Potential Antagonism between Bactericidal β-lactam Antibiotics and Bacteriostatic Antibiotics in an *in vitro* Pharmacodynamic Model of Septic Shock

By

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Abstract

Background

Septic shock caused by bacterial infections continues to be one of the main causes of death in the Intensive Care Units (ICU) of developed countries. *Staphylococcus aureus* and *Escherichia coli* are commonly isolated in septic individuals. Improving the outcome of septic shock has focused on accelerating microbiological burden clearance by using more potent antibacterials, optimization of pharmacokinetics and evaluating the additive and synergistic effects of combination therapy of antibiotics (AB).

Objective

This study assesses the antibacterial potency of a bactericidal β-lactam combined with a bacteriostatic agent against sensitive gram-positive and gram-negative bacteria in both a time kill (TK) assay and an *in vitro* pharmacodynamic assay (IVPD). These combinations of ABs are routinely administered to septic shock ICU patients.

Methods

Antibacterial susceptibility testing, TK assays, single and multiple drug IVPD assays were used to determine the effect of AB combinations on the rate of bacterial killing. Specifically, cefazolin with erythromycin or clindamycin against *S. aureus* clinical isolate 118324 and ceftriaxone with tigecycline or chloramphenicol against *E. coli* ATCC 700973 Serotype O18:K1:H7 (BORT) in cation adjusted Mueller-Hinton broth (Ca-MHB).

Results

S. aureus was susceptible to cefazolin, clindamycin and erythromycin with MIC's equal to 0.5, 0.5 and 1 µg/ml respectively. *E. coli* was susceptible to ceftriaxone, tigecycline and chloramphenicol with MIC's equal to 0.03, 0.25 and 8 µg/ml respectively. *E. coli* TK results showed antagonism after 12-hours of drug exposure, for combinations of ceftriaxone with tigecycline or chloramphenicol. *S. aureus* TK results showed antagonism by 12- and 24-hours for combinations of cefazolin with clindamycin or erythromycin. The results of *E. coil* and *S. aureus* IVPD assays demonstrated antagonism as well as a decrease in bacterial killing rate when a β-lactam was given in combination with a protein synthesis inhibitor.

Conclusion

This study demonstrated that clinically relevant serum concentrations of a β-lactam in combination with a bacteriostatic protein synthesis inhibitor, resulted in a reduced rate of bacterial killing for both *E. coli* and *S. aureus* in both TK and IVPD assays. A reduction in the rate of killing of pathogens could play an important role in clinical treatment for delay sensitive conditions such as septic shock.

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Contributions

Anton Kowalski performed the MIC screening of bacterial isolates until suitable bacterial strains were found with resistance to the ABs within the susceptible range. The time kill and IVPD experiments for the majority were also set up and performed by Anton. The thesis was also written by Anton.

Naresh Sharma helped perform set up time kill and IVPD experiments and also take samplings when Anton was unable. Naresh also played a vital role in data analysis, specifically with the statistics and graph making.

List of Abbreviations (in alphabetical order)

AB	Antibiotic
ABI	Antibiotic Interactions
AMR	Antibacterial Resistance
AUC	Area Under the Curve
CA-MHB	Cation Adjusted Mueller-Hinton Broth
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem Resistant Enterobacteriaceae
C_{max}	Maximum Concentration
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
ESBL	Extended spectrum β-lactamase
GLASS	Global Antibacterial Resistance and Use Surveillance System
ICU	Intensive Care Unit
IL-1	Interleukin-1
IV	Intravenous
IVPD	*In Vitro* Pharmacodynamic Model
LOD	Limit of Detection
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
PAE	Post Antibiotic Effect
PAF	Platelet Activating Factor
PBP	Penicillin Binding Protein
RPM	Revolutions Per Minute

SIRS	Systemic Inflammatory Response Syndrome
SMP	Sulfamonomethoxine
SOFA	Sequential Organ Failure Assessment
SSTI	Skin and Soft Tissue Infection
T>MIC	Time Greater than the MIC
TK	Time Kill
TLR -4	Toll Like Receptor-4
TMP	Trimethoprim
TNF	Tumor Necrosis Factor
TSST-1	Toxic Shock Syndrome Toxin 1
UTI	Urinary Tract Infection
VRE	Vancomycin Resistant *Escherichia coli*
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WHO	World Health Organization

1. Introduction

This introduction attempts to provide readers with pertinent background information on sepsis and septic shock, disease pathophysiology, commonly isolated pathogens, characterization of the classes of ABs used our experiments and rational for examining AB combination therapy in time-sensitive conditions such as septic shock.

1.1 Sepsis and septic shock definitions

Sepsis is currently defined as life-threatening organ dysfunction caused by a dysregulated host response to infection[1]. The definition of organ dysfunction is further characterized as an increase of ≥2 on the Sequential Organ Failure Assessment (SOFA) score. SOFA monitors a variety of physiological parameters and generates a score (ranging from 0 to 24) depending on the extent of deviation from normal physiological parameters[1]. An increase in score of ≥2 is associated with a mortality risk of approximately 10% and is consistent across all ages and sex[2].

Septic shock is defined as a subset of sepsis in which underlying circulatory and cellular metabolic abnormalities are profound enough to substantially increase mortality[1]. A major characteristic of septic shock is cardiovascular decompensation. Patients with septic shock can be clinically identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mmHg or greater and serum lactate level >2 mmol/L in the absence of hypovolemia. This combination is associated with hospital mortality rates >40% worldwide, across the range of young children to the elderly[2].

1.2 Clinical burden of septic shock

Sepsis and septic shock caused by a bacterial or fungal (*Candida* species) pathogens, continues to be a major cause of ICU mortality in developed countries and highlights a burden on already stressed healthcare systems worldwide. In 2017, the World Health Organization (WHO) estimated that approximately 48.9 million cases of sepsis resulted in an approximate 11 million sepsis related deaths. This estimation accounts for approximately 20% of all recorded deaths globally[3]. Individuals who survive sepsis or septic shock are reported to experience a lower quality of life compared to age and sex matched cohorts[4]**.**

Arefian *et al.* 2017 performed a systemic review of hospital costs to treat a septic individual and indicated that the mean hospital cost to treat sepsis was $32,421 United States Dollars (USD). Re-admission to hospital, within 30 days of treatment, was observed in approximately 20.4% of patients treated with sepsis[5]. Approximately 10.4% of all US intensive care unit (ICU) admissions were cases of septic shock with a mean mortality rate of 37.5%[5]. A prospective observation study by Martin *et al*. 2009 examined data on individuals with septic shock entering into the ICU and who stayed a duration greater than 24 hours. This study was collected across 12 Canadian community and teaching hospital critical care units in British Columbia, Manitoba, Ontario and Newfoundland. They concluded that approximately 20% of patients admitted to the ICU were considered to be septic and reported a mortality rate of 38.1%[2]. In a separate review by Husak *et al*. 2010 30,587 hospitalizations were recorded in Canada outside of Quebec. The median age of these individuals was 66 with 54.6% of patients being male. A 30.5% mortality rate was calculated within this cohort contributing to 10.9% of all deaths occurring in hospitals. A rise in the number of cases of sepsis and severe sepsis was also reported from 2004 – 2009[6].

There is a paucity of economic data pertaining to sepsis within Canada. Letarte *et al.* 2002 estimated that the province of Quebec spent $73 million CAD treating sepsis related conditions. Farrah *et al.* 2021 assessed the cost per patient to treat sepsis in the Ontario region. They calculated that each patient cost an estimated $28,568 – $29,913 CAD and had higher re-hospitalization rates when compared to the control[7]. Hospitalization of sepsis and septic shock patients proves to be an expensive treatment.

1.3 Pathophysiological models of septic shock

At present, a single, clear, broadly accepted pathophysiological paradigm of septic shock does not exist. This is due to the fact that as a syndrome with many different manifestations, rather than a specific disease, there are a multitude of associated pathogens which can be the cause of septic shock. Infection can occur at various anatomic sites in the human body presenting phenotypic variations related to the genetic make-up, underlying co-morbidities and differing host responses to a pathogen[1].

Three major models related to the pathophysiology of septic shock have been proposed over the past 40 years. Prior to the 90's, the pathophysiologic framework to explain sepsis and septic shock was based on the microbial burden on the body engendered by uncontrolled infection[8]. The key component in this model of sepsis is the presence of a microbial pathogen resulting in intense downstream systemic inflammatory responses. The primary treatment involved an effort to minimize the microbiological burden with AB therapies. This would reduce systemic inflammatory activity as a secondary benefit. This paradigm evolved into a model of sepsis that

dominated research through the 1990's to 2010. It remains a core element of pathophysiological models of sepsis and septic shock still being investigated today[8].

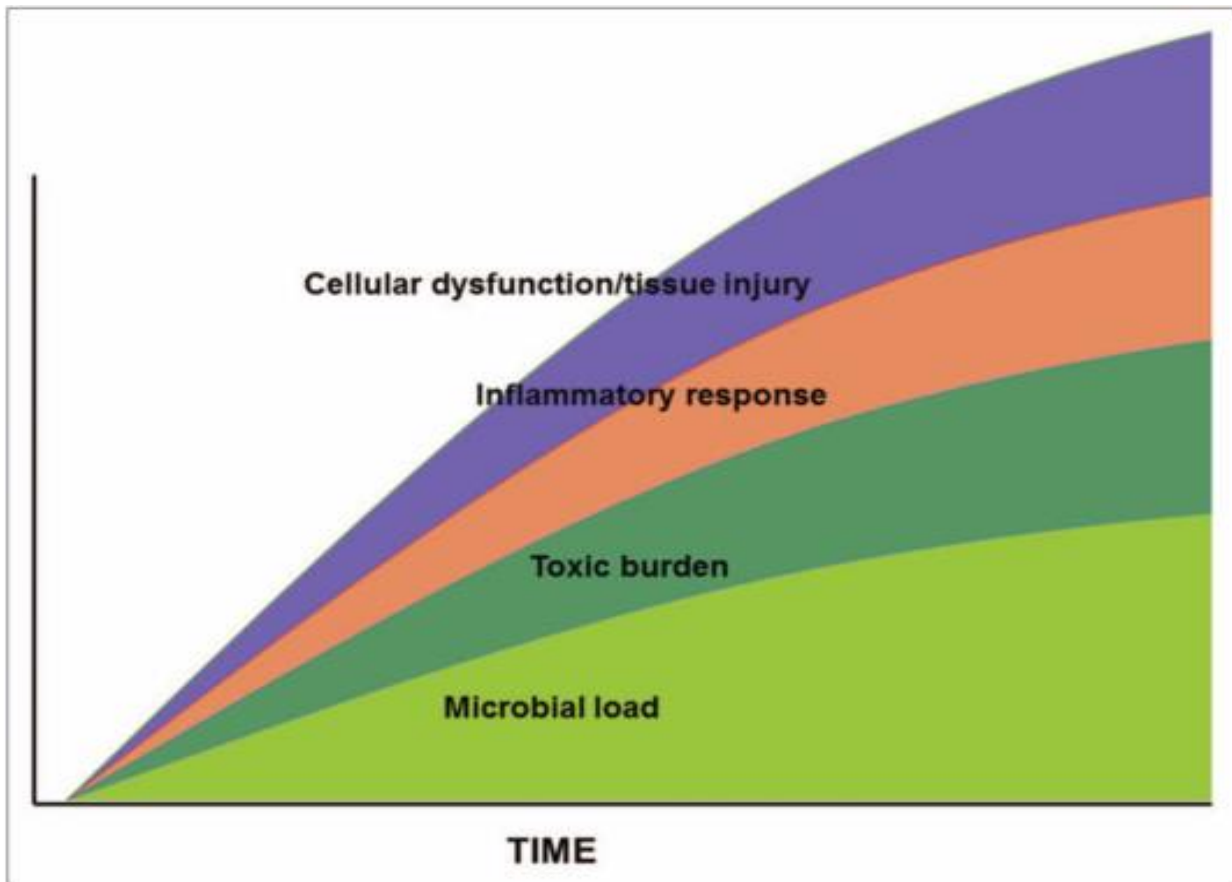


Figure 1.1 The classical microbiology model of septic shock. Pathophysiological model of septic shock places the microbial load as the source of a toxin burden which leads to an inflammatory response resulting in cellular dysfunction and tissue injury. This image copied with permission from [8].

By the 1990's, a new model of the dominant pathophysiologic processes underlying sepsis was proposed. The key component in this model was the concept that sepsis was driven by over activation of the patient's innate immune system, even following rapid elimination of the bacterial or fungal pathogen with potent antibacterial treatment. Experimental therapeutic research at this time consisted of targeting the overactive immune system responses with immuno-regulatory compounds. Targets of key immune system factors included: Tumor Necrosis Factor (TNF), Interleukin 1 (IL-1), Platelet Activating Factor (PAF), Toll Like Receptor 4 (TLR-4) and many others. There was little to no patient improvement in these experimental immunomodulatory clinical trials[9,10].

A recent model of septic shock presented by Kumar *et al*. 2014 combines elements of the microbiological and immunologic models of septic shock with an additional component known as "irreversible shock". This concept of "irreversible shock" was described by Wiggers *et al*. 1944 using a dog animal model where a state of hypovolemic shock was induced in dogs[11]. The dogs were allowed to remain in a shock state for various time periods before resuscitation was started. There was a critical time period identified after which supportive therapies proved ineffective and the dogs began an irreversible progression towards death. This became known as the "shock threshold" (Figure 3), or the point at which inflammatory stress to the cardiovascular system exceeds hemodynamic reserve. At this stage of sepsis, the entity progresses into shock or past the shock threshold. Hence, there is a finite period of time to intervene. The microbial burden, or the source which is causing the shock, must be eliminated before the patient is irreversibly injured and before irreversible shock has been established. Once the body has been exposed to a certain degree of shock, despite the intervention of supportive therapies, the individual will

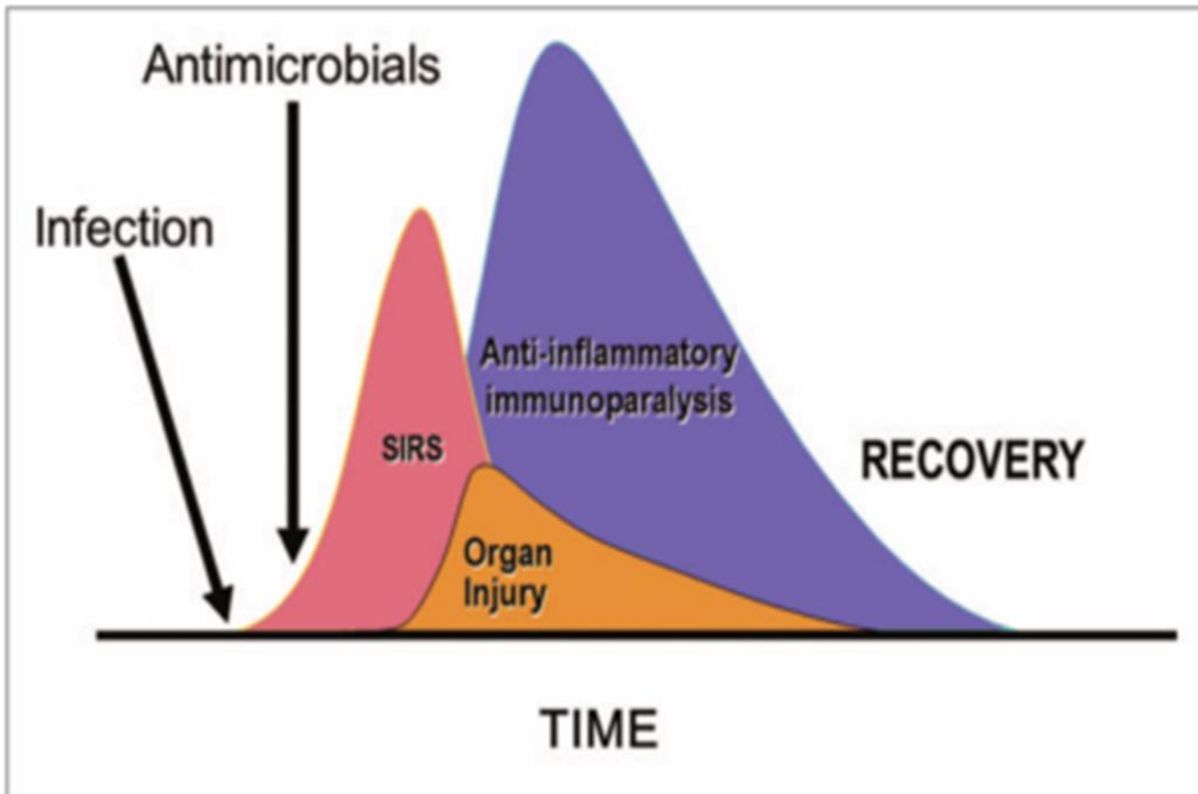


Figure 1.2 The immunological model of septic shock. Pathophysiological model of septic shock showing the overactivation of the immune system leading to SIRS, organ injury and an anti-inflammatory immunoparalysis. This image was copied from [8].

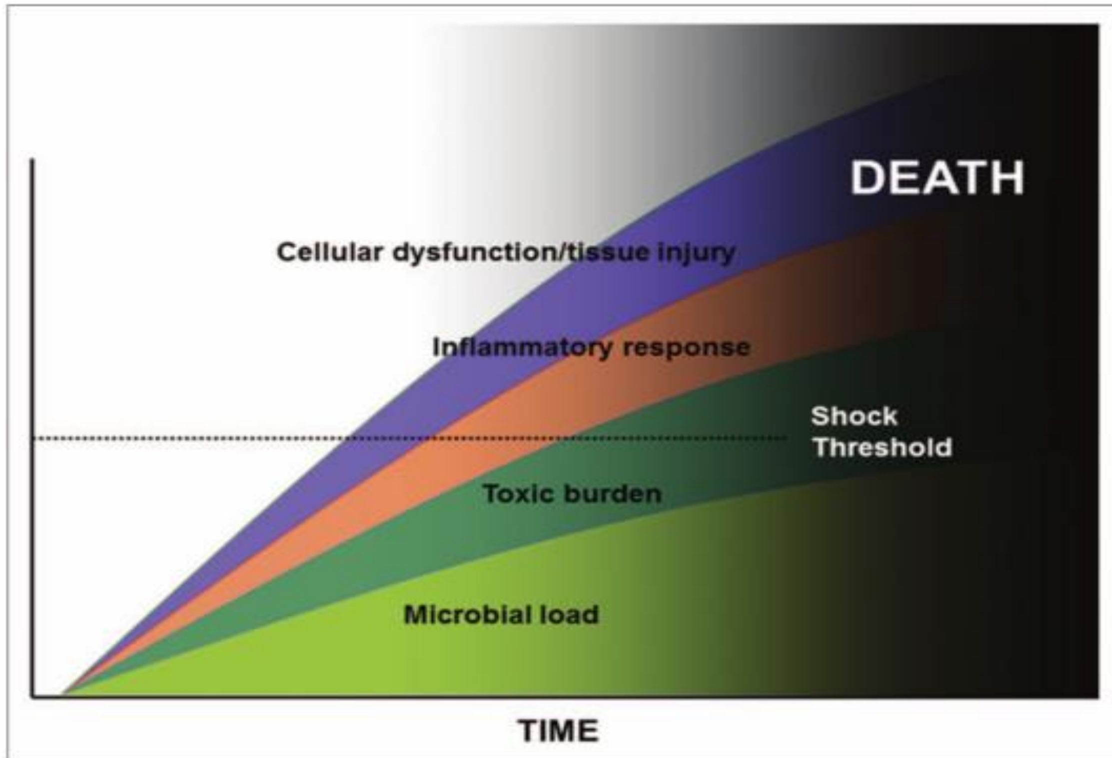


Figure 1.3. The composite model of septic shock. Pathophysiological model of septic shock which incorporates the notion of irreversible shock as depicted by a shock threshold. The microbial load drives the toxic burden, leading to an inflammatory response which causes cellular dysfunction/tissue injury. Once this shock threshold is passed, the individual has begun the irreversible progression towards death despite therapeutic and supportive therapies. This image was copied from [8].

will ultimately die[8]. This concept of septic shock can translate to other forms of shock such as hypovolemic, neurogenic, anaphylactic and cardiogenic shock[11,12]. In the composite model of septic shock, elements from the microbiological and immunologic models constitute the base, with the additional concept of irreversible shock proposed by Wiggers *et al.* 1944.

In the composite pathophysiological model of septic shock, the driving source of irreversible shock is the presence of a microbial load. This model specifically highlights the concept of irreversible shock if not reversed in a timely manner. The treatment which would theoretically result in the greatest influence on mortality would be related to the speed in which the microbial load can be reduced and eliminated[8,13]. The most currently accepted model of sepsis is the classical microbiological model.

1.4 Microbiology and infection sources of sepsis

The most commonly isolated site of infection in patients with sepsis, across all demographics, was the respiratory tract followed by the abdomen at 67.4% and 21.8% respectively[14]. Gram-negative bacteria are more commonly isolated when compared to gram-positive bacteria accounting for approximately two-thirds of isolated pathogens from septic patients[3].

A 2012 nationwide audit by the World Federation of Societies of Intensive and Critical Care Medicine data from ICUs in Europe, Asia, America, South America, Africa, and Oceania was collected and compiled. Sakr *et al*. 2018 examined specific ICU data pertaining to sepsis cases including 28-day mortality, SOFA scores, antibacterial therapy administered as well as the isolated pathogens. In 2012, the most commonly isolated gram-negative pathogens worldwide were *E. coli* (22.7%), *Klebsiella pneumoniae* (17.2%) and *Pseudomonas aeruginosa* (16.3%). The most commonly isolated gram-positive pathogens worldwide were coagulase negative *S. aureus*

(24.2%), Methicillin-susceptible *S. aureus* (MSSA) (12.4%) and Methicillin-resistant *S. aureus* (MRSA) (7.3%). There were regional differences in the epidemiology of sepsis with greater incidences of *E. coli* occurring in Europe when compared to North America and East and Southeast Asia[14]. Higher rates of MRSA were found in North America and the Middle East when compared to other countries[14]. The overall ICU mortality of all the countries comparing gram-positive and gram-negative bacteria was similar, at 26.2% and 26.6%, respectively. Within the worldwide gram-positive spectrum, infections caused by MSSA had the highest mortality at 28%. In the gram-negative spectrum, *Acinetobacter* species had the highest rate of mortality at 37% followed by *P. aeruginosa* at 30.1%[15]. *S. aureus* and *E. coli* will be the bacterial species further examined as they were tested in our experiments.

1.5 *Escherichia coli*

E. coli is a gram-negative facultative anaerobe that has become one of the most well studied microbes in science[15]. It has played a fundamental role in understanding transcription, translation, genetic code and cell replication. *E. coli* is a commensal gram-negative organism of the gut microbiome of mammals and for the most part, is harmless towards the host. The ability of *E. coli* to incorporate a variety of virulence factors into its genome can allow the bacterium to become an opportunistic pathogen[16]. Hence, *E. coli* can be divided into different pathotypes based on the mechanisms and type of diseases induced. Some of the varying pathotypes of *E. coli* include: enteropathogenic *E. coli* (EPEC), enterohamerrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and uropathogenic *E. coli* (UPEC)[17]. *E. coli* infections often result in diarrheal diseases, urinary tract infections (UTI), blood stream infections, and sepsis.

Treatment of *E. coli* infections is dependent on the location of the infection site and the AB resistance phenotype. Diarrheal diseases caused by *E. coli* are generally limited to 3-5 days with severe cases treated with fluid resuscitation therapy. *E. coli* UTIs can lead to further complicated conditions such as pyelonephritis and sepsis, where both are readily treated with ABs. AB monotherapy, as a first line of treatment, is often sufficient in the majority of infections, with Abs such as fosfomycin, cephalexin, nitrofurantoin and ceftriaxone being the most commonly prescribed. In cases of severe *E. coli* infection, such as sepsis, intravenous (IV) ABs will be administered. Kumar *et al*. 2006 suggested that combination therapy of a β-lactam with aminoglycosides, fluoroquinolones or macrolides is associated with a superior outcome as compared to AB monotherapies with a β-lactam[12,18] in the treatment of septic shock. The *E. coli* species utilized in our experiments is a well-known classified strain. It was chosen due to its low susceptibility profile as well as being a well characterized laboratory reference strain.

1.6 *Staphylococcus aureus*

S. aureus is a gram-positive, facultative anaerobe that is common to human flora often found in the nares, skin, and mucous membranes. *S. aureus* is an opportunistic pathogen that can cause a wide variety of diseases ranging from uncomplicated soft skin and tissue infection (SSTI), to life threatening conditions such as osteomyelitis, pneumonia, and sepsis[19]. *S. aureus* was originally sensitive to penicillin, however, strains with penicillin resistance, via penicillinase activity, have been increasingly identified and have rapidly rendered penicillin ineffective as an antimicrobial intervention. Treatment of mild *S. aureus* infections depends on the site of infection and severity of the disease. SSTIs may require the removal of the nidus of infection, followed by treatment with ABs. MSSA indicates *S. aureus* species that are sensitive to methicillin. Treatment of sepsis

caused by MSSA often requires source control, IV AB administration, including penicillinase-resistant semisynthetic penicillin and first-generation cephalosporins. MRSA isolates have a *mec* gene encoded within its chromosome that confers resistance to multiple ABs when expressed and includes β-lactams, methicillin, nafcillin and oxacillin[19]. Vancomycin, daptomycin, trimethoprim-sulfamethoxazole and linezolid are potential treatment options for sepsis caused by MRSA in Canada and worldwide. The *S. aureus* strain selected for study in our project is the clinical isolate 118324. It was selected after screening of multiple *S. aureus* isolates from the labs -80°C freezer and chosen due to its susceptibility towards all the ABs used in this study.

1.7 Antibacterial resistance

The WHO 2019 report update has classified antibacterial resistance (AMR) as one of the top 10 global public health concerns facing humanity[20]. Pathogens developing resistance to multiple classes of ABs pose a threat not only to the critically ill, but also to healthy individuals. Globally, pathogens such as *E. coli, K. pneumoniae,* and *S. aureus* have the broadest range of resistance to the most commonly used ABs[20].

In a report by the global antibacterial resistance and use surveillance system (GLASS), *E. coli* resistance rates of ciprofloxacin treatment ranged from 8.4% - 92.9%. Ciprofloxacin followed by meropenem and then ceftriaxone had the highest rates of AMR reported[21]. Extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae such as *E. coli,* are resistant to the majority of penicillins and cephalosporins currently prescribed. This results in carbapenem being one of the few remaining AB treatment options. However, carbapenem resistant Enterobacteriaceae (CRE) is an ever-growing concern, leading to colistin being the sole treatment option available for CRE

infections[22]. To emphasize the global concern of AMR, certain regions have already reported colistin resistance in CRE, leaving no available treatment[23]**.** The gene encoding for colistin resistance, *mcr-1* is rapidly transferable and could exacerbate the rate at which *E. coli* becomes resistant to the last line of AB available to treat MDR gram-negative bacteria[24].

MRSA is currently a widespread issue in communities and hospitals worldwide extending to North and South America, the greater Asia continent, Africa and Europe. MRSA can be resistant to penicillins, cephalosporins, chloramphenicol, lincomycin, aminoglycosides, tetracyclines, macrolides, quinophthalones, sulfonamides, and rifampicin[19]. Current treatment options for MRSA include vancomycin, daptomycin, linezolid and tigecycline. MRSA has also developed resistance to most ABs resulting in limited or no options for treatment. AMR can impact or complicate the treatment of sepsis and septic shock due to treatment if the pathogen is resistant to the ABs. The standard treatment for sepsis and septic shock is empiric AB therapy, fluid resuscitation as well as supportive therapies.

1.8 Bactericidal and bacteriostatic categories of antibiotics

The majority of ABs, such as β-lactams, are not exclusively bactericidal. Bacteriostatic ABs, such as protein synthesis inhibitors, strictly inhibit the bacterial growth, however, there can be a minor killing effect observed. Classification of ABs as either bactericidal or bacteriostatic is dependent on the *in vitro* effects of these drugs in relation to the microorganism being studied. The specific ratio of the minimum bactericidal concentration (MBC) is compared to calculated minimum inhibitory concentration (MIC)[25]. The MBC value represents the lowest concentration of AB required to result in reductions of bacterial inoculate of >99.9%, measured as CFU/ml, usually

over a 24-hour time period[26]. The MIC is defined as the lowest concentration of AB required to prevent the visible growth <99.9% clearance of a bacteria inoculum, usually over 24-hours[27]. The microbiological definition of a bactericidal AB is when the ratio of the MBC compared to the MIC is ≤4. Some bactericidal AB classes include aminoglycosides, β-lactams, fluroquinolones, glycopeptides, lipopeptides and nitrofurans.

Bacteriostatic AB are defined as having an MBC/MIC ratio >4. Some bacteriostatic AB classes include lincosamides, macrolides, sulfonamides, oxazolidinones and glycylcyclines[27]. This definition is strictly used for *in vitro* characterization of AB activity.

ABs can also be both bactericidal and bacteriostatic depending on the concentration administered and the infectious organism being treated. Tigecycline can be both bactericidal and bacteriostatic against *E. coli* depending on the concentration and the resistance profile of the strain[28]. The effects of certain ABs such as β-lactams are irreversible compared to bacteriostatic ABs. Once administered and given enough time to kill bacteria, removing the presence of the bactericidal AB will not result in the continued growth of bacteria, unless a highly resistant sub-population has appeared**.** Conversely, the removal of bacteriostatic AB from a treated population of bacteria will result in their continued growth[29].

1.9 Synergism and antagonism between antibiotics

There are 4 types of interactions which can occur between ABs 1) Indifferent interactions, where two ABs have no effect on each other. 2) Additive interactions, where the combined effects of the ABs are greater than either AB alone. 3) Synergistic interactions, where the combined effects of the ABs are vastly superior to either AB individually, more so than additive. 4) Antagonism

interactions, where the combined effects of the ABs result in a decreased effect compared to either AB individually[13,18]. These interactions are important considerations when determining combinations of ABs for treatment. When examining the combination of two bactericidal ABs, such as a β-lactam combined with an aminoglycoside, there have been reports of synergism[30]. β-lactam mechanisms of action involve disruption of cell wall peptidoglycan synthesis by binding PBP[31]. Aminoglycosides disrupt protein synthesis by irreversibly binding to the 30S ribosomal subunit[32]. Preventing the synthesis of the cell wall with the β-lactam, as well as the reduction in protein synthesis with the aminoglycosides, leads to a decrease in available PBP which results in a greater bacterial killing rate compared to monotherapy of either AB[33]. Studies have shown that the combination of a bactericidal agent with a bacteriostatic agent results in antagonism between the two ABs[13,30].

Once septic shock has occurred, there is a time sensitive window in the treatment of the infection source, particularly when the causative agent is unknown. Initially, empiric therapy often includes the administration of more than one broad-spectrum AB. Upon positive identification of the infectious agent(s), the AB regimen is typically adjusted from broad spectrum ABs to an AB which can specifically lead to the eradication of the pathogen[8]. This initial administration of combinations of ABs could potentially result in decreased rates of bacterial reduction if both bactericidal and bacteriostatic ABs are combined. β-lactam ABs will only affect growing populations of bacteria. Bacteriostatic ABs such as lincosamides and glycycylines result in the cessation of growth by inhibiting certain fundamental cellular processes such as protein synthesis. Therefore, administering a bactericidal β-lactam into a population of bacteria where

growth has been halted by a bacteriostatic AB, may result in antagonism as the killing mechanism of the bactericidal AB have been halted[29,30].

1.10 Bactericidal class - Cephalosporins

Cephalosporins are a group of chemically related molecules with a β-lactam ring core and a dihydrothiazine ring as seen in Figure 1.4. There are 6 generations of cephalosporins, distinguished by differences in the side chains attached to the β-lactam and the dihydrothiazine rings. These differences result in cephalosporins to treat a broad range of both gram-positive and gram-negative pathogens[34]. The antibacterial mechanism of action is related to the interaction with the Penicillin Binding Protein (PBP) which disrupts the crosslinking of peptidoglycan. Compromised integrity of peptidoglycan is the initial result of cephalosporin ABs, followed by the continued breakdown of the peptidoglycan wall through the bacterially produced autolysins. Cephalosporins, for the most part, demonstrates bactericidal activity *in vitro* against susceptible *E. coli* and MSSA strains[34].

First generation cephalosporins include cefazolin and are often used to treat infections caused by MSSA, Streptococcal species and some gram-negative bacilli. In the clinical setting, these could include conditions such as vertebral osteomyelitis, necrotizing fasciitis, pyomyositis, SSTI, bacteremia, and endocarditis[34].

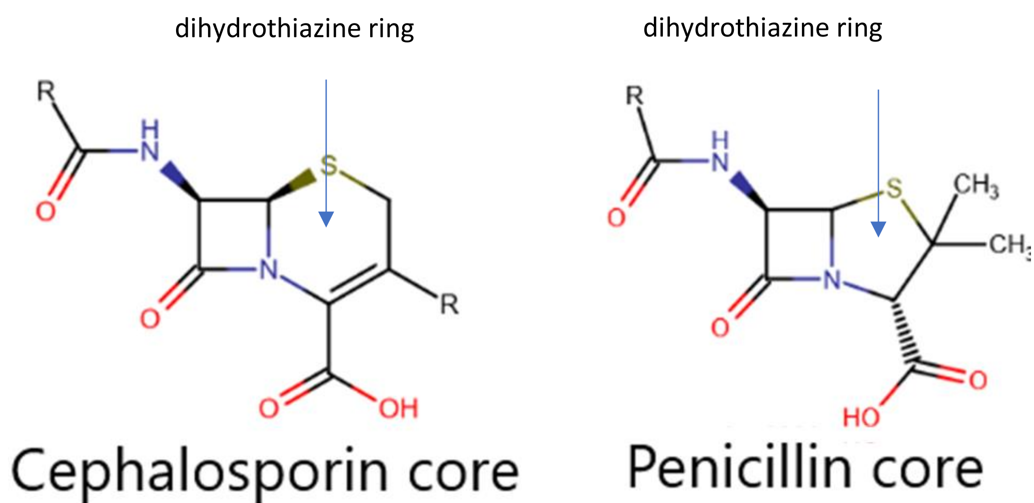


Figure 1.4. Comparison of cephalosporin and penicillin-based antibiotics which all are β-lactam category antibiotics due to the similar β-lactam ring structure. Chemical diagrams shown were obtained from the Chem Space website (https://chem-space.com).

Second generation cephalosporins include cefoxitin and cefotetan and both drugs have improved gram-negative bacterial species killing activity. They are often used to treat infections caused by *Haemophilus influenzae, Neisseria spp.,* and *Staphylococcus* species. Due to the ability of the second generation cephalosporins to be effective against anaerobic bacteria, this class of ABs are often used prophylactically in medical procedures such as amputations, head and neck, esophageal, gastroduodenal and colorectal surgeries[34,35].

Third generation cephalosporins include ceftriaxone and cefotaxime and both have improved killing activity against gram-negative pathogens, but retain activity for gram-positive Streptococci. Ceftriaxone has a wide range of bacterial killing activity including *E. coli, Enterobacteriaceae,* MSSA, *Neisseria* species, *Streptococcus* species, but limited activity against *Pseudomonas* species.

The pharmacokinetics of ceftriaxone describes AB absorption, distribution, protein binding and the rate of clearance from the body. Ceftriaxone pharmacokinetics include a volume of distribution that is dependent on dose upwards of 10.1L at a 2g dose. Serum protein binding of ceftriaxone is also dose related ranging from ~95% at >70mg/L to ~58% at 600mg/L. Ceftriaxone has an average half-life of 6-hours and is eliminated both hepatically and renally in humans[36]. Ceftriaxone shows bactericidal pharmacodynamic properties with its efficacy related to the time (T) that the plasma concentration exceeds the pathogen MIC (T>MIC). Post-antibiotic effects (PAE) were observed to last from 1 – 7.2 hours depending on the concentration and pathogen[37]. Clinical uses of ceftriaxone include the treatment of pyelonephritis, necrotizing fasciitis, SSTI, human and animal bites, Lyme disease, sexually transmitted infections and bacteremia[37].

Additionally, ceftriaxone and cefotaxime have good penetration into the central nervous system (CNS) and can be used to treat meningitis[28].

Adverse side effects observed with cephalosporins include anaphylaxis with potential cross-reactivity in those who have penicillin allergies due to the common β-lactam ring. Resistance to cephalosporins arise from structural alterations of the PBP structure and the cleavage of the β-lactam ring via a cephalosporinase enzyme[34].

Antagonism was observed by Ocampo *et al*. 2014 when a bacteriostatic agent was added to a cephalosporin in an *in vitro* checkerboard assay[20]. Conversely, synergism has been reported between cephalosporins and aminoglycosides in an *in vitro* checkerboard assay[30,32].

Fourth generation cephalosporins include cefepime which has coverage against MSSA, *P. aeruginosa, Streptococcus* species, and gram-negative bacilli such as *E. coli*. Cefepime is often used for the treatment of intra-abdominal infections, meningitis and cholangitis[34].

The fifth generation of cephalosporins includes ceftaroline and ceftibiprole. This is the only group of cephalosporins that have activity against MRSA[34]. There are also AB effects observed against *Streptococcus* species, *P. aeruginosa,* many Enterobacteriaceae *including E. coli* and *Acinetobacter baumanii.* The majority of infections treated with this generation are SSTI and community and hospital acquired MRSA infections[34].

The newest class of cephalosporin compounds includes combinations of cephalosporins with β-lactamase inhibitors. One example is ceftolozane/tazobactam[34]. This generation of cephalosporins has AB activity against anaerobes, many gram-positives including MSSA and Streptococci, and a wide range of gram-negative bacilli including *P. aeruginosa*[34].

1.11 Bacteriostatic AB classes

1.11.1 Lincosamides (clindamycin)

Lincosamides consist of clindamycin, lincomycin, and pirlimycin and are structurally characterized by a pyrrolidine ring bonded to a pyranose moiety[38] (Figure 1.5). The antibacterial mechanism of action consists of reversible binding to the 50S ribosomal subunit, interfering with the transpeptidation reaction and inhibition of protein chain elongation. Inhibition of alpha toxin expression has also been observed[39]. Clindamycin has a broad-spectrum of coverage for gram-positive bacteria such as Staphylococci species. This includes MSSA, certain strains of MRSA and *Streptococcus* species, many anaerobic bacteria, particularly microaerophilic Streptococci found above the diaphragm. Lincosamides are not effective at inhibiting growth of *Bacteroides fragilis* and *Clostridium difficile.* Clindamycin is not effective against Enterococci species, nor widely infectious gram-negative bacteria such as *E. coli* and *K. pneumoniae*[40].

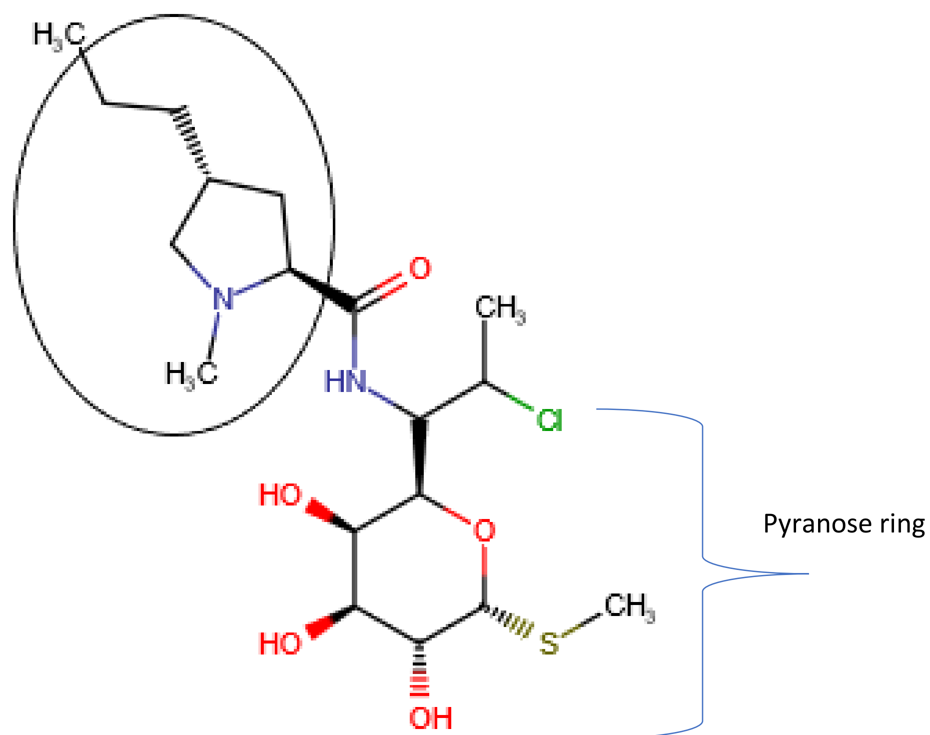


Figure 1.5. Chemical structure of the lincosamide back bone consisting of a pyrrolidine and pyranose rings which connect by an amine bond. Image drawn on https://chem-space.com

The pharmacokinetics of clindamycin includes high bioavailability at 90% and good penetration into a variety of tissues but excludes cerebrospinal fluid (CSF). The drug is hepatically excreted with a half-life between 2 and 2.5 hours[40]. Clindamycin exhibits bacteriostatic pharmacodynamics with a long PAE for many pathogens including *S. aureus.* The antibacterial efficacy is related to the ratio of the area under the curve (AUC) compared to the MIC (AUC/MIC)[40]. Clinical uses of clindamycin include treatment of intra-abdominal infections, lower respiratory infections, gynecological infections, bone and joint infections, SSTI's and sepsis. Schlievert *et al*. 1984 demonstrated that in the treatment of *Staphylococcus* infections, the use of clindamycin has led to significant decrease in alpha toxin expression which appears to be useful in treatment of *S. aureus*-associated toxic shock syndrome(TSS)[39].

Adverse effects of clindamycin include gastrointestinal upset, joint pain, heartburn, patchy white mouth plaques and vaginal irritation. Resistance mechanisms towards clindamycin include methylation of the ribosome site or mutation of the ribosome to prevent binding. There is efflux of clindamycin via specific multi-drug selective efflux pumps such as small multidrug resistance family, major facilitator superfamily and multidrug and toxic compound extrusion family[41]. Inactivation of clindamycin can occur via the structural modification of O-nucleotidyltransferase. Inducible resistance towards clindamycin has been observed in certain strains of *S. aureus* [42,43].

Clindamycin demonstrates antagonism *in vitro* against MSSA when given alongside a macrolide AB or erythromycin. This is due to the similar site of binding and mechanisms of action[40]. Synergism has been observed *in vitro* when clindamycin was administered alongside an aminoglycoside AB in gram-positive and gram-negative bacteria including *Prevotella melaninogenica*, *Bacteroides fragilis, Chlamydia trachomatis*[44,45]. Lincosamides such as

clindamycin are readily administered alongside other ABs especially when a *S. aureus* species capable of producing TSST-1 is the suspected causative pathogen.

1.11.2 Chloramphenicol

Chloramphenicol was widely used in the 1950's in much of the developed world. It was replaced by less toxic ABs such as ceftriaxone. The chemical structure of chloramphenicol consists of a nitrobenzene core with an attached propane group[46]. The mechanism of action occurs when chloramphenicol binds to the 50S ribosomal subunit, inhibiting the peptidyl transferase reaction and preventing protein chain elongation[46]. Chloramphenicol is a broad-spectrum AB with activity against a variety of gram-positive and gram-negative pathogens as well as *Streptococcus* species, MRSA and MSSA and *E. coli*. Chloramphenicol can penetrate into the CSF and it is bactericidal for the treatment of common childhood meningitis pathogens, such as *H. influenzae, S. pneumoniae, N. meningitidis* [46,47]. Chloramphenicol was used to treat meningitis until modern β-lactams such as cefazolin or ceftriaxone became available.

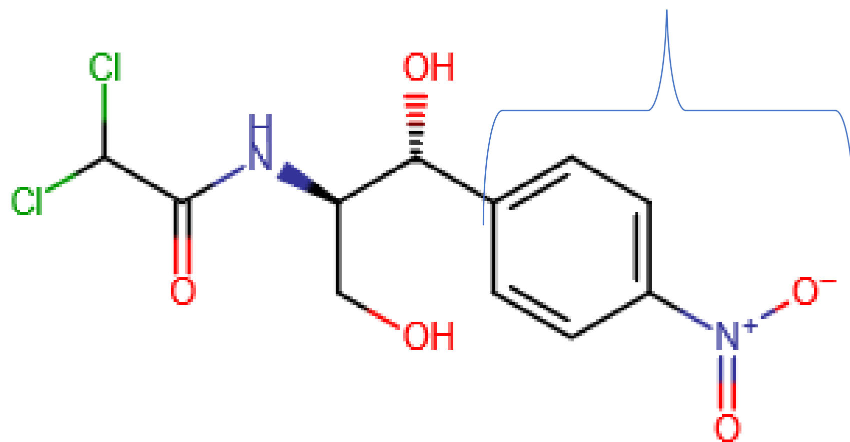


Figure 1.6. Chemical structure of chloramphenicol with the nitrobenzene core highlighted.

Image drawn on https://chem-space.com

The pharmacokinetics of chloramphenicol includes distributing evenly into most of the tissues including the CSF. Distribution into the CSF is an important consideration when targeting infections such as meningitis[47]. Protein binding of chloramphenicol is approximately 50-60% and is excreted renally. The pharmacodynamics of chloramphenicol can display either bactericidal or bacteriostatic effects depending on the pathogen and achieved concentration of the AB. When susceptible strains of *E. coli* were administered chloramphenicol, they demonstrated a cessation of growth indicating bacteriostatic activity. Chloramphenicol displays a C_{max}/MIC or the AUC/MIC pharmacodynamic property. The greater the concentration achieved and the longer the time the concentration of chloramphenicol remains above the MIC, the greater chloramphenicol's antibacterial effects[48].

In clinical settings, chloramphenicol is prescribed sparingly because of a variety of adverse effects. Due to the increasing amount of AMR infections, it is being re-visited for potential therapeutic use in developing countries such as India. Chloramphenicol was readily used in the 1950's for the treatment of conditions of salmonellosis, meningitis, brain abscesses, intraocular infections and cystic fibrosis[46]. There are a variety of adverse effects observed in patients treated with chloramphenicol, some being dose related and others being dependent on the duration of treatment. Adverse reactions can manifest as pancytopenia, suppression of bone marrow through the inhibition of mitochondrial protein synthesis and "grey baby syndrome" represented by vasomotor collapse. Gastrointestinal distress, neurological effects including delirium and suppression of lymphocytic transformation have also been observed[47].

Resistance to chloramphenicol occurs through a variety of mechanisms including alterations to the drug, efflux pumps that expel the drug from the cell, and alterations to the plasma

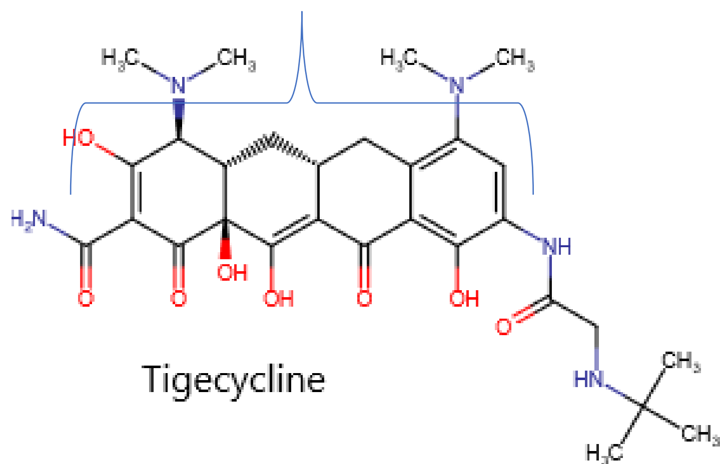
membrane[49]. Chloramphenicol acetyltransferases alter the chemical structure of the AB through acylation of the molecule. Alterations to the plasma membrane by the loss of the OmpF porin protein prevents chloramphenicol from entering into the cell[49,50].

Chloramphenicol, combined with β-lactam ABs, including ceftriaxone, has shown to be antagonistic towards bacteria including *S. aureus, Salmonella* spp. *K. pneumoniae, E. coli and Shigella typhimurium*[44,51]*.* Chloramphenicol, in combination with a β-lactam, has been studied previously[44,51]. This combination is being revisited in our study because of findings in large volume data reviews. The combination of chloramphenicol alongside a β-lactam resulted in an increased mortality in patients with septic shock. Chloramphenicol is sparingly used in developed countries, but due to the increase in AMR there is potential to see its return in clinical therapies.

1.11.3 Glycylcyclines (tigecycline)

Glycylcyclines are a class of tetracycline-based AB derivatives consisting of minocycline and tigecycline. The base chemical composition of glycylcyclines consists of four linear fused tetracyclic rings with varying functional groups at different positions[52]. The antibacterial mechanism of tigecycline action results from the reversible binding of the AB to a high affinity site within the 30S ribosome similar to that of tetracycline[53]. These binding blocks aminoacyl transfer RNA and prevents the incorporation of amino acids into the growing peptide chain. The overall outcome is the inhibition of protein synthesis[53]. Compared to tetracycline, tigecycline exhibits greater resistance to efflux pumps. Tigecycline is able to bind to both wild type and TetM protected tetracycline-resistant ribosomes which normally display resistance to tetracycline[51,53].

Four linear fused tetracyclic rings



Four linear fused tetracyclic rings

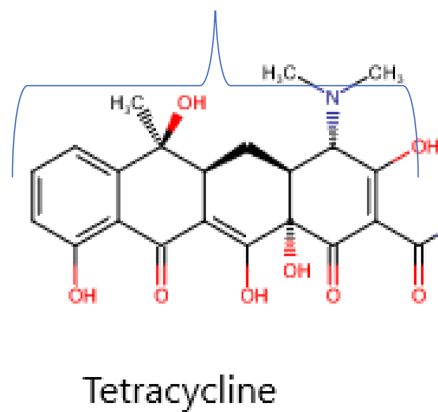


Figure 1.7. Chemical structures of two glycylcyclines, tigecycline and tetracycline with an emphasis on the four linear fused tetracyclic rings. Image drawn on https://chem-space.com

Consequently, tigecycline has greater potency against tetracycline resistant organisms[52]. Tigecycline shows activity *in vitro* against a wide variety of bacteria including both gram-positive and gram-negative aerobic and anaerobic isolates[53]. The gram-positive coverage includes *Staphylococcus* species including MSSA and MRSA, *Streptococcus* species as well as some *Enterococcus* species and *Listeria monocytogenes.* The gram-negative coverage includes *Acinetobacter, E. coli, Klebsiella* species, *Neisseria* species, *P. aeruginosa* and more[52].

The pharmacokinetics of tigecycline demonstrates 75-100% bioavailability via the oral route with an approximate 68% protein binding. Tigecycline displays a considerably large volume of distribution upwards to >10 L/kg. Tigecycline has a half-life of approximately 36-hours in healthy individuals and is primarily renally excreted. A PAE was observed to last between 5-9 hours depending on the pathogen examined[53].

Dependent on the pathogen, tigecycline exhibits both bactericidal and bacteriostatic activities with the activity being dependent on the pharmacodynamics of AUC/MIC at 24 hours. Bactericidal activity of tigecycline has been observed against MSSA, MRSA and *E. coli*, while bacteriostatic activity was observed against *Enterococcus faecalis, K. pneumoniae* and *Streptococcus pneumoniae*[28]. Clinical uses of tigecycline are generally reserved for drug resistant bacteria such as vancomycin-resistant *E. coli* (VRE) and MRSA. Several clinical trials have examined the efficacy of tigecycline in complicated SSTI's and intra-abdominal infections. Tigecycline monotherapy showed comparable results to other standard treatments[52,53]. Adverse patient reactions of tigecycline include itchiness, nausea and vomiting[52].

Resistance to tigecycline can be due to the efflux of the AB from the cell by efflux pumps and structural alterations to the binding site within the 30S ribosome. Enhanced over-expression of the resistance-nodulation-cell division-type efflux pumps have been observed in *Enterobacteriaceae* species, leading to reduced tigecycline susceptibility[53]. Alterations to the ribosomal S10 protein, by mutation of *rpsJ* gene, has also increased tigecycline resistance of bacteria such as *E. coli*[54].

When tigecycline was combined with a β-lactam AB, synergism was demonstrated against *P. aeruginosa* and ESBL-negative *K. pneumoniae*[55]. Tigecycline can be administered for infections caused by tetracycline resistant organisms providing another option for treatment. Tigecycline has a broad spectrum of activity including gram-positive and gram-negative pathogens such as MRSA and MDR *E. coli.* This drug was assessed in our experiments as Kumar found an increase in mortality in patients with septic shock who were treated with tigecycline and a β-lactam.

1.11.4 Macrolides (erythromycin/azithromycin)

Macrolides are a class of ABs which include erythromycin, azithromycin and clarithromycin. They have a macrocyclic lactone ring of 12 or more elements. Erythromycin was used in our experiments due to the increased distribution into human serum when compared to azithromycin. This allowed for tested serum concentrations to be greater than the measured MIC of the *E. coli* strain allowing for the tested macrolide to have a measurable effect against *E. coli*. Erythromycin has a mechanism of action involving the reversible binding to the nascent peptide exit tunnel of the 50S ribosome which modulates the synthesis of proteins. Protein synthesis can

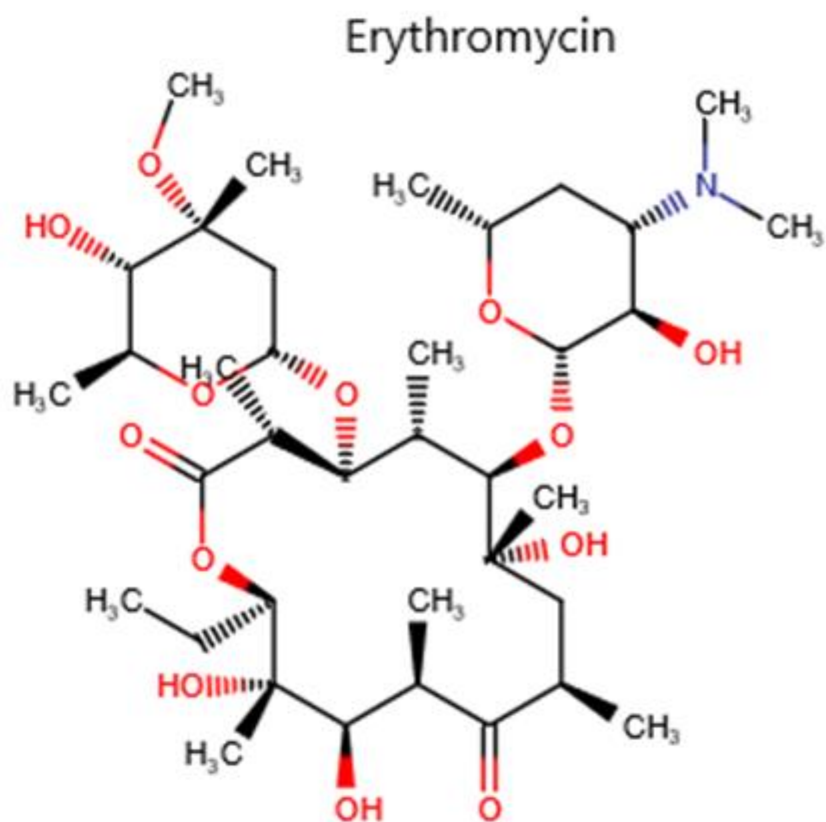


Figure 1.8. Chemical structure of erythromycin. Image drawn on https://chem-space.com

still occur even at extremely high concentrations of macrolides. However, it appears that macrolides prevent the formation of long protein chains from the ribosome[56]. Erythromycin has antibacterial effects against many gram-positive bacteria including *Streptococci* species, *Staphylococcus* species including MSSA, but not MRSA, *Neisseria meningitidis* nor *Clostridia* species[57].

The pharmacokinetics of erythromycin include 90% binding to serum proteins such as albumin and globulin and it is readily distributed into most parts of the body, including CSF. The half-life of erythromycin is approximately 1.9-hours and is renally excreted[58]. Erythromycin demonstrates a post-antibiotic leucocyte enhancement effect with bacteria being more susceptible to phagocytosis post-AB treatment[59].

Erythromycin has a broad spectrum of activity against *S. aureus,* enterococci, pneumococci, *Neisseria* species. Erythromycin demonstrates either a bacteriostatic or bactericidal effect depending on the microorganism and the dosing concentration[60]. *In vitro* erythromycin demonstrates bacteriostatic effects against susceptible strain of *E. coli* and MSSA. Erythromycin shows the greatest antibacterial effects when pharmacodynamic property of T>MIC is prolonged[61].

Clinically, erythromycin has been used to treat a variety of respiratory tract infections such as bronchitis, pneumonitis and pharyngitis, as well as cardiovascular and gastrointestinal tract infections[62]. Adverse effects observed for patients treated with erythromycin include general gastrointestinal discomfort. The most severe effect is the rare occurrence of cholestatic hepatitis

which can occur with prolonged usage between 10 to 20 days[62].

Erythromycin resistance mechanisms are similar to that of lincosamides[63]. Firstly, methylation of the 50S ribosome at the erythromycin binding site prevents this interaction. Secondly, resistance also occurs through the over-expression of efflux pumps. Thirdly, modification of erythromycin can occur resulting in drug inactivation. Esterases and phosphotransferases are the most common enzymes that can modify erythromycin[42].

An *in vitro* study by Ocampo *et al.* 2014 examined individual and combined interactions of bacteriostatic AB with bactericidal AB[30]. Erythromycin was observed to have varying levels of antagonism when combined with bactericidal agents such as ampicillin, fosfomycin, cefoxitin, lomefloxacin, ciprofloxacin, nitrofurantoin. This drug was being used in our experiments as Kumar found an increase in mortality in patients with septic shock who were co-administered erythromycin and a β-lactam.

1.12 Optimizing antibacterial therapy of septic shock

Based on the three pathophysiological models: 1) the classic microbiological paradigm[8] 2) the immunological model[8] and 3) composite model[8] discussed in section 1.3 of this thesis, three different therapeutic approaches have been used to treat septic shock. In the microbiological paradigm of septic shock[8], the goal was to reduce the presence of the microbial burden, decrease the immune response and lessen cellular and organ damage. Treatment in this pathophysiological model centered around reducing the microbial load through the use of monotherapy and combination therapies of ABs. This paradigm forms the basis for many antibacterial therapies for septic shock, where the primary goal is to reduce the microbial load[13].

However, this model does not take into account the many aspects of irreversible shock proposed by Wiggers *et al.* 1944[8]. Kumar *et al.* 2009 found that ABs were given without urgency and that one half of the initial AB doses were administered 6-hours following documentation of hypotension[12]. Consequently, the mortality rates of septic shock remained high in ICU's worldwide at approximately 50%[12].

In the immunological model of septic shock[8], varying immunomodulatory therapies were tested. These included treatments with anti-endotoxins, antagonists to specific immune modulators such as TNF, interleukins, reactive oxygen species, PAF[64]. The resounding underlying results of these clinical trials showed no significance in the overall outcome in treating sepsis. Similarly, corticosteroid therapy has been reviewed multiple times throughout history and initially regarded as an ineffective treatment for sepsis[65]. Currently, corticosteroids are being re-examined and many reports indicate benefits when they are administered to septic individuals. There have been correlations between the administration of low dose corticosteroids and decreased 28-day mortality. The findings also included reduction in the duration of time in the ICU, increased 7-day shock reversal rates and decreased 7-day SOFA scores[65–68].

Ultimately, neither the immunological nor microbiological model of septic shock was able to optimally address the driving factor of shock as it related to subsequent cardiovascular collapse. As a result, conditions such as septic shock, caused by uncontrolled infections have seen very little improvement in the survival rates of patients over several decades[69]. More recently, research by Kumar *et al*. 2009 provided a critical insight into the management and effective treatment of septic shock. Optimizing the speed of pathogen clearance can be achieved by: a) rapid administration of appropriate AB[12], b) pharmacokinetic dosing optimization[13], and

c) synergistic AB combinations[13]. The first step is access to ABs without delay and administrating ABs in an urgent and time sensitive manner, rather than routine or convenience[8,13]. AB treatment time has shown to tremendously impact survival rates in septic shock[70]. Kumar *et al.* 2010 concluded that every hour delay of AB administration resulted in a 7.6% increase in mortality to patients with septic shock. The quicker the reduction of microbial load achieved through optimized pathogen clearance led to higher likelihoods of survival in septic shock patients. Optimized pathogen clearance consists of pharmacokinetic dosing optimization and synergistic AB combinations. Optimizing the pharmacokinetic dosing depends on whether the drugs are time dependent or concentration dependent agents and the choice between using a bactericidal ABs or bacteriostatic ABs. In the composite model, the crucial and indispensable treatment is to provide timely and effective source control in order to accelerate pathogen clearance[13]. Decreasing the microbial load, decreases the toxic burden, which decreases inflammatory response and potentially prevents organ damage and dysfunction[8,13].

CHAPTER 2. Aim, Project Rational, Hypothesis and Objectives

2.1 Aim

This thesis seeks to assess the effect of antibacterial potency when a bactericidal β-lactam agent, that targets the cell wall (cefazolin, ceftriaxone), is combined with a bacteriostatic agent, which affects protein synthesis (clindamycin, chloramphenicol, erythromycin, tigecycline), against AB sensitive strains of *E. coli* and *S. aureus* using *in vitro* TK and an IVPD assays.

2.2 Project Rational

A cornerstone in the treatment of microbiological infections is the quick elimination of infection and its associated microbial burden. In 1942, after the introduction of penicillin into clinical practice, researchers quickly observed that AB resistance towards penicillin began to emerge[71]. The continued adaptation of bacteria and ever-increasing resistance to current AB treatment has led to the discovery and synthesis of more potent antibacterial agents, a greater understanding of the pharmacokinetics and pharmacodynamics of ABs, and the implementation of AB combination therapy[48]. Targeted AB combination therapy, where two different ABs can be co-currently administered to treat a pathogenic infection, has the potential to provide a more potent bacterial rate of bacterial killing compared to currently used ABs. This can occur by the complementation of different mechanism of actions of different ABs[29]. However, AB combination therapy can be a double-edged sword due to the lack of understanding of how combined interactions could potentially result in antagonism. Administering an antagonistic combination of ABs can ultimately lead to failure in therapeutic treatment[8,18]. Therefore, the rational of our

project was to investigate the antagonistic potential of classes of ABs which can be administered to critically ill patients in the ICU.

2.3 Hypothesis

We hypothesize that the combination of a β-lactam, either ceftriaxone for *E. coli* or cefazolin for *S. aureus,* will enhance AB antagonism when given in combination with either tigecycline or chloramphenicol for *E. coli,* and, clindamycin or erythromycin for *S. aureus,* using *in vitro* TK and IVPD assays.

2.4 Objectives

Here, single AB and combination AB treatments were tested against strains of *S. aureus* and *E. coli* using *in vitro* TK and IVPD assays. The antibacterial activity of ceftriaxone, chloramphenicol and tigecycline alone, and, the combinations of ceftriaxone with chloramphenicol or tigecycline were examined against *E. coli* ATCC 700973 Serotype O18:K1:H7 (BORT) in both TK and IVPD assays. The antibacterial activity of cefazolin, clindamycin and erythromycin were tested alone, and, the combinations of cefazolin with clindamycin or erythromycin were examined against *S. aureus* 118324 in both TK and IVPD assays.

TK assays were used to determine any preliminary antagonistic effects between combinations of ABs at concentrations equal to 1X, 2X, 4X and 8X MIC. The IVPD assays were representative of the pharmacokinetics of ABs administered to critically ill or septic shock patients. *In vitro* assays can provide preliminary insights into how ABIs act under ideal experimental conditions. The results generated are strictly related to the ABI.

Ultimately, this is only brief and focused experimental study of how drugs interact during defined *in vitro* bacterial culturing conditions which be helpful for those who study drug interactions clinically.

CHAPTER 3. Materials and Methods

3.1 Antibiotics used in this project

ABs Cefazolin (SteriMax INC., ON. CA), clindamycin (Sigma Aldrich Canada Ltd., ON. CA), erythromycin (Sigma Aldrich Canada Ltd., ON. CA), ceftriaxone (Sigma Aldrich Canada Ltd., ON. CA), chloramphenicol (Acros Organics^{TM}, ON. CA) and tigecycline (Sigma Aldrich Canada Ltd., ON. CA) were used in this study. All ABs chemical powders were kept in 4ºC and AB stock solutions were freshly prepared in small aliquots in either distilled water or anhydrous ethanol and stored at -80ºC to prevent AB degradation until thawed for use.

3.2 Bacterial strains

Two strains were examined in this project, the clinical isolate MSSA 118324 as well as *E. coli* ATCC 700973 Serotype O18:K1:H7 (BORT) were utilized in the experiments. Herein, the *E. coli* strain will be called *E. coli* BORT for simplicity. These strains were stored in vials at -80ºC in a 50/50 glycerol and CA-MHB mixture.

3.3 Minimum inhibitory concentration determination

MIC values were determined from the results of broth micro dilution methods according to the Clinical and Laboratory Standards Institute (CLSI) guidelines M07-A9[72] (Table 3.1). MICs of the AB were determined against the two bacterial strains used in this study. All AB stock solutions were prepared following the CLSI M7-A7 method[72] to a concentration of 2µg/ml.

Bacterial strains were sub-cultured from frozen cryo-stocks in 4 mL of CA-MHB (Difco) overnight. The optical density at 625nm of the overnight inoculum was adjusted to 0.080-0.085 units to

achieve an approximate bacterial concentration of $5 \times 10^5 - 1 \times 10^6$ CFU/ml using a Spectronics-20 spectrophotometer (Genesys, CA, USA). The inoculum was diluted 1:100 and 50µl of inoculum was dispensed into sterile 96 well microtiter plates already containing 50µl of cation adjusted (CA-MHB) broth with the desired concentration of AB to attain a bacterial concentration of 5×10^4 Colony Forming Units (CFU)/well. AB concentration ranges tested were 0.003, 0.006, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 µg/ml. Plates were incubated at 37^0C for 18 – 24 hours to grow cells. The lowest concentration of AB at which visible growth of bacteria was inhibited compared to the control was considered the MIC for that AB against the specific bacterial strain tested. MICs of *E. coli* and *S. aureus* were determined from the mean of three biological replicates.

3.4 Time kill assay for *E. coli* BORT and *S. aureus* 118324

TK assays were performed at 1X, 2X, 4X and 8X the MIC value for individual ABs and for AB combinations; concentrations are based on values previously stated for both *E. coli* BORT and *S. aureus* 118324 listed in Table 3.1 below. Each TK assay consisted of 50ml flasks containing 30ml of fresh sterile CA-MHB. The bacterial culture of *E. coli* BORT or *S. aureus* 118324 was grown as described in section 3.3 and a final bacterial inoculum of 1×10^6 CFU/ml was inoculated into each flask. Bacterial cultures previously grown overnight were introduced into the flask for 30 minutes prior to AB introduction to allow for adjustment to their new environment. The concentrations tested for each AB are described in Table 3.1.

Table 3.1. A summary of the 1X and 8X of MIC values for the antibiotic concentrations used in time kill assay for *E. coli* BORT and *S. aureus* 118324

Bacteria	Antibiotic	1X MIC (µg/ml)	8X MIC (µg/ml)
E. coli BORT	**Ceftriaxone**	0.03	0.24
	Chloramphenicol	8	64
	Tigecycline	0.25	2
S. aureus 118324	**Cefazolin**	0.5	4
	Clindamycin	0.5	4
	Erythromycin	1	8

Five minutes post-AB addition, the first sample was taken and considered the time 0 point. 100µl was removed at the following sample points 0, 0.75, 1.5, 3, 6, 9, 12 and 24 hours and incubated in a 37^0C shaking incubator set to 150 revolutions per minute (RPM) to ensure constant mixing and liquid aeration. The CFU/ml of each sampling time point was assessed by performing agar spread plating for colony count determination on CA-MH agar plates. Before spread plating, samples were diluted 10^1 to 10^4 to minimize the drug carry over. All dilutions were spread-plated on agar plates and incubated at 37^0C for 18-24 hours prior to counting measurements from collected data, CFU/ml time curves were prepared by plotting the sampled time points versus its respective log_{10} CFU/ml, and analyzed between each treatment, with an emphasis on the 12- and 24-hour time points to determine the overall effects of AB combinations. The comparison between single and combination regimens were performed. Antagonism was considered if there was a ≥2 log_{10} increase in the combination compared to the most potent individual AB. Three biological replicates were performed on different days. A two-way ANOVA statistical analysis was performed using the Prism – Graphpad software version 9.2.0.

3.5 *In vitro* pharmacodynamic model single drug for *E. coli* BORT and *S. aureus* 118324

The IVPD assay was performed as a one-compartment *in vitro* infection model described previously[43] and summarized in Figure 5. Briefly, the culture flask consisted of a 250 ml one-compartment central round bottom glass flask with two ports, for the addition and removal of CA-MHB and the other, covered with a rubber stopper that was aseptically removed for the addition of AB, bacterial inoculum, and during culture sampling. All IVPD assays were set up in a sterile condition. Connecting tubing of the flasks was made with sterile silicone tubing prepared in sterile conditions. A computerized peristaltic pump (Masterflex®, L/S®; Cole-Parmer Company,

Illinois, USA) was used to facilitate the flow of fresh media to the experimental flask at a rate representative of the half-life of the AB being tested calculated by the following formula listed below and half-life values (T1/2) listed in Tables 3.2 and 3.3.

Formula 1:

$$Cl = \frac{\ln 2 \; Vd \; (ml)}{T1/2(min)}$$

As seen in Figure 3.1, the primary culture containing central flask was filled with MHB broth to the required volume of 100 ml. Bacterial suspensions of either *E. coli* or *S. aureus* to be added was prepared and inoculated as 10^6 CFU/ml described in section 3.4. Bacteria were allowed to reach a logarithmic phase of growth prior to AB being injected as a bolus dose. Logarithmic phase of growth was determined by previous lab members working on similar projects. The central flask was kept in a water bath at 37^0C and magnetic stirrer was used to mix MHB continuously during the experiment. AB concentrations were simulated as free C_{max} (fC_{max}) in critically ill/septic humans as listed on Tables 3.4 and taking into account AB serum protein binding by only adding the fC_{max} fraction into the central flask.

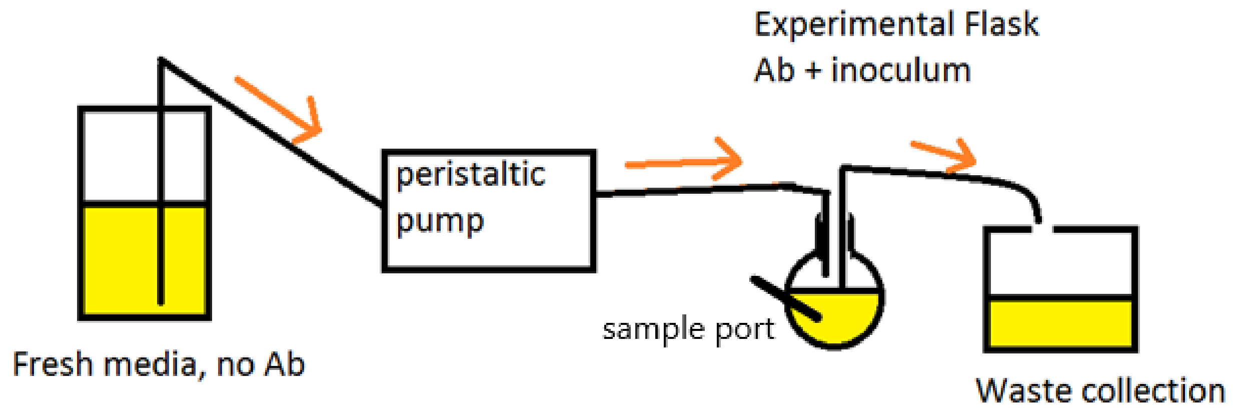


Figure 3.1. IVPD assay set up representing the measurements of a single AB half-life The one-compartment model is shown in the diagram with the peristaltic pump set to add fresh media at a rate equal to the half-life of the AB. The positive pressure in the experimental flask will remove antibiotic containing media and inoculum as fresh media is added.

Table 3.2. A summary of the *E. coli* pharmacokinetic parameters used in the IVPD assay

Drug	fC_{max} (µg/ml)	T1/2 (hrs)	Redose	References
Ceftriaxone	20.5	6.40 ±1.1	q24h	Joynt G.M et al. 2008
Tigecycline	0.794	44.9 ±13.5	q12h	MacGowan, A.P et al. 2002
Chloramphenicol	8	3.20	q6h	www.drugbank.com/ chloramphenicol

Abbreviations: fC_{max}; free maximum concentration, T1/2; half-life

Table 3.3. A summary of the *S. aureus* pharmacokinetic parameters used in the IVPD assay

Drug	fC_{max} (µg/ml)	T1/2 (hrs)	Redose	References
Cefazolin	28	2.7	q8h	Jordan et al. 2016
Clindamycin	1.8	2.7	q12h	Laplante et al. 2008
Erythromycin	1.37	1.9	q12h	Krasniqi S. et al. 2012

Abbreviations: fC_{max}; free maximum concentration, T1/2; half-life

3.6 *In vitro* pharmacodynamic assay two drugs

The IVPD assay was performed in a two-compartment *in vitro* model previously described by J.Blaser[73] and the set-up is shown in Figure 3.2. Compared to the single drug model described in section 3.5, analyzing two ABs with different half-lives has additional requirements. As seen in Figure 6, the first is a second peristaltic pump (Masterflex®, L/S®; Cole-Parmer Company, Illinois, USA) hooked up to another fresh media flask.

The two pumps have different flow rates, but ultimately the outflow from the set-up is equivalent to the AB with the shorter half-life (Cl_A). To compensate for the loss of the AB with the longer half-life, another round bottom flask is introduced which is connected to the experimental flask and the second peristaltic pump. This flask contains AB_B and is supplemented into the experimental flask at a rate equal to the difference between the two AB clearance rates (Cl_A – Cl_B) as summarized in Table 4. The set up ultimately supplements AB_B into the experimental flask at a rate which would be representative of its half-life while removing AB_A from the experimental flask at its respective half-life.

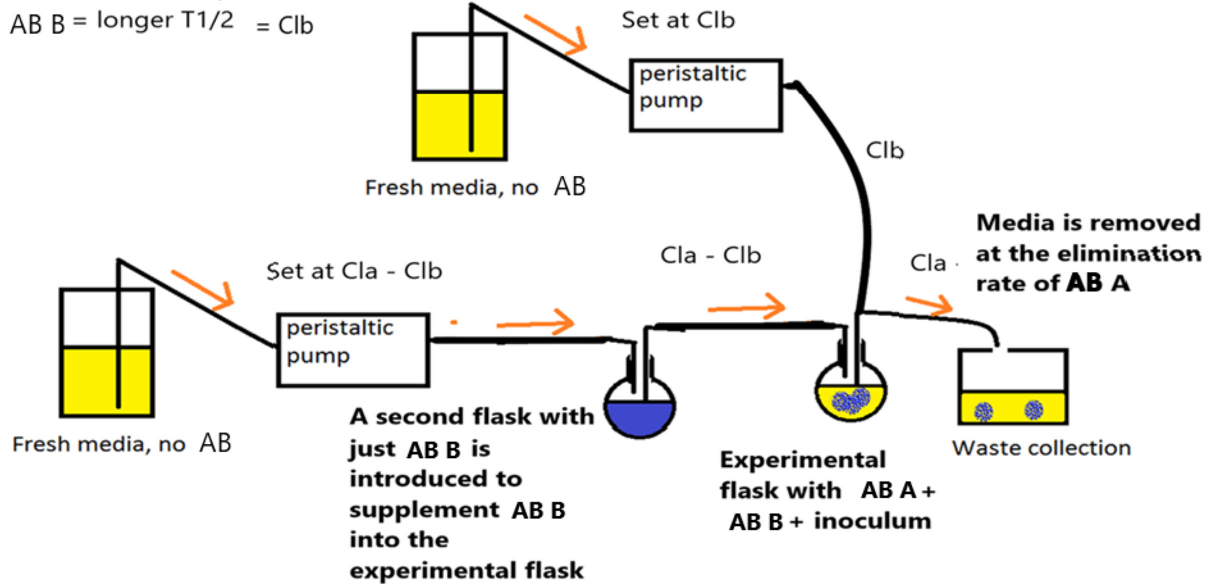


Figure 3.2 IVPD assay representing the different half-lives of two antibiotics. An IVPD assay capable of simulating two different half lives of two AB therapies. Two peristaltic pumps are used, one at the flow rate equal to the difference of the antibiotic with the longer half-life (Cl_A) and the second antibiotic with the shorter half-life (Cl_B) which is connected to a flask containing the antibiotic with the longer half-life (AbB). This flask is connected to the round bottom experimental flask which has both antibiotics present as well as bacterial culture. The second peristaltic pump, feeding fresh media is connected to the “experimental flask” at a flow rate equal to Cl_B. The overall result is the outflow of the experimental flask equal to Cl_A with AbB being supplemented at a rate representative of its half-life.

Table 3.4. Respective half-lives of the AB's used in the IVPD assay with its corresponding flow rate as determined by formula (2)

Antibiotic	$T_{1/2}$ (hrs)	Cl (ml/min)	Reference
Ceftriaxone	6.4	0.180	Joynt
Chloramphenicol	3.2	0.361	www.drugbank.com/ chloramphenicol
Tigecycline	44.9	0.030	April Barbour
Cefazolin	2.7	0.430	Jordan et al. 2016
Clindamycin	2.4	0.481	Laplante et al. 2008
Erythromycin	1.9	0.610	Krasniqi S et al. 2012

The volume required for the supplemental flask could be calculated by the following formula:

$$V_s = \frac{Cl_A - Cl_B}{Cl_B} V_c,$$

Formula 2

Where V_s: volume of the supplementary flask, Cl_A: clearance rate of AB_A, Cl_B: clearance rate of AB_B, V_c: Volume of the experimental flask

The central flask was filled with sterile CA-MHB to the volume of 100 ml. Bacterial suspensions of *E. coli* BORT or *S. aureus* 118324 were prepared and inoculated as 10^6 CFU/ml described in section 3.4. Bacteria were allowed to reach an early stage of logarithmic phase of growth as determined previously by other lab members performing similar experiments prior to AB being injected as a bolus dose. The central and supplemental flasks were kept in a water bath at 37^0C and magnetic stirrer were used to mix MHB continuously during the experiment. AB concentrations were simulated as fC_{max} in critically ill/septic humans and taking into account protein binding.

3.7 Time point sampling for both TK and IVPD *E. coli* BORT and *S. aureus* 118324 assays

Prior to the infusion of AB, 0.5 ml of samples were withdrawn from the TK or the IVPD experimental flask to determine the initial bacterial counts using spread plating experiments described in section 3.3. Once the flasks were injected with the respective AB, the flasks were left in their experimental environment for 5 minutes to allow the interaction of AB in the bacterial culture. 0.5ml of sample was collected from all the flasks after 5 minutes and this point was regarded as time 0 hours. The calibrated peristaltic pumps were set to a rate equal to that of the respective half-lives of the AB. The experiment ended after 24-hours.

3.8 Statistical analysis

Bacterial log_{10} CFU/ml reductions of the single AB treatment were assessed and compared to the combined AB treatment using a Student's t-test. For significant change, a p-value of ≤0.05 was considered. Three biological replicates were performed for *E. coli* BORT and *S. aureus* 118324 in both the TK and IVPD assays for individual and AB combinations.

CHAPTER 4. RESULTS

4.1 Minimum inhibitory concentration

MIC values were determined to assess the susceptibility of *E. coli* BORT and *S.* aureus 118324 against the ABs used and to determine AB concentrations to be used in the TK assay. MIC values determined from AST of *E. coli* BORT and *S.* aureus 118324 against each of the selected ABs for analysis and are tabulated in Table 3.1. All strains were deemed susceptible against the respective ABs as compared to the EUCAST clinical breakpoints.

4.2 The results of *E. coli* BORT and *S. aureus* 118324 AB Time kill assays shows antagonism

The TK assay was performed to assess any preliminary AB antagonism at a variety of concentrations related to the MIC of either *E. coli* BORT or *S. aureus* 118324. If at these concentrations antagonism or a reduction in the rate of bacterial killing can be observed, this provides a rational to test clinically relevant serum concentrations in the IVPD assay.

In the *E. coli* TK assays, shown in Figure 4.1 and values summarized in Table 4.1, a reduction in the rate of bacterial killing was observed for combinations of ceftriaxone with chloramphenicol, where significant reduction in the CFU/ml of bacterial kill was observed from 6-hours (p-value 0.005167) up to 24-hours (p-value 0.02026). The addition of ceftriaxone alone resulted in a 4 $\log_{10}$ CFU/ml *E. coli* reduction within 1.5-hours. The resulting combination of ceftriaxone with chloramphenicol only resulted in a 2 $\log_{10}$ CFU/ml reduction by the 24- hour point. The combination of ceftriaxone with chloramphenicol showed statistically significant different rates in the reduction of CFU/ml bacterial killing beginning at the 6-hour mark. Antagonism was observed at the 9- hour point in *E. coli* TK assays which continued to the 24- hour point, where the $\log_{10}$ CFU/ml differences between the two groups was >2 indicating antagonism.

In the *E. coli* TK assay, the combination of ceftriaxone with tigecycline, resulted in statistically significant reductions in the rate of bacterial killing by 3-hours up till 24-hours (Table A1). At the 12- hour time point, experiments of ceftriaxone alone addition demonstrated an approximate 4 $\log_{10}$ CFU/ml reduction of *E. coli*, while in the combination with tigecycline, an approximate 1.8 $\log_{10}$ CFU/ml (p-value = 0.000001) reduction was observed (Table A1). At the 24- hour time point, ceftriaxone alone demonstrated an approximate 4.2 $\log_{10}$ CFU/ml reduction of *E. coli* while in the combination of ceftriaxone with tigecycline a 3.5 $\log_{10}$ CFU/ml (p-value = 0.003912) reduction was observed. Detectable AB antagonism ceftriaxone with tigecycline was observed beginning at the 6-hour mark up to the 24-hour mark, where the $\log_{10}$ CFU/ml difference between the two groups was <2. These results suggest that there is antagonism and a reduction in the rate of kill of bacterial CFU/ml between ceftriaxone in combination with either tigecycline or chloramphenicol at AB concentrations equal to 8X *E. coli* BORT MIC.

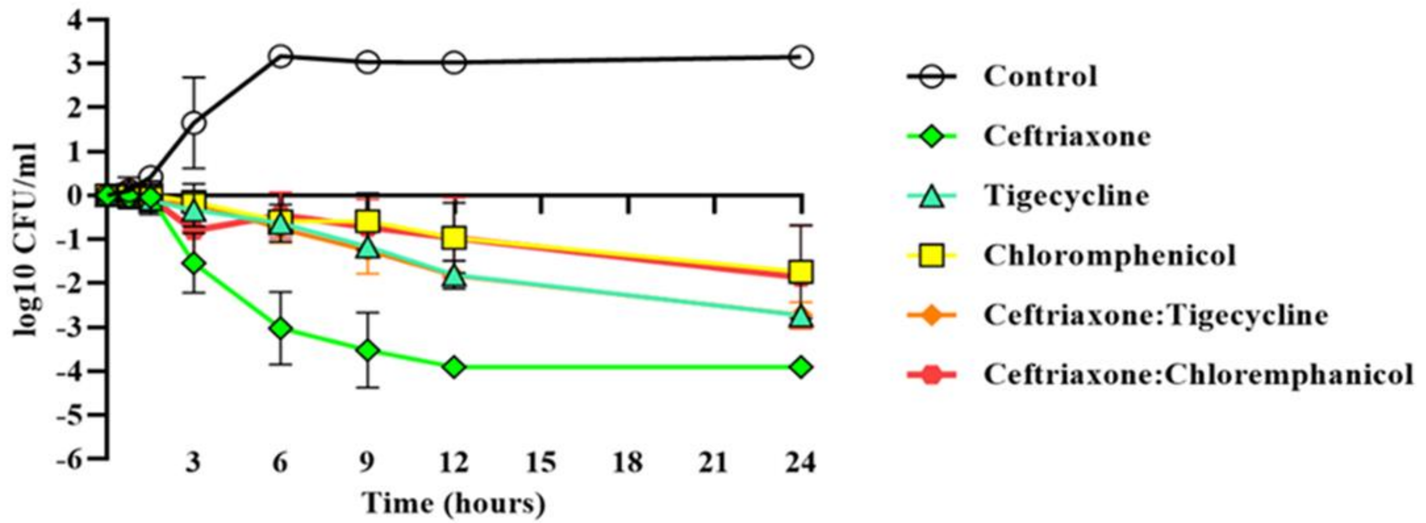


Figure 4.1. *E. coli* BORT TK assay depicting the mean $\log_{10}$ CFU/ml of triplicate biological replicates over the course of 24 hours with variance in sampling values depicted as bars. Associated p-values can be found in Table A1.

Table 4.1. *E. coli* BORT TK assay sampling points with the biological replicate $\log_{10}$ CFU/ml for the control and the ABs tested over 24-hours

Time(h)	**Control**			**Ceftriaxone**			**Tigecycline**		
0	6.10	5.90	6.15	5.90	5.70	6.12	6.12	5.65	6.17
0.75	6.15	6.20	6.13	5.90	5.90	5.80	5.95	6.10	6.18
1.5	6.40	6.50	6.50	6.10	5.75	5.75	6.14	5.75	5.70
3	7.20	8.75	7.16	4.90	3.40	4.80	5.55	5.75	5.65
6	9.20	9.20	9.25	2.00	3.45	3.20	5.35	5.50	5.20
9	9.10	9.20	8.95	2.00	3.15	2.00	4.90	4.70	4.85
12	9.20	9.14	8.90	2.00	2.00	2.00	4.13	4.21	4.18
24	9.30	9.20	9.11	2.00	2.00	2.00	3.15	3.10	3.50
Time(h)	**Chloramphenicol**			**Ceftriaxone and Tigecycline**			**Ceftriaxone and Chloramphenicol**		
0	6.11	5.80	6.11	6.14	5.85	6.24	6.11	5.75	6.13
0.75	5.95	6.10	6.00	5.95	5.95	6.10	6.10	6.10	6.11
1.5	6.12	6.13	5.80	6.12	5.70	5.95	6.11	5.60	6.10
3	5.65	6.12	5.80	5.95	5.65	6.10	5.60	5.55	4.45
6	5.40	5.25	5.60	5.60	5.25	5.10	5.35	5.90	5.40
9	5.11	5.95	5.19	4.90	5.12	4.45	4.90	5.75	5.17
12	4.35	5.65	5.12	4.33	4.24	4.18	4.25	5.70	5.15
24	3.30	5.11	4.40	3.10	3.15	3.80	3.15	5.13	4.12

The results from *S. aureus* TK assays are shown in Figure 4.2 and CFU/ml values are summarized in Table 4.2. *S. aureus* TK assays examining the combination of cefazolin with clindamycin, demonstrated statistically significant differences in rates in AB bacterial killing based on CFU/ml at 6-hours through the rest of the experiment. At the 12-hour time point, the addition of cefazolin alone to *S. aureus* cultures resulted in an approximate 3 log_{10} CFU/ml reduction from the starting inoculum, while the combination of cefazolin with clindamycin resulted in an approximate 0.5 log_{10} CFU/ml reduction (p-value 0.000022) (Table A3). At 24-hour time points, compared to the starting inoculum, while cefazolin with clindamycin only resulted in an approximate 0.7 log_{10} CFU/ml reduction (p-value 0.000336) (Table A3).

The combination of cefazolin with erythromycin in *S. aureus* TK assays demonstrated statistically significant differences in the rates of bacterial killing beginning at the 3-hour mark and continued through the rest of the experiment (Table A3) At the 12-hour time point, cefazolin alone represented an approximate 3 log_{10} CFU/ml reduction from the starting inoculum, while the cefazolin with erythromycin only resulted in an approximate 0.4 log_{10} CFU/ml reduction (p-value 0.000004). At the 24-hour time point, the addition of cefazolin alone to *S. aureus* cultures resulted in an approximate 4 log_{10} CFU/ml reduction from the starting inoculum, while the combination of cefazolin and erythromycin only resulted in an approximate 0.8 log_{10} CFU/ml (p-value 0.000191) reduction. These results suggest that in the TK assay there is antagonism and a reduction in the rate of bacterial killing between cefazolin in combination with either clindamycin or erythromycin at AB concentrations equal to 8X *S. aureus* 118324 MIC.

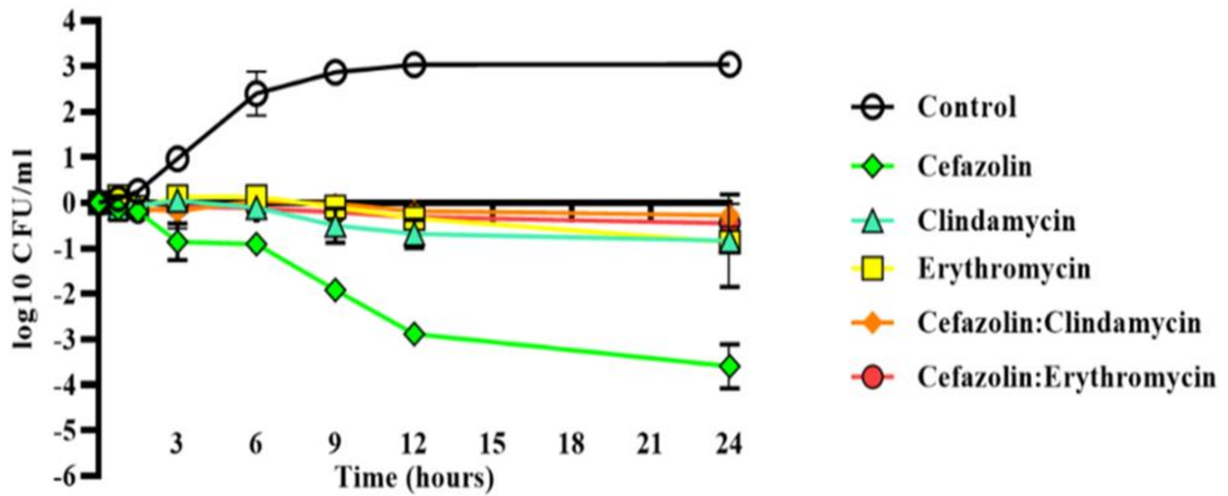


Figure 4.2. *S. aureus* 118324 TK assay depicting the mean $\log_{10}$ CFU/ml of triplicate biological replicates over the course of 24 hours with variance in sampling values depicted as bars. Associated p-values can be found in Table A3.

Table 4.2. *S. aureus* 118324 TK assay sampling points with the biological replicate log_{10} CFU/ml for the control and the ABs tested over 24-hours.

Time(h)	Control			Cefazolin			Clindamycin		
0	6.32	6.18	6.16	6.30	6.38	6.16	6.18	6.32	6.16
0.75	6.60	6.23	6.12	6.26	6.06	6.13	6.04	6.48	5.80
1.5	6.84	6.30	6.28	6.37	6.00	5.90	6.00	6.51	6.00
3	7.23	7.11	7.23	5.90	5.24	5.13	6.20	6.49	6.12
6	8.98	8.88	8.00	5.53	5.22	5.40	6.00	6.51	5.85
9	9.28	9.18	8.80	4.50	4.48	4.12	5.34	6.23	5.60
12	9.26	9.36	9.14	3.34	3.51	3.35	5.27	6.00	5.36
24	9.20	9.30	9.30	2.81	2.26	3.00	6.11	5.88	4.17
Time(h)	**Erythromycin**			**Cefazolin and clindamycin**			**Cefazolin and erythromycin**		
0	6.16	6.00	5.80	6.18	6.32	6.16	6.38	6.31	6.16
0.75	6.11	6.12	6.13	6.00	6.48	5.90	6.14	6.17	6.11
1.5	6.12	5.65	5.90	6.00	6.45	5.85	6.19	6.19	5.90
3	6.12	6.13	6.13	6.15	6.43	5.55	6.38	6.11	6.13
6	6.12	6.12	6.14	6.23	6.45	6.11	6.24	6.14	6.11
9	6.00	5.75	5.95	6.15	6.30	6.16	6.36	5.93	5.95
12	5.65	5.45	5.85	6.06	6.20	5.85	6.06	5.95	5.90
24	5.13	5.35	4.90	6.04	6.20	5.60	6.00	5.81	5.70

4.3 The results of *E. coli* BORT and *S. aureus* 118324 AB IVPD assays shows an initial reduction in the bacterial rate of kill (CFU/ml)

The IVPD experiments were designed to further assess the antagonism observed in the TK assay in a model which closer represents the pharmacokinetics observed in critically ill or septic patients. Half-lives of the drugs were simulated by adjusting clearance rates of the peristaltic pump, and, the concentrations of ABs were closer representative of those found in critically ill human serum concentrations.

The results from the *E. coli* IVPD assay shown in Figure 4.3 and Table 4.3, the combination of ceftriaxone with chloramphenicol demonstrated statistically significant reductions in the bacterial killing rate at the 6-hour time point until the 12-hour time point. At the 12-hour time point, ceftriaxone treatment alone demonstrated a 4 log_{10} CFU/ml reduction in *E. coli* when compared to the starting inoculum. The combination of ceftriaxone and chloramphenicol only resulted in an approximate 2.5 log_{10} CFU/ml (p-value 0.00002) reduction in *E. coli* log_{10} CFU/ml values. At the 24-hour mark, both treated groups achieved an approximate 4 log_{10} CFU/ml reduction reaching the LOD. These results suggest that the combination of ceftriaxone and chloramphenicol, when compared to ceftriaxone alone, initially resulted in a slower rate of kill against *E. coli* BORT. However, given enough time, similar levels of log_{10} CFU/ml reductions were observed in the combination involving chloramphenicol when compared to ceftriaxone alone.

The *E. coli* IVPD assay results, testing the combination of ceftriaxone with tigecycline showed statistically significant rates in bacterial CFU/ml reduction beginning at 1.5-hours lasting until the 6-hour time point (Table A2). At the 12-hour time point, the *E. coli* ceftriaxone treatment alone demonstrated a 4 log_{10} CFU/ml reduction when compared to time 0-hour, while this combination also resulted in 4 log_{10} CFU/ml reduction (p-value NaN). At the 24-hour time point, both ceftriaxone alone and in combination with tigecycline achieved an approximate 4 log_{10} CFU/ml (p-value NaN) reduction reaching the LOD (Table A2). These results suggest that the combination of ceftriaxone and tigecycline, when compared to ceftriaxone alone, initially resulted in a slower rate of bacterial killing against *E. coli* BORT. However, given enough time, similar levels of log_{10} CFU/ml reductions were observed in the combination involving tigecycline, when compared to ceftriaxone alone.

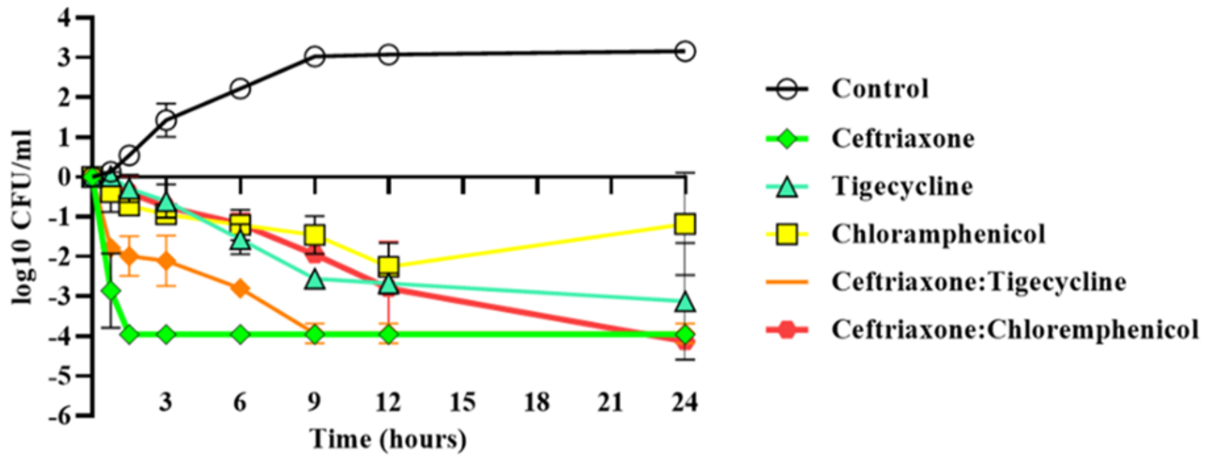


Figure 4.3. *E. coli* BORT IVPD assay depicting the mean log_{10} CFU/ml of triplicate biological replicates over the course of 24 hours with variance in sampling values depicted as bars. Associated p-values can be found in Table A2.

Table 4.3. *E. coli* BORT IVPD assay sampling points with the biological replicate log_{10} CFU/ml for the control and the ABs tested over 24-hours.

Time(h)	Control			Ceftriaxone			Tigecycline		
0	6.00	6.12	6.20	6.17	6.00	5.70	5.70	6.12	5.80
0.75	6.10	6.15	6.15	3.15	4.15	2.00	5.75	6.12	5.80
1.5	6.40	6.30	6.45	2.00	2.00	2.00	5.70	5.45	5.60
3	7.20	7.40	7.45	2.00	2.00	2.00	5.55	5.15	5.10
6	8.80	8.90	9.00	2.00	2.00	2.00	4.45	4.14	4.35
9	9.10	9.00	9.10	2.00	2.00	2.00	3.40	3.35	3.20
12	9.10	9.20	9.00	2.00	2.00	2.00	3.30	3.20	3.10
24	9.20	9.15	9.15	2.00	2.00	2.00	4.26	2.00	2.00
Time(h)	**Chloramphenicol**			**Ceftriaxone and Tigecycline**			**Ceftriaxone and Chloramphenicol**		
0	6.12	6.10	6.95	5.65	6.14	6.00	6.13	6.11	6.15
0.75	5.90	6.12	6.00	4.10	4.15	4.20	5.85	5.80	6.20
1.5	5.50	5.40	6.11	4.15	4.17	3.50	6.20	5.60	5.40
3	5.18	5.27	5.90	4.11	4.15	3.20	5.45	5.35	5.35
6	5.10	5.15	5.30	3.10	3.10	3.20	5.20	4.65	5.00
9	4.90	4.95	4.95	2.00	2.00	2.00	4.30	4.00	4.25
12	4.16	4.24	4.00	2.00	2.00	2.00	4.00	4.00	2.00
24	6.12	5.10	4.40	2.00	2.00	2.00	2.00	2.00	2.00

S. aureus IVPD assay results shown in Figure 4.4 and summarized in Table 4.4, demonstrate the outcomes from the combination of cefazolin with clindamycin, where statistically significant reductions in bacterial killing were observed at the 0.75-hour mark that continued until the 24-hour time mark. At the 12-hour time point, cefazolin treatment alone represented an approximate 3 $\log_{10}$ CFU/ml *S. aureus* 118324 reduction compared to the starting inoculum. By comparison, cefazolin with clindamycin resulted in an approximate 2.0 $\log_{10}$ CFU/ml (p-value 0.00035) reduction. At the 24-hour time point, cefazolin alone demonstrated an approximate 4 $\log_{10}$ CFU/ml reduction from the starting inoculum, while cefazolin with clindamycin showed an approximate 3 $\log_{10}$ CFU/ml (p-value 0.4737) reduction (Table A4). These results suggest that the combination of cefazolin with clindamycin, when compared to cefazolin alone, initially resulted in a slower rate of bacterial killing against *S. aureus* 118324. This reduction becomes less significant past the 12-hour time point. However, cefazolin alone still resulted in a greater $\log_{10}$ CFU/ml decrease at the 24-hour time point, when compared to cefazolin with clindamycin.

In the IVPD assay of *S. aureus* 118324, the added combination of cefazolin with erythromycin, resulted in statistically significant reductions beginning at the 0.75-hour timepoint until the 24-hour time point. At the 12-hour time point, cefazolin alone represented an approximate 3 $\log_{10}$ CFU/ml reduction from the starting inoculum, while cefazolin with erythromycin resulted in an approximate 2.0 $\log_{10}$ CFU/ml (p-value 0.001089) reduction (Table A4) At the 24-hour time point, cefazolin addition alone represented an approximate 4 $\log_{10}$ CFU/ml reduction from the 0-hour time point, while the combination showed an approximate 3 $\log_{10}$ CFU/ml reduction (p-value

0.000861) (Table A4). These results suggest that the combination of cefazolin with erythromycin, when compared to cefazolin alone against *S. aureus* 118324, resulted in a slower rate of kill and greater log_{10} CFU/ml at the 24-hour time point.

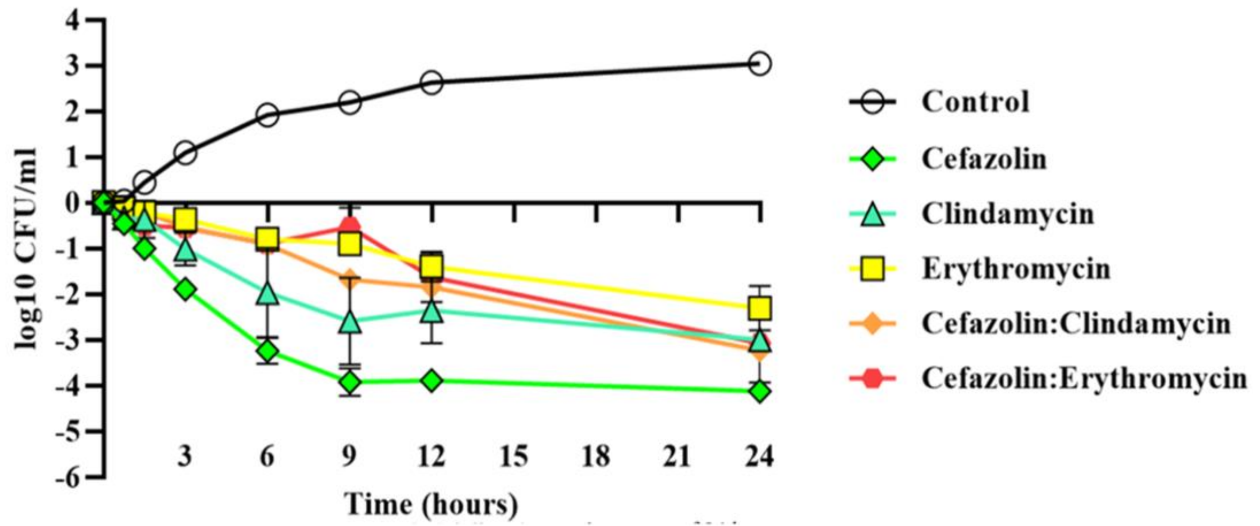


Figure 4.4. *S. aureus* 118324 IVPD assay depicting the mean $\log_{10}$ CFU/ml of triplicate biological replicates over the course of 24 hours with variance in sampling values depicted as bars. Associated p-values can be found in Table A4.

Table 4.4. *S. aureus* 118324 IVPD assay sampling points with the biological replicate log_{10} CFU/ml for the control and the ABs tested over 24-hours.

Time	**Control**			**Cefazolin**			**Clindamycin**		
0	6.17	6.32	6.12	6.20	6.00	6.17	6.40	6.20	6.00
0.75	6.20	6.23	6.30	5.70	5.60	5.70	6.00	5.80	5.80
1.5	6.55	6.80	6.60	5.20	5.00	5.17	5.90	5.50	6.10
3	7.23	7.37	7.30	4.15	4.11	4.45	5.15	5.00	5.40
6	8.11	8.12	8.15	2.65	3.00	3.00	4.00	3.55	5.15
9	8.40	8.30	8.50	2.00	2.40	2.20	3.16	3.18	4.50
12	8.80	8.80	8.90	2.20	2.30	2.20	3.48	3.60	4.45
24	9.15	9.20	9.40	2.00	2.00	2.00	3.30	3.30	3.00
Time	**Erythromycin**			**Cefazolin and clindamycin**			**Cefazolin and erythromycin**		
0	6.00	6.00	5.90	6.13	6.20	6.25	6.15	6.00	6.15
0.75	6.18	5.80	5.55	6.13	6.20	5.90	5.85	6.00	5.75
1.5	5.70	6.00	5.55	6.15	6.00	5.80	5.65	5.55	5.60
3	5.50	5.70	5.60	5.65	5.70	5.65	5.55	5.50	5.60
6	5.15	5.35	5.00	5.20	5.35	5.30	5.12	5.15	5.35
9	5.00	5.20	5.00	4.60	4.40	4.55	5.70	5.85	5.15
12	4.25	4.65	4.80	4.35	4.25	4.00	4.18	5.00	4.24
24	3.46	3.38	4.15	3.50	2.20	3.20	3.15	2.80	3.14

CHAPTER 5. Discussion

Optimizing pathogen clearance with antibiotic combination therapy (ABCT) can provide a greater spectrum of coverage in empiric therapy[18]. ABCT can also reduce emerging AMR and has the potential for synergism leading to even quicker pathogen clearance when compared to monotherapy[30]. Drug synergism has been extensively examined in combinations of β-lactams with aminoglycosides, and, β-lactams with fluroquinolones against both *E. coli* and MRSA[74]. However, antagonism can also be an adverse outcome of ABCT. Reducing the rate of bacterial killing by AB combinations can allow for the persistence of the microbial load and treatment failure in time sensitive conditions such as meningitis or septic shock[74].

Our study was designed to determine the effects on the rate of bacterial killing of ABCT against the *E. coli* BORT strain and the clinical isolate *S. aureus* 118324. Specifically, the combination of a protein synthesis inhibitor administered concurrently with a cell wall inhibiting AB in *in vitro* TK and IVPD assays of *E. coli* and *S. aureus*. The levels of ABs tested in the IVPD assay were akin to concentrations detected in human serum during treatment of septic critical illness. Antagonism, in TK and IVPD assays was defined by Doern *et al*. 2014 and was determined by a net loss of 2 $\log_{10}$ CFU/ml reduction compared to the most potent AB[75]. Antagonism was observed in our study in both the *E. coli* and *S. aureus* TK and IVPD assays. In the *E. coli* TK assay, antagonism was observed in the combination of ceftriaxone with chloramphenicol or tigecycline up to the 12-hour time point (Figure 7 and Table 5). In the *S. aureus* TK, antagonism was observed by in the combination of cefazolin with clindamycin or erythromycin at the 24-hour time point (Figure 4.4 and Table 4.4). In the *E. coli* IVPD assay, antagonism was observed up to the 3-hour time point for the combination of ceftriaxone with tigecycline and up to the 12-hour time point for the

combination of ceftriaxone with chloramphenicol (Figure 4.3 and Table 4.3). Antagonism, in the case of ABCT, is never a desired result. Compared to synergy, antagonism reduces the overall effectiveness of treatment and can lead to therapeutic failure.

The most potent AB treatment for *E. coli* by either TK or IVPD assays performed herein, was for ceftriaxone alone (Figure 4.1, 4.3 and Tables 4.1, 4.3). Ceftriaxone reached the LOD of 1×10^2 CFU/ml in our experiments at 12-hours in the TK assay and 1.5-hours in the IVPD assay. There was an approximate 4 $\log_{10}$ CFU/ml reduction. Similar ceftriaxone rates of bacterial killing were reported by Satta *et al*. 1988 where they showed an approximate 3.8 $\log_{10}$ CFU/ml reduction observed at 24-hours of ceftriaxone[76]. The MIC of the *E. coli* strain used in their studies were 0.06 and 4 µg/ml compared to 0.03 µg/ml for *E. coli* BORT. Despite having different volumes, different clearance rates, different MICs and different C_{max} values, there was the similarity in the optimal bacterial killing parameter for ceftriaxone. At a fT>MIC of 50-70%, bactericidal effects should be observed against gram-negative bacteria[77]. In both of these experiments the fT>MIC was above 70% for the entire duration.

A study by Asmar *et al.* 1988 investigated antagonism between ceftriaxone with chloramphenicol in a checkerboard and TK assay. A 4 $\log_{10}$ CFU/ml difference was observed at the 24-hour time point for the tested *E. coli* strain[51]. The tested *E. coli* strain had ceftriaxone and chloramphenicol MICs equal to *E. coli* BORT.

In our *E. coli* BORT TK assay, the combination of ceftriaxone with tigecycline showed antagonism at the 6-hour timepoint until the 24-hour point (Table 4.1). At the 24- hour point, the $\log_{10}$ CFU/ml of both treatment groups was <1 log apart (Table 4.1). This could suggest that ceftriaxone alone could lead to a greater initial decrease of bacteria when compared to a combination of

ceftriaxone with tigecycline. A study by Enteza *et al.* 2009 reviewed the literature for *in vitro* tests examining tigecycline in combination with other ABs[55]. The study assessing ceftriaxone in combination with tigecycline against *Enterococcus faecium* concluded indifference in the combination[55]. The majority of other Enterobacteriaceae studies involving other ABs yielded indifference. One *Enterococcus faecalis* IVPD study concluded antagonism with arbekacin[55]. Synergism was reported primarily with rifampicin, amikacin, cefepime, linezolid and vancomycin[55]. There are very few literature reports observing antagonism with tigecycline with a cephalosporin[55,78]. In examining tigecycline with a variety of other ABs[78], the consensus in the study indicated that antagonism occurred with β-lactams such as meropenem and imipenem. Both of these ABs have similar mechanisms of actions compared to ceftriaxone where they all inhibit cell wall synthesis. Based on the similar mechanisms of action as ceftriaxone, antagonistic effects might be expected when tigecycline is administered with a cell wall targeting AB.

The most potent AB for *S. aureus,* in either TK or IVPD assays, was cefazolin (Figures 4.2, 4.4 and Tables 4.2, 4.4). Cefazolin reached the LOD of 1 x 10^2 CFU/ml in our experiments at 24-hours in the TK assay (Figure 4.2) and 9-hours in the IVPD assay (Figure 4.4). There was an approximate 4 $\log_{10}$ CFU/ml reduction when compared to the starting inoculum, which is similar to the results obtained by Zelenitsky *et al.* 2018[79]. The MSSA strains used in the Zelenitsky study had equivalent or greater MIC values when compared to the *S. aureus* strain used in our studies.

The results from the *S. aureus* TK and the IVPD assays (Figures 4.2, 4.4 and Tables 4.2, 4.4) supports our thesis hypothesis that a β-lactam, specifically cefazolin alone, would result in a greater and quicker reduction of bacterial CFU/ml when compared to a β-lactam in combination with a protein synthesis inhibitor, either clindamycin or erythromycin. Defined antagonism was

detected in the *S. aureus* TK assay when compared to the IVPD assay (Tables 4.2, 4.4). In both assays, statistically significant reductions in the rate of bacterial killing were observed when cefazolin was administered with a bacteriostatic agent either clindamycin or erythromycin.

Similar results were achieved by Zelenitsky *et al*. 2018 when comparing ceftriaxone with cefazolin alone against clinical isolates of MSSA. A range of 1.84 – 3.51 $\log_{10}$ CFU/ml reductions was observed against the MSSA clinical strains over the course of a 24-hours IVPD assay when ceftriaxone was tested[79]. *In vitro* studies, examining AB antagonism have concluded similar results. For *S. aureus,* the rate of bacterial killing is reduced when a bacteriostatic AB, which inhibits protein synthesis, is combined with a bactericidal AB targeting the integrity of the cell wall[30,51].

Ultimately, it is not whether a drug has a bacteriostatic or bactericidal effect, but rather, the antibacterial mechanism which is driving an antagonistic effect[30]. Bactericidal and bacteriostatic are terms given to the observed effects *in vitro* against specific pathogens in ideal conditions. At times, bactericidal ABs can display bacteriostatic effects and vice versa depending on the pathogen and concentrations of the ABs. The mechanism of action of ABs is straightforward. A β-lactam will always target PBP's and an aminoglycoside will always target the 16S rRNA of the 30S ribosome. Examining the mechanistic actions of ABs may provide greater insight into determining ABI prior to testing. For example, cefazolin irreversibly binds to PBP and inhibits the structural formation of the cell wall, whereas cefazolin-PBP binding encourages the breakdown of existing peptidoglycan structures[31]. This effect can only occur if PBPs are being produced by the protein machinery of the cell. Clindamycin inhibits protein formation by reversibly binding to the 50S ribosomal subunit, thereby, preventing peptide bond formation[25]. Hence, if there is less

PBP being produced by a bacterium due to the antibacterial effects of clindamycin, there will be less PBP for cefazolin to bind. The driving antibacterial mechanism of cefazolin is effectively being prevented, resulting in decreased efficacy. We assume, this may be a factor of decreased efficacy of cefazolin when combined with clindamycin. By extension, β-lactam ABs are only able to exert their antibacterial effects on growing bacteria. Bacteriostatic drugs cease the growth of bacteria, ultimately decreasing the efficacy of the β- lactam ABs[27].

Alternatively, the differences between the TK and IVPD assay results herein for *E. coli* BORT and *S. aureus* 118324 could be due to the relative MBC/MIC ratios of the ABs observed and the driving pharmacodynamics related to each AB[80]. Bactericidal ABs, such as β-lactams, display the greatest benefit when the T>MIC is >50-70%. The majority of bacteriostatic drugs display the greatest efficacy when the pharmacodynamic parameters of T>MIC or AUC/MIC are considered[80]. Different concentrations of ABs were used in the TK and the IVPD assays and there was a greater concentration of clindamycin, erythromycin, tigecycline and chloramphenicol in the 8X MIC TK assay when compared to the IVPD assay (Tables 3.1, 3.2, 3.3). In the IVPD assays, there was a greater concentration of cefazolin and ceftriaxone. A potential explanation as to the greater antagonism observed in the TK compared to the IVPD assay, could relate to the concentrations of ABs used and their driving pharmacodynamic parameters. There was a greater concentration of bacteriostatic AB in the TK leading to a larger AUC/MIC when compared to the IVPD assays. The greater the AUC/MIC for bacteriostatic ABs, the greater the observed bacteriostatic effect. In the IVPD assays, there was a smaller AUC/MIC compared to the TK assay and a greater concentration of β-lactam leading to a larger T>MIC and a greater CFU/ml reduction in the combination experiments.

In the study by Ocampo *et al*. 2014 combinations of bactericidal and bacteriostatic ABs were examined against *E. coli* K-12 (BW25113) and several patterns of antagonism and synergy were noted. Synergism was often observed when a β-lactam was tested with an aminoglycoside[30]. Antagonism was observed in combinations of bactericidal ABs such as ampicillin, cefoxitin and fosfomycin with bacteriostatic ABs including chloramphenicol, erythromycin, tobramycin This supports the idea that bactericidal agents targeting the cell wall, not only β-lactams will have a decreased effect when combined with a protein synthesis inhibitor.

Asmar *et al.* 1988 examined chloramphenicol alone, and in combination with 3rd generation cephalosporins, cefotaxime or ceftriaxone, *in-vitro,* against a panel of bacteria including *E. coli.* In the TK assay by Asmar, antagonism was observed in the combination of cefotaxime or ceftriaxone with chloramphenicol against *E. coli*. A 4 $\log_{10}$ CFU/ml difference was observed at the 24-hour time point compared to either cephalosporin alone[51]. This study suggests that defined antagonism occurs between cefotaxime or ceftriaxone in combination with chloramphenicol. These results concur with our results from the *E. coli* BORT TK assay.

Nishino *et al*. 1975 also reported cases of antagonism in the 3rd generation cephalosporin, cephalexin, when combined with erythromycin against MSSA 209-P JC. When erythromycin was tested alone, thicker cell walls were observed[81]. When cephalexin was tested alone, cell protoplasts were observed suggesting that the cell wall integrity had been compromised due to the presence of endogenous autolytic enzymes. When both ABs were administered together and examined under an electron microscope, no protoplast formation was observed[81]. The authors suggested that erythromycin was responsible for decreasing protein production and decreasing the rate of cell wall degradation via the autolytic enzymes. In the TK assay, when cephalexin was

administered with erythromycin, there was antagonism and a 5 $\log_{10}$ CFU/ml difference by the end of their experiment. This study complements the results observed between cefazolin and erythromycin in our experiment. When cefazolin was administered alone, there was a marked decrease in the *S. aureus* $\log_{10}$ CFU/ml when compared to cefazolin in combination with erythromycin (Figures 4.2, 4.4). Extrapolating from the study by Nishirio *et al*. 1975, our experiments showed cefazolin alone most likely resulted in protoplast formation which compromised the integrity of the cell, causing death. When cefazolin was combined with erythromycin, protoplast formation was inhibited leading to an increased *S. aureus* cell viability, indicating an antagonistic effect between cefazolin and erythromycin.

Cohn *et al*. 1980 examined the ABI between erythromycin, ampicillin, gentamicin and cefamandole against a panel of commonly isolated respiratory pathogens. Erythromycin showed antagonistic effects when combined with the 2nd generation cephalosporin cefamandole against both *E. coli* and *S. aureus in vitro*[82]. His study results agreed with our own study showing that antagonism will occur between a cephalosporin such cefazolin alongside a protein synthesis inhibitor such as erythromycin.

In vitro ABI research examines detailed interactions that could occur between two ABs. However, the observed effects may not translate exactly when applied to the clinical setting. The most important clinical indicator of an improved therapeutic outcome would be a decrease in rate of morbidity and mortality. Kumar *et al*. 2010 through a meta-regression analysis examined the effects of combination therapy compared to monotherapy for individuals who were either septic or in septic shock[74]. Kumar *et al.* 2010 found that individuals who were in septic shock and at a high risk of death, significantly benefited from AB combination therapy as compared to

monotherapy. Across these clinical reviews[13,18,70,74], a decrease in mortality was reported when AB combinations consisted of two bactericidal agents, with similar mechanisms of action, were used.

There are clinical scenarios where antagonism observed in our study could be a major issue in serious or life-threatening infections. An example is the co-administration of cefazolin with clindamycin, which is often administered to individuals with MSSA bacteremia[83–85]. Clindamycin is often administered for serious *S. aureus* infections. Most *S. aureus* strains do not produce toxic shock syndrome toxin 1 (TSST-1). However, clindamycin is often added to decrease TSST-1 toxin production, should those genes be present in *S. aureus*[43,86]. According to Stryjewski *et al*. 2007 the bactericidal rate of killing of cefazolin would be reduced if clindamycin was co-administered. However, does the treatment with cefazolin alone result in a decrease in mortality due to the increased rate of bacterial killing? Or, would there be an increase in the rate of mortality due to the increased levels of TSST-1 being produced as there was no suppression from clindamycin. These questions require further analysis.

Lepper *et al*. 1951 reported clinically significant antagonism between the β-lactam penicillin and the bacteriostatic aureomycin[87]. An increase in mortality was observed in patients with bacterial meningitis when aureomycin with penicillin was employed as a treatment when compared to penicillin monotherapy[87].

Meningitis is another condition with a time sensitive nature to treatment. The persistent presence of the bacterial load is related to the outcome and mortality[88]. *In vitro* and *in vivo* meningitis mouse models have demonstrated that antagonism occurs when a β-lactam was given alongside a bacteriostatic agent leading to an increase in mortality[89].

Generally, *in vitro,* varying degrees of antagonism are often observed when bactericidal ABs are co-administered with bacteriostatic ABs[30,32,51,82,90] . Despite this known antagonism, there are clinical scenarios where a bactericidal and a bacteriostatic AB are co-administered. A prime example of is in the treatment of *S. aureus* infections. Piperacillin/tazobactam, a β-lactam with a β-lactamase inhibitor, and vancomycin, a bacteriostatic AB are co-administered because of the high incidence of MRSA in clinical settings. Another example would be the case of polymicrobial infections where two or more different pathogens coexist or suppression of toxin production is desired. Ultimately, continued research in animal models is required, to address the specifics where the host will have its own intrinsic effects on the ABs and pathogens. In addition, common clinical scenarios of potential antagonism between ABs should be retrospectively reviewed.

5.1 Study limitation

Several limitations were addressed in this study. Using an *in vitro* model provides insight into ABI in ideal conditions and does not take into consideration other factors such as the influence of the host and possible co-morbidities. Three biological repeats of all experiments were conducted, however, a greater sample size would provide more confidence to the results. Only one strain of *E. coli* and *S. aureus* was used leading to a very specific range of MICs being tested.

5.2 Future directions

Future directions for this study would include examining these AB combinations in a greater number of *E. coli* and MSSA strains, examining these combinations in other commonly isolated pathogens in septic shock such as *K. pneumoniae* and *P. aeruginosa* and ultimately, adopting these combinations to a more complex *in vivo* mouse model of septic shock.

CHAPTER 6. Conclusion

Our study showed that there was a reduction in the bacterial killing efficacy of a β-lactam AB. Specifically, ceftriaxone or cefazolin, when given concurrently with a bacteriostatic AB including tigecycline or chloramphenicol with ceftriaxone, and, clindamycin or erythromycin with cefazolin. Antagonism was observed in the following combinations for *E. coli*, ceftriaxone with chloramphenicol and ceftriaxone with tigecycline in both TK and IVPD assays. For *S. aureus*, antagonism was observed for the following combinations, cefazolin with clindamycin and cefazolin with erythromycin in both TK and IVPD assays. Antagonism was usually observed occurring up the 3, 6, 9, 12- hour time points, in either assay, for either bacterial strain, depending on the AB combination. These findings can have therapeutic implications when treating time-sensitive infections such as meningitis and septic shock. It would be important to determine if the use of these specific AB combinations have resulted in greater mortality in time sensitive infections.

CHAPTER 7. Appendix

Table A1. A summary of the calculated p-values for *E. coli* TK assays shown in Figure 4.1

Drug combination	Time (h)	p-value	significant?
Ceftriaxone vs ceftriaxone + chloramphenicol	0	0.630516	ns
Ceftriaxone vs ceftriaxone + tigecycline	0	0.370070	ns
Ceftriaxone vs ceftriaxone + chloramphenicol	0.75	0.002118	**
Ceftriaxone vs ceftriaxone + tigecycline	0.75	0.090734	ns
Ceftriaxone vs ceftriaxone + chloramphenicol	1.5	0.749742	ns
Ceftriaxone vs ceftriaxone + tigecycline	1.5	0.753949	ns
Ceftriaxone vs ceftriaxone + chloramphenicol	3	0.245340	ns
Ceftriaxone vs ceftriaxone + tigecycline	3	0.037850	*
Ceftriaxone vs ceftriaxone + chloramphenicol	6	0.005167	**
Ceftriaxone vs ceftriaxone + tigecycline	6	0.006689	**
Ceftriaxone vs ceftriaxone + chloramphenicol	9	0.003227	**
Ceftriaxone vs ceftriaxone + tigecycline	9	0.004802	**
Ceftriaxone vs ceftriaxone + chloramphenicol	12	0.001996	**
Ceftriaxone vs ceftriaxone + tigecycline	12	0.000001	****
Ceftriaxone vs ceftriaxone + chloramphenicol	24	0.020260	*
Ceftriaxone vs ceftriaxone + tigecycline	24	0.003912	**

Table A2. A summary of the calculated p-values for *E. coli* IVPD assays shown in Figure 4.3

Drug combination	Time (h)	p-value	significant?
Ceftriaxone vs ceftriaxone + chloramphenicol	0	0.27710700	ns
Ceftriaxone vs ceftriaxone + tigecycline	0	0.90050500	ns
Ceftriaxone vs ceftriaxone + chloramphenicol	0.75	0.01084840	*
Ceftriaxone vs ceftriaxone + tigecycline	0.75	0.16657100	ns
Ceftriaxone vs ceftriaxone + chloramphenicol	1.5	0.00010032	***
Ceftriaxone vs ceftriaxone + tigecycline	1.5	0.00091382	***
Ceftriaxone vs ceftriaxone + chloramphenicol	3	0.00000006	****
Ceftriaxone vs ceftriaxone + tigecycline	3	0.00421461	**
Ceftriaxone vs ceftriaxone + chloramphenicol	6	0.00005184	****
Ceftriaxone vs ceftriaxone + tigecycline	6	0.00000446	****
Ceftriaxone vs ceftriaxone + chloramphenicol	9	0.00322712	**
Ceftriaxone vs ceftriaxone + tigecycline	9	NaN	n/a
Ceftriaxone vs ceftriaxone + chloramphenicol	12	0.00001935	****
Ceftriaxone vs ceftriaxone + tigecycline	12	NaN	n/a
Ceftriaxone vs ceftriaxone + chloramphenicol	24	NaN	n/a
Ceftriaxone vs ceftriaxone + tigecycline	24	NaN	n/a

Table A3. A summary of the calculated p-values for *S. aureus* TK assays shown in Figure 4.2

Drug combination	Time (h)	p-value	significant?
Cefazolin vs cefazolin + clindamycin	0	0.503189	ns
Cefazolin vs cefazolin + erythromycin	0	0.972642	ns
Cefazolin vs cefazolin + clindamycin	0.75	0.907388	ns
Cefazolin vs cefazolin + erythromycin	0.75	0.877933	ns
Cefazolin vs cefazolin + clindamycin	1.5	0.967414	ns
Cefazolin vs cefazolin + erythromycin	1.5	0.985515	ns
Cefazolin vs cefazolin + clindamycin	3	0.154600	ns
Cefazolin vs cefazolin + erythromycin	3	0.037507	*
Cefazolin vs cefazolin + clindamycin	6	0.002791	***
Cefazolin vs cefazolin + erythromycin	6	0.001355	***
Cefazolin vs cefazolin + clindamycin	9	0.000158	***
Cefazolin vs cefazolin + erythromycin	9	0.000784	****
Cefazolin vs cefazolin + clindamycin	12	0.000022	****
Cefazolin vs cefazolin + erythromycin	12	0.000004	****
Cefazolin vs cefazolin + clindamycin	24	0.000336	****
Cefazolin vs cefazolin + erythromycin	24	0.000191	****

Table A4. A summary of the calculated p-values for *S. aureus* IVPD assays shown in Figure 4.4

Drug combination	Time (h)	p-value	significant?
Cefazolin vs cefazolin + clindamycin	0	0.382020	ns
Cefazolin vs cefazolin + erythromycin	0	0.784684	ns
Cefazolin vs cefazolin + clindamycin	0.75	0.013196	*
Cefazolin vs cefazolin + erythromycin	0.75	0.066612	*
Cefazolin vs cefazolin + clindamycin	1.5	0.001943	***
Cefazolin vs cefazolin + erythromycin	1.5	0.002258	***
Cefazolin vs cefazolin + clindamycin	3	0.000192	****
Cefazolin vs cefazolin + erythromycin	3	0.000293	****
Cefazolin vs cefazolin + clindamycin	6	0.000015	****
Cefazolin vs cefazolin + erythromycin	6	0.000025	****
Cefazolin vs cefazolin + clindamycin	9	0.000059	****
Cefazolin vs cefazolin + erythromycin	9	0.000155	****
Cefazolin vs cefazolin + clindamycin	12	0.000056	****
Cefazolin vs cefazolin + erythromycin	12	0.001089	****
Cefazolin vs cefazolin + clindamycin	24	0.069710	ns
Cefazolin vs cefazolin + erythromycin	24	0.000861	****

CHAPTER 8. References

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