacFaddin, 2000).

2.2.9.1.6 Ethidium Bromide:

The stock solution is ready by melting (0.05 gm) powder of ethidium bromide in 10 ml of D.W, in a sterile dark bottle, the solution being combined

with a fully dissolved vortex (Sambrook and Rusell, 2001). For electrophoresis it is used as popular DNA stain .

2.2.9.1.7 Tris-Borate-EDTA (TBE) Buffer

This buffer was used at 1X concentration (1:10 concentration stock dilution).D.W.have diluted the stock solution.And held at room temperature.

2.2.9.1.8 Tris-EDTA Buffer (TE)

This buffer was equipped by addition (0.05M) Tris-OH and (0.001 M) EDTA to (800ml) D.W;the pH is changed to 8 by D.W,then autoclaved for 15 minutes at 121°C ,and processed for use at 4°C .TE buffer also is used prepared by Bioneer (korea).

2.2.10 Biochemical Tests:

2.2.10.1 Catalase Test:

Catalase is an enzyme that catalyzes convert of H2O2 to H2O and O2.A lesser quantity of bacterial growth was transported via a sterile wooden stick to the superficial of a clean dry glass slide, producing a 3% drop of H2O2.Gas bubble production yielded a positive result (Collee *et al*., 1996).

2.2.10.2 Oxidase Test (Cytochrome C):

Soak aportion of filter paper in petridish with afew drops of tetramethyl-p-phenylene-diamine dihydrochloride,pick isolated colonies grown on nutrient agar with platinum loop or clean glass rod or stick and smear over the moist area and observe the inoculated area of the filter paper for color change when present the cytochrome oxidase(oxidize) the reagent tetramethyl-p-phenylene-diamine to indophenol,adeep blue or purple color product,when the enzyme is not present,the reagent remains in the reduced state and colorless(Forbes *et al.*, 2007).

2.2.10.3 Indole Test:

Inoculate peptone water media with suspected colonies from the nutrient agar plate,incubate at 35 °C for 18-24 hr under aerobic condition,add(6-8) drops of,Kovac s reagent to the inner wall of the tube,appositive indoletest

indicated by the formation of pink to red color in the reagent layer on top of the medium with in seconds of adding the reagent ,if indole negative,the reagent will remain yellow(McFaddin, 2000).

2.2.10.4 Methyl-Red Test:

Inoculated with small bacterial colonies, MR-VP broth tubes were incubated at 37°C for 24-48 hours. It was then replaced by five drops of reagent methyl red. Red color presence and observation mean a positive result(McFaddin,2000).

2.2.10.5 Vogues-Proskauer Test:

Selected bacterial colonies were inoculated in MR-VP broth tubes and incubated at 37°C for 24 hours. The results were then read by applying solutions of 0.6 ml of alpha nephthol (reactive A) and (0.2 ml of 40 per cent) KOH (reactive B).The look of pink color afterward 15 min showed a positive result owed to partial hydrolysis of glucose that creates acetone or acetyl-methyl-carbinol (McFaddin, 2000).

2.2.10.6 Citrate Utilization Test:

Inoculated Simmons citrate agar on the slant by touching 18-24 hr old culture from the nutrient agar and incubate at 35-37°C under aerobic condition and observed the growth and development of blue color denoting alkalinization. Appositive result indicates growth on the medium, with a color change of the indicator. Growth results in the bromothymol blue indicator turning from green to blue. Negative result indicates absence of growth (Winn , 2006).

2.2.10.7 Urease Test:

Streak the surface of a urea agar with well- isolated colony from the nutrient agar plate and Leave the cap loosely and incubate the tube at 35-37° C for 48hr Change in the color of slant from light orang to magenta indicates appositive reaction, while no color change indicates negative reaction (McFaddin, 2000).

2.2.11 Bacterial isolate Survival and Maintenance:

Bacterial isolates were maintained at 4°C on nutrient agar slant. Reculturing on fresh medium held the isolates monthly. For long-term survival, nutrient broth added with 15% glycerol was secondhand and the isolates were kept frozen at- 20°C (deep-freeze) for several months (Collee *et al*.,1996).

2.2.12 Subculture of Frozen Stock Cultures:

Frozen stock crops were sub-cultivated on fresh blood agar plates, then incubated for 24 hours at 37C° in aerobic condition (Thomas, 2007).

2.2.13 Genotyping Assay

2.2.13.1 DNA extraction/ G-spin^{TM} Genomic DNA Extractions Kit/gDNA Bacteria Kit:

Bacterial DNA extraction

Bacterial DNA was extracted from bacterial isolates samples by using G-spinTM Total DNA Extraction Kit, as following steps:

1.A 100ml overnight culture broth was transferred to sterile 1.5ml microcentrifuge tube, and then centrifuged at 10000rpm for 1minute.

2.The supernatant was discarded added 100µl lysozyme solution and mixed by vortex. and incubated at RT for 5 minutes.

3.After that, 200µl of Buffer BL was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 58℃ for 10 minutes, and inverted every 3 minutes through incubation periods.

4. 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.

5. DNA spin column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow.through were discarded and placed the column in a new 2 ml collection tube.

6.600µl WA buffer were added to the DNA spin column, then centrifuge at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.

7.600µl WB Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.

8.All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.

9.The dried DNA spin column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre.heated CE buffer were added to the center of the column matrix.

10.The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2.2.13.2 DNA Concentration Measurement:

The quantity and quality of DNA was measured using a spectrophotometer (nano-drop) as follows:

1- One micro litter of TE solution was applied to the lens to calibrate.

2- After that, one micro liter of DNA was applied to the lens and the absorbance was read at 260/280nm.DNA levels were registered at50µg/ml.

2.2.13.3 Preparation of Agarose Gel and DNALoading:

Agarose gel was prepared by adding (1gm) of agarose powder to 100 ml of TBE buffer previously prepared (90 ml D.W. were added to 10 ml TBE buffer l0X, the final concentration was 1 X and pH 8). The mixture was placed in boiling water bath until it become clear, then allowed to cool to 50°C, and ethidium bromide at concentration of 0.5 µg/ml was added. The agarose poured kindly in equilibrated gel tray earlier set with comb fixed in the end, and the ends of gel tray were sealed. The agarose allowed solidifying at room temperature for 30 min. The comb and the seal were removed gently from the tray. The comb made wells used for loading DNA samples.

2.2.13.4 Gel electrophoresis

PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- 1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.

5- Then 10µl of PCR product were added in to each comb well and 3µl of (100bp Ladder) in first well.

6- Then electric current was performed at 100 volt and 80 AM for 1hour.

2.2.13.5 Preparation of PCR primers

The primers are prepared depending on the manufacturing instruction by dissolving the lyophilized primers with TE (Tris-EDTA) buffer to make stock

solution of concentration of 100 pmole/Ml, On spinning down and stay overnight at 4°C , primers working solution were prepared by diluting the stock solution with TE buffer to get final working solution (10 pmole/Ml) for each primer

2.2.13.6 PCR master mix reaction preparation:

PCR master mix reaction was prepared by using (one taq quick-load) PCR Kit.PCR master mix reaction was shown in table (2-11).

Table(2-11):PCR-Master Mix Reaction

Standard PCR master mix:

PCR Master mix	Volume
DNA template 5-50ng	5µL
Forward primer (10pmol)	2µL
Reverse primer (10pmol)	2µL
Green Master Mix kit	12.5µL
PCR water	3.5µL

2.2.13.7 PCR Thermocycling Conditions:

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table (2-12)

Table (2-12)programs of PCR thermocycling condition

Genes	Temperature (°C) / Time					No. of cycles	Amplicon size(bp)
	Initial denaturation	Cyclinc condition			Final extention		
		Denaturation	Anealing	Extention			
blaOXA-51	94/5min	94/45 sec	52/40 Sec	72/45 sec	72/6min	30	353
16SrRN A	94/4min	94/35 sec	55/45 Sec	72/40 sec	72/4 min	30	150
carO	94/5 min	94/30 sec	55/45 sec	72/1 min	72/5 min	30	370
oprD	94/5 min	94/30 sec	55/45 sec	72/1 min	72/5 min	30	587

CHAPTER THREE

RESULTS

And DISCUSSION

3. Results and Discussion

3.1. Isolation and Identification of *Acinetobacter baumannii* isolates

In current study, 100 clinical specimens were taken from patients suffering from different infections. Out of 100 specimens, 20 isolates were recovered as *A. baumannii* (20%). By using chromagar (The specimens were 38 wounds , 37 burns and 25urine). However ,69 isolates out of 100(69%) were identified as other bacterial spp. While no growth (11%).

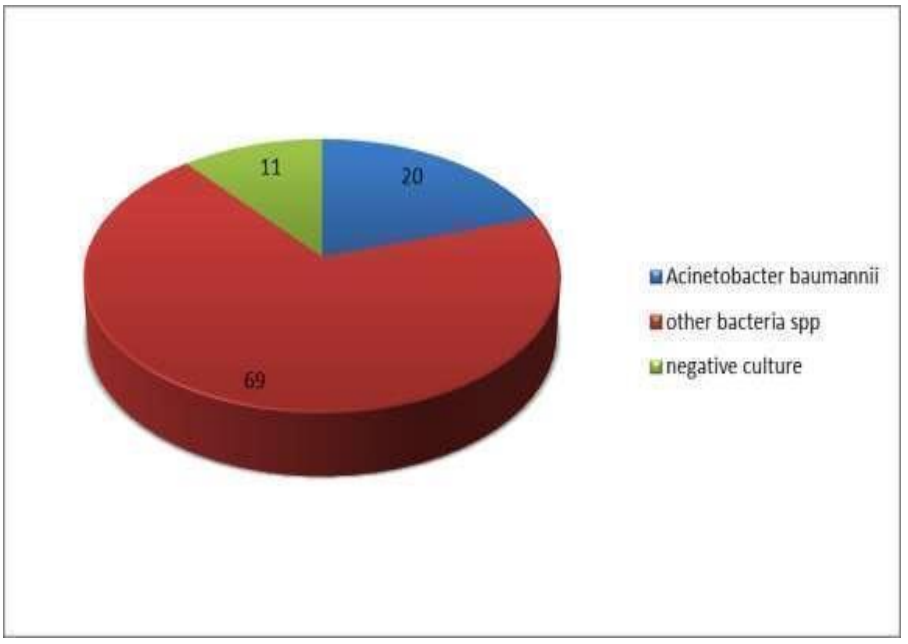
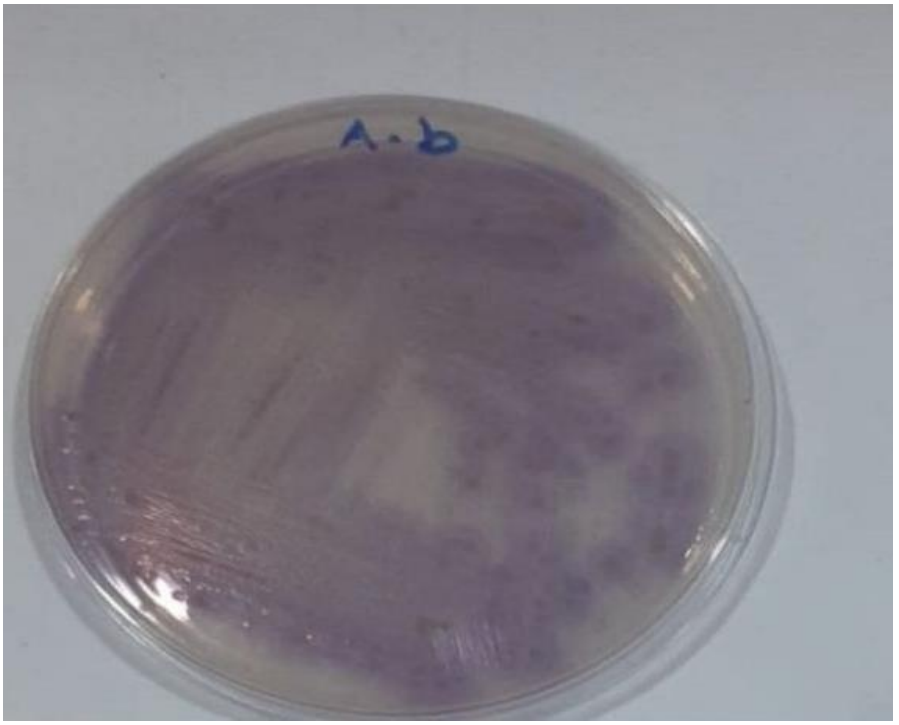
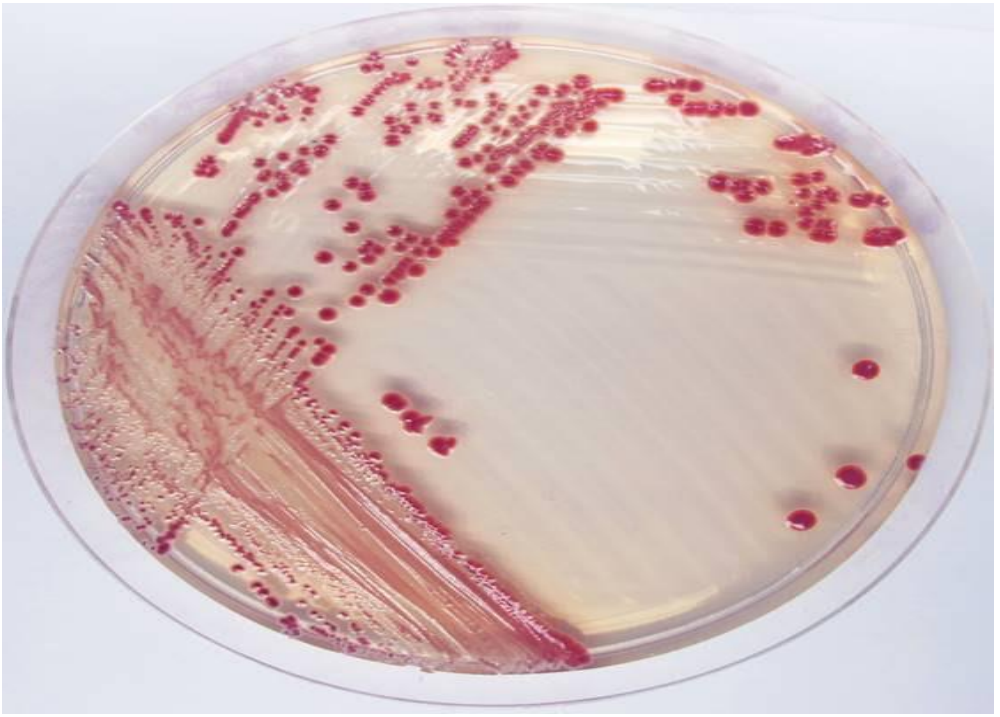


Figure (3-1): Percentage of Isolation rate in *A.baumannii* from Different Clinical specimens.

The detection of *A.baumannii* based on cultural characteristics, microscopic examination, and biochemical testing. Colonies of *A.baumanni* were convex and circular , non-hemolytic, elevated, opaque, smooth , and smaller than Enterobacteiaceae, On MacConkey agar non lactose fermenter, colonies were convex, smooth, oval, translucent to slightly opaque,complete margins and unpigmented, while on chrom agar colonies were oval,smooth, convex, translucent to slightly, and unpigmented with complete margins, and appeared circular and red ,violit colour as shown in figure (3-2A, 3-2B)



Figure(3.2 A) *Acinetobacter baumannii* in chromagar(violit colour)



Figure(3.2B) *Acinetobacter baumanni* in chromagar (red colour)

3.2. Distribution of *Acinetobacter baumannii* isolates Among Clinical Specimens

Acinetobacter baumannii isolates were recovered from different specimens with various percentages as shown in table (3-1).

Table (3-1): Distribution of *A.baumannii* isolates among clinical specimens.

Type of specimens	No. of specimens	No. of Isolates	Percentage
Burn swab	37	7	35 %
Wound swab	38	8	40 %
Urine	25	5	25 %
Total	100	20	100%

The results of this study revealed that out of 100 specimens, 20(20%) isolates were obtained belonged to *A. baumannii* collected from different clinical specimens , distributed in burn swabs7 (35%), wound swabs 8(40%) , urine 5 (25%). The results of current study were highest from studies by (Raheem, 2020; Rahi, 2021), established that isolation rate of *A.baumannii* in Al-Hilla (3.33%) isolates. However, Al-Masoudi, (2014) found that the isolation rate(15%), the results of current study were similar to other local studies (Al-Warid, 2014; Al-Harmoosh, 2015; Al-Kadmy et al.,2019) were found (21.5%), also the present study was consistant with the results of Hamza and Hadi, (2020) who found the isolation rate of *A. baumannii* was (20%), while Paul *et al*.,(2018) and Ribeiro *et al* .,(2021), established that isolation rate of *A. baumannii* was (84%, 55.6%) respectively. Furthermore, a study conducted in Karbala city by Al-Baroody and Al- Ghanimi,(2020), showed that the isolation rate was (2.40%). Despite that Al-Hilali, (2019) and recorded (8.3%) were belonged to *A. baumannii* out of 360 specimens, also, Al- Zubaibi, (2020), found that the isolation rate of *A. baumannii* was (9.23%). the other

hand, Al-Hasnawy *et al* ., (2018) found that the isolation rate of *A. baumannii* was (13%). Other studies presented by Wong *et al*.,(2019) and Mirzaei *et al*.,(2020), revealed that the isolation rate of *A. baumannii* was (3.09%, 9.51%) respectively. The disparity in the isolation rates may be due to many factors such as geographical distribution, seasonal variations and sample size. Many *Acinetobacter* infections varies according to the season. *Acinetobacter* infection is favored by *A. baumannii,* which develops in damp conditions with more humid ambient air. Several outbreaks have been traced to liquid or wet environmental sources that have aided *Acinetobacter* species spread. There are several factors that are a significant cause of *A. baumannii* including environments, wide variety of PH (Al- Baroody and Al-Ghanimi, 2020).

3.3 Molecular identification of *Acinetobacter baumannii* isolates

Molecular technique by PCR was used to confirm *A.baumannii* isolates using 16SrRNA gene as adiagnostic marker, it was found that out of 20 isolates detected by chromagar initialy, 19 isolates(95%) was confirmed as *A.baumannii* as shown in figure (3-3)

In regard to 16sRNA gene amplification based on PCR, the results showed positive isolates in 19 (95%) out of 20 isolates of Acinetobacter baumannii , in compartion with another study done by Abdul- Hussein *et al*., (2019), who showed that positive results for 45 isolates out of 61 isolates (73.77), different study made by (Qader,2021) showed positive results for 75 isolate out of 150 isolates (50%).

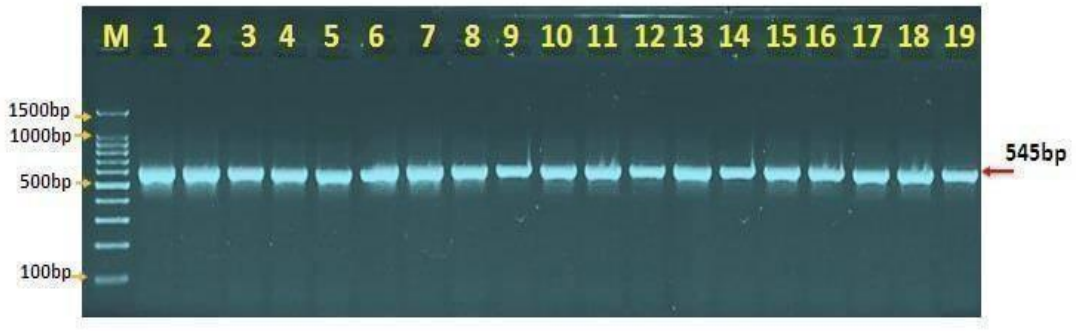


Figure (3-3) agarose gel electrophoresisimage that showed the PCR product analysis of 16sRNA gene in Acinetobacter baumannii isolates ,where lane (M) ladder marker (1500- 100bp) ,showed positive 16Srna gene in Acinetobacter baumannii at(545bp) PCR product size

3.4 Antibiotic Susceptibility Profile of *A .baumannii* isolates:

The susceptibility of 19 *A. baumannii* isolates towards 8 antibiotics were evaluated by using Kirby–Bauer method. Antibiotic susceptibility by disk diffusion (DDT) method on Muller Hinton Agar according to the clinical and laboratory standard institute (CLSI, 2021). The resulting zones of inhibition were measured and compared with the breakpoints standard value of CLSI(2021).

Figure(3-4), showed high level of resistance in *A. baumannii* isolates to most antibiotics used in the current study. All isolates (100%), were resistant to Piperacillin/Tazobactam, that similar to local studies in Babylon province by Al- Warid, (2014) and Rahi, (2021) ,found (100%) ,resistance for this antibiotic. Carbapenems group including Imipenem showed resistance rate in 15 isolates (75%), another study by Mshachal et al .,(2017), showed resistance rate (50%) ,for imipenem, which varied from different hospitals in Thailand.

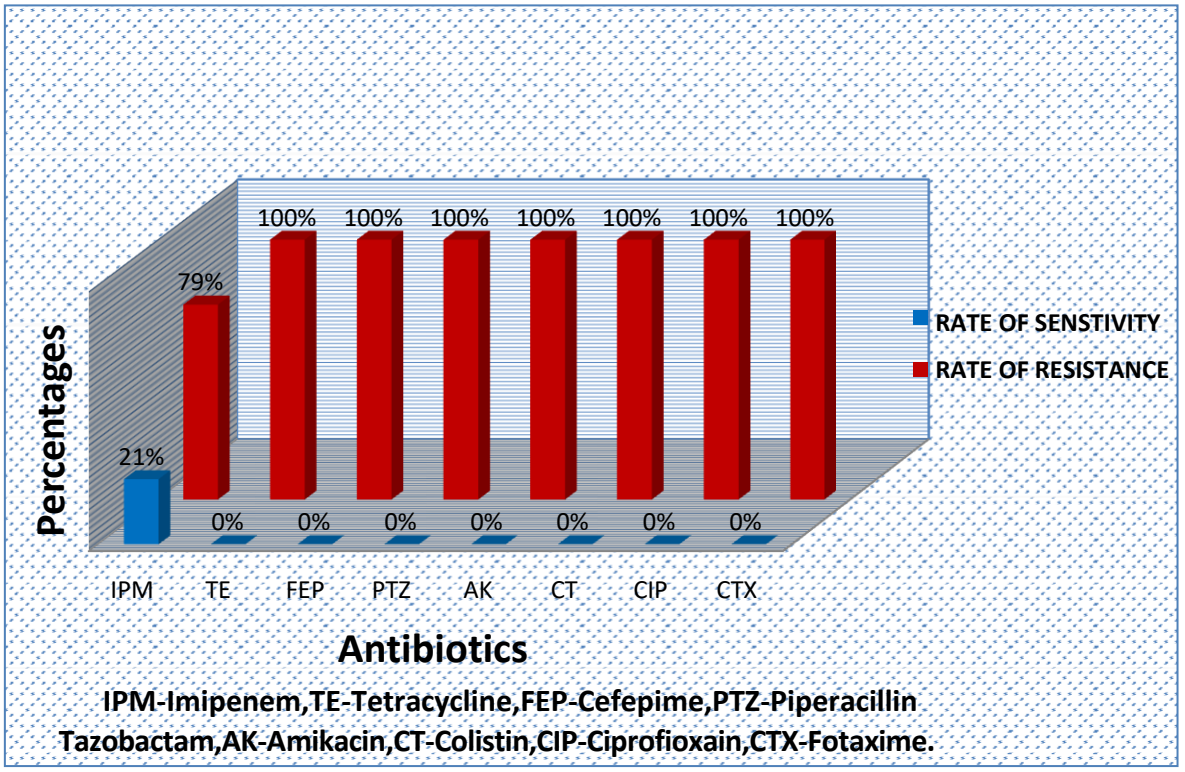


Figure (3-4): Percentages of Antibiotic Susceptibility Profile of Acinetobacter baumannii Isolates Detected by DDT(n=19)

However, Thirapanmethee *et al*., (2020), who found that *A.baumannii* isolates were resistant 100% for imipenem, and different from the study of Rahi, (2021) who found (100%) resistance rate to imipenem.

On the other hand, Cefotaxime showed highest resistance rate 20 (100%) which was simillar to local studies in different hospitals in Baghdad by (Al–Saleem, 2013;Al– Kadmy *et al*., 2019) whose found that *A.baumannii* isolates were resistant (100%) to Cefotaxime, while different from the study of Al-

Taliby and Al-Daraghi, (2019) whose showed resistance rate (80%).

Fourth-generation cephalosporin (Cefepime) also showed highest resistance rate 20(100%) which was similar to study in Babylon province by Kareem, (2020) who found resistance rate (100%) for Cefepime, in addition to, high resistant rate of *A .baumannii* for Amikacin and tetracycline (100%) for each one (figure 3-4).

This was similar to the results that conducted by Al- Baroody and Al-Ghanimi, (2020), they found the isolation rate of *A.baumannii* were resistant (100%),while different from the result of Sobouti *et al*., (2020), who showed resistance rate (60%).

In current study Ciprofloxacin, also showed high resistance rate 20(100%), which was higher than the result of Kareem, (2020), who found that the resistance rate (76%) for Ciprofloxacin. and different from results of Al- Zubaidi, (2020),(78.19%). Furthermore, the resistance rate to colistin showed (100%) which was higher than the result of Hussein, (2013) who found resistance rate (66.96%), and more than the result study of Rahi, (2021) who found the resistance rate (45%). The difference may be due to the variation in specimens and tests for susceptibility used in different studies.

In this study , most classes of antibiotics listed in CLSI ,2021 were used so that consider multy drug resistance MDR (resistance to at least one agent in three or more categories of antibiotics. most bacterial isolates were resistant in at least one agent in 3 or more antimicrobial agents class, which means they were MDR (Magiorakos *et al*., 2012). The appearance of MDR isolates in *A.bumannii* have caused many problems in the treatment of baterial infections.

These findings can be justified by inadequate adherence to infection control guidelines and also inappropriate use of antibiotics . The differences in resistance ratios in this and other studies may be due to the diversity of isolated sources and to the acquisition of resistance genes in the *A.baumannii* isolates(Kadom, 2018) .In Iraq, there is a significant increase in carbapenem resistant bacteria in last two decades, especially after 2003 war, this is may be due to Iraq opennesto the world and the entry of especially from the endemic area(Hamed *et al*., 2019)

3.5 Determination of Imipenem susceptibility by minimum inhibitory concentrations(MIC)

The determination of minimum inhibitory concentrations (MIC) for imipenem using agar dilution method was used in current study according to CLSI, (2021). The results showed that 5 isolates exhibited imipenem resistance (100%) at three concentrations (64, 128, 256) mg/ml, all 5 isolates showed resistance in concentrations (128,256) mg/ml while only 2 isolates out of 5 (40%) showed resistance in concentration(64) mg/ml, with comparable to a study conducted in Oxford university by pournaras,(2021) which indicated the MIC for imipenem resistance was at a concentration (64mg/ml). Another study done by Fonseca, (2013) who investigated (84) isolates and all isolates showed resistance for imipenem at concentrations ranging from (0,125-0,50) mg/ml.

This demonstrates the importance of agar dilution in that not only the identical distribution of imipenem concentrations in the agar plates and the accuracy of this method for MIC determination, but also the antibiotics good stability in Mueller Hinton agar when stored properly. In several studies (Moskowitz *et al*., 2010), agar dilution was shown to be reliable for determining MICs and was also successfully used for screening purposes (Nordmann *et al* ., 2016). MIC values for all isolates tested with agar dilution were highly reproducible, not only among spots on the same plate but also among replicates of fresh plates and stored ones.

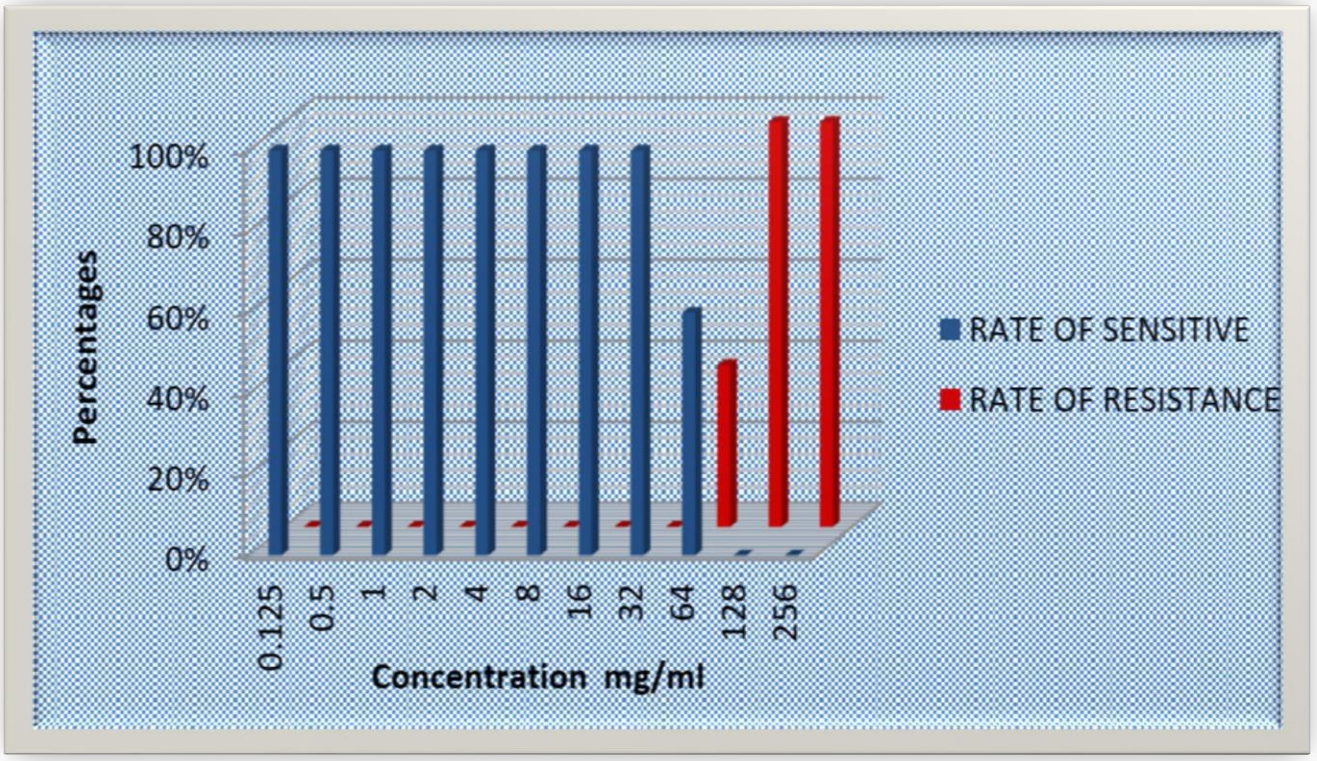
The agar dilution method was found to be superior in terms of reproducibility, robustness and, ease of use compared to imipenem MIC determination. These observations may justify more extensive validations of agar standardized protoco dilution, with the goal of developing a globally accepted for imipenem susceptibility testing(Turlej-Rogacka *et al*.,2018).

Table(3-2) showed the results in this study. the variation in the results may be

due to variations in the method used for MIC deautomated,number of specimens and geographical distributions

Table(3-2):Minimum inhibitory concentrations of imipenem in *A. baumannii* detected by agar dilution method(n=5).

No. of isolates	MIC concentration										
	0.125	0.5	1	2	4	8	16	32	64	128	256
1	S	S	S	S	S	S	S	S	R	R	R
2	S	S	S	S	S	S	S	S	S	R	R
4	S	S	S	S	S	S	S	S	S	R	R
6	S	S	S	S	S	S	S	S	S	R	R
7	S	S	S	S	S	S	S	S	R	R	R



Figure(3-5):Percentages of imipenem Susceptibility in Different Concentration of imipenem in *A. baumannii* Isolates Detected by Agar Dilution method(n=5).

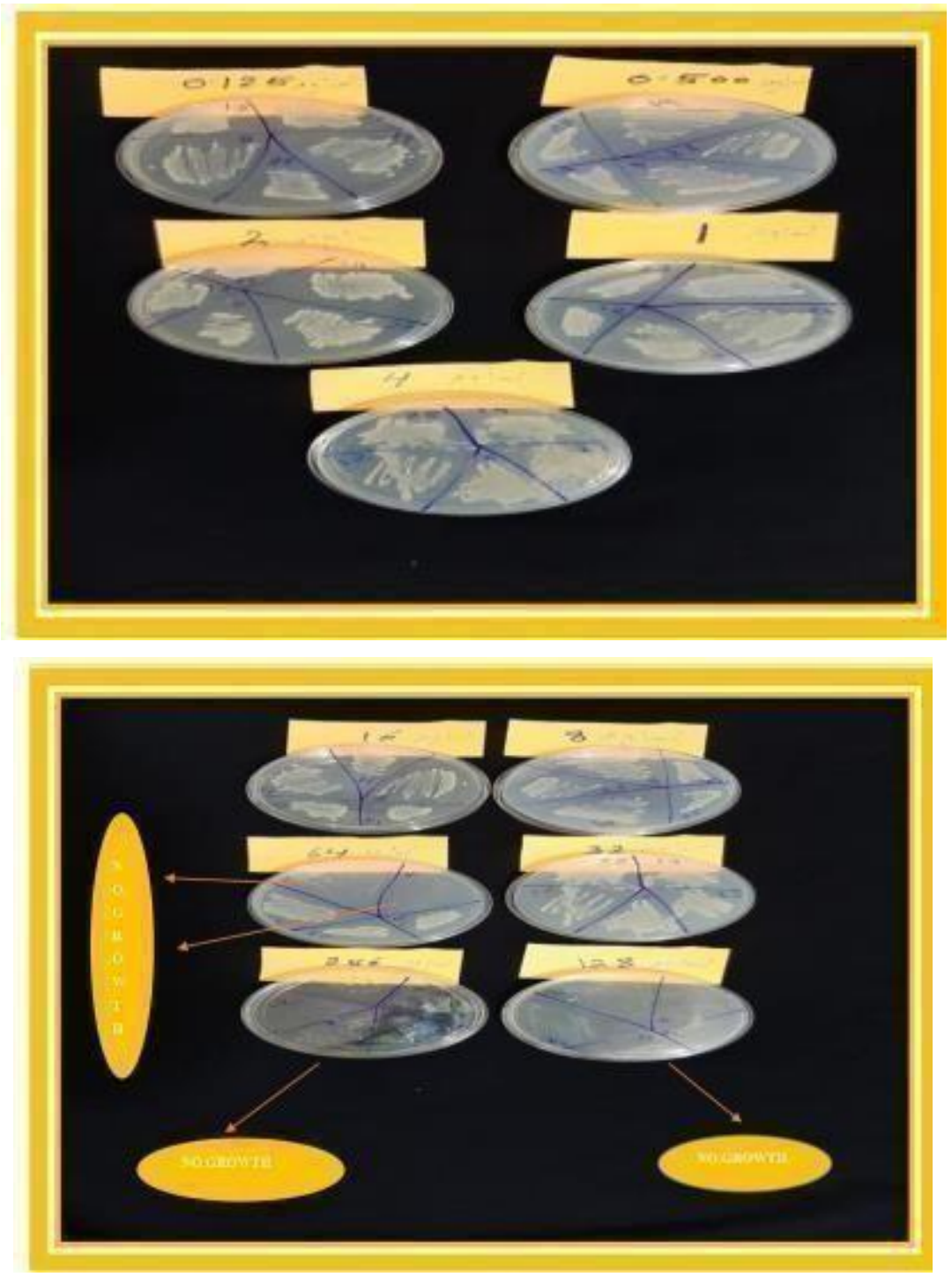


Figure (3-6): Agar plates showed the Minimum Inhibitory Concentrations of imipenem in A. baumannii isolates detected by agar dilution method (n=5).

3.6 Polymerase Chain Reaction Technique:

3.6.1. Detection of Bacterial genomic DNA profile by polymerase chain reaction technique:

The extracted DNA was electrophoresed on 1% agarose gel and the DNA band was visualized via ethidium bromide staining and UV detection. Figure(3-7)

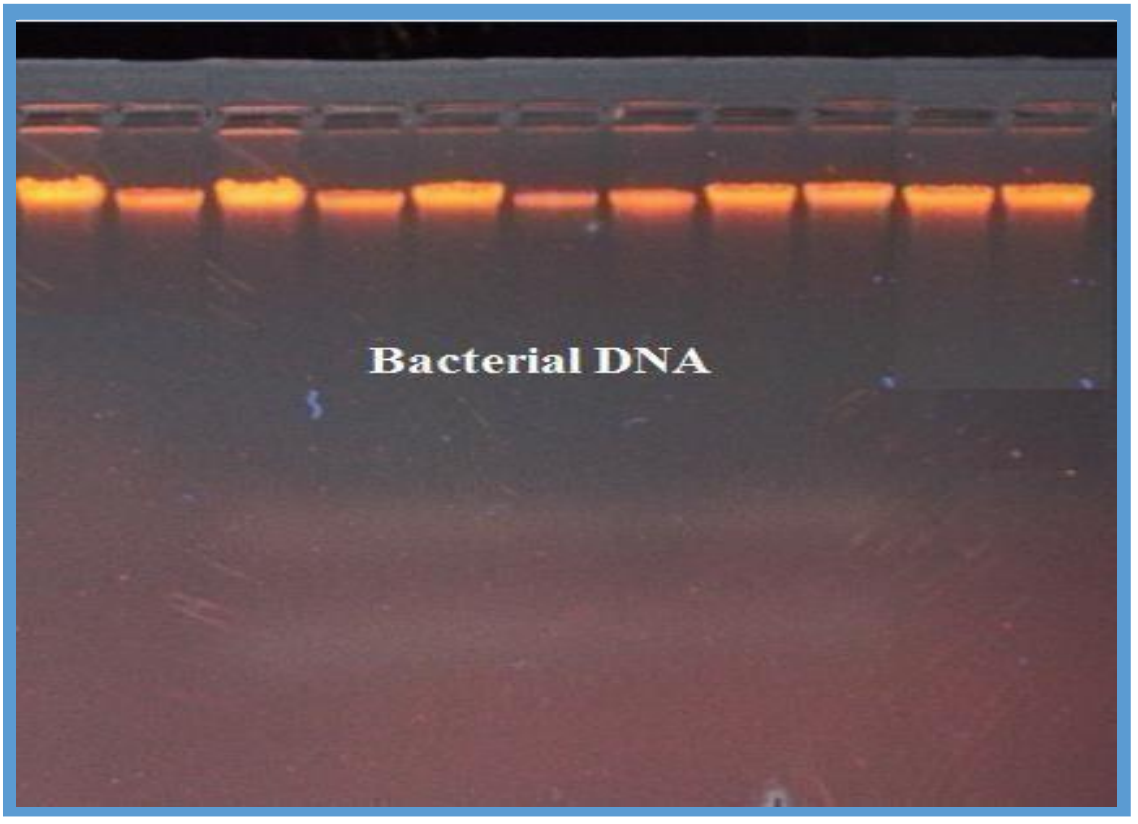


Figure (3-7): Ethidium Bromide stained agarose gel electrophoresis appearance that displays bacterial genomic DNA .

3.5.2 Molecular detection of carbapenem resistance gene(Intrinsic Carbapenemase Gene *bla*OXA-51- like) of *Acinetobacterbaumannii.*

In the current study PCR was performed for all *A.baumannii* isolates which were resistant to imipenem phenotypically in the present study. $bla_{\text{OXA-51}}$ was considered a major factor in the resistance to carbapenem in *A.baumannii* (Al- Harmoosh, 2015).

This gene was detected in 17 out of 19 isolates confirmed using 16sRNA by polymerase chain reaction (PCR) with specific primer for $bla_{\text{OXA-51}}$ like gene with a product of (353) bp figure (3-8). In the current study, bla_{OXA-51} was conducted for all isolates mentioned above, in *A.baumannii* isolates were 17(89%), this percentage was nearly to study of Al- masoudi,(2013), who showed (86%) for 13 isolates out of 15. Also another studies by Mohamed *et al* ., (2020), revealed that $bla_{\text{OXA-51}}$ gene was detected in 82.3% (14/17) in Eygpt.

Also Rahi, (2021) found resultes (95%) for this gene. Another study performed by Abdul- Hussein *et al.,* (2019), showed that *bla*OXA-51 was detected (73.77%) in 45 isolates among 61 carbapenem-resistant *A.baumannii* isolates. in Babylon hospitals, other study, Al- Hindawi and Jarallah ,(2018), *bla*OXA-51 was detected (100%) in *A.baumannii* isolates and comparable observations were made and other studies by Al- Hasnawy *et al*. ,(2018) recorded that *bla*OXA-51 was detected in (13%) A. *baumannii* isolates .

Also studies of Thirapanmethee *et al* ., (2020), suggested the presence of the *blaoxa-51*-like gene and detected in all clinical isolates 183(100%). While Al-Masoudi, (2015) , detected *bla*OXA-51 in *A.baumannii* isolates in percentage (80%) and Al –Baroody, (2020), recorded that *blaOXA-51* was detected in all isolates of *A. baumanii* 15 isolates (100%) However Catel-ferreira *at el* ., (2011), found that OXA carbapenemase gene was found to be the predominant carbapenemase gene, with 99% of the isolates harbouring

*bla*OXA-51-like carbapenemase genes. But Mekkey *et al* ., (2020), showed the presence of the *blaoxa-51*-like gene in 33 (66%) out of 50 *A.baumannii* isolates. Although Anane *et al* ., (2020), detected (15%) *bla*OXA-*51* gene in *A.baumannii* isolates. A study conducted by Rao *et al*., (2020), recorded that the *bla*OXA-51 was harboured in all of *A. baumanii* isolates (100%).

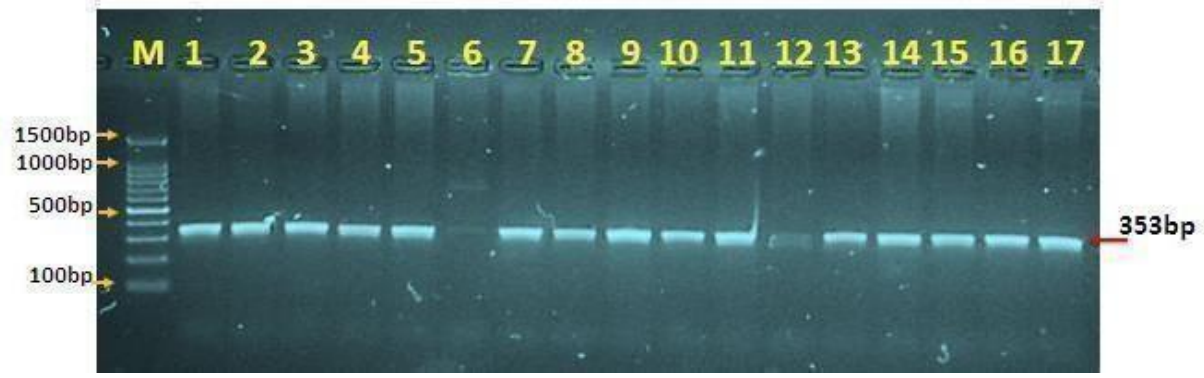


Figure (3-8) :- Gel electrophoresis for PCR product of (*blaOXA-51* gene) showed 353bp ,(Agarose 1%, 10min. at 100 voltage for 30min and then lowered to 50 volts for 45min).Visualized under U.V light after staining with ethidium bromide.

3.5.3. Molecular detection of outer membrane protiens (*opr*D and *car*O) genes by polymerase chain reaction technique

The current study investigated a possible porin-mediated mechanism relating to the carbapenem resistance-associated outer membrane protein *car*O and *opr*D to determine whether this porins may be a diffusion pathway for carbapenems in *A. baumannii*. In the current study, PCR was applied 17 isolates that were positive for *bla*OXA 51-like gene(carbapenemase enzyme) that related to carbapenem resistant isolates to investigate the presence of outer membrane protiens in these resistant *A.baumannii.*

For molecular detection of outer membrane protein (OMPs) **.**17 isolates thate give positive *bla*OXA-51 like gene out of 19 isolates were analayzed and found that 9 out of 17 were positive for *car*O gene (52.9%), and 8 out of 17 positive for *opr*D gene was (47.1%), in comparison with study of Zhu *et al*., (2019) who presented that all isolates observed were carrying both *car*O and *opr*D genes.

With the increased number of resistant *Acinetobacter baumannii* isolates, it is urgently required to study the molecular bases of outer membrane permeability protients (*opr*D, *car*O).

Furthermore Catel-ferreira *at el*., (2011), determined the first evidence that *car*O channels possess an imipenem (but not meropenem) binding site, and that their specificities depend on their primary structure. Any decrease in *car*O expression would thus reduce the susceptibility of *A. baumannii* to this antibiotic. Also, Uppalapati *at el* .,(2020) proved that decreased expression of the OMPs was significantly associated with carbapenem resistance.

One such weapon in the arsenal of *A. baumannii* is the outer membrane protein (OMP) gathering. OMPs in *A. baumannii* play distinctive roles in facilitating the bacterial adaptation to antibiotic- and host-induced stresses. OMPs are major immunogenic proteins in bacteria conferring bacteria host-fitness advantages including immune evasion, stress tolerance, and resistance to antibiotics and antibacterials(Uppalapati *at el* .,2020).

Figure (3-9) agarose gele electrophoresis image that showed the PCRproduct analysis of *car*O gene of *Acinetobacter baumannii* isolates .where,lane (M) marker ladder (1500-100bp) this lane showed positive *car*O gene in *Acinetobacter baumannii* isolate at (370bp) PCRproduct size.

Figure (3-10) agarose gel electrophoresis image that showed the PCR product analysis of oprD gene in *Acinetobacter baumannii* isolates.Where,Lane (M) Marker ladder(1500- 100bp) ,Lane(1-17):showed some positive *Opr*D gene in *Acinetobacter baumannii* isolate at(587bp),PCR product size

3.7 SequencingAnalysis:

3.7.1. Three dimensional structural modeling:

In the present study amino acid sequences were translated from *car*O gene sequences between imipenem resistant *A. baumannii* (IRAB) and imipenem sensitive *A.baumannii* (ISAB). As in figure (3-11) the resistance profile of IRAB is increasingly severe in clinical settings. Changes in the conformation of *car*O and *opr*D proteins were determined in the present study by sequence analysis of these proteins and compared with NCBI blast refrence strain of *A.baumannii* as listed in table(3-3). Conformational change in *car*O porin was identified as one of the important molecular mechanisms involved in imipenem resistance in *A baumannii*. *car*O and *opr*D participate in the resistance of imipenem with nonspecific and specific monomeric channel in *A baumannii,* respectively, (Dupton, 2005; Catel-ferreira, 2012 and Zahn *et al* ., 2015). The relationship between *car*O or *opr*D mutation and imipenem resistance has been the focus of controversy. Three-dimensional structural modeling showed that significant modifications such as deletion,subsatition, insertion, or polarity reversal of *car*O amino acids mostly occurred at the position of β folds. As in table(3-3) Conformational changes in porin *car*O caused by *car*O gene mutations eventually reduce the permeability of outer membrane and lead to drug resistance. This result is consistent with the reports by(Benmahmod *et at* ., 2019), but different from that of Moran- Barrio *et al* ., (2017). It may be due to different epidemic types of the bacteria and multiple drug resistance mechanisms involved in different regions.

3.7.2 Phylogenetic tree analysis:

The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). Figure (3-13) showed that the local *Acinetobacter baumannii* imipenem resistance isolates (*car*O No.4 , *car*O No. 5 and *car*O No.3) were showed closed genetic related to PBD:4rlb template imipenem resistance strain whereas, the local *Acinetobacter baumannii* imipenem resistance isolates (*car*O No.1) and local *Acinetobacter baumannii* imipenem isolates (*car*O No.2) were showed genetic different than PBD:4rlb template imipenem resistance strain. The variations between isolates could be due to the differences in source of specimens used in the current study despite that isolates No.2 express the same phenotypic imipenem susceptability pattern.

Furthermore, figure (3-16) revealed that the local *Acinetobacter baumannii* imipenem resistance isolates and local *Acinetobacter baumannii* imipenem sensitive isolates were showed genetic differences than PBD: 5dl5.1 *opr*D protein template strain. Isolate No. 4 showed separate lineage difference from the other isolates when compared with standard NCBI strain (PBD: 5dl5.1 *opr*Dprotein template strain). However this isolate expressed phenotypic imipenem resistance pattern. These results indicated that there was no differences were present between sensitive and resistant isolates in regard to phylogenetic analysis. For knowledge, Local studies were not found and this study could be considered the first one in Iraq.

However, Little studies were found regarded these two OMPs (*car*Oand *opr*D) to compare the current results, Zou *et al*., (2019) *car*O was identified as one of the molecular mechanisms involved in imipenem resistance in *A baumannii*.

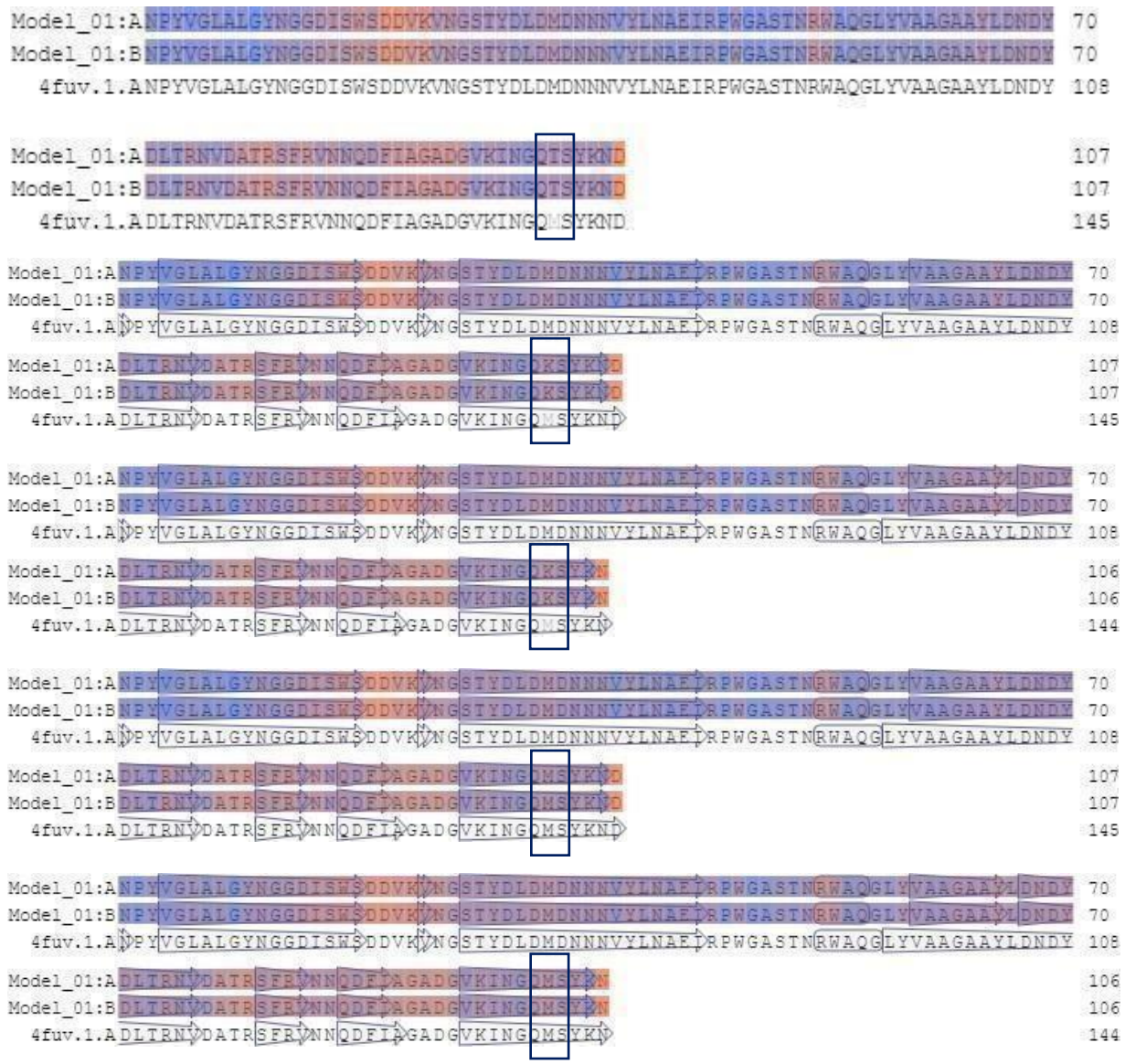
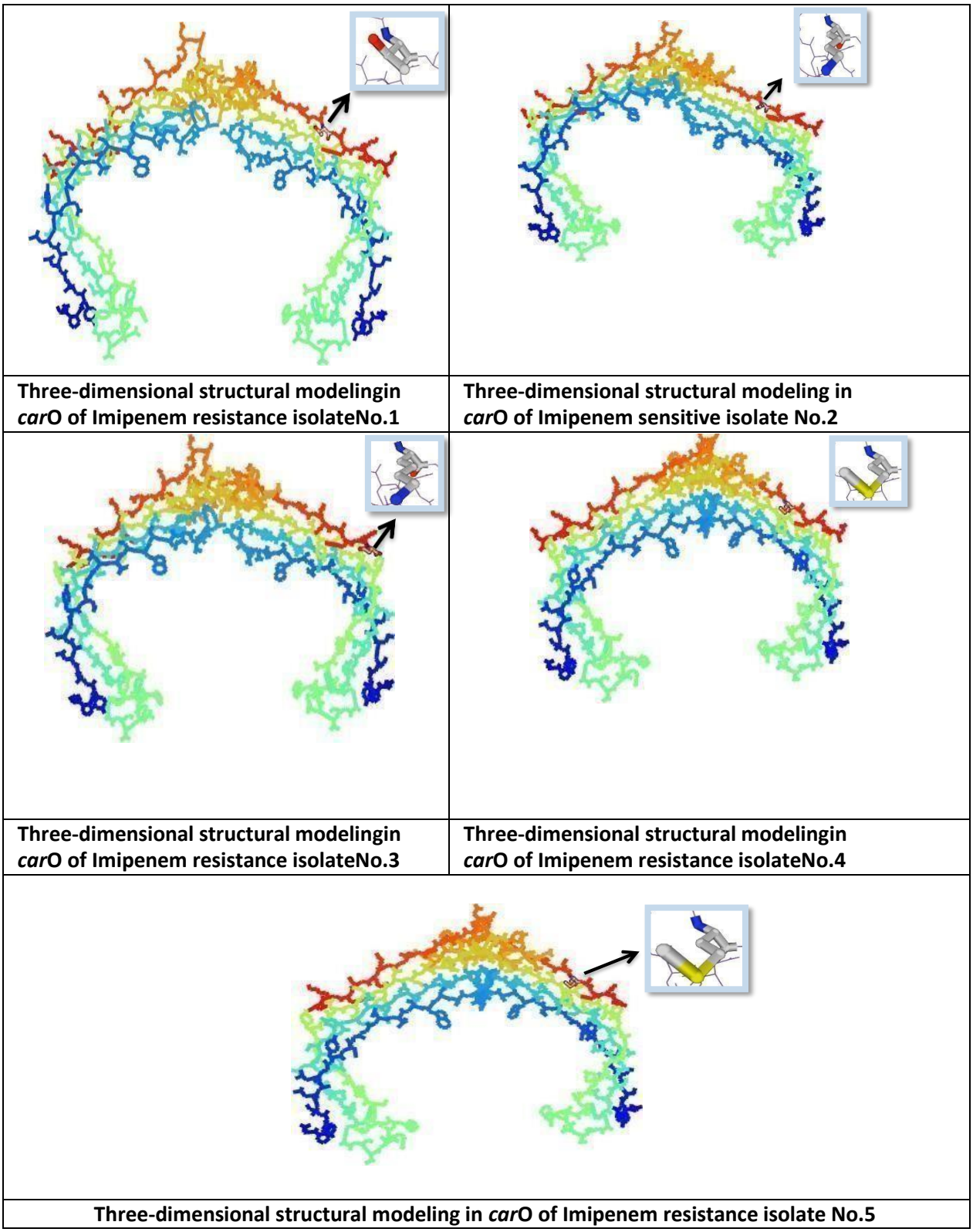


Figure (3-11) Multiple sequence alignment analysis of *car*O porin protein associated with imipenem resistance and sensitive *Acinetobacter baumannii* isolates as well as PBD:4rlb as a template(*car*O porin protein associated with imipenem resistance: peptide Chain A and Chain B). the showed some amino acids mutations between local imipenem resistance and sensitive *Acinetobacter baumannii* isolates and template strain



Figure(3-12) three dimentional structural modeling of *car*O protein in resistance and sensitive *Abaumannii* isolates.

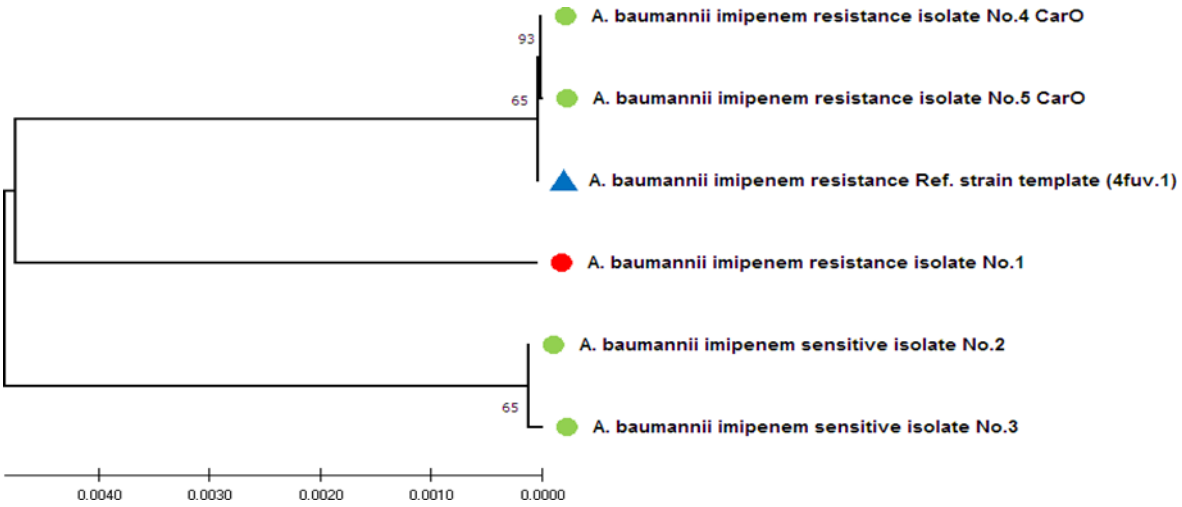


Figure (3.13): Phylogenetic tree analysis of *car*O porin protein associated with imipenem resistance and sensitive *Acinetobacter baumannii* isolates. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in(MEGA 6.0 version). The local *Acinetobacter baumannii* imipenem resistance isolates (*car*O.No.4 and *car*O. No.5) were showed closed genetic related to PBD:4rlb template imipenem resistance strain whereas, the local *Acinetobacter baumannii* imipenem resistance isolates(*car*O. No.1) and local *Acinetobacter baumannii* imipenem isolates (*car*O. No.2 and *car*O.No.3) were showed genetic different than PBD:4rlb template imipenem resistance strain.

Table (3.3) the Homology amino acid identity (%) between local imipenem resistance and sensitive *Acinetobacter baumannii* isolates and template strain.

Target amino acid sequence *car*O protein isolate No.	Accession number	Template strain	Identity	Amino acid substitution mutation
Imipenem resistance *car*O.No.1		4fuv.1: Crystal Structure of *car*O porin associated with imipenem resistance	99.07%	(K-M) lysine—methionine
Imipenem sensitive *car*O.No.2		4fuv.1: Crystal Structure of *car*O porin associated with imipenem resistance	99.07%	(K-M) lysine—methionine
Imipenem resistance *car*O.No.3		4fuv.1: Crystal Structure of *car*O porin associated with imipenem resistance	99.06%	(K-M) lysine—methionine
Imipenem resistance *car*O.No.4		4fuv.1: Crystal Structure of *car*O porin associated with imipenem resistance	100.00%	(M-M) methionine-methionine
Imipenem resistance *car*O.No.5		4fuv.1: Crystal Structure of *car*O porin associated with imipenem resistance	100.00%	(M-M) methionine-methionine

78- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.1 **compared with NCBI BLAST imipenem sensitive A. baumannii isolate.**

Score	Expect	Identities	Gaps	Strand
588 bits(318)	7e-173	320/321(99%)	0/321(0%)	Plus/Plus

```
Query  1    AACCCATATGTAGGTTTAGCATTGGGTTATAACGGCGGTGACATTTCTTGGTCTGATGAT  60
Sbjct  1    ............................................................  60

Query  61   GTAAAAGTCAATGGATCAACTTATGACCTTGATATGGATAATAACAACGTTTATTTAAAT  120
Sbjct  61   ............................................................  120

Query  121  GCTGAGATTCGTCCATGGGGTGCAAGCACTAACCGTTGGGCTCAAGGCTTATATGTAGCT  180
Sbjct  121  ............................................................  180

Query  181  GCTGGGGCGGCTTACCTTGATAACGATTATGATTTAACTCGTAACGTTGATGCGACTCGT  240
Sbjct  181  ............................................................  240

Query  241  TCATTCCGTGTAAATAACCAAGACTTTATTGCGGGTGCCGATGGTGTCAAAATTAACGGA  300
Sbjct  241  ............................................................  300

Query  301  CAAACGTCATATAAAAATGAT  321
Sbjct  301  ....A.................  321
```

79- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.2

Score	Expect	Identities	Gaps	Strand
593 bits(321)	2e-174	321/321(100%)	0/321(0%)	Plus/Plus

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Query  1    AACCCATATGTAGGTTTAGCATTGGGTTATAACGGCGGTGACATTTCTTGGTCTGATGAT  60
Sbjct  1    ............................................................  60

Query  61   GTAAAAGTCAATGGATCAACTTATGACCTTGATATGGATAATAACAACGTTTATTTAAAT  120
Sbjct  61   ............................................................  120

Query  121  GCTGAGATTCGTCCATGGGGTGCAAGCACTAACCGTTGGGCTCAAGGCTTATATGTAGCT  180
Sbjct  121  ............................................................  180

Query  181  GCTGGGGCGGCTTACCTTGATAACGATTATGATTTAACTCGTAACGTTGATGCGACTCGT  240
Sbjct  181  ............................................................  240

Query  241  TCATTCCGTGTAAATAACCAAGACTTTATTGCGGGTGCCGATGGTGTCAAAATTAACGGA  300
Sbjct  241  ............................................................  300

Query  301  CAAAAGTCATATAAAAATGAT  321
Sbjct  301  ....................  321
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80- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.3

Score	Expect	Identities	Gaps	Strand
571 bits(309)	7e-168	311/312(99%)	0/312(0%)	Plus/Plus

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Query  62   GTAAAAGTCAATGGATCAACTTATGACCTTGATATGGATAATAACAACGTTTATTTAAAT  121
Sbjct  61   ............................................................  120

Query  122  GCTGAGATTCGTCCATGGGGTGCAAGCACTAACCGTTGGGCTCAAGGCTTATATGTAGCT  181
Sbjct  121  ............................................................  180

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Sbjct  181  ............................................................  240

Query  242  TCATTCCGTGTAAATAACCAAGACTTTATTGCGGGTGCCGATGGTGTCAAAATTAACGGT  301
Sbjct  241  ............................................................A  300

Query  302  CAAAAGTCATAT  313
Sbjct  301  ............  312
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4- NCBI BLAST Multiple nucleotide sequence alignment analysis of *car*O of Imipenem resistance isolate No.4 compared with NCBI BLAST imipenem sensitive *Acinetobacter baumannii* isolates.

Score	Expect	Identities	Gaps	Strand
588 bits(318)	7e-173	320/321(99%)	0/321(0%)	Plus/Plus

```
Query  1    AACCCATATGTAGGTTTAGCATTGGGTTATAACGGCGGTGACATTTCTTGGTCTGATGAT  60
Sbjct  1    ............................................................  60

Query  61   GTAAAAGTCAATGGATCAACTTATGACCTTGATATGGATAATAACAACGTTTATTTAAAT  120
Sbjct  61   ............................................................  120

Query  121  GCTGAGATTCGTCCATGGGGTGCAAGCACTAACCGTTGGGCTCAAGGCTTATATGTAGCT  180
Sbjct  121  ............................................................  180

Query  181  GCTGGGGCGGCTTACCTTGATAACGATTATGATTTAACTCGTAACGTTGATGCGACTCGT  240
Sbjct  181  ............................................................  240

Query  241  TCATTCCGTGTAAATAACCAAGACTTTATTGCGGGTGCCGATGGTGTCAAAATTAACGGA  300
Sbjct  241  ............................................................  300

Query  301  CAAACGTCATATAAAAATGAT  321
Sbjct  301  ....A.................  321
```

5- NCBI BLAST Multiple nucleotide sequence alignment analysis of *car*O of Imipenem resistance isolate No.5 compared with NCBI BLAST imipenem sensitive *Acinetobacter baumannii* isolates.

Score	Expect	Identities	Gaps	Strand
593 bits(321)	2e-174	321/321(100%)	0/321(0%)	Plus/Plus

```
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Sbjct  1    ............................................................  60

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Sbjct  61   ............................................................  120

Query  121  GCTGAGATTCGTCCATGGGGTGCAAGCACTAACCGTTGGGCTCAAGGCTTATATGTAGCT  180
Sbjct  121  ............................................................  180

Query  181  GCTGGGGCGGCTTACCTTGATAACGATTATGATTTAACTCGTAACGTTGATGCGACTCGT  240
Sbjct  181  ............................................................  240

Query  241  TCATTCCGTGTAAATAACCAAGACTTTATTGCGGGTGCCGATGGTGTCAAAATTAACGGA  300
Sbjct  241  ............................................................  300

Query  301  CAAAAGTCATATAAAAATGAT  321
Sbjct  301  ....................  321
```

Table (3.4) Numbers and types of mutations in nucleotide sequence between local imipenem resistance and sensitive *Acinetobacter baumannii* isolates and template strain

Nucleotide sequence *car*O protein isolate No.	Accession number	No of Mutation	Nucleotide substitution mutation	Identity
Imipenem resistance *car*O.No.1		1	Missense mutation (T/A) (T/C)	99.38%
Imipenem sensitive *car*O.No.2		1	Missense mutation (T/A) and (T/C)	99.38%
Imipenem resistance *car*O.No.3		1	Missense mutation (T/A)	99.68%
Imipenem resistance *car*O.No.4		1	Missense mutation (T/A)	99.69%
Imipenem resistance *car*O.No.5		-	Missense mutation (T/A)	99.69%

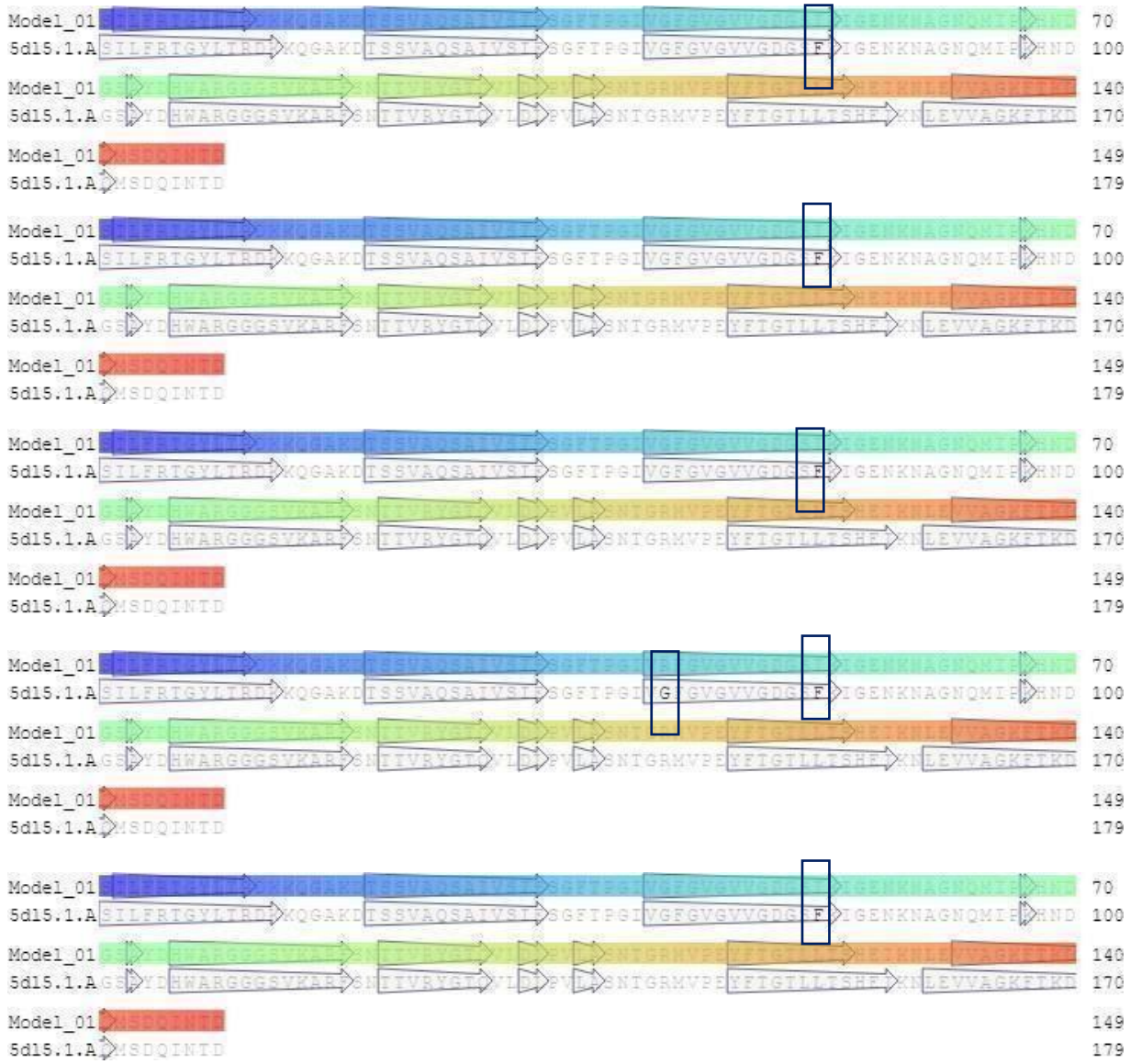
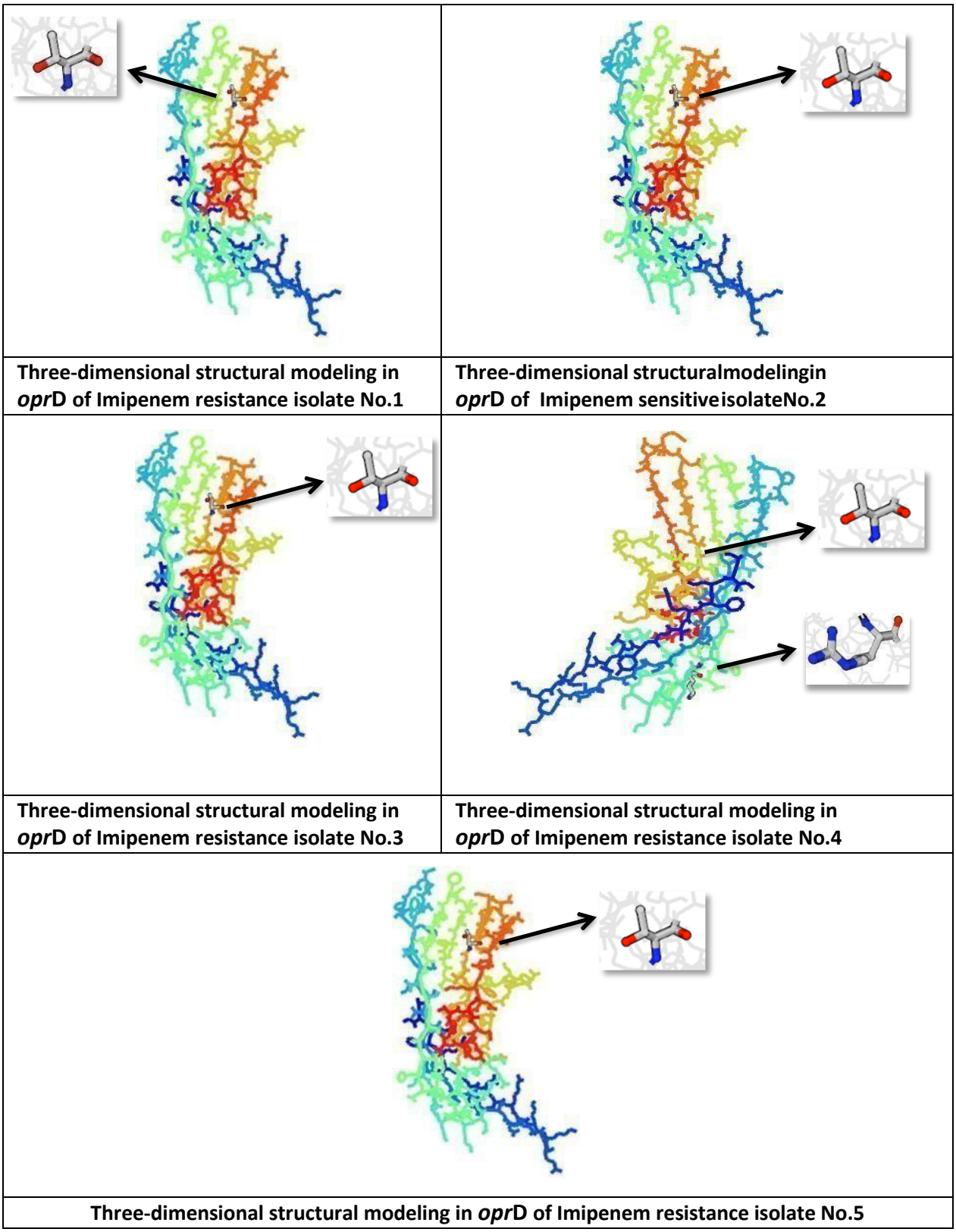


Figure (3.14): Multiple sequence alignment analysis of *opr*D protein associated with imipenem resistance and sensitive *Acinetobacter baumannii* isolates as well as PBD: 5dl5.1 as atemplate(*opr*D protein). the showed some amino acids mutations between local imipenem resistance and sensitive *Acinetobacter baumannii* isolates and template strain.



Figure(3-15)) three dimentional structural modeling of *opr*Dprotein in resistance and sensitive *A. baumannii* isolates.

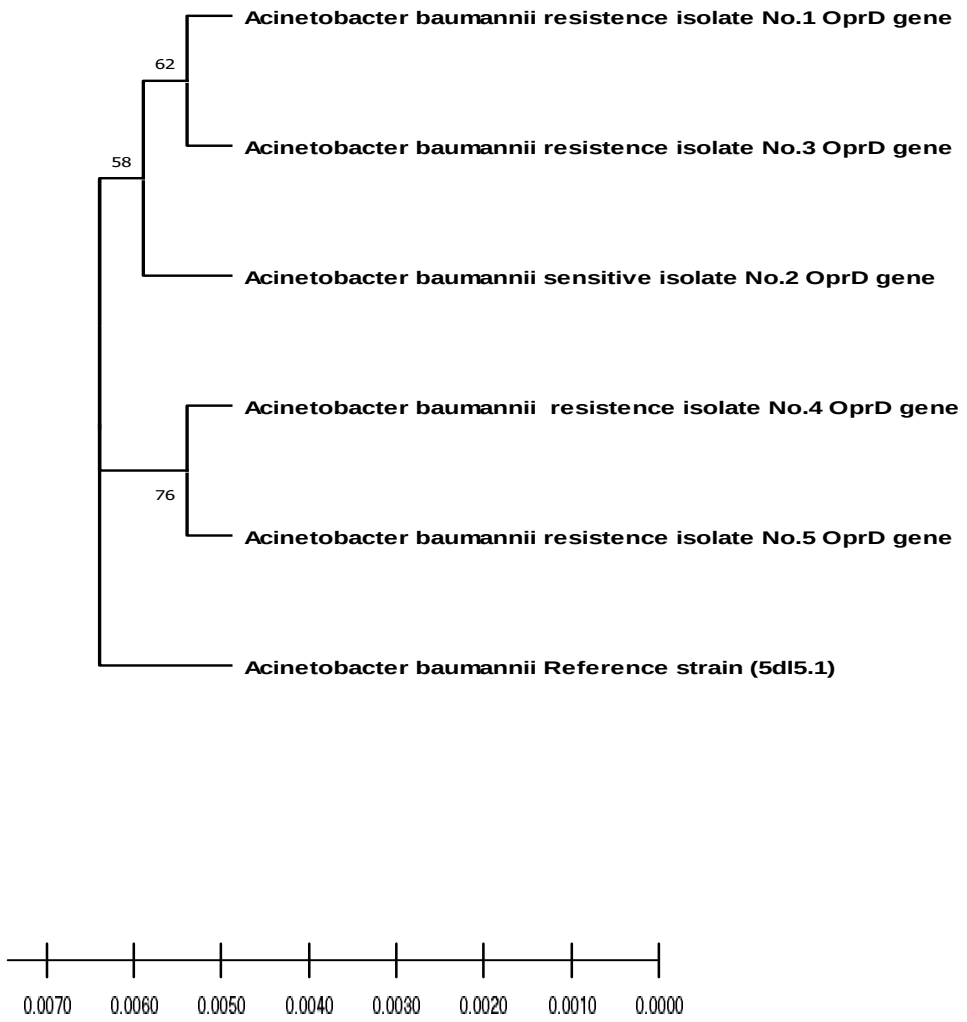


Figure (3.16): Phylogenetic tree analysis of *opr*D protein associated with imipenem resistance and sensitive *Acinetobacter baumannii* isolates. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).The local *Acinetobacter baumannii* imipenem resistance isolates and local *Acinetobacter baumannii* imipenem sensitive isolates were showed genetic different than PBD: 5dl5.1 *opr*Dprotein template strain.

Table (3.5) the Homology amino acid identity (%) between local imipenem resistance and sensitive *Acinetobacter baumannii* isolates and template strain.

Target amino acid sequence *opr*D protein isolate No.	Accession number	Template strain	Identity	Amino acid substitution mutation
Imipenem resistance *opr*D.No.1		5dl5.1.A Membrane protein Crystal structure of Acinetobacter baumannii	99.33%	(F-I) Phenylalanine — isoleucine
Imipenem sensitive *opr*D.No.2		5dl5.1.A Membrane protein Crystal structure of Acinetobacter baumannii	99.33%	(F-I) Phenylalanine — isoleucine
Imipenem sensitive *opr*D.No.3		5dl5.1.A Membrane protein Crystal structure of Acinetobacter baumannii	99.33%	(F-I) Phenylalanine — isoleucine
Imipenem resistance *opr*D.No.4		5dl5.1.A Membrane protein Crystal structure of Acinetobacter baumannii	98.66%	(F-I) Phenylalanine — isoleucine G-R) glycine —arginine
Imipenem resistance *opr*D.No.5		5dl5.1.A Membrane protein Crystal structure of Acinetobacter baumannii	99.33%	(F-I) Phenylalanine — isoleucine

Table (3.6) Numbers and types of mutations in nucleotide sequence between local imipenem resistance and sensitive *Acinetobacter baumannii* isolates and template strain.

Nucleotide sequence *opr*D protein isolate No.	Accession number	No of Mutation	Nucleotide substitution mutation	Identity
Imipenem resistance *opr*D.No.1		1	Missense mutation (T/A)	99.78%
Imipenem sensitive *opr*D.No.2		1	Missense mutation (T/A)	99.78%
Imipenem resistance *opr*D.No.3		1	Missense mutation (T/A)	99.78%
Imipenem resistance *opr*D.No.4		2	Missense mutation (T/A) (T/C)	98.88%
Imipenem resistance *opr*D.No.5		1	Missense mutation (T/A)	99.78%

89- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.1

Score	Expect	Identities	Gaps	Strand
821 bits(444)	0.0	446/447(99%)	0/447(0%)	Plus/Plus

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Query  121  GGTTTTGGTGTTGGTGTTGTTGGTGATGGTTCATTTAAAATTGGTGAAAATAAAAATGCA  180
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Sbjct  421  .........................  447
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90- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.2

Score	Expect	Identities	Gaps	Strand
821 bits(444)	0.0	446/447(99%)	0/447(0%)	Plus/Plus

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Query  121  GGTTTTGGTGTTGGTGTTGTTGGTGATGGTTCATTTAAAATTGGTGAAAATAAAAATGCA  180
Sbjct  121  ................................A...........................  180

Query  181  GGTAATCAAATGATTCCAAAACATAATGATGGTTCAGCATATGATCATTGGGCACGTGGT  240
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Query  241  GGTGGTTCAGTTAAAGCACGTTTTTCAAATACTACTGTTCGTTATGGTACTCAAGTTTTA  300
Sbjct  241  ............................................................  300

Query  301  GATTTACCAGTTTTAGCATCAAATACTGGTCGTATGGTTCCAGAATATTTTACTGGTACT  360
Sbjct  301  ............................................................  360

Query  361  TTATTAACTTCACATGAAATTAAAAATTTAGAAGTTGTTGCAGGTAAATTTACTAAAGAT  420
Sbjct  361  ............................................................  420

Query  421  CAAATGTCAGATCAAATTAATACTGAT  447
Sbjct  421  .........................  447
```

91- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.3

Score	Expect	Identities	Gaps	Strand
821 bits(444)	0.0	446/447(99%)	0/447(0%)	Plus/Plus

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Sbjct  1    ............................................................  60

Query  61   TCATCAGTTGCACAATCAGCAATTGTTTCAATTGAATCAGGTTTTACTCCAGGTATTGTT  120
Sbjct  61   ............................................................  120

Query  121  GGTTTTGGTGTTGGTGTTGTTGGTGATGGTTCATTTAAAATTGGTGAAAATAAAAATGCA  180
Sbjct  121  ................................A...........................  180

Query  181  GGTAATCAAATGATTCCAAAACATAATGATGGTTCAGCATATGATCATTGGGCACGTGGT  240
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Query  241  GGTGGTTCAGTTAAAGCACGTTTTTCAAATACTACTGTTCGTTATGGTACTCAAGTTTTA  300
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Query  301  GATTTACCAGTTTTAGCATCAAATACTGGTCGTATGGTTCCAGAATATTTTACTGGTACT  360
Sbjct  301  ............................................................  360

Query  361  TTATTAACTTCACATGAAATTAAAAATTTAGAAGTTGTTGCAGGTAAATTTACTAAAGAT  420
Sbjct  361  ............................................................  420

Query  421  CAAATGTCAGATCAAATTAATACTGAT  447
Sbjct  421  .........................  447
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92- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.4

Score	Expect	Identities	Gaps	Strand
798 bits(432)	0.0	445/447(98%)	0/447(0%)	Plus/Plus

```
Query  1    TCAATTTTATTTCGTACTGGTTATTTAACTCGTGATAAAAAACAAGGTGCAAAAGATACT  60
Sbjct  1    ............................................................  60

Query  61   TCATCAGTTGCACAATCAGCAATTGTTTCAATTGAATCAGGTTTTACTCCAGGTATTGTT  120
Sbjct  61   ............................................................  120

Query  121  GGTTTTGGTGTTGGTGTTGTTGGTGATGGTTCATTTAAAATTGGTGAAAATAAAAATGCA  180
Sbjct  121  ................................A...........................  180

Query  181  GGTAATCAAATGATTCCAAAACATAATGATGGTTCAGCATATGATCATTGGGCACGTGGT  240
Sbjct  181  ............................................................  240

Query  241  GGTGGTTCAGTTAAAGCACGTTTTTCAAATACTACTGTTCGTTATGGTACTCAAGTTTTA  300
Sbjct  241  ............................................................C...  300

Query  301  GATTTACCAGTTTTAGCATCAAATACTGGTCGTATGGTTCCAGAATATTTTACTGGTACT  360
Sbjct  301  ............................................................  360

Query  361  TTATTAACTTCACATGAAATTAAAAATTTAGAAGTTGTTGCAGGTAAATTTACTAAAGAT  420
Sbjct  361  ............................................................  420

Query  421  CAAATGTCAGATCAAATTAATACTGAT  447
Sbjct  421  .........................  447
```

93- NCBI BLAST Multiple nucleotide sequence alignment analysis of A. baumannii isolate No.5

Score	Expect	Identities	Gaps	Strand
821 bits(444)	0.0	446/447(99%)	0/447(0%)	Plus/Plus

```
Query  1    TCAATTTTATTTCGTACTGGTTATTTAACTCGTGATAAAAAACAAGGTGCAAAAGATACT  60
Sbjct  1    ............................................................  60

Query  61   TCATCAGTTGCACAATCAGCAATTGTTTCAATTGAATCAGGTTTTACTCCAGGTATTGTT  120
Sbjct  61   ............................................................  120

Query  121  GGTTTTGGTGTTGGTGTTGTTGGTGATGGTTCATTTAAAATTGGTGAAAATAAAAATGCA  180
Sbjct  121  ................................A...........................  180

Query  181  GGTAATCAAATGATTCCAAAACATAATGATGGTTCAGCATATGATCATTGGGCACGTGGT  240
Sbjct  181  ............................................................  240

Query  241  GGTGGTTCAGTTAAAGCACGTTTTTCAAATACTACTGTTCGTTATGGTACTCAAGTTTTA  300
Sbjct  241  ............................................................  300

Query  301  GATTTACCAGTTTTAGCATCAAATACTGGTCGTATGGTTCCAGAATATTTTACTGGTACT  360
Sbjct  301  ............................................................  360

Query  361  TTATTAACTTCACATGAAATTAAAAATTTAGAAGTTGTTGCAGGTAAATTTACTAAAGAT  420
Sbjct  361  ............................................................  420

Query  421  CAAATGTCAGATCAAATTAATACTGAT  447
Sbjct  421  .........................  447
```

Conclusion :

1. In present study, All *A. baumannii* isolates were found to be resistance nearly to all antibiotics.

2. 16SrRNA of *A. baumannii* isolates Used as simple method for identification.

3. In this study, agar dilution method, was more reliable, cheap, rapid and effective method for estimation of imipenem resistance in *A . baumannii* isolates.

4. Polymerase chain reaction technique was found to be simple and useful tool for detection of outer membrane protiens *car*O and*opr*D.

5. Sequencing of *car*O and *opr*D gene PCR product, revealed many mutations found in *A . baumannii*

Recommendation :

1. Powerful prevention and control of nosocomial infection is particularly important to avoid the situation of no medicine available.
2. clinical management of the use of antibiotics should be strengthened to slow or reduce the emergence of drug resistance.
3. Additionally, aseptic operation and disinfection isolation system should be strictly implemented to prevent the spread of drug-resistantbacteria.
4. Further experiments such as quantitative analysis of OMPs mRNA expression and structural and functional research of OMPs between IRAB and ISAB are necessary for the future.
5. Using bioinformatics to predict the resistance phenotype in *A.baumannii* local isolates.

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