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IN VITRO ACTIVITY OF POLYMYXIN B AND MEROPENEM ALONE AND IN COMBINATION AGAINST CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

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IN VITRO ACTIVITY OF POLYMYXIN B AND MEROPENEM ALONE AND IN COMBINATION AGAINST CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Pharmacy Practice and Science at the University of Kentucky

By

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2016

ABSTRACT OF THESIS

IN VITRO ACTIVITY OF POLYMYXIN B AND MEROPENEM ALONE AND IN COMBINATION AGAINST CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

Background: Infections caused by carbapenem-resistant Enterobacteriaceae such as *Escherichia coli* and *Klebsiella pneumoniae* are among the most urgent threats of the infectious disease realm. The incidence of these infections has only been increasing over the years and due to very limited treatment options, mortality is estimated at about 50%.

Methods: To evaluate the *in vitro* activity of meropenem and polymyxin B against carbapenem-resistant Enterobacteriaceae, antimicrobial susceptibility testing and time-kill studies were performed on *K. pneumoniae* clinical isolates representing a wide range of meropenem resistance (MICs 4 – 128 mg/L).

Results: Regrowth was observed at clinically relevant concentrations of meropenem alone (4, 16, and 64 mg/L) or polymyxin B alone (0.25 and 1 mg/L) within 24 hours. However, meropenem and polymyxin B in combination were consistently bactericidal, achieving synergistic activity in strains with lower meropenem resistance (MICs ≤32 mg/L).

Conclusions: Our findings are in agreement with the limited available literature, but we add that the synergistic interaction between meropenem and polymyxin B is dependent on the degree of meropenem resistance in KPC-producing *K. pneumoniae*. This data suggests that lower level resistance to carbapenems may be amenable to antimicrobial combinations involving a carbapenem and a polymyxin.

KEYWORDS: *Klebsiella pneumoniae*, time-kill, meropenem, polymyxin B, synergy

Brandon Kulengowski

4/23/16

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This thesis is dedicated to Dr. David Burgess whose passion for research and leadership is only surpassed by his love for teaching and mentorship of students like myself. I would not be where I am today without his vision, his patience, and his support of all that I have done. For this, I am most thankful and hope I have made him proud.

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Chapter One:

Antimicrobial Resistance

Infectious disease treatment and management in patients is made difficult when resistance to contemporary antimicrobial agents is involved. In fact, expression of resistance renders antimicrobial agents less effective and has been overwhelmingly associated with poor clinical outcomes, including increased mortality.[1-10] Unfortunately, resistance always follows the development of any novel antimicrobial, given enough time (Figure 1.1).[11] Commonly known pathogens such as *Escherichia coli* and *Klebsiella pneumoniae* are among the most notorious for expression of drug resistance because they exist as part of the normal flora in the gastrointestinal tract of humans. Frequent antimicrobial consumption fosters development of drug resistance among these enteric bacteria for which novel antimicrobials are dwindling and currently available antimicrobials are few.[12] With this in mind, efforts such as the present work contribute information to questions such as what antimicrobials alone or in combination provide patients with the greatest chance of survival when confronted by these highly resistant pathogens? Do particular agents work better together than others? How many antimicrobial agents are sufficient to ensure a high probability of recovery?

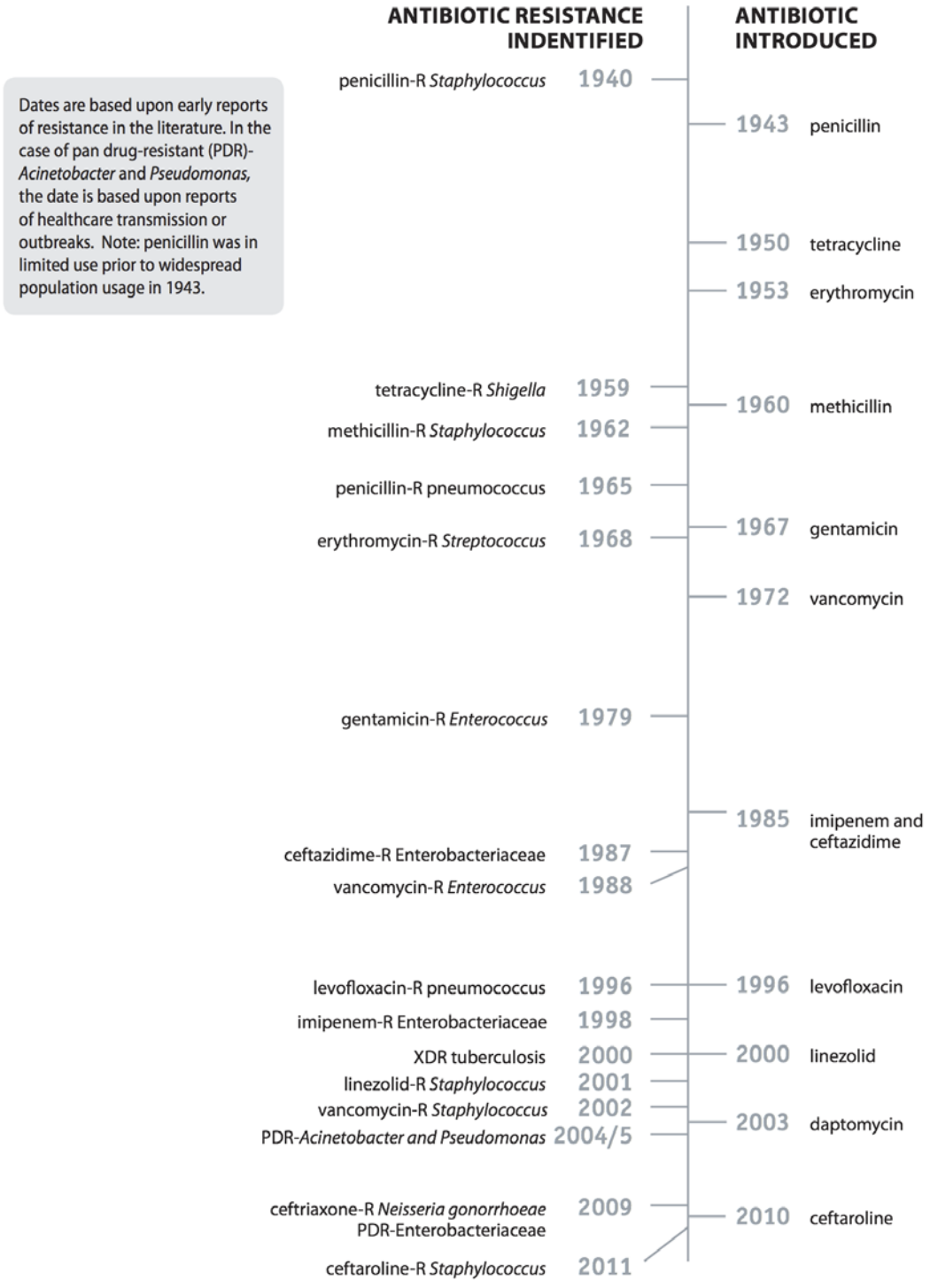


Figure 1.1: Antimicrobial Resistance Timeline. **Reprinted**[11]

In March 2015, the U.S. Department of Health and Human Services (HHS) established the President's Advisory Council on Combating Antibiotic-Resistant Bacteria (PACCARB) which is responsible for providing advice, information, and recommendations to the Secretary of Health and Human Services regarding programs and policies from the National Action Plan. Within this plan is the Centers for Disease Control and Prevention (CDC) 2013 report which categorized a variety of antimicrobial resistance problems based on seven factors associated with resistant infections – clinical impact, economic impact, incidence, 10-year projection of incidence, transmissibility, availability of effective antibiotics, and barriers to prevention.

Three threat levels (concerning, serious, and urgent) were identified using these seven factors (Figure 1.2). Serious threats include organisms such as extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug resistant *Pseudomonas aeruginosa*, and drug-resistant *Tuberculosis*, among others. Antimicrobial resistance in gram-negative organisms, specifically carbapenem-resistant Enterobacteriaceae (CRE), were among the highest of threat levels, designated as urgent (Figure 1.2).[11] The present work focuses on this group of urgent threat level pathogens.

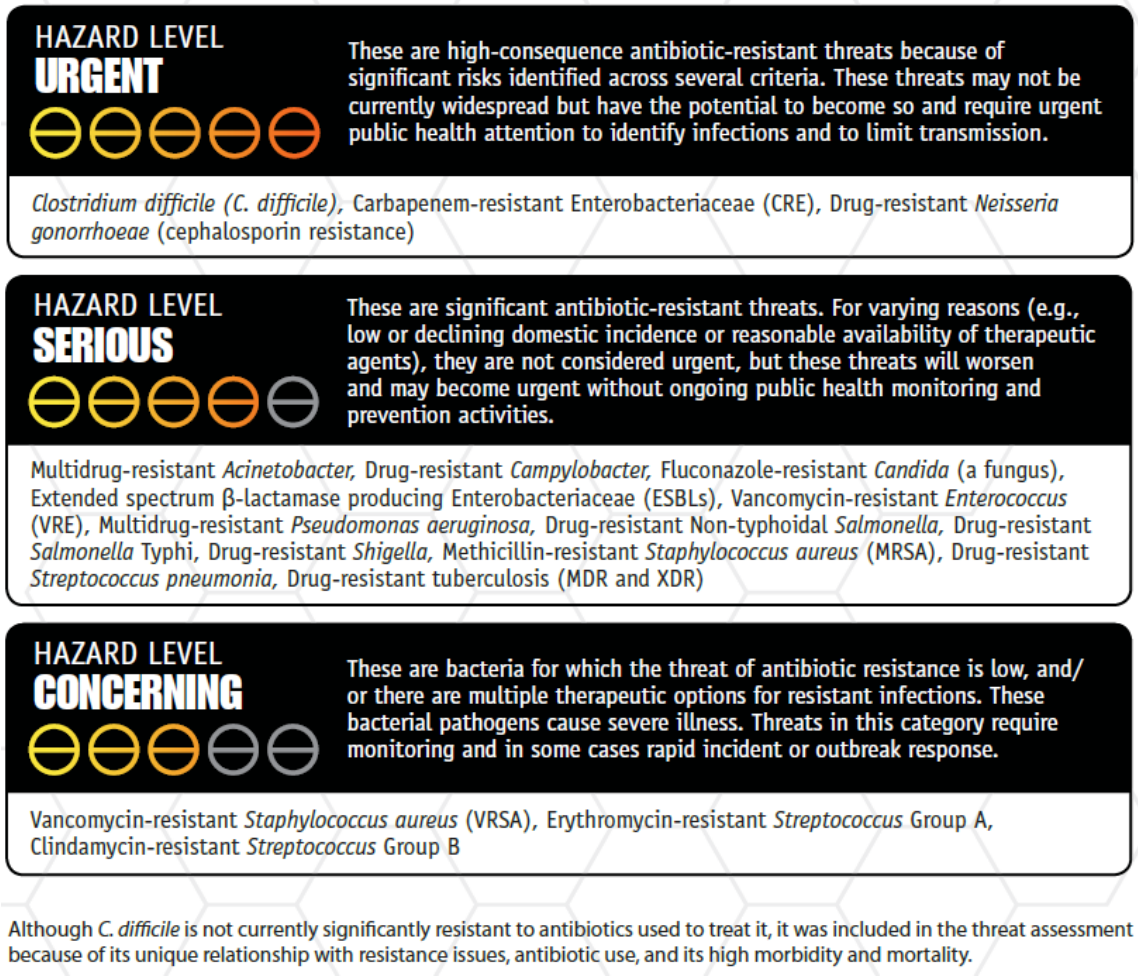


Figure 1.2: Antimicrobial Resistance Threat Levels. **Reprinted**[11]

MECHANISMS OF RESISTANCE

Understanding mechanisms of resistance is important because these shape the direction of research and also the choice of therapy. For example, in the interest of obtaining clinically relevant information, it may be more advantageous to focus on characterizing the more common mechanisms of resistance rather than the least common where particular knowledge about a certain mechanism of resistance may afford certain therapeutic advantages (e.g. the sustained activity of aztreonam against exclusively metallo-β-lactamase producing organisms). For this reason, a general discussion of mechanisms of resistance is valuable and specifics to the present work will be covered in Chapter 2.

Resistance mechanisms can be broadly grouped into four categories. 1) efflux pumps which actively remove antimicrobials from the target site of action, 2) enzymatic degradation of the antimicrobial agent (e.g. β-lactamases),[13] 3) changes in cell wall permeability which may slow or prevent the antimicrobial from reaching the target site (e.g. mutations in channels called porins), and 4) target site alterations that prevent the antimicrobial from binding (Figure 1.3). Bacteria are not limited to simply one mechanism and in fact often exhibit multiple mechanisms which may confer resistance to multiple classes of antimicrobials at once. For β-lactam antimicrobials, enzymatic degradation is the most common mechanism.[14]

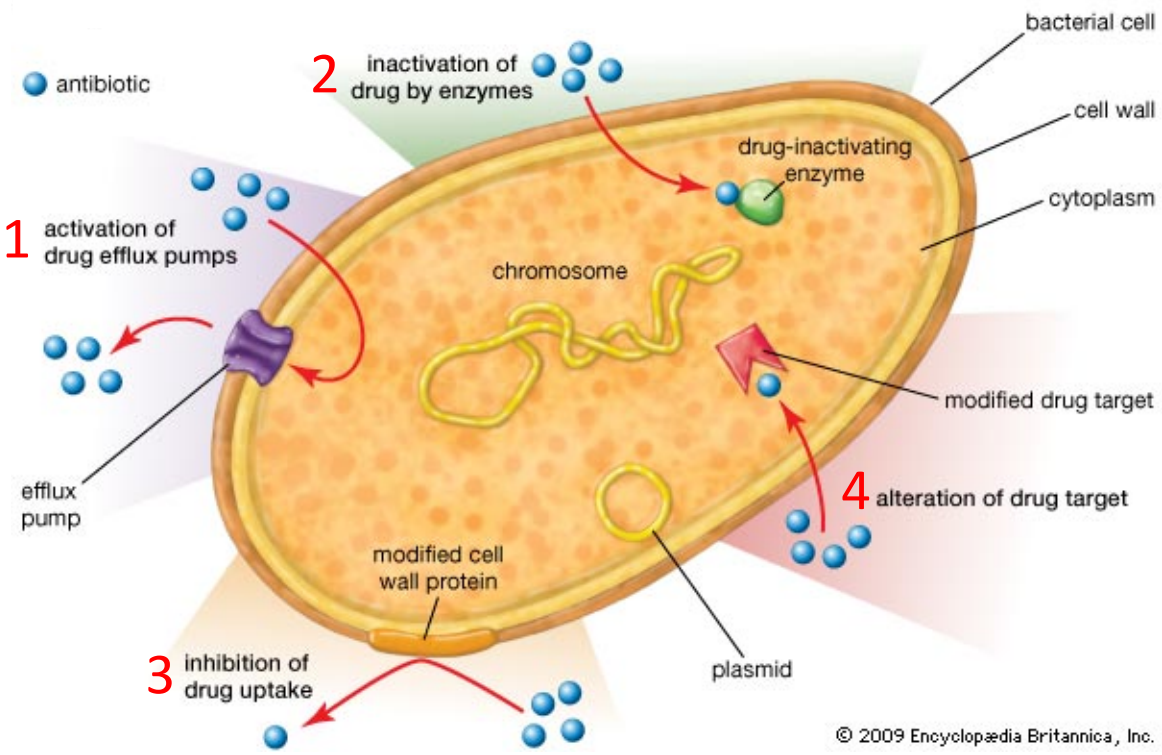


Figure 1.3: Mechanisms of Antibiotic Resistance. **Reprinted**[15]

HISTORY OF β-LACTAM RESISTANCE DEVELOPMENT

Penicillin, the original β-lactam, was first administered to Anne Miller in 1942 as treatment for a streptococcal bloodstream infection. However, bacteria have been evolving to survive long before the introduction of antibiotics to humans. In fact, Edward Abraham and Ernst Chain[16] identified a mechanism of penicillin resistance in 1940, two years before penicillin was even administered to Anne Miller. They discovered that a particular strain of *Escherichia coli* produced AmpC ("Classification of β-lactamases"), an enzyme capable of inactivating penicillin, and in later experiments it was found that previously penicillin-sensitive *Staphylococcus aureus* could be made penicillin resistant after continuous subculture in the presence of penicillin *in vitro.*[17] By 1943, one year following human introduction to penicillin, four penicillin-resistant staphylococci strains were isolated from patients during the course of treatment.[18] The predominant mechanism of resistance among *Staphylococcus aureus* at this time was discovered to be β-lactamase production,[19,20] termed "penicillinases."

In response, the dose of penicillin was increased to compensate for reduced susceptibility;[21] but by 1947, a majority of hospital *Staphylococcus aureus* isolates were entirely resistant to penicillin.[22] To counter the growing resistance rates, chemists developed anti-staphylococcal penicillins (e.g. methicillin) for gram-positive organisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis* and aminopenicillins (e.g. ampicillin) for gram-negative organisms such as *Escherichia coli, Klebsiella pneumoniae,* and *Serratia marcescens.*[23] These novel antimicrobials were not hydrolyzed by early penicillinase-producing organisms.

Primarily in gram-negative organisms, β-lactamases developed that conferred resistance to aminopenicillins (classified as TEM-1 and TEM-2 in organisms like *E. coli* and SHV-1 in *K. pneumoniae*). These enzymes were countered by the development of β-lactam/β-lactamase inhibitor combinations (e.g. ampicillin/sulbactam) and cephalosporins. These compounds functioned in the presence of early β-lactamases such as TEM-1, TEM-2, and SHV-1, but once again, resistance developed with AmpC and ESBL-production. At first, ESBLs in the U.S. were point mutations of the TEM and SHV families, of which there are now hundreds of different subtypes. However, other ESBL families also developed and spread from other parts of the world, such as the second-largest group, CTX-M, originally from the chromosome of *Kluyvera spp*.[24] OXA-type β-lactamases are another example of an ESBL, originally discovered in a *Pseudomonas aeruginosa*[25] isolate from Turkey. There are myriad other β-lactamase families (PER,[26,27] VEB,[28] GES,[29,30] BES,[31] TLA,[32] SFO,[33] and IBC[34,35]), discovered from diverse geographic locations, and even some chromosomally located ESBLs.[36]

Today, the CDC estimates that 19% of healthcare-associated Enterobacteriaceae infections in the U.S. are caused by ESBL-producing organisms. Of the top two Enterobacteriaceae, 23% of *Klebsiella pneumoniae* and 14% of *Escherichia coli* infections now produce ESBL.[11] For perspective, ten years ago, the National Healthcare Safety Network (NHSN) estimated 1% of *Klebsiella pneumoniae* infections and 0.5% of *E. coli* infections produced ESBLs. In other parts of the world, ESBL-producing bacteria are as high as 52% in Thailand,[37] and 70% in Egypt.[38] Eastern Europe has also reported rates as high as 25-50%.[39] Generally speaking, the rate of ESBLs in Europe is higher than that of the U.S., but lower than Latin American or Asia.[40]

The antimicrobial agents of choice for ESBL-producing organisms are the carbapenems. Currently there are four – imipenem, ertapenem, meropenem, and doripenem. However, we now enter the current era of the resistance mechanisms – carbapenemases. These will be discussed in more detail in "Chapter 2: Carbapenem Resistant Enterobacteriaceae (CRE)," but the two most common groups are *Klebsiella pneumoniae* carbapenemases (KPC; more common in the U.S.) and metallo-β-lactamases (MBL; more common in Europe and Southeast Asia;[13] Figure 1.4).

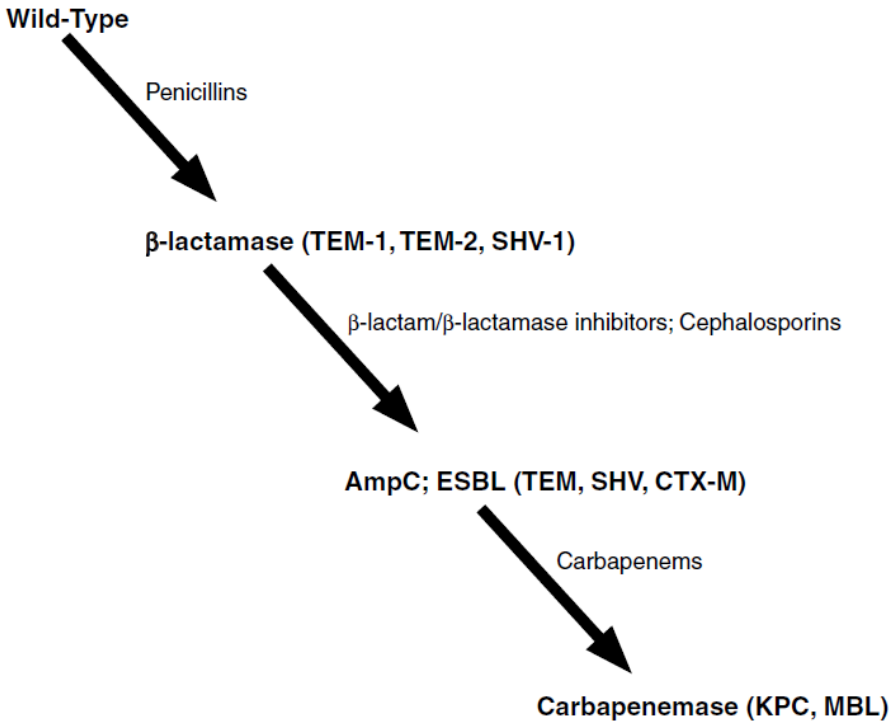


Figure 1.4: The Evolution of β-lactamases. **Reprinted**[13]
ESBL = extended-spectrum β-lactamase; KPC = *Klebsiella pneumoniae* carbapenemase
MBL = metallo-β-lactamase
TEM-1, TEM-2, SHV-1, TEM, SHV, CTX-M = types of β-lactamases

CLASSIFICATION OF β-LACTAMASES

To date, many attempts have been made to categorize β-lactamase enzymes, but these classification schemes can be summarized in two major approaches – classification based on biochemical and functional characteristics or classification based on molecular structure of the enzymes.[41] For the former, criteria such as the spectrum of antimicrobial substrates, hydrolysis rate (V_{max}), binding affinity (K_m), isoelectric focusing (pI), molecular weight, and amino acid composition[42] have been used to develop classes and many subclasses,[43] but will not be discussed here. The simpler, molecular classification scheme uses four classes (*Ambler class* A-D) which are described below.

Ambler class A is the broadest class and is most simply described as a catch-all class to enzymes not fitting one of the other classes, consisting of β-lactamase enzymes that are located on plasmids, transposons, or chromosomes (e.g. TEM, SHV, PER, PSE, hundreds of others).[41,44,45] Class A enzymes range from hydrolyzing a narrow spectrum of β-lactams (e.g. penicillinases) to a broad spectrum (e.g. ESBL or carbapenemases). A very high degree of sequence variability and kinetic properties exist for this class.

Ambler class B enzymes, also called metallo-β-lactamases (MBLs), require zinc to carry out their function. Common MBLs in CRE organisms are imipenem-type carbapenemases (IMP), Verona integron-encoded metallo-β-lactamases (VIM), and New Delhi metallo-β-lactamase (NDM). Class B enzymes hydrolyze all β-lactam antimicrobials except monobactams (e.g. aztreonam) and are not inhibited by any current β-lactamase inhibitors such as clavulanic acid, sulbactam, tazobactam, and avibactam.

Ambler class C enzymes, commonly known as the cephalosporinases[46] or AmpC enzymes, are chromosomally encoded with highly conserved sequences.[47,48] AmpC enzymes hydrolyze most extended spectrum β-lactams and β-lactam/β-lactamase inhibitor combinations such as ceftriaxone or piperacillin/tazobactam, but usually not carbapenems. However, CMY-10 is among the first AmpC enzymes that hydrolyze carbapenems.[49] Additionally, non-carbapenem hydrolyzing AmpC production in combination with porin channel mutations can also confer resistance to carbapenems.[41]

Ambler class D enzymes are also known as oxacillinases (OXA) due to their ability to hydrolyze isoxazolyl β-lactamases such as oxacillin and methicillin.[50] There is a lot of structural similarity between class D and class A enzymes which can make differentiation, or even detection, difficult.[41] Class D enzymes are usually not inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam, but they are inhibited *in vitro* by sodium chloride concentrations of 100mM.[51-55] Additionally, these enzymes are relatively inactive against cephalosporins.[14] Although the OXA enzyme family consists of more than a hundred unique subtypes, 9 are considered ESBL and 37 are considered to be carbapenemases.[56]

DEVELOPMENT OF RESISTANCE

Resistance to antimicrobials can be **inherent**, "natural" resistance such as inadequate uptake of an antimicrobial due to lack of transporters (e.g. *Pseudomonas aeruginosa* and tetracycline antimicrobials), lack of drug-activating mechanisms (e.g. metronidazole and aerobic organisms), or lack of target sites (e.g. penicillin binding proteins of enterococci and all cephalosporin class antimicrobials); or resistance to antimicrobials can be **acquired** through normal mutation, vertically through reproduction, or horizontally through transformation, transduction, and conjugation (Figure 1.5). Sometimes, observable resistance requires a combination of the above.

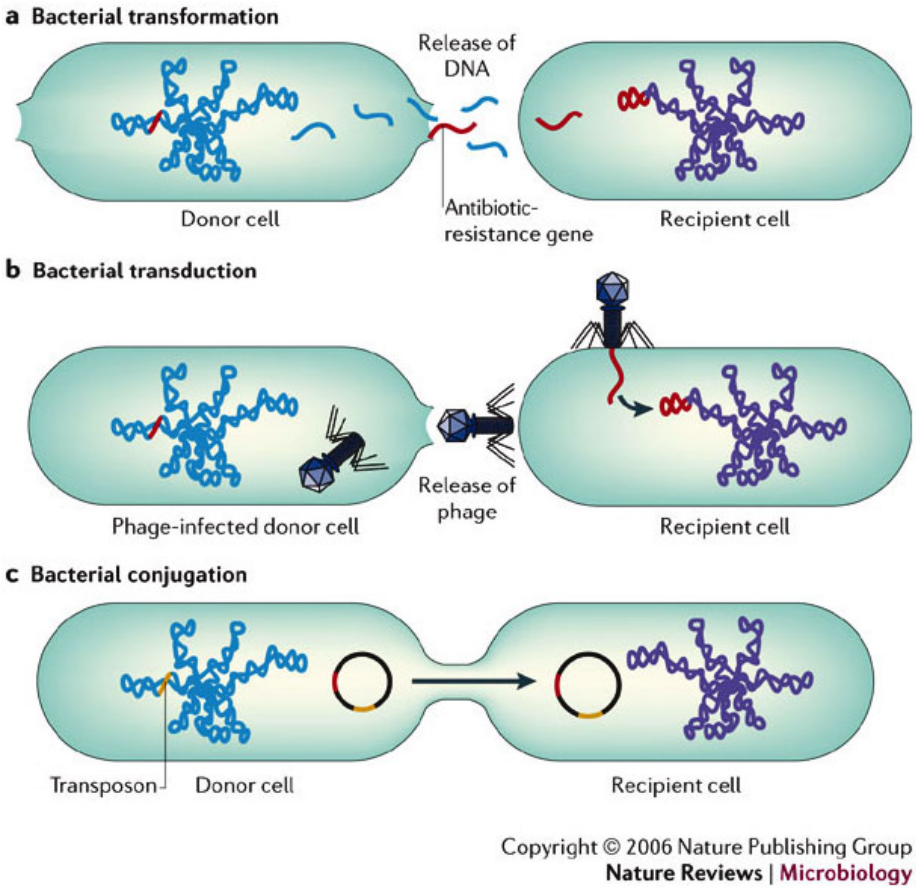


Figure 1.5: Horizontal Acquisition of Resistance. **Reprinted**[57]

Acquired resistance is an area of great concern because, unlike intrinsic resistance, these resistance profiles can be dynamic and unique for each species of bacteria and can change even during the course of therapy, making clinical decisions difficult and outcomes worse. Spontaneous mutation frequency that confers antimicrobial resistance is approximately on the order of 10^{-8} to 10^{-9} which means that one in every hundred billion to one trillion bacteria in an infection will develop resistance to an antimicrobial through random mutation.[58] Once exposed to an environment containing an antimicrobial, resistant organisms are preferentially selected for survival and this resistance can then be passed along through reproduction or through horizontal gene transfer.

In the setting of horizontal gene transfer, genetic material can be exchanged between individual bacteria of the same or different species.[58] One of the most common methods is conjugation where bacteria come into direct cell-to-cell contact and exchange small pieces of DNA called plasmids which may contribute to the explanation of why *Ambler class* A β-lactamases are so diverse in protein structure whereas class C β-lactamases (i.e. AmpC β-lactamases, which are typically located chromosomally) retain such highly conserved protein sequences. Another method of gene transfer is transformation where parts of DNA are taken up by bacteria from the environment which usually originated from the death or lysis of another bacterium.[58] The final method is transduction where bacteria-specific viruses called bacteriophages inject DNA into the bacteria cell (Figure 1.5).

Chapter Two:

Carbapenem-resistant Enterobacteriaceae (CRE)

This chapter will discuss epidemiology, therapeutic agents, and current literature on carbapenem-resistant Enterobacteriaceae – the group of drug resistant pathogens labeled an urgent threat by the CDC.[11] Enterobacteriaceae include organisms such as *Escherichia coli*, *Klebsiella spp.*, *Enterobacter spp.*, *Salmonella spp.*, *Shigella spp.*, *Proteus spp.*, *Serratia spp.*, *Citrobacter spp.*, and *Yersinia pestis.* CRE-specific resistance mechanisms are often more complex, not always entirely understood, and can be multifaceted. One commonality between CRE and other gram-negative organisms is that enzyme production (e.g. β-lactamase) is still the most common resistance mechanism, but can also be present in combination with changes in cell wall permeability (e.g. porin channel mutations), upregulation of efflux pumps, or target site alterations which can further contribute to carbapenem resistance.[14] Additionally, β-lactamases without intrinsic carbapenemase activity but with cephalosporinase activity (e.g. DHA, ACT, CMY, SHV-5, CTX-M-15) can contribute to carbapenemase resistance when combined with other non-enzyme mediated mechanisms of resistance.[59-62]

EPIDEMIOLOGY OF CARBAPENEM RESISTANCE

Understanding the epidemiology of CRE will attest to the relevance of the present work and help predict the ultimate direction of future studies. Interestingly, substantial geographic diversity exists for CRE. For example, in the United States, *Klebsiella pneumoniae* with KPC-2 or KPC-3 (two Ambler class A serine-based enzymes) comprise about 80% of CRE cases.[14,63-65] In contrast, MBL (Ambler class B) or OXA-like (Ambler class D*)* enzymes are more clinically significant world-wide such as in India and Pakistan (NDM),[66] Greece and Italy (VIM),[67] or Turkey, Spain and North Africa (OXA-48).[14,68-72] Some countries (e.g. China) have low CRE prevalence, but significant diversity of carbapenemase enzymes.[73,74] Coexistence of various sequence types of MBLs and KPCs within the same *Klebsiella pneumoniae* strains has also been observed.[67,75-77] Ultimately, no two countries are the same when it comes to CRE characteristics and the prevalence can be drastically different, even between acute-care centers within the same country.[14]

Regarding the U.S. specifically, there are no national requirements to report CRE, but requirements for reporting CRE do vary by state.[78] To facilitate data collection nationally, the CDC maintains two voluntary surveillance systems for CRE monitoring – Healthcare Associated Infections Community Interface (HAIC) which uses 10 sites across the nation for determining communities having or at risk for having CRE infections, and the National Healthcare Safety Network (NHSN) which tracks 17,000 facilities nationwide for healthcare-associated infections.[79] Figures 2.1-2.4 show the data reported to the CDC, separated by β-lactamase type (KPC, NDM, VIM, OXA-48) and by state.

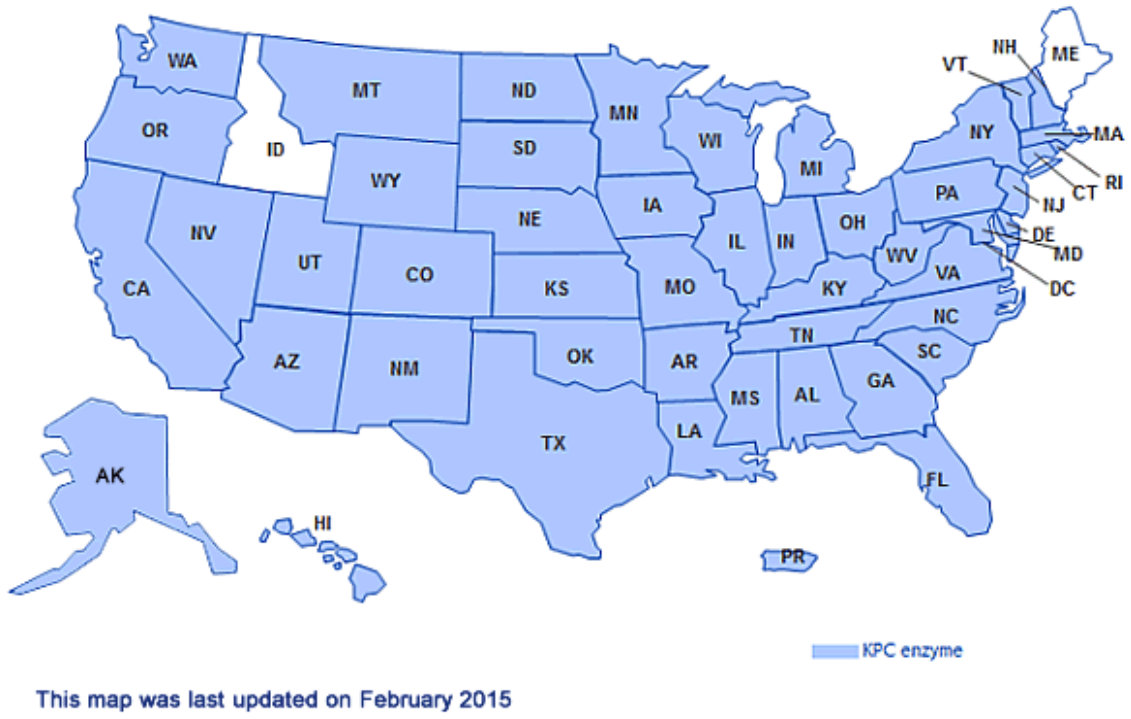


Figure 2.1: States with KPC-producing CRE Reported to the CDC. **Reprinted**[79]

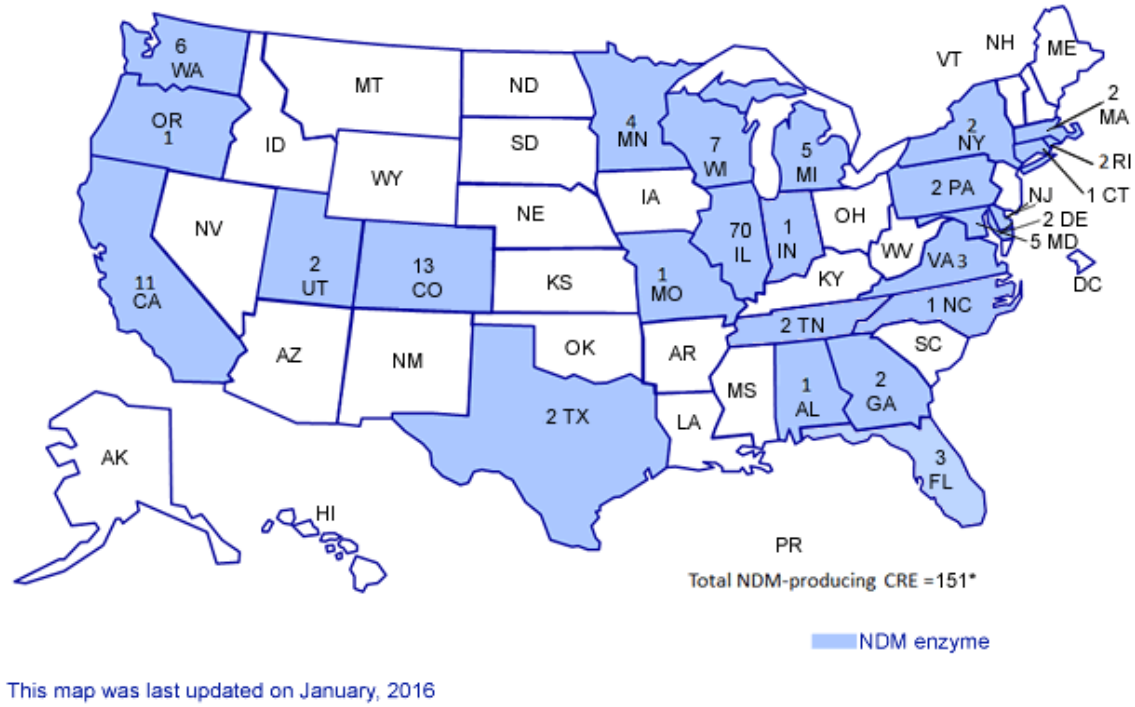


Figure 2.2: NDM-producing CRE Isolates Reported to the CDC. **Reprinted**[79]

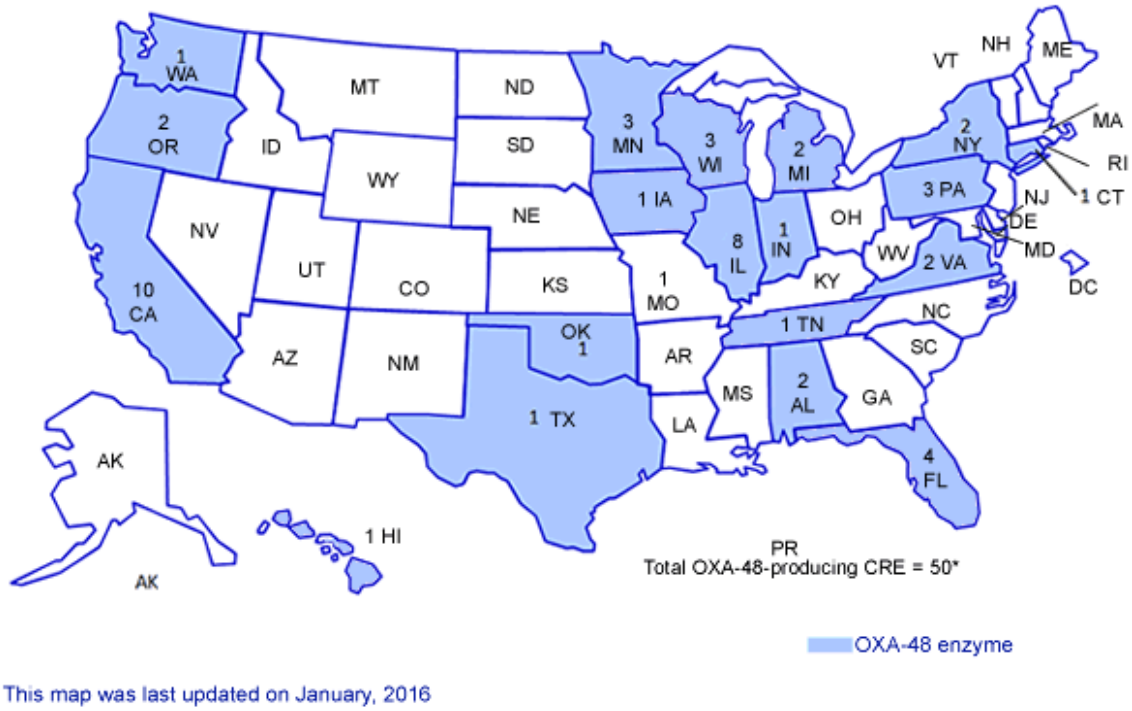


Figure 2.3: OXA-48-producing CRE Isolates Reported to the CDC. **Reprinted**[79]

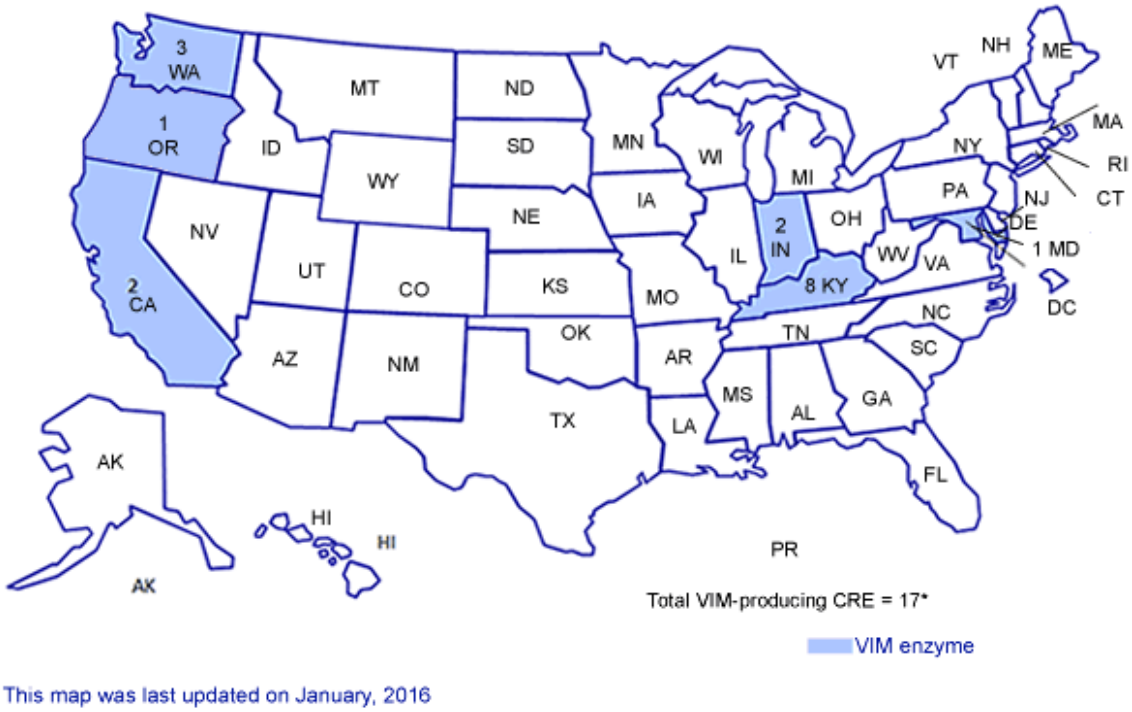


Figure 2.4: VIM-producing CRE Isolates Reported to the CDC. **Reprinted**[79]

Klebsiella pneumoniae Carbapenemase (KPC; Class A)

A KPC-producing CRE was first identified in the United States from *Klebsiella pneumoniae* cultured from a patient in North Carolina in 1996, but reported in 2001.[80] Thereafter named KPC-1, it was not the first carbapenemase to be reported in Enterobacteriaceae because MBLs had been identified in Enterobacteriaceae in Japan as early as 1991.[81-83] It was, however, the first Ambler class A, serine-based carbapenemase to be found in Enterobacteriaceae. Subtype variants KPC-2 (later identified as identical to KPC-1) and KPC-3, initially concentrated in eastern states such as New York and New Jersey,[3,80,84-90] but have since spread to all but two states, Idaho and Maine (Figure 2.1). Among CRE sent to the CDC, KPC has primarily been identified in *Klebsiella pneumoniae, Escherichia coli,* and *Enterobacter* spp.,[91] and the KPC-2 and KPC-3 subtype variants are the most common in the United States. As of July 2015, Lahey Clinic had 24 KPC subtypes reported,[92] but only subtypes 1/2, 3, 4, 6, 7, 8, 11 and 12 have been reported in *Klebsiella pneumoniae*.[93-95]

Outside the U.S., the first KPC-producing Enterobacteriaceae was identified in 2005 from a hospital in Paris, France where a patient had been recently hospitalized in New York.[96] Currently, 34 of 38 surveyed European countries have reported KPC-producing CRE (Figure 2.5),[97,98] as well as Israel[99] and China.[100]

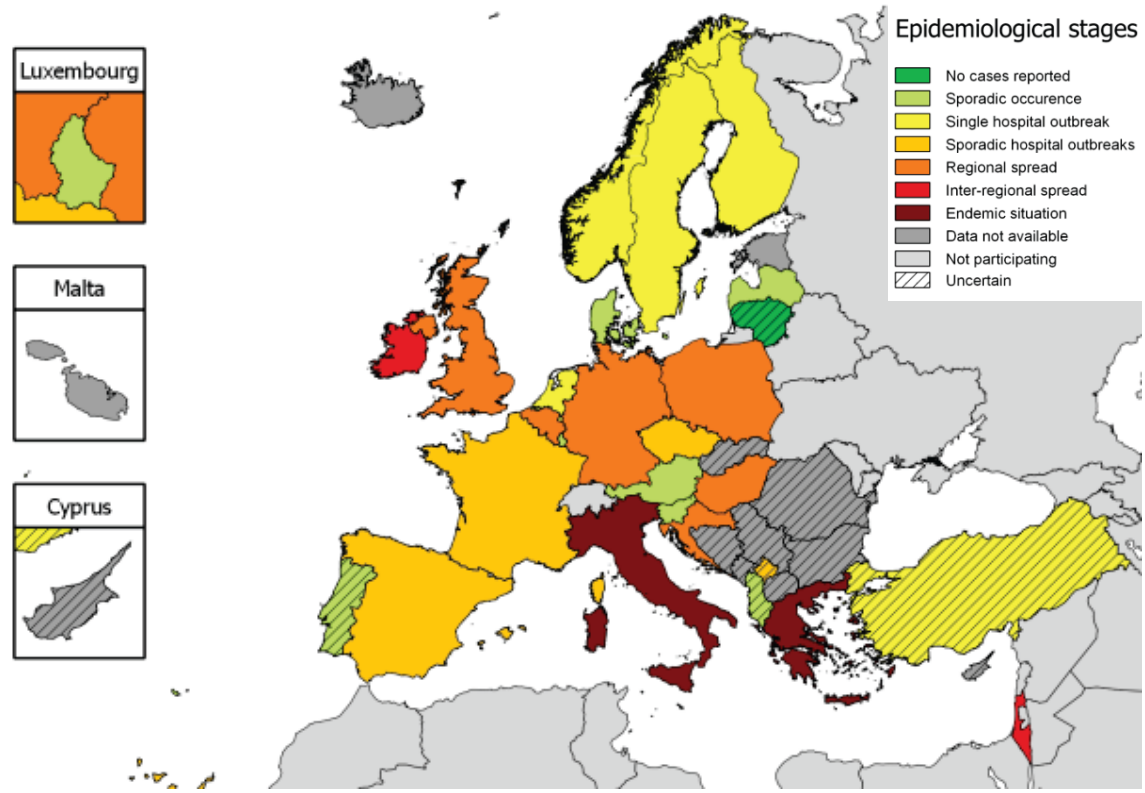


Figure 2.5: KPC-producing Enterobacteriaceae in 38 European Countries Based on Self-Assessment by National Experts. **Reprinted**[98]

Imipenem-like Carbapenemase (IMP; Class B)

In 1991, the very first MBL identified in Enterobacteriaceae was IMP-1, found in a *Serratia marcescens* clinical isolate in Japan.[81,82] IMP also emerged in Italy and Portugal in 1997 and 1998, respectively.[101] The differences in European IMP subtypes and Japanese IMP subtypes have led to the belief that European IMP-production emerged locally rather than global dissemination from Japan.[101] IMP has also been identified in Canada, China, Korea, Singapore, Taiwan and Australia to name a few more regions outside the U.S. IMP-producing CRE in Europe is depicted in Figure 2.6.[101]

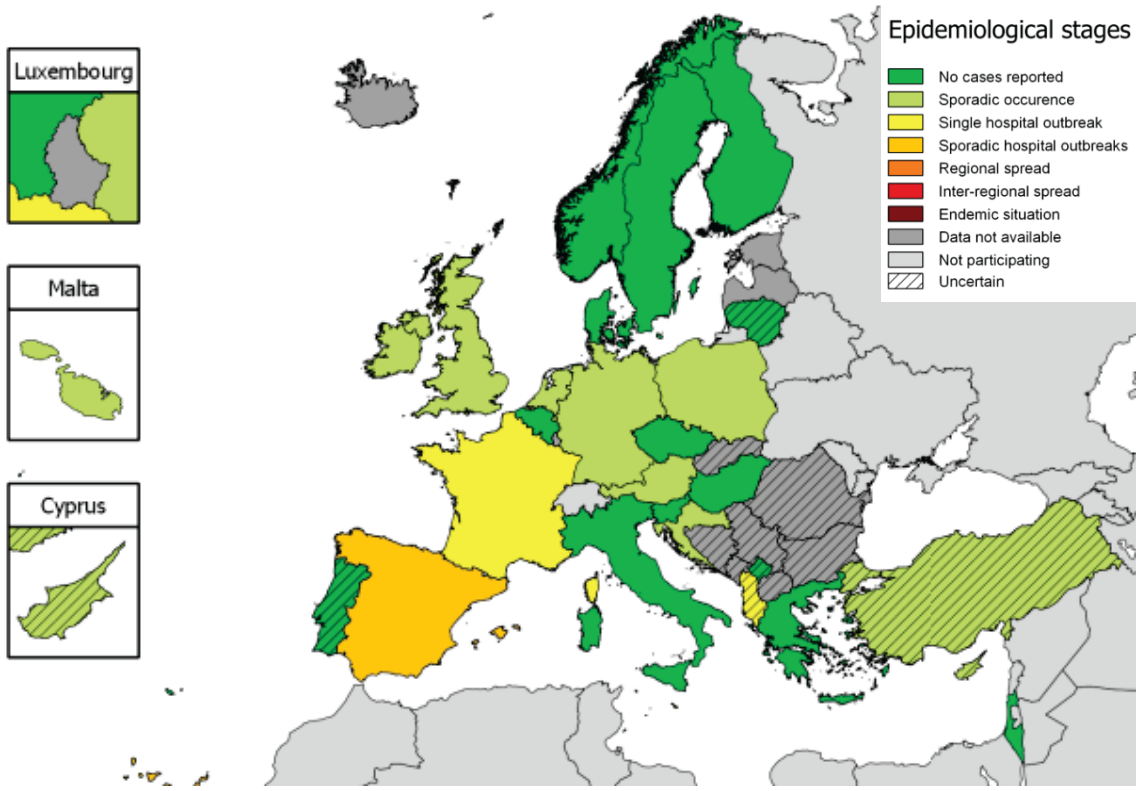


Figure 2.6: IMP-producing Enterobacteriaceae in 38 European Countries Based on Self-Assessment by National Experts. **Reprinted**[98]

From 2009 - 2010, the first IMP-producing CREs were isolated in the U.S. from three pediatric patients with no history of travel or receipt of medical care outside the United States.[102] Before this, the first IMP-producing isolate was *Pseudomonas aeruginosa*, reported in 2006.[103] Otherwise, there have been no other reports of IMP-producing CRE in the U.S. As of July 2015, Lahey Clinic had reported 53 subtypes of IMP-type β-lactamases.[92]

Verona Integron-encoded Metallo-β-lactamase (VIM; Class B)

A VIM-producing CRE was first identified in Greece from *Escherichia coli* in 2001,[104,105] and then later from other *E. coli* and *K. pneumoniae* isolates.[101,106] VIM-production has also been reported in Japan, South Korea, Portugal, Spain, Poland, Croatia, Chile, Venezuela, Argentina, Belgium and most recently in the United States.[107-113] Figure 2.7 depicts VIM-production in Europe whereas Figure 2.4 describes VIM-production in the United States.

The first VIM-producing CRE identified in the U.S. was in an adult patient with *Klebsiella pneumoniae* in 2006.[114] Additionally, a recent publication describes the first and only cluster of VIM-producing CRE in the U.S. Perirectal cultures of eight isolates (4 *E. cloacae*, 1 *Raoultella sp.*, 1, *E. coli*, 2 *Klebsiella pneumoniae*) from six patients were obtained – six from a neonatal intensive care unit, and two from an adult trauma and surgical intensive care unit.[115] To date, this is the only VIM-producing CRE colonization reported to include a neonatal population. Previous VIM-producing CRE have only involved a single species, and only one VIM-producing CRE-colonized patient had been reported (2013) in the same hospital. As of July 2015, Lahey Clinic had reported 46 subtypes of VIM-type β-lactamases.[92]

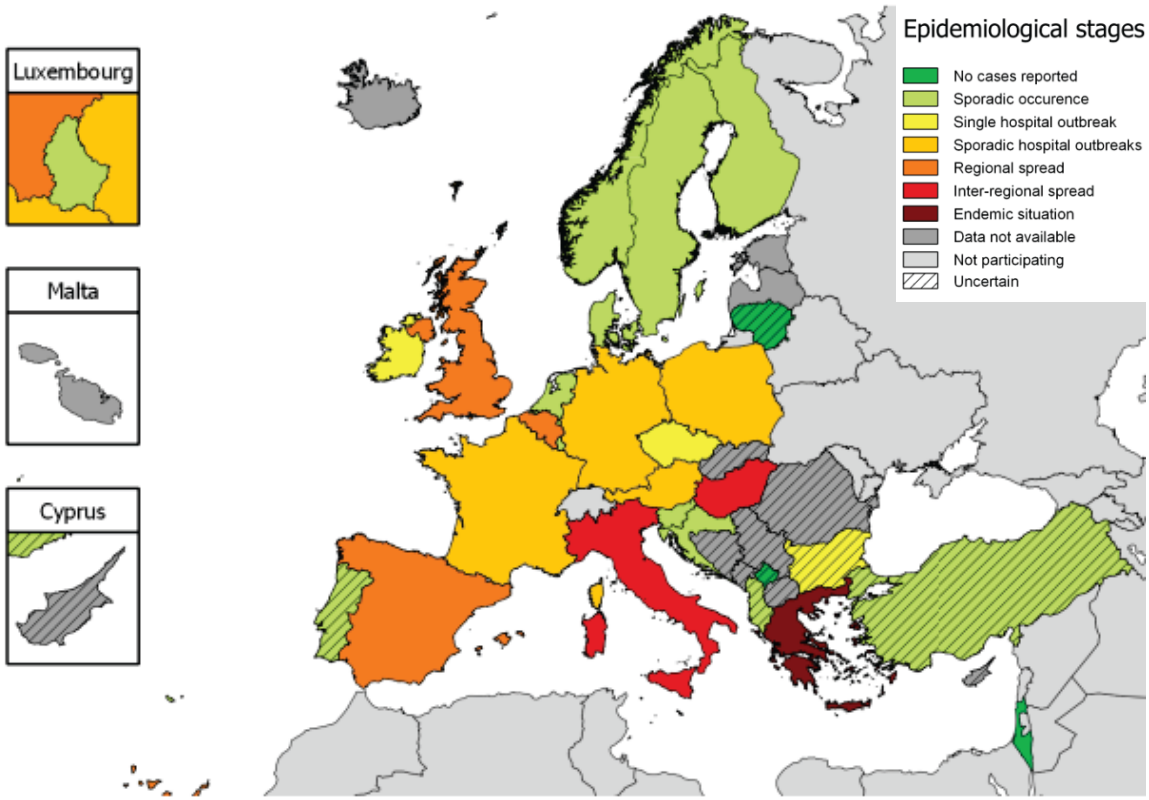


Figure 2.7: VIM-producing Enterobacteriaceae in 38 European Countries Based on Self-Assessment by National Experts. **Reprinted**[98]

Oxacillinase Group β-lactamase (OXA; Class D)

In 2001, OXA-48 was the first Ambler class D carbapenemase isolated in Enterobacteriaceae. It was first found in a *Klebsiella pneumoniae* isolate from Turkey.[54] Interestingly, this particular OXA enzyme has the highest hydrolysis rate of imipenem compared to all other published OXA enzymes.[56] OXA-48 has also been identified in Russia,[116] South Korea,[117] Argentina, India,[118] Taiwan,[119] North Africa,[65] and the U.S.[79] (Figure 2.3). Figure 2.8 depicts OXA-48 dissemination in Europe. Other OXA group carbapenemases include OXA-163 and OXA-181.[120]

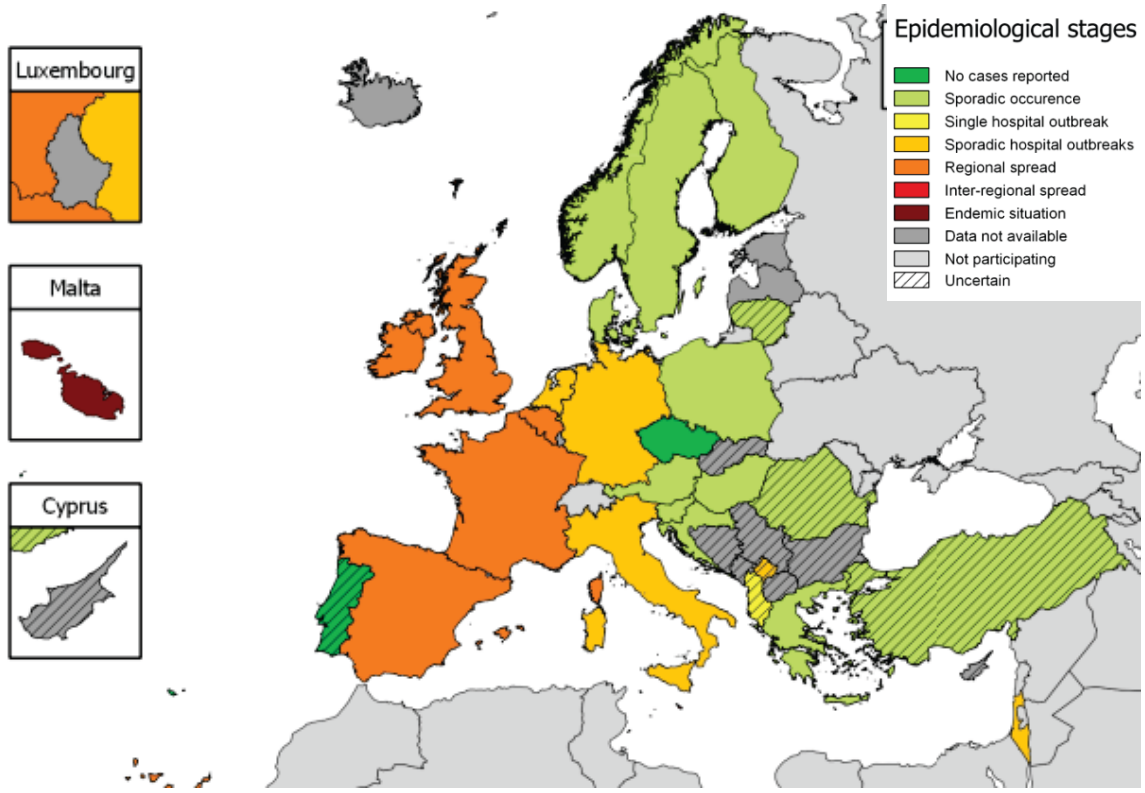


Figure 2.8: OXA-48-producing Enterobacteriaceae in 38 European Countries Based on Self-Assessment by National Experts . **Reprinted**[98]

New Delhi Metallo-β-lactamase (NDM; Class B)

In 2009, an NDM-producing CRE was first identified in Sweden from *Klebsiella pneumoniae*, cultured from a patient of Indian descent who had recently traveled to New Delhi, India and acquired a urinary tract infection (UTI).[121] This novel MBL was designated NDM-1. Currently, at least sporadic NDM-producing CRE has been reported in most European countries (Figure 2.9), but a more thorough description of NDM spread across Europe is described by Cantón *et al.*[122] As of July 2015, Lahey Clinic had reported 16 NDM-type β-lactamases.[92]

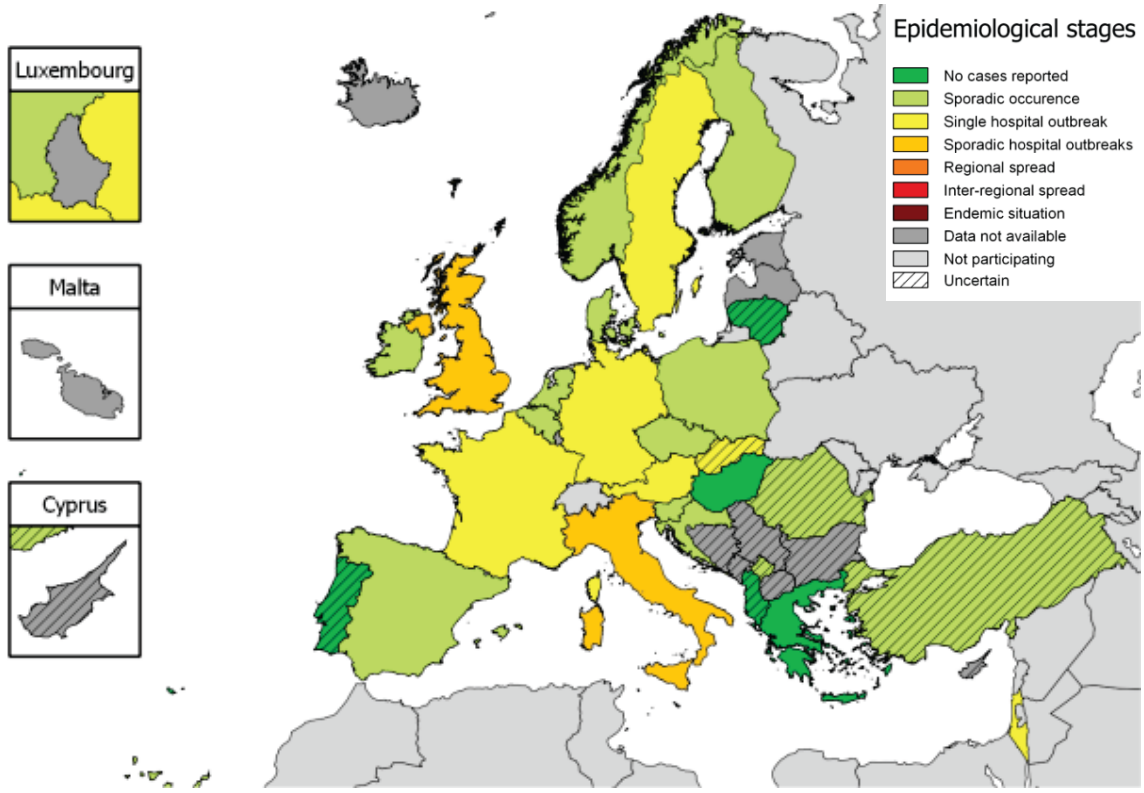


Figure 2.9: NDM-producing Enterobacteriaceae in 38 European Countries Based on Self-Assessment by National Experts. **Reprinted**[98]

In the U.S., the first NDM-producing Enterobacteriaceae was among nine isolated from 2009 - 2011 (5 *K. pneumoniae,* 2 *E. coli,* 1 *E. cloacae*, and 1 *Salmonella enterica*) from eight patients across five states (5 California, 1 Illinois, 1 Maryland, 1 Massachusetts, and 1 Virginia). All patients had recently been to India or Pakistan. Eight of these isolates were confirmed by the CDC to encode NDM-1, but the ninth isolate (*E. coli*) coded for what is now called NDM-6.[66] NDM-producing isolates were also being described in other parts of the world by this time, consistently in patients with recent travel to India or Pakistan.[91] Since 2012, the epidemiology in the U.S. appears to be changing as more NDM-producing CRE are being isolated from patients without recent travel outside country, suggesting local acquisition.[91]

CRE Incidence and Prevalence

Regarding the top 3 CRE reported in the U.S., 69% were *Klebsiella pneumoniae/oxytoca*, 18% were *E. coli*, and 13% were *Enterobacter* spp.[123] Nationwide, carbapenem resistance among *Klebsiella pneumoniae/oxytoca* was <1% in 2000,[124] but by 2010, the CDC reported carbapenem resistance up to 12.8% and 12.5% for central-line associated bloodstream infections (CLABSIs) and catheter-associated urinary tract infections (CAUTIs),[123] respectively. An academic medical center in New York reported carbapenem-resistant *Klebsiella pneumoniae* rates of 38% in 2008.[125] A collective report of 14 hospitals in New York also noted overall 38% *Klebsiella pneumoniae* carbapenem resistance in 2006, but has recently reported a decrease to 29% in 2009.[126] This study focuses on KPC-producing *Klebsiella pneumoniae* which will be further discussed later.

"TYPICALLY SUSCEPTIBLE" ANTIMICROBIALS

CRE are complex and diverse – what may work for some organisms may not be universally applicable to others. In general, CRE are resistant to all β-lactams and β-lactam/β-lactamase inhibitor combinations with the exception of ceftazidime/avibactam, a newly approved β-lactam/β-lactamase-inhibitor combination for KPC-producing gram-negative bacteria.[14] Additionally, exclusively MBL-producing CRE may be susceptible to aztreonam. However, due to the complexity and commonly multi-factored resistance that accompanies most CRE, this is seldom applicable.[127]

Regarding other classes of antimicrobials, CRE are typically only susceptible (>85%) to colistin, polymyxin B, tigecycline, fosfomycin, and variably susceptible (35-75%) to aminoglycosides. There are limitations with each of the antimicrobials for which CRE are typically susceptible, ranging from pharmacologic characteristics and rapid resistance development to toxicity and adverse events.[14]

Polymyxins

The polymyxin class of antibiotics was introduced in the mid-1950s, consisting now of two agents – polymyxin B and polymyxin E (colistin). Both agents are cationic polypeptides that share a ring of amino acids and a fatty acid tail (Figure 2.10). The structural difference of colistin involves a substitution of the phenylalanine in polymyxin B with D-leucine. Additionally, both polymyxins have two major components based on the fatty acid chain length – polymyxin B1 and B2 and colistin A and B.[14]

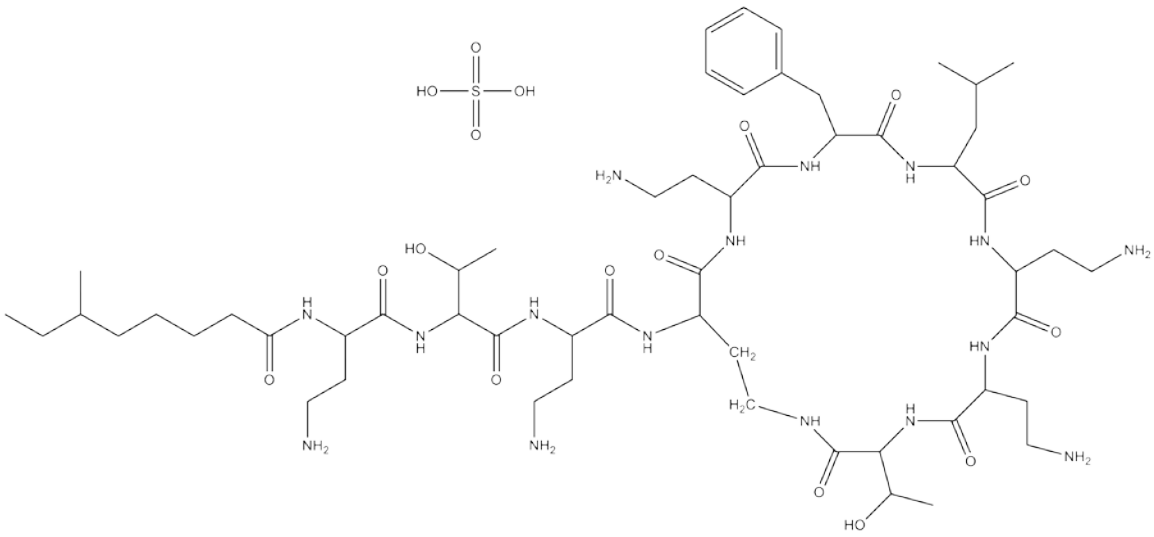


Figure 2.10: Structure of Polymyxin B. **Reprinted**[128]

The mechanism of action of the polymyxin class involves binding to negatively-charged moieties in the lipopolysaccharide (LPS) present in the outermost membrane of gram-negative bacteria (Figure 2.11). This interaction results in the loss of intracellular products, killing the bacteria.

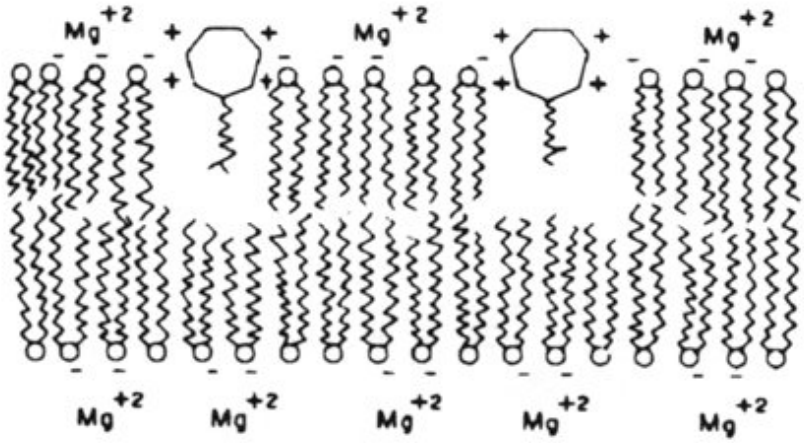


Figure 2.11: Polymyxin B/Colistin Mechanism of Action. **Reprinted**[129]

Polymyxins have a broad gram-negative spectrum of activity – including Enterobacteriaceae (except *Proteus spp.* and *Serratia spp.*), *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. However, their utility had been limited by the development of safer antimicrobials like the aminoglycosides and cephalosporins. In fact, this antimicrobial class was primarily reserved for cystic fibrosis patients, gastrointestinal (GI) tract decontamination, and topical antimicrobial therapy.[14] In the '90s, this class was "reintroduced" to address problems with carbapenem-resistant organisms. One of the first published successes of polymyxin treatment for CRE involved a critically ill patient with a carbapenem-resistant *Klebsiella pneumoniae* bloodstream infection.[130]

Polymyxin B and colistin differ significantly in their pharmacokinetics. However, routes of administration are similar between the polymyxins with the exception that polymyxin B cannot be administered orally. Otherwise, both polymyxins can be administered intravenously, intramuscularly, intrathecally, topically, or by inhalation. Clinically, colistin is administered as a prodrug (colistimethate sodium; CMS) which must be first hydrolyzed into various derivatives (e.g. colistin) before having any antimicrobial effect, whereas polymyxin B is administered as an active agent. The rates of hydrolysis are variable according to the physical environment within the patient (e.g. pH and temperature). Differences in rates and extents of hydrolysis have been observed brand-to-brand or even batch-to-batch.[131] Since there is no appreciable antimicrobial activity from the parent compound, rational dosing of CMS is very challenging.[132,133]

Additional challenges in CMS dosing exist in its elimination, which is primarily renal, and its conversion to colistin, which is non-renal. In fact, patients with normal renal function are often so efficient at eliminating CMS that a dose 4 to 5 times that which is

needed to attain required plasma concentrations of colistin must be administered.[133] Colistin can also be found concentrated in the urine, but this is primarily due to post-renal conversion of the parent compound because colistin is primarily eliminated through a non-renal mechanism. As a result, large interpatient and intrapatient variability exists for CMS dosing.[134] In stark contrast, polymyxin B, which is administered as an active agent, is eliminated mainly by non-renal mechanisms and very little polymyxin B can be found in the urine.[135,136] Figure 2.12 summarizes the different elimination pathways for colistin vs. polymyxin B.[133]

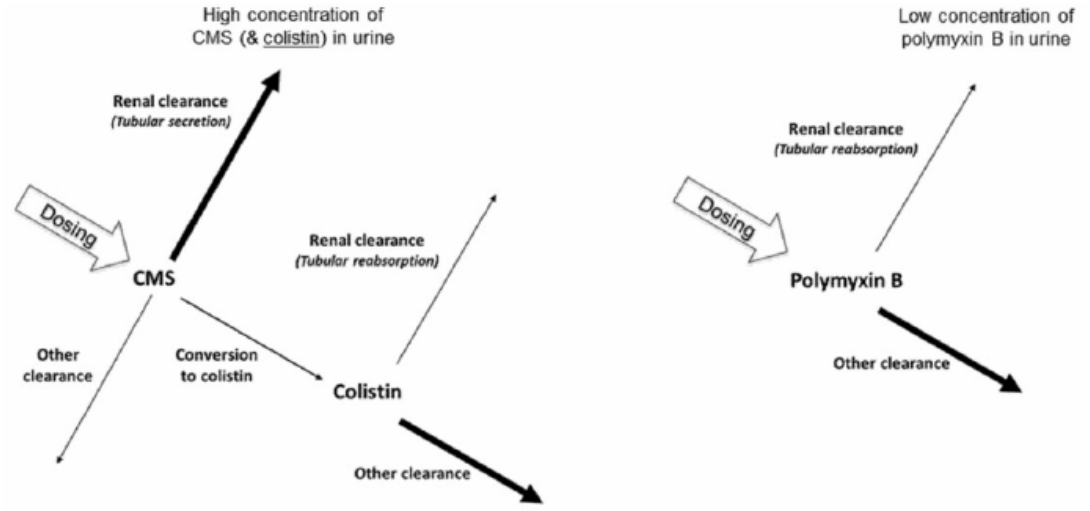


Figure 2.12: Elimination Pathways for Colistin and Polymyxin B. **Reprinted**[133]
Thickness of the arrows indicates relative magnitude of clearance mechanism

The clinical implication of these pharmacokinetic differences are that, even with a loading does of CMS, it takes several hours to achieve effective plasma levels of colistin and delay in appropriate antimicrobial therapy has been associated with increased mortality in critically ill patients.[137,138] Furthermore, low colistin plasma levels have been

associated with growth of colistin-resistant subpopulations[139-142] and as renal function improves (or declines), the rate and extent of conversion of parent drug to colistin changes, and dosing strategies accounting for this change have not yet been perfected, rending it impossible to reliably achieve effective steady-state plasma concentrations in patients with creatinine clearance above 80 mL/min.[134,143] For example, at the maximal approved dose of CMS (300 mg colistin base activity / day), patients with creatinine clearance > 80 mL/min achieved plasma concentrations < 2 mg/L.[134] Nephrotoxicity, another independent predictor of mortality, is the dose limiting adverse effect of colistin.[144] Polymyxin B, however, can rapidly achieve desired plasma levels that can be effectively maintained[136] and nephrotoxicity with polymyxin B has been reported to be lower than with CMS,[145,146] being closer to about 14%,[147] compared to colistin which has been reported closer to 45% using RIFLE criteria.[144]

Polymyxin-resistant CRE infections are another concern altogether. Rapid resistance development has been observed when colistin or polymyxin B are used alone.[139-141] Colistin-resistant (MIC >2 μg/mL) CRE have occurred in various parts of the globe including Italy,[148] Greece,[149] Spain,[72] and the United States.[150,151] In fact, a tertiary center in Spain reports an increase from 13.5% to 31.7% in colistin resistance among *Klebsiella pneumoniae* isolates.[72] A retrospective multi-center observational study in Italy showed a threefold increase in colistin-resistant KPC-producing *Klebsiella pneumoniae* from 2010 to 2013 (20% resistance overall), and colistin-resistance was determined to be an independent risk factor for 14-day mortality (47% vs. 31%; P = 0.001).[152]

Mechanisms that lead to resistance to polymyxins in Enterobacteriaceae are not fully understood. One proposal is that modifications in components that make up the LPS layer of gram-negative organisms, like lipid A, may play a role in polymyxin-resistance. Specifically, *phoP/phoQ* and *pmrA/pmrB* can be activated by environmental stimuli (e.g. low magnesium concentrations or polymyxin exposure), or can harbor mutations which typically lead to constitutive expression. One result is that phosphate head-groups in lipid A are substituted with 4-amino-4-deoxy-L-arabinose (L-Ara4N) which inhibits polymyxin binding. Figure 2.13 shows possible mutations (red star symbols) and how the *phoP/phoQ* and *pmrA/pmrB* system modifies the LPS layer, which ultimately leads to lower binding affinity (resistance) to polymyxin class antibiotics.

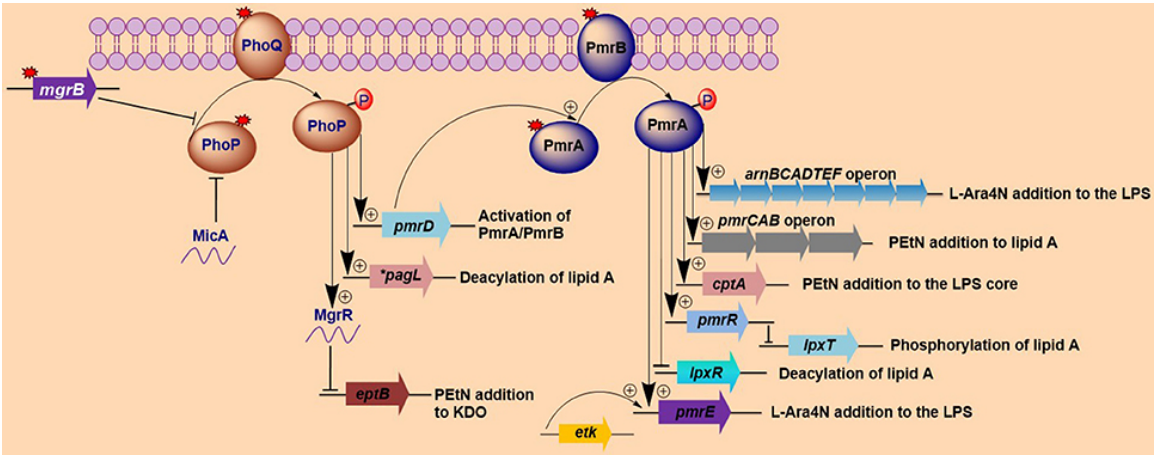


Figure 2.13: Proposed Polymyxin Resistance Pathway in Gram-negative Organisms. **Reprinted**[153]

Additional mechanisms of resistance include modification of outer membrane proteins (e.g. OprH) which can block polymyxin merger with the cell membrane as well as efflux pumps.[153] Regarding *K. pneumoniae* specifically, alterations in the mgrB gene (removal a negative feedback loop on the *phoP/phoQ* system) has been associated with an epidemic dissemination of colistin-resistant CRE in Italian hospitals[154,155] but has also been identified in other parts of Europe, Asia, Africa and the United States.[154] As a final addition, lipid A modifications in *K. pneumoniae* have been associated with cross-resistance to host defense systems as well,[156] which may contribute to the observed increase in mortality associated with colistin-resistant CRE.

Tigecycline

Tigecycline is a glycylcycline (Figure 2.14), a class related to the tetracyclines, that also inhibits protein synthesis by binding to the 30S ribosomal subunit.[157-159] The charged aminoacyl-tRNA can no longer bind to the ribosome in the presence of tigecycline due to the distorted ribosomal acceptor site, which halts the reproduction process of the bacteria. As a result, tigecycline is bacteriostatic and has a broad spectrum of activity including both gram-positive organisms and gram-negative organisms, even anaerobic and atypical organisms, but it does not clinically impact *Pseudomonas spp.* or *Proteus spp.*[160,161]

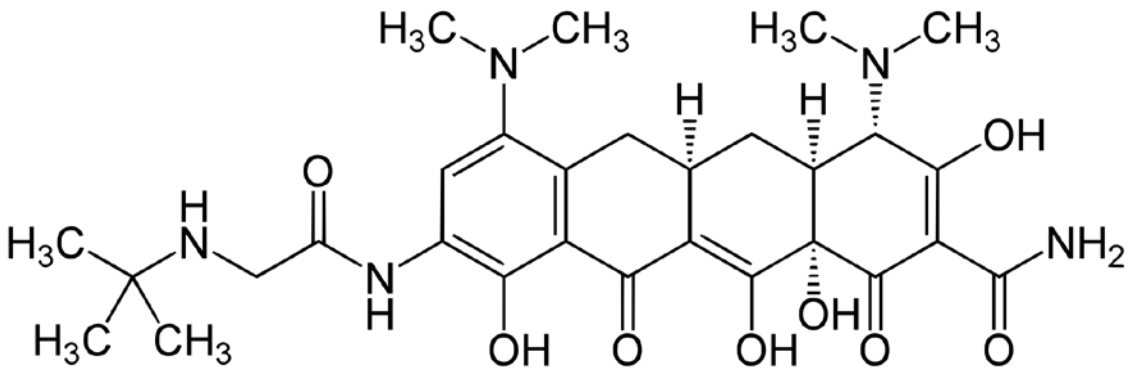


Figure 2.14: Structure of Tigecycline. **Reprinted**[162]

Clinical experience with tigecycline has set its role in complicated skin and skin structure infections (cSSSIs) and complicated intra-abdominal infections (cIAIs).[163] Tigecycline has been associated with increased mortality when compared to other antimicrobial agents and so is typically reserved after failure of other antimicrobials or in

situations where antimicrobial choices are limited such as in CRE or other multidrug resistant (MDR) organisms.[164,165] An AUC/MIC ratio > 12.5 correlates with clinical outcome in cSSSI, which suggests clinical breakpoints of 0.25-0.5,[166] but CLSI currently has not established a breakpoint for Enterobacteriaceae. The susceptibility breakpoint established by the FDA is ≤2 μg/mL.[167] Of note, peak serum concentrations of 0.60 μg/mL after a 100 mg infusion render tigecycline unable to effectively treat bloodstream infections.[14]

Resistance to tigecycline (MIC >2 μg/mL) is not common, but when it has been characterized, it is usually associated with mutations in the *ramA* gene which leads to upregulation of AcrAB-TolC, a multidrug efflux pump in Enterobacteriaceae.[168] Additionally, overproduction of *marA, rarA, acrAB,* and *oqxAB* genes can lead to tigecycline resistant phenotypes.[169] Tigecycline resistance has been recently reported in China,[170] Europe,[171-173] and the United States.[172,174]

Fosfomycin

Fosfomycin is bacterial cell-wall inhibitor, discovered in Spain in 1969,[175,176] and it is relatively unique in structure by containing an epoxide (Figure 2.15). Peptidoglycan synthesis is inhibited by fosfomycin which blocks the formation of N-acetylmuramic acid by competitively inhibiting phosphoenol pyruvate synthetase (Figure 2.16).[14]

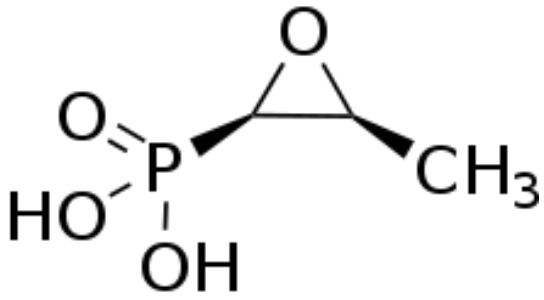


Figure 2.15: Structure of Fosfomycin. **Reprinted**[177]

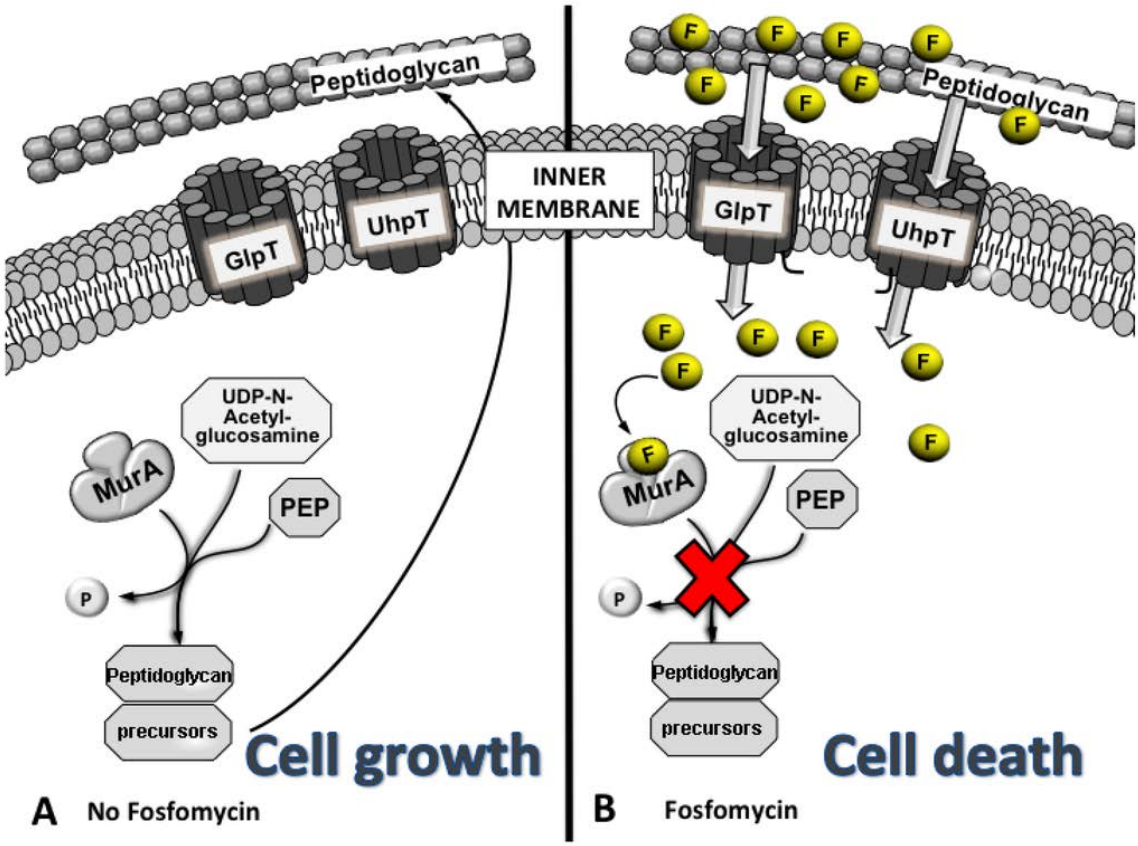


Figure 2.16: Fosfomycin Mechanism of Action. **Reprinted**[178]

Like other cell-wall inhibitors (e.g. β-lactam antibiotics), fosfomycin is bactericidal and has a broad spectrum of activity that includes both gram-positive and gram-negative bacteria. In Europe, fosfomycin is primarily used in combination with other antimicrobials for CRE treatment, particularly strains with reduced susceptibility to colistin and tigecycline.[179,180] In the U.S., fosfomycin is only approved orally for uncomplicated urinary tract infections due to its excellent genitourinary penetration. The optimal dosing strategy for treatment is still unclear. The FDA label indicates a single 3 gm daily dose whereas other clinical trials have evaluated 3 gm every 2 or 3 days for urinary tract infections.[181]

In CRE infections, a multicenter (11 ICUs), prospective case-series study from Greece showed favorable outcomes when fosfomycin was used in combination with another antimicrobial (usually colistin or tigecycline) in a majority of patients with fosfomycin-susceptible carbapenem-resistant infections, a majority of which (41 out of 68) were *Klebsiella pneumoniae*. Bacterial eradication was observed in 56.3% of cases overall and in 60% of cases caused by colistin-resistant CRE. Fosfomycin resistance developed during the course of treatment in three cases.[182]

Fosfomycin resistance has been characterized, and usually results from either mutations in the transport systems (GlpT and UhpT) that are located on the chromosome of bacteria or through inactivating enzymes (*fos*A family) located on bacterial plasmids. The chromosomal mutations prevent the uptake of fosfomycin into the cell and, although these mutations are relatively quickly acquired, a high fitness cost is observed in *E. coli* which limits fosfomycin resistance when not under direct antimicrobial pressure.[183] The same fitness cost has not been observed in *Klebsiella spp.* and *Enterobacter spp.*, and

therefore fosfomycin monotherapy may select for resistant isolates among these Enterobacteriaceae.[184] Regarding plasmid-mediated resistance, a plasmid carrying both bla_{KPC-2} and *fos*A3 is circulating among carbapenem-resistant *Klebsiella pneumoniae* in China and accounts for 60% of the observed fosfomycin resistance in that country.[185]

Aminoglycosides

The aminoglycoside class was introduced in the 1940s with streptomycin.[14] Today, three aminoglycosides are primarily used (four in the Europe)– tobramycin, gentamicin, amikacin and kanamycin (in Europe). Although generally similar in structure (Figure 2.17) and function, those structural differences that do exist among this class often confer differences in stability against a variety of aminoglycoside modifying enzymes that would inactive these antimicrobials.

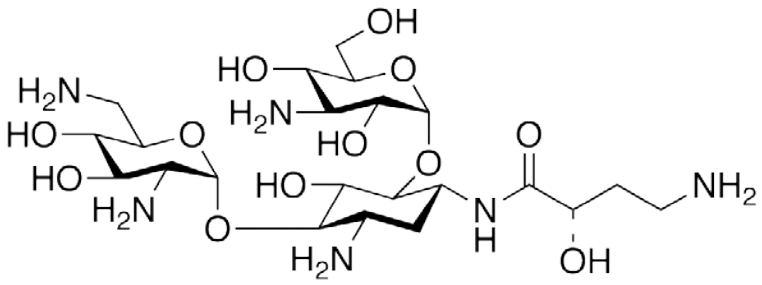


Figure 2.17: Structure of Amikacin. **Reprinted**[186]

Like tigecycline, aminoglycosides function by binding to the 30S ribosomal subunit, but these antimicrobials can additionally facilitate the insertion of incorrect amino acid sequences into proteins rather than only preventing their translation (Figure 2.18). As a result, aminoglycosides exhibit bactericidal activity against gram-positive and gram-negative organisms, including *Pseudomonas spp.*, but have little affect against anaerobes.

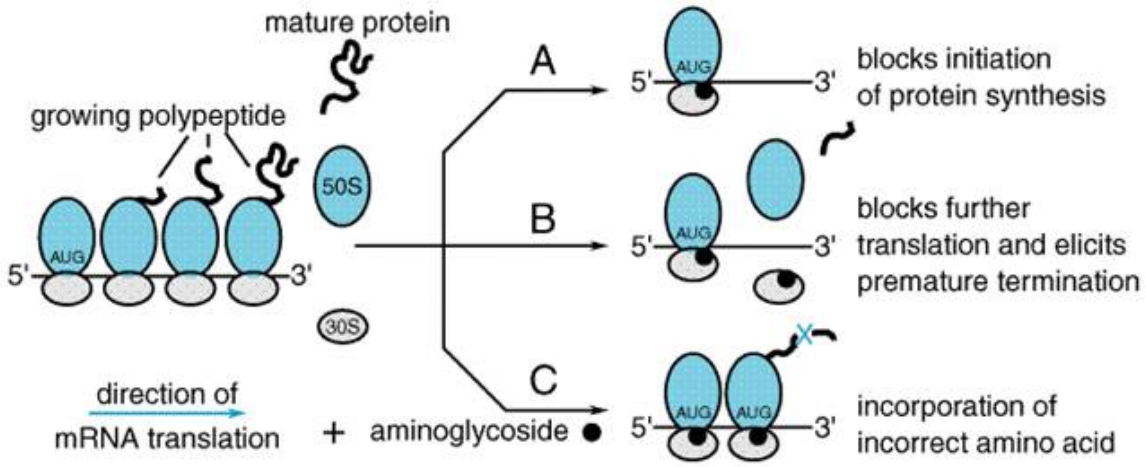


Figure 2.18: Mechanism of Action of Aminoglycosides. **Reprinted**[187]

Aminoglycosides are sometimes a viable option against aminoglycoside-susceptible CRE. In fact, a superior rate of microbiologic clearance was observed in a retrospective cohort study of CRE bacteriuria when an aminoglycoside was used (88%) compared to either polymyxin B (64%) or tigecycline (43%).[188] Additionally, aminoglycosides are associated with less nephrotoxicity (~10-20%)[189] than colistin (~45%),[144,190,191] but perhaps similar to polymyxin B (~14%),[147] while maintaining bactericidality and the ability to treat bloodstream infections over tigecycline.[14]

Resistance to aminoglycosides is primarily mediated by aminoglycoside-modifying enzymes (analogous to β-lactamase production) but is highly variable, showing regional dependence as well as differences among hospitals within the same geographic region. Resistance rates can even vary by strain, but in general, rates of non-susceptibility have been reported as ranging from 35% to 63% for gentamicin, 61% to 98% for tobramycin, and 16% to 82% for amikacin.[192-194] Other resistance mechanisms have been identified as well, including modification of the ribosome target,[195] reduced permeability of the bacterial cell wall, and also efflux pumps.[196,197]

ANTIMICROBIAL THERAPY FOR CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

A majority of cases, cases series, and the published experience of medical centers indicate that combination therapy might provide a mortality benefit when compared to monotherapy,[10,68,184,198-202] considering the antimicrobial agents available today. Additionally, polymyxin B, colistin, tigecycline, and fosfomycin have all demonstrated rapid selection for resistance when used as monotherapy against CRE.[120,184] There are no randomized-controlled clinical trials comparing single agents or their combinations, but rather retrospective or prospective (non-randomized) analyses which results in a significant limitation on analyzing such data. Some conclude that a systematic review and meta-analysis is not possible regarding CRE treatment due to the heterogeneity of available evidence.[203]

In Vitro Studies

Polymyxin combinations

The interaction between antimicrobial agents has primarily been characterized by time-kill methodology, where polymyxins are most frequently investigated in combination with either a carbapenem, tigecycline, fosfomycin, rifampin, an aminoglycoside, or sometimes with three or four agents from multiple drug classes.[120] The goal of *in vitro* testing in this setting is to quickly evaluate combinations of antimicrobials that might show synergistic interactions when used to treat CRE. Most *in*

vitro testing has been performed on KPC-producing CRE whereas MBL- and OXA-48-producing CRE have more limited data.[14]

In KPC-producing Enterobacteriaceae, time-kill studies have shown polymyxin B exhibiting synergistic activity ($\geq 10^2$ CFU/mL more killing than the more active agent alone at 24 hours) when in combination with rifampin and when in combination with imipenem.[204] Polymyxin B in combination with both doripenem and rifampin were determined to interact synergistically and exhibit bactericidal activity ($\geq 10^3$ CFU/mL killing at 24 hours).[205] Colistin and tigecycline have also been evaluated in combination together and determined to be synergistic.[206] In a broth microdilution checkerboard assay of 12 KPC-producing *Klebsiella pneumoniae* isolates, polymyxin B was synergistic in combination with either tigecycline, doxycycline or rifampin, but no synergy was detected for combinations with imipenem or gentamicin.[207]

In 42 VIM-producing *K. pneumoniae* isolates from Greece, colistin was found to be synergistic with imipenem in about 50% of colistin-susceptible isolates, regardless of imipenem MIC, and indifferent (CFU/mL killing is the same as the more active agent alone at 24 hours) in the rest. For the colistin-non-susceptible isolates, the combination was antagonistic (CFU/mL killing was less than the more active agent alone at 24 hours) for 56% of the isolates and synergistic for only 11%.[208]

In nine colistin-resistant KPC-producing *Klebsiella pneumoniae* and three colistin-susceptible KPC-producing *Klebsiella pneumoniae*, colistin in combination with two carbapenems (doripenem and ertapenem) showed synergy in 8 of 12 isolates. Colistin in combination with one carbapenem (doripenem) showed synergy in 6 of 12

isolates and colistin in combination with the other carbapenem (ertapenem) showed synergy in 5 of 12 isolates. Interestingly, the authors noticed an association between synergy of the triple combination of colistin-doripenem-ertapenem and porin expression levels. Specifically, the eight isolates showing synergy had the highest porin expression, and receiver operator characteristic (ROC) analysis designated this group of eight as significantly different from the remaining four in terms of porin expression (P = 0.002). The authors speculated that permeability for both carbapenems was limited by porin channel expression and that higher expression provided easier access to the sites of action of both carbapenems.[209]

A systematic review and meta-analysis of *in vitro* interactions between polymyxins and any carbapenem against polymyxin-susceptible CRE found an overall synergy rate of 55%. This analysis also indicated that Etest® and checkerboard synergy testing typically reported lower than did time-kill methodology, and that the use of combination therapy led to less resistance development *in vitro* when post-exposure resistance testing was performed.[210]

Colistin (COL), meropenem (MEM), and tigecycline (TIG) interactions were evaluated using a 3-D checkerboard assay in 20 carbapenem-resistant *K. pneumoniae* clinical isolates. Among these, 13 were resistant to colistin and 6 were resistant to tigecycline. Synergy rates were 10% for MEM and TIG; 30% for COL and MEM; 30% for COL and TIG; and 30% COL, MEM, and TIG. It was noted by the authors that synergy was correlated with higher TIG MICs (>2 µg/mL) and higher COL MICs (>8 µg/mL), there was no antagonism, and addition of a third antimicrobial agent did not contribute to synergy.[211] COL, MEM, and TIG were also evaluated in a time-kill study of

eight CRE clinical isolates (4 *K. pneumoniae*, 2 *E. coli*, 1 *E. cloacae*, 1 *S. marcescens*). MEM and TIG were not synergistic in any of the eight strains. TIG and COL showed synergy at concentrations above the MICs for most strains.[206]

Other combinations

Time-kill assays involving double and triple antimicrobial combinations of aztreonam, ciprofloxacin, colistin, daptomycin, fosfomycin, meropenem, rifampin, telavancin, tigecycline, and vancomycin against MBL-producing (2 VIM and 2 NDM) polymyxin-susceptible *K. pneumoniae* isolates were used to evaluate potential combination therapy against MBL-producing CRE. Sample times were 0, 1, and 24 hours. Synergy was found in double combinations of colistin with either aztreonam, fosfomycin, meropenem, or rifampin and in triple combinations with colistin and meropenem with either aztreonam, fosfomycin, or rifampin. The most effective combination was meropenem, colistin, and rifampin demonstrating bactericidal and synergistic activity throughout 24 hours for all four strains. Ciprofloxacin, tigecycline, daptomycin, telavancin, and vancomycin alone and in combination with colistin was without synergy or bactericidal activity at 24 hours.[212]

In KPC-producing *Klebsiella pneumoniae,* fosfomycin in combination with either meropenem or colistin was synergistic in 64.7% and 11.8% of isolates, respectively. Fosfomycin in combination with gentamicin was indifferent.[213] Synergy was evaluated in another study with fosfomycin in combination with imipenem (74%), meropenem (70%),

doripenem (74%), colistin (36%), netilmicin (42%), and tigecycline (30%) for 50 KPC-producing *Klebsiella pneumoniae*.[214]

Amikacin (AMK) 16 µg/mL was evaluated alone and in combination with ertapenem (ETP) 2 µg/mL, imipenem (IPM) 4 µg/mL, and meropenem (MEM) 4 µg/mL against four *K. pneumoniae* clinical isolates resistant to all four antimicrobials (MICs >8 µg/mL for ETP, IPM, and MEM; MIC 32 µg/mL for AMK). Alone, none of the antimicrobials achieved bactericidal activity. Synergy was found in combinations of AMK with either MEM or IPM throughout 24 hours in all isolates. Bactericidal activity was found in 2 of 4 isolates for MEM and AMK and 1 of 4 isolates for IPM and AMK. ETP with AMK was not synergistic or bactericidal in any isolate.[215]

In Vitro Pharmacodynamic Models

Human pharmacokinetics of meropenem were simulated to optimize meropenem dosing against carbapenemase-producing *Klebsiella pneumoniae* using a one-compartment, chemostat model. An advantage to the 0.5 hour infusion of 1 gm every 8 hours was found in a high dose/prolonged infusion regimen (3 hour infusion of 2 gm every 8 hours). Using this regimen, bactericidal activity ($\geq 10^3$ CFU/mL killing) was obtained by 6 hours against all KPC-producing *Klebsiella pneumoniae* isolates. However, regrowth was observed for 9 of 11 isolates with meropenem MICs ≥ 8 µg/mL, but not for two isolates whose meropenem MICs were 2 and 8 µg/mL. Measured meropenem levels were lower than expected using the model, but this was attributed to the production of carbapenemase enzymes by the *K. pneumoniae* isolate.[216]

Human pharmacokinetics of tigecycline (as 50 mg every 12 hours) in combination with either meropenem (as 2 gm infused over 3 hours every 8 hours)[216] or rifampin (as 600 mg every 12 hours) in lung-epithelial fluid were modeled using a one-compartment, chemostat model. Tigecycline alone and in combination with rifampin against carbapenemase-producing *Klebsiella pneumoniae* isolates showed little activity when used against isolates with meropenem MICs ≤ 2 μg/mL. However, when tigecycline was used in combination with meropenem, a synergistic, bactericidal effect was observed for isolates with tigecycline MICs up to and including 2 μg/mL and meropenem MICs up to and including 16 μg/mL. However, none of the regimens maintained bactericidal activity for the full 48-hour study period.[217]

Using time-kill methodology and a 3-dimensional response model, six 2-agent combinations of amikacin (AMI), doripenem (DOR), levofloxacin (LEV) and rifampin (RIF) were evaluated against a KPC-2-producing *K. pneumoniae* (MICs AMI: 64 μg/mL, DOR: 16 μg/mL, RIF: >64 μg/mL, LEV: 128 μg/mL) and a KPC-3-producing *K. pneumoniae* (MICs AMI: 32 μg/mL, DOR: 32 μg/mL, RIF: >256 μg/mL, LEV: 8 μg/mL). Clinically relevant concentrations (AMI: 4-80 μg/mL; DOR 4-32 μg/mL; LEV 0.5-10 μg/mL; RIF 0.25-6 μg/mL) were used in combinations to determine synergy based on the 3-D response model and 24-hour colony count. DOR and AMI was the only combination determined to be synergistic; DOR and RIF, DOR and LEV, and LEV and RIF were additive; AMI and RIF, and AMI and LEV were antagonistic. Murine pneumonia models were used to confirm results obtained through the time-kill experiments and the model for DOR and AMI, and AMI and LEV. As predicted by the model, DOR and AMI showed improved survival for both isolates whereas AMI and

LEV displayed inferior survival rates. Although limited in design, this study is one of few models that analyzed polymyxin-sparing regimens.[218]

Dual-Carbapenem therapy

Doripenem and ertapenem alone and in combination were evaluated against a carbapenemase-producing *K. pneumoniae* clinical isolate (doripenem MIC 4 µg/mL; ertapenem MIC 64 µg/mL) using a one-compartment chemostat model. The free doripenem concentrations simulated a 3-hour infusion of 2 gm every 8 hours in humans, and the free ertapenem concentrations simulated a dose of 1 gm every 24 hours in humans. Adding doripenem to ertapenem extended the bactericidal activity from 6h with monotherapy of either agent to 16h in the combination. Doripenem levels were above the MIC of the organism for a majority of the dosing interval.[219]

Animal Studies

KPC-producing Enterobacteriaceae

In both an immunocompetent and a neutropenic murine thigh model, doripenem was administered to simulate human administration of a 4-hour infusion of 1 gm and 2 gm doripenem every 8 hours. These regimens were evaluated against KPC-producing *Klebsiella pneumoniae* with doripenem MICs ranging from 4 to 32 µg/mL. 1- and 2- gm doses of doripenem achieved bacteristasis in both models against *K. pneumoniae* isolates with doripenem MICs up to and including 8 and 16 µg/mL, respectively. Expectedly,

there was significantly more killing (0.5-1 CFU/mL) in the immunocompetent murine model compared to the neutropenic murine model at 24 hours.[220]

Doripenem and ertapenem alone and in combination were evaluated against three KPC-producing *K. pneumoniae* clinical isolates (doripenem MICs 8, 16, and 32 μg/mL; ertapenem MICs >64 μg/mL) using a murine thigh model of both immunocompetent and neutropenic mice. The free doripenem concentrations simulated a 4-hour infusion of 2 gm every 8 hours in humans, and the free ertapenem concentrations simulated a dose of 1 gm every 24 hours in humans. Although a higher degree of bacterial killing was observed in the combination regimens when compared to monotherapy, only the combination against the lowest doripenem MIC isolate (MIC 8 μg/mL) in the immunocompetent mice was statistically significant, and only at 72 hours (not 24 or 48).[221]

Meropenem, tigecycline, and polymyxin B were evaluated in a rat model alone and in double and triple regimen combinations (n=10 for each regimen) against KPC-2-producing *Klebsiella pneumoniae*. Additionally, time-kill assays were performed on each agent alone and in combination. No pharmacokinetic studies were performed to verify equivalent human dosing, but all combinations involving polymyxin B showed significantly superior results in terms of mortality (Figure 2.19) and culture clearance. Interestingly, meropenem and tigecycline combinations were antagonistic by time-kill analysis, but this interaction was seemingly overcome by the addition of polymyxin B. This was observed in the rat model as well, but there was not an observable advantage in triple-combination therapy compared to polymyxin B in combination with either meropenem or tigecycline.[222]

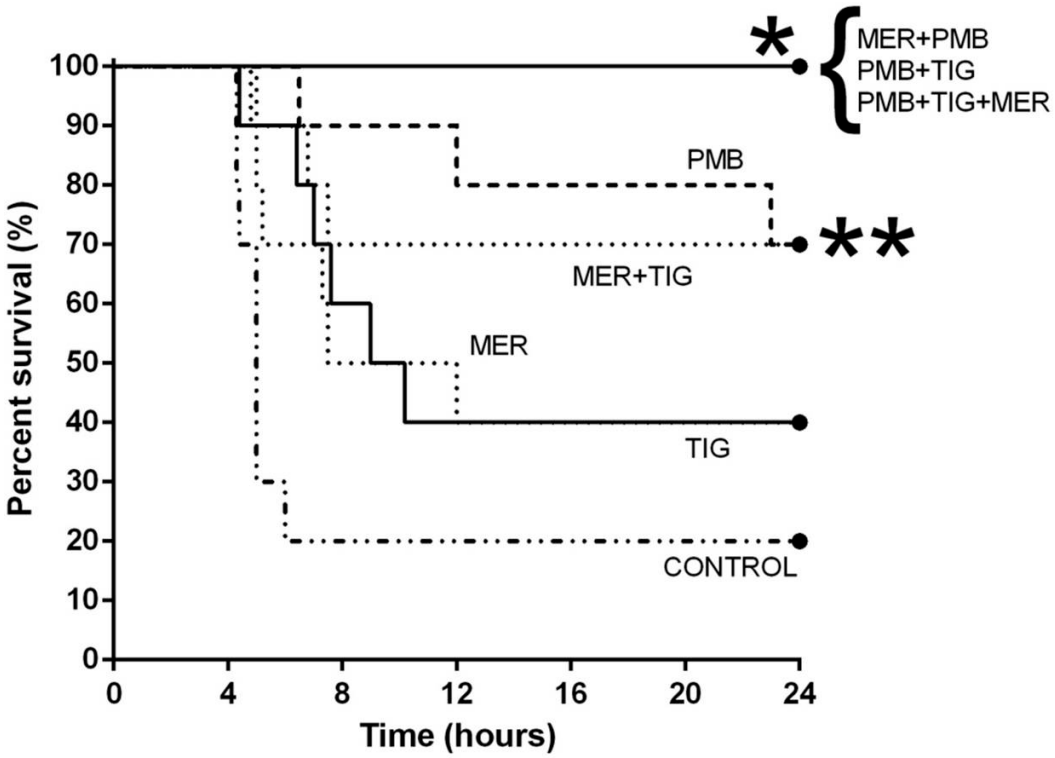


Figure 2.19: Survival curves of a rats infected with KPC-2-producing *Klebsiella pneumoniae*. **Reprinted**[222]
* P-value of <0.05 compared with other groups
** P-value of <0.05 compared with control

MBL-producing Enterobacteriaceae

Two animal models (one murine, one rabbit) have evaluated carbapenem monotherapy in VIM-1-producing *K. pneumoniae* or *E. coli*. In both studies, isolate MICs to carbapenems were relatively low (imipenem MICs ≤4 μg/mL for all but one isolate). Dosing regimens were selected to simulate dosing in humans and optimize T>MIC, the pharmacodynamic index correlating with clinical outcome.[223] In both studies, carbapenems were effective in significantly reduced colony counts (CFU/mL) compared to placebo, but were either not as effective as observed in the non-VIM producing isolate,[224] or were surpassed by aztreonam activity, which is relatively stable in the

presence of MBL enzymes.[225] These data suggest that while carbapenems are still active as monotherapy against MBL-producing CRE, there may be other factors than time above the MIC that play a role in optimizing treatment.

Doripenem and ertapenem as monotherapy were evaluated in a murine thigh model against a wild-type *K. pneumoniae*, and an isogenically derived NDM-1- and a KPC-2- producing *K. pneumoniae*. Four clinical isolates of NDM-1-producing *K. pneumoniae* were also included for comparison. Dosing regimens of doripenem and ertapenem simulated a 4-hour infusion of 2 gm every 8 hours and 1 gm every 24 hours, respectively. Interestingly, at least 10^1 CFU/mL killing was observed at 24 hours for the wild-type *K. pneumoniae,* the isogenic NDM-1- and the NDM-1-producing clinical isolates with doripenem MICs $\leq$ 8 μg/mL. However, the isogenic KPC-producing *K. pneumoniae* showed growth (Figure 2.20), despite a 4-fold lower MIC to ertapenem and doripenem.[226] The results of the KPC-producing isolate were consistent however with previous work performed in this lab.[221]

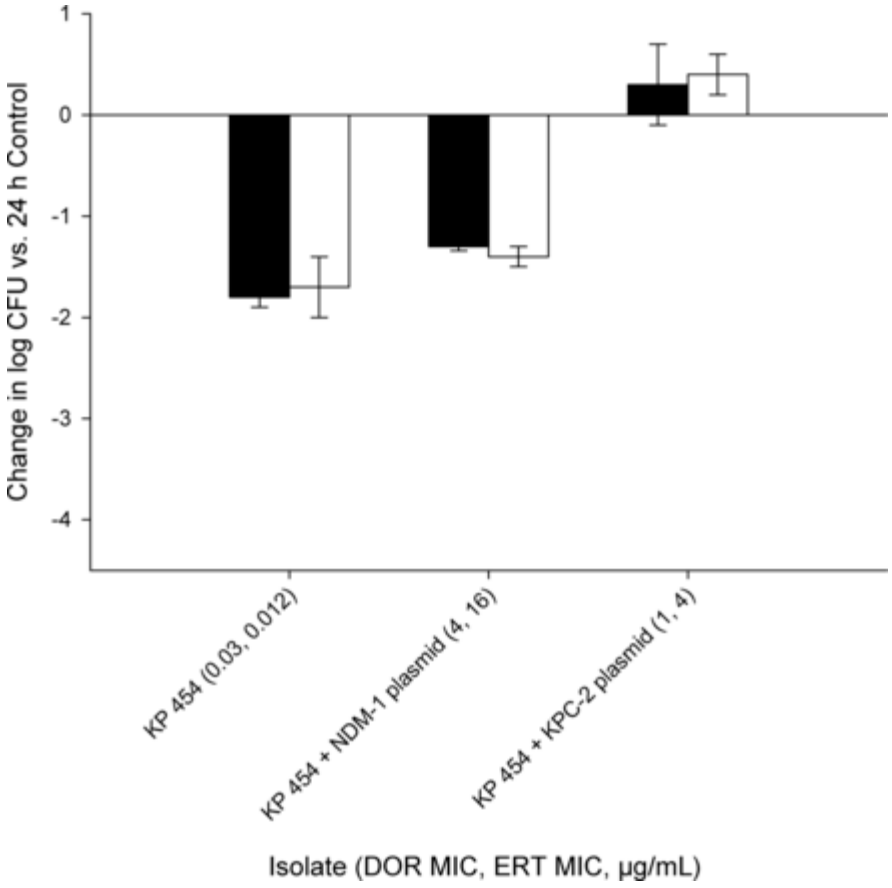


Figure 2.20: Change in $\log_{10}$ CFU/mL after 24 hours. **Reprinted**[226]
Wild-type *K. pneumoniae* strain and its derived isogenic strains harboring either an NDM-1 or a KPC-2 plasmid after treatment with either doripenem at 2 gm every 8 hours (black) or ertapenem at 1 gm every 24 hours (white) in an immunocompetent mouse thigh infection model. Each value is the mean ± standard deviation for infected thighs for each isolate.

OXA-48-producing Enterobacteriaceae

Doripenem, ertapenem, ceftazidime, and levofloxacin were evaluated in a murine thigh model against an isogenic pair of wild-type *K. pneumoniae* and OXA-48-producing *K. pneumoniae* as well as six OXA-48-producing Enterobacteriaceae clinical isolates, with and without other ESBLs (doripenem MICs 0.38 - 8 µg/mL). Levofloxacin, ertapenem and ceftazidime exhibited efficacy correlating with pharmacodynamic targets

and *in vitro* MIC. However, similar to experiments involving isolates producing NDM-1,[226] the observed efficacy of doripenem treatment was surprising. However, whereas doripenem seemed efficacious against low-MIC NDM-1-producing isolates,[226] there was variable efficacy observed by doripenem across all OXA-48-producing isolates, despite achieving the pharmacodynamic target of at least 40% T>MIC.[223] It was concluded by the authors that genotypic expression may be more important than phenotypic MIC and pharmacodynamic targets in selecting appropriate therapy.[227]

Other Studies

In a rather unique study, gene transcription levels of carbapenemase enzymes were analyzed in clinical isolates harboring either CTX-M-15 (ESBL; 1 *K. pneumoniae* and *1 E. coli*), OXA-48 (*E. coli)*, NDM-1 (*K. pneumoniae*) or KPC-2 (*Salmonella spp.*) after infecting mice or inoculating test tubes. The aim of the study was to determine carbapenemase enzyme induction, inhibition, or lack of effect by single antimicrobials or combinations. For the mice studies, rifampin alone, colistin alone and colistin in combination with ertapenem, meropenem, imipenem, fosfomycin, kanamycin, tigecycline, ceftazidime, or rifampin were evaluated. For *in vitro* studies, colistin, meropenem, rifampin and tigecycline alone were evaluated as well as colistin in combination with meropenem, fosfomycin, rifampin, or tigecycline.[228]

The authors listed likely beneficial combinations based on carbapenemase transcription levels observed *in vitro* and *in vivo* and mortality observed *in vivo* according to enzyme type. For OXA-48, colistin in combination with a carbapenem, rifampin,

fosfomycin, or tigecycline seemed most beneficial, but monotherapy with any agent was not recommended. For NDM-1, colistin in combination with rifampin, fosfomycin, or tigecycline were most effective, but again, monotherapy was not recommended with any agent. Finally, for KPC, colistin in combination with a carbapenem, fosfomycin or kanamycin were most beneficial.[228]

Human Studies

Reiterating, comparisons between monotherapy and combination therapy for CRE treatment in humans is limited, but most conclude that combination therapy is preferred. Perez *et al.* selectively compared retrospective reports of CRE bloodstream infections in hundreds of patients receiving either combination or monotherapy. Their analysis concluded a mortality risk reduction of approximately 50% when combination antimicrobials were used compared to monotherapy. These studies were primarily in KPC-producing *K. pneumoniae* and combinations were usually carbapenem-containing in addition to a polymyxin or tigecycline.[14]

Despite known resistance to carbapenems, when CRE are treated with combinations containing a carbapenem, there appears to be added benefit on top of the benefit for combination therapy, particularly in strains with lower carbapenem MIC's (MIC $\leq$ 8 μg/mL). Zouvelekis *et al.* evaluated studies using monotherapy with carbapenems (meropenem or imipenem) and determined that the failure rate of 50 CRE patients across 15 studies was found to be proportional to the MIC for the respective carbapenem used. Note, clinical failure definitions varied from physician to physician

and study to study. Some definitions were patient death, superinfection or reinfection with same organism, prolonged hospital stay, and resistance development while on antimicrobial therapy, but overall clinical failure was estimated to be 75% for CRE infections with carbapenem MICs above 8 µg/mL. This failure rate decreased to 33.3%, 28.6%, and 25% when carbapenem MICs were 8, 4, and 2 µg/mL or less, respectively.[120]

This observation fits in the context of the PK/PD studies in humans and the pharmacodynamic index for carbapenems – 40% to 50% time above the MIC (T>MIC) in that higher carbapenem MICs render target attainment of 50% T>MIC more difficult. It is estimated that for a meropenem MIC of 4 µg/mL, the probability of attaining 50% T>MIC is 69% for a dosing regimen of a 30 minute infusion of 1 gm every 8 hours. When a high dose/prolonged infusion is used (e.g. 3-hour infusion of 2 gm every 8 hours), the probability of target attainment increases to 100%. When the MIC is 8, the probability of attaining 50% T>MIC of a high dose/prolonged infusion of meropenem is 85%.[229]

Adding to the evidence of carbapenem-based combination regimens are two articles evaluating CRE treatment in Greece. For the first study, 103 *K. pneumoniae* isolates producing either VIM or KPC were treated with combination therapy (30% carbapenem-based) and 72 isolates were treated with monotherapy and mortality was significantly lower in the combination therapy group (27.2% vs. 44.4%, p=0.018). Lower mortality was observed for carbapenem-containing regimens when compared to regimens without carbapenems (19.3% vs. 30.6%).[202] For the second study, 132 VIM-producing *K. pneumoniae* and 102 KPC-producing *K. pneumoniae* isolates were included across nine studies where it was determined combination therapy was superior to monotherapy (p =

0.01; odds ratio 2.41; 95% confidence interval 1.2-4.7) and those regimens that included carbapenems were associated with a 6.7% failure rate compared to a 26.9% failure rate of those regimens without a carbapenem (P-value 0.04).[184]

In Italy, 14 day mortality was assessed in 661 patients with KPC-producing *K. pneumoniae*. Independent predictors of 14 day mortality were determined to be bloodstream infection, presentation with septic shock, inadequate empirical antimicrobial therapy, chronic renal failure, high APACHE III score, and colistin resistance. Combination therapy with at least two drugs showing *in vitro* activity against the isolate was associated with lower mortality (odds ratio 0.52; (95% confidence interval 0.35-0.77). Combinations that included meropenem were associated with significantly higher survival rates when the meropenem MIC was ≤ 8 μg/mL.[152]

A review article of published case reports and case series from 2001-2011 included 105 total cases of KPC-producing infections (101 of which were Enterobacteriaceae). Cases receiving monotherapy were 49 (47%) whereas cases receiving combination therapy were 56 (53%), 19 (34%) of which included a carbapenem. Treatment failure was associated more with monotherapy than combination therapy (49% vs. 25%; p = 0.01). Other significant differences were between monotherapy vs. combination therapy involving pulmonary infections, polymyxins, or carbapenems, (Table 2.1).[200]

Table 2.1 Treatment Failure: Monotherapy vs. Combination Therapy

	Monotherapy (%)	Combination (%)	P
Overall Treatment Failure	24/49 (49)	14/56 (25)	**0.01**
Source:			
Blood	12/24 (50)	9/32 (28)	0.09
Pulmonary	10/15 (67)	5/17 (29)	**0.03**
Urine	1/8 (13)	0/3 (0)	0.4
Polymyxin Treatment Failure	8/11 (73)	10/34 (29)	**0.02**
Carbapenem Treatment Failure	12/20 (60)	5/19 (26)	**0.03**
Tigecycline Treatment Failure	2/7 (29)	7/19 (37)	0.4
Aminoglycoside Treatment Failure	0/6 (0)	4/24 (17)	0.6

Reprinted[200]

Another review article systematically obtained CRE case reports, case series, and observational studies from the across the globe (e.g. U.S., Spain, Ireland, Columbia, China, Israel, Brazil, Taiwan, Switzerland, and Greece). A total of 301 patients infected with *Klebsiella pneumoniae* were identified, about half KPC-producing and half MBL-producing. Patients were stratified into seven groups based on treatment regimens (Figure 2.21). Once more, combination therapy with a carbapenem was significantly superior to alternative combinations analyzed.[120]

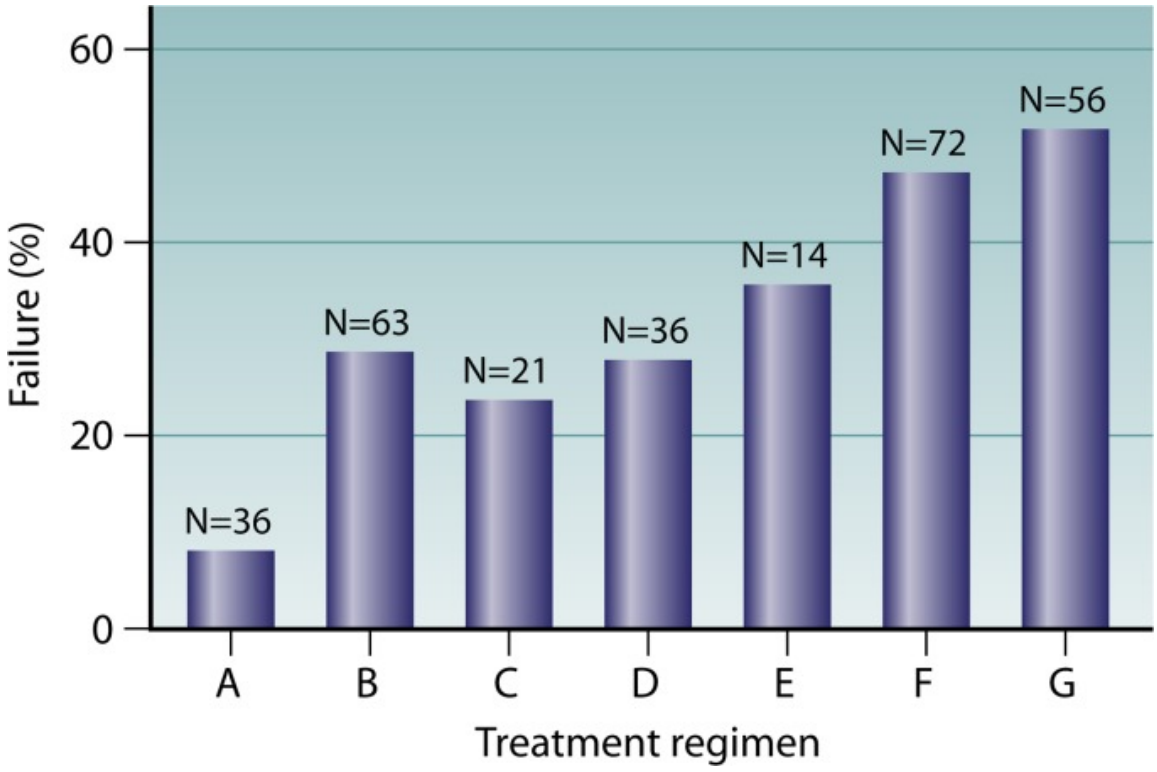


Figure 2.21: Outcomes of infections caused by carbapenemase-producing *Klebsiella pneumoniae*, according to treatment regimen. **Reprinted**[120]
Regimen A, combination therapy with ≥2 active drugs, one of which was a carbapenem; regimen B, combination therapy with ≥2 active drugs, not including a carbapenem; regimen C, monotherapy with an aminoglycoside; regimen D, monotherapy with a carbapenem; regimen E, monotherapy with tigecycline; regimen F, monotherapy with colistin; regimen G, inappropriate therapy. Regimen A was superior to regimens B, E, F, and G (for A versus B, E, F, and G, the *P* value was 0.02, 0.03, <0.0001, and <0.0001, respectively). Regimens B, C, and D were superior to regimen G (for B versus G, *P* = 0.014; for C versus G, *P* = 0.04; and for D versus G, *P* = 0.03).

Only two observational human studies have evaluated fosfomycin against CRE, both were prospective. The first was a multicenter case-series of 41 carbapenemase-producing *K. pneumoniae* and 17 carbapenemase-producing *P. aeruginosa*. Fosfomycin (median dose 24g/day) was usually combined with either colistin or tigecycline with a clinical success rate at day 14 of 54%. 28 day mortality was 37.5%. Interestingly, resistance to fosfomycin developed in only three cases.[182] The second study followed 11 ICU patients infected with fosfomycin susceptible, carbapenem-resistant *K. pneumoniae*

where fosfomycin was administered in combination with colistin (6 patients), gentamicin (3 patients), or piperacillin/tazobactam (1 patient). The combination used for the 11th patient was not mentioned by the authors. All-cause in-hospital mortality was 18.2% (2/11 ICU patients).[230]

Novel Antimicrobials against Carbapenem-resistant Enterobacteriaceae

Ceftazidime/avibactam (AVYCAZ®) is the first of few upcoming antimicrobials with activity against CRE. The FDA approved its use in complicated urinary tract infections (cUTIs) and complicated intra-abdominal infections (cIAIs) in February 2015. Its spectrum of activity is similar to ceftazidime (i.e. wild-type Enterobacteriaceae and *Pseudomonas spp.*) but avibactam (a diazabicyclooctanase)[231] adds Ambler class A[232] and D[233] carbapenemase-producing Enterobacteriaceae in addition to ESBL-producing Enterobacteriaceae with porin channel mutations to the spectrum.[232] However, limited activity against MBLs has been observed. Ceftazidime/avibactam has also been tested *in vitro* against KPC-producing isolates with OmpK36 porin channel mutations. All 72 KPC-producing isolates studied were resistant to ceftazidime (MICs >64 µg/mL) but tested susceptible (ceftazidime MICs <4 µg/mL) with the addition of avibactam.[234] Experience treating bacteremia caused by CRE is limited at this time, but cases of success have been reported.[232]

SUMMARY

1. CRE are among the top threats in infectious disease according to the CDC and the President's Advisory Council on Combating Antibiotic-Resistant Bacteria.[11]

2. CRE are present throughout the world, but the characteristics of carbapenem resistance can vary widely depending on the country, state, or even acute care center. Within the U.S., KPC-producing Enterobacteriaceae comprise 80% of CRE cases.[14]

3. Significant mortality is associated with CRE infection, ranging from 24-70%.[1-10]

4. CRE are challenging to treat, often only being susceptible to polymyxins, fosfomycin, or tigecycline, for which there are no randomized controlled trials directing antimicrobial therapy.

5. A review of the literature favors combination therapy, usually with a carbapenem and/or a polymyxin, but genotypic expression may yet play a larger role on optimal therapy than phenotypic expression and pharmacodynamic targets.

6. There are fewer and fewer antimicrobials in development, and there is not a "magic bullet" that will treat all CRE.

HYPOTHESES

The study hypotheses were: 1) meropenem and polymyxin B in combination would exhibit synergistic, bactericidal activity against carbapenem-resistant *Klebsiella pneumoniae* having low and high levels of carbapenem resistance, described by the minimum inhibitory concentration of meropenem and 2) meropenem and polymyxin B used alone against carbapenem-resistant *Klebsiella pneumoniae* would not prevent bacterial growth by 24 hours

SPECIFIC AIMS

The specific aims of this study were:

1. To evaluate the minimum inhibitory concentration of meropenem and polymyxin B using broth microdilution against carbapenem-resistant *Klebsiella pneumoniae* clinical isolates from the University of Kentucky Chandler Hospital

2. To describe the growth of carbapenem-resistant *Klebsiella pneumoniae* when exposed to meropenem and polymyxin B alone and in combination using time-kill methodology

3. To characterize the *in vitro* interaction of the combination of meropenem and polymyxin B against *Klebsiella pneumoniae* clinical isolates representing low and high levels of carbapenem resistance which is described by the minimum inhibitory concentration of meropenem

Chapter Three:

Methods

Bacterial Isolates

Clinical isolates of 229 non-duplicate, multidrug resistant (MDR), gram-negative organisms were collected between November 9, 2008 and December 31, 2016 from the Clinical Microbiology Laboratory at the University of Kentucky Chandler Medical Center in Lexington, Kentucky. All isolates were cultured and identified during routine testing in the clinical laboratory according to guidelines from the Clinical and Laboratory Standards Institute (CLSI).[235] All isolates were frozen at -80˚C in 10% glycerol in water solution until needed for study ("Storage of Bacterial Isolates").[236] Multidrug resistance was defined as non-susceptibility to at least one agent in three or more antibiotic classes.[165] Isolates were designated carbapenem-resistant Enterobacteriaceae (CRE) if they were Enterobacteriaceae with documented carbapenemase production or non-susceptibility to any of the carbapenem antimicrobials (ertapenem, imipenem, meropenem, or doripenem; Table 3.8).[79]

Each isolate was subcultured once in cation-adjusted Mueller-Hinton broth 8-12 hours prior to experiments. Inoculation of conical tubes (15 mL polypropylene conical centrifuge tubes; USA Scientific, Ocala, FL) containing about 5 mL of cation-adjusted

Mueller-Hinton broth was accomplished using a sterile loop applicator (Fisherbrand™; ThermoFisher Scientific, Waltham, MA). These cultures were then incubated at 35˚C in a shake incubator (Figure 3.1; MaxQ 6000; ThermoFisher Scientific, Waltham, MA) at 220 oscillations per minute until turbid. All isolate manipulations were performed in Biological-Pharmaceutical Complex (BPC) room 374B. Three ATCC® quality control (QC) organisms were used: *E. coli* ATCC® 25922, *P. aeruginosa* ATCC® 27853, and *K. pneumoniae* ATCC® 700603. The QC *E. coli* strain is recommended for antimicrobial susceptibility testing on Enterobacteriaceae for antimicrobials other than carbapenems and polymyxins for which QC *P. aeruginosa* is recommended. QC *K. pneumoniae* is a negative control for MBL testing by Etest®.[237]

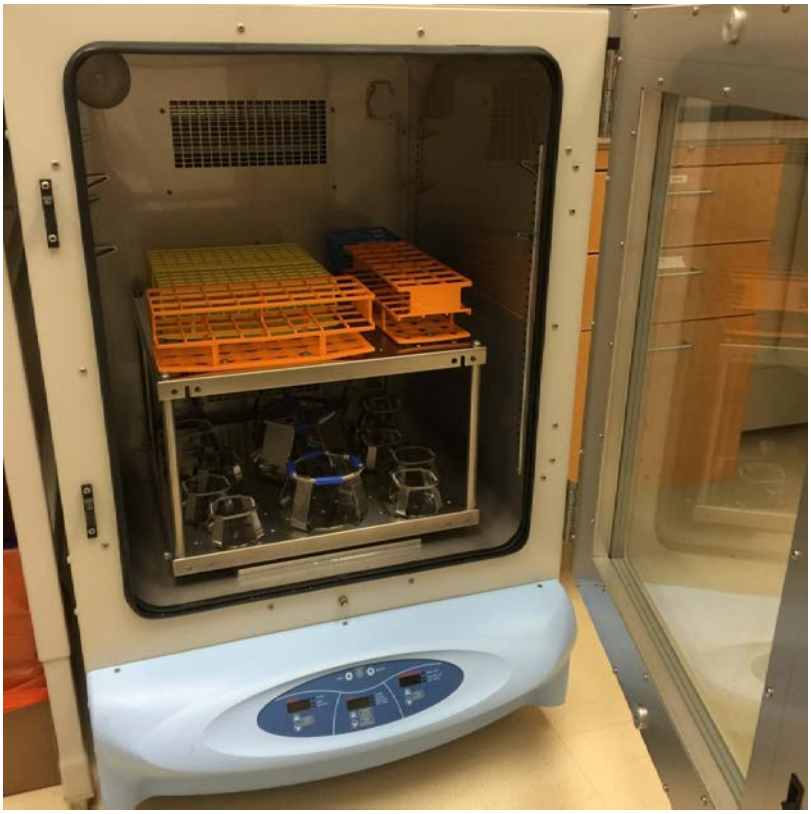


Figure 3.1: MaxQ 6000 (ThermoFisher Scientific, Waltham, MA)

Antimicrobial Agents

Antimicrobial powders were obtained from the manufacturers or supply companies listed in Table 3.1. After adjusting for potency, these powders were used in all studies.

Table 3.1: Sources of Antimicrobial Powders

Antimicrobial	Manufacturer/Supply Company	
Amikacin	Sigma-Aldrich®	St. Louis, MO
Cefepime	USP	Rockville, MD
Colistin	Sigma-Aldrich®	St. Louis, MO
Levofloxacin	Sigma-Aldrich®	St. Louis, MO
Meropenem	USP	Rockville, MD
Minocycline	Sigma-Aldrich®	St. Louis, MO
Piperacillin	Sigma-Aldrich®	St. Louis, MO
Polymyxin B	Sigma-Aldrich®	St. Louis, MO
Rifampin	Sigma-Aldrich®	St. Louis, MO
Tazobactam	LKT Laboratories, Inc.	St. Paul, MN
Tigecycline	TSZ Chem.	Framingham, MA

IN VITRO SUSCEPTIBILITY TESTING

Broth Microdilution MIC Testing

In vitro susceptibility testing was performed in duplicate using broth microdilution according to CLSI guidelines as isolates were identified.[235] Susceptibility testing was only performed on the first 24 *Klebsiella pneumoniae* received by the lab. MICs were determined for amikacin, cefepime, levofloxacin, meropenem, minocycline, polymyxin B, and tigecycline. Colistin, rifampin, and piperacillin/tazobactam were only evaluated for the first 12 *Klebsiella pneumoniae* isolates. Quality control organisms used in each experiment were *E. coli* ATCC® 25922 for antimicrobials other than carbapenems and polymyxins and *P. aeruginosa* ATCC® 27853 for carbapenems and polymyxins.

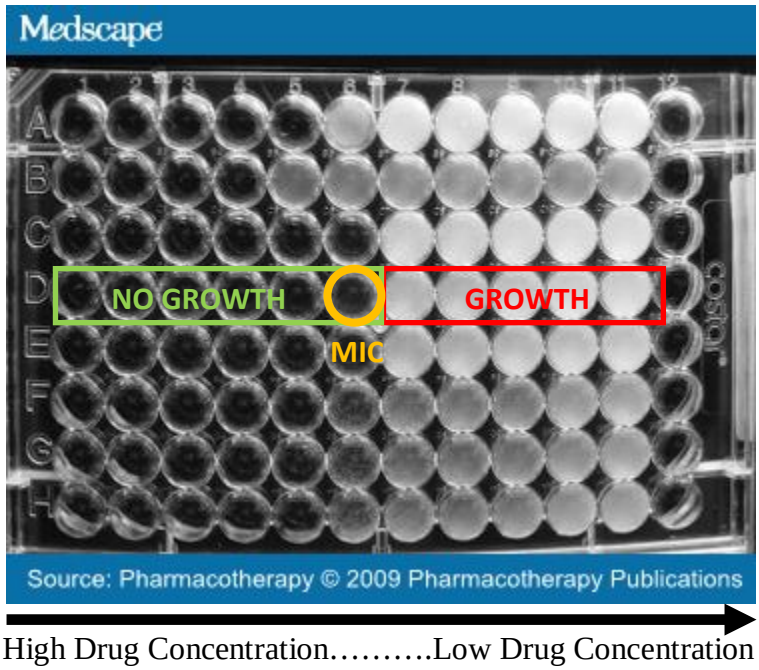


Figure 3.2: Example Broth Microdilution 96-well Tray

Automated MIC Testing

BD Phoenix™ is an automated system designed to identify the organism and perform antimicrobial susceptibility testing. The University of Kentucky Clinical Microbiology Laboratory evaluated each isolate with BD Phoenix™ in order to obtain MICs for amikacin, ampicillin, ampicillin/sulbactam, aztreonam, cefazolin, cefepime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, ertapenem, gentamicin, levofloxacin, meropenem, nitrofurantoin, piperacillin/tazobactam, tetracycline, tobramycin, and sulfamethoxazole/trimethoprim. This data was originally reported for clinical use in the management of patients infected with these isolates.

Figure 3.3: BD Phoenix™. **Reprinted**[238]

Kirby-Bauer Disk Diffusion

Kirby-Bauer disk diffusion was used to evaluate susceptibility of CRE isolates to ceftazidime/avibactam. This method does not measure MICs, but instead indicates susceptible, intermediate, or resistant to the antibiotic of choice based on the diameter of the zone of inhibition ("Kirby-Bauer Disk Diffusion Procedure" and Figure 3.4).

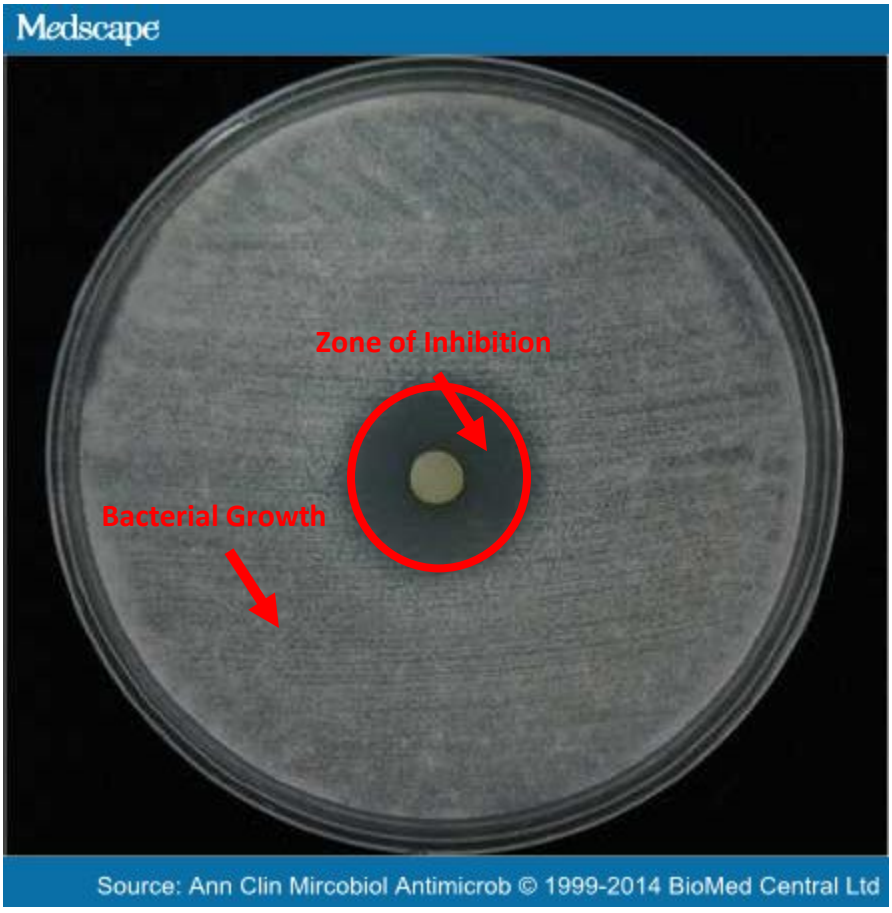
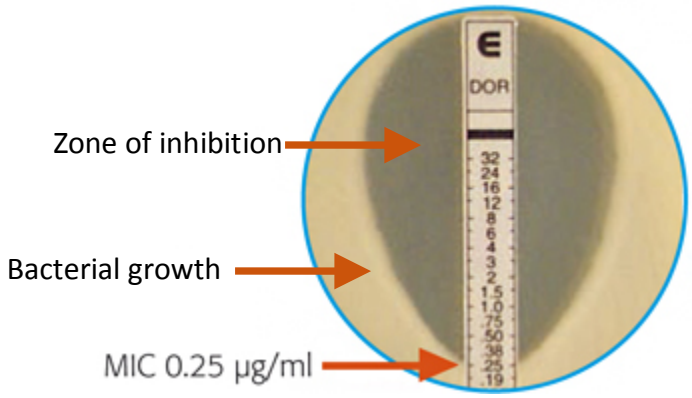


Figure 3.4: Kirby-Bauer Disk Diffusion

Etest®

Etest® strips (MBL MP/MPI 8/2; bioMérieux, Inc., Durham, NC) were primarily used to detect metallo β-lactamase (MBL) production in CRE isolates non-susceptible to ceftazidime/avibactam from Kirby-Bauer testing ("Kirby-Bauer Disk Diffusion Procedure"). However, Etest® strips can measure MICs (Figure 3.5) like broth microdilution and were used for determining fosfomycin MICs for the first 12 *Klebsiella pneumoniae* isolates identified by the University of Kentucky Clinical Microbiology Laboratory. *Klebsiella pneumoniae* ATCC® 700603 was used as a quality negative control for MBL Etest® strips and *E. coli* ATCC® 25922 was used as a quality control organism for fosfomycin MIC testing with Etest® strips.



Source: bioMérieux, Inc., Durham, NC

Figure 3.5: Example of Doripenem Etest®

Glassware, Plastic Tubing, and Pipette Tip Preparation

All glassware and autoclavable plastic were provided pre-sterilized by the manufacturer and were either disposable or sterilized by autoclave at 121°C for at least 20 minutes and verified by autoclave indicator tape (Fisherbrand™, ThermoFisher Scientific, Waltham, MA) prior to use in experiments. Equipment that could not be autoclaved (e.g. automated plate pourer or the laser colony counter) was sterilized by 70% ethanol in a spray bottle. A dispenser (Oxford®; Cole-Parmer®, Vernon Hills, IL) was sterilized by two 70% ethanol washes followed by two 0.22 µm filtered, distilled water washes and verified by negative growth of water dispensed onto an agar plate.

Media Preparation

Cation-adjusted Mueller-Hinton broth (Difco™; Becton Dickinson, Sparks, MD) was used for antimicrobial dilution and bacterial culture. Preparation involved dissolving 63 grams of broth powder in 3 liters of filtered, distilled water (Q-POD® Millipore using a 0.22 µm Millipak® 40 filter; Merck KGaA, Darmstadt, Germany). The solution was then autoclaved at 121˚C for 30 minutes. The manufacturer reports reconstituted Mueller-Hinton broth solutions are stable for up to one year after reconstitution (Becton, Dickinson and Company; personal communication, February 24, 2016). All broth was utilized within one to two weeks of reconstitution.

A 10 mg/mL stock solution of calcium and a 10 mg/mL stock solution of magnesium were prepared by adding 3.68 g of $CaCl_2*2H_2O$ to 100 mL of filtered, distilled water and adding 8.36 g of $MgCl_2*6H_2O$ to 100 mL of filtered, distilled water.

Each stock solution was filter sterilized again using a 0.22 micron filter (Corning® 150 mL Bottle Top Filter 0.22 µm; Corning Inc., Corning, NY). For every liter of Mueller-Hinton broth, 2.5 mL of calcium chloride stock solution and 1.25 mL of the magnesium sulfate stock solution were added for a final concentration of 25 mg/L calcium chloride and 12.5 mg/L magnesium sulfate.

Mueller-Hinton agar (Difco™; Becton Dickinson, Sparks, MD) was prepared by suspending 38 grams of agar powder in 1 L of filtered, distilled water. The suspension was then autoclaved at 121˚C for 30 minutes. Following sterilization, the suspension was poured by an automated machine (Figure 3.6; MP-1000 PourMatic 100mm; John Morris Scientific, Chatswood, Sydney, Australia) onto Petri dishes (Falcon® 100x15 mm sterile petri dishes; Corning Inc., Corning, NY) which were subsequently sealed in manufacturer supplied bags and stored in a walk-in refrigerator at <4°C until needed for use. The manufacturer reports Mueller-Hinton agar is stable for up to 3-5 months after reconstitution (Becton, Dickinson and Company; personal communication, February 24, 2016). All agar plates were utilized within one month of being reconstituted.

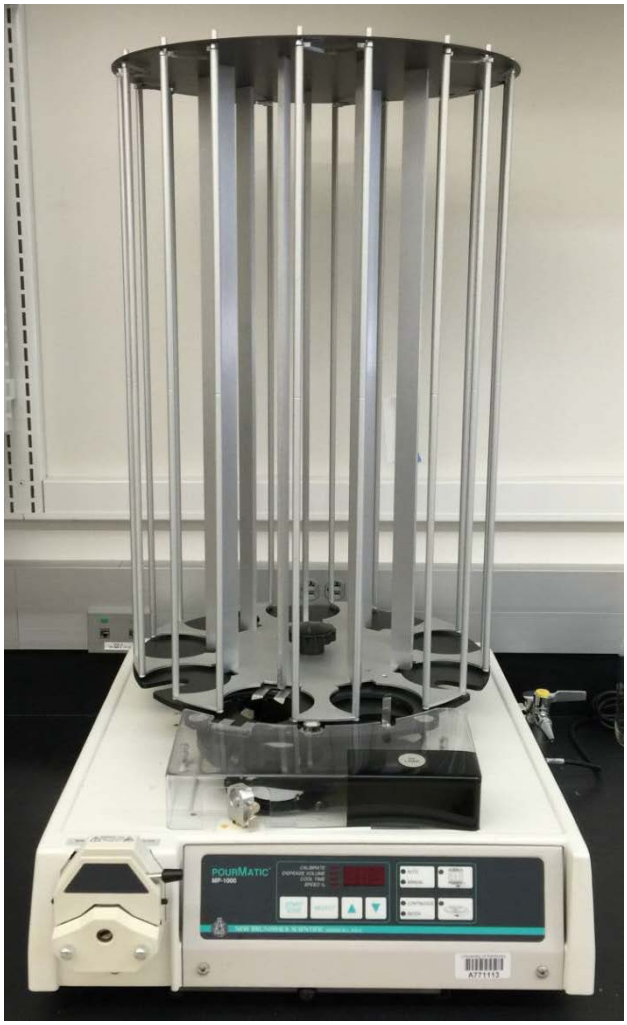


Figure 3.6: MP-1000 PourMatic® 100mm

For subpopulation analysis experiments, an extra step was added to the preparation of Mueller-Hinton agar plates. That is, after sterilization, but before the PourMatic® distributed the agar suspension onto petri dishes, antimicrobial agents were added to the agar suspension. Specifically, three unique types of antimicrobial plates were made – meropenem 16 µg/mL, meropenem 64 µg/mL, and polymyxin B 4 µg/mL.

Sterilized Mueller-Hinton agar was measured in a 1000 mL graduated cylinder (Fisherbrand™, ThermoFisher Scientific, Waltham, MA) to the 1 L mark. A stir bar

(Fisherbrand™, ThermoFisher Scientific, Waltham, MA) was dropped inside the cylinder and 1.56 mL of the 10.24 mg/mL meropenem stock solution ("Antimicrobial Solution Preparation") was added to the cylinder for a total concentration of 16 µg/mL meropenem. The agar was stirred on a hotplate (Fisherbrand™, ThermoFisher Scientific, Waltham, MA) for five minutes before being poured by the PourMatic® onto petri dishes which were subsequently sealed in manufacturer supplied bags and stored in a walk-in refrigerator at <4°C until needed for use. This process was repeated for the other two antimicrobial concentrations, but 6.25 mL of 10.24 mg/mL meropenem was used for the 64 µg/mL final concentration and 1.56 mL of the 2.56 mg/mL stock solution of polymyxin B was used for the 4 µg/mL final concentration plates.

Antimicrobial Solution Preparation

Stock solutions were prepared for each antimicrobial agent using CLSI recommended diluents (usually sterile water).[237] For water sterilization, filtered (Q-POD® Millipore using a 0.22 µm Millipak® 40 filter; Merck KGaA, Darmstadt, Germany) water was autoclaved at 121˚C for 15 minutes in 1 L batches. Primary stock solutions were prepared using the antimicrobial powders described under "Antimicrobial Agents." 10 mL of each antimicrobial agent was prepared using a volumetric flask (Kimax® 10 mL volumetric flask; Kimble; Cole-Parmer®, Vernon Hills, IL) and powder was weighed using an analytical balance (Mettler AE200, Figure 3.7; Marshall Scientific, Hampton, NH). Rifampin was prepared using 1.5 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich®, St. Louis, MO) which was added to aid in solubility into sterile water and a 50 mL

volumetric flask to avoid conical vial cracking and assist in dissolution of drug particles once frozen. The other exception to sterile water as a diluent was cefepime, which was prepared in a phosphate buffer at a pH of 6.0. A phosphate buffer stock solution at a pH of 6.0 was previously prepared from 13.2 mL of a 0.1M dibasic potassium phosphate solution (Dry Powder; Sigma-Aldrich®, St. Louis, MO) and 86.8 mL of a 0.1M monobasic potassium phosphate solution (Dry Powder; Sigma-Aldrich®, St. Louis, MO). Levofloxacin was completely soluble in sterile water at the stock concentration utilized. Primary stock solutions were frozen at -20˚C until needed for an experiment, frozen and thawed no more than 5 times, and were not used beyond 6 months after making.[239]

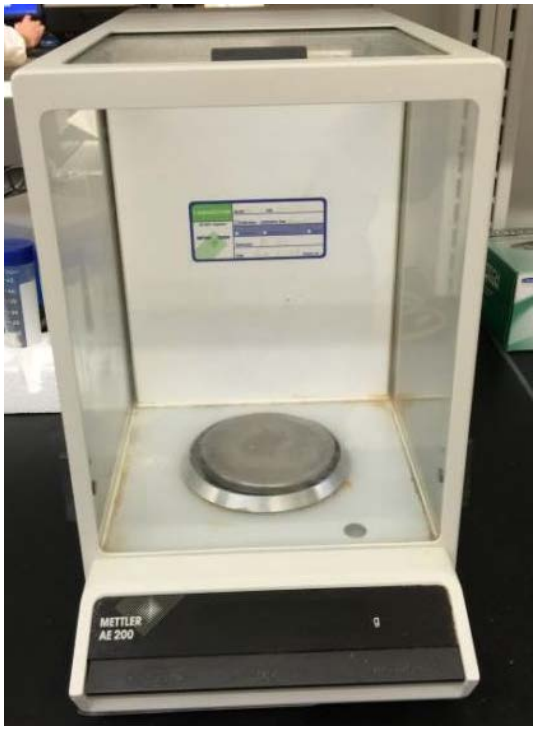


Figure 3.7: Analytical Balance

Table 3.2: Primary Antimicrobial Stock Solutions

Antimicrobial	Powder (mg)	Potency (mg/mg)	Concentration (mg/mL)
Amikacin	263.9	0.776	20.48
Cefepime	124.0	0.826	10.24
Colistin	32.7	0.7827	2.56
Levofloxacin	12.9	0.99	1.28
Meropenem	117.3	0.873	10.24
Minocycline	28.7	0.892	2.56
Piperacillin	216.5	0.946	20.48
Polymyxin B	34.4	0.7450	2.56
Rifampin*	66.0	0.97	1.28
Tazobactam	16.1	0.994	1.60
Tigecycline	25.8	0.9937	2.56

*10 mL volume primary stock prepared for each agent above except Rifampin, which was a 50 mL volume stock

All prepared antimicrobial stock solutions were stored in plastic conical vials (15 mL polypropylene conical centrifuge tubes; USA Scientific, Ocala, FL). Secondary stock solutions were prepared using broth instead of sterile water on the day of testing in similar plastic conical vials and used immediately following preparation. See "Broth Microdilution Procedure" for addition of antibiotics to the microtiter trays (Costar® non-treated, sterile, polystyrene 96-well; Sigma-Aldrich®, St. Louis, MO).

Table 3.3: Antimicrobial Secondary Stock Concentrations and testable MIC range

Antimicrobial	Concentration (mg/mL)	Concentration Range (mg/L)
Amikacin	2.048	0.25 – 512
Cefepime	1.024	0.125 – 256
Colistin	0.256	0.031 – 64
Levofloxacin	0.128	0.016 – 32
Meropenem	1.024	0.125 – 256
Minocycline	0.256	0.031 – 64
Piperacillin/Tazobactam	2.048 / 0.160	0.25/4 – 512/4
Polymyxin B	0.256	0.031 – 64
Rifampin	0.128	0.016 – 32
Tigecycline	0.256	0.031 – 64

Preparation of Inocula for Susceptibility Testing

Inocula were prepared by the McFarland Standard Method using 0.5 and 1 McFarland standards and a Wickerham Card (Figure 3.8 Remel™ McFarland Turbidity Standard; ThermoFisher Scientific, Waltham, MA). An actively growing bacterial suspension ("Bacterial Isolates") was added drop-wise using an ErgoOne micropipette (Figure 3.9; USA Scientific, Ocala, FL) to a glass test tube (Fisherbrand™ Disposable Culture Tubes 16x125mm Borosilicate Glass; ThermoFisher Scientific, Waltham, MA) containing about 5 mL 0.22 micron filtered, distilled water. Using the Wickerham Card, the turbidity of the glass test tube was matched as closely as possible to the 0.5

McFarland standard which is approximately equivalent to 1.0 - 1.5 x 10^8 CFU/mL. See Figure 3.8 for setup before inoculating the glass test tube.

Figure 3.8: Test Tube with Water, McFarland Standards and Wickerham Card

Figure 3.9: ErgoOne Micropipette

For susceptibility testing using broth microdilution, the McFarland-matched suspension was subsequently diluted 1:200 in two steps by first adding 100 µL to 9.9 mL cation-adjusted Mueller-Hinton broth, and then secondly adding 50 µL of this solution to 50 µL of broth and antimicrobial agent during the final inoculation step of the broth microdilution susceptibility testing ("Broth Microdilution Procedure"). The final bacterial concentration in each well was approximately 5.0 - 7.5 x 10^5 CFU/mL.

In preparing microtiter trays for susceptibility testing of piperacillin/tazobactam, a 1:200 dilution was made in two different steps by first adding 200 µL of McFarland-matched suspension to 9.8 mL cation-adjusted Mueller-Hinton Broth and then secondly adding 25 µL of this solution to 75 µL of broth and piperacillin/tazobactam ("Broth Microdilution Procedure"), resulting in an approximate final concentration of 5.0 - 7.5 x 10^5 CFU/mL.

For Kirby-Bauer disk diffusion and Etest®, the McFarland-matched suspension was not diluted prior to inoculating agar plates ("Inoculation of Agar Plates").

Broth Microdilution Procedure

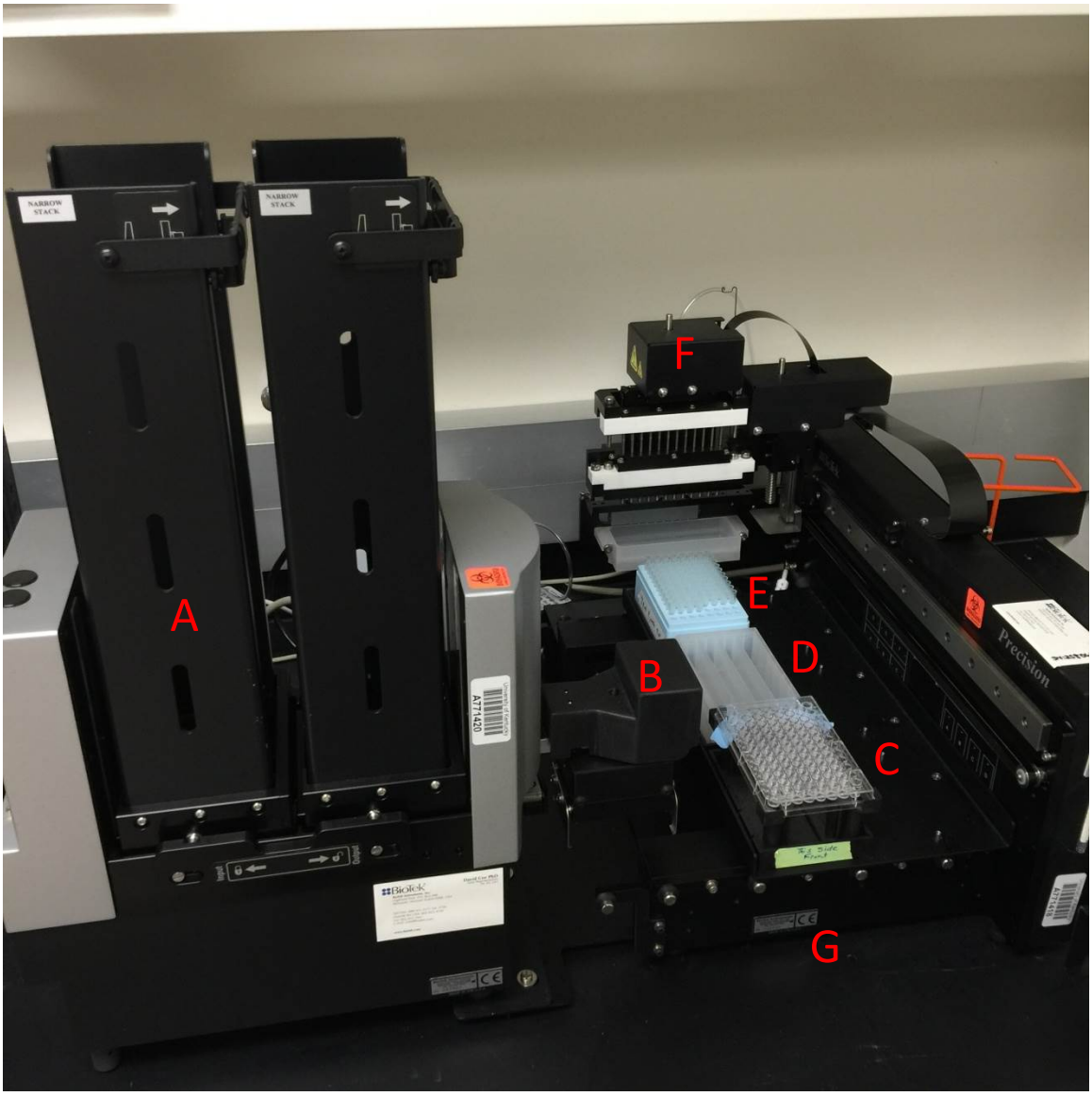


Figure 3.10: BioStack™ (Left) Attached to Precision™ Pipetting System (Right).
A) The BioStack™ consists of the two black columns (left) which hold the unfilled and filled 96-well trays. B) The arm is the mechanical device that transfers 96-well trays to and from deck C. C) This deck holds 96-well trays for broth, antimicrobial, and organism deposition. D) This deck holds the 4 row by 1 column (4x1) reservoir (shown) as well as the 1x6 reservoir (not shown). E) This deck holds the sterile pipette tips for the manifold F. F) The manifold transfers fresh broth first, antibiotic second, serially dilutes the 96-well tray third, and lastly, adds the inoculated suspension to the 96-well tray. G) The sharps disposal container is placed here to catch used pipette tips.

After all necessary preparations ("Media Preparation," "Antimicrobial Solution Preparation," and "Preparation of Inocula for Susceptibility Testing"), a stack of 96-well trays were added to the left column of the BioStack™ (Figure 3.10), sterile pipette tips were added to deck E, and the freshly prepared cation-adjusted Mueller-Hinton broth was added to each reservoir in the 4x1 reservoir. The computer that manages the Precision™ pipetting system is not shown in Figure 3.10, but the Precision Power™ software was launched and the program labeled "1 BTK Broth MIC testing (Initial 50 mcl broth only).PGM" was loaded and run. The arm then transferred a 96-well tray from the left column on the BioStack™ to deck C. The manifold picked up 12 pipette tips from deck E and extracted broth from the 4x1 reservoir into each. The manifold then deposited 50 µL into each well of the 96-well tray. The arm transferred the 96-well tray back to the right column of the BioStack™ and picked up a new plate from the left column of the BioStack™ to repeat this whole process for each and every 96-well tray.

Once all 96-well trays contained 50µL of broth, the manifold disposed of the pipette tips into container (G) and the program terminated. The right column of the BioStack™ was manually exchanged with the left column, hereafter always referred to by relative position to each other (i.e. the old right column is now the left column) because each program tells the arm to pull 96-well trays from the left column. The 4x1 reservoir was exchanged with another 4x1 reservoir that contained 1 compartment of broth without an antimicrobial agent and 3 out of 10 of the freshly prepared secondary stock solutions ("Antimicrobial Solution Preparation") which were at concentrations 4x what was needed in the first well of the 96-well trays. This would in the next step provide

the 8x12 tray with 2 rows of a growth control and 2 rows of each of the 3 antibiotics in the 4x1 reservoir with 12 serial dilutions (Figure 3.11).

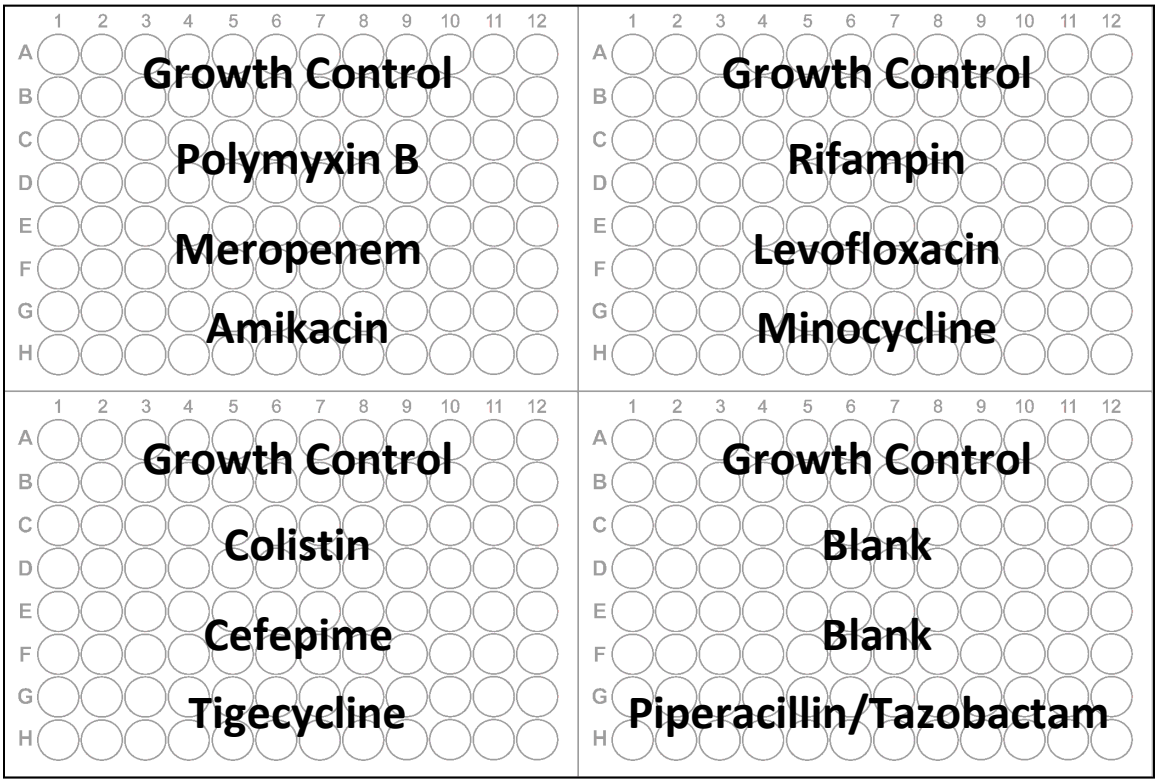


Figure 3.11: Complete set of 4 unique 96-well trays

At this point, all 96-well trays were filled with 50 µL of cation-adjusted Mueller-Hinton broth and were in the left column of the BioStack™, ready for serial dilution of antimicrobials. The program "2 BTK multiDrug MIC testing (12 dilutions).PGM" was run which, similar to the first program, instructed the arm to take a 96-well tray from the left column BioStack™ and place it on deck C. The manifold picked up 12 pipette tips, withdrew 50 µL of antimicrobials into each tip from the 4x1 reservoir, and deposited the contents into the first well. Following mixing, 50 µL were withdrawn from the first well

and added to the second well. This repeated from well to well until all remaining wells (12 total) had been serially diluted and mixed. The manifold disposed of the pipette tips into the sharps disposal container and picked up 12 more sterile pipette tips while the arm exchanged the antimicrobial filled 96-well tray with a new one, placing the former into the right column BioStack™. This process repeated until all 96-well trays were filled with the set of antimicrobials. This program was repeated for each antimicrobial set as in Figure 3.11, exchanging the 4x1 reservoir for a new set of antimicrobials as needed as well as adding new pipette tips and changing the contents of the BioStack so that 96-well trays without antibiotic were on the left and the freshly serially diluted trays were set aside (removed from the right column) in groups.

For the piperacillin/tazobactam group, only piperacillin was placed in the 4th row of the 4x1 reservoir to be serially diluted so that a constant concentration of tazobactam can be later placed into each well. Once this group of 96-well trays was finished, the columns of the BioStack™ were exchanged and another program was loaded and run, "2.5 BTK Drug PIP TAZO MIC testing.PGM." However, prior to running this, pipette tips were replaced if needed and a 1x6 reservoir replaced the 4x1 reservoir with the 1st row being filled with tazobactam at 4x the needed concentration, or 16 mcg/mL ("Antimicrobial Solution Preparation"). This program instructed the arm to retrieve a 96-well tray from the left column BioStack™ and place it on deck C. The manifold obtained 12 sterile pipette tips and aspirated tazobactam from the 1st row of the 1x6 reservoir. 25 µL of tazobactam were dispensed into each well, very similarly to the program that initially dispensed broth into each well before the serial dilutions. This program finished once all piperacillin group 96-well trays had a total of 75 µL of volume in each well.

Once all 96-well trays had antimicrobials serially diluted across all 12 rows, each well now contained 50 µL of broth with antimicrobial agent (except the piperacillin/tazobactam group trays) and the 96-well trays were placed by group of common antimicrobials (Figure 3.11), one group at a time, in the left column of the BioStack as before. At first, all of the growth control, polymyxin B, meropenem, and amikacin 96-well plates were placed in the left column BioStack while the other 3 groups were set aside. Pipette tips were replaced as necessary and the 4x1 reservoir was exchanged with a 1x6 reservoir which contained 6 unique 0.5 McFarland-matched bacterial suspensions that had been diluted 1:100 (step 1 of "Preparation of Inocula for Susceptibility Testing"). Note that the bacterial suspensions for the piperacillin/tazobactam 96-well trays were at the time diluted 1:50.

Before running the final program, a checklist was used to ensure that the left column of the BioStack contained only 1 group of 96-well trays and that the right column of the BioStack was empty to receive the completed trays. The pipette tips were replaced in deck E if needed and the 1x6 reservoir contained a different bacterial suspension in each of the 6 compartments. Furthermore, unless the group of piperacillin/tazobactam 96-well trays were being used, the 1:50 diluted suspensions were not needed yet.

"3 BTK multiBug MIC testing (Lay bug 50 mcl).PGM" was loaded and run in the software. This program instructed the arm to add a 96-well tray to deck C and instructed the manifold to pick up 12 sterile pipette tips, aspirate bacterial suspension from the 1st column of 6 of the 1x6 reservoir and to dispense 50 µL into each of the 96 wells, finishing the 2nd step of the 1:200 dilution and resulting in an initial bacterial concentration of approximately 5.0 - 7.5 x 10^5 CFU/mL in each well. The arm then

placed the complete 96-well tray into the right column BioStack™ and retrieved another 96-well tray. The manifold disposed of the previously used pipette tips and obtained new ones. Following this, the 2nd column of 6 of the 1x6 reservoir was aspirated and 50 µL of suspension was dispensed into each well. This repeated until 6 plates were completed with a unique suspension in each. The program was repeated after replacing pipette tips if needed and changing the 1x6 reservoir for 6 new bacterial suspensions. It was not necessary to change the BioStack™ columns at this time because there was an equivalent number of 96-well trays remaining as there were bacterial suspensions. Once all bacterial suspensions had been used for the first group of antimicrobial 96-well trays, these trays were placed in an incubator (Heratherm™ Incubator, Figure 3.12; ThermoFisher Scientific, Waltham, MA) at 35˚C and this entire processes was repeated again for the 2nd and 3rd group of a 96-well trays.

Figure 3.12: Heratherm™ Incubator

For the 4th group of 96-well trays, also known as the piperacillin/tazobactam group, a different program was needed because only 25 µL of a 1:50 bacterial suspension needs to be placed into each well. "3.5 BTK multiBug PIP TAZO MIC testing (Lay bug 25 mcl).PGM" was loaded and run. This program is similar to the one in the previous paragraph except that the 1x6 reservoirs contained 6 unique bacterial suspensions each at a 1:50 dilution and only 25 µL of this suspension was dispensed into each well.

In summary, every 96-well tray had 50 µL of cation-adjusted Mueller-Hinton broth and a group of antimicrobial agents (Figure 3.11) serially diluted across the 12 columns. 50 µL of a 1% 0.5 McFarland-matched bacterial suspension was added to each well for a total volume of 100 µL in each well. The only exception were the

piperacillin/tazobactam group trays which had 25 µL of 16 mcg/mL tazobactam added and 25 µL of a 2% 0.5 McFarland-matched bacterial suspension for a total volume of 100 µL in each well. See Table 3.3 for the concentration ranges of antimicrobial agent.

Incubation

Once inoculated, all 96-well microtiter trays and Mueller-Hinton agar plates were sealed using the manufacturer supplied lids and incubated at 35˚C for 18-24 hours in an incubator (Heratherm™ Incubator; ThermoFisher Scientific, Waltham, MA). Agar plates were inverted and stacked no more than four high.

Minimum Inhibitory Concentration (MIC) Determination

MIC is defined as the lowest concentration of antimicrobial agent required to completely inhibit the growth of the microorganism to the unaided eye. On the 96-well microtiter trays, this would be a complete absence of turbidity, individual colonies, and strings. An example is shown on Figure 3.2. The resulting growth by well was depicted on data sheets for each tray (Figure 3.13).

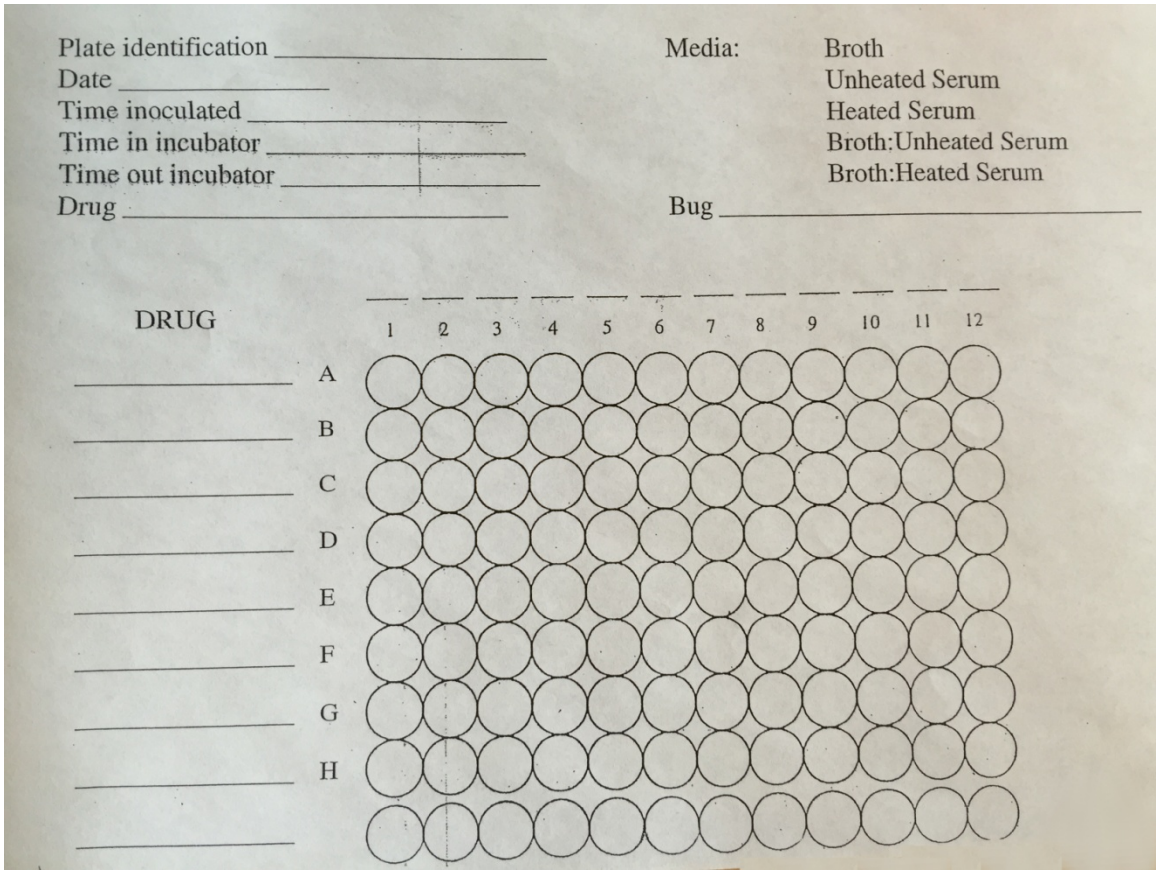


Figure 3.13: Antimicrobial Susceptibility 8x12 Microtiter Data Sheet

Inoculation of Agar Plates

After preparing the agar plates ("Media Preparation") and bacterial suspensions for inoculation ("Preparation of Inocula for Susceptibility Testing"), a wooden, sterile, cotton-tipped applicator (Fisherbrand™; ThermoFisher Scientific, Waltham, MA) was dipped into the 0.5 McFarland-matched bacterial suspension and then rolled on the side of the same glass tube to remove excess suspension. The agar plate was streaked in a back-and-forth motion as if painting the entire plate from top to bottom (Figure 3.14). The plate was rotated 90˚ and the same cotton swap was used to streak the plate again but without dipping into the bacterial suspension a second time. This coated the agar plate with a lawn of bacteria.

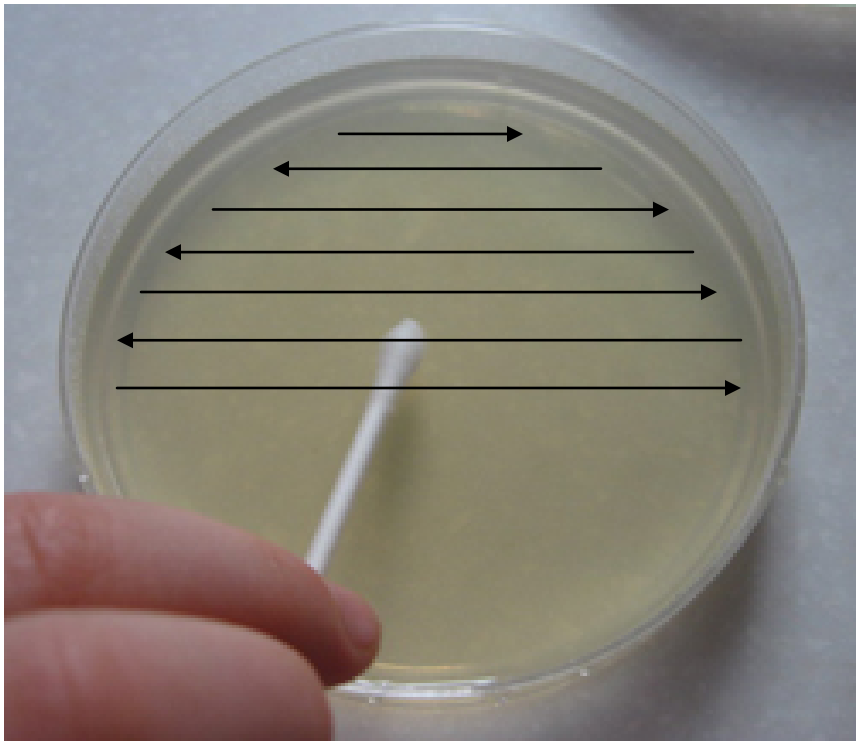


Figure 3.14: Inoculation of Mueller-Hinton Agar Plate

Kirby-Bauer Disk Diffusion Procedure

Following inoculation of the agar plate ("Inoculation of Agar Plates"), a ceftazidime/avibactam (30 µg / 20 µg) impregnated disk (Actavis; Parsippany, New Jersey) was placed on the plate using sterile forceps (Fisherbrand™, ThermoFisher Scientific, Waltham, MA). The plate was then incubated (Heratherm™ Incubator; ThermoFisher Scientific, Waltham, MA) at 35˚C for 18-24 hours and the diameter of the zone of inhibition (Figure 3.4) was measured in millimeters to the nearest whole number and recorded. Susceptibility was determined based on CLSI guidelines (≥21 mm susceptible; 18-20 mm intermediate; ≤17 mm resistant).[237]

At the time of experiment, 152 unique MDR isolates had been collected, consisting of 75 CRE (Enterobacteriaceae with an ertapenem MIC >0.5 or a meropenem MIC >1). All 75 isolates underwent Kirby-Bauer disk diffusion testing for susceptibility to ceftazidime/avibactam. Isolates that had a zone of inhibition ≤ 21mm (borderline susceptible, intermediate, or resistant) underwent a second test to verify resistance. If a discrepancy between the results occurred, the test was repeated once more.

Etest® Procedure

Following inoculation of the agar plate ("Inoculation of Agar Plates"), an Etest® strip was placed on the plate using sterile forceps. The plate was then incubated (Heratherm™ Incubator; ThermoFisher Scientific, Waltham, MA) at 35˚C for 18-24 hours.

MBL MP/MPI Etest® strips were interpreted as positive for MBL if 1) the MIC ratio of meropenem (MP) to meropenem with EDTA (MPI) was ≥8 (Figure 3.5 for MIC reading of Etest®), 2) if there was a phantom zone (i.e. an extra inhibition zone between the MP and MPI regions; Figure 3.15), or 3) if a deformation of the MP or MPI ellipses was present. All CRE isolates with ceftazidime/avibactam zones of inhibition measuring ≤ 21mm (borderline susceptible, intermediate, or resistant; "Kirby-Bauer Disk Diffusion Procedure") were tested for MBL production by Etest® except four isolates which were already known to produce MBL by PCR from previous work.

Fosfomycin Etest® strips were read for MIC and recorded (Figure 3.5). Only the first 12 *Klebsiella pneumoniae* isolates were tested with fosfomycin Etest®.

Different growth-inhibition patterns:

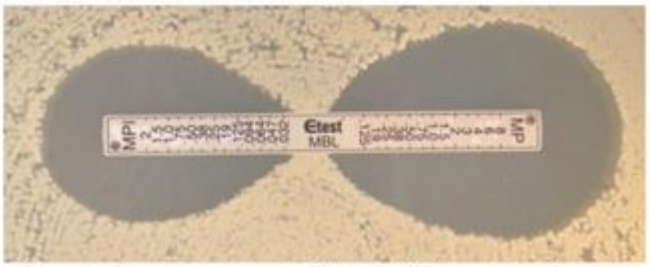


Figure 2. Clear cut MBL negative: MP/MPI IC <0.125/<0.032

Figure 3. Clear cut MBL positive: MP/MPI IC >8/0.19 = >42

Figure 4. Phantom zone between MP/MPI is indicative of MBL

Source: bioMérieux, Inc., Durham, NC

Figure 3.15: MBL MP/MPI Etest® Interpretation

Selection of Bacterial Isolates for Further Testing

Clinical isolates were selected for further testing based on the meropenem and polymyxin B MICs. For subsequent time-kill studies, four isolates showing polymyxin B susceptibility, defined as ≤2 mg/L based on CLSI breakpoints for *A. baumannii* and *P. aeruginosa*,[237] with varying degrees of meropenem resistance were chosen. In order of increasing meropenem resistance, those isolates selected were named KP 34 (4 mg/L), KP 22 (16 mg/L), KP 24 (32 mg/L), and KP 44 (128 mg/L).

TIME-KILL STUDIES

Time-kill experiments can be used to evaluate bacteria colony count at various time points during exposure to a set concentration of one or more antimicrobial agents. All time kill assays were performed at least in duplicate with a positive growth control and samples collected at 0, 1, 2, 4, 8, 24, and 48 hours, diluted as necessary, and aliquots (50µL) logarithmically plated onto Mueller Hinton agar using a spiral plater (Figure 3.16; AutoPlate® spiral plater; Advanced Instruments, Inc., Norwood, MA), which helped control for antibiotic carryover.[240] Colonies were counted using a laser colony counter (Figure 3.17; QCount Automated Colony Counter; Spiral Biotech, Advanced Instruments, Inc., Norwood, MA) with a lower limit of quantification of 10^2 CFU/mL.

Cation-adjusted Mueller-Hinton broth was used for growth media ("Media Preparation" and "Inocula Preparation"). Meropenem and polymyxin B alone were evaluated at three (4, 16, and 64 mg/L) and seven (0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/L) clinically relevant concentrations, respectively.[241,242] For combination studies, polymyxin B concentrations of 0.25 and 1 mg/L were evaluated with all three concentrations of meropenem against KP 22, KP 24, KP 32, and KP 44. However, for the highly meropenem resistant isolate (KP 44), polymyxin B at 4 mg/L was also evaluated in combination with the three concentrations of meropenem. Repeat MICs for polymyxin B were determined for regrowing bacteria at 24 hours ("Resistance Development Testing").

Figure 3.16: AutoPlate® spiral plater; Advanced Instruments, Inc., Norwood, MA

Figure 3.17: QCount Automated Colony Counter; Spiral Biotech, Advanced Instruments, Inc., Norwood, MA

Time-Kill Procedure

Meropenem, polymyxin B, or both agents in combination were added to cation-adjusted Mueller-Hinton broth ("Media Preparation") in conical vials (50 mL polypropylene conical centrifuge tubes; USA Scientific, Ocala, FL) up to a total volume of 30 mL measured by ErgoOne micropipettes and a Pipet-Aid® (Drummond Scientific Co., Figure 3.18; Broomall, PA). Portions of the primary stock vials of antimicrobials ("Antimicrobial Solution Preparation") were added directly to the 50 mL conical vial according to Table 3.4 and the desired concentrations.

Figure 3.18: Drummond Pipet-Aid®

Table 3.4: Time-Kill Volume Table

Meropenem Alone							
Antimicrobial Concentration (µg/mL)	**4**		**16**		**64**		
Volume of Stock Soln. (µL)	117		469		1875		
Volume of 0.5 McFarland Suspension (µL)	150		150		150		
Volume of Broth in 50 mL vial (mL)	29.8		29.4		28.0		
Total Volume (mL)	**30**		**30**		**30**		
Polymyxin B Alone							
Antimicrobial Concentration (µg/mL)	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**
Volume of Stock Soln. (µL)	2	4	7	15	29	59	117
Volume of 0.5 McFarland Suspension (µL)	150	150	150	150	150	150	150
Volume of Broth in 50 mL vial (mL)	29.8	29.8	29.8	29.8	29.8	29.8	29.8
Total Volume (mL)	**30**	**30**	**30**	**30**	**30**	**30**	**30**
Combination*							
Meropenem Concentration (µg/mL)	**4**		**16**		**64**		
Volume of Stock Soln. (µL)	117		469		1875		
Volume of 0.5 McFarland Suspension (µL)	150		150		150		
Volume of Broth in 50 mL Vial (mL)	29.7		29.4		28.0		
Total Volume (mL)	**30**		**30**		**30**		

*Polymyxin B volume contribution ignored

After the antimicrobial agents were added to their respective conical vials, a 1:200 dilution of the 0.5 McFarland matched suspension was made by adding 150 µL of the suspension to the conical vial. Immediately following this addition, the conical vial was mixed swiftly using the pipette tip of the ErgoOne micropipette and a 0.5 mL sample was drawn and serially diluted in 1:10 dilutions in glass test tubes (Fisherbrand™ Disposable Culture Tubes 16x125mm Borosilicate Glass; ThermoFisher Scientific, Waltham, MA) containing 4.5 mL sterile water. A dispenser (Oxford®; Cole-Parmer®, Vernon Hills, IL) was calibrated and used to equally measure 4.5mL volumes. Vials were then placed in a shake incubator (MaxQ 6000; ThermoFisher Scientific, Waltham, MA) at 35˚C and 220 oscillations per minute for the remainder of the experiment.

Samples were drawn by an ErgoOne micropipette at times 0, 1, 2, 4, 8, 24, and 48 hours. Since the laser colony counter (QCount Automated Colony Counter; Spiral Biotech, advanced Instruments, Inc., Norwood, MA) most optimally measures 10^3 - 10^5 CFU/mL, at time 0, a 1:10 and a 1:100 dilution were made from a 0.5 mL serially diluted sample due to an initial colony count of about 5.0 - 7.5 x 10^5 CFU/mL. At each time point, it was noted whether the conical vial contents were clear or turbid because the unaided eye can see turbidity at approximate 10^7 CFU/mL based on previous work performed in our laboratory. If clear, an undiluted 2 mL sample was drawn and a 1:100 dilution sample was made from a 0.5 mL serially diluted sample, both plated via spiral plater, and both placed in an incubator (Heratherm™ Incubator; ThermoFisher Scientific, Waltham, MA). If turbid, a 1:10^4 and a 1:10^6 dilution were made from a 0.5 mL serially diluted sample, both plated via spiral plater, and both placed in an incubator. Subsequent 0.5 mL samples drawn after a turbid time point were diluted 1:10^5 and 1:10^7, both plated via spiral plater, and both placed in an incubator.

As previously mentioned, the target measurement for the laser colony counter is 10^3-10^5 CFU/mL and dilution choices were made based on previous time-kill studies performed in the laboratory. All plates incubated for 18-24 hours and were read by a laser colony counter. The lower limit of quantification was 10^2 CFU/mL which was the value used for any time point reading less than this.

Resistance Development Testing

Following two separate time-kill studies, MIC determination by broth microdilution was performed on colonies growing on the 24-hour time point agar plates ("Time-Kill Procedure") but were tested only for changes in polymyxin B MIC ("*In vitro* Susceptibility Testing"). Resistance development was defined as a ≥4-fold increase in MIC from colonies growing at 24 hours when compared to the baseline MIC of the organism. An MIC > 2 was considered non-susceptible according to CLSI breakpoints for *A. baumannii* and *P. aeruginosa*.[237]

Subpopulation Analysis and Microfiltration

A modified time-kill procedure was used to evaluate subpopulations of the four *K. pneumoniae* isolates selected for time-kill studies ("Time-Kill Studies"). The results of this study would allow us to explain observed regrowth in the other time-kill experiments by quantifying the subpopulations of each isolate. These subpopulations often have a different MIC than the MIC of the majority or overall population.[142] Instead of adding antimicrobial agents to the 50 mL conical vials, 29.8 mL of cation-adjusted Mueller-Hinton broth ("Media Preparation") with 150 µL of 0.5 McFarland matched bacterial suspension ("Preparation of Inocula for Time-Kill") were used for a total of 30 mL of approximately 5.0 - 7.5 * 10^5 CFU/mL of bacteria. Additionally, instead of using Mueller-Hinton agar plates, the antimicrobial-impregnated plates were used ("Media Preparation"). Finally, sampling time points were 0, 2, 3, 4, 6, and 24 hours. All other aspects of this modified study were similar to the "Time-Kill Procedure."

It was hypothesized that the subpopulations we wished to quantify may be below the lower limit of quantification (10^2 CFU/mL) for the laser colony counter. To address this, two comparable approaches were used. First, instead of using aliquots of 50 µL logarithmically plated by the spiral plater, a uniform 500 µL setting was used. Second, we implemented a process called microfiltration which involved taking a specific sample volume at each time point (Tables 3.5 - 3.8), passing sample through a 0.22 µm filter, and then placing the filter (bacteria-side up) directly onto the antimicrobial-impregnated agar plate where nutrients could diffuse through the filter paper to the bacteria. In both cases, the plates were incubated (Heratherm™ Incubator; ThermoFisher Scientific, Waltham, MA) at 35˚C for 18-24 hours and then manually counted so that a colony count (CFU/mL) could be calculated based on the volume utilized for each sample. The lower limit of quantification associated with microfiltration ranges from 30-300 CFU/mL, decreasing as larger sample volumes are used.[243,244]

Figure 3.19: Vacuum Filter Apparatus

Table 3.5: Microfiltration Sample Volumes at Each Time Point for KP 34

Agar Plate	0 (hours)	2 (hours)	3 (hours)	4 (hours)	6 (hours)	24 (hours)
MEM 16 (µg/mL)	500 µL	500 µL	250 µL	100 µL	100 µL	No sample
PMB 4 (µg/mL)	1000 µL	1000 µL	500 µL	250 µL	250 µL	No sample

Table 3.6: Microfiltration Sample Volumes at Each Time Point for KP 22

Agar Plate	0 (hours)	2 (hours)	3 (hours)	4 (hours)	6 (hours)	24 (hours)
MEM 16 (µg/mL)	1000 µL	500 µL	250 µL	250 µL	250 µL	No sample
PMB 4 (µg/mL)	1000 µL	1000 µL	500 µL	250 µL	250 µL	No sample

Table 3.7: Microfiltration Sample Volumes at Each Time Point for KP 24

Agar Plate	0 (hours)	2 (hours)	3 (hours)	4 (hours)	6 (hours)	24 (hours)
MEM 64 (µg/mL)	1000 µL	500 µL	250 µL	100 µL	100 µL	No sample
PMB 4 (µg/mL)	1000 µL	1000 µL	500 µL	250 µL	100 µL	500 µL

Table 3.8: Microfiltration Sample Volumes at Each Time Point for KP 44

Agar Plate	0 (hours)	2 (hours)	3 (hours)	4 (hours)	6 (hours)	24 (hours)
MEM 64 (µg/mL)	100 µL	100 µL	No sample	No sample	No sample	No sample
PMB 4 (µg/mL)	1000 µL	1000 µL	500 µL	500 µL	250 µL	No sample

DATA ANALYSIS

In Vitro Susceptibility Testing

For each isolate, MIC results from within the same experiment as well as from duplicate or sometimes triplicate experiments were compared and evaluated for essential agreement (within one two-fold dilution).[237] If MIC results did not agree, broth microdilution was repeated for these strains. When MIC results were not the same, but were in agreement, the greater of the two results was accepted as the MIC. If three MIC results differed, the most common (modal) MIC was accepted. If there was not a modal MIC, the middle MIC was accepted if all results were in agreement with the middle MIC reading. Additionally, MIC range, MIC_{50}, and MIC_{90} were determined. Percent susceptible was calculated based on breakpoints established by CLSI (Table 3.8).[237]

Table 3.9: Susceptibility Breakpoints for Enterobacteriaceae

	Susceptible	Intermediate	Resistant
Amikacin	≤16	32	≥64
Ampicillin	≤8	16	≥32
Ampicillin/Sulbactam	≤8/4	16/8	≥32/16
Aztreonam	≤4	8	≥16
Cefazolin	≤2	4	≥8
Cefepime*	≤2	4-8	≥16
Cefoxitin	≤8	16	≥32
Ceftazidime	≤4	8	≥16
Ceftriaxone	≤1	2	4
Cefuroxime	≤8	16	≥32
Ciprofloxacin	≤1	2	≥4
Colistin[†]	≤2	4	≥8
Ertapenem	≤0.5	1	≥2
Fosfomycin	≤64	128	≥256
Gentamicin	≤4	8	≥16
Levofloxacin	≤2	4	≥8
Meropenem	≤1	2	≥4
Minocycline	≤4	8	≥16
Polymyxin B[†]	≤2	4	≥8
Nitrofurantoin	≤32	64	≥128
Piperacillin / Tazobactam	≤16/4	32/4 - 64/4	≥128/4
Tetracycline	≤4	8	≥16
Tigecycline[§]	≤2	4	≥8
Tobramycin	≤4	8	≥16
Sulfamethoxazole / Trimethoprim	≤2/38	-	≥4/76

*Cefepime does not have an intermediate susceptibility but instead has a susceptible dose-dependent designation

[†]Polymyxin B and colistin do not have CLSI breakpoints for Enterobacteriaceae. The breakpoints for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were used instead[237]

[§]Tigecycline does not have CLSI breakpoints for gram-negative organisms. An FDA-approved breakpoint of ≤ 2 μg/mL was considered susceptible[167]

Time-Kill Studies

Plots of colony count ($\log_{10}$ CFU/mL) versus time were constructed for each isolate and antimicrobial(s) studied. Furthermore, combined plots across multiple experiments were generated using geometric means of the colony counts and standard deviations for each time point. Additionally, plots of 24-hour change in $\log_{10}$ CFU/mL were constructed using the logarithm of the geometric mean of the initial (time = 0) colony count subtracted from the logarithm of the geometric mean of the 24-hour colony count with un-pooled standard deviations. Activity was evaluated as bactericidal, bacteriostatic, or growth where bactericidality was defined as a $\geq 10^3$ decrease in colony count at 24 hours, bacteriostatic was defined as a $< 10^3$ decrease in colony count at 24 hours, and growth was any positive change at 24 hours. Synergy was also evaluated for combinations, being defined as a $\geq 10^2$ CFU/mL lower colony count at 24 hours when compared to the most active agent used alone.

Subpopulation Analysis

A table of colony count ($\log_{10}$ CFU/mL) at time 0 was constructed which included each isolate studied in time-kill assays. Measurements from microfiltration were preferentially used when either microfiltration data were below the lower limit of quantification (10^2 CFU/mL) for the laser colony counter or when the laser colony counter data were below the lower limit of quantification. When the laser colony counter and microfiltration data were above 10^2 CFU/mL or if the microfiltration method produced too many colonies to count, the laser colony counter value was used.

Additionally, reported values were not rounded to the lower limit of quantification because higher error was accepted as a limitation for comparing colony counts that were expected to be so low.

Statistical Analyses

Chi-squared analyses with a Holm-Bonferroni correction were used to compare antimicrobial susceptibility data such as broth microdilution, BD Phoenix™, and nationally reported data.[245,246] However, statistical inferences are limited in that high sample sizes may confer statistical significance with a lack of clinical significance. For example, a difference in susceptibility of 62% compared to 60% may be statistically significant depending on the sample size, but a difference in susceptibility of 100% to 98% indicating first appearance of resistance may be more clinically significant, regardless of statistical significance.

Geometric means and standard deviations are most meaningful regarding time-kill and log-change studies due to the very high inter-experiment variability (heterogeneity) observed across studies. Therefore, statistical parameters describing intra-experiment variability (e.g. standard deviation and coefficients of variance) are a better indicator of valid results in the face of dynamically growing organisms where external factors are difficult to control without many samples. This has been a limitation and described by numerous meta-analyses and review articles[14,200,203,210] leading to standardized definitions for describing and comparing data (e.g. bactericidality and synergy), primarily by CLSI.[235,237]

Chapter Four:

Results

Bacterial Isolates

Clinical isolates of 229 non-duplicate, MDR,[165] gram-negative organisms were collected between November 9, 2008 and December 31, 2015 from the Clinical Microbiology Laboratory at the University of Kentucky Chandler Medical Center in Lexington, Kentucky. The most common MDR species in descending order were *Klebsiella pneumoniae* (28%), *Escherichia coli* (20%), *Enterobacter cloacae* (16%), and *Pseudomonas aeruginosa* (15%). CRE make up 48% of the MDR isolates obtained, and *Klebsiella pneumoniae* (40%) and *Enterobacter cloacae* (32%) make up the majority of this group. These isolates are described in Tables 4.1-2.

Table 4.1: All MDR Clinical Isolates

Organism	Number of Isolates	Percentage of Isolates
Acinetobacter baumannii	10	4.4%
Burkholderia cepacia	1	0.4%
Citrobacter amalonaticus	2	0.9%
Citrobacter freundii	10	4.4%
Citrobacter youngae	1	0.4%
Enterobacter aerogenes	4	1.7%
Enterobacter cloacae	37	16.2%
Enterobacter gergoviae	2	0.9%
Enterobacter hormaechei	1	0.4%
Enterobacter spp.	2	0.9%
Escherichia coli	46	20.1%
Escherichia vulneris	1	0.4%
Klebsiella oxytoca	5	2.2%
Klebsiella pneumoniae	64	27.9%
Klebsiella ozaenae	1	0.4%
Pantoea agglomerans	3	1.3%
Proteus mirabilis	2	0.9%
Pseudomonas aeruginosa	34	14.8%
Pseudomonas putida	2	0.9%
Sphingomonas paucimobilis	1	0.4%
TOTAL	229	

Table 4.2: Carbapenem-Resistant Enterobacteriaceae (CRE) Clinical Isolates

Organism	Number of Isolates	Percentage CRE Isolates
Citrobacter amalonaticus	2	1.8%
Citrobacter freundii	9	8.3%
Citrobacter youngae	1	0.9%
Enterobacter aerogenes	3	2.8%
Enterobacter cloacae	35	32%
Enterobacter gergoviae	2	1.8%
Enterobacter hormaechei	1	0.9%
Enterobacter spp.	2	1.8%
Escherichia coli	5	4.6%
Klebsiella oxytoca	4	3.7%
Klebsiella pneumoniae	44	40%
Klebsiella ozaenae	1	0.9%
TOTAL	109	

IN VITRO SUSCEPTBILITY TESTING

Minimum Inhibitory Concentrations (MICs)

All isolates underwent identification and antimicrobial susceptibility testing for clinical purposes through the University of Kentucky Clinical Microbiology Laboratory using BD Phoenix™ prior to collection by our lab. This data (Appendix A, Tables A.1-A.2) was provided to and verified by our lab using antimicrobial susceptibility testing by broth microdilution methodology, the gold standard for determination of MICs.[235] The primary objective of antimicrobial susceptibility testing through broth microdilution was to identify isolates that represented the majority of CRE observed in the U.S., namely carbapenem-resistant *Klebsiella pneumoniae* (CR-KP).[11] Resistance was described by the measured MIC value,[237] and these isolates exhibited low to high levels of resistance to meropenem – the most commonly used carbapenem antimicrobial at the University of Kentucky Chandler Medical Center. Furthermore, these experiments identified CRE susceptible to polymyxin B since polymyxin susceptibility is more frequently observed in CRE.[14]

The MICs for all 229 isolates are shown for the 20 antimicrobials tested by BD Phoenix™ in Appendix A, Tables A.1-A.2. The MIC_{50}, MIC_{90}, and percentage susceptible across MDR isolates numbering at least 30 according to CLSI guidelines for cumulative susceptibility reporting[245] are shown in Appendix B, Tables B.1-B.4. Since clinical isolates were continually being sent throughout the study from the University of Kentucky Clinical Microbiology Laboratory, only the first 24 received of ultimately 44 CR-KP organisms underwent antimicrobial susceptibility testing by broth microdilution

and MICs to the antimicrobials evaluated are shown in Appendix C, Table C.1. The MIC_{50}, MIC_{90}, and percentage susceptible for the first 24 (12 where noted) CR-KP organisms tested by broth microdilution and E-test are shown in Table 4.3.

Table 4.3: *In Vitro* Susceptibility Results for CRE *K. pneumoniae*

	MICs (µg/mL)			
Antimicrobial	**Range**	**MIC_{50}**	**MIC_{90}**	**% Susceptible**
Amikacin	0.5 – 128	4	128	67%
Cefepime	≤0.125 – >256	32	>256	17%
Colistin*	≤ 0.03 – 8	0.06	0.5	92%†
Levofloxacin	≤0.03 – 64	8	>32	29%
Meropenem	≤0.015 – 128	16	128	25%
Minocycline	1 – >64	8	32	42%
Piperacillin/ Tazobactam*	≤0.25/4 – >512/4	0.25/4	>512	50%
Polymyxin B	0.06 – >256	0.125	8	83%†
Rifampin*	16 – >32	32	>32	N/A
Tigecycline	0.125 – 4	1	4	88%†
Fosfomycin§	12 – >1024	32	>1024	75%

* Evaluated for the first 12 *K. pneumoniae* isolates only
† No CLSI breakpoint for Enterobacteriaceae. Therefore, FDA breakpoint of ≤ 2 µg/mL was used for tigecycline[167] and CLSI breakpoint for non-Enterobacteriaceae of ≤ 2 µg/mL was used for polymyxin B and colistin[237]
§ Evaluated for the first 12 *K. pneumoniae* isolates and by E-test only

Among the first 24 CR-KP clinical isolates, meropenem resistance (MIC ≥4 µg/mL)[237] ranged from 4 - 128 µg/mL. A majority of the CR-KP isolates were polymyxin B susceptible (MIC ≤2 µg/mL)[237] and had MIC values of 0.06 - 0.125 µg/mL (Appendix C, Table C.1). Other observations, although not the focus of the antimicrobial susceptibility testing, included higher susceptibility (≥75%) among colistin, polymyxin B, tigecycline, and fosfomycin; modest susceptibility to amikacin (67%); and relatively poor (≤50%) susceptibility to other antimicrobial agents evaluated.

Selection of Bacterial Isolates for Time-kill Studies

Four CR-KP isolates from lowest to greatest meropenem resistance (KP 34, KP 22, KP 24, and KP 44) were selected to represent the range of meropenem resistance described previously (4 - 128 µg/mL) while maintaining polymyxin B susceptibility. These isolates were later evaluated in time-kill studies with meropenem (MEM) and polymyxin B (PMB) alone and in combination. The isolates and their respective MIC values are in Table 4.4 (letter abbreviations indicate susceptible or resistant).

Table 4.4: Microdilution MICs for *K. pneumoniae* Isolates Selected for Time-Kill

Antimicrobial	KP 34 MIC(µg/mL)	KP 22 MIC(µg/mL)	KP 24 MIC(µg/mL)	KP 44 MIC(µg/mL)
Amikacin	1 **(S)**	64 **(R)**	4 **(S)**	4 **(S)**
Cefepime	2 **(S)**	32 **(R)**	64 **(R)**	256 **(R)**
Colistin*	0.125 **(S)**	0.06**(S)**	0.125 **(S)**	0.06 **(S)**
Levofloxacin	0.06 **(S)**	64 **(R)**	64 **(R)**	64 **(R)**
Meropenem	4 **(R)**	16 **(R)**	32 **(R)**	128 **(R)**
Piperacillin/Tazobactam	≤0.25 **(S)**	512 **(R)**	>512 **(R)**	>512 **(R)**
Polymyxin B*	0.125 **(S)**	0.06 **(S)**	0.125 **(S)**	0.06 **(S)**
Rifampin[+]	32	>32	32	>32
Tigecycline[§]	0.25 **(S)**	2 **(S)**	0.25 **(S)**	4 **(R)**
Fosfomycin[†]	12 **(S)**	16 **(S)**	24 **(S)**	48 **(S)**

*Breakpoint of *P. aeruginosa* and *A. baumannii* of ≤ 2µg/mL used[237]
[+]No CLSI breakpoints for gram-negative organisms[237]
[§]FDA breakpoint of ≤ 2µg/mL used[167]
[†]Evaluated by Etest®

TIME-KILL STUDIES

All time-kill curves (both individual and average), separated by organism and antimicrobial, are located in Appendix D, Figures D.1-D.24. However, in order to best compare antimicrobial agents within the same organism, the average time-kill results are displayed for each isolate at all concentrations tested in combination as well as the corresponding concentrations of agents used alone. Using these graphs, the killing activity of each antimicrobial alone and their combination can be described as growth – any increase in colony count (CFU/mL) from the previous time point, bacteriostatic – any decrease in colony count (CFU/mL) from starting inoculum that is $<10^3$ CFU/mL, and bactericidal – any decrease in colony count (CFU/mL) from starting inoculum that is $\geq 10^3$ CFU/mL. It is important to distinguish between describing antimicrobial activity as bactericidal (or bacteriostatic) overall (which implies $\geq 10^3$ CFU/mL killing compared to starting inoculum that persisted up to 24 hours) and describing an antimicrobial as exhibiting bactericidal activity for a small window of time, which is a more detailed description of killing activity over time.

Additionally, the interaction of the two antimicrobial agents can be described as synergistic – a $\geq 10^2$ CFU/mL lower colony count of the combination at 24 hours compared to the more active agent (the agent with a lower colony count) alone, additive/indifferent – an absolute difference in colony count of $< 10^2$ CFU/mL between the combination and the more active agent alone, or antagonistic – a $\geq 10^2$ CFU/mL higher colony count of the combination at 24 hours compared to the more active agent alone. In some cases, the interaction may not be determinable if one of the antimicrobial agents alone exhibits enough killing to be $<10^4$ CFU/mL at 24 hours because this is

within 10^2 CFU/mL (unable to determine synergy) of the lower limit of quantification (10^2 CFU/mL). A plot of log change in colony count from 0 to 24 hours facilitates evaluation of the interaction of meropenem and polymyxin B, which is described later.

Antimicrobial Activity in KP 34

Figure 4.1 describes KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL) and the activity of polymyxin B (0.25 and 1 µg/mL), meropenem (4 and 16 µg/mL), and their combination at clinically relevant concentrations. Specifically, both concentrations of polymyxin B exhibited bactericidal activity within 1 hour, but growth was observed by 4 hours.

Meropenem 4 µg/mL (1 x MIC) displayed bacteriostatic activity with growth observed by 8 hours. Meropenem 16 µg/mL (4 x MIC) displayed bactericidal activity within 2 hours, but growth was observed by 24 hours. Meropenem 64 µg/mL (16 x MIC) displayed bactericidal activity by 2 hours and maintained this activity throughout the 48 hour time period of testing (Appendix D, D.1).

All combinations tested were bactericidal by 2 hours and maintained this activity throughout the 48 hour time period of testing.

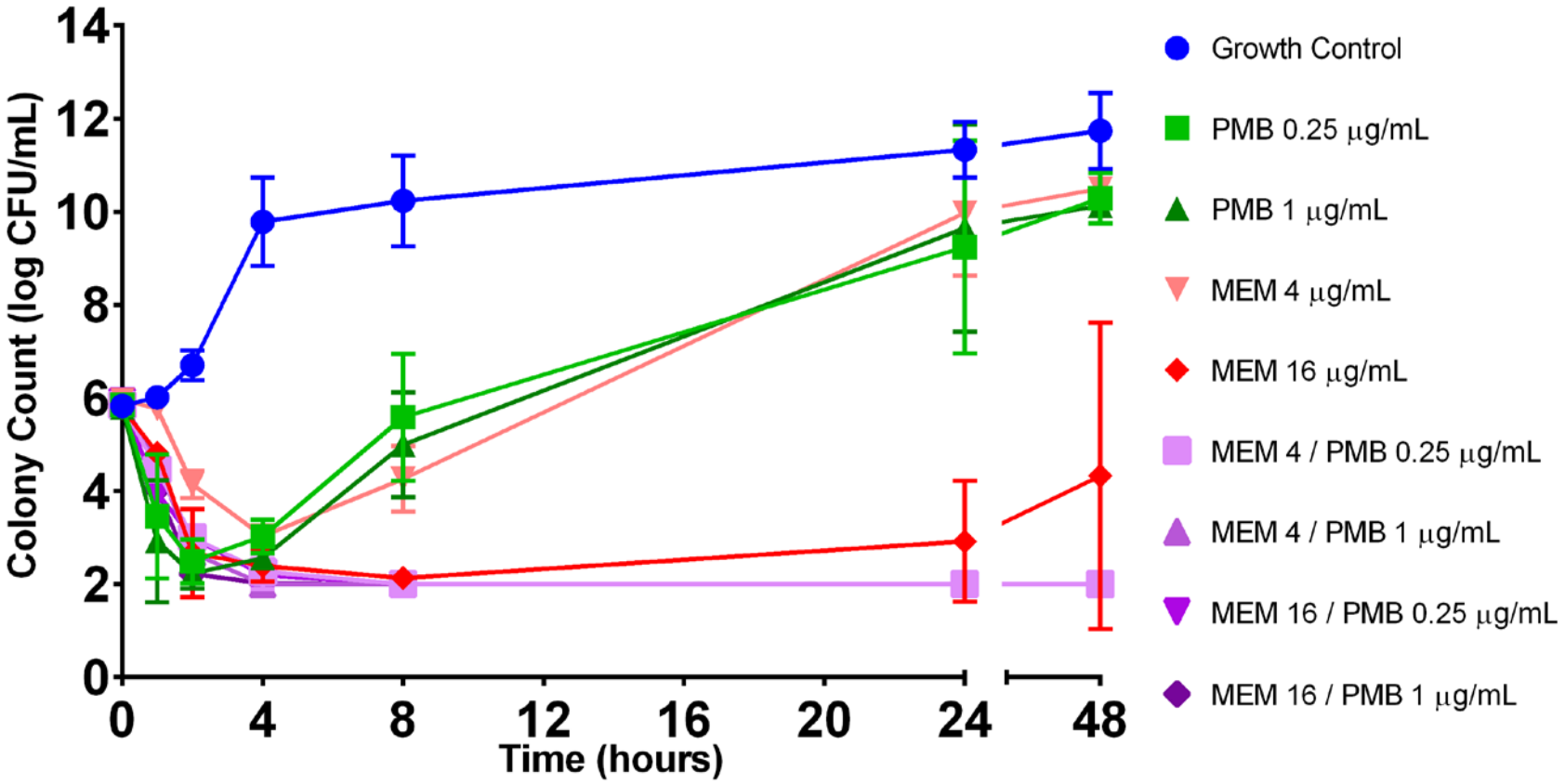


Figure 4.1: Time-kill curve against KP 34
Time-kill curve of meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). Data points are geometric means with error bars being one standard deviation of replicate experiments (n = 2). The lower limit of quantification was 10^2 CFU/mL.

Antimicrobial Activity in KP 22

Figure 4.2 describes KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL) and the activity of polymyxin B (0.25 and 1 µg/mL), meropenem (4, 16, and 64 µg/mL), and their combination at clinically relevant concentrations. Specifically, both concentrations of polymyxin B exhibited bactericidal activity within 1 hour, but growth was observed by 8 hours instead of 4 hours as seen in KP 34.

Meropenem 4 µg/mL (1/4 x MIC) displayed bacteriostatic activity with growth observed by 8 hours. Meropenem 16 µg/mL (1 x MIC) displayed bactericidal activity within 4 hours, but growth was observed by 8 hours. Meropenem 64 µg/mL (4 x MIC) displayed bactericidal activity by 2 hours and maintained this activity throughout the 48 hour time period of testing.

All combinations tested were bactericidal by 1 hour (compared to 2 hours observed in KP 34) and maintained this activity throughout the 48 hour time period of testing.

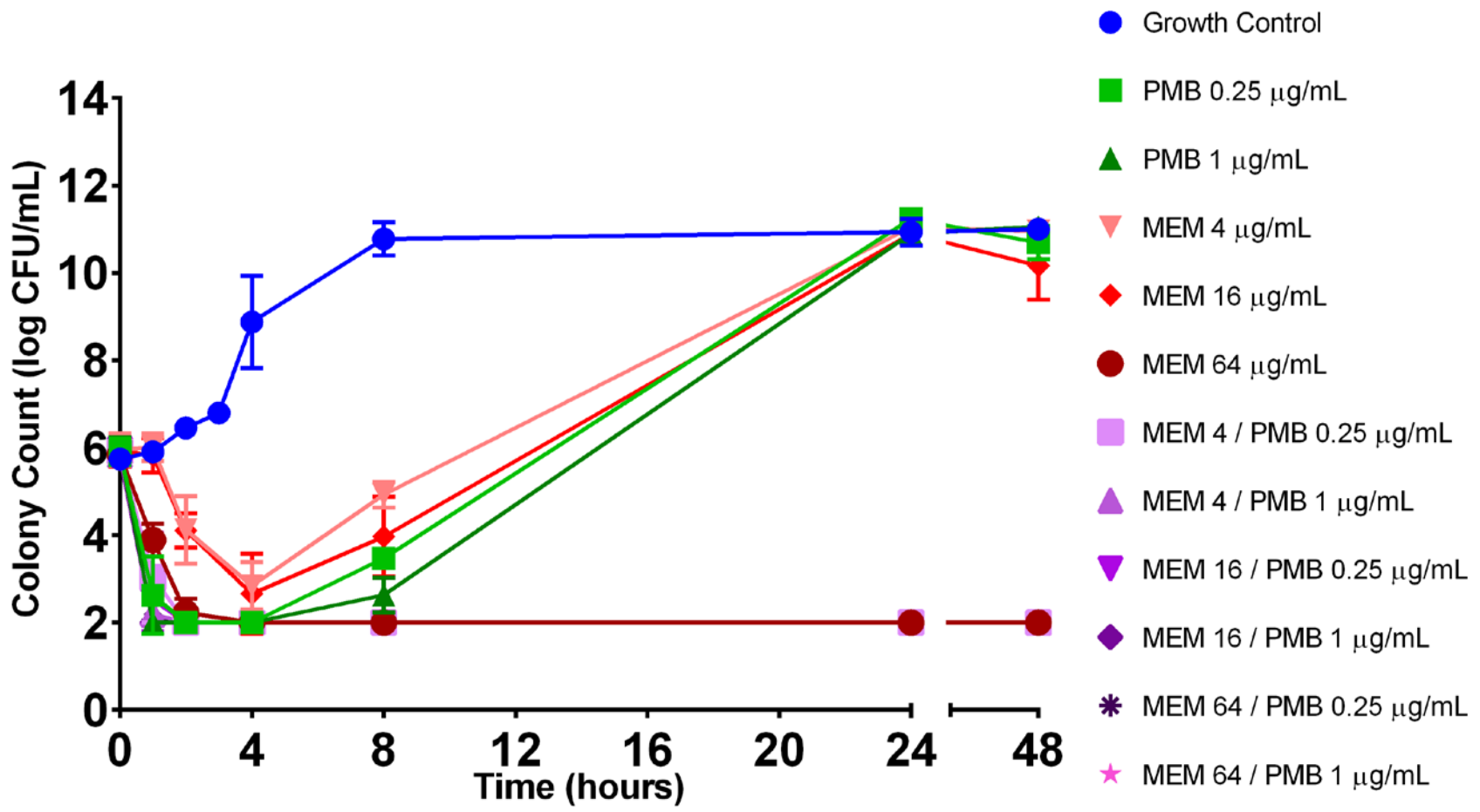


Figure 4.2: Time-kill curve against KP 22
Time-kill curve of meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 22 (MICs: MEM 16 μg/mL, PMB 0.06 μg/mL). Data points are geometric means with error bars being one standard deviation of replicate experiments (n = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

Antimicrobial Activity in KP 24

Figure 4.3 describes KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL) and the activity of polymyxin B (0.25 and 1 µg/mL), meropenem (4, 16, and 64 µg/mL), and their combination at clinically relevant concentrations. Specifically, polymyxin B 0.25 µg/mL (2 x MIC) exhibited bactericidal activity within 2 hours whereas polymyxin B 1 µg/mL (8 x MIC) exhibited bactericidal activity within 1 hour, but growth was observed for both concentrations by 8 hours, more similar to KP 22 than KP 34.

Meropenem 4 µg/mL (1/8 x MIC), 16 µg/mL (1/2 x MIC), and 64 µg/mL (2 x MIC) displayed bacteriostatic activity with growth observed by 8 hours.

All combinations with meropenem concentrations ≥16 µg/mL (≥1/2 x MIC) were bactericidal by 1 hour and maintained this activity throughout the 48 hour time period of testing. However, both combinations with meropenem concentrations 4 µg/mL (1/8 x MIC) were bactericidal by 1 hour with growth observed by 8 hours.

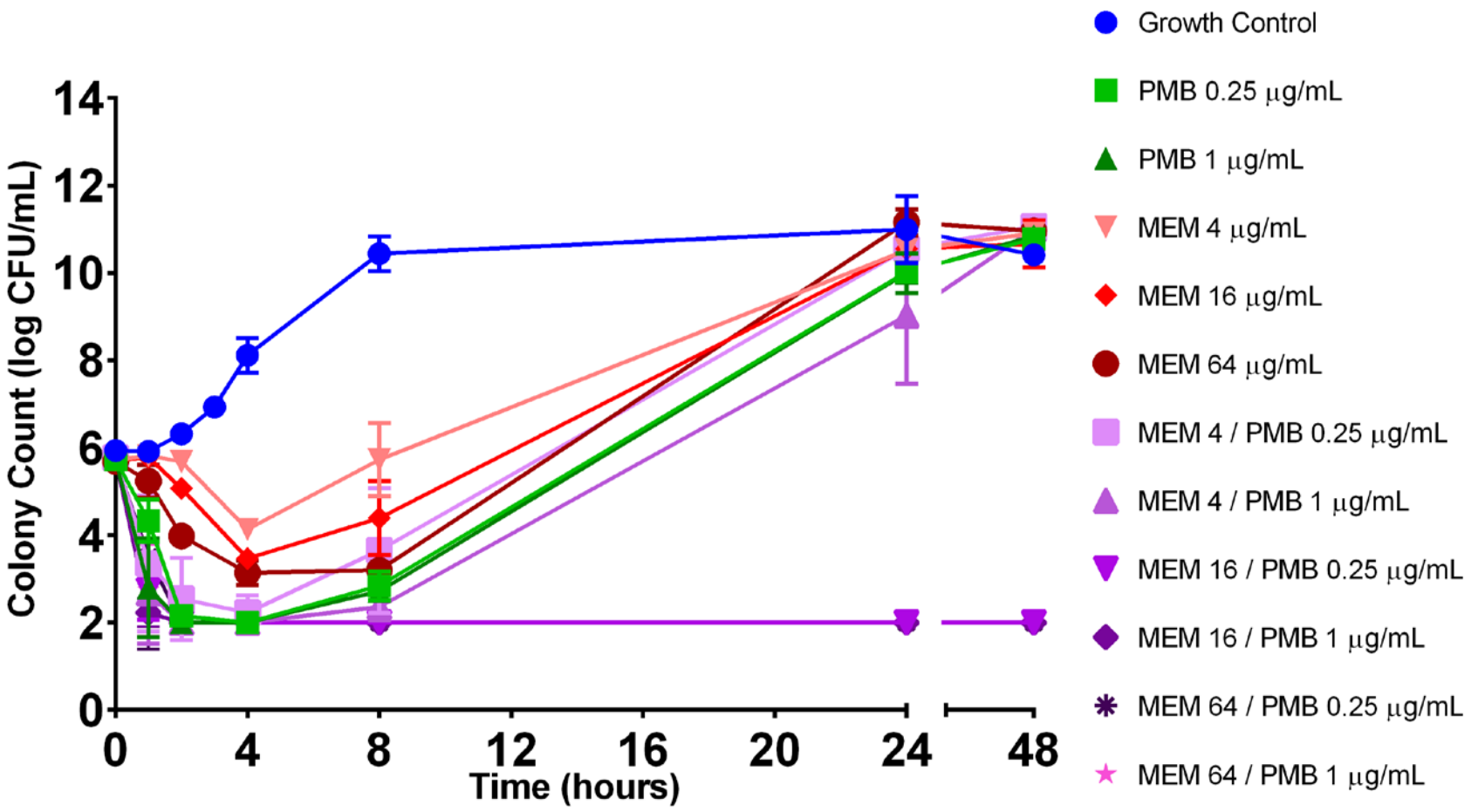


Figure 4.3: Time-kill curve against KP24
Time-kill curve of meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 24 (MICs: MEM 32 μg/mL, PMB 0.125 μg/mL). Data points are geometric means with error bars being one standard deviation of replicate experiments (n = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

Antimicrobial Activity in KP 44

Figure 4.4 describes KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL) and the activity of polymyxin B (0.25, 1, and 4 µg/mL), meropenem (4, 16, and 64 µg/mL), and their combination at clinically relevant concentrations. Specifically, all concentrations of polymyxin B exhibited bactericidal activity within 1 hour, but growth was observed by 8 hours for polymyxin B at 0.25 and 1 µg/mL (4 x MIC and 16 x MIC, respectively) and 24 hours for polymyxin B 4 µg/mL (64 x MIC), more similar to KP 22 and KP 24 than KP 34.

Meropenem 4 µg/mL (1/32 x MIC) displayed no activity, with growth observed by 1 hour. Meropenem 16 µg/mL (1/8 x MIC) exhibited bacteriostatic activity with growth observed by 8 hours. Meropenem 64 µg/mL (1/2 x MIC) displayed bacteriostatic activity with growth observed by 24 hours.

Combinations with polymyxin B concentrations ≥1 µg/mL (≥16 x MIC) were bactericidal by 1 hour whereas combinations with polymyxin B concentrations of 0.25 µg/mL (4 x MIC) were bactericidal by 2 hours. Growth was observed by 8 hours for combinations with meropenem 4 µg/mL (1/32 x MIC) whereas growth was observed by 24 hours for combinations with meropenem ≥16 µg/mL (≥1/8 x MIC). The only combination that maintained bactericidal activity throughout the 48 hour time period of testing was the combination with the highest concentrations of both antimicrobial agents – meropenem 64 µg/mL (1/2 x MIC) in combination with polymyxin B 4 µg/mL (64 x MIC).

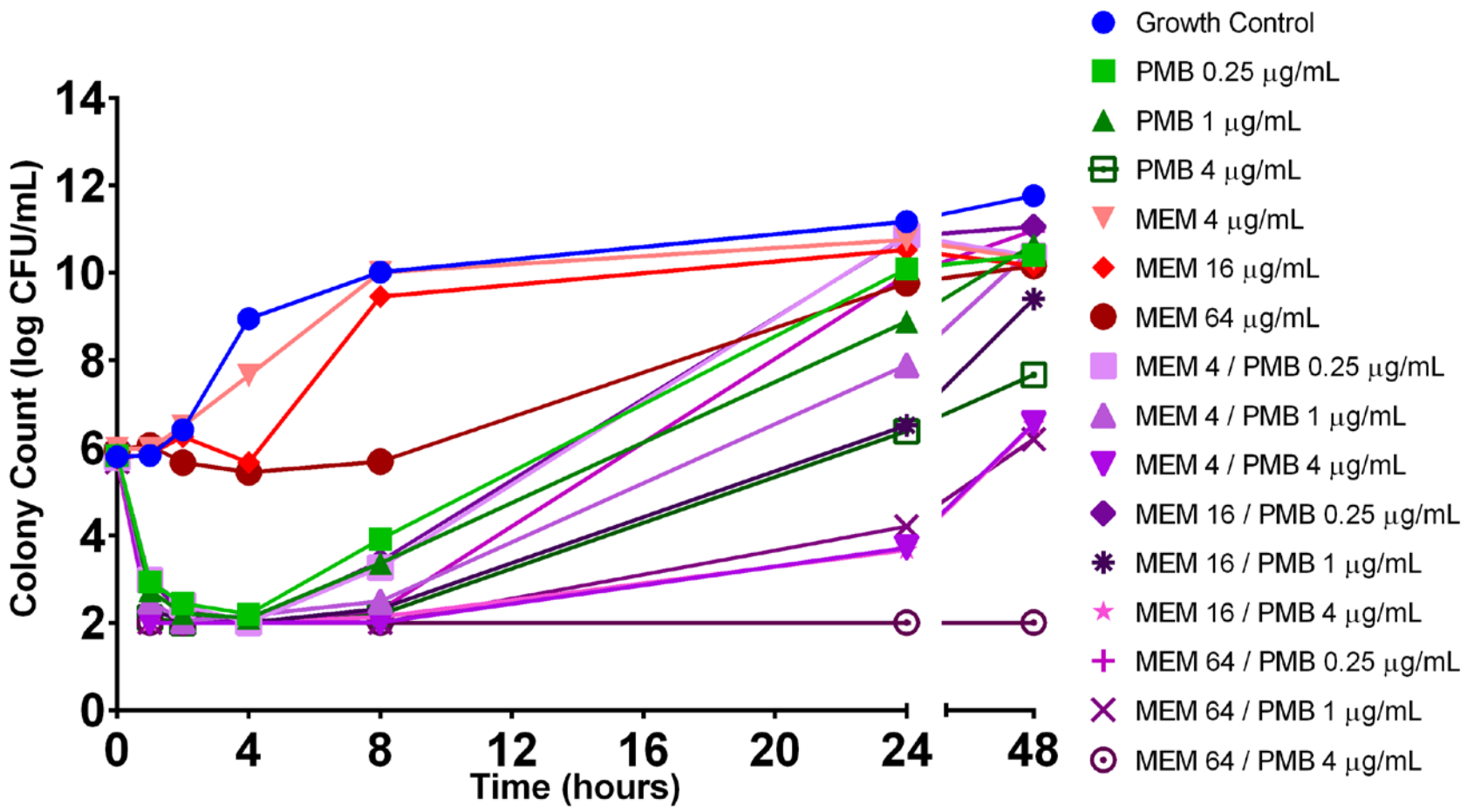


Figure 4.4: Time-kill curve against KP 44

Time-kill curve of meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 44 (MICs: MEM 128 μg/mL, PMB 0.06 μg/mL). Data points are geometric means of replicate experiments (n = 2 to 4). The lower limit of quantification was 10^2 CFU/mL.

Meropenem and Polymyxin B Interaction in KP 34

In addition to synergy, additivity/indifference, and antagonism, the definitions of growth, bacteriostatic, and bactericidal can be applied to further characterize the interaction of polymyxin B and meropenem at 24 hours. The activity of combinations with meropenem 4 µg/mL (1 x MIC) were all bactericidal and synergistic. Combinations with meropenem 16 µg/mL (4 x MIC) were all bactericidal, but the interaction was indeterminate because the activity of meropenem 16 µg/mL alone was too close to the lower limit of quantification to evaluate synergy among the corresponding combinations. Table 4.5 summarizes these results. Figure 4.5 describes the change in colony count from 0 to 24 hours for each antimicrobial tested alone and in combination.

Table 4.5: Meropenem and Polymyxin B Interaction for KP 34

	Meropenem 4 (µg/mL)	**Meropenem 16 (µg/mL)**	**Meropenem 64 (µg/mL)**
Polymyxin B 0.25 (µg/mL)	S / B	I / B	Not Tested
Polymyxin B 1 (µg/mL)	S / B	I / B	Not Tested
Polymyxin B 4 (µg/mL)	Not Tested	Not Tested	Not Tested

B - Bactericidal
I - Indeterminate
S - Synergistic

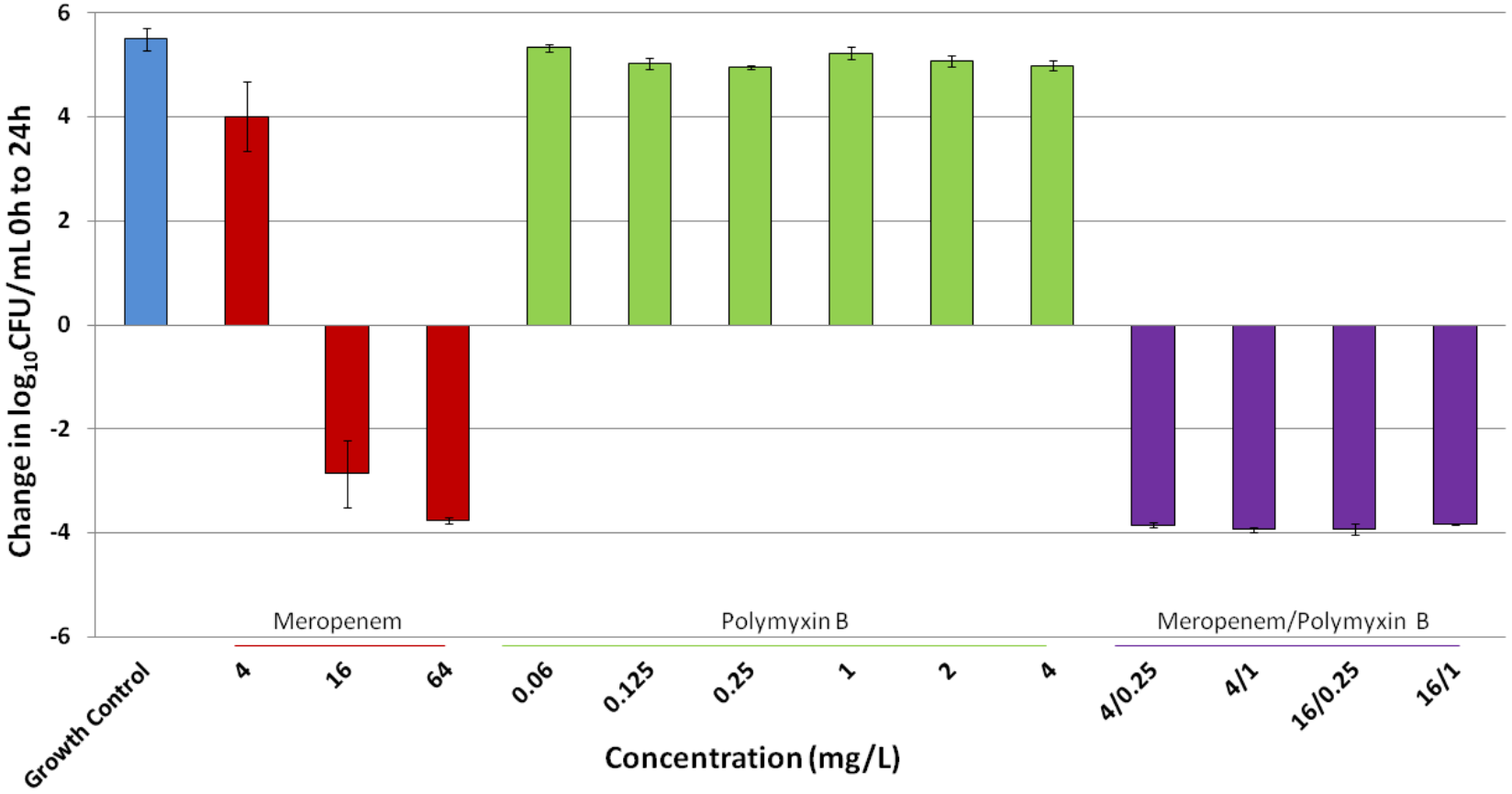


Figure 4.5: 24 hour change in colony count against KP 34
24 hour change in colony count for meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). Data are geometric means with un-pooled standard deviations of replicate experiments (n = 2).

Meropenem and Polymyxin B Interaction in KP 22

The activity of combinations with meropenem 4 µg/mL and 16 µg/mL (1/4 x MIC and 1 x MIC, respectively) were all bactericidal and synergistic. Combinations with meropenem 64 µg/mL (4 x MIC) were all bactericidal, but the interaction was indeterminate because the activity of meropenem 64 µg/mL alone was too close to the lower limit of quantification to evaluate synergy among the corresponding combinations. Table 4.6 summarizes these results. Figure 4.6 describes the change in colony count from 0 to 24 hours for each antimicrobial tested alone and in combination.

Table 4.6: Meropenem and Polymyxin B Interaction for KP 22

	Meropenem 4 (µg/mL)	**Meropenem 16 (µg/mL)**	**Meropenem 64 (µg/mL)**
Polymyxin B 0.25 (µg/mL)	S / B	S / B	I / B
Polymyxin B 1 (µg/mL)	S / B	S / B	I / B
Polymyxin B 4 (µg/mL)	Not Tested	Not Tested	Not Tested

B - Bactericidal
I - Indeterminate
S - Synergistic

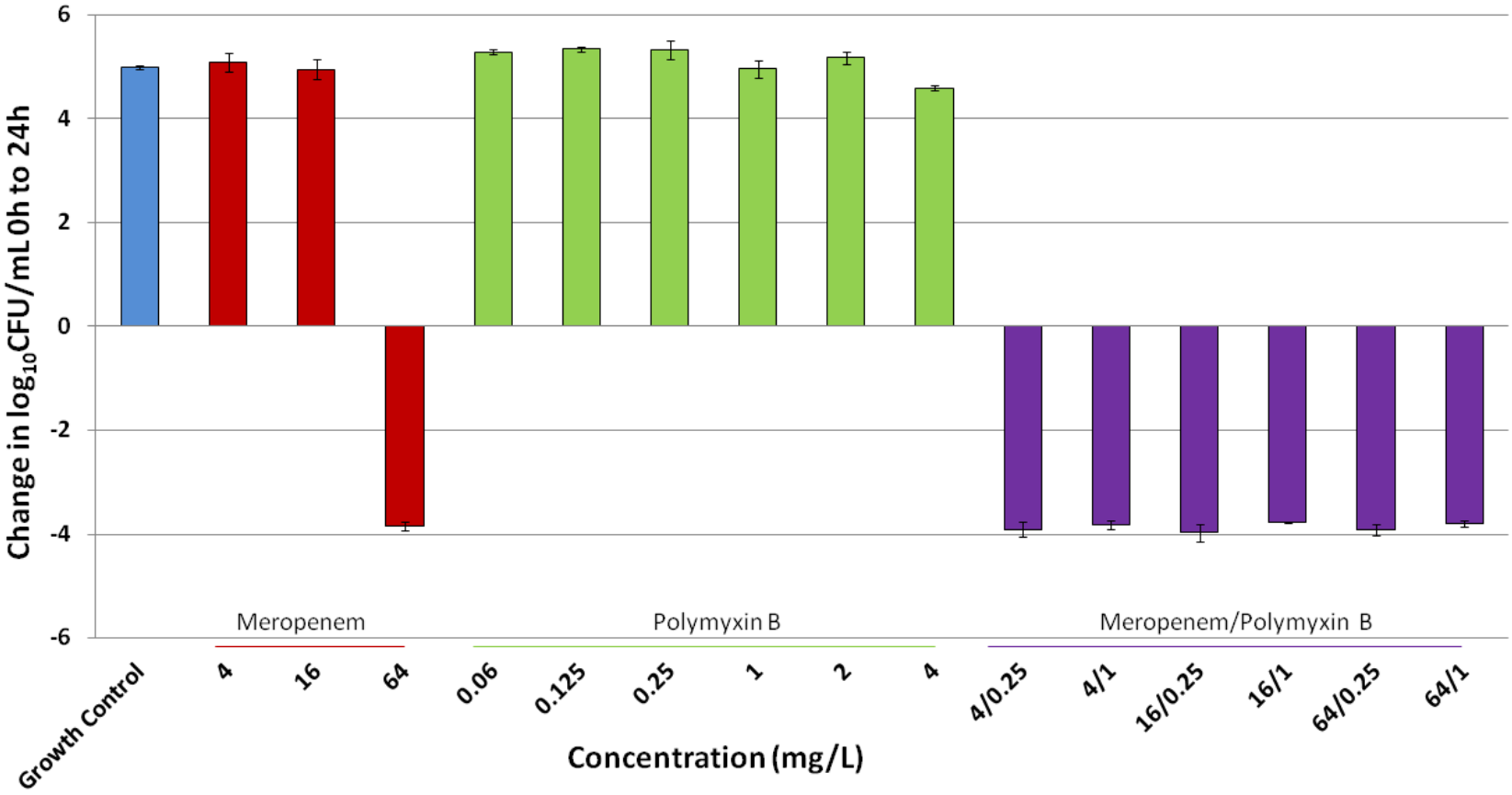


Figure 4.6: 24 hour change in colony count against KP 22
24 hour change in colony count for meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL). Data are geometric means with un-pooled standard deviations of replicate experiments (n = 2 to 3).

Meropenem and Polymyxin B Interaction in KP 24

The activity of combinations with meropenem 4 µg/mL (1/8 x MIC) were all additive/indifferent and growth was observed at 24 hours. In contrast, combinations with meropenem at 16 and 64 µg/mL (1/2 x MIC and 2 x MIC, respectively) were all bactericidal and synergistic. Table 4.7 summarizes these results. Figure 4.7 describes the change in colony count from 0 to 24 hours for each antimicrobial tested alone and in combination.

Table 4.7: Meropenem and Polymyxin B Interaction for KP 24

	Meropenem 4 (µg/mL)	**Meropenem 16 (µg/mL)**	**Meropenem 64 (µg/mL)**
Polymyxin B 0.25 (µg/mL)	A / G	S / B	S / B
Polymyxin B 1 (µg/mL)	A / G	S / B	S / B
Polymyxin B 4 (µg/mL)	Not Tested	Not Tested	Not Tested

A - Additive/Indifferent
B - Bactericidal
G - Growth
S - Synergistic

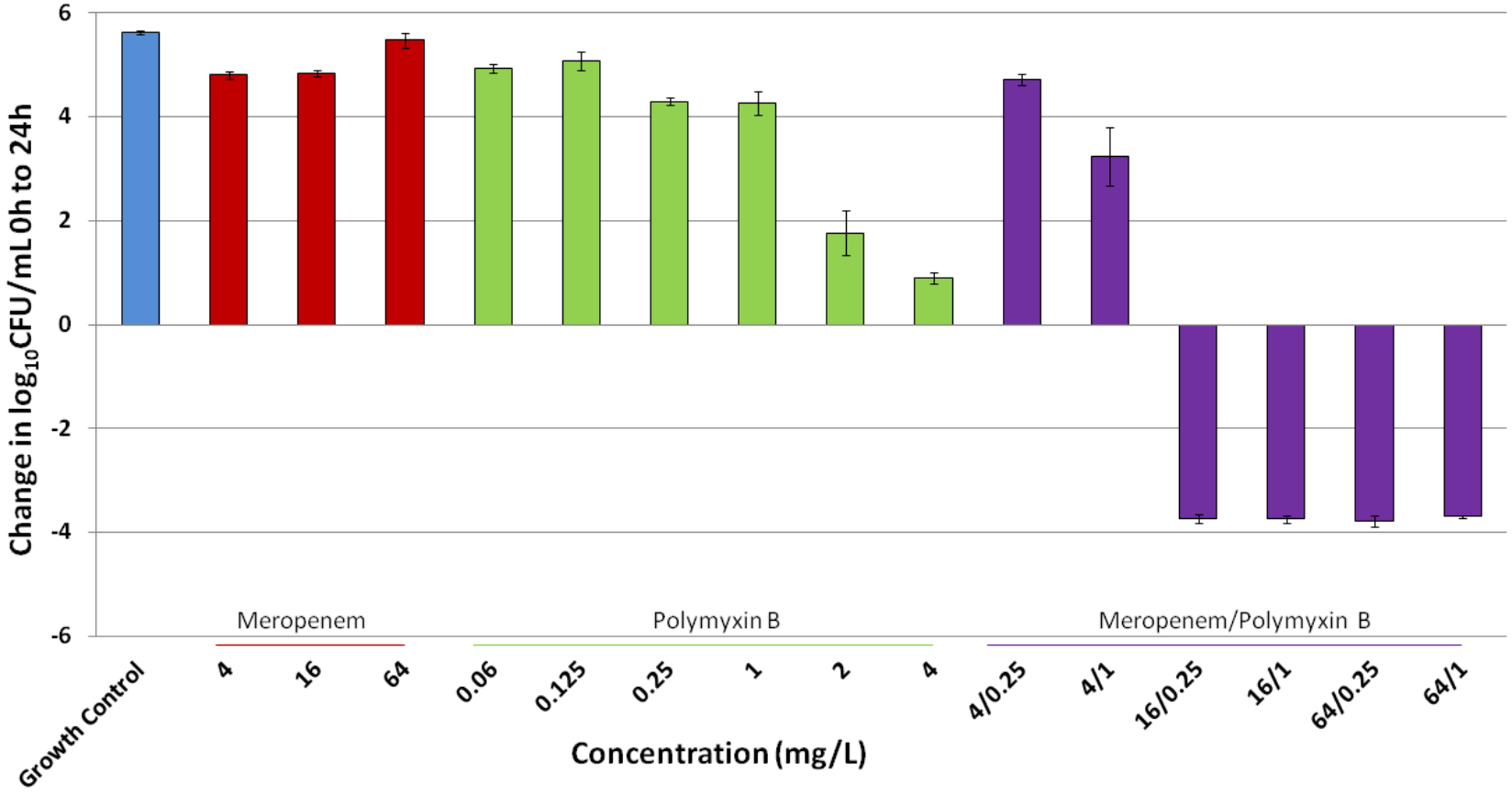


Figure 4.7: 24 hour change in colony count against KP 24

24 hour change in colony count for meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL). Data are geometric means with un-pooled standard deviations of replicate experiments (n = 2 to 3).

Meropenem and Polymyxin B Interaction in KP 44

The activity of meropenem 4 µg/mL (1/32 x MIC) in combination with polymyxin B at 0.25 or 1 µg/mL (2 x MIC and 8 x MIC, respectively) was additive/indifferent and growth was observed at 24 hours. However, meropenem 4 µg/mL in combination with polymyxin B 4 µg/mL (32 x MIC) was synergistic with bacteriostatic activity observed at 24 hours.

The activity of meropenem 16 µg/mL (1/8 x MIC) in combination with polymyxin B 0.25 µg/mL was additive/indifferent whereas in combination with polymyxin B at 1 or 4 µg/mL was synergistic. However, at 24 hours, growth was observed for the lower two polymyxin B combinations and bacteriostatic activity was observed for the combination with polymyxin 4 µg/mL.

The activity of meropenem 64 µg/mL (1/2 x MIC) in combination with polymyxin B 0.25 was additive/indifferent with growth observed. The combination with polymyxin B 1 µg/mL was synergistic with bacteriostatic activity. The only combination to produce synergistic, bactericidal activity was meropenem 64 µg/mL in combination with polymyxin B 4 µg/mL.

Table 4.8 summarizes these results. Figure 4.8 describes the change in colony count from 0 to 24 hours for each antimicrobial tested alone and in combination.

Table 4.8: Meropenem and Polymyxin B Interaction for KP 44

	Meropenem 4 (µg/mL)	**Meropenem 16 (µg/mL)**	**Meropenem 64 (µg/mL)**
Polymyxin B 0.25 (µg/mL)	A / G	A / G	A / G
Polymyxin B 1 (µg/mL)	A / G	S / G	S / BS
Polymyxin B 4 (µg/mL)	S / BS	S / BS	S / BC

A - Additive/Indifferent
BC - Bactericidal
BS - Bacteriostatic
G - Growth
S - Synergistic

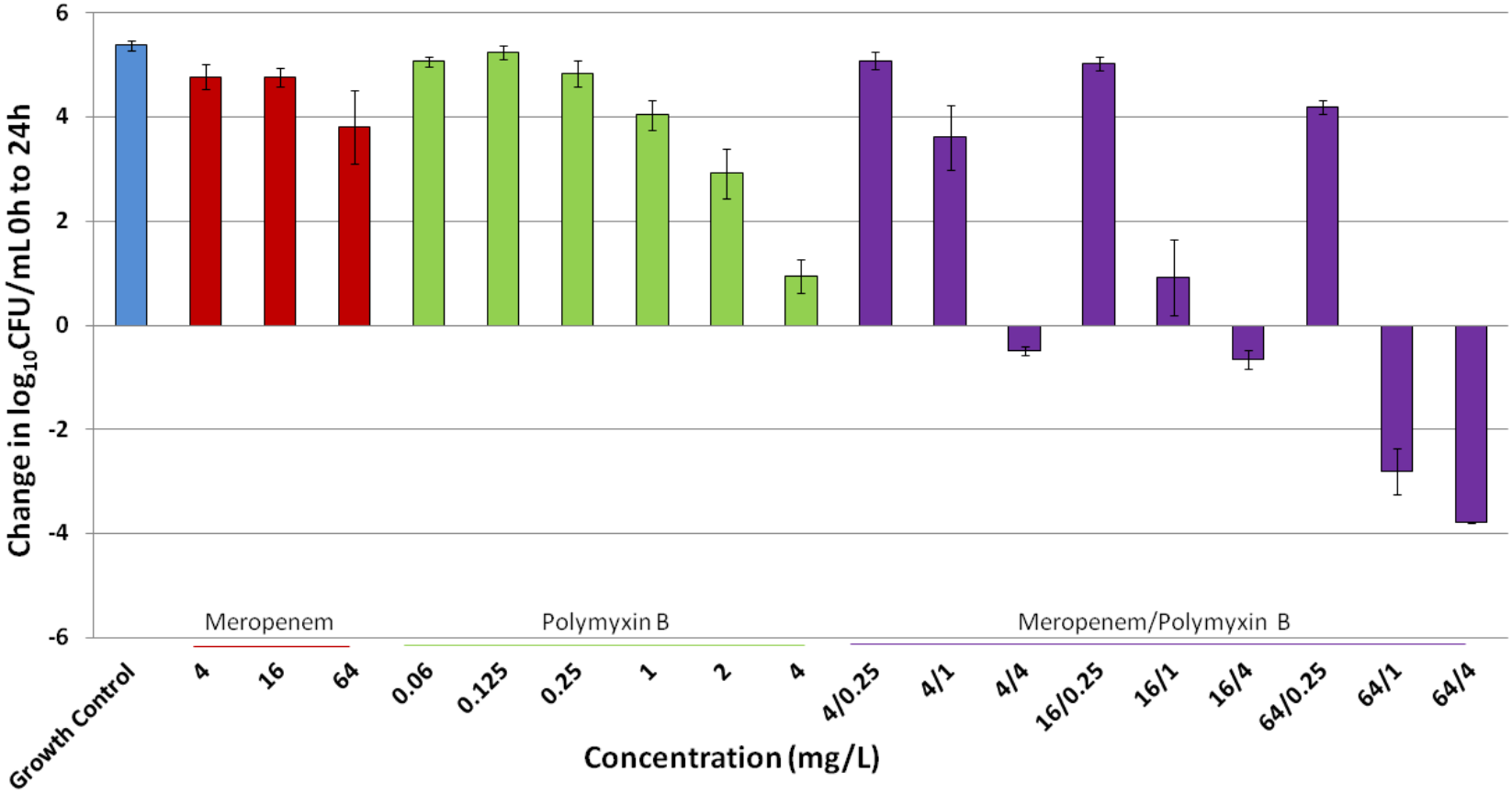


Figure 4.8: 24 hour change in colony count against KP 44
24 hour change in colony count for meropenem (MEM) and polymyxin B (PMB) alone and in combination against KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL). Data are geometric means with un-pooled standard deviations of replicate experiments (n = 2 to 4).

Meropenem and Polymyxin B Alone Summary

Meropenem alone, at all concentrations tested, achieved bactericidal activity ($\geq 10^3$ decrease in CFU/mL) within four hours for KP 34 (MEM MIC 4 µg/mL) and KP 22 (MEM MIC 16 µg/mL). Regrowth in these strains was observed for the two lowest (4 and 16 µg/mL) but not the highest (64 µg/mL) meropenem exposures (Figures 4.1 and 4.2). In contrast, meropenem alone produced only bacteriostatic activity ($< 10^3$ decline in CFU/mL) in KP 24 (MEM MIC 32 µg/mL) and KP 44 (MEM MIC 128 µg/mL; Figures 4.3 and 4.4). Regrowth for these two isolates began by 8 hours.

Polymyxin B alone produced bactericidal activity at all concentrations tested against all strains within 2 hours, but regrowth occurred within 8 hours in all instances (Figures 4.1-4.4).

Meropenem and Polymyxin B in Combination Summary

The interaction of meropenem with polymyxin B in combination was characterized by synergism and 24-hour bactericidality as described in Chapter 3: Methods "Data Analysis". Both combinations of meropenem 4 µg/mL and polymyxin B (0.25 or 1 µg/mL) concentrations achieved synergistic activity ($\geq 10^2$ decrease in CFU/mL at 24 hours compared to the most active agent alone) against KP 34 (MEM MIC 4 µg/mL; Figure 4.5), with no regrowth over 48 hours (Figure 4.1). Higher concentrations of meropenem alone (16 or 64 µg/mL) eradicated KP 34 and so synergism was indeterminate for these combinations.

All combinations of meropenem (4 or 16 µg/mL) and polymyxin B (0.25 or 1 µg/mL) concentrations achieved synergistic activity against KP 22 (MEM MIC 16 µg/mL; Figure 4.6) with no regrowth over 48 hours (Figure 4.2), but higher concentrations of meropenem alone (64 µg/mL) eradicated KP 22 which rendered synergism assessment indeterminate.

Meropenem 4 µg/mL in combination with polymyxin B 0.25 or 1 µg/mL produced additive/indifferent activity ($<10^2$ change in CFU/mL at 24 hours compared to the most active agent alone) against KP 24 (MEM MIC 32 µg/mL; Figure 4.7) with regrowth occurring by 8 hours (Figure 4.3), but all remaining combinations of meropenem 16 or 64 µg/mL with polymyxin B 0.25 or 1 µg/mL achieved synergistic activity with no regrowth over 48 hours (Figures 4.3 and 4.7).

Combinations of meropenem 4, 16, or 64 µg/mL with polymyxin B 0.25 µg/mL displayed additive/indifferent activity against KP 44 (MEM MIC 128 µg/mL; Figure 4.8) with variable regrowth (Figure 4.4). Combinations with polymyxin B at 1 or 4 µg/mL displayed synergy, but only the highest tested concentration of meropenem (64 µg/mL) and polymyxin B (4 µg/mL) also prevented regrowth against KP 44 (Figures 4.4 and 4.8).

Resistance Development to Polymyxin B

Surviving or regrowing bacteria from time-kill studies often exhibit higher MICs to the antimicrobials to which they were exposed. This can be as a result of antimicrobial pressure which selects for resistant subpopulations or reveals adaptable or development of resistance.[142] MICs were reevaluated for polymyxin B in all regrowing colonies for two time-kill studies through broth microdilution susceptibility testing because others have reported rapid resistance development, attributed mostly to the selection of subpopulations.[139,142]

For surviving colonies, the MIC to polymyxin B increased at least 256-fold (from 0.06 - 0.125 µg/mL to 16 - >64 µg/mL) following exposure to polymyxin B alone in concentrations from 0.06 to 4 µg/mL (Table 4.6). This observation will eventually lead to characterization of any resistant subpopulations ("Subpopulation Analysis").

Table 4.9: MIC Testing of Isolates Following Exposure to Polymyxin B Alone

	PMB 0.06 µg/mL	PMB 0.125 µg/mL	PMB 0.25 µg/mL	PMB 1 µg/mL	PMB 2 µg/mL	PMB 4 µg/mL
KP 34	16	64	*	*	>64	>64
KP 22	>64	0.125	64	>64	>64	>64
KP 24	64	64	32	32	>64	>64
KP 44	64	16	32	64	>64	>64

*Concentration not tested in particular time-kill studies, but growing colonies previously observed

Following exposure to meropenem alone, the polymyxin B MIC of surviving colonies remained in essential agreement (within one two-fold dilution) with the originally measured MIC of the corresponding isolate (0.06 µg/mL for KP 22 and KP 44 and 0.125 µg/mL for KP 34 and 24). This was expected given there was no antimicrobial pressure for selection of subpopulations resistant to polymyxin B. However, if there was cross-resistance or some other dependency relationship between meropenem and polymyxin B resistance, we may have seen a change. It appears that cross-resistance may not be significant enough to impact the MIC values of polymyxin B (Table 4.7).

Table 4.10: MIC Testing of Isolates Following Exposure to Meropenem Alone

	MEM 4 µg/mL	MEM 16 µg/mL	MEM 64 µg/mL
KP 34	*	*	§
KP 22	0.06	0.06	§
KP 24	≤0.03	0.125	0.06
KP 44	0.06	0.06	0.06

*Concentration not tested in particular time-kill studies, but growing colonies previously observed
§No growth of colonies during time-kill studies

Surviving colonies of meropenem and polymyxin B in combination were also evaluated for change in polymyxin B MIC. Results were similar to exposure to polymyxin B alone (≥256-fold increase in MIC) suggesting that polymyxin B-resistant subpopulations may be a correlating factor with treatment outcomes (Table 4.8).

Table 4.11: MIC Testing of Isolates Following Exposure to Combination

	MEM/PMB 4/0.25 µg/mL	MEM/PMB 4/1 µg/mL	MEM/PMB 16/0.25 µg/mL	MEM/PMB 16/1 µg/mL	MEM/PMB 64/0.25 µg/mL	MEM/PMB 64/1 µg/mL
KP 34	§	§	§	§	§	§
KP 22	§	§	§	§	§	§
KP 24	32	32	§	§	§	§
KP 44	16	16	16	64	16	16

[§]No growth of colonies during time-kill studies

In summary, the polymyxin B MICs of isolates exposed to polymyxin B alone or in combination with meropenem generally increased from 0.06 or 0.125 µg/mL at baseline to ≥16 µg/mL in all experiments where regrowth occurred. In contrast, the polymyxin B MICs for regrowing bacteria exposed only to meropenem remained at 0.06 or 0.125 µg/mL (Tables 4.6-4.8).

Subpopulation analysis

In order to assess for the presence of subpopulations with increased MIC values relative to the overall population, growing bacteria were cultured onto an agar plate impregnated with antimicrobials which would inhibit the growth of all colonies with MICs less than or equal to the antimicrobial concentration of the agar. Growing colonies were counted and then related to the overall population as described in Chapter 3: Methods "Subpopulation Analysis and Microfiltration". Table 4.9 summarizes the results of the subpopulation time-kill study. The lower limit of quantification for microfiltration was estimated to be 30 CFU/mL when 1000 µL samples were used, but the limitation of larger error was accepted to report measurements below this so as to better compare values between isolates.[243,244]

Table 4.12: Hetero-resistant Subpopulations of *Klebsiella pneumoniae* Isolates

	KP 34	KP 22	KP 24	KP 44
MEM MIC > 16	**7.2 CFU/mL**	**31.6 CFU/mL**	-	-
MEM MIC > 64	-	-	**6.7 CFU/mL**	**5.3 x 10^3 CFU/mL**
PMB MIC > 4	**<1 CFU/mL**	**2 CFU/mL**	**<1 CFU/mL**	**2.4 CFU/mL**

All reported values are proportionally corrected to a 10^6 CFU/mL overall population

For the isolates with meropenem MICs of 4 and 16 µg/mL, subpopulations with MICs > 16 µg/mL were 7 x 10^0 and 3 x 10^1 CFU/mL, respectively. For the isolates with meropenem MICs of 32 and 128 µg/mL, subpopulations with MICs > 64 µg/mL were 7 x 10^0 and 5 x 10^3 CFU/mL, respectively. Surprisingly, for all isolates, subpopulations with polymyxin B MICs > 4 µg/mL were almost non-detectable at < 3 CFU/mL.

CEFTAZIDIME-AVIBACTAM DISK DIFFUSION AND MBL ETEST®

Ceftazidime/Avibactam is a recently approved antimicrobial for the treatment of CRE in the absence of MBL-production. In order to contribute to the growing knowledge of this novel antimicrobial agent, we evaluated the susceptibility of all CRE that we had collected to date (75 out of 164 isolates).

Metallo-β-lactamase (MBL)-production information from the University of Kentucky Clinical Microbiology Laboratory was available for four of the 75 isolates tested (isolates 26, 40, 41, and 42). Since metallo β-lactamases are not inhibited by avibactam, it was expected that these isolates would be resistant to ceftazidime/avibactam, but only three of four were resistant, verified by duplicate experiments.

Excluding the four known MBL-producers, six isolates met criteria for MBL testing. Three isolates were borderline susceptible to ceftazidime/avibactam with a zone of inhibition of 21 mm (isolate 21, KP 24, and isolate 29), two isolates were resistant (isolates 53 and 134 with zones 17 mm and 15 mm, respectively) and one isolate was intermediate (KP 22; zone 20 mm). In a duplicate experiments, only the isolate testing as intermediate changed in interpretation with the second test and the third test being susceptible (23 mm and 24 mm respectively; Table 4.10). The designation of KP for two of the isolates is to indicate that isolate 22 and 24 are the same *Klebsiella pneumoniae* isolates that were tested in the time-kill studies.

Table 4.13: Ceftazidime / Avibactam Kirby Bauer Disk Diffusion Results

Organism	Isolate Number	Ceftazidime / Avibactam Zone of Inhibition (mm)	Interpretation (S/I/R)
Citrobacter amalonaticus	36	24	S
Citrobacter amalonaticus	91	25	S
Citrobacter freundii	27	29	S
Citrobacter freundii	50	22	S
Citrobacter freundii	54	30	S
Citrobacter freundii	101	27	S
Citrobacter freundii	127	27	S
Citrobacter freundii	145	31	S
Citrobacter freundii	147	30	S
Citrobacter youngae	136	26	S
Enterobacter aerogenes	97	30	S
Enterobacter cloacae	9	27	S
Enterobacter cloacae	10	28	S
Enterobacter cloacae	16	27	S
Enterobacter cloacae	17	27	S
Enterobacter cloacae	19	29	S
Enterobacter cloacae	20	27	S
Enterobacter cloacae	30	25	S
Enterobacter cloacae	39	27	S
Enterobacter cloacae*	**40**	**17, 16**	**R**
Enterobacter cloacae*	**41**	**18, 17**	**R**
Enterobacter cloacae	52	27	S
Enterobacter cloacae[§]	**53**	**17, 18**	**R**
Enterobacter cloacae	70	29	S
Enterobacter cloacae	96	26	S
Enterobacter cloacae	107	24	S
Enterobacter cloacae	121	25	S
Enterobacter cloacae	126	28	S
Enterobacter cloacae[§]	**134**	**15, 14**	**R**
Enterobacter cloacae	144	27	S
Escherichia coli	25	30	S
Escherichia coli	33	25	S
Escherichia coli	103	25	S
Enterobacter gergoviae	13	28	S
Enterobacter gergoviae	95	32	S
Enterobacter spp.	146	25	S
Klebsiella oxytoca	8	35	S
Klebsiella oxytoca	14	29	S
Klebsiella ozaenae	128	28	S
Klebsiella pneumoniae	7	32	S
Klebsiella pneumoniae[§]	21	21, 21	S

***Klebsiella pneumoniae*[§]**	KP 22	20, 23, 24	S
***Klebsiella pneumoniae*[§]**	KP 24	21, 27	S
Klebsiella pneumoniae*	26	27, 26	S
Klebsiella pneumoniae	28	22	S
***Klebsiella pneumoniae*[§]**	29	21, 21	S
Klebsiella pneumoniae	31	31	S
Klebsiella pneumoniae	32	33	S
Klebsiella pneumoniae	KP 34	28	S
Klebsiella pneumoniae	35	29	S
Klebsiella pneumoniae	37	26	S
Klebsiella pneumoniae*	**42**	**14, 14**	**R**
Klebsiella pneumoniae	43	25	S
Klebsiella pneumoniae	KP 44	24	S
Klebsiella pneumoniae	45	24	S
Klebsiella pneumoniae	46	27	S
Klebsiella pneumoniae	47	27	S
Klebsiella pneumoniae	48	25	S
Klebsiella pneumoniae	49	27	S
Klebsiella pneumoniae	51	25	S
Klebsiella pneumoniae	55	23	S
Klebsiella pneumoniae	69	25	S
Klebsiella pneumoniae	77	30	S
Klebsiella pneumoniae	93	27	S
Klebsiella pneumoniae	98	23	S
Klebsiella pneumoniae	99	25	S
Klebsiella pneumoniae	105	27	S
Klebsiella pneumoniae	116	27	S
Klebsiella pneumoniae	119	25	S
Klebsiella pneumoniae	123	28	S
Klebsiella pneumoniae	129	23	S
Klebsiella pneumoniae	130	27	S
Klebsiella pneumoniae	142	26	S
Klebsiella pneumoniae	143	24	S
Klebsiella pneumoniae	152	25	S
TOTAL	**75**		**93% S**

*MBL identified by PCR at University of Kentucky Clinical Microbiology Laboratory
[§]MBL identified by Etest®

MBL-production was identified in all but one isolate tested (KP 24 was indeterminate twice). MBL-production was identified in two borderline susceptible isolates (21 and 29), the initially intermediate isolate (KP 22), and both resistant isolates (53 and 134; Table 4.11). KP 24 was indeterminate upon initial testing because the resulting MIC ratio was ≥ 4 without a phantom zone or an ellipse deformation (Chapter 3: Methods "Etest® Procedure"). Isolate 21 was borderline negative after initial MBL testing, but tested positive upon retest. All other isolates were interpreted as positive for both tests (Table 4.11).

Table 4.14: MBL MP/MPI Etest® Results

Isolate	MP/MPI MIC (µg/mL)	Interpretation	MP/MPI MIC (µg/mL)	Interpretation
21	**0.5 / 0.064**	**Negative**	**1.5 / 0.094**	**Positive**
22	**> 8 / ≤ 0.032**	**Positive**	**3 / 0.032**	**Positive**
24	**> 8 / > 2**	**Indeterminate**	**> 8 / > 2**	**Indeterminate**
29	**> 8 / ≤ 0.032**	**Positive**	**> 8 / ≤ 0.032**	**Positive**
53	**> 8 / ≤ 0.032**	**Positive**	**> 8 / ≤ 0.032**	**Positive**
134	**4 / ≤ 0.032**	**Positive**	**2 / ≤ 0.032**	**Positive**
Negative Control	**≤ 0.0125 / ≤ 0.032**	**Negative**	**≤ 0.0125 / ≤ 0.032**	**Negative**
Positive Control	**1.5 / ≤ 0.032**	**Positive**	**1.5 / ≤ 0.032**	**Positive**

Positive Control - Isolate 42 (Confirmed MBL by PCR)
Negative Control - *Klebsiella pneumoniae* ATCC® 700603

Overall, five of the 75 CRE isolates were resistant to ceftazidime/avibactam (93% susceptibility across all CRE tested), and all five are associated with MBL-production. All four of the MBL-producing *E. cloacae* isolates (40, 41, 53, and 134) were resistant to ceftazidime/avibactam whereas one isolate (42) of the four MBL-producing *K. pneumoniae* isolates (21, 22, 29, and 42) were resistant to ceftazidime/avibactam. Divided by phenotypic (determined by Etest®) compared to genotypic MBL-production (determined by PCR from the University of Kentucky Clinical Microbiology Laboratory), two of five phenotypic MBL-producing isolates were resistant to ceftazidime/avibactam whereas three of four genotypic MBL-producing isolates were resistant to ceftazidime/avibactam.

Chapter Five:

Discussion and Conclusions

Carbapenem-resistant Enterobacteriaceae (CRE) are a growing national and international threat.[39,97,98,123-126] In fact, a "Call to Action" has been issues by various organizations (e.g. CDC, PACCARB, IDSA) hoping to raise awareness among the medical community as well as highlight the dwindling development of novel antimicrobial agents showing activity against these hard-to-treat infections.[12] Compounding this issue is the wide variability in types of carbapenem resistance among CRE (e.g. KPC, MBL, OXA-48, and ESBL or AmpC with porin mutations) and the large differences observed among nations or even among hospitals within the same country, state, or province.[14] There are no randomized, controlled clinical trials evaluating optimal therapy for CRE treatment, and treatment strategies may vary depending on the type of resistance. It has also been suggested that strain-to-strain differences or bacteria genotype may be more important to optimal therapeutic decision-making than MIC or pharmacodynamic indices alone.[227]

In order to contribute to the growing knowledge of CRE management, this study focused on KPC-producing *Klebsiella pneumoniae*, the most common CRE in the U.S., estimated to comprise up to 80% of CRE cases in some regions.[14] More specifically, we

evaluated the use of polymyxin B in combination with meropenem because, among the four "typically susceptible" antimicrobial agents, fosfomycin is only approved in the U.S. for uncomplicated urinary tract infections,[177] tigecycline is unable to reach effective serum concentrations for treatment of CRE bacteremia[167,247] where mortality is estimated to be around 50%,[11] and colistin is associated with higher rates of nephrotoxicity, cumbersome therapeutic drug monitoring, and more difficult rational drug dosing when compared to polymyxin B.[132,133,147] All of these agents are associated with rapid resistance development when used as monotherapy,[14] and previous data have indicated that combination therapy, in particular combinations including a carbapenem, have a mortality benefit over monotherapy.[10,68,184,198-202]

This study is among the first to evaluate the *in vitro* interaction of polymyxin B in and meropenem across a wide range of carbapenem resistance, testing multiple concentrations and for a longer duration (48 hours) than previous studies which have typically evaluated combinations involving colistin.[200,204,205,248-250] Meropenem and polymyxin B alone and in combination were evaluated against a total of four KPC-producing *K. pneumoniae* clinical isolates, representing polymyxin-susceptible (PMB MIC < 2 µg/mL) CRE of varying meropenem resistance (MEM MIC 4 – 128 µg/mL). These isolates were evaluated using CLSI-standardized *in vitro* laboratory methodology,[237] designed and approved to minimize variability between laboratories to facilitate more meaningful comparisons of results.

IN VITRO SUSCEPTIBILITY TESTING

The antimicrobial susceptibility summaries of KPC-producing *Klebsiella pneumoniae* isolates from the University of Kentucky Clinical Microbiological Laboratory (using BD Phoenix™ and broth microdilution) were compared against a similar report from isolates submitted to the SENTRY Antimicrobial Surveillance Program or the MYSTIC Program recovered from primarily U.S. medical institutions.[251] Chi-squared analysis was used to identify antimicrobial agents for which differences existed among U.S. isolates, University of Kentucky isolates tested by BD Phoenix™ and UK isolates tested by broth microdilution (Table 5.1). Significant differences between groups were identified after a Holm-Bonferroni correction.[246] Only one antimicrobial agent (amikacin) in the table below was not tested for in the SENTRY/MYSTIC isolates. Instead, a study evaluating 50 carbapenem-resistant *K. pneumoniae* isolates from the Pittsburg area was referenced which reported comparable gentamicin and tobramycin susceptibilies.[194]

Table 5.1: Antimicrobial Susceptibility Summary of CR-KP in the U.S.

Antimicrobial	U.S.[251] (n=123)	BD Phoenix™ (n=44)	Microdilution (n=24)*	P-value
Cefepime				
MIC_{50} (µg/mL)	>16	16	32	
MIC_{90} (µg/mL)	>16	>16	>256	
%S	11%	28%[§]	17%	**0.0376**
Ceftazidime				
MIC_{50} (µg/mL)	>16	>16	-	
MIC_{90} (µg/mL)	>16	>16	-	
%S	3%	0%	-	0.2312
Ceftriaxone				
MIC_{50} (µg/mL)	>32	>32	-	
MIC_{90} (µg/mL)	>32	>32	-	
%S	2%	2%	-	0.7955
Meropenem				

MIC_{50} (µg/mL)	>8	8	16	
MIC_{90} (µg/mL)	>8	>8	128	
%S	1%	30%[§]	25%[§]	**<0.0001**
Piperacillin/Tazobactam				
MIC_{50} (µg/mL)	>64/4	>64/4	0.25/4	
MIC_{90} (µg/mL)	>64/4	>64/4	>512/4	
%S	3%	2%	50%[§]	**<0.0001**
Amikacin (n=50)[194]				
MIC_{50} (µg/mL)	16	≤8	4	
MIC_{90} (µg/mL)	32	>32	128	
%S	84%	75%	67%	0.2300
Gentamicin				
MIC_{50} (µg/mL)	≤4	>8	-	
MIC_{90} (µg/mL)	>8	>8	-	
%S	61%	46%	-	0.0756
Tobramycin				
MIC_{50} (µg/mL)	>8	>8	-	
MIC_{90} (µg/mL)	>8	>8	-	
%S	6%	25%	-	**0.0004**
Ciprofloxacin				
MIC_{50} (µg/mL)	>2	>2	-	
MIC_{90} (µg/mL)	>2	>2	-	
%S	11%	34%	-	**0.0004**
Levofloxacin				
MIC_{50} (µg/mL)	>4	>4	8	
MIC_{90} (µg/mL)	>4	>4	>32	
%S	12%	36%[§]	29%	**0.0013**
Polymyxin B				
MIC_{50} (µg/mL)	≤1	-	0.125	
MIC_{90} (µg/mL)	>4	-	8	
%S	89%	-	83%	0.3909
Tigecycline				
MIC_{50} (µg/mL)	0.5	-	0.125	
MIC_{90} (µg/mL)	2	-	4	
%S	99%	-	88%	**0.0018**

* For piperacillin/tazobactam in broth microdilution studies, n=12 and there was only one experiment performed in duplicate

[§] Significant Holm-Bonferroni corrected p-value compared to U.S. isolates from literature

Compared to national data, susceptibilities to fluoroquinolones and tobramycin were significantly higher at the University of Kentucky (about 30% vs. 10% nationally) whereas tigecycline susceptibilities were significantly lower (88% vs. 99%; P = 0.0018). Interestingly, susceptibilities to cefepime and meropenem were also significantly higher at the University of Kentucky, despite similar carbapenemase production. Wide variability in susceptibility patterns has been reported by others, even at the regional level between acute care centers.[14] Since lower carbapenem MICs are associated with lower mortality,[120] combinations with antimicrobials associated with less toxicity may be equally effective but have improved safety profiles. Specifically, combinations with a carbapenem and an agent other than a polymyxin may be worth investigating for use at the University of Kentucky. For example, in a setting of higher fluoroquinolone or aminoglycoside susceptibility, a susceptible agent in combination with a carbapenem may show improved morbidity or mortality not solely due to clinical cure of the infection but also avoidance of the limiting toxicities associated with the polymyxins (nephrotoxicity or neurotoxicity) or tigecycline (severe nausea, vomiting, diarrhea).

Additionally, with the exception of piperacillin/tazobactam, susceptibilities reported by BD Phoenix™ for antimicrobial agents were consistently higher than those reported by broth microdilution, however none of these differences were significant. Concerning the difference in piperacillin/tazobactam observed between broth microdilution, BD Phoenix™, and national data, a number of possible explanations exist. Broth microdilution was performed once (in duplicate) on only 12 isolates where such a small sample size could have led to selection bias. An alternative hypothesis is that an error occurred in the selection of the piperacillin/tazobactam program in Chapter 3:

Methods "Broth Microdilution Procedure". If the tazobactam laying program were run instead of serially diluting piperacillin across the 96-well tray, then this would explain the polar interpretation of either >512/4 or ≤0.25/4 (all growth in wells vs. no growth observed at all) since all wells would have actually contained roughly piperacillin/tazobactam 400/3.2 µg/mL in 125 µL (Appendix C, Table C.1). The way to confirm this would be to retest these isolates. Finally, similar to national data, we observed relatively high susceptibilities among clinical isolates of KPC-producing CRE to polymyxin B, tigecycline, colistin, and fosfomycin.

In order to characterize the *in vitro* interaction of polymyxin B and meropenem, we selected four KPC-3-producing, polymyxin-susceptible, carbapenem-resistant, *Klebsiella pneumoniae* isolates to represent the spectrum of carbapenem resistance most commonly encountered Enterobacteriaceae in the United States. The subtype of the KPC enzyme was previously evaluated by PCR at the University of Kentucky Clinical Microbiology Laboratory for clinical use but the information was released to us for research purposes. Table 4.4 displays the MIC data from broth microdilution susceptibility testing for each of the four clinical isolates (KP 34, KP 22, KP 24, and KP 44) and Appendix A, Tables A.1 - A.2 display the MIC data from BD Phoenix™ susceptibility testing for these isolates. For most isolates tested, polymyxin susceptibility was most frequently observed at polymyxin B/colistin MICs of 0.06 - 0.125 µg/mL whereas meropenem resistance ranged from 4 µg/mL - 128 µg/mL. The four isolates selected for time-kill studies had MICs within the previously mentioned ranges (Table 4.4).

IN VITRO ACTIVITY OF MEROPENEM AND POLYMYXIN IN TIME-KILL STUDIES

Polymyxin B alone against polymyxin-susceptible, KPC-producing *Klebsiella pneumoniae* generally exhibited quick bactericidal activity, with rapid regrowth observed whereas meropenem alone generally exhibited bacteriostatic activity initially with growth observed as well. These results are consistent with *in vitro* data from other groups.[210,252] When used in combination, results were often bactericidal, synergistic, and maintained this activity throughout 48 hours unless resistance to meropenem was high (≥32 μg/mL) in which case higher levels of antimicrobial agents were shown to overcome the strains with elevated MICs, but such regimens may have limited feasibility in a patient where antimicrobial concentrations are not static but change as drug is eliminated. Therefore, additional *in vitro* or animal (or even human) models are needed to elucidate the impact of pharmacokinetics and the degree of meropenem resistance on the activity of meropenem and polymyxin B in combination against KPC-producing *K. pneumoniae*.

A 2013 meta-analysis on *in vitro* synergy of polymyxins and carbapenems highlighted that most data for comparison involves non-Enterobacteriaceae such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii*. In fact, the authors included only three studies[204,248,253] that evaluated polymyxin B in combination with a carbapenem (imipenem, doripenem, meropenem, or ertapenem) across a total of 34 unique isolates of *K. pneumoniae*, most of which were polymyxin-susceptible. Synergy rates for polymyxin B and a carbapenem were higher than synergy rates for colistin and a carbapenem (64% vs. 40%; P = 0.04), but substantial heterogeneity among these studies was present (I^2 = 51%).[210] Since the publication of the meta-analysis, only one other study has compared

polymyxin B in combination with a carbapenem *in vitro* against KPC-producing *Klebsiella pneumoniae.*[252]

Among the four studies evaluating the *in vitro* activity of polymyxin B in combination with a carbapenem against KPC-producing *K. pneumoniae*, two evaluated exclusively KPC-2-producing *K. pneumoniae* (18 isolates total),[204,252] one evaluated exclusively KPC-3-producing *K. pneumoniae* (4 isolates),[253] and the last evaluated both KPC-2- and KPC-3-producing *K. pneumoniae* (8 and 6 isolates, respectively). Our study evaluated KPC-3-producing *K. pneumoniae* (4 isolates). Although KPC-2 is considered the ancestral enzyme, KPC-3 has also been frequently observed in the United States. KPC-3 is very similar to KPC-2 in both structure and phenotypic resistance expression, differing only by a single nucleotide polymorphism and therefore also a single amino acid substitution of histidine for tyrosine (H272Y).[254] To date, there is no data to suggest distinguishing between KPC-2 or KPC-3 correlates with differences in phenotypic resistance or clinical outcome, and so this difference among studies was accepted. Finally, definitions of synergy and bactericidality among studies was consistent except when noted.

Polymyxin B or a Carbapenem Alone

Comparing results of monotherapy was not possible among all studies because complete time-kill data was only provided by Lee *et al.*[253] Data for 0 hours and 24 hours was provided by Bratu *et al.*,[204] but the other studies only provided the difference from 0 hours to 24 hours.[248,252] Lee *et al.* studied the four KPC-3 isolates most similar to this

study, exhibiting polymyxin B MICs ranging from 0.125 - 0.25 µg/mL (this study: MICs 0.06 - 0.125 µg/mL) and doripenem (DOR) MICs ranging from 16 - 32 µg/mL (this study: MEM MICs 4 - 128 µg/mL). Polymyxin B at 2 x MIC displayed similar killing to the present study, but we observed regrowth sooner (4 hours) than did Lee *et al.* (8 hours), despite our use of higher concentrations relative to the MIC (Figure 5.1 vs. Figures 4.1-4.4). Concerning carbapenem therapy, the bacteriostatic activity and growth observed with doripenem used alone (Figure 5.1) was similar to the present study, despite our use of meropenem instead.[253]

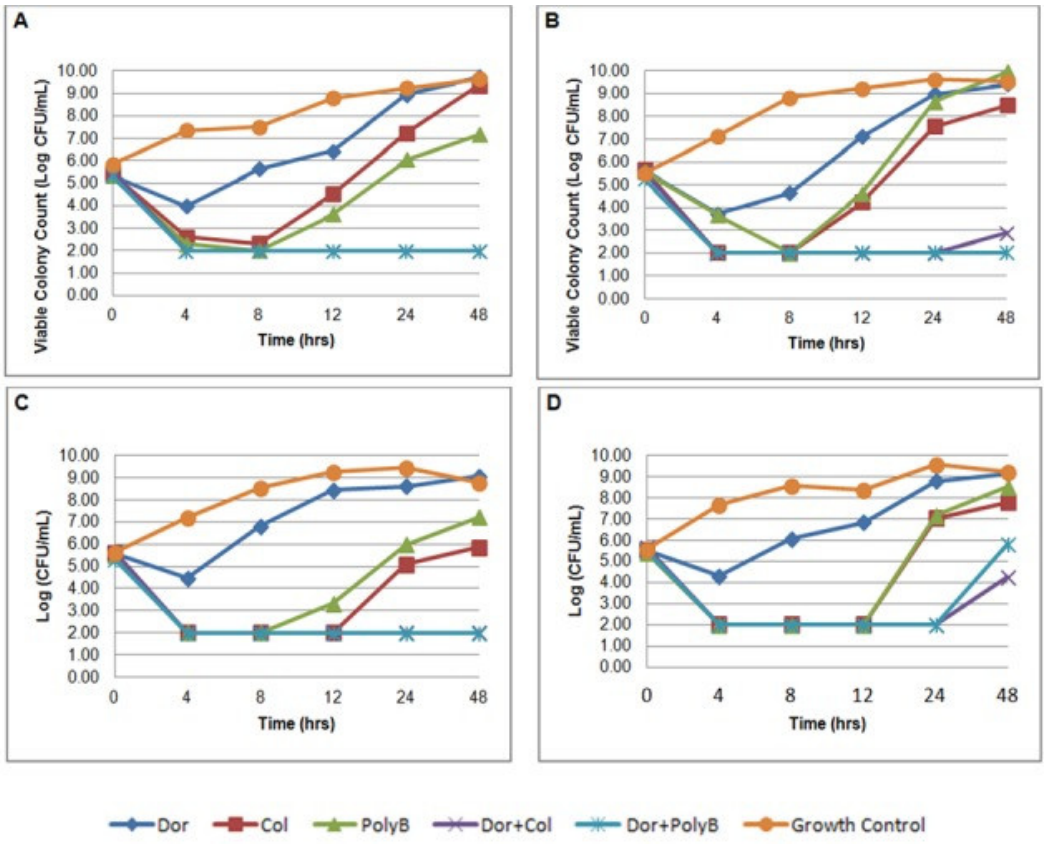


Figure 5.1: Time-kill curves for four KPC-3-producing *K. pneumoniae*. **Reprinted**[253]
Abbreviations: Dor - Doripenem, Col - Colistin, PolyB - Polymyxin B

Comparing the data provided by Bratu *et al.*, polymyxin B alone at 0.5, 1, 2, and 4 µg/mL remained bactericidal at 24 hours for 2, 7, 12, and 13 isolates, respectively, out of 16 total. Interestingly, isolates from this study had at least 4-fold lower polymyxin B MICs than those studied by Bratu *et al.* (0.06 - 0.125 µg/mL vs. 0.5 - 16 µg/mL), but we, in contrast, observed regrowth in all polymyxin B concentrations tested alone before 24 hours. Comparing carbapenem therapy, imipenem (IPM) 4 µg/mL alone displayed growth in all 16 isolates. For the present study, the three isolates most similar to those evaluated by Bratu *et al.* (IPM MICs 8 - >32) were KP 22 (MEM MIC 16 µg/mL), KP 24 (MEM MIC 32 µg/mL), and KP 44 (MEM MIC 128 µg/mL). Like Bratu *et al.*, meropenem alone showed growth at 4 and 16 µg/mL.[204]

Polymyxin B and a Carbapenem in Combination

Lee *et al.* evaluated colistin or polymyxin B at 2 x MIC in combination with doripenem 6 µg/mL against four polymyxin-susceptible, KPC-3-producing *Klebsiella pneumoniae* (DOR MIC 16 - 32 µg/mL). Bactericidal, synergistic activity was observed throughout 24 hours for all isolates with some regrowth observed only at 48 hours for 1 of 4 isolates with polymyxin B and 2 of 4 with colistin.[253] Polymyxin B and meropenem showed similar activity against KP 22 and KP 24 (MEM MICs 16 and 32 µg/mL, respectively) in the present study at comparable concentrations (MEM 4 µg/mL and 16 µg/mL). One notable difference compared to our study was that meropenem 4 µg/mL in combination with polymyxin B at 0.25 or 1 µg/mL (2 and 8 x MIC, respectively) did not maintain bactericidal or synergistic activity by 24 hours against KP 24. However,

meropenem concentrations ≥1 x MIC in combination with polymyxin B did regain bactericidal and synergistic interaction. The difference between meropenem and doripenem in combination with polymyxin B cannot be explained with good evidence, but clinically, others have observed equivalent efficacy of doripenem compared to other carbapenems when being used in lower doses but at extended infusions.[255]

Pankey *et al.* evaluated polymyxin B at 1/4, 1/2, and 1 x MIC in combination with meropenem 1 x MIC against 14 KPC-producing *Klebsiella pneumoniae* (MER MIC 16 - >32 μg/mL, PMB MIC ≤2 for 11 of 14 isolates). Synergy was observed for 9 of 14 isolates for all concentrations tested with meropenem and polymyxin B. Non-synergistic isolates showed indifferent/additive activity, but only 1 of 5 was resistant to polymyxin B (PMB MIC 32 μg/mL) before study. The authors did not comment on the killing activity (bactericidal vs. bacteriostatic) of meropenem and polymyxin B in combination against these isolates. Compared to the present study, we observed a loss of synergistic activity between meropenem and polymyxin B related to increasing meropenem MIC. A similar assessment is difficult to make in the study by Pankey *et al.* because detection of synergy did not depend on meropenem MIC, but a much smaller range of carbapenem resistance was evaluated (MEM MIC 16 - >32 μg/mL). Similarly, carbapenem MIC did not change the observation of synergy in the study by Lee *et al.*, but again, a smaller range (16 - 32 μg/mL) of carbapenem MIC was evaluated whereas we evaluated MEM MICs 4 - 128 μg/mL.[248]

Bratu *et al.* evaluated polymyxin B at 1 μg/mL and 1/2 x MIC in combination with imipenem 4 μg/mL against 16 KPC-2-producing *Klebsiella pneumoniae* that were mostly polymyxin-susceptible (14 of 16 isolates). Imipenem MICs were all >32 μg/mL

except for one isolate which had an imipenem MIC of 8 µg/mL. Data were unavailable for each isolate tested individually, but synergy was reported for 10 of 16 isolates with polymyxin B at 0.5 x MIC in combination with imipenem. Interestingly, antagonism was observed in 3 of 16 isolates. Although antagonism was not observed with polymyxin B and meropenem in the present study, the antagonism reported by Bratu *et al.* is consistent with our observed loss or reduction of synergy as the carbapenem MIC increases. It is important to note that a majority of isolates evaluated by Bratu *et al.* had imipenem MICs > 32 µg/mL while we observed loss of synergy at lower concentrations of meropenem (4 µg/mL) when meropenem MICs were at least 32 µg/mL. It is also interesting that synergy was still observed in a majority of highly carbapenem-resistant isolates (10 of 16 isolates) at polymyxin B concentrations close to 1 µg/mL in combination with imipenem 4 µg/mL when the present study required meropenem concentrations ≥ 16 µg/mL in combination with polymyxin B 1 µg/mL to maintain synergy.[204]

In the most recently published study, Barth *et al.* evaluated polymyxin B at 0.5, 1, and 2 µg/mL in combination with meropenem or imipenem at 4 µg/mL against two KPC-2-producing *Klebsiella pneumoniae*. Both isolates had a polymyxin B MIC of 2 µg/mL and a meropenem MIC of 32 µg/mL, but had different imipenem MICs of 8 and 32 µg/mL. Synergy was observed in both strains at all combinations studied which is in contrast to this study where polymyxin B at 0.5 and 1 µg/mL in combination with meropenem 4 µg/mL was not synergistic for KP 24, despite having a lower polymyxin B MIC (MEM MIC 32 µg/mL, PMB MIC 0.125 µg/mL). Although not as directly comparable, but interesting, Barth *et al.* also evaluated two strains each of *Escherichia coli* and *Serratia marcescens* for which synergy was also found in all the same

concentrations of polymyxin B in combination with either meropenem or imipenem. This result was most surprising for *S. marcescens* which is intrinsically resistant to polymyxins. The *E. coli* strains had meropenem MICs of 64 µg/mL whereas the *S. marcescens* strains had meropenem MICs of 128 and 256 µg/mL. The polymyxin B MICs for *E. coli* were 2 µg/mL whereas the *S. marcescens* stains had polymyxin B MICs of 64 and >64 µg/mL. The author did characterize the killing of these antimicrobial agents against the strains, however their definition of bactericidality was different from most studies, including the present one. The authors assessed bactericidal activity based on a 10^3 CFU/mL difference between the colony count of the combination and the colony count of the most active agent alone, which, while appropriate for synergy, could actually mean that growth (as defined in this and most studies) occurred but to a lesser extent than the most active agent. Since only this difference was reported, it is not possible to compare the killing activity in the experiments by Barth *et al.* to the killing activity observed in this study.[252]

Overall, other studies evaluating polymyxin B in combination with a carbapenem by time-kill assay observed bactericidal activity and synergistic interaction maintained throughout 24 hours most of the time[204,248,252,253] which is in agreement with our findings. Antagonism was rarely observed, and it was only seen in a minority of isolates with high carbapenem MICs (IPM MICs > 32)[204] which is also similar to our findings because a lower extent of synergy was also observed in this study when carbapenem MICs were elevated (MEM MICs ≥ 32 µg/mL). A carbapenem alone exhibited similar activity in this study as compared to the Lee *et al.*[253] and Bratu *et al.*[204] Polymyxin B alone exhibited variable activity depending on the study. Our results were more similar to Lee *et al.*

where growth was consistently observed, but at a slower rate.[253] In contrast, Bratu *et al.* observed growth in only a fraction of the isolates, depending on the concentration of polymyxin B. This might best be explained by variable heteroresistant subpopulations among KPC-producing *K. pneumoniae* isolates,[142] however this has not been well characterized and was not discussed by Bratu *et al.*[204]

Heteroresistant Subpopulations

KP 34, 22, 24, and 44 underwent preliminary characterization of the meropenem and polymyxin B MICs of any heteroresistant subpopulations. Table 4.9 displays the exact colony counts with respect to a 10^6 CFU/mL total population concentration. For a more complete subpopulation analysis, studies with higher inocula would provide higher sensitivity but would reduce the internal validity of comparing observations from the subpopulation study to our time-kill results, which was the primary purpose. The most interesting result was observing subpopulations with MICs at least 32 x the MIC of the total population (e.g. a strain with a population MIC of 0.125 µg/mL growing on an agar plate with 4 µg/mL polymyxin B). Better characterization of these heteroresistance isolates may elucidate the cause of regrowth observed throughout our experiments when polymyxin B was used alone or when combination therapy failed to prevent regrowth.

Other studies have also described polymyxin heteroresistance.[142] Among the four studies recently discussed, only Lee *et al.* reported post-exposure susceptibility testing on their regrowing isolates from time-kill studies. Similar to the present study, Lee *et al.* observed an increase in colistin or polymyxin B MIC from 0.125 - 0.25 µg/mL to 8 - 128

μg/mL whereas we observed changes from 0.06 - 0.125 μg/mL to 16 - >64 μg/mL. Lee *et al.* also observed no change in doripenem MICs following polymyxin exposure whereas we did not look at change in meropenem MICs. However, we observed no change in polymyxin MICs following meropenem exposure, suggesting insignificant (if any) cross-resistance between these antimicrobial classes. However, both studies observed polymyxin B and colistin MICs correlating very strongly together.[253]

Meletis *et al.* performed more thorough subpopulation studies on 16 carbapenemase-producing *K. pneumoniae* clinical isolates. In that study, subpopulations growing on agar plates impregnated with up to 8 μg/mL colistin had population MICs ranging from 1 - 4 μg/mL. Susceptibility testing on colonies growing on these agar plates ranged from 16 - 64 μg/mL, which was similar to both Lee *et al.* and our study, although we analyzed polymyxin B rather than colistin. The colony counts growing on agar plates containing colistin 8 μg/mL ranged from 3×10^0 to 4×10^3 CFU/mL whereas the colony counts we observed on agar plates containing 4 μg/mL polymyxin B was closer to 2×10^0 CFU/mL. Accounting for this difference may be the difference in the MIC of the populations since this study analyzed strains with much lower polymyxin B/colistin MICs (0.06 - 0.125 μg/mL) whereas Meletis *et al.* analyzed strains with colistin MICs ≥ 1 μg/mL. In other words, more similar colony counts may be observed in our isolates if we were to utilize agar plates impregnated with similar proportions of polymyxin B/colistin such as 0.5 - 1 μg/mL (approximately 4 - 8 x MIC).

Meletis *et al.* noticed that about 8 of 16 isolates did not exhibit heteroresistance which was demonstrated by a lack of growth on agar plates with colistin concentrations exceeding the colistin MIC of the population.[142] This observation may explain the

differences observed between Lee *et al.*, our study, and Bratu *et al.* concerning regrowth with regimens containing either polymyxin alone or in combination. If heteroresistance impairs the synergistic interaction between polymyxins and carbapenems, then a lack of heteroresistance would explain the complete killing or indeterminate synergy observed by Bratu *et al.* since polymyxins alone seemed to be sufficient in preventing growth at concentrations above the MIC.[142]

In conclusion, agar plates with lower polymyxin concentrations would better characterize the heteroresistance exhibited by KP 34, 22, 24, and 44, but we as well as others have observed wide variability in polymyxin MICs, even among the same strains.[142,253] The clinical role of polymyxin heteroresistance is not known at this time, but it is suspected to impair the synergistic interaction between carbapenems and polymyxins. Finally, not all carbapenem-producing *Klebsiella pneumoniae* have observable polymyxin heteroresistance.[142]

CEFTAZIDIME-AVIBACTAM *IN VITRO* SUSCEPTIBILITY AND MBL-PRODUCTION

Among the 75 CRE tested for susceptibility to ceftazidime-avibactam (CZA), only five were non-susceptible (7%). All non-susceptible isolates were found to produce MBL either phenotypically by Etest® or genotypically by PCR (performed by the University of Kentucky Clinical Microbiology Laboratory). Since MBLs are not inhibited by avibactam[256] but still hydrolyze 3rd-generation cephalosporins such as ceftazidime, these results were not surprising. The most interesting results were that the three isolates phenotypically positive for MBL-production and the one isolate genotypically positive for VIM-production were susceptible to ceftazidime-avibactam when all four (isolates 21, KP 22, 26 and 29) were resistant to ceftazidime (MIC >16 µg/mL; Appendix A, Table A.1). This may warrant further inquiry.

Two other large studies have evaluated the activity of ceftazidime/avibactam against CRE. In 2015, Castanheira *et al.* reported 98% susceptibility (CZA MIC ≤ 8/4 µg/mL) among 153 CRE isolates collected from 71 U.S. medical centers as part of the International Network for Optimal Resistance Monitoring (INFORM) program (P = 0.07346 when compared to this study). The MIC_{50} was 0.5 µg/mL and the MIC_{90} was 2 µg/mL. Non-susceptibility was observed in two *Klebsiella pneumoniae* strains isolated from a Colorado medical center and one *Proteus mirabilis* strain of an unnamed source. All three CRE had ceftazidime-avibactam MICs > 32 µg/mL. Similar to our non-susceptible isolates, the *K. pneumoniae* isolates were found to harbor NDM-1 but the *P. mirabilis* isolate tested negative for CTX-M subgroups 1, 2, 8, 9, and 25; TEM wild type and ESBL; SHV wild type and ESBL; AmpC; KPC; and NDM-1 but positive only for

TEM-212, a narrow-spectrum β-lactamase inhibitor (e.g. tazobactam, clavulanic acid, sulbactam) resistant β-lactamase.[257] The authors did not discuss the *P. mirabilis* strain further, but TEM-212 may also be resistant to the β-lactamase inhibitor, avibactam, and high ceftazidime MICs (≥32 μg/mL) have been observed in two *Providencia stuartii* isolates.[258] However, alternative explanations may exist in non-β-lactamase mediated mechanisms of resistance that still warrant exploration.

By 2016, de Jonge *et al.* evaluated ceftazidime-avibactam against 961 meropenem-non-susceptible Enterobacteriaceae collected from Europe, Asia/Pacific, Latin America, and the Middle East/Africa as part of the INFORM program. Of these, susceptibility to ceftazidime-avibactam was reported in 83.5%. Upon excluding MBL-producing isolates, 97.7% susceptibility was observed among 816 isolates, which is not significantly different from our study upon also excluding MBL-producing isolates (P=0.2113). A most interesting result, however, was the decreased susceptibility observed among 207 carbapenemase-negative meropenem-non-susceptible Enterobacteriaceae compared to 609 carbapenemase-positive, MBL-negative, meropenem-non-susceptible Enterobacteriaceae (94.7% vs. 98.7%; P=0.0009). Among those 207, AmpC, ESBL or both genes were identified in only 195 isolates. Among the 12 remaining isolates, 8 (67%) were susceptible to ceftazidime/avibactam. Although a small subgroup, this suggests that non-enzyme mediate resistant may play a role in ceftazidime-avibactam non-susceptibility. In total, the authors identified only 19 of 961 isolates for which ceftazidime-avibactam non-susceptibility couldn't be explained by the presence of MBLs.[259] Target site modifications[260] and other MBLs not yet identified by PCR were among the most suspected whereas upregulation of efflux pumps were considered less

likely after direct testing.[261] Finally, among 145 MBL-producing isolates, susceptibility to ceftazidime-avibactam was 3.4% whereas in the present study, 4 of 9 MBL-producing isolates were susceptible (44%; P<0.0001). The implications of this are not understood, but regional differences in resistance patterns combined with non-β-lactamase mediated resistance provide one hypothesis. Further studies are warranted to better understand this observation.

Back in the U.S., the first case report of ceftazidime-avibactam resistance in a KPC-3-producing *Klebsiella pneumoniae* clinical isolate was published in October 2015. The exact resistance mechanism is still being investigated, but it is not suspected to be related to KPC-3-production since the amino acid sequence encoded by $bla_{\text{KPC-3}}$ in this isolate was unaltered. This adds to the growing evidence that there may be a non-β-lactamase mediated resistance mechanism to ceftazidime-avibactam. The clinical implications of these reports are that susceptibility testing of ceftazidime-avibactam may still be warranted, even in the setting of MBL-negative carbapenem resistance.[262]

FUTURE WORK

Data from retrospective human studies have been compelling regarding the advantage of combination therapy, especially those containing a carbapenem, for the treatment of CRE. However, questions such as which antimicrobials, at what doses, for how long, and are there other factors that might determine clinical outcome remain. Additionally, significant limitations such as heterogeneity and correlative evidence begs for randomized controlled trials evaluating antimicrobial combinations head-to-head. However, cost, coordination, and design hurdles nearly render this undertaking infeasible, which leads to *in vitro* and animal studies to scratch at the answers needed. So far, time-kill studies have identified numerous combinations of antimicrobials with high rates of synergy, among the top are polymyxins in combination with a carbapenem.

Since this study is among the first to suggest that meropenem MICs may correlate with synergy between polymyxin B and meropenem, additional time-kill studies using this hypothesis against additional isolates could validate this observation. Furthermore, Enterobacteriaceae species other than *Klebsiella pneumoniae* could be evaluated to see if synergy dependence on carbapenem MIC still holds across species. Whether or not similar results are obtained, this data would fuel the construction of models that could describe new pharmacodynamic indices or targets of antimicrobials used in combination. As a hypothetical example, if meropenem and polymyxin are used in combination, perhaps a pharmacodynamic target of 50% time above the MIC for meropenem is not necessary but instead only 25% time above the MIC when the AUC/MIC ratio of the combination drug is at least 4. Such data, when modeled, may establish "hybrid" breakpoints where agents used in combination have altered susceptibility definitions from

traditional single agent breakpoints. This may translate to clinical reports directing clinicians about which combination therapies to employ against a particular CRE strain rather than trusting that single agent susceptibility data predicts successful clinical outcome of a combination.

Genetic testing has been suggested by others to play a bigger role than previously understood in CRE treatment.[227] Genetic testing of the four strains studied in addition to future strains undergoing *in vitro* study may better describe the proposed dependence of synergy on carbapenem MIC or could lead to alternative categorization. For example, instead of carbapenem MIC being the primary variable describing synergy (or hopefully clinical success), it may be enzyme copy number, number of unique carbapenemase enzymes, enzyme expression level, or some other genetic variable that may describe clinical success. *In vitro* work incorporating genetic variability in the study design is certainly opportunity for investigation.

Data with *in vitro* pharmacodynamic and animal models more closely emulating human pharmacokinetics and the immune system are also warranted. Using the time-kill data from this study, models should be constructed which can guide therapeutic drug targets of polymyxin B and meropenem based on the level of carbapenem resistance. These models can be adjusted to include other factors such as drug elimination through the use of bioreactors that mimic dynamic rather than static drug levels. Murine models can be used to incorporate the effects of an immune system and other factors of a living, infected host. Previous data, although limited, can serve as a foundation for experimental design and also for comparison once data is obtained.

CONCLUSIONS

Infections caused by carbapenem-resistant Enterobacteriaceae are a growing international threat where treatment options are very limited and mortality is extremely high (about 50%).[14] Preventative measures and antimicrobial stewardship may provide much needed time to evaluate treatment strategies, but already CRE are receiving attention from the European and U.S. government as well as receiving calls to action from organizations like the CDC and IDSA. Treatment strategies to date have shown the highest reductions in mortality when a carbapenem is used in combination with one or two additional antimicrobials, among which polymyxins are the most common.

This study has corroborated the findings of others regarding the synergy and effectiveness of a carbapenem in combination with polymyxin B, but the impact that the degree of carbapenem resistance may have on clinical outcome needs to be addressed because synergy is just a surrogate endpoint that may not always correlate with clinical success. Furthermore, evaluation of traditionally "non-susceptible" antimicrobials such as fluoroquinolones and aminoglycosides, especially in areas where susceptibilities to these antimicrobials are maintained, may improve clinical outcomes. Finally, this study has evaluated local susceptibility of CRE to ceftazidime-avibactam – a recently approved antimicrobial treatment for KPC-producing Enterobacteriaceae, but clinical data regarding the use of this agent, especially as monotherapy against carbapenem-resistant organisms is limited.

Appendix A:

Minimum Inhibitory Concentrations Determined by BD Phoenix™

Tables A.1-A.2 provide the results of *in vitro* susceptibility testing performed by the University of Kentucky Clinical Microbiology Laboratory using BD Phoenix™.

The following abbreviations are used in Table A.1: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

The following abbreviations are used in Table A.2: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Acinetobacter baumannii	63	>16	*	>16	>16	>16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	75	>16	*	>16	>16	>16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	81	>16	*	>16	>16	>16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	83	>16	*	>16	>16	>16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	86	>16	*	>16	>16	16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	191	>16	*	>16	>16	>16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	194	>16	*	>16	>16	>16	>16	>16	>32	>16	*	>8	*
Acinetobacter baumannii	215	>16	>16/8	>16	>16	>16	>16	>16	>32	*	*	>8	*
Acinetobacter baumannii	219	>16	>16/8	>16	>16	>16	>16	>16	>32	*	*	>8	*
Acinetobacter baumannii	226	>16	>16/8	>16	>16	>16	>16	>16	>32	*	*	>8	*
Burkholderia cepacia	67	>16	>16/8	>16	>16	*	>16	4	32	>16	>4	4	32/4
Citrobacter amalonaticus	36	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	4	4	>64/4
Citrobacter amalonaticus	91	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	4	>64/4
Citrobacter freundii	6	>16	>16/8	>16	>16	8	>16	4	>32	>16	2	≤1	>64/4
Citrobacter freundii	27	>16	>16/8	>16	>16	8	>16	8	>32	>16	2	≤1	>64/4
Citrobacter freundii	50	>16	>16/8	>16	>16	>16	16	>16	>32	>16	4	≤1	>64/4
Citrobacter freundii	54	>16	>16/8	>16	>16	4	>16	8	>32	>16	4	≤1	>64/4
Citrobacter freundii	101	>16	>16/8	>16	>16	2	>16	>16	32	>16	2	≤1	>64/4
Citrobacter freundii	127	>16	>16/8	>16	>16	2	>16	>16	>32	>16	>4	2	>64/4
Citrobacter freundii	135	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	≤0.5	4	>64/4
Citrobacter freundii	145	>16	>16/8	>16	>16	2	>16	>16	>32	>16	2	≤1	>64/4
Citrobacter freundii	147	>16	>16/8	>16	>16	8	>16	>16	>32	>16	2	≤1	>64/4
Citrobacter freundii	225	>16	>16/8	>16	>16	*	16	>16	>32	*	≤0.25	≤0.5	>64/4
Citrobacter youngae	136	>16	>16/8	>16	>16	16	>16	8	>32	>16	>4	4	>64/4
Enterobacter aerogenes	97	>16	>16/8	>16	>16	2	>16	>16	>32	>16	1	≤1	>64/4
Enterobacter aerogenes	163	>16	>16/8	4	>16	≤1	≤4	8	>32	>16	≤0.5	≤1	≤2/4
Enterobacter aerogenes	179	>16	>16/8	≤2	>16	≤1	>16	>16	>32	>16	4	>8	>64/4
Enterobacter aerogenes	187	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	2	≤1	>64/4

Abbreviations: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Enterobacter cloacae	1	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	8	>64/4
Enterobacter cloacae	3	>16	>16/8	>16	>16	8	>16	>16	>32	>16	>4	≤1	>64/4
Enterobacter cloacae	4	>16	>16/8	>16	>16	4	>16	>16	>32	>16	>4	≤1	>64/4
Enterobacter cloacae	5	>16	>16/8	>16	>16	16	>16	8	>32	>16	>4	2	>64/4
Enterobacter cloacae	9	>16	>16/8	>16	>16	16	>16	>16	>32	>16	4	>8	>64/4
Enterobacter cloacae	10	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Enterobacter cloacae	12	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Enterobacter cloacae	15	>16	>16/8	>16	>16	4	>16	>16	>32	>16	2	≤1	>64/4
Enterobacter cloacae	16	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	≤1	>64/4
Enterobacter cloacae	17	>16	>16/8	>16	>16	4	>16	>16	>32	>16	>4	≤1	>64/4
Enterobacter cloacae	19	>16	>16/8	>16	>16	8	>16	>16	>32	>16	2	≤1	>64/4
Enterobacter cloacae	20	>16	>16/8	>16	>16	8	>16	>16	>32	>16	>4	8	>64/4
Enterobacter cloacae	23	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	2	>64/4
Enterobacter cloacae	30	>16	>16/8	>16	>16	*	>16	>16	>32	>16	>4	≤1	>64/4
Enterobacter cloacae	39	>16	>16/8	>16	>16	8	>16	16	>32	>16	>4	≤1	>64/4
Enterobacter cloacae	40	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	4	8	>64/4
Enterobacter cloacae	41	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	4	>8	>64/4
Enterobacter cloacae	52	>16	>16/8	>16	>16	16	>16	8	>32	>16	>4	>8	>64/4
Enterobacter cloacae	53	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	8	>64/4
Enterobacter cloacae	61	>16	>16/8	>16	>16	≤1	>16	>16	16	>16	≤0.5	≤1	4/4
Enterobacter cloacae	70	>16	>16/8	>16	>16	2	>16	>16	>32	>16	2	≤1	8/4
Enterobacter cloacae	73	>16	>16/8	>16	>16	≤1	>16	>16	8	>16	≤0.5	≤1	4/4
Enterobacter cloacae	96	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	1	>8	64/4
Enterobacter cloacae	107	>16	>16/8	>16	>16	8	>16	8	>32	>16	>4	8	>64/4
Enterobacter cloacae	121	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	8	>64/4
Enterobacter cloacae	126	>16	>16/8	>16	>16	>16	>16	4	>32	>16	>4	>8	>64/4
Enterobacter cloacae	134	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	2	8	>64/4
Enterobacter cloacae	144	>16	>16/8	>16	>16	2	>16	16	>32	>16	>4	4	>64/4
Enterobacter cloacae	167	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	2	4	>64/4
Enterobacter cloacae	168	>16	>16/8	>16	>16	8	>16	16	>32	>16	>4	>8	>64/4

Abbreviations: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Enterobacter cloacae	169	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	2	4	>64/4
Enterobacter cloacae	171	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Enterobacter cloacae	175	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	2	4	>64/4
Enterobacter cloacae	189	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	4	4	>64/4
Enterobacter cloacae	200	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	4	≤1	>64/4
Enterobacter cloacae	203	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Enterobacter cloacae	209	>16	>16/8	>16	>16	>16	>16	16	>32	>16	>4	>8	>64/4
Enterobacter gergoviae	13	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Enterobacter gergoviae	95	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	≤1	>64/4
Enterobacter hormaechei	186	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Enterobacter sp.	146	>16	>16/8	>16	>16	8	>16	16	>32	>16	>4	>8	>64/4
Enterobacter sp.	210	>16	>16/8	>16	>16	>16	>16	16	>32	>16	>4	>8	>64/4
Escherichia coli	25	>16	>16/8	4	>16	≤1	8	4	>32	>16	4	≤1	>64/4
Escherichia coli	33	>16	>16/8	>16	>16	8	>16	>16	>32	>16	4	≤1	>64/4
Escherichia coli	58	>16	16/8	4	>16	8	8	1	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	60	>16	>16/8	>16	>16	>16	≤4	16	>32	>16	≤0.5	≤1	4/4
Escherichia coli	64	>16	>16/8	>16	>16	>16	8	4	>32	>16	≤0.5	≤1	>64/4
Escherichia coli	71	>16	>16/8	>16	>16	16	16	>16	>32	>16	≤0.5	≤1	16/4
Escherichia coli	72	>16	>16/8	16	>16	16	≤4	2	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	78	>16	>16/8	>16	>16	>16	16	>16	>32	>16	≤0.5	≤1	>64/4
Escherichia coli	79	>16	>16/8	>16	>16	>16	≤4	16	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	84	>16	>16/8	>16	>16	>16	16	>16	>32	>16	≤0.5	≤1	32/4
Escherichia coli	85	>16	>16/8	>16	>16	>16	≤4	16	>32	>16	≤0.5	≤1	8/4
Escherichia coli	87	>16	>16/8	16	>16	4	≤4	4	>32	>16	≤0.5	≤1	>64/4
Escherichia coli	90	>16	16/8	≤2	>16	2	≤4	1	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	94	>16	>16/8	>16	>16	16	16	16	>32	>16	≤0.5	≤1	8/4
Escherichia coli	103	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	1	≤1	32/4
Escherichia coli	106	>16	>16/8	>16	>16	4	≤4	16	>32	>16	≤0.5	≤1	16/4
Escherichia coli	111	>16	>16/8	>16	>16	>16	≤4	16	>32	>16	≤0.5	≤1	>64/4
Escherichia coli	112	>16	>16/8	4	>16	2	8	2	>32	>16	≤0.5	≤1	16/4

Abbreviations: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Escherichia coli	113	>16	>16/8	>16	>16	8	8	8	>32	>16	≤0.5	≤1	16/4
Escherichia coli	114	>16	>16/8	>16	>16	>16	8	>16	>32	>16	≤0.5	≤1	4/4
Escherichia coli	115	>16	>16/8	>16	>16	≤1	≤4	>16	16	16	≤0.5	≤1	8/4
Escherichia coli	117	>16	2/4	8	>16	8	8	2	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	118	>16	16/8	4	>16	>16	≤4	2	>32	>16	≤0.5	≤1	4/4
Escherichia coli	120	>16	8/4	>16	>16	8	8	8	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	124	>16	>16/8	>16	>16	>16	16	16	>32	>16	≤0.5	≤1	8/4
Escherichia coli	125	>16	>16/8	>16	>16	16	≤4	16	>32	>16	≤0.5	≤1	8/4
Escherichia coli	131	>16	>16/8	8	>16	8	8	2	>32	>16	≤0.5	≤1	>64/4
Escherichia coli	132	>16	>16/8	>16	>16	>16	8	>16	>32	>16	≤0.5	≤1	4/4
Escherichia coli	137	>16	8/4	≤2	16	≤1	16	8	≤2	>16	≤0.5	≤1	16/4
Escherichia coli	138	>16	>16/8	>16	>16	>16	≤4	16	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	139	>16	>16/8	>16	>16	>16	≤4	16	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	140	>16	>16/8	>16	>16	>16	8	>16	>32	>16	≤0.5	≤1	4/4
Escherichia coli	141	>16	>16/8	>16	>16	16	≤4	16	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	151	>16	>16/8	>16	>16	16	16	16	>32	>16	≤0.5	≤1	4/4
Escherichia coli	158	>16	8/4	8	>16	4	≤4	4	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	160	>16	>16/8	16	>16	16	8	2	>32	>16	≤0.5	≤1	4/4
Escherichia coli	172	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	2	8	>64/4
Escherichia coli	176	>16	>16/8	>16	>16	16	16	>16	>32	>16	>4	≤1	>64/4
Escherichia coli	188	>16	>16/8	>16	>16	>16	≤4	>16	>32	>16	≤0.5	≤1	64/4
Escherichia coli	190	>16	>16/8	16	>16	8	≤4	8	>32	>16	≤0.5	≤1	8/4
Escherichia coli	195	>16	8/4	16	>16	4	≤4	8	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	201	>16	8/4	16	>16	8	≤4	8	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	206	>16	>16/8	16	>16	16	8	2	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	213	>16	16/8	>16	>16	>16	8	8	>32	>16	≤0.5	≤1	≤2/4
Escherichia coli	216	>16	>16/8	>16	>16	>16	≤4	16	>32	*	≤0.25	≤0.5	≤2/4
Escherichia coli	223	>16	>16/8	>16	>16	>16	≤4	>16	>32	*	≤0.25	≤0.5	>64/4
Escherichia vulneris	155	>16	4/2	8	>16	≤1	≤4	>16	16	16	≤0.5	≤1	≤2/4
Klebsiella oxytoca	8	>16	>16/8	>16	>16	≤1	8	4	16	>16	>4	≤1	>64/4

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Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Klebsiella oxytoca	14	>16	>16/8	>16	>16	8	8	8	>32	>16	>4	≤1	>64/4
Klebsiella oxytoca	166	>16	>16/8	>16	>16	8	≤4	2	>32	>16	>4	8	64/4
Klebsiella oxytoca	177	>16	>16/8	>16	>16	≤1	8	8	>32	>16	4	2	>64/4
Klebsiella oxytoca	197	>16	>16/8	16	>16	≤1	≤4	≤0.5	≤2	>16	≤0.5	≤1	>64/4
Klebsiella ozaenae	128	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	2	>16	>16/8	8	>16	2	≤4	4	32	>16	4	≤1	>64/4
Klebsiella pneumoniae	7	>16	>16/8	>16	>16	>16	16	8	>32	>16	>4	8	>64/4
Klebsiella pneumoniae	11	>16	>16/8	>16	>16	8	>16	4	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	18	>16	>16/8	>16	>16	8	>16	16	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	21	>16	>16/8	16	>16	2	>16	>16	32	>16	2	≤1	>64/4
Klebsiella pneumoniae	KP 22	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	KP 24	>16	>16/8	>16	>16	16	>16	>16	>32	>16	>4	2	>64/4
Klebsiella pneumoniae	26	>16	>16/8	>16	>16	≤1	>16	>16	8	>16	>4	8	>64/4
Klebsiella pneumoniae	28	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	29	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	31	>16	>16/8	8	>16	4	≤4	4	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	32	>16	>16/8	>16	>16	>16	>16	4	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	KP 34	>16	>16/8	≤2	>16	≤1	≤4	2	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	35	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	37	>16	>16/8	16	>16	*	16	16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	42	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	2	≤1	>64/4
Klebsiella pneumoniae	43	>16	>16/8	>16	>16	16	>16	16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	KP 44	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	45	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	46	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	47	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	48	>16	>16/8	>16	>16	>16	16	>16	>32	>16	>4	≤1	>64/4
Klebsiella pneumoniae	49	>16	>16/8	>16	>16	16	>16	16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	51	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	55	>16	>16/8	>16	>16	8	8	>16	>32	>16	>4	>8	>64/4

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Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Klebsiella pneumoniae	65	>16	>16/8	>16	>16	>16	16	>16	>32	>16	≤0.5	≤1	8/4
Klebsiella pneumoniae	66	>16	>16/8	>16	>16	>16	>16	16	>32	>16	≤0.5	≤1	>64/4
Klebsiella pneumoniae	69	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	2	≤1	8/4
Klebsiella pneumoniae	76	>16	>16/8	≤2	>16	≤1	≤4	8	≤2	8	≤0.5	≤1	>64/4
Klebsiella pneumoniae	77	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	2	>64/4
Klebsiella pneumoniae	92	>16	16/8	≤2	>16	4	≤4	≤0.5	>32	>16	≤0.5	≤1	≤2/4
Klebsiella pneumoniae	93	>16	>16/8	>16	>16	>16	>16	16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	98	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	2	≤1	>64/4
Klebsiella pneumoniae	99	>16	>16/8	>16	>16	8	>16	8	>32	>16	>4	8	>64/4
Klebsiella pneumoniae	102	>16	>16/8	≤2	>16	≤1	≤4	8	≤2	≤4	≤0.5	≤1	>64/4
Klebsiella pneumoniae	104	>16	>16/8	≤2	>16	≤1	≤4	8	≤2	≤4	≤0.5	≤1	>64/4
Klebsiella pneumoniae	105	>16	>16/8	>16	>16	8	8	16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	116	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	8	>64/4
Klebsiella pneumoniae	119	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	122	>16	>16/8	≤2	>16	2	≤4	8	≤2	8	≤0.5	≤1	>64/4
Klebsiella pneumoniae	123	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	129	>16	>16/8	>16	>16	8	8	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	130	>16	>16/8	>16	>16	>16	8	>16	>32	>16	>4	8	>64/4
Klebsiella pneumoniae	142	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Klebsiella pneumoniae	143	>16	>16/8	>16	>16	≤1	>16	>16	>32	>16	4	2	>64/4
Klebsiella pneumoniae	148	>16	16/8	≤2	>16	>16	≤4	4	>32	>16	≤0.5	≤1	≤2/4
Klebsiella pneumoniae	149	>16	>16/8	8	>16	>16	≤4	2	>32	>16	≤0.5	≤1	16/4
Klebsiella pneumoniae	150	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	≤0.5	≤1	>64/4
Klebsiella pneumoniae	152	>16	>16/8	≤2	>16	16	>16	16	≤2	>16	4	≤1	>64/4
Klebsiella pneumoniae	154	>16	>16/8	>16	>16	*	*	>16	>32	>16	≤0.5	≤1	>64/4
Klebsiella pneumoniae	159	>16	>16/8	>16	>16	>16	≤4	>16	>32	>16	≤0.5	≤1	32/4
Klebsiella pneumoniae	164	>16	>16/8	>16	>16	8	≤4	16	>32	>16	≤0.5	≤1	16/4
Klebsiella pneumoniae	165	>16	>16/8	>16	>16	8	>16	16	>32	>16	>4	8	>64/4
Klebsiella pneumoniae	170	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	4	>8	>64/4
Klebsiella pneumoniae	173	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	2	8	>64/4

Abbreviations: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Klebsiella pneumoniae	174	>16	>16/8	≤2	>16	>16	>16	>16	>32	>16	2	>8	>64/4
Klebsiella pneumoniae	180	>16	>16/8	>16	>16	>16	≤4	>16	>32	>16	≤0.5	≤1	4/4
Klebsiella pneumoniae	193	>16	>16/8	>16	>16	16	≤4	>16	>32	>16	≤0.5	≤1	16/4
Klebsiella pneumoniae	196	>16	>16/8	8	>16	>16	≤4	16	>32	>16	≤0.5	≤1	>64/4
Klebsiella pneumoniae	198	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	≤0.5	≤1	32/4
Klebsiella pneumoniae	204	>16	>16/8	8	>16	16	>16	2	>32	>16	≤0.5	≤1	16/4
Klebsiella pneumoniae	212	>16	>16/8	>16	>16	>16	≤4	>16	>32	>16	≤0.5	≤1	4/4
Klebsiella pneumoniae	217	>16	>16/8	>16	>16	≤1	8	>16	>32	*	0.5	≤0.5	>64/4
Klebsiella pneumoniae	230	>16	>16/8	>16	>16	>16	>16	>16	>32	*	>1	>8	>64/4
Pantoea agglomerans	178	>16	16/8	≤2	>16	4	>16	2	32	16	≤0.5	≤1	≤2/4
Pantoea agglomerans	185	>16	>16/8	≤2	>16	4	16	1	32	>16	≤0.5	≤1	≤2/4
Pantoea agglomerans	214	>16	16/8	≤2	>16	2	16	2	32	16	≤0.5	≤1	≤2/4
Proteus mirabilis	162	>16	*	≤2	>16	>16	8	2	>32	>16	≤0.5	≤1	≤2/4
Proteus mirabilis	227	>16	>16/8	≤2	>16	>16	8	2	>32	*	≤0.25	*	8/4
Pseudomonas aeruginosa	56	>16	>16/8	16	>16	>16	>16	>16	>32	>16	>4	≤1	>64/4
Pseudomonas aeruginosa	57	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	4	>64/4
Pseudomonas aeruginosa	62	>16	>16/8	16	>16	16	>16	4	>32	>16	>4	>8	32/4
Pseudomonas aeruginosa	68	>16	>16/8	>16	>16	16	>16	16	>32	>16	>4	8	64/4
Pseudomonas aeruginosa	74	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	64/4
Pseudomonas aeruginosa	80	>16	>16/8	>16	>16	16	>16	>16	>32	>16	4	≤1	>64/4
Pseudomonas aeruginosa	82	>16	>16/8	8	>16	16	>16	>16	>32	>16	>4	8	>64/4
Pseudomonas aeruginosa	88	>16	>16/8	16	>16	>16	>16	>16	>32	>16	>4	>8	>64/4
Pseudomonas aeruginosa	100	>16	>16/8	>16	>16	16	>16	>16	>32	>16	>4	8	>64/4
Pseudomonas aeruginosa	108	>16	>16/8	16	>16	16	>16	16	>32	>16	>4	≤1	64/4
Pseudomonas aeruginosa	109	>16	>16/8	16	>16	16	>16	8	>32	>16	>4	>8	32/4
Pseudomonas aeruginosa	110	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	2	>64/4
Pseudomonas aeruginosa	133	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	32/4
Pseudomonas aeruginosa[†]	153	*	*	R	*	R	*	*	*	*	*	R	R
Pseudomonas aeruginosa	157	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	64/4

Abbreviations: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

Table A.1: Minimum Inhibitory Concentration of β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMP	AMS	AZT	CFZ	CPM	FOX	CAZ	CAX	CRM	ETP	MEM	PTC
Pseudomonas aeruginosa	161	>16	>16/8	>16	>16	>16	>16	4	>32	>16	>4	>8	16/4
Pseudomonas aeruginosa	181	>16	>16/8	>16	>16	16	>16	4	32	>16	>4	>8	32/4
Pseudomonas aeruginosa	182	>16	>16/8	>16	>16	8	>16	16	>32	>16	>4	8	64/4
Pseudomonas aeruginosa	183	>16	>16/8	16	>16	16	>16	8	>32	>16	>4	8	32/4
Pseudomonas aeruginosa	184	>16	>16/8	>16	>16	16	>16	>16	>32	>16	>4	>8	>64/4
Pseudomonas aeruginosa	192	>16	>16/8	16	>16	16	>16	>16	>32	>16	>4	2	>64/4
Pseudomonas aeruginosa	199	>16	>16/8	>16	>16	>16	>16	>16	>32	>16	>4	>8	64/4
Pseudomonas aeruginosa	202	>16	>16/8	>16	>16	16	>16	>16	>32	>16	>4	8	64/4
Pseudomonas aeruginosa	205	>16	>16/8	>16	>16	16	>16	8	>32	>16	>4	≤1	32/4
Pseudomonas aeruginosa	207	>16	>16/8	>16	>16	16	>16	8	>32	>16	>4	>8	16/4
Pseudomonas aeruginosa	208	>16	>16/8	16	>16	16	>16	16	>32	>16	>4	>8	>64/4
Pseudomonas aeruginosa	211	>16	>16/8	16	>16	16	>16	>16	>32	>16	>4	8	>64/4
Pseudomonas aeruginosa	218	>16	>16/8	16	>16	16	>16	>16	>32	*	>1	8	>64/4
Pseudomonas aeruginosa	220	>16	8/4	8	>16	16	>16	8	>32	*	>1	>8	>64/4
Pseudomonas aeruginosa	221	>16	>16/8	>16	>16	>16	>16	16	>32	*	>1	>8	64/4
Pseudomonas aeruginosa	222	>16	>16/8	>16	>16	16	>16	>16	>32	*	>1	2	>64/4
Pseudomonas aeruginosa	224	>16	>16/8	>16	>16	16	>16	16	>32	*	>1	>8	32/4
Pseudomonas aeruginosa	228	>16	>16/8	16	>16	16	>16	16	>32	*	>1	4	64/4
Pseudomonas aeruginosa	231	>16	>16/8	>16	>16	16	>16	16	>32	*	>1	>8	64/4
Pseudomonas putida	38	>16	>16/8	>16	>16	8	>16	>16	>32	>16	*	>8	64/4
Pseudomonas putida	156	>16	>16/8	8	>16	8	>16	1	>32	>16	>4	>8	64/4
Sphingomonas paucimobilis[†]	59	*	*	R	*	R	*	*	*	*	*	R	R

*Not tested by University of Kentucky Microbiology Laboratory

[†]Organism tested by Etest® and reported only as S, I, or R

Abbreviations: AMP - Ampicillin; AMS - Ampicillin/Sulbactam; AZT - Aztreonam; CFZ - Cefazolin; CPM - Cefepime; FOX - Cefoxitin; CAZ - Ceftazidime; CAX - Ceftriaxone; CRM - Cefuroxime; ETP - Ertapenem; MEM - Meropenem; PTC - Piperacillin/Tazobactam

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Acinetobacter baumannii	63	>32	>2	>8	>4	>64	>8	>8	>2/38
Acinetobacter baumannii	75	>32	>2	>8	>4	>64	>8	>8	>2/38
Acinetobacter baumannii	81	≤8	>2	8	>4	>64	>8	≤2	>2/38
Acinetobacter baumannii	83	>32	>2	>8	>4	>64	>8	>8	>2/38
Acinetobacter baumannii	86	≤8	>2	8	>4	>64	>8	≤2	>2/38
Acinetobacter baumannii	191	≤8	>2	≤2	>4	>64	>8	8	>2/38
Acinetobacter baumannii	194	>32	>2	>8	>4	>64	>8	>8	>2/38
Acinetobacter baumannii	215	>32	>2	>8	>4	>64	>8	>8	>2/38
Acinetobacter baumannii	219	>32	>2	>8	>4	>64	>8	>8	>2/38
Acinetobacter baumannii	226	>32	>2	>8	>4	>64	>8	>8	>2/38
Burkholderia cepacia	67	>32	2	>8	4	>64	>8	>8	≤0.5/9.5
Citrobacter amalonaticus	36	≤8	>2	>8	>4	32	4	>8	>2/38
Citrobacter amalonaticus	91	≤8	>2	>8	>4	64	4	>8	>2/38
Citrobacter freundii	6	≤8	>2	≤2	4	≤16	4	≤2	>2/38
Citrobacter freundii	27	>32	>2	>8	>4	≤16	≤2	>8	>2/38
Citrobacter freundii	50	≤8	>2	>8	4	≤16	4	>8	>2/38
Citrobacter freundii	54	≤8	>2	>8	>4	≤16	4	>8	≤0.5/9.5
Citrobacter freundii	101	≤8	>2	8	>4	≤16	>8	4	>2/38
Citrobacter freundii	127	≤8	>2	>8	>4	≤16	>8	>8	>2/38
Citrobacter freundii	135	≤8	≤0.5	≤2	≤1	≤16	≤2	8	>2/38
Citrobacter freundii	145	≤8	>2	≤2	>4	≤16	4	≤2	≤0.5/9.5
Citrobacter freundii	147	≤8	≤0.5	≤2	≤1	≤16	≤2	≤2	≤0.5/9.5
Citrobacter freundii	225	≤8	2	≤2	2	≤16	>8	≤2	≤0.5/9.5
Citrobacter youngae	136	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Enterobacter aerogenes	97	≤8	≤0.5	≤2	≤1	≤16	≤2	≤2	≤0.5/9.5
Enterobacter aerogenes	163	≤8	1	≤2	≤1	32	≤2	≤2	>2/38
Enterobacter aerogenes	179	≤8	≤0.5	8	≤1	>64	≤2	>8	>2/38
Enterobacter aerogenes	187	≤8	≤0.5	≤2	≤1	>64	>8	≤2	≤0.5/9.5

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Enterobacter cloacae	1	≤8	>2	>8	>4	>64	>8	>8	>2/38
Enterobacter cloacae	3	≤8	1	>8	≤1	64	≤2	>8	>2/38
Enterobacter cloacae	4	≤8	1	>8	≤1	64	≤2	>8	>2/38
Enterobacter cloacae	5	≤8	≤0.5	>8	≤1	64	≤2	>8	>2/38
Enterobacter cloacae	9	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter cloacae	10	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter cloacae	12	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter cloacae	15	≤8	1	8	≤1	32	≤2	>8	≤0.5/9.5
Enterobacter cloacae	16	≤8	>2	>8	>4	32	8	>8	>2/38
Enterobacter cloacae	17	≤8	>2	>8	>4	64	4	>8	>2/38
Enterobacter cloacae	19	≤8	1	8	≤1	64	≤2	>8	≤0.5/9.5
Enterobacter cloacae	20	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter cloacae	23	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter cloacae	30	≤8	>2	>8	>4	>64	>8	>8	>2/38
Enterobacter cloacae	39	≤8	>2	>8	2	32	≤2	>8	≤0.5/9.5
Enterobacter cloacae	40	≤8	>2	>8	>4	>64	4	>8	>2/38
Enterobacter cloacae	41	16	1	>8	≤1	64	>8	>8	>2/38
Enterobacter cloacae	52	≤8	>2	8	>4	32	8	>8	≤0.5/9.5
Enterobacter cloacae	53	≤8	>2	>8	>4	>64	>8	>8	>2/38
Enterobacter cloacae	61	≤8	>2	>8	>4	>64	8	8	>2/38
Enterobacter cloacae	70	≤8	>2	>8	>4	>64	>8	8	>2/38
Enterobacter cloacae	73	≤8	≤0.5	>8	≤1	>64	≤2	8	>2/38
Enterobacter cloacae	96	≤8	≤0.5	≤2	≤1	≤16	≤2	≤2	≤0.5/9.5
Enterobacter cloacae	107	≤8	>2	>8	>4	32	4	>8	≤0.5/9.5
Enterobacter cloacae	121	≤8	1	>8	≤1	>64	4	>8	>2/38
Enterobacter cloacae	126	≤8	>2	>8	>4	32	8	>8	≤0.5/9.5
Enterobacter cloacae	134	≤8	1	>8	≤1	>64	≤2	>8	>2/38
Enterobacter cloacae	144	≤8	>2	>8	2	≤16	≤2	>8	≤0.5/9.5
Enterobacter cloacae	167	≤8	1	≤2	*	64	≤2	8	>2/38
Enterobacter cloacae	168	≤8	>2	8	>4	64	8	>8	≤0.5/9.5

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Enterobacter cloacae	169	≤8	1	≤2	≤1	64	≤2	8	>2/38
Enterobacter cloacae	171	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter cloacae	175	≤8	1	>8	≤1	32	≤2	>8	>2/38
Enterobacter cloacae	189	≤8	≤0.5	≤2	≤1	64	≤2	8	>2/38
Enterobacter cloacae	200	≤8	>2	>8	>4	64	8	>8	>2/38
Enterobacter cloacae	203	≤8	1	>8	≤1	64	≤2	>8	>2/38
Enterobacter cloacae	209	≤8	>2	>8	>4	>64	8	>8	≤0.5/9.5
Enterobacter gergoviae	13	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter gergoviae	95	≤8	≤0.5	≤2	≤1	64	>8	≤2	≤0.5/9.5
Enterobacter hormaechei	186	≤8	>2	>8	4	>64	8	>8	>2/38
Enterobacter sp.	146	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Enterobacter sp.	210	≤8	>2	8	>4	≤16	4	>8	≤0.5/9.5
Escherichia coli	25	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	33	≤8	>2	>8	>4	>64	≤2	8	≤0.5/9.5
Escherichia coli	58	≤8	>2	≤2	>4	32	>8	≤2	>2/38
Escherichia coli	60	≤8	1	≤2	≤1	≤16	>8	≤2	>2/38
Escherichia coli	64	≤8	>2	≤2	>4	≤16	≤2	≤2	>2/38
Escherichia coli	71	≤8	>2	≤2	>4	64	>8	>8	>2/38
Escherichia coli	72	≤8	>2	≤2	>4	≤16	≤2	≤2	>2/38
Escherichia coli	78	>32	>2	>8	>4	≤16	>8	>8	>2/38
Escherichia coli	79	≤8	>2	≤2	>4	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	84	≤8	>2	>8	>4	≤16	>8	>8	≤0.5/9.5
Escherichia coli	85	≤8	>2	≤2	>4	>64	>8	>8	>2/38
Escherichia coli	87	16	>2	≤2	>4	>64	>8	>8	>2/38
Escherichia coli	90	≤8	>2	≤2	>4	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	94	≤8	>2	>8	>4	≤16	>8	>8	≤0.5/9.5
Escherichia coli	103	≤8	>2	≤2	>4	64	>8	>8	>2/38
Escherichia coli	106	≤8	>2	≤2	>4	≤16	>8	>8	>2/38
Escherichia coli	111	≤8	>2	>8	>4	≤16	≤2	8	>2/38
Escherichia coli	112	≤8	>2	≤2	>4	≤16	≤2	>8	>2/38

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Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Escherichia coli	113	≤8	>2	≤2	>4	≤16	≤2	>8	>2/38
Escherichia coli	114	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	115	>32	≤0.5	>8	≤1	≤16	>8	>8	>2/38
Escherichia coli	117	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	118	≤8	>2	≤2	>4	32	≤2	≤2	≤0.5/9.5
Escherichia coli	120	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	124	≤8	>2	>8	>4	≤16	>8	>8	>2/38
Escherichia coli	125	≤8	>2	>8	>4	≤16	>8	>8	≤0.5/9.5
Escherichia coli	131	≤8	>2	>8	>4	≤16	≤2	8	>2/38
Escherichia coli	132	≤8	>2	≤2	>4	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	137	≤8	≤0.5	≤2	≤1	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	138	≤8	>2	≤2	>4	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	139	≤8	>2	≤2	>4	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	140	≤8	>2	8	>4	≤16	≤2	8	≤0.5/9.5
Escherichia coli	141	≤8	>2	≤2	>4	≤16	≤2	≤2	≤0.5/9.5
Escherichia coli	151	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	158	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	160	≤8	>2	>8	>4	≤16	>8	8	>2/38
Escherichia coli	172	≤8	1	≤2	≤1	≤16	≤2	>8	>2/38
Escherichia coli	176	≤8	>2	≤2	>4	≤16	≤2	≤2	>2/38
Escherichia coli	188	≤8	>2	≤2	>4	≤16	>8	>8	>2/38
Escherichia coli	190	≤8	>2	>8	>4	≤16	>8	>8	>2/38
Escherichia coli	195	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	201	≤8	≤0.5	≤2	≤1	≤16	>8	≤2	≤0.5/9.5
Escherichia coli	206	≤8	>2	>8	>4	≤16	≤2	8	≤0.5/9.5
Escherichia coli	213	≤8	≤0.5	≤2	≤1	≤16	>8	≤2	≤0.5/9.5
Escherichia coli	216	≤8	>2	≤2	>4	≤16	>8	≤2	>2/38
Escherichia coli	223	≤8	>2	≤2	>4	≤16	>8	≤2	≤0.5/9.5
Escherichia vulneris	155	≤8	≤0.5	≤2	≤1	64	≤2	≤2	≤0.5/9.5
Klebsiella oxytoca	8	≤8	≤0.5	≤2	≤1	≤16	≤2	≤2	1/19

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Klebsiella oxytoca	14	≤8	2	>8	2	≤16	4	>8	>2/38
Klebsiella oxytoca	166	≤8	≤0.5	≤2	≤1	32	≤2	≤2	≤0.5/9.5
Klebsiella oxytoca	177	≤8	≤0.5	>8	≤1	≤16	≤2	>8	≤0.5/9.5
Klebsiella oxytoca	197	≤8	≤0.5	≤2	≤1	32	≤2	≤2	≤0.5/9.5
Klebsiella ozaenae	128	≤8	>2	4	>4	≤16	4	4	>2/38
Klebsiella pneumoniae	2	≤8	≤0.5	>8	≤1	64	>8	>8	>2/38
Klebsiella pneumoniae	7	≤8	≤0.5	≤2	≤1	64	≤2	≤2	>2/38
Klebsiella pneumoniae	11	≤8	≤0.5	≤2	≤1	>64	8	≤2	2/38
Klebsiella pneumoniae	18	≤8	≤0.5	≤2	≤1	>64	8	≤2	>2/38
Klebsiella pneumoniae	21	≤8	1	4	≤1	≤16	≤2	>8	>2/38
Klebsiella pneumoniae	KP 22	32	>2	>8	>4	>64	>8	>8	>2/38
Klebsiella pneumoniae	KP 24	≤8	>2	8	>4	>64	4	>8	≤0.5/9.5
Klebsiella pneumoniae	26	>32	>2	>8	>4	>64	≤2	>8	>2/38
Klebsiella pneumoniae	28	32	>2	4	>4	>64	8	>8	>2/38
Klebsiella pneumoniae	29	32	>2	4	>4	>64	4	>8	2/38
Klebsiella pneumoniae	31	≤8	≤0.5	>8	≤1	>64	≤2	>8	2/38
Klebsiella pneumoniae	32	≤8	>2	≤2	4	64	8	≤2	≤0.5/9.5
Klebsiella pneumoniae	KP 34	≤8	≤0.5	≤2	≤1	64	≤2	≤2	≤0.5/9.5
Klebsiella pneumoniae	35	≤8	>2	>8	>4	>64	8	>8	≤0.5/9.5
Klebsiella pneumoniae	37	>32	≤0.5	>8	≤1	32	≤2	>8	≤0.5/9.5
Klebsiella pneumoniae	42	≤8	2	>8	≤1	≤16	≤2	>8	>2/38
Klebsiella pneumoniae	43	≤8	>2	>8	>4	64	4	>8	≤0.5/9.5
Klebsiella pneumoniae	KP 44	≤8	>2	>8	>4	>64	>8	>8	1/19
Klebsiella pneumoniae	45	≤8	>2	>8	>4	>64	4	>8	2/38
Klebsiella pneumoniae	46	>32	>2	>8	>4	>64	4	>8	>2/38
Klebsiella pneumoniae	47	>32	>2	>8	>4	>64	≤2	>8	>2/38
Klebsiella pneumoniae	48	>32	>2	>8	>4	>64	4	>8	≤0.5/9.5
Klebsiella pneumoniae	49	≤8	>2	>8	4	32	4	>8	≤0.5/9.5
Klebsiella pneumoniae	51	≤8	>2	8	>4	64	4	8	≤0.5/9.5
Klebsiella pneumoniae	55	16	>2	≤2	>4	64	>8	>8	>2/38

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Klebsiella pneumoniae	65	≤8	>2	≤2	>4	>64	8	≤2	>2/38
Klebsiella pneumoniae	66	≤8	≤0.5	≤2	≤1	64	4	≤2	≤0.5/9.5
Klebsiella pneumoniae	69	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Klebsiella pneumoniae	76	≤8	≤0.5	≤2	≤1	32	>8	≤2	≤0.5/9.5
Klebsiella pneumoniae	77	≤8	>2	>8	4	>64	≤2	8	≤0.5/9.5
Klebsiella pneumoniae	92	≤8	1	>8	≤1	64	>8	>8	>2/38
Klebsiella pneumoniae	93	≤8	>2	>8	>4	64	4	>8	≤0.5/9.5
Klebsiella pneumoniae	98	≤8	≤0.5	4	≤1	≤16	>8	>8	≤0.5/9.5
Klebsiella pneumoniae	99	≤8	≤0.5	≤2	≤1	>64	>8	≤2	2/38
Klebsiella pneumoniae	102	≤8	≤0.5	≤2	≤1	32	≤2	≤2	≤0.5/9.5
Klebsiella pneumoniae	104	≤8	≤0.5	≤2	≤1	>64	>8	≤2	>2/38
Klebsiella pneumoniae	105	≤8	≤0.5	≤2	≤1	64	≤2	≤2	1/19
Klebsiella pneumoniae	116	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Klebsiella pneumoniae	119	16	>2	>8	>4	>64	4	>8	>2/38
Klebsiella pneumoniae	122	≤8	≤0.5	≤2	≤1	>64	>8	≤2	>2/38
Klebsiella pneumoniae	123	≤8	>2	>8	>4	>64	4	>8	>2/38
Klebsiella pneumoniae	129	16	>2	≤2	>4	32	≤2	>8	>2/38
Klebsiella pneumoniae	130	≤8	1	>8	≤1	>64	≤2	>8	≤0.5/9.5
Klebsiella pneumoniae	142	≤8	≤0.5	≤2	≤1	64	≤2	≤2	≤0.5/9.5
Klebsiella pneumoniae	143	≤8	>2	>8	>4	>64	>8	8	>2/38
Klebsiella pneumoniae	148	≤8	≤0.5	≤2	≤1	32	≤2	≤2	≤0.5/9.5
Klebsiella pneumoniae	149	≤8	1	>8	≤1	>64	>8	≤2	>2/38
Klebsiella pneumoniae	150	≤8	>2	8	>4	>64	4	8	>2/38
Klebsiella pneumoniae	152	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Klebsiella pneumoniae	154	≤8	>2	>8	>4	64	>8	>8	2/38
Klebsiella pneumoniae	159	≤8	>2	>8	>4	>64	≤2	8	≤0.5/9.5
Klebsiella pneumoniae	164	≤8	>2	≤2	≤1	32	≤2	8	>2/38
Klebsiella pneumoniae	165	≤8	>2	>8	>4	64	8	>8	≤0.5/9.5
Klebsiella pneumoniae	170	≤8	>2	>8	4	32	>8	>8	>2/38
Klebsiella pneumoniae	173	≤8	1	≤2	≤1	32	≤2	8	>2/38

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Klebsiella pneumoniae	174	≤8	1	>8	≤1	≤16	>8	>8	>2/38
Klebsiella pneumoniae	180	≤8	1	≤2	≤1	64	≤2	≤2	>2/38
Klebsiella pneumoniae	193	≤8	>2	>8	≤1	64	>8	>8	>2/38
Klebsiella pneumoniae	196	≤8	>2	>8	≤1	>64	>8	>8	>2/38
Klebsiella pneumoniae	198	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Klebsiella pneumoniae	204	≤8	>2	>8	>4	>64	>8	8	>2/38
Klebsiella pneumoniae	212	≤8	2	≤2	≤1	>64	≤2	≤2	>2/38
Klebsiella pneumoniae	217	≤8	>2	>8	>4	>64	>8	>8	>2/38
Klebsiella pneumoniae	230	≤8	>2	4	>4	>64	8	>8	>2/38
Pantoea agglomerans	178	≤8	≤0.5	≤2	≤1	>64	≤2	≤2	≤0.5/9.5
Pantoea agglomerans	185	≤8	≤0.5	≤2	≤1	>64	≤2	≤2	≤0.5/9.5
Pantoea agglomerans	214	≤8	≤0.5	≤2	≤1	>64	≤2	≤2	≤0.5/9.5
Proteus mirabilis	162	≤8	>2	≤2	>4	>64	>8	>8	>2/38
Proteus mirabilis	227	16	>2	>8	>4	>64	>8	>8	≤0.5/9.5
Pseudomonas aeruginosa	56	32	>2	>8	>4	>64	>8	4	>2/38
Pseudomonas aeruginosa	57	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	62	16	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	68	≤8	2	≤2	2	>64	>8	≤2	*
Pseudomonas aeruginosa	74	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	80	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	82	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	88	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	100	≤8	≤0.5	≤2	≤1	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	108	>32	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	109	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	110	≤8	>2	≤2	*	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	133	>32	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa[†]	153	R	*	R	R	*	*	R	*
Pseudomonas aeruginosa	157	≤8	>2	>8	>4	>64	>8	>8	>2/38

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Table A.2: Minimum Inhibitory Concentration of Non-β-lactam Antimicrobials for University of Kentucky Clinical Isolates by BD Phoenix™

ORGANISM	ISOLATE	AMK	CIP	GEN	LEV	NIT	TET	TOB	SXT
Pseudomonas aeruginosa	161	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	181	≤8	>2	4	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	182	≤8	>2	4	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	183	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	184	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	192	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	199	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	202	≤8	≤0.5	≤2	≤1	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	205	≤8	2	≤2	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	207	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	208	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	211	≤8	>2	>8	>4	>64	8	8	>2/38
Pseudomonas aeruginosa	218	≤8	>2	8	>4	>64	8	≤2	>2/38
Pseudomonas aeruginosa	220	≤8	>2	>8	4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	221	32	>2	8	>4	>64	>8	4	>2/38
Pseudomonas aeruginosa	222	≤8	2	≤2	4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	224	≤8	>2	4	>4	>64	>8	≤2	>2/38
Pseudomonas aeruginosa	228	≤8	>2	>8	>4	>64	>8	>8	>2/38
Pseudomonas aeruginosa	231	≤8	>2	≤2	>4	>64	>8	≤2	>2/38
Pseudomonas putida	38	≤8	2	4	4	>64	>8	4	*
Pseudomonas putida	156	≤8	2	8	4	>64	>8	4	*
Sphingomonas paucimobilis[†]	59	R	*	R	*	*	*	R	S

*Not tested by University of Kentucky Clinical Microbiology Laboratory

[†]Organism tested by Etest® and reported only as S, I, or R

Abbreviations: AMK - Amikacin; CIP - Ciprofloxacin; GEN - Gentamicin; LEV - Levofloxacin; NIT - Nitrofurantoin; TET - Tetracycline; TOB - Tobramycin; SXT - Sulfamethoxazole/Trimethoprim

Appendix B:

Cumulative Antimicrobial Susceptibility Summary Report from BD Phoenix™

Tables B.1-B.4 provide the cumulative summary antimicrobial susceptibility results from the *in vitro* susceptibility testing performed by the University of Kentucky Clinical Microbiology Laboratory using BD Phoenix™.

Reports were only generated for species with testing data for ≥ 30 isolates.[245] Duplicate isolates were not collected so that only the first isolate (i.e. one species per patient per collection period) is represented in the tables. Percentage intermediate or resistant were not included in the report.

The following abbreviations are used in Tables B.1-B.4: MIC_{50} - Minimum Inhibitory Concentration (MIC) for 50% of isolates, MIC_{90} - Minimum Inhibitory Concentration (MIC) for 90% of isolates, %S - percentage susceptible.

Table B.1: Cumulative Antimicrobial Susceptibility Summary Report for all 64 *Klebsiella pneumoniae* Clinical Isolates

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range	%S
Ampicillin	>16	>16	>16	0%
Ampicillin/Sulbactam	>16/8	>16/8	16/8 - >16/8	0%
Aztreonam	>16	>16	≤2 - >16	19%
Cefazolin	>16	>16	>16	0%
Cefepime	16	>16	≤1 - >16	27%
Cefoxitin	>16	>16	≤4 - >16	33%
Ceftazidime	>16	>16	≤0.5 - >16	14%
Ceftriaxone	>32	>32	≤2 - >32	8%
Cefuroxime	>16	>16	≤4 - >16	6%
Ertapenem	>4	>4	≤0.5 - >4	31%
Meropenem	≤1	>8	≤1 - >8	52%
Piperacillin/Tazobactam	>64/4	>64/4	≤2/4 - >64/4	16%
Amikacin	≤8	32	≤8 - >32	88%
Ciprofloxacin	>2	>2	≤0.5 - >2	38%
Gentamicin	8	>8	≤2 - >8	47%
Levofloxacin	4	>4	≤1 - >4	45%
Nitrofurantoin	>64	>64	≤16 - >64	22%
Tetracycline	4	>8	≤2 - >8	52%
Tobramycin	>8	>8	≤2 - >8	34%
Sulfamethoxazole/Trimethoprim	>2/38	>2/38	≤0.5/9.5 - >2/38	44%

Abbreviations: PMB - Polymyxin B; COL - Colistin; RIF - Rifampin; AMI - Amikacin; MIN - Minocycline; TIG - Tigecycline; MEM - Meropenem; LEV - Levofloxacin; CPM - Cefepime; PTC - Piperacillin/Tazobactam; FOS - Fosfomycin

Table B.2: Cumulative Antimicrobial Susceptibility Summary Report for all 37 *Enterobacter cloacae* Clinical Isolates

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range	%S
Ampicillin	>16	>16	>16	0%
Ampicillin/Sulbactam	>16/8	>16/8	>16/8	0%
Aztreonam	>16	>16	≤2 - >16	3%
Cefazolin	>16	>16	>16	0%
Cefepime	>16	>16	≤1 - >16	14%
Cefoxitin	>16	>16	>16	0%
Ceftazidime	>16	>16	4 - >16	3%
Ceftriaxone	>32	>32	8 - >32	0%
Cefuroxime	>16	>16	>16	0%
Ertapenem	>4	>4	≤0.5 - >4	5%
Meropenem	4	>8	≤1 - >8	32%
Piperacillin/Tazobactam	>64/4	>64/4	4/4 - >64/4	8%
Amikacin	≤8	≤8	≤8 - 16	100%
Ciprofloxacin	>2	>2	≤0.5 - >2	41%
Gentamicin	>8	>8	≤2 - >8	11%
Levofloxacin	>4	>4	≤1 - >4	44%
Nitrofurantoin	64	>64	≤16 - >64	24%
Tetracycline	8	8	≤2 - >8	49%
Tobramycin	>8	>8	≤2 - >8	3%
Sulfamethoxazole/Trimethoprim	>2/38	>2/38	≤0.5/9.5 - >2/38	43%

Abbreviations: PMB - Polymyxin B; COL - Colistin; RIF - Rifampin; AMI - Amikacin; MIN - Minocycline; TIG - Tigecycline; MEM - Meropenem; LEV - Levofloxacin; CPM - Cefepime; PTC - Piperacillin/Tazobactam; FOS - Fosfomycin

Table B.3: Cumulative Antimicrobial Susceptibility Summary Report for all 46 *Escherichia coli* Clinical Isolates

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range	%S
Ampicillin	>16	>16	>16	0%
Ampicillin/Sulbactam	>16/8	>16/8	4/2 - 16/8	13%
Aztreonam	>16	>16	≤2 - >16	15%
Cefazolin	>16	>16	16 - >16	0%
Cefepime	16	>16	≤1 - >16	11%
Cefoxitin	8	16	≤4 - >16	76%
Ceftazidime	16	>16	1 - >16	28%
Ceftriaxone	>32	>32	≤2 - >32	2%
Cefuroxime	>16	>16	16 - >16	0%
Ertapenem	≤0.5	1	≤0.5 - >4	89%
Meropenem	≤1	≤1	≤1 - 8	98%
Piperacillin/Tazobactam	4/4	>64/4	≤2/4 - 64/4	72%
Amikacin	≤8	≤8	≤8 - >32	93%
Ciprofloxacin	>2	>2	≤0.5 - >2	13%
Gentamicin	≤2	>8	≤2 - >8	72%
Levofloxacin	>4	>4	≤1 - >4	13%
Nitrofurantoin	≤16	64	≤16 - >64	89%
Tetracycline	>8	>8	≤2 - >8	41%
Tobramycin	≤2	>8	≤2 - >8	52%
Sulfamethoxazole/Trimethoprim	>2/38	>2/38	≤0.5/9.5 - >2/38	37%

Abbreviations: PMB - Polymyxin B; COL - Colistin; RIF - Rifampin; AMI - Amikacin; MIN - Minocycline; TIG - Tigecycline; MEM - Meropenem; LEV - Levofloxacin; CPM - Cefepime; PTC - Piperacillin/Tazobactam; FOS - Fosfomycin

Table B.4: Cumulative Antimicrobial Susceptibility Summary Report for all 34 *Pseudomonas aeruginosa* Clinical Isolates

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range	%S
Ampicillin	>16	>16	>16	*
Ampicillin/Sulbactam	>16/8	>16/8	8/4 - >16/8	*
Aztreonam	>16	>16	8 - >16	6%
Cefazolin	>16	>16	>16	*
Cefepime	16	>16	8 - >16	6%
Cefoxitin	>16	>16	>16	*
Ceftazidime	>16	>16	4 - >16	9%
Ceftriaxone	>32	>32	32 - >32	*
Cefuroxime	>16	>16	>16	*
Ertapenem	>4	>4	>1	*
Meropenem	8	>8	≤1 - >8	21%
Piperacillin/Tazobactam	64/4	>64/4	16/4 - >64/4	6%
Amikacin	≤8	32	≤8 - >32	85%
Ciprofloxacin	>2	>2	≤0.5 - >2	6%
Gentamicin	>8	>8	≤2 - >8	41%
Levofloxacin	>4	>4	≤1 - >4	9%
Nitrofurantoin	>64	>64	>64	*
Tetracycline	>8	>8	8 - >8	*
Tobramycin	4	>8	≤2 - >8	50%
Sulfamethoxazole/Trimethoprim	>2/38	>2/38	>2/38	*

*No CLSI or FDA breakpoints

Abbreviations: PMB - Polymyxin B; COL - Colistin; RIF - Rifampin; AMI - Amikacin; MIN - Minocycline; TIG - Tigecycline; MEM - Meropenem; LEV - Levofloxacin; CPM - Cefepime; PTC - Piperacillin/Tazobactam; FOS - Fosfomycin

Appendix C:

Minimum Inhibitory Concentrations Determined by Broth Microdilution Susceptibility Testing and Etest®

Table C.1 provide the results of *in vitro* susceptibility testing performed by broth microdilution and Etest®.

The following abbreviations are used in Table C.1:

PMB - Polymyxin B

COL - Colistin

RIF - Rifampin

AMI - Amikacin

MIN - Minocycline

TIG - Tigecycline

MEM - Meropenem

LEV - Levofloxacin

CPM - Cefepime

PTC - Piperacillin/Tazobactam

FOS - Fosfomycin

Table C.1: Minimum Inhibitory Concentration (MIC) Values for 24 *Klebsiella pneumoniae* Clinical Isolates Determined by Broth Microdilution

Isolate	PMB	COL*	RIF*	AMI	MIN	TIG	MEM	LEV	CPM	PTC	FOS†
7	0.06	≤0.03	32	1	2*	0.125*	0.015	≤0.03	≤0.125*	≤0.25/4	32
21	0.5	0.5	32	1	4*	0.25*	1	≤0.03	4*	≤0.25/4	48
KP 22	0.06	0.06	>32	64	16*	2*	16	64	32*	512/4	16
KP 24	0.125	0.125	32	4	4*	0.25*	32	64	64*	>512/4	24
28	8	8	>32	64	32	2	128	64	>256	>512/4	64
29	2	-	-	32	8*	2*	32	>64	32*	-	-
31	0.25	0.125	32	4	4*	0.125*	0.015	≤0.03	≤0.125*	≤0.25/4	12
32	0.125	0.06	16	2	4*	0.25*	8	2	256*	≤0.25/4	256
KP 34	0.125	0.125	32	1	2*	0.25*	4	0.06	2*	≤0.25/4	12
35	0.125	0.06	>32	2	32*	2*	16	4	32*	>512/4	>1024
37	0.125	0.25	>32	128	4*	0.25*	16	≤0.03	>256*	>512/4	32
43	0.06	0.06	>32	4	16*	1*	16	8	32*	≤0.25/4	>1024
KP 44	0.06	0.06	>32	4	1*	4*	128	64	256*	>512/4	48
45	>256	-	-	4	16	4	128	>32	>256	-	-
46	0.25	-	-	128	8	2	32	>32	>256	-	-
47	128	-	-	128	8	2	32	>32	>256	-	-
48	2*	-	-	64	8	1*	8*	>32*	16*	-	-
49	0.125*	-	-	1	8*	1*	16*	4*	8*	-	-
51	0.125*	-	-	0.5	16*	4*	32*	8*	32*	-	-
55	0.125*	-	-	128	4	0.5	8*	16	1	-	-
69	0.25*	-	-	0.5	>64	2	≤0.125*	32	256	-	-
77	0.125*	-	-	2	4	1	0.25*	8	16	-	-
93	4*	-	-	2	32	2	8*	32	32	-	-
98	0.5*	-	-	1	8*	2*	0.06*	0.125*	4*	-	-

MIC values expressed in the table are in units of µg/mL
*Only a single experiment performed, but in duplicate
†Etest® only
- Not tested

Abbreviations: PMB - Polymyxin B; COL - Colistin; RIF - Rifampin; AMI - Amikacin; MIN - Minocycline; TIG - Tigecycline; MEM - Meropenem; LEV - Levofloxacin; CPM - Cefepime; PTC - Piperacillin/Tazobactam; FOS - Fosfomycin

Appendix D:

Time-kill Studies

Figures D.1-D.24 illustrate four carbapenem-resistant *Klebsiella pneumoniae* clinical isolates exposed to polymyxin B and meropenem alone and in combination. Graphs are separated by antimicrobial used and by isolate. The order begins with the least resistant isolate and ends with the most resistant isolate by meropenem MIC. For each isolate, the time-kill curves for meropenem, polymyxin, and then their combination are displayed. Ultimately, each antimicrobial and isolate combination (12 total combinations) is represented by two graphs, the first showing all experimental data for comparison, and the second showing the geometric mean of replicate experiments with error bars indicating one standard deviation.

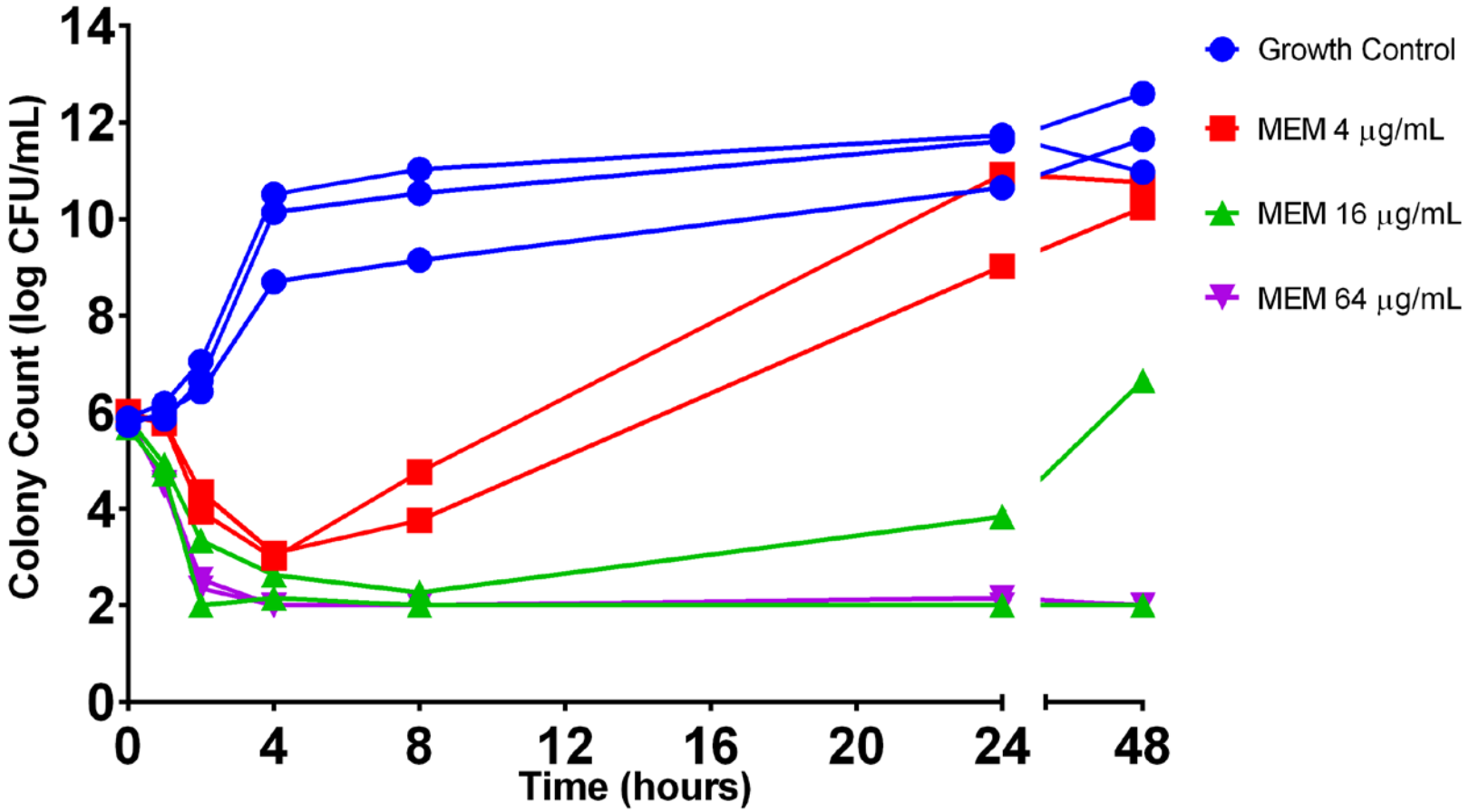

Figure D.1: Time-kill curve of meropenem (MEM) against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

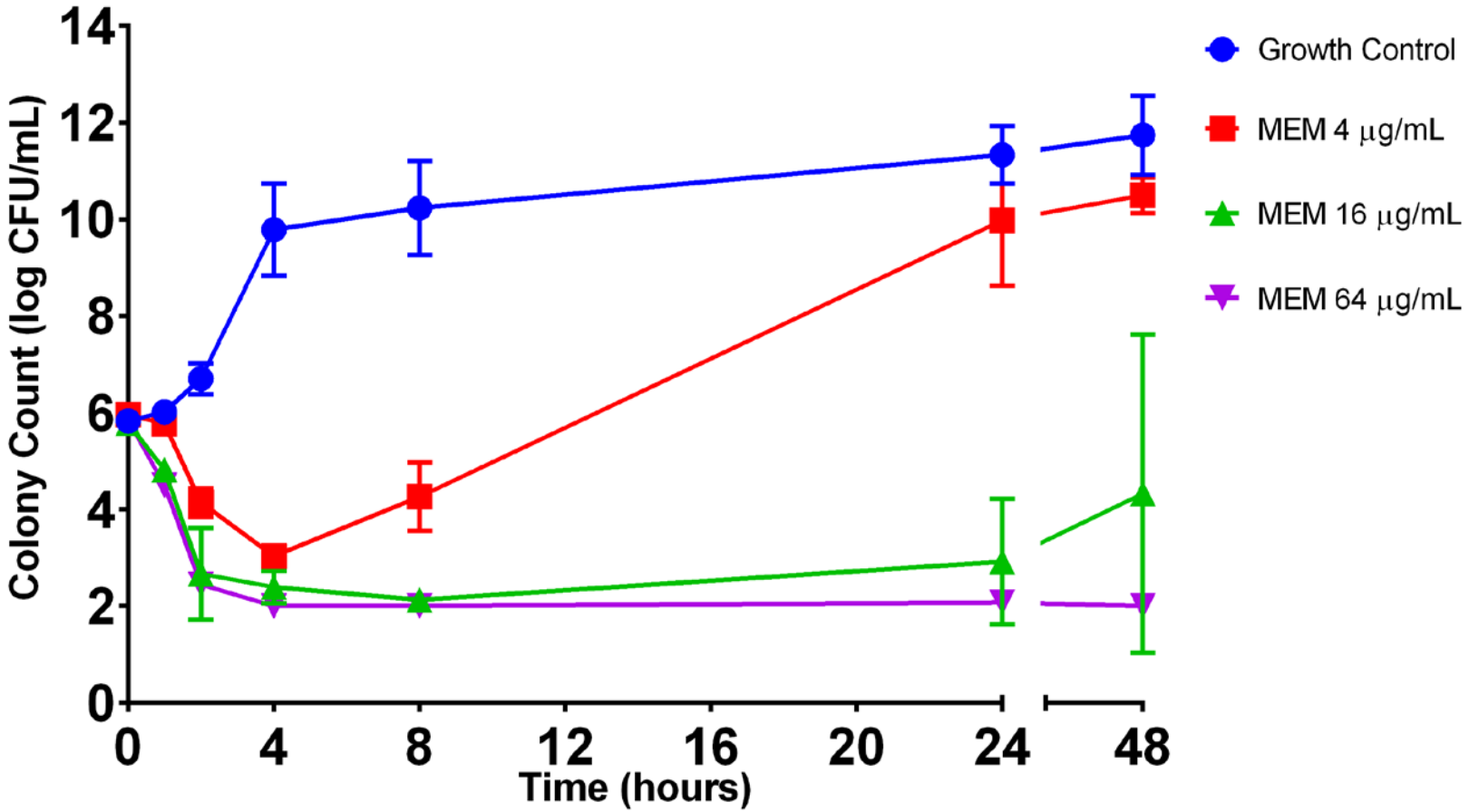

Figure D.2: Time-kill curve of meropenem (MEM) against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

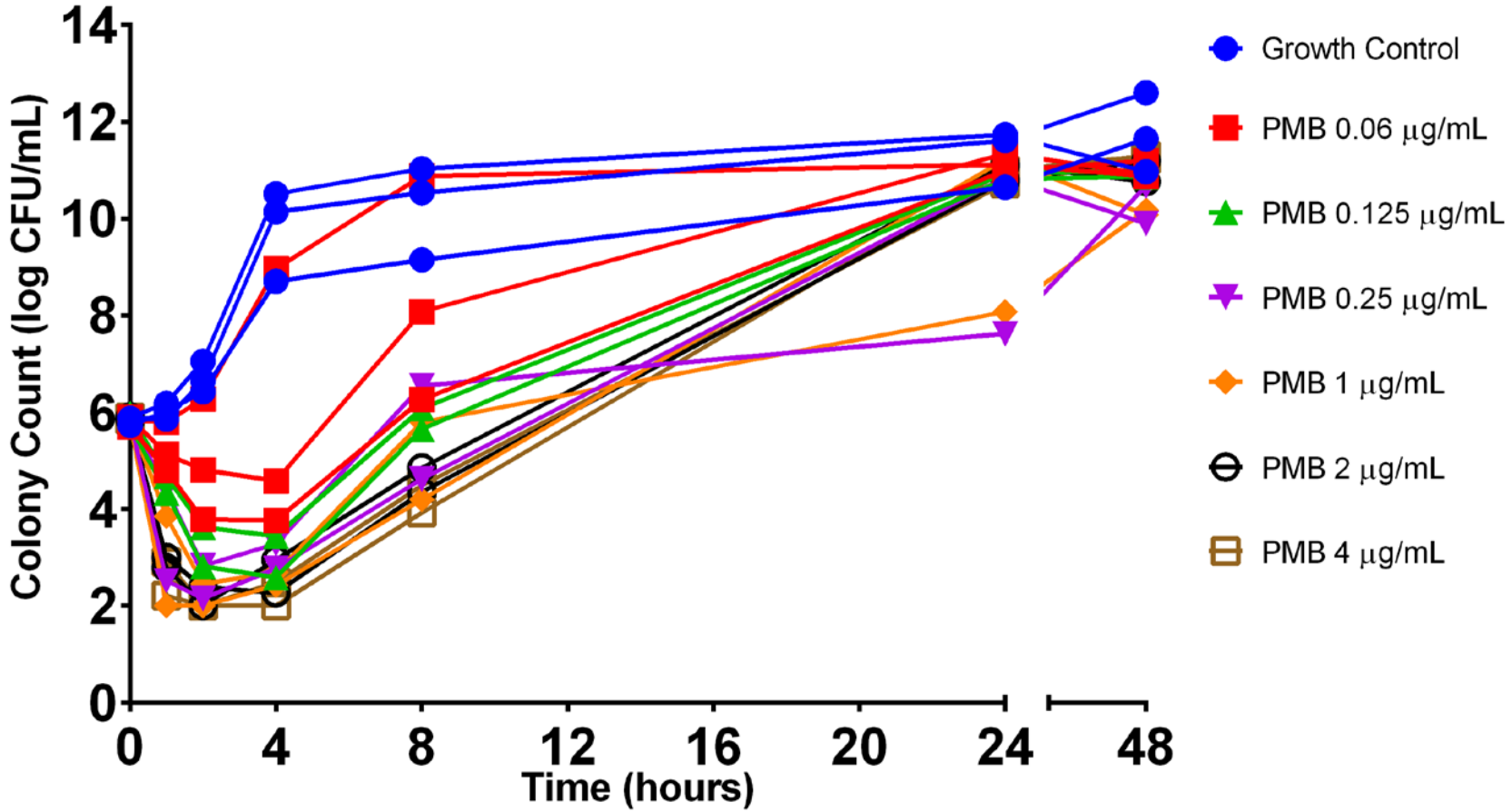

Figure D.3: Time-kill curve of polymyxin B (PMB) against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

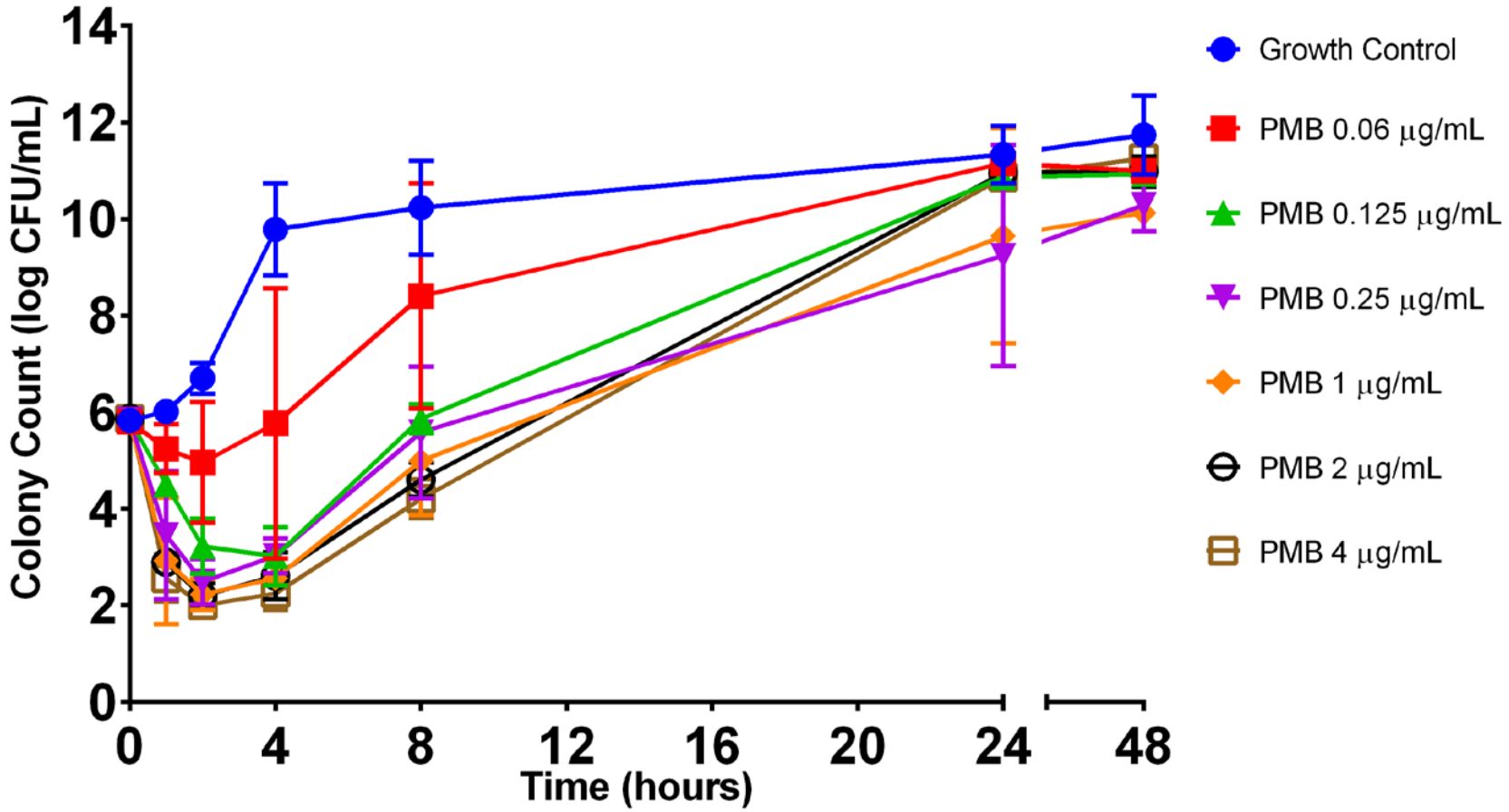

Figure D.4: Time-kill curve of polymyxin B (PMB) alone against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

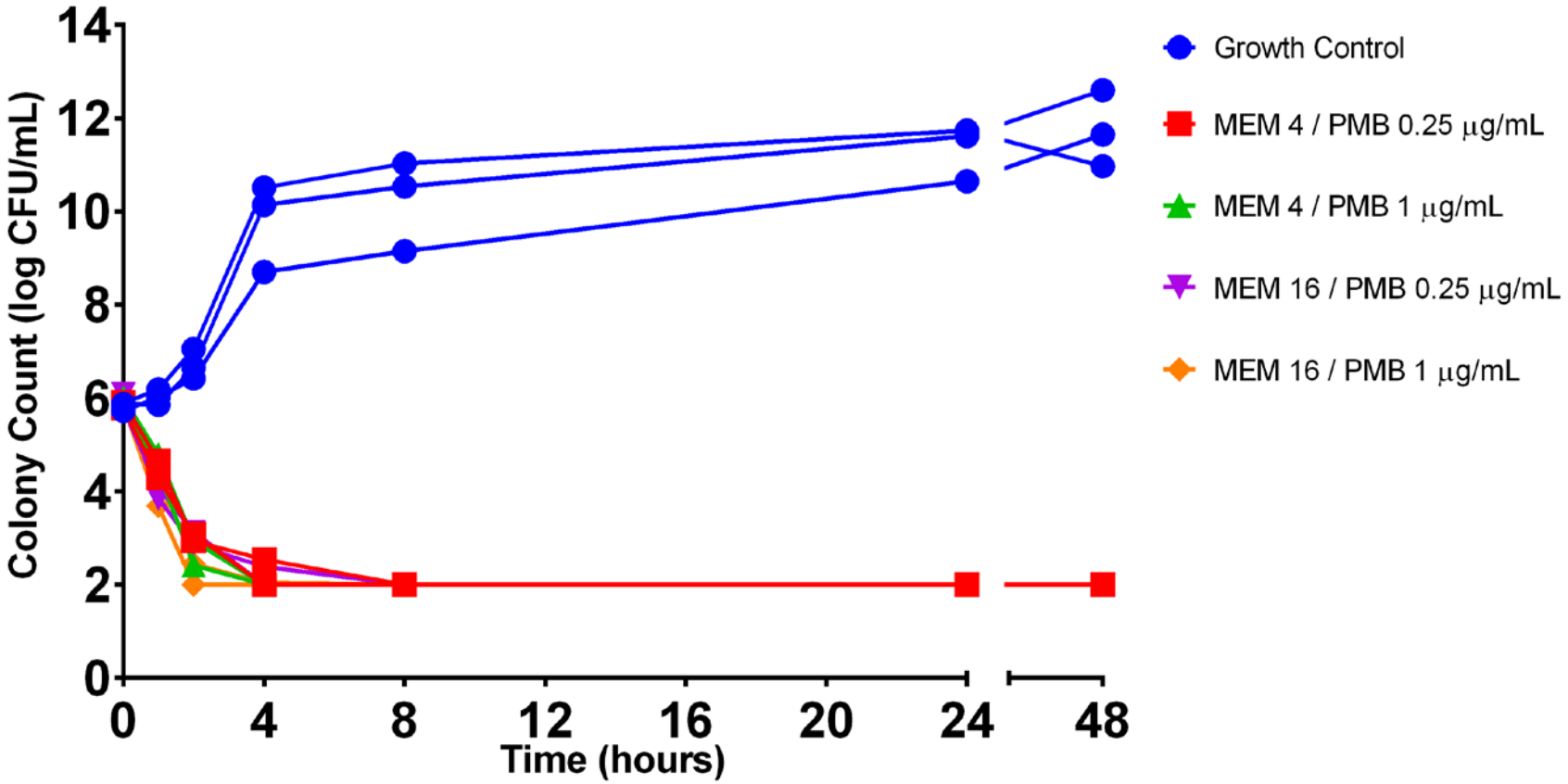

Figure D.5: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

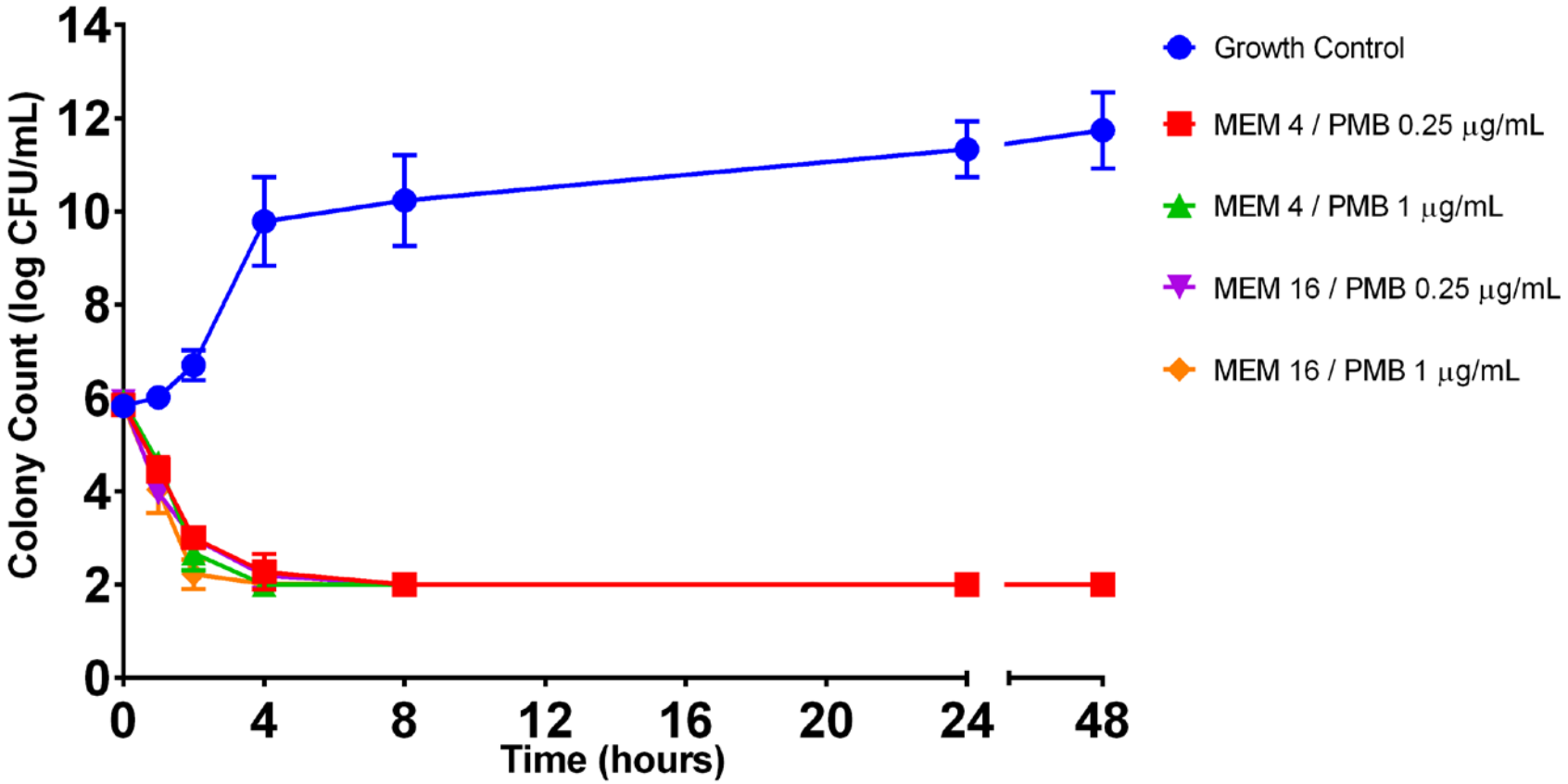

Figure D.6: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 34 (MICs: MEM 4 µg/mL, PMB 0.125 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

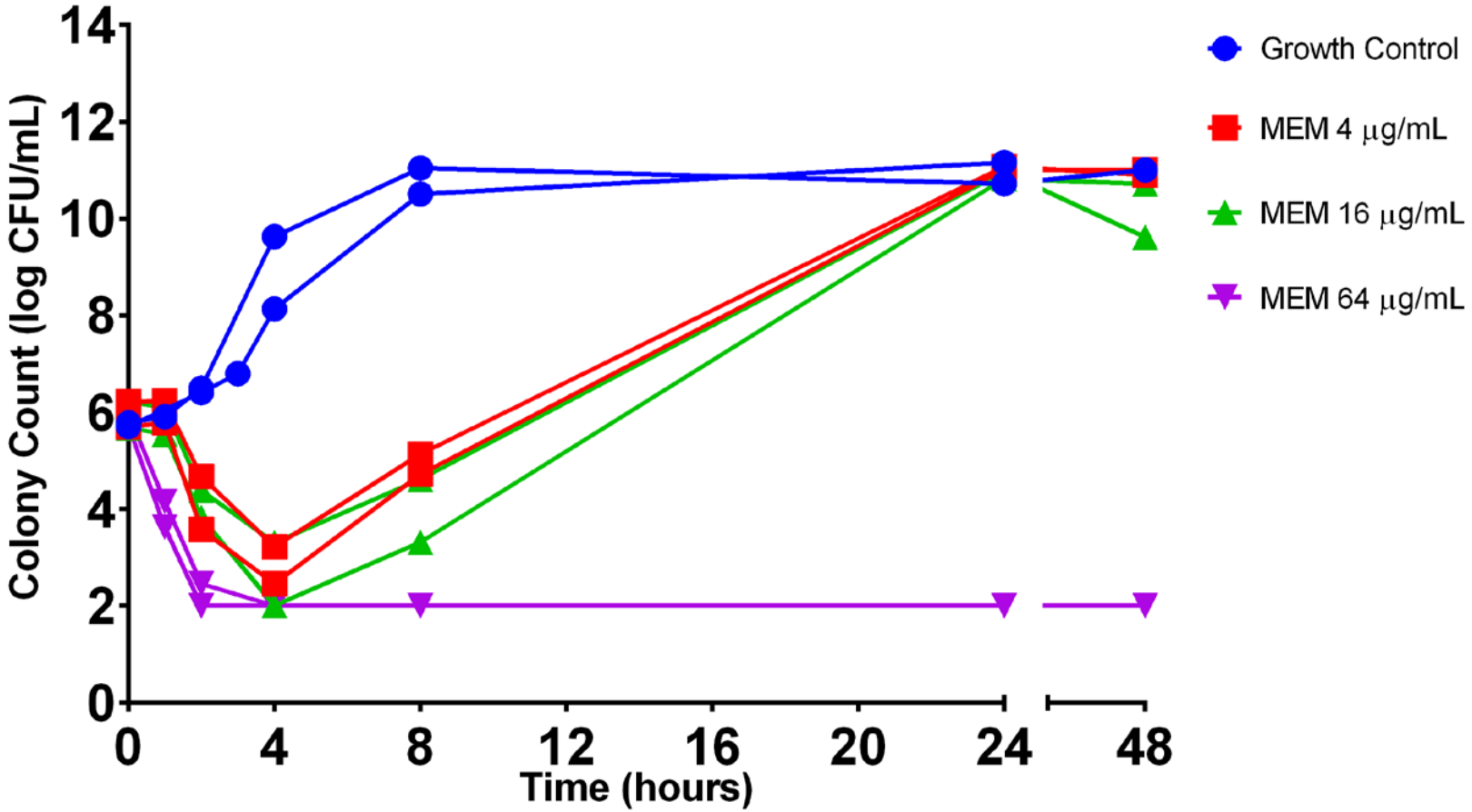

Figure D.7: Time-kill curve of meropenem (MEM) against KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

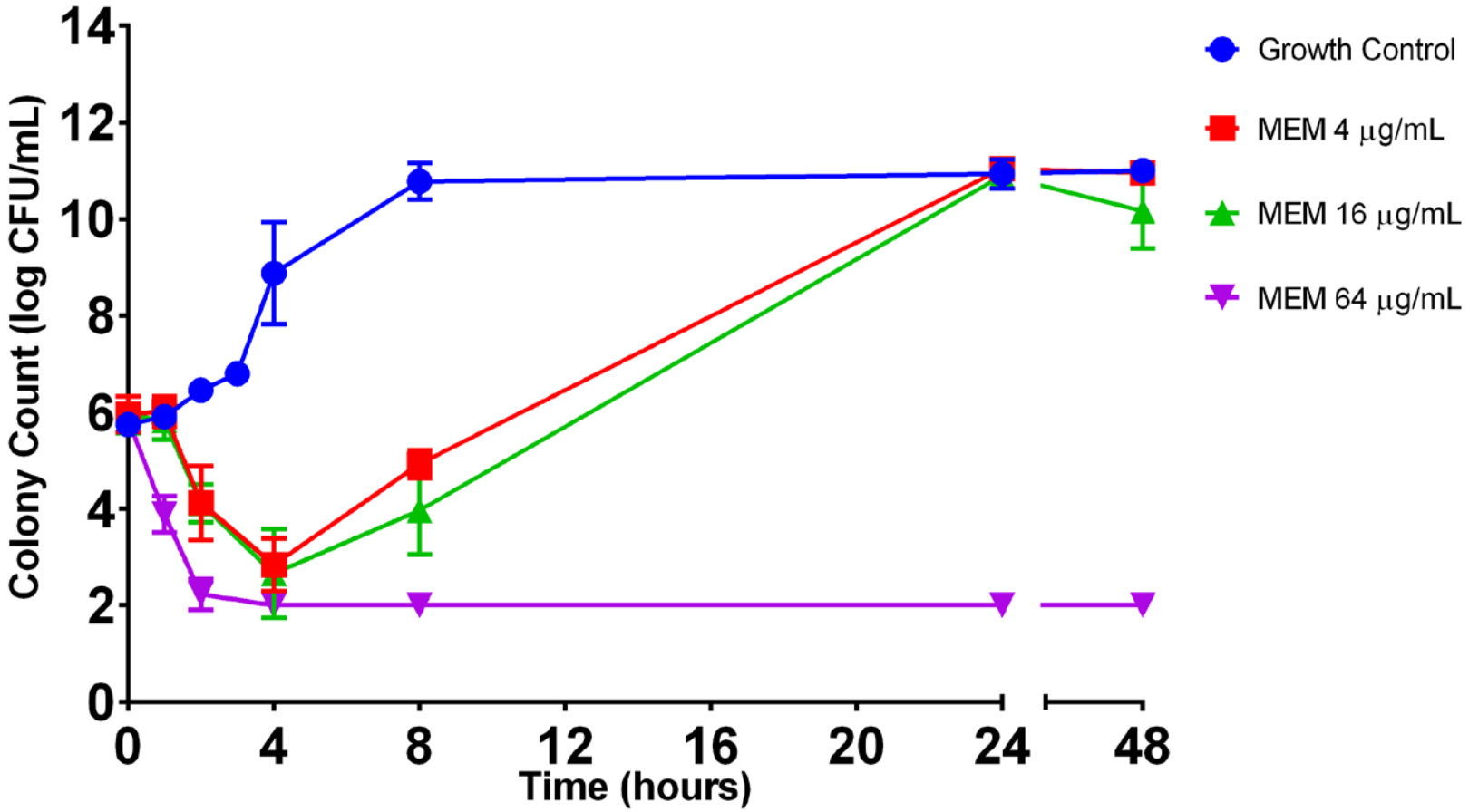

Figure D.8: Time-kill curve of meropenem (MEM) against KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2). The lower limit of quantification was 10^2 CFU/mL.

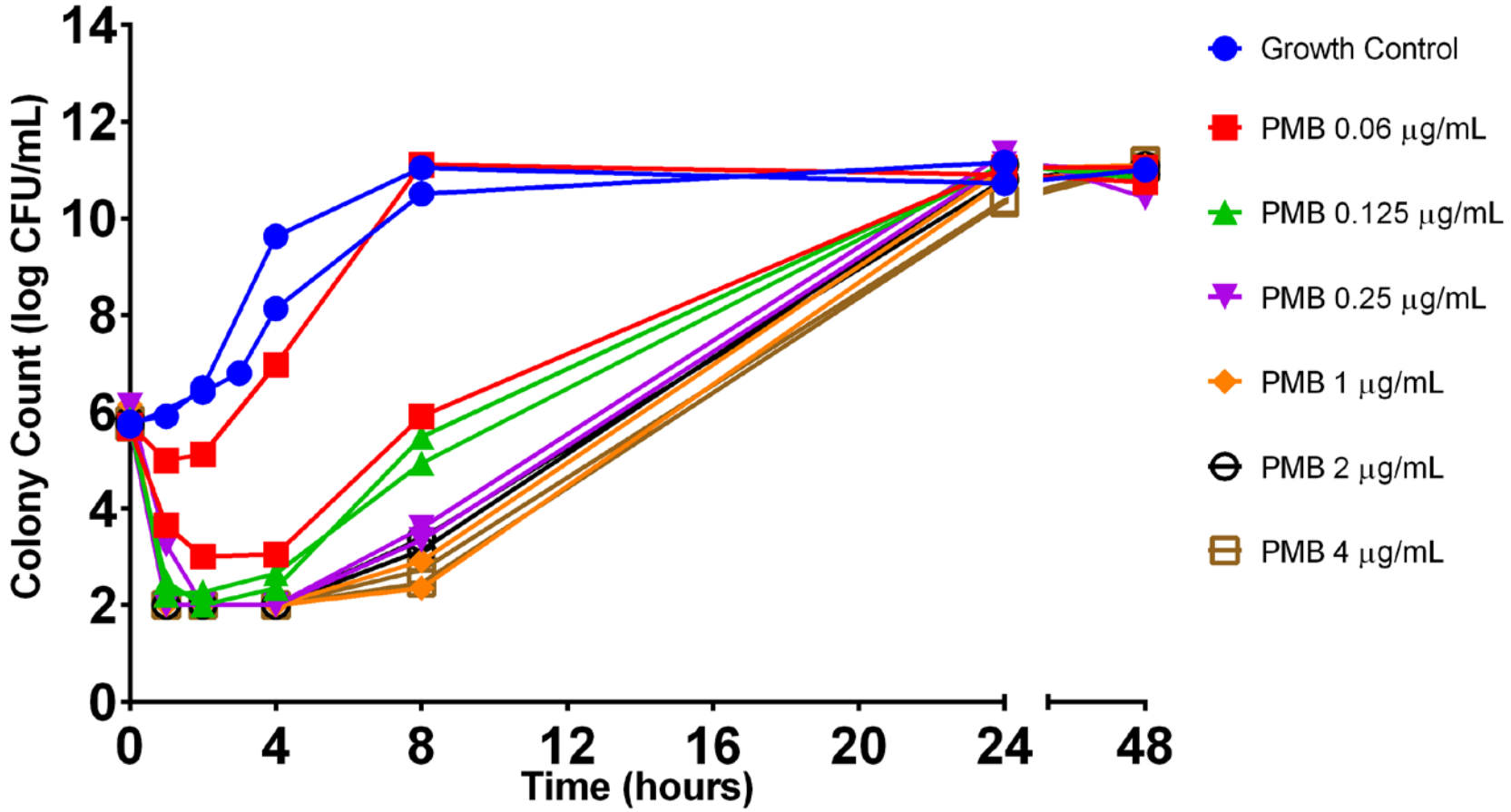

Figure D.9: Time-kill curve of polymyxin B (PMB) against KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

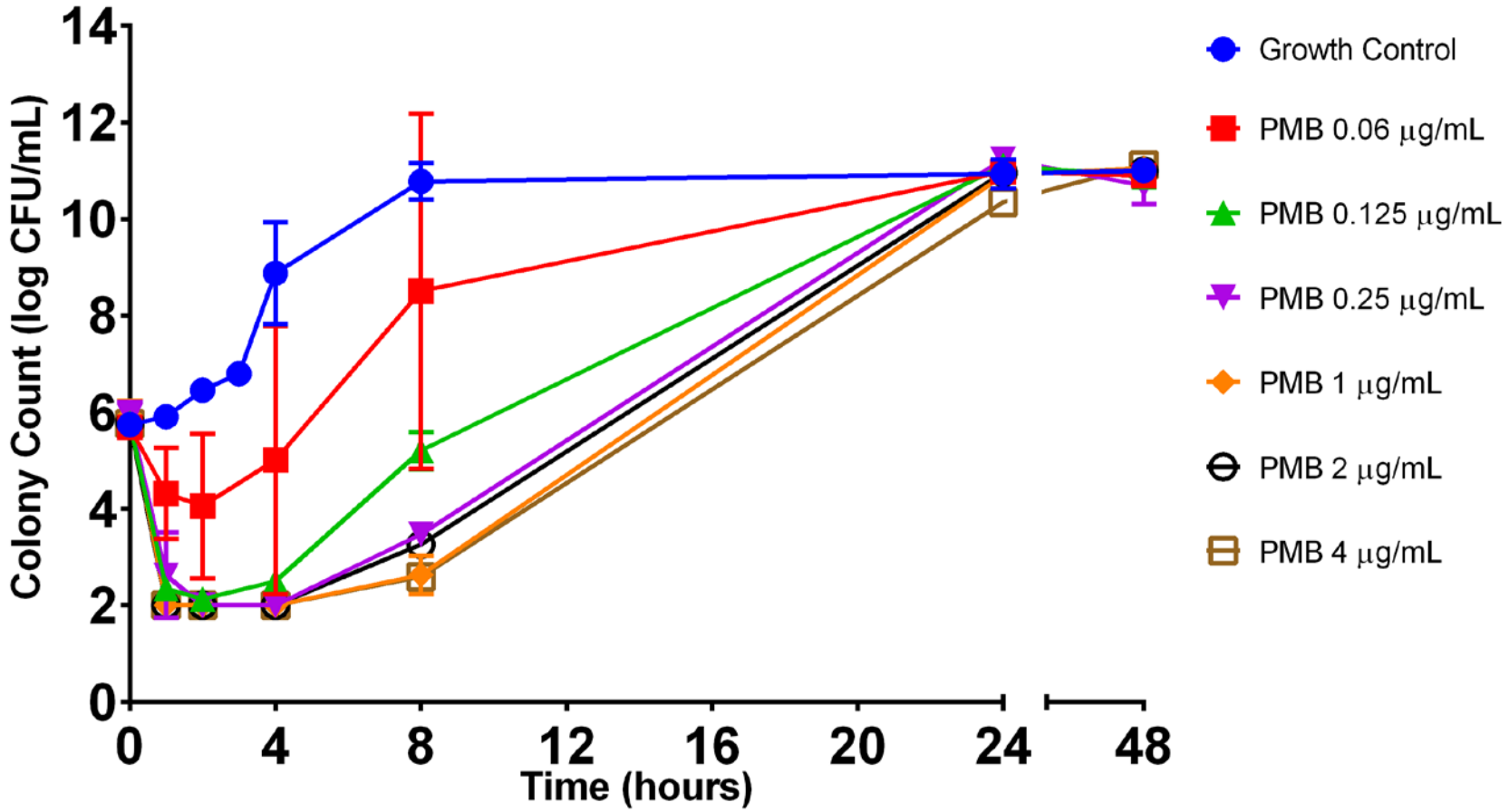

Figure D.10: Time-kill curve of polymyxin B (PMB) against KP 22 (MICs: MEM 16 μg/mL, PMB 0.06 μg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2). The lower limit of quantification was 10^2 CFU/mL.

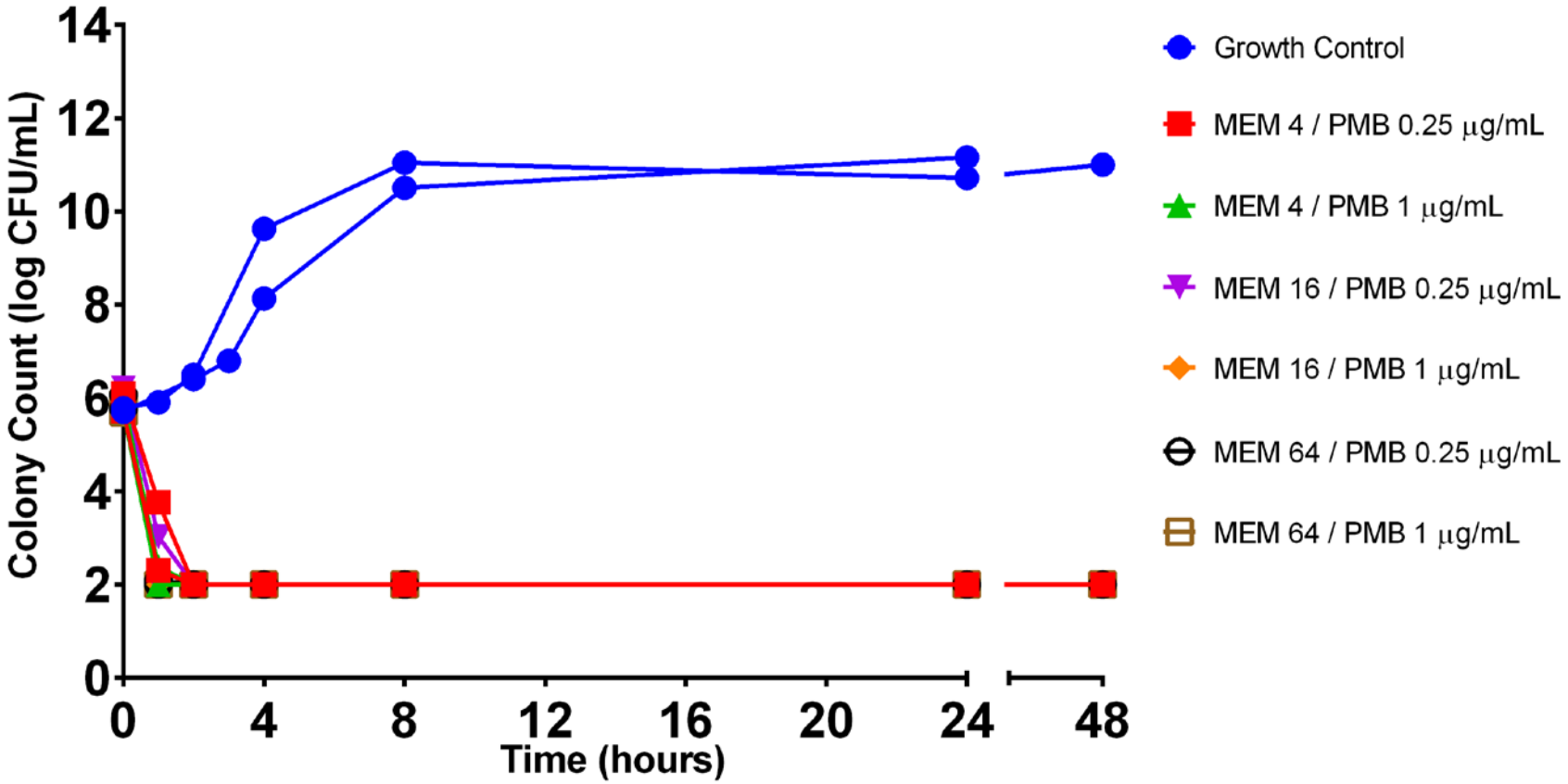

Figure D.11: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

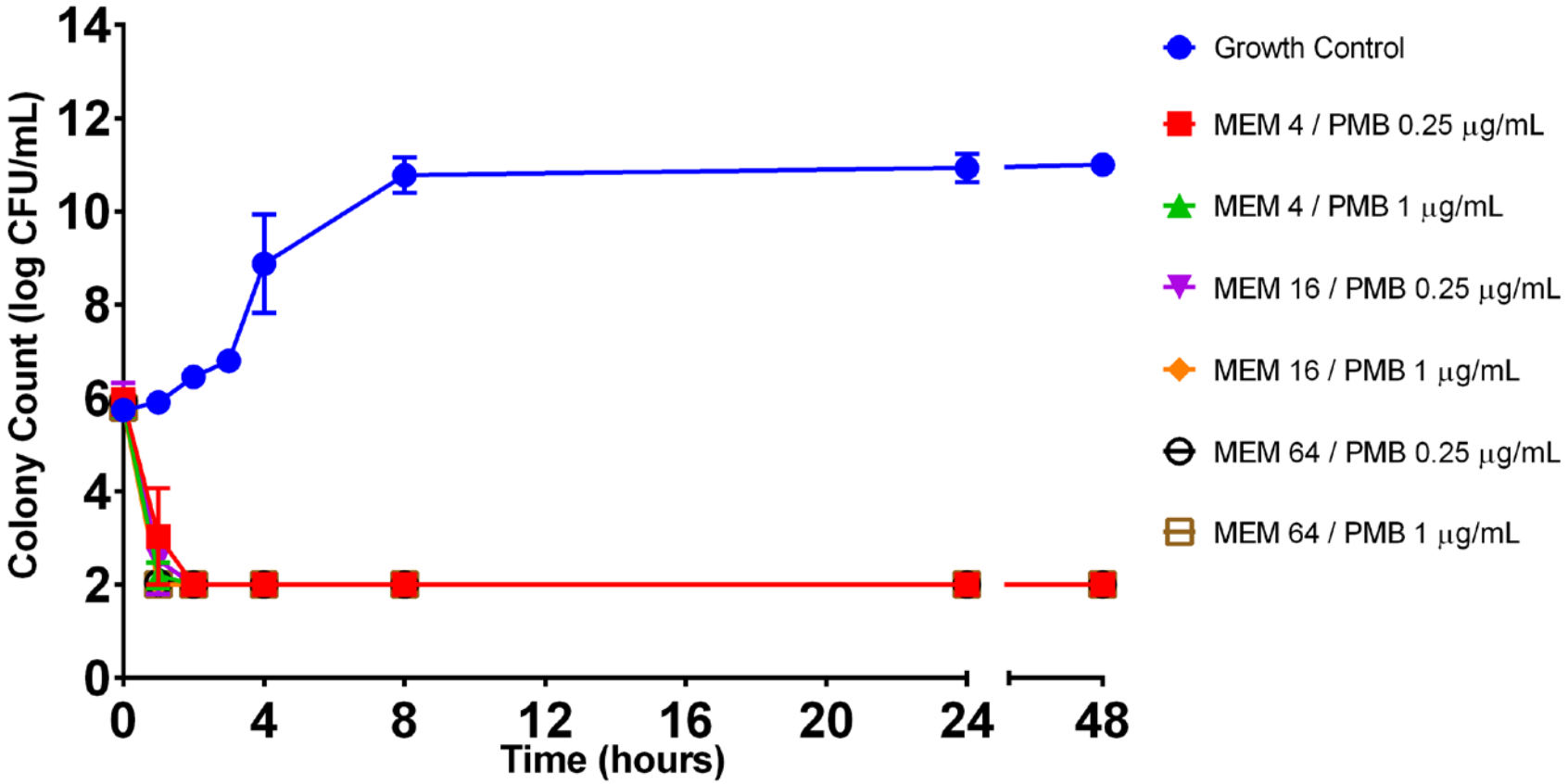

Figure D.12: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 22 (MICs: MEM 16 µg/mL, PMB 0.06 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2). The lower limit of quantification was 10^2 CFU/mL.

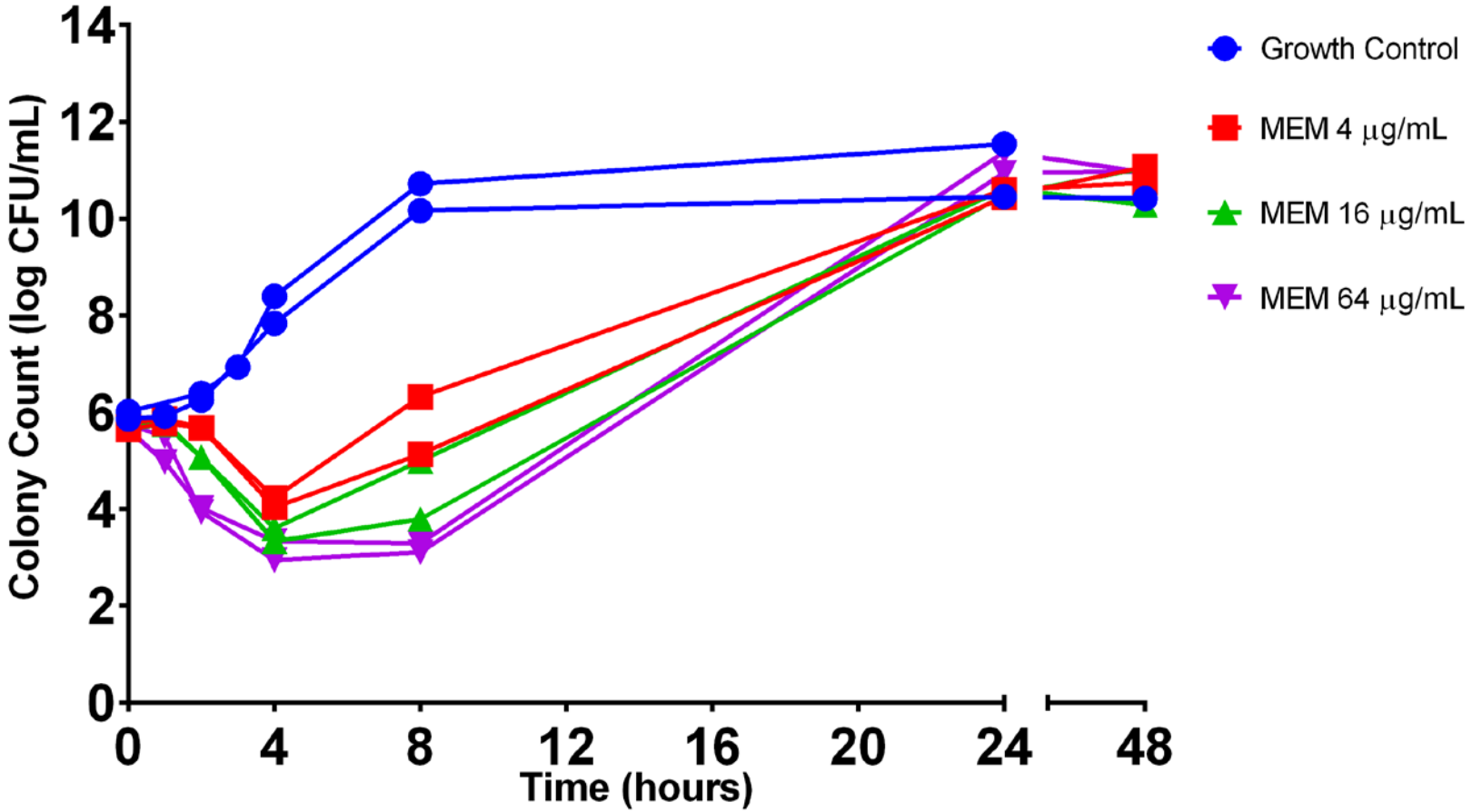

Figure D.13: Time-kill curve of meropenem (MEM) against KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

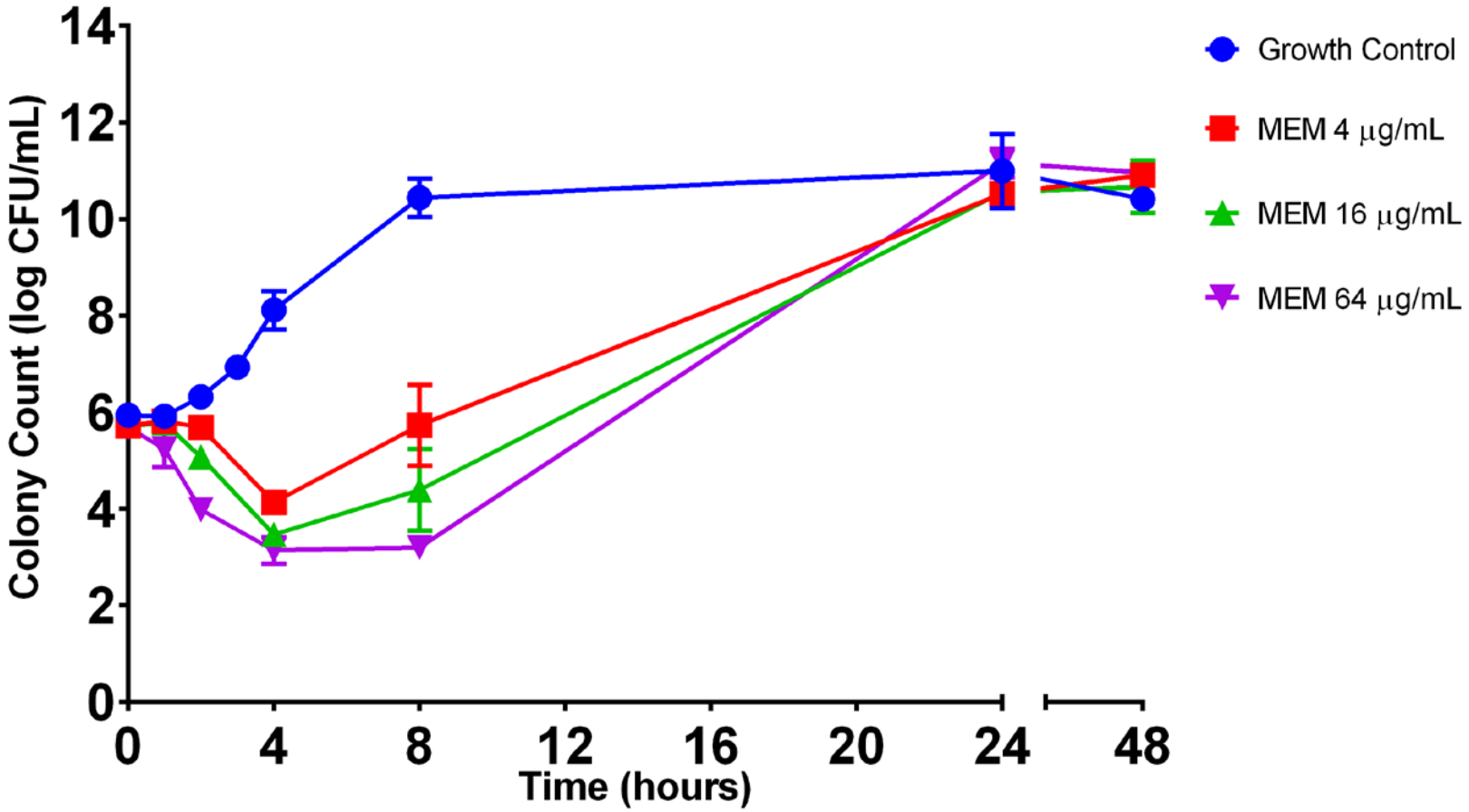

Figure D.14: Time-kill curve of meropenem (MEM) against KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

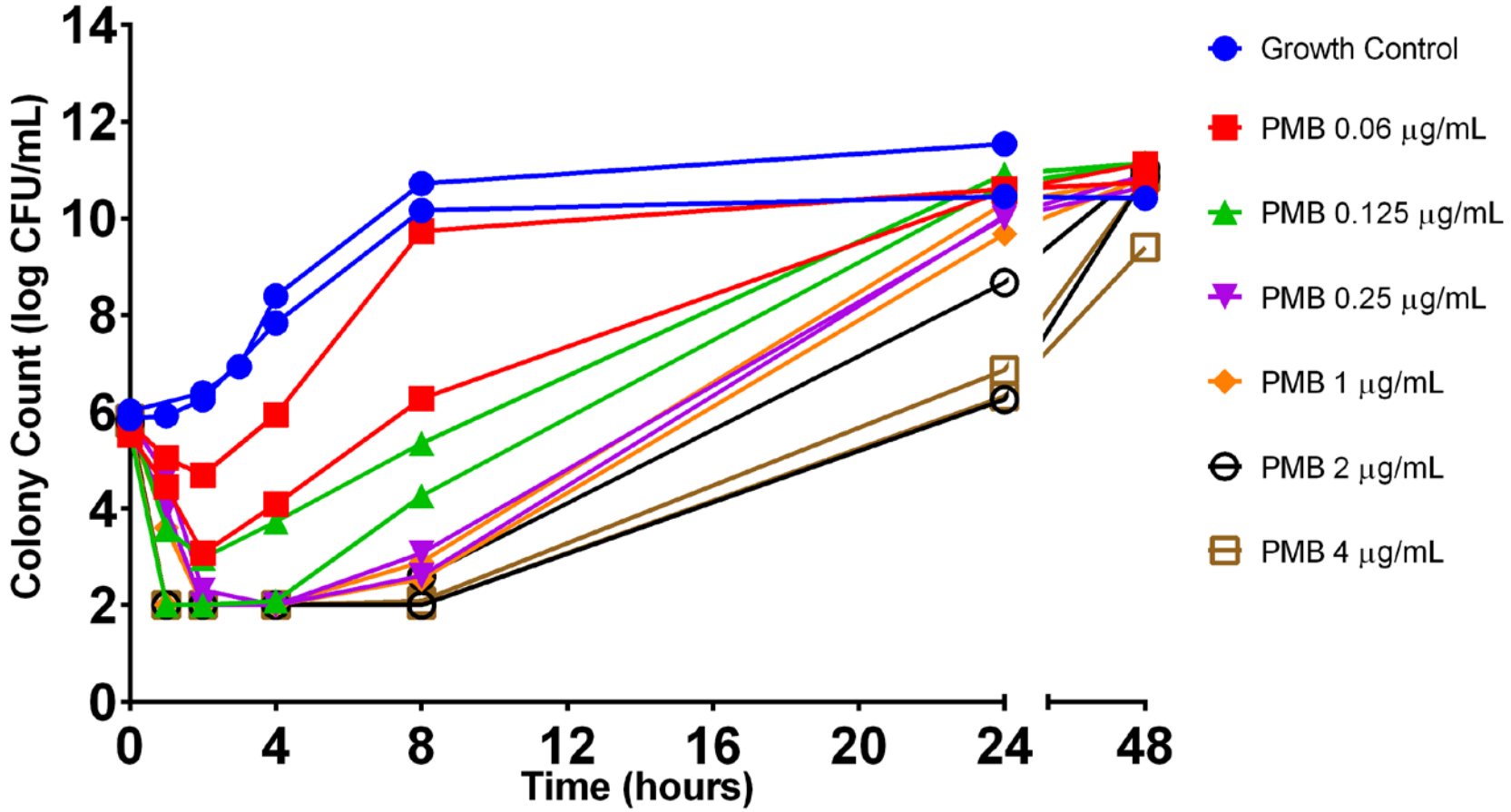

Figure D.15: Time-kill curve of polymyxin B (PMB) against KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

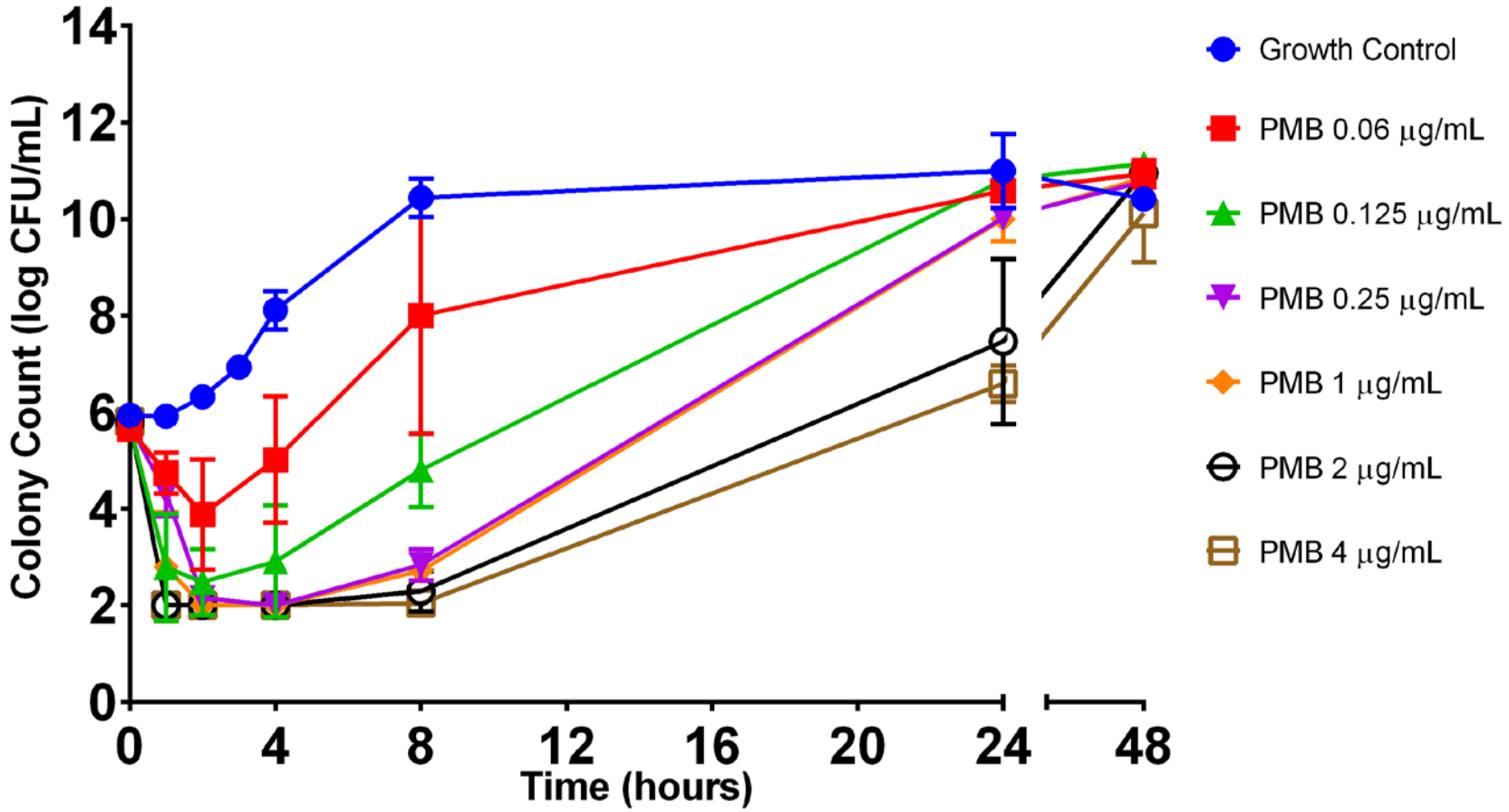

Figure D.16: Time-kill curve of polymyxin B (PMB) against KP 24 (MICs: MEM 32 μg/mL, PMB 0.125 μg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

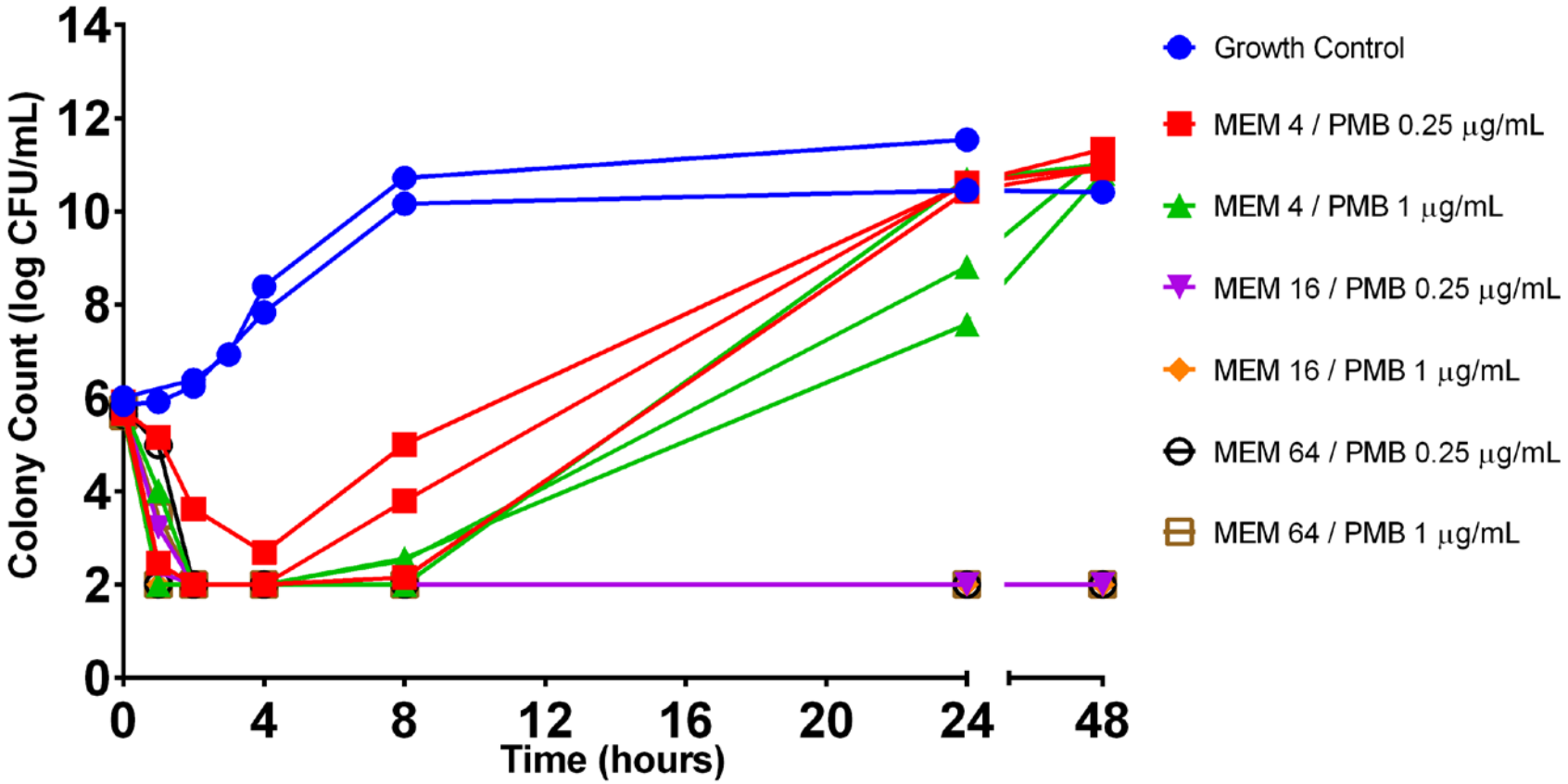

Figure D.17: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

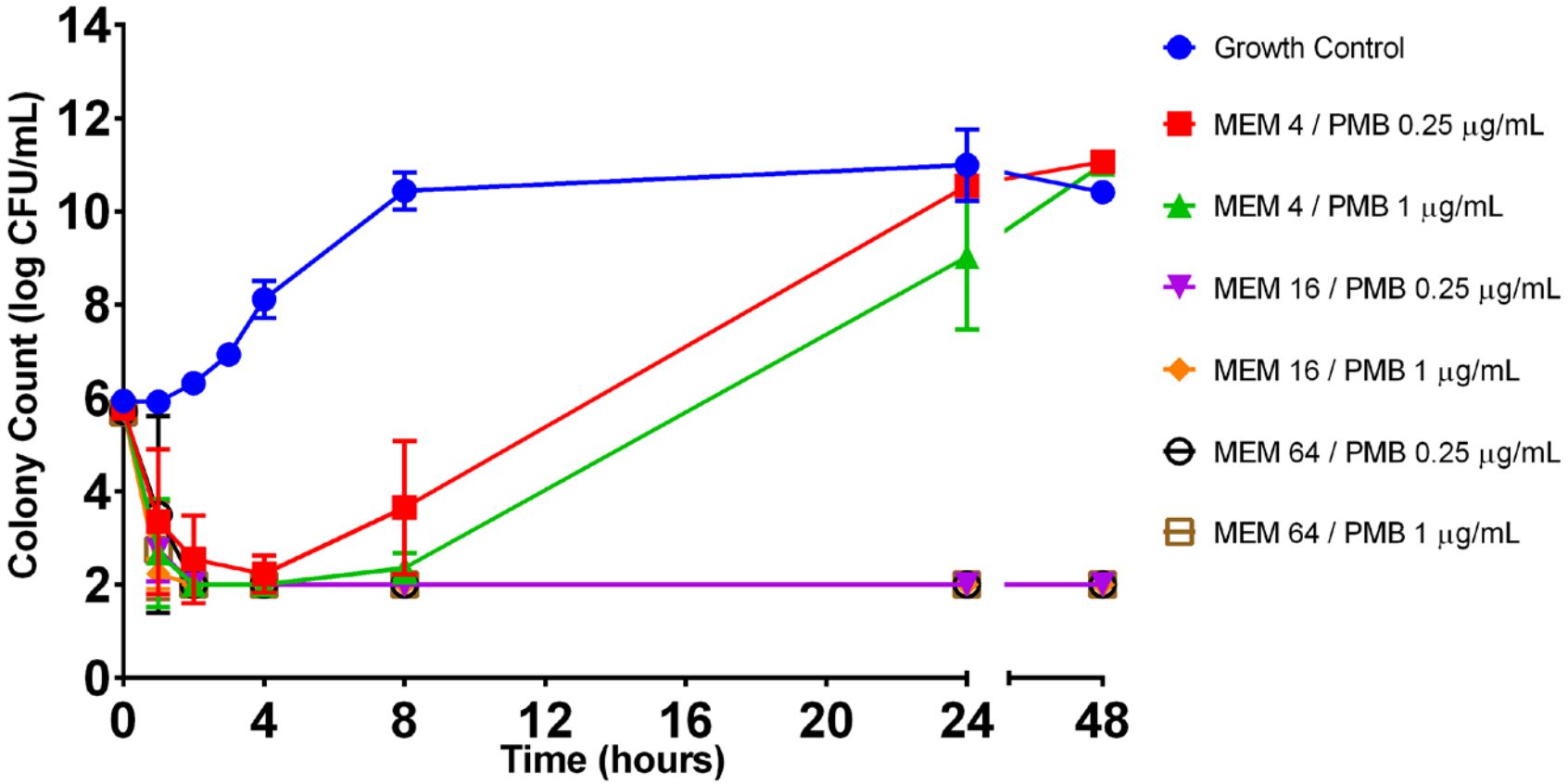

Figure D.18: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 24 (MICs: MEM 32 µg/mL, PMB 0.125 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

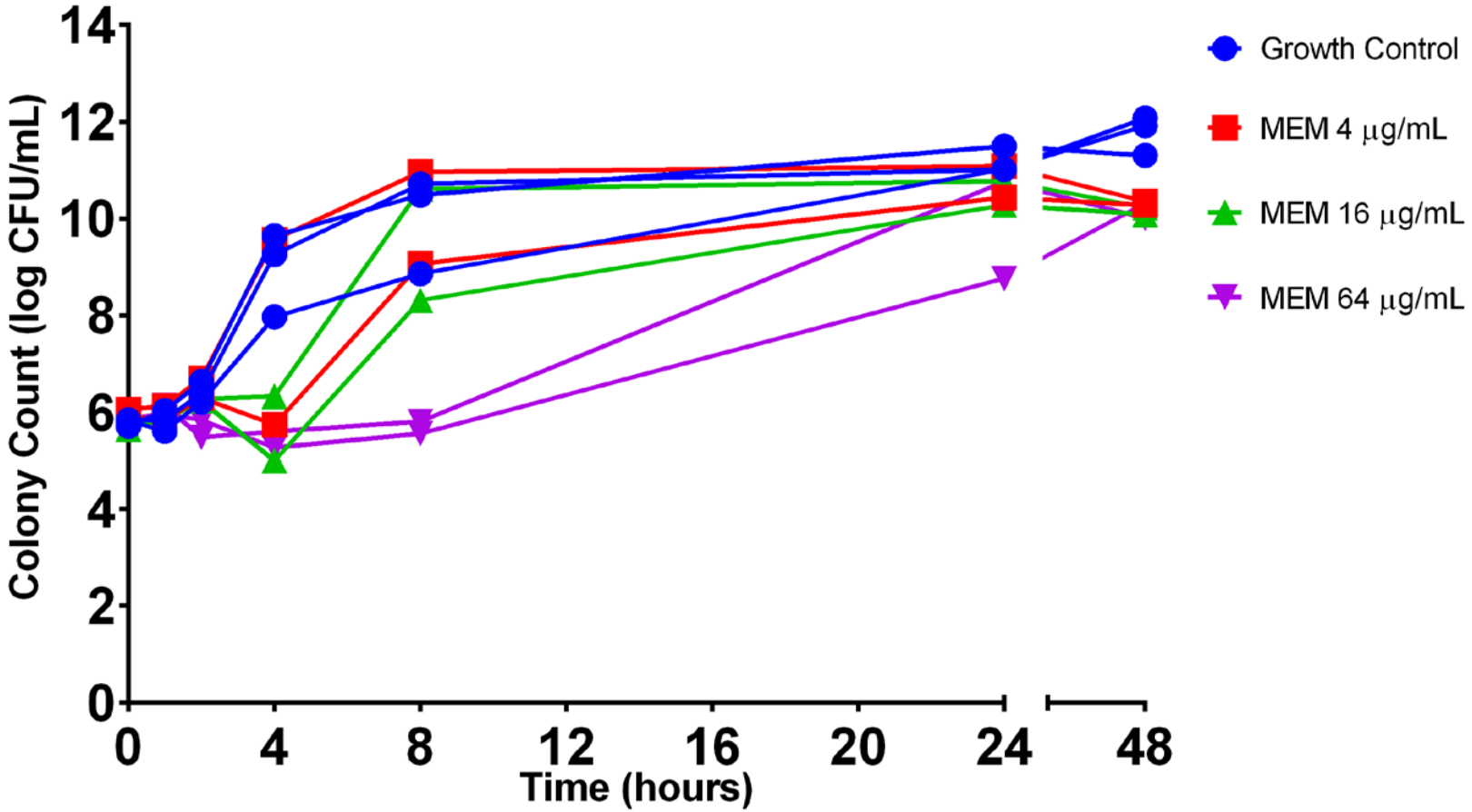

Figure D.19: Time-kill curve of meropenem (MEM) against KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

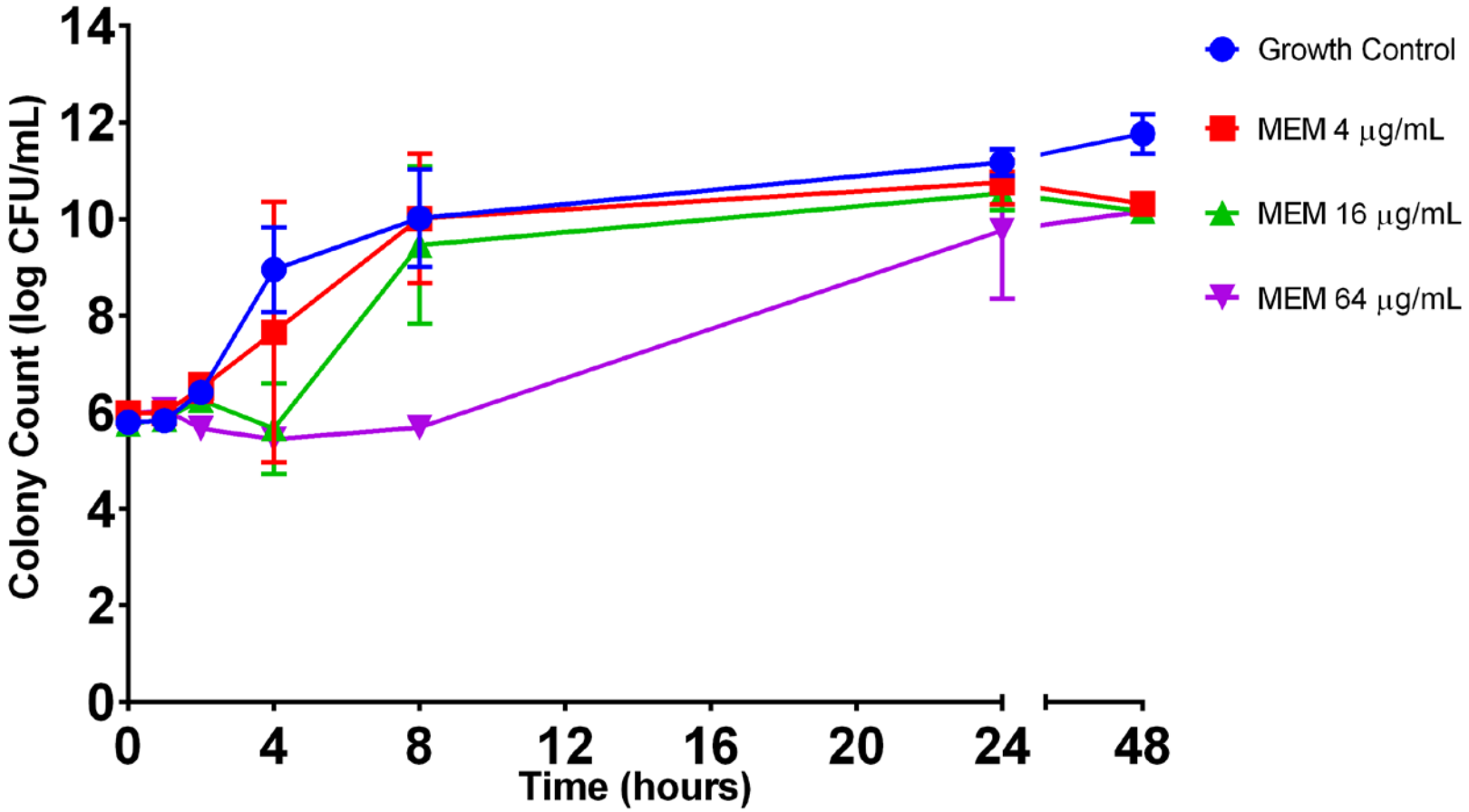

Figure D.20: Time-kill curve of meropenem (MEM) against KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 3). The lower limit of quantification was 10^2 CFU/mL.

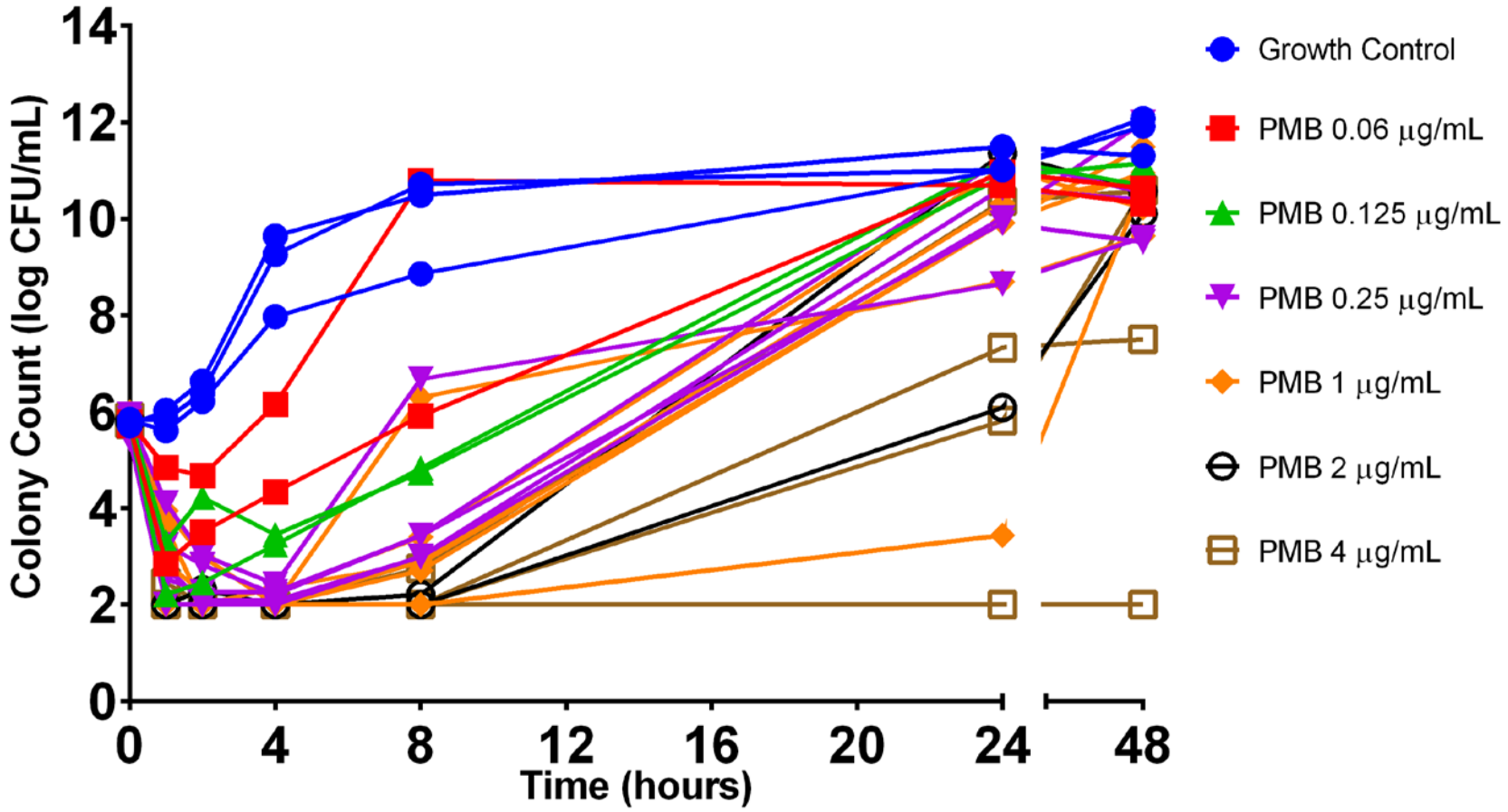

Figure D.21: Time-kill curve of polymyxin B (PMB) against KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

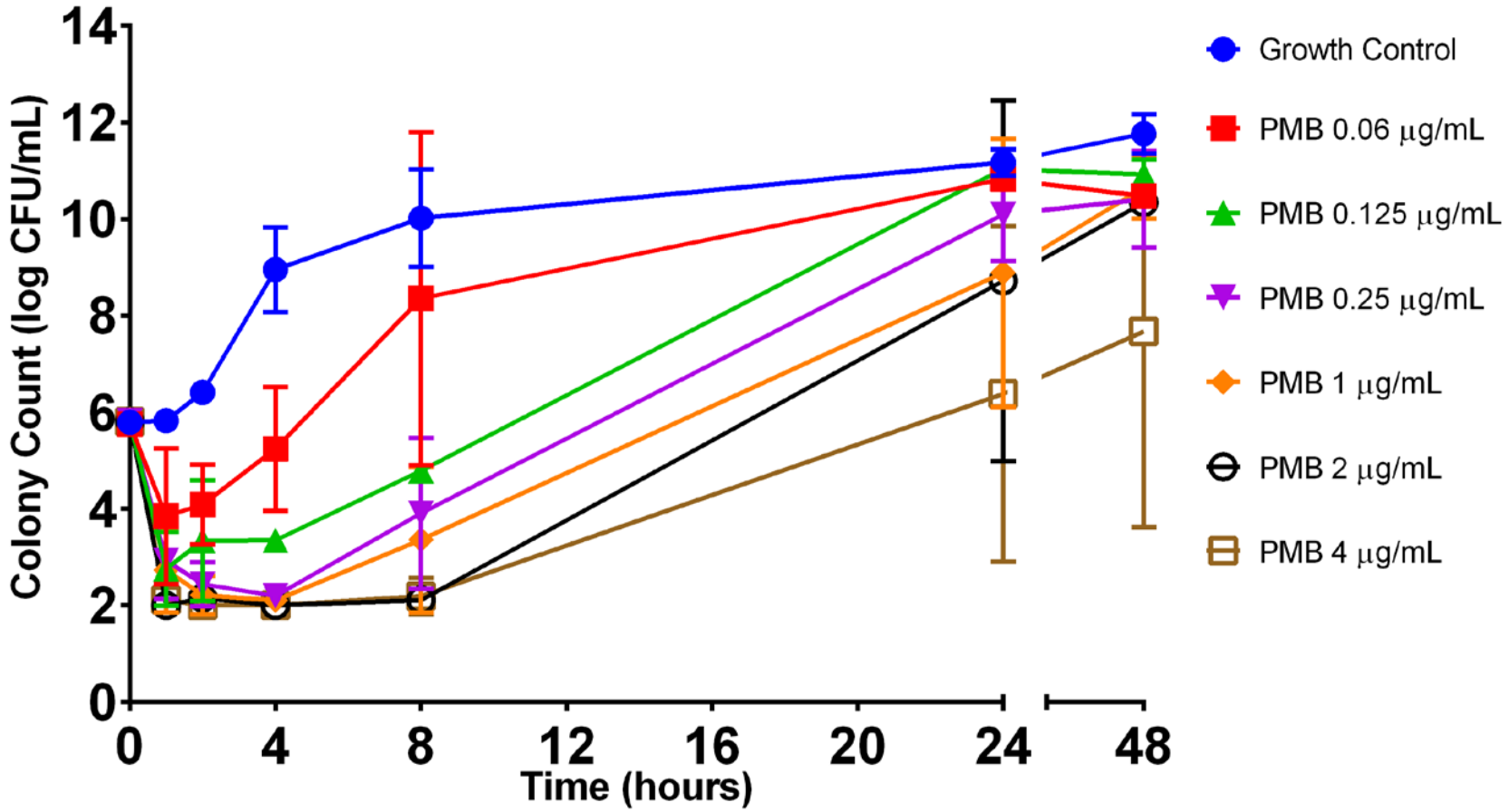

Figure D.22: Time-kill curve of polymyxin B (PMB) against KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 6). The lower limit of quantification was 10^2 CFU/mL.

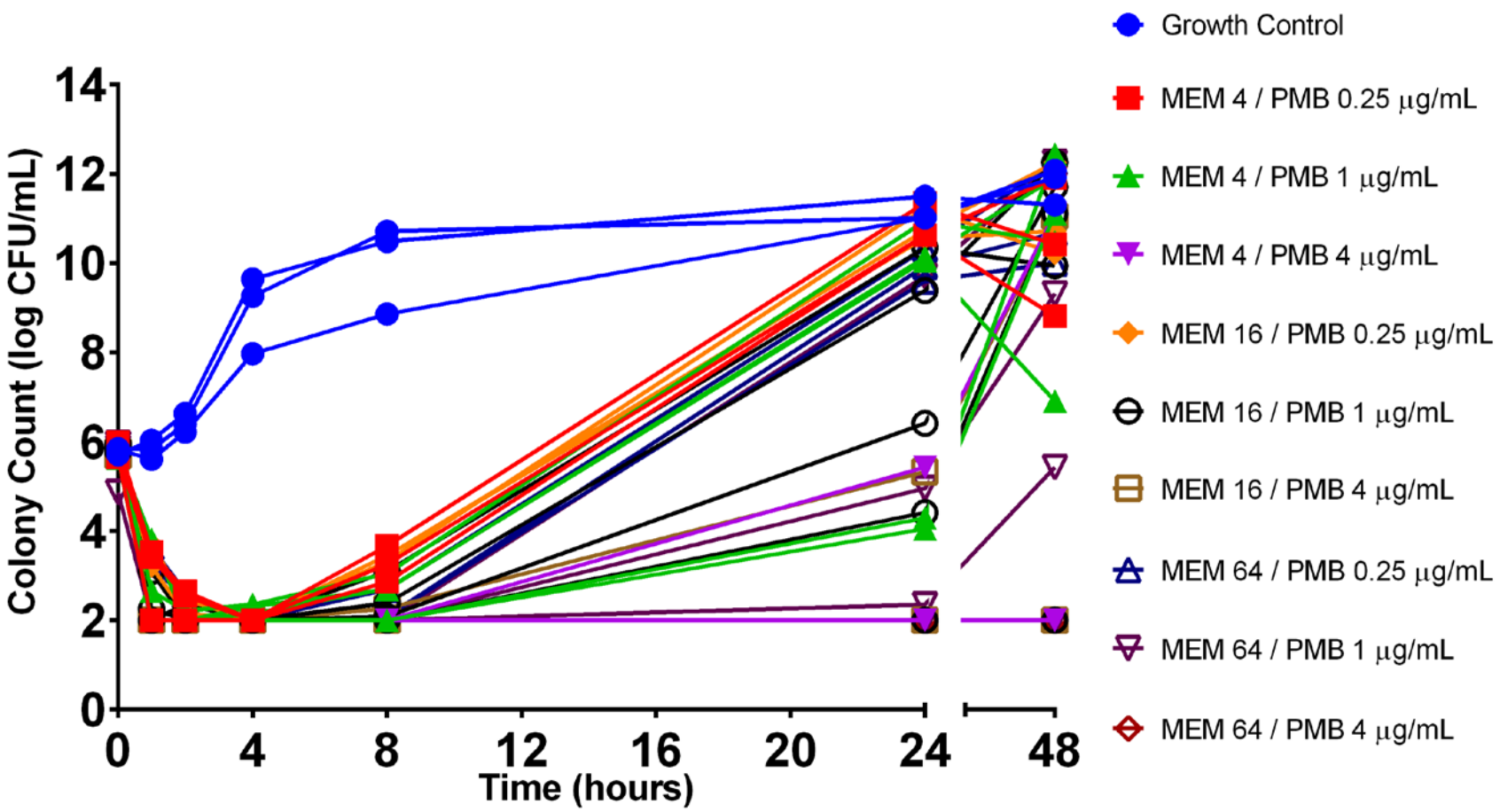

Figure D.23: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 44 (MICs: MEM 128 µg/mL, PMB 0.06 µg/mL). The lower limit of quantification was 10^2 CFU/mL.

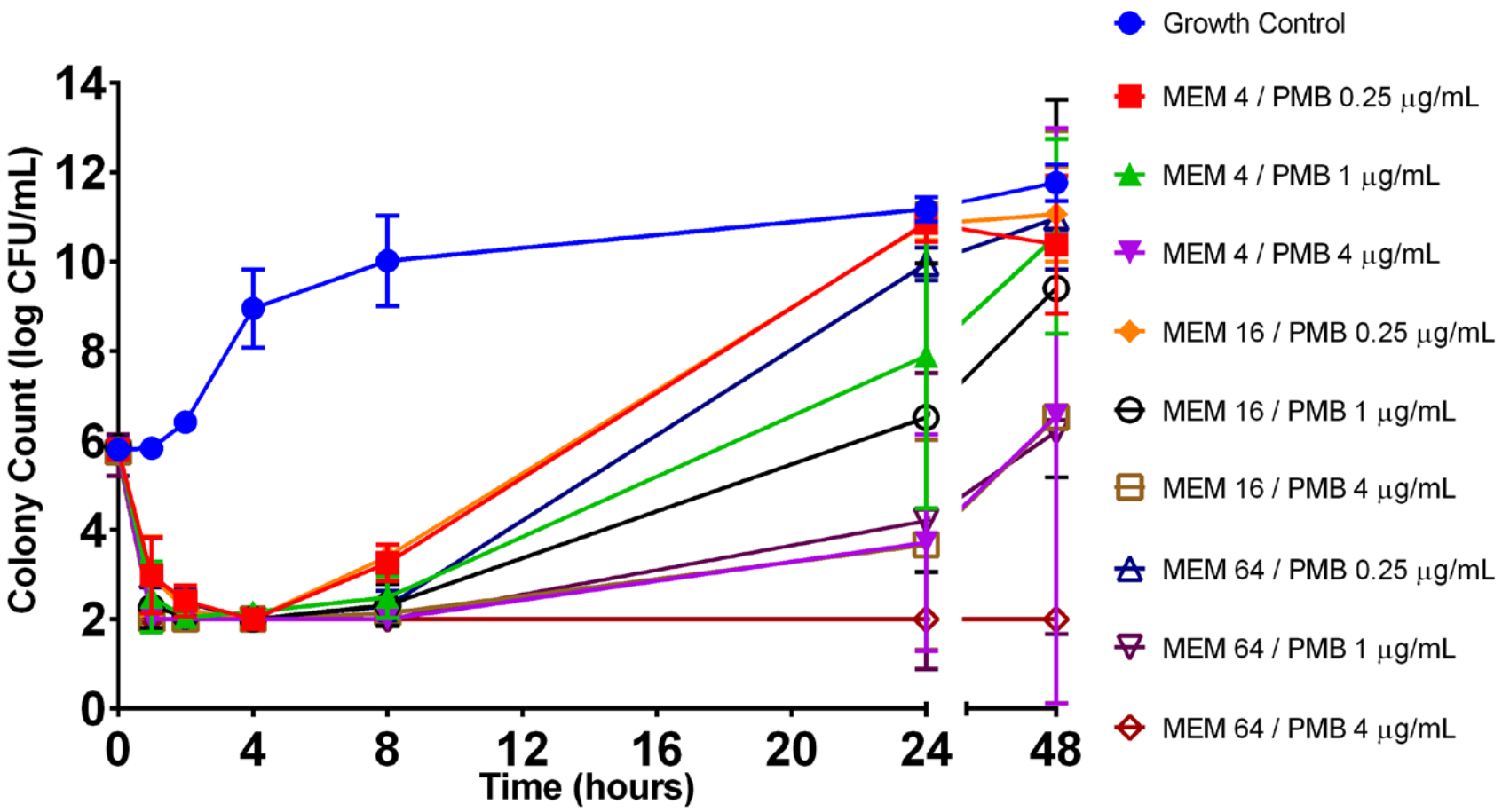

Figure D.24: Time-kill curve of meropenem (MEM) and polymyxin B (PMB) in combination against KP 44 (MICs: MEM 128 μg/mL, PMB 0.06 μg/mL). Data are geometric means of replicate experiments with standard deviation error bars (*n* = 2 to 5). The lower limit of quantification was 10^2 CFU/mL.

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VITA

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EDUCATION

Master of Science in Pharmaceutical Science, University of Kentucky, Lexington, KY.
Expected May 2016
Major field: Infectious Disease.
Thesis: *In Vitro Activity of Polymyxin B and Meropenem Alone and in Combination against Carbapenem-resistant Enterobacteriaceae*.

Doctor of Pharmacy, University of Kentucky, Lexington, KY.
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PUBLICATIONS

Beller JA, Kulengowski B, Kobraei EM, et al. Comparison of Sensory Neuron Growth Cone and Filopodial Responses to Structurally Diverse Aggrecan Variants, *In Vitro*. *Exp Neurol.* 2013; 247:143-57. **Published**

Landon J, Gao X, Kulengowski B, Neathery JK, Liu K. Impact of Pore Size Characteristics on the Electrosorption Capacity of Carbon Xerogel Electrodes for Capacitive Deionization. *J Electrochem Soc.* 2012; 159:61-66. **Published**

Kulengowski B, Campion J, Feola D, Burgess D. Effect of the degree of meropenem resistance on the killing activity of meropenem and polymyxin B in combination against KPC-producing *Klebsiella pneumoniae*. *Clin Inf Dis.* **In preparation**

PRESENTATIONS

Podium:

Kulengowski B, Burgess DS. Susceptibility of Multi-drug Resistant *Pseudomonas aeruginosa* against Ceftolozane/Tazobactam, a Novel Antibiotic. Astronaut Scholarship Foundation. Kennedy Space Center, FL. 30 May. 2015.

Kulengowski B, Burgess DS. Developing Bacterial Resistance Outpacing Development of Antibiotics. Astronaut Scholar Technical Conference. Astronaut Scholarship Foundation. Kennedy Space Center, FL. 3 May 2014.

Kulengowski B, Landon J. Waste Water Cleanup through Capacitive Deionization (CDI). Astronaut Scholar Technical Conference. Astronaut Scholarship Foundation. Marriott, Cocoa Beach, FL. 20 Apr. 2013.

Poster:

Kulengowski B, Mattingly C, Feola DJ, Campion J, Burgess DS. *In Vitro* Characterization of Meropenem and Polymyxin B Alone and in Combination against Carbapenemase Producing *Klebsiella pneumoniae.* Rho Chi Research Day. University of Kentucky College of Pharmacy, Lexington, KY. Apr. 2015.

Kavuluru R, Rios A, Kulengowski B, McNamara P. A Knowledge-Based Collaborative Clinical Case Mining Framework. AMIA annual symposium. Washington Hilton, Washington, DC. Nov. 2014.

Kulengowski B, Mattingly C, Feola DJ, Campion J, Burgess DS. *In Vitro* Characterization of Meropenem/Polymyxin B Combination Therapy against Carbapenemase Producing *Klebsiella pneumoniae.* Drug Discovery and Development Symposium. University of Kentucky College of Pharmacy, Lexington, KY. Sep. 2014.

Kulengowski B, Mattingly C, Feola DJ, Campion J, Burgess DS. *In Vitro* Characterization of Meropenem/Polymyxin B Combination Therapy against Carbapenemase Producing *Klebsiella pneumoniae.* Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). American Society for Microbiology. Walter E. Washington Convention Center, Washington, DC. 7 Sep. 2014.

Campion J, Kulengowski B, Mattingly C, Feola DJ, Burgess DS. Modeling the Selection of Resistant Subpopulations in Carbapenemase-Producing *Klebsiella pneumoniae* Exposed to Polymyxin B and Meropenem Alone and in Combination. Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). American Society for Microbiology. Walter E. Washington Convention Center, Washington, DC. 7 Sep. 2014.

Kulengowski B, Calulot C, Snow D. A Single Filopodial Contact with Inhibitory Chondroitin Sulfate Proteoglycans Induces Behavioral Changes in Sensory Neurons *In Vitro.* International Symposium on Neural Regeneration. NIH. Asilomar Conference Center, Pacific Grove, CA. 7 Dec. 2011.

Kulengowski B, Calulot C, Snow D. A Single Filopodial Contact with Inhibitory Chondroitin Sulfate Proteoglycans Induces Behavioral Changes in Sensory Neurons *In Vitro.* Spring Neuroscience Day. Bluegrass Society for Neuroscience. Student Center Grand Ballroom, Lexington, KY. Spring 2011.

SCHOLASTIC HONORS

Astronaut Scholarship Foundation Scholarship	2012-2013
University of Kentucky Commonwealth Scholarship	2009-2013
Ralph and Janice Young Scholarship	2009-2012
National Eagle Scout Scholarship	2009

PROFESSIONAL POSITIONS

University of Kentucky HealthCare Pharmacy Intern