AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT OF COMBINATION THERAPY IN BALB/c MICE INFECTED BY MULTIDRUG RESISTANT *ENTEROBACTERIACEAE*

by
NOOR ALI SALLOUM

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Experimental Pathology, Immunology and Microbiology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
May 2014

AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT OF COMBINATION THERAPY IN BALB/c MICE INFECTED BY MULTIDRUG RESISTANT *ENTEROBACTERIACEAE*

by
NOOR ALI SALLOUM

Approved by:

Ghassan Matar, Ph.D., Professor — Advisor
Department of Experimental Pathology, Immunology and Microbiology

Alexander Abdelnoor, Ph.D., Professor — Member of Committee
Department of Experimental Pathology, Immunology and Microbiology

Ghassan Al-Awar, MD, Assistant Professor — Member of Committee
Department of Internal Medicine

Elias Rahal, Ph.D., Assistant Professor — Member of Committee
Department of Experimental Pathology, Immunology and Microbiology

Date of thesis defense: May 5th, 2014

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: Salloum (Last) Noor (First) Ali (Middle)

☑ Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☑ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, **three years after the date of submitting my thesis, dissertation, or project,** to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

Signature May 12, 2014
Date

ACKNOWLEDGMENTS

I would like to express my gratitude and appreciation to my advisor Dr. Ghassan Matar for his support, guidance and valuable input throughout the period of my thesis work and writing. Special thanks also go to Dr. Alexander Abdelnoor, Dr. Elias Rahal, and Dr. Ghassan Al-Awar for their much appreciated comments as members of my committee. And I would also like to thank Dr. Rahal for his help in the statistical analysis. I would also like to thank the Research Assistants, especially Ms. Kohar Annie Kissoyan and Ms. Katia Cheaito for their much valued and appreciated assistance.

I would like to thank my friends, Sukayna Fadlallah, Nathaline Haidar Ahmad, Helene Hajjar, Souraya Sayegh, and Dalal Jaber for making this a memorable journey. A special thanks goes to Ibrahim Abdel Nour for the exceptional support and encouragement throughout this period.

Last but not least, I am grateful for my parents, and my sister for their endless support, love, and motivation to keep going on and doing my best. As my mom would say, “just do your best, you can’t do more than that!” I would also like to thank my extended family, for their overseas love and support, and for believing in me.

P.S. I would like to thank the mice, all 540 of them, for being a major part of my work!

AN ABSTRACT OF THE THESIS OF

Noor Ali Salloum for Master of Science
Major: Microbiology and Immunology

Title: Assessment of Combination Therapy in BALB/c Mice Infected by Multidrug Resistant *Enterobacteriaceae*

Background: Due to the increase in resistance to third generation cephalosporins in extended spectrum β-lactamases (ESBLs) producing *Enterobacteriaceae,* carbapenems are administered as an alternative treatment for infections caused by *Enterobacteriaceae*. However, the extensive use of carbapenems led to the emergence of carbapenem resistance among these organisms. Subsequently, treatment options for patients with infections caused by carbapenem resistant *Enterobacteriaceae* are limited to new drug tigecycline, and the older drugs: rifampicin, colistin, or fosfomycin, although these antibacterial agents proved to be notorious. It is currently debatable whether using these antibacterial agents in combination therapy is more advantagous than monotherapy. To that purpose we attempted to assess *in vitro* and *in vivo,* the efficacy of combination therapy on ESBL producing and carbapenem resistant *Enterobacteriaceae* harboring various ESBL and carbapenemase encoding genes.
Methods: Four carbapenem resistant isolates were selected from previous studies; two *Klebsiella pneumoniae* (one isolate harboring the ESBL *bla*CTX-M-15 gene and one harboring the carbapenemase *bla*NDM-1gene), one *Escherichia coli* isolate harboring the carbapenemase *bla*OXA-48 gene and one *Salmonella* isolate harboring the carbapenemase *bla*KPC-2 gene. Antibacterial susceptibility testing was performed by the disc diffusion method. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were determined by the broth dilution method. Antibacterial synergism between the combination of different antibacterial agents was assessed by the double disc diffusion method. Carbapenemase production was tested by the Modified and Re-modified Hodge Tests (MHT and RMHT). Assessment of gene transcript levels was determined by RT-qPCR, in response to selected treatment options (combination and monotherapy) carried out *in vitro* and *in vivo*. The efficacy of combination therapy was assessed *in vivo* by monitoring weight and survival rates in female BALB/c mice (n=339).
Results: The various antibacterial agents assessed by the double disc diffusion showed synergistic effects. Both MHT and/or RMHT detected carbapenemase production in all isolates harboring carbapenemase encoding genes. Assessment by RT-qPCR of the effect of combination therapy on genes transcript levels, revealed in all isolates a decrease in the ESBL and carbapenemase encoding genes transcript levels when rifampicin and tigecycline were used singly, or in combination with colistin. However, when colistin was used singly or in combination with meropenem or fosfomycin, variable genes transcript levels were observed by RT-qPCR in all isolates. *In vivo* assessment of antibacterial combination therapy on weight and survival rate of BALB/c mice demonstrated that all combinations used were effective for isolates harboring the ESBL encoding gene *bla*CTX-M-15, and the carbapenemase encoding genes *bla*OXA-48 and *bla*NDM-1. Conversely, the most

significant antibacterial combination treatment regimen for the isolate harboring the *bla*KPC-2 gene was colistin with either fosfomycin, kanamycin, ertapenem or meropenem. **Conclusion**: Based on the results from this study, it can be concluded that antibacterial combination therapy appears to be as efficient as monotherapy. However, in certain situations combination therapy regimens appear to be more efficient and may provide a less toxic treatment regimen. Moreover, based on gene transcript levels and mouse survival rates, appropriate treatment regimens should be administered for carbapenem resistant isolates based on their ESBL and/or carbapenemase encoding genes.

CONTENTS

ILLUSTRATIONS

Figure Page

TABLES

CHAPTER I

INTRODUCTION

During the past decade, the occurrence of multi-drug resistant *Enterobacteriaceae* increased significantly. Previously, cephalosporins were the treatment of choice for infections with *Enterobacteriaceae*. However, with the increase of extended spectrum β-lactamases (ESBLs) in these microorganisms, carbapenems or the "last- resort drugs" represented the alternative treatment.

Carbapenems, the broadest spectrum β-lactam antibacterial agents, are effective against both Gram positive and Gram negative bacteria. They are considered the primary choice of treatment for ESBL producing bacteria. Nevertheless, the overuse of these antibacterial agents led to the development of resistance by *Enterobacteriaceae*.

Resistance to carbapenems is often convoyed with resistance to other classes of antibacterial agents, narrowing the treatment options against these multi-drug resistant bacteria. Subsequently, treatment alternatives are limited to colistin, tigecycline, rifampicin, and fosfomycin. However, reports of associated nephro/neuro-toxicity and increased emergence of resistance marked their use as controversial. Nevertheless, some studies highlight the importance of using these antibacterial agents in combination, in the attempt to overcome carbapenem resistance, although still controversial. Moreover, the development of new antibacterial agents capable of overcoming carbapenem resistance is challenging, which adds to the important use of combination therapy.

Due to the recent emergence of carbapenem resistance at the American University of Beirut Medical Center (AUBMC) in ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*, two previous studies were conducted at the Department of Experimental Pathology, Immunology and Microbiology. These studies aimed at determining the predominant molecular mechanisms of carbapenem resistance in the collected specimens. The findings of the previous studies indicated that carbapenem resistance in *E. coli* was due to either production of carbapenemases, and /or ESBL production in conjunction with efflux pump, and outer membrane impermeabilities. However, resistance to carbapenem in *K. pneumoniae* was due to production of carbapenemases, and / or ESBL production with porin loss.

The importance of the current study derives from the fact that carbapenem resistance is on the rise in Lebanon, the region, and worldwide, and more importantly the lack of a recognised efficient treatment.

This study attempted to assess the effect of combination therapy, both *in vitro* and *in vivo*, in order to recommend potential establishment of effective regimens against carbapenem resistance in *Enterobacteriaceae*. To that purpose, four isolates were selected from previous studies, harboring the prevalent ESBL encoding gene, *bla*CTX-M-15 and three carbapenem resistance encoding genes, namely, *bla*OXA-48, *bla*NDM-1, and *bla*KPC-2, and aimed at assessing:

1. The efficacy of combination therapy on the selected multi-drug resistant *Enterobacteriaceae*, both *in vitro* and *in vivo*.

2. The effective dose to be administered for each antibacterial agent (whether Minimal Inhibitory Concentration or Minimal Bactericidal Concentration).
3. The transcript levels of the carbapenem resistance encoding genes in response to various selected treatment options, from *in vitro* and *in vivo* samples, in order to determine whether certain carbapenemase encoding genes are inducible by the administered antibacterial agents singly and in combination.

In short, this study may shed light on the status of combination therapy and its efficacy in treating multi-drug resistant *Enterobacteriaceae* associated infections.

CHAPTER II

LITERATURE REVIEW

A. General characteristics of *Enterobacteriaceae*

The *Enterobacteriaceae* is a large family of Gram negative bacteria that includes more than 50 genera, consisting but not limited to *Escherichia, Klebsiella, Salmonella, Shigella, Yersinia,* and *Citrobacter* (1). In general, species that belong to the *Enterobacteriaceae* family are rod-shaped facultative anaerobes, and non-spore forming. Some exhibit motility by peritrichous flagella, while others do not. Similarly, they may or may not be lactose fermenters (2). Many species of *Enterobacteriaceae* can be part of the normal flora of the intestinal tract (2), thus termed as "enterics", as they aid in the digestion process (3). In addition, they are part of the normal flora of the skin, and can be found in the environment such as water and soil (2).

Very few species of *Enterobacteriaceae* are implicated in disease. However, those known to cause infections in humans are namely *Escherichia coli, Klebsiella pneumoniae*, and *Salmonella* spp in addition to others (2).

1. *Pathogenicity of* Escherichia coli

E. coli is the most commonly isolated microorganism in clinical laboratories (2). It is also considered as one of the primary causes of enterobacterial nosocomial infections, as well as community acquired infections (2, 4). As long as these bacteria do not acquire virulence factors that are encoded by genetic factors, they remain benign commensals (2). However,

strains that acquire genetic elements encoding for virulence factors such as enterotoxins or exotoxins, by horizontal gene transfer through transduction, conjugation or transformation, will acquire the potential to cause diseases (2). The primary site of infection is both the enteric and the urinary tract systems (3, 5). The route of entry for *E. coli* is mainly through fecal-oral transmission, through unhygienic food preparation and consumption of contaminated water or food (3). Infections with *E. coli* can be asymptomatic, however if symptomatic, symptoms arise 1-5 days after infection and last a few days. For instance, *E. coli* can cause enteric infections, ranging from an asymptomatic infection or a watery diarrhea, to an inflammatory diarrhea or dysentery (2, 3). In addition, *E. coli* can cause urinary tract infections (UTI) such as pyelonephritis and prostatitis. Moreover, more than 90% of UTI are caused by *E. coli* (6). *E. coli* may also be the cause of deep tissue infections by invading the blood and causing septicemia (7).

2. ***Pathogenicity of*** **Klebsiella pneumoniae**

K. pneumoniae is a colonizer of the intestinal tract and the skin and implicated in community acquired and nosocomial acquired infections (4, 8). The route of entry of *K. pneumoniae* is by contact or through the aspiration of the colonizing oropharyngeal microbes into the lower respiratory tract where it may lead to pneumonia (8). However, most cases of pneumonia are not via infection with *K. pneumoniae* (2). *K. pneumoniae* affects the middle aged individuals and the elderly with debilitating diseases (diabetes, malignancy, alcoholism, etc.). Healthy patients are not often affected by *K. pneumoniae* (5); rather people with prolonged hospital stays, requiring devices like ventilators or intravenous or urinary catheters, and patients taking long courses of certain antibacterial agents are mostly at risk for klebsiellar infections (8).

Such healthcare-associated infections include: pneumonia (in the form of bronchopneumonia or bronchitis), bloodstream infections, wound or surgical site infections, meningitis, septicemia, and UTI (especially in patients having catheters) (2).

3. ***Pathogenicity of*** **Salmonella**

About 100-140 million years ago, the *Escherichia* and *Salmonella* genera diverged from a mutual ancestor (9). Comparable to *E. coli*, the route of entry of *Salmonella* spp is usually fecal-oral with the consumption of contaminated food and water. (10, 11). Depending on the serotype of *Salmonella*, it can cause two types of diseases: typhoid fever and nontyphoidal salmonellosis. Typhoid fever is more serious than nontyphoidal salmonellosis with a higher mortality rate. Whereas, nontyphoidal salmonellosis, is a self-limiting disease in immunocompetent individuals, although it can be life-threatening in some cases, and may require medical treatment (11). *Salmonella* spp infections are associated with intestinal symptoms and can cause intestinal inflammations associated with diarrhea that is often mucopurulent and bloody. However, sepsis does not usually occur except in patients that are immunocompromised. Other symptoms include fever, vomiting, abdominal cramps 12-72hrs post infection and usually last 4-7 days. Patients displaying these symptoms usually recover without important medical treatment. On the other hand, patients at risk (immunocompromised, children, and infants) require immediate antibacterial treatment (12).

B. Treatment of Bacterial Infections:

Antibacterial agents are specialized for the treatment and prevention of bacterial infections (13, 14), which are widely used for improving human, animal and even plant health for the

prevention of bacterial infection. Antibacterial agents can be classified as either bacteriostatic or bactericidal (15). Either category can be either synthetic or natural (13,14). However, they are frequently categorized based on their mode of action (15). Antibacterial agents can act on any of the following based on their mode of action:

a) Inhibition of cell wall synthesis, with interference of the peptidoglycan synthesis, e.g. β-lactams (such as penicillin, cephalosporins and carbapenems).
b) Disruption of the bacterial cell membrane structure e.g. polymyxins (colistin).
c) Inhibition of RNA synthesis e.g. rifampin (rifampicin).
d) Inhibition of DNA synthesis e.g. fluoroquinolones (ciprofloxacin and levofloxacin).
e) Inhibition of protein synthesis e.g. aminoglycoside (such as kanamycin and gentamycin).
f) Inhibition of a metabolic pathways e.g. sulfonamides (such as sulfamethoxazole).

1. *Treatment of Enterobacterial Infections:*

Antibacterial agents are distributed into different classes, such as β-lactams, quinolones, tetracyclines, macrolides, sulfonamides, and aminoglycosides (14). The most common antibacterial agents used for the treatment of enterobacterial infections are the β-lactams. However, fluoroquinolones, sulfonamides, and aminoglycosides are often used as treatment options as well (16, 17).

In recent years, due to the extensive use of these antibacterial agents for treatments (14, 17), some bacterial strains have shown resistance to some of these agents (17, 19). Therefore, according to the resistance of each bacteria, the antibacterial treatment regimens would be administered. To date, carbapenem is still the most effective antibacterial agent used against

ESBL producing Gram-negative bacilli (17, 20, 21). Carbapenems have the broadest spectrum of activity against Gram positive and Gram negative organisms (17, 21); however, some organisms such as *Stenotrophomonas maltophilia*, *Flavobacterium*, *Chryseobacterium* spp. and *Aeromonas hydrophila* are intrinsically resistant to carbapenems (22).

Recently, emerging resistant strains against carbapenems have been reported (22-24), and have caused serious concerns that have led to the reuse of old and controversial antibacterial agents (25). An example of an alternative treatment would be colistin, tigecycline, or fosfomycin (26), or a combination therapy if extensive drug resistance is found (19, 27). These treatment options will be discussed later.

A bacterial isolate is considered as multi-drug resistant when it is resistant to at least one agent in at least three classes of the antibacterial agents. However, the term extensively-drug resistant is used when a bacterial isolate is resistant to all but one or two classes. Finally, pan-drug resistant refers to a bacterial isolate that is resistant to *all* available classes of antibacterial agents (28, 29).

2. *β-lactams:*

β-lactams, along with their derivatives, are the most regularly used antibacterial agents (30). They compose the largest group of antibacterial agents that share a similar structure of a four-membered, nitrogen containing, β-lactam ring at the core of their structure. The β-lactam family consists of four major groups: penicillins, cephalosporins, monobactams, and carbapenems (17, 30).

3. *Carbapenems:*

Carbapenems, part of the β-lactam antibacterial agents, have an exceptionally broad spectrum activity (14, 31). They are considered the "last resort drugs" for many Gram negative bacteria causing serious nosocomial infections (23). Carbapenems have a bactericidal effect and are stable against most β-lactamases including AmpC β-lactamases and extended-spectrum β-lactamases (31).

The basic structure of the peptidoglycan layer of the bacterial cell wall is acquired by the cross linkage of alternating molecules of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). To each NAM unit, a pentapeptide chain with a D-alanine-D-alanine sequence is attached. This crosslinking of two D-alanine-D-alanine is catalyzed by Penicillin-Binding-Protein (PBP). The crosslinking is what confers the integrity and the stability of the bacterial cell wall. The β-lactam ring resembles the D-alanine-D-alanine sequence of the pentapeptide sequence on the NAM molecule. During cell wall synthesis, the PBP "mistakenly" uses the β-lactam as a building block. The β-lactams bind irreversibly to PBP; this causes the acylation of the PBP, making it inactive and incapable of further transpeptidation reactions. (17, 21, 31). This action disrupts the cell wall synthesis by weakening the peptidoglycan layer and ultimately causing the cell to burst due to osmotic pressure and eventually cell death (21).

The current carbapenems are derived from thienamycin, the first carbapenem to be produced in the 1970s. Its use was ceased and eventually served as the parent model of the current carbapenems (21). Thienamycin is a compound produced by *Streptomyces cattelya*, an organism found in the soil (21, 31). This could explain why some bacteria are intrinsically resistant to carbapenems as they were found in nature, long before the antibacterial agent era

(18). Carbapenems approved for clinical use include ertapenem, imipenem, meropenem, and the most recent, doripenem (32).

While carbapenems remain effective against susceptible organisms, there have been many cases of emergence of resistance to this class of antibacterial agents. There are several mechanisms of resistance to carbapenems and these include:

a) The production of a β-lactamase enzyme, such as the production of a carbapenemase that hydrolyzes the carbapenem.
b) An outer porin loss/decreases permeability in the outer membrane along with an ESBL or an AmpC β-lactamase.
c) Alteration of the carbapenem target, PBPs, or production of low affinity PBP
d) Increase in efflux pumps (4, 33-35).

C. Transmission of Resistance

Genes encoding for an enzymatic resistance can be either chromosomally through a point mutation (vertical gene transfer), or by horizontal gene transfer through the mobile elements like plasmids or transposons that can be disseminated among various bacteria by conjugation (16, 30, 36).

D. Beta-Lactamase

The major mode of resistance to the β-lactam antibacterial agents is by the production of a β-lactam hydrolyzing enzyme, known as β-lactamase (16, 30, 37). β-lactamases affect the β-lactam ring of antibacterial agents by hydrolyzing the amide bond of the four-membered β-lactam ring (16).

There are two major classification schemes for the categorization of the β-lactamase enzymes (17, 19, 34), the Ambler classification, and the Bush-Jacoby-Medeiros classification. The Ambler classification scheme, classes A through D, categorizes the enzymes based on their amino acid sequence homology; while the Bush-Jacoby-Medeiros classification scheme, groups 1 through 4, categorizes the enzymes based on the substrate and the inhibitor profile (38, 39). Nonetheless, the Ambler classification scheme is the most commonly used. According to the Ambler classification scheme classes A, C and D have a serine molecule at their active site; while class B, a metallo-β-lactamase, requires a zinc ion at the active site (17, 19, 34, 39).

E. Extended Spectrum Beta Lactamases

After the introduction of penicillins in the 1940s followed by penicillin derivatives, many bacteria gained the ability to resist such antibacterial agents due to the production of β-lactamases from either Ambler Class A (TEM-1 or SHV-1) or Ambler Class C (AmpC) in response to their overuse. This can be constitutive or inducible, chromosomal or plasmid mediated mode of resistance (40, 41). This steered the direction to the development of new antibacterial agents that can overcome this resistance and are termed second-, third- and fourth-generation oxyimino-cephalosporins (42). Additionally, this led to the development of β-lactamase inhibitors; there are three clinically approved β-lactamase inhibitors including clavulanic acid, sulbactam, and tazobactam that can be used, along with a β-lactam, as substitute treatment option (40, 43).

However, with the sustained and prolonged use of the extended spectrum oxyimino-cephalosporins for the treatment of severe infections with Gram negative organisms producing

β-lactamase selection pressure induced the production of extended spectrum beta-lactamases (ESBLs) by the bacteria (44, 45). Just within a few years of their production, some cases of emergence of resistance were reported (44, 46), where in Germany 1983, the first reported case of ESBL production was isolated from a *K. pneumoniae* isolate (44, 45) that conferred resistance to the extended spectrum cephalosporins.

1. *TEM-1 and SHV-1 Enzymes*

The vast majority of ESBLs are derived from genes that encode for TEM-1 or SHV-1 enzymes. TEM-1 and SHV-1 β-lactamases can hydrolyze and inactivate ampicillin, second generation cephalosporins, but not the third-generation cephalosporins (44, 47). The initial cases of ESBL production were by point mutations in the parent *bla*TEM and *bla*SHV genes that led to a single amino acid change in the β-lactamase (42, 44, 46-48); thus extending the spectrum of activity to include third-generation cephalosporins and monobactams (41, 44, 47).

Nowadays, ESBLs are typically plasmid mediated β-lactamases (49), which attained the ability to hydrolyze the oxyimino group of the third-generation cephalosporins (48, 50). However, ESBLs typically remain susceptible to the β-lactamase inhibitors, cephamycins, and carbapenems (40-43, 47, 51, 52). Moreover, along with the genes on the plasmid that confer resistance to the oxyimino-cephalosporins, other genes might also be found on these plasmids (42, 49, 53). This appears to extend the spectrum of resistance to several classes of antibacterial agents such as fluoroquinolones and aminoglycosides (34, 41, 42, 49, 53, 54). Recently, this mode of resistance has even extended to include resistance to carbapenems, a primary course of therapy for ESBL producing bacterial infections (34, 41, 54).

Usually ESBLs are not limited to one class, but belong to a variety of classes, e.g. Ambler class A (derivates of the plasmid mediated TEM and SHV penicillinases (41-43, 45, 55, 56), CTX-M β-lactamases recently the most prevalent ESBL (45, 54-56), Ambler Class C (AmpC), and Ambler class D (OXA) (40, 44).

2. *CTX-M β-lactamases:*

During the past decade, CTX-M β-lactamases, the most recent β-lactamases belonging to Ambler class A, have become the most prevalent and rapidly spreading ESBLs worldwide, whereas TEM and SHV type-ESBLs have been mainly in North America (17, 45, 54, 57).

CTX-M β-lactamase was first discovered in a European patient in the 1980s (57). They are plasmid mediated enzymes (42, 44, 49, 56). The *bla*CTX-M genes are commonly found on large plasmids that often harbour other genes conferring resistance to other antimicrobial agents including aminoglycosides, fluoroquinolones, chloramphenicols, and tetracyclins (42, 45, 47, 49). In addition, CTX-M encoded ESBLs have a resistance profile similar to the other ESBLs (44, 57).

3. *AmpC Cephaloporinase Enzymes*

Resistance to the third generation cephalosorins can be also mediated by the Ambler class C enzymes, which are also known as the AmpC cephaloporinase enzymes (40, 43). AmpC enzyme producers confer resistance to penicillins, cephamycin, oxyimino-cephalosporins, and azteonam, but not to cefepim (22), and are not inhibited by β-lactamase inhibitors or Ethylenediaminetetraacetic acid (EDTA) (43, 58, 59).

In contrast to ESBLs, genes for AmpC enzymes are predominantly chromosomal. Upon exposure to β-lactams, where single or multiple mutations occur in the regulatory genes of the bacteria, AmpC enzyme production might be induced (59, 60). Moreover, AmpC enzymes may even become over expressed upon the exposure to third- and fourth-generation cephalosporins (43, 59). In addition, resistance can be mediated through plasmid transfer, such as ACC, ACT, CMY, FOX, LAT, or MOX, etc (43, 58-60).

F. Carbapenem Resistance:

Even though primary reports describe resistance to carbapenems amongst *Enterobacteriaceae* due to the production of the chromosomally mediated AmpC-mediated β-lactamases or ESBL in association with a porin mutation/loss, new emerging reports describe new methods of carbapenem resistance. Another mode of resistance is by the production of carbapenem hydrolyzing enzymes, known as carbapenemases, mediated by chromosomes or plasmids (33, 40, 61). As carbapenems are no longer an effective treatment in case of resistance, very few treatment options are left (20). Colisin, fosfomycin or tigecycline are the remaining treatment options left (26, 27, 62, 63). The broad spectrum range of hydrolysis for carbapenemases generate some bacterial isolates resistant to all carbapenems, cephamycins, and the majority to the β-lactamase inhibitors (26, 64).

The detection of the first carbapenemase producer in *Enterobacteriaceae* (NmcA) was in 1993; in the meantime, there have been numerous identifications of carbapenemase encoding genes in *Enterobacteriaceae* (64). Carbapenem hydrolyzing β-lactamases belong to 3 classes of the Ambler classification, classes A, B and D (34, 64). The clinically foremost essential carbapenemases in *Enterobacteriaceae*, and especially *K. pneumoniae* and *E.coli*

consist of the serine KPC(Class A)- and OXA(Class D)-type carbapenemases and the metallo VIM-, IMP- and NDM-type-β-lactamases (Class B) (65-67).

G. *Carbapenemase Detection*

Detection of carbapenemase production can be done by several methods, which include but are not limited to, determination of the susceptibility profile through disc diffusion, E-test, and broth dilution methods, in addition to other phenotypic and molecular tests.

Broth Dilution Method and E-test method, can both be implied for the quantification of the Minimal Inhibitory Concentration (MIC) of an organism (68). However, carbapenemase detection, especially in case of KPC, might not show a resistance profile, however, the MIC levels can be elevated but within the susceptible ranges according to the current Clinical and Laboratory Standards Institute (CLSI) breakpoints (67).

In response to that issue, CLSI has recommended performing the Modified Hodge test (MHT) for organisms showing an elevated but susceptible MIC levels. MHT is the only screening test for carbapenemase production recommended by CLSI (69).

However, the problem with phenotypic tests is that they are subjective and are not always accurate. False positives and negatives have been identified by this method due to low carbapenemase production, or in cases of CTX-M production with reduced outer membrane permeability (70). MHT would best detect carbapenemases belonging to class A and D (69-71), for example KPC producing organism; however, it is not as sensitive for MBL producing organisms (70, 72). As a modification, the Remodified hodge Test (RMHT), the addition of zinc sulfate to the disc in will improve the results for MBL producers (70, 73, 74).

Polymerase Chain Reaction is the gold standard method for the detection and the

determination of the exact carbapenemase produced. Although this technique is quick and sensitive, it is mostly used for research; however, its use clinically has been increasing to overcome the problems of the phenotypic tests (73).

H. Classes of Carbapenemases:

1. *Class A enzymes:*

Ambler class A is subdivided into 4 families: NMC/IMI, SME, GES, and KPC enzymes. These enzymes require a serine at their active site for their hydrolytic mechanisms. A wide number of class A carbapenemases have been identified, some are chromosomally encoded (NmcA, Sme, IMI-1) while others are plasmid encoded (KPC, IMI-2, GES derivatives) (43, 46, 64). Generally, Class A enzymes effectively hydrolyze carbapenems, cephalosporins, penicillins, and monobactams. They, however, seem to be inhibited by clavulanic acid and tazobactam (24, 43, 46, 75).

a. Chromosomally Encoded Enzymes: SME, NMC, and IMI

The resistance profile for the strains that express chromosomally encoded resistance gene is distinctive as they hydrolyze the penicillins, early cephalosporins, aztreonam, and carbapenems, but are susceptible to extended-spectrum cephalosporins and cefoxitin (75). The genes for these β-lactamases are chromosomally located, a fact that may have contributed to their rarity (43).

i. *Serratia marcescens* Enzyme

The SEM enzymes have only been identified in *S. marcescens* (75, 76). SME-1 (*Serratia marcescens* Enzyme) was first detected in England in 1982 from two *S. marcescens* isolates (76). However, recently other SME derivatives have been found sporadically in the United States (75, -76).

ii. Non-Metalloenzyme Carbapenemase and Imipenem-Hydrolyzing β-lactamase

The NMC-A (Non-Metalloenzyme Carbapenemase) and the IMI (Imipenem-Hydrolyzing β-lactamase) enzymes have been detected in rare clinical isolates of *E. cloacae* in the United States, France, and Argentina (75, 76). Although the genes for these enzymes are mediated chromosomally, genes encoding IMI-2 β-lactamases were located on plasmids in *Enterobacter asburiae* (United States rivers) and an *E. cloacae* isolate (China) (75, 76).

b. Plasmid-Encoded Enzymes: KPC and GES

i. *Klebsiella pneumoniae* Carbapenemase

While all the enzymes are important clinically, the *K. pneumoniae* Carbapenemase (KPC) remains to be the most clinically common (34, 64, 76, 77).

The *bla*-KPC gene is encoded by a plasmid, and was first identified in North Carolina, United States, in 1996 (64). Later in 2003, another KPC enzyme, as a result of a point mutation that led to 2 amino acid changes, was isolated and termed as KPC-2. KPC producing organisms hydrolyze β-lactams of all the classes, including third generation cephalosporins, carbapenems, and monobactams (34, 78). They hydrolyze more efficiently the penicillins and narrow-spectrum cephalosporins than the carbapenems, cefotaxime, and aztreonam, and weakly hydrolyze cephamycins and ceftazidime. The hydrolysis acitivity of the KPC enzymes

are slightly inhibited by the β-lactamase inhibitors (75, 76, 70). KPC encoding genes are frequently found on large plasmids that can also harbour aminoglycoside-resistance determinants (61, 79, 80), or the most widespread ESBL gene, *bla*CTX-M-15 (79). Accordingly, they may be considered as multidrug resistant, especially to all the β-lactams, as a result making treatment options more restricted (64, 79).

However, the detection of KPC production might be unrecognized by routine susceptibility laboratory tests (4, 55). Minimal Inhibitory Concentration (MIC) testing on KPC producing isolates show a varying profile of moderately increased MIC (2-4μg/ml) to resistant (≥32μg/ml) (46, 75). This can be explained by the fact that when KPCs are found alone, they might not confer carbapenem resistance, yet only slightly increase carbapenem MICs (67, 79). Hence, they may also be mistaken for ESBLs using certain phenotypic tests (79). When a KPC enzyme is combined with an alteration in outer membrane permeability, the carbapenem MICs becomes consistent with resistant profiles (4, 79, 81).

ii. Guiana Extended Spectrum

Guiana extended spectrum (GES) β-lactamases were originally identified as ESBLs, however, over time, GES variants showed low, but measurable imipenem hydrolysis (75). It was first isolated in *K. pneumoniae* in a French hospital in 1998, where the patient had been previously treated in French Guiana; hence, the name (76). All GES enzymes except GES-7 are carried by a plasmid or an integron. GES enzymes, although rare, have been identified worldwide (75, 76).

2. *Class B enzymes:*

Class B enzymes are referred to as Metallo-β-lactamases as they require a zinc ion at the active site for an efficient hydrolysis of carbapenems, thus, a different hydrolytic mechanism from the other serine class carbapenemases (55). MBLs likely evolved separately from the other serine requiring Ambler classes (46). This class of enzymes has a broad substrate spectrum. In addition to carbapenems, they efficiently hydrolyze cephalosporins and penicillins. Moreover, they are resistant to the β-lactamase inhibitors, but lack the ability to hydrolyze aztreonam (46, 55, 83). They seem to be inhibited by EDTA which is a divalent cation chelator (24). The *bla*MBL genes can either be located on chromosomes, plasmids or transposons (46).

a. Chromosomal Metallo-β-lactamases:

The chromosomal MBLs were first identified in certain environmental and opportunistic organisms such as *Bacillus cereus, Aeromonas* species, and *S. maltophilia* as much as 40 years ago (46, 75). Isolated from *Bacillus cerues,* BCII was the first metallo-β-lactamase identified (84). Fortuitously, with the exclusion of *S. maltophilia*, these bacteria have not been regularly associated with serious nosocomial infections (46, 75), as they are generally opportunistic pathogens, and the chromosomal metallo-β-lactamase genes are not easily transferred (75).

b. Acquired or transferrable Metallo-β-lactamases

The most important and most widespread MBLs include the VIM and IMP families, which have been detected in strains of *P. aeruginosa, A. baumannii*, and members of the family E*nterobacteriaceae* (19, 55, 61, 64).

i. Imipenem Hydrolyzing Enzyme and Verona integron-encoded metallo-β-lactamase

IMP stands for active on imipenem (75). IMP enzymes were first reported in Japan in 1990 (75, 83) in *P. aeruginosa* isolate, and remain predominantly there. VIM-1 was first isolated in 1997 in Verona, Italy, followed by the subsequent report of VIM-2 from France isolated in 1996. VIM enzymes are the most prevalent carbapenemases in Europe. The *bla*IMP and *bla*VIM genes are carried on mobile gene cassettes inserted into chromosomal- or plasmid-borne isolated in Korea; all belong to the MBL class and have been prevalent but confined within their countries of origin (86, 87).

ii. SPM, GIM, and SIM

Sao Paulo metallo-β-lactamase (SPM), first detected in Brazil; German imipenem metallo-β-lactamase (GIM), detected in Germany; Seoul imipenem metallo-β-lactamase (SIM) first isolated in Korea; all belong to the MBL class and have been prevalent but confined within their countries of origin (86, 87).

iii. New Delhi Metallo-β-lactamases

The IMP and the VIM enzymes were the most predominant MBLs worldwide until the isolation and the detection of the New Delhi Metallo-β-lactamase (NDM) (34, 61). NDM-1 enzyme was first isolated from a Swedish patient, previously hospitalized in New-Delhi, thus the name (28, 61, 64, 85). Its geographic distribution is worldwide, however, it is mainly found in India, Pakistan, United Kingdom, and the Balkan region (28).

The *bla*NDM-1 gene is encoded by a plasmid, thus making it easier to disseminate to other isolates (29, 34, 64). NDM-1 enzymes hydrolyse not only carbapenems, but also

oxyimino-cephalosporins and penicillins. As a matter of fact, the plasmids carrying the *bla*NDM-1 gene are diverse and can harbour numerous resistant genes which might include resistance to other antibacterial classes (17, 64), with some isolate only remaining susceptible to colistin, tigecycline and fosfomycin (17, 64).

3. *Class D enzymes:*

Class D enzymes are referred to "oxacillinases" because of their ability to effectively hydrolyze oxacillins at a higher rate than penicillins (34, 46). In general, OXA enzymes are capable of hydrolyzing penicillins, some cephalosporins, oxacillin and cloxacillin, and are resistant to the β-lactamase inhibitors (18, 75). However, they have poor hydrolyzing activity on extended spectrum cephalosporins and carbapenems (89).

Resistance to carbapenems by OXA-type carbapenemases increases considerably when an organism expressing the enzyme also shows alterations in outer membrane permeability or alterations in the affinity/production of PBPs (75, 89).

The first OXA β-lactamase with carbapenem hydrolysis activity was isolated from an *A. baumannii* strain that was isolated from a patient in Scotland in 1985 (75). The majority of the clinically relevant class D β-lactamases are acquired enzymes through plasmids, however, the most predominant oxacillinases are chromosomally encoded (89, 90).

The enzymes OXA-48 was isolated for the first time in Turkey 2003, in a *K. pneumoniae* isolate (64, 77, 90). This OXA variant was plasmid encoded (77) and had less than 50% amino acid similarity to the other OXA members (91).The *bla*OXA-48 gene encoding plasmid can encode resistance for other genes as well, and thus confer multi-resistance (90).

I. The role of the outer membrane: porin channels and efflux pumps

The outer membrane, the peptidoglycan cell wall, and the inner membrane compose the basic cell envelope structure of the Gram negative bacteria (92). Outer membranes of Gram negative bacteria must allow the influx of nutrients and efflux of waste products (93).

The porin proteins, often referred to as outer membrane porins, exist on the outer membrane as trimers; these outer membrane porins act as water-filled protein channels that allow the transport of small hydrophilic molecules - such as iron, nutrients, and even antibacterial agents into the periplasm of the bacteria (92, 94).

Up to the present time, most antibacterial agents target the intracellular processes; therefore, they must penetrate the bacterial cell envelope. The two fundamental pathways through which the antibacterial agents can enter are: a lipid-mediated pathway for hydrophobic antibacterial agents, and general diffusion porins for hydrophilic antibacterial agents. (94).

1. *Porin channels:*

There are three main porins produced by *E. coli*, which are OmpF, OmpC and PhoE, with comparable homologues found in most Gram negative bacteria (17, 95). The major porin proteins OmpF and OmpC in *K. pneumoniae* are referred to as OmpK35 and OmpK36, respectively (92, 95). Overall, porins are non-specific but have preferred substrates, such as PhoE prefering inorganic phosphate and anions, while OmpF and OmpC prefering cationic substrates (17, 92). In comparison, OmpF has a larger porin channel size than OmpC (94). With variable osmotic changes, low osmotic conditions prefer the expression of OmpF while high osmotic conditions prefer the expression of OmpC (94).

Most antibacterial agents cross the outer membrane of bacteria through the OmpF and OmpC porin proteins; this holds true for the *Enterobacteriaceae* family and the β-lactams as well (92, 94, 95). Since the β-lactams enter through these porins, it is easily understandable that when the porin impermeability decreases, the entry of the β-lactams would decrease as well. Impermeability results when the porin expression is modified, attributable to a decrease in the level of expression of a porin, or a mutation in the porin channel resulting in its non-functionality (95). As a result in the decreased entrance of the β-lactams to the inner cellular space, decreased susceptibility ranges for cephalosporins and carbapenems in *Enterobacteriaceae* can occur.

There have been reports for two principal porin-based mechanisms for antibacterial resistance in clinical isolates: 1) an altered function due to specific mutations reducing permeability, or 2) alterations of outer membrane profiles, including either the replacement of one or two major porins by another, or by the loss/severe reduction of porins (94).

a. <u>Mutations in porin proteins</u>

Mutations or alterations in porin proteins themselves have also been recorded. This can be due to several reasons, which include: point mutations in the coding sequence, point mutations in the promoter region that may affect transcription, or interruption of the coding sequence by insertion sequences that result in early termination of translation. The end result is an altered function caused by specific mutations reducing permeability of the agents (96).

b. <u>Alteration in Porin expression:</u>

Ordinarily, the total quantity of OmpF and OmpC proteins is relatively stable, but what varies is the relative proportion of the two (97). The replacement of the constitutively expressed large general diffusion porins with a smaller porin is another strategy for acquiring antibacterial resistance, and even a quiescent porin, such as OmpK37 in some *K. pneumoniae* clinical isolates, a smaller pore, expressing lower susceptibility to β-lactams (94). A study showed a multi-drug resistant profile was attributable to a decrease in the expression of OmpK35, and an increase in the expression of OmpK36 (98).

2. *Extended spectrum β-lactamases and porin alterations*

Many studies have discussed and associated the presence of a Class A ESBL (e.g. CTX-M) or an AmpC cephalosporinase, along with porin alterations in the implication of carbapenem resistance in *E. coli* and *K. pneumoniae* isolates (33, 99). Other than the ability of these organisms to hydrolyze cephalorsporins, imperiability decreases the antibacterial agent concentration in the periplasmic region, resulting in the emergence of carbapenem resistance (100, 101).

A study showed that when conjugation experiments were performed, plasmids carrying ESBLs were isolated from the carbapenem resistant isolates with altered porins and transferred to recipient *E. coli* strains with unaltered porins, carbapenem resistance was found to be non-transferrable; rather, carbapenem MICs were significantly decreased and consistent with a susceptible profile in transconjugants (102).

Resistance to ertapenem in *Enterobacteriaceae*, with alterations/loss in the membrane porins and drug efflux pumps accompanied by a Class A ESBL or an AmpC enzyme have

been reported (96, 103, 104). Resistance to imipenem in *Enterobacter aerogenes* was also reported due to loss of porin in the outer membrane, or active efflux pumps (101).

Porin loss also reduces the susceptibility, not just to β-lactams, but also to other non-β-lactam antibacterial agents, such as fluoroquinolones, of ESBL-producing organisms. (94, 103).

J. Efflux pumps:

Although they can be found in several species belonging to *Enterobacteriaceae*, however, efflux pumps are predominantly found in *P. aeruginosa.* Resistance to carbapenems due to efflux pump activity has been observed in *Enterobacteriaceae* (98, 104, 105).

These efflux pumps confer resistance as they are responsible for exporting the antibacterial agents out of the cell (106) including tetracycline, chloramphenicol, quinolones, β-lactams, and macrolides (98, 103, 106). Efflux pumps have very broad spectrums of activity, and the over-expression of a single efflux pump may result in the resistance to a number of classes of antimicrobial agents (108, 109).

K. Alteration of the carbapenem target: the PBPs

One of the mechanisms used by the bacteria to resist carbapenems is by alterating in the carbapenem target, the Penicillin-binding-protien (PBP). However, this mode of resistance is rarely reported in Gram negative bacteria, especially in *Enterobacteriaceae* (110, 111). This mechanism of alteration is by the decreased production of the initial PBP-1, which carbapenems have a higher affinity to, and the increased production of an alterated PBP-2, which carbapenems have little affinity to (112, 113).

L. Treatment of Carbapenem Resistant Organisms

Currently, a small number of antibacterial agents are available for the treatment of carbapenem resistant organisms (22, 65, 114). Plasmids carrying β-lactam resistance genes, can carry other resistance genes, which leads to a reduced susceptibility to the non-β-lactams (65). Treatments remaining available are polymyxins (colistin), tigecycline, rifampicin, and fosfomycin (19, 22, 28, 65). However, the susceptibility to these agents are not predictable (115).

1. *Colistin*

As a matter of fact, the absence of novel antibacterial agents for carbapenem resistant Gram negative bacteria, has forced clinicians to re-evaluate the clinical value of colistin (115-117). Although polymyxins, both polymyxin B and colistin, were developed long time ago, they are being reused in the era of carbapenem resistance (22). Colistin, is now often considered the first line choice treatment for carbapenem resistant organisms (65, 114, 118).

Colistin, a lipopeptide antibacterial agent, (25), has been shown to have bactericidal effect, *in vitro* (117). Colistin acts on the surface and penetrates into the bacterial cell membrane (22). Colistin binds to the anionic lipopolysaccharide molecules, thus displacing cations from the Gram negative bacterial outer cell membrane. This leads to permeability changes in the cell envelope, leakage of cell contents, and eventually cell death (25, 117). The concern with colistin is its nephrotoxicity and neurotoxicity (22, 25, 28).

Many studies show that colistin has a higher rate of success when used in combination therapy but not as a monotherapy (22, 27). Although the superiority of colistin-based combination therapy over monotherapy is still in its infancy, a genuine interest in combination

therapy persists due to the concern of heteroresistance (65, 115, 118). However, colistin heteroresistance has not been proven at this time (119). Nonetheless, heteroresistance should be taken into consideration; thus, colistin, should not be used as monotherapy, rather in combination to prevent the risk of emergence of resistance (65, 118, 119).

However, several studies have reported that treatment with colistin was associated with higher mortality than with treatment with imipenem or meropenem in cases of severe infections with strains susceptible to carbapenem (34, 65).

2. *Tigecycline*

Tigecycline, a semi-synthetic drug, is the first member of the glycylcycline class of antibacterial agents (19, 120). It is not bactericidal, rather bacteriostatic, which may limit its effectiveness (120); however, it has a broad spectrum of activity against Gram negative and Gram positive bacteria (121). Tigecycline inhibits protein translation by binding to the 30S ribosomal subunit, preventing the incorporations of the amino acid residues into the elongating peptide chains (22, 120).

Another limitation for the use of tigecycline is the low bloodstream and urinary tract concentration levels which are inadequate for treatment for bacteremias and UTIs (28, 34, 122). As a result, it has been approved for complicated skin, community-acquired pneumonia, soft tissue and intra-abdominal infections, where higher drug concentrations are found (34).

Pooled analysis from several studies have revealed higher mortality for tigecycline compared with other regimens, particularly for the treatment of hospital-acquired and ventilator associated pneumonia (28, 34, 65). If tigecycline were to be used due to extended resistance, experts do not advise its use as monotherapy for blood stream or urinary tract

infections (27, 28, 122). Moreover, tigecycline monotherapy is only to be used when there are no alternative drugs (65).

3. *Fosfomycin*

Fosfomycin, a phosphonic acid derivative, is a broad-spectrum antibacterial agent with bactericidal activity against both Gram positive and Gram negative organisms. Fosfomycin acts by inhibiting the peptidoglycan association; thus, disrupting the bacterial cell wall synthesis (19, 123). It is well tolerated, and does not have adverse effects (124), which makes it a good candidate for use with other antibacterial agents with some toxicity. Fosfomycin can be a substitute treatment for extended-drug resistant organisms (124).

Fosfomycin, in contrast to tigecycline, may be highly efficient and effective for UTIs (26, 28). However, if the infection is not localized to the genitourinary tract, its use is not advisable as monotherapy for other body site infections (26). Moreover, concerns regarding emergence of resistance, by mutation, increase the importance of its uses alongside other antibacterial agents as part of combination therapy (26, 34, 65, 125).

4. *Aminoglycosides*

Aminoglycosides bind the 16S rRNA constituent of the 30S ribosome subunit. This interaction induces an alteration in the conformation of the complex formed between an mRNA codon and the aminoacyl tRNA promoting tRNA mismatching; this leads to protein mistranslation, thereby contributing to cell death (15).

Aminoglycosides are suboptimal therapies, especially as monotherapy (34, 65), because of high rates of nephrotoxicity and otovestibular toxicity (34). It should be used as a

combination therapy, to decrease the dosage, and to increase efficacy against carbapenem resistant organisms (65). As previously mentioned above, plasmids carrying encoding genes for carbapenemases, often carry other resistance genes to aminoglycoside, which is another reason why aminoglycoside should not be used as monotherapy (34, 49).

5. ***Rifampicin***

Rifampicin is a semisynthetic bacteriostatic antibacterial drug of the rifamycin group. Rifampicin inhibits gene expression (126) by constraining and inhibiting the DNA-dependent RNA synthesis through the inhibition of the bacterial DNA-dependent RNA polymerase (15).

Studies suggest that rifampicin has a synergistic activity when combined with another antibacterial agent, against carbapenemase producing bacteria (19). Rifampicin is also advised not to be used alone as monotherapy for its high chance of resistance (15). Rifampicin monotherapy in some studies showed it had no bactericidal effect (82), but when added in combination with another antibacterial agent, mainly colistin, its activity was enhanced (127).

M. Combination therapy versus Monotherapy

The "effectiveness" of combination therapy, whether colistin based or not, has been inconclusive. Some experts advocate the use of combination therapy, while others think it has a negative effect.

1. *Monotherapy*

Monotherapy, or the use of a single-drug therapy during the treatment regimen, would lead to less toxicity exposure as a consequence of the antibacterial agent itself (19, 34). One of the

major concerns in combination therapy is the increased toxicity with the use of two drugs, thereby increasing the chances of toxicity reactions (19, 34, 116). For instance, many studies have reported the toxicity disadvantages of colistin with neurotoxicty and nephrotoxicity, and aminoglycoside with nephrotoxicity (122). Another major concern, besides toxicity effects of the antibacterial agents, is the effect of the two drugs when used together. Some drugs might not show synergistic or additive effect, rather show antagonism (the effect of the two drugs (or more) is smaller than the effect of the single-drug treatment) (15).

Other advocates for monotherapy would include the additional costs of using two or three drugs for the whole treatment course (19, 116). Monotherapy would lead to less exposure to unnecessary antibacterial agents for treatment, therefore prolonging emergence of resistance (125).

In short, there is no solid proof of the superiority of combination therapy over monotherapy, which is a good reason why some clinicians find combination therapy debatable (128, 129).

2. *Combination therapy*

Although clinically combination therapy has not shown clear evidence of its efficacy, however, more and more clinicians are tending to use combination therapy in attempt to treat carbapenemase producing organisms (27, 125). Despite the fact that there are several disadvantages, there are numerous advantages for the treatment with more than one drug. Colsitin, tigecycline, fosfomycin, rifampicin, and aminoglycosides have some limitations to their use alone (34, 125, 129, 130). Combination therapy has been proliferating as an interesting strategy to overcome the potential limitations of the previous drugs (125). Moreover, resistance to each of the previous drugs has been increasingly reported amongst

carbapenemase producing bacteria (34). In the light of such considerations, combination therapy for such organisms should be considered to increase the efficacy of the treatment (34), and prolong resistance (34, 125, 131). In addition, if synergism is detected between two antibacterial agents, their use in combination would lead to an increased death rate for carbapenem resistant bacteria (125, 131).

Another underlying principle for the use of combination therapy is using antibacterial agents with diverse mechanisms of action and/or resistance to ensure the coverage of a broad spectrum of activity (19, 131). An additional prospective benefit of combination therapy is the increased possibility of the infecting organism to be susceptible to at least one of the combination regimen (128).

Combination therapy is usually based on a cornerstone antibacterial agent for which the organisms presents susceptibility *in vitro*, and another adjuvant antibacterial agent for which the organism does not necessarily have to be susceptible to (125).

The most commonly used cornerstone antibacterial agents are the polymyxins, and specifically colistin (125), which probably increases the permeability of other drugs through the unstablized outer cell membrane (65, 115). The most frequently used adjuvant antibacterial agents are carbapenems, rifampicin, tigecycline, fosfomycin, and aminoglycosides (65, 116, 118, 125, 131).

Combination therapy with rifampicin and polymyxins is one of the most discussed alternatives for the treatment of MDR Gram negative bacterial infections (115, 116, 118). It has been demonstrated, *in vitro*, that the activity of colistin in combination with rifampicin was increased significantly (127). Some studies show that combination therapy with rifampicin has a synergistic effect (the effect of the two drugs (or more) is greater than the

effect of the single-drug treatment) on both drugs, where neither one alone was able to induce a bactericidal effect, until given in combination (116, 132) even if the organisms were resistant to rifampicin (34, 116).

Additionally, other studies showed that treatment with colistin and carbapenems to be the most effective regimen (34, 125). These combinations have shown to be competent even if the organisms were resistant to carbapenem (34, 116), while others report antagosistic effect of the colistin and carbapenem combination (131). Furthermore, some studies do not show synergism with the use of tigecycline and meropenem in combination, but a synergistic and bactericidal effect of tigecycline and colistin in combination (131). Likewise, studies found that better bactericidal activity was achieved when fosfomycin was used in combination with colistin, rather than fosfomycin monotherapy (131).

Other studies show a synergistic activity of colistin with other antimicrobial agents (ceftazidime, aztreonam) against extended-drug resistant organisms, and showed to be more effective than colistin monotherapy (27, 28, 116, 117). When compared to other drugs, colistin monotherapy may be inferior in the treatments of such resistant bacterial infections (125)

Moreover, most clinicians currently would hesitate to give a colistin monotherapy to treat severe infections caused by extended-drug resistant bacteria, because of the emergence of heteroresistance (116). Colistin should be used as combination to preserve its activity against such resistant organisms (118).

While several reports propose advantages of combination therapy over monotherapy, the clinical data are scarce, and indecisive. Most experiments that demonstrate the superiority of combination therapy were done *in vitro*; hence, more *in vivo* experiments and clinical trials are needed to better translate the effectiveness of the different combination regimens in

patients (131). Meanwhile, until a clear understanding of the disadvantages of combination therapy, rationally optimized combination therapy is highly promising and continues to be the standard of care in carbapenem resistant bacteria (125).

CHAPTER III

Materials and Methods

A. Collection of Bacterial Isolates:

Two carbapenem resistant *K. pneumoniae* isolates (IMP33 and IMP216) and one carbapenem resistant *E. coli* isolate (IMP53) previously collected at the Clinical Microbiology Laboratory (Department of Pathology and Laboratory Medicine) at AUBMC were used in this study. In addition, a fourth isolate, a *Salmonella* spp. (KPC) received from the Centers for Disease Control and Prevention (CDC) was included in this study. Isolates IMP33, IMP53, IMP216, and KPC harbor the *bla*CTX-M-15, *bla*OXA-48 and *bla*CTX-M-15, *bla*NDM-1, and *bla*KPC-2 genes respectively.

The isolates were previously stored at -80˚C in Brucella Broth containing 15% Glycerol, until the date of use. These isolates were cultured on MacConkey Agar (BBL, Becton, Dickinson and Company, Sparks, Maryland) plates and incubated at 37˚C under aerobic conditions for 24 hours. Subsequently, API20E (Biomérieux, SA69280 Marcy-l'Etoile, France) was performed to confirm the identity of the bacteria to species level.

B. Antimicrobial susceptibility testing:

1. ***Materials***

Antimicrobial susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The Kirby-Bauer Disk Diffusion agar method was used to determine resistance profiles on Mueller Hinton II Agar (MHA) (BBL, Becton, Dickinson

and Company, Sparks, Maryland). The antimicrobial susceptibility discs were chosen as follows: imipenem (10µg), meropenem (10µg), ertapenem (10µg), ceftazidime (30µg), cefepime (30µg), aztreonam (30µg), tigecycline (15µg), colistin (10µg), kanamycin (30µg), and fosfomycin (50µg).

2. *Procedure:*

Overnight cultured bacteria were inoculated in Mueller Hinton II Broth (MHB) (BBL, Becton, Dickinson and Company, Sparks, Maryland). The Optical Density (OD) was adjusted to 0.5 McFarland. Then, a confluent lawn of bacteria was swiped across the plate, and were allowed to dry for 5 minutes after which the antibacterial susceptibility discs was dispensed. The plates were incubated for 24 hours at 37˚C, then the zone of inhibition was measured around each disc. The disc susceptibility results were interpreted according to the CLSI breakpoints (133), except for tigecycline which was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (134). According to CLSI and EUCAST breakpoint for colistin and rifampicin have not been established yet. This test was performed in duplicates.

C. Synergy by Disc Susceptibility

After the results of each of the antimicrobial discs were measured, each combination used had its respective discs separated from each other by a distance of 20mm from center to center, (135, 136) to observe if there was an increase in the zone of inhibition. Afterwards, the distance was modified based on the previous results. The increase in the zone of inhibition between the 2 discs or the appearance of a line of inhibition of growth where the two

antibacterial diffusions meet, was considered as synergy (135, 136).

D. Modified Hodge Test:

Modified Hodge Test (MHT), was carried out as recommended by the CLSI guidelines. The OD of ATCC 25922 *E. coli* strain (indicator strain) was adjusted to 0.5 McFarland, followed by a 1:10 dilution to be used for the inoculation of a confluent lawn. The plates were allowed to dry for 5 minutes. Both Ertapenem and Meropenem discs were used in this test. The isolates in test were streaked in a straight line from the center of the disc to the edge of the plate. The results were observed after an overnight culture at 37˚C, and were examined for a "clover-leaf shape" zone of inhibition, that is a visible growth of the ATCC 25922 reference strain towards the carbapenem inhibition zone along the inoculum line of isolate which characterises a possible carbapenemase producer. The test was done in duplicates.

E. Remodified Hodge Test

Remodified Hodge Test (RMHT) was performed in parallel to the MHT, following the same procedure. However, the difference was the addition of 10µl of 50mM zinc sulfate to the carbapenem discs. The observation of the "clover-leaf shape" in the RMHT, but not in the MHT indicated a positive test for MBL production (69, 79, 154, 155). The test was done in duplicates.

F. Minimum Inhibitory Concentration Determination

The Broth dilution method was used to determine the MIC of the drugs used. Each drug dilution process included 18 tubes, tube#1 having the highest concentration and tube#17

having the lowest concentration, whereas tube#18 contained only bacteria and served as a positive control.

After the addition of 1ml of MHB into the tubes 1-17, each antibacterial agent was subjected to a two-fold serial dilution process as follows:

1) 1ml of the tested antibacterial agent was added in tube#1 and vortexed.
2) 1ml was withdrawn from tube#1 and added to tube#2.
3) Then, 1ml was withdrawn from tube#2 and added to tube#3.
4) Steps 2 and 3 were repeated till tube#17.
5) Lastly, 1ml was discarded from tube#17.

To prepare the bacterial solution, fresh bacterial cultures were inoculated in 2ml of MHB, adjusted to 0.5 McFarland ($\approx 10^8$CFU). This was followed by a 1:100 dilution, for a final bacterial count of 10^6CFU/ml. Next, 1ml was added to tube#1 till tube#18, reaching a total volume of 2ml and a final concentration of $5x10^5$ CFU (1:2 dilution). The tubes were incubated for 24 hours at 37˚C. The lowest concentration of the antimicrobial agent that did not show visible bacterial growth/turbidity in the tubes was considered as the MIC.

G. Minimal Bactericidal Concentration:

For each antibacterial agent, all tubes that did not show visible turbidity in the MIC assay, were used for the determination of the Minimal Bactericidal Concentration (MBC). A volume of 0.5ml was withdrawn from all the "clear" tubes and added to 2ml of fresh MHB. Afterwards, 0.2ml was withdrawn from each tube and inoculated onto MacConkey Agar plate and incubated overnight at 37˚. The above volumes were chosen taking into account that the

bacterial count in the tubes was $5x10^5$ CFU/ml. Thus, a final count of approximately $2x10^4$ CFU were present in the 0.2ml inoculated onto the plates.

MBC is defined as the lowest concentration of the antibacterial agent that kills 99.9% of the bacteria present in the original inoculum. Consequently, the agar plate for each antimicrobial agent that contained 20 colonies or less (0.1% of 2×10^4 CFU/ml) was considered as the MBC.

H. Genomic DNA extraction:

The illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, UK Limited Little Chalfont Buckinghamshire) was used for extraction of DNA, according to the protocol for "Purification of Genomic DNA from Gram negative Bacteria".

1. ***Materials used (provided by the kit)****:*

- Lysis buffer type 2
- Lysis buffer type 3
- Lysis buffer type 4
- Wash buffer type 6
- Elution buffer type 5
- Proteinase K enzyme

2. ***Protocol:***

a. Collection of bacterial culture

1. Few colonies from overnight grown bacteria were inoculated in Trypticase Soy Broth (Becton, Dickinson and Company, Sparks, Maryland) and were incubated overnight.

2. 1 ml from overnight cultured broth was transferred to 1.5ml microcentrifuge tube.
3. Afterwards, the tubes were spun for 30 seconds at 16000g, and the supernatant was removed without disturbing the pellet.

b. Lysis Stage:

1. 40µl of lysis buffer type 2 was added to each sample and immediately vortexed, until all the bacterial pellet was resuspended.
2. Then, 10µl of proteinase K was added to each sample, and was vortexed for 10 seconds.
3. After that, 10µl of lysis buffer type 3 was added to each tube, and was vortexed for 10 seconds, followed by a 5 second spin to collect the sample at the bottom of the tube.
4. Afterwards, the samples were incubated at 55ºC for 15 minutes; the samples were vortexed and spun for 5 seconds, halfway through (t=7mins) and at the end of the incubation time (t=15mins).

c. Purification Step:

1. 500µl of lysis buffer type 4 was added to each sample and was vortexed for 10 seconds.
2. Afterwards, the samples were incubated for 10 minutes at room temperature the samples half-through the incubation time and at the end were vortex and spun for 5 seconds at 1000xg.
3. Each sample was applied onto a mini spin column placed inside a collection tube, and was centrifuged for 1 minute at 11000xg.
4. The flow-through was discarded, and the column was placed inside a new collection tube.

d. Wash and Dry Step:

1. A 500µl of lysis buffer type 4 was added to the column and spun for 1 minute at 11000xg.
2. The flow-through was discarded, and the column was placed back in the collection tube.
3. Then, 500µl of wash buffer type 6 was added to the column, and was centrifuged for 3 minutes at 16000xg.
4. The collection tube was discarded and the column was transferred to a fresh DNase-free 1.5ml micro centrifuge tube.

e. Elution Step:

1. 200µl of low ionic strength Elution Buffer type 5, preheated to 70˚C, was added to the center of the column, and was incubated for 1 minute at room temperature.
2. To recover the purified genomic DNA as flow through, the samples were spun for 1 minute at 11000xg.

f. DNA concentration measurement and storage

- Using a spectrophotomer, the concentration of each DNA sample (20 µl of DNA diluted in 480 µl distilled water) was measured at an absorbance of 260 nm.
- The purified eluted genomic DNA was aliquoted and stored at -20˚C until further use.

I. Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR) amplifications were carried out to confirm the presence of the genes of interest. These genes were *bla*NDM-1, *bla*KPC-2, *bla*OXA-48, *bla*CTX-M-15,

*bla*TEM-1, *omp*C, and *omp*F. The PCR amplifications for these genes were done separately on the extracted genomic DNA, using standard PCR protocol.

1. *Materials used:*

- 10x Taq DNA polymerase buffer with $(NH4)_2SO_4$ (Fermentas, USA)
- 25 µM Magnesium chloride (Fermentas, USA)
- 2 mM dNTPs (Fermentas, USA)
- Nuclease free water (Amresco, USA)
- 5U/µl Taq DNA polymerase stored in buffer containing 0.1mM EDTA, 50mM tris HCL, 5mM dithiothereitol stabilizers and 50% glycerol (Fermentas, USA)
- The extracted DNA (diluted to become 10 µg/ml)
- The following primers:

-TEM-1 Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5'-ATGAGTATTCAACATTTCCG-3', and reverse primer: 5'-CCAATGCTTAATGAGTGAGG-3', with an amplicon size of 836 base pair (bp).

-OMP-C Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5'-GTTAAAGTACTGTCCCTCCTG-3', and reverse primer: 5'-GAACTGGTAAACCAGACCCAG-3', with an amplicon size of 1086 bp.

-OMP-F Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5'-CAGGTACTGCAAACGCTGC-3', and reverse primer: 5'-GTCAACATAGGTGGACATG-3', with an amplicon size of 953 bp.

-OXA-48 Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5‘-TTGGTGGCATCGATTATCGG-3‘, and reverse primer: 5‘-GAGCACTTCTTTTGTGATGGC-3‘, with an amplicon size of 744 bp.

-NDM-1 Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5‘-GGAAACTGGCGACCAACG-3‘, and reverse primer: 5‘-ATGCGGGCCGTATGAGTGA-3‘, with an amplicon size of 678 bp.

-CTX-M-15 Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5‘-GGTTAAAAAATCACTGCGTC-3‘, and reverse primer: 5‘-TTACAAACCGTCGGTGACGA-3‘, with an amplicon size of 874 bp.

-KPC-2 Primer (Thermo Scientific Inc., USA):

Forward primer had a sequence of: 5’-GCAGCGGCAGCAGTTTGTTGATT-3’, and reverse primer: 5’-GTAGACGGCCAACACAATAGGTGC-3’, with an amplicon size of 184 bp.

2. *Reaction mixtures:*

For each of the PCR amplifications the following conditions were used (except for the *bla*NDM-1 gene amplification):

- 2.5µl of DNA template, with a final concentration of 10µg/l
- 2.5µl of each primer (forward and reverse), with a final concentration of 1µM in solution
- 0.25µl of taq DNA polymerase with a final concentration of 1.25U in the 50µl volume
- 5µl of 10X taq polymerase buffer (Fermentas Life Sciences)
- 4µl of MgCl2 with a final concentration of 2.5mM

- 5µl of deoxynucleoside triphosphate (dNTP) with a final concentration of 0.25 mM (Fermentas Life Sciences)
- 28.25µl of Nucleasefree water

As for *bla*NDM-1, in order to increase the sensitivity of the reaction, the conditions were optimized as follows:

- 5µl of DNA template, with a final concentration of 10µg/l
- 5µl of each primer (forward and reverse) with a final concentration of 2µM in solution
- 0.25µl of taq DNA polymerase
- 5µl of 10X taq polymerase buffer (Fermentas Life Sciences)
- 4µl of MgCl2 with a final concentration of 2.5mM
- 5µl of deoxynucleoside triphosphate (dNTP) with a final concentration of 0.25 mM (Fermentas Life Sciences)
- 20.75µl of Nuclease-free water

3. *PCR conditions:*

PCR reaction mixtures for each gene were done in a PCR thermal cycler (PCR Sprint Thermal Cycler, Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions for all the genes were taken from previous in-house optimizations and were as follows:

- The cycling program for the *bla*NDM-1 gene: initial denaturation at 95ºC for 7mins for 1 cycle, 35 cycles of denaturation at 95ºC for 45secs, annealing at 60ºC for 45secs, elongation at 72ºC for 45secs, and a final extension cycle at 72ºC for 7mins.

- The cycling program for the *bla*KPC-2 gene: initial denaturation at 95ºC for 5mins for 1 cycle, 35 cycles of denaturation at 95ºC for 1min, annealing at 58ºC for 30secs, elongation at 72ºC for 30secs, and a final extension cycle at 72ºC for 10mins.
- The cycling programs for the *omp*C and *omp*F genes respectively were: initial denaturation at 95ºC for 7mins for 1 cycle, 35 cycles of denaturation at 95ºC for 45secs, annealing at 50ºC for 45secs for *omp*C and 56ºC for 45secs for ompF, elongation at 72ºC for 1min, and a final extension cycle at 72ºC for 10mins for *omp*C and 72ºC for 7mins for *omp*F.
- The cycling programs for the *bla*CTX-M-15 and *bla*TEM-1 genes were: initial denaturation at 94ºC for 10mins for 1 cycle, 35 cycles of denaturation at 94ºC for 30secs, annealing at 50ºC for 40secs, elongation at 72ºC for 1min, and a final extension cycle at 72ºC for 10mins.
- The cycling program for the *bla*OXA-48 gene was as follows: initial denaturation at 94ºC for 7mins for 1 cycle, 35 cycles of denaturation at 94ºC for 45secs, annealing at 56ºC for 45secs, elongation at 72ºC for 1min, and a final extension cycle at 72ºC for 10mins.

The PCR products were stored at 4˚C until they were used.

J. Gel Electrophoresis:

1. *Materials used:*

- 10x TBE (Tris Base (108g) + Boric acid (55g) + EDTA disodium (9.3g), added to 1 L of distilled water) (Amresco, USA)

- Seakem Agarose Powder (Lonza, USA)
- 0.625 mg/ml Ethidium bromide (Amresco, USA)
- 6x Loading dye (Fermentas, USA)
- 100 bp DNA ladder (Fermentas, USA)
- 50 bp DNA ladder (Fermentas, USA)

2. *Procedure:*

The gel was prepared by mixing 1.5g of agarose powder Seakem Agarose Powder (Lonza, USA) into 100 ml of 1×TBE buffer diluted from the 10x buffer. Afterwards, the gel was microwaved for approximately 2 minutes, in order to completely dissolve the agarose powder and 2 drops of 0.625 mg/ml ethidium bromide (Amresco, Solon, OH 44139, USA) were added. Then, the liquid gel was poured into the casting tray placed in the electrophoretic chamber and allowed to solidify for about 30 minutes. Then, 900ml of 1xTBE was added into the chamber to fully submerge the solidified gel, before loading the samples into the wells.

A ladder was run in parallel to the samples serving as a size marker for the amplicons. In the first column of the gel, a 100bp ladder was used with the PCR products greater than 300bp, while PCR products less than 300bp, both 100bp and 50bp ladders were used. 2µl of the ladder, 2µl of loading dye (Fermentas USA), and 8µl of 1xTBE were mixed together before loading into the well. However, to load the samples, 10µl of PCR product was mixed with 2µl of loading dye. Afterwards, the gel was run for approximately 45 minutes at 120V. Ultraviolet (UV) transilluminator (Haake buchler Instruments inc., USA) and Olympus digital

camera using the Digi-Doc it Program were used for visualizing and photographing the bands respectively.

K. RNA extraction

RNA extraction was done using the IIustra RNAspin Mini RNA Isolation Kit (GE healthcare, UK) according to the manufacturer's specifications for bacterial cells.

1. *Materials used:*

- TE buffer (Amresco,USA)
- Lysozyme (USB, USA)
- β-mercaptoethanol
- 70% Ethanol
- Ribolock RNase inhibitor (Fermentas, USA)
- DNase I
- Buffer RA1 (with kit)
- Membrane Desalting Buffer (MDB) (with kit)
- DNase Reaction Buffer (with kit)
- Buffer RA2 (with kit)
- Buffer RA3 (with kit)
- RNase free water (with kit)

2. *Preparation of samples for RNA extraction:*

RNA extraction, for *in vitro* samples, was performed on the 4 isolates. Each isolate had 3 different protocols for the preparation of the bacterial suspension. The extractions were run in duplicates.

a) Protocol 1: In tube #1 for each of the isolates, 3ml of the bacterial suspension was inoculated. The bacterial suspension was prepared by a 1:100 dilution of 0.5 McFarland, to reach a total of 1×10^6 CFU/ml, which was used for the rest of the protocols.
b) Protocol 2: In tubes #2, 3, 4 and 5; 1.5ml of the bacterial suspension was added to 1.5ml of the MIC dose of colistin, rifampicin, tigecycline, and meropenem respectively.
c) Protocol 3: In tubes #6, 7, 8, and 9; 1ml of the bacterial suspension was added to 1ml of the MIC dose of colistin, in addition to 1ml of the MIC dose of meropenem, fosfomycin, rifampicin, and tigecycline respectively.

Then, the tubes were incubated for 18 hours at 37˚C. The samples were adjusted to have the same bacterial concentrations in all the tubes, a total of 3.33×10^5 CFU/ml.

Note: The MIC levels were diluted by half, and therefore the total concentration was MIC/2.

RNA extraction, for *in vivo* samples, was performed on the 4 isolates as well. Each isolate had 4 different protocols for the preparation of the bacterial suspension. A total number of 32 mice

were used. Each mouse was injected with 3xLD_{50} dose. The protocols for each of the isolates were as follows:

a) Protocol 1: 2 mice were injected with 0.2 ml of the bacterial suspension.
b) Protocol 2: 2 mice were injected with 0.2 ml of the bacterial suspension, followed by 0.1ml of the MIC dose of colistin.
c) Protocol 3: 2 mice were injected with 0.2 ml of the bacterial suspension, followed by 0.1ml of MIC doses of each of colistin and meropenem.
d) Protocol 4: 2 mice were injected with 0.2 ml of the bacterial suspension, followed by 0.1ml of MIC doses of each of colistin and fosfomycin.

For all the protocols, all bacterial injections were given at t=0hrs, while the drug was given at t=1hr. After 4 hours from the antibacterial agent injection (t=5hrs), the mice were euthanized, dissected, and blood was collected from the heart. The 2 mice from the same protocol had their blood pooled into one centrifuge tube. Afterwards, the tubes were centrifuged for 20 minutes at 4˚C, serum was collected and the extraction was run according to the manufacturer's specifications for bacterial cells. The extractions were run in duplicates.

3. *RNA extraction protocol:*

a. Cell lysis and Homogenization

- For the tubes that showed turbidity, 1.5ml was withdrawn and transferred to a microcentifuge tube. While for the tubes that did not show any turbidity, the whole volume was withdrawn and placed in centrifuge tubes. Afterwards, the tubes where centrifuged at maximum speed for 15 minutes.

- The supernatant from each sample was discarded without disturbing the bacterial cell pellet. The latter was resuspended in 100µl of TE buffer containing 0.2mg/ml lysozyme. After applying vigorous vortexing to each of the tubes, the tubes were incubated at 37˚C for 10 minutes.
- Afterwards, 350µl of RA1 buffer and 3.5µl of β-mercaptoethanol were added to each tube.

b. Filtration of lysate

- The mixture was transferred to a violet RNAspin Mini filter unit placed in a collection tube and was centrifuged for 1 minute at 11,000xg (equivalent to 12,800 rpm).
- The filtrate was then transferred to a new 1.5ml microcentrifuge tube and the RNAspin Mini filter unit was discarded.

c. RNA binding in adjusted conditions

- 350µl of previously prepared 70% ethanol was added to each filtrate. The mixture was mixed by pipetting up and down prior to the transfer to a Blue RNAspin Mini column placed in a new collection tube.
- The samples were centrifuged for 30 seconds at 8,000xg and the column was placed in a new collection tube.

d. Desalt silica membrane and DNA digestion

- To each column, 350µl of Membrane Desalting Buffer (MDB) was added. Next, the samples were centrifuged for 1min at 11,000xg to dry the membrane.
- Afterwards, the filtrate was discarded and the column was returned to the same collection tube.

- A DNase reaction mixture was prepared by adding 10µl reconstituted DNase I to 90µl DNase reaction buffer (per sample). The solution was mixed by flicking the tube several times.
- For each sample, 95µl of the DNase reaction mixture was added directly to the center of the column. The samples were incubated at room temperature for 30 minutes.

e. Washing and Drying

- To each of the RNA spin Mini column, 200µl of buffer RA2 was added, followed by centrifugation for 1 minute at 11,000xg. The column was placed into a new collection tube.
- A 600µl of buffer RA3 was added to each RNA spin Mini column, and then centrifuged for 1 minute at 11,000xg. The filtrate was discarded and the column was returned to the same collection tube.
- A 200µl of buffer RA3 was added to each RNA spin Mini column, the samples were centrifuged for 2 minute at 11,000xg. The column of each sample was transferred into a nuclease free 1.5 ml microcentrifuge tube.

f. Elution and Aliquoting

- A 60 µl RNase free water was added to the samples, followed by centrifugation for 1 minute at 11,000xg to elute the RNA.
- Immediately after the elution of the RNA, the tubes were placed on ice to prevent potential degradation.
- 1µl Ribolock RNase inhibitor was added to each sample. Then, 3 aliquots of 20µl were prepared and stored at -80˚C for further use.

g. RNA concentration determination

- Using a spectrophotomer, the concentration of the RNA samples (20 µl of RNA diluted in 480 µl distilled water) was measured at an absorbance of 260 nm.

N. Reverse Transcription and cDNA synthesis

The QuantiTect Reverse Transcription Kit (Qiagen, Germany) was used, according to the manufacturer's specifications, for the production of cDNA from the previously extracted RNA. The cDNA was synthesized to be used in RT-qPCR (Reverse Transcriptase – qualitative PCR).

1. *Materials used:*

- Extracted RNA
- gDNA Wipeout Buffer (with kit)
- RNase free water (with kit)
- RT primer Mix, containing oligo-dT dissolved in water (with kit)
- Quantiscript RT Buffer, containing dNTPs and Mg^{2+} (with kit)
- Quantiscript Reverse Transcriptase, containing RNase inhibitor (with kit)

2. *Protocol:*

The 2 major steps involved in this protocol are: elimination of genomic DNA (gDNA) and reverse transcription. All the reagents and the RNA samples were kept on ice, while performing the procedure.

a. Elimination of genomic DNA (gDNA)

A concentration of 0.1µg of RNA was chosen to produce the cDNA. The RNA samples were diluted using RNase free water, so that the volume of RNA and RNase free water in total was 12µl. Afterwards, 2µl of gDNA wipeout buffer was added to each samples, hence a total reaction volume of 14µl. The samples were then incubated at 42ºC for 2 minutes afterwards they were placed immediately on ice.

b. Reverse Transcription Reaction

The preparation of a master mix which contained 1µl of RT Primer Mix (per sample), 1µl of Quantiscript reverse Transcriptase (per sample), and 4µl of Quantiscript RT buffer (per sample), and was carried out on ice. Next, 6µl of the master mix was mixed with the 14µl mixture of each sample prepared from the previous step. In total, the reaction volume for each sample was 20µl.

Afterwards, the samples were placed in a thermal cycler to manage incubation conditions of: 15 minutes at 42ºC followed by 3 minutes at 95ºC. Lastly, the cDNA were stored at -80ºC until further use.

O. Real time Polymerase Chain Reaction (RT-qPCR)

To examine the relative expression of the *bla*NDM-1, *bla*OXA-48, *bla*KPC-2, and *bla*CTX-M-15 genes, *in vitro* and *in vivo*, the QuantiFast™ SYBER® green PCR kit (Qiagen, Germany) was used. The expression of the *bla*CTX-M-15, *bla*OXA-48 and *bla*CTX-M-15, *bla*NDM-1, and *bla*KPC-2 genes were tested in the isolates IMP33, IMP53, IMP216, and KPC respectively.

The primers that were used for this PCR were reconstituted in specific volumes of TE according to the manufacture's guidelines.

1. ***Materials used:***

- cDNA
- RNase free water (with kit)
- The Real time primers with a concentration of 5 µM:

  -CTX-M-15 Primer (Thermo Scientific Inc., USA):

  Forwards Primer: 5'-GCGTGATACCACTTCACCTC-3', Reverse Primer: 5'-TGAAGTAAGTGACCAGAATC-3', with an amplicon size of 260 bp.

  -OXA-48 Primer (Thermo Scientific Inc., USA):

  Forwards Primer: 5'- TTCGGCCACGGAGCAAATCAG-3', Reverse Primer: 5'-GATGTGGGCATATCCATATTCATCGCA-3', with an amplicon size of 240 bp.

  -NDM-1 Primer (Thermo Scientific Inc., USA):

  Forwards Primer: 5'- TTGGCGATCTGGTTTTCC-3', Reverse Primer: 5'-GGTTGATCTCCTGCTTGA -3', with an amplicon size of 195 bp.

  -KPC-2 Primer (Thermo Scientific Inc., USA):

  Forwards Primer: 5'-GCAGCGGCAGCAGTTTGTTGATT-3', Reverse Primer: 5'-GTAGACGGCCAACACAATAGGTGC -3', with an amplicon size of 184 bp.

  -rpoB Primer (Thermo Scientific Inc., USA) (reference gene):

  Forwards Primer: 5'- TCGAAACGCCTGAAGGTC-3', Reverse Primer: 5'-TTGGAGTTCGCCTGAGC -3', with an amplicon size of 184 bp.

- QuantiFast SYBR Green PCR master mix (with kit) included:

  - dNTP mix

  - SYBR Green I

  - HotStar Taq® Plus DNA polymerase which will be activated by the 5 minute 95˚C incubation step

  - QuantiFast SYBR Green PCR buffer containing Tris-Cl, KCl, NH_4Cl, $MgCl_2$, and additives.

2. *Protocol:*

- All the samples and reagents used in this experiment were thawed on ice.
- In order to have equal concentrations between all the samples, the concentration of the cDNA samples for each run was adjusted using PCR grade water.
- Two different Master Mixes for each RT-qPCR run were prepared, one for the gene in question (*bla*CTX-M-15, *bla*OXA-48, *bla*NDM-1, *bla*KPC-2) and another for the housekeeping gene (*rpoB* gene). Each master mix included: 10µl of QuantiFast SYBR Green PCR master mix, 4µl of RNase free water, 2µl Forward primer, and 2µl Reverse primer, per sample. The total volume of master mix per sample was 18µl.
- A 96-well-plate was used for each run. In each well, 18µl from the master mix for the corresponding gene. Afterwards, 2 µl of the cDNA samples was added into the wells, for a total volume of 20 µl. Moreover, each sample was run in duplicates for the gene in inquiry and the housekeeping gene.

- As a final step, the wells were sealed and the plate was centrifuged for 5 seconds to get rid of bubbles.
- The Bio-Rad CFX96 Real Time System C1000 Thermal Cycler (Bio-Rad, Germany) was used for the Real time runs; and the cycling condition steps for each primer were as follows:
    1. 1 cycle of 95ºC for 15 minutes (for initial sample denaturation and enzyme activation)
    2. 45 cycles of : 95ºC for 10 seconds (denaturation)

       Ta for 30 seconds (annealing)

       72ºC for 20 seconds (elongation/extension)
    3. Melt curve 40˚C to 95˚C, increment 0.5˚C for 5 seconds (melt curve analysis)
    4. 12˚C for 5 minute.
- The expression levels of the genes in question were calculated for the samples treated with antimicrobial agents compared to samples without antimicrobial agent, employing the reference gene *rpoB* as a standard, and using the Bio Rad CFX manager software.

3. *Statistical Analysis:*

Statistical analysis was done for the transcript levels of the *bla*CTX-M-15, *bla*OXA-48, *bla*NDM-1, *bla*KPC-2 genes using the unpaired student t- test. All p-values ≤ 0.05 were considered to be statistically significant.

N. Determination of LD_{50} of IMP33, IMP53, IMP216, and KPC isolates in BALB/c Mice:

The mice used in this study were obtained from the Animal Care Facility, after the approval from the Institutional Animal Care and Use Committee (IACUC) at AUB. The mice in use were BALB/c, adult female, ranging between 6-8weeks old and weighing between 20-30g. A total number of 339 mice were used. The mice were cared for and handled according to "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources Committee on Life Sciences Nation Research Council (137). In addition, the mice were allowed to consume food and water as much as desired without any restrictions.

The LD_{50} of IMP33, IMP53, IMP216, and KPC in BALB/c mice was identified using the method described by Nowotny (138). For each isolate, a total of 24 mice were distributed into 6 groups, which received incremental doses of the respective bacteria. The doses included were: 10^3, 10^4,10^5,10^6,10^7, and 10^8 CFU suspended in 0.2 ml TSB. The mice were monitored for survival for a period of 7 days. The LD_{50} was determined using the formula:

Log LD_{50} = log (highest dose tested) + (log D)[(1/2) – (ΣR/N)] where D is the fold difference between successive doses, ΣR is the total number of dead mice after 7 days of monitoring, and N is the number of animals per group.

O. Treatment of infected BALB/c mice using antimicrobial agents for assessment of efficacy

1. *Mice groups*

A total number of 85 mice was used for each of IMP33 and IMP53 isolates, 75 mice for IMP216 isolate, and 94 mice for the KPC isolate. The groups were numbered as Groups I-IV respectively. The varying number of mice between groups was due to obtaining equivalent MIC and MBC doses with some of the antibacterial agents. The mice were divided so that

each group contained 3 mice. The injections were calculated so as the maximum total volume to be administered per mouse would not exceed 0.5 ml. Those injection volumes were: 0.2 ml TSB containing 3x LD_{50} of either IMP33, IMP53, IMP216, or KPC, 0.1ml TSB for the negative control injections, and 0.1 ml for all the antibacterial injections. The antibacterial agents that were used were, ceftazidime, colistin, ertapenem, imipenem, meropenem, fosfomycin, kanamycin, tigecycline, rifampicin, aztreonam, and the β-lactamase inhibitor Tazobactam. In addition, all the injections were administered intraperitoneally. The first injection administered was the bacterial injection at t=0hr. Followed by a second injection, which was the treatment regimen, given at t=1hr. The mice were monitored for weight loss and survival for a period of 10 days. Table 6 shows the distribution of mice into various groups with different treatment regimens for each subgroup.

2. *Preparation of bacterial suspension injections:*

The bacterial dose administered for infection in the mice was 3xLD_{50} of either IMP33, IMP53, IMP216, or KPC. To prepare the bacterial injections, fresh isolated colonies were inoculated in 3ml TSB and incubated overnight at 37ºC. On the next day, a turbidity meter was used to determine the concentration (CFU/ml) of the bacterial suspension. A volume was transferred to a centrifuge tube and spun for 20 minutes at 3500 rpm. Following that, the supernatant was discarded, and the pellet was resuspended in a volume of TSB corresponding to the total volume of bacterial injections needed (0.2 ml/bacterial injection). Afterwards, 1.0ml fine syringes were filled with 0.2 ml each.

3. *Preparation of antibacterial agents' injections:*

The therapeutically relevant *in vivo* MIC equivalent dose of the antimicrobial agents used was extrapolated from their *in vitro* MIC according to the following formula:

-Antimicrobial agent *in vivo* MIC dose (µg) = [Antimicrobial agent *in vitro* MIC (µg/µl) x *in vitro* MIC broth volume (µl) x concentration (CFU) of the isolate administered *in vivo*] / concentration (CFU) of the isolate per *in vitro* MIC reaction.

Similarly, therapeutically relevant *in vivo* MBC equivalent dose of the antimicrobial agents used was extrapolated from their *in vitro* MBC according to the following formula:

-Antimicrobial agent *in vivo* MBC dose (µg) = [Antimicrobial agent *in vitro* MBC (µg/µl) x *in vitro* MBC broth volume (µl) x concentration (CFU) of the isolate administered *in vivo*] / concentration (CFU) of the isolate per *in vitro* MBC reaction.

4. ***Mice Dissection and API***

During the monitoring period, 1 dead mouse from each group was dissected. Blood was collected from the heart, and then cultured on MacConkey agar plates to check for the presence of the respective isolate. After that, the colonies grown on the plates were identified to the species level using API20E kit, which allowed the verification of the cause of death being due to the particular isolate.

5. *Statistical Analysis*

Statistical analysis using the Kaplan-Meier curves (PASW Statistics 18), was done on the mouse survivals rates for each of the groups injected with the different isolate.

CHAPTER IV

RESULTS

A. Disc Diffusion, MIC, and MBC:

The results for the disc diffusion susceptibility profiles for all the isolates are shown in Table 1. Whereas, the results for the Broth dilution method susceptibility profiles for all the isolates are shown in Table 3.

B. Synergy by Disc Diffusion

The synergy results for all the combinations tested by the disc diffusion method are shown in Table 2. The most frequent synergies were detected between the combinations of colistin with either meropenem, fosfomycin, or tigecycline, and between the combination of ertapenem with either imipenem or meropenem. There were no detected antagonisms between the different combinations.

C. Modified Hodge Test

There was a visible growth of the ATCC 25922 reference strain towards the carbapenem inhibition zone along the inoculum line of isolates IMP53 and KPC, indicating positive results for these isolates. Both isolates IMP33 and IMP216 did not show any indentation or visible growth along the inoculum line. The results for MHT are shown in Table 4.

D. Remodified Hodge Test

Likewise, there was a visible growth of the ATCC 25922 reference strain towards the carbapenem inhibition zone along the inoculum line of isolates IMP53 and KPC, and more importantly IMP216 (an NDM-1 producer) indicating positive results for these isolates. The results for RMHT are shown in Table 4.

E. Detection of resistance and porin encoding genes by PCR

The presence of the expected respective genes were confirmed:

- IMP 33: *bla*CTXM-15, *bla*TEM-1, and *omp*C were detected.
- IMP53: *bla*CTXM-15, *bla*OXA-48, *bla*TEM-1, *omp*C, and *omp*F were detected.
- IMP216: *bla*NDM-1, *bla*TEM-1, and *omp*F were detected.
- KPC: *bla*KPC-2, *bla*TEM-1, and *omp*C were detected.

The results for detection of resistance and porin genes for each isolate are shown in Table 5. Whereas the images of the gels for each gene are shown in Figures 1.a-1.f.

F. Gene Transcript Levels by RT-qPCR

The *in vitro* transcript levels for the ESBL and the carbapenemase encoding genes *bla*CTX-M-15, *bla*OXA-48, *bla*NDM-1, and *bla*KPC-2 in the isolates IMP33, IMP53, IMP216, and KPC tested with antibacterial agents singly, in comparison to their control (bacteria grown alone) respectively, are shown below;

- For testing with colistin, the transcript levels were: 4.94 ($p=0.025$), 0 ($p=0.875$), 10.20 ($p=0.072$), 1.23 ($p=0.005$) times respectively.

- For testing with meropenem, the transcript levels were: 15.54 (p=0.376), 0.36 (p=0.746), 16.79 (p=0.046), 9.89 (p=0.198) times respectively.
- For testing with rifampicin, the transcript levels were: 0 (p=0.002), N/A, 1.47 (p=0.049), 0.23 (p=0.001) times respectively.
- For testing with tigecycline, the transcript levels were: 0.07 (p=0.038), 0.04 (p=0.001), 2.60 (p=0.002), 0.56 (p=0.043) times respectively.

The *in vitro* transcript levels for the ESBL and the carbapenemase encoding genes *bla*CTX-M-15, *bla*OXA-48, *bla*NDM-1, and *bla*KPC-2 in the isolates IMP33, IMP53, IMP216, and KPC tested with combination of antibacterial agents, in comparison to their control (bacteria grown alone), respectively, are shown below:

- For testing with colistin and meropenem, the transcript levels were: 16.95 (p=0.12), 0 (p=0.841), 5.14 (p=0.054), 0 (p=0.011) times respectively.
- For testing with colistin and fosfomycin, the transcript levels were: 9.87 (p=0.487), 0 (p=0.007), 14.91 (p=0.002), 4.93 (p=0.032) times respectively.
- For testing with colistin and rifampicin, the transcript levels were: 0 (p=0.001), 0 (p=0.045), 4.14 (p=0.002), 0 (p=0.012) times respectively.
- For testing with colistin and tigecycline, the transcript levels were: 0 (p=0.125), 0 (p=0.001), 1.38 (p=0.372), 0 (p=0.085) times respectively.

The *in vivo* transcript levels of the ESBL and the carbpenemase encoding genes *bla*CTX-M-15, *bla*OXA-48, *bla*NDM-1, and *bla*KPC-2 in the isolates IMP33, IMP53, IMP216, and KPC treated with antibacterial agents singly or in combination, in comparison to their control (bacteria grown alone) respectively;

- Treatment with colistin, the transcript levels were: 5.40 (p=0.593), 3.53 (p=0.822), 2.22 (p=0.430), 0.53 (p=0.216) times respectively.
- Treatment with colistin and meropenem, the transcript levels were: 1.38 (p=0.938), 0 (p=0.841), 3.10 (p=0.217), 1.49 (p=0.980) times respectively.
- Treatment with colistin and fosfomycin, the transcript levels were: 0.19 (p=0.124), 0 (p=0.996), 1.44 (p=0.052), 0.75 (p=0.429) times respectively.

The levels for each of the carbapenemase encoding gene, for the respective isolates are shown in Figure 2.a-2.e.

G. LD_{50}

The LD_{50} of the 4 isolates was determined to be $5.62x10^5$ CFU, $1x10^7$ CFU, $1.47x10^7$ CFU, and $1x10^8$ CFU for isolates IMP33, IMP53, IMP216, and KPC respectively.

H. Mice Observations

The survivals and the average weight loss for each group, during the 10 day monitoring period, injected with $3\times LD_{50}$ and exposed to the different antibacterial therapies are shown in Appendix 1-4.

All the mice in the negative control subgroups survived the monitoring period. On the other hand, the death rate in the positive control subgroups was 66% for Group I injected with isolate IMP33, 33% for Group III injected with isolate IMP216, and 100% in Groups II and IV injected with IMP53 and KPC respectively.

During the monitoring period, the highest survival rate of 100% was observed in Group I subgroups injected with isolate IMP33 followed by treatment (Figure 3.a.). Similarly, 97.1% survival rate in Group III subgroups injected with isolate IMP216 followed by treatment (Figure 3.c.). On the other hand, the survival rate for Group II subgroups injected with isolate IMP53 followed by treatment was 68% (Figure 3.b.), while the lowest survival rate of 60.7% was observed in the subgroups that were injected with the KPC isolate followed by treatment (Figure 3.d.).

During the monitoring period, the average weight of the mice in the negative control subgroups for the four isolates increased, while that of the positive control subgroups decreased within the first four days, after which they started gaining weight till the endpoint. For Group I, II and IV, the mice that survived showed weight gain after 5 days of receiving the respective bacterial and treatment injections (Figures 4.a, b & d). As for Group III, the mice that survived showed stability and increase in their weight (Figure 4.c).

The API testing confirmed that the cause of death was due to the respective bacterial injections; i.e. *K. pneumonia* for isolates IMP33 and IMP216, *E. coli* for isolate IMP53, and *Salmonella* spp for KPC isolate.

Table 1: Antimicrobial susceptibility profiles of IMP33, IMP53, IMP216, and KPC isolates as determined by Disk Diffusion Method (values are in mm); FEP: cefepime, CAZ: ceftazidime, ATM: aztreonam, ETP: ertapenem, MEM: meropenem, IPM: imipenem, DOR: doripenem, FOS: fosfomycin, K: kanamycin, TGC: tigecycline, CT: colistin, RO: rifampicin.

	FEP		CAZ		ATM		ETP		MEM		IPM		DOR		FOS		K		TGC		CT		RO	
IMP33	27	S	15	R	6	R	18	R	33	S	30	S	31	S	28	S	13	R	27	S	15	NE	6	NE
IMP53	6	R	6	R	6	R	15	R	19	I	20	I	24	S	32	S	13	R	26	S	16	NE	19	NE
IMP216	6	R	6	R	6	R	6	R	6	R	6	R	6	R	18	S	6	R	21.8	S	8	NE	6	NE
KPC	20	S	13	R	6	R	18	R	35	S	25	S	25	S	34	S	26	S	35	S	16	NE	6	NE

S: susceptible, I: intermediate resistance, R: resistant, NE: not established.

Table 2: Synergy results of IMP33, IMP53, IMP216, and KPC isolates as determined by Double Disc Diffusion Method. CAZ: ceftazidime, ATM: aztreonam, ETP: ertapenem, MEM: meropenem, IPM: imipenem, DOR: doripenem, FOS: fosfomycin, K: kanamycin, TGC: tigecycline, CT: colistin, RO: rifampicin. "+": an increase in zone of inhibition between the two discs, or a line of inhibition were the antibacterial diffusions meet, "-": no increase in zone of inhibition between the two discs, or a line of inhibition were the antibacterial diffusions meet.

Comb / Isolate	CT + DOR	CT + IPM	CT + ETP	CT + MEM	CT + ATM	CT + CAZ	CT + FOS	CT + K	CT + RO	CT + TGC	FOS + K	IPM + ETP	IPM + MEM	ETP+ MEM	K + CAZ
IMP33	+	+	+	+	-	-	+	+	+	+	-	+	+	+	-
IMP53	-	-	-	-	-	-	+	-	-	+	+	+	-	+	-
IMP216	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-
KPC	-	+	-	+	+	+	-	-	+	-	+	+	+	+	+

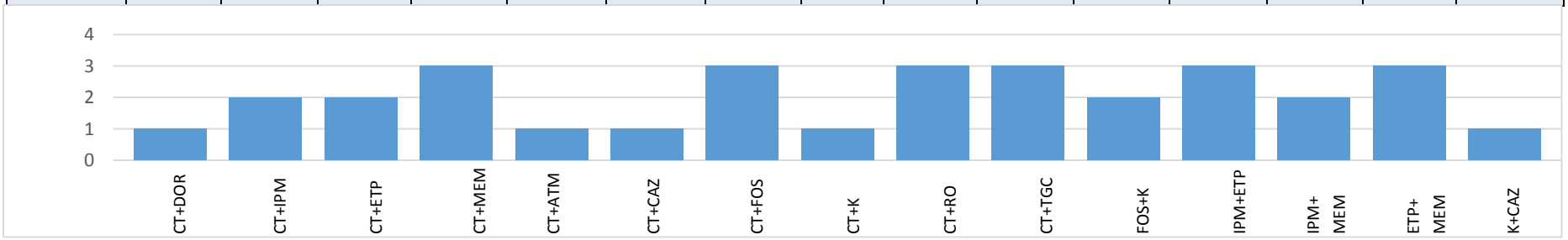

Table 3: MICs and MBCs (in µg/ml) of IMP33, IMP53, IMP216, and KPC isolates as determined by Broth Dilution Method. TZ: ceftazidime, ETP: ertapenem, IPM: imipenem, MEM: meropenem, ATM: aztreonam, FM: fosfomycin, K: kanamycin, CO: colistin, RO: rifampicin, TGC: tigecycline

ATB / Isolate	Dose	TZ		ETP		IPM		MEM		ATM		FM		K		CO		RO		TGC	
IMP33	MIC	128	R	2	R	2	I	0.125	S	2048	R	4096	R	64	R	2	S	32	N	8	R
	MBC	512	R	16	R	4	R	0.125	S	4096	R	8192	R	256	R	2	S	256	N	512	R
IMP53	MIC	1024	R	32	R	8	R	4	R	4096	R	512	R	128	R	2	S	8	N	0.5	S
	MBC	2048	R	32	R	16	R	4	R	>4096	R	512	R	256	R	2	S	8	N	128	R
IMP216	MIC	4096	R	512	R	1024	R	64	R	1024	R	>1024	R	>16384	R	128	R	128	N	4	R
	MBC	>4096	R	1024	R	2048	R	64	R	2048	R	>1024	R	>16384	R	128	R	1024	N	1024	R
KPC	MIC	128	R	4	R	4	R	4	R	128	R	256	R	32	I	2	S	8	N	0.5	S
	MBC	2048	R	8	R	16	R	16	R	256	R	256	R	32	I	4	R	32	N	128	R

S: susceptible, I: intermediate resistance, R: resistant, NE: not established.

Table 4: MHT and RMHT results of the four isolates IMP33, IMP53, IMP216, and KPC isolates. "-": no indentation, "+": positive indentation, "++": enhanced indentation.

MHT	Result
IMP33	-
IMP53	+
IMP 216	-
KPC+	+

RMHT	Result
IMP33	-
IMP53	+
IMP 216	++
KPC+	+

Table 5: Gene distribution among the isolates, IMP33, IMP53, IMP216, and KPC isolates; "pos": positive amplification for the corresponding gene, "neg": no amplification.

Gene / Isolate	NDM-1	CTXM-15	KPC-2	OXA-48	TEM-1	OMPF	OMPC
IMP33	neg	pos	neg	neg	pos	neg	pos
IMP53	neg	pos	neg	pos	pos	pos	pos
IMP216	pos	neg	neg	neg	pos	pos	neg
KPC	neg	neg	pos	neg	pos	neg	pos

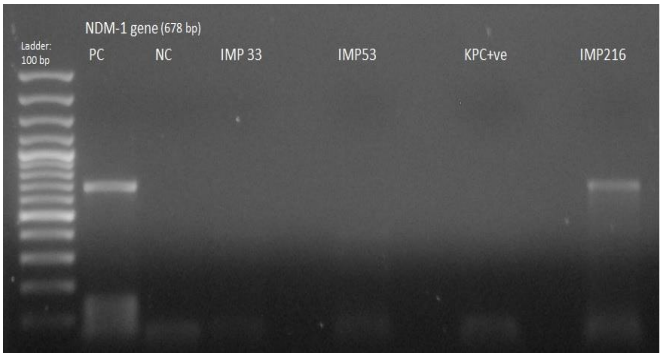

Figure 1.a.: PCR results for the detection of the *bla*NDM-1 gene, in the four isolates, IMP33, IMP53, IMP216, and KPC. (NC: negative control; PC: positive control).

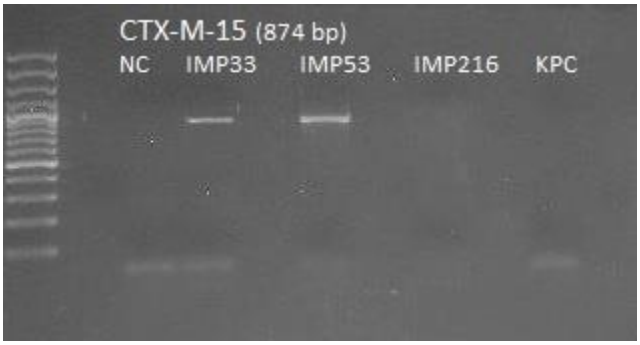

Figure 1.b: PCR results for the detection of the *bla*CTX-M-15 gene, in the four isolates, IMP33, IMP53, IMP216, and KPC. (NC: negative control).

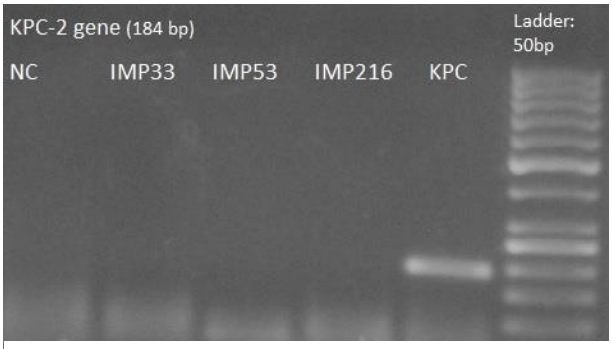

Figure 1.c: PCR results for the detection of the *bla*KPC-2 gene, in the four isolates, IMP33, IMP53, IMP216, and KPC. (NC: negative control).

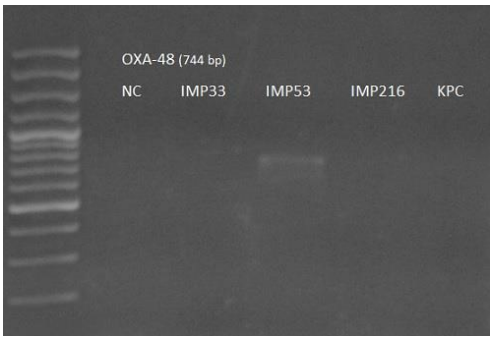

Figure 1.d: PCR results for the detection of the *bla*OXA-48 gene, in the four isolates, IMP33, IMP53, IMP216, and KPC. (NC: negative control).

Figure 1.e: PCR results for the detection of the *bla*TEM-1 gene, in the four isolates, IMP33, IMP53, IMP216, and KPC. (NC: negative control).

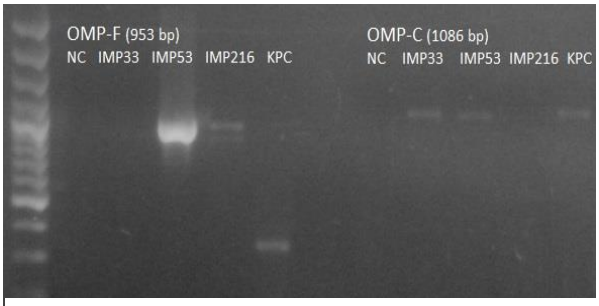

Figure 1.f: PCR results for the detection of the *omp*F and *omp*C genes, in the four isolates, IMP33, IMP53, IMP216, and KPC. (NC: negative control).

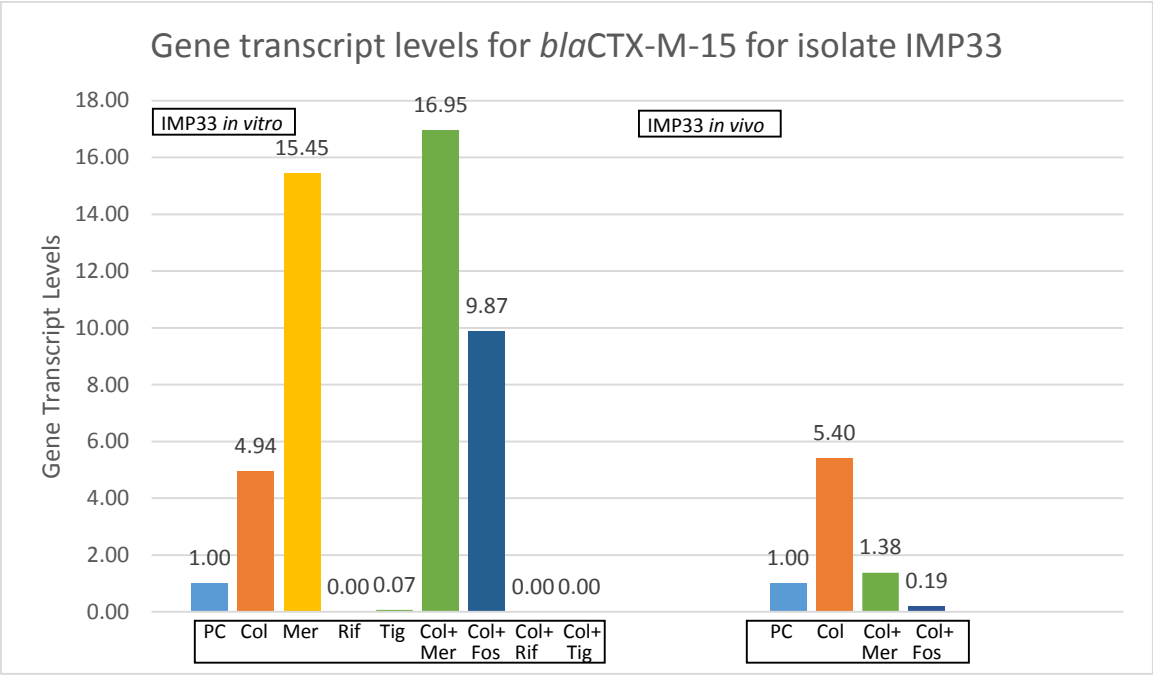

Figure 2.a: Transcript levels of the *bla*CTX-M-15 gene in *K. pneumoniae* for isolate IMP33 test with colistin, meropenem, rifampicin, tigecycline, colistin+meropenem, colistin+fosfomycin, colistin+rifampicin, colistin+tigecycline at the MIC levels in vitro; while in vivo, isolate IMP33 treated with colistin, colistin+meropenem, colistin+fosfomycin, at the MIC levels. PC: Positive Control, Col: colistin, Rif: rifampicin, Tig: tigecycline, Mer: Meropenem, Fos: fosfomycin.

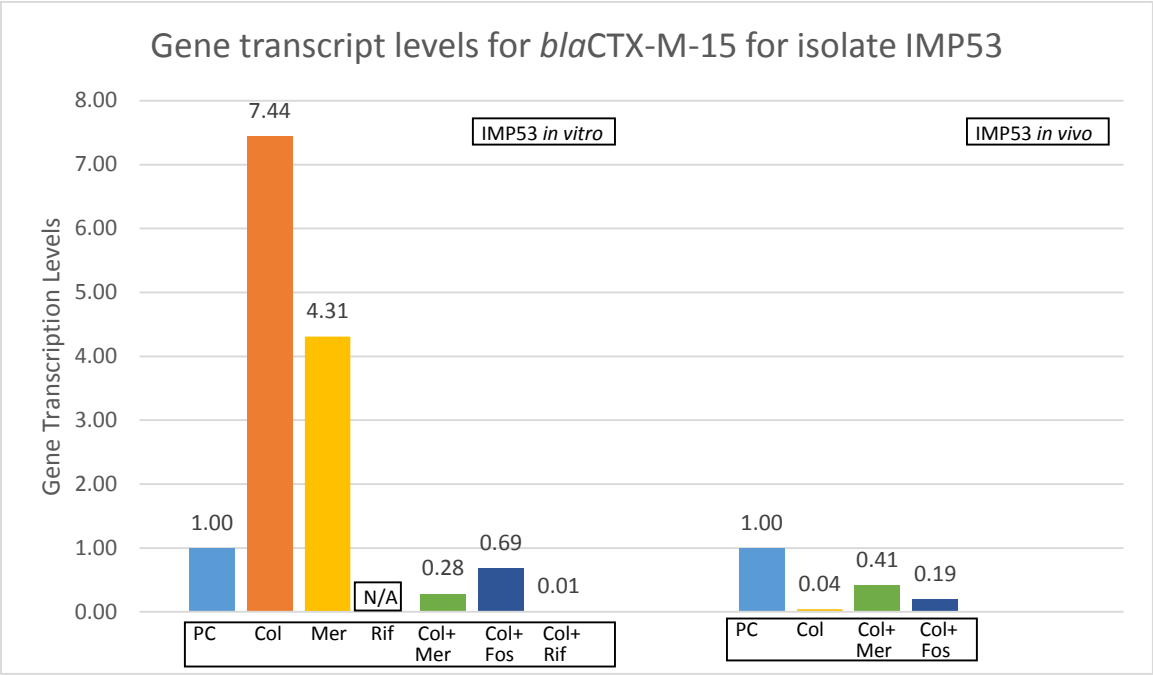

Figure 2.b: Transcript levels of the *bla*CTX-M-15 gene in *E. coli* for isolate IMP53 tested with colistin, colistin+meropenem, colistin+fosfomycin, colistin+rifampicin, at the MIC levels in vitro; while in vivo, isolate IMP53 treated with colistin, colistin+meropenem, colistin+fosfomycin, at the MIC levels. PC: Positive Control, Col: colistin, Rif: rifampicin, Mer: Meropenem, Fos: fosfomycin.

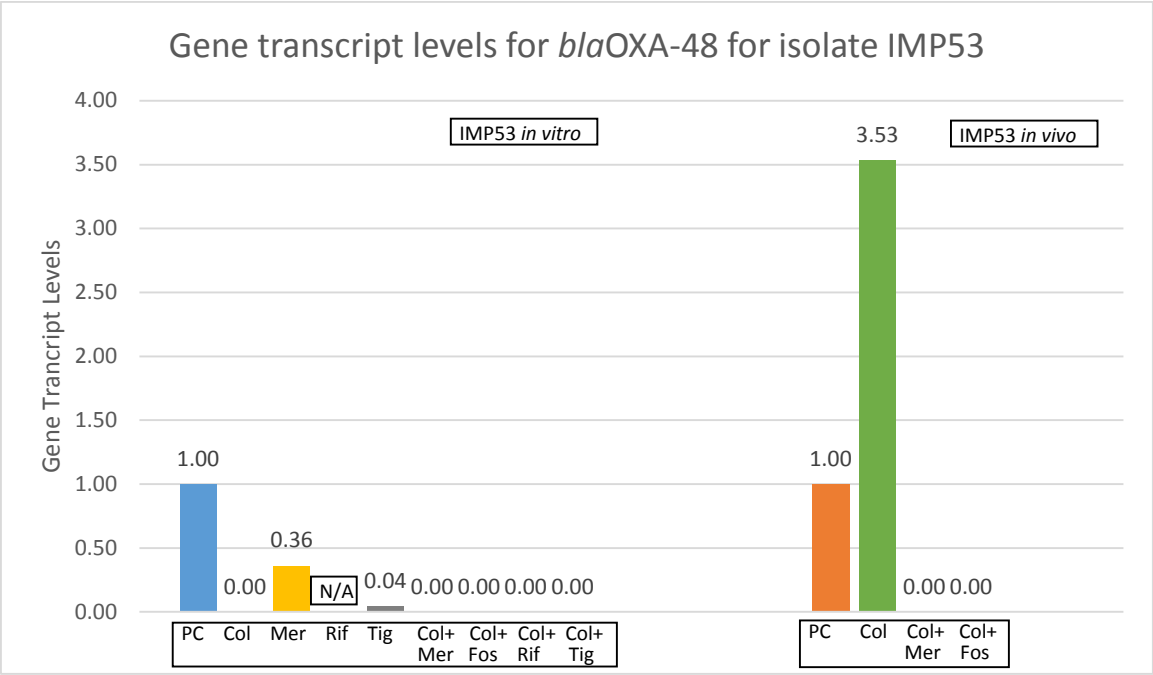

Figure 2.c: Transcript levels of the *bla*OXA-48 gene in *E. coli* for isolate IMP53 tested with colistin, rifampicin, tigecycline, colistin+meropenem, colistin+fosfomycin, colistin+rifampicin, colistin+tigecycline at the MIC levels in vitro; while in vivo, isolate IMP53 treated with colistin, colistin+meropenem, colistin+fosfomycin, at the MIC levels. . PC: Positive Control, Col: colistin, Rif: rifampicin, Tig: tigecycline, Mer: Meropenem, Fos: fosfomycin.

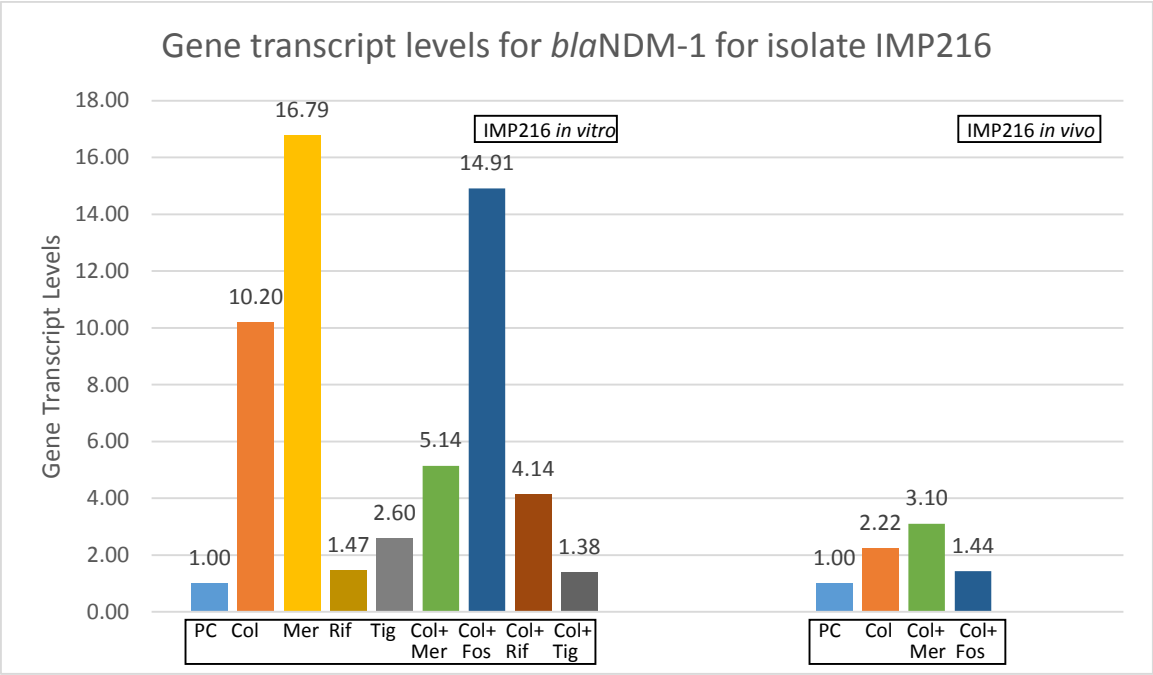

Figure 2.d: Transcript levels of the *bla*NDM-1 gene in *K. pneumoniae* for isolate IMP216 tested with colistin, rifampicin, tigecycline, colistin+meropenem, colistin+fosfomycin, colistin+rifampicin, colistin+tigecycline at the MIC levels in vitro; in vivo, isolate IMP216 treated with colistin, colistin+meropenem, colistin+fosfomycin, at the MIC levels. . PC: Positive Control, Col: colistin, Rif: rifampicin, Tig: tigecycline, Mer: Meropenem, Fos: fosfomycin.

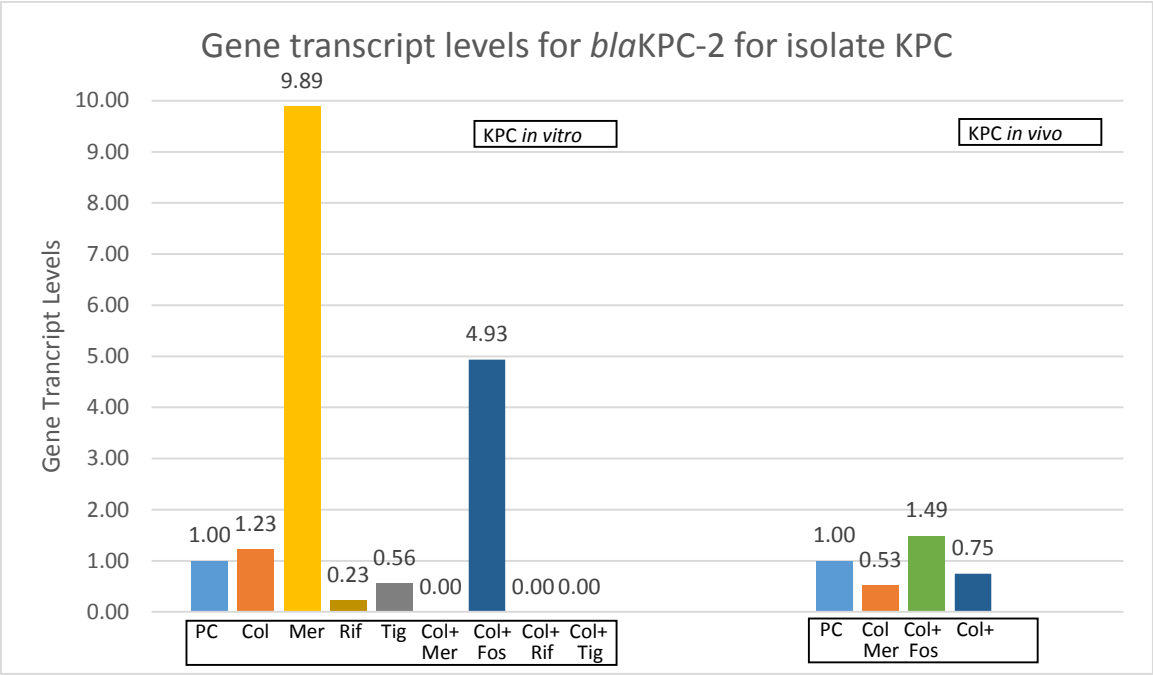

Figure 2.e: Transcript levels of the *bla*KPC-2 gene in *Salmonella* for isolate KPC tested with colistin, rifampicin, tigecycline, colistin+meropenem, colistin+fosfomycin, colistin+rifampicin, colistin+tigecycline at the MIC levels in vitro; in vivo, isolate KPC treated with colistin, colistin+meropenem, colistin+fosfomycin, at the MIC levels. . PC: Positive Control, Col: colistin, Rif: rifampicin, Tig: tigecycline, Mer: Meropenem, Fos: fosfomycin.

Table 6: Mouse groups and treatment regimens of the four isolates, IMP33, IMP53, IMP216, and KPC. TSB: Trpticase Soy Broth, MIC: Minimal Inhibitory Concentration, MBC: Minimal Bactericidal Concentration

IMP33(ESBL)	
Grp 1	TSB
Grp 2	Bacteria
Grp 3	Colistin
Grp 4	Ceftazidime
Grp 5	Colistin+ Meropenem
Grp 6a	Colistin+ Ertapenem (MIC)
Grp 6b	Colistin+ Ertapenem (MBC)
Grp 7a	Colistin+ Imipenem (MIC)
Grp 7b	Colistin+ Imipenem (MBC)
Grp 8a	Colistin+ Tigecyclin (MIC)
Grp 8b	Colistin+ Tigecyclin (MBC)
Grp 9a	Colistin+ Fosfomycin (MIC)
Grp 9b	Colistin+ Fosfomycin (MBC)
Grp 10a	Colistin+ Kanamycin (MIC)
Grp 10b	Colistin+ Kanamycin (MBC)

IMP53(OXA-48)	
Grp 20	TSB
Grp 21	Bacteria
Grp 22	Colistin
Grp 23	Ceftazidime
Grp 24	Colistin+ Meropenem
Grp 25	Colistin+ Ertapenem
Grp 26	Meropneme+ Ertapenem
Grp 27a	Colistin+ Imipenem (MIC)
Grp 27b	Colistin+ Imipenem (MBC)
Grp 28a	Colistin+ Tigecyclin (MIC)
Grp 28b	Colistin+ Tigecyclin (MBC)
Grp 29	Colistin+ Fosfomycin
Grp 30	Colistin: 2doses
Grp 31a	Colistin+ Kanamycin (MIC)
Grp 31b	Colistin+ Kanamycin (MBC)

IMP216(NDM-1)	
Grp 41	TSB
Grp 42	Bacteria
Grp 43	Colistin
Grp 44	Ceftazidime
Grp 45	Colistin+ Meropenem
Grp 46a	Colistin+ Ertapenem (MIC)
Grp 46b	Colistin+ Ertapenem (MBC)
Grp 47a	Colistin+ Imipenem (MIC)
Grp 47b	Colistin+ Imipenem (MBC)
Grp 48a	Colistin+ Tigecyclin (MIC)
Grp 48b	Colistin+ Tigecyclin (MBC)
Grp 49	Colistin+ Fosfomycin
Grp 50	Colistin+Kanamycin
Grp 51	Kanamycin+ Tazobactam
Grp 52	Kanamycin

KPC(KPC-2)	
Grp 61	TSB
Grp 62	Bacteria
Grp 63a	Colistin (MIC)
Grp 63b	Colistin (MBC)
Grp 64	Ceftazidime
Grp 65a	Colistin+ Ertapenem (MIC)
Grp 65b	Colistin+ Ertapenem (MBC)
Grp 66a	Colistin+ Imipenem (MIC)
Grp 66b	Colistin+ Imipenem (MBC)
Grp 67a	Colistin+ Meropenem (MIC)
Grp 67b	Colistin+ Meropenem (MBC)
Grp 68a	Colistin+ Tigecylcin (MIC)
Grp 68b	Colistin+ Tigecylcin (MBC)
Grp 69a	Colistin+ Fosfomycin (MIC)
Grp 69b	Colistin+ Fosfomycin (MBC)

Grp 11a	Kanamycin+ Tazobactam (MIC)
Grp 11b	Kanamycin+ Tazobactam (MBC)
Grp 12a	Aztreonam+ Tazobactam (MIC)
Grp 12b	Aztreonam+ Tazobactam (MBC)
Grp 13a	Aztreonam (MIC)
Grp 13b	Aztreonam (MBC)
Grp 14	Ceftazidime+ Colistin
Grp 15	Ceftazidime+ Ertapenem
Grp 16	Ceftazidime+ Imipenem
Grp 17	Ceftazidime+ Meropenem
Grp 18	Rifampicin
Grp 19	Rifampicin+ Colistin

Grp 32a	Kanamycin+ Tazobactam (MIC)
Grp 32b	Kanamycin+ Tazobactam (MBC)
Grp 33a	Aztreonam+Tazobactam (MIC)
Grp 33b	Aztreonam+ Tazobactam (MBC)
Grp 34a	Aztreonam (MIC)
Grp 34b	Aztreonam (MBC)
Grp 35	Ceftazidime+ Colistin
Grp 36	Ceftazidime+ Ertapenem
Grp 37	Ceftazidime+ Imipenem
Grp 38	Ceftazidime+ Meropenem
Grp 39	Rifampicin
Grp 40	Rifampicin+ Colistin

Grp 53a	Aztreonam+ Tazobactam (MIC)
Grp 53b	Aztreonam+ Tazobactam (MBC)
Grp 54a	Aztreonam (MIC)
Grp 54b	Aztreonam (MBC)
Grp 55	Ceftazidime+ Colistin
Grp 56	Ceftazidime+ Ertapenem
Grp 57	Ceftazidime+ Imipenem
Grp 58	Ceftazidime+ Meropenem
Grp 59	Rifampicin
Grp 60	Rifampicin+ Colistin

Grp 70a	Colistin+ Kanamycin (MIC)
Grp 70b	Colistin+ Kanamycin (MBC)
Grp 71	Kanamycin+ Tazobactam
Grp 72	Kanamycin
Grp 73a	Aztreonam+ Tazobactam (MIC)
Grp 73b	Aztreonam+ Tazobactam (MBC)
Grp 74a	Aztreonam (MIC)
Grp 74b	Aztreonam (MBC)
Grp 75	Ertapenem (MIC)
Grp 76	Ceftazidime+ Colistin
Grp 77	Ceftazidime+ Ertapenem
Grp 78	Ceftazidime+ Imipenem
Grp 79	Ceftazidime+ Meropenem
Grp 80	Rifampicin
Grp 81	Rifampicin+ Colistin

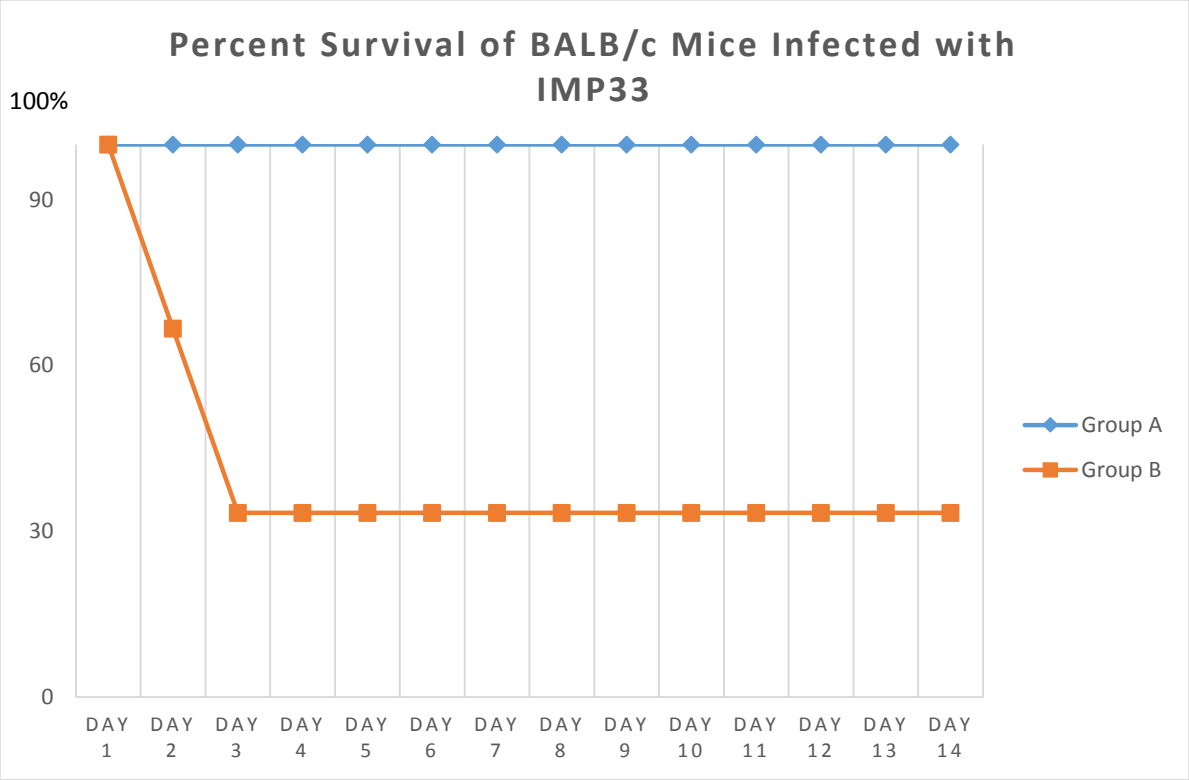

Figure 3.a: Percentage of survivals of Group I injected with isolate IMP33, during the monitoring period.

Group A refers to the following groups	Group B refers to the following groups
TSB (negative control)	Bacterial Injection (positive control)
Colistin	
Ceftazidime	
Colistin+Meropenem	
Colistin+Ertapenem (MIC & MBC)	
Colistin+Imipenem (MIC & MBC)	
Colsitin+Tigecycline(MIC & MBC)	
Colistin+Fosfomycin (MIC & MBC)	
Colistin+Kanamycin (MIC & MBC)	
Kanamycin+Tazobactam (MIC & MBC)	
Aztreonam+Tazobactam (MIC & MBC)	
Aztreonam (MIC & MBC)	
Ceftazidime+Colistin	
Ceftazidime+Ertaoenem	
Ceftazidime+Imipenem	
Ceftazidime+Meropenem	
Rifampicin	
Rifampicin+Colistin	

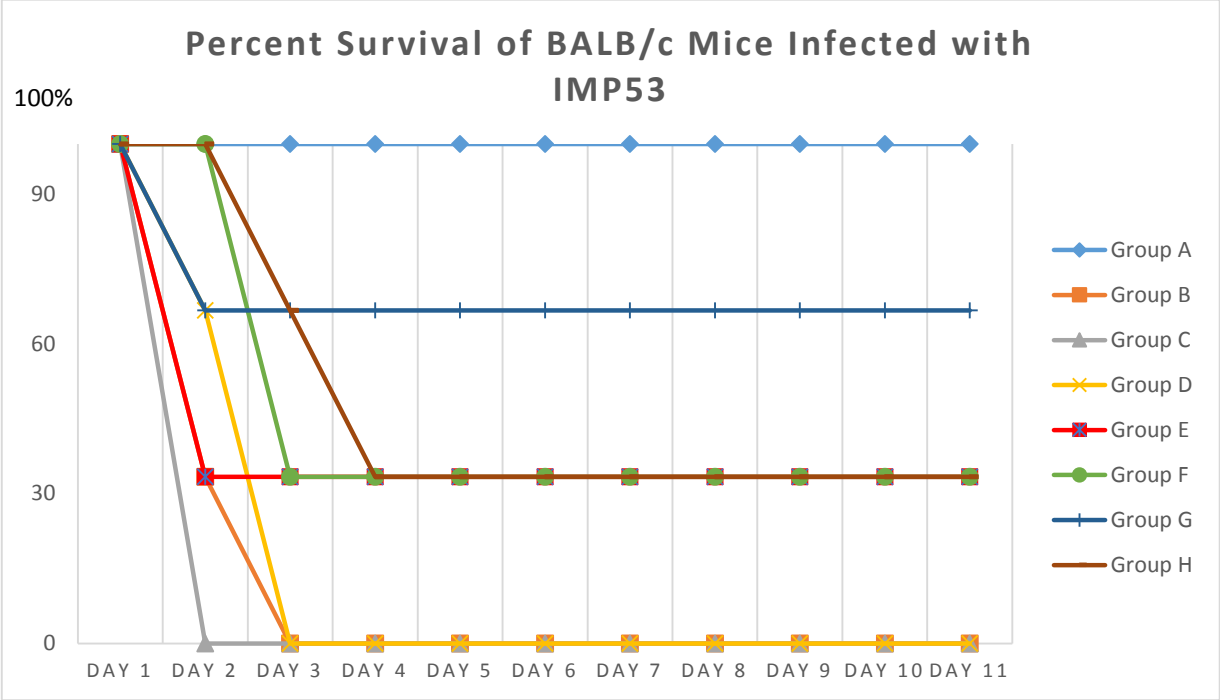

Figure 3.b: Percentage of survivals of Group II injected with isolate IMP53, during the monitoring period

Group A refers to:	Group B refers to:	Group C refers to:	Group D refers to:	Group E refers to:	Group F refers to:	Group G refers to:	Group H refers to:
-TSB -Colistin -Col+Erta -Col+Mero -Mero+Erta -Col+Imi (MIC&MBC) -Col+Tig (MIC&MBC) -Col+Fosfo -Colx2 doses -Col+Kan (MIC&MBC) -Kan+Tazo (MIC&MBC) -Cefta+Col	-Bacterial Injection -Cefta -Rif	-Az+Tazo (MIC)	-Az+tazo (MBC)	-Az (MIC & MBC)	-Cefta+ Erta	-Cefta+ Mer -Rif+Col	Cefta+Imi

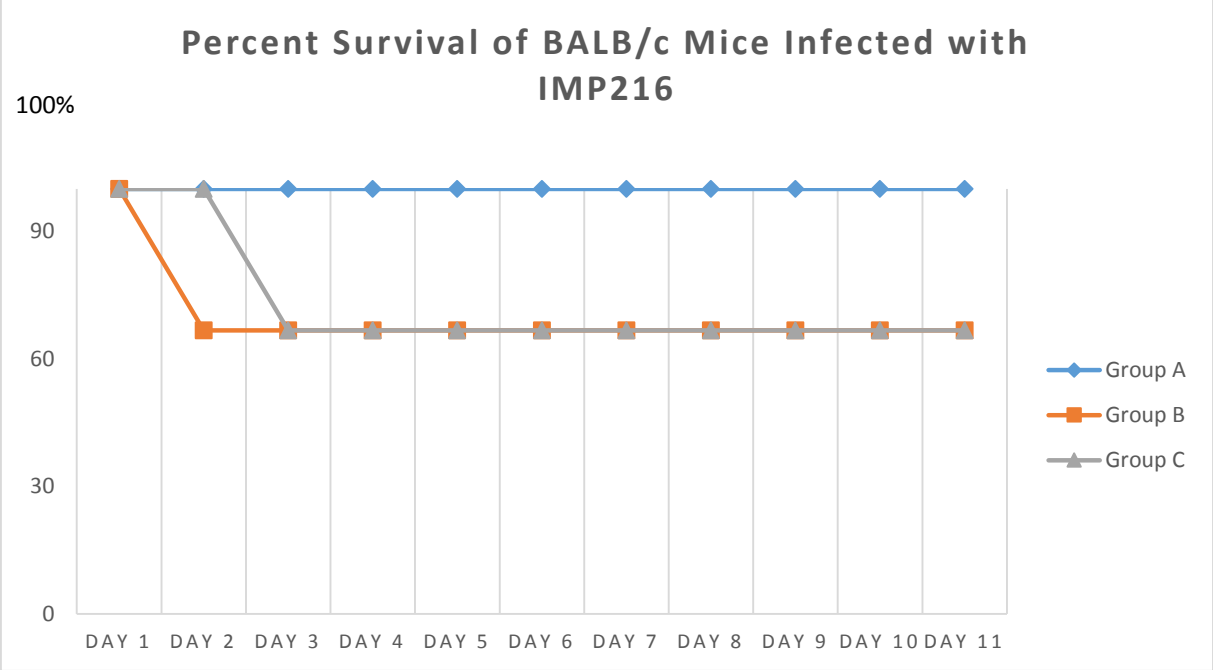

Figure 3.c: Percentage of survivals of Group III injected with isolate IMP216, during the monitoring period

Group A	Group B	Group C
-TSB (negative control) -Colistin -Ceftazidime -Colistin+Meropenem -Colistin+Ertapenem (MIC & MBC) -Colistin+Imipenem (MIC & MBC) -Colsitin+Tigecycline(MIC & MBC) -Colistin+Fosfomycin (MIC & MBC) -Colistin+Kanamycin (MIC & MBC) -Kanamycin+Tazobactam (MIC & MBC) -Aztreonam+Tazobactam (MIC & MBC) -Aztreonam (MBC) -Ceftazidime+Colistin -Ceftazidime+Ertapenem -Ceftazidime+Meropenem -Rifampicin -Rifampicin+Colistin	-Bacterial Injection	-Aztreonam (MIC) -Ceftazidime+Imipenem

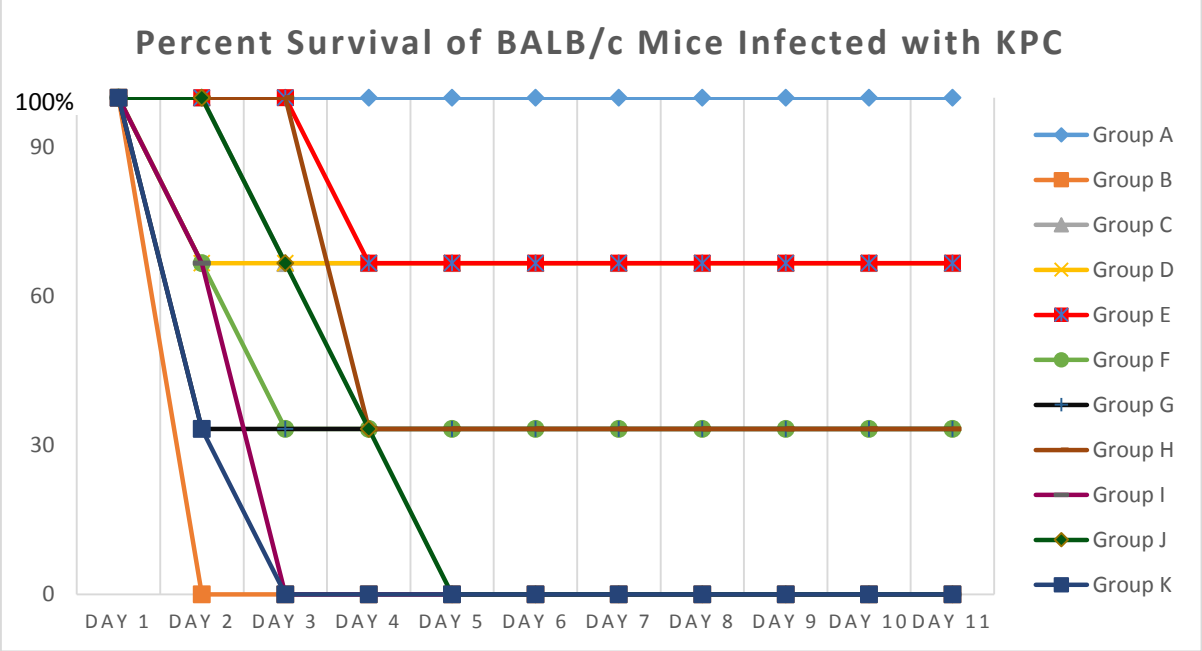

Figure 3.d: Percentage of survivals of Group IV injected with isolate KPC, during the monitoring period

Grp A	Grp B	Grp C	Grp D	Grp E	Grp F	Grp G	Grp H	Grp I	Grp J	Grp K
-TSB -Col+Erta (MIC&MBC) -Col+Mer (MIC&MBC) -Col+Tig (MBC) -Col+Fos (MIC&MBC) -Col+Kan (MIC&MBC) -Cefta+Col	-Col+Tig (MIC) -Az+Tazo (MIC&MBC)	-Col (MIC) -Kan -Cefta+ Erta	-Col+Imi (MBC) -Cefta+Imi -Rif+ Col	-Col (MBC) -Cefta	-Az (MIC&MBC) Col+Imi (MIC)	Cefta+Mero	Kan+Tazo	Erta	Rif	Bacteria

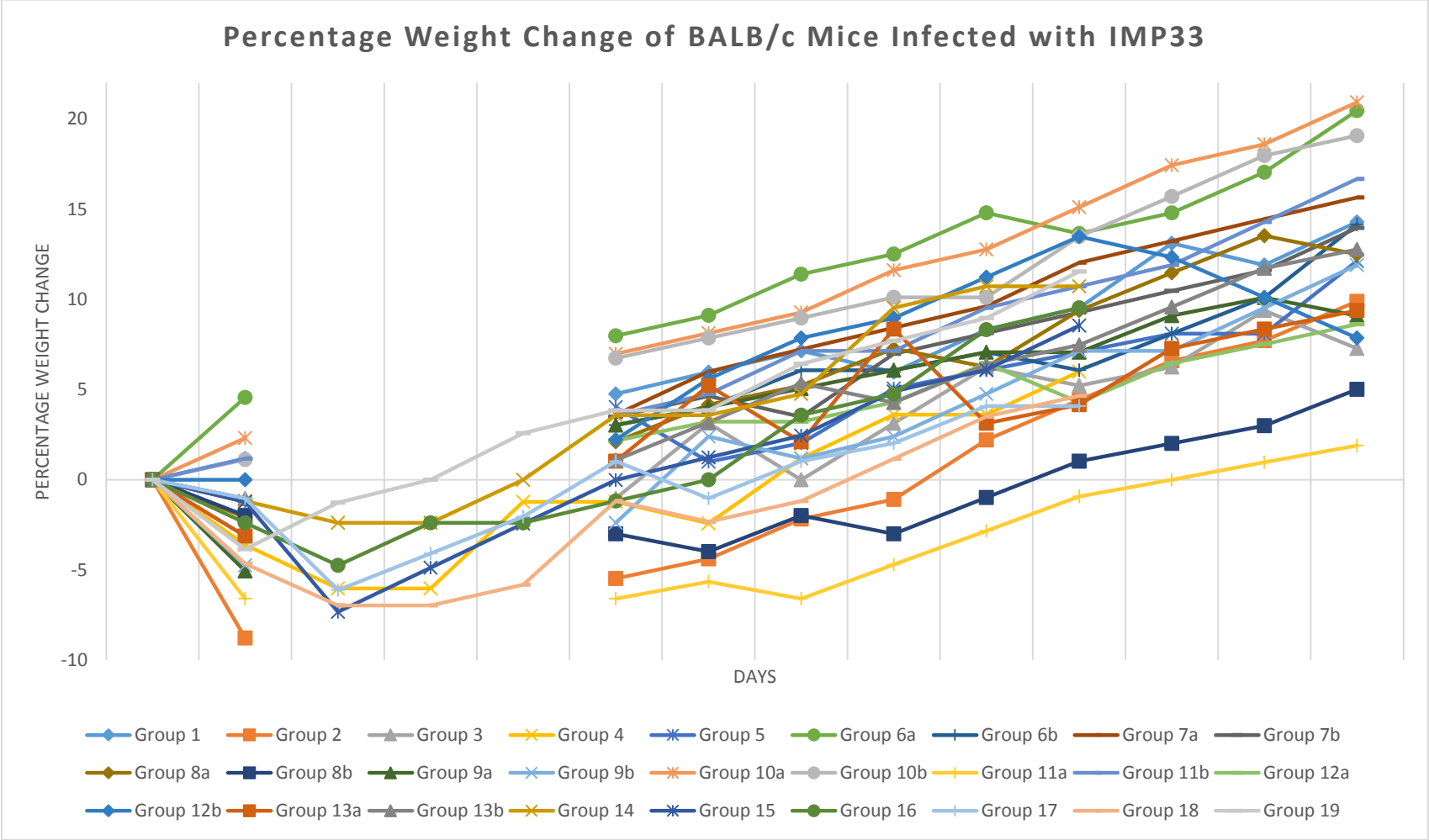

Figure 4.a: Percent weight change for BALB/c mice infected with IMP33 and treated with different combination therapies. Blank lines= groups not weighed.

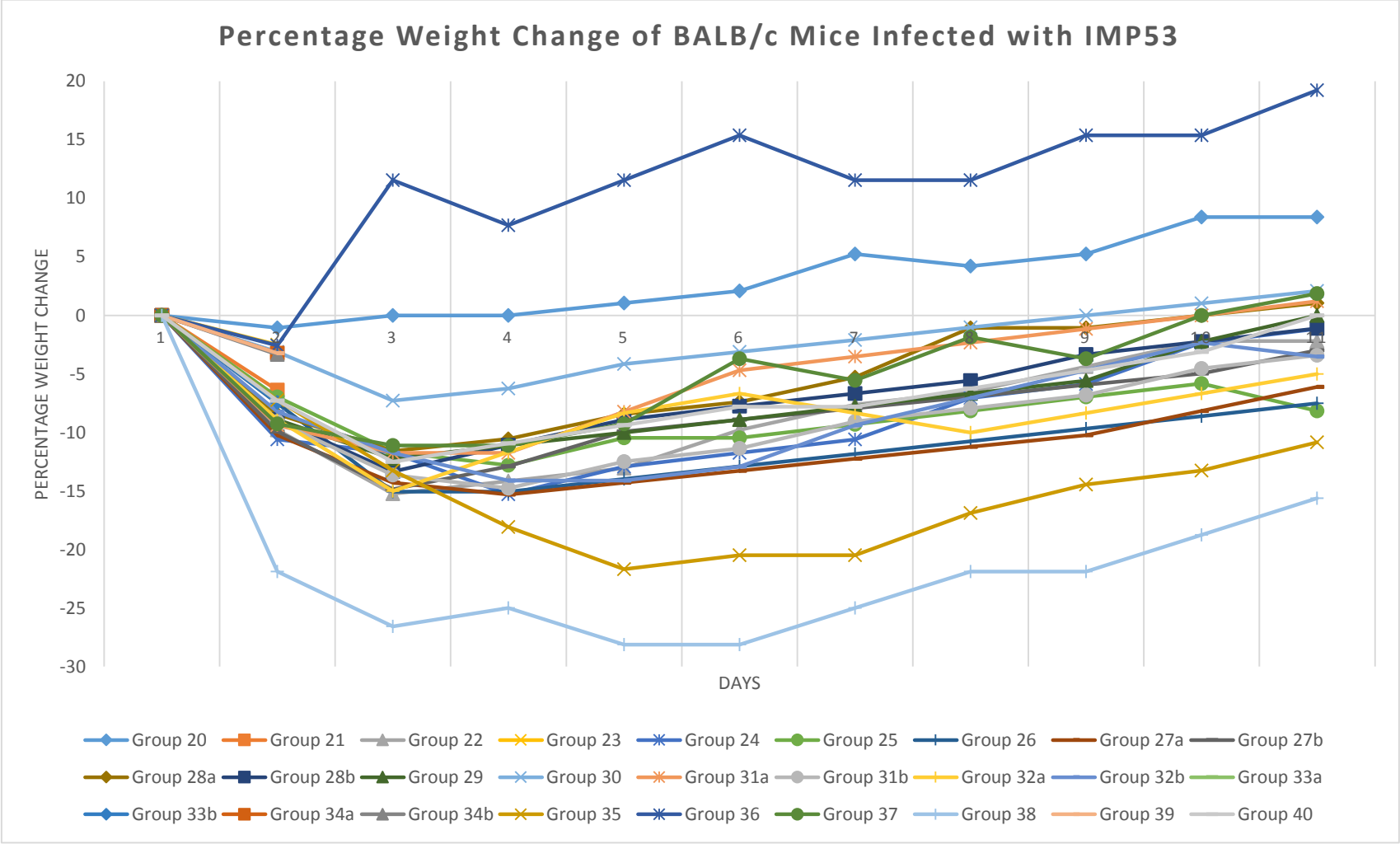

Figure 4.b: Percent weight change for BALB/c mice infected with IMP53 and treated with different combination therapies.

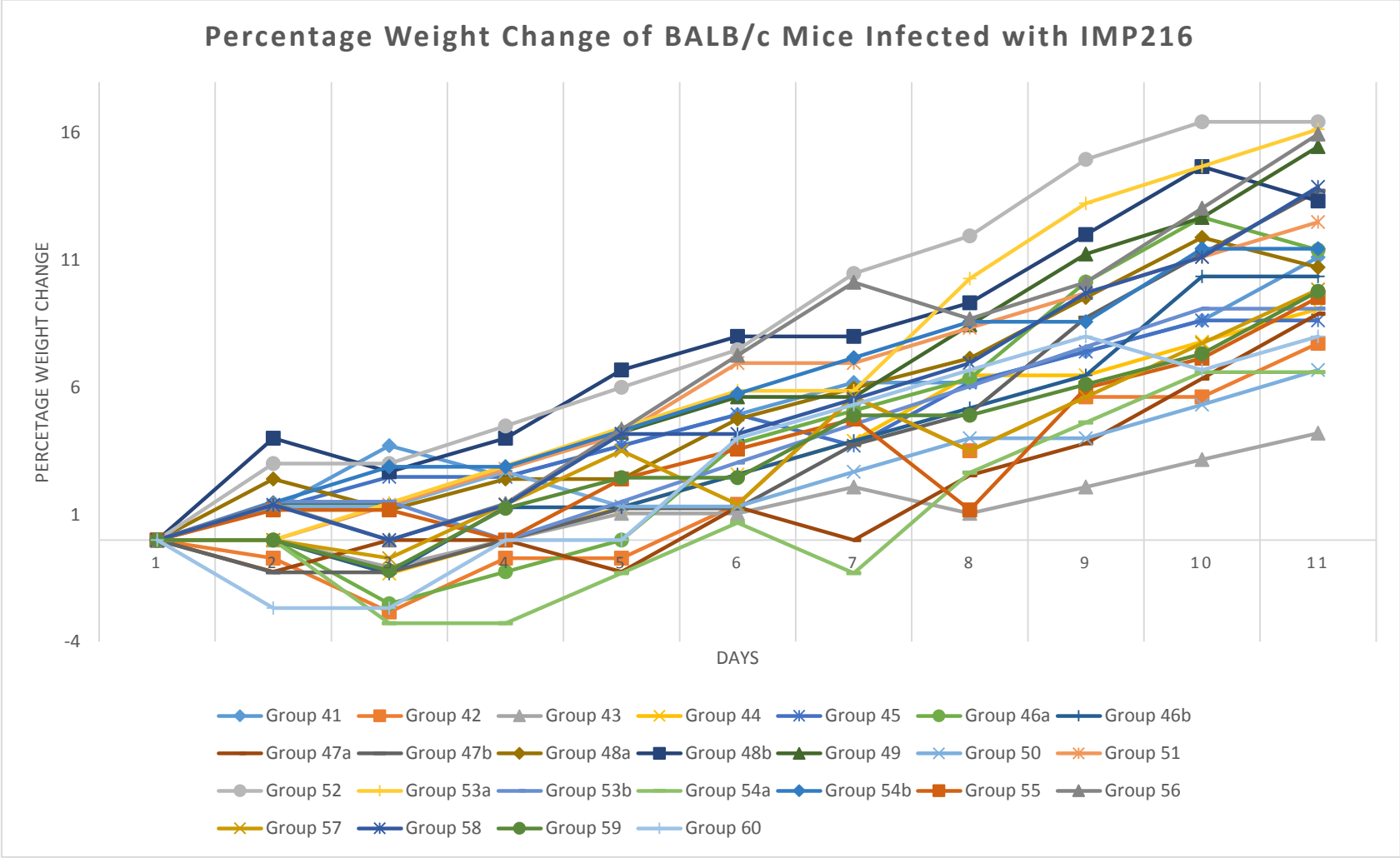

Figure 4.c: Percent weight change for BALB/c mice infected with IMP216 and treated with different combination therapies.

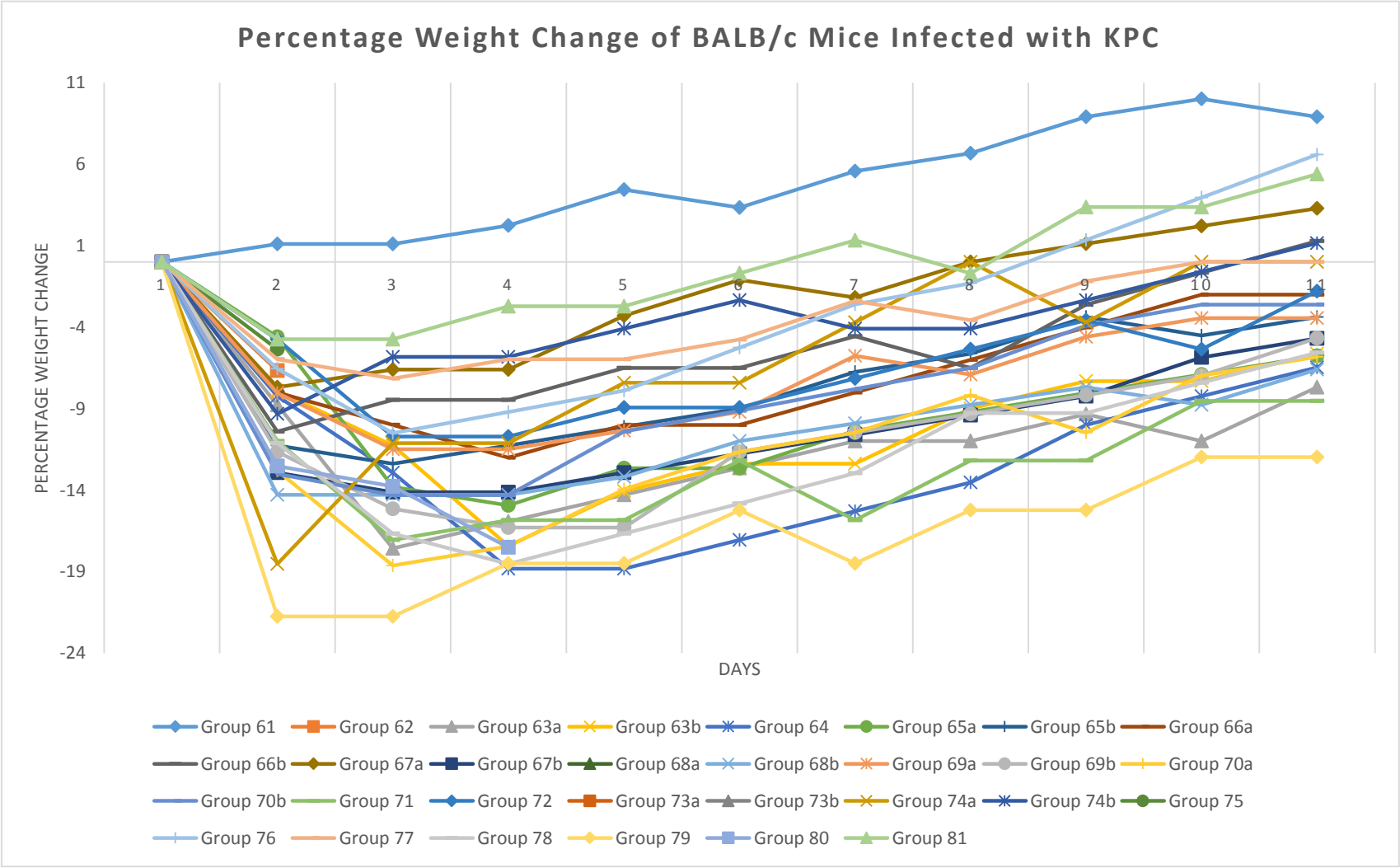

Figure 4.d: Percent weight change for BALB/c mice infected with KPC and treated with different combination therapies.

Chapter V

Discussion

This study attempted to assess the effect of combination therapy, both *in vitro* and *in vivo*, in order to recommend potential effective regimens against carbapenem resistant *Enterobacteriaceae*.

Carbapenemase production in *Enterobacteriaceae* (such as those of Classes A or D), is not always associated with a high level of carbapenem resistance, rather it can be linked with elevated carbapenem MICs but within the susceptible range, or with low level resistance to carbapenems (4, 67, 70, 75). This was observed in isolates IMP33, IMP53, and KPC, as they showed low level resistance to ertapenem; in addition, IMP53 was determined as intermediately resistant to both imipenem and meropenem. Whereas isolate IMP216 was determined as extensively-drug resistant, since it showed resistance to all antibacterial agents except colistin and fosfomycin. This was expected since this isolate harbors both *bla*NDM-1 and *bla*TEM-1 genes, in addition to porin mutations, and the fact that it may harbor other genes that were not tested in this study. The differences in susceptibility ranges, between these isolates, can be explained by the hydrolyzing effectiveness of the enzymes they produce (75).

Moreover, RT-qPCR was performed to assess the effect of the treatment options on the gene transcript level of the carbapenemase encoding genes. The antibacterial agents that led to the most noticeable decrease in the transcript levels were either rifampicin monotherapy or in combination with colistin. This decrease in the transcript levels was expected since rifampicin inhibits gene transcription (126) by constraining and inhibiting the DNA-dependent RNA

synthesis (15). Although rifampicin monotherapy resulted in the most efficient transcript inhibition, its use as monotherapy is not recommended due to the high rate of emerging resistance (15). Consequently, the combination of colistin and rifampicin seems to be the treatment of choice.

Similarly, a decline in the transcript levels of the carbapenemase encoding genes was observed upon the use of either tigecycline monotherapy or in combination with colistin, in most of the isolates. Moreover, since the transcript levels of tigecycline and colistin in combination led to lower results than their monotherapies, it can be concluded that their use in combination can be more effective.

In vitro testing with colistin in combination with either meropenem or fosfomycin, or the monotherapies of either colistin or meropenem, led to different gene transcript levels in isolates IMP33, IMP216, and KPC when compared to their controls, and when compared to their *in vivo* gene transcript levels. This may be explained by the fact that these antibacterial agents do not inhibit gene transcription like rifampicin, rather they act on the bacterial cell wall or could be inducing the survival mode in the bacteria where the production of the carbapenemase increases to counteract the carbapenem introduced into the periplasm (139).

The difference between the *in vitro* and *in vivo* results may limit the understanding of the efficacy of combination therapy. These discrepancies can be explained by the fact that *in vivo* conditions can trigger different mechanisms in the bacteria (154, 157), in addition to the immunity factor *in vivo*, the pharmacokinetics and the pharmacodynamics parameters of the antibacterial agents (145, 150).

Isolate IMP53, harboring *bla*OXA-48 gene, responded differently than the other isolates in terms of gene transcript levels. An evident decrease was observed in all treatment

modes, both *in vitro* and *in vivo*, except with colistin monotherapy. This might be explained by the fact that the plasmid harboring the *bla*OXA-48 gene, is associated with insertion sequences (81, 91), which provide the carbapenemase encoding gene with a promoter region that controls its transcription level (91, 140). The promoter involved in this isolate might not be efficient, reflecting a decrease in the transcript levels of the carbapenemase encoding gene (141). Furthermore, even though OXA enzymes confer carbapenem resistance, they exhibit poor carbapenem and cephalosporin hydrolysis activities (75, 89). In fact, the MIC levels of IMP53 are reported as intermediate resistance to meropenem, and susceptible to both fosfomycin and colistin. As a result, these antibacterial treatments might be effective in killing the bacteria, thus explaining the low carbapenemase encoding gene transcript levels.

Concerning the efficacy of combination therapy *in vivo*, it was noticed that all the mice in Group I injected with isolate IMP33 followed by treatment survived. However, evident weight loss was observed after receiving the injections, which reflects acquisition of disease. Their survival can be due to the administration of efficient treatment regimens since it was susceptible to most of the antibacterial agents used. Moreover, carbapenem resistance in this isolate is due to ESBL production and porin loss, which represents less clinical threat than carbapenemase production (146). In addition, not all carbapenem resistant bacteria are highly pathogenic or virulent, which may further explain the 100% survival rate (147).

The total survival rate for Group II injected with the IMP53 isolate followed by a treatment was 68%. Moreover, it was noticeable that colistin based treatment regimens whether monotherapy or as combination led to a 100% survival rates in these subgroups.

Whereas, the total survival rate for Group III injected with isolate IMP216 was 97.10%. Isolate IMP216 was defined as extensively-drug resistant as it remained susceptible

only to colistin and fosfomcyin; nevertheless, 66% of the mice in the positive control subgroup survived. Moreover, there was no significant weight loss observed in the subgroups. In fact, several studies have reported colonization of organisms harboring the *bla*NDM-1 gene as part of the fecal flora, without displaying any symptoms (148-150, 155). This can be explained by the fact that antibacterial resistance can be associated with both a decreased fitness, expressed by impairment of the bacterial growth in the infected host (151, 153, 156) and a decreased virulence, represented by diminished invasiveness and higher clearance rates (149, 151, 153, 156).

In Group IV, the subgroups that received the KPC isolate injections followed by treatment, revealed the lowest survival rates (60.7%). However, the mice that survived presented clinical symptoms of illness with clear weight loss and diarrhea, demonstrating its high virulence.

Based on the pooled results of this study and the literature, few treatment options can be considered as potential effective regimens against carbapenem resistant organisms. The treatment regimens for infections caused by ESBL producing isolates and porin loss, extend to include colistin in combination with an adjuvant antibacterial agent such as a carbapenem, rifampicin, or kanamycin (fosfomycin or tigecycline remain for severe infections).

As for infections caused by organisms harboring *bla*OXA-48 gene, colistin in combination with an adjuvant such as a carbapenem, rifampicin, tigecycline or fosfomycin at both MIC concentrations are useful and advisable.

Furthermore, the most effective treatment option for infections caused by organisms harboring the *bla*NDM-1 gene include, and might be limited to, combination therapy of colistin with either rifampicin, fosfomycin, or tigecycline at the MBC concentration.

Last but not least, the best treatment options for organisms encoding the *bla*KPC-2 gene include the use of colistin with either a carbapenem, rifampicin, kanamycin, or tigecycline at the MBC concentration as a combination therapy.

However, the treatment regimens that are not recommended for infections caused by carbapenemase producing *Enterobacteriaceae,* include the monotherapies of either rifampicin, tigecycline, aztreonam, carbapenem, or colistin, or the combination therapy of a carbapenem with a third generation cephalosporin, or aztreonam in combination with tazobactam.

The selection of the appropriate adjuvant as the choice of therapy for the particular bacterial infection should be based on the antibacterial susceptibility testing.

Based on the survival rates in all the groups, it can be concluded that combination therapy is not associated with increased death rates, as opposed to previous reports, especially since the selected cornerstone antibacterial agent colistin, which has high reports of neuro/nephro-toxicities.

Moreover, based on the gene transcript levels and mouse survival rates, one generalized treatment regimen cannot be administered for an effective treatment for the various carbapenem resistant isolates; thereby, stressing the importance of the antibacterial susceptibility testing and the determination of resistance encoding genes for each isolate to secure the administration of an appropriate regimen. The use of rationally optimized combination therapy might lead to better results than monotherapy especially in virulent strains. Indeed, additional studies are needed to further assess the efficacy of combination therapy, using larger number of mice per group.

Bibliography

1. Murray, PR., Rosenthal KS, Pfaller, MA. Medical Microbiology, 5th ed. Elsevier Inc. 2005 p. 323-330
2. Guentzel NM. *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, and *Proteus*. In Baron S, editor. Medical Microbiology. 4th ed. Galveston: University of Texas Medical Branch at Galveston; 1996.
3. Ingerson-Mahar M. and Reid A. A report from the American Academy of Microbiology *E. coli*: good, bad, & deadly. American Society for Microbiology 2011
4. Baroud M, Dandache I, Araj GF, Wakim R, Kanj S, Kanafani Z, et al. Underlying mechanisms of carbapenem resistance in extended-spectrum beta-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. Int J Antimicrob Agents 2013 Jan;41(1):75-79.
5. Rai S, Das D, Niranjan DK, Singh NP, Kaur IR. Carriage prevalence of carbapenem-resistant Enterobacteriaceae in stool samples: A surveillance study. Australas Med J 2014 Feb 28;7(2):64-67.
6. Amin M., Mehdinejad M., Pourdangchi Z. Study of bacteria isolated from urinary tract infections and determination of their susceptibility to antibiotics. Jundishapur Journal of Microbiology (2009); 2(3): 118-123
7. Schaechter M. *Escherichia coli*. In Schaechter M, editor. Encyclopedia of Microbiology. San Diego: Academic Press, Oxford; 2009. p. 125-132.
8. Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 1998 Oct;11(4):589-603.
9. Battistuzzi FU, Feijao A, Hedges SB. A genomic timescale of prokaryote evolution: insights into the origin of methanogenesis, phototrophy, and the colonization of land. BMC Evol Biol 2004 Nov 9;4:44.

10. Kidgell C, Reichard U, Wain J, Linz B, Torpdahl M, Dougan G, et al. Salmonella typhi, the causative agent of typhoid fever, is approximately 50,000 years old. Infect Genet Evol 2002 Oct;2(1):39-45.
11. Lampel K., Al-Khaldi S., Cahill M., Food and Drug Administration. Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins. Second Edition; 2012; [Gram negative Bacteria, pp. 10-13].
12. FSANZ (2013) Agents of Foodborne Illness. 2nd ed, Food Standards Australia New Zealand, Canberra
13. Davison HC, Low JC, Woolhouse ME. What is antibiotic resistance and how can we measure it? Trends Microbiol 2000 Dec;8(12):554-559.
14. Bouki C, Venieri D, Diamadopoulos E. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. Ecotoxicol Environ Saf 2013 May;91:1-9.
15. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 2010 Jun;8(6):423-435.
16. Gold HS, Moellering RC,Jr. Antimicrobial-drug resistance. N Engl J Med 1996 Nov 7;335(19):1445-1453.
17. Nicolau DP. Carbapenems: a potent class of antibiotics. Expert Opin Pharmacother 2008 Jan;9(1):23-37.
18. Bush K. Proliferation and significance of clinically relevant beta-lactamases. Ann N Y Acad Sci 2013 Jan;1277:84-90.
19. Kanj SS, Kanafani ZA. Current concepts in antimicrobial therapy against resistant gram-negative organisms: extended-spectrum beta-lactamase-producing Enterobacteriaceae, carbapenem-resistant Enterobacteriaceae, and multidrug-resistant Pseudomonas aeruginosa. Mayo Clin Proc 2011 Mar;86(3):250-259.
20. El-Herte RI, Araj GF, Matar GM, Baroud M, Kanafani ZA, Kanj SS. Detection of carbapenem-resistant Escherichia coli and Klebsiella pneumoniae producing NDM-1 in Lebanon. J Infect Dev Ctries 2012 May 14;6(5):457-461.
21. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. Carbapenems: past, present, and future. Antimicrob Agents Chemother 2011 Nov;55(11):4943-4960.

22. El-Herte RI, Kanj SS, Matar GM, Araj GF. The threat of carbapenem-resistant Enterobacteriaceae in Lebanon: an update on the regional and local epidemiology. J Infect Public Health 2012 Jun;5(3):233-243.
23. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 2012 Sep;18(9):1503-1507.
24. Dortet L, Poirel L, Nordmann P. Rapid identification of carbapenemase types in Enterobacteriaceae and Pseudomonas spp. by using a biochemical test. Antimicrob Agents Chemother 2012 Dec;56(12):6437-6440.
25. Srisupha-olarn W., Polymyxins Revisited: Carbapenem-Resistant Gram-negative Bacteria. Pharmacotherapy Conference; April 30, 2010.
26. Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N. What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. Int J Antimicrob Agents 2011 May;37(5):415-419.
27. van Duin D, Kaye KS, Neuner EA, Bonomo RA. Carbapenem-resistant Enterobacteriaceae: a review of treatment and outcomes. Diagn Microbiol Infect Dis 2013 Feb;75(2):115-120.
28. Perez F, Van Duin D. Carbapenem-resistant Enterobacteriaceae: a menace to our most vulnerable patients. Cleve Clin J Med 2013 Apr;80(4):225-233.
29. Savard P, Perl TM. A call for action: managing the emergence of multidrug-resistant Enterobacteriaceae in the acute care settings. Curr Opin Infect Dis 2012 Aug;25(4):371-377.
30. Samaha-Kfoury JN, Araj GF. Recent developments in beta lactamases and extended spectrum beta lactamases. BMJ 2003 Nov 22;327(7425):1209-1213.
31. Zhanel GG, Wiebe R, Dilay L, Thomson K, Rubinstein E, Hoban DJ, et al. Comparative review of the carbapenems. Drugs 2007;67(7):1027-1052.
32. Jones RN, Sader HS, Fritsche TR. Comparative activity of doripenem and three other carbapenems tested against Gram-negative bacilli with various B-lactamase resistance mechanisms. Diagnostic Microbiology and Infectious Disease. 2005; 52: p. 71–74.

33. Stapleton PD, Shannon KP, French GL. Carbapenem resistance in Escherichia coli associated with plasmid-determined CMY-4 beta-lactamase production and loss of an outer membrane protein. Antimicrob Agents Chemother 1999 May;43(5):1206-1210.
34. Satlin MJ, Jenkins SG, Walsh TJ. The global challenge of carbapenem-resistant enterobacteriaceae in transplant recipients and patients with hematologic malignancies. Clin Infect Dis 2014 May;58(9):1274-1283.
35. Hong T, Moland ES, Abdalhamid B, Hanson ND, Wang J, Sloan C, et al. Escherichia coli: development of carbapenem resistance during therapy. Clin Infect Dis 2005 May 15;40(10):e84-6.
36. Osano E, Arakawa Y, Wacharotayankun R, Ohta M, Horii T, Ito H, et al. Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of Serratia marcescens that shows imipenem resistance. Antimicrob Agents Chemother 1994 Jan;38(1):71-78.
37. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 1995 Jun;39(6):1211-1233.
38. Ambler RP. The structure of β-lactamases. Phil. Trans. R. Soc. Lond. B. 1980; 289: p. 321–331.
39. Bush K, Jacoby GA, Medeiros AA. A Functional Classification Scheme for β-lactamase and its Correlation with Molecular Structure Antimicrobial Agents and Chemotherapy. 1995; 39(6): p. 1211-1233.
40. Livermore DM, Woodford N. The β-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. TRENDS in Microbiology. 2006; 14(9): p. 412-420.
41. Paterson DL. Recommendation for treatment of severe infections caused by Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs). Clin Microbiol Infect 2000 Sep;6(9):460-463.
42. Matar GM, Al Khodor S, El-Zaatari M, Uwaydah M. Prevalence of the genes encoding extended-spectrum beta-lactamases, in Escherichia coli resistant to beta-lactam and non-beta-lactam antibiotics. Ann Trop Med Parasitol 2005 Jun;99(4):413-417.

43. Payne DJ, Du W, Bateson JH. beta-Lactamase epidemiology and the utility of established and novel beta-lactamase inhibitors. Expert Opin Investig Drugs 2000 Feb;9(2):247-261.
44. Bradford PA. Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. Clinical Microbiology Reviews. 2001; 14(4): p. 933–951.
45. Baroud M., Araj G., and Matar G. Spread of CTX-M-15 Extended Spectrum β-lactamases Encoding Genes Among Enterobacteriaceae in the Middle Eastern Region. The International Arabic Journal of Antimicrobial Agents; 2011; 1(4)
46. Drawz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev 2010 Jan;23(1):160-201.
47. Naas T, Poirel L, Nordmann P. Minor extended-spectrum beta-lactamases. Clin Microbiol Infect 2008 Jan;14 Suppl 1:42-52.
48. Matar GM, Kattar MM, Khairallah MT, Abi-Rached R, Mokhbat J. Detection of plasmid-encoded bla-CTX-M-15 and bla-TEM-1 genes in a Lebanese Salmonella isolate that produces extended-spectrum beta-lactamase. Ann Trop Med Parasitol 2008 Oct;102(7):651-653.
49. Harajly M, Khairallah MT, Corkill JE, Araj GF, Matar GM. Frequency of conjugative transfer of plasmid-encoded ISEcp1 - blaCTX-M-15 and aac(6')-lb-cr genes in Enterobacteriaceae at a tertiary care center in Lebanon - role of transferases. Ann Clin Microbiol Antimicrob 2010 Jul 20;9:19-0711-9-19.
50. Matar GM, Khairallah MT, Dandache I, Sabra A, Mokhbat J. Further evidence of plasmid-encoded bla-(CTX-M-15) and bla-(TEM-1) genes in Lebanese isolates of Salmonella enterica serovar Typhimurium that produce extended-spectrum beta-lactamase. Ann Trop Med Parasitol 2010 Jan;104(1):91-94.
51. Mammeri H, Nordmann P, Berkani A, Eb F. Contribution of extended-spectrum AmpC (ESAC) beta-lactamases to carbapenem resistance in Escherichia coli. FEMS Microbiol Lett 2008 May;282(2):238-240.

52. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum beta-lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect 2008 Jan;14 Suppl 1:90-103.
53. Kanj SS, Corkill JE, Kanafani ZA, Araj GF, Hart CA, Jaafar R, et al. Molecular characterisation of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella spp. isolates at a tertiary-care centre in Lebanon. Clin Microbiol Infect 2008 May;14(5):501-504.
54. Cheaito K. and Ghassan M. The Mediterranean Region: A Reservoir for CTX-M-ESBL Producing Enterobacteriacae. JJBS March 2014 7(1) P: 1-6
55. Thomson KS. Extended-spectrum-beta-lactamase, AmpC, and Carbapenemase issues. J Clin Microbiol 2010 Apr;48(4):1019-1025.
56. Sabra AH, Araj GF, Kattar MM, Abi-Rached RY, Khairallah MT, Klena JD, et al. Molecular characterization of ESBL-producing Shigella sonnei isolates from patients with bacilliary dysentery in Lebanon. J Infect Dev Ctries 2009 May 1;3(4):300-305.
57. Wang G, Huang T, Surendraiah PK, Wang K, Komal R, Zhuge J, et al. CTX-M beta-lactamase-producing Klebsiella pneumoniae in suburban New York City, New York, USA. Emerg Infect Dis 2013 Nov;19(11):1803-1810.
58. Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC beta-lactamases among Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis isolates at a veterans medical center. J Clin Microbiol 2000 May;38(5):1791-1796.
59. Jacoby GA. AmpC β-Lactamases. Clinical Microbiology Reviews. 2009; 22(1): p. 161–182.
60. Bradford PA, Urban C, Mariano N, Projan SJ, Rahal JJ, Bush K. Imipenem resistance in Klebsiella pneumoniae is associated with the combination of ACT-1, a plasmid-mediated AmpC beta-lactamase, and the foss of an outer membrane protein. Antimicrob Agents Chemother 1997 Mar;41(3):563-569.
61. Gupta N, Limbago BM, Patel JB, Kallen AJ. Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. Clin Infect Dis 2011 Jul 1;53(1):60-67.
62. Brink, A., Coetzee, J., Clay, C., Corcoran, C., van Greune, J., Deetlefs, J., Nutt, L., Feldman, C., Richards, G., Nordmann, P., Poirel,L. The spread of carbapenem-resistant

Enterobacteriaceae in South Africa: Risk factors for acquisition and prevention. South African Medical Journal 2012;102(7).

63. Martin ET, Tansek R, Collins V, Hayakawa K, Abreu-Lanfranco O, Chopra T, et al. The carbapenem-resistant Enterobacteriaceae score: a bedside score to rule out infection with carbapenem-resistant Enterobacteriaceae among hospitalized patients. Am J Infect Control 2013 Feb;41(2):180-182.
64. Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 2011 Oct;17(10):1791-1798.
65. Tängdén T. Multidrug-Resistant *Escherichia coli* and *Klebsiella pneumonia* Treatment, Selection and International Spread [PhD thesis]. Sweden: ACTA; Universitatis Upsaliensis Uppsala 2012
66. Benouda A, Touzani O, Khairallah MT, Araj GF, Matar GM. First detection of oxacillinase-mediated resistance to carbapenems in Klebsiella pneumoniae from Morocco. Ann Trop Med Parasitol 2010 Jun;104(4):327-330.
67. Matar GM, Cuzon G, Araj GF, Naas T, Corkill J, Kattar MM, et al. Oxacillinase-mediated resistance to carbapenems in Klebsiella pneumoniae from Lebanon. Clin Microbiol Infect 2008 Sep;14(9):887-888.
68. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 2001 Jul;48 Suppl 1:5-16.
69. Girlich D, Poirel L, Nordmann P. Value of the modified Hodge test for detection of emerging carbapenemases in Enterobacteriaceae. J Clin Microbiol 2012 Feb;50(2):477-479.
70. Rai S, Manchanda V, Singh NP, Kaur IR. Zinc-dependent carbapenemases in clinical isolates of family Enterobacteriaceae. Indian J Med Microbiol 2011 Jul-Sep;29(3):275-279.
71. Hatipoglu M, Turhan V. Notice of first NDM-1 expressing isolates in Turkey and the use of modified Hodge test. Mikrobiyol Bul 2014 Jan;48(1):188-189.
72. Nordmann P, Poirel L, Carrer A, Toleman MA, Walsh TR. How to detect NDM-1 producers. J Clin Microbiol 2011 Feb;49(2):718-721. Balan K Modified Hodge Test

and Remodified Hodge Test for Carbapenemase Detection: Better Indicator INDIAN JOURNAL OF APPLIED RESEARCH. 2013 March 1(3); p. 179-180

73. Jain S., Rai S., Prasad K., Dhole T., Maurya A. (Lucknow, IN) Detection of carbapenemase enzymes in clinical isolates of Pseudomonas aeruginosa by Re-modified Hodge Test and other phenotypic methods. P. 768
74. Balan K Modified Hodge Test and Remodified Hodge Test for Carbapenemase Detection: Better Indicator INDIAN JOURNAL OF APPLIED RESEARCH. 2013 March 1(3); p. 179-180
75. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev 2007 Jul;20(3):440-58, table of contents.
76. Walther-Rasmussen J, Høiby N. Class A Carbapenemases. Journal of Antimicrobial Chemotherapy. 2007; 60: p. 470-482.
77. Matar GM, Dandache I, Carrer A, Khairallah MT, Nordmann P, Sabra A, et al. Spread of OXA-48-mediated resistance to carbapenems in Lebanese Klebsiella pneumoniae and Escherichia coli that produce extended spectrum beta-lactamase. Ann Trop Med Parasitol 2010 Apr;104(3):271-274.
78. Hirsch EB, Tam VH. Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. J Antimicrob Chemother 2010 Jun;65(6):1119-1125.
79. Nordmann P, Cuzon G, Naas T. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis 2009 Apr;9(4):228-236.
80. Patel G, Huprikar S, Factor SH, Jenkins SG, Calfee DP. Outcomes of carbapenem-resistant Klebsiella pneumoniae infection and the impact of antimicrobial and adjunctive therapies. Infect Control Hosp Epidemiol 2008 Dec;29(12):1099-1106.
81. Anderson KF, Lonsway DR, Rasheed JK, Biddle J, Jensen B, McDougal LK, et al. Evaluation of methods to identify the Klebsiella pneumoniae carbapenemase in Enterobacteriaceae. J Clin Microbiol 2007 Aug;45(8):2723-2725.
82. Lee HJ, Bergen PJ, Bulitta JB, Tsuji B, Forrest A, Nation RL, et al. Synergistic activity of colistin and rifampin combination against multidrug-resistant Acinetobacter

baumannii in an in vitro pharmacokinetic/pharmacodynamic model. Antimicrob Agents Chemother 2013 Aug;57(8):3738-3745.

83. Lagatolla C, Tonin EA, Monti-Bragadin C, Dolzani L, Gombac F, Bearzi C, et al. Endemic carbapenem-resistant Pseudomonas aeruginosa with acquired metallo-beta-lactamase determinants in European hospital. Emerg Infect Dis 2004 Mar;10(3):535-538.
84. Rasmussen BA, Bush K. Carbapenem-hydrolyzing beta-lactamases. Antimicrob Agents Chemother 1997 Feb;41(2):223-232.
85. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 2009 Dec;53(12):5046-5054.
86. Castanheira, M., M. A. Toleman, R. N. Jones, F. J. Schmidt, and T. R. Walsh. Molecular characterization of a ß-lactamase gene, *blaG*IM-1, encoding a new subclass of metallo-ß-lactamase. Antimicrob. Agents Chemother. 2004. 48:4654-4661.
87. Lee, K., J. H. Yum, D. Yong, H. M. Lee, H. D. Kim, J.-D. Docquier, G. M. Rossolini, and Y. Chong. Novel acquired metallo-ß-lactamase gene, *bla*SIM-1, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob. Agents Chemother. 2005. 49:4485-4491.
88. Struelens MJ, Monnet DL, Magiorakos AP, Santos O'Connor F, Giesecke J, European NDM-1 Survey Participants. New Delhi metallo-beta-lactamase 1-producing Enterobacteriaceae: emergence and response in Europe. Euro Surveill 2010 Nov 18;15(46):19716.
89. Walther-Rasmussen J, Høiby N. OXA-type carbapenemases. Journal of Antimicrobial Chemotherapy. 2006; 57: p. 373-383.
90. Poirel L, Castanheira M, Carrer A, Rodriguez CP, Jones RN, Smayevsky J, et al. OXA-163, an OXA-48-related class D beta-lactamase with extended activity toward expanded-spectrum cephalosporins. Antimicrob Agents Chemother 2011 Jun;55(6):2546-2551.

91. Poirel L, Heritier C, Tolun V, Nordmann P. Emergence of Oxacillinase-Mediated Resistance to Imipenem in *Klebsiella pneumoniae*. Antimicrobial Agents and Chemotherapy. 2004; 48(1): p. 15-22.
92. Tsai YK, Fung CP, Lin JC, Chen JH, Chang FY, Chen TL, et al. Klebsiella pneumoniae outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. Antimicrob Agents Chemother 2011 Apr;55(4):1485-1493.
93. Nikaido H, Vaara M. Molecular basis of bacterial outer membrane permeability. Microbiol Rev 1985 Mar;49(1):1-32.
94. Delcour AH. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta 2009 May;1794(5):808-816.
95. Pagès JM, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nature Reviews: Microbiology. 2008; 6: p. 893-903.
96. Doumith M, Ellington MJ, Livermore DM, Woodford N. Molecular mechanisms disrupting porin expression in ertapenem-resistant Klebsiella and Enterobacter spp. clinical isolates from the UK. J Antimicrob Chemother 2009 Apr;63(4):659-667.
97. Liu X, Ferenci T. Regulation of porin-mediated outer membrane permeability by nutrient limitation in Escherichia coli. J Bacteriol 1998 Aug;180(15):3917-3922.
98. Hasdemir UO, Chevalier J, Nordmann P, Pages JM. Detection and Prevalence of Active Drug Efflux Mechanism in Various Multidrug-Resistant *Klebsiella pneumoniae* Strains from Turkey. Journal of Clinical Microbiology. 2004; 42(6): p. 2701–2706.
99. Jacoby GA, Mills DM, Chow N. Role of β-Lactamases and Porins in Resistance to Ertapenem and Other β-Lactams in *Klebsiella pneumoniae*. Antimicrobial Agents and Chemotherapy. 2004; 48(8): p. 3203–3206.
100. Charrel RN, Pages JM, De Micco P, Mallea M. Prevalence of outer membrane porin alteration in beta-lactam-antibiotic-resistant Enterobacter aerogenes. Antimicrob Agents Chemother 1996 Dec;40(12):2854-2858.
101. Biendo M, Canarelli B, Thomas D, Rousseau F, Hamdad F, Adjide C, et al. Successive emergence of extended-spectrum beta-lactamase-producing and carbapenemase-

producing Enterobacter aerogenes isolates in a university hospital. J Clin Microbiol 2008 Mar;46(3):1037-1044.

102. Bidet P, Burghoffer B, Gautier V, Brahimi N, Mariani-Kurkdjian P, El-Ghoneimi A, et al. In Vivo Transfer of Plasmid-Encoded ACC-1 AmpC from *Klebsiella pneumoniae* to *Escherichia coli* in an Infant and Selection of Impermeability to Imipenem in *K. pneumoniae*. Antimicrobial Agents and Chemotherapy. 2005; 49(8): p. 3562–3565.
103. Martinez-Martinez L. Extended-spectrum beta-lactamases and the permeability barrier. Clin Microbiol Infect 2008 Jan;14 Suppl 1:82-89.
104. Grobner S, Linke D, Schutz W, Fladerer C, Madlung J, Autenrieth IB, et al. Emergence of carbapenem-non-susceptible extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates at the university hospital of Tubingen, Germany. Journal of Medical Microbiology. 2009; 58: p. 912–922.
105. Szabo D, Silveira F, Hujer AM, Bonomo RA, Hujer KM, Marsh JW, et al. Outer Membrane Protein Changes and Efflux Pump Expression Together May Confer Resistance to Ertapenem in *Enterobacter cloacae*. Antimicrobial Agents and Chemotherapy. 2006; 50(8): p. 2833–2835.
106. Srikumar R, Li XZ, Poole K. Inner membrane efflux components are responsible for beta-lactam specificity of multidrug efflux pumps in Pseudomonas aeruginosa. J Bacteriol 1997 Dec;179(24):7875-7881.
107. Li XZ, Zhang L, Srikumar R, Poole K. Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of Pseudomonas aeruginosa. Antimicrob Agents Chemother 1998 Feb;42(2):399-403.
108. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, et al. Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. Antimicrob Agents Chemother 2001 Jan;45(1):105-116.
109. Webber MA, Piddock LJV. The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy. 2003; 51(1): p. 9–11.

110. Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clinical Microbiology and Infection. 2006; 12: p. 826-836
111. Livingstone D, Gill MJ, R W. Mechanisms of resistance to the carbapenems. Journal of Antimicrobial Chemotherapy. 1995; 35(1): p. 1-5.
112. Neuwirth C, Siebor E, Duez JM, Pechinot A, Kazmierczak A. Imipenem resistance in clinical isolates of *Proteus mirabilis* associated with alterations in penicillin-binding proteins. Journal of Antimicrobial Chemotherapy. 1995; 36(2): p. 335-342.
113. Williams RJ, J YY, Livermore DM. Mechanisms by which imipenem may overcome resistance in Gram-negative bacilli. Journal of Antimicrobial Chemotherapy. 1986; 18(Supplement E): p. 9-13.
114. Poudyal A, Howden BP, Bell JM, Gao W, Owen RJ, Turnidge JD, et al. In vitro pharmacodynamics of colistin against multidrug-resistant Klebsiella pneumoniae. J Antimicrob Chemother 2008 Dec;62(6):1311-1318.
115. Cai Y, Chai D, Wang R, Liang B, Bai N. Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. J Antimicrob Chemother 2012 Jul;67(7):1607-1615.
116. Petrosillo N, Ioannidou E, Falagas ME. Colistin monotherapy vs. combination therapy: evidence from microbiological, animal and clinical studies. Clin Microbiol Infect 2008 Sep;14(9):816-827.
117. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis 2005 May 1;40(9):1333-1341.
118. Lim TP, Tan TY, Lee W, Sasikala S, Tan TT, Hsu LY, et al. In-vitro activity of polymyxin B, rifampicin, tigecycline alone and in combination against carbapenem-resistant Acinetobacter baumannii in Singapore. PLoS One 2011 Apr 21;6(4):e18485.
119. Hawley JS, Murray CK, Jorgensen JH. Colistin heteroresistance in acinetobacter and its association with previous colistin therapy. Antimicrob Agents Chemother 2008 Jan;52(1):351-352.

120. Tellis R., Rao S., Lobo A. An In-Vitro Study of Tigecycline Susceptibility Among Multidrug Resistant Bacteria in a Tertiary Care Hospital. IJBR 2012 3(4):192-195
121. Kelesidis T, Karageorgopoulos DE, Kelesidis I, Falagas ME. Tigecycline for the treatment of multidrug-resistant Enterobacteriaceae: a systematic review of the evidence from microbiological and clinical studies. J Antimicrob Chemother 2008 Nov;62(5):895-904.
122. El-Srougy R., Kalabalik J., Kim C., Song A., Carbapenem-Resistant Enterobacteriaceae: An Emerging Threat. US Pharm. 2013;38(12) HS2-HS5.
123. Njoku J, Van Schooneveld T. Fosfomycin: Review and Use Criteria. The Nebraska Medical Center [Online].; 2014 [cited 2014 April 20]. Available from: http://www.nebraskamed.com/
124. Raz R. Fosfomycin: an old--new antibiotic. Clin Microbiol Infect 2012 Jan;18(1):4-7.
125. Zavascki AP, Bulitta JB, Landersdorfer CB. Combination therapy for carbapenem-resistant Gram-negative bacteria. Expert Rev Anti Infect Ther 2013 Dec;11(12):1333-1353.
126. Rahal EA, Kazzi N, Kanbar A, Abdelnoor AM, Matar GM. Role of rifampicin in limiting Escherichia coli O157:H7 Shiga-like toxin expression and enhancement of survival of infected BALB/c mice. Int J Antimicrob Agents 2011 Feb;37(2):135-139.
127. Bassetti M, Repetto E, Righi E, Boni S, Diverio M, Molinari MP, et al. Colistin and rifampicin in the treatment of multidrug-resistant Acinetobacter baumannii infections. J Antimicrob Chemother 2008 Feb;61(2):417-420.
128. Pena C, Suarez C, Ocampo-Sosa A, Murillas J, Almirante B, Pomar V, et al. Effect of adequate single-drug vs combination antimicrobial therapy on mortality in Pseudomonas aeruginosa bloodstream infections: a post Hoc analysis of a prospective cohort. Clin Infect Dis 2013 Jul;57(2):208-216.
129. Johnson K, Boucher HW. Editorial commentary: imminent challenges: carbapenem-resistant enterobacteriaceae in transplant recipients and patients with hematologic malignancy. Clin Infect Dis 2014 May;58(9):1284-1286.
130. Brink, A., Coetzee, J., Clay, C., Corcoran, C., van Greune, J., Deetlefs, J., Nutt, L., Feldman, C., Richards, G., Nordmann, P., Poirel,L. The spread of carbapenem-resistant

Enterobacteriaceae in South Africa: Risk factors for acquisition and prevention. South African Medical Journal 2012;102(7).

131. Kmeid JG, Youssef MM, Kanafani ZA, Kanj SS. Combination therapy for Gram-negative bacteria: what is the evidence? Expert Rev Anti Infect Ther 2013 Dec;11(12):1355-1362.
132. Tascini C, Urbani L, Biancofiore G, Rossolini GM, Leonildi A, Gemignani G, et al. Colistin in combination with rifampin and imipenem for treating a blaVIM-1 metallo-beta-lactamase-producing Enterobacter cloacae disseminated infection in a liver transplant patient. Minerva Anestesiol 2008 Jan-Feb;74(1-2):47-49.
133. CLSI. Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA. 2013.
134. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0, 2014. [Online].; 2014 [cited 2014 April 20]. Available from: http://www.eucast.org
135. Soltani R., Khalili H., Shafiee F. Double disc synergy test for detection of synergistic effect between antibiotics against nosocomial strais against staphylococcus aureus. Journal of Research in Pharmacy Practice 2012 February 1(1)
136. Tsering DC, Das S, Adhiakari L, Pal R, Singh TS. Extended Spectrum Beta-lactamase Detection in Gram-negative Bacilli of Nosocomial Origin. J Glob Infect Dis 2009 Jul;1(2):87-92.
137. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. 2011.
138. Nowotny, A. Determination of toxicity, in Basic Exercises in Immunochemistry Springer 1971; p.303-305.
139. Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev 2007 Jan;20(1):79-114.
140. Xu J, McCabe BC, Koudelka GB. Function-based selection and characterization of base-pair polymorphisms in a promoter of Escherichia coli RNA polymerase-sigma(70). J Bacteriol 2001 May;183(9):2866-2873.

141. Ma L, Siu LK, Lu PL. Effect of spacer sequences between bla(CTX-M) and ISEcp1 on bla(CTX-M) expression. J Med Microbiol 2011 Dec;60(Pt 12):1787-1792.
142. Potron A, Poirel L, Nordmann P. Derepressed transfer properties leading to the efficient spread of the plasmid encoding carbapenemase OXA-48. Antimicrob Agents Chemother 2014 Jan;58(1):467-471.
143. Hook-Barnard I, Johnson XB, Hinton DM. Escherichia coli RNA polymerase recognition of a sigma70-dependent promoter requiring a -35 DNA element and an extended -10 TGn motif. J Bacteriol 2006 Dec;188(24):8352-8359.
144. Davies SN, Martin D, Millar JD, Aram JA, Church J, Lodge D. Differences in results from in vivo and in vitro studies on the use-dependency of N-methylaspartate antagonism by MK-801 and other phencyclidine receptor ligands. Eur J Pharmacol 1988 Jan 12;145(2):141-151.
145. Polak S. In vitro to human in vivo translation - pharmacokinetics and pharmacodynamics of quinidine. ALTEX 2013;30(3):309-318.
146. Opazo A, Dominguez M, Bello H, Amyes SG, Gonzalez-Rocha G. OXA-type carbapenemases in Acinetobacter baumannii in South America. J Infect Dev Ctries 2012 Apr 13;6(4):311-316.
147. Centers for Disease Control and Prevention. Carbapenem Resistant Enterobacteriaceae [Online].; 2014 [cited 2014 April 20]. Available from: http://www.cdc.gov.
148. Wilson ME, Chen LH. NDM-1 and the Role of Travel in Its Dissemination. Curr Infect Dis Rep 2012 Jun;14(3):213-226.
149. Randall LP, Bagnall MC, Karatzas KA, Coldham NC, Piddock LJ, Woodward MJ. Fitness and dissemination of disinfectant-selected multiple-antibiotic-resistant (MAR) strains of Salmonella enterica serovar Typhimurium in chickens. J Antimicrob Chemother 2008 Jan;61(1):156-162.
150. Chen TL, Fung CP, Lee SD. Spontaneous eradication of a NDM-1 positive Klebsiella pneumoniae that colonized the intestine of an asymptomatic carrier. J Chin Med Assoc 2011 Feb;74(2):104.
151. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 2010 Apr;8(4):260-271.

152. Fuursted K, Scholer L, Hansen F, Dam K, Bojer MS, Hammerum AM, et al. Virulence of a Klebsiella pneumoniae strain carrying the New Delhi metallo-beta-lactamase-1 (NDM-1). Microbes Infect 2012 Feb;14(2):155-158.
153. Andersson DI. The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol 2006 Oct;9(5):461-465.
154. Davies SN, Martin D, Millar JD, Aram JA, Church J, Lodge D. Differences in results from in vivo and in vitro studies on the use-dependency of N-methylaspartate antagonism by MK-801 and other phencyclidine receptor ligands. Eur J Pharmacol 1988 Jan 12;145(2):141-151. Hatipoglu M, Turhan V. Notice of first NDM-1 expressing isolates in Turkey and the use of modified Hodge test. Mikrobiyol Bul 2014 Jan;48(1):188-189.
155. Samuelsen O, Thilesen CM, Heggelund L, Vada AN, Kummel A, Sundsfjord A. Identification of NDM-1-producing Enterobacteriaceae in Norway. J Antimicrob Chemother 2011 Mar;66(3):670-672.
156. Skurnik D, Roux D, Cattoir V, Danilchanka O, Lu X, Yoder-Himes DR, et al. Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas aeruginosa revealed through high-throughput sequencing. Proc Natl Acad Sci U S A 2013 Dec 17;110(51):20747-20752.
157. Small details between 'in vivo' and 'in vitro' studies make for big differences. [Online]: December 2013 [cited 2014 April 20]. Available from: http://phys.org/

Appendix 1

Average weight and survival of Group I, injected with isolate IMP33, during the 10 day monitoring period. Note: Some groups were not monitored for 3 days (marked as "x"), therefore, prolonging their monitoring period by 3 days. "-" marks as no survivors in that group.

Group	Details		Days Post Infection													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group 1	TSB	Average Weight	28.00	28.33	x	x	x	29.33	29.67	30.00	29.67	30.33	30.67	31.67	31.33	32.00
		Survivors	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Group 2	Bacteria	Average Weight	30.33	27.67	x	x	x	28.67	29.00	29.67	30.00	31.00	31.67	32.33	32.67	33.33
		Survivors	6	4	2	2	2	2	2	2	2	2	2	2	2	2
Group 3	Colistin	Average Weight	32.00	31.00	x	x	x	31.67	33.00	32.00	33.00	34.00	33.67	34.00	35.00	34.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 4	Ceftazidime	Average Weight	27.67	26.67	26.00	26.00	27.33	27.33	27.00	28.00	28.67	28.67	29.33	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			
Group 5	Colistin+ Meropenem	Average Weight	33.00	32.33	x	x	x	34.33	33.33	33.67	34.67	35.00	35.33	35.67	35.67	37.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 6a	Colistin+ Ertapenem MIC	Average Weight	29.33	30.67	x	x	x	31.67	32.00	32.67	33.00	33.67	33.33	33.67	34.33	35.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 6b	Colistin+ Ertapenem MBC	Average Weight	33.00	32.33	x	x	x	34.00	34.33	35.00	35.00	35.33	35.00	35.67	36.33	37.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Group 7a	Colistin+ Imipenem MIC	Average Weight	27.67	27.33	x	x	x	28.67	29.33	29.67	30.00	30.33	31.00	31.33	31.67	32.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 7b	Colistin+ Imipenem MBC	Average Weight	28.67	28.33	x	x	x	29.67	30.00	29.67	30.67	31.00	31.33	31.67	32.00	32.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 8a	Colistin+ Tigecyclin MIC	Average Weight	32.00	31.33	x	x	x	32.67	33.33	33.67	34.33	34.00	35.00	35.67	36.33	36.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 8b	Colistin+ Tigecyclin MBC	Average Weight	33.33	32.67	x	x	x	32.33	32.00	32.67	32.33	33.00	33.67	34.00	34.33	35.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 9a	Colistin+ Fosfomycin MIC	Average Weight	33.00	31.33	x	x	x	34.00	34.33	34.67	35.00	35.33	35.33	36.00	36.33	36.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 9b	Colistin+ Fosfomycin MBC	Average Weight	28.00	26.67	x	x	x	27.33	28.67	28.33	28.67	29.33	30.00	30.00	30.67	31.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 10a	Colistin+ Kanamycin MIC	Average Weight	28.67	29.33	x	x	x	30.67	31.00	31.33	32.00	32.33	33.00	33.67	34.00	34.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 10b	Colistin+ Kanamycin MBC	Average Weight	29.67	30.00	x	x	x	31.67	32.00	32.33	32.67	32.67	33.67	34.33	35.00	35.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 11a	Kanamycin+ Tazobactam MIC	Average Weight	35.33	33.00	x	x	x	33.00	33.33	33.00	33.67	34.33	35.00	35.33	35.67	36.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 11b	Kanamycin+ Tazobactam MBC	Average Weight	28.00	28.33	x	x	x	29.00	29.33	30.00	30.00	30.67	31.00	31.33	32.00	32.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Group 12a	Aztreonam+Tazobactam MIC	Average Weight	31.00	30.67	x	x	x	31.67	32.00	32.00	32.33	33.00	32.33	33.00	33.33	33.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 12b	Aztreonam+ Tazobactam MBC	Average Weight	29.67	29.67	x	x	x	30.33	31.33	32.00	32.33	33.00	33.67	33.33	32.67	32.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 13a	Aztreonam MIC	Average Weight	32.00	31.00	x	x	x	32.33	33.67	32.67	34.67	33.00	33.33	34.33	34.67	35.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 13b	Aztreonam MBC	Average Weight	31.33	31.00	x	x	x	31.67	32.33	33.00	32.67	33.33	33.67	34.33	35.00	35.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Group 14	Ceftazidime+ Colistin	Average Weight	28	27.67	27.33	27.33	28.00	29.00	29.00	29.33	30.67	31.00	31.00	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			
Group 15	Ceftazidime+ Ertapenem	Average Weight	27.33333	27.00	25.33	26.00	26.67	27.33	27.67	28.00	28.67	29.00	29.67	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			
Group 16	Ceftazidime+ Imipenem	Average Weight	28	27.33	26.67	27.33	27.33	27.67	28.00	29.00	29.33	30.33	30.67	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			
Group 17	Ceftazidime+ Meropenem	Average Weight	32.66667	32.33	30.67	31.33	32.00	33.00	32.33	33.00	33.33	34.00	34.00	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			
Group 18	Rifampicin	Average Weight	28.66667	27.33	26.67	26.67	27.00	28.33	28.00	28.33	29.00	29.67	30.00	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			
Group 19	Rifampicin+ Colistin	Average Weight	26	25.00	25.67	26.00	26.67	27.00	27.00	27.67	28.00	28.33	29.00	x	x	x
		Survivors	3	3	3	3	3	3	3	3	3	3	3			

Appendix 2

Average weight and survival of Group II, injected with isolate IMP53, during the 10 day monitoring period. "-" marks as no survivors in that group.

Group	Details		Days Post Infection										
			1	2	3	4	5	6	7	8	9	10	11
Group 20	TSB	Average Weight	31.67	31.33	31.67	31.67	32.00	32.33	33.33	33.00	33.33	34.33	34.33
		Survivors	4	4	4	4	4	4	4	4	4	4	4
Group 21	Bacteria	Average Weight	31.33	29.33	-	-	-	-	-	-	-	-	-
		Survivors	6	2	0	0	0	0	0	0	0	0	0
Group 22	Colistin	Average Weight	30.67	27.67	26.00	26.33	26.67	27.67	28.33	28.67	29.33	30.00	30.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 23	Ceftazidime	Average Weight	26.67	26.00	-	-	-	-	-	-	-	-	-
		Survivors	3	1	0	0	0	0	0	0	0	0	0
Group 24	Colistin+ Meropenem	Average Weight	28.33	25.33	25.00	24.00	24.67	25.00	25.33	26.33	26.67	27.67	28.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 25	Colistin+ Ertapenem	Average Weight	28.67	26.67	25.33	25.00	25.67	25.67	26.00	26.33	26.67	27.00	26.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 26	Meropneme+ Ertapenem	Average Weight	31.00	28.67	26.33	26.33	26.67	27.00	27.33	27.67	28.00	28.33	28.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 27a	Colistin+ Imipenem MIC	Average Weight	32.67	29.33	28.00	27.67	28.00	28.33	28.67	29.00	29.33	30.00	30.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3

Group 27b	Colistin+ Imipenem MBC	Average Weight	33.67	30.33	28.67	29.33	30.33	30.67	31.00	31.33	31.67	32.00	32.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 28a	Colistin+ Tigecyclin MIC	Average Weight	31.67	29.00	28.00	28.33	29.00	29.33	30.00	31.33	31.33	31.67	32.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 28b	Colistin+ Tigecyclin MBC	Average Weight	30.00	27.33	26.00	26.67	27.33	27.67	28.00	28.33	29.00	29.33	29.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 29	Colistin+ Fosfomycin	Average Weight	30.00	27.33	26.33	26.67	27.00	27.33	27.67	28.00	28.33	29.33	30.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 30	Colistin: 2doses	Average Weight	32.00	31.00	29.67	30.00	30.67	31.00	31.33	31.67	32.00	32.33	32.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 31a	Colistin+ Kanamycin MIC	Average Weight	28.33	25.67	25.00	25.00	26.00	27.00	27.33	27.67	28.00	28.33	28.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 31b	Colistin+ Kanamycin MBC	Average Weight	29.33	26.67	25.33	25.00	25.67	26.00	26.67	27.00	27.33	28.00	28.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 32a	Kanamycin+ Tazobactam MIC	Average Weight	30.00	27.33	25.50	26.50	27.50	28.00	27.50	27.00	27.50	28.00	28.50
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 32b	Kanamycin+ Tazobactam MBC	Average Weight	28.33	26.00	25.00	24.33	24.33	24.67	25.67	26.33	27.00	27.67	27.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 33a	Aztreonam+ Tazobactam MIC	Average Weight	31.33	-	-	-	-	-	-	-	-	-	-
		Survivors	3	0	0	0	0	0	0	0	0	0	0

Group 33b	Aztreonam+ Tazobactam MBC	Average Weight	31.33	29	-	-	-	-	-	-	-	-	-
		Survivors	3	2	0	0	0	0	0	0	0	0	0
Group 34a	Aztreonam MIC	Average Weight	31	30	-	-	-	-	-	-	-	-	-
		Survivors	3	1	0	0	0	0	0	0	0	0	0
Group 34b	Aztreonam MBC	Average Weight	30	29	-	-	-	-	-	-	-	-	-
		Survivors	3	1	0	0	0	0	0	0	0	0	0
Group 35	Ceftazidime+ Colistin	Average Weight	27.67	25.67	24.00	22.67	21.67	22.00	22.00	23.00	23.67	24.00	24.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 36	Ceftazidime+ Ertapenem	Average Weight	26.00	25.33	29.00	28.00	29.00	30.00	29.00	29.00	30.00	30.00	31.00
		Survivors	3	3	1	1	1	1	1	1	1	1	1
Group 37	Ceftazidime+ Imipenem	Average Weight	27.00	24.50	24.00	24.00	24.50	26.00	25.50	26.50	26.00	27.00	27.50
		Survivors	3	2	2	2	2	2	2	2	2	2	2
Group 38	Ceftazidime+ Meropenem	Average Weight	32.00	25.00	23.50	24.00	23.00	23.00	24.00	25.00	25.00	26.00	27.00
		Survivors	3	3	2	1	1	1	1	1	1	1	1
Group 39	Rifampicin	Average Weight	31.00	30.00	-	-	-	-	-	-	-	-	-
		Survivors	3	1	0	0	0	0	0	0	0	0	0
Group 40	Rifampicin+ Colistin	Average Weight	32.00	29.67	28.00	28.50	29.00	29.50	29.50	30.00	30.50	31.00	32.00
		Survivors	3	3	2	2	2	2	2	2	2	2	2

Appendix 3

Average weight and survival of Group III, injected with isolate IMP216, during the 10 day monitoring period. "-" marks as no survivors in that group.

Group	Details		Days Post Infection										
			1	2	3	4	5	6	7	8	9	10	11
Group 41	TSB	Average Weight	27.00	27.33	28.00	27.67	28.00	28.33	28.67	28.67	29.00	29.33	30.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 42	Bacteria	Average Weight	23.67	23.50	23.00	23.50	23.50	24.00	25.00	24.50	25.00	25.00	25.50
		Survivors	3	2	2	2	2	2	2	2	2	2	2
Group 43	Colistin	Average Weight	31.67	31.67	31.33	31.67	32.00	32.00	32.33	32.00	32.33	32.67	33.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 44	Ceftazidime	Average Weight	25.67	25.67	25.33	25.67	26.00	26.33	26.67	27.33	27.33	27.67	28.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 45	Colistin+ Meropenem	Average Weight	27.00	27.33	27.67	27.67	28.00	28.33	28.00	28.67	29.00	29.33	29.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 46a	Colistin+ Ertapenem MIC	Average Weight	26.33	26.33	25.67	26.00	26.33	27.33	27.67	28.00	29.00	29.67	29.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 46b	Colistin+ Ertapenem MBC	Average Weight	25.67	25.67	25.33	26.00	26.00	26.33	26.67	27.00	27.33	28.33	28.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 47a	Colistin+ Imipenem MIC	Average Weight	26.33	26.00	26.33	26.33	26.00	26.67	26.33	27.00	27.33	28.00	28.67

		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 47b	Colistin+ Imipenem MBC	Average Weight	26.67	26.33	26.33	26.67	27.00	27.00	27.67	28.00	29.00	29.67	30.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 48a	Colistin+ Tigecyclin MIC	Average Weight	28.00	28.67	28.33	28.67	28.67	29.33	29.67	30.00	30.67	31.33	31.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 48b	Colistin+ Tigecyclin MBC	Average Weight	25.00	26.00	25.67	26.00	26.67	27.00	27.00	27.33	28.00	28.67	28.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 49	Colistin+ Fosfomycin	Average Weight	23.67	24.00	24.00	24.33	24.67	25.00	25.00	25.67	26.33	26.67	27.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 50	Colistin+ Kanamycin	Average Weight	25.00	25.33	25.33	25.67	25.33	25.33	25.67	26.00	26.00	26.33	26.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 51	Kanamycin+ Tazobactam	Average Weight	24.00	24.00	24.33	24.67	25.00	25.67	25.67	26.00	26.33	26.67	27.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 52	Kanamycin	Average Weight	22.33	23.00	23.00	23.33	23.67	24.00	24.67	25.00	25.67	26.00	26.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 53a	Aztreonam+ Tazobactam MIC	Average Weight	22.67	22.67	23.00	23.33	23.67	24.00	24.00	25.00	25.67	26.00	26.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 53b	Aztreonam+ Tazobactam MBC	Average Weight	22.00	22.33	22.33	22.00	22.33	22.67	23.00	23.33	23.67	24.00	24.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3

Group 54a	Aztreonam MIC	Average Weight	25.33	25.33	24.50	24.50	25.00	25.50	25.00	26.00	26.50	27.00	27.00
		Survivors	3	3	2	2	2	2	2	2	2	2	2
Group 54b	Aztreonam MBC	Average Weight	23.33	23.67	24.00	24.00	24.33	24.67	25.00	25.33	25.33	26.00	26.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 55	Ceftazidime+ Colistin	Average Weight	28.00	28.33	28.33	28.00	28.67	29.00	29.33	28.33	29.67	30.00	30.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 56	Ceftazidime+ Ertapenem	Average Weight	23.00	23.33	23.00	23.33	24.00	24.67	25.33	25.00	25.33	26.00	26.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 57	Ceftazidime+ Imipenem	Average Weight	23.67	23.67	23.50	24.00	24.50	24.00	25.00	24.50	25.00	25.50	26.00
		Survivors	3	3	2	2	2	2	2	2	2	2	2
Group 58	Ceftazidime+ Meropenem	Average Weight	24.00	24.33	24.00	24.33	25.00	25.00	25.33	25.67	26.33	26.67	27.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 59	Rifampicin	Average Weight	27.33	27.33	27.00	27.67	28.00	28.00	28.67	28.67	29.00	29.33	30.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 60	Rifampicin+ Colistin	Average Weight	25.00	24.33	24.33	25.00	25.00	26.00	26.33	26.67	27.00	26.67	27.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
			1	2	3	4	5	6	7	8	9	10	11
Group			Days Post infection										

Appendix 4

Average weight and survival of Group IV, injected with isolate KPC, during the 10 day monitoring period. "-" marks as no survivors in that group.

Group	Details		Days Post Infection										
			1	2	3	4	5	6	7	8	9	10	11
Group 61	TSB	Average Weight	30.00	30.33	30.33	30.67	31.33	31.00	31.67	32.00	32.67	33.00	32.67
		Survivors	4	4	4	4	4	4	4	4	4	4	4
Group 62	Bacteria	Average Weight	30.00	28.00	-	-	-	-	-	-	-	-	-
		Survivors	6	2	0	0	0	0	0	0	0	0	0
Group 63a	Colistin MIC	Average Weight	30.33	27.67	25.00	25.50	26.00	26.50	27.00	27.00	27.50	27.00	28.00
		Survivors	3	3	2	2	2	2	2	2	2	2	2
Group 63b	Colistin MBC	Average Weight	29.67	27.33	26.33	24.50	25.50	26.00	26.00	27.00	27.50	27.50	28.00
		Survivors	3	3	3	2	2	2	2	2	2	2	2
Group 64	Ceftazidime	Average Weight	28.33	26.00	24.67	23.00	23.00	23.50	24.00	24.50	25.50	26.00	26.50
		Survivors	3	3	3	2	2	2	2	2	2	2	2
Group 65a	Colistin+ Ertapenem MIC	Average Weight	29.00	27.67	25.00	24.67	25.33	25.33	26.00	26.33	26.67	27.00	27.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3

Group 65b	Colistin+ Ertapenem MBC	Average Weight	29.67	26.33	26.00	26.33	26.67	27.00	27.67	28.00	28.67	28.33	28.67
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 66a	Colistin+ Imipenem MIC	Average Weight	25.00	23.00	22.50	22.00	22.50	22.50	23.00	23.50	24.00	24.50	24.50
		Survivors	3	2	1	1	1	1	1	1	1	1	1
Group 66b	Colistin+ Imipenem MBC	Average Weight	25.67	23.00	23.50	23.50	24.00	24.00	24.50	24.00	25.00	25.50	26.00
		Survivors	3	2	2	2	2	2	2	2	2	2	2
Group 67a	Colistin+ Meropenem MIC	Average Weight	30.33	28.00	28.33	28.33	29.33	30.00	29.67	30.33	30.67	31.00	31.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 67b	Colistin+ Meropenem MBC	Average Weight	28.33	24.67	24.33	24.33	24.67	25.00	25.33	25.67	26.00	26.67	27.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 68a	Colistin+ Tigecylcin MIC	Average Weight	32.33	-	-	-	-	-	-	-	-	-	-
		Survivors	3	0	0	0	0	0	0	0	0	0	0
Group 68b	Colistin+ Tigecylcin MBC	Average Weight	30.33	26.00	26.00	26.00	26.33	27.00	27.33	27.67	28.00	27.67	28.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 69a	Colistin+ Fosfomycin MIC	Average Weight	29.00	26.67	25.67	25.67	26.00	26.33	27.33	27.00	27.67	28.00	28.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3

Group 69b	Colistin+ Fosfomycin MBC	Average Weight	28.67	25.33	24.33	24.00	24.00	25.33	25.67	26.00	26.33	26.67	27.33
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 70a	Colistin+ Kanamycin MIC	Average Weight	28.67	25.00	23.33	23.67	24.67	25.33	25.67	26.33	25.67	26.67	27.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 70b	Colistin+ Kanamycin MBC	Average Weight	25.67	22.33	22.00	22.00	23.00	23.33	23.67	24.00	24.67	25.00	25.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 71	Kanamycin+ Tazobactam	Average Weight	27.33	24.33	22.67	23.00	23.00	24.00	23.00	24.00	24.00	25.00	25.00
		Survivors	3	3	3	1	1	1	1	1	1	1	1
Group 72	Kanamycin	Average Weight	28.00	26.67	25.00	25.00	25.50	25.50	26.00	26.50	27.00	26.50	27.50
		Survivors	3	3	2	2	2	2	2	2	2	2	2
Group 73a	Aztreonam+ Tazobactam MIC	Average Weight	27.67	-	-	-	-	-	-	-	-	-	-
		Survivors	3	0	0	0	0	0	0	0	0	0	0
Group 73b	Aztreonam+ Tazobactam MBC	Average Weight	26.00	-	-	-	-	-	-	-	-	-	-
		Survivors	3	0	0	0	0	0	0	0	0	0	0
Group 74a	Aztreonam MIC	Average Weight	27.00	22.00	24.00	24.00	25.00	25.00	26.00	27.00	26.00	27.00	27.00
		Survivors	3	2	1	1	1	1	1	1	1	1	1

Group 74b	Aztreonam MBC	Average Weight	28.67	26.00	27.00	27.00	27.50	28.00	27.50	27.50	28.00	28.50	29.00
		Survivors	3	2	1	1	1	1	1	1	1	1	1
Group 75	Ertapenem	Average Weight	28.00	26.50	-	-	-	-	-	-	-	-	-
		Survivors	3	2	0	0	0	0	0	0	0	0	0
Group 76	Ceftazidime+ Colistin	Average Weight	25.33	23.67	22.67	23.00	23.33	24.00	24.67	25.00	25.67	26.33	27.00
		Survivors	3	3	3	3	3	3	3	3	3	3	3
Group 77	Ceftazidime+ Ertapenem	Average Weight	28.00	26.33	26.00	26.33	26.33	26.67	27.33	27.00	27.67	28.00	28.00
		Survivors	3	3	2	2	2	2	2	2	2	2	2
Group 78	Ceftazidime+ Imipenem	Average Weight	27.00	24.00	22.50	22.00	22.50	23.00	23.50	24.50	24.50	25.00	25.50
		Survivors	3	2	2	2	2	2	2	2	2	2	2
Group 79	Ceftazidime+ Meropenem	Average Weight	30.67	24.00	24.00	25.00	25.00	26.00	25.00	26.00	26.00	27.00	27.00
		Survivors	3	1	1	1	1	1	1	1	1	1	1
Group 80	Rifampicin	Average Weight	26.67	23.33	23.00	22.00	-	-	-	-	-	-	-
		Survivors	3	3	2	1	0	0	0	0	0	0	0
Group 81	Rifampicin+ Colistin	Average Weight	24.67	23.50	23.50	24.00	24.00	24.50	25.00	24.50	25.50	25.50	26.00
		Survivors	3	2	2	2	2	2	2	2	2	2	2