Molecular Characterization of Carbapenem Resistance in Clinical Isolates of *Acinetobacter baumannii* and Therapeutic Options

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Dedication

I dedicate this work

To my father, my mother my wife Isra', my sun

suliman, my sisters and brothers

for always supporting and encouraging me.

I love you all.

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Abstract

Acinetobacter baumannii is gram negative coccobacilli characterized by being non-motile, oxidase negative. It has been recognized to colonize hospitalized patients particularly the elderly, infants and the immunocompromised thus causing severe infections with high morbidity and mortality. *A. baumannii* plays a predominant role in causing nosocomial pneumonia particularly the ventilation-related pneumonia seen among patients in intensive care units. *A. baumannii* has been classified as one of the six most important multidrug-resistant microorganisms in hospitals worldwide. Although its resistance to all known antibiotics has appeared, most strains remain susceptible to carbapenems. Therefore, treatment options are limited to carbapenems, colistin and Tigecycline. The aim of this study was to screen for carbapenemase producing *A. baumannii* clinical isolates.

The study has been conducted on 59 hospitalized patients and outpatients attending clinics throughout the West Bank, Palestine. The samples were collected between October 2016 and November 2017. *Acinetobacter baumannii* clinical isolates were obtained from different sources including urine, wound, blood, sputum and tracheal aspirates. Antibiotic susceptibility testing by the disk diffusion method and minimal inhibitory concentration by the microbroth dilution method was performed following the guidelines of the Clinical and Laboratory Standards Institute. Polymerase gene reaction was performed to determine the genes involved in carbapenem resistance. The genes tested include the OXA-b-lactamases; OXA 51, OXA 23, OXA 24 and OXA 58.

The results obtained indicated that all isolates were resistant to Meropenem. Susceptibility to colistin and Tigecycline were 84.7% (50/59) and 88.1% (52/59) respectively.

PCR results showed that all *bla*$_{OXA51}$ gene was carried by all isolates while *bla*$_{OXA523}$ gene was carried by 54.2% (32/59) of isolates. None of the *Acinetobacter* isolates tested carried the *bla*$_{OXA24}$ and *bla*$_{OXA58}$ genes.

bla$_{OXA51}$ gene has been found to be carried by all strains of *A. baumannii* and usually used to confirm the identification of the clinical isolates. Regarding the *bla*$_{OXA24}$ and *bla*$_{OXA58}$ genes,

It has been reported that *A. baumannii* carrying these two genes show have resistance to carbapenems. However, these two genes are are not universally spread but reported in certain areas in the world such as Spain and France. Regarding *bla*$_{\text{OXA-23,}}$ our finding is consistent with published literature regarding the spread of carbapenem resistance in *A. baumannii* isolates carrying this gene.

الخلاصة

A. baumannii عصيات سلبية الصبغة تتميز بعدم الحركة ، oxidase سالبة. هذه الجرثومة لها القدرة على اصابة المرضى في المستشفيات وخاصة كبار السن والرضع والذين يعانون من نقص المناعة مما يسبب التهابات شديدة مع حدة الاعراض وارتفاع معدلات الوفيات. *A. baumannii* لها دور بارز في التسبب في الالتهاب الرئوي لمرضى المستشفيات خاصة بين المرضى في وحدات العناية المركزة. تم تصنيف *A. baumannii* كواحدة من أهم ستة كائنات دقيقة مقاومة للأدوية المتعددة في المستشفيات في جميع أنحاء العالم. على الرغم من مقاومتها لجميع المضادات الحيوية المعروفة ، إلا أن معظم السلالات تظل حساسة للكاربابينيمات. لذلك تقتصر خيارات العلاج على الكاربابينيمات ، الكوليستين و Tigecycline . الهدف من هذه الدراسة هو الكشف عن العزلات الإكلينيكية المنتجة للانزيمات التي تبطل مفعول الكاربابينيمات.

أجريت الدراسة على 59 مريضًا من مستشفيات وعيادات خارجية في جميع أنحاء الضفة الغربية في فلسطين. تم جمع العينات بين أكتوبر 2016 ونوفمبر 2017. تم الحصول على عزلات *A. baumannii* السريرية من اماكن مختلفة من المرضى بما في ذلك البول والجروح والدم والبلغم ونضح القصبة الهوائية. تم إجراء اختبار الحساسية المختلفة للمضادات الحيوية باتباع مبادئ CLSI. كما تم إجراء PCR لتحديد وجود الجينات التي لها علاقة في مقاومة الكاربينيمات. الجينات التي تم اختبارها تشمل OXA 51 beta lactamases و OXA 23 و OXA 24 و OXA 58.

أشارت النتائج أن جميع العزلات كانت مقاومة لميروبينيم. بينما كانت نسبة الحساسية للكوليستين واالتايجيسايكلين 84.7% (50/59) و 88.1% (59/52) على التوالي.

أظهرت نتائج PCR أن جميع الجينات blaOXA51 كانت تحملها جميع العزلات بينما كانت نسبة blaOXA23 تعادل

54.2 ٪ (59/32). لم يحمل أي من عزلات *Acinetobacter* جينات blaOXA24 و blaOXA58.

من المعروف ان جين blaOXA51 يستخدم للتاكد من صحة التعرف على *A. baumannii*. فيما يتعلق بالجينات blaOXA24 و blaOXA58 ، فمن خلال المقالات العلمية المنشورة، فان عزلات *A. baumannii* التي تحمل أيا من هذين الجينين تظهر مقاومة للكاربابينيمات. مع ان الجدير بالذكر بان انتشارهما محدود في مناطق معينة في العالم مثل إسبانيا وفرنسا. فيما يتعلق بـ blaOXA-23 ، تتفق نتائج هذا البحث مع المقالات المنشورة المتعلقة بانتشار مقاومة الكاربابينيمات في عزلات *A. baumannii* التي تحمل هذا الجين

Chapter One

Introduction and literature review.

Acinetobacter baumannii

The Dutch bacteriologist Beijerinck was the first to isolate *Acinetobacter baumannii* from soil in 1911. He designated it as *Micrococcus calcoaceticus* (Asif, Alvi et al. 2018). The genus Acinetobacter is glucose non-fermentative microorganism classified in the family Moraxellaceae. It has a microscopic gram-negative coccobacillus morphology. Although there are more than 20 Acinetobacter species identified so far, *A.baumannii* is the most commonly isolated from clinical specimen (Doi, Murray et al. 2015).

Recent advances in molecular biology has resulted in major taxonomic changes due to the genetic make-up of this group of microorganisms. Recently, *A. baumannii* has been classified as gamma Proteobacteria in the order of Pseudomonadales and the family Moraxellaceae. The most recent taxonomical classification is as follows:

Domain: ***Bacteria***.

Phylum: ***Proteobacteria***.

Class: ***Gamma Proteobacteria***.

Order: ***Pseudomonadales***.

Family: ***Moraxellaceae***.

Genus: ***Acinetobacter***.

As aerobic gram-negative coccobacilli, *Acinetobacter spp.* are ubiquitous in nature and can be encountered in the environments in soil and water (Kim, Kim et al. 2014). The morphology and

size of gram stained smear of *Acinetobacter spp*. During the lgarthmic phase of growth Appear as short, plump, 1.0–1.5 by 1.5–2.5 μm but appears as more coccoid during the stationary phase. It may pleomorphic appearing in pairs or longer chains (Almasaudi 2018) as shown in Figure 1 below.

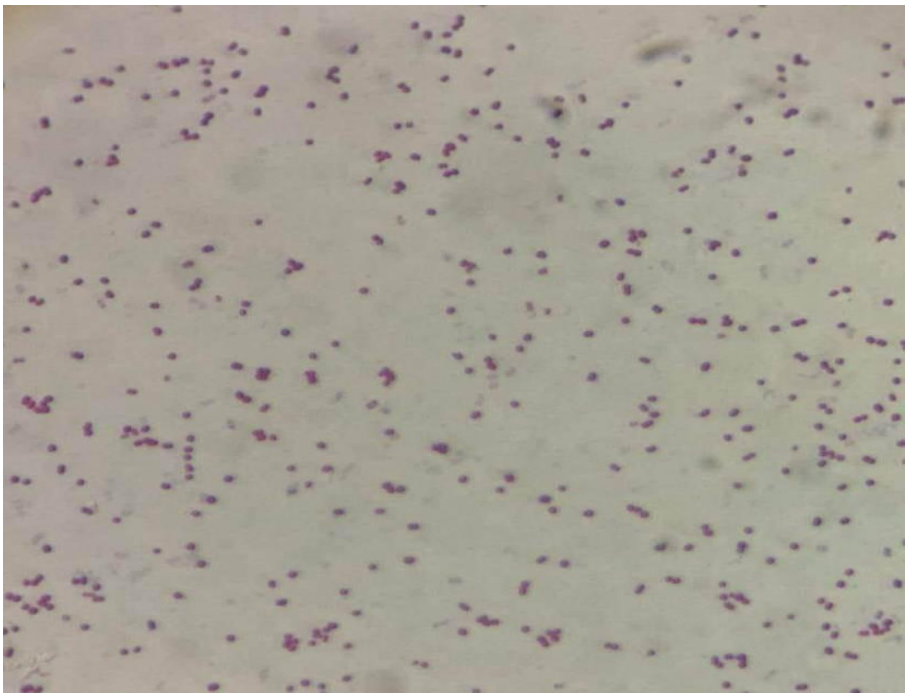

Figure 1: Gram staining for an *A. baumannii* isolate taken from growth on MacConkey agar. Note the coccobacilli appearance.

Other features of *Acinetobacter baumannii* that is frequently used in rapid or presumptive identification is motility, oxidase reaction and susceptibility to penicillin. *A. baumannii* is nonmotile, oxidase negative and resistant to penicillin. In addition, it is non-fastidious and can grow on regular laboratory media such as Blood and MacConkey agars . On the blood agar plates, the colonies show typical shape and size, it is colorless (white or creamy), smooth, milky, 1–2 mm in diameter (when incubated at 37^{o} C for18–24 hours). On MacConkey agar, the

colonies appear as light lavender in color characteristic of non lactose fermenting organisms (Almasaudi 2018).

Figure 2: Typical growth of *A. baumannii* on MacConkey agar, note the colonial morphology, color and negative lactose fermentation.

As mentioned earlier, *A.baumannii* is similar to other *Acinetobacter spp.*, It is not fastidious organism, which grows on commonly used laboratory media at various temperatures and pH levels. The versatility of this organism is in its utilizing various carbon sources to generate its

energy requirements. These properties are key features for this organism to survive in both moist and dry atmospheres particularly in the hospital environment, thereby facilitating its transmission and spread. The above mentioned features of *A. baumannii* when combined with its intrinsic resistance to many antimicrobial agents, contribute to its adaptability as well as ability to persist and spread in hospitals contributing to its pathogenicity in ICUs among immune devbilitated people (Abbo, Navon-Venezia et al. 2005).

In addition to the most commonly encountred *Acinetobacter spp*., *Acinetobacter baumannii***,** there are other species that are of clinical significance and have the ability (less frequently) to cause disease in humans; *Acinetobacter haemolyticus* and *Acinetobacter calcoaceticus*.(Jung and Park 2015).

Predisposing factors for *Acinetobacter* infections include the presence of prosthesis, endotracheal intubation, intravenous (I.V.) catheters and prior antibiotic therapy in a seriously ill-patient in hospital. Such infections are often extremely difficult to treat because of widespread resistance to the major groups of antibiotics and long-term survival of bacteria in the hospital environment (Tripathi, Gajbhiye et al. 2014) . Up to 25% of healthy ambulatory adults exhibit cutaneous colonization by Acinetobacter and are the most common Gram-negative bacteria carried on the skin of hospital personnel (Tripathi, Gajbhiye et al. 2014).

A. baumannii has been classified by the Infectious Diseases Society of America as one of the six most important multidrug-resistant (MDR) microorganisms in hospitals worldwide (Antunes, Visca et al. 2014). Resistance to all known antibiotics has now appeared in *Acinetobacter spp.*, most strains are still susceptible to carbapenems. Acinetobacter multidrug-resistant (MDR) are

associated with increased mechanical ventilation time, the intensive care unit, and the hospital environment.

Treatment options are severely limited; carbapenems and colistin are the preferred agents. More research and more emphasis on the prevention of Acinetobacter MDR infection-related medication are important (Tripathi, Gajbhiye et al. 2014).

Various human infections are caused by Acinetobacter species, including pneumonia (most commonly associated with endotracheal tubes or tracheostomy), endocarditis, meningitis, infections of the skin and wounds, peritonitis (in patients receiving peritoneal dialysis) and urinary tract infections. Some cases of conjunctivitis and osteomyelitis have also been reported.

Acinetobacter spp. has been recognized to colonize hospitalized patients particularly the elderly, infants and the immunocompromised and thus have a significant role in causing severe infections with high morbidity and mortality. Although *Acinetobacter spp.* have been implicated in causing several nosocomial infections such as bacteremia, urinary tract infection, and secondary meningitis, Its predominant role is in causing nosocomial pneumonia, especiparticularly the ventilation-related pneumonia seen commonly among patients in intensive care units. (Nazir 2019)

Acinetobacter baumannii is the most commonly found species in human clinical specimens, followed by *Acinetobacter lwoffii, Acinetobacter haemolyticus* and *Acinetobacter johnsonii. Acinetobacter baumannii* is the species most often responsible for hospital-acquired infections, and is increasingly being reported as a significant pathogen causing sepsis, wound infections,

and pneumonia in both hospitalized patients and in the community setting. *A. baumannii* has also been associated with combat injuries in Iraq and Afghanistan. A fatal outbreak of extensively drug-resistant *A.baumannii* with enhanced virulence (so-called Clade B strains) has been reported in a cohort of relatively immunocompetent patients raising concerns that the low virulence potential of A. baumannii may need to be reevaluated. A bio typing system for differentiating 17 biotypes of *A. baumannii* based on the utilization of six substrates has been established and may be useful for epidemiologic studies.(Tripathi, Gajbhiye et al. 2014)

The spread of antimicrobial resistance among Acinetobacter strains has become a public health concern worldwide. Therefore, therapeutic options are extremely limited or not available. This results in increasing costs, hospital stay and morbidity, mortality rates. Recent continuous emergence of antibiotic resistance among pathogenic microorganisms and its dynamics are due to evolutionary processes, partially fueled by anthropogenic activity (Da Silva and Domingues 2016).

Infections Caused by Acinetobacter baumannii

Acinetobacter baumannii is a major cause of nosocomial infections. The sources of *A. baumannii* include soil and foods; it colonizes the skin of healthy humans in low density for a short time. The nature of the organism and its durability as well as its resistance to antibiotics makes it a successful infectious agent in hospitals. Infections with *A. baumannii* most often occur in critically ill hospitalized patients; mainly in advanced age, immunosuppression and burn injuries. It has been observed that most infections caused by *A. baumannii* involve organ systems

containing high amounts of fluids. These systems include the urinary tract and respiratory tract, peritoneal cavity, and are associated to a great degree to indwelling devices (Almasaudi 2018). The two most commonly reported clinical manifestations of *A. baumannii* are nosocomial pneumonia and bacteremia (Wong, Nielsen et al. 2017). In hospital settings, the mortality rates is about 26% which increases drastically to reach 43% in ICUs. In addition, the motality rate of ventilator-associated pneumonia caused by *A. baumanni* accounts for nearly 15% of all hospital-acquired infections. The morbidity and mortality is reported to be the higest in medical wards particularly in the ICUs. It accounts for approximately 50% of the total antibiotics used in the ICUs (Asif, Alvi et al. 2018).

Nosocomial Pneumonia

Nosocomial pneumonia caused by *A. baumannii* has been associated with aspiration processes involving the respiratory tract. It has been reported that endotracheal aspiration tubes create the ideal mean for the environmental transmission of Acinetobacter. It strongly adheres to inanimate surfaces including the aspirator plastic and forms a biofilms which I extremely difficult to remove by disinfectants (Wong, Nielsen et al. 2017). As reported by many references in literature, the highest frequency (3-5%) of nosocomial pneumonia occurs in intensive care units (ICUs) with and with rough death rates of 30–75% (Almasaudi 2018).

Bacteremia (Blood Stream Infection)

It has been emphasized that *A. baumannii* infections including the bloodstream infections are primarily associated with the central venous catheter and secondarily due to extensive pneumonia (Wong, Nielsen et al. 2017). Surgical wounds burns and urinary tract infections as well as endocarditis do not commonly contribute to bacteremia. It has been noted that in 21-70% of reported bacteremia result from unknown etiligy and un identified origin. *A. baumannii* has

been incriminated in causing higher rates of bacteremia in the ICU units as compared to - non-ICU-ward infections. It has been reported that the overall mortality rate from bacteremia caused by *A. baumannii* ranged from 34.0% to 43.4% at the ICU compared to 16.3% in non-ICU units (Peleg, Seifert et al. 2008, Almasaudi 2018).

Wound Infection

A.baumannii has also been incriminated in causing skin/soft tissue infections among non-military population, with a rate of 2.1% in ICU-acquired skin/soft tissue infections.(Guerrero, Perez et al. 2010) However, rate of casualties inflicted among US combatant soldiers in Iraq and Afghanistan was significantly higher accounting for 32.5% of cases. *A. baumannii* was the most frequently isolated organisms from soldiers who had tibia fractures in particular (Peleg, Seifert et al. 2008, Almasaudi 2016).

UTI

Among outpatients observed in the urinary clinics, *A. baumannii* was not involved in causing complicated UTIs. However, this organisms is primarily incriminated in causing UTIs in association with urinary catheters or percutaneous nephrostomy tubes (Peleg, Seifert et al. 2008, Wong, Nielsen et al. 2017, Almasaudi 2018).

Meningitis

The ability of *A.* baumannii in causing meningitis is limited to trauma or post neurosurgical surgeries. Multidrug-resistant *A. baumannii* has been increasingly incriminated in causing acute meningitis in adults. Meningitis caused by Acinetobacter spp., accounted for about 10% of Gram-negative bacilli and 4% of all nosocomial meningitis. It is noteworthy that the mortality

rate of meningitis due Acinetobacter may reach as high as 70% (Basri, Zueter et al. 2015, Wong, Nielsen et al. 2017).

Epidemiology

A. baumannii is commonly found in water and soil as well as in hospital environment. It is known that this organism is capable of colonizing hospitalized patients. A wealth of literature has designated *A. baumannii* as a primary a healthcare-associated pathogen. It is also notorious for causing a large number of outbreaks and nosocomial infections mainly bacteremia, pneumonia associated with ventilators, urinary tract and wound infections..

As multidrug resistant nosocomial pathogen, *A. baumannii* infections has been frequently reported world wide including Europe, North America, South America, South East Asian countries and others.

MDR *A. baumannii* has also been implicated in causing in causing nosocomial infections in several Arab countries such as the United Arab Emirates, Bahrain, Saudi Arabia, Palestine and Lebanon. A retrospective study conducted in the intensive care unit of Riyadh Military Hospital, Saudi Arabia, showed a high rate of MDR *A. baumannii* isolates of 40.9%. Another retrospective study conducted in an adult ICU tertiary care Hospital in Riyadh, Saudi Arabia, showed that *A. baumannii* was the most frequently isolated organism (Kamolvit, Sidjabat et al. 2015, Almasaudi 2018).

A. baumannii Virulence

Although MDR *A.baumannii* has been increasingly implicated in causing serious infections, the pathogenesis mechanisms are unclear and need to be elucidated (Doi, Murray et al. 2015). The virulence of Acinetobacter is considered to be low. The expected virulence factors include verotoxins, cell surface hydrophobicity, outer membrane proteins (OMPs) and toxic slime polysaccharides. The surface hydrophobicity of *Acinetobacter spp*. plays an important role in adhesion to cell surface and protect the organism from being phagocytosed by phagocytic cells (Almasaudi 2018).

Several outer membrane proteins (OMPs) belonging to the OmpA family have been investigated in many *Acinetobacter* strains. Outer membrane proteins are an important component in the outer membrane of the gram negative bacteria. OMPs contribute to the pathogenicity of gram negative bacteria in general and *A.baumannii* in particular. OMPs play an important role in antibiotic resistance in Acinetobacter spp. (Kanafani and Kanj 2015, Almasaudi 2018).

The lipid A component (endotoxin) of the lipopolysaccharide (LPS) layer of *A.baumannii* contribute to an inflammatory response through the stimulation of circulating white blood cells to release pro-inflammatory substances. LPS affect the function of neutrophils by inhibiting their migration and impair their phagocytic abilities. The pathogenesis of *A.baumannii* can be mediated by the production of extracellular enzymes, cytotoxins and secreted vascular permeability. These factors have been implicated in playing an important role in the pathogenesis of *A.baumannii* clinical isolates. They cause damage to host tissues in general

particularly in infections involving the respiratory tract. (Kanafani and Kanj 2015, Almasaudi 2018).

Risk Factors for Acquiring *A. baumannii*

It has been reported that *A.baumannii* have the potential to adhere to dry surfaces and survive under nutrient limiting conditions. These characteristics facilitates the persistence of the organism and its transmission and spread in the medical environment. In addition, its ability to colonize inanimate surfaces includes, medical devices and other hospital equipment which could serve as reservoir during prolonged nosocomial outbreaks (Kanafani and Kanj 2015). Compared to other gram negative bacteria such as *E. coli*, most strains of *A.baumannii* persist much longer than *E coli* on dry surfaces. It has been reported that Acinetobacter durability may keep them viable on the dry surfaces for up to four months. In addition, it was found that *A.baumannii* isolates were able to survive more than 20 days on glass at room temperature and persisted on both moist and dry surfaces. This specific characteristic is of particular importance in enabling the organism to survive in hospital surfaces and equipments and its ability to spread infections.

There is no doubt that *A.baumannii* is the most medically significant *Acinetobacter spp. A. baumannii* infections have particular clinical importance for causing high rates of morbidity and mortality particularly among critically ill patients. On one hand *A.baumannii* is considered to have low virulence among immunocompetent individuals only. On the other hand, it has high virulence among the critically ill and the immunocompromised patients. Therefore, Acinetobacter is common in the hospital environment and most frequently associated with

nosocomial rather than community-acquired infections. The characteristics that enable *A. baumannii* to acquire the genes and become multidrug resistance added to its ability to persist in unfavorable environmental conditions results in severe infections. The severity of infections are observed in patients with major surgeries, have malignant diseases, burns, immunosuppressed patients particularly the elderly, neonates with low birth weights, and patients with chronic or prolonged illnesses. Multidrug resistant strains are rather acquired by patients using mechanical ventilation devices for extended periods of time, prolonged hospital stay particularly in the ICU units and have been exposed to infected or colonized patients inside the hospital are at increasing risk for acquiring MDR-outbreak strains (Almasaudi 2018).

Antimicrobial Susceptibility

It has been known that *Acinetobacter* is capable of developing antimicrobial resistance through several diverse mechanisms and eventually leading to the emergence of multi-drug resistance strains.

Definitions

In 2011, the European and the US centers for disease control (ECDC, CDC), have jointly proposed specific terms to define and characterize antimicrobial resistance in microorganisms involved in causing health care-associated infections. Based on the extent of resistance in *Acinetobacter*, to various classes of antibiotics (cephalosporins, fluoroquinolones and carapenems) the following definitions have been established:

- **Multidrug-Resistant**: isolate is non-susceptible to at least one agent in three or more antibiotic classes

●**Extensively Drug-Resistant**: isolate is non-susceptible to at least one agent in all but two or fewer antibiotic classes

●**Pan Drug-Resistant**: isolate is non-susceptible to all agents

This has set the criteria for defining and designating antimicrobial resistance to one of the terms propsed in the previos paragraph. This has also eliminated ambiguieties and established a universal consensus in properly using the terms such as "multidrug resistance", in clinical studies evaluating various regimens for drug resistant *Acinetobacter* infections(Kanafani and Kanj 2015).

Carbapenem resistance of *A.baumannii* occurs by the production of carbapenemases. These enzymes can be either intrinsic to the organism or acquired. Intrinsically, *A.baumannii* naturally carries the bla_{OXA51} on its chromosome thus produces chromosomally encoded OXA-51-group carbapenemase at a low level, and acquisition of a stronger promoter by transposition of an insertion sequence, upstream of the OXA-51-group gene may lead to elevation of carbapenem MICs. Carbapenem resistance in *A. baumannii* is mediated by acquiring plasmids carrying certain OXA-group β-lactamase genes. Four groups of the Class D carbapenem-hydrolyzing enzymes, including OXA-23-like, OXA-24-like, OXA-58-like, and OXA-51-like enzymes, have been identified in *A. baumannii*. OXA-23 group has been found to be the most prevalent worldwide.(Doi, Murray et al. 2015)

Recent research showed that non-OXA group carbapenemases commonly spread in the *Enterobacteriaceae* have also been acquired by *A.baumannii*. One of he most alarming genes among these is the metallo-β-lactamase NDM-1. Since 2011, Carbapenem-resistant *A.*

baumannii strains producing NDM-1 has been isolated and identified worldwide. Recenly, *A. baumannii* strains producing KPC-group carbapenemase has been reported only in Puerto Rico, with no records indicating its spread in any other part in the world (Doi, Murray et al. 2015).

Acinetobacter baumannii Resistance to Antibiotic

A.baumannii has been implicated in causing nosocomial pneumonia and bacteremia among hospitalized patients particularly the critically ill ones in the intensive care unit (ICU) setting worldwide [Vahdani, 2011, Fournier 2006]. The emergence of nosocomial infections caused by multidrug resistant *A.baumannii* has created a great deal of concern. *A.baumannii* has been found to be the most important pathogen associated with nosocomial infections worldwide [Lin and Lan, 2014].

One of the early encounters with multidrug resistant *A.baumannii* was detected in large number of US veterans returning from the Iraq-Iran war more than two decade ago [Hujer KM et.al., 2006]. Therefore, it became essential to identify the mechanisms of *Acinetobacter spp*. resistance and identify the responsible genes. According to the WHO, *A.baumannii* is one of the most serious ESKAPE organisms (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* and *Enterobacter* species) that effectively escape the effects of antibacterial drugs (Boucher et al., 2009).

Enzyme-mediated degradation (beta-lactamases), genetic manipulations (mutations, acquiring or leaving a gene, upregulation or downregulation of gene expression), and efflux pumps are different strategies adopted by *Acinetobacter* to escape from destruction of antibiotics (Asif, Alvi et al. 2018)[Lee et al., 2017, Lin and Lan, 2014].

The Figure below summarizes Acinetobacter resistance to various antimicrobial classes/agents and therapeutic options. Resistance of A. baumannii to carbapenems has been extensively studied. Resistance to carbapenems is commonly mediated by the production of carbapenemases, class B metallo-b-lactamases (MBLs) including New Delhi metallo-b-lactamase-1 (NDM-1) and class D oxacillinase (OXAs) [Walsh, 2010].

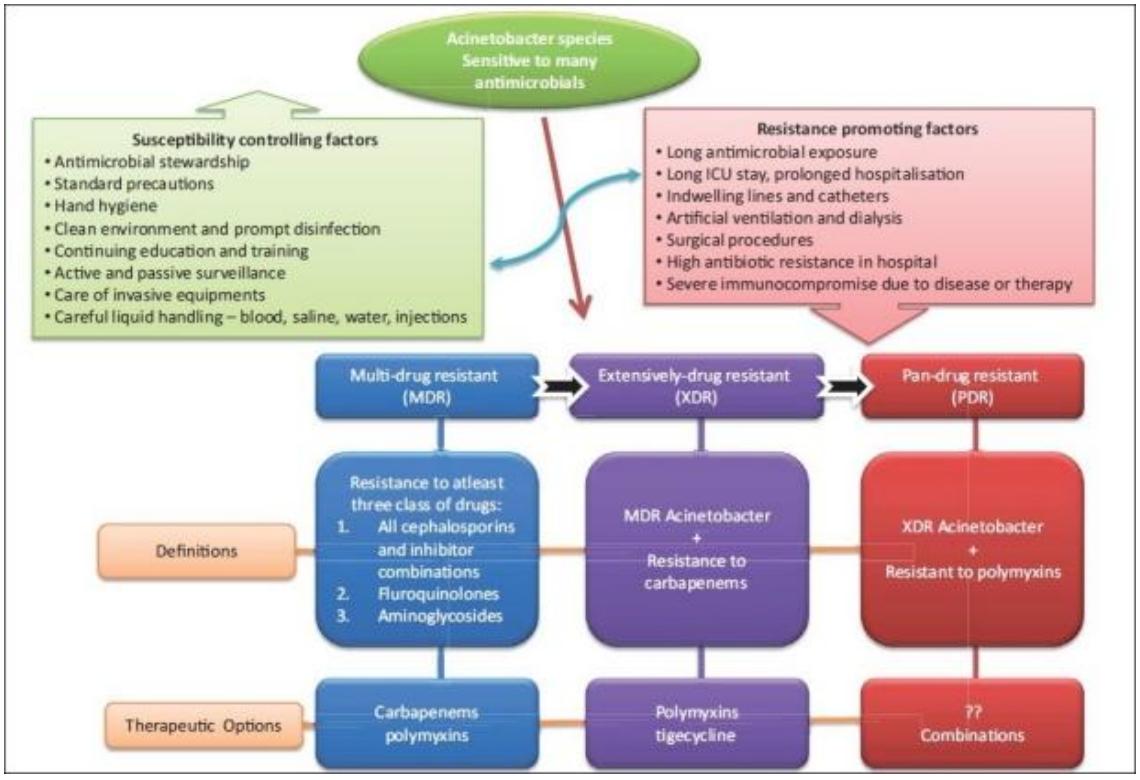

Figure 3: Definition of drug resistant Acinetobacter species along with therapeutic options. Resistance promoting factors and susceptibility controlling factors has been summarized. Courtesy [Manchanda V. et. Al., 2010].

Prevention

Isolation: Patient-isolation precautions are designed to prevent transmission of microorganisms by common routes in hospitals. Because agent and host factors are more difficult to control, interruption of the spread of microorganisms is directed primarily at transmission.

Handwashing: Handwashing is frequently called the single most important measure to reduce the risks of transmitting microorganisms from one person to another or from one site to another on the same patient. Washing hands as promptly and thoroughly as possible between patient contacts and after contact with blood, body fluids, secretions, excretions, and contaminated equipment or articles is an important component of infection control and isolation precautions. Although handwashing may seem like a simple procedure, it is often performed incorrectly. Healthcare settings must continually remind practitioners and visitors on the proper procedure in washing their hands to comply with effective hand-washing practices.

Visitors: In order to adequately control the spread of infections, all visitors must follow the same precautionary measures as hospital staff. Visitors and healthcare personnel are equally to blame in transmitting infections. Moreover, multi-drug resistant infections can leave the hospital and become part of the community flora if the necessary steps are not taken in consideration to curb their transmission.

Gloves: In addition to handwashing, gloves play an important role in reducing the risks of transmission of microorganisms. Gloves provide a protective barrier and prevent gross contamination of the hands when touching blood, body fluids, secretions, excretions, mucous

membranes and skin. They can reduce the chance that microorganisms present on the hands of the medical staff will be transmitted to patients during invasive or other patient-care procedures that involve touching a patient's mucous membranes and nonintact skin. Gloves can also reduce the chance of cross contamination between the medical staff and patients. Gloves must be changed between patient's contacts and hands must be washed after their removal. Gloves does not replace the need for handwashing, they may have small, non-apparent pores where hands can become contaminated. Failure to change gloves between patient contacts is an infection control hazard.

Aprons: Wearing an apron during patient care reduces the risk of infection. The apron should either be disposable or be used only when caring for a specific patient under specific circumstances.

Chapter Two

Aims of the Study

The primary aim of this study is to screen for carbapenemase producing *A. baumannii* in clinical isolates from patients in three main governmental hospitals in the West Bank, Palestine. In addition, to determine the mechanisms of resistance.

Objectives

1- To evaluate the presence of carbapenemase resistant genotypes by PCR.

2- To determine the presence of the oxacillinase genes OXA-23, OXA-24, OXA-51 and OXA-58.

3- Perform the antimicrobial susceptibility testing using the disk diffusion method and micro broth dilution method for the antibiotics used to treat multidrug resistant isolates of *A. baumannii*; Meropenem, Tigecycline and colistin.

Chapter Three

Materials and Methods

Sample Collection

A total of 59 samples were collected from hospitalized patients and outpatients attending clinics at Palestine Medical complex, Ramallah (30), Beit Jala hospital, Bethlehem (15) and Rafidia surgical hospital, Nablus (14) figure 1. The majority of the specimens were wound (20), Respiratory (20), blood (7) and others (12). The samples were collected from October 2016 to November 2017. Acinetobacter baumannii clinical isolates were obtained from different specimens including: urine, wound, blood, sputum as well as tracheal aspirates. Information related to the type of specimen, the hospital ward and other relevant information are shown in Table1. Its noteworthy to see that all isolates were multidrug resistant including the carbapenems.

Specimen type	Ward/Clinic	number	Total
Wound swab	ICU Orthopedic Pediatric Cardiac Surgery ER	3 9 1 1 5 1	20
Respiratory tract	ICU	20	20
Blood	ICU Pediatric	6 1	7
urine	ER ICU	5 1	6
Fluid	ER	1	1
Rectal swab	Pediatric Clinic	2	2
Burns	Burn Department	3	3
Total number of samples			**59**

Table 1: Types of *A. baumannii* specimen's ward or clinic and number collected.

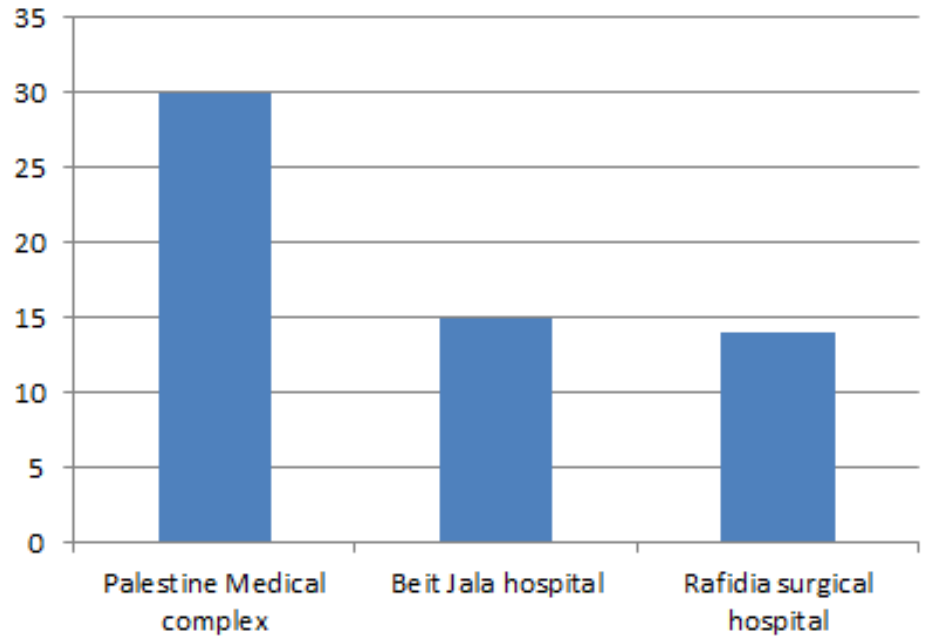

Figure 4: Distribution of clinical sample between three hospitals.

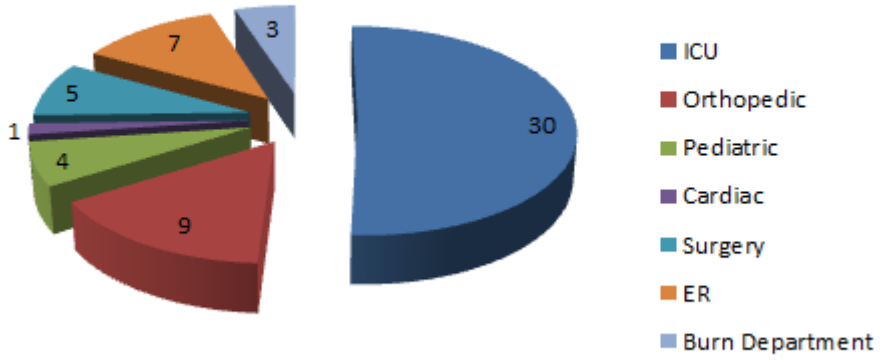

Figure 5: Distribution of *A. baumannii* isolates according to hospital wards.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed by the disk diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017).

Suspension of 0.5 McFarland were made for the isolates, using sterile cotton swab to surface of a Muller Hinton Agar (MHA) plate by streaking the swab over the entire sterile agar surface. Between 5 to 15 min should be added the antimicrobial disks to the surface of the MHA plate. Each disk must be pressed down to ensure complete contact with the agar surface. Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so they are no closer than 24 mm from center to center. Ordinarily, no more than 12 disks should be placed on one 150-mm plate, or more than five disks on a 100-mm plate. In all cases, however, it is best to place disks that give predictably small zones (eg, gentamicin, vancomycin) next to those that give larger zones (eg, cephalosporins) in an effort to avoid overlapping zones.

The antibiotics tested were obtained from oxoid (England) and include: Cefotaxime 30 μg, ciprofloxacin 5 μg, ceftriaxone 30 μg, Piperacillin-tazobactam 100/10 μg, Amikacin 30 μg, gentamycin 10 μg, Meropenem 10 μg, imipenem 10 μg, colistin 10 μg and Tigecycline 15 μg. The interpretation of antimicrobial susceptibility was done according to the recommendations of CLSI 2017.

All the isolates are multidrug resistant Acinetobacter baumannii which is resistant to Meropenem 10 μg and two other classes of antibiotic.

Minimal Inhibitory Concentration (MIC)

MIC in this research project was performed by the microbroth dilution method following the guidelines of CLSI. This method was applied to Meropenem, colistin and Tigecycline.

The Meropenem trihydrate powder was obtained from Anfarm Hellas, it has a potency of 74%, Tigecycline was obtained from Sigma-Aldrich with a potency of 98% and colistin was obtained from Sigma-Aldrich with a potency of 1500 U/mg. The stock of each antibiotic was prepared in sterile distilled water at 1mg/ml according to the following formula:

Weight = (1000/P) x V x C

Broth Microdilution method

All the 56 isolates were tested for the sensitivity of three main antibiotic was the drug of choice for treatment of Acinetobacter species.

Meropenem

Carbapenems were the basis of antibacterial Therapy against *A. baumannii* infections since 1990. first discovered resistance to carbapenems in 1985, the opening year of imipenem, announcing that mechanisms of antibiotic resistance existed even before first use.(Asif, Alvi et al. 2018)

After doing the sensitivity of Meropenem using disk diffusion method the isolates show a completely resistance for carbapenem group.

Antibiotic standard powders of Meropenem with potency of 74% were used. The concentrations tested for Meropenem were: 0.5. 1, 2, 4, 8, 16, 32, 64, 128 and 246 μg/ml

Tigecycline

Tigecycline, being the first member of Glycycline, is a novel drug approved by the US Food and Drug Administration in June 2005 for the treatment of complicated skin infections, community-acquired pneumonia, and intra-abdominal infections.(Asif, Alvi et al. 2018)

It is also being used in the treatment of bacteremia and UTIs by multidrug-resistant (MDR) Gram-negative bacteria. In Palestine doctors use Tigecycline for treatment of infection caused by Acinetobacter, according to this the sensitivity test for this antibiotic done using dick diffusion method and broth microdilution method both procedure shown almost the same results.

Colistin sulfate

Colistin or Polymyxin E is a bactericidal drug that disrupts cell membrane like a detergent. Its positively charged cationic region binds to negatively charged hydrophilic portion of LPSs. The resulting loss of integrity causes cell death.45 The current panic situation of antibiotic resistance in Acinetobacter infections had led to the use of historically discarded drug, colistin. Colistin has shown high nephrotoxicity, ranging from 11% to 76% in various retrospective and prospective studies.(Asif, Alvi et al. 2018).

There are unclear situation when we talking about colistin sensitivity test, and the result is not the same between two deferent methods. Take into consideration the potency of antibiotic as provided on the documentation received with the powder, when weighing the powder. Note the unit of potency used IU or μg.

According to SIGMA Company the potency of colistin sulfate is > 15000 U/mg. The concentrations tested for colistin sulfate were: 0.5. 1, 2, 4, 8, 16, 32, 64, 128 and 246 μg/ml.

Molecular Diagnosis

In our study we are focusing in the genes that are responsible for formation of resistance in Acinetobacter, and there are four genes were chosen in this study

1- OXA 51
2- OXA 23
3- OXA 24
4- OXA 58

The first group of carbapenem-resistant OXA-type -lactamases to be identified in A.baumannii was the OXA-23 group

After identification and isolation of isolates all of 56 sample were inoculated from mac agar to non-specific agar (nutrient agar), and after incubation 18-24 hours at 37c incubator the DNA extraction was done using boiling method.

DNA Extraction

1- One loop full of each isolate was taken and put in 100 µl of sterile distilled water in a 0.2 ml Eppendorf tube
2- Transfer the tubes to 95c water path for 10 minutes.
3- Centrifuge at 12000 rbm for 2 min to form the pellet.
4- Transfer supernatant into a properly labeled eppendorf tube (with sample name).
5- Store it at -20 to 4°C.

PCR

Amplification was then performed with 5 µl of the supernatant as the DNA template in 25 µl reaction volume. The complete mix consisted of the following components:12.5µl Go Taq Green Master Mix, 2X (Promega), 0.3 µl upstream primer (0.1µM), 0.3 µl downstream primer (0.1µM), and 6.9µl nuclease free water All primers used for PCR amplification are listed in the table.

The amplification conditions were as follows: an initial denaturation step at 95o C, for 4minutes, denaturation 95 for 30 sec, 35 cycles of: 94o C for 40 seconds, annealing for 53 seconds at primer set specific temperature and extension at 72o C for 40 seconds and final extension 72 for 5min. This was followed by a final extension step at 72o C for 10 minutes. A negative control of sterile distilled water was included with every PCR. Positive controls used were selected from strains tested in this project. A 100-bp DNA ladder (Thermo) was used to assess the size of PCR products. PCR products were resolved on 2.0 % agarose gels, stained with ethidium bromide and visualized on UV transilluminator, and selected gels were photographed.

Primer	Sequence 5’ to 3’	Annealing temp ^{o}C	Target gene	Amplicon bp
Oxa51-F	TAATGCTTTGATCGGCCTTG	52	*blaOXA-51*	*353bp*
Oxa51-R	TGGATTGCACTTCATCTTGG	52	*blaOXA-51*	
Oxa23-F	GATCGGATTGGAGAACCAGA	50	*blaOXA-23*	*501bp*
Oxa23-R	ATTTCTGACCGCATTTCCAT	50	*blaOXA-23*	
Oxa24-F	CAAGAGCTTGCAAGACGGACT	56	*blaOXA-24*	*420bp*
Oxa24-R	TCCAAGATTTTCTAGCRACT	56	*blaOXA-24*	
Oxa58-F	TCGATCAGAATGTTCAAGCGC	54	*blaOXA-58*	530bp
Oxa58-R	ACGATTCTCCCCTCTGCGC	54	*blaOXA-58*	

Table 2: Primers and annealing temperatures used for the amplification of genes in *Acinetobacter baumannii* isolates.

Ethical Considerations

This study was approved by the Medical Research Committee at Birzeit University, Palestine. The recommendation of the ethical committee at Birzeit University were fully implemented throughout this project.

Chapter Four

Results

This study was conducted on *Acinetobacter baumannii* implicated in causing hospital acquired infections. A total of 59 samples were collected from governmental hospitals throughout the West Bank, Palestine. The rate of male patients was 71% (42/59), and 29% females (17/59). The age distribution for the participants ranged from < 20, 21-30, 31-40, 41-50, to >50 as shown in Figure 7 below. The distribution of patients on the hospital wards is shown in Table 1 and Figure 5. The rate of hospital acquired infections was 84.7% (50/59) while the rate of possible community acquired infection (patients seen in outpatient clinics) was 15.2% (9/59). The rate of the isolates obtained from the ICU units was 50.8% (30/59). Most of the isolates of A. baumannii were taken from wounds (20), respiratory tract (20), blood (7) and urine (6) as shown in Table 1. The rate of isolates obtained from these four sites represented 89.8% (53/59).

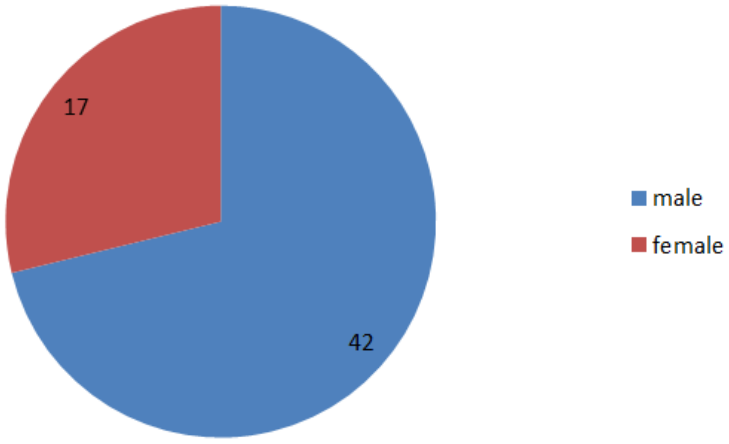

Figure 6: Male and female distribution of the patients.

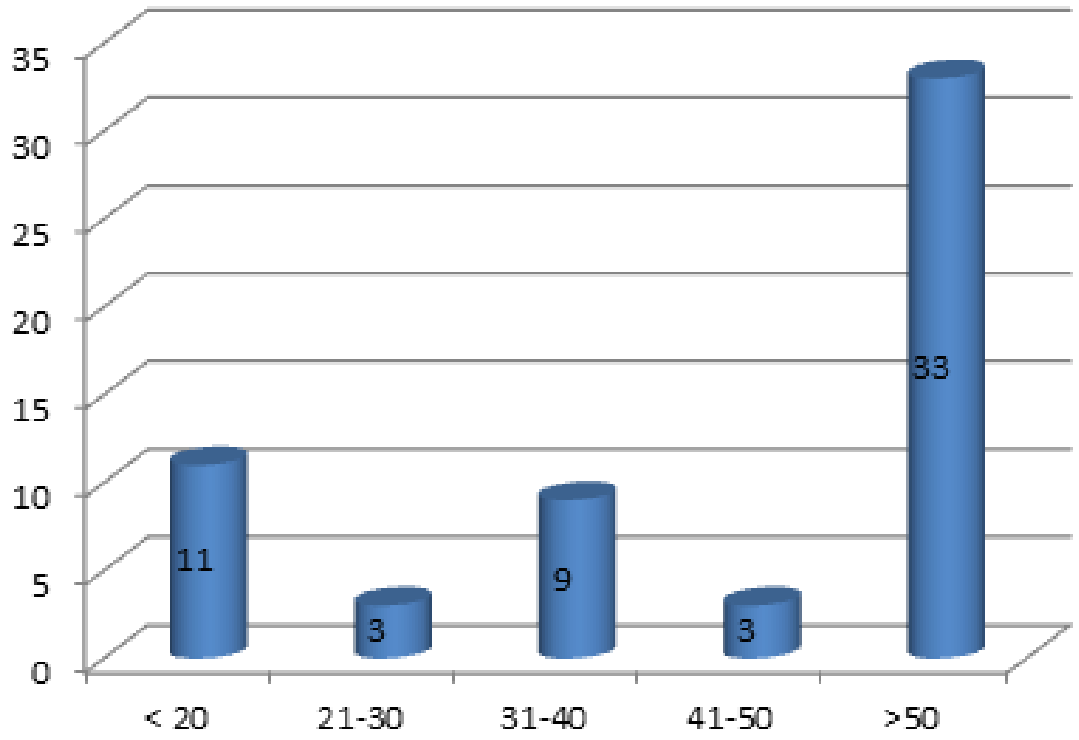

Figure 7: Age distribution of the patients included in the study.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed for all isolates by the disc diffusion method following the guidelines of CLSI 2017. The minimal inhibitory concentrations (MIC) for *A. baumannii* isolates against Meropenem, Tigecycline and colistin were performed using the micro broth dilution method. The results obtained are summarized in Tables 3 below. It is apparent from Tables 4, 5 and 6 that all isolates were resistant to Meropenem. However, susceptibility rates of these isolates as shown in these Tables to colistin and Tigecycline were 84.7% (50/59) and 88.1% (52/59) respectively.

	MIC ug/mL (n=59 isolates)									
Antibiotic	0.5	1	2	4	8	16	32	64	128	256
Colistin[1]	16	21	13	5	4	0	0	0	0	0
Meropenem[2]	0	0	0	0	15	21	5	9	9	0
Tigecycline[3]	2	9	34	7	3	4	0	0	0	0

Table 3: MIC values for *Acinetobacter baumannii* according to the guidelines of CLSI

1. MIC $\leq$ 2 (S), $\geq$ 4 (R)
2. MIC $\leq$ 2 (S), 4 (I), $\geq$ 8 (R)
3. MIC $\leq$ 4 (S), 8 (I), $\geq$ 16 (R)

	MIC µg/ ml for Meropenem										
MIC	0.5	1	2	4	8	16	32	64	128	265	total
Number	0	0	0	0	15	21	5	9	9	0	59
%	0	0	0	0	25%	35%	8.4%	15%	15%	0	100%
Interpretation	S	S	S	I	R	R	R	R	R	R	

Table 4: *A. baumannii* (n=59) MIC ranges and distributions for Meropenem.

S: sensitive, I: intermediate, R: resistant

	MIC µg/ ml for Tigecycline										
MIC	0.5	1	2	4	8	16	32	64	128	265	total
Number	2	9	34	7	3	4	0	0	0	0	59
%	3.3%	15.2%	57%	11.8%	5%	6.7%	0	0	0	0	100%
Interpretation	S	S	S	S	I	R	R	R	R	R	

Table 5: *A. baumannii* (n=59) MIC ranges and distributions for Tigecycline.

S: sensitive, I: intermediate, R: resistant

	MIC µg/ ml for Colistin sulfate										
MIC	0.5	1	2	4	8	16	32	64	128	265	total
Number	16	21	13	5	4	0	0	0	0	0	59
%	27.1%	35.5%	22.0%	8 %	6.7%	0	0	0	0	0	100%
Interpretation	S	S	S	R	R	R	R	R	R	R	

Table 6: *A. baumannii* (n=59) MIC ranges and distributions for Colistin sulfate.

S: sensitive, I: intermediate, R: resistant

Interpretations of the results were done according to the CLSI recommendations. The MIC for Meropenem was considered sensitive at ≤2 µg /ml, intermediate at 4 µg /ml and resistant at ≥8 µg /ml as shown in Table 4. The MIC for Tigecycline was considered sensitive at ≤4 µg/ml, intermediate at 8 µg /ml and resistant at ≥16 µg /ml as shown in Table 5. At present, there is no agreement on how to interpret resistance for colistin using disc diffusion method, and the CLSI

recommend that do not using this method for the **antimicrobial susceptibility testing, and only use the MIC.** The MIC for colistin was considered sensitive at ≤2 μg /ml, and resistant at ≥4 μg /ml.

	Meropenem		**Tigecycline**		**Colistin sulfate**	
	Disk diffusion	MIC	Disk diffusion	MIC	Disk diffusion	MIC
Sensitive %	0 %	0%	84.9 %	88.3 %	---	85.3%
Intermediate %	0 %	0 %	6.7 %	5 %	---	---
Resistant %	100 %	100 %	8.4 %	6.7 %	---	14.7 %

Table 7: *A. baumannii* isolates susceptibility percentages to Meropenem. Tigecycline and Colistin sulfate for two type of susceptibility testing.

The correlation of the disk diffusion results vs. the MIC results are compared and shown in Table 7. It is obvious from Table 7 that the MIC and disc diffusion results are compatible for meropenem. Minor variations were noted between the two methods for tigecycline. The rate of susceptible results was slightly higher (84.9% vs. 88.3%) for tigecycline but slightly lower for the intermediate and resistant isolates. As for the colistin sulfate, the disk diffusion is not recommended by the CLSI and instead only MIC was applied for the isolates (CLSI 2017).

PCR Amplification for Carbapenemases

Agarose gel electrophoresis was performed on all PCR amplification results to determine the carriage of oxacillinase genes by the *A.baumannii* isolates. PCR was performed to determine the carriage of the Acinetobacter isolates to the class D β-lactamase genes (Oxacillinase): OXA-51, OXA-23, OXA 24 and OXA 58. Our results showed that all the isolates tested carried the bla_{OXA51} gene as shown in Figure 9, a band of 353 bp. bla_{OXA523} gene was carried in 54.2% (32/59) of isolates as shown in Figure 10, a band of *501bp*, None of the Acinetobacter isolates tested carried the genes for OXA-24 and OXA-58.

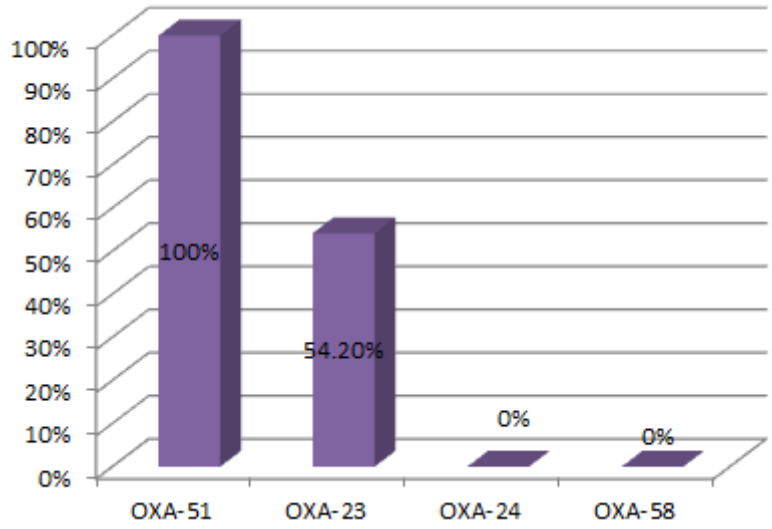

Figure 8: Class D beta lactamase (oxacillinase) genes carried by the clinical isolates of *A. baumannii* that was resistant to Meropenem.

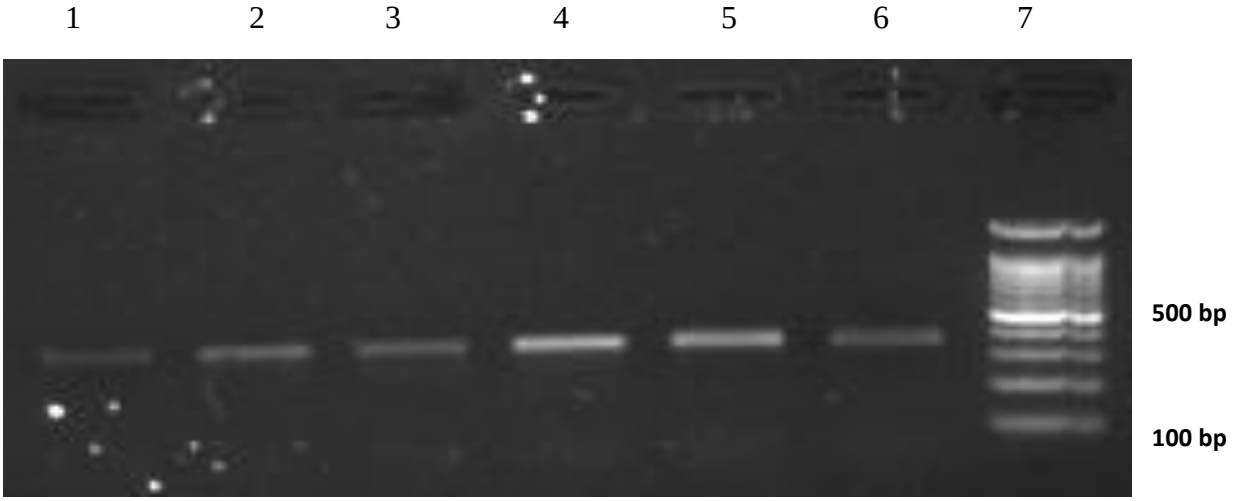

Figure 9: Acinetobacter baumannii isolates (well 1 to 6) carrying the Oxa 51gene (365 bp)

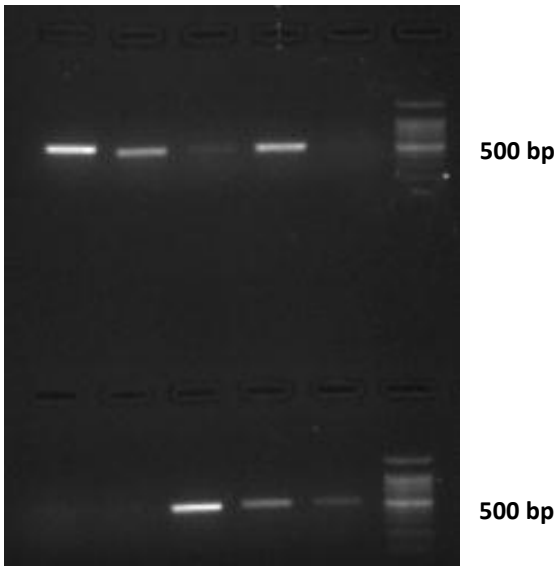

Figure 10: Acinetobacter baumannii isolates (well 1 to 6) carrying the Oxa23 gene (501 bp).

Chapter Five

Discussion

A. baumannii has been responsible for a high rate of mortality and morbidity among Palestinian patients. The disastrous outcomes of this multidrug resistant microorganism were evident mainly in hospitals in ICU and to a lesser extent among the outpatients, particularly the elderly. The therapeutic options for this highly resistant organism was extremely limited to polymyxin E class of antibiotics (colistin) and the glycylcycline (tigecycline), both administered by the intravenous route with severe adverse or unpleasant side effects(Chen and Shi 2018). However, the carbapenems that used to be the drug of choice for treatment are no longer useful to treat infections caused by this organism.

Antimicrobial susceptibility testing by both the disc diffusion and MIC methods showed that the *A. baumannii* is fully resistant to this class of antibiotics restricting the options for successful treatment. Therefore, the options to treat this organism became limited to colistin and tigecycline.

As for colistin, (the ultimate treatment option), our results showed that 84.7% of the isolates were sensitive to this antibiotic. It is noteworthy that all clinical samples (39 samples) received and tested before 2017 were 100% susceptible to this antibiotic. However, there were 20 samples received during the year 2017. We noted that the emergence of resistant strains was included in this group of samples. We found that 45% (9/20) of these samples were resistant to colistin as

shown in Table 6. Unfortunately, this has resulted in major crisis in hospitals, since this was the first encounter for colistin resistance.

AS for tigecycline, the alternative option for treating colistin resistant isolates, the rate of susceptibility was considerably high (88.3%). Fortunately, the 9 resistant colistin isolates were susceptible to tigecycline.

Interpretations of the results were done according to the CLSI recommendations. The MIC for Meropenem was considered sensitive at ≤2 ug/ml, intermediate at 4 ug/ml and resistant at ≥8 ug/ml as shown in Table 4. The MIC for Tigecycline was considered sensitive at ≤4 ug/ml, intermediate at 8 ug/ml and resistant at ≥16 ug/ml as shown in Table 5. At present, there is no agreement on how to interpret resistance for colistin using disc diffusion method, and the CLSI recommend that do not using this method for the antimicrobial susceptibility testing, and only use the MIC. The MIC for colistin was considered sensitive at ≤2 ug/ml, and resistant at ≥4 ug/ml.

Our susceptibility results showed that the rate of multidrug isolates was higher in males than females 71% to 29% respectively. However, there may be controversy in these rates since the number of female patients was only 17 as compared to 42 male patients. For this comparison to be reasonable, equal number of male and female patients' results should be compared.

It is known that for older age, the status of the immune system and length of hospitalization plays a major role in the pathogenicity of this organism(Howard, O'Donoghue et al. 2012). Our results showed that most of the infected patients were above 50 years of age, a rate of 55.9% (33/59). Furthermore, there were a considerable number of patients less than 20 suffered from

Acinetobacter infection, a rate of 18.6 % (11/59). These younger patients suffered from low immunity and had a prolonged hospital stay.

In this project, we tested the presence of class D beta-lactamases genes, the oxacillinases (OXA23, OXA 24, OXA 51 and OXA 58). The OXA type carbapenem resistance hydrolytic enzymes are common in in *A. baumannii* clinical isolates. OXA 51 has been found in all strains of *A. baumannii* and hence, it is usually used to confirm the identification of the clinical isolates. In this work, all the clinical isolates tested (59 isolates) carried the OXA 51 genes, indicating correct identification of the isolates. Originally, *A. baumannii* as well as other gram negative bacteris belonging to the enterobacteriaceae family were identified by the API20e (Biomeieux, France Paris). Published literature also indicated that although most *A. baumannii* carriying the OXA 51 gene were resistant to carbapenems, several isolates remaind susceptible to this group of antibiotics.(Lin, Hsia et al. 2010)

Our results showed that OXA 24 and OXA 58 genes were not carried by the tested clinical isolates of *A. baumannii*. It has been reported that *A. baumannii* carrying the OXA 24 gene was responsible for an outbreak in Spain in 1997. OXA 58 gene was first reported in France in 2003. It has been known that OXA 24/58 genes carried by *A. baumannii* showed high resitance to carbapenems. These two genes are not universally spread, indicating a type of geographic distribution. That could the reason why OXA 24/58 genes are found in certain areas in the world such as Spain and France and not found in others as in China.(Chen, Gao et al. 2017) This may also explains the absence of these two genes (OXA24/58) in the clinical isolates tested in this project.

However, OXA 23 was carried by 54.2% (32/59) of the isolates. This finding is consistent with published literature regarding the spread of carbapenem resistance in *A. baumannii* isolates carrying the OXA 23 gene. (Chen, Gao et al. 2017)It has been shown that introducing of $bla_{\text{OXA-23}}$ or into *A. baumannii* ATCC 15151 has resulted in the overexpression of OXA-23 gene. This finding has been related to the significant increase in resistance to imipenem, and meropenem (Lin, Hsia et al. 2010)

Recommendations

1- Further studies should be conducted by PFGE to determine the relatedness of the strains and their correlation with the resistance genotypes

2- Strict rules must be implemented in hospitals to curb the spread of MDR *A.baumannii.*

Table 8: List of all specimen collected and related information.

Sample Number	Site of infection	Department	Sensitivity	Age	Gender
1	Wound swab	ICU	MDR	85 year	Female
2	Sputum culture	ICU	MDR	75 year	Female
3	Wound swab	Orthopedic	MDR	72 year	Female
4	Sputum culture	ICU	MDR	63 year	Female
5	Blood culture	ICU	MDR	19 year	Male
6	Wound swab	Orthopedic	MDR	81 year	Female
7	Sputum culture	ICU	MDR	70 year	Female
8	Sputum culture	ICU	MDR	40 year	Female
9	Wound swab	Surgical word	MDR	52 year	Male
10	Urine culture	Emergency	MDR	38 year	Female
11	Sputum culture	ICU	MDR	63 year	
12	Sputum culture	ICU	MDR	58 year	Female
13	Blood culture	ICU	MDR	33 year	Male
14	Wound swab	Orthopedic	MDR	24 year	Male
15	Wound swab	Orthopedic	MDR	20 year	Male
16	Wound swab	Orthopedic	MDR	39 year	Male
17	Wound swab	Emergency	MDR	32 year	Female

18	Sputum culture	ICU	MDR	72 year	Male
19	Urine culture	ICU	MDR	14 year	Female
20	Sputum culture	ICU	MDR	77 year	Male
21	Blood culture	ICU	MDR	77 year	Male
22	Sputum culture	ICU	MDR	14 year	Female
23	Blood culture	ICU	MDR	57 year	Male
24	Wound swab	Pediatric	MDR	52 year	Male
25	Sputum culture	ICU	MDR	66 year	Male
26	Urine culture	Out clinic	MDR	66 year	Male
27	Urine culture	Out clinic	MDR	85	Male
28	Blood culture	Pediatric ICU	MDR	6 month	Male
29	Burn wound swab	Burn Department	MDR	56 year	Male
30	Wound swab	Orthopedic	MDR	42 year	Male
31	Sputum culture	ICU	MDR	20 year	Male
32	Sputum culture	ICU	MDR	58 year	Male
33	Sputum culture	ICU	MDR	74 year	Male
34	Sputum culture	ICU	MDR	53 year	Male
35	Sputum culture	ICU	MDR	18 year	Male
36	Blood culture	ICU	MDR	18 year	Male
37	Wound swab	Orthopedic	MDR	74 year	Male
38	Wound swab	Cardiac	MDR	66 year	Male
39	Wound swab	ICU	MDR	28 year	Male

40	Wound swab	Orthopedic	MDR	17 year	Male
41	Wound swab	Surgical word	MDR	40 year	Male
42	Rectal swab	Pediatric ICU	MDR	3 month	Male
43	Sputum culture	ICU	MDR	78 year	Male
44	Urine culture	Out clinic	MDR	73 year	Female
45	Sputum culture	ICU	MDR	59 year	Male
46	Sputum culture	ICU	MDR	52 year	Male
47	Blood culture	ICU	MDR	69 year	Male
48	Wound swab	ICU	MDR	66 year	Male
49	Sputum culture	ICU	MDR	67 year	Male
50	Wound swab	Orthopedic	MDR	50 year	Male
51	Urine culture	Emergency	MDR	35 year	Female
52	Fluid culture	Emergency	MDR	49 year	Female
53	Wound swab	Surgical word	MDR	26 year	Male
54	Wound swab	Surgical word	MDR	40 year	Female
55	Wound swab	Surgical word	MDR	55 year	Male
56	Tip culture	ICU	MDR	39 year	Female
57	Rectal swab	Pediatric ICU	MDR	4 month	male
58	Burn wound swab	Burn Department	MDR	66 year	Male
59	Burn wound swab	Burn Department	MDR	64 year	Male

Chapter Six

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