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EVALUATING THE EFFECTS OF CARBAPENEM/β-LACTAMASE INHIBITOR COMBINATIONS IN TREATING CARBAPENEM-RESISTANT GRAM-NEGATIVE BACTERIAL INFECTIONS

by
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AN ABSTRACT OF THE THESIS OF

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Title: Evaluating the Effects of Carbapenem/β-Lactamase Inhibitor Combinations in Treating Carbapenem-Resistant Gram-Negative Bacterial Infections

Background: Gram-negative bacteria are common human pathogens that may cause various complicated infections and can become resistant to carbapenems. This form of resistance is clinically relevant as carbapenems are the last line of safe antimicrobials to be used before resorting to toxic drugs such as colistin. Carbapenem-hydrolyzing enzymes are the β-lactamases responsible for carbapenem resistance. Class A β-lactamases include KPC, Class B β-lactamases include NDM, and Class D β-lactamases include OXA-type carbapenemases. When treating carbapenem-resistant infections, antimicrobial combination therapy has been the solution to avoid using colistin extensively, but its benefit over monotherapy remains controversial. The use of β-lactam/β-lactamase inhibitor (BL/BLI) combinations is an alternative approach to treat carbapenem-resistant infections. In this study, we assessed the effect of three carbapenemase inhibitors: calcium-EDTA (Ca-EDTA), avibactam, and relebactam, were combined with each of imipenem, meropenem, and ertapenem against several bacterial isolates that harbor carbapenem-resistance genes.
Methods: Six *Acinetobacter baumannii* isolates were screened for *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24/40}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, and *bla*$_{\text{OXA-143-like}}$ genes, and eight *Enterobacteriaceae* isolates were screened for *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM-1}}$, and *bla*$_{\text{KPC-2}}$ via PCR amplification. Minimal inhibitory concentrations of carbapenems with corresponding β-lactamase inhibitors for each isolate were determined using broth antimicrobial microdilution testing. Efficacy of the most suitable *in vitro* treatment regimen of an isolate that harbors *bla*$_{\text{OXA-48}}$ was tested on 30 BALB/c mice by infecting them with the isolate and treating them appropriately, then monitoring their survival for seven days. Reverse transcription real-time PCR analysis was performed to assess the molecular response of the isolate that harbors *bla*$_{\text{OXA-48}}$ to the selected treatment regimen under both *in vitro* and *in vivo* conditions.
Results: PCR amplification detected the target genes in most of the tested samples, mainly *bla*$_{\text{OXA-48}}$ in five *Enterobacteriaceae* isolates, *bla*$_{\text{NDM-1}}$ in two *Enterobacteriaceae* isolates, and *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-51-like}}$ genes in all *A. baumannii* isolates. The use of imipenem, meropenem, or ertapenem in combination with the corresponding β-lactamase inhibitors restored the isolates' susceptibility to those antimicrobial agents in 64.3%, 71.43%, and 14.3% of the tested isolates, respectively. Survival studies in murine animal models revealed a marked increase in survival rates of mice treated with meropenem in combination with avibactam, compared to those without the dual therapy. Meanwhile, RT-qPCR studies for both *in vitro* and *in vivo*

settings respectively showed a significant increase in *bla*$_{\text{OXA-48}}$ expression upon treating the bacterial isolate with meropenem alone or in combination with avibactam.
Conclusion: Based on the results of this study, it can be concluded that the use of β-lactams in combination with novel β-lactamase inhibitor**s** is a viable alternative to antimicrobial combination therapy as it showed high efficacy *in vitro* and *in vivo*. However, a bigger sample size that includes several bacterial species harboring different carbapenemases should be tested for the β-lactam/β-lactamase inhibitor combinations used in this study before potentially being translated into clinical trials.

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CHAPTER I

INTRODUCTION

Carbapenem resistant Gram-negative bacteria have been gradually increasing in prevalence in recent years, with US estimates of 9,000 annual infections caused by carbapenem-resistant *Enterobacetriaceae* (CRE) and 7,300 annual infections caused by multidrug-resistant *A. baumannii* (1). In Lebanon, the most recent nation-wide data indicates that around 2% of *Enterobacteriaceae* isolates identified over the past few years were imipenem-resistant, while that percentage was much higher among *Acinetobacter spp*. isolates, at 82.4% (2).

Carbapenem resistance has been associated with several mechanisms, including the production of extended-spectrum β-lactamases (ESBLs) or AmpC-type enzymes in addition to efflux pump activity and porin loss (3). However, the main mode of resistance to carbapenems is through the expression of carbapenem-hydrolyzing β-lactamases, such as KPC, OXA-type carbapenemases, and NDM-1.

Treating bacterial infections that are resistant to carbapenems poses a major health threat as carbapenems are the last-resort antimicrobial agents to be used. As such, the current alternative treatment to carbapenem-resistant bacteria involves the use of antimicrobial combination therapy (4) that incorporates the highly nephrotoxic polymyxins with other antimicrobial agents such as fosfomycin and tetracyclines (5). Previous work has addressed the issue of antimicrobial combination therapy and concluded that such treatment requires the tailored use of antimicrobial agents based on the phenotypic/genotypic characteristics of the carbapenem-resistant infectious agents

(6). However, despite the results of that study as well as numerous published works that outline the benefits of combination therapy, it remains a topic of controversy when compared to antimicrobial monotherapy; therefore, an alternative approach that addresses the issue of carbapenem-resistance would be the use of β-lactam/β-lactamase inhibitor combinations.

In the present study, we aimed to:

- Evaluate the *in vitro* effects of three β-lactamase inhibitors: avibactam, Ca-EDTA, and relebactam, when combined with three carbapenems: imipenem, ertapenem, and meropenem, against Gram-negative bacteria that produce the carbapenem-hydrolyzing OXA-type carbapenemases, *K. pneumoniae* carbapenemases (KPC), and New Delhi Metallo-β-lactamases (NDM).
- Evaluate the efficacy of meropenem in addition to avibactam against an *Enterobacteriaceae* isolate that harbors $bla_{\text{OXA-48}}$ in a mouse model via survival experiments.
- Assess the molecular response of that bacterial isolate to meropenem and avibactam under both *in vitro* and *in vivo* settings using reverse transcription real-time polymerase chain reaction (RT-qPCR).

CHAPTER II

LITERATURE REVIEW

A. *Acinetobacter spp*.

1. General Characteristics:

Acinetobacter is a genus of Gram-negative coccobacilli belonging to the *Moraxellaceae* family (7). Generally, *Acinetobacter* are non-motile, non-fermentative, non-fastidious, obligately aerobic, oxidase-negative, and catalase-positive bacteria (8, 9). On growth media, *Acinetobacter spp*. form pale-yellow to gray smooth colonies that are domed and approximately 2 mm in diameter (9). To date, more than thirty species of *Acinetobacter* have been identified, including *A. baumannii*, *A. lwoffii*, *A. pittii*, *A. calcoaceticus*, and *A. nosocomialis* (10). Identification of the different species of *Acinetobacter* can be challenging as the traditional biochemical techniques and even modern automated systems and commercial kits occasionally fail to assign the microorganism to its appropriate species. Namely, API® 20NE (bioMérieux, Marcy l'Etoile, France) only identifies 54% of the genus into species, while VITEK 2 (bioMérieux, Marcy l'Etoile, France) identifies 53% of it (7). As such, genetic sequence analysis using the 16S rRNA gene provides a more accurate identification method since it can assign 92% of the genus into species (7). From a clinical perspective, *A. nosocomialis*, *A. pittii*, *A. lwoffii*, *A. ursingii*, *A. junii*, *A. soli*, and *A. parvus* have been

implicated in human infections; however, *A. baumannii* remains the most encountered member of the genus and accounts for higher mortality rates than the rest (7, 11).

2. Acinetobacter baumannii:

A. baumannii is the most clinically relevant member of the *Acinetobacter* genus with its main implications in nosocomial infections and war-related or natural disaster incidents (12).

a. Epidemiology and Clinical Manifestations:

Acinetobacter baumannii experiences a worldwide distribution in a generally clonal manner (11). A survey of 492 Imipenem-resistant *A. baumannii* isolates collected in the mid-2000s from 139 centers across 32 countries from North and Latin America, Asia, Australia, Europe, and Africa employed rep-PCR to investigate the molecular epidemiology of those isolates and found that nearly half of them originally belong to the Worldwide Clone 2 lineage, whereas most of the remaining isolates belong to Worldwide Clone 1 and 3-8 lineages (13).

In the environment, *A. baumannii* uses soil, water, sewage, and animals as reservoirs (7), allowing for community-acquired infections. Such modes of infection are especially observed in war-torn countries or areas affected by natural disasters like earthquakes and tsunamis, with victims of these incidents having *A. baumannii* isolated from their wounds (7, 12, 14). In addition, *Acinetobacter* infections have also been reported as sources of severe community-acquired pneumonia in East and Southeast Asia as well as tropical areas of Australia, especially during wet seasons, with risk factors for such

transmission including excessive alcohol intake and smoking (7, 15-17). Despite that, nosocomial acquisition of *A. baumannii* remains the primary encountered route to be reported. A 2010 survey that collected 514 non-duplicate clinical *Acinetobacter spp*. isolates from 65 different locations across the United States and Puerto Rico found that 94.6% of the isolates were *A. baumannii* with specimen sources including blood (23.9%), upper and lower respiratory tract (58.8%), wounds (7.6%), urine (3.3%), and cerebrospinal fluid (0.2%) (18). In Lebanon, a retrospective nationwide study revealed an increase in the count of *Acinetobacter spp*. retrieved from patient samples, with 242 isolates in 2011 to 1463 isolates in 2013 (2), while at the American University of Beirut Medical Center (AUBMC), more than 97% of *Acinetobacter spp*. identified since 2012 are *A. baumannii* (19-23).

b. Virulence and Pathogenesis:

Acinetobacter baumannii employs a diverse array of virulence factors that play a role in its pathogenicity. Firstly, *A. baumannii* transmission is supported by the organism's ability to form biofilms on environmental surfaces and medical equipment such as urinary catheters, central line catheters, and tracheal tubes; thus, prolonging its survival by preventing its desiccation, as well as, protecting it from external stressors that include antibacterial chemicals (11). Factors involved in biofilm formation include pilus production, outer membrane protein A (OmpA), extracellular polysaccharides such as poly-β-1,6-N-acetylglucosamine for biofilm development, and biofilm-associated protein (Bap) for biofilm maturation and maintenance (11, 24). Once transmitted, the bacterium binds to host cells via Bap, OmpA, Omp33-36, and autotransporter Ata that

can also bind to eukaryotic extracellular matrix components (11, 25). Following the adherence to target cells, *A. baumannii* can then enter the cell in order to evade the host's immune system, and it accomplishes that through OmpA, Omp33-36 and phospholipase D, a secreted enzyme that can breakdown lipids; thus, damaging cell membrane and allowing entrance into the cell (11, 24). Once inside, *A. baumannii* favors its survival within the host cell through the action of Omp33-36 that interferes with host cell autophagy, acinetobactin, which is a siderophore that sequesters iron from host cell in order to allow for bacterial survival under iron-deficient conditions, and OmpA, that can translocate to the host cell's mitochondria leading to the release of cytochrome *c*, an initiator of apoptosis (11, 24). Following cell death and release of the bacterium back into circulation, *A. baumannii* can evade serum complement by, first, having OmpA bind to Complement Factor H (24); hence, degrading C3-convertase and interrupting the alternative complement pathway (26), and secondly, through the protective properties of the K1 polysaccharide capsule (27).

B. *Enterobacteriaceae*:

The family *Enterobacteriaceae* comprises a group of Gram-negative bacilli that share a common polysaccharide core component of the lipopolysaccharide (LPS) in their cell wall, known as the enterobacterial common antigen (28).

1. General Characteristics:

Generally, they are non-spore forming, facultatively anaerobic, catalase-positive, oxidase-negative, and glucose-fermenting bacteria (28, 29). Members of the *Enterobacteriaceae* family can be motile through peritrichous or polar flagella, though

not all of them exhibit that characteristic (29). Other appendages that are often found on the members of the *Enterobacteriaceae* family include fimbriae, that assist the bacteria in adhering to surfaces and cellular receptors, and conjugative pili, that permits the transfer of genetic material between competent bacterial cells (28).

The *Enterobacteriaceae* family encompasses more than 50 genera that are classified according to their biochemical, structural, and genetic properties (10, 28). They are widely spread in the environment and constitute a significant portion of human and animal intestinal microflora (28, 29). As such, identification of bacterial isolates that belong to the *Enterobacteriaceae* family is vital when diagnosing infections caused by them. A variety of molecular and phenotypic laboratory tests can be employed for that purpose. At the molecular level, genetic sequence analysis using the 16S rRNA gene is a reliable technique, whereas at the phenotypic level, a key identification method is determining the biochemical profile of the isolate, and that can be achieved through the traditional biochemical tests (30) or through API® 20E. And based on those biochemical properties, *Enterobacteriaceae* can be classified into lactose-fermenters (LF) and non-lactose-fermenters (NLF) (28). Genera that are LF include *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*, while NLF genera include *Salmonella*, *Shigella*, *Proteus*, *Yersinia*, and *Serratia* (30).

2. Virulence Factors:

Members of the *Enterobacteriaceae* family possess common features that make them pathogenic (28):

- Endotoxin: It is a virulence factor that is dependent on the Lipid A constituent of the lipopolysaccharide (LPS) layer. It is associated with systemic manifestations such as cytokine release, serum complement activation, fever, disseminated intravascular coagulation (DIC), and septic shock.
- Capsule: It is a protective layer that can delay phagocytosis due to its hydrophilic antigens that repel hydrophobic interfere with the binding of antibodies to the bacteria. In addition, it prevents serum killing to prolong bacteremia.
- Antigenic variation: It is a feature that alters the expression of somatic O, capsular K, and flagellar H antigens to protect from the antibody-mediated immune response.
- Siderophores: They are molecules that sequester iron from host cells since iron is an essential factor for bacterial growth.
- Secretion systems: An example is the Type III Secretion System, which allows the bacteria to transfer their virulence factors into the host cell.

3. Escherichia coli

In addition to being part of the human intestinal microflora, *E. coli* can be pathogenic upon relocating to other organ systems or by gaining virulence factors through its transformation with bacteriophagic DNA (28).

E. coli can be grouped into six pathotypes that cause diarrheal diseases: Shiga-toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*

(EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (31).

a. Pathogenesis:

Each of the six pathotypes express specialized virulence factors that suite their clinical manifestations. ETEC express heat-labile and heat-stabile endotoxins as well as colonization factor antigens, EPEC express bundle-forming pili as well as the outer membrane antigen intimin, EAEC express heat-stable endotoxins as well as aggregative adherence fimbriae (AAF), STEC express Shiga toxins, EIEC express invasive plasmid antigen and hemolysin, and DAEC express afimbrial and fimbrial adhesins (28, 32). Heat-labile and heat-stable endotoxins respectively increase the levels of cAMP and cGMP in intestinal epithelia, leading to the excess secretion of fluids within the intestinal lumen (32). Intimin binds to its receptor on host epithelial cell membrane, causing actin rearrangement within the cell, which leads to cytoskeleton instability and consequent cell death (28). Aggregative adherence fimbriae attach *E. coli* to the intestinal cell and induce the secretion of interlukin-8 (32). Shiga toxins interrupt protein synthesis within the cell to cause cell death (33). Afimbrial and fimbrial adhesins assist *E. coli* in forming diffused attaching patterns on enterocytes (32).

b. Clinical Manifestations:

E. coli infections can have several manifestations depending on the affected organ system (28):

- Gastroenteritis – ETEC, EPEC, EAEC, and EIEC manifest as watery diarrhea with abdominal cramps, dehydration, nausea, and vomiting, with EIEC possibly developing into dysentery. On the other hand, STEC causes hemorrhagic colitis that can progress into hemolytic uremic syndrome.
- Urinary Tract Infections (UTIs) – Most *E. coli* strains that cause cystitis are originally located in the colon, but contaminate the urethra and migrate upwards into the urinary bladder to bind to the cells that line the urinary tract and initiate inflammation.
- Complicated intra-abdominal Infections – Due to intestinal leakage.
- Sepsis – Following intra-abdominal or urinary tract infections, *E. coli* might move into circulation to cause sepsis, leading to a systemic infection.
- Neonatal Meningitis – After its perinatal acquisition, *E. coli* can move from the intestines into the bloodstream and then pass through the blood-brain barrier into the central nervous system to establish a meningeal infection (32).

4. Klebsiella pneumoniae

K. pneumoniae possess a prominent capsule that is responsible for the mucoid appearance of the bacterial colonies on solid growth media as well as its enhanced virulence within the host (28). It is a commonly isolated member of the *Enterobacteriaceae* family (2) and can cause several infections.

a. Pathogenesis

The polysaccharide capsule of *K. pneumoniae* has been proven to resist neutrophil-mediated phagocytosis (34). The hyper-mucoid phenotype and lipopolysaccharide layer of *K. pneumoniae* have made it more resistant to complement-mediated serum killing with LPS preventing complement system components from binding to the bacterial cell (35, 36). Type 3 fimbrial adhesion proteins assist *K. pneumoniae* in attachment to host cells (37).

b. Clinical Manifestations

K. pneumoniae is associated with several diseases including:

- Pneumonia – It can either be community- or hospital-acquired and results in alveolar necrosis and cavitation, as well as hemoptysis (28).
- Bloodstream Infections – Nosocomial bloodstream infections of *K. pneumoniae* are more common than the community-acquired ones (38).
- Genitourinary Tract Infections – Including cystitis, pyelonephritis, prostatitis, and renal abscess (39).
- Meningitis – Mostly nosocomial rather than community-acquired (39).
- Liver abscess and bacterial peritonitis (35).

5. Salmonella spp.

Salmonella spp. is a commonly acquired infectious pathogen that can colonize animals and cause diseases in humans (28) while being the cause of several outbreaks

associated with contaminated food items (40). Among the various species and subspecies that the *Salmonella* genus comprises, *S. enterica* subsp. *enterica*, is the most clinically relevant, and contains more than 2500 serotypes, including Enteritidis, Typhi, Paratyphi, and Typhimurium (28, 41, 42).

a. Pathogenesis

Salmonella establishes an infection following its ingestion by attaching to the intestinal mucosa and invading M (microfold) cells of the Peyer's patches where it replicates inside an endocytic vacuole then translocates to the mesenteric lymph nodes before spreading throughout the body via systemic circulation (28, 43). In order to invade enterocytes, *Salmonella* uses its type III secretion system to inject salmonella-secreted invasion proteins (Ssps) into its target. In addition, *Salmonella* mediates cAMP production, prostaglandin secretion, and proinflammatory cytokines release (28, 43).

b. Clinical Manifestations

Salmonellosis can be clinically manifested in three forms (28):

- Gastroenteritis – It is due to the consumption of contaminated food or water and is the most common disease caused by *Salmonella spp*. It is characterized by nausea, vomiting, and non-bloody diarrhea, as well as fever and myalgia
- Enteric fever – It is also known as typhoid fever if caused by *Salmonella* Typhi, or paratyphoid fever if caused by *Salmonella* Paratyphi. It is a febrile illness with gastrointestinal involvement that persists for a week.

- Bacteremia – It can be caused by all *Salmonella spp*., leading to systemic infections. It is more common among immunocompromised patients and patients at extremes of age.

C. Treatment of Gram-Negative Bacterial Infections:

When targeting Gram-negative bacteria, antimicrobial agents of different classes may be effective. According to the type of bacterial infection, antimicrobial agents like fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole, and β-lactams can be used (4, 23). Among those options, the β-lactams are the most diverse and widely prescribed class (44).

1. β-lactams:

The β-lactams constitute a large class of commonly-prescribed antimicrobial agents that possess a mutual structural element, the β-lactam ring, and share a mechanism of action that targets the inhibition of bacterial cell wall synthesis (5). Antibiotic groups that are classified as β-lactams include penicillins, cephalosporins, monobactams, and carbapenems (45). Mechanistically, β-lactams exert their antibacterial effect by binding to a transpeptidase penicillin-binding protein (PBP) since their chemical structure is analogous to the D-alanyl-D-alanine segments of the peptide polymers involved in the peptidoglycan layer formation. Upon binding to the transpeptidase PBP, β-lactams hinder the function of the enzyme; thus, preventing the cross-linking of peptide polymers, and consequently disrupting cell wall synthesis (45).

Carbapenems have the broadest spectrum of activity compared to the other β-lactams and β-lactam/β-lactamase inhibitor combinations, covering both Gram-positive and Gram-negative bacteria while being resistant to enzyme-mediated degradation by most β-lactamases (45, 46). However, clinically relevant bacterial pathogens have developed mechanisms of resistance to these carbapenems.

D. Carbapenem Resistance:

The Gram-negative bacteria's adaptive capacities have allowed them to develop resistance to carbapenems, with the first clinical isolate reported in 1991 in Japan (47). Since then, carbapenem non-susceptibility has been gradually spreading to other countries and continents until it reached worldwide prevalence around a decade after its initial identification (5).

In the United States, the CDC reports that more than 9,000 nosocomial infections are caused by carbapenem-resistant *Enterobacteriaceae* each year, with 11% being due to *K. pneumoniae* and 2% being due to *E. coli* (1). Moreover, around 12,000 *Acinetobacter* infections are reported annually in the US, with 63% of them being due to multidrug resistant *A. baumannii* (1). In Lebanon, a compilation of antimicrobial susceptibility data from 16 hospitals across the country indicated that between 2011 and 2013, there were 3,343 *Acinetobacter spp*. isolates identified and only 17.6% of them were susceptible to imipenem (2). On the other hand, the same study reported that during the same time period, *E. coli* isolates (n=30,411) had a 0.3% decrease (p=0.145) in susceptibility to imipenem, while *K. pneumoniae* isolates (n=7883) experienced a significant 1.3% decrease ($p<0.05$) in susceptibility to imipenem (2).

Carbapenem resistance can be achieved through several mechanisms. First, the combined effect of bacterial efflux pumps overexpression, decreased cell permeability due to outer membrane porin loss, and production of extended-spectrum β-lactamases can result in resistance to carbapenems (3). However, the more clinically relevant mechanism is the production of plasmid- or chromosomally-encoded carbapenem-hydrolyzing enzymes (5). Gram-negative bacteria can harbor these carbapenemases that render the isolates resistant to virtually all β-lactam antimicrobial agents; thus, treatment options against infections caused by these isolates become severely limited (5).

1. Carbapenem-hydrolyzing β-lactamases:

Carbapenem-hydrolyzing β-lactamases are grouped according to the Ambler molecular classification, that accounts for amino acid sequences and tertiary structure, into 4 classes (48). An interesting aspect of these enzymes is that some of them are chromosomal while others are plasmid-encoded (48). Carbapenemases that belong to Classes A, B, and D have been of increasing importance in recent years.

a. <u>Class A β-lactamases:</u>

At the active site of the enzyme, a serine moiety resides and is involved in the enzyme's hydrolytic activity against carbapenems; thus, this class of carbapenemases can also be labelled as Serine β-lactamases (48). Mechanistically, these enzymes pass through an acyl-enzyme tetrahedral intermediate before dissociating that complex and degrading the β-lactam substrate in the process (48). This class of β-lactamases comprises chromosomal and plasmid-based carbapenemases. Among the

chromosomally-encoded enzymes are the NMCs (non-metallo-carbapenemases), SMEs (*Serratia marcescens* enzymes), and IMIs (imipenem-hydrolyzing β-lactamases), while among the plasmid-encoded enzymes are the GESs (Guiana extended spectrums) and KPCs (*K. pneumoniae* carbapenemases) (49).

Historically, KPC, encoded by bla_{KPC}, was first identified in 1996 in the United States, but quickly spread to other regions of the world, including South America, Europe, and China (50). To date, 20 different variants of KPCs have been identified, with KPC-2 and KPC-3 being the most commonly encountered, amassing for a death rate of 25-69% (48, 49). A multicenter epidemiological study from the New York/New Jersey area revealed that 92% of carbapenem-resistant *Klebsiella pneumoniae* isolates in 2103 produced KPC, with 48% of them producing KPC-3 and 44% of them producing KPC-2 (51).

b. Class B β-lactamases:

Present at the active site of these β-lactamases is a divalent ion(s), usually Zn^{2+}, that allows for β-lactam hydrolysis. Therefore, this class of carbapenemases is also referred to as metallo-β-lactamases, which are further classified into B1, B2, and B3 subclasses based on amino acid sequence and the number of Zn^{2+} ions needed at the active site, with B1 and B3 enzymes requiring two ions, whereas B2 enzymes only requiring one ion (48). Mechanistically, these enzymes hydrolyze the β-lactam antimicrobial by having a deprotonated water molecule bind to the zinc ion at the active site and attack the carbonyl group of the β-lactam ring to form a tetrahedral intermediate, that is further stabilized by the zinc ion, before having the β-lactam ring C-N bond break to inactivate the antimicrobial agent (52, 53). Similarly to Class A β-

lactamases, the Class B enzymes can also be chromosomal or plasmidic. Chromosomal metallo-β-lactamases have been characterized in environmental and opportunistic organisms such as *Chryseobacterium*, *Stenotrophomonas*, *Aeromonas*, and *Bacillus cereus* (54, 55). Transferrable metallo-β-lactamases are more clinically relevant and include the Veron integron-encoded metallo-β-lactamases (VIMs), IMP-type metallo-β-lactamases, São Paulo metallo-β-lactamases (SPMs), German imipenemases (GIMs), and New Delhi metallo-β-lactamases (NDMs) (48-50).

Historically, VIMs and IMPs are the most commonly encountered metallo-β-lactamases with a worldwide distribution (49); however, the recent discovery of NDM-1, encoded by $bla_{\text{NDM-1}}$, from an Indian patient and its subsequent spread across several geographic regions, conferring high rates of resistance, have made it the most important member of the metallo-β-lactamase family (50). NDM-1 has been reported in Southeast Asia, Far East Asia, Eastern and Western Europe, the Middle East, and Northern America (51, 56-63). In Lebanon, NDM-1 detection has been demonstrated in *Enterobacteriaceae* isolates among patients coming from neighboring countries (64, 65) as well as a single case report that identified NDM-1 in an *A. pitti* isolate that infected a pediatric patient in the north (66). However, nation-wide data remains unavailable.

c. Class D β-lactamases:

This class of carbapenemases can also be labelled as Serine β-lactamases due to the presence of a serine residue in the enzyme's active site; however, they are colloquially referred to as OXA-type carbapenemases because of their ability to hydrolyze oxacillin (48), among other antimicrobial agents. Mechanistically, they degrade carbapenems in a way similar to other serine β-lactamases, with the enzyme

forming a carbapenem-enzyme acylate which, upon dissociation, hydrolyzes the β-lactam (48). OXA-type carbapenemases are grouped into 9 clusters based on their amino acid sequence, with clusters 1, 2, 3, and 4 being associated with *Acinetobacter spp.*, while cluster 6 being associated with *Enterobacteriaceae* (67). Cluster 1 comprises the subfamily OXA-23 and is encoded by $bla_{\text{OXA-23-like}}$ genes; cluster 2 comprises the subfamily OXA-24/40 and is encoded by $bla_{\text{OXA-24/40}}$; cluster 3 comprises the subfamily OXA-51 and is encoded by $bla_{\text{OXA-51-like}}$ genes; cluster 4 comprises OXA-58 and is encoded by $bla_{\text{OXA-58}}$; and cluster 6 comprises the subfamily OXA-48 and is encoded by $bla_{\text{OXA-48}}$ (67, 68). On the other hand, the subfamily OXA-143, encoded by $bla_{\text{OXA-143-like}}$ genes, is also associated with *Acinetobacter spp*. (69), but has not been classified as one of the clusters, yet. The chromosomally-located genes of OXA-type carbapenemases include $bla_{\text{OXA-51-like}}$ genes, while the plasmid-based genes of OXA-type carbapenemases include $bla_{\text{OXA-48}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-24/40}}$, $bla_{\text{OXA-143-like}}$, $bla_{\text{OXA-51-like}}$, and $bla_{\text{OXA-23-like}}$ genes (49).

Historically, the first OXA-type carbapenemase to be identified was OXA-23, named ARI-1 at the time (70), and since then, OXA-23 and OXA-51 have been identified in several nosocomial outbreaks and sporadic cases of *Acinetobacter* infections worldwide (49). In Lebanon, a recent survey that collected 142 non-duplicate *A. baumannii* isolates from nine hospitals across the country found that OXA-23 was prevalent in 141 of them while OXA-51 was detected in all isolates (71). As for OXA-48, it was initially characterized in 2001 and has since been extensively detected in several *Enterobacteriaceae* isolates from countries spanning the Mediterranean region, including Turkey, Lebanon, Egypt, Tunisia, Spain, France, Italy, and Greece (72-79). In

Lebanon, OXA-48 was first identified in 2008 (75) and has since been repeatedly isolated from patient samples between 2008 and 2014 with a prevalence of 36% among *E. coli* and *K. pneumoniae* isolates at AUBMC (80).

E. Combination Therapy Against Carbapenem-Resistant Bacterial Infections

The current recommendation for treating carbapenem-resistant Gram-negative bacterial infections is the use of combination antimicrobial therapy (4). The reliance on this approach to therapy is mostly guided by the lack of new antimicrobial agents that the bacteria might still be susceptible to since carbapenem-resistant Gram-negative bacteria usually degrade all beta-lactam antimicrobial agents while also harboring genes that confer fluoroquinolone and aminoglycoside resistance (5). Therefore, older antimicrobials like polymyxins or fosfomycin, and newer antimicrobials like tetracyclines, have to be used (5). Choices for combination antimicrobial therapy can either be with or without a carbapenem (81). Combination therapy without a carbapenem can be a double or triple combination that includes an aminoglycoside, colistin, tigecycline, fosfomycin, rifampicin, and ampicillin/sulbactam (82). On the other hand, carbapenem-based combinations include imipenem-tobramycin, meropenem-minocycline, meropenem-fosfomycin, carbapenem-ampicillin/sulbactam, and ertapenem-doripenem in which ertapenem acts as a suicide drug (82, 83).

The efficacy of combination therapy compared to monotherapy has been evaluated. One study established that combination therapy improved survival rates of patients suffering from bloodstream infections and decreased mortality rates from 54.3% to 34.1% ($p=0.02$) when compared against monotherapy (84). Another study conducted on 205

patients infected with KPC-producing *K. pneumoniae* concluded that combination therapy decreased patient mortality rate from 40% to 19.4% when a carbapenem is used in addition to other antimicrobials (85). However, despite the reported benefits of combination therapy, there remains controversy regarding its advantages over monotherapy due to the sources of bias in the existing combination therapy reports since a lot of studies include both carbapenem-resistant and carbapenem-susceptible isolates as well as disregard towards empirical treatment that the patient might have taken prior to being enrolled in the study, which might affect the combination therapy outcomes (86). In addition, the use of colistin as a last-resort antimicrobial agent against carbapenem-resistant bacteria, whether solely or in combination with other antimicrobials, causes nephrotoxicity in more than 40% of the patients who receive it (87). Not to mention, there exists discrepancies in the literature regarding combination therapy, where certain studies report that the use of carbapenems as part of the double or triple therapy is recommended when the MIC needed against the isolate is ≤8μg/mL (85, 88) whereas other studies assign that breakpoint at ≤4μg/mL (81, 82). Finally, combination therapy increases the cost of treatment (83) and exposes the bacteria to several antimicrobials that it might develop resistance to. As such, combination therapy use remains off-label and largely biased. Therefore, an alternative approach to treating carbapenem-resistant bacterial infections is the use of β-lactam/β-lactamase inhibitor combinations.

F. Beta-Lactamase Inhibitors:

β-lactamase inhibitors are molecules that can hinder the action of β-lactamases, thereby protecting the β-lactam antimicrobial agents from early degradation (45). The widely commercial β-lactamase inhibitors include clavulanic acid, sulbactam, and tazobactam. They are all β-lactam-based β-lactamase inhibitors, meaning their chemical backbone comprises a β-lactam ring and they act as "suicide" drugs that occupy the active site of an enzyme while its actual substrate is left to exert its effect; thus, extending the spectrum of antimicrobial activity (45). Clavulanic acid is commercially coupled with amoxicillin (Augmentin®, GlaxoSmithKline, NC, USA), sulbactam is commercially coupled with ampicillin (UNASYN®, Pfizer, NY, USA), and tazobactam is commercially coupled with piperacillin (ZOSYN®, Wyeth®, PA, USA).

As helpful as these β-lactam/β-lactamase inhibitor combinations have been in treating Gram-negative infections, resistance to them has been documented. Among *E. coli* isolates collected from 16 tertiary care centers across Lebanon, amoxicillin-clavulanate and piperacillin-tazobactam susceptibilities decreased from 66.7% to 58.5% ($p<0.05$) and from 89.2% to 78.9% ($p<0.05$) between 2011 and 2013, respectively (2). Similarly, *Klebsiella spp*. susceptibility to the same antimicrobials from the same hospitals decreased from 71.1% to 64.6% ($p<0.05$) and from 83.4% to 79.5% ($p<0.05$) between 2011 and 2013, respectively (2). As for *Acinetobacter spp*. isolates from the same study, the resistance to piperacillin-tazobactam increased from 69.4% to 88.9% ($p<0.05$) during the same time period (2). In addition, a total of 214 extended-spectrum β-lactamase-producing *E. coli* and *K. pneumoniae* isolates from 9 hospitals in China exhibited 5.4% and 2.1% ampicillin-sulbactam susceptibility, respectively (89).

Therefore, the commonly available β-lactam/β-lactamase inhibitor combinations are no longer viable options to a lot of Gram-negative bacterial infections; thus, new β-lactamase inhibitors need to be tested.

1. Avibactam:

Previously known as NXL104, avibactam is a novel non-β-lactam-based β-lactamase reversible inhibitor that inactivates serine carbapenemases through the covalent acylation of the enzyme followed by a slow deacylation step that restores avibactam's chemical structure (90, 91) without inducing the production of additional β-lactamases on its own (92). Avibactam has previously shown efficacy against extended spectrum beta-lactamase producers in a murine model (93) as well as KPC-producing *K. pneumoniae* (94, 95); however, decreased susceptibility to ceftazidime/avibactam has been reported in KPC-producing *K. pneumoniae* with OmpK35 deficiency (96). Currently, avibactam is approved by the U.S. Food and Drug Administration (FDA) to be combined with ceftazidime (AVYCAZ®, Forest Pharmaceuticals, Inc, Cincinnati, OH, USA) for the treatment of complicated intraabdominal and urinary tract infections (97). In a phase III clinical trial, ceftazidime/avibactam coupled with metronidazole were as efficacious as meropenem alone in treating patients suffering from complicated intraabdominal infections caused by ceftazidime-resistant Gram-negative bacteria, with cure rates of approximately 94% for each treatment option (98). Another phase III clinical trial where patients with complicated urinary tract or intraabdominal infections were either treated with ceftazidime/avibactam or the best-available therapy, which consisted of a carbapenem 97% of the time, found that the clinical cure rates were approximately 91% for either treatment option (99). In conclusion,

ceftazidime/avibactam has proven to be a successful β-lactam/β-lactamase inhibitor combination; however, it does not seem to have been clinically tested against carbapenem-resistant bacteria as most of the isolates infecting patients involved in the current clinical trials are ESBL-producers (100).

2. Edetate Calcium Disodium (Ca-EDTA):

Ethylenediaminetetraacetic acid (EDTA) is known to be an inhibitor of metallo-β-lactamases (MBLs) as well as a chemical agent used in the phenotypic detection of metallo-β-lactamases in the double disc synergy test or gradient diffusion Etest® (bioMérieux, Marcy l'Etoile, France) (48). An enhanced zone of inhibition with EDTA added to an antimicrobial agent compared to the zone of inhibition of the antimicrobial alone indicates the production of metallo-β-lactamases (55). EDTA can be used as described due to its metal-chelating properties; thus, it sequesters the divalent cation found at the active site of metallo-β-lactamases and renders them inactive (101). In addition, EDTA can neutralize bacterial toxins and enzymes as well as act on the outer membrane of Gram-negative bacteria by disrupting its lipopolysaccharide structure (101). Despite the beneficial properties of EDTA, it has not been used in clinical settings due to its adverse effects that include severe hypocalcemia (102), which may lead to tetany, seizures, and heart failure (103). To allow for clinical use, EDTA was coupled with calcium to form a calcium-EDTA (Ca-EDTA). Calcium disodium versanate (Edetate calcium disodium) is currently approved by the U.S. Food and Drug Administration (FDA) for intravenous injection to treat acute or chronic lead poisoning (104). As such, Ca-EDTA may be tested for possible clinical use against MBL-

producing bacterial infections. Evidently, calcium-EDTA has be proven to enhance the antibacterial effects of β-lactams against *Pseudomonas aeruginosa* and *E. coli* isolates that express MBLs *in vivo* by decreasing the bacterial burden of the mice injected with the infectious pathogens (101, 105).

3. Relebactam:

Previously known as MK7655, relebactam is a novel non-β-lactam-based β-lactamase inhibitor that targets Class A and Class C β-lactamases (106). Earlier studies that combined relebactam with imipenem/cilastatin have shown how successful this combination is at restoring imipenem susceptibility to imipenem-resistant bacteria (107-109). A recent phase II clinical trial that tested imipenem/cilastatin with relebactam at different doses against imipenem alone in treating patients with complicated intraabdominal infections found that combining 125mg or 250mg of relebactam to 500mg of imipenem yielded clinical response rates that were comparable to 500mg of imipenem alone (98.8% or 96.3% vs. 95.2%, respectively) (110). Consequently, two phase III clinical trials are currently in progress. The first one tests the efficacy of imipenem/cilastatin with relebactam against the combination of colistimethate sodium with imipenem/cilastatin and relebactam in treating imipenem-resistant bacterial infections (111). And the second one tests imipenem/cilastatin with relebactam against piperacillin/tazobactam in treating bacterial pneumonia (112).

CHAPTER III

MATERIALS AND METHODS

A. Isolate Collection:

Bacterial isolates included in this study were provided by the Department of Pathology and Laboratory Medicine at the American University of Beirut Medical Center (AUBMC) and the Centers for Disease Control and Prevention (CDC). A total of fourteen isolates were used, including: six *A. baumannii*, five *K. pneumoniae*, two *E. coli*, and one *Salmonella spp*. isolates. The labelling of each isolate can be found in Table 1.

B. Minimal Inhibitory Concentration (MIC) Determination:

Each of the provided isolates was tested for its MIC to 3 carbapenems: Imipenem, Ertapenem, and Meropenem. The MICs were determined through Etest® (bioMérieux, Marcy l'Etoile, France), and the technique used followed the Clinical and Laboratory Standards Institute® (CLSI) guidelines (113). Quality control strain *Escherichia coli* (ATCC® 25922™) was tested in parallel with the isolates, as that strain is implicated in Gram-negative non-fastidious bacteria (114).

1. Materials Needed:

- Mueller-Hinton agar (Difco™, BD, Franklin Lakes, NJ, USA)
- Imipenem, Ertapenem, and Meropenem Etest® strips (bioMérieux, Marcy l'Etoile, France)

- Sterile cotton swabs
- Sterile forceps
- Double distilled water

2. Procedure:

- Colonies of bacterial isolates, previously cultured overnight at 37ºC on MacConkey agar (Scharlau, Barcelona, Spain), were inoculated with a loop in sterile distilled water and the suspension adjusted with a turbidimeter to reach a turbidity standard of 0.5 McFarland (1-2 × 10^8 McFarland).
- A sterile cotton swab was used to transfer a sample of the prepared suspension to a Mueller-Hinton agar plate.
- Plate streaking was done in 3 different directions to uniformly cover the entire surface of the agar plate with the bacterial culture.
- Etest® strips of Imipenem, Ertapenem, and Meropenem were placed on the streaked plate using forceps.
- The Mueller-Hinton plate was then incubated at 37ºC for 16-18 hours.

C. Total DNA Extraction:

Bacterial isolates were cultured in LB broth (Difco™, BD, Franklin Lakes, NJ, USA) overnight at 37ºC prior to their DNA extraction.

1. Materials Needed:

- QIAamp® DNA Mini Kit (QIAGEN, Germany):
  - Buffer ATL

  - Proteinase K
  - Buffer AL
  - Buffer AW1
  - Buffer AW2
  - Buffer AE
  - QIAamp® Mini spin column
  - 2mL collection tube
- 95% Ethanol
- Sterile 1.5mL microcentrifuge tube

2. Procedure:

Total DNA extraction was performed according to manufacturer's instructions (115) with minor modifications that best suit the available equipment; thus, the following protocol was used:

- 1mL of bacterial suspension was added to a sterile 1.5mL microcentrifuge tube and centrifuged at 5,000 x *g* for 5 minutes.
- The supernatant was discarded and 170µL of Buffer ATL was added to the pellet.
- 20µL of Proteinase K were then added and the microcentrifuge tube was vortexed for 10 seconds then incubated in a heating block at 56˚C for 1 hour. The microcentrifuge tube was vortexed 3 times for a few seconds during the incubation period to dissolve the pellet.
- 200µL of Buffer AL were then added and the microcentrifuge tube was vortexed for 15 seconds and then incubated in a heating block at 70˚C for 10 minutes.

- 200µL of 95% ethanol were added and the microcentrifuge tube was vortexed for 15 seconds.
- The mixture was then added to the center of a QIAamp® Mini spin column with a 2mL collection tube without touching the rim and the spin column was centrifuged at 6,000 x *g* for 1 minute.
- The spin column was then transferred to a new 2mL collection tube and 500µL of Buffer AW1 (with 70% ethanol) were added to the center of the spin column without touching the rim and it was centrifuged at 6,000 x *g* for 1 minute.
- The spin column was then transferred to a new 2mL collection tube and 500µL of Buffer AW2 (with 70% ethanol) were added to the center of the spin column without touching the rim and it was centrifuged at 20,000 x *g* for 3 minutes.
- The spin column was then transferred to a new 2mL collection tube and centrifuged at 20,000 x *g* for 1 minute to get rid of residual Buffer AW2.
- The spin column was then transferred to a 1.5mL microcentrifuge tube and 200µL of Buffer AE were added to its center and it was incubated at room temperature for 5 minutes.
- Following incubation, the spin column with the microcentrifuge tube were centrifuged at 6,000 x *g* for 1 minute, then the spin column was discarded and the DNA extracts were left in the microcentrifuge tube with Buffer AE.

The microcentrifuge tube was then stored at –20˚C until needed.

D. Polymerase Chain Reaction (PCR):

PCR was performed to detect and amplify specific genes of carbapenem resistance in each isolate. For the *E. coli* and *K. pneumoniae* isolates, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ genes were tested. For the *A. baumannii* isolates, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24/40}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, and *bla*$_{\text{OXA-143-like}}$ genes were tested. The *Salmonella spp*. isolate was not screened by PCR since it was confirmed that it harbors *bla*$_{\text{KPC-2}}$.

1. Materials Needed:

- Forward and reverse primers (TIB Molbiol, Berlin, Germany)
- 10X TopTaq™ PCR Buffer (QIAGEN, Germany)
- TopTaq™ DNA Polymerase (QIAGEN, Germany)
- 2mM dNTP mixture (Thermo Scientific™, Waltham, MA, USA)
- Sterile, nuclease-free, water (AMRESCO®, Solon, OH, USA)
- Sterile 0.2mL PCR tubes
- C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA)

2. Primers Used:

Primers sequences for each gene along with their target sizes are listed below:

bla$_{\text{OXA-48}}$: (744bp)	Forward: 5'-TTGGTGGCATCGATTATCGG-3'	(6)
	Reverse: 5'-GAGCACTTCTTTTGTGATGGC-3'	
bla$_{\text{NDM-1}}$: (678bp)	Forward: 5'-GGAAACTGGCGACCAACG-3'	(6)
	Reverse: 5'-ATGCGGGCCGTATGAGTGA-3'	
bla$_{\text{OXA-23-like}}$: (501bp)	Forward: 5'-GATCGGATTGGAGAACCAGA-3'	(68)

	Reverse: 5’-ATTTCTGACCGCATTTCCAT-3’	
bla$_{\text{OXA-24/40}}$: (246bp)	Forward: 5’-GGTTAGTTGGCCCCCTTAAA-3’	(68)
	Reverse: 5’-AGTTGAGCGAAAAGGGGATT-3’	
bla$_{\text{OXA-51-like}}$: (353bp)	Forward: 5’-TAATGCTTTGATCGGCCTTG-3’	(68)
	Reverse: 5’-TGGATTGCACTTCATCTTGG-3’	
bla$_{\text{OXA-58}}$: (599bp)	Forward: 5’-AAGTATTGGGGCTTGTGCTG-3’	(68)
	Reverse: 5’-CCCCTCTGCGCTCTACATAC-3’	
bla$_{\text{OXA-143-like}}$: (149bp)	Forward: 5’-TGGCACTTTCAGCAGTTCCT-3′	(116)
	Reverse: 5’-TAATCTTGAGGGGGCCAACC-3′	

3. Reaction Composition:

The used PCR reaction compositions were based on those recommended for TopTaq™ DNA Polymerase (117) with minor modifications. Each PCR reaction mixture had a volume of 50µL and contained the following:

- 5µL of 10X TopTaq™ PCR Buffer containing 15mM $MgCl_2$ for a final PCR buffer concentration of 1X.
- 5µL of dNTP mixture containing 2mM of each dNTP for a final concentration of 0.2mM of each dNTP.
- 0.25µL of TopTaq™ DNA Polymerase for a final concentration of 1.25 units.
- 0.1µL of 100µM forward primer for a final concentration of 0.2µM.
- 0.1µL of 100µM reverse primer for a final concentration of 0.2µM.
- 34.55µL of nuclease-free sterile water.
- 5µL DNA sample.

4. Cycling Conditions:

Cycling conditions for *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24/40}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-143-like}}$, and *bla*$_{NDM-1}$ genes were generated by using the Protocol AutoWriter feature of the C1000™ Thermal Cycler, which takes into account primer sequences and target gene size when calculating annealing temperatures and setting cycling conditions. As for *bla*$_{\text{OXA-48}}$, pre-existing cycling settings were followed (118). Thus, the conditions used are listed below:

- For *bla*$_{\text{OXA-23-like}}$ genes: Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 55.7°C for 30 seconds, and elongation at 72°C for 36 seconds, then a final extension at 72°C for 5 minutes.
- For *bla*$_{\text{OXA-24/40}}$: Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 55.9°C for 30 seconds, and elongation at 72°C for 30 seconds, then a final extension at 72°C for 5 minutes.
- For *bla*$_{\text{OXA-51-like}}$ genes: Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 56.8°C for 30 seconds, and elongation at 72°C for 30 seconds, then a final extension at 72°C for 5 minutes.
- For *bla*$_{\text{OXA-58}}$: Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 56.4°C for 30 seconds, and elongation at 72°C for 36 seconds, then a final extension at 72°C for 5 minutes.

- For *bla*$_{\text{OXA-143-like}}$ genes: Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 58.2°C for 30 seconds, and elongation at 72°C for 30 seconds, then a final extension at 72°C for 5 minutes.
- For *bla*$_{\text{NDM-1}}$: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 61°C for 35 seconds, and elongation at 72°C for 1 minute, then a final extension at 72°C for 10 minutes.
- For *bla*$_{\text{OXA-48}}$: Initial denaturation at 94ºC for 7 minutes, followed by 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 56ºC for 45 seconds, and elongation at 72ºC for 1 minute, then a final extension at 72ºC for 10 minutes.

DNA concentrations and purity were measured using the NanoDrop™ (Thermo Scientific™, Waltham, MA, USA).

All PCR products were then stored at 4ºC until later use.

E. Gel Electrophoresis:

Gel electrophoresis was used to detect the PCR amplification product of each target gene. Visualizing the resulting gel was accomplished by using the Gel Doc™ XR+ System (Bio-Rad, Hercules, CA, USA), and a digital copy of its image was saved using Quantity One® 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

1. Materials Needed:

- 1X Tris-Borate-EDTA (TBE) buffer (AMRESCO®, Solon, OH, USA)
- Agarose-Molecular Biology Grade (GeneDireX®, Taiwan)

- 0.625mg/ml ethidium bromide (AMRESCO®, Solon, OH, USA)
- 100bp DNA Ladder Ready-to-Load (Solis BioDyne, Tartu, Estonia)
- GeneRuler™ 50bp DNA ladder (Thermo Scientific™, Waltham, MA, USA)
- 6X DNA Loading Dye (Thermo Scientific™, Waltham, MA, USA)

2. Procedure:

- 10X TBE buffer was prepared by dissolving 108g of Tris base, 57g of boric acid, and 9.3g of EDTA disodium in 1L of distilled water, then autoclaving the resulting solution.
- 1X TBE buffer was then prepared by diluting the 10X TBE buffer 10 times in sterile distilled water for further usage in the experiment.
- 1.5% agarose gel was prepared by dissolving 1.5g of agarose powder for every 100mL of 1X TBE buffer needed and then heating the mixture for a few minutes using a microwave to allow the agarose powder to completely dissolve.
- 2 drops of 0.625 mg/ml ethidium bromide were then added to the liquid gel and it was poured into the electrophoresis casting tray that contained a comb to create wells. The liquid was left for 30 minutes to solidify.
- Afterwards, the casting tray was placed in the electrophoresis chamber and 2.5L of 1X TBE buffer were poured over it to allow for current conduction.
- Loading the samples into the wells of the gel was performed by adding 2µL of loading dye to 10µL PCR product and then pipetting the 12µL mixture into the well. A 50bp ladder size marker was run in parallel with the samples targeting *bla*$_{\text{OXA-24/40}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, and *bla*$_{\text{OXA-143-like}}$ genes, whereas a 100bp

ladder size marker was run in parallel with the samples targeting *bla*$_{\text{OXA-23-like}}$ genes, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{NDM-1}}$.

- After loading the wells, an electric current with a voltage of 120V was allowed to pass through the gel, and the run was set to 1 hour for samples with the 100bp ladder, and 2 hours for samples with the 50bp ladder.
- The resulting gel was then visualized and a digital record of it was saved.

F. Broth Microdilution Antimicrobial Susceptibility Testing:

Broth microdilution antimicrobial susceptibility testing was performed to determine the minimal inhibitory concentrations of imipenem (IPM), ertapenem (ETP), and meropenem (MEM) when the selected β-lactamase inhibitors are added to each one of them. The first β-lactamase inhibitor, avibactam (AVI), was added to each of the 3 carbapenems and tested against isolates harboring *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24/40}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, and *bla*$_{\text{OXA-143-like}}$ genes. The second β-lactamase inhibitor, edetate calcium disodium (Ca-EDTA), was added to each of the 3 carbapenems and tested against isolates harboring *bla*$_{\text{NDM-1}}$. The third β-lactamase inhibitor, relebactam (REL), was added to each of the 3 carbapenems and tested against the isolate that harbors *bla*$_{\text{KPC-2}}$.

The procedure followed in performing this assay adhered to CLSI guidelines (119); however, minor modifications to broth volumes were made in order to accommodate for the presence of the β-lactamase inhibitors; which were not accounted for in the procedure described by the CLSI guidelines. As such, adjustments were made to keep the concentrations of all constituents consistent with CLSI recommendations.

Testing of the isolates for the new MICs was done in duplicates and *Escherichia coli* (ATCC® 35218™) was used as a quality control strain since it is suggested by the CLSI for β-lactam/β-lactamase inhibitor combination testing (120, 121).

1. Materials Needed:

- 96-well polystyrene microtiter plates (Costar®, Corning Inc, NY, USA)
- Cation-Adjusted Mueller-Hinton Broth (BBL™, BD, Franklin Lakes, NJ, USA)
- Imipenem/Cilastatin (Tienam®, Merck & Co. Inc, Whitehouse Station, NJ, USA)
- Ertapenem (Invanz®, Merck & Co. Inc, Whitehouse Station, NJ, USA)
- Meropenem (Meronem®, AstraZeneca, Wilmington, DE, USA)
- Avibactam (MedChem Express, Monmouth Junction, NJ, USA)
- Relebactam (MedChem Express, Monmouth Junction, NJ, USA)
- Ethylenediaminetetraacetic acid calcium disodium salt (Ca-EDTA) (Sigma®, St. Louis, MO, USA)

2. Procedure:

- Powders of each of the 3 carbapenems (IPM/CS, ERT, and MEM), avibactam (AVI), relebactam (REL), and Ca-EDTA were dissolved in sterile distilled water to achieve stock solution concentrations of 160µg/mL, 160µg/mL, 160µg/mL, and 1280µg/mL, respectively. Aliquots were then made and stored at –80°C for further usage.

- Colonies of each tested isolate were taken from a MacConkey plate that was cultured overnight at 37°C, and a 0.5 McFarland turbidity standard was prepared to attain a bacterial concentration of 1 to 2 × 10^8 CFU/mL.
- Cation-adjusted Mueller-Hinton broth (CA-MHB) was added to all wells of the 96-well microtiter plate and an equal volume of each antimicrobial agent was added from the aliquot stock solutions to wells 1 and 12 (negative control) of each row.
- Two-fold serial dilutions were then made between wells 1 and 10 of each row. For the *Enterobacteriaceae* isolates, the highest concentration of each antimicrobial agent was 16µg/mL, and the lowest concentration was 0.03125µg/mL, at a 1:512 dilution. For the *A. baumannii* isolates, the highest concentration of each antimicrobial agent was 32µg/mL, and the lowest concentration was 0.0625µg/mL, also at a 1:512 dilution.
- The corresponding β-lactamase inhibitor was then added to wells 1 till 10 and well 12 (negative control) of each row so that each well contains a fixed concentration of the corresponding β-lactamase inhibitor: Avibactam and Relebactam at 4µg/mL (95, 122-124), while Ca-EDTA at 32µg/mL (101, 105).
- Within 15 minutes of preparing the 0.5 McFarland turbidity standard, the bacterial suspension was diluted 1:200 in CA-MHB before being added to wells 1 till 11 (positive control) of each row so that the final concentration of bacteria in those wells was 5 × 10^5 CFU/mL (± 3 × 10^5).
- Plates were then covered, to prevent broth evaporation, and incubated at 37°C for 16-20 hours.

- Each run was accompanied by testing *Escherichia coli* (ATCC® 35218™) quality control strain under the same conditions as the tested isolate.

Overall, each microtiter plate tested a single isolate against 3 antimicrobial agents combined with the appropriate β-lactamase inhibitor that corresponds to the gene of resistance that the tested isolate harbors.

Interpreting the results of this assay to determine the MIC was done visually, where the well with the lowest antimicrobial agent concentration that does not show growth counted as the MIC. However, to eliminate human error, the results were also read on a plate reader at a wavelength of 630nm.

In addition, each isolate was further tested against the β-lactamase inhibitor alone. To do so, each bacterial isolate was incubated at a concentration of 5×10^5 CFU/mL ($\pm 3 \times 10^5$) in CA-MHB containing the appropriate β-lactamase inhibitor at concentrations of 4µg/mL for avibactam or relebactam, and 32µg/mL for Ca-EDTA. A positive control, containing bacteria only, and a negative control, containing reagents only, were also set up with each tested isolate.

G. Determination of LD_{50} in BALB/c Mice:

The median lethal dose of IMP57 was determined. Animals used in this experiment were purchased from the Animal Care Facility (ACF) at the American University of Beirut (AUB) and their use was approved by the Institutional Animal Care and Use Committee (IACUC) at AUB.

The procedure followed to determine the LD_{50} of the selected isolate was based on an earlier protocol (125), but with an extended monitoring period.

1. Mice Characteristics:

In this experiment, 20 BALB/c male mice were used. They were all 7-8 weeks old and weighed 40-45g each.

2. Materials Needed:

- BALB/c mice
- Sterile 1mL syringes with needles (BD™, Franklin Lakes, NJ, USA)
- Trypticase™ Soy Broth (TSB) (BBL™, BD, Franklin Lakes, NJ, USA)

3. Procedure:

All animals were handled and cared for as instructed by the ACF and were allowed to consume food and water ad libitum.

The mice were divided into 5 groups containing 4 mice each. Then, 5 concentrations of bacterial doses were prepared: 10^8, 10^7, 10^6, 10^5, and 10^4 CFU, and each mouse was intraperitonially injected with 0.2mL of the bacterial dose that its group is assigned to. The 10^8 CFU concentration was prepared by culturing the selected bacterial isolate in TSB overnight at 37ºC and then adjusting its turbidity to 0.5 McFarland by adding fresh TSB the next day. Once the turbidity was adjusted, 6mL of the suspension were withdrawn and centrifuged at 3500 rpm for 20 minutes. The resulting supernatant was discarded and the pellet was resuspended in 1.2mL of fresh TSB, then divided to fill up two syringes. The 1.2mL (6 mice × 0.2mL/mouse) added is relevant to the number of mice to be injected in the group, with 2 extra injections as backup, so that each mouse is

administered 10^8 CFU per 0.2mL. Preparations of the subsequent concentrations were done similarly, but were first diluted in TSB to the desired concentrations from the 10^8 CFU suspension.

Following infection, the mice were monitored for survival over 7 days and the average weight of each group was recorded daily.

At the end of the monitoring period, the LD_{50} of the selected isolate was calculated based on the Spearman-Karber method, using the formula (125) stated below:

$$\log(LD_{50}) = \log(LD_{100}) + [\log(D) \times \left(\frac{1}{2} - \frac{\sum R}{N}\right)]$$

in which LD_{100} = The highest dose tested that killed all test subjects injected with it.

D = The fold difference between consecutive doses.

$\sum R$ = The total number of dead test subjects.

N = The number of test subjects per group.

H. Survival Experimentation with BALB/c Mice:

BALB/c mice were infected with IMP57 and an attempt to treat those mice using meropenem (MEM) with avibactam (AVI) was made.

1. Mice Characteristics:

In this experiment, 30 BALB/c male mice were used. They were all 7-8 weeks old and weighed 40-45g each.

2. Materials Needed:

- BALB/c mice
- Sterile 1mL syringes with needles (BD™, Franklin Lakes, NJ, USA)
- Trypticase™ Soy Broth (TSB) (BBL™, BD, Franklin Lakes, NJ, USA)
- Meropenem (Meronem®, AstraZeneca, Wilmington, DE, USA)
- Avibactam (MedChem Express, Monmouth Junction, NJ, USA)

3. Experimental Scheme:

The mice were divided into five groups of six each. The experimental setup is outlined in Table 2.

4. Procedure:

- All animals were handled and cared for as instructed by the ACF and were allowed to consume food and water ad libitum.
- The bacterial dose injected into the tested mice was $3\times LD_{50}$ of the selected carbapenem-resistant isolate.
- To determine the meropenem (MEM) dose needed for each injection, the following formula (126) was used to extrapolate the dose from its *in vitro* MIC obtained through the broth microdilution antimicrobial susceptibility testing assay:

$$\text{Meropenem } (\mu g) = \frac{\text{MIC of MEM with AVI on tested isolate } \left(\frac{\mu g}{mL}\right) \times \text{Broth volume used in MIC testing (mL)} \times \text{Bacterial dose injected } \textit{in vivo} \text{ (CFU)}}{\text{Bacterial concentration used in MIC testing (CFU)}}$$

- The used concentration of the β-lactamase inhibitor avibactam (AVI) was at a 4:1 ratio with the antimicrobial agent as it has previously shown to be efficacious (93, 94).
- All injections were performed intraperitonially and followed the framework outlined in Table 2, with each mouse receiving the treatment course once, daily.
- Mice in each group were monitored for 7 days for survival, weight change, physical appearance, and physical activity.
- Mice that expired prior to the end of the monitoring period had their blood cultured and API® 20E (bioMérieux, Marcy l'Etoile, France) performed on the colonies isolated to verify if the cause of death were the administered agent.

I. Reverse Transcription Real-Time Polymerase Chain Reaction (RT-qPCR):

The molecular aspect of the *in vitro* and *in vivo* experiments with IMP57 was tackled via RT-qPCR.

1. in vitro Aspect:

The same bacterial isolate that was moved to animal experimentation had its bacterial suspension collected from the broth microdilution susceptibility assay at the minimal inhibitory concentrations of MEM only & MEM+AVI. In addition, a bacterial suspension without adding any antimicrobial agents was also collected to act as a positive control. RNA extraction was then performed on these samples.

2. in vivo Aspect:

10 BALB/c mice with the same physical characteristics as those used in the survival study were divided into five groups of two and intraperitonially administered with the same bacterial isolate before receiving treatment an hour later as outlined in Table 2. The mice were then sacrificed the next day and blood was collected from groups 1, 2, and 3 via cardiac puncture. The blood was centrifuged at 1500 rpm for 30 minutes and the separated serum was retrieved for RNA extraction. In this case, group 1 acted as positive control with IMP57 only, group 2 contained IMP57 treated with MEM only, and group 3 contained IMP57 treated with MEM and AVI.

3. RNA Extraction:

RNA extraction was performed on both *in vitro* and *in vivo* samples.

a. <u>Materials Needed:</u>

- illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare UK Limited, Buckinghamshire, UK):
  - Lysis Solution (RA1)
  - Desalting Buffer (MDB)
  - DNase I
  - Wash Buffer I (RA2)
  - Wash Buffer II (RA3)
  - RNase-free H_2O
  - RNAspin Mini Filters
  - RNAspin Mini Columns

  - RNAspin Mini Collection Tubes
- TE Buffer (AMRESCO®, Solon, OH, USA)
- Lysozyme from chicken egg white (Sigma®, St. Louis, MO, USA)
- β-Mercaptoethanol (β-ME)
- 70% Ethanol
- RiboLock RNase Inhibitor (Thermo Scientific™, Waltham, MA, USA)
- Sterile 1.5mL microcentrifuge tubes

b. Procedure:

RNA extraction was performed according to manufacturer's instructions (127) and was as follows:

- 1.5mL of culture broth or 500µL of serum were added to a 1.5mL microcentrifuge tube and centrifuged at maximum speed for 15 minutes.
- The supernatant was removed and 100µL of TE buffer containing 0.2mg/mL of lysozyme were added to the pellet and vortexed before incubating the microcentrifuge tube at 37°C for 10 minutes.
- 350µL of Lysis solution (RA1) and 3.5µL of β-ME were then added and the microcentrifuge tubes were vortexed for a few seconds.
- The RNAspin Mini Filter (purple colored) was placed in a collection tube and the lysate was added to the filter then centrifuged at 11,000 x *g* for 1 minute.
- The filter was discorded and the lysate transferred into a 1.5mL microcentrifuge tube without disturbing the pellet.

- 350µL of 70% ethanol were then added and the microcentrifuge tubes were vortexed for 5 seconds.
- The lysate was mixed with pipette (up and down) then transferred to an RNAspin Mini Column with collection tube (blue colored) and centrifuged at 8,000 x *g* for 30 seconds.
- The spin column was transferred to new collection tube and 350µL of Desalting Buffer were added.
- The spin columns were centrifuged at 11,000 x *g* for 1 minute then the filtrate was discarded and the spin columns were returned to the same collection tube.
- 95µL of DNase I in TE buffer were added to the center of the spin column and it was incubated at room temperature for 15 minutes.
- 200µL of Wash Buffer I (RA2) was then added to the spin column and it was centrifuged at 11,000 x *g* for 1 minute.
- The spin columns were then transferred to a new collection tube and 600µL of Wash Buffer II (RA3) were added before centrifuging the spin columns at 11,000 x *g* for 1 minute.
- The filtrate was discarded and the spin column was placed back into the collection tube before adding to it 250µL of Wash Buffer II (RA3) and centrifuging it at 11,000 x *g* for 2 minutes.
- The spin columns were then transferred to a 1.5mL microcentrifuge tube and 100µL of RNase-free water were added before centrifuging them at 11,000 x *g* for 1 minute.

- The spin column was discarded and 1µL of RiboLock RNase Inhibitor was added to the extracted RNA that was immediately placed on ice.

Extracted RNA concentrations and purity were measured using the DS-11 FX Spectrophotometer (DeNovix®, Wilmington, DE, USA).

All RNA extracts were then placed in the freezer at –80°C.

4. cDNA Synthesis:

Following RNA extraction, each sample had to be converted to complementary DNA (cDNA) for usage in further real-time PCR studies.

a. Materials Needed:

- iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA)
  - 5x iScript™ Reaction Mix
  - iScript™ Reverse Transcriptase
  - Nuclease-free water
- Sterile 0.5mL PCR tubes

b. Reaction Composition:

The reaction composition was based on the recommendations by the kit manufacturer (128) and it contained 20µL of:

- 4µL of 5x iScript™ Reaction Mix
- 1µL of iScript™ Reverse Trasncriptase
- 5µL of nuclease-free water
- 10µL of RNA template

cDNA concentrations and purity were measured using the DS-11 FX Spectrophotometer (DeNovix®, Wilmington, DE, USA).

All cDNA synthesis products were then stored at –20°C until later use.

5. Real-Time Polymerase Chain Reaction (qPCR):

Real-time PCR permits the quantification of *bla*$_{\text{OXA-48}}$ (target gene) and *rpoB* (housekeeping gene) expression levels in all cDNA synthesis products.

a. Materials Needed:

- iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA)
- Forward and reverse primers (TIB Molbiol, Berlin, Germany)
- Sterile, nuclease-free, water (AMRESCO®, Solon, OH, USA)
- 0.2mL 8-Tube PCR Strips (Bio-Rad, Hercules, CA, USA)

b. Primers Used:

Primer sequences for each gene along with their target sizes are listed below:

bla$_{\text{OXA-48}}$: (240bp)	Forward: 5'-TTCGGCCACGGAGCAAATCAG-3'	(6)
	Reverse: 5'-GATGTGGGCATATCCATATTCATCGCA-3'	
rpoB: (184bp)	Forward: 5'-TCGAAACGCCTGAAGGTC-3'	(6)
	Reverse: 5'-TTGGAGTTCGCCTGAGC-3'	

c. Reaction Composition:

The used qPCR reaction compositions were based on those recommended for iTaq™ Universal SYBR® Green Supermix (129) with minor in-house modifications for

optimal performance. Each qPCR reaction mixture was run in duplicates and had a volume of 10µL that consisted of:

- 5µL of iTaq™ Universal SYBR® Green Supermix
- 0.25µL of 20µM forward primer for a final concentration of 0.5µM
- 0.25µL of 20µM reverse primer for a final concentration of 0.5µM
- 3.5µL of nuclease-free water
- 1µL of cDNA template with a final concentration of 400ng/reaction

d. Cycling Conditions:

The used qPCR cycling conditions were based on those recommended for iTaq™ Universal SYBR® Green Supermix (129) with minor modifications. As such, they were as follows: Polymerase activation and initial DNA denaturation at 95°C for 30 seconds, followed by 45 cycles of DNA denaturation at 95°C for 5 seconds, annealing at 55°C for 30 seconds, and extension at 60°C for 30 seconds, then melt curve analysis from 65-95°C with 0.5°C increments at 5 seconds/step.

6. Statistical Analysis:

The relative gene expression of *bla*$_{\text{OXA-48}}$ was analyzed using the $2^{-\Delta\Delta CT}$ (Livak) method (130). In addition, the unpaired student's t-test was used for statistical analysis, in which p-values ≤ 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Determining Carbapenem Resistance:

Etest® results showed that all fourteen isolates included in this study were resistant to imipenem, ertapenem, and meropenem when their MICs were compared to the antimicrobial agents' MIC breakpoints set by the CLSI (120, 121). The clinical isolates provided by the Department of Pathology and Laboratory Medicine at AUBMC, comprising *A. baumannii*, *E. coli*, and *K. pneumoniae*, had an MIC >32µg/mL against the 3 tested carbapenems. On the other hand, the *Salmonella spp*. isolate, supplied by the Centers for Disease Control and Prevention, had MICs of 32µg/mL, 16µg/mL, and 6µg/mL for ertapenem, imipenem, and meropenem, respectively.

B. Detecting Carbapenem-Resistance Genes:

Following gel electrophoresis of the PCR amplicons, it was determined that the tested isolates harbored most of the genes of carbapenem resistance they were screened for. Consequently, $bla_{\text{OXA-48}}$ was present in IMP53, IMP57, IMP197, IMP215, and IMP223, while $bla_{\text{NDM-1}}$ was present in IMP216 and IMP217. As for the *A. baumannii* isolates, $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ genes were detected in all isolates while $bla_{\text{OXA-24/40}}$ was not detected in any of them. On the other hand, $bla_{\text{OXA-58}}$ was additionally detected in ACN2090 and ACN3630, and $bla_{\text{OXA-143-like}}$ was detected in ACN2285 and ACN3630. The results are organized in Table 3 and the digital records of all gel electrophoresis results are displayed in Figure 1 (a-g).

C. Broth Microdilution Antimicrobial Susceptibility Testing:

Considering that the CLSI does not currently have published MIC breakpoints for carbapenems in combination with any of the β-lactamase inhibitors used in this study, the MIC breakpoints of the antimicrobial agents alone were used to interpret the susceptibility testing results except for the quality control strain *Escherichia coli* (ATCC® 35218™) since the CLSI has published MIC ranges for ceftazidime-avibactam and imipenem-relebactam for it (131). As such, those MIC ranges were respectively used to assess the broth microdilution assay of *Escherichia coli* (ATCC® 35218™) when any of the carbapenems was tested in combination with either avibactam or relebactam.

Among the 5 isolates that harbor *bla*$_{\text{OXA-48}}$, the addition of avibactam to either meropenem or imipenem restored the susceptibility of the tested isolates to both antimicrobial agents with MICs ≤1µg/mL. On the other hand, the addition of avibactam to ertapenem only restored susceptibility in 1 isolate, IMP223 with an MIC of 0.25µg/mL, but managed to lower the MIC in the remaining isolates from >32µg/mL to 2µg/mL in IMP53, 8µg/mL in IMP215, and 16µg/mL in IMP57 and IMP197.

Among the 2 isolates that harbor *bla*$_{\text{NDM-1}}$, the addition of Ca-EDTA to imipenem, ertapenem, and meropenem restored the susceptibility of both isolates to imipenem and meropenem, with MICs ≤1µg/mL, while only restoring the susceptibility of IMP217 to ertapenem, with an MIC of 1µg/mL.

Among the 6 *A. baumannii* isolates that harbor OXA-type carbapenemases, mainly *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-51-like}}$ genes, the addition of avibactam to the 3 carbapenems did not managed to restore susceptibility to any of the isolates; however, it did lower the

MIC of imipenem and meropenem to 8µg/mL for ACN2273 and ACN3630, while the rest of the isolates remained resistant with MICs >32µg/mL.

As for the *Salmonella spp*. isolate that harbors bla_{KPC-2}, the addition of relebactam to the 3 carbapenems only restored the isolate's susceptibility to ertapenem and meropenem with an MIC of 0.0625µg/mL for either of the two antimicrobial agents.

The susceptibility results for each isolate are displayed in Table 4 and Figure 2 (a-d). None of the inhibitors used in this study could inhibit the growth of bacteria when added solely to the culture.

D. Determination of LD_{50} in BALB/c Mice:

Following the infection of 20 BALB/c mice with decreasing concentrations of IMP57 and monitoring the mice's survival over a period of 7 days, it was observed that a total of 5 mice expired on the first day, corresponding to 3 mice from the group infected with 10^8 CFU and 2 mice from the group infected with 10^7 CFU, while the remaining 15 mice survived the entire monitoring period. As such, not all mice died from the group receiving the highest dose; therefore, according to the followed protocol (125), the next highest bacterial concentration of 10^9 CFU had to be considered as the LD_{100}. Consequently, the LD_{50} calculated was 1.78×10^8 CFU.

E. Survival Experimentation with BALB/c Mice:

After determining that the $3\times LD_{50}$ concentration of the bacterial isolate IMP57 was 5.34×10^8 CFU, the *in vivo* experimental study commenced as described earlier. The following observations were made by the end of the monitoring period:

- Figure 3a displays the survival rates in a line graph and Figure 3b displays the same rates as a Kaplan-Meier plot; indicating that out of the 30 mice, a total of 17 mice died, leaving 13 survivors by the end of the experiment.
- By the second day, 83.3% of the mice in Group 1 (positive control) and 100% of the mice in Groups 2 (MEM only) and 4 (AVI only) did not manage to survive.
- Throughout the entire monitoring period, one mouse from Group 1 and all the mice in Groups 3 (MEM+AVI) and 5 (negative control) managed to survive.
- Concerning the monitored weight changes, Figure 4 shows that as time progressed, the average weight of the mice that survived in Groups 1, 3, and 5 slightly fluctuated around the initial value without a marked change. It is important to note that starting with day 2, the recorded weight value for Group 1 is that of one mouse only, as it was the only one that survived.
- Concerning physical activity and behavioral changes, it was observed that following the infection with bacteria, the mice in groups 1, 2, 3, and 4 became very docile and less inquisitive; however, on the second day, the mice that survived showed improvement in physical activity and had resumed their inquisitive behavior. Those parameters kept on showing improvement for the mice in groups 1, 2, 3, and 4 as time progressed. Mice in group 5 neither had altered activity nor behavior throughout the entire experiment.

F. Reverse Transcription Real-Time Polymerase Chain Reaction:

Quantifying the *in vitro* levels of $bla_{\text{OXA-48}}$ expression in IMP57 following the addition of meropenem only and meropenem with avibactam when compared to the

positive control indicated a 6-fold increase (p=0.0024) in *bla*$_{\text{OXA-48}}$ expression when meropenem was added, and a 10-fold increase (p=0.00072) in expression when meropenem and avibactam were added. Moreover, there was a statistically significant difference (p=0.028) in expression levels when comparing meropenem to meropenem with avibactam. Table 5a displays the *in vitro* results.

Quantifying the *in vivo* levels of *bla*$_{\text{OXA-48}}$ expression in IMP57 following the treatment with meropenem only and meropenem with avibactam when compared to the positive control indicated a 3-fold increase (p=0.046) in *bla*$_{\text{OXA-48}}$ expression when meropenem was administered, and a 4-fold increase (p=0.024) in expression when meropenem and avibactam were administered. However, there difference in *bla*$_{\text{OXA-48}}$ expression when comparing meropenem to meropenem with avibactam was not statistically significant (p=0.091). Table 5b displays the *in vivo* results.

Table 1: Labels of each isolate included in the study.

Enterobacteriaceae			*Acinetobacter spp.*
E. coli (2 isolates)	*K. pneumoniae* (5 isolates)	*Salmonella spp.* (1 isolate)	*A. baumannii* (6 isolates)
IMP 53	IMP 197	KPC	ACN 2090
IMP 57	IMP 215		ACN 2209
	IMP 216		ACN 2273
	IMP 217		ACN 2285
	IMP 223		ACN 2493
			ACN 3630

Table 2: Mice groups and injections used in survival experimentation.

Days		Group 1 (P.C)	Group 2	Group 3	Group 4	Group 5 (N.C)
Day 1	t = 0hr	Bacterial injection	Bacterial injection	Bacterial injection	Bacterial injection	TSB
	t = 1hr	-	Administer Meropenem	Administer Meropenem + Avibactam	Administer Avibactam	-
Day 2						
Day 3						
Day 4						
Day 5						
Day 6						
Day 7						

Table 3: Detected genes of carbapenem resistance among all tested isolates.

Bacterial Species	Isolate ID	Genes of Resistance
Escherichia coli	IMP53	*bla*$_{\text{OXA-48}}$
	IMP57	
Klebsiella pneumoniae	IMP197	
	IMP215	
	IMP223	
	IMP216	*bla*$_{\text{NDM-1}}$
	IMP217	
Acinetobacter baumannii	ACN2090	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$
	ACN2209	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$
	ACN2273	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$
	ACN2285	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-143-like}}$
	ACN2493	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$
	ACN3630	*bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-143-like}}$

Table 4: MIC of the carbapenems with and without inhibitor against the tested isolates. The inhibitor for *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-23-like}}$, and *bla*$_{\text{OXA-51-like}}$ is avibactam. The inhibitor for *bla*$_{\text{NDM-1}}$ is Ca-EDTA. The inhibitor for *bla*$_{\text{KPC-2}}$ is relebactam.

Gene of Resistance	Isolate ID	MIC (µg/mL)					
		IPM	IPM + Inhibitor	MEM	MEM + Inhibitor	ERT	ERT + Inhibitor
bla$_{\text{OXA-48}}$	IMP 53	>32	0.25	>32	0.03125	>32	2
	IMP 57	>32	1	>32	0.25	>32	16
	IMP 197	>32	1	>32	1	>32	16
	IMP 215	>32	0.5	>32	0.125	>32	8
	IMP 223	>32	0.25	>32	0.03125	>32	0.25
bla$_{\text{NDM-1}}$	IMP 216	>32	0.25	>32	0.0625	>32	2
	IMP 217	>32	0.5	>32	0.03125	>32	1
bla$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-51-like}}$	ACN 2090	>32	>32	>32	>32	>32	>32
	ACN 2209	>32	>32	>32	>32	>32	>32
	ACN 2273	>32	8	>32	8	>32	>32
	ACN 2285	>32	>32	>32	>32	>32	>32
	ACN 2493	>32	>32	>32	>32	>32	>32
	ACN 3630	>32	8	>32	8	>32	>32
bla$_{\text{KPC-2}}$	KPC	16	4	6	0.0625	32	0.0625

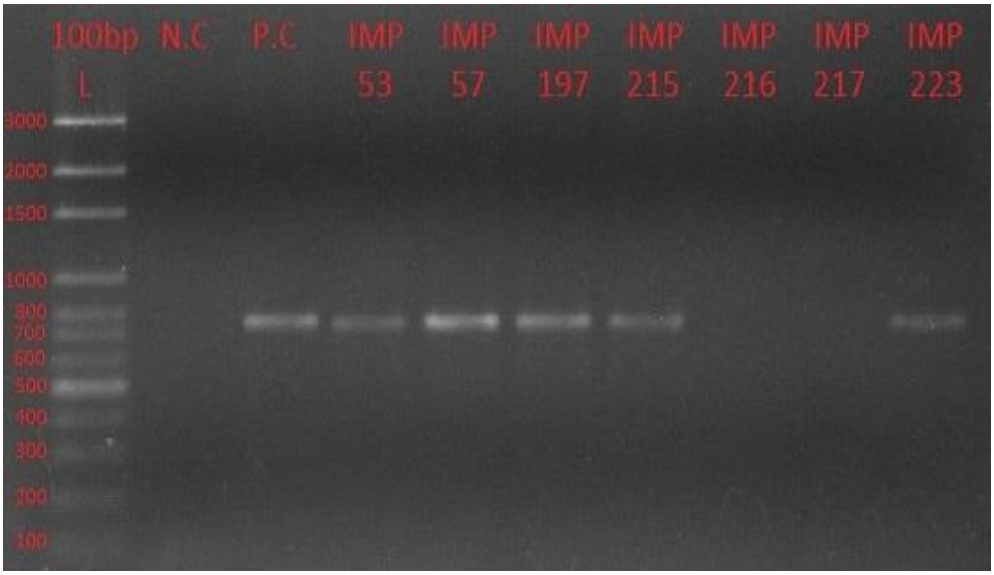

Figure 1.a: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{OXA-48}}$ showing amplification in IMP53, IMP57, IMP197, IMP215, and IMP223

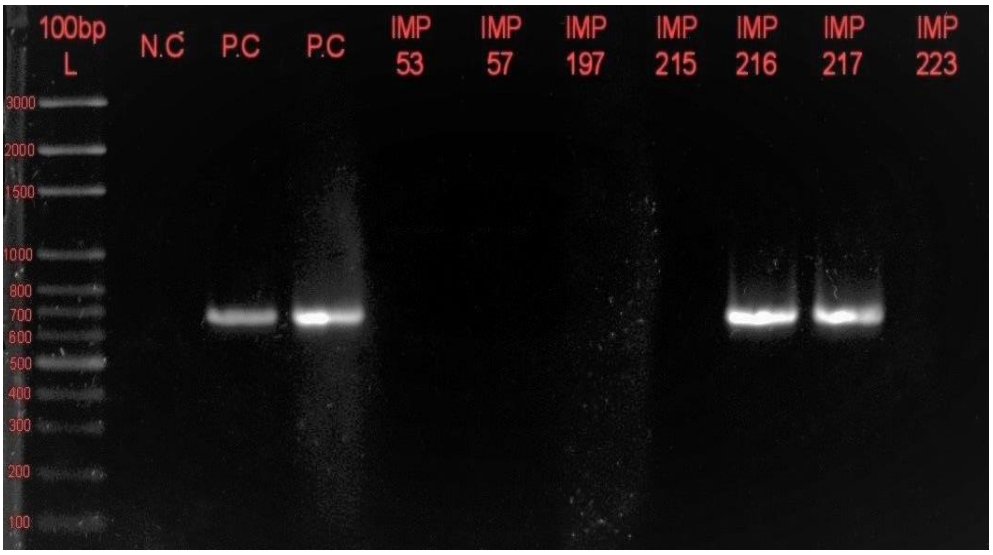

Figure 1.b: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{NDM-1}}$ showing amplification in IMP216 and IMP217.

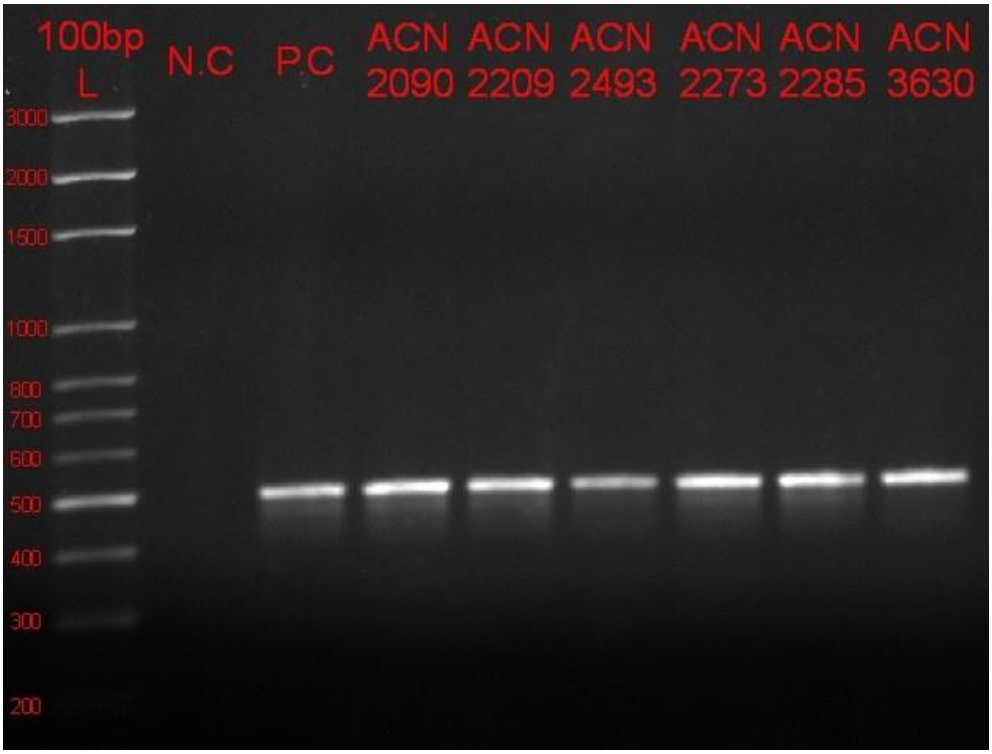

Figure 1.c: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{OXA-23-like}}$ showing amplification in all *A. baumannii* isolates.

Figure 1.d: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{OXA-24/40}}$ does not show amplification in any of the *A. baumannii* isolates.

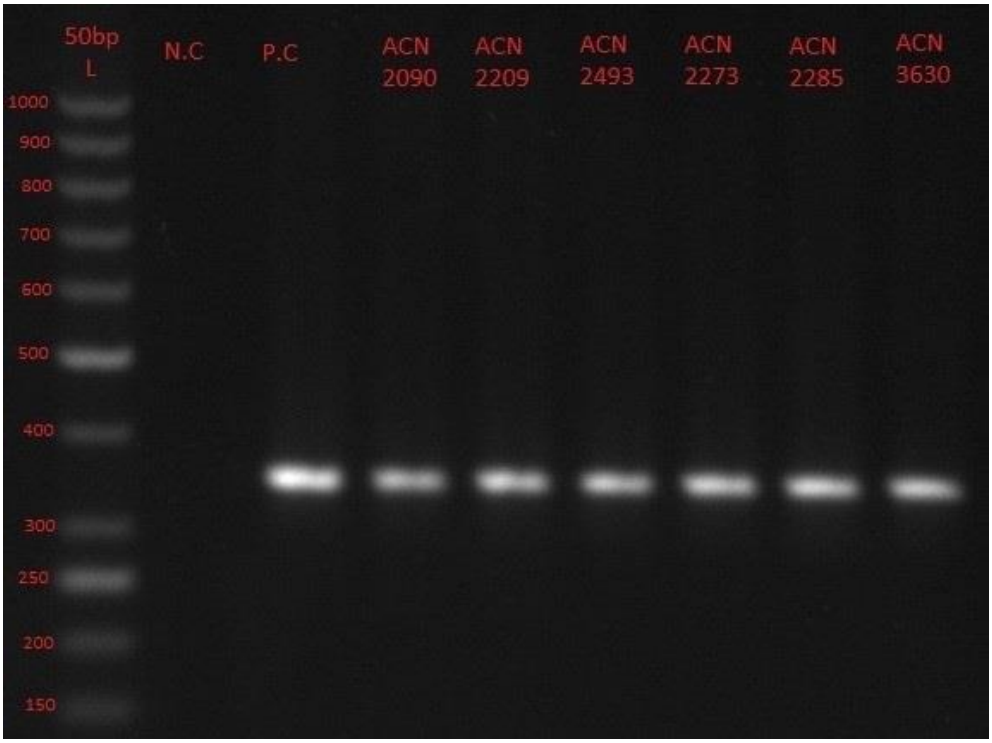

Figure 1.e: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{OXA-51-like}}$ genes showing amplification in all *A. baumannii* isolates.

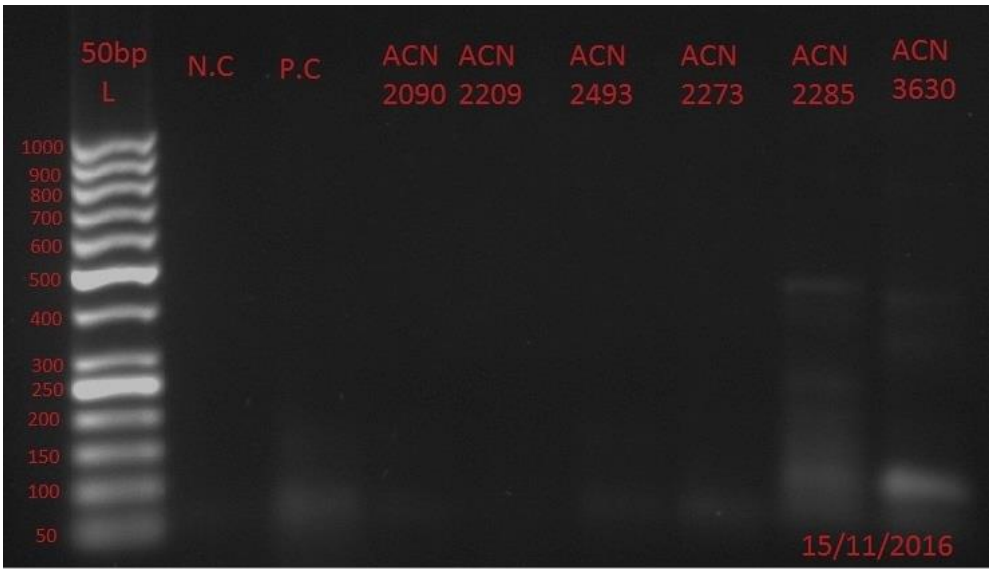

Figure 1.f: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{OXA-143-like}}$ genes showing amplification in ACN2285 and ACN3630.

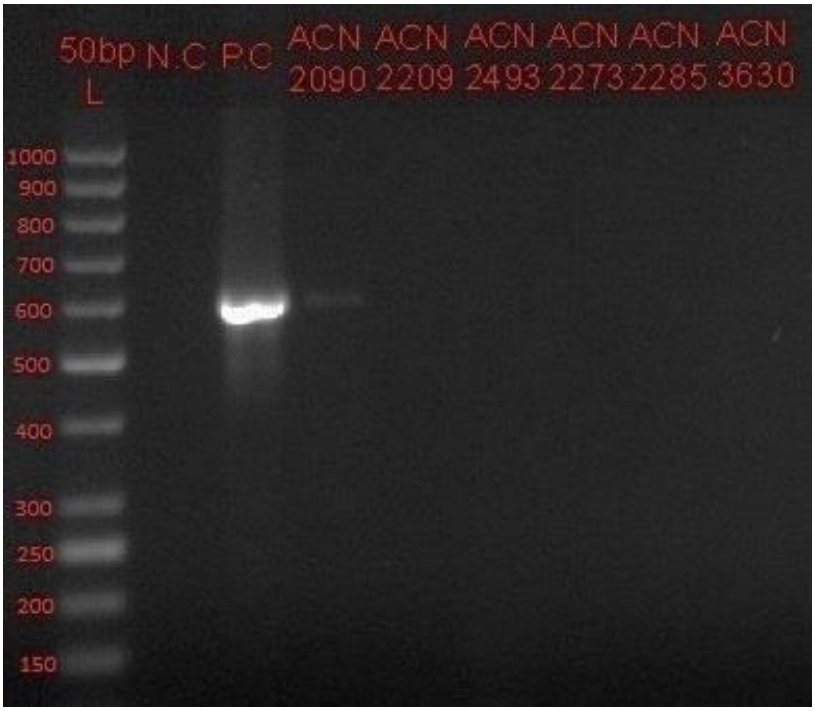

Figure 1.g: Gel electrophoresis of the PCR amplicons for the detection of *bla*$_{\text{OXA-58}}$ showing amplification in ACN2090 and ACN3630 with faint bands.

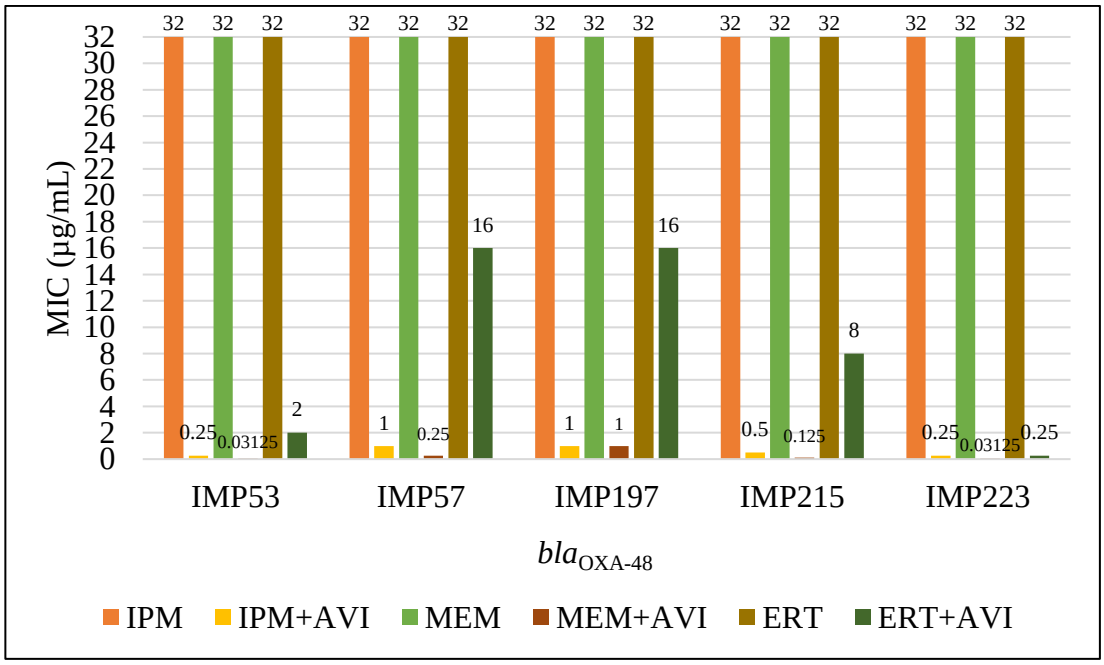

Figure 2a: MIC of carbapenems with and without avibactam against *Enterobacteriaceae* isolates that harbor *bla*$_{\text{OXA-48}}$

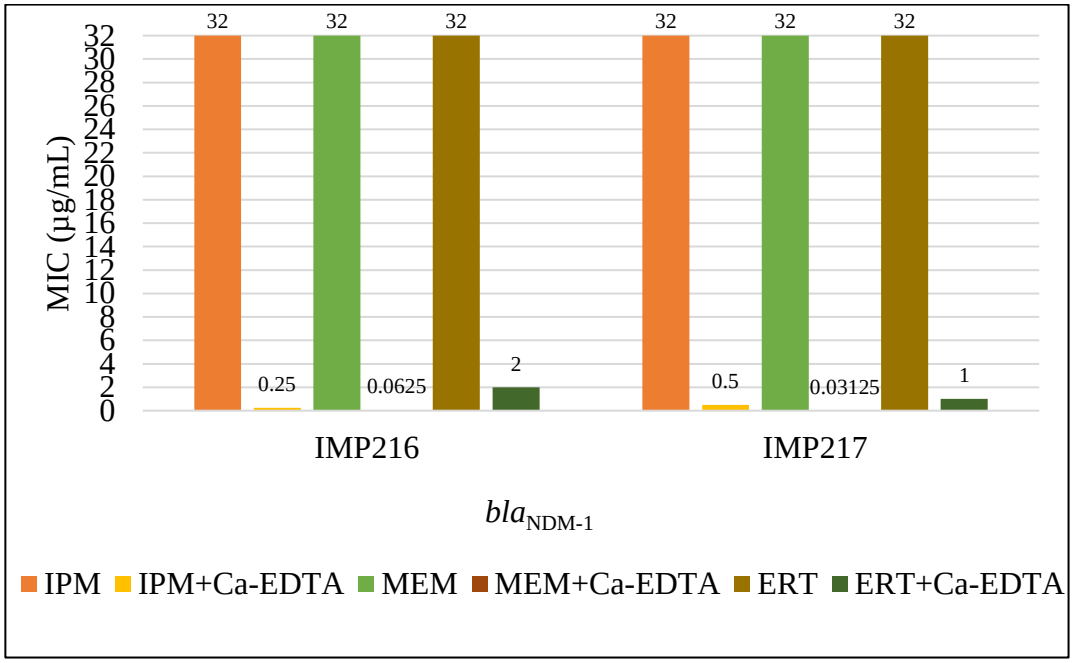

Figure 2b: MIC of carbapenems with and without Ca-EDTA against *Enterobacteriaceae* isolates that harbor *bla*$_{\text{NDM-1}}$

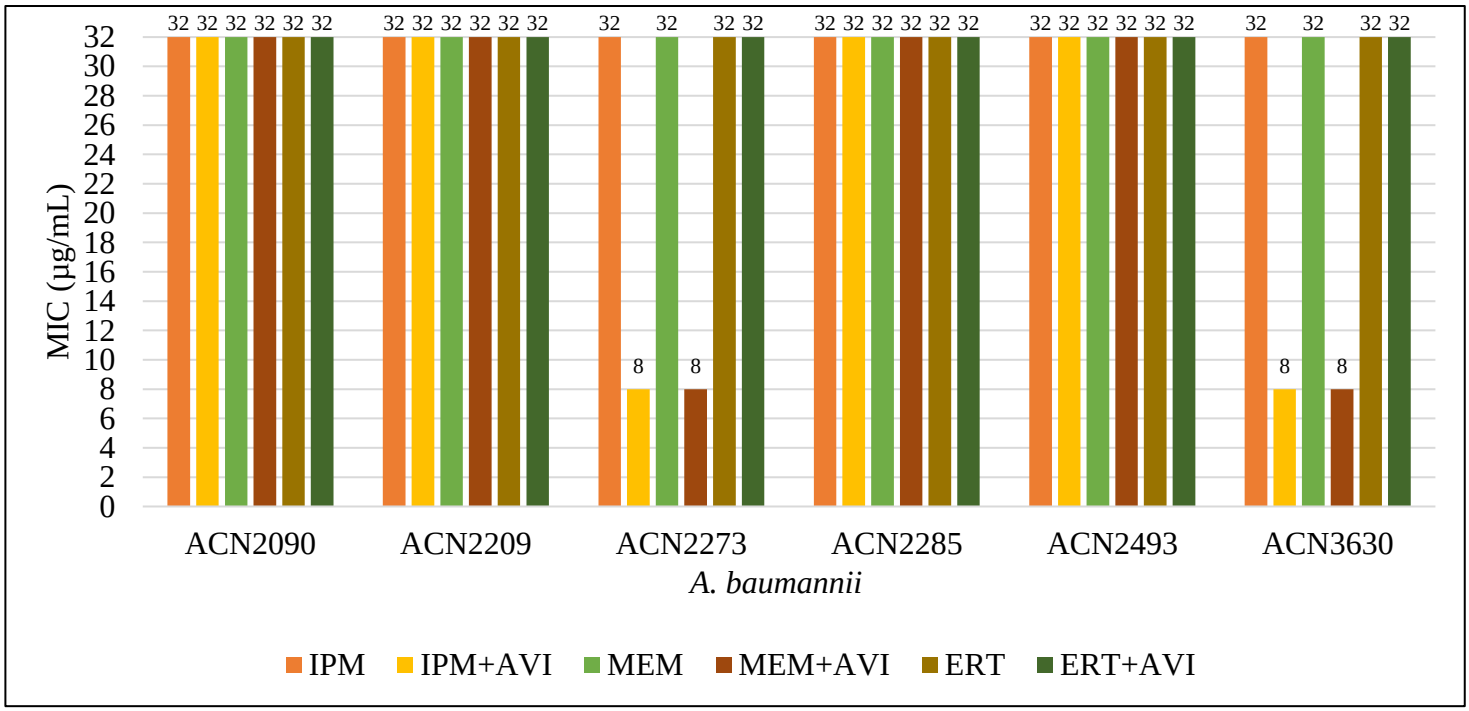

Figure 2c: MIC of carbapenems with and without avibactam against *A. baumannii* isolates

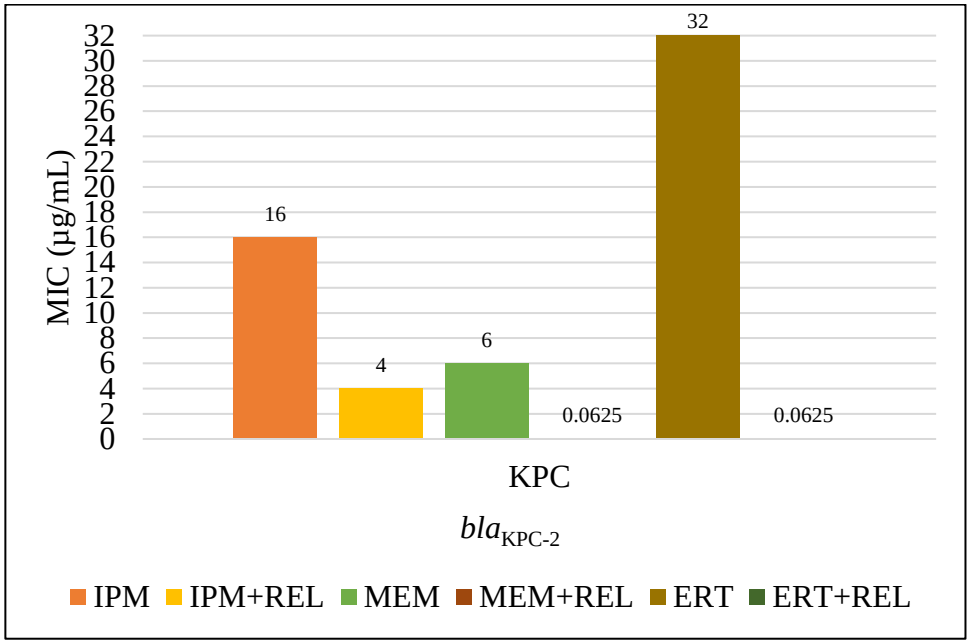

Figure 2d: MIC of carbapenems with and without relebactam against a *Salmonella spp*.

isolate that harbors bla_{KPC-2}

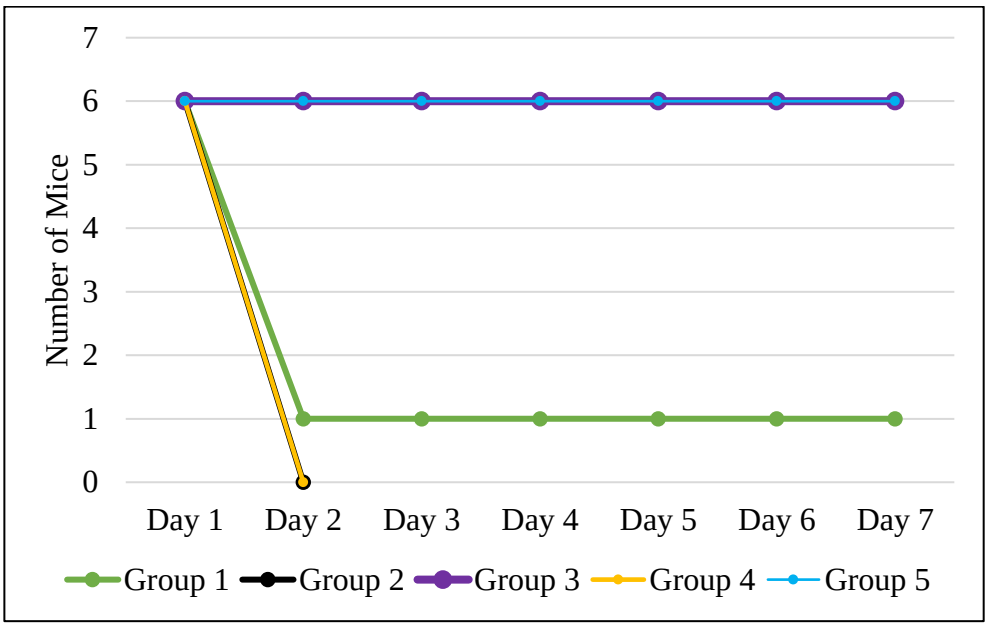

Figure 3a: Survival rates of treated and untreated BALB/c mice infected with IMP57

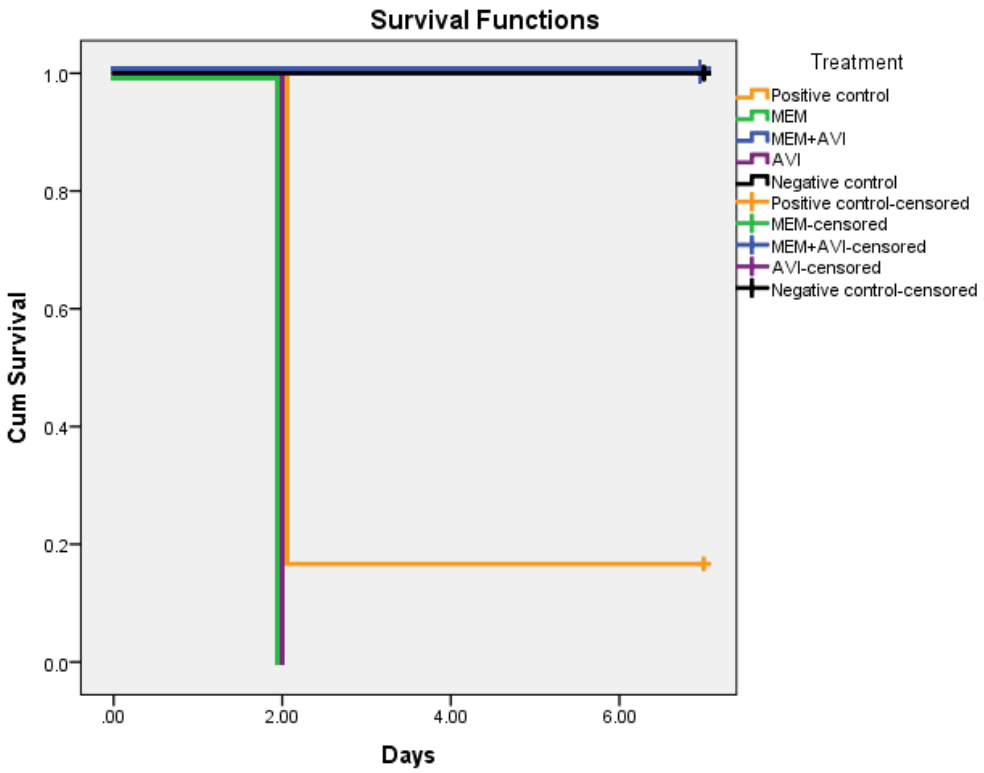

Figure 3b: Survival rates of treated and untreated BALB/c mice infected with IMP57 in a Kaplan-Meier graph.

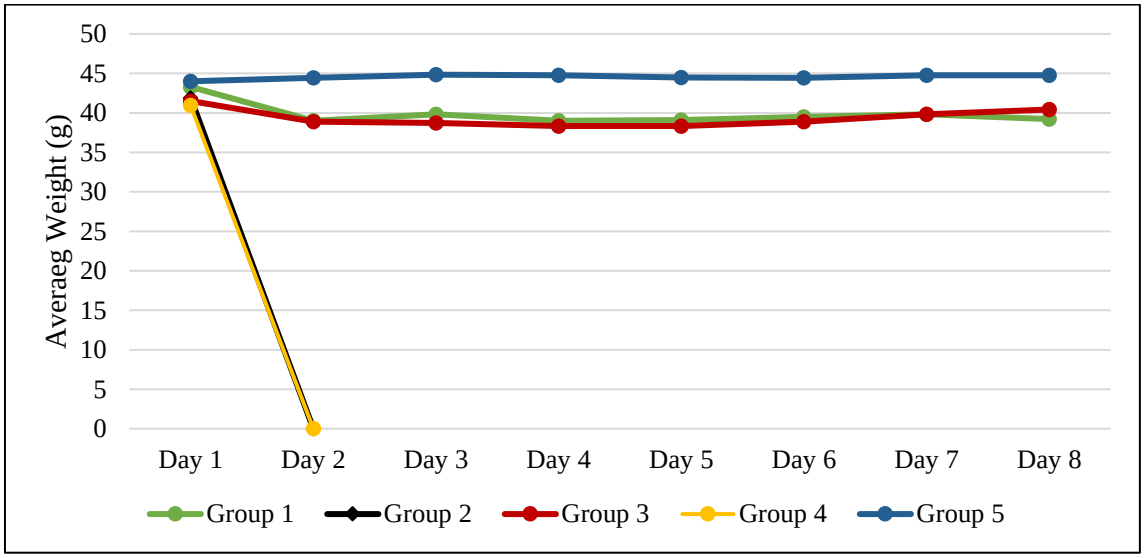

Figure 4: Average weight of tested BALB/c mice

NOTE: The average weight of the mice in group 1 starting day 2 is the weight of 1 mouse that survived. All mice in groups 2 and 4 died by the second day.

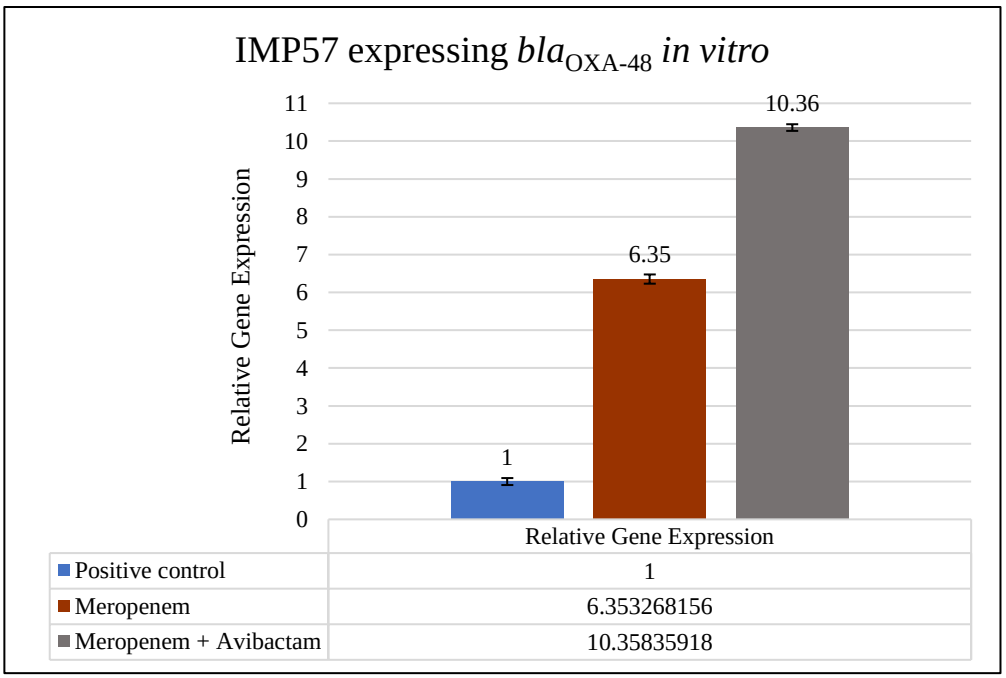

Figure 5a: The *in vitro* relative expression levels of bla_{OXA-48} in IMP57 following the addition of meropenem or meropenem + avibactam

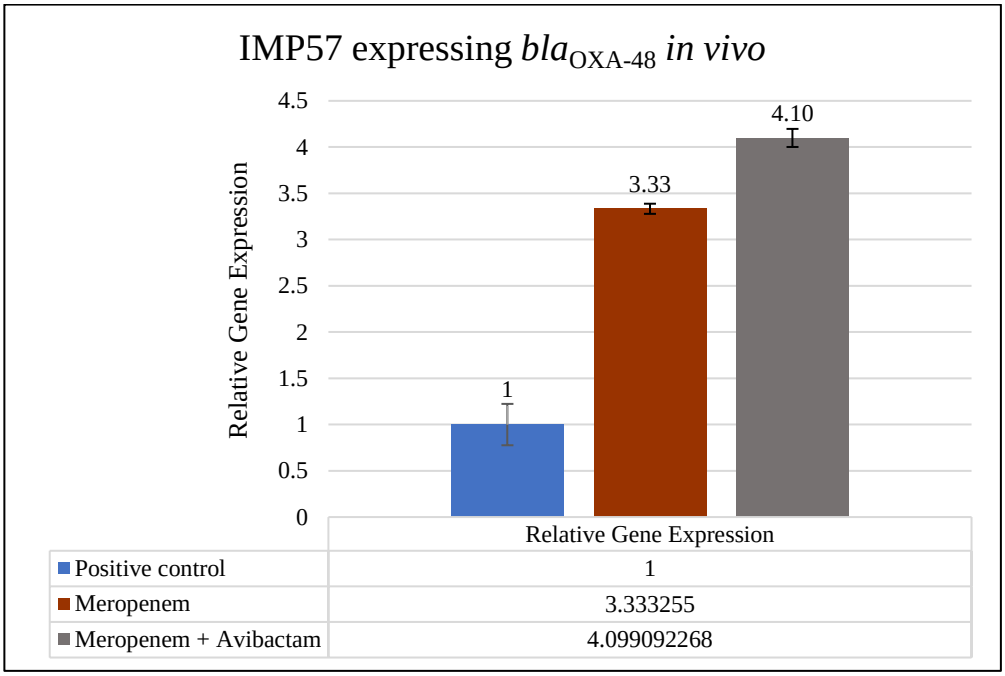

Figure 5b: The *in vivo* relative expression levels of *bla*$_{\text{OXA-48}}$ in IMP57 following treatment with meropenem or meropenem + avibactam

CHAPTER V

DISCUSSION

Antimicrobial resistance is a major health threat with unpredictable imminence. Bacteria can develop resistance to new antimicrobial agents within a few years after their commercialization (1). Among the pathogens that have become resistant to antimicrobial agents, the World Health Organization (WHO) ranks carbapenem-resistant *Enterobacteriaceae* (CRE) and *Acinetobacter baumannii* as the highest priority pathogens for research and drug discovery (132). Similarly, the CDC classifies CREs as an urgent health hazard, while multidrug-resistant *A. baumannii* is classified as a serious health hazard (1). Evidently, we are in need for a constant supply of alternative solutions to treat carbapenem-resistant bacterial infections. Combination therapy has proven to be useful; however, its benefits over monotherapy remain debatable. Therefore, β-lactam/β-lactamase inhibitor (BL/BLI) combinations were chosen as potential alternative solutions to antimicrobial combination therapy.

A. Broth Microdilution Antimicrobial Susceptibility Assay:

The addition of novel β-lactamase inhibitors avibactam, relebactam, and Ca-EDTA to carbapenems has successfully restored most of the tested isolates' susceptibility to that class of antimicrobial agents.

Concerning avibactam, most of the literature reports the combination of ceftazidime and avibactam against antimicrobial-resistant bacteria; however, Aktas et al. (122) have found that the combination of imipenem/avibactam managed to restore the susceptibility of 26 *Enterobacteriaceae* isolates with OXA-48 to imipenem. Although those findings

coincide with the results of imipenem/avibactam in this study since avibactam managed to restore the susceptibility of all *Enterobacteriaceae* isolates that harbor OXA-48 to imipenem, it was observed that the addition of meropenem/avibactam displayed considerably lower MIC values than imipenem/avibactam (Table 4); thus, highlighting the fact that meropenem/avibactam is the most efficacious BL/BLI combination against the tested *Enterobacteriaceae* isolates that harbor OXA-48. However, none of the BL/BLI combinations used in this study managed to restore the susceptibility of any of the *A. bauamnnii* isolates that mainly harbor OXA-23 and OXA-51 (Table 4). These finding are consistent with reported literature that certain Class D β-lactamases such as OXA-23 are resistant to inhibition by avibactam (133). Ertapenem was not as effective as imipenem or meropenem when combined with avibactam against OXA-type carbapenemases as it only managed to restore the susceptibility of one isolate out of 11 tested.

Concerning Ca-EDTA, the results generated from this work validate an earlier study (105) that assessed the efficacy of imipenem and meropenem in combination with Ca-EDTA against *K. pneumoniae* and *E. coli* isolates that harbor NDM-1 since the susceptibility to imipenem was restored in both studies upon the addition of imipenem/Ca-EDTA (Table 4); however, in this study, meropenem/Ca-EDTA was also a successful combination that restored isolate susceptibility. This is in contradiction with the study by Yoshizumi et al. (105) that found that meropenem/Ca-EDTA only managed to lower the MIC of one sample to a level below the susceptibility breakpoint. It is important to note that meropenem/Ca-EDTA were also the most efficacious combination against NDM-1-producing *Enterobacteriaceae* in the present study. The combination of ertapenem/Ca-EDTA was not as successful as the other two

combinations since it failed to restore the susceptibility of one isolate and only managed to lower the MIC to the breakpoint of susceptibility in the second isolate.

Concerning relebactam, the results observed in this study demonstrate that the combination of meropenem and ertapenem successfully restored the KPC-producing isolate's susceptibility to both antimicrobial agents; however, the combination of imipenem/relebactam failed to restore susceptibility. These findings are conflicting since imipenem/relebactam are currently in phase III clinical trials that target imipenem-resistant bacterial infections as well as ventilator-associated pneumonias (111, 112). In addition, an earlier study (124) that tested imipenem/relebactam against 451 *Bacteroides spp.* isolates failed to lower the percent of imipenem-resistance in the tested sample size. On the other hand, a second study reported that imipenem/relebactam managed to restore the susceptibility of 97% of KPC-producing *K. pneumoniae* isolates (109).

B. Survival Experiments:

The current regional prevalence of OXA-48 (72-80) prompted further *in vivo* investigation of BL/BLI combinations against the bacterial isolate IMP57 that harbors the carbapenemase. Meropenem showed the highest *in vitro* efficacy when combined with avibactam against OXA-48-producing *Enterobacteriaceae*; thus, that treatment regimen was selected for the animal model experiments.

Assessing the survival rate of animal models following their infection with a Gram-negative bacterial isolate that harbors $bla_{\text{OXA-48}}$ and attempting to treat the animals with meropenem/avibactam has not been documented in the literature yet. As such, experimental design and dosage determinations were guided by earlier studies with similar target parameters (6, 93, 125, 126).

The findings of this study translated the *in vitro* results into the *in vivo* setting with minimal discrepancy. The group of mice that was infected with IMP57 and administered meropenem/avibactam showed a 100% survival rate, while the groups that received meropenem or avibactam only resulted in 0% survival by the end of the monitoring period (Figure 3). These findings are proof of the efficacy that meropenem/avibactam possess against enterobacterial isolates that harbor OXA-48, and the concentration of meropenem to avibactam at a 4:1 ratio (93) is effective for ceftazidime/avibactam as well as meropenem/avibactam. As for the positive control group, the survival of one mouse that was infected with IMP57 without receiving treatment might have been due to a rare technical error during the injection of the bacterial inoculum, which might have resulted in the inoculum being injected into the lumen of the mouse's stomach or its intestines instead of its intraperitoneal cavity (134). Finally, the negative control group that was only injected with tryptic soy broth showed a predictable outcome without any abnormalities in mice behavior or health status.

C. Relative *bla*$_{\text{OXA-48}}$ Expression:

Concerning the reverse transcription real-time polymerase chain reaction, the quantification of *bla*$_{\text{OXA-48}}$ expression levels following the addition of meropenem/avibactam has also not yet been documented in the literature.
Both *in vitro* and *in vivo* results showed a similar trend in relative *bla*$_{\text{OXA-48}}$ expression, which signifies the consistency of meropenem/avibactam activity despite the difference of environment between *in vitro* and *in vivo* settings. Such findings provide insight on the availability of the drugs in the organism and their ability to cross cellular linings to reach the blood and act on the infectious pathogen in this sepsis model of infection.

The resulting increase in relative *bla*$_{\text{OXA-48}}$ expression upon the addition of meropenem can be explained by considering the antimicrobial agent an inducer of β-lactamase production by the bacterial cell to degrade the antimicrobial agent. On the other hand, the statistically significant overexpression of *bla*$_{\text{OXA-48}}$ upon the addition of meropenem/avibactam can be explained by considering the synergic relationship between meropenem and avibactam. It has been previously proven that avibactam does not induce the production of β-lactamases when administered by itself (135), and that feature might be attributed to the reversible inhibition that avibactam displays (90); thus, had there not been synergism between meropenem and avibactam, then the level of *bla*$_{\text{OXA-48}}$ expression upon the addition of meropenem/avibactam would be similar to the level of gene expression following the addition of meropenem alone; however, due to the potentiating effect that avibactam exerts on meropenem, the efficacy of the antimicrobial agent exceeded its initial limit and lead to the overexpression of the carbapenemase gene in order to compensate for the effect of the drug. Phenotypically, this potentiating effect is observed through 1) the decrease in the MIC value of meropenem as determined by the broth microdilution assay and 2) the increase in BALB/c mice survival rates. It is important to note that regardless of the overexpressed carbapenemase, a low concentration of avibactam was sufficient to inhibit the enzymes and allow meropenem to exert its effect. Such low concentration of avibactam is sufficient since the mechanism of action that it follows restores its chemical structure after it detaches from the enzyme it is reversibly inhibiting (90).

In conclusion, the use of carbapenems, namely meropenem, with novel β-lactamase inhibitors avibactam, relebactam, and Ca-EDTA have proven to be capable of restoring the susceptibility of bacteria that are resistant to carbapenems due to the expression of *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC-2}}$, and *bla*$_{\text{NDM-1}}$. However, a larger sample size with

greater genetic and species diversity needs to be tested for both phenotypic and genotypic parameters before establishing recommendation guidelines or moving into clinical trials. It is important to not restrict our scope to data from *in vitro* tests only.

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