Master Program in Clinical Laboratory Science

Molecular characterization, genotyping and Combination susceptibility testing for colistin, rifampin and fosfomycin of *Acinetobacter baumannii*

M.Sc. Thesis

Sanaa Ghalib Alkhatib

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Molecular characterization, genotyping and Combination susceptibility testing for colistin, rifampin and fosfomycin of *Acinetobacter baumannii*

التوصيف الجزيئي والتنميط الجيني والجمع في اختبارات الحساسية بين الكوليستين والريفامبين والفوسفوميسين على الأسينيتوباكتر بوماني

Submitted by

Sanaa Ghalib Alkhatib

1195003

Supervisor

Dr. Mohammad A. Farraj

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This thesis was successfully defended and approved on January 20, 2022

Committee Members	Signature
Mohammad Farraj, Ph.D., Supervisor	_____
Emilia Rapocciolo, Ph.D., Internal Examiner	_____
Hani shtaya, Ph.D., Internal Examiner	_____

Dedication

There are a number of people without whom this thesis might not have been written, and to whom I am greatly indebted.

This thesis is wholeheartedly dedicated to the Almighty God, thank you for the strength, power of mind and protection and for giving us a healthy life.

My beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide their moral, spiritual and emotional support.

My beloved grandma Fatima who always believed in me, may her soul rest in peace.

My sisters and brothers, your patience and love helped me overcome all obstacles that I came across.

I would also like to dedicate this thesis with deepest gratitude to Smaheen for his presence, support and love that I never failed to find. Dedication goes to his family, too.

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Finally, I like to place on record my genuine appreciation to anyone and everyone who directly and indirectly have lent me their time, wise words and encouragement in this adventure.

Sana'a Alkhatib

Declaration

I hereby certify that all work provided in this thesis, unless otherwise referenced, is my own work, submitted in partial fulfillment of the requirements for the master degree in clinical laboratory science at Birzeit University. This thesis has not been submitted elsewhere for any other degree or qualification or any other university or institute of learning. I am aware of and understand the university's policy on plagiarism.

Electronic signature: Sanaa G. S. Alkhatib

Date: January 2022

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List of Abbreviations

WHO	World Health Organization
ICU	Intensive Care Unit
MIC	Minimal Inhibitory Concentrations
MBC	Minimal Bactericidal Concentrations
RAPD	Randomly amplified polymorphic DNA
PCR	Polymerase Chain Reaction
COPD	Chronic Obstructive Pulmonary Disease
OMP	Outer Membrane Protein
LPS	Lipopolysaccharides
PBPs	Penicillin-binding proteins
ESBL	Extended Spectrum beta- Lactamase
MRSA	Methicillin Resistant S. aureus
MBL	Metallo beta Lactamases
MDR	Multi Drug Resistant
EPS	Extracellular Polymeric Substances
TAE	Tris-Acetate-EDTA
PBS	Phosphate Buffered Saline
CLSI	Clinical Laboratory Standard Institute
FIC	Fractional Inhibitory Concentration
FICi	Fractional Inhibitory Concentration Index

DNTP	Deoxynucleotide Triphosphate
CPO	The capsular exopolysaccharide
gyrA	DNA gyrase subunit A
parC	Topoisomerase IV subunit C
VIM	The Verona Integron encoded Metallo-beta-lactamase
IMP	Imipenem metallo-beta-lactamase
NDM	New Delhi Metallo-beta-lactamase
SIM	Seoul Imipenemase
OXAs	Oxacillinases
MHB	Mueller Hinton Broth
TSB	Tryptone Soy broth
DNTP	Deoxynucleotide triphosphate
COPD	Chronic obstructive pulmonary disease
GC	Guanine-cytosine
UTIs	Urinary tract infections
BHI	Brain Heart Infusion
DW	Distilled Water
dH_2O	Deionized Water
EDTA	Ethylenediaminetetraacetic acid
DMSO	Dimethylsulfoxide
BSA	Bovine Serum Albumin

EDTA.Na2.2H2O. Disodium salt of EDTA

الملخص

تم جمع 29 عزلة سريرية من ال *Acinetobacter baumannii* من مجمع فلسطين الطبي في رام الله ومؤسسة المقاصد الخيرية في القدس. كانت جميع العزلات مقاومة للعديد من الأدوية بما في ذلك مقاومتها الكاربابينيمات. وقد تم تحديد أقل تركيز مثبط (MIC) من المضادات الحيوية الكوليستين والريفامبين والفوسفوميسين لجميع العزلات. بالإضافة إلى ذلك، تم فحص مزيج من كوليستين مع ريفامبين ومزيج من كوليستين مع فوسفوميسين لتحديد احتمال وجود تآزر بينهما. علاوة على ذلك، فقد تم تقييم آلية مقاومة البكتيريا لمجموعة الكاربابينيمات بواسطة تفاعل البوليمير المتسلسل (polymerase chain reaction) لجينات بيتا لاكتام (carbapenemases) مصنفة في مجموعة آمبلر A و B و C و D. وقد تم اختيار الجينات التالية: *bla*KPC-2, *bla*NDM-1, *bla*OXA-23, *bla*OXA-24, *bla*OXA-48, *bla*OXA-51, *bla*OXA-58 . بخصوص تكوين البيوفيلم، فقد تم عمله على العزلات النامية في أنابيب وصبغها بالبنفسج الكريستالي وكذلك بالسفرانين و قد تم تحديد النميط الجيني لعزلات *A. baumannii* بواسطة التضخيم العشوائي للحمض النووي متعدد الأشكال بتفاعل البوليمير المتسلسل Random Amplified Polymorphic DNA- polymerase chain reaction (RAPD-PCR).

كان MIC للكوليستين 2 ug/mL ≥ بنسبة 89.7٪ (29/26) في العزلات مما يثبت حساسيتها للكوليستين ، و وجدنا ٣ عزلت كان MIC لها 4≥ ug/mL بنسبة 10.3% (29/3) أي انها مقاومة للكوليستين. أظهر مزيج الكوليستين مع الريفامبين وجود تآزر في 5 عزلات حيث كان مؤشر التثبيط الجزئي (Fractional Inhibitory Index) اقل من 0.5 (FIC < 0.5)، اما بقية العزلات فكان التأثير في تقليل ال MIC بنسبة كبيرة مما يساعد في تخفيف الضغط الانتقائي ومنع تطوير مقاومة من البكتيريا ضد الكوليستين. أدى هذا التأثير التآزري إلى تقليل MICsللكوليستين بحيث ان MIC≥4 ug/ml أصبحت MIC=0.25 ug/ml. كذلك الامر للريفامبين بحث كانت MIC>4 ug/ml أصبحت MIC<1 ug/ml ، فتحولت العزلات المقاومة الى عزلات حساسة.

بالنسبة للفوسفومايسين، كانت MICs> 64 ug/ml ولم تكن أي من العزلات حساسة له، لكن بعد دمجه مع الكوليستين أصبحت جميع العزلات ما عدا واحدة حساسة للفوسفوميسين (MIC ≤ 4 ug/ml). كما كان تأثير

الدمج فعالا اذ أدى الى تقليل تركيز MIC للكوليستين من 4≥ ug/ml قبل الدمج الى 1.0 ug/ml بعده وهذا تم رؤيته في واحدة من العزلات، اذ انه قد يؤثر إيجابيا باستخدام المزيج للعلاج ومنع تطور مقاومة البكتيريا للكوليستين.

لدراسة حمل البكتيريا لبعض جينات البيتا لاكتام وعلاقتهم بمقاومتها للكاربابينيمات، فقد أظهرت النتائج وجود جين $bla_{\text{OXA-23}}$ بنسبة 100% و جين $bla_{\text{OXA-48}}$ بنسبة 93.1%، وجين $bla_{\text{NDM-1}}$ بنسبة 79.3%، وجين $bla_{\text{KPC-2}}$ بنسبة 48.3%.

حملت جميع العزلات السريرية الجينات $bla_{\text{OXA-23}}$ و $bla_{\text{OXA-51}}$ بمعدل 100٪ وهذا قريب جدًا من النسب المذكورة في الأدبيات ، كما كان جين$bla_{\text{OXA-48}}$ موجودًا في 93.1٪ من العزلات وهذه كانت أيضا نسبة قريبة جدا من الادبيات. لكن لم تحمل أي من العزلات $bla_{\text{OXA-24}}$ و $bla_{\text{OXA-58}}$ في هذا البحث، وكذلك كانت نسب حمل هذين الجينين منخفضة في الأدبيات. وحملت العزلات المفحوصة جينات $bla_{\text{NDM-1}}$ و $bla_{\text{KPC-2}}$ بمعدلات عالية في بحثنا، ولكن بمعدلات منخفضة في الأدبيات.

لقد توصلنا ان جين $bla_{\text{OXA-23}}$ هو الجين السائد في العزلات المفحوصة لدينا متبوعا بجين $bla_{\text{OXA-48}}$ من ثم جين $bla_{\text{NDM-1}}$ وأخيرا جين $bla_{\text{KPC-2}}$.

بالنسبة للبيوفيلم فقد كانت نسبة تكوينه من عزلات *A. baumannii* هي 65.5٪. وقد كان مثيرا للاهتمام ان جميع العزلات التي كونت البيوفيلم كانت تحمل جين $bla_{\text{NDM-1}}$ بمعدل 100٪ (19/19) والتي كانت تحمل جين $bla_{\text{KPC-2}}$ كانت نسبة البيوفيلم فيها 63.2٪ (19/12).

تم وضع نتائج RAPD-PCR في 9 فئات مختلفة بناءً على حجم وعدد الحزم الناتجة (النطاقات). معظم العزلات صنفت في المجموعة الأولى بنسبة 58.6٪ (17/29) وكان 58.8% منها ماخوذ من مرضى العناية المكثفة، اما بقية العزلات فقد وزعت على بقية المجموعات وكان عدد الحزم يتراوح من 2 الى 5 حزم. كانت نسبة الحزمة ال 1500 bp موجودة بأعلى نسبة (28/29) 96.6%، تبعتها حزمة ال bp2500 وحزمة 1000 bp بنسبة 93.1% (27/29) لكل منهما. وحزمة ال 600 bp بنسبة 75.9% (22/29) ، ووضعت بقية العزلات (650, 500, 400, and 250 bp) في المجموعات الأخرى بنسب تتراوح من 3.4% الى 6.9% .

في الختام، *A. baumannii* من الجراثيم الشديدة العدوى والتي تسبب العديد من الالتهابات في جسم الانسان وتودي بحياة الكثير من المرضى من كبار السن وذوي نقص المناعة المكتسبة وغيرهم من المقيمين في غرف العناية المكثفة، وخوفا من نشوء جراثيم مقاومة للكوليستين المضاد الحيوي الأخير لعلاج هذه الجرثومة فمن المفضل عدم استعماله لوحده بل مع مضادات حيوية أخرى مثل ريفامبين وفوسفومايسين حيث أظهرت نتائج هذه الدراسة نجاح استعمال هذه المضادات معا.

Abstract

A total of 29 clinical isolates of *Acinetobacter baumannii* were collected from Palestine Medical Complex in Ramallah and Al-Makassed Charitable Foundation in Jerusalem. All the isolates were multidrug resistant including resistance to carbapenems. The minimal inhibitory concentration (MIC) for all isolates was determined for colistin, rifampin and fosfomycin. In addition, combination of colistin-rifampin and colistin-fosfomycin were tested using the checkerboard method to determine the possible presence of synergy. Furthermore, the mechanism of resistance for carbapenems was evaluated by Polymerase Chain Reaction (PCR). Genes encoding for beta lactamases (carbapenemases) from Ambler group A, B, C, and D were selected and tested by PCR. The following beta lactamase genes were tested; *bla*$_{\text{KPC-2}}$ (group A), *bla*$_{\text{NDM-1}}$ (group B), and *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-51}}$, and *bla*$_{\text{OXA-58}}$ (Group D). Biofilm formation was also determined after growing the isolates in tubes and stained with crystal violet and confirmed by repeating the experiment and staining with safranine. The epidemiologic genotyping of the *A. baumannii* isolates was determined by the Random Amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR) fingerprinting method.

The MIC for colistin was $\leq$ 2 ug/mL in 89.7% (26/29) indicating that these isolates were susceptible to colistin. However, an MIC of $\geq$4 ug/mL was found in 10.3% of the isolates (3/29). The combination of colistin-rifampin showed the presence of synergy

in 5 isolates where the Fractional Inhibitory Index (FIC) for the combination was <0.5, other isolates showed additive effect that could help relief the selective pressure and prevent the evolution of colistin resistance. This synergistic effect has resulted in the reduction of the MICs of colistin (MIC≥4 became 0.25)

and rifampin (MIC>4 became <1) when combined with each other's where resistant isolates turned to sensitive. As for fosfomycin, the MICs were >64 ug/mL and none of the isolates tested were susceptible to fosfomycin. However, the MIC results showed that most of isolates became fosfomycin susceptible with an MIC of ≤4 ug/ml after combination with colistin (MIC >64 became ≤4)**.** There was one isolate only that remained resistant to fosfomycin after combination with colistin at MIC >64. It is also interesting to note that the MIC result showed that one of the isolate that was colistin resistance (MIC=32ug/ml) became sensitive to colistin when combined with fosfomycin (MIC was reduced to 1 ug/ml). There was no synergy detected in colistin - fosfomycin combination. However, the effect was additive (FIC is 0.5-4). Since the MICs for fosfomycin were significantly lowered, it may be useful to use it in combination with colistin to treat infections caused by *A. baumannii* which may prevent evolution of resistance to colistin due to selective pressure.

The mechanisms of carbapenem resistance in *A. baumannii* are the acquisition of Class D beta lactamases OXa-23, OXA51 and OXa-48, followed by Class B metallo beta lactamase NDM-1 then Class A KPC-2. All of the clinical isolates carried the genes

that encoded for OXA-23 and OXA-51 at rate of 100% and this is too close to literature. OXA-48 was present in 93.1% of the isolates, a finding that is also very close to that reported in literature. However, OXA-24 and OX-58 were not found in our research, but was found in low rates in literature. NDM-1 and KPC-2 was found in high rates in our research (79.3% and 48.3%), but in low rates in literature. We found out that OXA-23 is the most prominent OXA-type in our study, followed by OXA-48, NDM-1 and KPC-2, respectively.

Biofilm was formed by 65.5% of the *A. baumannii* isolates tested. It was interesting to note that all *A. baumannii* isolates that formed a biofilm are positive for $bla_{\text{NDM-1}}$ and most of them has the $bla_{\text{KPC-2 gene}}$ .In the absence of $bla_{\text{NDM-1}}$, no biofilm was formed where the presence of $bla_{\text{NDM-1}}$ gene in isolates that formed biofilm has a rate of 100% (19/19) .The presence of $bla_{\text{KPC-2}}$ gene in isolates that formed biofilm has a rate of 63.2% (12/19). Biofilm formation could be correlated with the presence of $bla_{\text{NDM-1}}$.

The results of the RAPD-PCR were put in 9 different classes based on the size and number of these bands. Most of the isolates (58.6%, 17/29) were located in one main classe (I), the remaining isolates were placed in separate classes. It is of interest to note that 58.8% (10/17) of the isolates in class I pattern came from the intensive care unit (ICU). Our data showed that four main bands of 2500, 1500, 1000 and 600 were observed in the gel, only 5 isolates had one different band than these mentioned above (650, 500, 400,250 bp). The band of 2500 bp and 1000 bp have been seen in 93.1%

(27/29) of the isolates, the band of 1500 bp has the highest rate of 96.6% (28/29) among the isolates and the band of 600 bp has a rate of 75.9% (22/29). The band of 500bp has a rate of 6.9% (2/29) while bands 400, 650 and 250 bp each has a rate of 3.4% (1/29).

In conclusion, *A. baumannii* is an organism that causes many infections and claims the lives of many patients. It has evolved to become multidrug resistant and can only be treated with colistin. The emergence of colistin resistant isolates has been observed in this research and reported in literature. It is of utmost importance to implement measures to prevent the emergence of colistin resistance in this pathogen. We recommend based on our finding to use combination therapy such as rifampin which proved to be synergistic when combined with colistin. Combination with fosfomycin is also recommended where reduction in MIC was observed.

Chapter I

I.1 Background

Acinetobacter baumannii has been described as Gram negative, non-fermentative, nonmotile, obligate aerobic coccobacilli. Since its discovery in 1911 by the Dutch microbiologist Beigerinck, Acinetobacter became recognized as a significant pathogen for causing nosocomial infections during the 1970s particularly in the intensive care units [Towner, 2009]. However, it was susceptible to most available antibiotics at that time and easily treated. In the 1980s, In the 1980s, an initial landmark classification based on DNA-DNA hybridization helped to group the *Acinetobacter* genus and distinguished 12 DNA groups or genospecies, some of them were given formal names like *A. baumannii, A. haemolyticus, A. calcoaceticus, A. junii, A. johnsonii, and A. lwoffii*. As of 2019 and according to a review published by Vijayakumar et al., *Acinetobacter* has 59 species, where 15 of them have a tentative description and 11 have defined names. *Acinetobacter* species that are pathogenic and associated with human diseases includes *A. baumannii, A. nosocomialis, A. pittii, A. dijkshoorniae and A. seifertti.* [Moubareck and Halat, 2020, Vijayakumar et al., 2019]. Since then, *A. baumannii* has emerged as a significant pathogen that continuously developed resistance to antibiotics [Smiline and Priyadharsini, 2019]. The World Health

Organization (WHO) has declared that *A. baumannii* is a serious pathogen that requires high priority because it is considered as significant threat to human health and the need for new antibiotics is urgent [WHO, 2019].

A. baumannii became notorious in causing nosocomial infections particularly in the intensive care units. The most commonly affected patients were the ones suffering from burns and those requiring mechanical respiratory devices (ventilator). Risk factors predisposing infections by this pathogen were immunosuppression, chronic obstructive pulmonary disease (COPD) and diabetes [Moubareck and Halat, 2020, Gong et al., 2021]. In immunocompetent individuals, *A. baumannii* poses no risks other than colonizing the skin without causing infections [Al-Anazi and Al-Jasser 2014].

The scope of infections caused by *A. baumannii* in the immunocompromised patients in the ICU include ventilator-associated pneumonia, skin, soft-tissue infections, meningitis, urinary tract infections, wound infections, burn infections, blood infection, endocarditis, and surgical site infections [Al-Anazi Al-Jasser 2014]. It carries several virulence factors which include adhesion and attachment on dry surfaces, ability to obtain nutrients as iron, ability to enzymatically destroy host tissues and biofilm formation [Zeighami et al., 2019].

Biofilm formation is a process where bacteria live and aggregate together in a community. The bacterial adherence in the biofilm is due to the production of matrix composed of extracellular DNA, proteins, and polysaccharides. Biofilm formation is a

significant process that protects the bacteria from disinfectants, inhibit antibiotics from reaching their targets and enhance persistence of the organism. Biofilm formation in the urinary bladder causes recurrent bacterial infection since the antibiotics used for treatment will inhibit the growth or kill planktonic forms. *A. baumannii* biofilm formation plays a significant role in bacterial colonization. Biofilm forming cells differ from the planktonic cells. Biofilm is a virulence factor that contributes to persistence and resistance to disinfectants and antibiotics [Gedefie et al., 2021].

The evolution of multi drug resistant (MDR) *A. baumannii* is a main concern for health care. According to CDC, MDR bacterial isolates are resistant to at least three different classes of antibiotics. There are several mechanisms where *A. baumannii* have to combat the antibiotics which include enzymatic and non-enzymatic.

The aims of this research have been focused on carbapenem resistant *A. baumannii* isolates. We used broth microdilution method to determine the minimal inhibitory and bactericidal concentrations (MIC, and MBC) to colistin, the ultimate therapeutic agent. We also tried to determine the presence of synergistic effects when colistin was combined at different concentrations with other antimicrobial agents. In addition, it has been known that the primary mechanisms of resistance of *A. baumannii* to carbapenems has been mediated by Ampler class D beta lactamases. Therefore, we determined by PCR which class was predominant among the tested isolates. The results can provide information about the correlation of the isolates with beta lactamases and may provide

geographic distribution of these enzymes in this region. We evaluated biofilm formation among the isolates by using the crystal violet and safranine dyes. Finally, we applied RAPD-PCR test to type the isolates.

I.2. Introduction

The genus Acinetobacter was first described in 1911, by Beijernick, the Dutch microbiologist who isolated it from the soil using minimal medium supplemented with calcium acetate [Peleg et al., 2008]. It can be described as strictly aerobic, non-glucose fermenting, non-oxidizing, non-fastidious, non-motile, catalase positive and oxidase negative Gram-negative coccobacillus that is widely distributed in nature. The guanine-cytosine (GC) content in the DNA of this genus is 39% to 47% [Asif et al., 2018]. *Acinetobacter spp.* are found in soil, water, and food products of animal origin. In addition, it infrequently colonizes the skin of healthy individuals in low density for short periods of time, and can usually be isolated from several clinical specimens including sputum, urine, feces, and vaginal secretions [Peleg et al., 2008, Asif et al., 2018].

Acinetobacter is routinely cultured and isolated in the clinical laboratory on blood agar supplemented with 5% sheep blood and MacConkey agar. Visible colonies can be seen after 24 hours of incubation at 37^{o} C in 5% carbon dioxide atmosphere or ambient air. On blood agar medium, the colonies are smooth, opaque, raised, white or creamy. On MacConkey agar, the colonies are purple but do not ferment lactose [Almasaudi, 2018]. *Acinetobacter* isolates are usually identified based on colonial morphology and

characteristic negativity for the oxidase test and immotility. In Addition, biochemical identification is usually made by the reference API20 method or by automated vitek-2 compact. However, the *bla*$_{\text{OXA-51}}$ gene is present in all isolates, therefore detection of this gene by PCR can provide a definitive identification *of A. baumannii* [Fallah et al., 2019].

*Acinetobacter baumanni*i is an emerging pathogen in hospitals. It causes various infections in hospitalized patients which include bacteremia, pneumonia, meningitis, urinary tract infection and wound infection. The risk factors that may contribute to colonization and/or infection with *A. baumannii* include prolonged hospital stay, stay in intensive care units (ICU), use of mechanical respirators, selective antibiotic pressure, surgery or invasive procedures and severe underlying illness [Gong, et al., 2021].

I.3 Pathogenicity

A. baumannii, previously a low-category pathogen has recently emerged as a major cause of nosocomial infections worldwide with an increasing frequency as cause of community acquired infections [Lin and Lan, 2014]. The factors that contribute to its persistence in hospitals are related to its ability to resist many antimicrobial agents and disinfectants and its survival on dry surfaces and in desiccants [Benjamin et al., 2013]. The majority of *A. baumannii* infections has been reported in patients in the intensive care unit (ICU) who are critically ill. The rate of infections caused by *A. baumannii* in intensive care units reaches 20% worldwide [Ayobami et al., 2019].

A. baumannii cause various infections including pneumonia, blood infection with the development of endocarditis, meningitis, skin infections and urinary tract infections. Infections usually occur due to the exposure of patients to *A. baumannii* contaminating hospital equipment or by infected healthcare employees [Rodriguez-Bano et al., 2009]. Hospital acquired pneumoniae has been attributed to several factors including the use of contaminated ventilation devices, prolonged hospital stays, presence in the ICU, invasive procedures, surgical procedures, and comorbidities as chronic obstructive pulmonary disease (COPD) [Li et al., 2017]. There are increased mortality rates of nosocomial pneumoniae caused by *A. baumannii* infection in critically ill patients due to ventilator use has been found to range between 40% to 70% worldwide. The extended use of broad-spectrum antibiotics contributes to the higher rates of infection [Sileem, 2017].

A. baumannii is a common cause of bloodstream infections in the ICU setting. The source of this infection has been found to be caused from lower respiratory tract infections and intravascular devices [Garnacho-Montero et al., 2015] and from wound and urinary tract infections to a lower extent [Falgas et al., 2015]. Blood stream infections caused by *A. baumannii* have been associated with immunosuppression, ventilator use, wide spectrum antibiotic therapy, as well invasive procedures [Islahi et al., 2015]. The bloodstream infection of the bacteria ranges from transient bacteremia to severe diseases with septic shock and high mortality. [Garnacho-Montero et al.,

2015]. According to a study involved 52 hospitals in the United States, the overall mortality rate among patients with bloodstream infection caused by *A. baumannii* was 36.9% and comparable to previously reported crude mortality rates associated with *A. baumannii* bloodstream infection that may be as high as 52% [Wisplinghoff et al., 2012].

Acinetobacter baumannii is an important cause of burn infections, although it can be difficult to differentiate between infection and colonization of burn sites [Jung and Park, 2015]. Because of the high rates of multidrug resistance and the poor penetration of some antibiotics into burn sites, these infections can be extremely challenging for clinicians [Shoja et al., 2017]. Recent studies reporting high incidences of *A. baumannii* infection in burn units have underscored the importance of *A. baumannii* in this patient population.

Record review performed on the prevalence of MDR pathogens in surgical research burn center, dedicated to US military personnel reported that *A. baumannii* was the most prevalent bacteria recovered (22%) during the study period of 2003–2008 [Keen, et al., 2010]. Burn site infection and colonization can progress to infection of the underlying tissue and subsequent systemic spread of the bacteria. Multidrug resistant *A. baumannii* has been found to cause meningitis in patients after neurosurgical procedures. A retrospective study was done on *A. baumannii* nosocomial meningitis

patients from a Brazilian hospital showed a mortality rate of of 72.7% [Tuon et al., 2010].

The virulence factors are associated with persistence of *A. baumannii* in the environment, interaction and subsequent host cell destruction. The most studied virulence factors are the outer membrane proteins (porins), OmpA and the Omp33-36. OmpA is the main and most abundant porin protein that contributes to the pathogenesis of this organism. OmpA plays a role in transporting antibiotics out of the periplasmic space and hamper the diffusion of negatively charged β-lactam antibiotics thus contributing to resistance. It has also been found that OmpA induces apoptosis by inducing the release of cytochrome C and other factors in the mitochondria which mediate the damage of human cells [Smani, et al., 2012 and 2013, Fahmy et al., 2018]. In addition, OmpA mediates adherence and invasion of epithelial cells leading to the dissemination of *A. baumannii* during infection. OmpA protein in the patient's serum can also neutralize the alternative complement pathway and avoid complement mediated killing. The persistence and survival of *A. baumannii* is due to the biofilm formation and surface motility mediated by additional functions of the OmpA [Smani, et al., 2013]. The Omp 33-36-kDa protein forms a channel for water. Its expression is associated with resistance to carbapenems. Strains with deficient Omp 33–36 showed reduction in the growth rate of the organism and significant reduction in adhesion and virulence [Smani, et al., 2013]. This natural permeability barrier is enhanced with the collaboration of the porins present on the outer membrane.

The lipopolysaccharides (LPS) of *A. baumannii* contributes significantly to its virulence. LPS consists of the lipid A, the carbohydrate core, and the repetitive O-antigen as found in other Gram-negative bacteria. It has been found the LPS play a key role in activating the innate immune response. Furthermore, it induces an inflammatory response and contributes to cell mortality [Moubareck and Halat, 2020].

Hepta-acylated lipid A plays an important role in bacterial desiccation survival and the loss of fatty acids in the outer membrane is detrimental to the bacterial persistence on inanimate objects in the hospital [Garnacho-Montero et al., 2015].

The capsular exopolysaccharide (CPO) are found in almost all isolates of *A. baumannii* has been considered a universal virulence factor [Edward and Isberg, 2015]. The survival of this pathogen in dry environments, its desiccation resistance and persistence are due to the presence of CPOs [Boll, et al, 2015, Harding et al., 2018]. Mutants deficient in the genes that encode for the capsular polysaccharides showed reduced growth in human serum, and were cleared within 24 hours [Russo et al., 2013]**.** Therefore, it is obvious that the capsular polysaccharides in this pathogen protects it from the action of the innate immune response.

A. baumannii produces phospholipases, enzymes that catalyze and cleave the ester bonds of phospholipids in eukaryotic cell membranes. Degradation of phospholipids by phospholipases contribute to the pathogenesis of this organism. Therefore, phospholipases have been considered as virulence factor for their role in lysing the host cell membrane, as the product release by the hydrolysis of phospholipids by phospholipases plays an important role in the penetration of the host cell and its lysis.

Phospholipases are active component of bacterial toxins and has cytotoxic effects on human macrophage. They also promote colonization and increasing the level of host cell invasion and spread of the bacteria [Kaur et al., 2018, Stahl et al., 2015].

Resistance mechanisms in *A. baumannii* have been focused on impermeability of the outer membrane, efflux pump and production of beta lactamases. However, Penicillin binding proteins (PBPs) contribute to bacterial survival for their role in the synthesis of peptidoglycan layers.

PBPs catalyze the transglycosylation and cross-linking by transpeptidation of peptidoglycan. PBPs constitute a crucial mechanism for the organism's resistance to carbapenems. Alteration in PBPs have been associated with beta-lactam resistance in Gram negative bacteria and may play a role in in carbapenem resistance in *A. baumannii* [Jitendra et al. 2011, Zrvosen et al., 2012].

I.4 Scope of Resistance of *A. baumannii* to antimicrobial agents

The scope of resistance of *A. baumannii* to various classes of antibiotics makes it a successful pathogen in the present day. It develops resistance against major classes of antibiotics such as aminoglycoside, where it produces aminoglycoside modifying enzymes to inactivate aminoglycoside. Most strains of *A. baumannii* are also resistant to the fluoroquinolones. The emergence of *A. baumannii* strains resistant to all beta lactam antibiotics including carbapenems is due to selective pressure [Lee C-R, et al, 2017]. *A. baumannii* produces several classes of beta lactamases. Ambler class A beta-lactamases which include the development of extended spectrum beta lactamases

(ESBL). Ambler class B β-lactamases also referred to as metallo β-lactamases contains metal ions in their active site. Ambler class C β-lactamases are cephalosporinases or the chromosomally encoded AmpC Ambler class D β-lactamases are oxacillinases primarily involved in resistance to carbapenems [Lowe et al., 2018].

I.5 Antimicrobial agents:

I.5-1 Beta-lactam antibiotics

Beta-lactam antibiotics are characterized by the presence of beta lactam ring in their structure. These antibiotics include penicillins, cephalosporins, carbapenems and monobactams as shown in Figures 1-4 below. The Beta-lactam ring is a four membered lactam containing one nitrogen atom attached to the beta carbon next to the carbonyl.

The targets of the β-lactam antibiotics are the PBPs. It has been found that the β-lactam ring mimics the D-alanyl D-alanine portion of the pentapeptide chain normally bound by PBP. PBPs function in the synthesis of peptidoglycan layers in the bacterial cell wall. Therefore, the interaction of PBPs and the beta-lactam ring inhibits the synthesis the peptidoglycan layers leading to its disruption and lysis of the bacteria.

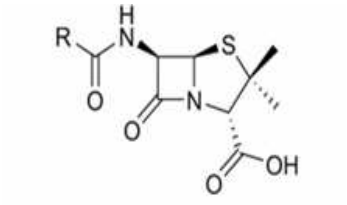


Figure 1. Structure of Penicillin

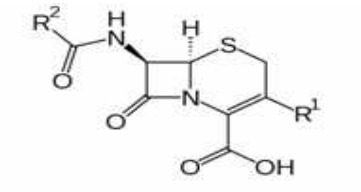


Figure 2. Structure of Cephalosporin

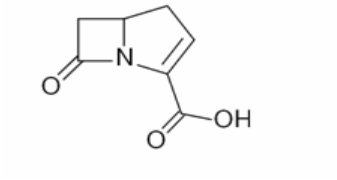


Figure 3. Structure of Carbapenem.

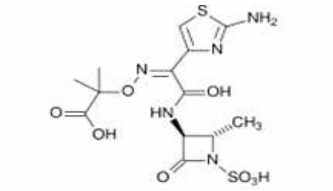


Figure 4. Structure of Monobactams

I.5-2 Penicillin

The antibacterial activity of penicillin was first observed by Alexander Fleming in 1929. It was secreted by the fungus *Penicillium notatum* that accidentally contaminated an agar plate cultured with *S. aureus*, and subsequently inhibited its growth. Penicillin inhibits cell wall synthesis leading to bacterial lysis [Etebu and Arikekpar, 2016].

I.5-3 Cephalosporins

Cephalosporins are synthesized by the fungus *Cephalosporium acremonium*. It was observed early in 1964 that cephalosporin has antibacterial effects because it inhibits the growth of *S. aureus* and other bacteria. The structure of cephalosporins consists of a β-lactams ring attached to a dihydrothiazine ring (6 carbon ring) as shown in Figure 2.

Cephalosporins inhibit bacterial cell wall synthesis by blocking the transpeptidases and other PBPs during the synthesis and cross-linking of peptidoglycan. Resistance to cephalosporins may result from mutations in the PBPs resulting in altered binding site or due to the production of extended-spectrum beta-lactamases or both.

Cephalosporins have been classified into five generations based on the spectrum of their activity. The first generation cephalosporins such as cephalexin, are more effective against Gram positive and some gram-negative bacteria. The second generation represented by cefuroxime are effective against both gram positive and gram-negative bacteria. The third generation represented by ceftazidime and cefotaxime, have extended spectrum activity against gram negative bacteria including those that are not affected by first or second generations. The fourth generation such as cefepime, are effective against both gram positive and gram-negative bacteria. They are used for the treatment of severe infections particularly in the immunosuppressed patients. Fifth-generation cephalosporin such as ceftaroline are useful to treat organisms that are resistant to other antibiotics such as methicillin resistant *S. aureus* (MRSA) [Etebu and Arikekpar, 2016].

I.5-4 Carbapenems

The Carbapenems have a beta-lactam ring and a thiazolidone where the sulfur atom (in penicillin) ring as penicillin is replaced by a carbon atom as shown in Figure 3. Examples for carbapenems include meropenem, imipenem and ertapenem. Although they are synthetic β-lactams, they were derived from thienamycin, an antibiotic produced by *Streptomyces cattleya* [Etebu and Arikekpar, 2016]. Similar to other beta lactams they inhibit peptidoglycan synthesis in the cell wall by binding to PBPs, and are effective against Gram negative bacteria. However, resistance to carbapenems has been observed as seen in *A. baumannii*. Resistance to carbapenems is mediated by the

production of carbapenemases that may be intrinsic or acquired. Lack of outer membrane proteins (OMP) is another mechanism of resistance where the transport of carbapenems is prevented.

I.5-5 Monobactams

The Monobactams such as aztreonam contain a single β-lactam ring only as shown in Figure 4.

Aztreonam was originally obtained from *Chromobacterium violaceum* but is currently synthetically made. Similar to other beta lactam antibiotics, aztreonam inhibits peptidoglycan synthesis in the bacterial cell wall by binding to the penicillin-binding protein (PBP) of Gram-negative bacteria, leading to the lysis of the cell [Etebu and Arikekpar, 2016].

I.5-6 Aminoglycosides

The name aminoglycosides implies that these molecules are composed of amino-sugars as shown in Figures 5 and 6. These are natural products where *Micromonospora purpurea* produced gentamicin and *Streptomyces griseus* produced streptomycin. The inhibitory effects of aminoglycosides on microorganisms are mediated by binding to the negative charges in the outer phospholipid membrane and displacing the cations that link the phospholipids causing disruption of the inner cell membrane. Aminoglyscosides can also inhibit protein synthesis by irreversible binding to the 30S ribosome subunit which causing bacterial death [Etebu and Arikekpar, 2016].

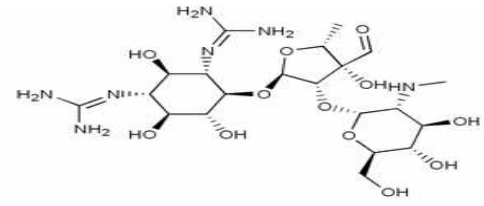


Figure 5. Structure of Streptomycin

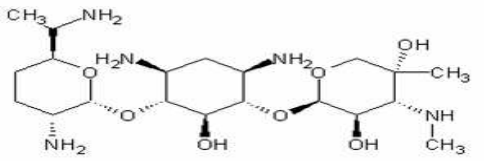


Figure 6. Structure of Gentamicin

I.5-7 Quinolones

Quinolones are broad-spectrum antibiotics that are used to treat infections caused by Gram-positive and Gram-negative bacteria. The general structure of quinolones is shown in Figure 7. They inhibit DNA synthesis by disrupting the topoisomerase IV and DNA gyrase enzymes. They also damage the bacterial chromosome. Bacteria in general and *A. baumannii* in particular have acquired resistance to quinolones due to overuse causing selective pressure. The mechanisms for quinolone resistance include chromosomal mutations, plasmid gene uptake which alter the topoisomerase targets, modification of the quinolone, and decreased accumulation by reduced antibiotic uptake or increased efflux [Etebu and Arikekpar, 2016].

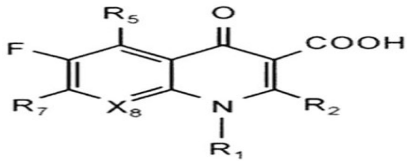


Figure 7. Structure of Quinolones

I.5-8 Polymyxins

Polymyxins are produced by *Bacillus polymyxa*. They disrupt the lipopolysaccharide bilayer.

Colistin or Polymyxin E structure is shown in Figure 8. It is a polypeptide antibiotic comprised of colistin A and B that have high toxicity. Colistin is now considered to be the last resort to treat serious infections caused by multi-drug resistant isolates of *A. baumannii, Pseudomonas aeruginosa*, or *Klebsiella pneumoniae* [Etebu and Arikekpar, 2016].

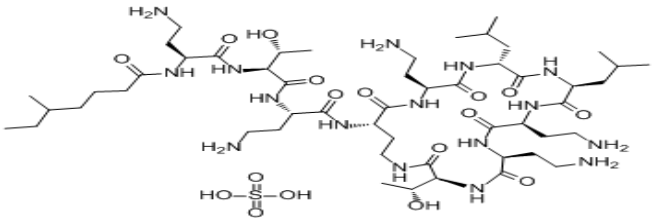


Figure 8. Structure of Colistin Sulfate

I.5-9 Tigecycline

Tigecycline consists of minocycline with an N-alkyl-glycylamido group substituted at position nine which provides it with broad spectrum of activity and protection from resistance mechanisms. Its structure is shown in Figure 9. Tigecycline is a bacteriostatic antibiotic that binds to the 30S ribosomal subunit of bacteria and blocks entry of aminoacyl t-RNA into the A site of the ribosome [Etebu and Arikekpar, 2016].

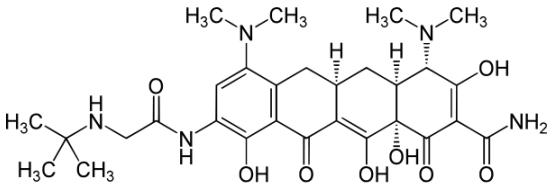


Figure 9. Structure of Tigecycline

I.5-10 Fosfomycin

Fosfomycin is a phosphonic acid antibiotic that has a broad-spectrum activity. It was first isolated from cultures of *Streptomyces fradiae*. This antibiotic has a low molecular weight and behaves as a bactericidal. It's been used as an oral treatment for urinary tract infections (UTIs) and it works against Gram-negative and Gram-positive bacteria, including multidrug-resistant strains. Fosfomycin is an inhibitor of MurA (UDP-N-acetylglucosamine enolpyruvyl transferase) enzyme that catalyzes the first step in peptidoglycan synthesis. It blocks the first step in this synthesis in UDP-GlcNAc enolpyruvyl-transferase leading to an inhibition of the cell wall synthesis [Ramos and Lletí, 2019 and Silver, 2017].

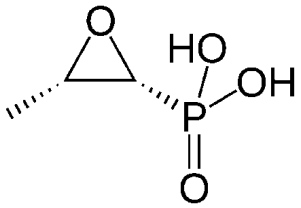


Figure 10. Structure of Fosfomycin

I.5-11 Rifampin

This antibiotic has a wide antibacterial spectrum. It is a semisynthetic derivative of the rifamycins and is a fermentation product of *Nocardia mediterranei*. It is effective in treating tuberculosis.

Rifampin inhibits bacterial RNA polymerase enzyme that is responsible for DNA transcription, while mammalian enzymes are not affected by rifampin. The drug forms a stable drug- enzyme complex and the bacteria could produce resistance to rifampin by having mutations leading to a change in the structure of the β subunit of RNA polymerase [Wehrli, 1983]

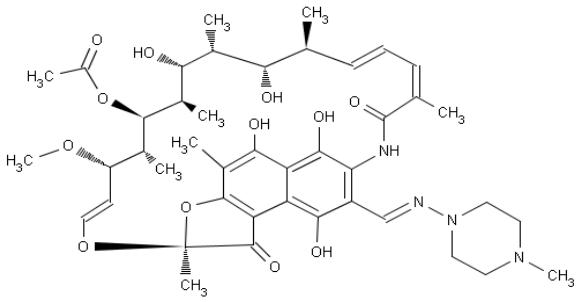


Figure 11. Structure of Rifampin

I.6 Combination Therapy

Combination of antibiotics is usually done in a checkerboard to determine the presence of synergy between two antibiotics. It is used to compare the potency of antibiotics in

combination to their individual activities. Combination therapy has been used to prevent the evolution of resistant bacterial strains. When synergy is identified, it can be used to treat life threatening infections as those caused by *A. baumannii*. Furthermore, the use of combination therapy, as in colistin with meropenem, or tigecycline could be useful for reducing and adjusting the antibiotic dose in order to avoid or minimize it toxic effects [Karakonstantis, et al., 2021]. Synergy occurs when the effects of combined antibiotics is greater than the sum of the effects of the individual ones. Antagonism occurs when one antibiotic, usually the one with the least effect, interferes with the effects of another antibiotic [Pachon-Ibanez, et al., 2010].

I.7 Mechanisms of Antimicrobial Resistance

There are several mechanisms of bacterial resistance to antimicrobial agents. The most common mechanisms of bacterial resistance include degradation of antibiotics by specific enzymes, target modification, decreased permeability, and/or efflux of antibiotics (Coyne et al., 2011). *A. baumannii* has been considered a successful pathogen due to its ability to develop resistance to antimicrobial agents. Some strains have developed resistance to several classes of the available antibiotics (Lin and Lan, 2014). There are several mechanisms of resistance utilized by *A. baumannii* which include innate and acquired mechanisms.

I.8 Innate Mechanisms of Resistance

A. baumannii have innate mechanisms that enable it to resist a number of antibiotics. One of the innate mechanisms of resistance is the presence of chromosomal *AmpC*-type beta-lactamases. *AmpC* belongs to the Ambler Class C cephalosporinase. This enzyme provides *A. baumannii* with the ability to resist the broad spectrum cephalosporins. In addition, the presence of the insertion sequence IS*Aba1* upstream of the *AmpC* b-lactamase gene provides it with a strong promotor that results in increased expression of gene and decreased susceptibility to beta lactam antibiotics and the main resistance for third generation cephalosporins [Perichon et al, 2014].

A. baumannii naturally harbors the Ambler Class D oxacillinase (OXA type beta lactamase), $bla_{\text{OXA-51}}$. This OXA is not normally associated with resistance to antibiotics. However, if over-expressed after association with IS*Aba1*, it will contribute to antibiotic resistance [Perichon et al, 2014]. Another innate mechanism of resistance in *A. baumannii* is associated with reduced permeability of the cell membrane or increased efflux of the antibiotic, thus preventing the entrance of antimicrobial agents to their targets [Blair et al., 2015].

This natural permeability barrier is enhanced with the collaboration with outer membrane porin proteins (OMPs) present on the outer membrane. OMP A plays a role in transporting antibiotics out of the periplasmic space. Efflux pumps is another mechanism that contribute to innate resistance [Kyriakdis et al, 2021].

I.9 Acquired Mechanisms of Resistance

Microorganisms can acquire resistance to antibiotics by various ways. Gene mutations are significant in affecting gene expression. Some of these mutations have been seen in the over-expression of the genes of the efflux systems, and under-expression of the genes in the outer membrane porins [Coyne *et al.,* 2011]. Mutations could also lead to the alteration of the antibiotic target making it ineffective. One of the important mechanisms is the horizontal transfer of mobile genetic elements (plasmids and transposons), which carry genes that lead to the acquisition and dissemination of antibiotic resistance [Vrancianu et al., 2020]. These mobile genetic elements may be incorporated into *A. baumannii* genome forming islands containing many antibiotic resistant genes. In *A. baumannii*, it has been observed that these islands can carry genes that makes resistance to all antimicrobial agents [Vrancianu et al., 2020].

I.10 Non-Enzymatic Mechanisms of Resistance

I.11 Efflux Pumps

Efflux pumps genes and proteins are not only present in *A. baumannii* but in all organisms, and causes reduced susceptibility to antibiotics. There are five super-families of efflux pumps that have been associated with drug resistance [Coyne et al., 2011]. The clinical significance of the efflux pumps families lies in the way they cross the inner and outer membranes and are capable to expel various substances. Efflux pumps over expression is primarily associated with pumping out antibiotics as well as

other harmful substances [Coyne et al., 2011]. Resistance to some antibiotics such as quinolones, macrolides and tetracycline is associated with efflux pumps overexpression. Over expression of efflux pumps is also responsible for resistance to aminoglycosides, chloramphenicol, tetracyclines and trimethoprim, carbapenems and tigecycline [Abdi et al., 2020].

I.12 Down Regulation of Porins

Down regulation of porin proteins works in synergy with the up-regulation of efflux pumps. Down regulation of porins reduces the permeability of the membrane and prevents expelled antibiotics from reentering the cell. Porins are outer membrane proteins that form channels through which substances including the carbapenems enter the bacterial cell. Down-regulation of porins in *A. baumannii* has been associated with carbapenem resistance [Zahn et al., 2016].

I.13 Antibiotic Target Modification

Antibiotic target modification is a mechanism of resistance present in *A. baumannii.* Point mutations in the DNA gyrase subunit A and B and topoisomerase IV subunit C genes (*gyrA, gyrB* and *parC genes*) can lead to modification in the target on fluoroquinolones resulting in resistance to these antibiotics [Park et al, 2011]. Mutations and modifications in *gyrA* or *parC* genes have been found to be responsible for fluoroquinolones resistance where alterations in the drug target occur [Park et al,

2011]. Single mutation in the *gyrA* has been found to be the most frequent (48.7%) as reported in a study in China followed by single mutation in the *parC* gene (21.6%) and 5.4% in both genes [Sun *et al.,* 2015]. Aminoglycosides are usually used to treat infections caused by *A. baumannii*. The main mechanism of resistance of *A. baumannii* to aminoglycosides is by enzymatic modification by the production of aminoglycoside-modifying enzymes. The enzymes responsible for this modification include acetyltransferases, phosphotransferases and nucleotidyl transferases. Resistance to aminoglycosides can also occur by other non-enzymatic mechanisms which modifies the target on the 16S ribosomal RNA unit [Toda et al, 2013, Wong et al. 2017].

I.14 Enzymatic Mechanisms of Resistance

This mechanism of resistance is mediated by the production of enzymes which enable microorganisms to degrade and inactivate the antimicrobial agent. There is a large number of enzymes that have been identified in pathogenic bacteria particularly *A. baumannii*. In addition, clinical isolates of *A. baumannii* have even acquired several enzymatic and non-enzymatic mechanisms to become multidrug resistant [Antunes et al., 2014].

I.15 Beta - Lactamases

In Gram-negative bacteria, production of beta lactamase enzymes that hydrolyze the drug is the most common mechanism of resistance. Beta-Lactamases hydrolyze the beta-lactam ring present in several classes of antibiotics by breaking the amide bond of

the beta lactam ring [Xia *et al.,* 2016]. This has been considered a major mechanism of resistance in *A. baum*annii. Based on amino acid homology, b- lactamases have been classified by Ambler into four molecular classes A, B, C, and D. Classes A, C and D are serine dependent β-lactamases while group B are metallo β-lactamases (MBLs) that require zinc or another metal ion for their activity [Ozturk et al., 2015]. The extended spectrum beta lactamases (ESBLs) have the potential to hydrolyze many beta-lactam antibiotics except the carbapenems, that are hydrolyzed only by carbapenemases[Wilson et al., 2018].

In the following sections, the prevalence of the different beta-lactamases, with an emphasis on carbapenemases, among *A. baumannii* isolates will be discussed. The beta lactamases according to Ambler molecular classification are shown in Table 1 below.

Table 1. Acquired beta-lactamases detected in *A. baumannii* according to the Ambler classification system

* This gene is used for the identification of *A. baumannii*

Ambler Class A Enzymes							
PER	**VEB**	**GES**	**KPC**	**SHV**	**TEM**	**CTXM**	**CARB**
PER-1	VEB-1	GES-11	KPC-2	SHV-5	TEM-92	CTXM-2	CARB-10
PER-2	VEB-3	GES-12	KPC-3	SHV-12	TEM-116	CTXM-15	
PER-3		GES-14	KPC-4			CTXM-43	
PER-7			KPC-10				

Ambler Class B Enzymes					
	NDM	**SIM**	**VIM**	**IMP**	
	NDM-1	SIM-1	VIM-1	IMP-1	
	NDM-2		VIM-2	IMP-2	
			VIM-4	IMP-4	
			VIM-6	IMP-5	
			VIM-11	IMP-6	
				IMP-8	
				IMP-11	
				IMP-14	
				IMP-19	
Ambler Class C Enzymes					
			AMPC		
			ADC-33		
			ADC-56		
Ambler Class D Enzymes					
***bla*$_{\text{OXA-51}}$**	**OXA-23**	**OXA-24**	**OXA-58**	**OXA-143**	**OXA-235**
		OXA-24	OXA-58	OXA-143	OXA-235
	OXA-27	OXA-25	OXA-96	OXA-182	OXA-237
	OXA-49	OXA-26	OXA-97	OXA-231	OXA-238
		OXA-72	OXA-164	OXA-253	

I.15-1 Ambler Class A β-lactamase Enzymes

Class A beta-lactamases enzymes hydrolyze beta-lactam antibiotics by forming an acyl enzyme and have serine in their active sites. Although abundant in the family

Enterobacteriaceae, few types have been found in *A. baumannii*. The first ESBL identified in *A. baumannii* was PER-type ESBL, PER-1. PER-1 confers resistance to ceftazidime but has limited carbapenemase activity. PER-1 has been detected in *A. baumannii* isolates in many countries worldwide. PER-2 enzyme detected in *A. baumannii* has 86% amino acid homology with PER-1 but its occurrence was limited to South America [Potron *et al.,* 2015]. PER-3 is an ESBL found in *A. baumannii* isolates from Egypt [Al-Hassan et al., 2013]. Another ESBL variant, PER-7, was identified in *A. baumannii* isolates in France, Sweden and the United Arab Emirates [Potron et al., 2015].

VEB beta-lactamase, the Vietnamese ESBL, shares 38% amino acid homology with PER-1. It was first identified in France, then disseminated in neighboring European countries. VEB-1 positive *A. baumannii* isolates were detected in Belgium, Iran and Argentina. One isolate producing theVEB-3 variant was detected in Taiwan [Potron et al., 2015].

GES beta lactamase, the Guiana ESBL, has hydrolytic activity against carbapenems. GES-11, was first identified in an *A. baumannii* clinical isolate in France and Belgium. GES-12 and GES-14 are two variants of this enzyme can hydrolyze ceftazidime and imipenem [Potron et al., 2015]. Interestingly, in Kuwait, *A. baumannii* isolates carried all three GES genes on plasmids. This may indicate that this region could be a reservoir for this ESBL. Furthermore, all *A. baumannii* isolates carried the GES-11 gene. GES-

12 was found in *A. baumannii* isolates in Sweden and Tunisia. The GES-14 carbapenemase was detected in *A. baumannii* isolates in France and Turkey [Potron et al., 2015].

A class A beta-lactamase (carbapenemase), commonly found in the Enterobacteriaceae in general and *Klebsiella pneumoniae* in particular that has the KPC. KPC has high activity against carbapenems. It has been found that the dissemination of KPC to *A. baumannii* is rare. The presence of KPC in *A. baumannii* has been limited to certain geographic areas in Puerto Rico for example, all isolates carried all variants of KPC enzymes including KPC-2, KPC-4, and KPC-10 [Martinez *et al.,* 2016]. However, the presence of KPC in *A. baumannii* seems to still be scarce and limited to one geographical area.

The class A β-lactamases that have been identified in *A. baumannii* include TEM, SHV, GES, CTX-M, SCO, PER, VEB, KPC, and CARB. Some of these enzymes are narrow spectrum β-lactamases such as TEM-1, CARB-4, and SCO-1. The remaining b-lactamases which include 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. ESBLs are characterized by their ability to hydrolyze third-generation cephalosporins and aztreonam but are inhibited by b-lactamase inhibitors such as clavulanic acid and others. ESBLs are plasmid-mediated, rapidly evolving and spreading, and are a major therapeutic challenge to treat

hospitalized patients and community- patients as well. ESBL producers cause uncomplicated infections as seen in the urinary tract infections or life-threatening ones.

I.15-2 Ambler Class B β-lactamases

Ambler class B beta-lactamases, also referred to as metallo beta lactamases (MBLs) contain zinc ions in their active site. These enzymes are characterized by their ability to hydrolyze carbapenems while not being inhibited by the beta lactam inhibitors such as clavulanic acid and tazobactam. In *A. baumannii*, other metalloenzymes are also involved in its resistance to carbapenems. These MBLs include the following:

- The Verona Integron encoded Metallo-beta-lactamase (VIM)
- Imipenem metallo-beta-lactamase (IMP)
- New Delhi Metallo-beta-lactamase (NDM)
- Seoul Imipenemase (SIM)

These MBLs have been identified in several areas worldwide. There are five variants of the VIM enzyme as shown in Table 1. *A. baumannii* positive isolates for VIM-1 were detected in Greece, VIM-2 in South Korea, Kuwait and China. VIM 4, VIM-6, and VIM-11 variants were produced by *A. baumannii* isolates in Italy, India and Taiwan. The SIM-1 carbapenemase has first been detected in South Korea. It was reported that its dissemination has been limited only to that country. NDM-1 was first identified and reported in *A. baumannii* isolates in India and later in China and Japan. Later, it was disseminated into worldwide countries such as Algeria, France and

Lebanon. NDM-2 variant was detected in Egypt, United Arab Emirates and Spain [Potron *et al.,* 2015].

I.15-3 Ambler Class C β-lactamases

The clinical significance of class C beta lactamases, also known as AmpC beta lactamases, needs further clarification. Ambler Class C b-lactamases are characterized by their wide spectrum activity against cephalodporins. *A. baumannii* producing class C enzymes are resistant to second generation cephalosporins (cephamycins) which include cefoxitin and cefotetan. Their scope of resistance includes penicillins, cephalosporins and b-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam [Potron et al., 2015]. The genes responsible for *A. baumannii* production of class C enzymes are intrinsic and acquired [Potron et al., 2015]. *A. baumannii* intrinsically produce AmpC, a class C cephalosporinase usually at low level which does not contribute to resistance against broad spectrum cephalosporins. Upon insertion of IS*Aba1* upstream of the AmpC genes, it causes an increase in the expression of these enzymes and subsequent acquiring resistance to broad spectrum cephalosporins, but not to carbapenems.

I.15-4 Ambler Class D β-lactamases

Class D b-lactamases are also referred to as oxacillinases (OXAs) due to their ability to hydrolyze isoxazolyl penicillin oxacillin much faster than benzylpenicillin but are

not inhibited by clavulanic acid [Jeon et al., 2015]. There have been more than 400 types of OXA β-lactamases identified some of which carry carbapenemase activity. Class D beta lactamases are the main cause of carbapenem resistance in *A. baumannii* [Nowak and Paluchowska, 2016]. Class D carbapenem hydrolyzing β-lactamases and MBLs are the major mechanisms in *A. baumannii* for Carbapenem resistance. [Lin and Lan, 2014].

The OXA enzymes that mediate resistance to carbapenems in *A. baumannii* are divided into six families: OXA-51-like, OXA-23-like, OXA-40/24-like, OXA-58-like, OXA-143-like, and OXA-235-like families [Evans and Amyes, 2014]. It is important to mention that OXA-24-like and OXA-40-like enzymes are identical, OXA-24-like is commonly used. The gene expressing the OXA-51-like enzymes is chromosomally located and intrinsically expressed. However, its expression alone does not cause carbapenem resistance. Overexpression and resistance to carbapenems occur after the insertion of ISAba1 upstream the gene promoter sequence. There are many enzymes identified chromosomally encoded belong to the OXA-51-like family. Nevertheless, carbapenem resistance resulting from over-expression of OXA-51-like is relatively infrequent [Nowak and Paluchowska, 2016].

The gene encoding the OXA-23 enzymes is located on transposons. The OXA-23-like family is the most prevalent among nosocomial isolates and contains the enzymes; OXA-23, OXA-27, and OXA-49 [Merino *et al.,* 2014 and Potron *et al.,* 2015]. Similar

to OXA-51, the gene encoding for the OXA-23 enzymes has intrinsic activity against carbapenems. However, enhanced carbapenemase activity is associated with the presence of IS*Aba1* upstream of $bla_{\text{OXA-23}}$ genes [Vijayakumar et al., 2016]. Several studies have reported the detection of OXA-23 at high rates in *A. baumannii* in many countries in the world. In the Gulf area (Saudi Arabia, United Arab Emirates, Qatar, Oman, Bahrain and Kuwait) for example, 91.5% of the isolates were positive for OXA-23 [Zowawi et al., 2015]. Furthermore, in Iran, the prevalence of this group of enzymes reached 88.7% [Sohrabi et al., 2012]. Another study in Iran showed that all the isolates tested harbored $bla_{\text{OXA-23}}$ [Salimizand et al., 2015].

It has been confirmed that carbapenem resistance in *A. baumannii* is mainly associated with the production of OXA-23, OXA-24, and OXA-58 carbapenem-hydrolyzing class D b-lactamases [Merino et al., 2013]. Although OXA-23- and OXA-58-producing *A. baumannii* are widely disseminated, OXA-24 producers have been endemic mainly in the Iberian Peninsula **[**Grosso et al., 2011]. In other countries in the world, the frequency of carbapenem resistant cases caused by OXA-24 is very little [Ji et al., 2014]. This warrants further studies to clarify the epidemiology and spread of this beta lactamase in *A. baumannii*. There are sporadic reports about cases of carbapenem resistance among *A. baumannii* isolates caused by the production of OXA-24 like enzymes. A recent multicenter study in hospital in Thailand by PCR and sequencing showed that *A. baumannii* isolates carried acquired class D carbapenemase genes, including $bla_{\text{OXA-23}}$ (82.6%), $bla_{\text{OXA-24}}$ (0.3%), and $bla_{\text{OXA-58}}$ (6.5%) [Udomluk et al.,

2018]. Carbapenem resistance *A. baumannii* isolates carrying the $bla_{\text{OXA-24}}$ gene has been detected in low rates in Algeria (Benamrouche et al, 2020), Egypt, [Al- Agamy *et al.,* 2014]. Moreover, Arab States in the Gulf [Zowawi et al., 2015] and Iran, [Sohrabi et al., 2012].

In Spain, OXA-24 was identified in high prevalence. Studies in Spain reported that the prevalence of OXA-24-like was 48.7% in 2000 and 51.6% in 2010. A long-term study from Spain has showed the prevalence of OXA-24-like among carabapenem resistant *A. baumannii* in a single hospital was 40% [Mosqueda et al., 2014].

The OXA-58-like family consists of OXA-58, OXA-96, OXA-97, and OXA-164. The genes for $bla_{\text{OXA-58}}$ are either carried on the chromosome or a plasmid. High expression of the genes is associated with the insertion sequences near their promotor conveying high level resistance to carbapenems [Nowak and Paluchowska, 2016]. Most of the cases of OXA-58-like positive carbapenem resistant *A. baumannii* have been reported from Europe and the Mediterranean countries. OXA-58 in *A. baumannii* was first discovered in France in 2003 [Poirel et al., 2005].

I.16 Epidemiology of *A. baumannii*

A. baumannii is a healthcare-associated pathogen and a major cause of outbreaks and nosocomial infections with high mortality rates. It causes various serious infections in patients which include ventilator-associated pneumonia, wound infections, burn

infections, septicemia, bacteremia, endocarditis, meningitis, and urinary tract infections. Outbreaks are commonly seen among patients using mechanical ventilation residing in intensive-care units [Kanafanin et al., 2018]. It rarely causes infections in healthy people including healthcare workers and close relatives of patients.

The natural habitat of *A. baumannii* is soil and water as well as healthcare facilities. Multi drug resistance (MDR) *A. baumannii* infections particularly nosocomial infections have been reported in many world regions which include Europe, North America, South America and South East Asia. Community acquired pneumonia usually occurs in warm areas with high humidity as in the tropics and Middle Eastern areas [Kanafani and Kanj, 2014]. There was high rate of infections of resistant *A. baumannii* strains among wounded American and British soldiers deployed to Iraq and Afghanistan [Almasaudi, 2018].

In the Middle East, many cases of MDR *A. baumannii* was found in hospitals in the United Arab Emirates, Bahrain, Saudi Arabia, Palestine and Lebanon. A retrospective study conducted on MDR bacterial isolates recovered from nosocomial infections in the intensive care unit in a military hospital in Saudi Arabia found that *A. baumannii* was the most commonly recovered at a rate of 40.9% [Kamolvit et al., 2015].

A. baumannii has been found to be notorious in causing outbreaks due to its high antimicrobial resistance and its ability to withstand desiccation. Multidrug resistance *A. baumannii* outbreaks were reported in Middle East hospitals and worldwide, this is

consistent with the increase in the incidence of infections caused by *A. baumannii* which was reported in Palestinian and Israeli hospitals. According to a Palestinian study done on hospitalized patients with MDR *A. baumannii* from all over Palestine, except Gaza. All the isolates were resistant to all the β–lactam antibiotics including the carbapenems [Handal et al., 2017].

I.17 Biofilm Formation

Biofilms are assemblages of microorganisms, encased in a matrix, that function as a cooperative consortium to provide a protected growth environment for microorganisms. Biofilms that form on contaminated medical implants and prosthetic devices are difficult to eradicate due to high tolerance to antibiotics [Khatoon et al., 2018]. The formation of biofilms begins with pioneer cells that attach to a surface via specific adhesion molecules as fimbriae, or non–specifically by extracellular polymeric substances (EPS). EPS are biopolymers of microbial origin in which biofilm microorganisms are embedded. It consists of polysaccharides, proteins, nucleic acids and lipids. The cells initially grow and divide to produce microcolonies, with time they coalesce to produce a biofilm and become enveloped in a matrix of EPS and other macromolecules. Biofilm resistance to antibiotics and phagocytosis, and recurrent infections after treatment are major concerns [Khatoon et al., 2018].

A. baumannii is capable of forming biofilms on biotic and abiotic surfaces. It has the ability to form biofilms on surfaces such as stainless steel, catheters, and host epithelial cells [Greene et al., 2016, and Cheng-Hong et al., 2019]. Biofilm formation is mainly

mediated by pili that promotes and enhance attachment. The *bap* gene has been found to play a significant role in intercellular adhesion, accumulation of bacterial cells, and establishment of biofilm. Furthermore, it has been reported that the outer membrane protein A (OmpA), of *A. baumannii* contributes partially to the development of biofilms in plastic surfaces such as catheters and others. Biofilm formation contributes to this pathogen's survival and rapid spread in the hospital environment [Cheng-Hong et al., 2019].

I.18 Aims of the study

A. baumannii became notorious in causing nosocomial infections particularly in the intensive care units. The scope of infections caused by *A. baumannii* in the immunocompromised patients in the ICU include ventilator-associated pneumonia, skin, soft-tissue infections, meningitis, urinary tract infections, wound infections, burn infections, blood infection, endocarditis, and surgical site infections. The evolution of multi drug resistant (MDR) *A. baumannii* is a main concern for health care. The aims of this research have been focused on carbapenem resistant *A. baumannii* isolates. The specific aims were:

- Confirm the identity of the collected isolates by PCR using primers for *bla*$_{\text{OXA-51}}$ gene.
- Determine the minimal inhibitory concentration for colistin, rifampin and fosfomycin. This information is necessary to use when doing the combination

testing of the antibiotics. In addition, it will detect the presence of colistin resistant isolates.

- Perform combination antibiotics testing using colistin-rifampin and colistin-fosfomycin combinations to determine the presence of synergy. This will contribute to reduce or eliminate the evolution of colistin resistance on one hand, and help reduce the dosage administered to patients and thus reduce the level of toxicity of this antibiotic.
- To determine the rate of carriage of Ambler class A, B and D beta lactamases in the isolates collected by PCR. We tested the isolates for the presence of *bla*$_{\text{KPC-2}}$ (Class A), *bla*$_{\text{NDM-1}}$ (class B) and *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-51}}$, and *bla*$_{\text{OXA-58}}$, (Class D). In addition, it will provide important information about the predominant class and the geographic distribution of these genes in the region.
- The isolates were tested for biofilm formation (virulence factor) by staining the tubes with crystal violet and safranine.
- We applied the RAPD PCR to provide information about the relatedness of the collected isolate and to determine the epidemiology of infectious agents and outbreaks.

Chapter II

Materials and Methods

II.1 *A. baumannii* isolates Collection

A total of 29 *A. baumannii* clinical isolates were obtained from Palestine Medical Complex in Ramallah and Makassed Charitable Foundation in Jerusalem. All the isolates were carbapenem and multidrug resistant. All the patients were treated with colistin. The demographic information about the bacterial isolates, hospital ward, gender and age of the patients are summarized in Table2.

Table 2. Demographic information about patients whose samples were tested in this project

		Gender	
Age Group (years)	**Number of patients**	**Male**	**Female**
20-39	7	4	3
40-59	11	10	1
>60	11	6	5
Total	29	20	9
Ward			
Intensive Care (ICU)	14		
Surgical	5		
Clinics	10		

II.2 Ethical Consideration

All the samples were coded and the identity of the patients were not shown in order to maintain confidentiality of patients. The study was approved by the Medical Research Committee at Birzeit University and got an ethical approval from the two hospitals.

Table 3. Materials used in the project

Product	**Lot Number**	**Company**
Mueller Hinton broth	0000378294	HiMedia
Tryptone Soy broth	0000237500	HiMedia
Brain Heart Infusion Agar	0000072347	HiMedia
Colistin Powder	113439	Rafa Labrotaries LTD
Rifampin Powder	13292461	Medochemie LTD
Fosfomycin	374060	Rafa Labrotaries LTD
Distilled Water	203538001	B.Braun Melsungen AG
Cotton Swap	160218	CiToSwAB
Cryotubes Vials	117675	nunc
Blue Tips	7383-718cC-718D	LabCon
Yellow Tips	141004	LabCon
Agarose	7031-17B	Life Gene
10X Loading dye	A276	TakaRa
Go Taq Green Master Mix 2X	0000270865	Promega
Sterile Aerosol pipet tips	40507-823C4-823D	LabCon

Primer Oxa 23 F	1785383	hylabs
Primer Oxa 23 R	1785384	hylabs
Primer Oxa 24 F	1785385	hylabs
Primer Oxa 24 R	1785449	hylabs
Primer Oxa 58 F	1785389	hylabs
Primer Oxa 58 R	1785390	hylabs
Primer Oxa 51 F	1785387	hylabs
Primer Oxa 51 R	1785388	hylabs
Primer Oxa 48 A	4417400	Metabion International AG
Primer Oxa 48 B	4417402	Metabion International AG
Primer NDM F	4417396	Metabion International AG
Primer NDM R	4417397	Metabion International AG
Primer KPC F	4417398	Metabion International AG
Primer KPC R	4417399	Metabion International AG
100bp Ladder RTU	110623120903	Gene Direx
QIAamp DNA Mini Kit 50	166051766	QIAGEN
PCR Thermal cycler	-	Bio-RAD
Molecular imager	-	Bio-RAD
Centrifuge tubes	160218	LabCon
Balance	-	TRAVELER
Microplate	195389	Greiner Bio
Serological pipettes	07182018	Sigma
Pipettes	-	ThermoFisher Scientific
Centrifuge tubes	181302	ThermoFisher Scientific
Inoculation Loops	181802	ThermoFisher Scientific

Glass tubes	-	Kimble
Ethidium Bromide	-	Sigma
Nanodrop Lite	269-275200	ThermoFisher Scientific
AP-ARB11 primer	6569633	Metabion International AG
AP- PG05 Primer	6569634	Metabion International AG
1KB DNA Ladder	1120201909	hy.labs

II.3 Preparation of Materials:

II.3.1 Mueller Hinton Broth (MHB)

MHB is a general-purpose medium. It was prepared by dissolving 21.0 g of MHB powder in 1 L of deionized water (dH_2O). Hot plate was used to dissolve the mixture in a pre-cleaned flask, and then the mixture was sterilized by autoclaving at 121 °C for 15 min [Alrashidi, 2020].

II.3.2 Soybean casein digest medium (Tryptone Soy broth, TSB)

TSB is a general-purpose broth. It was prepared by dissolving 30 g of TSB powder in 1 L of dH_2O. Hot plate was used to dissolve the mixture in a pre-cleaned flask, and then autoclaved at 121 °C for 15 min. After cooling, the medium was stored at 4 °C until further use [Alrashidi, 2020].

II.3.3 Brain Heart Infusion Agar (BHI Agar)

It is a general-purpose enriched non-selective media used to culture molds, yeasts and bacteria. It was prepared by dissolving 52 g of the powder in 1 L of dH_2O. Hot plate was used to dissolve the mixture in a pre-cleaned flask, and then autoclaved at 121 °C for 15 min. After autoclaving, the medium was cooled in a water bath to 50º C, then poured into sterile petri dishes. After solidification, the agar plates were marked and stored at 4º C until used [Alrashidi, 2020].

II.3.4 Freezing Medium

15% glycerol solution was prepared by adding 15 ml glycerol to 85 ml TSB medium to make a total of 100 ml freezing solution. The solution was then autoclaved, and dispensed in 1.5 ml aliquots in sterile cryofuge tube. 2-3 isolated colonies were taken from the fresh culture for each isolate (18-24 hours at 37º C), and suspended in the freezing medium and then placed at -20º C freezer until needed [Cold Spring Harb Protocols, 2006].

II.3.5 Agarose Gels

2% Agarose gels were prepared by adding 0.5 g agarose to 25 ml TAE in a clean beaker. The agarose was then melted in a microwave for 10 to 15 seconds, mixed and cooled to about 50º C. 1.5 ul of Ethidium bromide (a dye used for fluorescent DNA

visualization) was then added, mixed and poured into a gel tray containing proper size comb [Lee et al., 2012].

II.3.6 Tris-Acetate EDTA (TAE) electrophoresis buffer

This buffer is commonly used for DNA separation in agarose gel electrophoresis. It contains Tris base, acetic acid, and EDTA (Ethylenediaminetetraacetic acid). Tris-acetate maintains solution pH and provides electrical conductivity, while EDTA protects the DNA from nucleases during the run by chelating the divalent cations (Mg2+, Ca2+) that are required for nucleic acid modifying enzymes such as DNases. This chelation leads to an inhibition of these metal-dependent nucleases.

To prepare 50x TAE buffer, 0.5M EDTA is prepared first, by dissolving 186.12 g of Disodium salt of EDTA (EDTA.Na2.2H2O) in water to a final volume of 1000 ml. Stock TAE buffer (50X) is then prepared by dissolving 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA in dH_2O to a final volume of 1000 ml. Working solution (1X) is prepared by adding 20 ml from 50X TAE stock solution to 980 ml of dH_2O. This working Solution has a pH of 8.2 – 8.4 and contains 40 mM Tris base, 20 mM Acetic acid and 1 mM EDTA [Cold Spring Harb Protocols, 2006].

II.3.7 Phosphate Buffered Saline (PBS)

PBS was used to wash the crystal violet and safranine dyes used in the biofilm formation experiment. 1 liter of 1X PBS working solution was prepared by adding 8g

NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 to 800 mL dH_2O. The pH was measured and adjusted to 7.4 using HCL and enough dH_2O was then added to make a total volume of 1 liter [Cold Spring Harb Protocols, 2006 , Alrashidi, 2020].

II.3.8 0.1% Crystal violet

Crystal violet stain was prepared by completely dissolving 0.1g of crystal violet powder in 100 mL distilled water [Azimi et al., 2019].

II.3.9 1% Safranin stain solution

Safranine was prepared by completely dissolving 1.0g of safranine powder in 100 distilled water [Kırmusaoğlu, 2019].

II. 4 Antimicrobial susceptibility by broth dilution

II.4.1 Minimal Inhibitory Concentration (MIC)

The MIC for *A. baumannii* was determined for colistin, rifampin and fosfomycin antibiotics. Subsequently, Combinations of colistin-rifampin, and colistin-fosfomycin were performed on all the isolates to determine the possible presence of synergy. In this project, MIC was performed by the microbroth dilution method using 96 well microtiter plate, following the guidelines of CLSI. All the 29 isolates were tested for

the sensitivity of the three antibiotics which are the drugs of choice for testing against *Acinetobacter baumannii*.

II.4.2 Stock concentration preparation of antibiotics used

The antibiotics used in this project were prepared according to the recommendations of Clinical and Laboratory Standards Institute (CLSI), [M100, 2017 and M07-A9, 2012]. It is recommended that stocks are usually prepared of at least 1280 ug/ml or at least 10 times higher than the concentration to be tested. For long storage, colistin and rifampin stocks were prepared at a concentration of 2.6 mg/ml and fosfomycin at 2.56 mg/ml which is approximately 10 times higher than the working concentration. The antibiotics were weighed using the following formula recommended by CLSI:

Weight (mg) = Volume (ml) X Concentration (µg/ml) / Potency (µg/mg)

or

Volume (ml) = Weight (mg) X Potency (µg/mg) / Concentration (µg/ml)

We had colistin vials obtained from Rafa laboratories LTD. We used two vials of colistin powder with a total weight of 130 mg to make colistin stock. Using 50mL centrifuge tube, the 130 mg powder was dissolved in 50 ml distilled water (DW), making a final concentration of colistin stock, 2.6 mg/mL (130mg/50ml). Same weight (130mg) was taken from 300 mg rifampin pills obtained from Medochemie LTD (pills

was grinded) and dissolved in the same amount of DW, making the same concentration of rifampin stock 2.6 mg/ml.

A weight of 128 mg was taken from 3000 mg fosfomycin pills obtained from Rafa laboratories LTD (pills was grinded) and dissolved in 50ml DW using 50ml centrifuge tube, making a final concentration of fosfomycin stock 2.56 mg/ml. Stocks were kept in the refrigerator at 4^{o} C until use.

II.4.3 Broth Microdilution Method for MIC determination

We calculated MIC by performing broth microdilution method using 96-well microtiter plate.

First, we prepared working solutions from the antibiotic stocks according to this equation (C1V1= C2V2), and then we used this working solution to prepare dilutions as the follows:

In sterile tubes, 4 mL of working solution was added in the first tube and 8 other tubes were filled with 2 mL of MHB. 2ml was transferred from the first tube that contained the antibiotic working solution and added into the second tube to give a 50% of the concentration, mixed and serially diluted to remaining tubes.

These steps were done on the three antibiotics to prepare their dilutions. Colistin has dilutions of 64 ug/ml to 0.25 ug/ml, rifampin has dilutions of 64 ug/ml to 1 ug/ml and Fosfomycin has dilutions of 128 ug/ml to 2 ug/mL.

From these tubes, 100 µl of colistin diluents were added into wells of the first row (A2 to A10) and 100 ul of rifampin or fosfomycin dilutions were added to wells of the first column (B to H) of the 96-well plate. A combination of the two antibiotics was added to the combination wells (50 ul from each antibiotic was added to each well). See table 4.

The inoculum (bacterial culture) was prepared by picking few isolated colonies from a fresh bacterium (cultured on BHI agar) using a sterile cotton swab and added to a tube containing sterile saline (0.9%) or MHB. The tube was vortexed and the suspension turbidity was adjusted to 0.5 McFarland using a 0.5 McFarland standard tube. This suspension contained approximately 1.5 x 10^8 CFU/mL.

The inoculum was diluted 1:100 using MH broth. A total of 0.3 mL of the 0.5 equivalent McFarland suspension was added to 9.7 mL MHB to prepare an inoculum of 10^6 CFU/mL which is the appropriate inoculum size for standard MIC.

100 µL volume of the diluted microbial suspension was added to all wells to make a total volume of 200 µl. A1 had the bacterial suspension alone (200 ul) and was considered as a positive control, while A11 had 200 ul MH broth only (negative control).

The 96 well plate was incubated at 37 °C for 24 h. After incubation the MIC was determined visually. The MIC was obtained in accordance to the guidelines of CLSI twenty-fourth informational supplement: Performance standards for antimicrobial susceptibility testing (2014).

Adding the inoculum to the wells reduces their concentration to the half, meaning that if MIC was 8ug/ml, it becomes 4ug/ml.

II.4.4 Combination Board

Table 4. Colistin and Rifampin microtiter plate with volumes and concentrations added to each well. Note: 100 ul of inoculum is added to each well with combined antibiotics. NC=Negative control, PC = Positive control, I = Inoculum, Colistin (C) and Rifampin (R)

	Ug/mL	**Colistin**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	
	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**
	PC	**64**ug/mL	**32**ug/mL	**16**ug/mL	**8**ug/mL	**4**ug/mL	**2**ug/mL	**1**ug/mL	**0.5**ug/mL	**0.25**ug/mL	**NC**
	200uL	100uL C	100ul C	100uL C	100uL C	100uL C	100uL C	100uL C	100uL C	100uL C	**200uL**
	I	100uL I	I00uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	**MHB**
	B										
	64ug/ml	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	
R	100uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	
I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	
F	**C**										
A	**32ug/ml**	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	
M	100uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	
P	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	
I	**D**										
N	**16ug/ml**	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	50uL C	
	100uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	50uL R	
	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	100uL I	

	E **8ug/ml** 100uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	
	F **4ug/ml** 100uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	
	G **2ug/ml** 100uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	
	H **1ug/ml** 100uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	50uL C 50uL R 100uL I	

Table 5. Colistin and Fosfomycin microtiter plate with volumes and concentrations added to each well. Note: 100 ul of inoculum is added to each well with combined antibiotics. NC=Negative control, PC = Positive control, I = Inoculum, Colistin (C) and Fosfomycin (F)

	Ug/mL	**Colistin**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	
	A1 **PC** **200uL** **I**	**A2** **64**ug/mL 100uL C 100uL I	**A3** **32**ug/mL 100ul C I00uL I	**A4** **16**ug/mL 100uL C 100uL I	**A5** **8**ug/mL 100uL C 100uL I	**A6** **4**ug/mL 100uL C 100uL I	**A7** **2**ug/mL 100uL C 100uL I	**A8** **1**ug/mL 100uL C 100uL I	**A9** **0.5**ug/mL 100uL C 100uL I	**A10** **0.25**ug/mL 100uL C 100uL I	**A11** **NC** **200uL** **MHB**

FOSFOMYCIN	**B** **128**ug/ml 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	
	C **64ug/ml** 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	
	D **32ug/ml** 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	
	E **16ug/ml** 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	
	F **8ug/ml** 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	
	G **4ug/ml** 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	
	H **2ug/ml** 100uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	50uL C 50uL F 100uL I	

II.4.5 Synergy determination by checkerboard analysis

This method has been used to determine the effect of antibiotic combinations as compared to their individual activity. This can be obtained by calculating the fractional inhibitory concentration index (FIC). The FIC index evaluates the combination of antibiotics that produces the most significant change from the individual antibiotic MIC. The interaction between the two antibiotics tested is calculated by the following equation:

FIC index = MIC_A in combination/MIC_A alone + MIC_B in combination/MIC_B alone

II.4.6 Interpretation:

Synergy:

When the FIC is <0.5, then the combination of the compounds increases the inhibitory activity (MIC decreased) of one or both compounds than the compounds alone.

Additive or indifference:

When the FIC value is 0.5 – 4, the combination has no increase in inhibitory activity from the additive effect of both antibiotics combined.

Antagonism:

When the FIC is >4, the combination of the compounds increases the MIC, or lowers the activity of the antibiotics.

Minimal bactericidal concentration (MBC) is 99.9% killing of the bacteria and it's the lowest concentration of the antibiotic required to kill the bacteria. To determine the

MBC, we subcultured 3 dilutions higher than the MIC. The MBC is the first dilution higher than the MIC and has less than 10 colonies growing on nutrient agar . MBC was done on colistin, the drug of choice to treat infections caused by MDR gram negative bacteria such as *A. baumannii.* The antimicrobial agent is usually considered bactericidal if the MBC is no more than four dilutions above the MIC. MBC can be determined from broth dilution of MIC by subculturing to agar plates. According to preestablished protocols and standards for MIC and MBC. MBC will be the lowest concentration of the antibiotic that reduces viability of the initial inoculum by ≥ 99.9% or 3 log reductions [Jenkins, 2012].

II.5 DNA extraction

II.5.1 Boiling

The DNA was extracted from all *A. baumannii* isolates by the boiling method. Briefly, a loopful (about 5–7 colonies) of the freshly cultured on BHI agar medium was dissolved in 500 ul sterile dH2O in microtubes. Cell suspension was centrifuged for 5 mins at 4,500 rpm, and the pellets obtained were placed into a tube containing 500 µL nuclease-free water, then boiled at 100 °C for 10 minutes. Immediately after boiling, the mixture was placed on ice for 10 minutes and then centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant containing the DNA was transferred to an Eppendorf tube [Falah et al., 2019, Ahmad and Dablool, 2017].

II.5.2 DNA concentration and purity by NanoDrop Lite Spectrophotometer

The DNA concentration and purity was determined by using NanoDrop Lite spectrophotometer. DNA concentration was obtained by measuring the absorbance of the extracted DNA at 260 nm wavelength, as both DNA and RNA have an absorbance maximum at 260 nm. The Purity of extracted DNA was assessed from the ratios of the absorbance values of 260 nm vs 280 nm (A260/A280). The mean concentration for all samples was 117.10±73.45 ng/ul, and the mean ratio for all samples was 1.87±0.15 . The purity of the DNA was checked by agarose gel electrophoresis and by obtaining the 260/280 wavelength ratio, (≥ 1.6 is acceptable).

Table 6. Concentration and purity of DNA measured by NanoDrop Lite Spectrophotometer

Isolate	DNA (ng/ul)	Purity (A260/A280)	Isolate	DNA (ng/ul)	Purity (A260/A28)	Isolate	DNA (ng/ul)	Purity (A260/A280)
1	71.3	1.77	11	50.8	2.03	21	100.0	2.01
2	94.4	1.60	12	65.0	1.98	22	60.7	1.97
3	135.3	1.71	13	63.4	1.92	23	97.6	1.92
4	104.7	1.66	14	28.5	2.01	24	143.7	1.98
5	176.3	1.65	15	70.2	1.98	25	255.8	1.75
6	171.2	1.73	16	87.6	2.03	26	225.7	1.93
7	98.7	1.62	17	54.6	2.00	27	187.0	1.90
8	167.3	1.75	18	159.2	2.02	28	126.5	1.88
9	78.3	1.64	19	93.1	2.02	29	354.1	1.96
10	20.0	2.11	20	55.1	1.97			
						Mean ±SD	117.10±73.45	1.87± 0.15

II.5.3 Quality of DNA evaluated by agarose gel electrophoresis

The quality and integrity of the extracted DNA was evaluated by agarose gel electrophoresis. All extracted samples were electrophoresed on 0.8% agarose gel and 80 volts and 10X loading dye was added to the extracted DNA template to provide it with density so it won't float and diffuse in the running buffer. It also gives DNA the color to monitor its migration in gel electrophoresis. All extracted DNA was intact and not fragmented with high quality. The concentrations of the extracted DNA were then standardized at a certain concentration and stored at -20 °C for performing PCR.

II.5.4 PCR Amplification

Amplification of OXA genes, *bla*$_{\text{oxa-23}}$, *bla*$_{\text{oxa-24}}$, *bla*$_{\text{oxa-51}}$, *bla*$_{\text{oxa-58}}$ *and bla*$_{\text{oxa-48}}$, *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{KPC-2}}$ were performed by Taq PCR Master Mix (2X) . Amplification was performed in 25µl reaction volume using 5 µl of extracted DNA template. According to the usage information of GoTaq® Green Master Mix (2X) from Promega, the 2X-Master Mix used contains 0.1U/ul of *Taq* DNA polymerase, PCR buffer that has a pH of 8.5 and contains 3mM $MgCl_2$, 400 µM of each deoxynucleotide triphosphate (DNTP) and two loading dyes (yellow and blue dyes). The PCR mixture (20ul) consisted of the following: 12.5µl Master Mix (2X), 0.5µl Forward primer, 0.5ul Reverse primer, and 6.5 µl nuclease free water. The primers used for PCR amplification are listed in Table 7. The amplification conditions used for PCR were as follows: initial

denaturation at 95º C, for 4 minutes followed by 35 cycles of denaturation at 95º C for 30 seconds, annealing at (each primer has its own annealing as shown in Table 7) for 40 seconds, extension at 72º C for 40 seconds and a final extension step at 72º for 5 min [Shoja et al., 2013]. Amplification mixture tube lacking the DNA template was included with each amplification cycle as a negative control. Positive controls used were selected from previously tested samples expressing the gene of interest.

The PCR amplicons were evaluated by 2% agarose gel electrophoresis stained with ethidium bromide. A 100-bp DNA ladder was used to assess the size of PCR products and find genes of interest . UV transilluminator was used for visualization and selected gels were photographed.

Table 7. Primer sequences used to amplify the different classes of the beta lactamase genes [Chenggong Hou, 2015]

Gene	**Primer sequence**	**size (bP)**	**Annealing**
bla$_{\text{OXA-23}}$	F: 5'-GATCGGATTG- GAGAACCAGA-3' R: 5'-ATTTCTGA- CCGCATTTCCAT-3	501	50ºC
bla$_{\text{OXA-51}}$	F: 5'-TAATGCTTTGATCGGCCTTG-3' R: 5'-TGGATTGCACTTCATCTTGG-3'	353	52ºC
bla$_{\text{OXA-58}}$	F: 5'- AAGTATTGGGGCTTGTGCTG -3' R: 5'- CCCCTCTGCGCTCTACATAC -3'	507	50ºC

bla$_{OXA-24}$	F:5'- GGTTAGTTGGCCCCCTTAAA-3' R:5'- AGTTGAGCGAAAAGGGGATT-3'	246	54ºC
bla$_{OXA-48}$	F5'-TTGGTGGCATCGATTATCGG-3' R5'-GAGCACTTCTTTTGTGATGGC-3'	743	58ºC
bla$_{NDM-1}$	F: 5'GGGCCGTATGAGTGA3' R: 5'GAAGCTGAGCACCGCATTAG3'	758	60ºC
bla$_{KPC-2}$	F: 5'ATGTCACTGTATCGCCGTC-3' R: 5'AATCCCTCGAGCGCGAGT-3'	862	58ºC

II.5.5 Isolation of DNA using QIAamp DNA Mini Kit for RAPD-PCR Analysis

The procedure of QIAamp DNA Mini kit consists of 4 steps: sample lysis, DNA in the lysate binds to the silica membrane of the QIAamp Mini spin column, the membrane is then washed and the DNA is eluted from the membrane (see Figure 9) [QIAamp® DNA Micro Handbook, 2014].

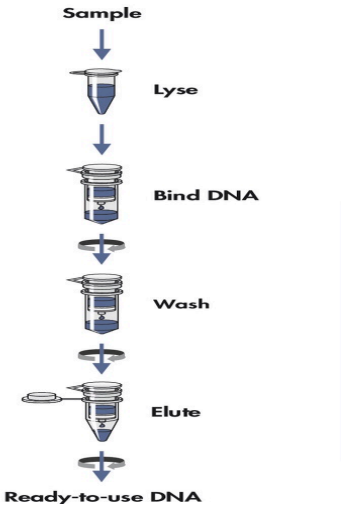


Figure 12. QIAamp DNA Mini Kit procedure [QIAamp® DNA Micro Handbook, 2014]

We followed the Quick-Start Protocol of QIAamp® DNA Mini Kit (2018) provided by QIAGEN to isolate the DNA. The lysis of the samples occurred under highly denaturing conditions at high temperatures in the presence of Buffer ATL and proteinase K.

A Loopful of bacteria was added to 180µl Buffer ATL in a 1.5mL Eppendorf tube and 20µl of Proteinase K was added and vortexed. The mixture was incubated at 56°C in a water bath for about 2 hours with occasional vortexing during incubation until completely lysed. 200µl of Buffer AL was added to the lysate to allow optimal binding of DNA to the silica membrane and vortexed to mix, then we incubated the tubes for 10 minutes at 70°C in a dry block heater. After that, drops on the lids of the tubes were removed by brief centrifugation and 200µl of 100% ethanol was added, then mixed by

vortexing. Another briefly centrifugation was applied to remove drops from the lids of the tubes.

Lysates were transferred onto the QIAamp Mini spin column in 2mL collection tube, and then centrifuged for 1 minute at 8000 rpm. In this step, the DNA is adsorbed onto the silica-gel membrane as the lysate is drawn through by centrifugation. Collection tube with the flow-through is removed and the QIAamp Mini spin column was placed into a new collection tube.

PCR inhibitors such as proteins and others are not retained on the QIAamp column due to the pH and salt conditions [QIAamp® DNA Micro Handbook, 2014].

Buffer AW1 (500µl) is added to cleanup of genomic DNA, then centrifugation for 1 minute at 8000 rpm was applied, and collection tube was then discarded. QIAamp column was placed into new collection tube and 500µl Buffer AW2 was added. Centrifugation at 14000 rpm was applied for 3 minutes and the collection tube with the flow-through was discarded. Washing with Buffer AW1 and then Buffer AW2 removes the contaminants while DNA remains bound to the membrane of the column.

QIAamp Mini spin column was placed in 1.5mL Eppendorf tube and 200µl Buffer AE was added. Tubes were Incubated for 1 minute at room temperature and then centrifuged for 1 minute at 8000 rpm to elute the DNA.

After extracting the DNA by the QIAamp DNA Mini Kit, NanoDrop Lite was used to determine the DNA concentration and purity (see Table 8). The mean concentration for all samples was 133.29±64.96 ng/ul, and the mean ratio for all samples was 1.98±0.11.

Table 8. Concentration and purity of DNA measured by NanoDrop Lite for RAPD analysis

Isolate	**DNA (ng/ul)**	**Purity (A260/A280)**	**Isolate**	**DNA (ng/ul)**	**Purity (A260/A28)**	**Isolate**	**DNA (ng/ul)**	**Purity (A260/A280)**
1	159.2	2.05	11	90.4	1.99	21	89.1	1.89
2	125.1	2.00	12	76.0	1.96	22	95.6	1.96
3	94.6	2.01	13	217.3	2.13	23	257.5	2.14
4	180.3	2.03	14	71.4	1.97	24	86.0	1.55
5	144.2	1.97	15	251.9	2.14	25	92.5	1.84
6	209.2	1.92	16	77.3	1.96	26	78.4	1.96
7	139.2	1.90	17	72.3	1.95	27	122.9	1.89
8	86.5	1.97	18	87.7	1.98	28	182.7	2.14
9	111.8	2.02	19	73.0	1.93	29	224.1	2.13
10	286.5	2.14	20	82.9	2.00			
						Mean +SD	**133.29±64.96**	**1.98± 0.11**

Quality of DNA was evaluated by agarose gel electrophoresis where all extracted samples were electrophoresed on 0.8% agarose gel and 80 volts using 10X loading dye. All extracted DNA was intact with high quality and not fragmented. The concentrations of the extracted DNA were then standardized at a certain concentration and stored at -20 °C for performing RAPD-PCR.

II.6 Molecular Genotyping of *A. baumannii* by the Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

This method generates DNA fragments from PCR amplification of random segments of genomic DNA. The advantage of using this method lies in that it doesn't require knowledge of the exact sequences of the target microorganism where a random primer sequence binds unknown genomic sequence, and the random parts amplified are expected to be identical amongst related species, and having the same banding patterns in gel electrophoresis.

Some gradient optimization on the annealing temperature and other modification on the amplification program were made. The RAPD procedure adopted in this research has been taken from Sadeghifard N, et al., 2010 and Abozahra R et al., 2021. The RAPD-PCR fingerprinting was carried out in a total reaction volume of 50uL containing 25ul GoTaq Green Master Mix (2X), 1uL of 10uM AP-ARB11 primer (5'-CTAGGACCGC-3') and 1uL of 10uM AP-PG05 primer (5'-AGCCCAGCTATGAAC-3') and 5ul of the DNA template and enough sterile dH2O (18ul) to make a total volume of 50 ul.

Amplification was carried out with denaturation at 94°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, annealing at 32°C for 2 minutes, extension at 62°C for 2 minutes, and a final extension of 10 minutes at 62°C to complete the partial polymerizations. The PCR product was run and visualized in 2% agarose gel stained with ethidium bromide, with the use of 1Kb ladder.

II.7 Tube Method of Biofilm Staining

II.7.1 Biofilm Staining with Safranin

A qualitative assay using a tube method was used for detecting visible biofilm formation by *A. baumannii* [Christensen et al., 1985]. The *A. baumannii* isolates were inoculated in polystyrene test tubes containing Tryptic soy broth (TSB) and incubated at 37º C for 24 hours. The biofilm formed on the sides of the tube was then stained with safranin for one hour. After rinsing twice with PBS to remove stain, the tubes were air dried. The occurrence of stained biofilm on sides and bottom of the tubes was assessed and scored [Kırmusaoğlu, 2019].

II.7.2 Biofilm Staining with Crystal Violet

A loopful of tested isolates was inoculated in 1mL of trypticase soy broth (TSB) with 1% glucose in test tubes, then tubes were incubated for 24 hours at 37°C. After incubation, tubes were washed with PBS (pH 7.3) and were air dried, and then they were stained with 0.1% crystal violet. Deionized water was used to rinse excess stain and tubes were then dried in an inverted position and results were scored [Azimi et al., 2019].

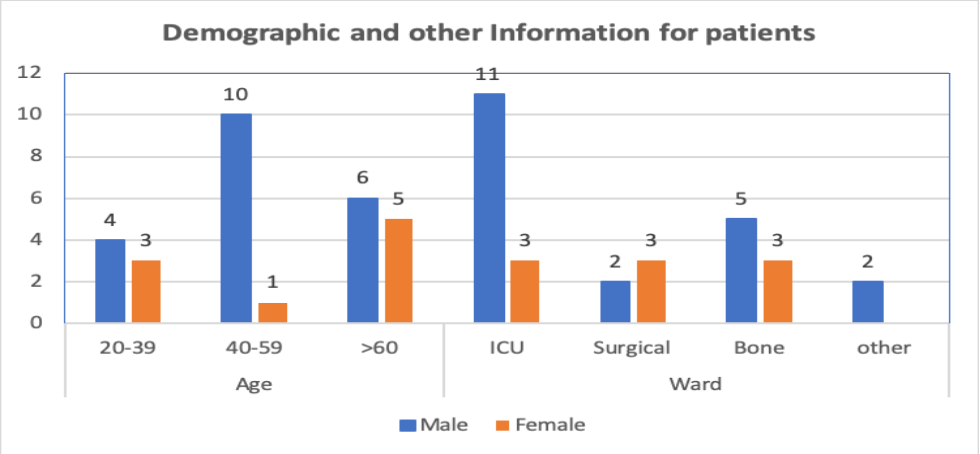
Chapter III

Results

III.1 Demographic and other information

A total of 29 A. baumannii isolates were collected from MCF in Jerusalem and PMC in Ramallah. The demographics (gender, age) and other related information (ward) are shown in Figure 13.

Figure 13. Demographic and related patients' information



The results showed that male to female ratio is 2:1 and male patients at age 40-59 years predominated over females at a rate of 91% (10/11) as well as residents in the ICUs 78.6% (11/14).

According to the patient's information taken from the two hospitals, the isolates were tested for bacterial identification using Vitek-2 compact machine and for antibiotic

susceptibility testing. All 29 isolates of *A. baumannii* collected were multidrug resistance. They were resistant to carbapenems (imipenem and meropenem), aminoglycosides (amikacin and gentamicin), third generation cephalosporins (ceftazidime, ceftriaxone and cefotaxime), fluoroquinolone (ciprofloxacin and levofloxacin) and piperacillin /tazobactam (beta-lactamase inhibitors). They all tested susceptible to colistin treated with it.

III.2 Results of MIC, MBC and the synergy testing

Broth microdilution method was used to test in vitro the antimicrobial activity of several agents against *A. baumannii* following the guidelines of CLSI. This method was used to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for the 29 clinical isolates. The MIC is the lowest antimicrobial concentration in μg/ml that inhibits bacterial growth. A lower value of MIC indicates that less drug is needed for inhibiting growth of the bacteria, so drugs with lower MIC scores are more effective antimicrobial agents. Results of MIC help to take measures to prevent evolution of drug-resistant strains of bacteria and improve outcomes for patients [LibreTexts, 2021].

The MIC determination for colistin was done twice. Since we used the microdilution method, it was important to repeat the MICs to confirm the accuracy of the results. The breakpoints for colistin have been obtained from the laboratory standards for antimicrobial susceptibility testing recently published by the Clinical laboratory

standard institute (CLSI) [M100, 2021]. The acceptable break points for the three antibiotics tested are shown in Table 9.

Table 9. Acceptable break points according to CLSI for the interpretation of the susceptibility results for colistin, rifampin and fosfomycin

MICs ug/ml				
Antibiotic	Sensitive (S)	Intermediate (I)	Resistant (R)	Comments
Colistin	≤ 2	-	≥ 4	
Rifampin	≤ 4	-	>4	Varshochi *et al*. 2019
Fosfomycin	≤ 64	128	≥ 256	

Our results of the MIC and MBC for colistin, rifampin and fosfomycin are summarized and shown in Table 10.

Table 10. Results for the MICs of colistin, rifampin and fosfomycin alone

	Colistin	Rifampin	Fosfomycin
#Sensitive (%)	26 (89.7%)	15 (51.7%)	None
#Intermediate (%)	None	None	Not done
#Resistant (%)	3 (10.3%)	14 (48.3%)	Not done
Total #	29	29	29

Results of the MIC and MBC for colistin alone, MIC for rifampin alone and MIC for Fosfomycin alone are shown is details in Table 11.

Table 11. Results of the MIC and MBC for colistin and, MICs for rifampin and for fosfomycin.

	Colistin				**Rifampin**	**Fosfomycin**
Isolate	**MIC-1 ug/ml**	**MIC-2 ug/ml**	**MBC-1 ug/ml**	**MBC-2 ug/ml**	**MIC ug/ml**	**MIC ug/ml**
1	1	1	4	4	16	>64
2	1	0.5	4	2	8	>64
3	0.5	0.5	2	2	4	>64
4	1	0.25	2	0.5	8	>64
5	0.5	0.25	2	1	8	>64
6	0.5	0.5	4	4	4	>64
7	0.5	0.5	2	2	8	>64
8	2	1	4	4	4	>64
9	0.5	0.5	2	1	2	>64
10	0.25	0.25	1	1	2	>64
11	0.25	0.25	1	1	2	>64
12	1	0.5	2	2	4	>64
13	0.25	0.5	2	1	2	>64
14	0.25	0.25	2	2	2	>64
15	0.5	0.5	1	1	2	>64
16	16	32	>32	>32	2	>64
17	0.25	0.5	2	4	4	>64

18	0.25	0.5	1	2	2	>64
19	0.25	0.5	2	4	8	>64
20	0.25	0.25	0.5	0.5	8	>64
21	1	1	2	2	8	>64
22	0.5	0.5	2	4	8	>64
23	4	16	16	16	8	>64
24	32	32	>32	>32	8	>64
25	0.5	0.5	2	2	4	>64
26	0.5	0.5	2	2	8	>64
27	0.25	0.25	1	1	8	>64
28	0.5	0.5	2	2	8	>64
29	0.5	0.5	2	2	4	>64
# of Resistant isolates	3	3			14	
Resistance%	10.3%	10.3%			48.3 %	

The results of *A. baumannii* susceptibility to colistin revealed a rate of 89.7% (26/29) and resistance at a rate of 10.3% (3/29) as seen in the Table 11 above. As for rifampin, the susceptibility rate was 51.7% (15/29) and the resistance rate was 48.3% (14/29). None of the isolates were susceptible to Fosfomycin since the MICs were >64 ug/ml. The MICs for the intermediate or resistant were out of the range tested. The variations observed between the MICs for colistin in Table 11 are within the recommendations allowed by the CLSI which is $\pm$ one dilution factor.

Table 12. Determination of the presence of synergy between Colistin and Fosfomycin (MICs ug/ml)

MIC of colistin alone	MIC of colistin in combination with fosfomycin	FIC of colistin (MIC in combination/ MIC alone)	MIC of fosfomycin alone	MIC of fosfomycin in combination with colistin	FIC of fosfomycin (MIC in combination/ MIC alone)	FIC total (FIC of colistin / FIC of fosfomycin)
1	0.5	0.5	64	1	0.02	0.52
0.5	0.5	1	64	1	0.02	1.02
0.5	0.5	1	64	1	0.02	1.02
0.25	0.25	1	64	1	0.02	1.02
0.25	0.25	1	64	1	0.02	1.02
0.5	0.5	1	64	1	0.02	1.02
0.5	0.5	1	64	1	0.02	1.02
1	1	1	64	1	0.02	1.02
0.5	0.25	0.5	64	1	0.02	0.52
0.25	0.25	1	64	1	0.02	1.02
0.25	0.25	1	64	1	0.02	1.02
0.5	0.5	1	64	1	0.02	1.02
0.5	1	2	64	2	0.03	2.03
0.25	0.25	1	64	1	0.02	1.02

0.5	0.5	1	64	1	0.02	1.02
32	16	0.5	64	4	0.06	0.56
0.5	1	2	64	1	0.02	2.02
0.5	0.5	1	64	1	0.02	1.02
0.5	1	2	64	1	0.02	2.02
0.25	0.5	2	64	2	0.03	2.03
1	0.5	0.5	64	2	0.03	0.53
0.5	1	2	64	1	0.02	2.02
16	16	1	64	64	1.00	2.00
32	1	0.03125	64	1	0.02	0.05
0.5	1	2	64	1	0.02	2.02
0.5	1	2	64	1	0.02	2.02
0.25	1	4	64	1	0.02	4.02
0.5	2	4	64	1	0.02	4.02
0.5	2	4	64	2	0.03	4.03

As for Fosfomycin, there was no synergy detected in combination with colistin. However, the effect was additive (FIC is 0.5-4). None of the isolates tested were susceptible to fosfomycin as the MIC was >64 ug/ml. However, it is interesting to note that the MIC results showed that most of isolates became fosfomycin susceptible with

an MIC of ≤4 ug/ml after combination with colistin colistin (MIC >64 became ≤4). There was one isolate only that remained resistant to fosfomycin after combination with colistin at MIC >64. It is also interesting to note that the MIC result showed that one of the isolates that was colistin resistance (MIC=32ug/ml) became sensitive to colistin when combined with fosfomycin (MIC was reduced to 1 ug/ml). Since the MICs for fosfomycin were significantly lowered, it may be useful to use it in combination with colistin to treat infections caused by *A. baumannii* which may prevent evolution of resistance to colistin due to selective pressure. Table 12 shows the results of colistin and fosfomycin each alone and in combination, also the FIC index of them to determine the presence of synergy.

Our results showed the presence of synergy between colistin and rifampin only in 5 isolates (FIC<0.5). Other isolates showed additive effect that could help relief the selective pressure and prevent the evolution of colistin resistance. In the cases where *A.baumannii* isolates were resistant to colistin alone, the MIC was reduced to become susceptible to colistin after combination with rifampin (MIC≥4 became 0.25). It was also observed that rifampin resistant isolates became susceptible to rifampin after combining with colistin (MIC>4 became <1). The results of this combination is shown in the table 13.

Table 13. Determination of the presence of synergy between Colistin and Rifampin

MIC of colistin alone	MIC of colistin in combination with rifampin	FIC of colistin (MIC in combination/ MIC alone)	MIC of rifampin alone	MIC of rifampin in combination with colistin	FIC of rifampin (MIC in combination/ MIC alone)	FIC total (FIC of colistin / FIC of rifampin)
1	1	1	16	1	0.0625	1.0625
1	0.5	0.5	8	1	0.125	0.625
0.5	1	2	4	0.5	0.125	2.125
1	0.5	0.5	8	0.5	0.0625	0.5625
0.5	0.25	0.5	8	0.5	0.0625	0.5625
0.5	0.5	1	4	0.5	0.125	1.125
0.5	0.25	0.5	8	0.5	0.0625	0.5625
2	2	1	4	0.5	0.125	1.125
0.5	0.25	0.5	2	0.5	0.25	0.75
0.25	0.25	1	2	0.5	0.25	1.25
0.25	0.25	1	2	0.5	0.25	1.25
1	0.25	0.25	4	0.5	0.125	0.375
0.25	0.25	1	2	0.5	0.25	1.25
0.25	0.25	1	2	0.5	0.25	1.25
0.5	0.25	0.5	2	0.5	0.25	0.75

16	0.25	0.0156	2	0.5	0.25	0.2656
0.25	0.25	1	4	0.5	0.125	1.125
0.25	0.25	1	2	0.5	0.25	1.25
0.25	0.5	2	8	0.5	0.0625	2.0625
0.25	0.25	1	8	0.5	0.0625	1.0625
1	0.25	0.25	8	0.5	0.0625	0.3125
0.5	0.5	1	8	0.5	0.0625	1.0625
4	0.25	0.0625	8	0.5	0.0625	0.125
32	0.25	0.0078	8	0.5	0.0625	0.0703
0.5	0.25	0.5	4	0.5	0.125	0.625
0.5	0.25	0.5	8	0.5	0.0625	0.5625
0.25	0.25	1	8	0.5	0.0625	1.0625
0.5	0.25	0.5	8	0.5	0.0625	0.5625
0.5	0.25	0.5	4	0.5	0.125	0.625

Minimum Bactericidal Concentration (MBC):

The MIC has been defined as the lowest concentration of an antimicrobial agent that inhibits visible growth of bacteria (bacteriostatic). However, the MBC is the lowest concentration of an antimicrobial agent that results in bacterial death. The antimicrobial agent is usually considered Bactericidal if the MBC is no more than four times higher than the MIC.

MBC can be determined from broth dilution of MIC by subculturing on agar plates. According to pre-established protocols and standards for MIC and MBC. MBC will be the lowest concentration of the antibiotic that reduces viability of the initial inoculum by ≥ 99.9% or 3 log reduction [Jenkins, 2012].

In this study, the MBC was considered as the plate that has <10 colonies or no growth. As shown in Table 11. In our project, we had 29 isolates, 2 of them were resistant to colistin (MIC ≥4), so their MBC couldn't be determined, while the MBCs of the other 27 isolates were observed and were either 1, 2 or 3 dilutions above the MIC at the rates of 22.2% (6/27) for 1 dilution higher than the MIC, 55.5% (15/27) for 2 dilutions higher than the MIC, and 18.5% (5/27) for 3 dilutions higher than the MIC.

III.3 Literature review regarding the results of MIC and the synergy testing

Colistin-Fosfomycin combination:

According to a research done in 2021 in Thailand to study colistin plus Sulbactam or Fosfomycin against Carbapenem-Resistant *A. baumannii*. 50 isolates were tested for synergistic effect of colistin-fosfomycin combination. They had a synergistic rate of 22% (11/50 isolates had FIC<0.5) and additive effects against 33 isolates 66% (33/50 with 0.5-4 FIC). There were no antagonistic effect against any of the strains [Saelim et al., 2021]. In our project, we had zero synergy (none <0.5) and 100% additive effect (0.5-4) between colistin and Fosfomycin.

In their research, the colistin MIC range (1 - 16 µg/mL) was reduced to (0.0625 – 2 µg/mL) for 5 isolates when combined with Fosfomycin [Saelim et al., 2021]. In our project, we had one isolate that was colistin resistance (MIC=32ug/ml) and became sensitive to colistin when combined with fosfomycin (MIC was reduced to 1 ug/ml) and most of Fosfomycin resistant isolates (28/29) became susceptible to Fosfomycin with an MIC of ≤4 ug/ml after combination with colistin. There was one isolate only that remained resistant to fosfomycin after combination (MIC >64).

Colistin-Rifampin combination:

In a study conducted in China in 2014 regarding the invitro activity of colistin-rifampin combination against multidrug resistant *A. baumannii*. The synergism test results showed 56% synergetic effect and 36% additive effect [Dong et al., 2014]. In our project, we had 17.2% (5/29), 82.8% (24/29) and 0%, relative to synergism, additive effect and antagonism.

In their study, the average MICs of each antibiotic were decreased when they were used in combination, which was in accordance with the changing trend in FICI values [Dong et al., 2014]. In our project, the MIC of the colistin resistant isolates was reduced and they became susceptible to colistin after combination with rifampin, also the MIC of rifampin resistant isolates was reduced and they became susceptible to rifampin after combining with colistin.

MIC of colistin, rifampin and Fosfomycin alone

In a study conducted in China in 2014, from the 25 strains of multi-drug resistant *A. baumannii* 3 strains were resistant to colistin (12% or 3/25) and 22 strains were sensitive (88% or 22/25) [Dong et al., 2014] . These results are similar to our results as we had a resistance rate of 10.3% (3/29) and a susceptibility rate of 89.7% (26/29 to colistin.

A study conducted in Brazil in 2016 about the antimicrobial combinations against Resistant *A.baumannii*. All of the 20 isolates of *A. baumannii* were resistant to rifampicin and fosfomycin (100% resistance and 0% susceptibility)[Leite et al., 2016]. In our project, we had the same result regarding the sensitivity to fosfomycin (0%) as none of the isolates were sensitive, but we couldn't determine whether they were resistant or intermediate because the MIC we had was >64 as we didn't work on higher concentrations. As for rifampin, we had 48.3% (14/29) resistance rate and 51.7% (15/29) susceptibility rate in our project.

III.4 DNA Purification

DNA was isolated by boiling method, then it concentration and purity was measured by NanoDrop Lite Spectrophotometer. The mean concentration for all samples was 117.10$\pm$73.45 ng/ul, and the mean ratio for all samples was 1.87$\pm$0.15. Quality and integrity of the extracted DNA was evaluated by agarose gel electrophoresis where all extracted DNA was intact and not fragmented with high quality.

III.5 PCR Results for beta-Lactamases

PCR on beta-lactamase genes was conducted to characterize the molecular mechanisms of carbapenem resistance in *A. baumannii* isolates. The results of PCR are shown in Table 14 below.

Table 14. Summary of results obtained by PCR of beta lactamases present in *A. baumannii* clinical isolates

	OXA-51	OXA-23	OXA-24	OXA-48	OXA-58	NDM-1	KPC
Positive	29	29	0	27	0	23	14
Negative	0	0	29	2	29	6	15
%Positive	100%	100%	0.0%	93.1%0	0.0%	79.3%	48.3%

All *A. baumannii* isolates tested had $bla_{\text{OXA-23}}$ carbapenemase gene and the naturally occurring $bla_{\text{OXA-51}}$ gene in rate of 100%. The $bla_{\text{OXA-23}}$ gene was tested to monitor carbapenem resistance in *A. baumannii* because this gene confers high level carbapenem resistance. The $bla_{\text{OXA-51}}$ gene is present in all isolates of *A. baumannii*. Detection of $bla_{\text{OXA-51}}$ gene in *A. baumannii* has been considered to be a more reliable identification method than biochemical identification or by the most commonly used API method. The $bla_{\text{OXA-48}}$ gene was tested to confirm resistance of *A. baumannii* to both carbapenem and imipenem. This gene is most frequent among the *Enterobacteriaceae* isolates in Mediterranean countries. It could have been acquired by *A. baumannii* during gene exchange with *Enterobacteriaceae* (mainly *K.*

pneumoniae). The rate of *bla*$_{\text{OXA-48}}$ gene in the isolates tested was significantly high of 93.3% (27/29). As for the *bla*$_{\text{OXA-58}}$ gene and *bla*$_{\text{OXA-24-}}$ gene also called *bla*$_{\text{OXA-40}}$, none of the isolates tested in this project had them.

The *bla*$_{\text{NDM-1}}$ gene encodes for the NDM beta lactamase, a class B metallo-beta lactamase (MBL) which contains zinc ions in the active site. These enzymes are characterized by their ability to hydrolyze carbapenems. The *bla*$_{\text{NDM-1}}$ gene was present in *A*. *baumannii* isolates tested at a rate of 79.3% (23/29). As mentioned earlier, the class A beta-lactamases (carbapenemase) are commonly found in the *Enterobacteriaceae* family. *Klebsiella pneumoniae* particularly harbors the *bla*$_{\text{KPC-2}}$ gene which encodes for beta lactamases that have high activity against carbapenems. This gene as the *bla*$_{\text{OXA-48}}$ has been disseminated to *A. baumannii* from *K. pneumoniae*. The rate of this gene in the *A. baumannii* isolates tested here was 48.3% (14/29). The presence of KPC in *A. baumannii* has been limited to certain geographic areas. The results of all beta-lactamase genes tested in this project are summarized in Table 14.

III.6 Literature review regarding the PCR Results for beta-Lactamases

According to a Palestinian study done in 2017 on hospitalized patients with MDR *A. baumannii* from all over Palestine, except Gaza. Of the 69 isolates, 100% were positive for *bla*$_{\text{OXA-51,}}$ 82.6% were positive for *bla*$_{\text{OXA-23}}$, 14.5% were positive for *bla*$_{\text{OXA-24}}$, and 3% were positive for *bla*$_{\text{OXA-58}}$. In addition, 5.8% were positive for *bla*$_{\text{NDM}}$ and 0% were positive *bla*$_{\text{KPC}}$, respectively [Handal

et al., 2017]. A multicenter study on *A. baumannii* was done to investigate the molecular epidemiology and genetic background of carbapenem resistance of *A. baumannii* isolates collected from 2014–2017 in Austria. They found out that the most prominent OXA-types were *bla*$_{\text{OXA-58}}$ (46.5%) and *bla*$_{\text{OXA-23}}$ (41.2%), followed by *bla*$_{\text{OXA-24}}$ (10.5%). The isolates had no *bla*$_{\text{NDM}}$ nor *bla*$_{\text{KPC}}$ [Grisold et al., 2021]. Another study done in 2020 in Tehran, Iran to evaluate the antimicrobial resistance patterns and molecular frequency of *bla*$_{\text{OXA-48}}$ gene in *A.baumannii* strains found *bla*$_{\text{OXA-48}}$ gene in 92% of the *A. baumannii* isolates they tested [Tarafdar et al., 2020] and this is close to the rate we had for *bla*$_{\text{OXA-48}}$. A study was carried out in 2013 in Algeria about the prevalence of carbapenemase-encoding genes including New Delhi metallo-β-lactamase in *Acinetobacter* species. Among the 113 isolates, The *bla*$_{\text{OXA-23}}$ gene was detected in 50% (40/80) of the isolates, the *bla*$_{\text{OXA-24}}$ gene was detected in 21.2% (17/80), the metallo-β-lactamase *bla*$_{\text{NDM-1}}$ was detected in 5 isolates (6.2%) of the isolates [Mesli et al.,2013]. A study was carried out in Iran in 2015 about the characterization of carbapenemases in extensively drug resistance *A.baumannii* in a burn care center. They found that 9.23% of the isolates carries *bla*$_{\text{KPC}}$ gene [Azimi et al., 2012]. In our project: the rate of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-51}}$ genes was 100%, *bla*$_{\text{OXA-48}}$ gene had a rate of 93.1%, while *bla*$_{\text{OXA-58}}$ and *bla*$_{\text{OXA-24}}$ genes had a rate of 0%. *bla*$_{\text{NDM-1}}$ had a rate of 79.3% and *bla*$_{\text{KPC-2}}$ gene had a rate of 48.3%.

All of the clinical isolates carried the genes that encoded for OXA 23 and OXA51 in rate of 100% and this is too close to literature. OXA-48 was present in 93.1% of the

isolates, a finding that is also very close to that reported in literature. However, OXA-24 and OX-58 were not found in our research, but was found in low rates in literature. NDM-1 and KPC-2 was found in high rates in our research, but in low rates in literature.

The *bla*$_{\text{OXA-51}}$ is intrinsic, we found out that *bla*$_{\text{OXA-23}}$ is the most prominent OXA-type in our study, followed by *bla*$_{\text{OXA-48}}$ then *bla*$_{\text{NDM-1}}$ then *bla*$_{\text{KPC-2}}$. These genes are responsible for the resistance against carbapenems in our isolates.

We observed that all *A. baumannii* isolates showed two bands on the gel electrophoresis for the amplicons of *bla*$_{\text{OXA-48}}$ as seen in Figure 19. We have seen the expected band of 743 bp and smaller band of 250 bp which could represent non-specific binding of the primer. However, this situation occasionally occurs even with the presence of control when testing some genes by PCR. Unfortunately, we are unable to get a positive control at this stage. In order to deal with such situation, experts recommend raising the annealing temperature by 1-2 degrees, or incorporating DMSO, or Bovine Serum Albumin (BSA) in the PCR mix. Since these suggestions don't always work, they suggested to cut the band from the gel and send for sequencing. We would like to emphasize that the amplicon representing this gene gave a band size as expected and therefore we reported the results as seen.

III.7 Gel electrophoresis for beta lactamase genes

A representative gel for all the beta lactamase genes tested is shown in Figure 14. It is obvious that *A. baumannii* isolates were negative for *bla*$_{\text{OXA-58}}$, and *bla*$_{\text{OXA-24}}$ in wells number 6 and 7 as shown in the Figure.

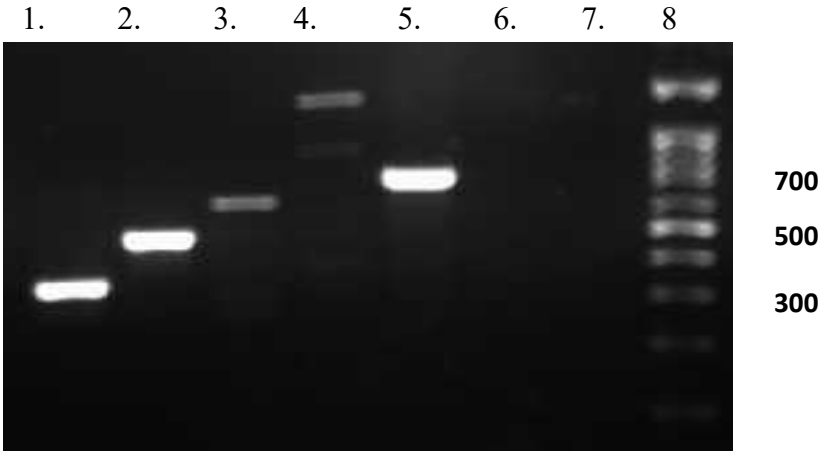


Figure 14. A representative gel for the different beta lactamases tested on *A. baumannii* clinical isolates. Lanes; 1. *bla*$_{\text{OXA-51 (353bp)}}$, 2. *bla*$_{\text{OXA-23 (501 bp)}}$, 3. *bla*$_{\text{OXA-48 (743bp)}}$, 4. *bla*$_{\text{KPC-2 (862bp)}}$, 5. *bla*$_{\text{NDM-1 (758bp)}}$, 6. *bla*$_{\text{OXA-58 (507bp)}}$, 7. *bla*$_{\text{OXA-24 (246bp)}}$, 8. 100bp ladder

1. 2. 3. 4. 5. 6. 7. 8

Figure 15: Lane 1 to 7 represents the absence of *bla*$_{\text{OXA-58}}$ (507 bp), Lane 8 has the 100 bp ladder

1. 2. 3. 4. 5. 6. 7. 8

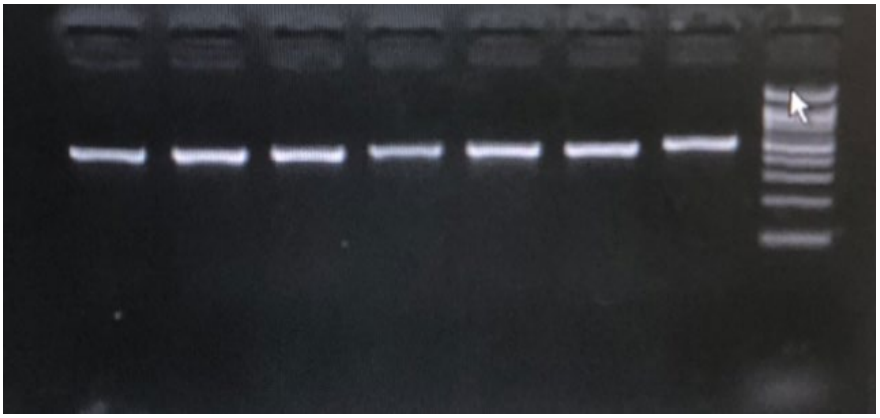


Figure 16: Lane 1 to 7 represents *bla*$_{\text{OXA-23}}$ (501 bp), Lane 8 has the 100 bp ladder

1. 2. 3. 4. 5. 6. 7. 8

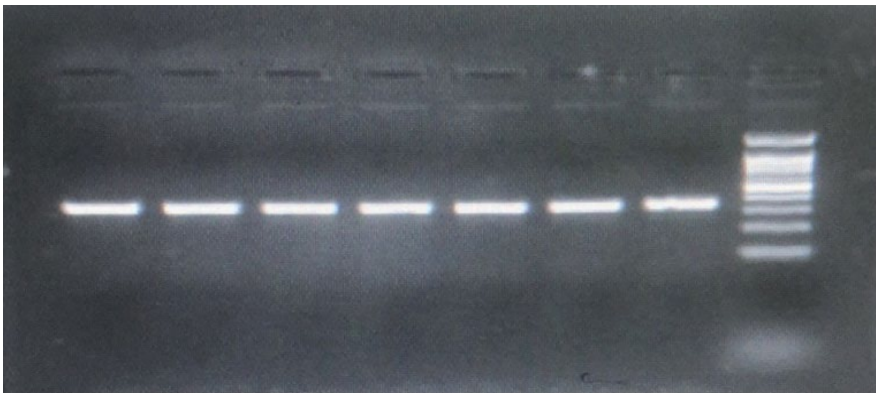


Figure 17: Lane 1 to 7 represents *bla*$_{\text{OXA-51}}$ (353 bp), Lane 8 has the 100 bp ladder

1. 2. 3. 4. 5. 6. 7. 8

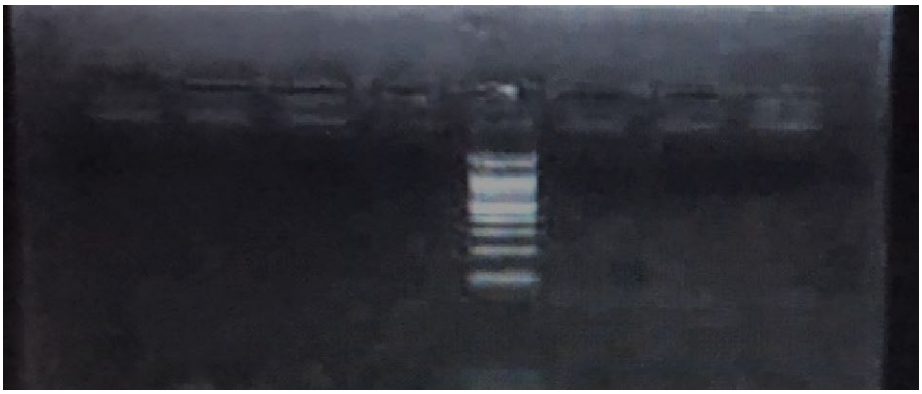


Figure 18: Lane 1 to 8 represents the absence of *bla*$_{\text{OXA24}}$ (246 bp), Lane 5 has the 100 bp ladder

1. 2. 3. 4. 5. 6. 7. 8

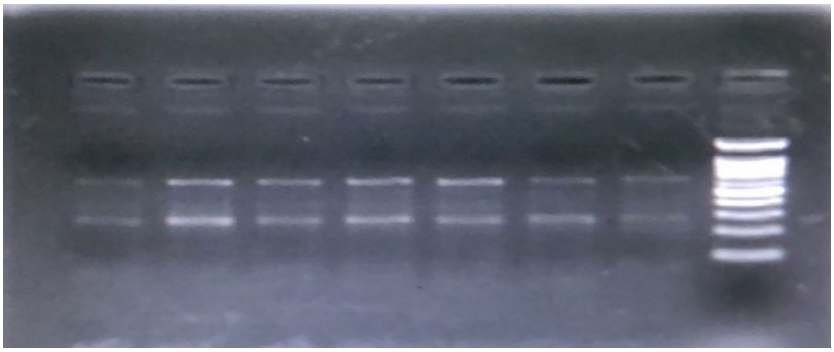


Figure 19: Bands above in lane 1 to 7 represent *bla*$_{OXA48}$ (743 bp), Lane 8 has the 100 bp ladder, all bands are present but some of them are faint

1. 2. 3. 4. 5. 6. 7. 8

Figure 20: Lane 1 to 7 represents *bla*$_{NDM-1}$ (758 bp), Lane 8 has the 100 bp ladder, all lands are positive for *bla*$_{NDM-1}$ except for lane 5 which is negative

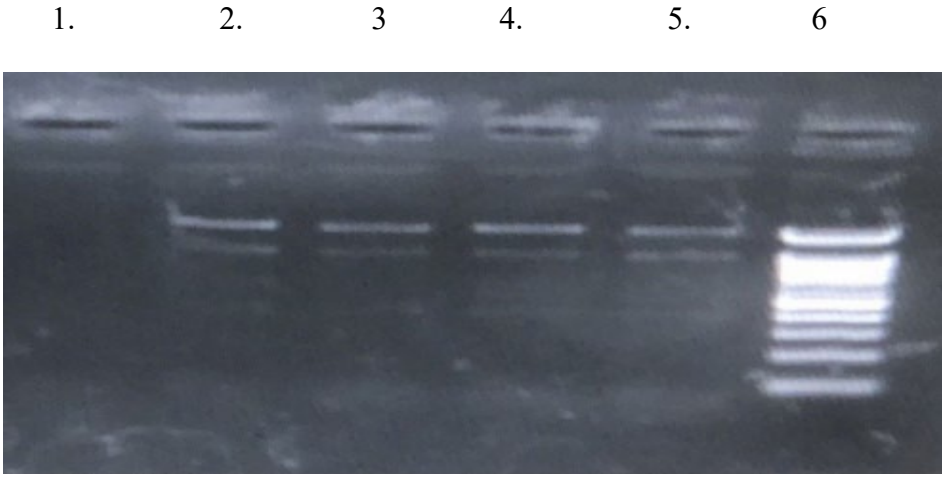


Figure 21: Bands below in lane 2 to 5 represent *bla*$_{KPC-2}$ (862 bp), Lane 6 has the 100 bp ladder, lane 1 is negative for this gene, bands below are present and positive, but faint

III.8 RAPD-PCR Analysis

There are many methods that can be used for molecular typing for epidemiologica purposes. In this study we used the random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) to determine the relatedness of the isolates. The results of the RAPD-PCR analysis are shown in Table 15 and Figures 22 and 23. Our data showed that four main bands of 2500, 1500, 1000 and 600 were observed in most of the isolates in the gel, Only 5 isolates had one band different than the above mentioned ones (650, 500, 400,250 bp). The band of 2500 bp and 1000 bp have been seen in 93.1% (27/29) of the isolates, the band of 1500 bp has the highest rate of 96.6% (28/29) among the isolates and the band of 600 bp has a rate of 75.9% (22/29). The

band of 500bp has a rate of 6.9% (2/29) while bands 400, 650 and 250 bp each has a rate of 3.4% (1/29).

Table 15. RAPD PCR results showing the different classes of *A. baumannii* according the size of the amplicons

Class	**No. isolates**	**Percent**	**Isolates**	**No. of Bands**	**Size bp**
I	17	58.6%	1, 2, 4, 5, 8, 11, 12, 16,17,18, 20, 21, 22, 23, 25, 26, 27	4	2500, 1500, 1000, 600
II	4	13.8%	3, 6, 14, 19	3	2500, 1500, 1000
III	1	3.4%	7	2	2500, 1500
IV	2	6.9%	13, 29	5	2500, 1500, 1000, 600, 500
V	1	3.4%	15	3	1500, 1000, 600
VI	1	3.4%	24	2	1500, 600
VII	1	3.4%	28	5	2500, 1500, 1000, 600, 400
VIII	1	3.4%	10	3	2500, 1000, 650
IX	1	3.4%	9	4	2500, 1500, 1000, 250
Total	29	99.9%			

The RAPD-PCR gels for the isolates are shown in Figures 22 and 23 below. We would like to mention that the estimation of the size and number of the bands was done manually. However, few small bands of 250 bp seems to be not precise. For precise size determination, we could have used a 50 bp ladder. Since these bands are rare in the isolates, our estimation of their size seems to be satisfactory for the purpose.

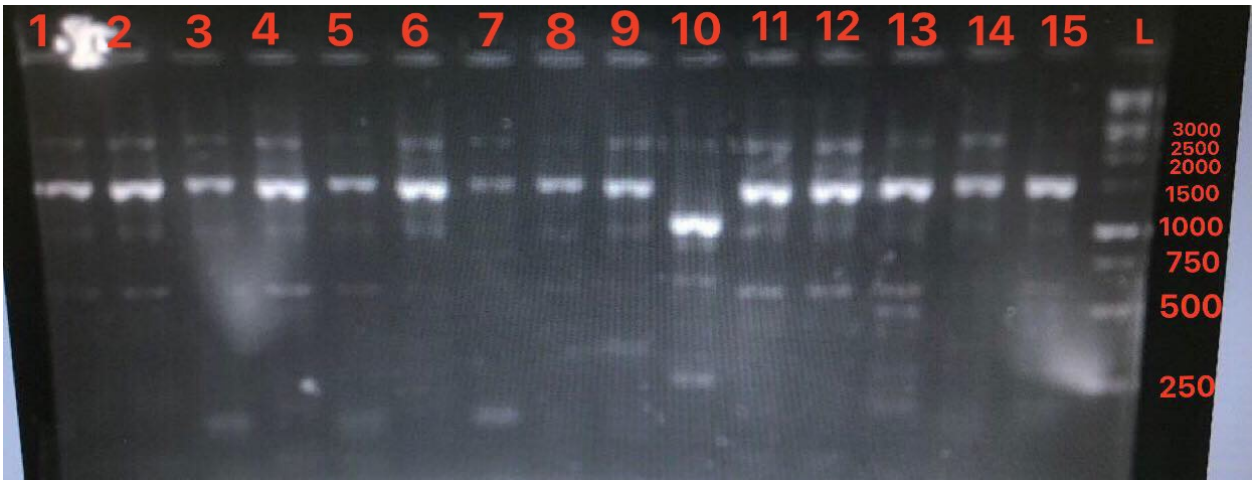


Figure 22. Representative RAPD-PCR gel-1. Lanes 1 to 15 are *A. baumannii* isolates. Lane 16 has 1kb ladder (size marker)

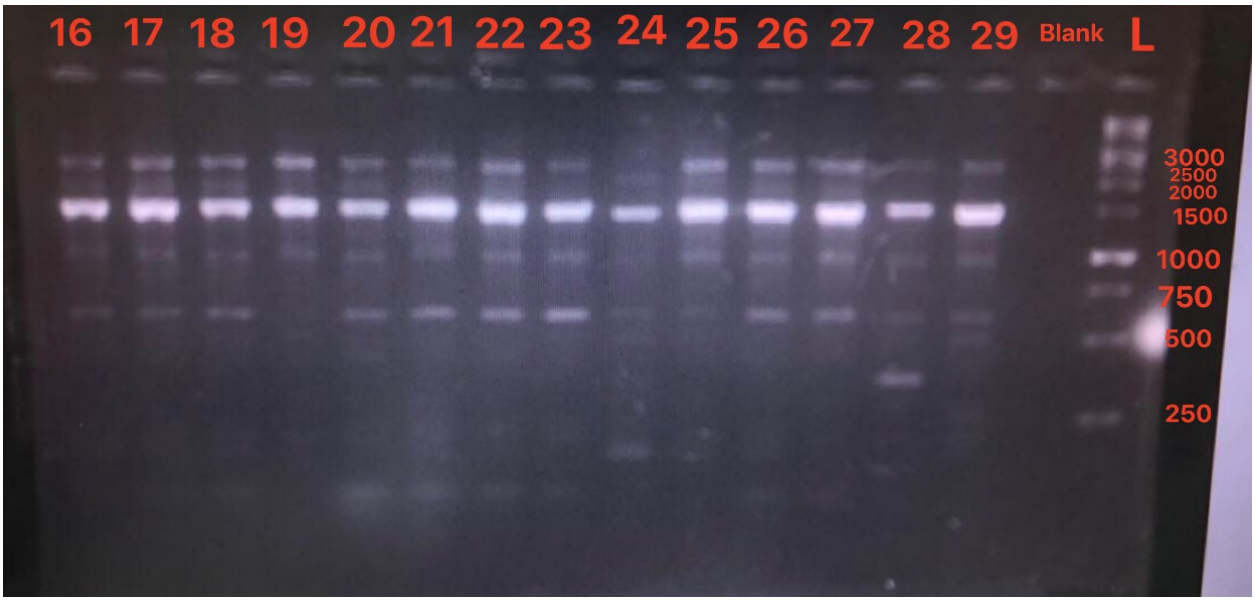


Figure 23. Representative RAPD-PCR gel-2. Lanes 16 to 29 are *A. baumannii* isolates. Lane 15 has the blank, lane 16 has 1kb ladder

The results of the RAPD PCR were put in 9 different classes based on the size and number of the amplicons. Most of the isolates (58.6%, 17/29) were located in one main classe (I), the remaining isolates were placed in separate classes. It is of interest to note that 58.8% (10/17) of the isolates in class I pattern came from the intensive care unit (ICU).

III.9 Biofilm Formation Testing

Biofilm formation testing was performed using the crystal violet and the safranine methods. The results obtained by these two methods were similar. The rate of biofilm

formation was 65.5% (19/29). The results of biofilm formation are summarized in Tables 16 and 17.

Table 16. Biofilm formation using crystal violet and safranine methods

	Crystal Violet	Safranine
Positive	19	19
Negative	10	10
% positive	65.5%	65.5%

It was observed that biofilm was formed in most isolates positive for *bla*$_{KPC}$, and *bla*$_{NDM-1}$ genes. However, in the absence of *bla*$_{NDM-1,}$ no biofilm was formed. The *bla*$_{NDM-1}$ gene was present in all 19 isolates that formed biofilm. The presence of *bla*$_{NDM-1}$ gene in isolates that formed biofilm has a rate of 100% (19/19) and *bla*$_{KPC}$ has a rate of 63.2% (12/19). Biofilm formation could be correlated with the presence of *bla*$_{NDM-1}$ gene. The results are summarized in Table 17.

Table 17. Correlation between biofilm formation and the carriage of NDM and KPC genes

Biofilm	NDM+ KPC+	NDM+ KPC-	NDM- KPC+	NDM- KPC-	Total (%)
Formed (n=19)	12	7	0	0	65.5% (19/29)
Not Formed (n=10)	1	3	1	5	34.5% (10/29)
% Biofilm formed	63.2% (12/19)	36.8% (7/19)	0%	0%	100% (19/19)
% No biofilm	10% (1/10)	30% (3/10)	10% (1/10)	50% (5/10)	100% (10/10)
Total	13	10	1	5	29

Chapter IV

Discussion

IV.1 *A. baumannii* and resistance to antibiotics

A. baumannii is intrinsically resistant to several classes of antibiotics and easily acquires resistance to others. Antibiotic resistance as well as resistance to desiccation and survival on surfaces make *A. baumannii* persist in hospital environment and spread of MDR worldwide. Infections caused by MDR *A. baumannii*, particularly carbapenem-resistant, have been associated with considerable rates of mortality and elevated hospital costs [Doi et al., 2015].

A. baumannii belongs to a group of clinical pathogens known for their extreme virulence and antibiotic resistance. Carbapenem resistant *A. baumannii* is of major concern because of the extremely limited therapeutic options. MDR *A. baumannii* has been regarded by the World Health Organization as the greatest threat to health. Colistin is the ultimate therapeutic agent prescribed to treat MDR *A. baumannii,* but colistin resistant *A. baumannii* has been increasingly reported with low rates. The rate of colistin resistance among the isolates tested in our project was 10.3% (3/29) while susceptibility rate to colistin was 89.7% (26/29). A study conducted in Saudi Arabia reported the emergence of colistin resistance in *A. baumannii* at a rate of 8.6% (14/163) [Elham et al., 2019]. Another study conducted in China in 2014, out of the 25 multidrug

resistant *A. baumannii strains,* 3 strains were resistant to colistin (12% or 3/25) and 22 strains were sensitive (88% or 22/25) [Dong et al., 2014], these results are similar to our results. Meta-analysis of 150 studies from many countries reported the rate of resistance to colistin as highest in Lebanon (17.5%) and China (12%) and lowest in Germany (0.2%) [Promohammad et al. 2020].

Mechanisms of colistin resistance have also been shown to occur through the addition of sugar moieties to lipid A component of the LPS. This causes a decrease in the net negative charge of the outer membrane [Maifiah et al., 2016]. Colistin resistance is also associated with mutations associated with genes (*lpxC*, *lpxA*, or *lpxD*) responsible for the LPS synthesis [Moffatt et al., 2010 and 2011].

According to a study conducted in Brazil in 2016, all 20 isolates of *A. baumannii* were resistant to rifampicin and fosfomycin (100% resistance and 0% susceptibility) [Leite et al., 2016]. In our project, we had the same result regarding the sensitivity to fosfomycin (0%), as none of the isolates were sensitive. As for rifampin, we had 48.3% (14/29) resistance rate and 51.7% (15/29) susceptibility rate.

The interpretation of the synergy results between colistin and rifampin was made by calculating the fractional inhibitory concentration (FIC) which had been considered the gold standard [Bonapace et al., 2002]. The colistin-rifampin combination showed synergistic effect only in 5 isolates (FIC<0.5), other isolates showed additive effect that could help relief the selective pressure and prevent the evolution of colistin resistance.

In the cases where *A.baumannii* isolates were resistant to colistin, the MIC was reduced drastically to become susceptible to colistin after combination with rifampin (MIC≥4 became 0.25 ug/ml), it was also observed that rifampin resistant isolates became susceptible to rifampin after combining with colistin (MIC>4 became <1 ug/ml). This significant reduction in the MIC can be important and useful in treating carbapenem resistant (also multidrug resistant) *A. baumannii* which may contribute in reducing or preventing the emergence of colistin resistance in this pathogen. In other studies, the combination of colistin and rifampin showed the highest synergy effect against colistin resistant *A. baumannii* [Leite et al. 2016]. This combination of colistin and rifampin have also been suggested for treating multidrug resistant *A. baumannii* [Aydemir et al. 2013]. The synergism test results of a study conducted in China in 2014 showed 56% synergetic effect and 36% additive effect between colistin and rifampin. They also found that the average MICs of each antibiotic were decreased when they were used in combination, which was in accordance with the changing trend in FICI values [Dong et al., 2014]. In our project, we had synergism of 17.2% (5/29), additive effect of 82.8% (24/29) and 0% antagonism.

As for fosfomycin, there was no synergy detected in combination with colistin. However, the effect was additive (FIC is 0.5-4). None of the isolates tested were susceptible to fosfomycin as the MIC was >64 ug/ml. However, it is interesting to note that the MIC results showed that most of isolates became fosfomycin susceptible with an MIC of ≤4 ug/ml after combination with colistin (MIC >64 became ≤4). There was

one isolate only that remained resistant to fosfomycin after combination with colistin at MIC >64. It is also interesting to note that the MIC result showed that one of the isolates that was colistin resistance (MIC=32ug/ml) became sensitive to colistin when combined with fosfomycin (MIC=32 was reduced to 1 ug/ml). According to a research done in 2021 in Thailand, the synergistic rate found was 22% (11/50 isolates had FIC<0.5) and the additive effect was against 33 isolates 66% (33/50 with 0.5-4 FIC). There were no antagonistic effect against any of the strains [Saelim et al., 2021]. In the same research, the colistin MIC range (1 - 16 µg/mL) was reduced to (0.0625 – 2 µg/mL) for 5 isolates when combined with Fosfomycin [Saelim et al., 2021]. Since the MICs for fosfomycin were significantly lowered, it may be useful to use it in combination with colistin to treat infections caused by *A. baumannii* which may prevent evolution of resistance to colistin due to selective pressure. A Brazilian study found that fosfomycin gave synergistic effects when tested with aminoglycosides. However, fosfomycin was not evaluated in clinical trials to treat patients with infections caused by *A. baumannii*. The reason is due to lack of approval to use fosfomycin as systemic treatment. Other studies using fosfomycin in combination with colistin showed significant results [Santimaleeworagun etal, 2011 and Wei et al, 2015]. The treated patients had more favorable clinical outcomes with effective microbiological response and reduced mortality. These effects were better than using colistin alone and possibly prevent the emergence of colistin resistance by this dreadful pathogen [Leite et al, 2016]. Despite limitations, the use of combination of colistin with rifampin and fosfomycin as shown in this study could be useful.

Differences found in rates of synergy in our results and literature are due to the sample size and the genotype of strains.

IV.2 Beta-Lactamases

A. baumannii clinical isolates collected for this project were identified by the Vitek 2 system (bioMerieux VITEK) with the GN card which is used for the identification of Gram-negative fermenting and non-fermenting bacilli. The isolates were also streaked on MacConkey agar where they produced pinkish colonies after 24 hours. They were further tested for oxidase and motility to confirm that all isolates were oxidase negative and non-motile. Furthermore, it has been confirmed that *A. baumannii* chromosomally carries the carbapenem hydrolyzing class D beta lactamase *bla*$_{\text{OXA-51}}$ genes which is intrinsically expressed. Since this gene is consistently found and unique to this species, their detection by PCR, could provide a simple, rapid and convenient method to confirm the identity of *A. baumannii.* Biochemical identification by API or Vitek systems are most commonly used [Shoukui et al., 2019, Gokmen et al., 2016]. All the clinical isolates collected for this research project (29/29) carried the *bla*$_{\text{OXA-51}}$ gene, and therefore their identity as *A. baumannii* has definitely been confirmed by PCR.

The most common mechanism of resistance in *A. baumannii* against carbapenems is the production of Ambler group D beta lactamases, the OXA type carbapenemase which include acquired OXA-23, OXA-24, OXA-48, OXA-58 and intrinsic OXA-51

[Dahdouh, 2017]. The expression of the beta lactamase *bla*$_{\text{OXA-51}}$ gene alone does not cause carbapenem resistance. However, overexpression and resistance to carbapenems usually occur after the insertion of ISAba1 upstream the gene promoter sequence (Nowak and Paluchowska, 2016). Nevertheless, carbapenem resistance resulting from over-expression of OXA-51-like is relatively infrequent (Nowak and Paluchowska, 2016). Although the *bla*$_{\text{OXA-51}}$ beta lactamase gene is usually located on the chromosome, it could also be carried in the plasmids of other species. It has been shown that this gene has moved by transposition from the chromosome to plasmids and further by conjugation from *A. baumannii* to other species. Therefore, this may affect the use of *bla*$_{\text{OXA-51}}$ gene in the differentiation between *A. baumannii* from others that acquired this gene [Lee, 2012].

All the 29 isolates tested in this project carried the *bla*$_{\text{OXA-23}}$ gene (100%). This gene is located on transposons and has intrinsic activity against carbapenems. The encoded OXA-23 enzyme has been found to be the most prevalent among nosocomial isolates [Merino et al., 2014]. The enhanced activity of OXA-23 beta lactamase is associated with the insertion of IS*Aba1* sequences upstream of the *bla*$_{\text{OXA-23}}$ gene [Vijayakumar et al., 2016].

According to a Palestinian study done in 2017 on hospitalized patients with MDR *A. baumannii* from all over Palestine, except Gaza. The isolates were 100% positive for *bla*$_{\text{OXA-51}}$, 82.6% were positive for *bla*$_{\text{OXA-23}}$,14.5% were positive for *bla*$_{\text{OXA-24}}$, 3% were positive for *bla*$_{\text{OXA-58}}$, 5.8% were positive for *bla*$_{\text{NDM}}$ and 0% were positive for

bla$_{KPC}$ [Handal et al.,2017]. A multicenter study on *A. baumannii* isolates collected from 2014–2017 in Austria found that the most prominent OXA-types were *bla*$_{OXA-58}$ (46.5%) and *bla*$_{OXA-23}$ (41.2%), followed by *bla*$_{OXA-24}$ (10.5%). The isolates had no *bla*$_{NDM}$ nor *bla*$_{KPC}$ (0%) [Grisold et al., 2021].

Our results showed that the *bla*$_{OXA-24}$ gene has not been expressed in any of the tested isolates. An Egyptian study reported a low rate of 7.5% [Al-Agamy, 2014]. In Thailand, the rate of carriage of *bla*$_{OXA-24}$ gene by *A. baumannii* isolates was 0.3%, similar to our results [Udomluk et al., 2018]. In China, the rate of carriage of this gene was also very low at 2.1% [Xiaohong Shi, 2021]. However, reports indicated that carriage and expression of this gene is endemic mainly in the Iberian Peninsula [Grosso et al., 2011]. In Spain, OXA-24 has the highest prevalence. A study conducted in a Spanish hospital showed that the prevalence of *bla*$_{OXA-24}$ carbapenemase gene was the most prevalent at a rate of 62.7% [Dahdouh et al., 2017]. Several studies from Spain have reported a prevalence of OXA-24 enzymes between 40% and 51.6% in the years 2014 and 2015 [Mosqueda *et al.,* 2014, Villalon *et al.,* 2015]. However, the epidemiology and spread of this carbapenemase need further evaluation [Ji et al., 2014].

OXA-48 beta-lactamase was first identified in a *K. pneumoniae* isolate in Turkey, in 2001, and widespread to other *Enterobacteriacea* thereafter including *A. baumannii* [Poirel 2004, Goncalves, 2013]. It is interesting to see that *bla*$_{OXA-48}$ gene was highly prevalent among the tested isolates in this project at a rate of 93.1%. This is definitely

a significant finding since this gene was not detected among *A. baumannii* isolates tested in China [Xiaohong Shi, 2021] and other countries. In Spain, an outbreak of OXA-48 producing *A. baumannii* at a low rate of 4.8% in an intensive care unit was reported [Robustillo-Rodela, 2017]. However, in *Enterobacteriaceae*, OXA-48 producing strains have been extensively reported as sources of nosocomial outbreaks in many parts of the world notably in Mediterranean countries. The Middle East and North Africa are considered as reservoirs of OXA-48 producers [Nassima, 2014]. A study in Saudi Arabia found that *bla*$_{\text{OXA-48}}$ was not detected in any of the tested *A. baumannii* isolates [El Badawy 2019]. Another study done in 2020 in Tehran, Iran on *A.baumannii* strains found *bla*$_{\text{OXA-48}}$ gene in 92% of the *A. baumannii* isolates they tested [Tarafdar et al., 2020].

The gene for *bla*$_{\text{OXA-58}}$ is either carried on chromosome or a plasmid. The high expression is associated with insertion sequences near the promotor inducing high level resistance to carbapenems (Nowak and Paluchowska, 2016). Most of the cases of OXA-58 positive carbapenem resistant *A. baumannii* have been reported from Europe and the Mediterranean countries. OXA-58 in *A. baumannii* was first discovered in France in 2003 (Poirel *et al.,* 2005). The *bla*$_{\text{OXA-58}}$ carbapenemase gene was not expressed in any of the *A. baumannii* isolates tested in this project. Contradictory to our results, a study conducted in Pakistan reported a prevalence of this gene in *A. baumannii* at a rate of 19.4% [Ejaz H, 2021]. An Egyptian study reported a low rate of

this gene at 5% [Al-Agamy, 2014]. A Tunisian study reported a major finding of co-expression of *bla*$_{\text{OXA-58}}$ and *bla*$_{\text{OXA-23}}$ in one *A. baumannii* isolate [Mathhlouthi, 2018]. A study in Saudi Arabia found that *bla*$_{\textit{OXA-58}}$ was not detected in any of the tested *A. baumannii* isolates [El Badawy 2019]. Although there are not enough literature reports addressing the expression of this gene, available literature indicates low rates of expression in *A. baumannii*.

Ambler class B beta-lactamases, or metallo beta lactamases (MBLs) contain zinc ions in their active site. These enzymes are characterized by their ability to hydrolyze carbapenems without being inhibited by beta lactam inhibitors such as clavulanic acid and tazobactam. NDM-1 was first identified and reported in *A. baumannii* isolates in India (Karthikeyan et al., 2010). In this study, the Ambler class B NDM-1 was detected at a high rate of 79.3% (23/29) in tested *A. baumannii* isolates. A study in Saudi Arabia confirmed this alarming high rate of *bla*$_{\text{NDM}}$ where it was detected in 62.5% of the tested isolates [El Badawy 2019]. However, A study conducted in Egypt reported a much lower rate than that detected in this study where *bla*$_{\text{NDM}}$ was only 12.1% of their isolates [Abouelfetouh, 2019]. NDM was first identified in *A. baumannii* in India in 2010 where it coexisted with OXA-23 [Karthikeyan,2010]. In this study, we found that the co-expression of OXA-23 and NDM-1 was high at a rate of 79.3% (23/29). It has been reported that NDM positive bacteria including *Acinetobacter* cause infections with high mortality rates [Rahman, 2018], and hence their rapid and sensitive

identification is required. A study was carried out in 2013 in Algeria, detected *bla*$_{\text{OXA-23}}$ gene in 50% (40/80) of the isolates, *bla*$_{\text{OXA-24}}$ in 21.2% (17/80) and *bla*$_{\text{NDM-1}}$ in 6.2% (5/80) of the isolates [Mesli et al., 2013].

The Ambler class A beta-lactamase (carbapenemase) commonly found in the *Enterobacteriaceae* in general and in *Klebsiella pneumoniae* particularly the KPC which has high activity against carbapenems. Although the dissemination of KPC to *A. baumannii* is rare, its presence has been limited to certain geographic areas [Martinez et al., 2016]. The most common mechanism of resistance to carbapenems in *A. baumannii* is due to Ambler groups B and D. Reports exist related to the association of *A. baumannii* resistance to some Ambler group A enzymes [Nowak 2016].

In this study, 48.3% (14/29) of *A. baumannii* isolates carried the *bla*$_{\text{KPC}}$ gene. Studies from Saudi Arabia [El Badawi, 2019] and Egypt [Abouelfetouh, 2019] found that *bla*$_{\text{KPC-2}}$ was not detected in any of the *A. baumannii* isolates tested. A study was carried out in Iran in 2015 found that 9.23% of the isolates carries *bla*$_{\text{KPC}}$ gene [Azimi et al., 2012].

- Our result for *bla*$_{\text{OXA-51}}$ (100%) was similar to that found in literature (100%- Palestinian study, 2017)
- Our result for *bla*$_{\text{OXA-48}}$ (93.1%) was very close to that found in literature (92%- Tahran- Iran study, 2020)

- Our result for *bla*$_{\text{OXA-23}}$ (100%) was high as same as that found in literature (82.6% - Palestinian, 2017 / 50% - Algeria, 2013 / 41.2% - Austria, 2014-2017)
- Literature had low rates of *bla*$_{\text{OXA-24}}$ (10.5% - Austria, 2014-2017 / 14.5% - Palestinian, 2017 / 21.2% - Algeria, 2013), we had rate of 0% as same as a study done in Thailand (0.3%)
- Literature had low rates of *bla*$_{\text{OXA-58}}$ (3% -Palestinian, 2017 / 46.5% - Austria, 2014-2017 / 5% - Egyptian study) , we had rate of 0% as same as a study in Saudi Arabia (0%)
- Literature had low rates of *bla*$_{\text{NDM-1}}$ (5.8% - Palestinian, 2017 / 6.2%- Algeria, 2013/ 12.1% -Egyptian study/ 0% - Austria, 2014-2017) , we had high rate 79.3% which is close to a study in Saudi Arabia (62.5%)
- Literature had low rates of *bla*$_{\text{KPC-2}}$ (0% Austria, 2014-2017 / 9.23% - Iran, 2015), but we had high rates of this gene (48.3%)

IV.3 RAPD-PCR

We chose the Random amplification of polymorphic DNA - polymerase chain reaction (RAPD-PCR). We did an optimization to determine the annealing temperature since the 40^{o} C suggested was not satisfactory for our initial experiments. We found that 32^{o} C gave better results than the suggested one. In addition, we used this method because it is cost effective, rapid and simple to perform generating the desired fingerprinting pattern.

In this study, we demonstrated that the majority of the isolates 58.6% (17/29) are identical with a pattern containing four major bands as shown. However, considering the bands individually, the *A. baumannii* isolates tested demonstrated a band pattern of 2500, 1500, 1000 and 600 base pairs at a rate of 93.1%, 96.6%, 93.1% and 75.9% respectively. It is interesting to note that 58.8% (10/17) of the isolates in class I pattern came from the intensive care unit (ICU). This classification is important since it is the first to be done on *A. baumannii* isolates in this country. However, in the absence of outbreaks, this information remains significant epidemiological profile. Furthermore, it can serve as a reference for *A. baumannii* strains that may be involved in future nosocomial outbreaks.

V. Conclusion

This study showed the presence of synergy between colistin and rifampin and additive result when combined with fosfomycin. However, the MICs in both combinations resulted in drastic reduction of the MICs. This may contribute to reduce the dose of colistin, thus reducing toxicity and avoid subjecting this pathogen to selective pressure which may results in developing resistance to colistin. Most of the strains tested had the ability to form biofilms particularly those who carried the NDM-1 gene. This may suggest an association between NDM-1 carriage and biofilm formation. By RAPD-PCR typing, we could assign the isolates into 9 classes. About half of the isolates tested (58.6%, 17/29) were assigned to class I, while the rest of the isolates had less similarities and assigned to the remaining 8 classes.

This study showed that the intrinsic *bla*$_{\text{OXA-51}}$ gene can be useful in the rapid identification and or confirmation of the identity of *A. baumannii*. The encoded OXA-23 enzyme has been found to be the most prevalent among nosocomial isolates tested and can be considered as the main mechanism of this pathogen resistance to carbapenems. The genes encoding for OXA48, NDM-1 and KPC-2 enzymes were also significantly expressed in the tested isolates and contribute to carbapenem resistance.

VI. Recommendations

We recommend the following for future research on *A. baumannii*:

1. To increase the sample size to an extent where all the results become significant, since this can be considered a pilot study.

2. Stop using the colistin alone to treat patients infected with *A. baumannii*, combinations with rifampin and fosfomycin will contribute to mor effective treatment with lower dosages. In addition, we recommend testing some of the newly developed antibiotics such as the Siderophore, cephalosporin (cefiderocol), the fluorocycline (eravacycline) and the newly developed beta lactam inhibitors such as ceftazidime-avibactam. Initial testing of these antibiotics showed high activity against multidrug resistant gram-negative bacteria.

3. To molecular characterize biofilm formation in *A. baumannii* by using related genes such as *bap*, $bla_{per\text{-}1}$, *ompA* and csuE and correlate that with antibiotic resistance phenotypes.

4. we also recommend sequencing of the beta lactamase genes tested in this study and compare the sequences with those stored in Genebank. If variations encountered, we can deposit our sequences in the Genebank as a reference for regional research involving these genes.

5. Further testing of *bla*$_{OXA-48}$ on additional *A. baumannii* isolates. This gene was highly expressed in the isolates tested in this project. Since literature reports indicate significant variation of this gene, further testing could be used as a geographical distribution of this gene in this country.

6. Farther testing of *bla*$_{NDM}$ and its correlation with biofilm formation.

7. We recommend to perform PCR to check for the presence of *MCR-1* gene in the 3 isolates that were colistin resistant and also in 3 sensitive isolates to colistin.

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