Novel *in vitro* Susceptibility Measurement of Vancomycin and Other Antibiotics Against Blood Stream Isolates of Methicillin-resistant *Staphylococcus aureus*

A Thesis Submitted to the College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the School of Public Health

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

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ABSTRACT

Staphylococcus aureus is a versatile pathogen causing mild to moderate to severe and life threatening infections including blood stream infections. Methicillin resistant *Staphylococcus aureus* (MRSA) has evolved as an important nosocomial pathogen and multi-drug resistance limits the available antibiotics for therapy. In many healthcare facilities, vancomycin remains the preferred agent for treating serious MRSA infections; however, other drugs like linezolid and tedizolid are also useful agents. Tedizolid is a newly approved drug and currently only available for clinical use in some countries. Some data suggests that as the minimum inhibitory concentration (MIC) of vancomycin for MRSA increases beyond 2 μg/ml, therapeutic failures become more common. We studied 60 blood culture of MRSA and determined MIC and mutant prevention concentration (MPC) values to vancomycin, linezolid and tedizolid. In separate experiments, we performed *in vitro* kill studies.

The minimum inhibitory concentration (MIC) is the lowest drug concentration required to inhibit or block the growth of 10^5 colony forming units per milliliter (CFU/ml) of bacteria and is the international standard for determining susceptibility/resistance in clinical laboratories. The mutant prevention concentration (MPC) defines the antimicrobial drug concentration blocking growth of the least susceptible cell present in high density ($\geq 10^9$ CFU) bacterial populations.

For vancomycin, MIC values ranged from 0.5 to 1.0 μg/ml as compared to 1 – 4 μg/ml and 0.125 - 0.5 μg/ml for linezolid and tedizolid respectively. By MPC testing, vancomycin MPC values ranged from 2 to ≥32 μg/ml as compared to 2 – 4 μg/ml for linezolid and 0.25 – 0.5 μg/ml for tedizolid. Such high vancomycin MPC values have not been previously reported.

To further characterize strains showing high vancomycin MPC values, we compared pulsed field gel electrophoresis (PFGE) profiles on strains with high vancomycin MPC values to rule in or out, the presence of a single clone. A single clone was not detected. We also compared PFGE profiles on select strains with high vancomycin MPC values to the profiles of the wild type (parental) strains and found the profiles were identical. Cell wall thickness of strains with high vancomycin MPC values was investigated as a possible explanation for the higher MPC values. For MRSA cells taken directly from agar plates containing high vancomycin

drug concentrations, increased cell wall thickness was seen. Amplification of cell wall accessory genes by polymerase chain reaction (PCR) failed to identify a genetic marker that could explain the high MPC values and the presence or absence of the Panton-Valentine leukocidin (*PVL*) gene was not more frequent in strains with high vancomycin MPC values. Additionally, serial passage experiments increasing vancomycin drug concentrations did not result in a strain of MRSA with a stably resistant clone. These experiments did not identify a common characteristic that could be associated with the survival of MRSA bacterial cells in high vancomycin drug concentrations. Therefore, further investigations to determine the mechanism of this observation are necessary.

MIC and MPC testing with MRSA strains tested against linezolid and tedizolid gave values consistent with expectations based on values previously generated with linezolid. For tedizolid, the MPC_{100} was 0.5 µg/ml. MPC values for tedizolid have not been previously reported.

In vitro kill experiments were conducted using a range of bacterial densities from 10^6 - 10^9 CFU/ml. Previously reported kill experiments used bacterial densities approximating 10^5-10^6 CFU/ml and as such the definition of bactericidal and bacteriostatic may not be relevant when higher bacterial densities are used. For our measurements and depending on the bacterial density tested, linezolid, tedizolid and vancomycin showed both bactericidal and bacteriostatic activity against select strains of MRSA.

In summary, high vancomycin MPC values are concerning and may impact the clinical use of the drug and be responsible for clinical failure in some patients. The low MPC values for tedizolid could potentially indicate a low propensity for resistance selection with this drug.

ACKNOWLEDGEMENTS

Dedication

First and foremost, I would like to express my most sincere and most profound gratitude as well as my immeasurable appreciation to all the individuals that contributed to making this study a reality.

I dedicate this thesis to my advisor, Professor Blondeau, for his continuous support over the last few years of my PhD. I cannot express my thanks and gratitude in a few words that could match his encouragement and guidance during all the time of my study. The completion of this thesis could not have been accomplished without his assistance, and I could never have imagined a better advisor, mentor and professor for my graduate study.

I am also taking the time to extend my gratitude to a significant number of people whose help were precious and essential. They each played a role in my research and my academic accomplishment.

Special thanks and acknowledgements

I am incredibly grateful to Dr. Deneer for the area he offered me in his lab to accomplish the molecular experiments needed as an essential part of this project as well as providing me with all the necessary tools and guidance for the lab techniques. I especially appreciated his availability; he always took on his time to answer all my questions and improve my suggestions.

I will also express my thanks to the committee members, Dr. Sanche, Dr. Rubin, Dr. Chirino-Trejo and Dr. Tikoo for their advice, comments and guidance throughout my research process.

I would also like to acknowledge the contribution of Ms. Fitch who taught me to strive for perfection when it came to lab techniques as well as her help and support not only during lab work but also outside of it, in my personal life.

My deepest and sincere thanks to Ms. Hills who helped me during this thesis with her steady clerical work.

Finally, I have to thank my parents for their moral and material assistance as well as their love. Thank you both for the countless times you supported me through my journey in college as well as your continuous backing when I faced difficulties, you gave me the strength to carry on.

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ABSSSIs	Acute bacterial skin and skin structure infections
Amik	Amikacin
AST	antimicrobial susceptibility testing
ATCC	American Type Culture and Collection
AUC	Area under the concentration–time curve
Azithro	Azithromycin
bp	Base Pairs
CDC	Centre for Disease Control and Prevention
cfu	Colony Forming Units
Chloro	Chloramphenicol
Cipro	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
C_{max}	Maximum (peak) serum concentration of a drug
EM	Electron Microscopy
ETBr	Ethidium Bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
GABHS	Group A Beta-Hemolytic Streptococcus
Gati	Gatifloxacin
Genta	Gentamicin
hVISA	Hetero Vancomycin intermediate S. aureus
Linz	Linezolid
LMP	Low Melting Point
MAO	Monoamine oxidase enzyme
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
Moxi	Moxifloxacin
MPC	Mutant Prevention Concentration

MRSA	Methicillin-resistant S. aureus
MSSA	Methicillin-susceptible S. aureus
MSW	Mutant-Selection Window
PAE	Post antibiotic effect
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PD	Pharmacodynamic
PFGE	Pulsed Field Gel Electrophoresis
PK	Pharmacokinetic
PSMs	Phenol-soluble modulins
PVL	Panton-Valentine leukocidin
SCC	Staphylococcal cassette chromosome
SSTIs	Skin and soft-tissue infections
TBE	Tris-HCl Boric Acid EDTA Buffer
TE	Tris-HCl EDTA Buffer
Ted	Tedizolid
Tig	Tigecycline
$Tiss_{max}$	Maximum tissue concentration of a drug
T_{max}	Time to peak serum concentration
TMP/SMX	Trimethoprim/sulfamethoxazole
Tobra	Tobramycin
TSA	Tryptic Soy Agar
Vanco	Vancomycin
VISA	Vancomycin Intermediate S. aureus
VRSA	Vancomycin resistant S. aureus

1.0 INTRODUCTION

1.1. *Staphylococcus aureus*

1.1.1 Laboratory Identification

Staphylococci are aerobic Gram-positive cocci (0.5 – 1.0 µm), grow in clusters, pairs and at times grow in short chains. *Staphylococcus aureus* (*S. aureus*) is the most pathogenic species and characterized by golden pigment production.

The catalase test is the simplest laboratory method to differentiate staphylococci, which are characterized by being catalase positive, from streptococci which are catalase-negative. The coagulase test, on the other hand, distinguishes *S. aureus* (coagulase-positive) from coagulase-negative staphylococci. *S. aureus* can also be identified or confirmed by applying polymerase chain reaction (PCR) methods. The 16S rRNA gene is typically considered to be the most useful taxonomic marker molecules [Becker*, et al*, 2004].

1.1.2 Opportunistic Infections

S. aureus is the most virulent species of staphylococci. It is an opportunistic pathogen colonizing the human epithelia and causes nosocomial and community-acquired infections. Research has shown that around 30% of healthy individuals are colonized with *S. aureus*, including both persistent and non-persistent carriers [Hamdan-Partida*, et al*, 2010]. Colonization with *S. aureus* can potentially increase the risk of incurring invasive infections if the bacterium penetrates the subcutaneous tissues through skin cuts, abrasions or intravascular access devices [Aung*, et al*, 2017].

The pathogenic capacity of *S. aureus* is strongly related to its capability of producing exoproteins and toxins, and thereby causing serious diseases, such as bacteremia, pneumonia, myocarditis, acute endocarditis, osteomyelitis and meningitis [Peshattiwar*, et al*, 2018]. Historical data collected through the National Nosocomial Infection Surveillance System (NNIS) showed *S. aureus* as the leading cause of nosocomial pneumonia, as well as being the second most common cause of bloodstream infections in the USA [National Nosocomial Infections Surveillance (NNIS), 1996; Stevens*, et al*, 2002]. Staphylococcal

bacteremia was also reported to be a major cause of human morbidity and mortality in hospitals [Klevens*, et al*, 2007].

1.1.2.1 Pathogenicity

S. aureus is characterized by the ability to adapt to volatile environments, including selective pressure posed by antibiotics. Also, it has the ability to spread and evolve new antibiotic resistance, which resulted in the emergence of methicillin-resistant *S. aureus* (MRSA).

Both MRSA and methicillin susceptible *S. aureus* (MSSA) can cause the same range of infections. Nevertheless, infections caused by MRSA usually result in worse outcomes compared with MSSA infections. A wider range of antibiotic options are available for MSSA infections [Pottinger, 2013]. Although there is insufficient evidence in the literature supporting the possibility that MRSA strains have a greater probability of causing invasive infections than MSSA [Watkins*, et al*, 2012], infections caused by MRSA are commonly severe and sometimes life-threatening. Also, they are the most frequently occurring infections among other antibiotic resistant species [Ventola, 2015]. This pathogen has demonstrated exceptionally versatility in terms of gradually arising and spreading in various epidemiological setting, including hospitals, community and animals, thereby posing a challenge for controlling and preventing associated infections.

In 2009, CANWARD data drawn from blood, wounds, urine and respiratory tract specimens revealed that *S. aureus* was the most commonly isolated organism overall and the second most common organism found in blood cultures. The Canadian Ward Surveillance Study, also known as CANWARD, is an ongoing national study focused on pathogens isolated from Canadian hospitals (inpatients and outpatients) and communities to determine their degree of antimicrobial resistance [Hoban & Zhanel, 2013]. Royal University Hospital in Saskatoon has been contributing to this surveillance program annually since its inception.

1.2. MRSA

1.2.1 Associated Risks

MRSA infections are associated with increased morbidity, prolonged hospitalization longer durations of antibiotic treatment, higher health care costs and an increased risk of death. Mortality risk of MRSA bacteremia is approximately two-fold higher than MSSA bacteremia [van Hal*, et al*, 2012]. In the USA, approximately 19,000 patients are killed every year by MRSA infections. This number is equivalent to the mortality rate caused by AIDS, tuberculosis, and viral hepatitis combined [Boucher & Corey, 2008].

1.2.2 Origin and Epidemiology

MRSA had initially been considered to be a rare phenomenon that evolved and spread only within hospitals. The first strain was reported in the United Kingdom in 1961 soon after methicillin was first administered into clinical practice to overcome penicillin-resistant *S. aureus* in 1959 [Hardy*, et al*, 2004]. Within a couple of years, MRSA had become widespread in Japan, Europe and Australia. In 1968, the first case of MRSA was reported in the USA. The frequency of related infections continued increasing in hospitals and has since been persistent in American health care institutions for more than twenty years [National Nosocomial Infections Surveillance (NNIS), 2004; Panlilio*, et al*, 1992].

Data collected from American intensive care units (ICU) showed the MRSA proportion among *S. aureus* isolates increased by 3% per year from 1992 to 2003. MRSA accounted for more than 60% of isolates in these ICUs [Klevens*, et al*, 2006].

In a case study conducted by Graffunder and Venezia, the risk factors that most strongly associated with MRSA infections were the use of the fluoroquinolone antimicrobial levofloxacin and macrolide antibiotics (macrolide antibiotic OR 4.06 was higher than enteral feeding 2.55) [Graffunder & Venezia, 2002]. Other risk factors include extensive previous exposure to antibiotics, admission to ICU, presence of an endotracheal tube or a catheter, long hospitalization and poor hand hygiene among healthcare workers [Pottinger, 2013].

Apart from the previously discussed incidents of the outbreak in hospitals, substantial outbreaks of MRSA infections were reported in the early 1980s among non-recently-hospitalized subjects in Detroit, USA [Pottinger, 2013]. It was determined that 85% of hospital patients were diagnosed with community-associated (CA)-MRSA and 47.5% of healthy community members were found to be subjected to at least one health-care associated risk

factor or had contact with persons with risk factors. In other words, the prevalence of MRSA among healthy community individuals was mainly due to health-care associated risk factors. Identifiable risk factors include recent hospitalization, outpatient visits, nursing home admission, antibiotic exposure, chronic illness, drug injections, and close contact with people subjected to risk factor [Beam & Buckley, 2006].

1.2.3 CA-MRSA VS HA-MRSA

Community MRSA strains are more likely to be susceptible to classes of antibiotics other than β-lactams, whereas hospital-associated (HA) strains are typically multi-drug resistant. This is probably due to the presence of higher selective pressure in hospitals. Genomic analysis of isolates from CA-MRSA and HA-MRSA cases indicates molecular differences beyond their drug susceptibilities. For example, the chromosomal elements for CA-MRSA are smaller and more mobile than those commonly found in HA-acquired MRSA (*SCCmec* types I–III) [Cameron*, et al*, 2011; David & Daum, 2010]. The larger gene elements found in HA-MRSA strains are characterized by having reduced bacterial fitness and decreased toxin production [Collins*, et al*, 2010]. For example, the Panton-Valentine leukocidins (*PVL*) toxin is more prevalent in CA-MRSA strains. There is also an increased expression of certain virulence determinants in CA-MRSA, such as phenol-soluble modulins (PSMs), which act as an aggravating factor causing more severe diseases [Wang*, et al*, 2007].

Although there are common diseases caused by both CA and HA MRSA strains, distinction can be identified to some degree by the types of infections observed. For example, CA-MRSA is most commonly associated with skin and soft-tissue infections (SSTIs), e.g., abscesses and cellulitis whereas HA-MRSA causes more invasive infections, e.g., pneumonia, bacteremia and osteomyelitis [Watkins*, et al*, 2012]. The prevalence of CA-MRSA SSTIs in the United States has been reported to constitute 15-74% of all known SSTIs [Khawcharoenporn*, et al*, 2010; Moran*, et al*, 2006], however, 80% of the invasive infections were caused by HA-MRSA [Dantes*, et al*, 2013].

1.2.3.1 Virulence Factors

CA-MRSA, particularly the USA 300 genotype, are well known for transmitting virulence genes including the *PVL* toxin through the staphylococcal cassette chromosome (*SCC*) which

confer a survival advantage to CA-MRSA strains. It has been demonstrated *in vitro* that β-lactam antibiotics induce the production of *PVL* as opposed to antibiotics like linezolid that block bacterial protein synthesis in which decreases the production of *PVL* [Bernardo*, et al*, 2004].

PVL has been extensively studied as a virulence factor used by CA-MRSA to escape the host's immune response. It is a bi-component exotoxin, encoded by *lukF-PV* and *lukS-PV* genes, and destroys the host's leukocytes by forming pores in their membranes. The genes encoding *PVL* were found in fewer than 5% of clinical *S. aureus* isolates before the emergence of CA-MRSA in the mid-1990s [David & Daum, 2010; Ma*, et al*, 2006]. During the early stages of MRSA epidemic development, researchers believed that *PVL* was the leading cause of the high-level virulence of CA-MRSA strains [Vandenesch*, et al*, 2003]. More recent evidence has questioned whether *PVL* is truly a major virulence determinant, considering that clones lacking *PVL* genes still show significant virulence [Otto, 2010]. Some experimental data including a meta-analysis study demonstrated that *PVL* expression is independently associated with skin and SSTI, but not necessarily with invasive infections [Shallcross*, et al*, 2013] which have raised arguments/ controversy in regard to the role of *PVL* in pathogenicity. Additional virulence factors that have been recently scrutinized and may contribute to the pathogenesis of both CA-MRSA and HA-MRSA are α-toxin, arginine catabolic mobile element, superantigens and biofilms [Watkins*, et al*, 2012].

1.2.4 Evolution of MRSA

Resistance to methicillin and other β-lactam derivatives in MRSA is caused by the acquisition of the *mecA* gene on a transmissible genetic element (*SCC*). This gene encodes an alternative penicillin-binding protein that has lower/reduced affinity for β-lactam antibiotics and consequently prevents these antibiotics from binding and inhibiting the synthesis of the bacterial cell wall. In turn, this transpeptidase facilitates cell wall synthesis and growth of MRSA in the presence of β-lactam antibiotics.

Diversity of *SCCmec* has evolved through the horizontal transfer of *mecA* gene in independent situations. To date, at least 11 types have been identified [Ito*, et al*, 2014]. It has been demonstrated that *SCCmec* (type I-VII) cause resistance to β-lactam antibiotics,

whereas *SCCmec* types II and III encode additional drug resistance genes to multiple classes of antibiotics include macrolide, lincosamide and streptogramin agents [Deurenberg & Stobberingh, 2009].

1.2.4.1 MRSA Clonal Theory

Two opposing theories have been proposed to describe the relationship between the first MRSA evolved and MRSA clones that recently have been identified. The single-clone theory suggests the presence of a common ancestor for all MRSA clones and that the *SCCmec* was introduced once into *S. aureus* at one specific time. On the other hand, the multi-clone theory hypothesizes that the *SCCmec* was introduced into multiple *S. aureus* lineages at different times [Deurenberg & Stobberingh, 2009].

It was believed that horizontal transfer would rarely occur due to the relatively large size of the *mecA* gene, and thereby all MRSA strains descended from a single clone and the *SCCmec* was acquired by *S. aureus* only once. Clonal similarity between all MRSA isolates supports this theory. However, modern molecular typing has revealed that the *mecA* gene is associated with multiple lineages of *S. aureus* and not restricted to a particular subgroup, which justifies the hypothesis that multiple horizontal transfers occurred in different clonal groups. It has been reported that at least 20 occasions of SCC*mec* integration have occurred in different lineages of MRSA [Deurenberg*, et al*, 2007]. This multi-clone theory has since received more widespread support [Deurenberg & Stobberingh, 2009; Matouskova & Janout, 2008].

The presence of multiple genotypes of MRSA colonizing European swine herds has led to the theory that swine may have served as a type of mixing carrier vessel between *S. aureus* and coagulase-negative Staphylococci before this form of bacteria was transmitted to humans thereafter[Pottinger, 2013].

1.2.5 MRSA Susceptibility to Vancomycin

Vancomycin had been the only available effective intravenous therapy for serious MRSA infections for decades. This glycopeptide was discovered in 1952 and approved in 1958 by

the USA Food and Drug Administration (FDA) for treating MRSA and penicillin-resistant *S. aureus* infections in humans [Levine, 2006]. To date, vancomycin remains to be the first-line treatment for MRSA infections for over forty years. However, the increasing prevalence of MRSA worldwide and the consequent increased consumption of vancomycin has since led to the emergence of MRSA phenotypes with reduced susceptibility to vancomycin. These phenotypes include vancomycin-intermediate *Staphylococcus aureus* (VISA), hetero-resistant vancomycin-intermediate *Staphylococcus aureus* (hVIS), and vancomycin-resistant *Staphylococcus aureus* (VRSA). Subsequently, reports of vancomycin clinical failures began to appear, thereby raising global alarms [Di Gregorio*, et al*, 2017; Hiramatsu, 1998; Howden*, et al*, 2010].

1.2.5.1 MRSA phenotypes with reduced susceptibility to vancomycin

The first reported *S. aureus* with reduced susceptibility to vancomycin was isolated in Japan in 1996 [Hiramatsu*, et al*, 1997]. This particular strain, known as Mu50, had an MIC of 8 µg/ml vancomycin which interpreted as intermediate (non-susceptible) according to the Clinical Laboratory Standards Institute (CLSI) breakpoints [Clinical and Laboratory Standards Institute, 2015; Conly & Johnston, 2002]. Since 1997, a number of infections caused by VISA strains have been reported in France, Japan, the United States, Germany, and South Korea. These strains were recovered from patients who failed prolonged therapy (six to eighteen weeks) with vancomycin [Denis*, et al*, 2002; Weinstein & Fridkin, 2001].

A different category of vancomycin resistance in *S. aureus* was reported in Japan in 1997 and given the term hVISA [Conly & Johnston, 2002; Hiramatsu*, et al*, 1997]. These strains are susceptible to vancomycin (MIC <4 µg/ml), but also contain subpopulations having the capability to grow at higher concentration of vancomycin (>4 µg/ml) and with a MIC of ≥8 µg/ml. Cases of hVISA have been reported in Europe, Asia and Brazil [Denis*, et al*, 2002].

Although the increased MIC to vancomycin falls within the susceptible range, clinical failures among patients with infections caused by MRSA isolates with MICs of 4 µg/ml have been reported [Holmes*, et al*, 2011; Lodise*, et al*, 2008; Park*, et al*, 2013]. This raised concerns about the clinical relevance/outcome of reduced susceptibility to vancomycin in *S. aureus*. CLSI, therefore, reduced vancomycin breakpoints for *S. aureus* from 4 µg/ml to 2 µg/ml in

2006 [Clinical and Laboratory Standards Institute, 2006; Holmes*, et al*, 2012]. Currently, VRSA and VISA are defined as MIC ≥16 µg/ml and 4-8 µg/ml respectively by the broth microdilution method.

Due to difficulties in confirming hetero resistance and the probability of treatment failure of vancomycin when MIC >2 µg/ml, both the British Society for Antimicrobial Chemotherapy and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) consider *S. aureus* to be resistant to vancomycin when MIC is above 2 µg/ml [Holmes*, et al*, 2012].

1.2.5.2 VISA and hVISA

Both VISA and hVISA strains belong to a restricted range of epidemic MRSA genotypes [Denis*, et al*, 2002]. The genetic and biochemical mechanisms behind their reduced susceptibility to vancomycin remain unknown. However, VISA strains demonstrate thickened cell walls, less penicillin-binding protein and a slower growth rate [Hiramatsu*, et al*, 1997]. It has been suggested that intermediate susceptibility to vancomycin is mediated by cell wall thickening and reduced cross-linking, which thereby facilitates trapping the drug within the peptidoglycan layers of cell wall. This prevents vancomycin from reaching the peptidoglycan precursor attached to the cell membrane [Maor*, et al*, 2007].

HVISA are known to be phenotypically susceptible by routine laboratory methods, such as broth micro-dilution, but contain subpopulations of vancomycin-intermediate "daughter" cells with proportions ranging from $1x10^5$ to $1x10^6$ cells [Maor*, et al*, 2007]. HVISA strains represent the first-step mutants that serve as precursors of VISA strains in patients undergoing prolonged therapy with vancomycin. Recent studies have reported the rate of hVISA resistance among MRSA isolates ranged between 6% and 11% [Garnier*, et al*, 2006; Maor*, et al*, 2007].

Vancomycin creates selective pressure favorable for the outgrowth of hVISA clones; continued exposure yields a uniform population of VISA clones [Liu & Chambers, 2003]. Yet, the lack of standardized criteria for identifying hVISA strains complicates the determination of their clinical significance and role in treatment failures. However, uncontrolled studies on patients with hVISA infections demonstrated complicated clinical courses as well as high rates

of mortality attributed to the infection [Ariza*, et al*, 1999; Denis*, et al*, 2002; Howden*, et al*, 2004]. One study comparing hVISA bacteremia to MRSA bacteremia indicated that patients with hVISA bacteremia were at a greater risk of having prolonged bacteremia and being less responsive to vancomycin [Charles*, et al*, 2004]

1.2.5.3 Vancomycin Resistant *S. aureus* (VRSA)

VRSA is a separate entity that entails the acquisition of the *vanA* gene, thereby conferring complete resistance to vancomycin in *S. aureus*. *VanA* encodes an altered structure of peptidoglycan precursor, replacing the d-alanine-d-alanine structure with a d-alanine-d-lactate. This alteration precludes vancomycin from binding to the peptidoglycan precursor.

The earliest documented case of clinical infection caused by a fully vancomycin-resistant *S. aureus* (MIC >32 µg/mL) was published in Michigan, USA in 2002. Fortunately, VRSA infections have remained relatively rare. To date, fourteen cases have been reported in the USA [McGuinness*, et al*, 2017]with additional cases documented in India and Iran [Azimian*, et al*, 2012; Banerjee & Anupurba, 2012; Chang*, et al*, 2003; Emaneini*, et al*, 2007; Saha*, et al*, 2008; Sievert*, et al*, 2008]

The first reported case of VRSA in the USA was isolated in April 2002 from a catheter exit site from a forty-year-old diabetic patient suffered from peripheral vascular disease and chronic renal failure, which necessitated hemodialysis. This patient had chronic foot ulcerations which were treated in 2001 with multiple antibiotics including vancomycin. In April 2002, the patient developed MRSA bacteremia following the amputation of his gangrenous toe. He was treated with vancomycin for three weeks and rifampin for ten days. Two months later, he developed a catheter exit-site infection caused by *S. aureus*. This strain was resistant to vancomycin (MIC >128 µg/mL) and oxacillin (MIC >16 µg/ml). After 7 days, the patient developed an infection in his foot ulcer. Multiple organisms were recovered from the ulcer culture including *Klebsiella oxytoca, vancomycin-resistant Enterococcus faecalis* (VRE) and VRSA. The VRSA isolate had the *vanA* (vancomycin resistance gene) and *mecA* (oxacillin-resistance gene). MIC were >128 *µ*g/ml, 32 *µ*g/ml and >16 *µ*g/ml for vancomycin, teicoplanin and oxacillin respectively by broth microdilution testing [Chang*, et al*, 2003].

The number of VRSA and VISA cases that have been reported thus far remain relatively small. The associated risk factors and epidemiology of theses infection are still unclear. However, there is evidence that patients who are colonized with MRSA and have a prior exposure to vancomycin are at higher risk of developing a *S. aureus* infection with reduced susceptibility to vancomycin [Giri, 2012].

1.2.5.4 MIC Creep

Further recent cases of poor clinical outcomes after vancomycin treatment have been reported in patients with *S. aureus* infections, where the vancomycin MIC is in the upper limit of the susceptible range (2 µg/ml) [Holmes*, et al*, 2012; van Hal*, et al*, 2012]. The existing literature provides inconsistent information in regard to the clinical outcome of vancomycin in patients infected by MRSA with high 'susceptible' compared to MRSA with 'low susceptible' MICs [Jacob & DiazGranados, 2013]. Some studies ruled out any significant association between higher MICs and poor outcomes [Crompton*, et al*, 2010; Liao*, et al*, 2008], whereas others have suggested an association between higher MICs and reported treatment failure [Maclayton*, et al*, 2006; Moise-Broder*, et al*, 2004; Neoh*, et al*, 2007; Sakoulas*, et al*, 2004; Soriano*, et al*, 2008]. A study undertaken by Moise *et al* indicated a 21% success rate of vancomycin treatment in bacteremia caused by MRSA with 'high susceptible' MICs (2g/ml) compared with 77% success rate of vancomycin treatment for bacteremia caused by MRSA with low 'susceptible' MICs (0.5 g/ml) [Moise*, et al*, 2007].

Between 2002 and 2006 some clinical centers observed a changing pattern of increased MICs within the susceptible range to vancomycin and cited this phenomenon as "MIC creep" [Sader*, et al*, 2009]. Creep is defined as a "gradual and unnoticed movement or shift" i.e., the mean of vancomycin MICs is gradually increasing for the dominant wild-type population [Sader*, et al*, 2009]. This observation demonstrates a drift in clinical isolates of *S. aureus* towards reduced susceptibility to vancomycin, but not necessarily a corresponding amount of resistance. It also varies depending on the associated epidemiological and clinical factors of the study location. Variable conditions include the differences in medical therapy of *S. aureus* infections, dosing of vancomycin, the severity of illness, and the used method of susceptibility testing. Also, since susceptibility testing methods evaluate the MIC on a base of

2 logarithmic scale, this gradual shift may not be identified until the mode shifts to the next highest tested dilution.

Although MIC creep is limited and emerging very slowly [Sader*, et al*, 2009], its occurrence would raise a concern because such gradual loss of vancomycin activity may compromise the drug's efficacy immediately and over time [Hidayat*, et al*, 2006; Kollef, 2007; Neoh*, et al*, 2007; Soriano*, et al*, 2008].

Published studies that have reported instances of vancomycin MIC creep in MRSA have indicated conflicting results [Sader*, et al*, 2009]. Data analysis in most studies was based on the measurement of the proportion of strains with vancomycin MIC of 1-1.5 µg/ml or on the comparison of MICs means during specified periods. However, the high frequency of elevated MICs to vancomycin in the isolates tested does not reflect an increase in vancomycin MIC for the wild-type. Moreover, the higher proportion of strains with vancomycin MICs of 1- 1.5 µg/ml may be caused by an extensive use of vancomycin or dissemination of clones with reduced susceptibility to vancomycin when dealing with *S. aureus.* Therefore, it is critical to differentiate between MIC creep and increased frequency of certain epidemic clones with an elevated MIC to vancomycin. False perceptions of vancomycin MIC creep could be caused by an escalated emergence of clonal strains with elevated MICs (>1 µg/ml). Rapid spread of these MRSA clones has been documented by Sader *et al* [Sader*, et al*, 2009]. In addition, combining data from multiple centers can obscure potential trends within a given institution or region as a result of differences in patient populations and drug usage patterns [Joana*, et al*, 2013].

1.3 Antimicrobial Resistance

The principles of evolution make the rise of antibiotic resistance in microbial populations unavoidable. Antibiotic resistance can evolve naturally through random mutation and amplified by applying an evolutionary stress, like antibiotics, on a population [Gaude & Hattiholli, 2013]. Antibiotics eliminate drug-sensitive competitors from the population which then serves to leave the resistant residual bacteria to survive and proliferate, and thereby remain as the surviving population due to natural selection [Ventola, 2015]. In such a

scenario, a single bacterial mutant cell can benefit from the selection pressure applied by an antibiotic and resistance emerges.

Antibiotic resistance is considered when a drug loses its ability to effectively prevent the growth of pathogenic bacteria; thus, resistant bacteria continue to multiply in the presence of the drug and may result in greater illness severity or complications. Moreover, bacteria may collect multiple resistance traits over time, in the course of which they gain resistance towards multiple classes of antibiotics. It is a matter of concern that the problem of resistance is steadily increasing globally and the available therapeutic options for certain infections have become limited [Zaman*, et al*, 2017].

1.3.1 Surveillance Data

An estimated 25,000 patients die every year in Europe due to infections caused by multi-drug resistance bacteria [Colomb-Cotinat*, et al*, 2016]. Similarly in the USA, at least 23,000 people die from similar infections every year in addition to a substantial economic impact of an extra $20 billion yearly [Demirjian*, et al*, 2015]. Recent reports estimate that deaths from drug-resistant infections will increase from 700,000 to 10 million by 2050, with an associated cost of $100 trillion [Jasovsky*, et al*, 2016].

1.3.2 Selection of Resistance

Developing resistance to a particular antibiotic may also select resistance (cross-resistance) to other compounds belonging to the same class. For instance, resistance to tetracycline may cause resistance to oxytetracycline, chlortetracycline, doxycycline and minocycline [Zaman*, et al*, 2017]. The level of antibiotic-resistance is also strongly correlated with the dosage and duration of antibiotic therapy. Development of antibiotic resistance is expedited by the overuse and misuse of antibiotics. This includes unnecessary prescription, suboptimal dosing, inappropriate drug choice, self-medication, and non-compliance with the (prescribed) antibiotic regimen.

Antibiotics used in food animals may also select for resistance to antibiotics used in humans because resistance to antibiotics in both humans and animals occurs by similar mechanism, which constitutes a grave concern to the World Health Organization [World Health Organization, 2017]. There is a demonstrable association between antibiotic

consumption in animals and the presence of commensal organisms resistant to the same antibiotic class in humans [Chantziaras*, et al*, 2014]. A recent report issued in Europe in 2017 identified that resistance to fluoroquinolones in humans in *Salmonella* and *Campylobacter* bacteria was due to the consumption of fluoroquinolones in animals with the potential of other factors being included as well [European Medicines Agency, 2017]. Although many antibiotics are solely designed for veterinary use the majority have similar structures to antibiotics used in humans [Heuer*, et al*, 2009; Swann, 1969]. In the USA, 62% of the antibiotics used in animals represent structurally related compounds that are considered "medically important" for human therapy [Laxminarayan*, et al*, 2013].

Moreover, large amounts of antibiotics are used in the agricultural sector worldwide to supply the needs (eggs, meat, dairy products) of a rapidly growing human population [Rassow & Schaper, 1996; Roura*, et al*, 1992; Vazquez-Moreno*, et al*, 1990]. In 2015, the FDA estimated the nation's annual antimicrobial consumption in food animals to be approximately 80%, with 74% being administered non-therapeutically as growth promotors [Food and Drug Administration, 2016].

In the 1940s, it was observed that animals that consumed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues increased their rate of growth; thus, the growth promoting effect of antibiotics was recognized [Modi*, et al*, 2011]. In 1951, the FDA approved the use of antibiotics, without prescriptions, as additives for animal feed [Jones & Ricke, 2003]. In 1969, the British government started to restrict the use of antibiotic growth promoters (AGPs) in order to reduce the risk of resistance developing to human antibiotics. That said, in the 1990s, vancomycin-resistant Enterococcus (VRE) was identified in European patients due to the use of avoparcin as a growth promoter. As a result, the European Union (EU) banned avoparcin and between 1999 - 2006 they banned additional AGPs that are medically important in human medicine [Cogliani*, et al*, 2011]. In 1986, Sweden became the first country to withdraw AGPs in food animal production.

More recently, the FDA established new regulations in 2017 to limit the use of antibiotics in farm animals and banned the use of "medically important" antibiotics as AGPs in animal husbandry [Brussow, 2017]. The increasing concern about antimicrobial resistance

in recent years has likewise led Health Canada to apply certain changes in regard to the accessibility to veterinary antibiotics. As of December 1st, 2018, a prescription will be required from all livestock producers in order to buy antibiotics for therapeutic use in animals. Moreover, veterinary drug manufacturers will voluntarily be removing growth promotion claims on their antimicrobial products. Health Canada made these policy changes to reduce the development of antimicrobial resistance in animals and to promote the responsible use of antimicrobials in animals [Canadian Animal Health Institute, 2018; Food and Drug Administration, 2016].

1.3.3 Development of Resistance

Some bacteria are naturally resistant, through their inherent characteristics, to certain antimicrobial agents. This natural ability is known as intrinsic resistance and is mediated by innate mechanisms. For example, mycoplasma species are naturally resistant to β-lactams due to the absence of the drugs' target site (peptidoglycan),i.e. mycoplasmas do not have a cell wall [Marinescu*, et al*, 2017].

Bacteria can also exhibit antimicrobial resistance through spontaneous mutations (*de novo*) in genes encoding drug targets or acquire resistance through horizontal gene transfer (encoding a resistance gene) from donor bacteria, phages, or free DNA [Sharma*, et al*, 2016]. Brown reported horizontal gene transfer as a major factor for the acquisition of antibiotic resistance in clinical isolates [Brown*, et al*, 2003]. Foreign DNA can also enter the bacterium on plasmids via cell-to-cell contact (conjugation) by bacteriophage (transduction) or by cellular uptake of naked DNA (transformation). Conjugation has been considered the most important mechanism for the dissemination of antibiotic resistance genes.

Acquired resistance involves genetic exchanges that could lower susceptibility to the extent that resistance arises in a single step, unlike *de novo* resistance which develops in a gradual and stepwise manner by the accumulation of mutations that lower susceptibility by slight increments [Drlica, 2003].

1.3.3.1 Mechanisms of Resistance in Bacteria

Both the acquired and *de novo* resistance enable a bacterium to resist the antibiotic through one of the following mechanisms: 1) by acquiring a mutation that limits the access of

drug via down-regulating porin genes,2) by acquiring genes encoding destructive enzymes which inactivate the drug, 3) by acquiring genes encoding efflux pumps that expel the antibiotic before reaching the target site or 4) by acquiring genes encoding altered binding sites [Tenover, 2006].

1.4 Tolerance

In contrast to acquired resistance, non-inherited resistance is phenotypic, transient and reversible, where bacterial subpopulations become transiently resistant to the drug's action even though they are genetically homogeneous and susceptible to the antibiotic. Different forms of non-inherited resistance, such as persistence and tolerance, could extend the duration of antimicrobial therapy and cause treatment failure in patients undergoing treatment [Levin & Rozen, 2006]

1.4.1 Tolerance versus Resistance

Resistance describes the ability of bacteria to grow and multiply at high/lethal concentrations of an antibiotic and it is measured by the MIC whereas tolerance describes the ability of bacteria to survive at high concentrations of drug while the MIC remains the same. Tolerance, as defined by Kester and Fortune "enables bacterial cells to survive a transient exposure to antibiotics at lethal concentrations" [Kester & Fortune, 2014]. Tolerance can occur against bactericidal antibiotics, but not with bacteriostatic agents where bacteria arrest their growth and are expected to survive the transient exposure to a non-lethal drug [Brauner*, et al*, 2016].

Tolerance may be acquired through a genetic mutation or encouraged by environmental conditions such as poor growth conditions or antibiotic administration. Lederberg and Zinder utilized the poor growth environment to generate tolerance and isolate auxotrophic mutants, where non-growing auxotrophs only survived after exposing the bacterial population to penicillin in a medium lacking a necessary amino acid for bacterial growth [Lederberg & Zinder, 1948]. Antibiotic application can also induce a non-growing state (drug-induced tolerance), which protect the bacteria from the bactericidal activity of the drugs.

This phenomenon has been observed in a number of bacterial species, such as *S. pneumoniae* and *S. aureus*, against antibiotics that interfere with cell-wall synthesis such as glycopeptides and β-lactams [Levin & Rozen, 2006]. Studies show that vancomycin tolerance often occurs in staphylococci and more often in strains with reduced vancomycin susceptibility, such as VISA and hVISA [Jones, 2006; Rose*, et al*, 2012].

1.4.2 Persistence

Persistence is observed when the majority of the bacterial population are killed rapidly, while a small subpopulation persists for a much longer period of time due to slow growth or dormancy [Brauner*, et al*, 2016]. In 1994, Bigger observed this property and coined the term 'persisters' to indicate the survivors. He recognized that when treating *S. aureus* with high concentrations of penicillin a small fraction survived ($\leq 10^6$) [Bigger, 1944] and when the drug was removed, this fraction re-grew and was as sensitive to penicillin as the wild type. He demonstrated the persisters' dormancy by exposing the bacterial population to penicillin in non-nutritive medium, where the drug did not effectively kill all the cells and their regrowth in a rich medium was delayed.

1.5 Antimicrobial Agents

1.5.1 Mechanism of Antibiotics

Antimicrobial agents are categorized based on their principal action mechanism. Antibiotics target essential cellular processes causing growth delay and death of bacterial cells using various mechanisms. These mechanisms include interference with cell wall synthesis, interference with nucleic acid synthesis, inhibition of intermediary metabolic pathways, inhibition of protein synthesis and disruption of the cytoplasmic membrane [Richardson*, et al*, 2001].

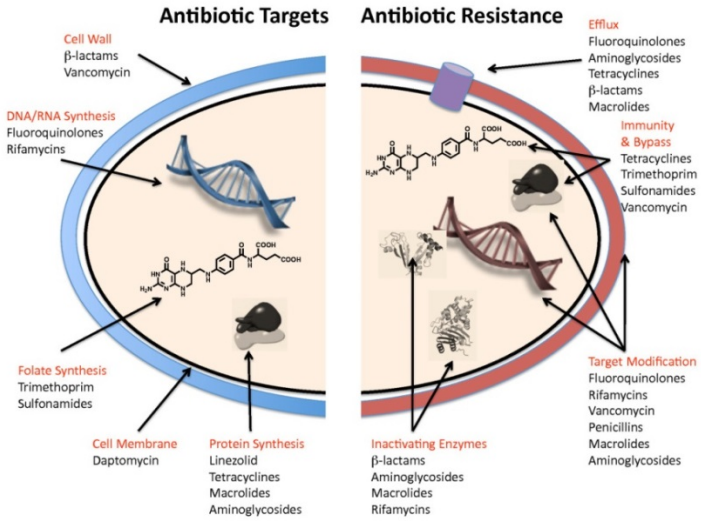


Figure 1.5.1.1 Antibiotic targets and mechanisms of action [Richardson, *et al*, 2001].

1.5.2 Glycopeptides

Glycopeptide antibiotics are frequently used in the treatment of life-threatening infections caused by MRSA and other Gram-positive pathogens. First generation glycopeptide antibiotics, including teicoplanin and vancomycin, are natural products produced by *Actinomycetes* species and composed of glycosylated non-ribosomal heptapeptides. The antibacterial activity of this class is initiated by the interaction with dipeptidyl residues of peptidoglycan precursors in the bacterial cell wall to inhibit its formation.

1.5.2.1 Vancomycin

Vancomycin is a relatively large glycopeptide, with a molecular weight of 1,485.7 Da and is derived from *Nocardia orientalis*. It has time-dependant bactericidal activity against most Gram-positive bacteria such as *Streptococci, Staphylococci, Corynebacteria, Clostridia, Listeriae and* Bacillus species. Unlike β-lactams antibiotics, vancomycin does not inhibit the enzymatic activity involved in cell wall synthesis but rather blocks the substrates for cell wall-synthesizing machinery, i.e., the D-alanyl-D-alanine residue (DDR) of the lipid II precursor. Therefore, it inhibits the use of the substrates by the glycosyltransferase enzyme, preventing the production of the nascent peptidoglycan chain [Hiramatsu, 2001].

For over five decades, vancomycin has been considered as the cornerstone and first-line agent for treating MRSA, Gram-positive β-lactams–resistant bacteria, and sensitive Gram-positive bacteria in patients allergic to penicillin.

Although vancomycin has limited bacterial resistance to vancomycin among Gram-positive bacteria [Geraci*, et al*, 1958] has been seen to date, there are major concern about the decreased susceptibility of this agent in *S. aureus* [Choo & Chambers, 2016]. In addition, suboptimal results (i.e., a slower bactericidal activity) have been demonstrated in patients with MSSA infections being treated with vancomycin in comparison to treatment with β-lactams [Crotty*, et al*, 2016]. Further issues include the challenges of dose adjustments, drug monitoring and associated toxicity that complicate the use of vancomycin in certain settings [Crotty*, et al*, 2016].

Vancomycin can be administered intravenously for treatment of systemic infections through home IV programs or hospitalization. The standard infusion time for its administration is set to minimize infusion-related adverse effects such as Red Man's Syndrome [Levine, 2006]. During this reaction, histamine is released from mast cells leading to generalized flushing, pruritus and an erythematous rash.

The pharmacokinetic profile of vancomycin is complex as it has a variable tissue distribution and may be influenced by the patient's conditions such as renal function, age, body weight and type of infection. This agent is eliminated primarily by glomerular filtration (renal route), with over 80% recovery in urine during the first 24 hours of a single dose, therefore, renal dysfunction is a major factor affecting the pharmacokinetics of vancomycin. Toxicity/nephrotoxicity is another major issue due to the narrow-therapeutic index for vancomycin i.e., the therapeutic and toxic doses are in close proximity. For this reason, vancomycin therapy requires weekly drug monitoring of serum trough concentrations along with monitoring of serum creatinine, blood urea nitrogen and a complete blood count [Bauer, 2008].

Achieving the appropriate dose of vancomycin to treat *S. aureus* infections could be difficult considering the pharmacokinetic/pharmacodynamic complexity in addition to the

clinical impact of vancomycin MIC creep and hetero resistance subpopulations among MRSA strains.

1.5.3 Oxazolidinones

The oxazolidinones (linezolid and tedizolid) represent a novel class of antibacterial agents that have a unique structure (containing 2-oxazolidine) and potent activity against susceptible and multidrug-resistant Gram-positive bacteria. Oxazolidinones exhibit their antibacterial effects by binding to the bacterial ribosome and preventing the formation of the 70S initiation complex; thus, inhibiting bacterial protein biosynthesis.

1.5.3.1 Linezolid

Linezolid was the first oxazolidinone that served as an alternative to vancomycin. This agent inhibits the synthesis of bacterial proteins by binding to 23S rRNA in the catalytic site of the 50S ribosome. It is classified as bacteriostatic with significant post antibiotic effect (PAE), i.e. continuous effect of drug's action when its concentration dropped below the MIC. In April 2000, it was approved by the FDA for treating community-acquired and nosocomial pneumonias and SSTIs caused by MRSA [Watkins*, et al*, 2012]. It has shown superior efficacy over vancomycin in treating complicated skin and soft-tissue infections (SSTIs) and was also evaluated for treating bacteremia as an alternative to vancomycin [Shorr*, et al*, 2005].

Due to the unique formulation and full bioavailability of linezolid, it offers the option of being administrated orally. It is characterized by good penetration and accumulation in various tissues including skin, bone, lung, hematoma and cerebrospinal fluid which increases the range of infections that can be treated by linezolid. Moreover, patient conditions such as age and sex have no significant effect on the pharmacokinetics of this drug. Its clearance is not dependent on hepatic enzyme action and thereby dosage adjustments are unnecessary for patients with hepatic insufficiency [Birmingham*, et al*, 2003].

Side effects of linezolid are reported to be mild such as diarrhea, nausea and headaches. Laboratory abnormalities include anemia and thrombocytopenia, which might be aggravated by liver disease. Longer treatments (over 28 days) can be associated with more serious adverse events such as lactic acidosis, irreversible peripheral neuropathy and optic neuritis.

It is also associated with serotonin toxicity when used concomitantly with other serotonin releasing agents [Watkins, *et al*, 2012].

Resistance to linezolid is rare, though the involvement of the *cfr* gene in conferring resistance was recently discovered [Zhanel, *et al*, 2015]. This finding is a matter of concern because this gene is horizontally transferable and therefore, a high potential for its widespread dissemination exists. However, another study reported the rate of linezolid resistance among MRSA strains in the USA to be as low as 1%, which has been stable since 2006 [Flamm, *et al*, 2012; Watkins, *et al*, 2012]. A recently published surveillance study has reported <1% resistance rates to linezolid observed in Africa, Asia, Europe, North America and South America between 2014 and 2016 [Seifert, *et al*, 2018].

1.5.3.2 Tedizolid

Tedizolid is a second generation oxazolidinone that has been engineered to improve the efficacy and bioavailability over linezolid with less toxicity. Tedizolid inhibits protein synthesis in a similar mechanism to linezolid. In terms of their structural difference, linezolid lacks the D-ring found in tedizolid, which offers greater interaction with additional sites on bacterial ribosomes and consequently increases of antimicrobial potency of tedizolid [Shaw & Barbachyn, 2011; Shaw, *et al*, 2008]. Additionally, it has an added phosphate which increases its bioavailability and water solubility.

Tedizolid has notable activity against Gram-positive bacteria including MRSA including linezolid-resistant strains. It has been approved in Canada, USA, EU and some other countries for treating acute bacterial skin and skin structure infections (ABSSSI) [McCool, *et al*, 2017]. Choi and colleagues showed *in vitro* that tedizolid has four-fold greater potency compared to linezolid against penicillin-resistant *S. pneumoniae* [Bassetti, *et al*, 2013; Choi, *et al*, 2012]. It has also been demonstrated *in vitro* that tedizolid has a 4-16 fold higher activity than linezolid against MSSA, MRSA, Streptococci and Enterococci [Burdette & Trotman, 2015].

The pharmacokinetic characteristics of tedizolid allow for a single dose per day, which offers similar efficacy and a better safety profile than does twice daily linezolid. It has been published that a 6-day course of tedizolid (200 mg once daily) is as effective as a 10- day course (600 mg twice daily) of linezolid in the treatment of ABSSSI [Joseph, *et al*, 2017]. Furthermore,

tedizolid is metabolized predominantly in the liver with 82% eliminated in feces and 12% in urine. For this reason, no dosage adjustment is needed for patients with renal or hepatic impairment [Crotty*, et al*, 2016].

Apart from nausea and headache, no major side effects were reported to tedizolid in clinical trials. Also, no hematological adverse effects have been reported for the therapeutic dose of tedizolid. Additionally, it does not inhibit monoamine oxidase *in vitro*, and therefore there are no potential interactions with serotonergic drugs which provides a safer dosage regimen [Crotty*, et al*, 2016].

Although such newer drugs may offer some advantages over vancomycin, they generally are bacteriostatic against MRSA and have significant limitations. Time-kill studies have indicated bacteriostatic activity of tedizolid against enterococci, staphylococci and streptococci [Crotty*, et al*, 2016]. Superior efficacy of newer agents compared to vancomycin, as far as treatment of serious MRSA infections is concerned, has only been demonstrated in limited studies. Therefore, vancomycin remains the treatment of choice for severe MRSA infections.

1.5.4 Pharmacodynamic Approach

Pharmacokinetic (PK) and pharmacodynamic (PD) principles are important to optimize the antimicrobial therapy through investigating various factors include the onset, magnitude and duration of the drug response [Finberg & Guharoy, 2012]. The pharmacokinetic profile of a drug outlines the time course of drug concentration in the body and describes its absorption, distribution, metabolism and elimination, whereas pharmacodynamics describes the impact of the drug plasma concentration on the pathogen and patient by monitoring the drug concentrations in plasma at different time intervals and also measuring the volume of distribution and clearance of the drug. The integration of PK/PD helps to assess the interactions between a pathogen, host and antimicrobial agent as well as to define the effective dosage regimens and required duration of antimicrobial therapy [Finberg & Guharoy, 2012].

Some drugs such as quinolones and aminoglycosides are known as concentration-dependent agents because as the drug concentration increases, the rate and extent of the

bactericidal activity increase correspondingly [Ambrose*, et al*, 2007]. In addition, these agents have a prolonged post-antibiotic effect (PAE). The efficacy of these antibiotics is determined by the peak concentration (C_{max}) and area under the concentration curve (AUC). For this group, concentrations of ten times the MIC (at least) have been suggested to achieve optimal bactericidal effect [Quintiliani, 2004].

On the other hand, agents like glycopeptides and β-lactams display a time-dependent pattern of bactericidal activity that occurs over a narrow range of drug concentrations (2 to 4 times the MIC) and depends largely on the exposure periods. The optimal bactericidal effect of these agents obtained when drug concentrations are maintained above the minimum inhibitory concentration (MIC) for prolonged periods of the dosing intervals [Barger*, et al*, 2003]. This group also tends to have minimal PAE [Levison, 2004].

A third group consists of bacteriostatic agents, like linezolid, and is characterized by moderate to prolonged PAE. Their efficacy is determined by the area under the drug concentration curve (AUC) and AUC to MIC ratio [Al-Dorzi*, et al*, 2014].

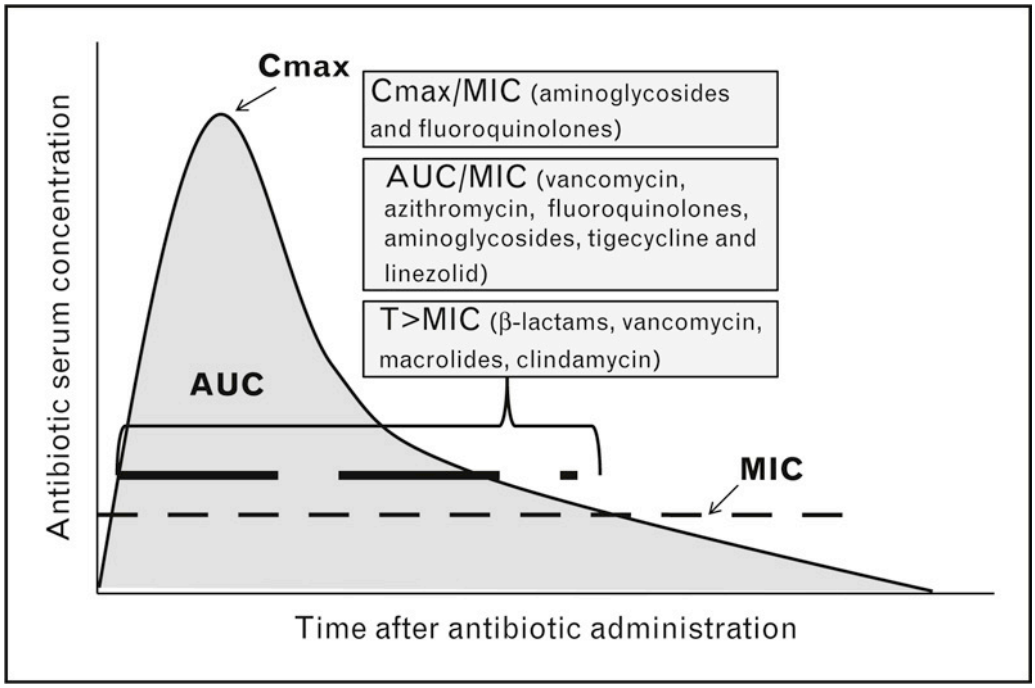


Figure 1.5.4.1: Pharmacokinetic and pharmacodynamic parameters [Al-Dorzi*, et al*, 2014].
AUC= area under the curve; C_{max}=peak antibiotic concentration; MIC=minimum inhibitory concentration. AUC/MIC ratio measurement for time and concentration dependent antibiotics; C_{max}/MIC ratio measurement for concentration-dependent antibiotics; T>MIC measurement for time-dependent antibiotics.

1.6 Susceptibility Testing

Antimicrobial susceptibility testing (AST) methods are standardized and updated annually by the CLSI in the USA and the EUCAST in Europe [Schofield, 2012]. These methods serve as therapeutic guidance tool to confirm susceptibility to drugs of choice for particular pathogens and to detect possible drug resistance, especially with species that may possess acquired resistance mechanisms (e.g., members of the *Enterobacteriaceae*, *Pseudomonas* species, *Staphylococcus* species, *Enterococcus* species and *Streptococcus pneumoniae*) [Jorgensen & Ferraro, 2009]. According to CLSI standards, only clinically problematic pathogens are tested.

The AST can be performed using qualitative methods, like conventional disk diffusion (Kirby-Bauer) or quantitative methods such as broth dilution, agar dilution, antimicrobial gradient (e.g., the E-test, AB Biodisk) or by employing semi-automated instrumentation. Dilution methods are used to determine the MICs of antimicrobial agents and are considered the reference method for testing antimicrobial susceptibility [Jorgensen & Ferraro, 2009]. They measure the micro-organisms' ability to produce visible growth in broth (so called broth dilution) or on agar plates (agar dilution) against various concentrations of the drug, usually in doubling dilutions. Broth microdilution is the most widely used method; however, for fastidious bacteria such as *Campylobacter* species that require special culture media, agar dilution is applied [Sykes & Rankin, 2012].

1.6.1 Minimum Inhibitory Concentration (MIC)

MIC is defined as "the lowest drug concentration that will inhibit the visible growth of an organism in broth" based on utilizing a standardized bacterial inoculum of 10^5 CFU/ml [Andrews, 2001]. MIC testing is the 'gold' standard of susceptibility testing and serves as an indicator for the accuracy of all other methods of susceptibility testing. It assists physicians to make better decisions in selecting ideal treatments. It is also used to confirm unusual resistance or to confirm a borderline result of other methods of testing. Data studies and trials on MIC determination have been used to determine susceptibility breakpoints and have served as a guidance for the management of most patients with infectious diseases [Andrews, 2001].

The broth micro-dilution technique is performed in microtiter plates with a capacity of <500 µl per well. Bacteria are inoculated into a broth culture medium in the presence of different concentrations of an antimicrobial agent (doubling dilutions). Visual growth of bacteria is assessed after overnight incubation (16-20 h) under ambient conditions and the MIC value is defined [Wiegand*, et al*, 2008]. Similarly, agar dilution involves the incorporation of different concentrations of the drug into agar medium, followed by inoculating plates with a standardized number of bacterial cells of interest.

The MIC value is designated as being susceptible, intermediate or resistant based on the established breakpoints. Results are generally accepted within one doubling dilution (i.e., + or - dilution of the end point). MIC values below the breakpoint typically indicate an organism's susceptibility to the drug which represents a good prediction for a favourable outcome. On the contrary, high MIC values represent resistance and predict unfavourable outcomes, although the correlation of *in vitro* susceptibility to clinical outcomes is not 100% accurate.

1.6.2 Mutant Prevention Concentration (MPC)

MPC determines the drug concentration that is required to block the growth of the least susceptible (single-step mutant) cell in a bacterial population based on an inocula of $\geq 10^9$ CFU. The rational for selecting $\geq 10^9$ cells is to ensure that the bacterial population is large enough for mutant subpopulations to be detected. Also, it's rare for infections to contain more than 10^{10} organisms, and testing a higher number of cells is often difficult [Drlica, 2003]. In the MPC method, 10^9 CFU of a bacterial suspension is applied to multiple blood agar plates prepared with different antimicrobial concentrations. The MPC is identified as the lowest drug concentration preventing 100% visible growth and recorded following 24 and 48 hours of incubation under ambient conditions.

This novel measurement of *in vitro* antimicrobial activity was described by Dong *et al* as "minimal concentration of antibiotic that will prevent the selection of first step resistant mutants from a large bacterial inoculum" [Dong*, et al*, 1999]. It has been published that spontaneous mutations evolve in bacterial inocula that range between 10^7- 10^9 bacterial cells

[Komp Lindgren, *et al*, 2003]. Therefore, spontaneous mutations are expected to occur in bacterial populations ≥10^9 CFU. However, it would normally not be detected by the lower inoculum used in traditional susceptibility testing.

Consequently, isolates considered susceptible by MIC testing may contain an undetected subpopulation of resistant cells. Blondeau *et al* demonstrated that first-step resistant mutants may be present in a bacterial population despite the fact that the organism was deemed susceptible by routine susceptibility testing methods [Blondeau, *et al*, 2001; Hansen, *et al*, 2003; Metzler, *et al*, 2004].

Blondeau *et al* reported MPC results for *Streptococcus pneumoniae* isolates that were fluoroquinolone susceptible by MIC testing. Greater than 20% of more than 100 strains indicated higher MPC values than the susceptible breakpoints [Blondeau, *et al*, 2001]. An observation was reported by Lim *et al* who showed 59% (48/82) of pneumococcal strains with MICs of 2 µg/ml (susceptible) to levofloxacin contained a first step *parC* mutation [Blondeau, *et al*, 2006; Lim, *et al*, 2003].

A group of select studies comparing MIC and MPC values for various pathogens and antibiotics is summarized in Table 1.6.2.1

Table 1.6.2.1: Comparison studies of MIC and MPC values for various pathogens and antibiotics.

Antibiotic	N	MIC_{90}	MPC_{90}	References
	Streptococcus pneumoniae			
Moxifloxacin	100	0.25	4	[Blondeau, *et al*, 2001]
Levofloxacin	100	1	4	
Azithromycin	177	0.125	4	[Blondeau, *et al*, 2006]
Clarithromycin	206	0.063	1	
Erythromycin	201	0.125	2	
Tigecycline	47	0.031	0.5	[Hesje, *et al*, 2015]
	Staphylococcus aureus MSSA			
Levofloxacin	1	0.125	1	[Allen, *et al*, 2004]
Moxifloxacin	1	0.015	0.25	

Cefazolin	26	2	64	[Blondeau & Metzler, 2005]
Cloxacillin	26	0.25	2	
Vancomycin	26	1	4	
Tigecycline	50	0.125	1	[Hesje, *et al*, 2015]
Ciprofloxacin	4	0.5	2	[Hedlin & Blondeau, 2004]
	Staphylococcus aureus MRSA			
Ciprofloxacin	1	0.125	1	[Allen, *et al*, 2004]
Levofloxacin	1	0.125	0.5	
Moxifloxacin	1	0.063	0.125	
Cefazolin	24	16	512	[Blondeau & Metzler, 2005]
Cloxacillin	24	32	>512	
Vancomycin	24	1	8	
Tigecycline	50	0.5	4	[Hesje, *et al*, 2015]
	Haemophilus influenzae			
Ciprofloxacin	31	0.016	0.5	[Metzler, *et al*, 2004]
Ofloxacin	31	0.031	0.5	
Levofloxacin	31	0.016	0.125	
Gatifloxacin	31	0.031	0.125	
Moxifloxacin	40	0.031	0.25	[Blondeau & Borsos, 2007]
Gemifloxacin	40	0.008	0.125	
Azithromycin	40	2	32	
Telithromycin	40	2	16	
Clarithromycin	40	8	≥64	
Cefuroxime	40	16	≥16	
	Citrobacter freundii			
Ciprofloxacin	20	0.125	2	[Hansen & Blondeau, 2005]
Levofloxacin	20	0.5	2	

Garenoxacin	20	4	8	
	Enterobacter cloacae			
Ciprofloxacin	20	≤0.06	1	[Hansen & Blondeau, 2005]
Levofloxacin	20	0.125	4	
Garenoxacin	20	1	>8	
	Klebsiella pneumoniae			
Ciprofloxacin	20	≤0.06	1	[Hansen & Blondeau, 2005]
Levofloxacin	20	1	2	
Garenoxacin	20	0.25	4	
Moxifloxacin	18	0.25	≥2	[Blondeau, *et al*, 2007]
	Pseudomonas aeruginosa			
Ciprofloxacin	20	1	4	[Blondeau, *et al*, 2007]
Levofloxacin	20	4	16	
	Escherichia coli			
Ciprofloxacin	20	≤0.06	0.05	[Sievert, *et al*, 2008]
Levofloxacin	20	≤0.06	1	

In all instances MPC values are higher than MIC values. Differences are noted between antibiotics within the same drug class. Therefore, MPC cannot be assumed from an MIC measurement. Drlica *et al* showed that R^2 values were usually below 0.8, when MIC and MPC values for a number of bug-drug combinations were compared [Drlica, *et al*, 2006]. As such, an MPC must be measured and not assumed or predicted.

Even though Smith *et al* suggested that MPC testing does not apply to many antimicrobial drug classes, in which horizontal gene transfer represents the main mechanism of resistance [Smith, *et al*, 2003], MPC can be measured for all bug-drug combinations to determine the drug concentrations blocking novel resistance that may arise by point mutations. Moreover, multiple reports have documented differences in MIC and MPC values for antimicrobial agents against bacteria where the major mechanism of resistance was a transmissible resistance element [Blondeau, 2009; Hesje, *et al*, 2007].

Furthermore, high bacterial burdens have been identified in humans during various infectious such as meningitis, respiratory and urinary tract infections. Frisch *et al* confirmed these findings by reporting a bacterial load of 10^{10}-10^{12} organisms to be present during community-acquired pneumonia. Another report by Fagon *et al* identified bacterial densities of *H. influenzae* and *S. pneumoniae* to exceed 10^7 CFU/ml during bronchitis [Fagon*, et al*, 1990; Frisch*, et al*, 1942]. Accordingly, resistant subpopulations are most likely to be present in these infections and these could be enriched during treatment [Metzler*, et al*, 2013].

Dosing choices based on MPC drug concentrations would theoretically prevent the selective amplification of the resistant subpopulation during infections. Hence, MPC testing may offer better guidance for optimal antimicrobial therapy. Yet there is no clinical study evaluating the quantitative relationship between *in vivo* and *in vitro* values [Drlica, 2003]. Besides, some MPC values for various drug-bug combinations are beyond realistically achievable drug concentrations with the currently approved dosing and higher drug dosages may result in unacceptable toxicities.

1.6.2.1 Mutant Selection Window (MSW)

The mutant-selection window (MSW) defines the drug concentration zone, or "danger zone", where selective amplification of resistant mutants may occur [Blondeau, 2009]. The MPC value represents the upper boundary of this MSW, whereas the lower boundary is defined by the MIC, or lowest drug concentration inhibiting the growth of susceptible cells.

It has been demonstrated *in vitro* that when drug concentrations exceed the MPC, both susceptible and mutant organisms are inhibited. Above this concentration, cell growth requires at least the presence of two simultaneous resistance mutations and growth is therefore rarely expected to occur [Iseman, 1994; Zhao*, et al*, 1997]. Consequently, maintaining the dose above the MPC during the entire dosing interval would prevent resistance selection. However, concentrations above the MPC for certain drugs cannot be reached due to toxicity limits [Olofsson & Cars, 2007].

On the other hand, susceptible cells are likely to be inhibited when drug concentrations fall within the MSW, as the drug concentration is in excess of the MIC. However, mutant cells will continue to proliferate while the drug concentration is below the MPC, which suggests

that therapeutic drug concentrations would possibly induce the amplification of mutant cells during infections [Hesje*, et al*, 2007]. The longer the antibiotic remains within the MSW, the greater likelihood for resistant mutants to be selected and amplified. Therefore, reducing this time by choosing compounds with narrower MSWs, for instance, may reduce the likelihood for resistance selection during therapy.

For time-dependent antimicrobial agents, doses are commonly adjusted to achieve antibiotic plasma concentrations above the MIC for the respective pathogen throughout the dosing interval [Mueller*, et al*, 2004]. Yet, the traditional recommendations concerning dosing, which is to exceed the MIC, would probably place drug concentrations within the selection window where they will act for the enrichment of resistant mutant subpopulations. Low drug concentrations, on the other hand, allow for pathogen population to multiply, and consequently promote the generation of new mutants over time.

1.6.3 Time Kill Curve

Previous methods (MIC and MPC) were established to measure the inhibition of bacterial growth, rather than the degree of bacterial killing. Time kill assays are used to assess the killing activity of an antimicrobial agent against a particular pathogen over time and provides a detailed assessment of the PK-PD relationships. This method is standardized and has been described in multiple papers [Blondeau*, et al*, 2006; Blondeau*, et al*, 2012; Blondeau*, et al*, 2015], where a bacterial culture is grown at 5×10^5 - 5×10^6 CFU/ml and treated with antibacterial agents. Viable cell numbers are then determined at different time intervals. Bactericidal activity is defined as ≥3 $\log_{10}$ decrease in CFU/ml at 24 h following drug exposure [Haas*, et al*, 2010].

Time-kill i*n vitro* models consider both drug concentration and time course of antibacterial effects and by this approach can be conducted with constant antibiotic concentrations or variable antibiotic concentrations. Determination of the degree of killing at various bacterial densities provides more in-depth information about the *in vitro* performance of an agent, especially when the bacterial burden varies throughout the course of infection [Blondeau*, et al*, 2006]. Such a dynamic PK-PD approach is more rational in providing a description of drug-bacteria interactions [Mueller*, et al*, 2004].

Measuring *in vitro* killing is also important for differentiating bacteriostatic from bactericidal antimicrobial which is critical when determining how to treat complicated infections and infections in immunosuppressed patients. Bacterial eradication has been linked to clinical outcomes in respiratory infections [Dagan*, et al*, 2001]. Complete and rapid killing of bacteria may impact clinical outcome significantly by reducing duration of therapy, probability for relapse, and occurrence of selection for resistance [Blondeau & Shebelski, 2016].

1.7 Objectives

The objectives of this thesis were as follows:

1. To determine mutant prevention concentration (MPC) values for blood culture isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) to linezolid, tedizolid and vancomycin.
2. To characterize the genetic and/or biological basis for high vancomycin MPC values in some MRSA isolates.
3. To assess the *in vitro* killing of MRSA strains by linezolid, tedizolid and vancomycin.

2.0 MATERIALS AND METHODS

2.1 Standard Laboratory Methods

2.1.1 Isolate Collection and Identification

Bloodstream isolates of Methicillin Resistance *Staphylococcus aureus* (MRSA) and Methicillin Susceptible *Staphylococcus aureus* (MSSA) used in this study were collected through the Clinical Microbiology Laboratory at Royal University Hospital, Saskatoon, SK. Identification of *S. aureus* was determined by the Vitek II (BioMerieux, St. Laurent, QC). Isolates were confirmed as being methicillin-resistant by the Mueller-Hinton oxacillin screen plate [Demir*, et al*, 2016] or by PCR amplification of the *mecA* determinant gene [Pournajaf*, et al*, 2014]. MRSA Isolates were collected from sixty patients with bacteremia from January (2011) to August (2012). No pre-selection criteria were used that would favor the inclusion of isolates with specific susceptibilities to vancomycin or other agents. American Type Culture Collection (ATCC) strains used included methicillin-susceptible *Staphylococcus aureus* (MSSA) (ATCC 29213), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *E. faecalis* (ATCC 29212) and were obtained from the American Type Culture Collection. ATCC strains were used as controls for susceptibility testing and were tested each time a susceptibility test was performed.

2.1.2 Storage of the Bacterial Isolates

MRSA isolates were streaked for isolated colonies on blood agar plates and incubated in ambient air (O_2) at 35-37° for 18-24 hours. Isolated colonies were then picked from the agar plate and inoculated into 1.2 ml Corning cryo vials containing 0.5 ml of skim milk. The vials were stored at -70°C.

2.2 Antimicrobial Compounds

The antimicrobial agents used for *in vitro* experiments were obtained from their respective manufacturers or purchased commercially and used in accordance with the

manufacturers' instructions. Sources of antimicrobials are listed in Appendix D.

2.3 Susceptibility Testing

2.3.1 Broth Micro-dilution

Broth micro-dilution is the reference method used by diagnostic laboratories to determine MIC values. In our experiments, MIC values were determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommended procedure. Ninety-six well flat bottom microtiter panels were filled with 100 µl of Mueller-Hinton Broth (MHB) (Becton Dickinson and Co, Sparks, MD) in each well of columns 2 to 12. The antimicrobial agent was then serially diluted across the panel with column 1 containing the highest concentration of the drug and column 12 receiving no drug (growth control). Isolates were sub-cultured onto blood agar plates and incubated overnight in ambient air (O_2) at 35-37°C. Each isolate was transferred to MHB and the concentration standardized to a 0.5 McFarland (~1.0 X 10^8 CFU/ml) standard using a colorimeter. The bacterial suspension was then diluted with MHB to reach approximately 1.0X 10^6 CFU/ml. One hundred µl of diluted cells were added to each well in the panel achieving a final bacterial density of ~1.0 x10^5 CFU/ml in a total volume of 200 µl. Purity of the bacterial suspensions was confirmed by plating each sample onto blood agar plates. Purity plates and inoculated panels were then incubated in ambient air at 35-37°C for 16-20 hr. The ATCC strain *S. aureus* 29213 was used as a control to confirm the accuracy of each MIC test. The MIC value was recorded as the lowest drug concentration at which there was no visible growth of organism. Susceptible, intermediate, or resistant phenotypes were evaluated based on CLSI breakpoints [Clinical and Laboratory Standards Institute, 2015].

2.3.2 Mutant Prevention Concentration (MPC)

MPC values for *S. aureus* were determined by a protocol (Metzler *et al.*, 2004) developed in Dr. J. Blondeau's laboratory at Royal University Hospital, Saskatoon, SK. This protocol was established based on modification to the protocol for *S. pneumoniae* [Blondeau, et al, 2001].

2.3.3 Inoculum

Each *S. aureus* isolate was streaked from thawed skim milk onto a blood agar plate (Tryptic Soy Agar [TSA - Fisher Scientific, Toronto, ON] with 5% Sheep red blood cells [Oxoid, Nepean, ON]) and incubated in ambient air at 35-37°C for 18 -24hours. Bacteria were then collected from each plate with a sterile swab and sub-cultured onto two blood agar plates. One plate was entirely covered with the bacteria and the second one was streaked for isolated colonies. All plates were then incubated in ambient air at 35-37°C for 24 hours. Sterile swabs were used to transfer the content of the plates to sterile glass bottles containing 100 ml of MHB and incubated overnight as described. A spectrophotometric absorbance measurement (at 600 nm) was done for each sample to ensure a reading of ≥0.3 which corresponds to a bacterial density of $\geq 10^9$ CFU/ml (previously determined in Dr. Blondeau's lab). From this 100 µl were taken and streaked onto blood agar plates containing various concentrations (in doubling dilutions) of antimicrobial agent. The range of drug concentrations tested included the MIC value, one doubling dilution below the MIC and five doubling dilutions above the measured MIC value. Inoculated plates were incubated for 48 hours in ambient air at 35-37°C and screened for growth at both 24 hours and 48 hours. The MPC was recorded as the lowest drug concentration that had no visible bacterial growth following 48 hours of incubation.

2.3.4 Antimicrobial plates

The range of drug concentrations used varied depending on the behavior of the organism, i.e. MPC drug concentration range was lowered or extended based on what drug concentrations the organism grew at. Antimicrobial agents were prepared as described in Appendix B. TSA was prepared according to the manufacturer's instructions as described in Appendix A. Addition of 5% sheep blood cells to the TSA was necessary for proper growth of the isolates. The agar was kept at 55°C in a water bath (Polyscience, Burlington, ON) until it was ready to pour into sterile petri plates (Fisher Scientific, Toronto, ON). The following formula was used to determine the amount of antimicrobial agent to be added.

$C_1V_1=C_2V_2$

C_1= stock concentration of antimicrobial agent

V_1= volume of antimicrobial agent to be added in order to obtain the desired drug concentration

C_2= desired concentration

V_2= total volume

Approximately 20 ml of agar containing the drug was poured into each sterile petri plate and each plate.

2.3.5 Viable Counts

For each measurement of MPC, viable counts were performed. The initial bacterial suspensions were diluted to 10^7, 10^8 and 10^9; 100µl of each dilution were then plated in triplicate on blood agar plates. The plates were incubated in ambient air for 24 hr at 35-37°C. Colony counts were then performed on each plate. Finally, colony forming units per ml was calculated by multiplying the average of three plates count by the reciprocal of the dilution factor and then multiplying by 10.

2.3.6 Time - Kill Experiments

The method used for kill studies was performed in accordance with the kill protocol published for *Staphylococcus pseudintermedius* [Blondeau & Shebelski, 2016]. Bacterial isolates were grown overnight on blood agar plates. The inoculum from one-half plate was transferred to 9ml MHB and incubated in ambient air for 2 hours at 35-37^{0}C. Spectrophotometric readings of ≥0.3 at 600 nm were recorded to verify a cell density of ≥10^9 cells/ml. To achieve lower cell densities (10^5-10^8 CFU/ml), further dilutions in MHB were performed and then the antimicrobial agent was added. The drug concentrations used were based on the measured MIC and MPC for each antimicrobial agent in addition to the published maximum serum and tissue drug concentrations. Measurement of kill ($\log_{10}$ reduction in viable cells and percentage of organism killed) was recorded at 0, 0.5, 1, 2, 3, 6, 12 and 24 hours after drug exposure by culturing 100 µl aliquots of each dilution to drug-free blood agar plates in triplicate. Plates were incubated overnight at 35-37°C in ambient air. The number of colonies from three plates at each time interval was averaged; the $\log_{10}$ reduction and percent killing of viable cells were calculated and recorded.

2.4 Characterization of Bacterial Isolates

2.4.1 Polymerase Chain Reaction

2.4.1.1 Primer preparation

Primers used in this study were supplied by Integrated DNA Technologies, Inc. (Coralville, Iowa) and are listed in Table 2.4.1.1.

Table 2.4.1.1: Various primers used for PCR.

Gene	Primer	Sequence (5' – 3')	Amplicon size (bp)	Reference
agrI	Forward	ATGCACATGGTGCACATGC	441	[Cazares-Dominguez, *et al*, 2015]
	Reverse	GCTACAAGTACTATAAGCTGCGAT		
agrII	Forward	ATGCACATGGTGCACATGC	575	
	Reverse	TATTACTAATTGAAAAGTGGCCATAGC		
agrIII	Forward	ATGCACATGGTGCACATGC	323	
	Reverse	GTAATGTAATAGCTTGTAAAAAGTGGCCATAGC		
agrIV	Forward	ATGCACATGGTGCACATGC	659	
	Reverse	CGATAATGCCGTAATACCCG		
*SCCmec*I	Forward	GCTTTAAAGAGTGTCGTTACAGG	613	
	Reverse	GTTCTCTCATAGTATGACGTCC		
*SCCmec*II	Forward	CGTTGAAGATGATGAAGCG	398	
	Reverse	CGAAATCAATGGTTAATGGACC		
*SCCmec*III	Forward	CCATATTGTGTACGATGCG	280	
	Reverse	CCTTAGTTGTCGTAACAGATCG		
SCCmec IV	Forward	GCCTTATTCGAAGAAACCG	776	
	Reverse	CTACTCTTCTGAAAAGCGTCG		
SCCmec V	Forward	GAACATTGTTACTTAAATGAGCG	325	
	Reverse	TGAAAGTTGTACCCTTGACACC		
MecA	Forward	AAAATCGATGGTAAAGGTTGGC	533	[Maina, *et al*, 2013]
	Reverse	AGTTCTGCAGTACCGGATTTGC		
PVL	Forward	ATCATTAGGTAAAATGTCTGGACATGATCCA	432	
	Reverse	GCATCAASTGTGTTGGATAGCAAAAGC		
ClpP	Forward	GGATCCGACATTGCGGGATTCTCT	1300	[Springer, *et al*, 2016]
	Reverse	AAGCTTACCAAGTCTTGCAATGCGTC		

Each primer was dissolved at a concentration of 100 µM by adding the appropriate volume of TE buffer (10mM *Tris*: 0.1mM EDTA; pH 8.0) to generate the primer stock solution. This solution was then diluted to 20 µM with TE buffer to generate a working primer solution. Equal volumes of the upstream and downstream primer were added to a single tube and stored at -20^{0}C.

2.4.1.2 **Polymerase Chain Reaction** (PCR)

Bacterial isolates were plated on blood agar plates and incubated overnight in ambient air at 35-37°C for 18-24 hours. From each plate, a visible loop of bacteria was added to 100µl of InstaGene™ Matrix (Bio-Rad, Hercules, CA) in a 1.5 ml Eppendorf tube (Fisher Scientific, Toronto, ON). Specimens were then resuspended by vortexing and boiled for 10 min. After 10 min, tubes were vortexed again and centrifuged for 30 seconds at high speed. Three µl of the upstream plus downstream primer solution was added to each tube of PuReTaq Ready-To-Go PCR Beads (Fisher Scientific, Toronto, ON), followed by adding 19.5µl of sterile distilled water. Finally, 2.5µl of supernatant from each specimen was then added to the appropriate PCR reaction tubes resulting in a final volume of 25µl. PCR reaction tubes were then vortexed and transferred immediately to a thermal cycler (Applied Biosystem 2720). The thermal cycling program shown in Table 2.4.1.3 was then begun. Upon completion of the cycling program, reaction products were visualized by agarose gel electrophoresis. A 1% agarose gel was prepared as described in Appendix A. Five µl of tracking dye (Fisher Scientific, Toronto, ON) was added to each reaction tube and a sample volume of 20µl was loaded into a designated lane of the gel. Six µl of 100 base pair DNA ladder (Fisher Scientific, Toronto, ON) was loaded in the first lane of each gel as a molecular weight marker. Electrophoresis was at 117 volts for 20 minutes. Separated PCR products were then visualized under UV light and was captured by a gel documentation system (Syngene GeneGenius, UK) [Hookey, *et al*, 1998].

2.4.1.3 Thermocycler Setting

Settings for the thermocycler varied, depending on the primers that were used. The following table summarizes the thermocycler settings that were used for each primer set.

Table 2.4.1.3: Thermocycler settings.

Gene	Initial Denaturation		Denaturation		Annealing		Elongation		Number of cycles	Final Elongation	
	Temp	**Time (min)**	**Temp**	**Time (sec)**	**Temp**	**Time (sec)**	**Temp**	**Time (sec)**		**Temp**	**Time (min)**
MecA* & *PVL	95°C	5	94	35	57	35	72°C	35	37	72°C	7
agr* & *SCCmec	94°C	5	94	35	51	35	72°C	40	38	72°C	7
ClpP	95°C	5	94	40	55	40	72°C	40	38	72°C	7

2.5 Pulsed field gel electrophoresis (PFGE)

2.5.1 Preparation of samples

A few (3-5) isolated colonies were picked from a pure culture of MRSA streaked on blood agar plates and inoculated into 3 ml MHB. Samples were then placed in a shaking water bath at 37°C for 16-18hr. Each sample was then centrifuged at 14,000rpm for 1 min. Each pellet was re-suspended in 150µl of cell suspension buffer (CSB) described in Appendix A [Prevost*, et al*, 1992]. PFGE conditions are described in Appendix B.

2.5.2 Casting Plug

Preparation of a 2.0% low melting point agarose was in CSB was used for the casting plugs (Appendix A). Two µl of lysostaphin at 1mg/ml was added to each tube followed by the immediate addition of 150µl of 2% LMP agarose and mixed well with a pipetter. The mixture was then transferred to the casting mold. The casting plugs were left to solidify for 15 min at room temperature and another 15 min at 4^0C.

2.5.3 Lysis of Cells in LMP Agarose

Lysis Buffer (Appendix A) was prepared fresh and 900 µl was added to a clean tube for each sample. The plugs were gently transferred from the mold to their corresponding labeled tubes containing lysis buffer. Tubes were then incubated in a water bath at 37°C for 1 hr. The lysis buffer was then aspirated off with a 1 ml pipette (VWR, Mississauga, ON). Then, 900 ml of proteinase K/PK buffer solution (Appendix A) was added to each tube containing the plugs. The tubes were then incubated in a shaking water bath at 50°C for 24hr.

2.5.4 Washing LMP Plugs

Tubes were removed from the water bath and the proteinase K solution was aspirated off. The plugs were rinsed once with 1.4 ml of wash buffer (Appendix A). Three additional 30 minute washes with 1 ml of fresh wash buffer (each time) was performed. The wash buffer was removed after the final wash and 1 ml of fresh wash buffer was added for long-term storage of the plugs at 4°C.

2.5.5 Restriction Enzyme Digestion of LMP Plugs

Plugs were removed from the microfuge tubes and placed on sterile Petri dishes (Fisher Scientific, Toronto, ON). One-third of each plug was cut and placed into a new 1.5 ml microfuge tube. Three hundred µl of 1 X buffer A were added to each tube containing plugs

and incubated at room temperature (20°C-25°C) for 10 min. *Sma I* enzyme (New England BioLabs Inc., Whitby, ON) was used to restrict DNA in the plugs. A total of 25 Units were used per sample. The volume required was calculated as follows:

$$\frac{\text{Total enzyme required (Units}}{\textit{SmaI}\text{ concentration (Units/ul}} = \text{Volume required}$$

Buffer A was removed from the plugs and 150µl of buffer A/*Sam I* mixture were added to each tube and incubated overnight at 25C.

2.5.6 Casting Agarose Gel

The samples were run on 1% pulsed field agarose gels prepared as described in Appendix A. Two liters of 0.5X Tris-Borate-EDTA Buffer (TBE) was added to the Counter Clamped Homogenous Electric Field (CHEF) chamber. The cooling mold was set at 14°C and the pump was turned on to allow the buffer to cool. The enzyme/buffer solution was aspirated from each sample and the plugs were melted in a water bath at 70°C for 10min. A lambda DNA ladder (New England BioLabs Inc.) was run in at least one lane as a marker to help determine molecular weight sizes of the resulting bands. Forty-five µl of each sample (melted plug) were loaded into designated lanes. The gel was then placed into the electrophoresis chamber.

2.5.7 Staining the Gel

After 18 hours of electrophoresis, the gel was placed into a plastic container of ethidium bromide (ETBr) (Appendix A). The gel was stained at room temperature for 30 min on a slowly rocking surface while prohibiting its exposure to light. The ETBr solution was then drained and de-staining was performed by adding 500 ml of distilled water to the container and placing it back on the rocking surface at low speed for another 30 min. Finally, the gel was examined and photographed after ultraviolet light illumination (Gel Doc 1000).

2.6 Electron Microscopy (EM)

MRSA isolates for EM were incubated under the selective pressure of the antimicrobial agent (vancomycin) at 32 µg/ml on agar plates and serially passaged three times on MHB agar plates containing 32 µg/ml of vancomycin. Colonies were collected and suspended in 300 µl saline and centrifuged at high speed for 1 min. Saline was then

decanted and 300 µl of 3% glutaraldehyde in 1.1M NaCAC (sodium cacodylate buffer) was added immediately to each eppendorf tube as a fixative. Samples were then sent to the Western College of Veterinary Medicine (WCVM) Imaging Center at the University of Saskatchewan to be processed. Briefly, after three hours in fixation, pellets were rinsed. Fixed cells were pelleted and the fixative was aspirated; the pellet was then mixed with warm 1% agarose and the cells re-pelleted. The centrifuge tubes were placed in the refrigerator at 4° C to allow the agar to firm up. The agar was then removed from the centrifuge tubes and the pelleted cells cut away from the excess agar. The pellet was cut into several pieces and placed in wash buffer (0.1 M sodium cacodylate buffer pH 7.2) and stored at 4° C overnight. Various fields of view containing 3-4 cells were photographed at 50,000X. Measurements were taken for multiple intact cells walls. For measurements of cell wall thickness, 3 random locations around the bacterial cell were selected and then applied to each subsequent measurement and results averaged.

3.0 RESULTS

3.1 Description of the MRSA and MSSA blood culture Isolates

A total of 61 MRSA blood culture isolates collected from 2011 to 2012 were used in this study. All isolates were collected from specimens submitted to the clinical microbiology laboratory, Royal University Hospital, Saskatoon, Saskatchewan, Canada. All isolates were confirmed as *S. aureus* by Vitek II (BioMerieux, St. Laurent, QC) by the clinical microbiology service.

Table 3.1.1 summarizes characteristics of 37 selected patients with blood cultures positive for MRSA. For the *S. aureus* isolates from these 21 patients, vancomycin MPC values ranged from 2-≥32 µg/ml with 14/21 (66.7%) having MPC values ≥8 µg/ml. A total of 19/21 (90%) patients were being treated with vancomycin at or around the time of blood culture collections or following the report of MRSA bacteremia. The MRSA isolates from patients with a history of vancomycin use had vancomycin MPC values of ≥8 µg/ml (1 isolate had an MPC value of 4 µg/ml).

Table 3.1.1: Characteristics of patients with MRSA bacteremia.

Patient	Age	Vanco MPC	Acute Diagnosis	Past Medical History	Abx History*	Current Abx + Dose
1	35	≥32	MRSA Endocarditis (TV) Septic emboli (lung, kidney	HIV (CD4 10) on ARVs (2010) IVDU	Azithromycin (2010) Fluconazole (2010) Bactrim (2010)	Clindamycin 300mg IV q8h x 1d Vancomycin 1-1.5g IV q8-12h x 60d Azithromycin 1250mg po weekly x 60d Septra I po BD x 9d Fluconazole 100mg po OD x 1d Gentamicin 60mg IV q8h x 5d PipTaz 3.375g IV q6h x 7d
2	20	8	MRSA Bacteremia Abscess right thigh/buttock	Type 1 DM Frequent DKA (2006, 2008, 2009, 2010x2)	No documented/available	Vancomycin 1-1.5g IV q8-12h x 13d
3	27	8	MRSA Endocarditis (TV) Septic PE	HIV (2009) HCV (2009) IVDU MRSA + (2009) Pneumonia (2011) Multiple dental abscesses (2008-2010) Breast abscess (2009)	Clindamycin (2009) Azithromycin (2010) Amoxil (2009, 2010) SMX-TMP (2010, 2011) ARVs (2010, 2011)	PipTaz 3.375g IV q12h x 2d Vancomycin 1g IV q12h x 2d Septra ii po OD x 6d Cefotaxime 2g IV STAT Zithromax 500mg po STAT
4	48	≥32	Hemodialysis	CRF secondary to post-infectious GN HPT EtOH, cannibas, IVDU Asthma/COPD	Moxifloxacin (2008)	Nil on record
5	27	8	MRSA Endocarditis (TV) Septic PE	HIV (2009) HCV (2009) IVDU MRSA + (2009) Pneumonia (2011) Multiple dental abscesses (2008-2010) Breast abscess (2009)	Clindamycin (2009) Azithromycin (2010, 2011) Amoxil (2009, 2010) SMX-TMP (2010, 2011) ARVs (2010, 2011) PipTaz (2011) Vancomycin (2011) Cefotaxime (2011)	Vancomycin 750mg-1g IV q12h x 35d
6	34	4	MRSA Endocarditis (TV) Septic emboli Persistent ankle wound	HIV (not on Rx) HCV IVDU Osteomyelitis R ankle (2010) Pneumonia (2011) Ankle injury (2010) Assault with brain injury (2007)	Polysporin (2010) Ciprofloxacin (2010) Bactrin (2010,2011) Clindamycin (2010) Levofloxacin (2011)	Vancomycin 1.5-2g IV q12-8h x 60d CTX 2g IV q12h x 2d Rifampin 600mg po OD x 14d
7	34	2	Endocarditis (TV) with septic emboli Persistent ankle wound	HIV (not on Rx) HCV	Polysporin (2010) Ciprofloxacin (2010)	Vancomycin 1.5-2g IV q12-8h x 60d CTX 2g IV q12h x 2d

				IVDU Osteomyelitis R ankle (2010) Pneumonia (2011) Ankle injury (2010) Assault with brain injury (2007)	Bactrin (2010,2011) Clindamycin (2010) Levofloxacin (2011)	Rifampin 600mg po OD x 14d
8	34	16	Hemorrhagic R frontal CVA	HCV (not on Rx) Decompensated cirrhotic liver dx MRSA+ (2010) End stage CRF on dialysis (FSGS) IVDU, EtOH Endocarditis Osteomyelitis, epidural abscess Cardiac tamponade (2010)	Vancomycin (2011) with dialysis PipTaz (2011)	Vancomycin 500mg IV 3x/week x 14d CTX 1g IV q24h x 1d
9	65	4	Necrotising pneumonia	Rheumatoid arthritis COPD Brain tumour (benign) Appendicectomy Laproscopic Cholecystectomy UGITB (hematemesis)	Erythromycin (2011) Amoxil (2011)	Vancomycin 1.25g IV q24-36h x 13d PipTaz 3.375g IV q6h x 7d Meropenem 1g IV q12h x 6d Erythromycin 250mg IV q6h x 2d
10	52	≥16	MRSA Bacteremia secondary to line infection	Seizure disorder with frontal lobectomy Multiple strokes (most recent 2011) CAD & MI (2007, 2010) PVD with amputation L toe DM (insulin requiring) HPT ESRD on HD MRSA+	Cotrimox (2011) Nitrofurantoin (2011) Clotrimazole cream 1% (2011)	Cefotaxime 2g IV STAT CTX 2g IV q24h x 2d Vancomycin 500mg - 1.5g IV q72h x 13d
11	14	16	Osteomyelitis L wrist post-fracture	Dental procedure @ 4y Born full term, no complications	Nil known	Cefazolin 2g IV q8h x 2d Vancomycin 1g IV q12h x 4d Clindamycin 600mg IV q8h x 16d
12		8	Metastatic uterine sarcoma Febrile Neutropenia Thrombocytopenia R arm DVT (PICC line) with thrombophlebitis	CML (2008)	Amoxil (2011 x 2) Amoxiclav (2011) Flagyl (2011) Moxifloxacin drops 0.5% (2011) Bactroban (2011)	Vancomycin 1.25g IV q16-24h x 24d PipTaz 3.375 - 4.5g IV q6h x 1d Bactroban 2% ointment x 10d
13	46	8	Osteomyelitis Diabetic foot Sepsis	IVDU HCV DM with chronic foot ulcers	Multiple abx regimens previously Cephalex (2011) Amoxiclav (2011)	PipTaz 3.375g IV q6h x 2d, missed 2d, then x 24d Cloxacillin 2g IV q4h x 1d Vancomycin 1-1.5g IV q8-12h x 28d Moxifloxacin 400mg po OD x 2d

15	41	8	Hepatorenal syndrome Intracranial hemorrhage CHF with pneumonia & sepsis UGITB	Autoimmune hepatitis with cirrhosis Oesophageal varices CRF	Amoxiclav (2011)	PipTaz 2.25-3.375g IV q6-8h x 5d Flagyl 500mg IV q8h x 2d Vancomycin 750mg - 1.5g IV q12-36h x 12d Linezolid 600mg IV q12h x 2d
16	41	8	Hepatorenal syndrome Intracranial hemorrhage CHF with pneumonia & sepsis UGITB	Autoimmune hepatitis with cirrhosis Oesophageal varices CRF	Amoxiclav (2011)	PipTaz 2.25-3.375g IV q6-8h x 5d Flagyl 500mg IV q8h x 2d Vancomycin 750mg - 1.5g IV q12-36h x 12d Linezolid 600mg IV q12h x 2d
17	41	8	Hepatorenal syndrome Intracranial hemorrhage CHF with pneumonia & sepsis UGITB	Autoimmune hepatitis with cirrhosis Oesophageal varices CRF	Amoxiclav (2011)	PipTaz 2.25-3.375g IV q6-8h x 5d Flagyl 500mg IV q8h x 2d Vancomycin 750mg - 1.5g IV q12-36h x 12d Linezolid 600mg IV q12h x 2d
18	57	8	MRSA Bacteremia Staph pneumonia	Lumbar spinal stenosis Previous facial # with plates in-situ	Clarithromycin (2011) Amoxil (2011)	Ciprofloxacin 400mg IV STAT Cloxacillin 2g IV q6h x 3d Cefuroxime 500mg IV po q12h x 2d Azithromycin 250mg po q24h x 2d Clindamycin 600mg IV q8h x 3d Vancomycin 1.75g IV q8-12h x 10d
19	41	8	Hepatorenal syndrome Intracranial hemorrhage CHF with pneumonia & sepsis UGITB	Autoimmune hepatitis with cirrhosis Oesophageal varices CRF	Amoxiclav (2011)	PipTaz 2.25-3.375g IV q6-8h x 5d Flagyl 500mg IV q8h x 2d Vancomycin 750mg - 1.5g IV q12-36h x 12d Linezolid 600mg IV q12h x 2d
20	27	4	MRSA Endocarditis	HIV (2008) - CD4 140, no Rx HCV IVDU MRSA+ Endocarditis (2010) TB Multiple Admissions with D/C AMA	Vancomycin IV (2010) x 9d, left AMA Clindamycin po (2010) Cephalexin (2011) Cotrimox (2011) Metronidazole (2011) Fucidin 2% cream (2011) Doxycycline po (2011) x 7d	Cefotaxime 2g IV x 1 dose Zithromax 500mg IV x 1 dose
21	26	4	Kidney transplant failure	Neurogenic bladder Intellectual delay Kidney transplant (1986) Polycystic kidney disease (congenital) Birth Injury Epilepsy HTN Depression	Miconazole cream 2% (2010) Ciprofloxacin (2010, 2011) Gentamicin (2011) Amoxil (2011) Doxycycline (2011) Nystatin (2011) Fluconazole (2011)	Gentamicin (bladder) OD x 150d Bactroban 2% BD x 17d Vancomycin 500mg IV q72h x 40d Tobramycin 40mg IV q72h x 2d Cefazolin 2g IV q72h STAT Nystatin 500,000 IU po qid x 14d

23	37	8	LRTI Cellulitis thigh	IVDU HCV (2009) HIV (2009) Smoker Recurrent bronchitis	Cefazolin IV (2009) Valacyclovir (2009) Clarithromycin IV (2009) Amoxicillin (2010) Nitrofurantoin (2010, 2011) Moxifloxacin (2011) Cephalex (2011)	Clindamycin 400mg po qid x undocumented duration
24	59	8	Diabetic foot Sepsis	DM HTN GERD CRF on peritoneal dialysis MRSA+ Severe CHF	Tobramycin (2011) Cefazolin (2011) Cotrimox (2011) Bactroban cream (2011) Vancomycin IV (2011, q5/7)	PipTaz 2.23g IV q6-8h x 40d Vancomycin 1.5-2g IV q96h x 34d
26	35	8	Cellulitis (L) arm MRSA Bacteremia	IVDU - methadone program HCV HTN Smoker (20PYH)	Not available	Vancomycin 1-1.75g IV q8h x 8d Ancef 2g IV STAT
27	37	8	Sepsis L Acromioclavicular jnt septic arthritis	IVDU HCV HIV Asthma	Amoxil (2011)	CTX 1g IV q12h x 1d Vanco 1-2g IV q8-12h x 10d Linezolid 600mg po BD x 19d
28	37	16	Sepsis L Acromioclavicular jnt septic arthritis	IVDU HCV HIV Asthma	Amoxil (2011)	CTX 1g IV q12h x 10d Vanco 1-2g IV q8-12h x 10d Linezolid 600mg po BD x 19d
29	37	16	Sepsis L Acromioclavicular jnt septic arthritis	IVDU HCV HIV Asthma	Amoxil (2011)	CTX 1g IV q12h x 10d Vanco 1-2g IV q8-12h x 10d Linezolid 600mg po BD x 19d
30	22	4	General malaise Treated as an out pnt	IVDU - on methadone program HCV (2009) HIV (2009) Known MRSA (2009) EtOH Endocarditis (2010) Septic arthritis (2010)	Cotrimox (2009) Clindamycin (2010 x 2) Vancomycin (2010) Cefotaxime (2010) CTX (2010) Amoxil (2010, 2011)	D/Chome with no Abx
41	35	4	Sepsis	HIV IE (TV) (2011) Hemolytic anemia (drug induced) Neutropenia (drug induced)	Fluconazole (2011) x 10d Vancomycin (2011) x 6w PipTaz (2011) STAT CTX (2011) x 10d	Fluconazole 100mg po OD x 2d CTX 1g IV q12h x 3d Vancomycin 500mg IV q12-16h x 9d, missed 17d, then x 14d PipTaz 3.375g IV q6h x 21d, missed 4d, then x 5d

				MRSA cellulitis R finger CMV colitis (Nov 2011)	Azithromycin (2011) x 2m Meropenem (2011) x 19d Keflex (2011) x 2w Gancyclovir (2011) Valgancyclovir (2011) Dapsone (2011) Caspofungin (2011) Pentamidine (2011) Foscarnet (2011)	Clarithromycin 500mg po BD x 10d Ethambutol 600mg po OD x 10d Linezolid 600mg IV q12h x 15d Azithromycin 1250mg weekly x 180d Nystatin 500,000 IU po qid x 2d, missed 37d, then x9d Caspofungin 50mg IV OD x 22d Foscarnet 1800mg IV q8h 23d Valganciclovir 900mg po OD x 13d Acyclovir 150mg IV q8h x 7d
43	30	4	Infective Endocarditis (TV) Newly dx HIV (CD4 34) Opioid dependence Anaemia	IVDU, EtOH HCV (2004) G6P3M1 GBS+ Pancreatitis (2005)	Ampicillin IV (2007) x 3d Erythromicin IV (2007) x 3d Cefazolin IV (2007,2008) STAT (C/S, NVD) Moxifloxacin IV (2011) x 9d	Vancomycin 1.25-1.5g IV q8-16h x 42d CTX 2g IV q12-24h x 16d Metronidazole 500mg IV q8h x 2d SMX-TMP 400/800 ii po 3x/week x 90d Azithromycin 1250mg po weekly x 90d
44	33	8	Spetic L knee	HIV on ARVs (2010) HCV (2010) Known MRSA IVDU (quit 2009) Spetic arthritis L knee (2008 - Strep) Pneumonia (2010)	Penicillin G IV (2008) x 1m Vancomycin IV (2008) x 1d Tazocin IV (2008) x 1d Azithromycin (2010) Px Cefotaxime IV (2010) x 2w Septra (2010) Px Vancomycin IV (2010) x 10d	Vancomycin 1g IV q8-12h x 7d Moxifloxacin 400mg IV/po q24h x 4d Septra I po OD x 5d
49	86	32	Sepsis UTI Aspiration Malnutrition	AF Left ACA Stroke (1994, 2009, 2010) Orbital lymphoma (2012) Type 2 DM HPT OSA ITP (refractory) Bilateral TKR Prostate surgery R leg DVT (2011) Bilateral Cataracts Kidney stones	Ciprofloxacin (16-Feb-2012) Erythromycin eye ointment 0.5% (2010) Ciprofloxacin (2010) Flagyl (2011) Cefotaxime (2011)	Cefotaxime 2g IV q8h x 6d Ciprofloxacin 400mg IV q12h x 1d Azithromycin 250mg po q24h x 5d Clindamycin IV q8h x 6d Tobradex drops x 13d Clotrimazole cream 1% x 5d Ciprofloxacin 400mg IV q12h x 2d PipTaz 3.375g IV q6h x 2d Vancomycin 1.5gIV q16h x 2d
51	33	4	Open perineal wounds post APR Pyoderma gangrenosum	Crohn's disease (18y) 2 x colostomies, APR Recurrent perianal abscesses (I&D) Psoriasis Endocarcitis (TV) (2008)	Significant # courses Metronidazole (last Jan 2012) Cipro (last Jan 2012) (Course duration & # no documented)	Ciprofloxacin 500mg po BD x 8d Metronidazole 500mg po BD x 8d Ciprofloxacin 400mg IV q12-24h x 20d Metronidazole 500mg IV q8h x 70d Ciprofloxacin 500mg po BD x60d, missed 4d, then x9d

				MRSA+ (2007) IVDU		Vancomycin 1.25g IV q12h x 10d
52	33	8	Open perineal wounds post APR Pyoderma gangrenosum	Crohn's disease (18y) 2 x colostomies, APR Recurrent perianal abscesses (I&D) Psoriasis Endocarcitis (TV) (2008) MRSA+ (2007) IVDU	Significant # courses (2006, 2008, 2011) Metronidazole (last Jan 2012) Cipro (last Jan 2012) (Course duration & # no documented) Nystatin (2006) Vancomycin + PipTaz (2007, 2008, 2011) Ketoconazole cream (2010) Ancef (2010)	Ciprofloxacin 500mg po BD x 8d Metronidazole 500mg po BD x 8d Ciprofloxacin 400mg IV q12-24h x 20d Metronidazole 500mg IV q8h x 70d Ciprofloxacin 500mg po BD x60d, missed 4d, then x9d Vancomycin 1.25g IV q12h x 10d
57	34	16	T/F to RUH	HCV IVDU Known MRSA (2012) Bronchopneumonia (2011) Cellulitis /L hand L clavicular # (2010) Neutropenia (drug use) Asthma Antiphospholipid syndrome	Bactrin (2010) Clindamycin (2010)	Cefotaxime 2g IV STAT
58	64	32	Post op seroma/hematoma with facial swelling	Pituitary tumour (transphenoidal resection, 2012) MI with stents x 5 HTN Pancreatitis Type 2 DM CVA Degenerative disc disease, lumbar	Cefazolin IV (2012) STAT Pnt from BC - no further hx available	CTX 1g IV q8h STAT
59	47	4	Osteomyelitis with septic emboli (lung, psoas)	Assault (2012) Osteoporosis + Scoliosis Smoker EtOH HCV IVDU (stopped 2007) Previous CVA	Macrobid (2010) x 1w	Vancomycin 1.25-1,75g IV q8-24h x 35d PipTaz 3.375g IV q6h x 4d Rifampin 600mg po OD x 30d
60	40	2	Septic arthritis L shoulder Endocarditis (TV)	HIV (2009) HBV, HCV (2009)	Doxycycline (2012) Fucidin ointment (2012)	Vancomycin 1.25-1.5g IV q8-12h x 21d CTX 2g IV q24h x 2d

				IVDU Smoker 20PYH Known MRSA	Moxifloxacin (2012) x 10d	Septra ii po TDS x 21d ARVs (since 2011)

3.1.1 *In vitro* Susceptibility Measurements of MSSA Blood Isolates

MPC values for tedizolid, linezolid and vancomycin had not previously been published for MSSA isolates. A total of 20 MSSA clinical isolates were tested for susceptibility to vancomycin, linezolid and tedizolid. Table 3.1.1.1 shows the MIC and corresponding MPC values for each isolate. For vancomycin, MIC values ranged from 0.25-1μg/ml as compared to 0.5 μg/ml for tedizolid and 1-2 μg/ml for linezolid. MPC values were similar to MIC values at 0.5 μg/ml for tedizolid. For linezolid, MPCs were similar to MIC values or 1-2 fold higher (2-4 μg/ml). For vancomycin, however, MPC values were 3-5 fold higher than MIC values and ranged between 4-16 μg/ml with 16/20 (80%) having MPC values $\geq$8 μg/ml and 2/20 (10%) having MPC values of 16 μg/ml.

Table 3.1.1.1: MIC and MPC values for 20 MSSA strains against 3 antimicrobial agents.

Isolate #	Vancomycin		Tedizolid		Linezolid	
	MIC	MPC	MIC	MPC	MIC	MPC
1	1	8	0.5	0.5	2	4
2	0.5	4	0.5	0.5	1	2
3	0.5	4	0.5	0.5	2	4
4	0.5	8	0.5	0.5	1	4
5	0.5	8	0.5	0.5	1	4
6	0.5	8	0.5	0.5	1	2
7	0.5	8	0.5	0.5	1	4
8	0.5	8	0.5	0.5	1	2
9	1	8	0.5	0.5	1	4
10	0.5	8	0.5	0.5	1	2
98	0.5	4	0.5	0.5	2	2
93	0.25	8	0.5	0.5	2	2
80	0.5	16	0.5	0.5	2	2
72	0.5	8	0.5	0.5	2	2
92	0.5	16	0.5	0.5	2	2
83	0.5	8	0.5	0.5	2	2
70	0.5	8	0.5	0.5	1	4
65	0.5	4	0.5	0.5	2	2
63	0.5	8	0.5	0.5	2	4
45	0.25	8	0.5	0.5	2	2

The distribution of MIC and MPC values, their percentage and mutant prevention index are summarized in Table 3.1.1.2. MIC_{50} and MPC_{50} values represent the drug

concentrations inhibiting the growth of 50% of isolates tested by MIC and MPC testing, respectively. Similarly, MIC_{90} and MPC_{90} are the drug concentrations at which 90% of isolates were inhibited from growth. Such data are useful for comparing *in vitro* antimicrobial potency between drugs. MIC_{90} values were equal to MIC_{50} values for all three drugs against the MSSA strains tested. The MPC_{90} value was one fold higher than the MPC_{50} value for linezolid and equal to the MPC_{50} values for tedizolid and vancomycin. MPC/MIC ratios were higher for vancomycin at 1:16 compared to linezolid (1:2) and tedizolid (1:1). The lower ratio (selection/prevention index) is the better ability of the drug to curb the resistant mutant subpopulations growths.

Table 3.1.1.2: MIC and MPC distribution data for 20 MSSA strains tested against 3 antimicrobial agents.

MIC											**Mutant Prevention Index**	
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**MIC_{50}**	**MIC_{90}**	**MPC_{50}/ MIC_{50}**	**MPC_{90}/ MIC_{90}**
Linz				9 (45%)	11 (55%)				2	2	1	2
Vanco		2 (10%)	16 (80%)	2 (10%)					0.5	0.5	16	16
Ted			20						0.5	0.5	1	1
MPC												
									MPC_{50}	**MPC_{90}**		
Linz					12 (60%)	8 (40%)			2	4		
Vanco						4 (20%)	14 (70%)	2 (10%)	8	8		
Ted			20 (100%)						0.5	0.5		

The Clinical and Laboratory Standards Institute (CLSI) is a multidisciplinary committee of experts that set minimal standards and recommendations for diagnostic laboratories in a number of testing areas including antimicrobial susceptibility testing. The susceptibility breakpoints as recommended by CLSI for *Staphylococcus aureus* against the drugs we tested are summarized in Table 3.1.1.3. Based on CLSI recommended breakpoints, all 20 MSSA strains were susceptible to linezolid and tedizolid by both MIC and MPC testing. However, for vancomycin all strains were susceptible by MIC testing but only 25% were susceptible ($\leq$4

µg/ml) by MPC testing; 65% were non-susceptible (at 8 µg/ml) and 10% were resistant at 16-32µg/ml.

Table 3.1.1.3: Comparative susceptibility of 20 MSSA strains against 3 antibiotics.

Drug	Vancomycin	Tedizolid	Linezolid
Susceptibility Breakpoint	≤ 4	≤ 0.5	≤ 4
Susceptibility % by MIC	100%	100%	100%
Susceptibility % by MPC	20%	100%	100%

3.1.2 *In vitro* Susceptibility Measurements of MRSA Blood Isolates

Susceptibility testing was performed against a variety of antimicrobial agents to provide a comprehensive susceptibility/resistance profile of the MRSA isolates investigated. Table 3.1.2.1 summarizes individual MIC values for 60 blood culture MRSA strains tested against 13 anti-microbial agents. All isolates were susceptible to vancomycin, linezolid and tedizolid; the majority (90-95%) were susceptible to tigecycline, gentamicin and amikacin. Fewer isolates (40-70%) were susceptible to the other drugs tested including ciprofloxacin, moxifloxacin, azithromycin and trimethoprim/sulfamethoxazole. None of the tested isolates were susceptible to chloramphenicol.

Table 3.1.2.1: Individual MIC values for 60 MRSA strains against 13 antimicrobial agents*.

	Vanco	Tig	Linz	Genta	Tobra	Amik	Ted	Gati	Azith	Cipro	Moxi	Chloro	TMP/SMX
1	0.5	0.063	1	1	0.5	4	0.25	2	≥8	8	2	≥16	0.125/3.375
2	1	0.063	2	0.5	1	4	0.25	0.125	1	0.5	0.016	≥16	0.5/9.5
3	0.5	0.063	2	0.5	1	8	0.25	0.125	1	0.5	0.016	≥16	0.125/3.375
4	0.5	0.063	2	0.5	1	4	0.25	0.125	≥8	0.25	0.016	≥16	0.063/1.158
5	1	0.063	2	1	2	4	0.25	0.5	4	0.5	0.063	≥16	0.5/9.5
6	1	0.063	2	1	0.5	4	0.25	0.125	≥8	0.5	0.016	≥16	0.125/3.375
7	0.5	0.063	2	0.5	0.5	4	0.25	0.063	≥8	0.25	0.016	≥16	0.125/3.375
8	0.5	0.063	2	1	0.5	2	0.25	4	≥8	≥8	2	≥16	0.125/3.375
9	0.5	0.125	2	1	1	8	0.25	4	≥8	≥8	1	≥16	0.125/3.375
10	0.5	2	2	1	≥32	16	0.25	≥8	≥8	≥8	8	≥16	0.125/3.375
11	0.5	0.063	2	1	0.5	2	0.125	0.125	1	0.25	0.031	≥16	0.125/3.375
12	0.5	0.125	2	0.5	0.5	2	0.25	0.125	1	0.25	0.031	≥16	0.25/4.75
13	0.5	0.125	2	0.5	0.5	4	0.125	0.125	≥8	8	1	≥16	0.063/1.158
15	0.5	0.25	2	0.5	0.25	2	0.25	4	≥8	8	2	≥16	0.125/3.375
16	0.5	0.125	2	1	0.25	2	0.25	4	≥8	8	2	≥16	0.125/3.375
17	0.5	0.125	2	0.5	0.5	4	0.25	4	≥8	8	2	≥16	0.125/3.375

18	0.5	0.063	2	0.5	0.5	2	0.125	0.063	1	0.125	0.016	≥16	0.063/1.158
19	1	0.063	2	0.5	1	4	0.125	0.125	1	0.25	2	≥16	0.125/3.375
20	0.5	0.063	2	1	1	2	0.125	0.125	0.5	0.25	0.031	≥16	0.125/3.375
21	1	0.063	2	1	1	8	0.25	0.063	≥8	0.125	0.016	≥16	0.063/1.158
22	1	0.125	2	0.5	≥32	8	0.25	8	≥8	≥8	4	≥16	0.125/3.375
23	0.5	0.25	2	0.5	0.5	8	0.25	4	≥8	8	2	≥16	0.125/3.375
24	0.5	0.063	4	1	≥32	8	0.25	≥8	≥8	≥8	0.016?	≥16	0.125/3.375
25	0.5	0.063	2	0.5	≥32	4	0.25	8	≥8	≥8	4	≥16	0.5/9.5
26	0.5	0.063	2	32	8	4	0.125	0.125	≥8	0.25	0.063	≥16	1/19
27	0.5	0.125	2	1	2	4	0.25	0.125	1	0.25	0.016	≥16	0.125/3.375
28	0.5	0.125	2	2	2	4	0.25	1	> 8	0.5	0.016	≥16	0.125/3.375
29	0.5	0.125	2	1	2	4	0.125	0.125	1	0.5	0.016	≥16	0.25/4.75
30	0.5	0.125	2	1	0.5	2	0.25	0.125	1	0.25	0.016	≥16	0.25/4.75
31	1	0.125	2	0.25	0.5	4	0.125	2	≥8	4	1	≥16	0.063/1.158
32	0.5	0.25	2	1	0.5	8	0.125	0.125	1	0.5	0.031	≥16	0.125/3.375
33	0.5	0.125	2	0.25	0.5	2	0.125	0.063	≥8	0.25	0.031	≥16	0.125/3.375
34	0.5	0.063	4	0.5	≥32	8	0.25	1	≥8	≥8	4	≥16	0.063/1.158
35	0.5	0.063	2	0.25	4	4	0.125	4	≥8	8	1	≥16	0.063/1.158

36	0.5	0.063	2	0.5	0.5	4	0.125	4	≥8	8	1	≥16	0.125/3.375
37	0.5	0.063	2	0.5	≥32	16	0.25	8	≥8	≥8	4	≥16	0.25/4.75
38	0.5	0.125	2	0.5	1	8	0.125	2	≥8	8	1	≥16	0.063/1.153
39	0.5	0.063	2	0.25	0.5	2	0.25	0.125	≥8	0.25	0.016	≥16	0.125/3.375
40	0.5	0.125	2	0.5	≥32	8	0.5	≥8	≥8	> 8	≥4	≥16	0.125/3.375
41	0.5	0.25	2	32	16	4	0.25	0.081	≥8	0.25	0.008	≥16	0.125/3.375
42	0.5	0.125	2	0.5	1	4	0.5	0.125	2	0.5	0.031	≥16	0.5/9.5
43	0.5	0.125	2	0.5	0.5	2	0.25	8	0.5	≥8	2	≥16	0.125/3.375
44	0.5	0.125	2	1	0.5	4	0.5	0.125	1	0.25	0.031	≥16	0.25/4.75
45	1	1	2	0.5	≥32	16	0.5	≥8	≥8	≥8	4	≥16	0.25/4.75
46	0.5	0.125	2	0.25	0.5	4	0.25	0.125	1	0.25	0.016	≥16	0.125/3.375
47	1	0.125	2	0.5	0.25	4	0.5	2	≥8	4	0.016	≥16	0.125/3.375
48	0.5	0.125	2	0.5	1	8	0.25	2	≥8	4	2	≥16	0.125/3.375
49	0.5	1	2	1	≥32	16	0.5	8	≥8	≥8	4	≥16	0.125/3.375
50	0.5	0.063	1	0.5	32	4	0.25	2	≥8	4	2	≥16	0.125/3.375
51	1	0.5	4	1	≥32	16	0.5	8	8	≥8	4	≥16	0.125/3.375
52	0.5	2	2	2	≥32	16	0.5	≥8	8	≥8	4	≥16	0.125/3.375
53	0.5	0.063	2	1	1	8	0.25	2	≥8	8	1	≥16	0.125/3.375

54	0.5	0.125	2	4	>32	16	0.5	≥8	≥8	≥8	≥4	≥16	0.25/4.75
55	0.5	0.125	2	0.5	0.5	2	0.25	0.063	4	0.25	2	≥16	0.25/4.75
56	0.5	1	2	0.5	≥32	16	0.5	8	≥8	≥8	4	≥16	0.125/3.375
56-	0.5	1	2	0.5	≥32	16	0.25	8	≥8	≥8	4	≥16	0.125/3.375
57	0.5	0.125	2	1	1	2	0.25	2	0.5	8	1	≥16	0.125/3.375
58	0.5	0.125	2	1	1	2	0.25	0.031	1	0.125	0.063	≥16	0.125/3.375
59	1	0.125	2	1	1	2	0.5	4	≥8	8	4	≥16	0.125/3.375
60	1	0.125	2	2	1	4	0.25	0.125	1	0.25	1	≥16	0.25/4.75

* amik=amikacin; azith=azithromycin; chloro=chloramphenicol; Cipro=ciprofloxacin; gati=gatifloxacin; genta=gentamicin; linz=linezolid; moxi=moxifloxacin; ted=tedizolid; tig= tigecycline; TMP/SMX=trimethoprim/sulfamethoxazole; tobra=tobramycin; vanco=vancomycin.

MIC distribution data for investigated antimicrobial agents against MRSA are summarized in Table 3.1.2.2. MIC values for the tested drugs ranged as follows; vancomycin 0.5-1 µg/ml, tigecycline 0.63-2 µg/ml, linezolid 1-4 µg/ml, gentamicin 1-4 µg/ml, tobramycin 0.25-32 µg/ml, amikacin 2-16 µg/ml, tedizolid 0.125-0.25 µg/ml, gatifloxacin 0.63-8 µg/ml, azithromycin 1-8 µg/ml, ciprofloxacin 0.125-8 µg/ml, moxifloxacin 0.016-8 µg/ml, chloramphenicol >16 µg/ml and trimethoprim/sulfamethoxazole. The MIC_{90} value was one dilution higher than the MIC_{50} value for vancomycin (1 µg/ml versus 0.5 µg/ml), tedizolid 0.25 µg/ml versus 0.125 µg/ml), tigecycline (0.25 µg/ml versus 0.125 µg/ml), gentamicin (1 µg/ml versus 0.5 µg/ml) and ciprofloxacin (8 µg/ml versus 4 µg/ml), 2 dilutions higher for moxifloxacin 4 µg/ml versus 1 µg/ml), amikacin (16 µg/ml versus 4 µg/ml) and gatifloxacin (8 µg/ml versus 2 µg/ml) and 4 dilutions higher for tobramycin ($\geq$16 µg/ml versus 1 µg/ml).

Table 3.1.2.2: Comparative MIC distribution data for 13 antimicrobial agents*.

	MIC													
	0.016	**0.031**	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**≥4**	**≥8**	**≥16**	**≥32**	**MIC_{50}**	**MIC_{90}**
Linz							2 (3%)	55 (92%)	3 (5%)				2	2
Vanco						48 (80%)	12 (20%)						0.5	1
Ted				13 (22%)	36 (60%)	11 (18%)							0.125	0.25
Tig			23 (38%)	26 (43%)	4 (7%)	1 (2%)	4 (7%)	2 (3%)					0.125	0.25
Genta					5 (8%)	27 (45%)	22 (37%)	3 (5%)	1 (2%)			2 (3%)	0.5	1
Tobra					3 (5%)	20 (33%)	15 (25%)	4 (6%)	1 (2%)	1 (2%)	1 (2%)	15 (25%)	1	>16
Amik								15 (25%)	24 (40%)	12 (20%)	9 (15%)		4	16
Gati		1 (3%)	6 (10%)	19 (31%)			3 (5%)	8 (13%)	9 (15%)	14 (23%)			2	8
Azithro						2 (3%)	14 (23%)	2 (3%)	2 (3%)	40 (67%)			>8	>8
Cipro					17 (28%)	8 (13%)			6 (10%)	29 (48%)			4	8
Moxi	16 (26.5%)	7 (11.5%)	3 (5%)				9 (15%)	11 (18%)	13 (21%)	1 (3%)			1	4
Chloro											60 (100%)		>16	>16
	0.063/1.158		**0.125/3.375**		**0.05/4.75**		**0.5/9.5**		**1/19**		**MIC_{50}**		**MIC_{90}**	
TMP/SMX	8		38		9		4		1		0.125/3.375		0.25/4.75	

*amik=amikacin; azith=azithromycin; chloro=chloramphenicol; Cipro=ciprofloxacin; gati=gatifloxacin; genta=gentamicin; linz=linezolid; moxi=moxifloxacin; ted=tedizolid; tig= tigecycline; TMP/SMX=trimethoprim/sulfamethoxazole; tobra=tobramycin; vanco=vancomycin.

Table 3.1.2.3 summarizes individual MPC values for blood culture MRSA strains tested against 13 antimicrobial agents. The numbers of strains tested by MPC were lower than the number of strains tested by MIC for some antimicrobial agents summarized. MPC testing was only performed on strains tested as susceptible by MIC testing following CLSI susceptibility testing criteria and breakpoints. For example, MPC testing was applied to 44 MRSA strains against tobramycin, 27 strains for gatifloxacin, 23 strains for moxifloxacin, 27 stains for ciprofloxacin and 18 strains for azithromycin.

Table 3.1.2.3: Individual MPC values for 60 MRSA strains tested against 12 antimicrobial agents.

	Vanco	Tig	Linz	Genta	Tobra	Amik	Ted	Gati	Azithro	Cipro	Moxi	TMP/SMX
1	≥16	0.25	2	8	≥16	≥32	0.25	X*	X	X	X	≥32/609
2	8	0.25	2	≥16	8	≥32	0.25	0.25	2	2	0.125	≥32/609
3	8	0.25	2	8	≥16	≥32	0.25	0.25	8	4	0.125	≥32/609
4	≥16	0.25	2	≥16	8	≥32	0.25	0.25	X	2	0.125	≥32/609
5	8	0.25	2	8	≥16	≥32	0.25	0.25	X	4	0.125	≥32/609
6	4	0.25	2	8	8	≥32	0.25	0.25	X	2	0.125	≥32/609
7	2	0.25	2	8	8	≥32	0.25	0.25	X	2	0.125	≥32/609
8	≥16	0.25	2	8	8	≥32	0.25	X	X	X	X	≥32/609
9	4	0.5	4	8	X	≥32	0.25	X	X	X	X	≥32/609
10	≥16	1	2	8	X	≥32	0.25	X	X	X	X	≥32/609
11	≥16	0.25	2	8	8	≥32	0.25	0.25	8	2	0.125	≥32/609
12	8	0.5	2	8	8	≥32	0.25	0.25	8	2	0.125	≥32/609
13	8	0.5	2	8	≥16	≥32	0.25	0.25	X	X	X	≥32/609
15	8	0.25	2	4	8	32	0.25	X	X	X	X	≥32/609
16	8	0.25	2	≥16	8	32	0.25	X	X	X	X	≥32/609
17	8	0.25	2	8	8	32	0.25	X	X	X	X	≥32/609
18	8	0.25	2	≥16	≥16	≥32	0.25	0.25	8	2	0.125	≥32/609
19	8	0.25	2	8	8	≥32	0.25	4	8	8	X	≥32/609
20	4	0.25	2	≥16	≥16	≥32	0.25	4	8	8	1	≥32/609
21	4	0.25	2	8	≥16	≥32	0.25	1	X	4	1	≥32/609
22	8	0.25	2	8	X	≥32	0.25	X	X	X	X	≥32/609
23	8	0.25	2	8	≥16	≥32	0.25	X	X	X	X	≥32/609
24	8	0.25	4	≥16	X	≥32	0.25	X	X	X	X	≥32/609
25	4	0.25	2	8	X	≥32	0.25	X	X	X	X	≥32/609
26	8	0.25	2	≥16	≥16	≥32	0.25	2	X	2	1	≥32/609
27	8	0.25	2	8	16	≥32	0.25	0.25	8	8	1	≥32/609
28	≥16	0.25	2	8	≥16	≥32	0.25	X	X	2	0.5	≥32/609
29	≥16	0.25	2	8	≥16	≥32	0.25	0.25	8	8	0.125	≥32/609
30	4	0.25	2	8	≥16	≥32	0.25	0.25	8	2	0.125	≥32/609
31	≥16	0.25	2	8	≥16	≥32	0.5	X	X	X	X	≥32/609
32	≥16	0.25	4	8	≥16	≥32	0.5	0.25	8	2	1	≥32/609
33	8	0.5	2	8	8	32	0.5	0.25	X	2	1	≥32/609
34	≥16	0.25	4	8	X	≥32	0.5	X	X	X	X	≥32/609
35	≥16	0.25	2	8	≥16	≥32	0.5	X	X	X	X	≥32/609

36	4	0.25	4	4	8	≥32	0.5	X	X	X	X	≥32/609
37	8	0.25	4	8	X	≥32	0.5	X	X	X	X	≥32/609
38	8	0.25	2	8	≥16	≥32	0.5	X	X	X	X	≥32/609
39	≥16	0.25	2	4	8	32	0.5	0.25	X	4	1	≥32/609
40	≥16	0.25	4	8	X	≥32	0.5	X	X	X	X	≥32/609
41	4	0.5	2	≥16	X	≥32	0.25	0.25	X	2	0.125	≥32/609
42	8	0.25	4	≥16	≥16	≥32	0.5	0.25	8	2	0.125	≥32/609
43	4	0.25	2	16	16	≥32	0.5	X	8	X	X	≥32/609
44	8	0.5	4	8	8	≥32	0.5	4	8	X	1	≥32/609
45	8	1	4	8	X	≥32	0.5	X	X	X	X	≥32/609
46	≥16	0.5	2	8	8	≥32	0.5	0.25	8	2	0.125	≥32/609
47	32	0.25	2	8	8	≥32	0.5	X	X	X	0.125	≥32/609
48	32	0.25	2	≥16	≥16	≥32	0.5	X	X	X	X	≥32/609
49	32	2	4	8	≥16	≥32	0.5	X	X	X	X	≥32/609
50	4	0.5	2	8	X	≥32	0.25	X	X	X	X	≥32/609
51	4	1	4	≥16	X	≥32	0.5	X	X	X	X	≥32/609
52	8	1	4	8	X	≥32	0.5	X	X	X	X	≥32/609
53	32	0.25	2	8	≥16	≥32	0.5	X	X	X	X	16/304
54	≥16	0.25	2	8	X	≥32	0.5	X	X	X	X	≥32/609
55	≥16	0.25	2	8	8	≥32	0.5	0.25	X	2	X	≥32/609
56-1	≥16	1	4	8	X	≥32	0.5	X	X	X	X	≥32/609
56-2	≥16	1	4	8	X	≥32	0.5	X	X	X	X	≥32/609
57	≥16	0.5	2	8	≥16	≥32	0.5	X	8	X	X	≥32/609
58	32	0.25	2	8	8	≥32	0.5	0.25	8	4	X	≥32/609
59	4	0.25	2	8	≥16	≥32	0.5	X	X	X	X	≥32/609
60	2	0.25	2	8	≥16	≥32	0.25	4	8	8	X	≥32/609

***X=Not tested by MPC as MIC was the resistance breakpoint.**

The number of strains tested by MPC differs for each drug based on prior MIC testing. As summarized in Table 3.1.2.4 for the strains tested, MPC values for vancomycin ranged from 4-≥32 µg/ml as compared to tigecycline 0.25-1 µg/ml, linezolid2-4 µg/ml, gentamicin 4->16 µg/ml, tobramycin8-≥16 µg/ml, amikacin ≥32 µg/ml, tedizolid 0.25-0.5 µg/ml, gatifloxacin 0.25-4 µg/ml, azithromycin 2-≥4 µg/ml, ciprofloxacin 2-≥8 µg/ml, moxifloxacin 0.125-≥1 µg/ml and trimethoprim/sulfamethoxazole ≥16 µg/ml.

Table 3.1.2.4: Comparative MPC distribution data for 60 MRSA strains tested against 11 antimicrobial agents.

MPC														
Drug/N*	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**	**32**	**≥32**	**MPC_{50}**	**MPC_{90}**	**MPC_{90}/MIC_{90}**
Linz 60						45 (75%)	15 (25%)					2	4	2
Vanco 60						2 (3%)	12 (20%)	22 (37%)	24 (40%)			8	≥16	≥16
Ted 60			32 (53%)	28 (47%)								0.25	0.5	2
Tig 60			44 (73%)	9 (15%)	6 (10%)	1 (2%)						0.25	1	4
Genta 60							3 (5%)	45 (75%)	12 (20%)			8	16	16
Tobra 44								19 (43%)	25 (57%)			≥16	≥16	1
Amik 60										7 (12%)	53 (88%)	32	32	2
Gati 27			21 (77%)		1 (4%)	1 (4%)	4 (15%)					0.25	4	0.5
Moxi 23		15 (65%)		1 (4%)	7 (30%)							0.125	1	0.25
Cipro 27						17 (63%)	5 (18.5%)	5 (18.5%)				2	16	2
Azithro 18					1 (6%)			17 (94%)				8	8	1

***N=total isolates tested by MPC (susceptible by MIC).**

* amik=amikacin; azith=azithromycin; cipro=ciprofloxacin; gati=gatifloxacin; genta=gentamicin; linz=linezolid; moxi=moxifloxacin; ted=tedizolid; tig= tigecycline; tobra=tobramycin; vanco=vancomycin.

Published CLSI MIC breakpoints for *S. aureus* against the tested drugs are summarized in Table 3.1.2.5. Based on CLSI recommended breakpoints, the percentage of strains susceptible by MIC and MPC testing were calculated for each drug. By MIC testing, all strains (100%) were susceptible to vancomycin, trimethoprim/sulfamethoxazole, linezolid, tedizolid and amikacin. The majority (95%) of strains were susceptible to gentamicin, 90% were susceptible to tigecycline 90% and 73% were susceptible to tobramycin. Fewer than half of the strains were susceptible to gatifloxacin (45%), moxifloxacin (42%), ciprofloxacin (45%) and azithromycin (30%). Unlike linezolid and tedizolid which showed 100% susceptibility by MPC testing, none of the strains were susceptible to amikacin, ciprofloxacin or tobramycin. Only 5-6% of strains were susceptible to azithromycin and gentamicin. Between 60-70% of strains were susceptible to gatifloxacin and moxifloxacin and over 80% were susceptible to tigecycline by MPC testing. None of the strains were susceptible to chloramphenicol either by MIC or MPC testing. None of the 60 MRSA strains were at or below the susceptibility breakpoint for TMP/SMX.

Table 3.1.2.5: Comparative susceptibility data for MRSA strains tested against 13 drugs.

Drug	Genta	Gati	Moxi	Vanco	TMP/SMX	Tobra	Cipro	Azithro	Tig	Linz	Chloro	Amik	Ted
MIC S.BP	≤ 4	≤ 0.5	≤ 0.5	≤ 4	≤ 2/38	≤ 4	≤ 1	≤ 2	≤ 0.5	≤ 4	≤ 8	≤ 16	≤ 0.5
Susceptibility % by MIC	95%	45%	42%	100%	100%	73%	45%	30%	90%	100%	0%	100%	100%
susceptibility% by MPC	5%	78%	67%	20%	NT	0%	0%	6%	88%	100%	NT	0%	100%

NT= not tested.

MIC S.BP=MIC susceptibility breaking point.

amik=amikacin; azith=azithromycin; chloro=chloramphenicol; Cipro=ciprofloxacin; gati=gatifloxacin; genta=gentamicin; linz=linezolid; moxi=moxifloxacin; ted=tedizolid; tig= tigecycline; TMP/SMX=trimethoprim/sulfamethoxazole; tobra=tobramycin; vanco=vancomycin;

The following experiments were performed to determine if viable colonies of MRSA isolated from agar plates containing ≥16 µg/ml of vancomycin retained an elevated MIC value upon retesting. Data summarized in Table 3.1.2.6 is based on experiments where colonies were collected from vancomycin-containing agar plates (≥16 µg/ml) and tested by the MIC assay to determine if the recovered MIC was elevated from the original parental MIC value. Parental MICs were compared to recovered MICs (from drug-containing plates and drug-free plates) for MRSA strains with high MPC values of vancomycin. All recovered MICs retained the same value as the parental MIC at 0.5 µg/ml. This data suggests some type of tolerance effect, which is potentially critical for patients being treated with vancomycin and with high bacterial burdens whereas organisms survive in the presence of high vancomycin concentrations.

Table 3.1.2.6: MIC results for 24 MRSA strains comparing wildtype parental MIC values to those recovered from MPC plates containing ≥16 µg/ml of vancomycin.

Isolate#	MPC	MIC original	MIC rec/direct	MIC rec/sub
32	16	0.5	0.5	0.5
35	≥16	0.5	0.5	0.5
40	≥16	0.5	0.5	0.5
48	16	0.5	0.5	0.5
49	≥16	0.5	0.5	0.5
53	≥16	0.5	0.5	0.5
54	16	0.5	0.5	0.5
55	16	0.5	0.5	0.5
57	16	0.5	0.5	0.5
58	≥16	0.5	0.5	0.5
46	16	0.5	0.5	0.5
1	16	0.5	0.5	0.5
8	16	0.5	0.5	0.5
10	16	0.5	0.5	0.5
11	16	0.5	0.5	0.5
28	16	0.5	0.5	0.5
29	16	0.5	0.5	0.5
31	16	0.5	0.5	0.5
34	16	0.5	0.5	0.5
47	16	0.5	0.5	0.5
48	16	0.5	0.5	0.5
56	16	0.5	0.5	0.5
4	≥16	0.5	0.5	0.5
56-b	16	0.5	0.5	0.5

3.2 MIC and MPC at Various Bacterial Densities

MPC values for 6 MRSA strains were determined against vancomycin at different bacterial densities ranging from 10^5-10^9 CFU/ml as shown in Table 3.2.1. MPC values at 10^8 CFU/ml was $\leq$ 3 double dilutions lower when compared to the 10^9 CFU/ml density while the 10^5-10^7 CFU/ml densities showed no growth at all drug concentrations tested. The following experiments were to determine how the MPC for vancomycin against MRSA strains changed by altering the cell density of the bacteria being tested.

Table 3.2.1: Inhibition of 6 MRSA strains by different concentrations of vancomycin exposed to varying bacterial densities.

Isolate #	Density	Vancomycin concentration					
		2	**4**	**8**	**16**	**32**	**64**
35	10^9	+*	+	+	-**	-	-
	10^8	+	+	-	-	-	-
	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-
40	10^9	+	+	+	-	-	-
	10^8	+	-	-	-	-	-
	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-
28	10^9	+	+	-	-	-	-
	10^8	+	+	-	-	-	-
	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-
29	10^9	+	+	+	-	-	-
	10^8	+	+	-	-	-	-
	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-
8	10^9	+	+	+	+	-	-
	10^8	+	-	-	-	-	-
	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-
11	10^9	+	+	-	-	-	-
	10^8	+	-	-	-	-	-

	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-
Control SA	10^9	+	+	+	-	-	-
	10^8	+	+	-	-	-	-
	10^7	-	-	-	-	-	-
	10^6	-	-	-	-	-	-
	10^5	-	-	-	-	-	-

*+ = growth
**- = no growth.

Isolate #5 was picked from a vancomycin containing plate at 32, 16 and 8 µg/ml and the MIC was determined at densities of 10^4-10^9 CFU/ml and compared to recovered MIC values from drug-free plates. In all instances the recovered MIC values were the same or within 1 doubling dilution of each other.

The following experiments summarized in Table 3.3.2 investigated if recovery of MRSA colonies from vancomycin-containing plates at different drug concentrations affected the recovered MIC value when tested using different bacterial cell densities.

Table 3.2.2: Comparative MIC values for 1 MRSA strain exposed to varying concentrations of vancomycin.

Drug concentration in plate subbed to	**32 µg/ml**		**16 µg/ml**		**8 µg/ml**	
	DC* plate	**DF** plate**	**DC plate**	**DF plate**	**DC plate**	**DF plate**
	MIC (µg/ml)		**MIC (µg/ml)**		**MIC (µg/ml)**	
10^9	0.5	0.5	0.5	0.5	0.5	0.5
10^8	1	1	1	1	1	1
10^7	1	1	1	1	1	1
10^6	1	1	0.5	0.5	0.5	0.5
10^5	1	0.5	0.5	0.5	0.5	0.5
10^4	1	1	1	1	1	1

***DC = drug-containing;**
****DF = drug-free**

For MIC testing, the MIC is read as the lowest drug concentration without visible growth. As an MIC assay is not a measurement of bacterial killing, viable organisms may still be present at the MIC drug concentration.

3.3 Visual End Point vs Actual End Point

In this next series of experiments, we were interested in determining if the visible endpoint for MPC assays had viable organisms. Negative (no visible colonies) MPC plates were sub-cultured to drug-containing plates (same drug concentration as isolated from) and to drug-free plates. MRSA (Tables 3.3.1. to 3.3.3) and group A β-hemolytic streptococci (GABHS) or *Streptococcus pyogenes* (Tables 3.3.4 to 3.3.7) were tested at different drug concentrations and against different antimicrobial agents including linezolid, vancomycin, moxifloxacin, azithromycin and penicillin. Growth was recovered when negative plates (with no visible bacterial growth) were sub-cultured to drug-free plates but not when sub-cultured to drug-containing plates. Therefore, visual end points do not necessarily reflect the end point defining the drug concentration in which no viable organisms remained. This might be expected when considering testing of bactericidal or bacteriostatic agents.

Table 3.3.1: Comparison of visual versus actual MPC endpoints for 15 MRSA strains exposed to linezolid.

MRSA - Linezolid									
Isolate #	MPC	Re-sub							
		4 µg/ml		8 µg/ml		16 µg/ml		32 µg/ml	
		4	DF	8	DF	16	DF	32	DF
1	4	-	+	-	+	-	+	-	+
2	4	-	+	-	+	-	+	-	+
3	4	-	+	-	+	-	+	-	+
4	4	-	+	-	+	-	+	-	+
5	4	-	+	-	+	-	+	-	+
6	4	-	+	-	+	-	+	-	+
9	4	-	+	-	+	-	+	-	+
22	4	-	+	-	+	-	+	-	+
30	4	-	+	-	+	-	+	-	+
34	4	-	+	-	+	-	+	-	+
44	4	-	+	-	+	-	+	-	+
45	4	-	+	-	+	-	+	-	+
51	4	-	+	-	+	-	+	-	+
52	4	-	+	-	+	-	+	-	+
56	4	-	+	-	+	-	+	-	+

DF = drug-free; - = no growth; + = growth.

Table 3.3.2: Comparison of visual versus actual MPC endpoints for 9 MRSA strains exposed to vancomycin.

MRSA-Vancomycin

Isolate #	MPC	Re-submit					
		4 µg/ml		8 µg/ml		16 µg/ml	
		4	DF	8	DF	16	DF
6	4	-	+	-	+	-	+
7	4	-	+	-	+	-	+
16	16	-	+	-	+	-	+
20	8	-	+	-	+	-	+
21	8	-	+	-	+	-	+
25	8	-	+	-	+	-	+
29	4	-	+	-	+	-	+
30	8	-	+	-	+	-	+
50	16	-	+	-	+	-	+

DF = drug-free; - = no growth; + = growth.

Table 3.3.3: Comparison of visual versus actual MPC endpoints for 8 MRSA strains exposed to moxifloxacin.

MRSA-Moxifloxacin							
Isolate #	MPC	Re-sub					
		0.125 µg/ml		0.25 µg/ml		0.5 µg/ml	
		0.125	DF	0.25l	DF	0.5	DF
6	0.125	-	+	-	+	-	+
7	0.125	-	+	-	+	-	+
4	0.125	-	+	-	+	-	+
5	0.125	-	+	-	+	-	+
11	0.125	-	+	-	+	-	+
12	0.125	-	+	-	+	-	+
41	0.125	-	+	-	+	-	+
42	0.125	-	+	-	+	-	+

DF = drug-free; - = no growth; + = growth.

Table 3.3.4: Comparison of visual versus actual MPC endpoints for 10 GABHS strains exposed to azithromycin.

GABHS-Azithromycin										
Isolate #	MIC	MPC	Re-sub							
			0.125 µg/ml		0.25 µg/ml		0.5 µg/ml		1 µg/ml	
			0.125	DF	0.25	DF	0.5	DF	1	DF
1	0.063	0.125	-	+	-	+	-	+	-	+
2	0.125	0.25	-	+	-	+	-	+	-	+
3	0.125	0.25	-	+	-	+	-	+	-	+
4	0.125	0.125	-	+	-	+	-	+	-	+

5	0.125	0.125	-	+	-	+	-	+	-	+
6	0.125	0.125	-	+	-	+	-	+	-	+
7	0.125	0.125	-	+	-	+	-	+	-	+
8	0.125	0.125	-	+	-	+	-	+	-	+
9	0.125	0.125	-	+	-	+	-	+	-	+
10	0.125	0.25	-	+	-	+	-	+	-	+

DF = drug-free; - = no growth; + = growth.

Table 3.3.5: Comparison of visual versus actual MPC endpoints for 10 GABHS strains exposed to penicillin.

GABHS-penicillin									
Isolate #	MIC	MPC	Re-submit						
			0.16 µg/ml		0.31 µg/ml		0.63 µg/ml		
			0.16	DF	0.31l	DF	0.63	DF	
1	0.008	0.125	-	+	-	+	-	+	
2	0.008	0.25	-	+	-	+	-	+	
3	0.004	0.25	-	+	-	+	-	+	
4	0.004	0.125	-	+	-	+	-	+	
5	0.004	0.125	-	+	-	+	-	+	
6	0.004	0.125	-	+	-	+	-	+	
7	0.008	0.125	-	+	-	+	-	+	
8	0.008	0.125	-	+	-	+	-	+	
9	0.008	0.125	-	+	-	+	-	+	
10	0.004	0.25	-	+	-	+	-	+	

DF = drug-free; - =no growth; + = growth.

Table 3.3.6: Comparison of visual versus actual MPC endpoints for 10 GABHS strains exposed to moxifloxacin.

GABHS-Moxifloxacin										
Isolate #	MIC	MPC	Re-submit							
			0.25 µg/ml		0.5 µg/ml		1 µg/ml		2 µg/ml	
			0.25	DF	0.5	DF	1	DF	2	DF
1	0.25	0.25	-	+	-	+	-	+	-	+
2	0.25	0.25	-	+	-	+	-	+	-	+
3	0.25	0.25	-	+	-	+	-	+	-	+
4	0.25	0.25	-	+	-	+	-	+	-	+
5	0.25	0.25	-	+	-	+	-	+	-	+
6	0.25	0.25	-	+	-	+	-	+	-	+
7	0.25	0.5	-	+	-	+	-	+	-	+
8	0.25	0.25	-	+	-	+	-	+	-	+

9	1	1	-	+	-	+	-	+	-	+
10	0.125	0.5	-	+	-	+	-	+	-	+

DF = drug-free; - = no growth; + = growth.

Table 3.3.7: Comparison of visual versus actual MPC endpoints for 10 GABHS strains exposed to vancomycin.

GABHS-Vancomycin										
Isolate #	**MIC**	**MPC**	**Re-submit**							
			0.5 µg/ml		**1 µg/ml**		**2 µg/ml**		**4 µg/ml**	
			0.5	**DF**	**1**	**DF**	**2**	**DF**	**4**	**DF**
1	0.25	0.125	-	+	-	+	-	+	-	+
2	0.25	0.25	-	+	-	+	-	+	-	+
3	0.25	0.25	-	+	-	+	-	+	-	+
4	0.25	0.125	-	+	-	+	-	+	-	+
5	0.25	0.125	-	+	-	+	-	+	-	+
6	0.25	0.125	-	+	-	+	-	+	-	+
7	0.25	0.125	-	+	-	+	-	+	-	+
8	0.25	0.125	-	+	-	+	-	+	-	+
9	0.25	0.125	-	+	-	+	-	+	-	+
10	0.5	0.25	-	+	-	+	-	+	-	+

DF = drug-free; - =no growth; + = growth.

The MIC determination utilized 10^5 CFU/ml against vancomycin, linezolid and tigecycline. Negative wells (showing now growth) were sub-cultured to drug-free blood agar plates to test if viable organisms could be recovered. For vancomycin, the majority of negative wells showed no growth when sub-cultured to drug-free plates. For tigecycline and linezolid, most negative wells had bacterial growth on drug-free blood agar plates as shown in Table 3.3.8-3.3.10.

In the following experiments shown in Tables 3.3.8-3.3.10, sub-culturing of visually negative wells was performed to determine if viable organism existed in the MIC assay in wells with no visual growth.

Table 3.3.8: Subculture results from negative MIC wells for MRSA strains tested against tigecycline.

Tig	8	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	0.008	µg/ml
1	-	-	-	-	-	-	-	-	-	+	+	+
4	-	-	-	-	-	-	-	-	-	+	+	+
8	-	-	-	-	-	-	-	-	+	+	+	+
10	-	-	-	-	-	-	+	+	+	+	+	+
11	-	-	-	-	-	-	-	-	+	+	+	+
35	-	-	-	-	-	-	-	-	+	+	+	+
40	-	-	-	-	-	-	-	-	+	+	+	+
44	-	-	-	-	-	-	-	-	+	+	+	+
Negative wells sub-cultured to drug-free agar plates												
1	-	-	+	+	+	-	-	+	+			
4	-	-	-	-	+	+	+	+	+			
8	+	+	+	-	+	+	+	+				
10	-	+	+	+	+	+						
11	-	+	-	-	-	+	-	+				
35	+	+	+	+	+	-	+	+				
40	-	+	+	-	+	+	+	+				
44	+	-	+	-	+	+	+	+				

- = no growth; + = growth.

Table 3.3.9: Subculture results from negative MIC wells for MRSA strains tested against linezolid.

Linz	8	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	0.008	µg/ml
1	-	-	-	-	+	+	+	+	+	+	+	+
4	-	-	-	+	+	+	+	+	+	+	+	+
8	-	-	-	+	+	+	+	+	+	+	+	+
10	-	-	-	+	+	+	+	+	+	+	+	+
11	-	-	-	+	+	+	+	+	+	+	+	+
35	-	-	-	+	+	+	+	+	+	+	+	+
40	-	-	-	+	+	+	+	+	+	+	+	+
44	-	-	-	+	+	+	+	+	+	+	+	+
Negative wells sub-cultured to drug-free agar plates												
1	+	+	+	+								
4	+	-	+									
8	+	+	+									
10	-	+	+									
11	-	+	+									
35	-	+	+									
40	+	+	+									
44	+	+	+									

- = no growth; + = growth.

Table 3.3.10: Subculture results from negative MIC wells for MRSA strains tested against vancomycin.

vanco	16	8	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	µg/ml
1	-	-	-	-	-	-	+	+	+	+	+	+
4	-	-	-	-	-	+	+	+	+	+	+	+
8	-	-	-	-	-	+	+	+	+	+	+	+
10	-	-	-	-	-	+	+	+	+	+	+	+
11	-	-	-	-	-	+	+	+	+	+	+	+
35	-	-	-	-	-	-	+	+	+	+	+	+
40	-	-	-	-	-	-	+	+	+	+	+	+
44	-	-	-	-	-	-	+	+	+	+	+	+
9	-	-	-	-	-	-	+	+	+	+	+	+
10	-	-	-	-	-	-	+	+	+	+	+	+
11	-	-	-	-	-	-	+	+	+	+	+	+
12	-	-	-	-	-	+	+	+	+	+	+	+
13	-	-	-	-	-	-	+	+	+	+	+	+
15	-	-	-	-	-	-	+	+	+	+	+	+
16	-	-	-	-	-	-	+	+	+	+	+	+
17	-	-	-	-	-	-	+	+	+	+	+	+
Negative wells sub-cultured to drug-free agar plates												
1	-	-	-	-	-	-						
4	-	-	+	-	-							
8	-	-	-	-	-							
10	-	-	-	-	-							
11	-	-	-	-	-							
35	-	-	-	-	-	-						
40	-	-	-	-	-	-						
44	-	+	-	-	-	-						
9	-	-	-	-	-	-						
10	-	+	-	-	-	-						
11	-	-	-	-	-	-						
12	-	-	-	-	-							
13	-	+	-	-	-	-						
15	-	+	-	-	-	-						
16	-	+	-	-	-	-						
17	-	+	-	-	-	-						

- = no growth; + = growth.

The MIC was investigated at bacterial densities ranging from 10^5-10^8 CFU/ml for 6 MRSA strains tested against vancomycin. Negative wells (8, 16 & 32 µg/ml) were sub-cultured

to drug-free agar plates and to drug containing agar plates (same concentration as isolated from) (Table 3.3.11). Bacteria from the latter plates – if negative – were then sub-cultured to drug-free plates to test if growth could be recovered (Table 3.3.12). Isolates from the 10^6 - 10^8 CFU/ml wells showed growth when sub-cultured to drug-free plates, but no growth was recovered from 10^5 CFU/ml density wells on drug-free plates. Growth also was not recovered when sub-culturing isolates (10^5-10^8 CFU/ml) to drug-containing plates (Table 3.3.11). When these negative drug containing plates were sub-cultured to drug-free plates, growth was recovered from 10^7-10^8 CFU/ml wells but not from 10^5 – 10^6 CFU/ml wells as summarized in Table 3.3.12.

Table 3.3.11: Subculture results of negative wells following MRSA MIC testing against vancomycin at varying bacterial densities.

Drug-containing plates				Drug free plates		
MRSA isolate #	**10^8**					
	32	**16**	**8**	**32**	**16**	**8**
8	-	-	-	+	+	+
11	-	-	-	+	+	+
39	-	-	-	+	+	+
47	-	-	-	+	+	+
48	-	-	-	+	+	+
49	-	-	-	+	+	+
10^7						
8	-	-	-	+	+	+
11	-	-	-	+	+	+
39	-	-	-	+	+	+
47	-	-	-	+	+	+
48	-	-	-	+	+	+
49	-	-	-	+	+	+
10^6						
8	-	-	-	+	+	+
11	-	-	-	-	-	+
39	-	-	-	-	+	+
47	-	-	-	+	+	+
48	-	-	-	+	+	+
49	-	-	-	+	+	+
10^5						
8	-	-	-	-	-	-
11	-	-	-	-	-	-

39	-	-	-	-	-	-
47	-	-	-	-	-	-
48	-	-	-	-	-	-
49	-	-	-	-	-	+

- = no growth; + = growth.

Table 3.3.12: Subculture results of negative drug-containing plates to drug-free plates.

Negative drug-containing plates				drug free plates		
isolate #	10^8					
	32	16	8	32	16	8
8	-	-	-	+	+	+
11	-	-	-	+	+	+
39	-	-	-	+	+	+
47	-	-	-	+	+	+
48	-	-	-	+	+	+
49	-	-	-	+	+	+
10^7						
8	-	-	-	+	+	+
11	-	-	-	-	-	+
39	-	-	-	-	+	-
47	-	-	-	+	+	+
48	-	-	-	-	+	+
49	-	-	-	-	+	+
10^6						
8	-	-	-	-	-	-
11	-	-	-	-	-	-
39	-	-	-	-	-	-
47	-	-	-	-	-	-
48	-	-	-	-	-	-
49	-	-	-	-	-	-
10^5						
8	-	-	-	-	-	-
11	-	-	-	-	-	-
39	-	-	-	-	-	-
47	-	-	-	-	-	-
48	-	-	-	-	-	-
49	-	-	-	-	-	-

- = no growth; + = growth.

3.4. Serial Passage

Serial passage experiments were performed in an attempt to isolate an MRSA strain with a stable elevated vancomycin MIC or MPC. In order to investigate the serial passage effect on the susceptibility level, MRSA colonies were picked from high vancomycin concentration plates (32 µg/ml) and sub-cultured 7 times on 32 µg/ml drug containing plates to select for a stable subpopulation with elevated MIC, if possible. Each passage was then subbed to a drug-free plate to compare with recovered MIC values to parental MIC values (Table 3.4.1). These experiments failed to produce a strain with a stably elevated MIC.

Table 3.4.1: Comparison of MIC values for 1 MRSA strain following serial passage on vancomycin (32 µg/ml) containing plates.

32 µg/ml	MIC from DC plate	MIC from DF plate
1st passage	0.125	0.063
2nd passage	0.125	0.063
3rd passage	0.125	0.125
4th passage	0.125	0.125
5th passage	0.125	0.063
6th passage	0.125	0.063
7th passage	0.125	0.063

DC = drug containing; DF = drug free.

3.5 Molecular Characterization

3.5.1. Polymerase Chain Reaction (PCR) Analysis

3.5.1.1 *mecA* and Panton-Valentine Leukocidin (*PVL*)

mecA and *PVL* testing were performed to confirm MRSA (by confirming the presence of the *mecA* gene) and to determine if *PVL* positive strains were a marker for high vancomycin MPC values. PCR testing for the *mecA* gene was performed for all isolates to confirm its presence in our MRSA isolates (Figure 3.5.1.1.1). PCR for the *PVL* gene was also performed to determine if there was a correlation between its presence and high MPC values (Figure 3.5.1.1.1). All MRSA strains were positive for the *mecA* gene. However, only some strains were *PVL* positive which did not correlate with high vancomycin MPC values (Table 3.5.1.1.1).

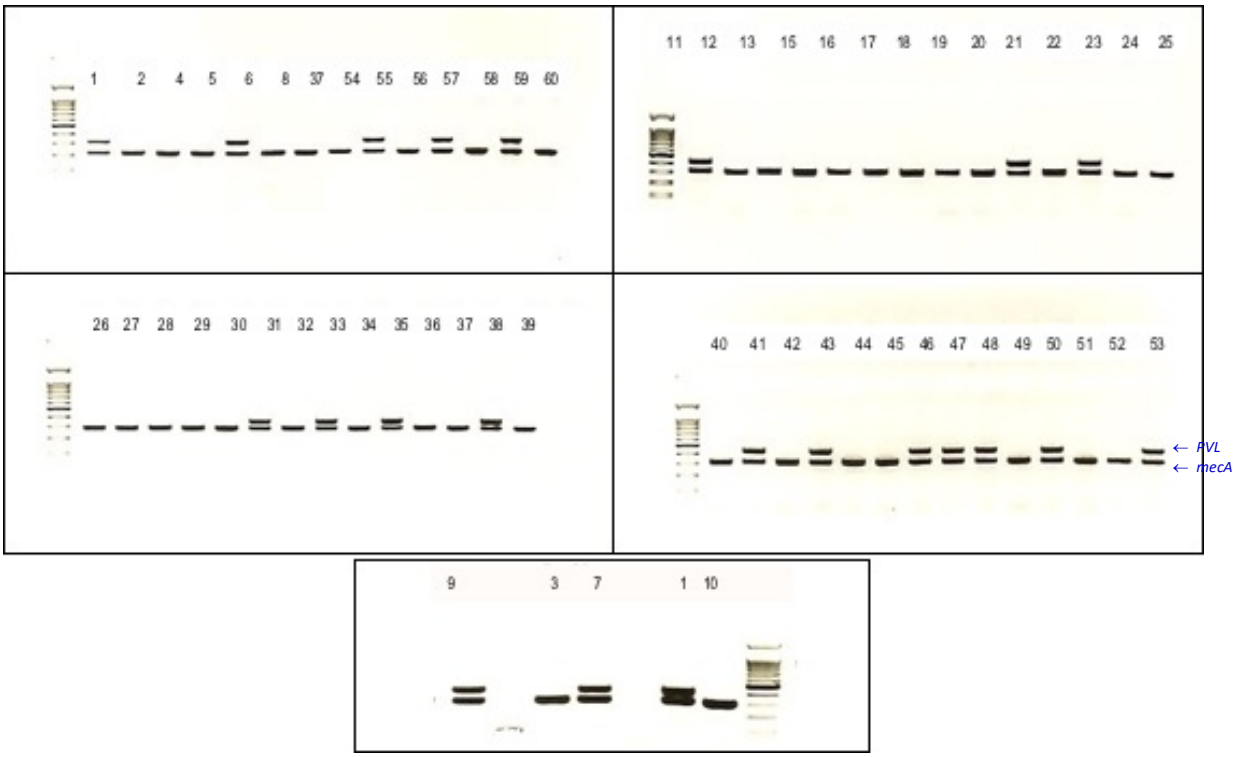


Figure 3.5.1.1.1: PCR amplification of *mecA* and *PVL* genes.

See Table 3.5.1.1.1 for interpretation of results.

Table 3.5.1.1.1: Individual *mecA* and *PVL* results for the 60 MRSA strains investigated.

Isolate #	*mecA*	*PVL*
1	+	+
2	+	-
3	+	-
4	+	-
5	+	-
6	+	+
7	+	+
8	+	-
9	+	+
10	+	-
11	+	-
12	+	-
13	+	-
15	+	-
16	+	-
17	+	-
18	+	-
19	+	-

20	+	-
21	+	+
22	+	-
23	+	+
24	+	-
25	+	-
26	+	-
27	+	-
28	+	-
29	+	-
30	+	-
31	+	-
32	+	-
33	+	-
34	+	-
35	+	-
36	+	-
37	+	-
38	+	-
39	+	-
40	+	-
41	+	-
42	+	-
43	+	-
44	+	-
45	+	-
46	+	+
47	+	+
48	+	+
49	+	-
50	+	+
51	+	-
52	+	-
53	+	+
54	+	-
55	+	+
56	+	-
57	+	+
58	+	+
59	+	-
60	+	-

3.5.1.2 *CLP*

As cell wall changes might be an explanation for MRSA strains with high vancomycin MPC values, we investigated select cell wall genes. Caseinolytic protease (*CLP*) gene is required for stress resistance and growth recovery in *S. aureus*. PCR for *Clp* gene on 6 random patients isolates of MRSA and 6 isolates of MSSA were conducted (Figure 3.5.1.2.1). There were no consistent signals observed for the presence of this gene in the isolates tested.

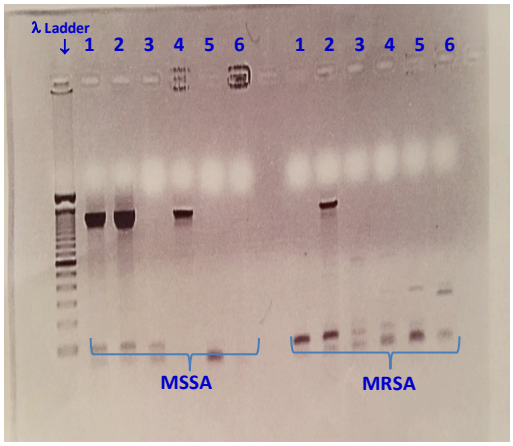


Figure 3.5.1.2.1: *ClpP* in *S. aureus* versus MRSA.

See Table 3.5.1.2.1 for interpretation of results.

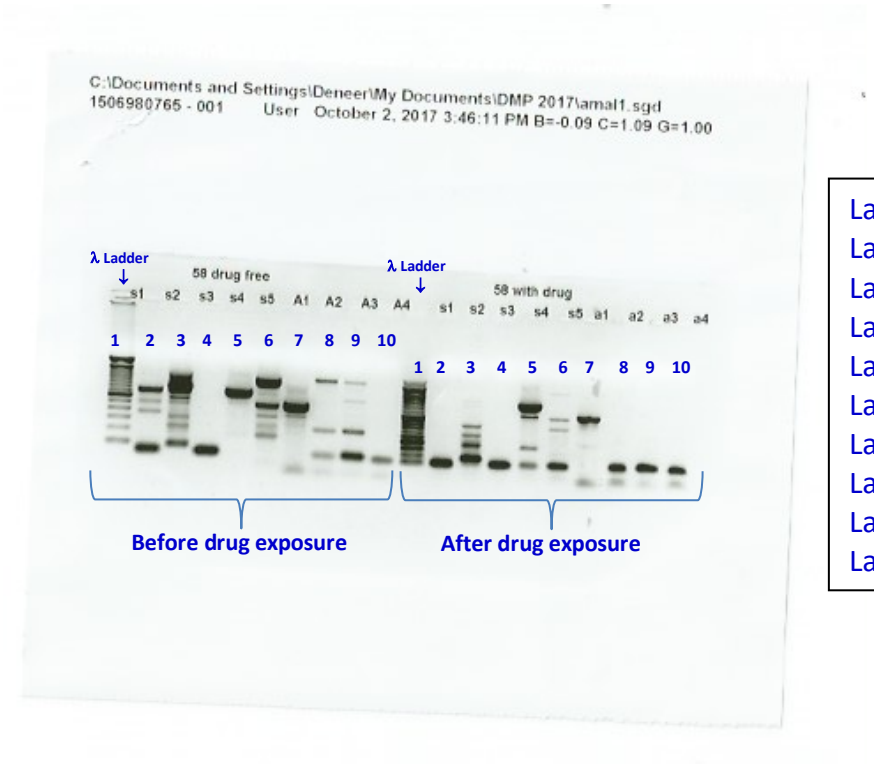
Table 3.5.1.2.1: Comparison of 6 MSSA and 6 MRSA strains for the presence or absence of the *CLP* gene.

Isolate #	MSSA	MRSA
1	+	-
2	+	+
3	-	-
4	+	-
5	-	-
6	-	-

3.5.1.3 *SCCmec* and *agr*

A PCR assay for *agr* (accessory gene regulator) and *SCC mec* (staphylococcal cassette chromosome *mec*) was carried out before and after vancomycin exposure. The *agr* locus is a regulator of virulence factors and controls a large set of genes including the genetic element carrying the β-lactam resistance gene *mecA*. Although the presence of PCRC artifacts in some

of the lanes made interpretation difficult (Figure 3.5.1.3.1), no reproducible differences in gene profiles were noted.



Lane 1 – Lambda ladder
Lane 2 – *SCCmecI*
Lane 3 – *SCCecII*
Lane 4 – *SCCmecIII*
Lane 5 – *SCCmecIV*
Lane 6 – *SCCecV*
Lane 7 – *agrI*
Lane 8 – *agrII*
Lane 9 – *agrIII*
Lane 10 – *agrIV*

Figure 3.5.1.3.1: Comparison of MRSA strain #58 for detection of *agr* and *SCCmec* genes before and after exposure to vancomycin.

3.5.2 Pulse Field Gel Electrophoresis (PFGE)

To rule out that a single clone might be responsible for the strains with high vancomycin MPC values, strain comparisons were made. PFGE profiles (Figure 3.5.2.1) show that a single dominant clone is not associated with the high vancomycin MPC values and similar profiles were observed between parental strains and strains recovered from drug containing plates. Related or unrelated strains were determined using the criteria as summarized by Tenover *et al* (2006) where strains were considered genetically indistinguishable if this restriction patterns have the same apparent size. For strains that were considered possible related, closely related and unrelated, comparison were based on differences in the number and sizes of bands in the banding profile. In our study, no single banding pattern was identified and differences were seen in banding profiles between low and high molecular weight bands.

Figure 3.5.2.1: Pulsed field gel electrophoresis profiles for 9 MRSA strains.
***Lane 1 – molecular weight markers; Lanes 2-10 – independent MRSA isolates.**

It was important to determine that colonies isolated off agar plates with high vancomycin drug concentration were the same as colonies from the parental strains. PFGE analysis for recovered strains (MPC plates) and parental strains were identical for each pair as shown in Figure 3.5.2.2.

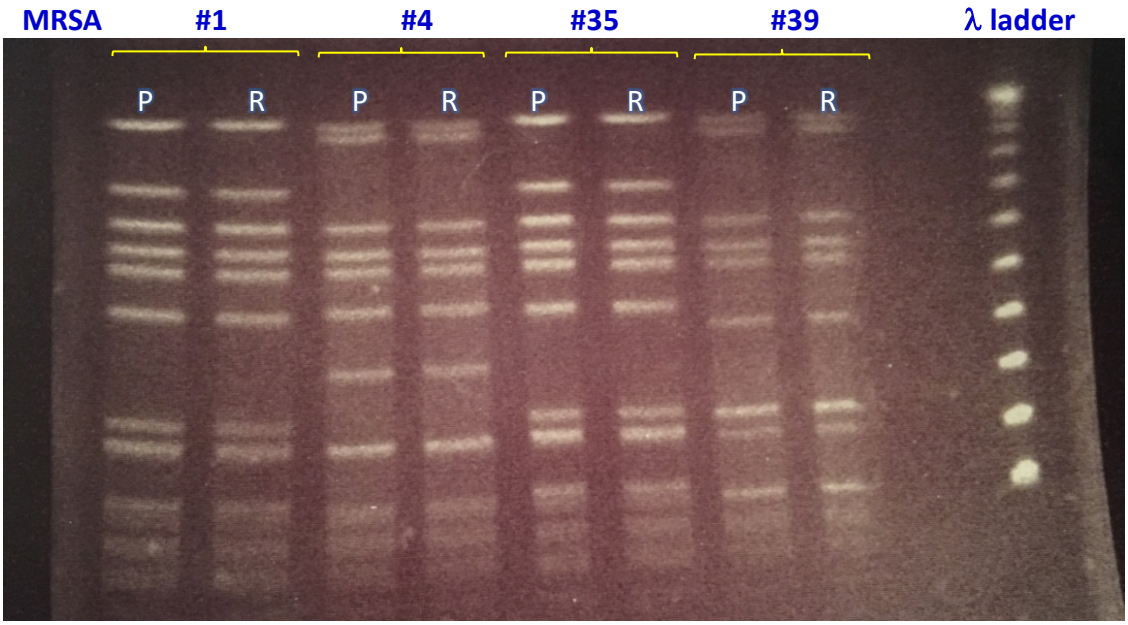


Figure 3.5.2.2: Pulsed field gel electrophoresis profiles of MRSA strains comparing parental (P) strains to organism recovered (R) from MPC plates containing ≥16 µg/ml of vancomycin.

3.6 Electron Microscopy

Cell wall thickening has been previously shown with vancomycin resistant strains. As such, we investigated if cell wall thickening was present in MRSA strains recovered from vancomycin containing plates. Electron microscopy examination was done to compare cell wall thickness of isolates that survived at high vancomycin drug concentrations (32 µg/ml) to parental isolates. Colonies were collected from 32 µg/ml plate and serially passaged 3 times on 32 µg/ml vancomycin plates. Some cells showed an increase in cell wall thickening after their exposure to high concentrations of vancomycin (Table 3.6.1). Also, cell replication as indicated by septum formation was observed in the presence of vancomycin. In order to eliminate the possibility of the agar interference with the drug getting to the cell, isolates were collected from Mueller-Hinton Broth containing vancomycin and TEM was repeated (Table 3.6.1). As summarized in the table, cells wall were on average 7-11 nm thicker in the presence of vancomycin.

Table 3.6.1: Comparison of cell wall thickness for 6 MRSA strains prior to and after exposure

to vancomycin.

MSRA #	MIC	MPC	TEM Picked From Plate at	Before adding drug (Range)	Average (3 cells)	After adding drug (Range)	Average (3 cells)
				Measurement in nanometer (nm)			
48	0.5	16	32µg/ml	18-23	21	27-30	29
49	0.5	>16	32µg/ml	22-25	23	30	30
32	0.5	16	32µg/ml	17-20	17	27-31	28
47	1	16	32µg/ml	18-24	21	27-32	30
11	0.5	16	32µg/ml	21-25	23	27-30	29
8	0.5	16	32µg/ml	22-25	**23**	26-31	**29**

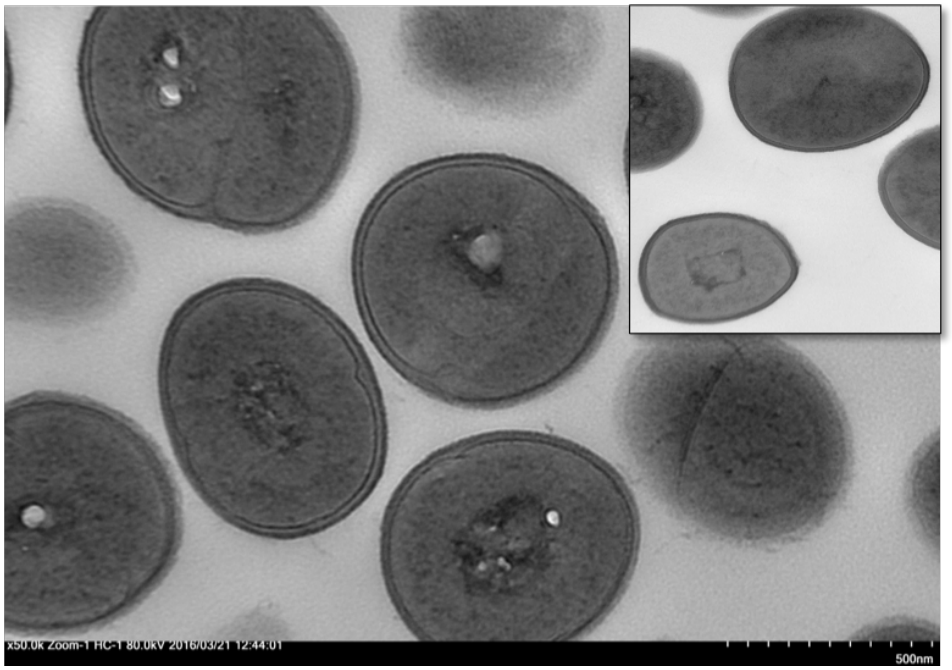


Figure 3.6.1: Electron micrograph of MRSA strain #8 at x50.0 k magnification showing thicker cell walls following vancomycin exposure compared with non-treated cells (upper right corner). Magnification is the same for picture and insert.

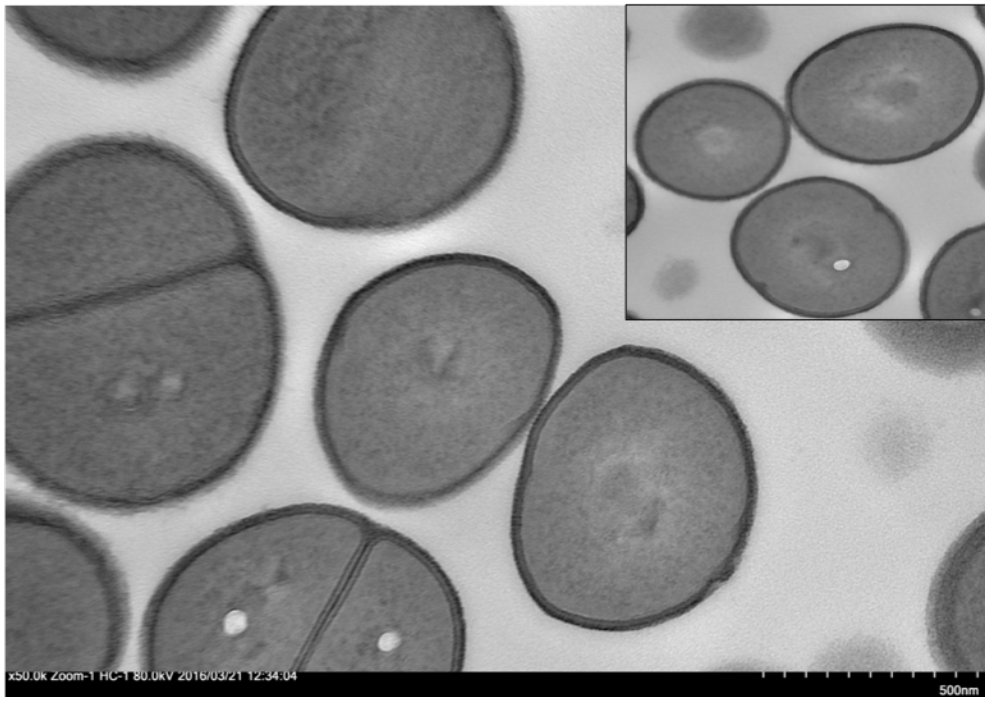
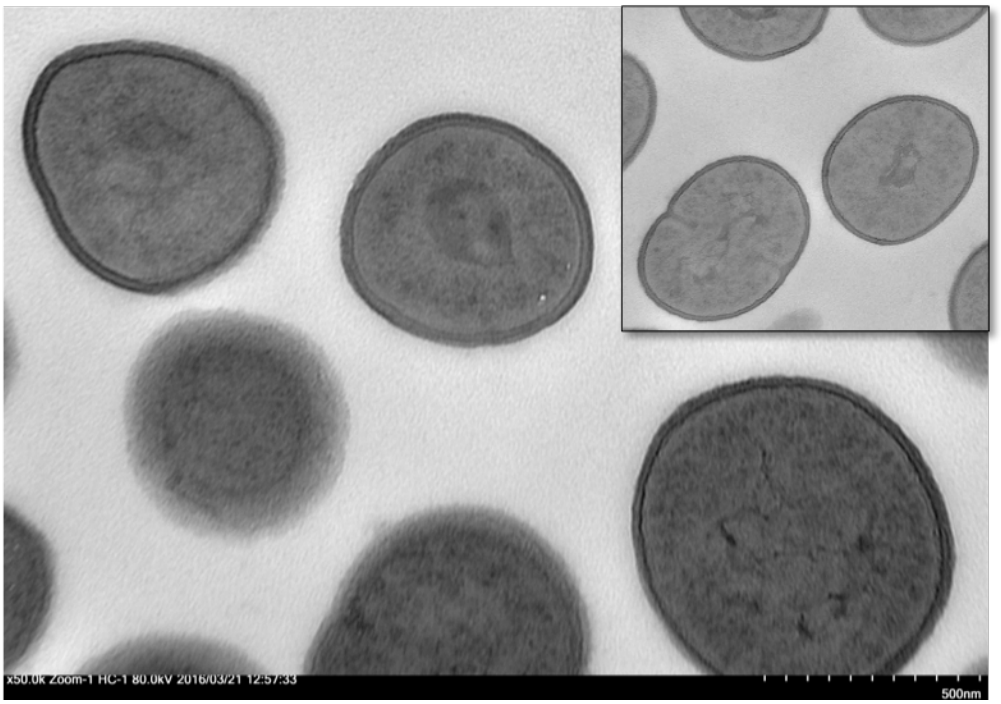
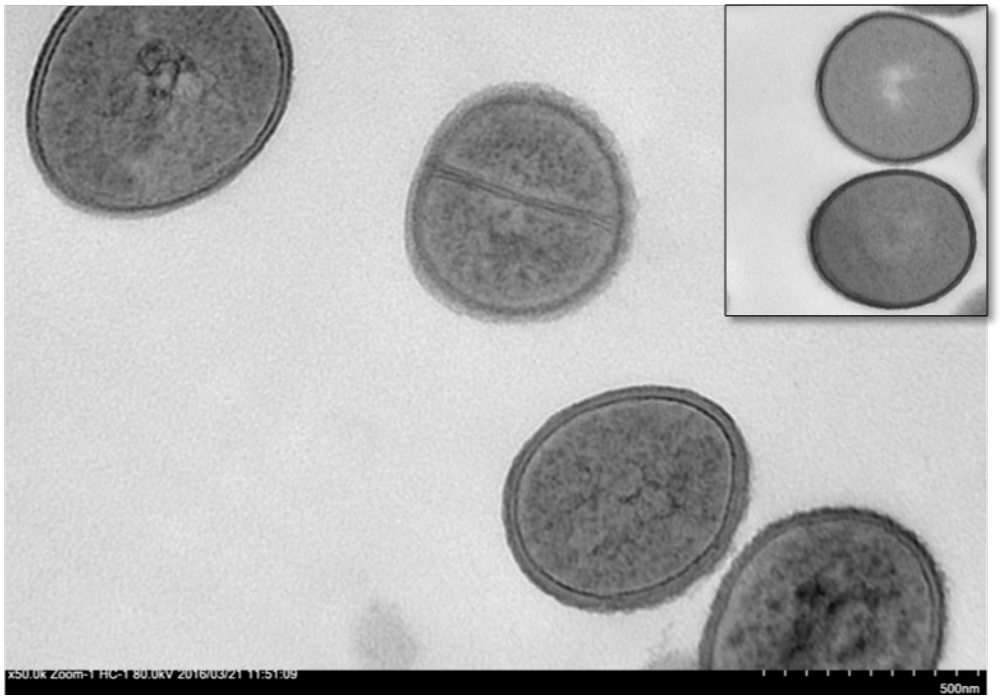


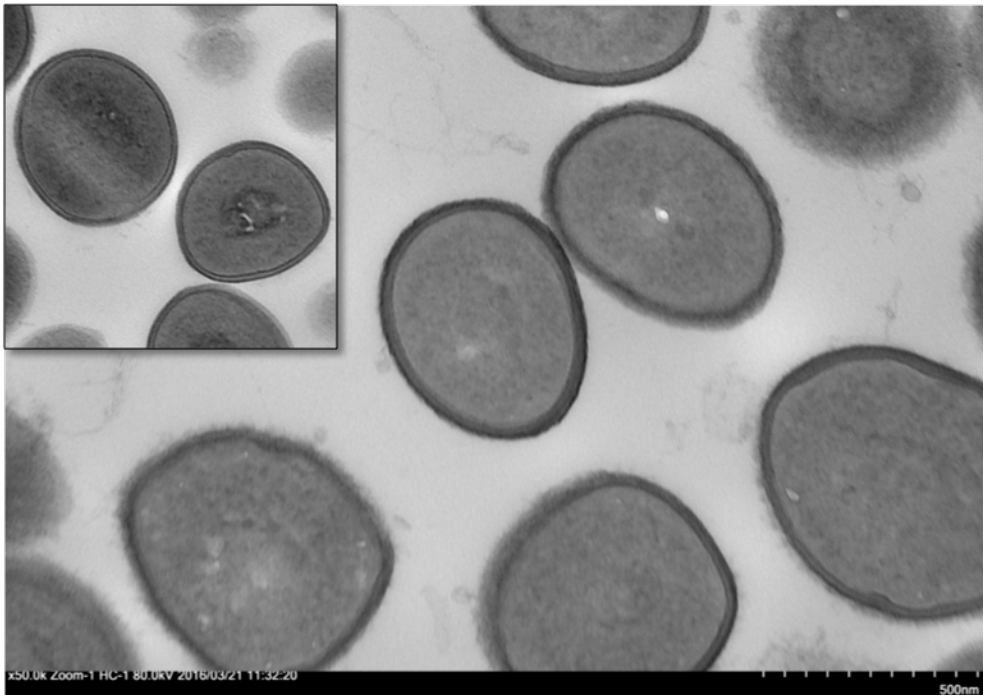
Figure 3.6.2: Electron micrograph of MRSA strain #11 at x50.k magnification showing thicker cell walls and septum formation following vancomycin exposure compared with non-treated cells (upper right corner). Magnification is the same for picture and insert.



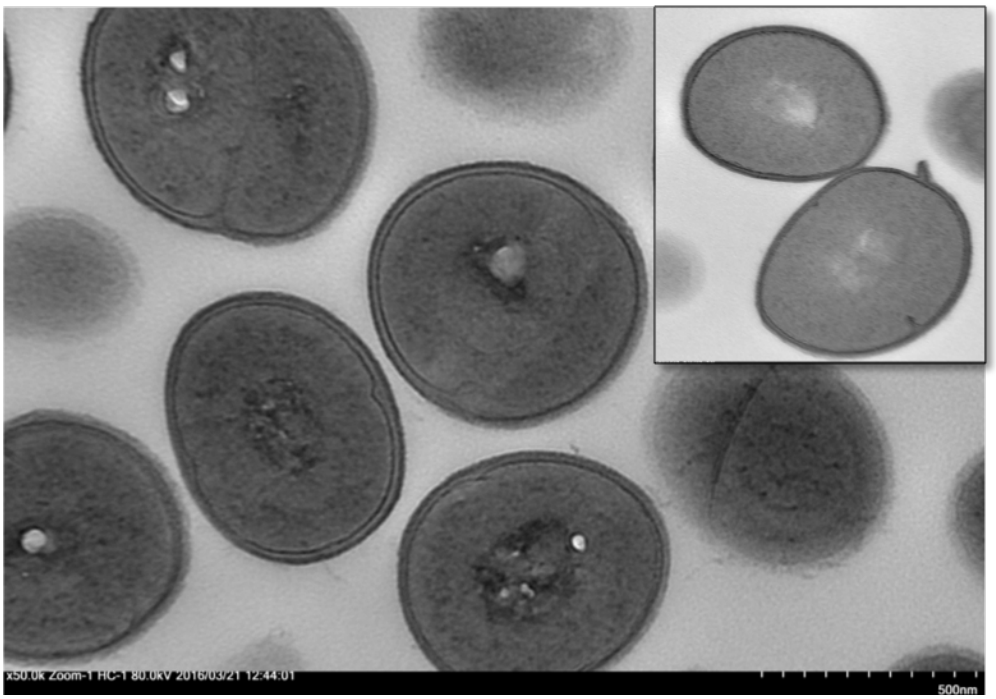
Fig*ur*e 3.6.3: Electron micrograph of MRSA strain #32 at x50.0 K magnification showing thicker cell walls following vancomycin exposure compared with non-treated cells (upper right corner). Magnification is the same for picture and insert.



Fig*ur*e 3.6.4: Electron micrograph of MRSA strain #47 at x50.0 K magnification showing thicker cell walls and septum formation following vancomycin exposure compared with non-treated cells (upper right corner). Magnification is the same for picture and insert.



Fig*ur*e 3.6.5: Electron micrograph of MRSA strain #48 at x50.0 K magnification showing thicker cell walls following vancomycin exposure compared with non-treated cells (upper left corner). Magnification is the same for picture and insert.



Fig*ur*e 3.6.6: Electron micrograph of MRSA strain #49 at x50.0 K magnification showing thicker cell walls following vancomycin exposure compared with non-treated cells (upper right corner). Magnification is the same for picture and insert.

During the experiments investigating cell wall thickness as a possible explanation for strains with high vancomycin MPC values, it was noticed in some EM micrographs, that organisms were potentially dividing (replication) as evidenced by septum formation. To further investigate that cell replication might be occurring in the presence of high vancomycin drug concentrations, the following experiments were conducted. For the first set of experiments, colonies were picked from agar plates containing 32 µg/ml of vancomycin, picking from the drug-containing plate occurred after serial passage 3 times on plates containing 32 µg/ml of vancomycin. These experiments are shown in EM micrographs – Figure 3.6.7 to 3.7.12. Strains 11, 47 and 48 selected for these experiments as colony growth occurred on plates containing 32 µg/ml of vancomycin. As shown in the figures, septum formation was seen in a number of bacterial cells and at different stages of formation. Multiple EM micrographs are shown depicting multiple different cells with septum formation and the beginnings of cell separation. Septum formation at various stages is also shown in the micrographs. The following figures show bacterial cell reproduction when recovered from blood agar plates after drug exposure: 11- Figures 3.6.7 and 3.6.8; 47-Figures 3.6.9 and 3.6.10; 48-Figures 3.6.11 and 3.6.12 respectively.

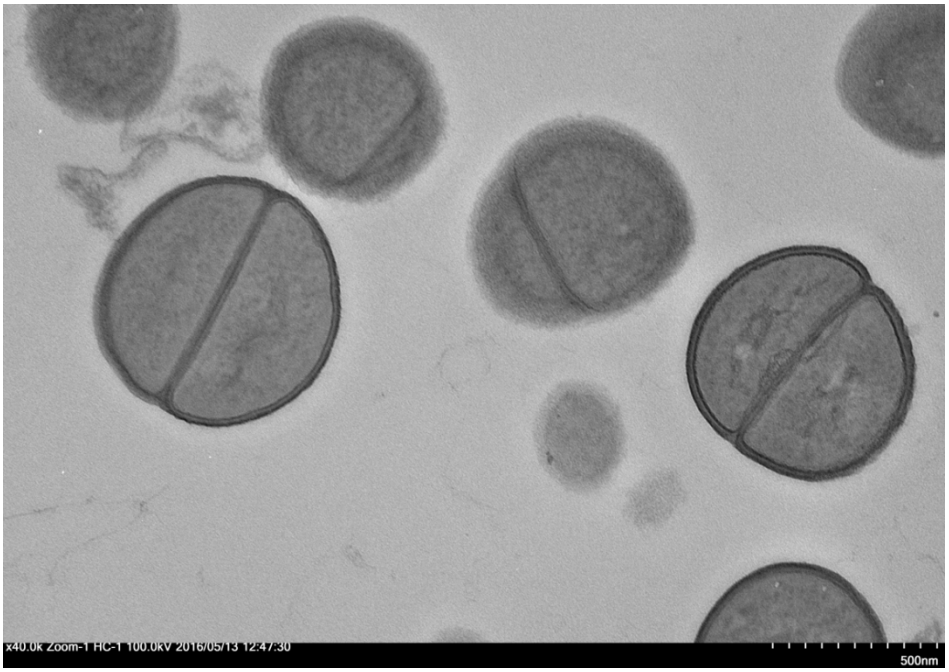


Figure 3.6.7: EM micrograph (strain 11) showing septum formation in multiple bacterial cells.

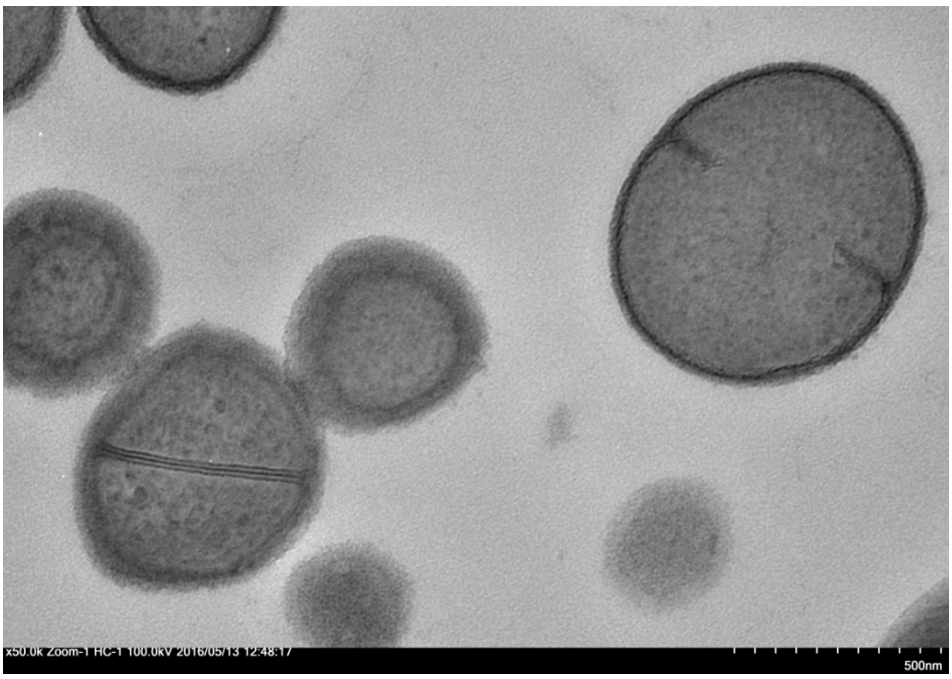


Figure 3.6.8: EM micrograph (strain 11) showing 2 bacterial cells with septum formation at different stages.

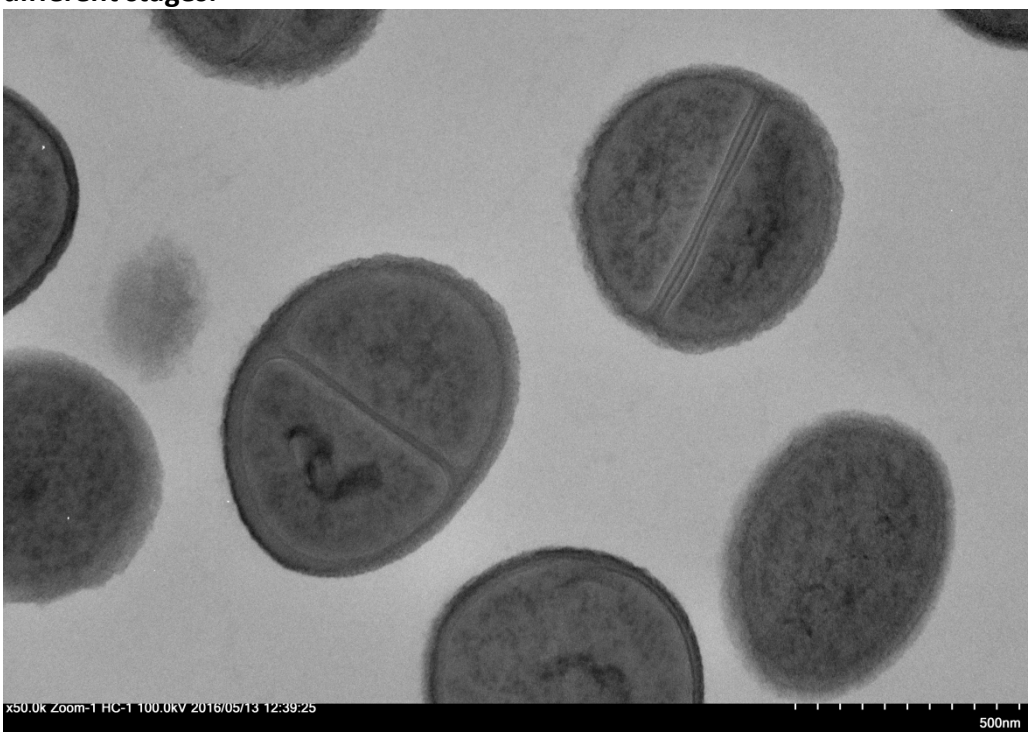


Figure 3.6.9: EM micrograph (strain 47) showing septum formation in multiple bacterial cells.

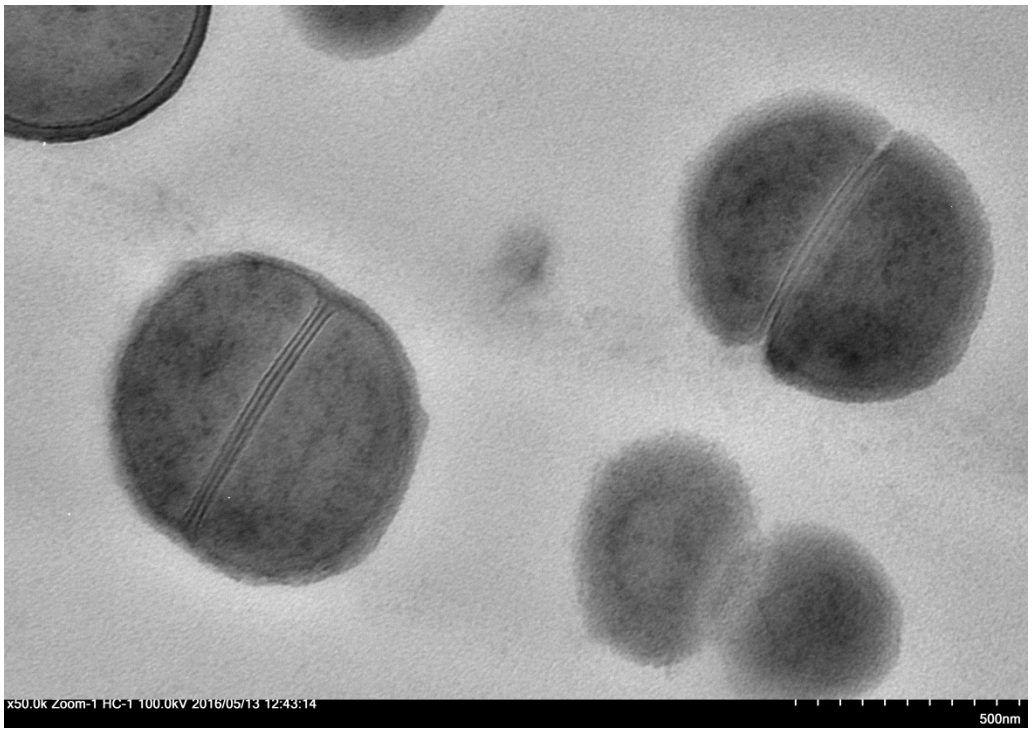


Figure 3.6.10: EM micrograph (strain 47) showing septum formation in 2 bacterial cells.

The cell to the top right appears to be beginning the separation of the replicated cells.

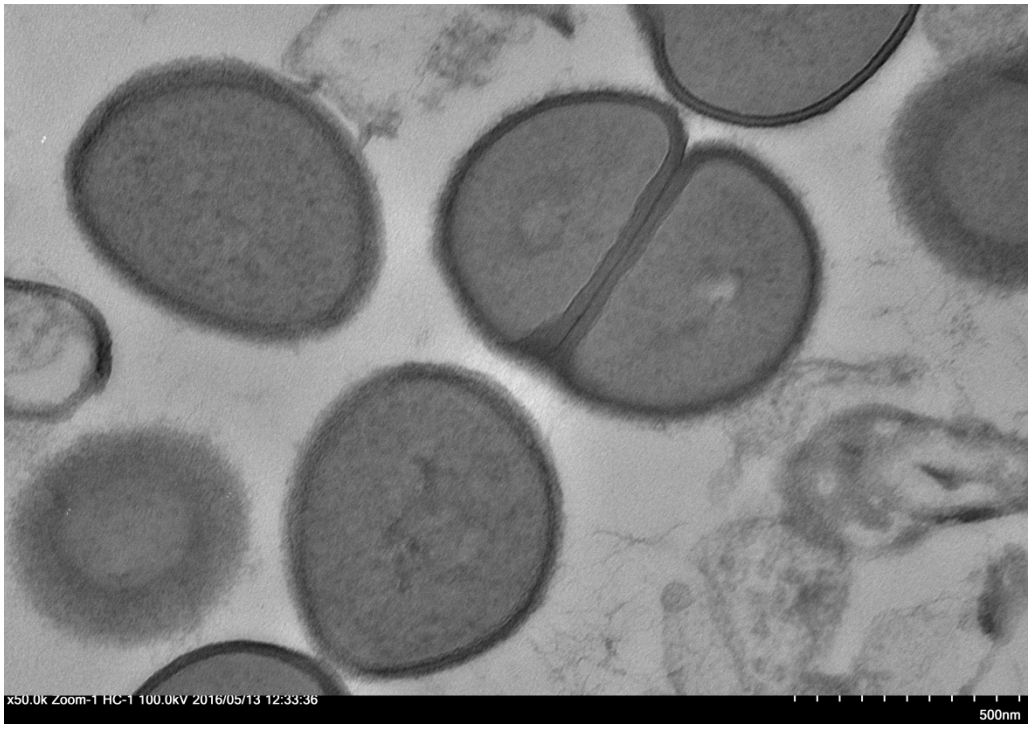


Figure 3.6.11: EM micrograph (strain 48) showing advanced septum formation in 1 cell.

"Pinching" on both sides of the cell suggest cell separation has begun.

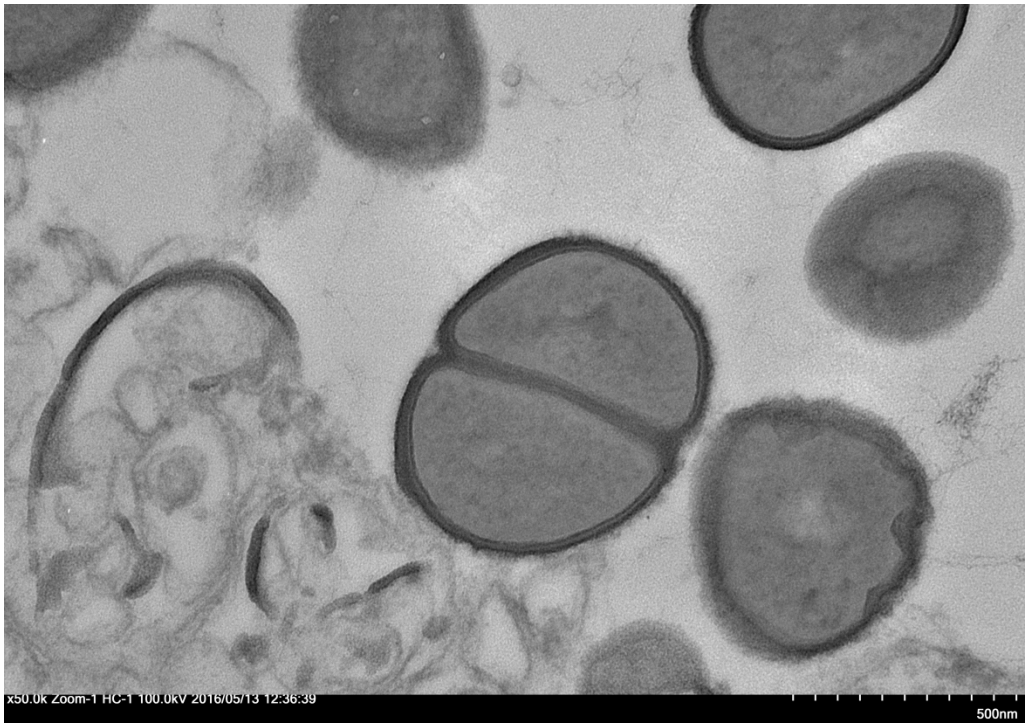


Figure 3.6.12: EM micrograph (strain 48) showing septum formation and the beginning of cell separation for 1 bacterial cell.

To address the possibility that some bacterial cells recovered from agar plates containing vancomycin might not be exposed to the drug and this could therefore be an explanation for organism growth in the presence of the drug, a second set of experiments were conducted. In the following experiments, 6 strains (8, 11, 39, 47, 48, 49) were picked from MHB containing 32 µg/ml of vancomycin. Examination of multiple strains reduced the probability of a single strain phenomenon and the possibility that the observation is related to lack of drug exposure.

The following figures show bacterial cell reproduction when recovered from MHB media after drug exposure: Figures 3.6.13 and 3.6.14; 11-Figures 3.6.15 and 3.6.16; 39-Figures 3.6.17 and 3.6.18; 47-Figures 3.6.19 and 3.6.20; 48-Figures 3.6.21 and 3.6.22; 49-Figures 3.6.23 and 3.6.24 respectively.

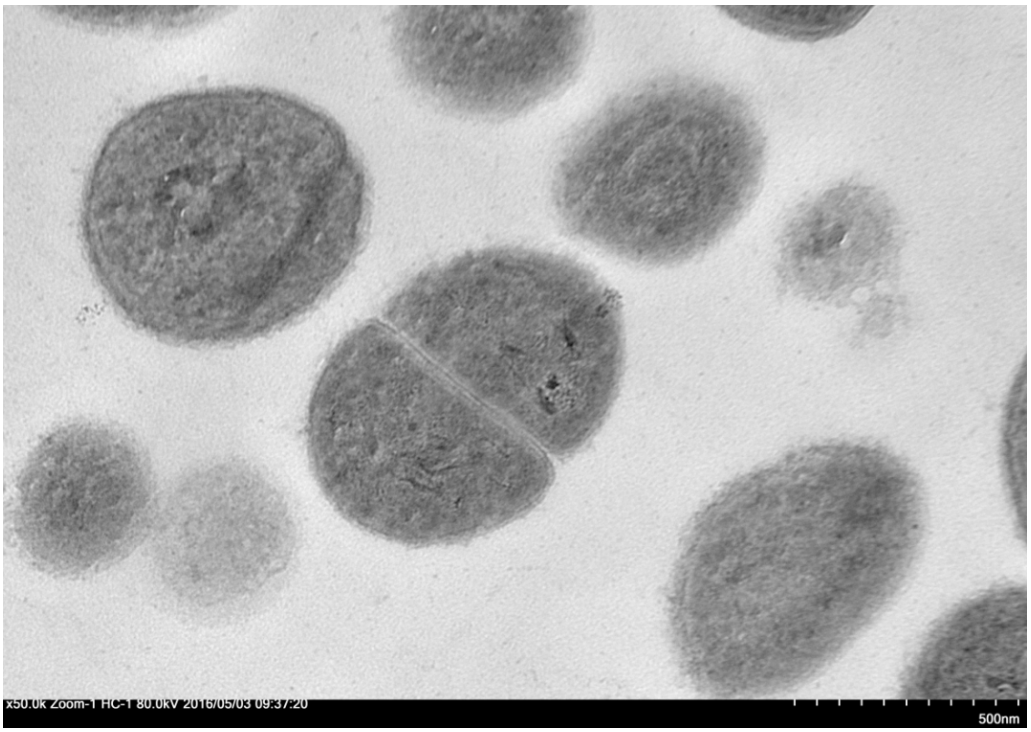


Figure 3.6.13: EM micrograph (strain 8) showing septum formation in 1 bacterial cell.

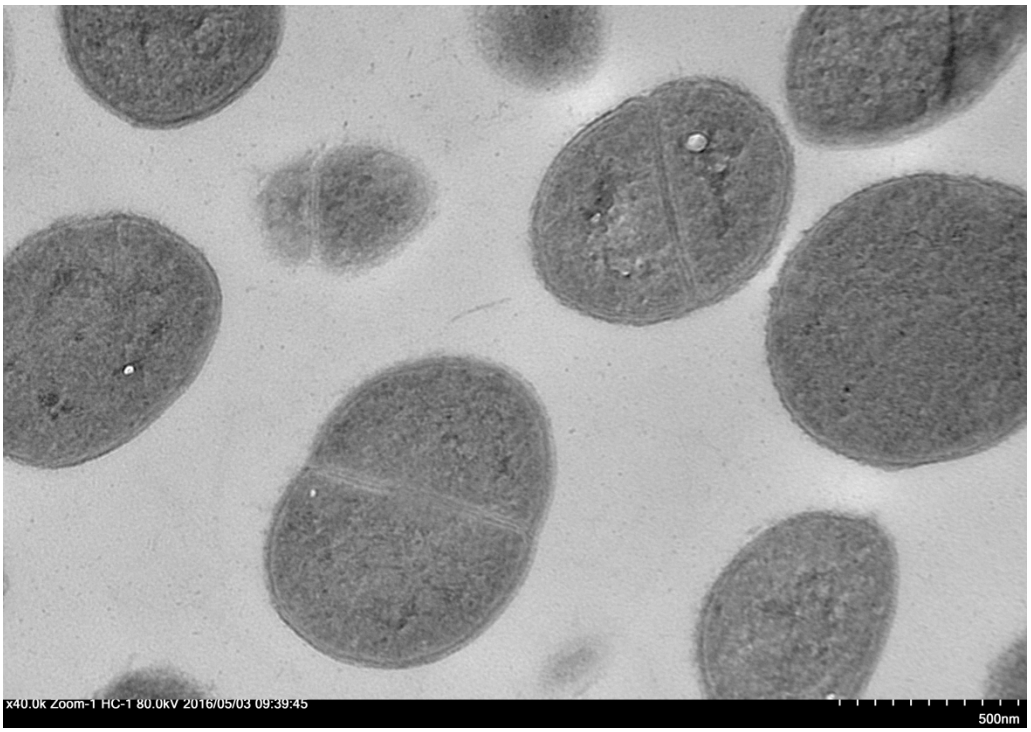


Figure 3.6.14: EM micrograph (strain 8) showing septum formation in multiple bacterial cells.

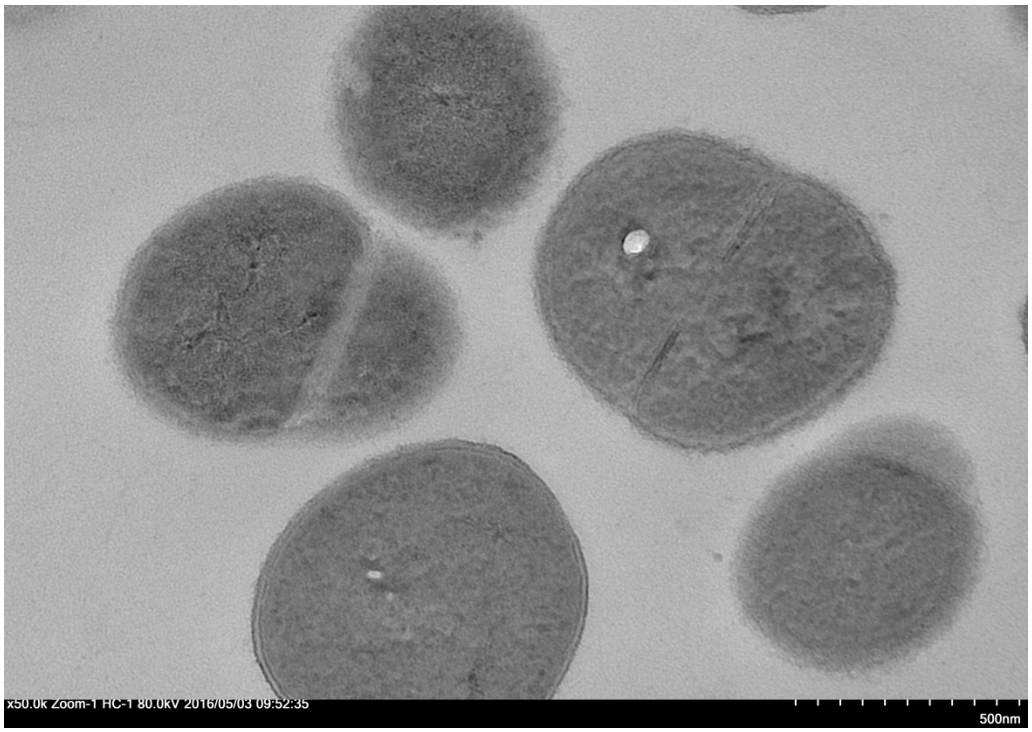


Figure 3.6.15: EM micrograph (strain 11) showing various stages of septum formation in 2 cells.

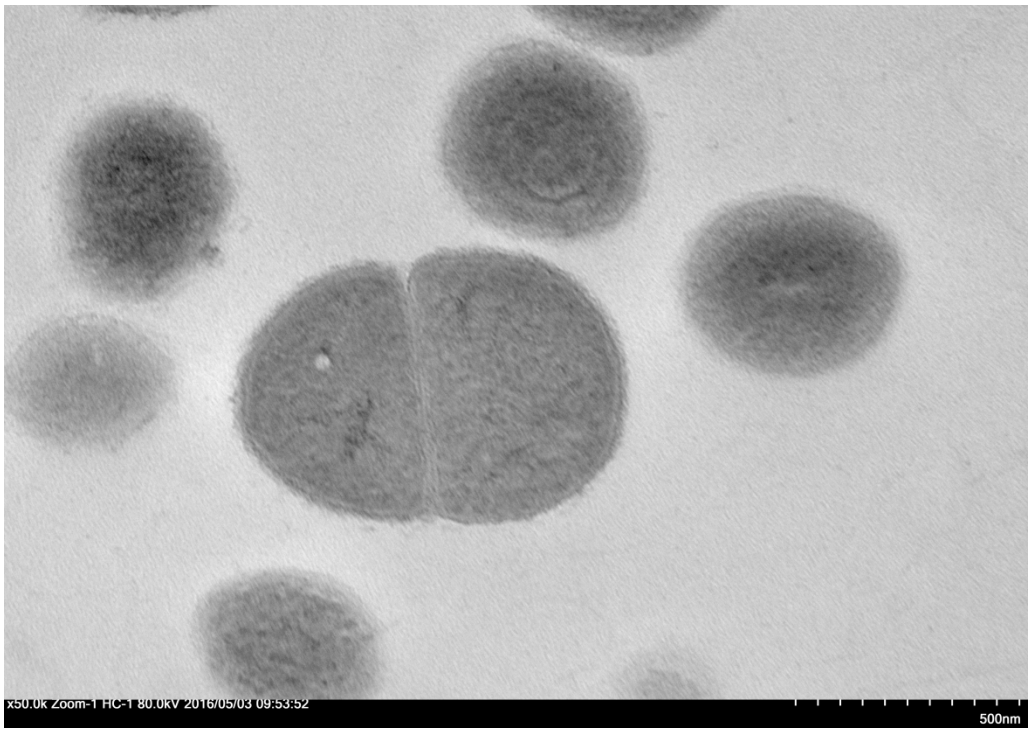


Figure 3.6.16: EM micrograph (strain 11) showing advanced septum formation and "pinching" indicating cell separation in 1 bacterial cell. Note increased size of the dividing cell.

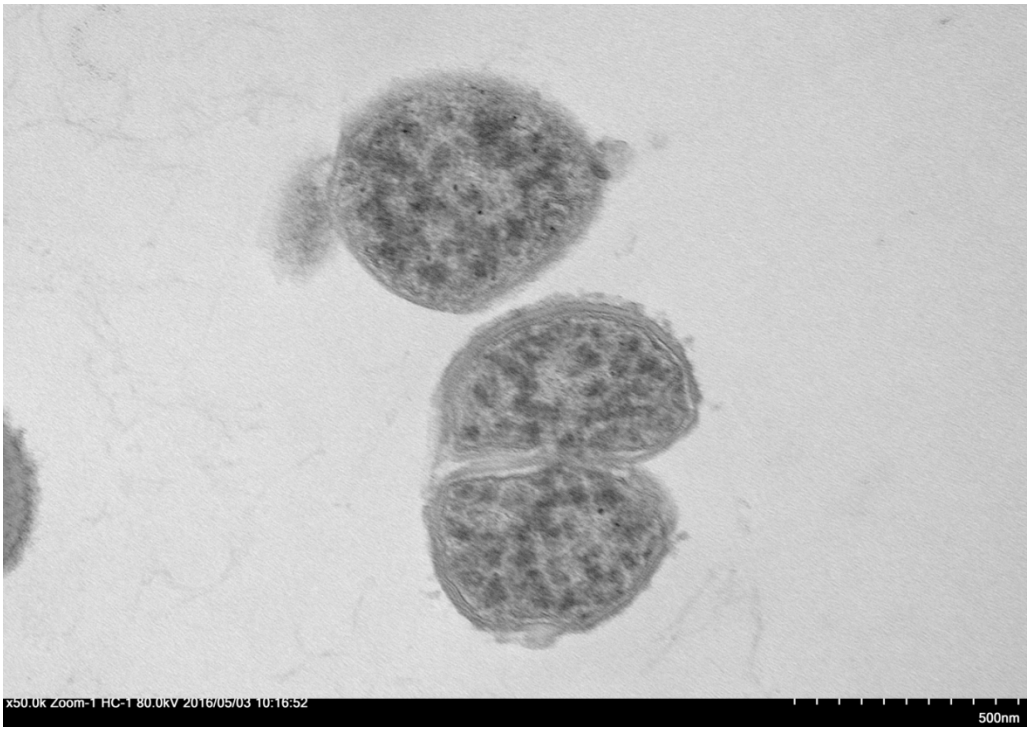


Figure 3.6.17: EM micrograph (strain 39) showing the final stages of cell separation for a dividing cell.

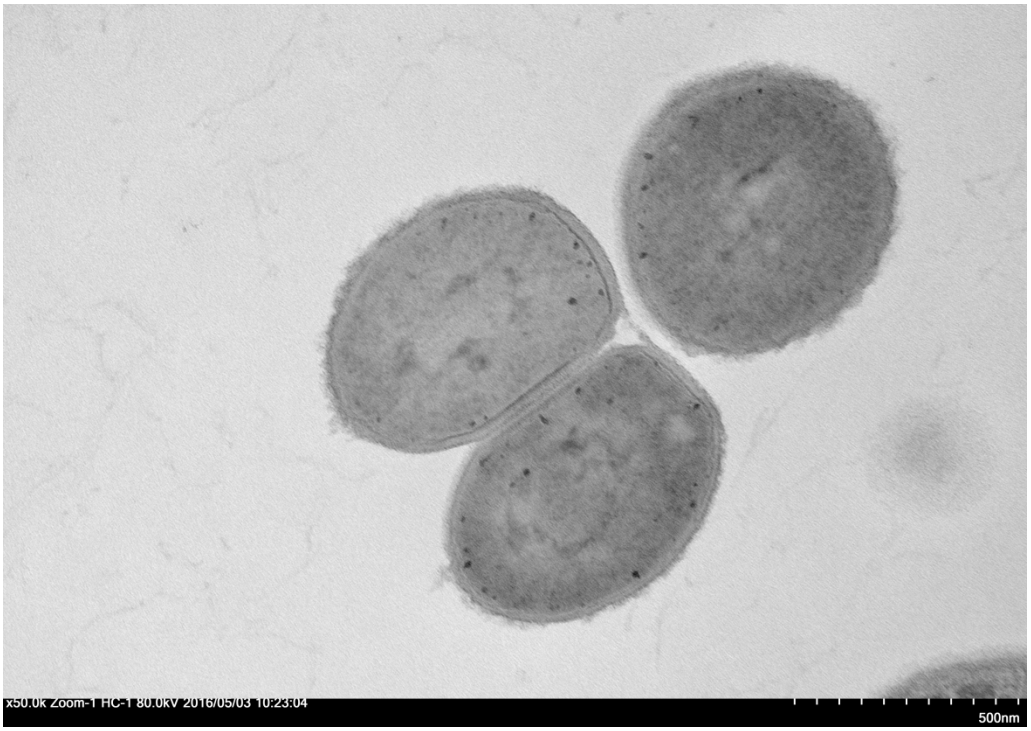


Figure 3.6.18: EM micrograph (strain 39) showing a dividing cell near its final stages of cell division.

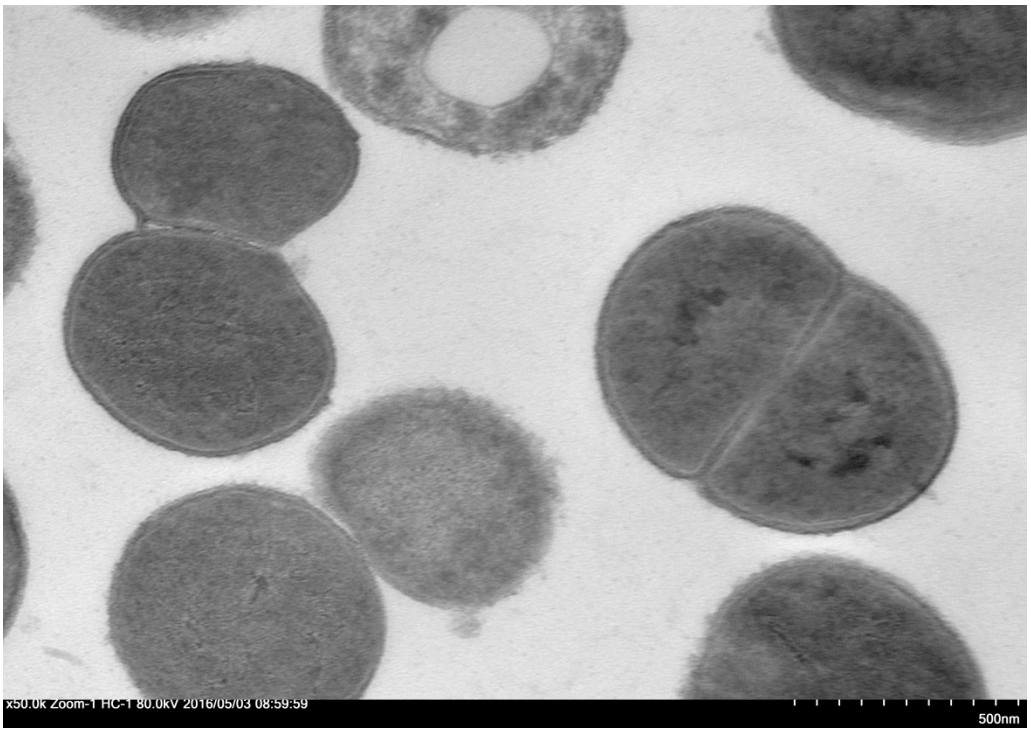


Figure 3.6.19: EM micrograph (strain 47) showing septum formation in 1 bacterial cell and the final stages of cell separation for 1 bacterial cell.

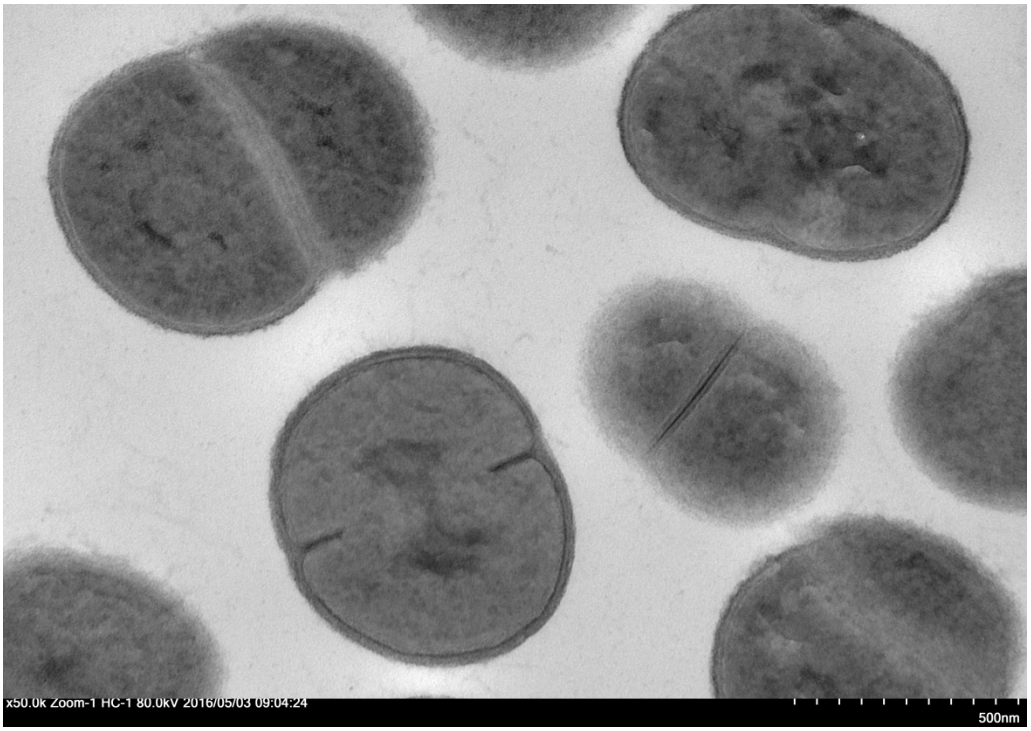


Figure 3.6.20: EM micrograph (strain 47) showing advanced septum formation and the start of septum formation in bacterial cells.

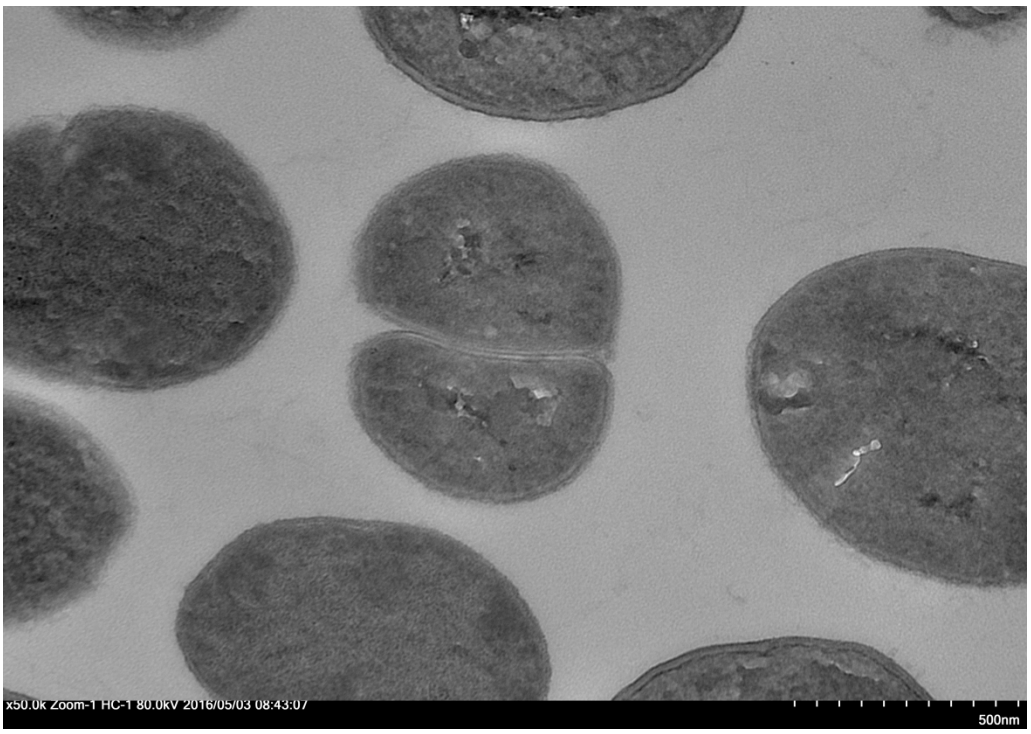


Figure 3.6.21: EM micrograph (strain 48) showing septum formation with bacterial cells.

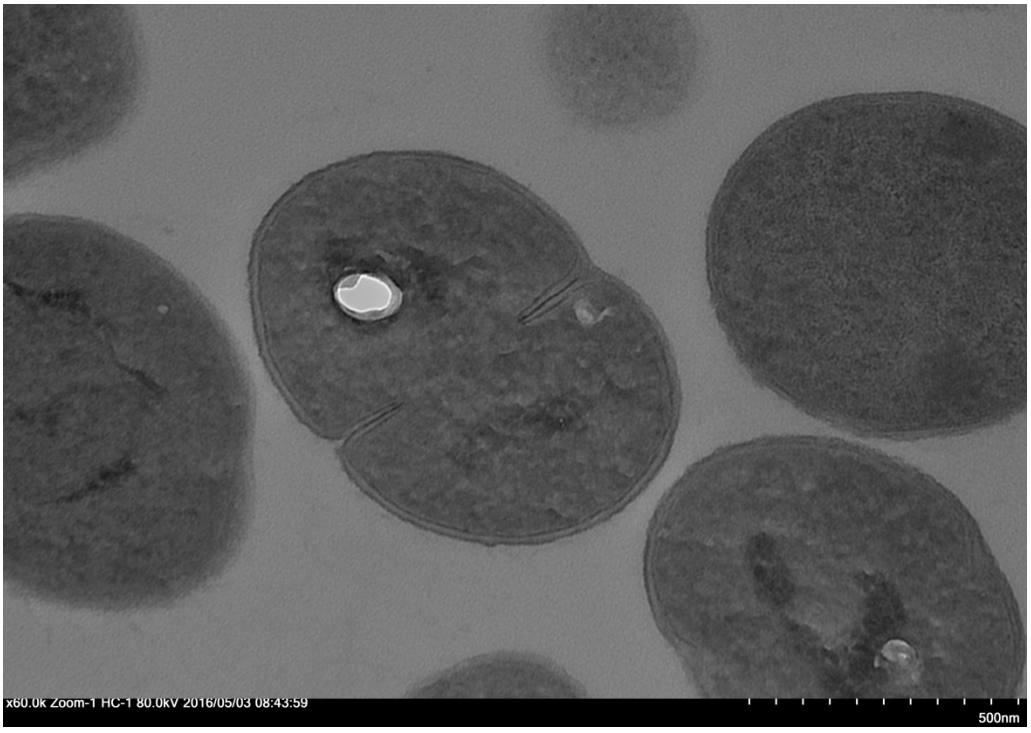


Figure 3.6.22: EM micrograph (strain 48) showing the early stages of septum formation in bacterial cells.

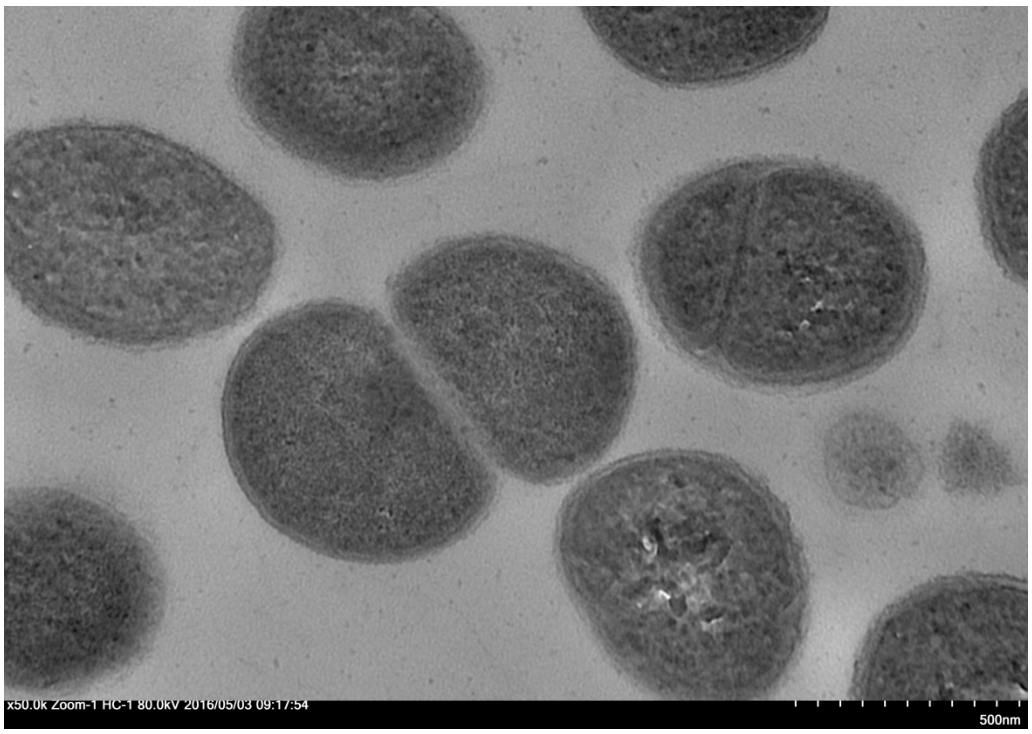


Figure 3.6.23: EM micrograph (strain 49) showing septum formation and the final stages of cell separation in bacterial cells.

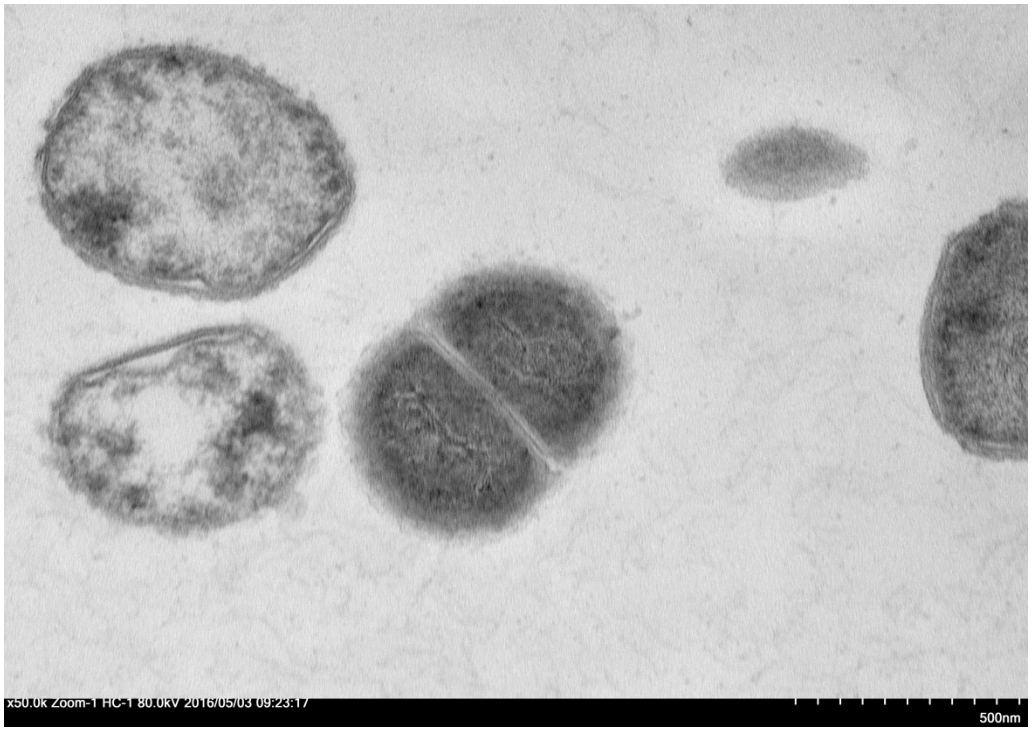


Figure 3.6.24: EM micrograph (strain 49) showing 1 bacterial cell with a fully formed septum.

3.7 Time-Kill Assay

The definition of bactericidal versus bacteriostatic is undergoing a restructuring. It is based on testing of 10^5 CFU/ml of organisms and with a reduction of viable cells ≥ 3 $\log_{10}$ being bactericidal and ≤ 2 $\log_{10}$ being bacteriostatic. Log_{10} reductions ≥ 2 $\log_{10}$ and ≤ 3 $\log_{10}$ are considered a gray zone. In previous publications [Blondeau & Shebelski, 2016; Blondeau*, et al*, 2015; Blondeau*, et al*, 2015] where higher bacterial densities were used in kill assays, some agents thought of as bacteriostatic actually showed substantial reductions in viable cells and additionally, for bactericidal agents, $\log_{10}$ reduction ≤ 3 were associated with >99% kill of bacterial cells. In light of the above published observations, we were interested in determining if linezolid and tedizolid inhibited bactericidal activity (these agents are currently considered as bacteriostatic by conventional measurements) when tested against higher bacterial densities. Vancomycin kill studies were also performed against varying bacterial densities. The following series of figures summarize the kill experiment for each drug.

For these kill experiments, MIC, MPC, maximum serum (C_{max}) and maximum tissue ($Tiss_{max}$) drug concentrations were used against bacterial densities ranging from 10^6-10^9 CFU/ml. The graphs are arranged in pairs with the $\log_{10}$ data in the first graph which is then followed by the graph expressed as percentage kill. Kill data is shown for each strain individually and 3 summary calculations: all isolates, strain 41 excluded from the summary analysis and strains 28 and 41 excluded from the summary analysis. Strains 28 and 41 consistently responded differently than the other 2 strains in the kill assay and as such, were included and excluded from the averaged comparisons.

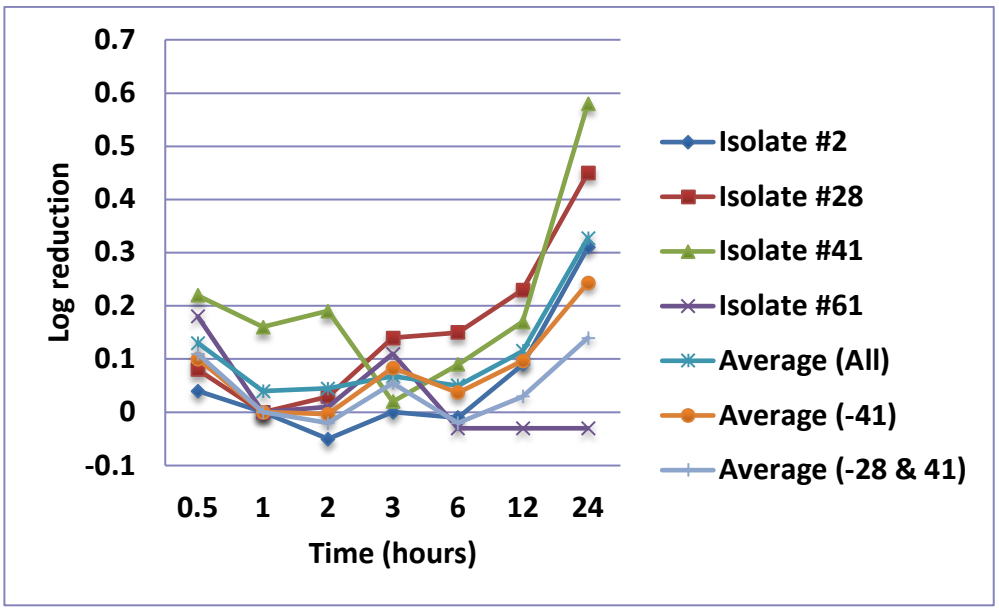


Figure 3.7.1: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

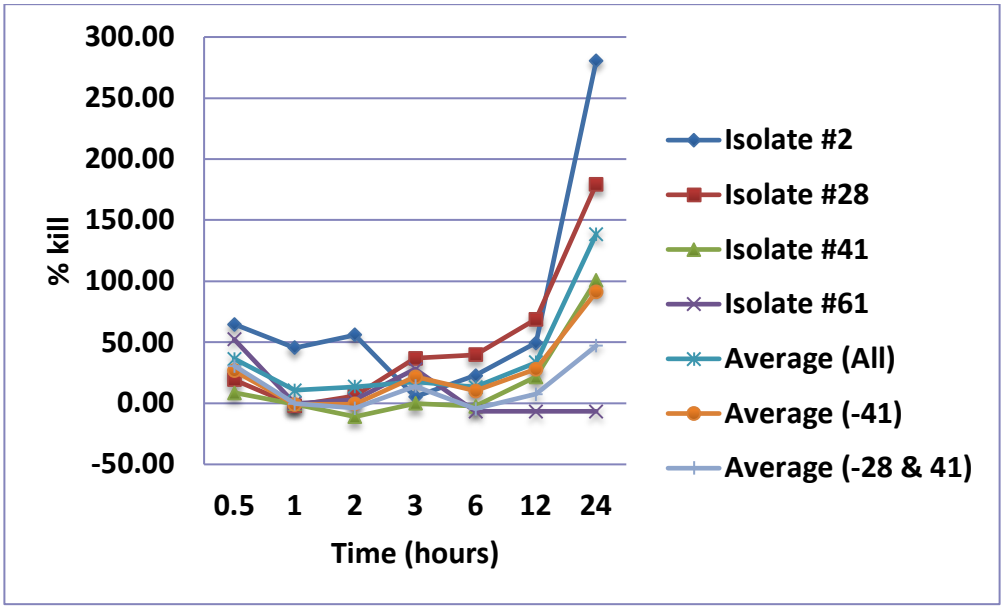


Figure 3.7.2: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

At a 10^6 CFU/ml inocula, MIC and MPC drug concentrations failed to achieve substantial killing against any of the 4 strains tested.

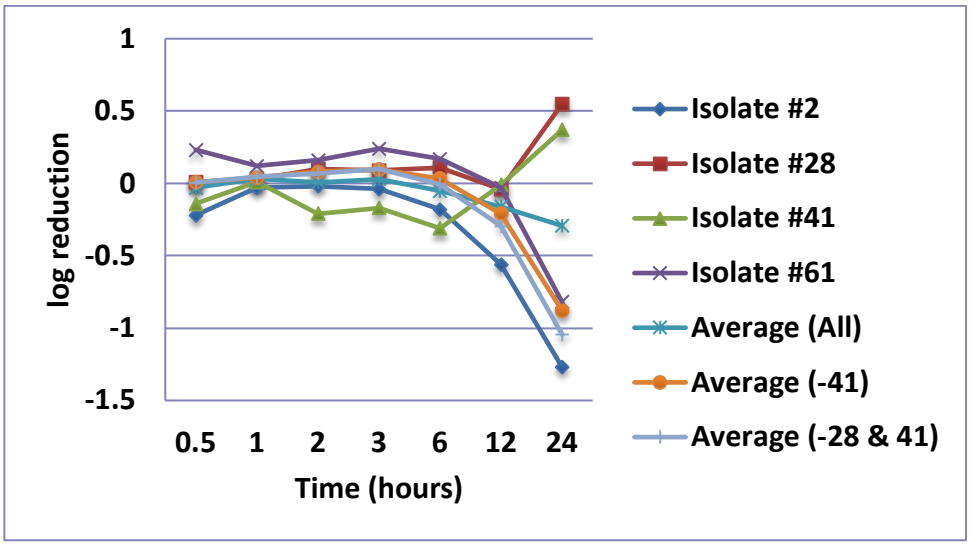


Figure 3.7.3: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

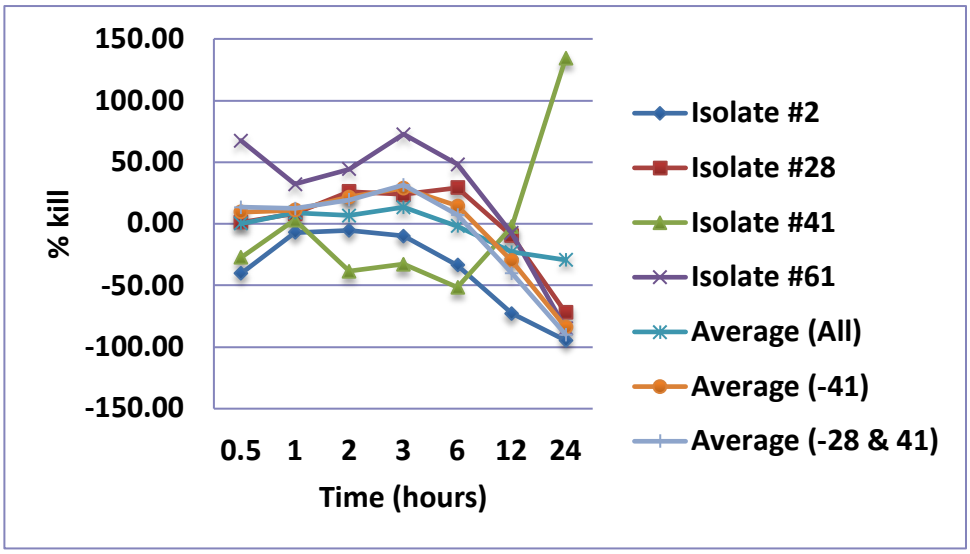


Figure 3.7.4: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

Exposure of the 4 MRSA strains to the $Tiss_{max}$ drug concentration of linezolid using 10^6 CFU/ml showed substantial reductions in viable cells for strains 2 and 61 with >90% kill following 24 hours of drug exposure. Approximately 75% of cells from strain 28 were killed following 12 hours of drug exposure. Note that 24 hours following drug exposure, >90% of viable cells were killed for some strains and this was based on a 1.3 $\log_{10}$ reduction.

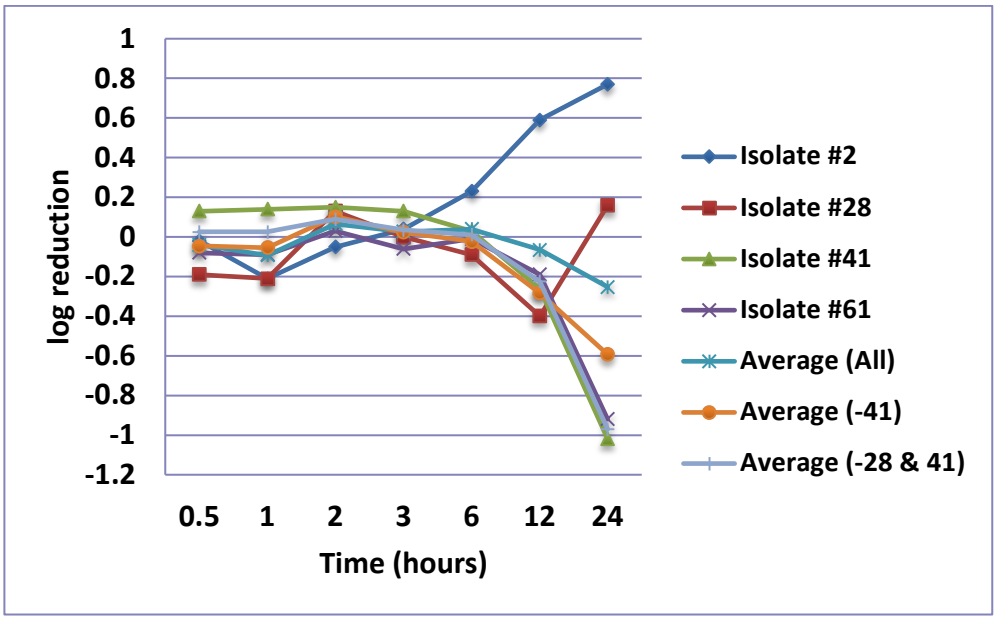


Figure 3.7.5: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the C_{max} drug concentration of linezolid.

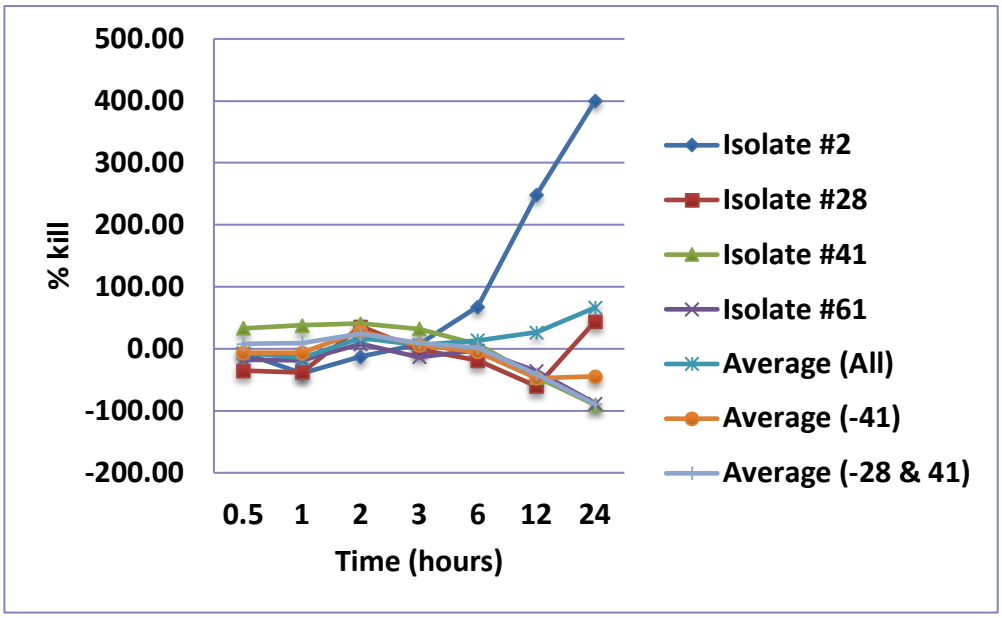


Figure 3.7.6: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the C_{max} drug concentration of linezolid.

Exposure of 10^6 CFU/ml to the C_{max} drug concentration of linezolid yielded a <1 to >1 $\log_{10}$ reduction for strains 41 and 61 which corresponded to a >90% kill.

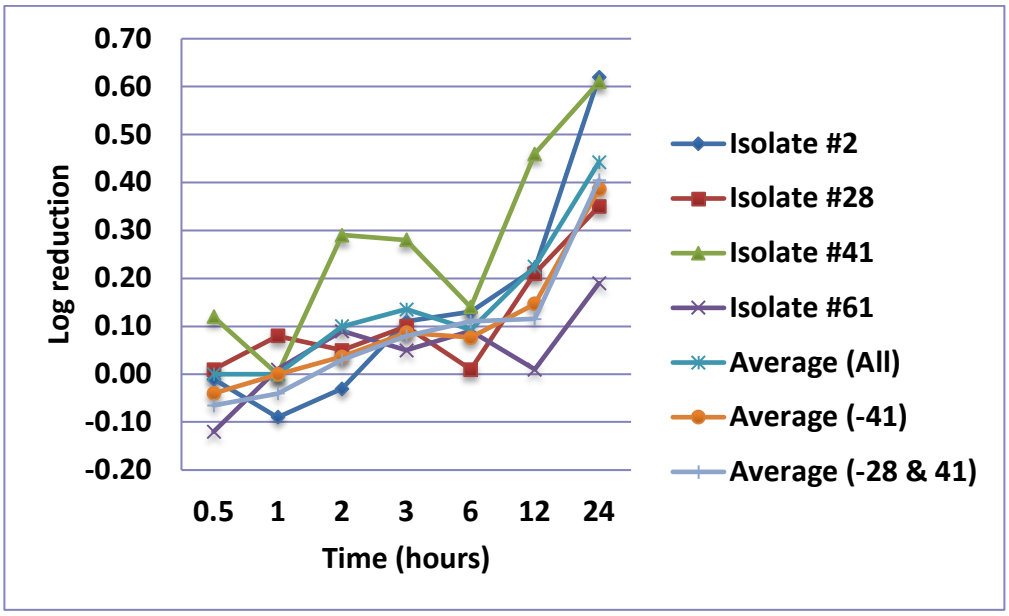


Figure 3.7.7: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

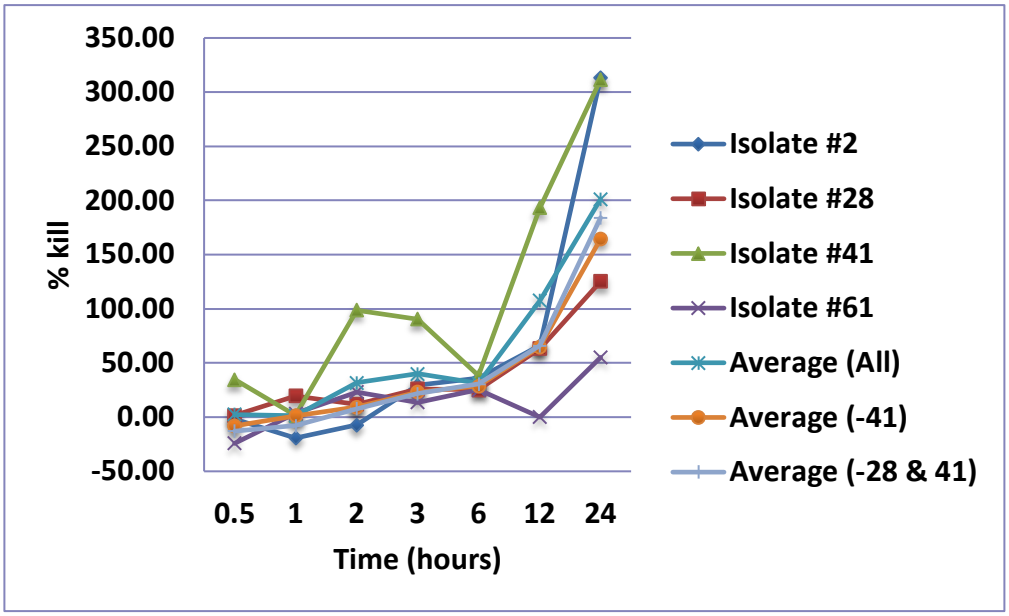


Figure 3.7.8: Percent kill of MRSA strain at 10 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

Exposure of 10^7 CFU/ml to the MIC or MPC drug concentration of linezolid failed to yield significant killing for any strain tested.

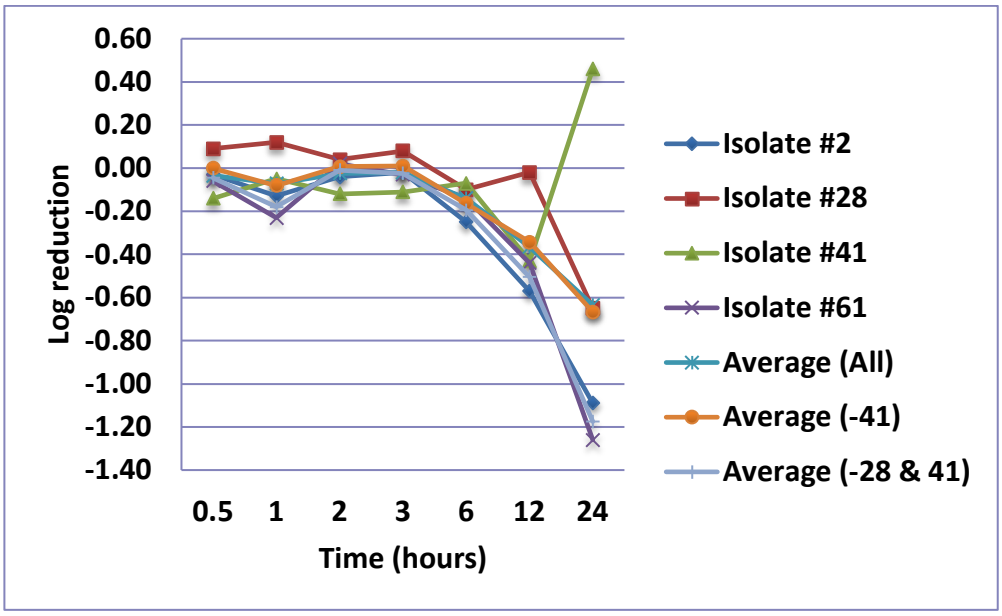


Figure 3.7.9: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

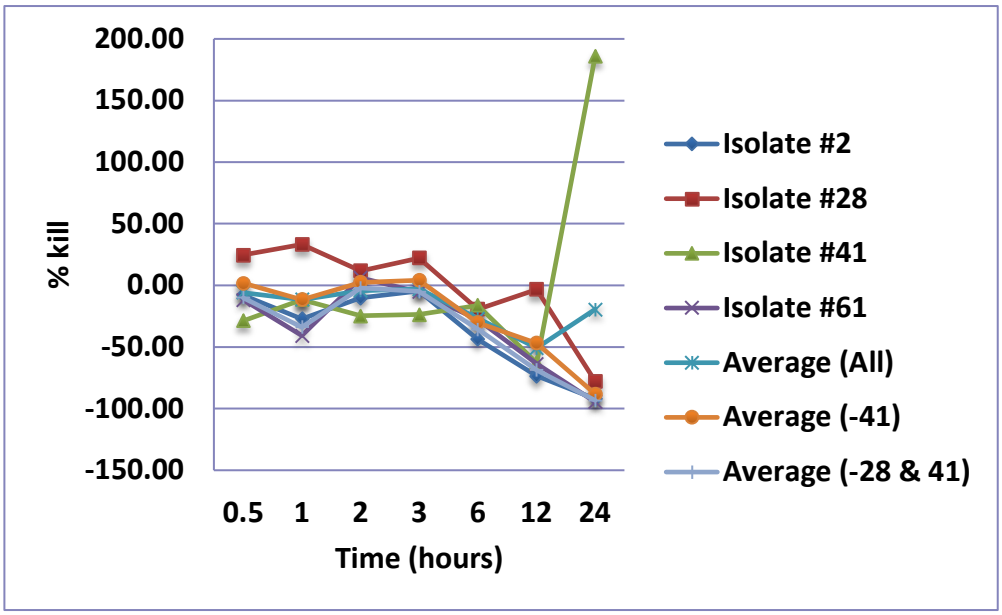


Figure 3.7.10: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

Exposure of 10^7 CFU/ml to the $Tiss_{max}$ drug concentration of linezolid resulted in log_{10} reduction of 0.4 to >1.0 following 12 and 24 hours of drug exposure respectively for strains 2 and 61 or >50 to >90% kill. Strain 41 showed initial decline in the presence of linezolid but regrew following 12 hours of drug exposure.

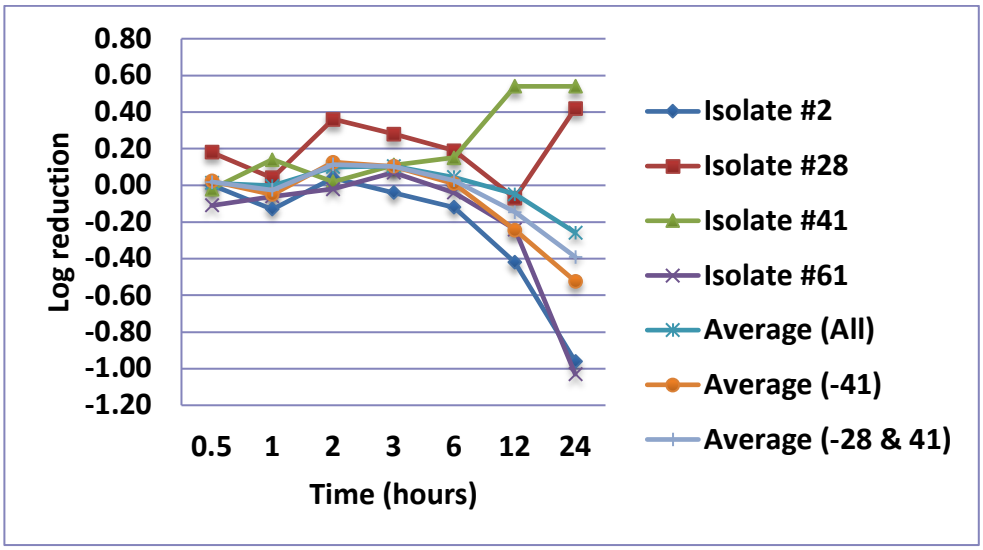


Figure 3.7.11: Log reduction of MRSA strain at 10 CFU/ml inocula using the C_{max} drug concentration of linezolid.

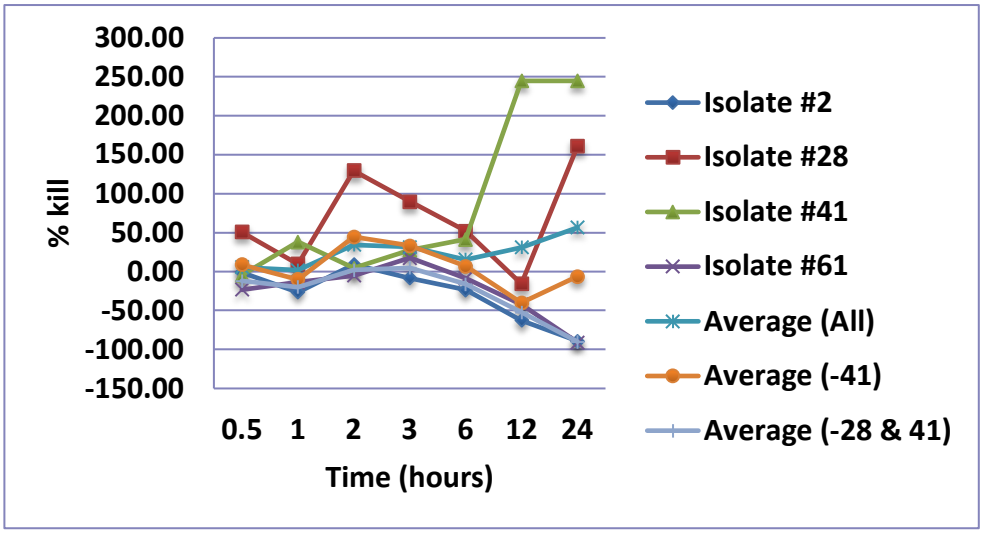


Figure 3.7.12: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the C_{max} drug concentration of linezolid.

Exposure of 10^7 CFU/ml to the C_{max} linezolid drug concentration showed a 0.2-0.5 $\log_{10}$ reduction in viable cells for strains 61 and 2 following 12 hours of drug exposure and an approximately 1 $\log_{10}$ reduction following 24 hours of drug exposure. Greater than 50% of viable cells (strains 2 and 61) were killed following 12 hours and >90% following 24 hours of drug exposure. For strains 28 and 41 growth in the presence of drug occurred.

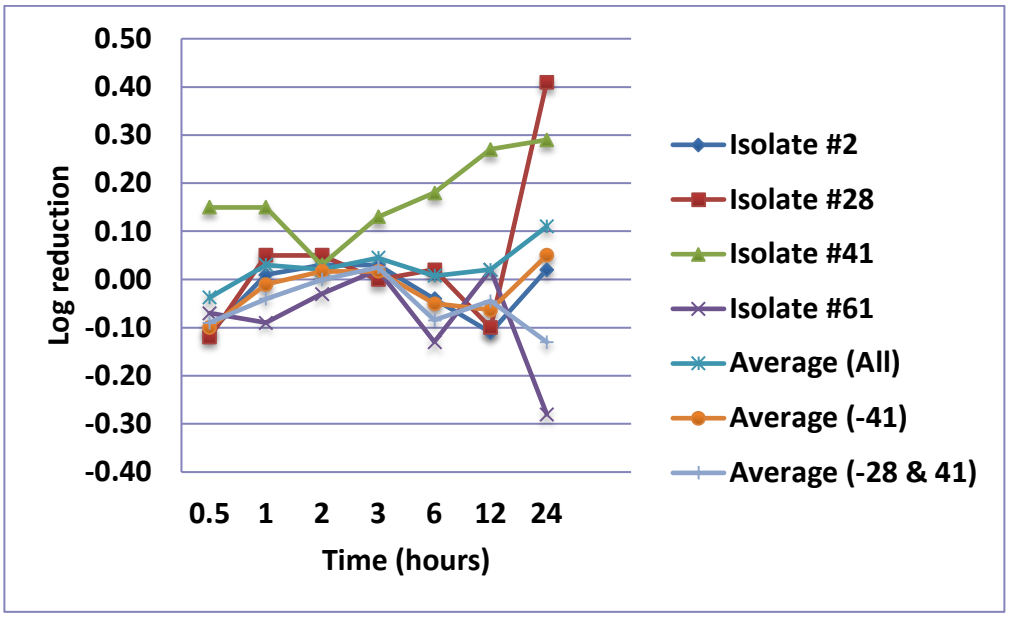


Figure 3.7.13: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

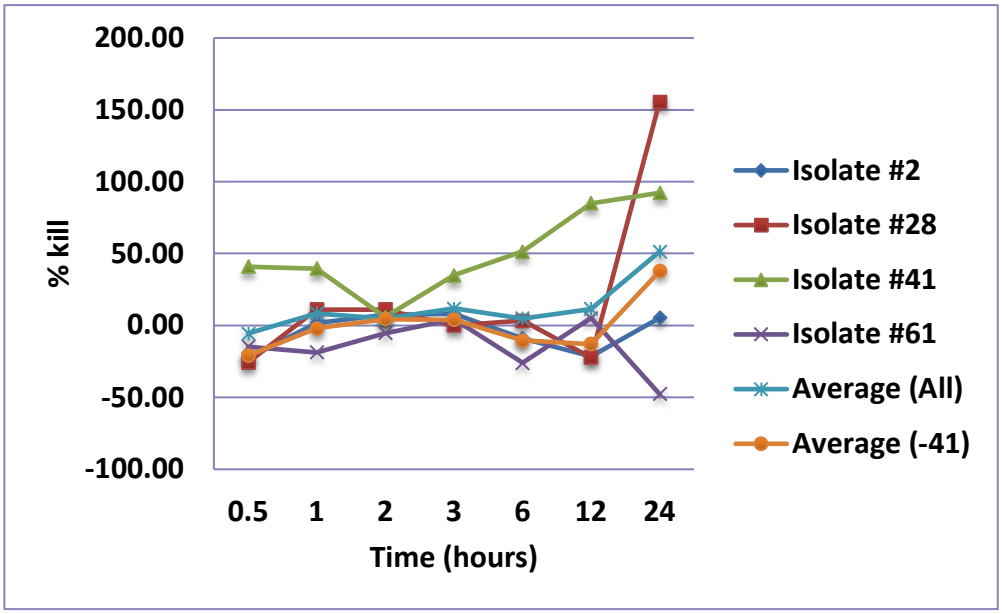


Figure 3.7.14: Percent kill of MRSA strain at 10 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

Exposure of 10^8 CFU/ml to the MIC and MPC drug concentration for linezolid failed to result in substantial killing for any strain tested, however, for strain 61 a 0.28 $\log_{10}$ reduction (approximately 50% kill) was seen following 24 hours of drug exposure.

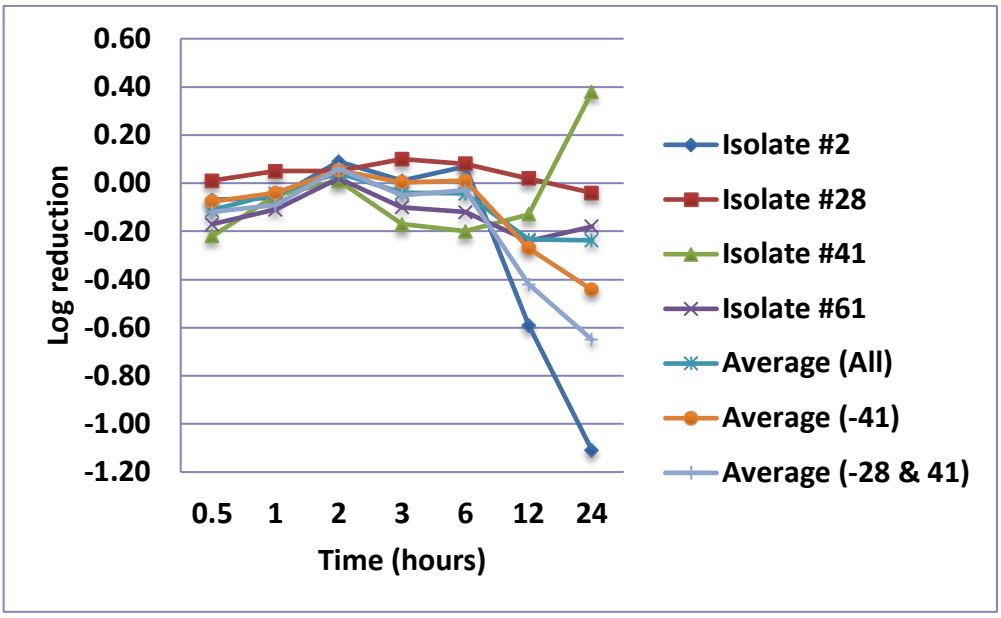


Figure 3.7.15: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

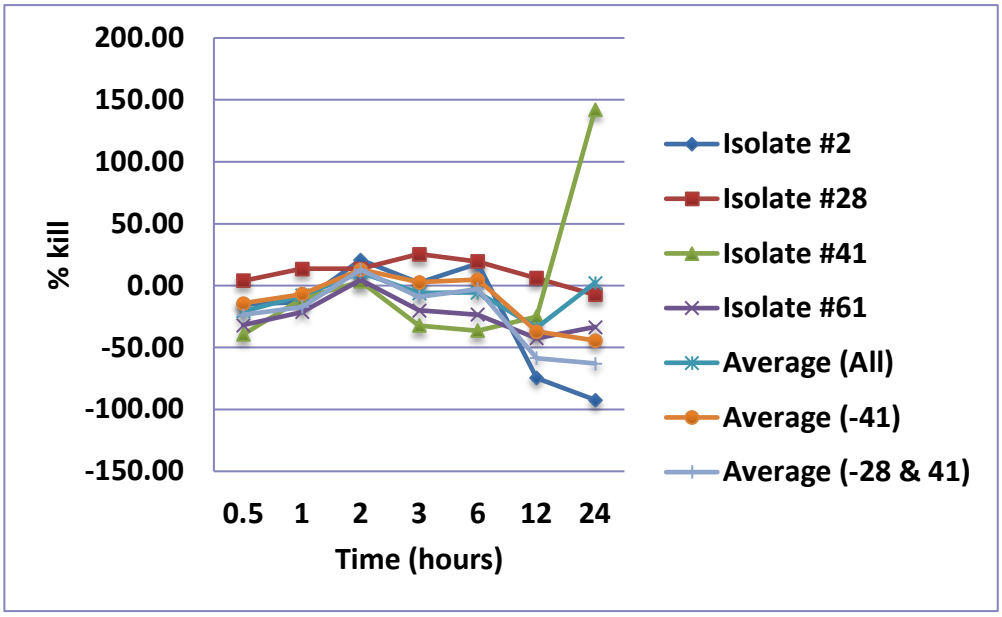


Figure 3.7.16: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

Exposure to 10^8 CFU/ml to the linezolid $Tiss_{max}$ drug concentration resulted in a 0.6-1.1 log_{10} reduction for strain 2 following 12 and 24 hours respectively of drug exposure, 70% and 79% kill. For the other strains either growth occurred or killing was <50% following 24 hours of drug exposure.

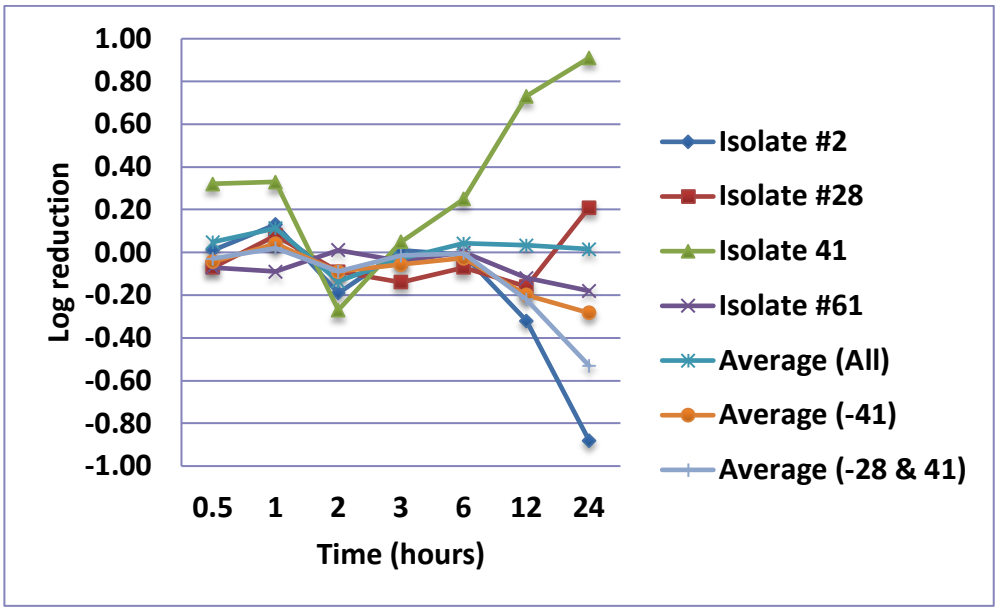


Figure 3.7.17: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the C_{max} drug concentration of linezolid.

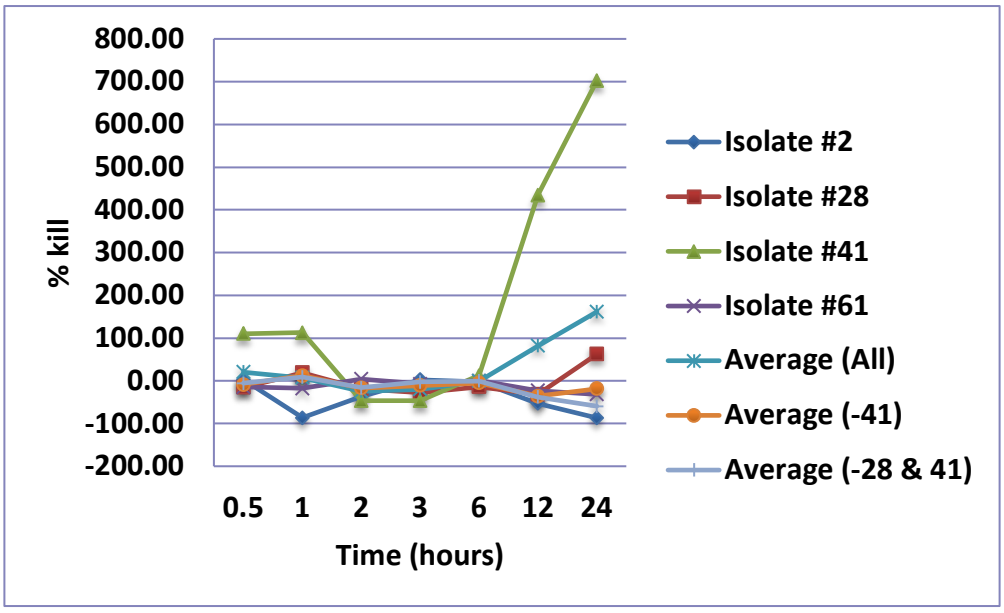


Figure 3.7.18: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the C_{max} drug concentration of linezolid.

Exposure of 10^8 CFU/ml to the linezolid C_{max} drug concentration yielded a 0.3 $\log_{10}$ reduction (~50% kill) following 12 hours of drug exposure for strain 2 as compared to a 0.9 $\log_{10}$ reduction (>90% kill) following 24 hours of drug exposure. Strain 61 saw a 0.2 $\log_{10}$ reduction in viable cells following 24 hours of drug exposure. Growth was seen for strains 41 and 28.

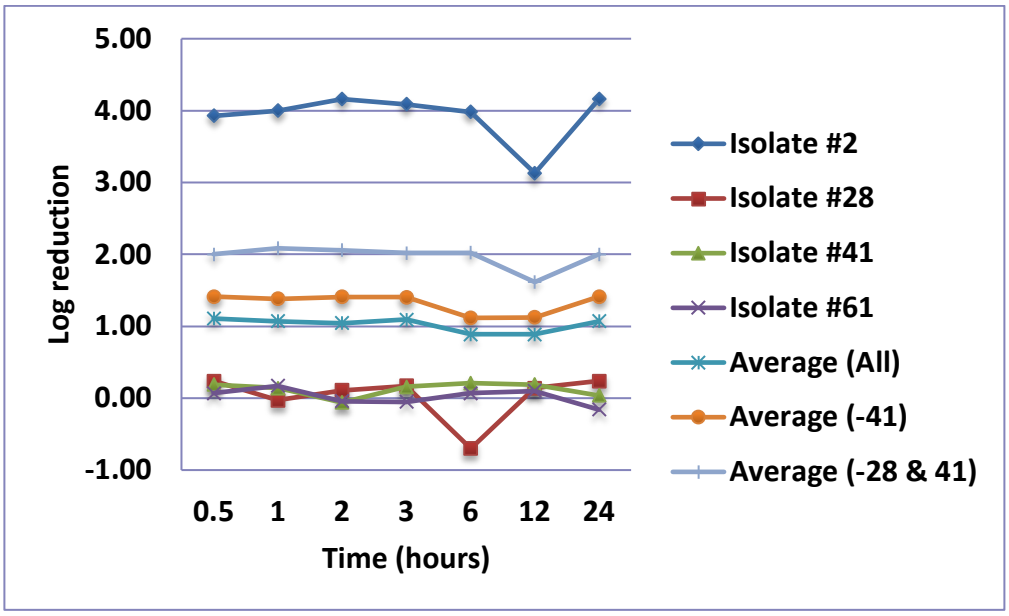


Figure 3.7.19: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

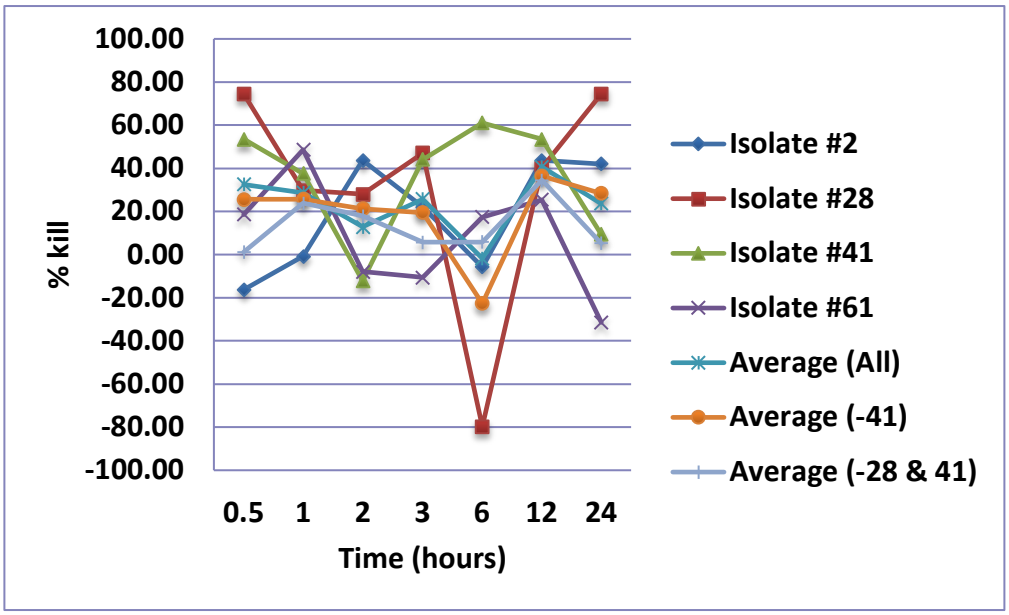


Figure 3.7.20: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

Exposure of 10^9 CFU/ml to the MIC or MPC drug concentration of linezolid yielded inconsistent results with the overall trend being growth in the presence of the drug for all strains at some time points measured. For strain 61, only 30% of viable cells were killed following 24 hours of drug exposure.

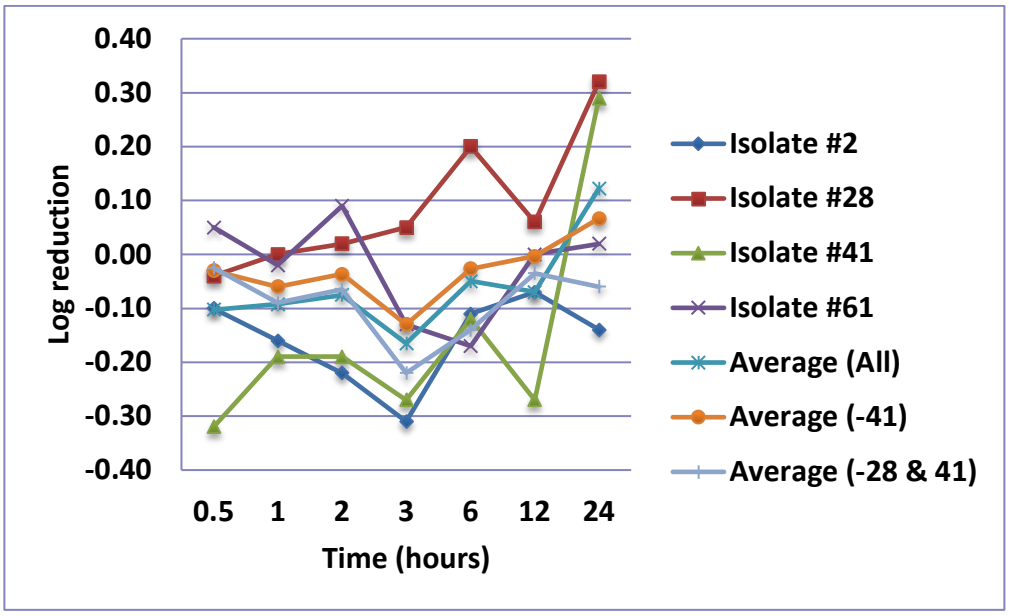


Figure 3.7.21: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

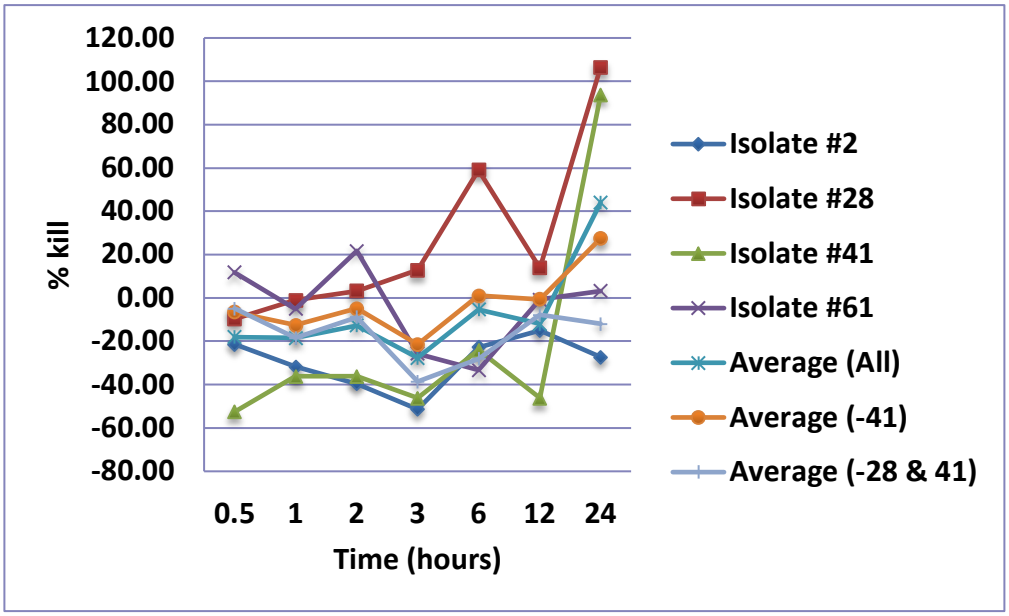


Figure 3.7.22: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

Exposure of 10^9 CFU/ml to the linezolid $Tiss_{max}$ drug concentration showed some initial reduction in viable cells within the first 3 hours of drug exposure followed by growth at the 6, 12 and 24 hour intervals. No more than 25% of viable cells were killed for strain 2 following 24 hours of drug exposure.

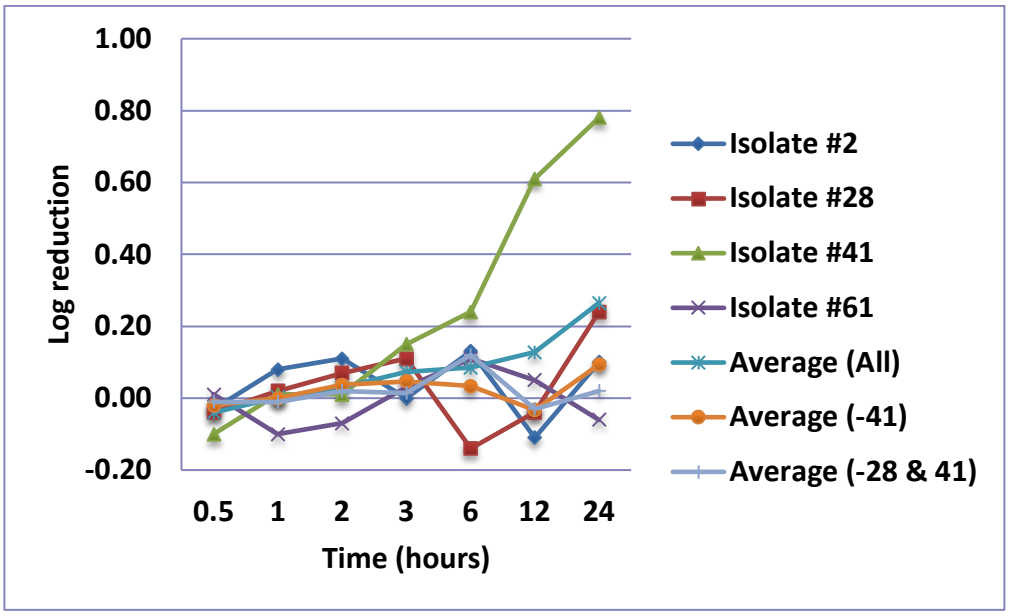


Figure 3.7.23: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the C_{max} drug concentration of linezolid.

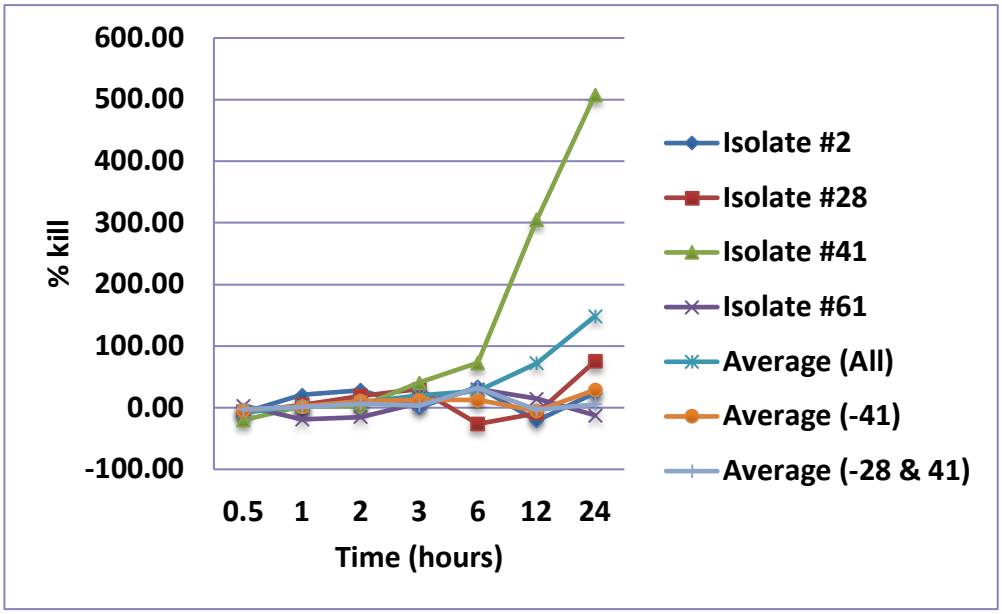


Figure 3.7.24: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the C_{max} drug concentration of linezolid.

Exposure of 10^9 CFU/ml of the linezolid C_{max} drug concentration failed to result in substantial killing of any strain tested. Substantial growth occurred for strain 41.

Tedizolid

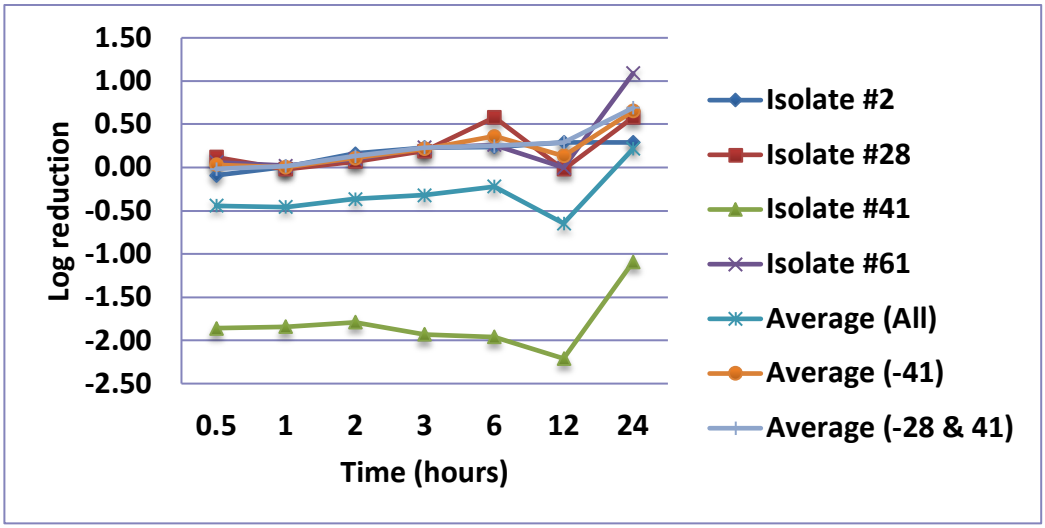


Figure 3.7.25: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

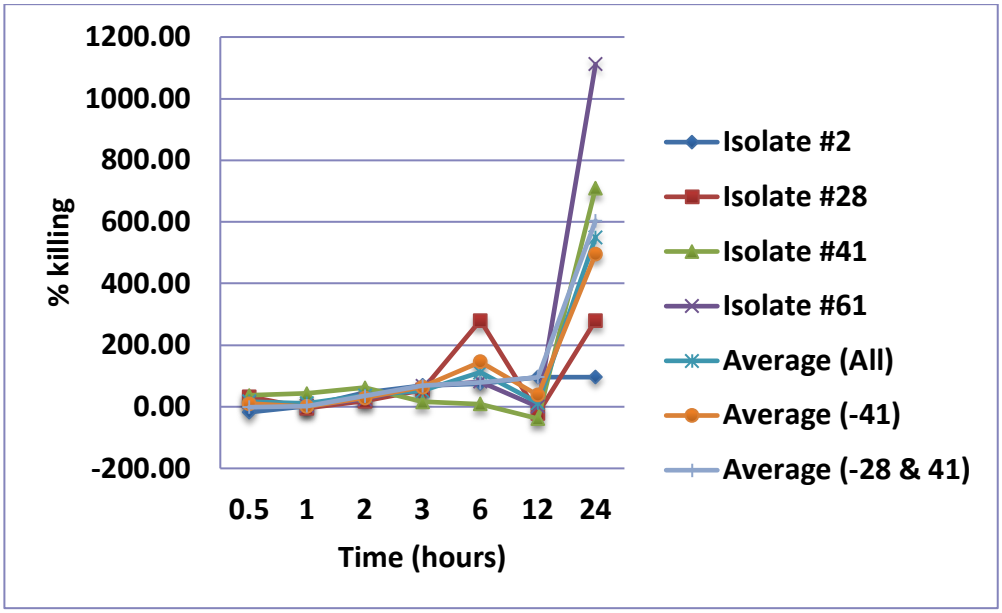


Figure 3.7.26: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

Exposure of 10^6 CFU/ml to the MIC or MPC tedizolid drug concentration failed to result in substantial killing for most strains tested. For strain 41, 1 1.8-2.3 $\log_{10}$ reduction was seen following 0.5-12 hour of drug exposure, however, regrowth occurred between 12 and 24 hours of drug exposure.

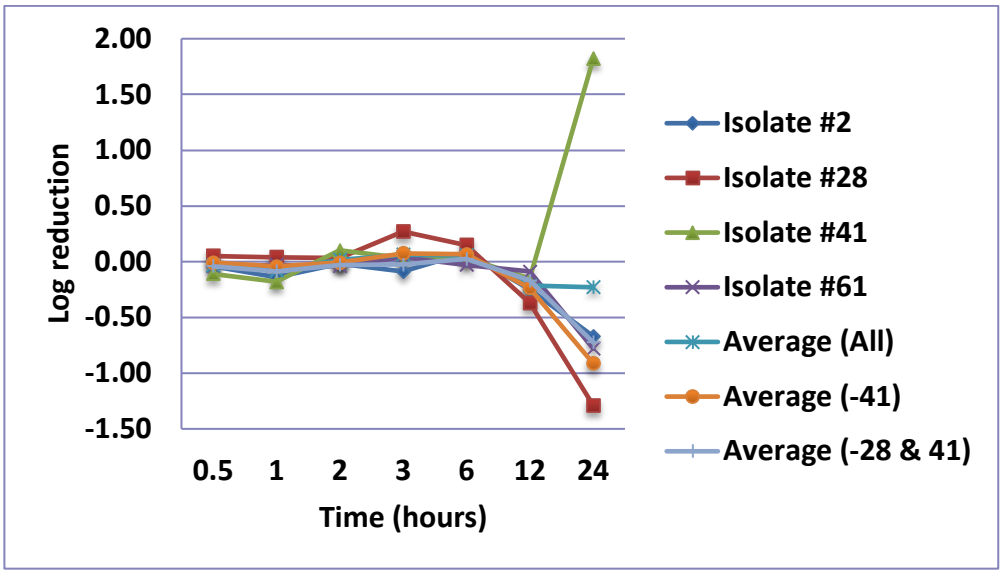


Figure 3.7.27: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

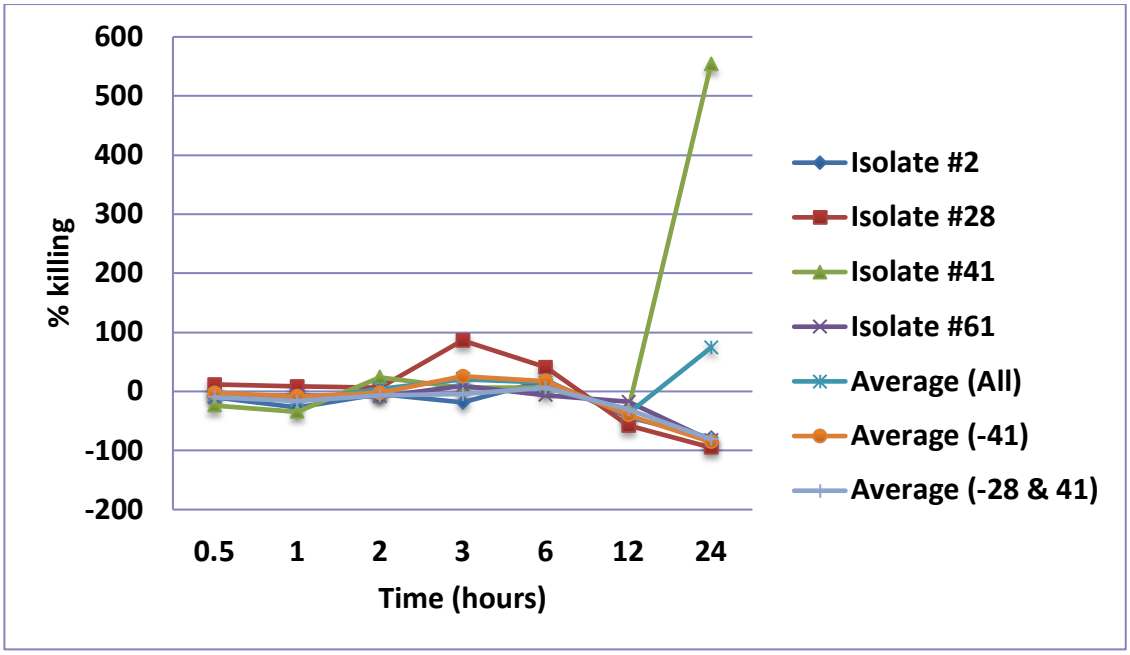


Figure 3.7.28: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

Exposure of 10^6 CFU/ml to the tedizolid $Tiss_{max}$ drug concentration resulted in a 1.3 log_{10} reduction for strain 28 following 24 hours of drug exposure which translated to a >99% kill. Reductions were also seen for strains 2 and 61 with >50% kill. Strain 41 showed substantial growth following 12 hours of drug exposure.

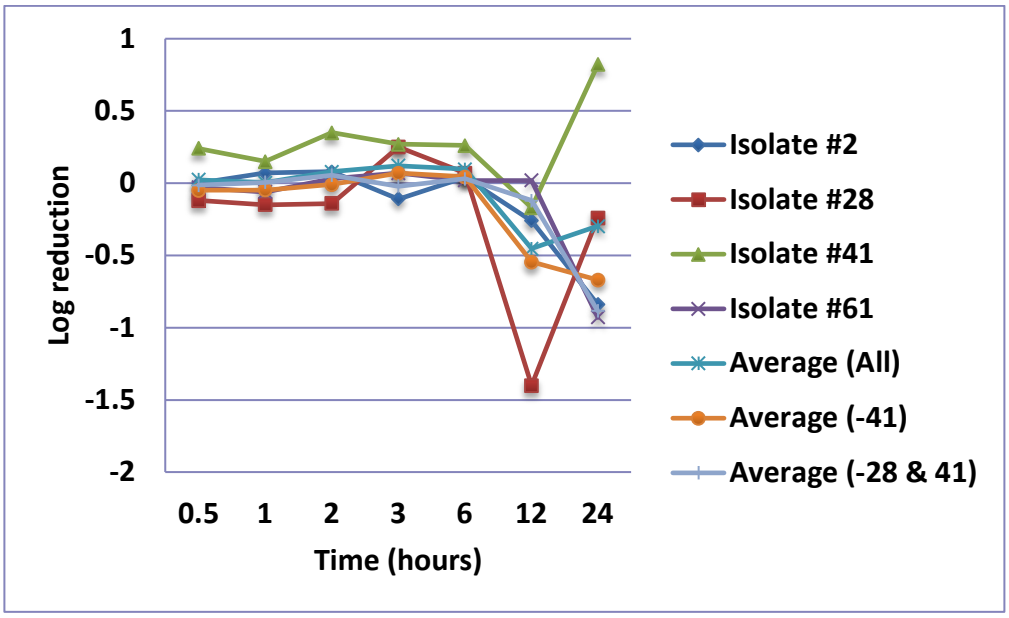


Figure 3.7.29: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

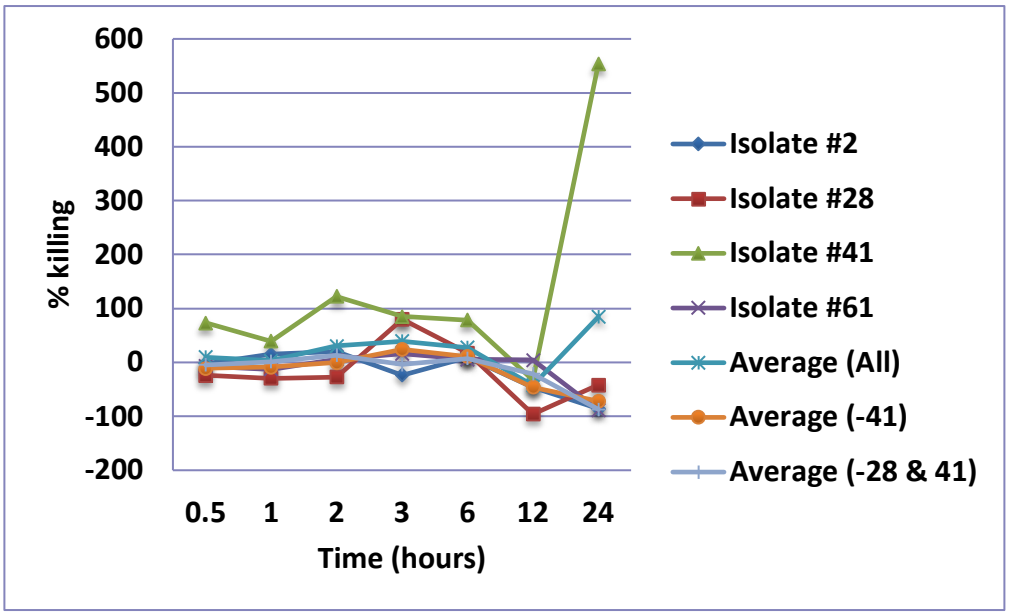


Figure 3.7.30: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

Exposure of 10^6 CFU/ml to the tedizolid C_{max} drug concentration resulted in $\log_{10}$ reduction of <1 following 24 hours of drug exposure for strains 2 and 61. Strain 28 showed>90% kill following 12 hours of drug exposure but had increased growth in the presence of the drug between 12 and 24 hours. Strain 41 was not inhibited by the drug.

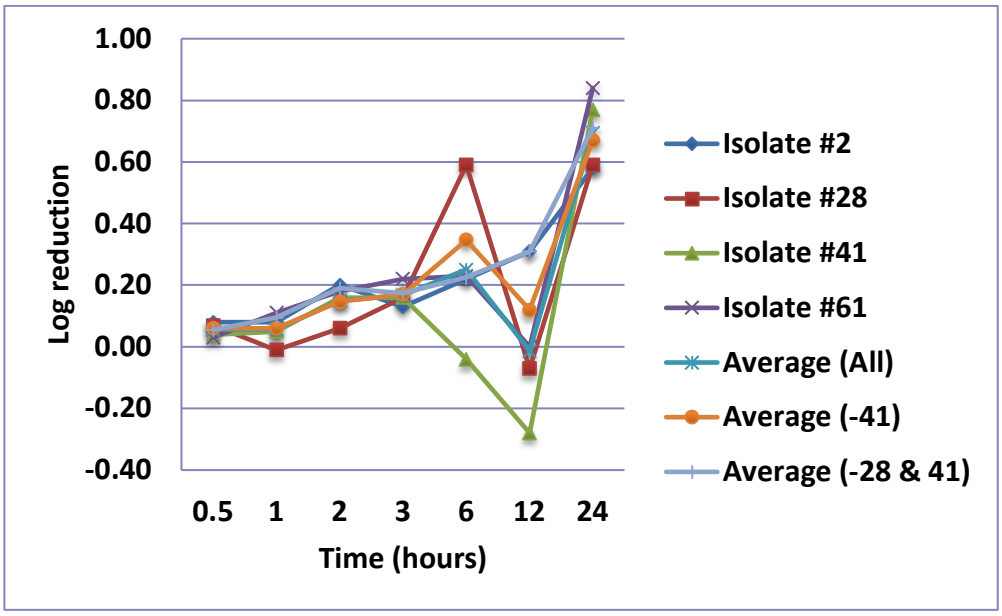


Figure 3.7.31: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

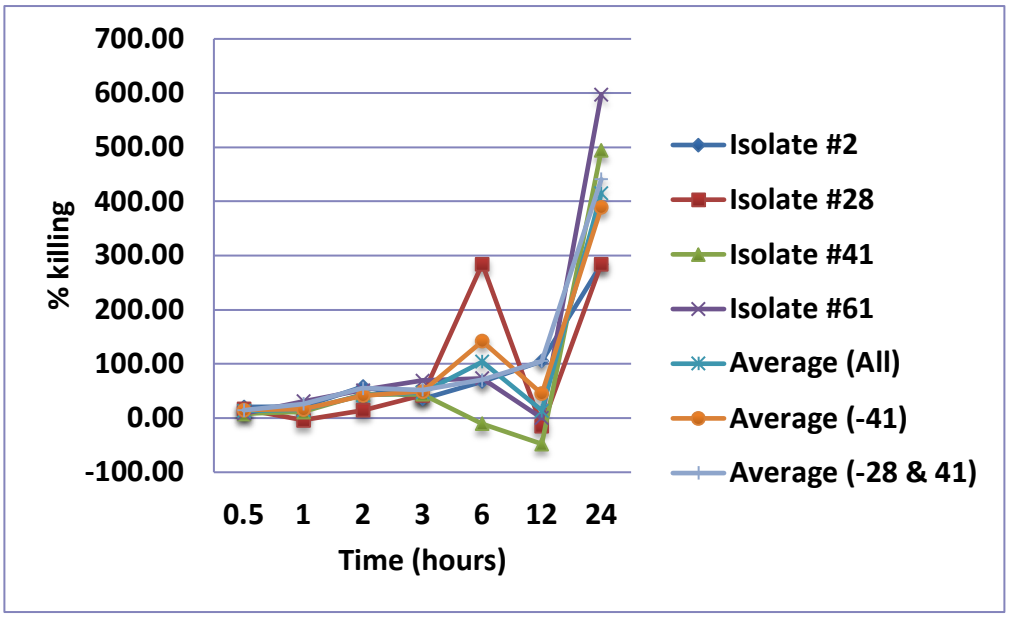


Figure 3.7.32: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

Exposure of 10^7 CFU/ml to the MIC or MPC drug concentration for tedizolid did not result in substantial killing for any strains. For all strains, substantial growth occurred between 12-24 hours of drug exposure.

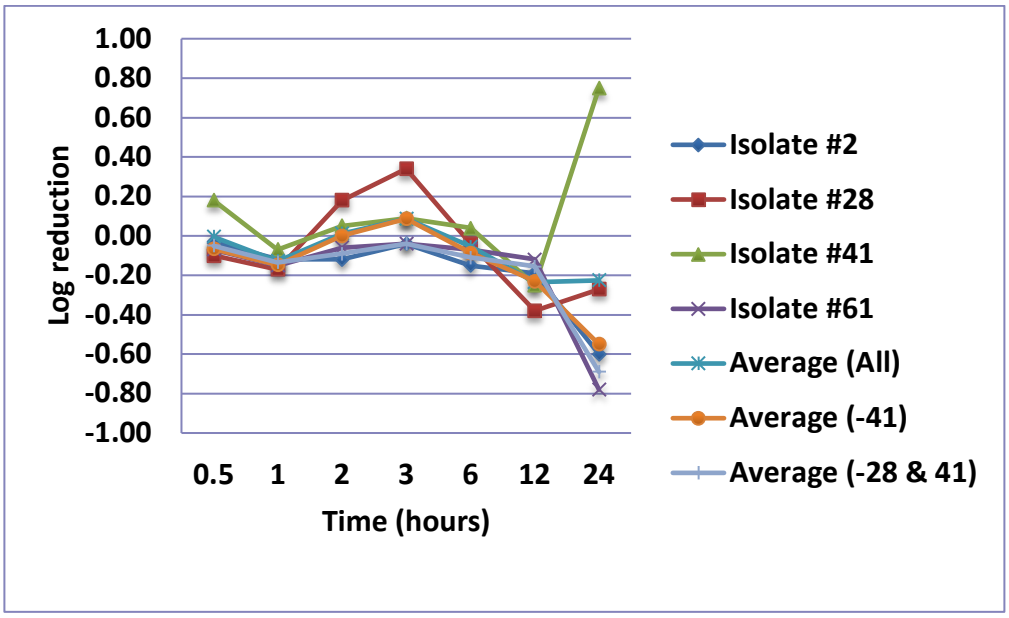


Figure 3.7.33: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

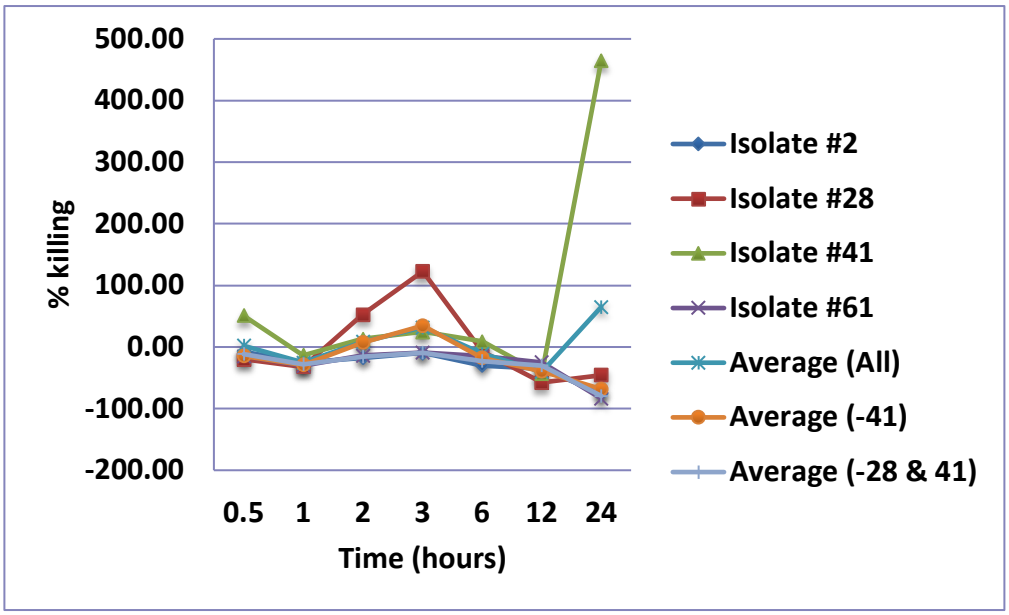


Figure 3.7.34: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

Exposure of 10^7 CFU/ml to the tedizolid $Tiss_{max}$ drug concentration yielded reduction in viable cells of 0.6-0.8 log_{10} for strains 2 and 61 following 24 hours of drug exposure. Strain 41 showed substantial growth between 12 and 24 hours of drug exposure.

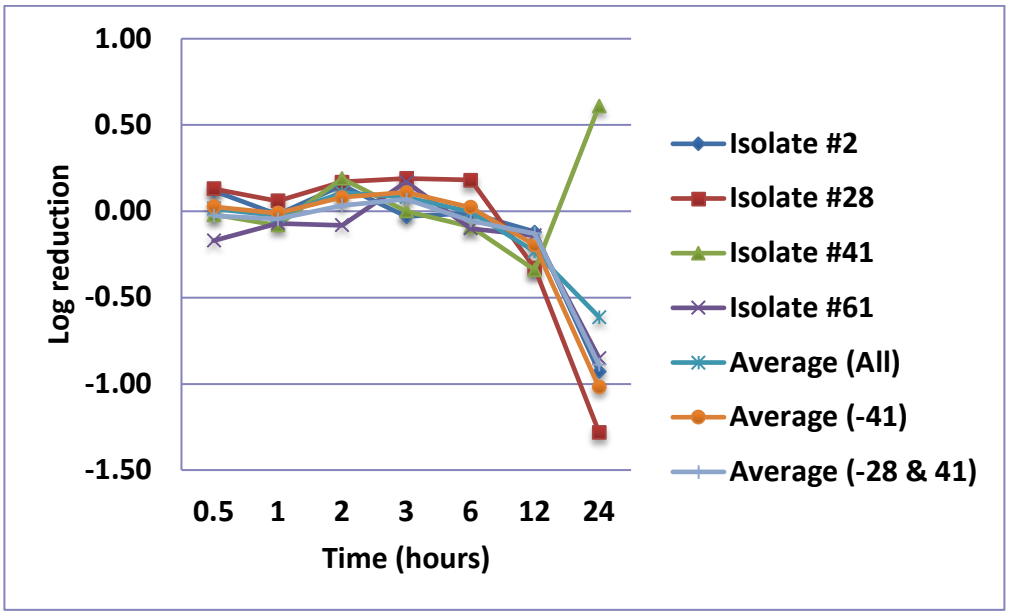


Figure 3.7.35: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

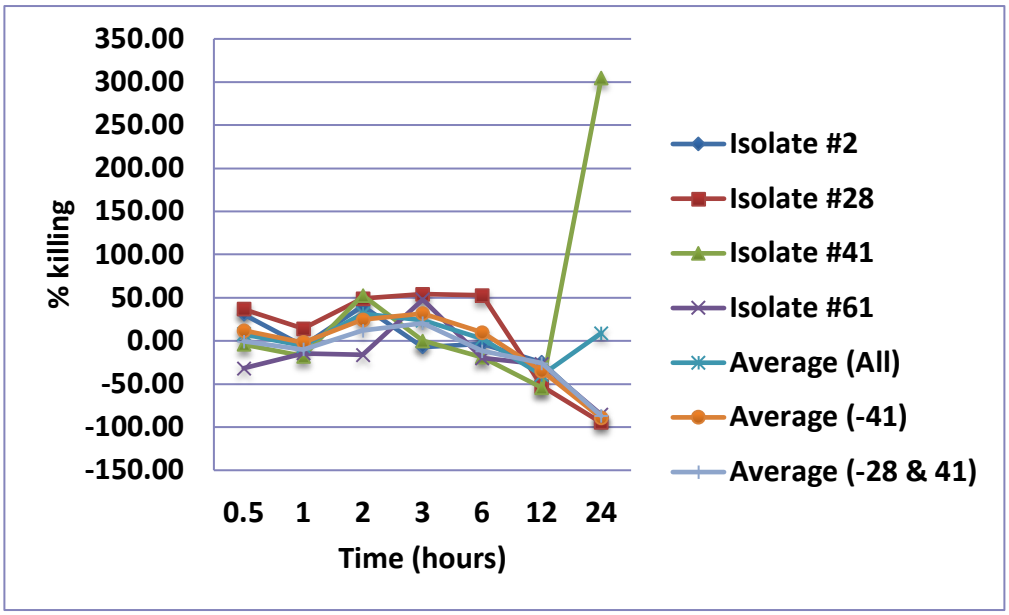


Figure 3.7.36: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

Exposure of 10^7 CFU/ml to the tedizolid C_{max} drug concentration resulted in 0.8 to 1.3 $\log_{10}$ reduction for strains 2, 28 and 61 following 24 hours of drug exposure. This translated to 80-95% killing. Substantial growth occurred with strain 41 between 12-24 hours.

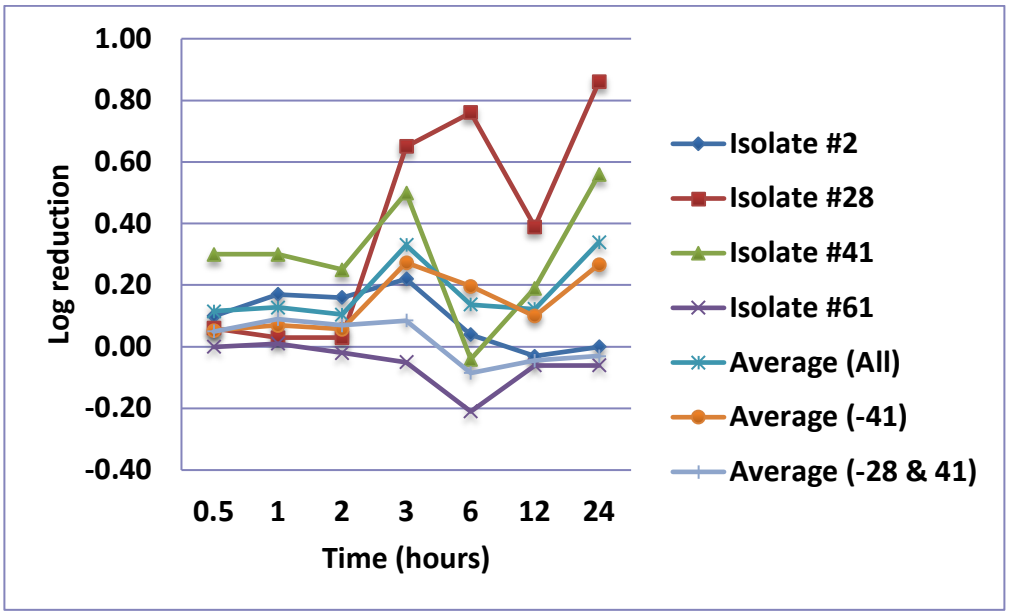


Figure 3.7.37: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

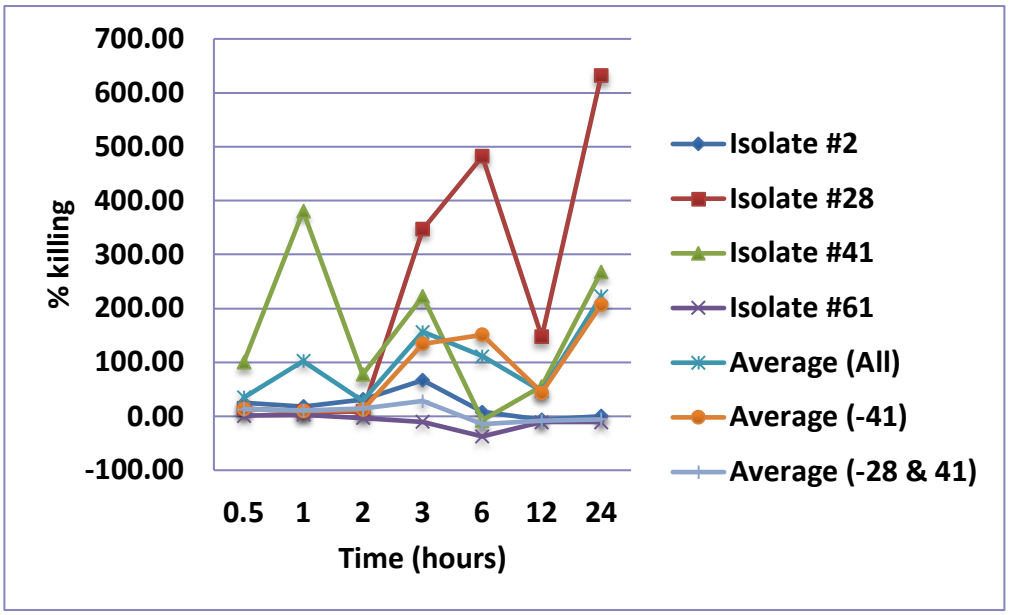


Figure 3.7.38: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

Exposure of 10^8 CFU/ml to the MIC or MPC tedizolid drug concentration resulted in minimum killing for strain 61 following 6 hours of drug exposure following regrowth occurred.

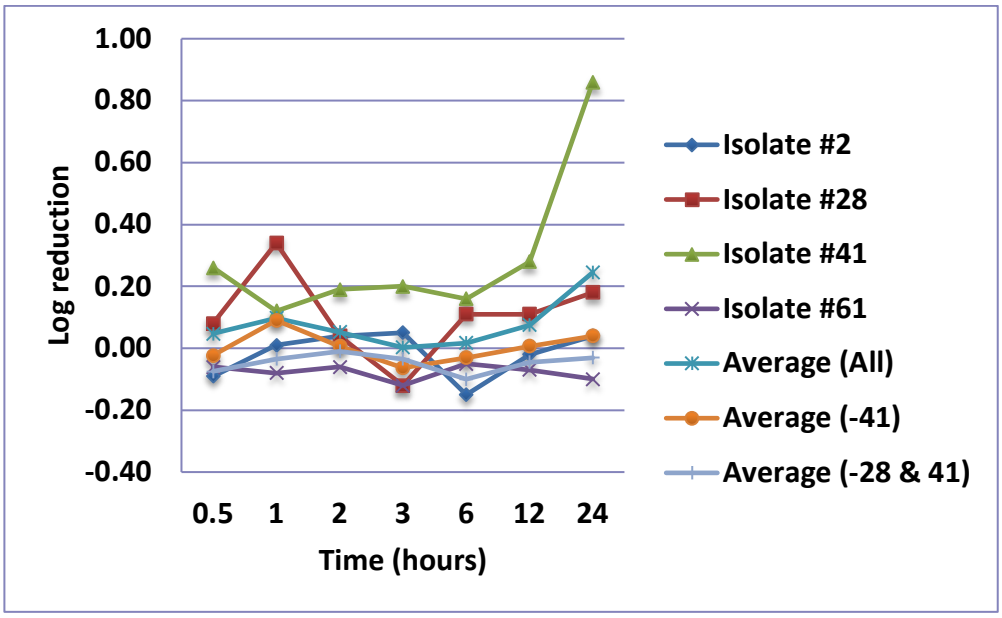


Figure 3.7.39: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

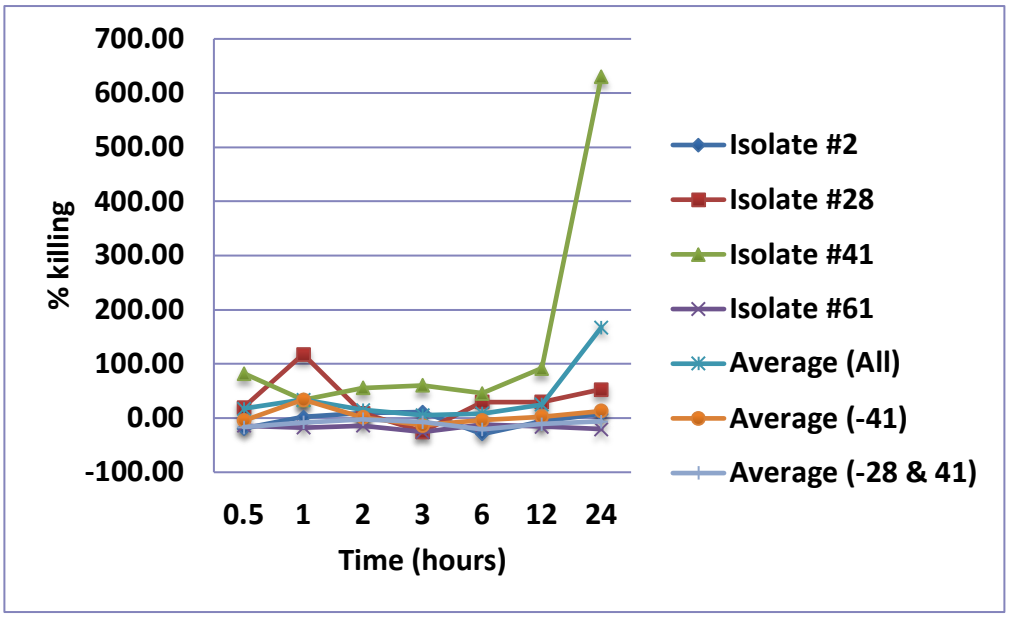


Figure 3.7.40: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

Exposure of 10^8 CFU/ml to the $Tiss_{max}$ drug concentration of tedizolid showed minimal killing of any strains. For strains 2 and 28, a 0.01 $\log_{10}$ reduction was seen at 3 and 6 hours after drug exposure but regrowth occurred. Strain 41 was not inhibited by tedizolid.

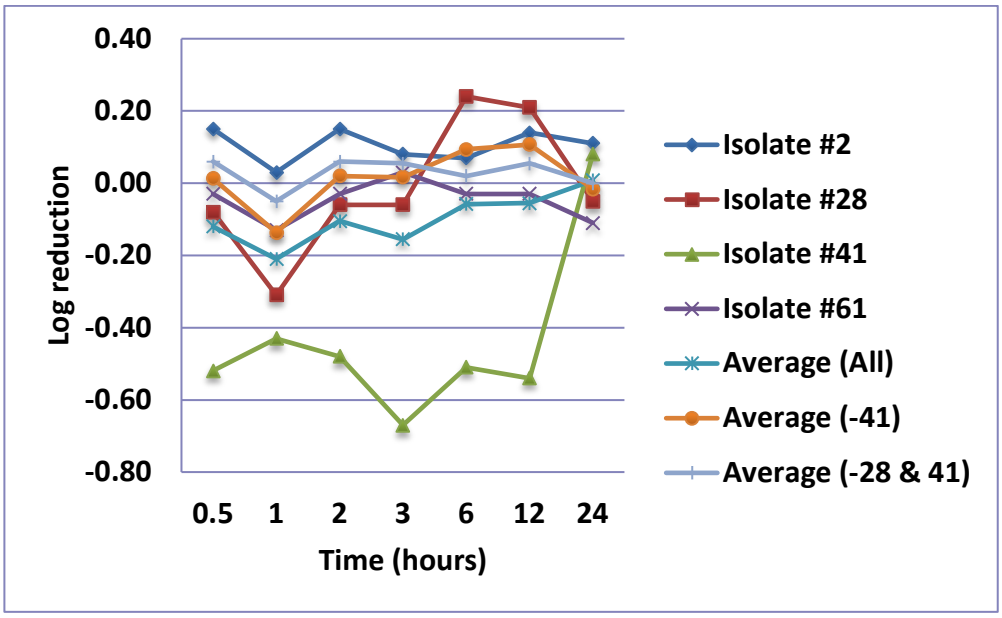


Figure 3.7.41: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

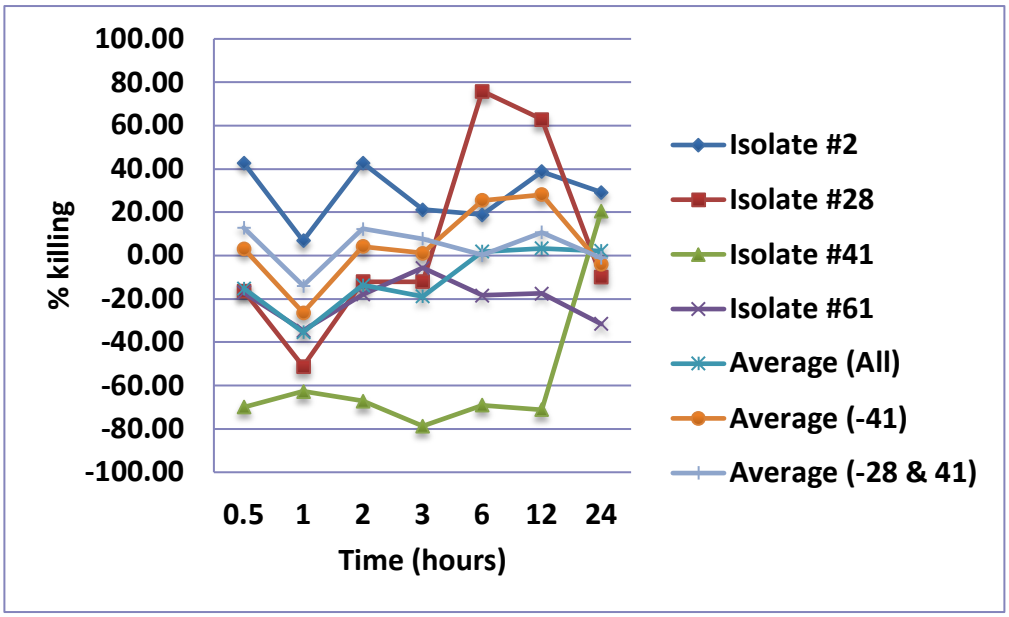


Figure 3.7.42: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

Exposure of 10^8 CFU/ml for the tedizolid C_{max} drug concentration showed initial killing of strain 41 (0.5-0.7 $\log_{10}$ reduction; 70-80% kill) between 0.5 and 3 hours, however, growth occurred following 12-24 hours of drug exposure. Killing did not exceed 30% for strain 61 following 24 hours of drug exposure.

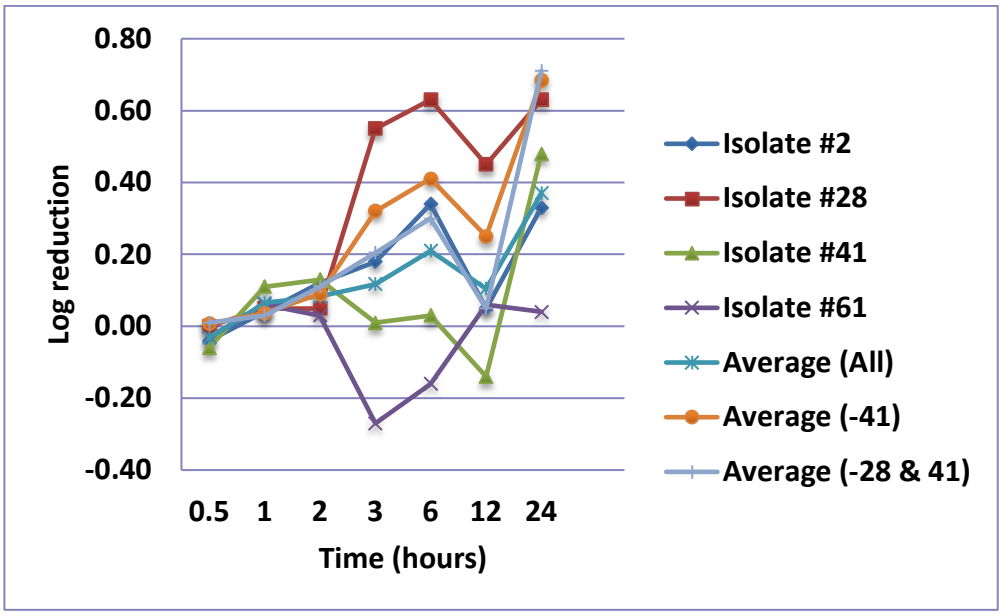


Figure 3.7.43: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

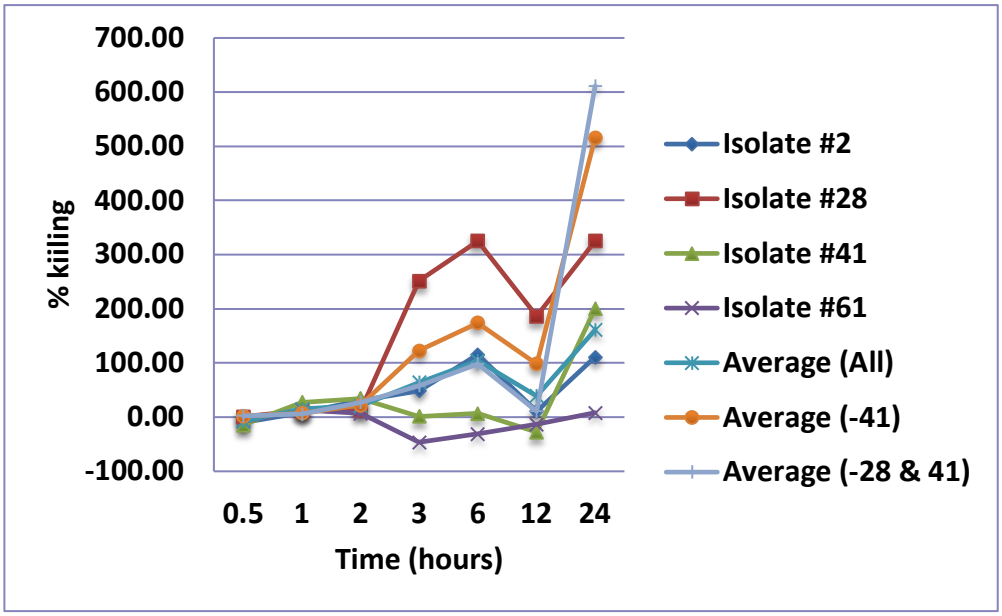


Figure 3.7.44: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

Exposure of 10^9 CFU/ml to the MIC or MPC drug concentration for tedizolid yielded a 0.25 $\log_{10}$ reduction (approximately 50% kill) following 3 hours of drug exposure but growth occurred thereafter.

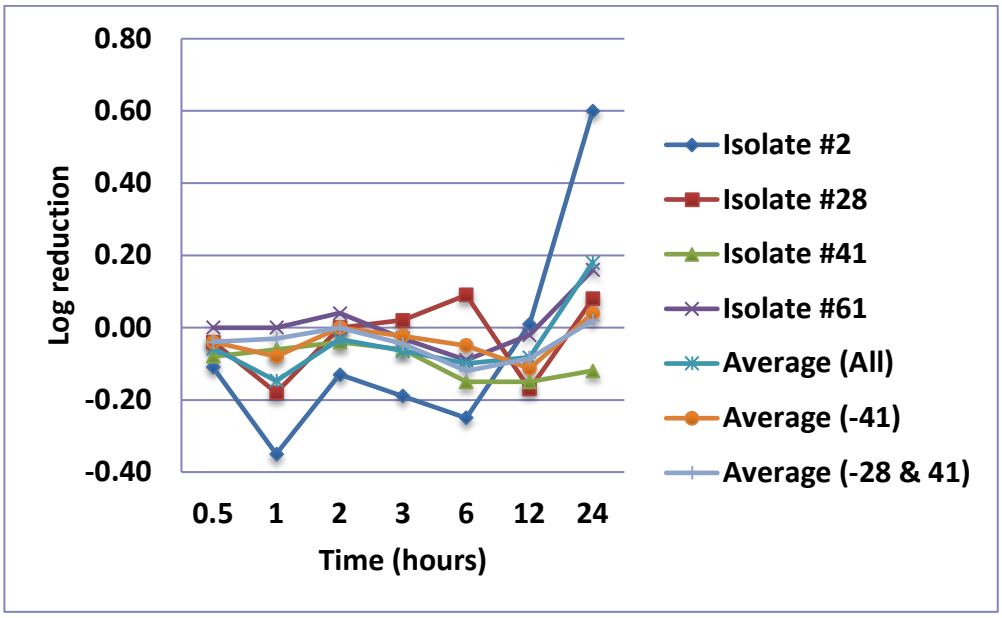


Figure 3.7.45: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

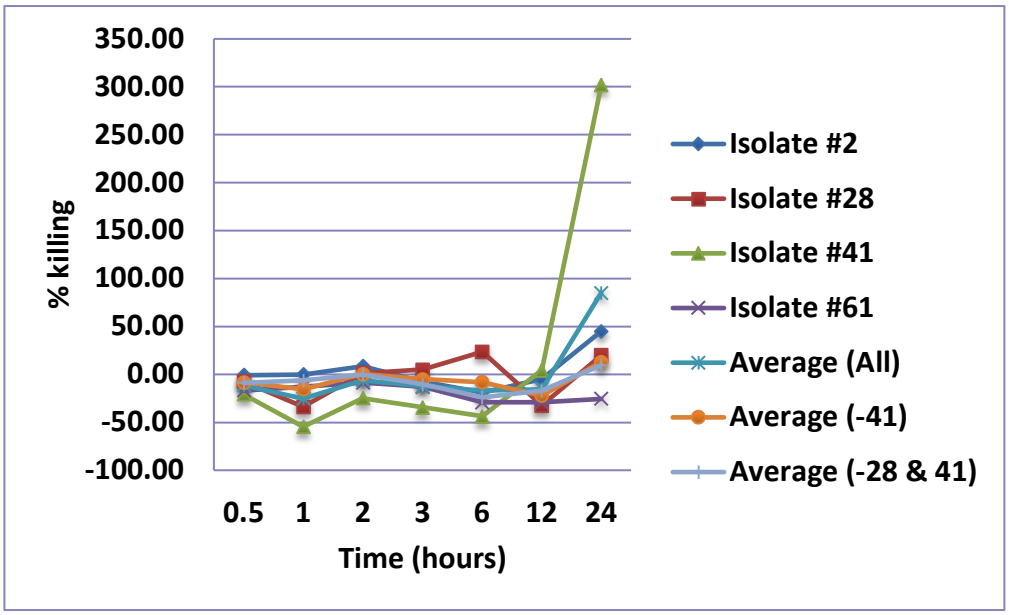


Figure 3.7.46: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

Exposure of 10^9 CFU/ml to the $Tiss_{max}$ drug concentration for tedizolid did not result in greater than 50% killing for any strain and strain 41 showed substantial growth between 12 and 24 hours of drug exposure.

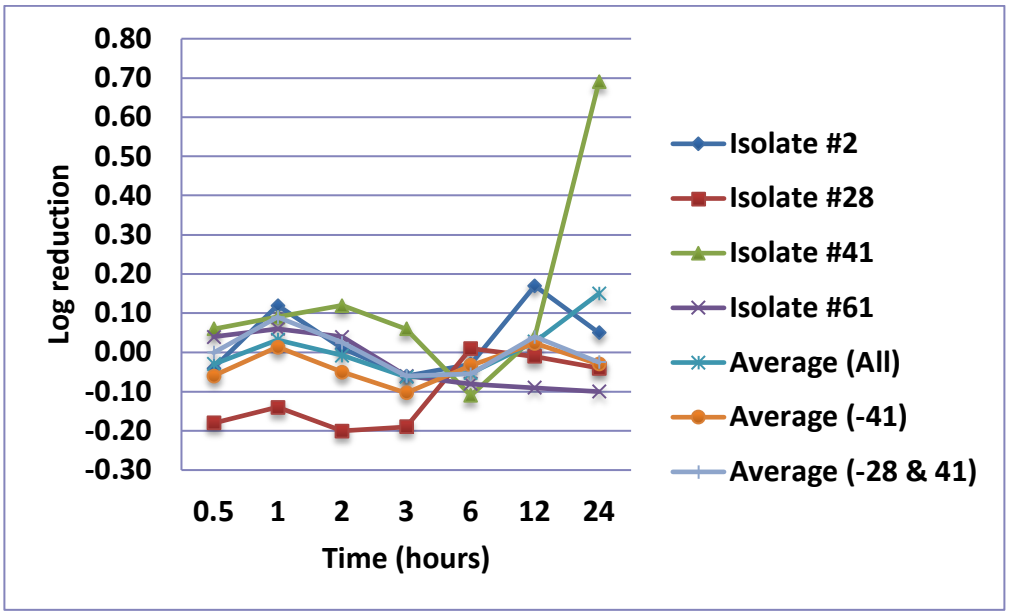


Figure 3.7.47: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

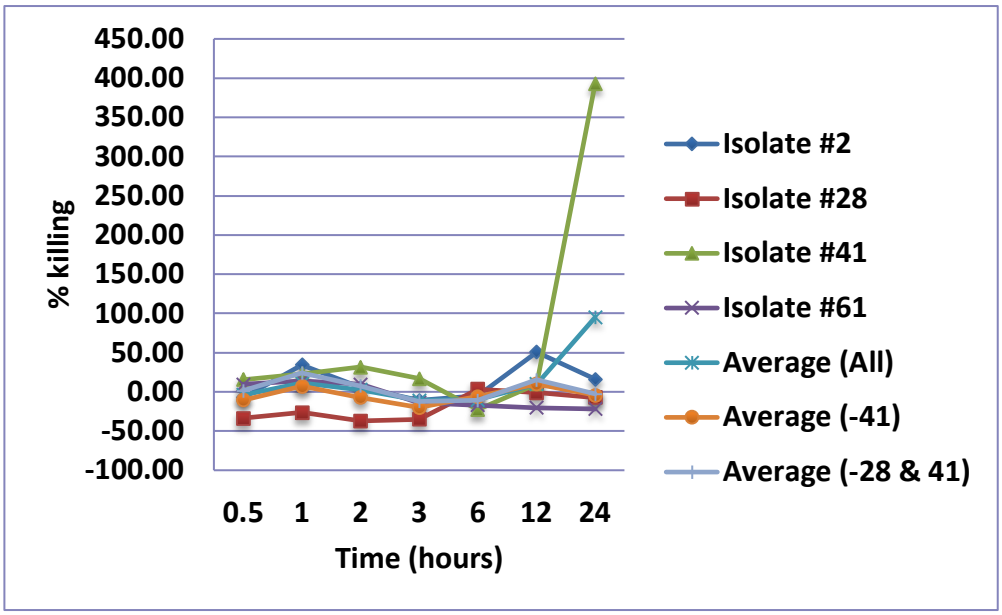


Figure 3.7.48: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

Exposure of 10^7 CFU/ml to the tedizolid C_{max} drug concentration failed to result in a >0.2 (<50% kill) $\log_{10}$ reduction for any strain. Substantial growth occurred with strain 41 between 12 and 24 hours of drug exposure.

Vancomycin

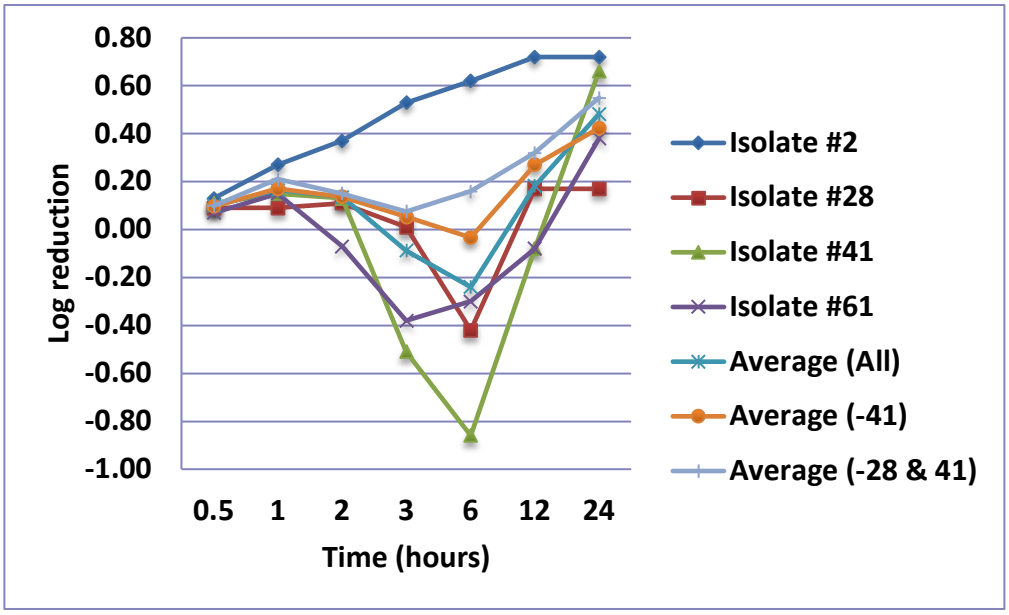


Figure 3.7.49: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the MIC drug concentration of vancomycin.

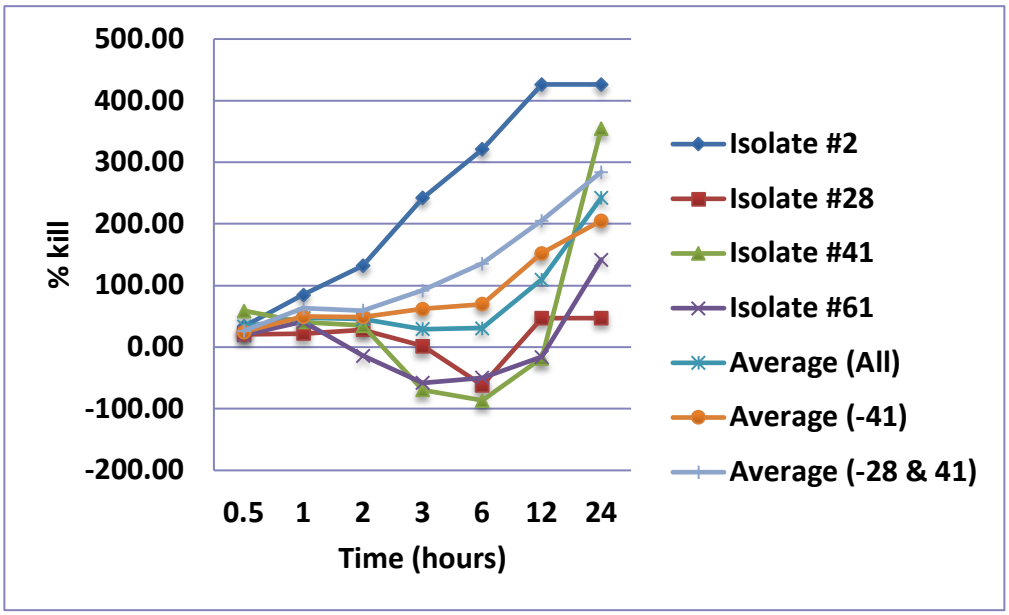


Figure 3.7.50: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the MIC drug concentration of vancomycin.

Exposure of 10^6 CFU/ml to the MIC drug concentration for vancomycin showed a 0.4 $\log_{10}$ (70% kill) of strain 28 and a 0.85 $\log_{10}$ (>90% kill) reduction for strain 2 following 6 hours of drug exposure after which growth occurred in the presence of the drug.

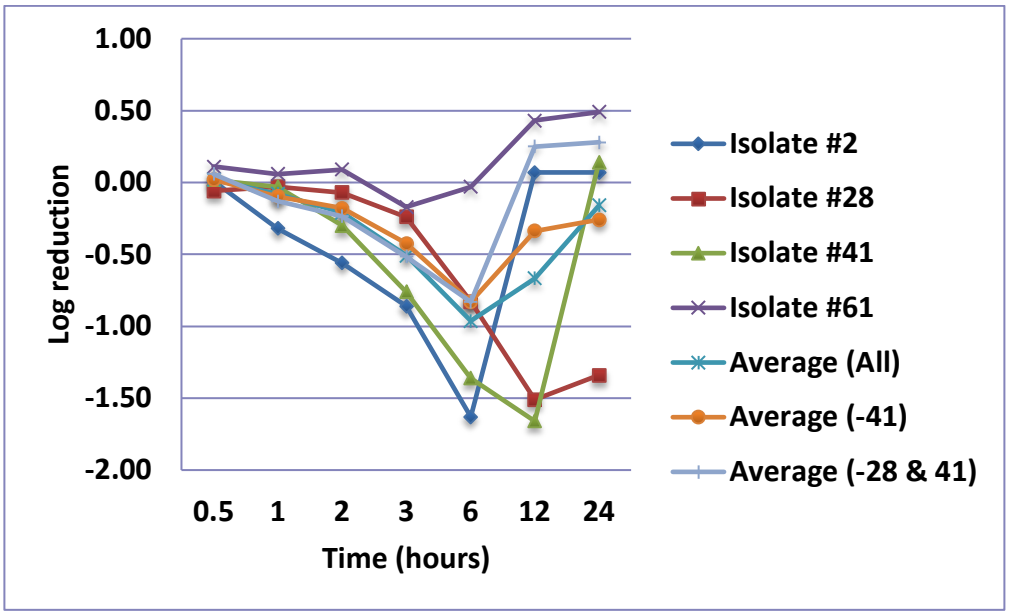


Figure 3.7.51: Log reduction of MRSA strain at 10^6 CFU/ml inocula using theMPC drug concentration of vancomycin.

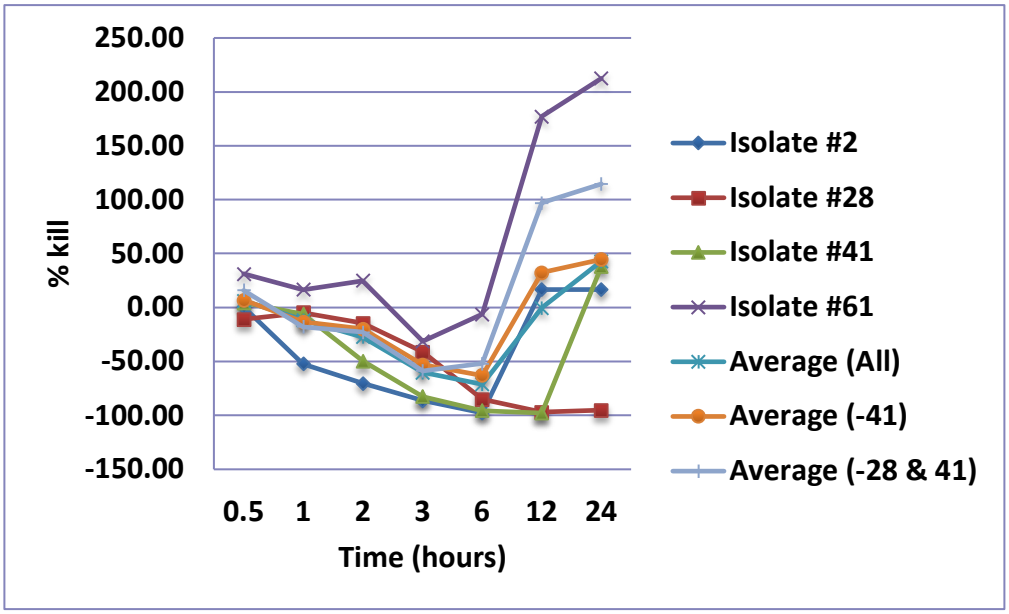


Figure 3.7.52: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the MPC drug concentration of vancomycin.

Exposure of 10^6 CFU/ml to vancomycin MPC drug concentration resulted in viable cell reduction for all strains (1-1.6 $\log_{10}$ reduction) with killing being >90% following 6-12 hours of drug exposure. Growth occurred for strains 2, 41 and 61 following 6-12 hours of drug exposure. Continued killing of strain 28 continued to 24 hours of drug exposure.

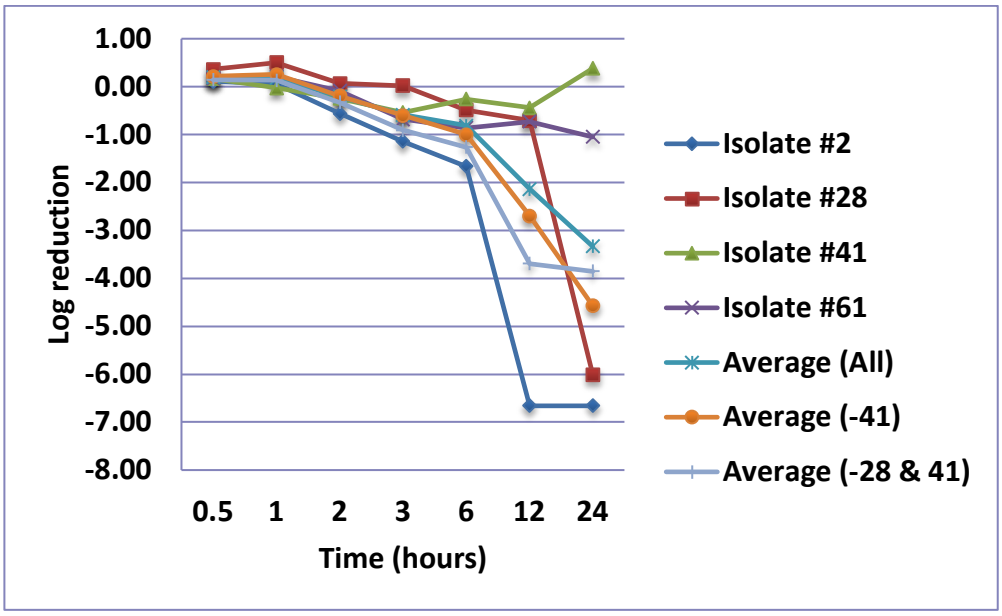


Figure 3.7.53: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

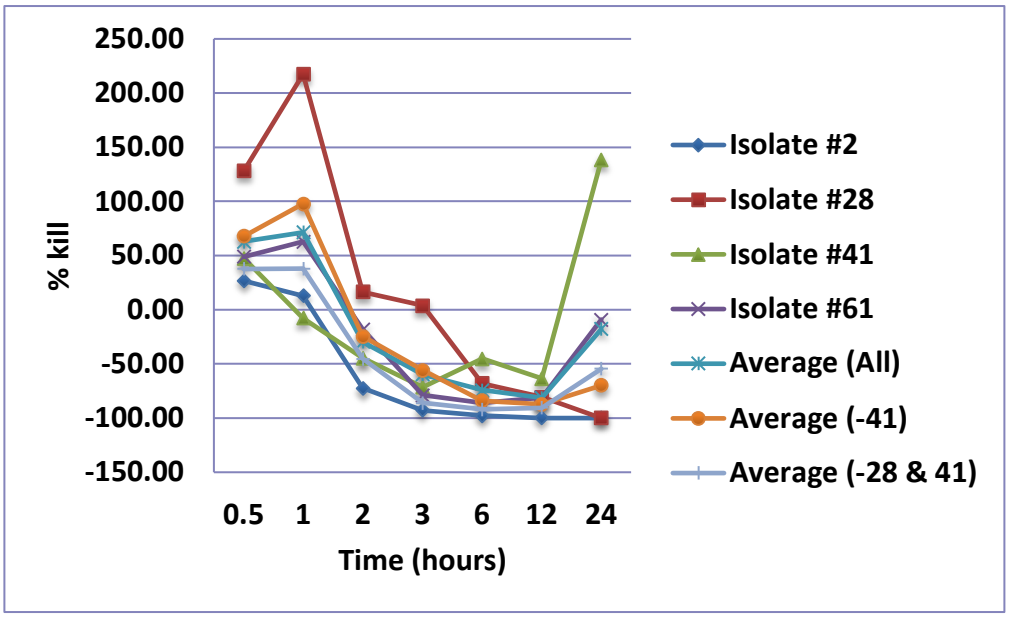


Figure 3.7.54: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

Exposure of 10^6 CFU/ml to the vancomycin $Tiss_{max}$ drug concentration showed substantial killing of strains 2, 28 and 61 with 4-6.5 log_{10} reduction. The percent kill was 81-100% for strains 2, 28 and 61 following 12 hours of drug exposure.

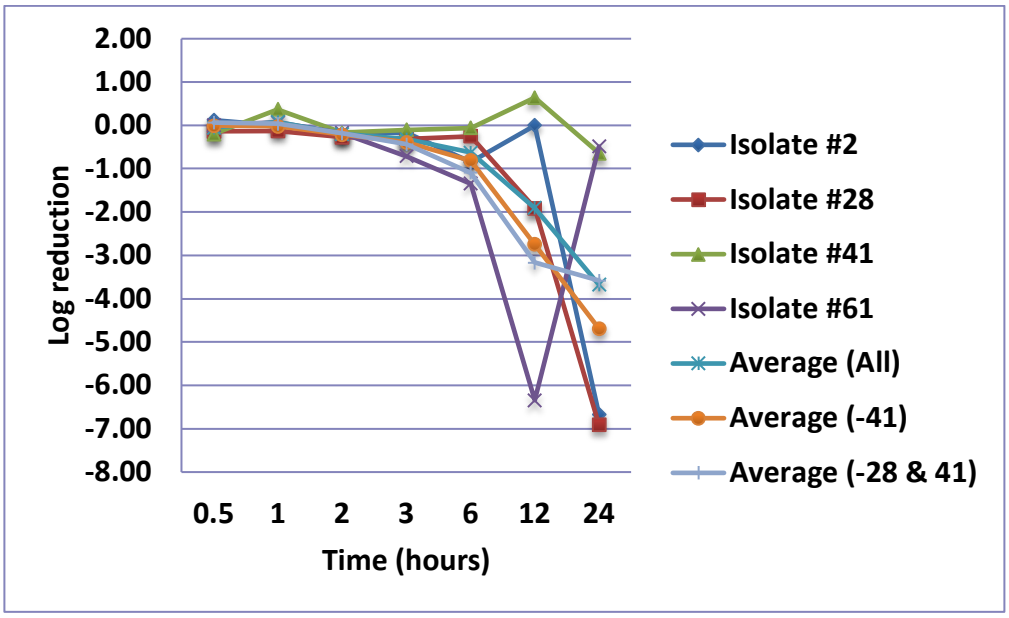


Figure 3.7.55: Log reduction of MRSA strain at 10^6 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

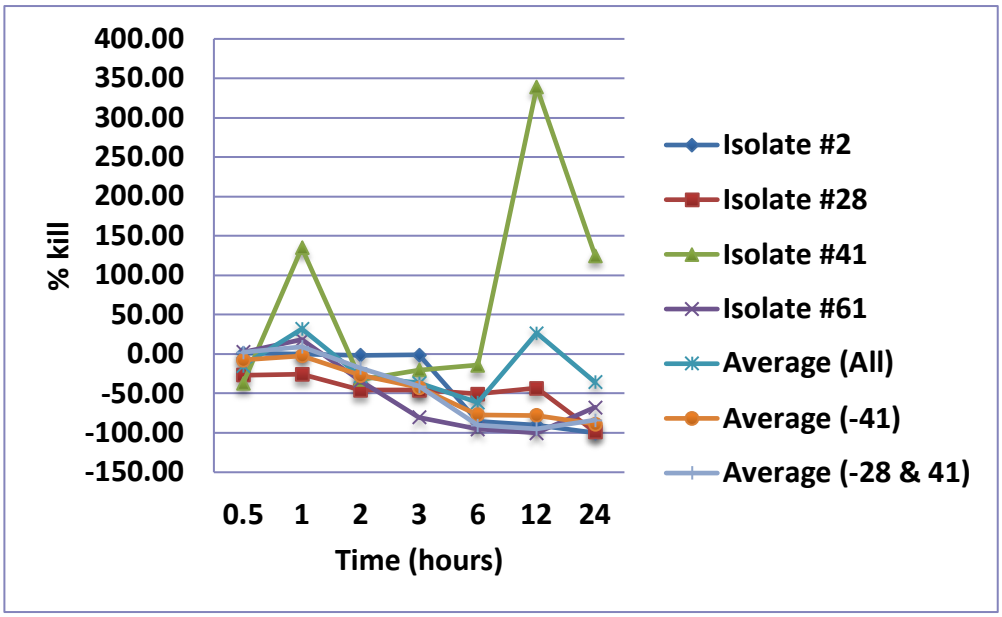


Figure 3.7.56: Percent kill of MRSA strain at 10^6 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

Exposure of 10^6 CFU/ml to the vancomycin C_{max} drug concentration resulted in killing of all strains (except 4) within the first 30 minutes of drug exposure. Following 24 hours of drug exposure a 6-7 $\log_{10}$ reduction in viable cells was seen. For strain 61, >99% of cells were killed (6.34 $\log_{10}$) by 12 hours of drug exposure.

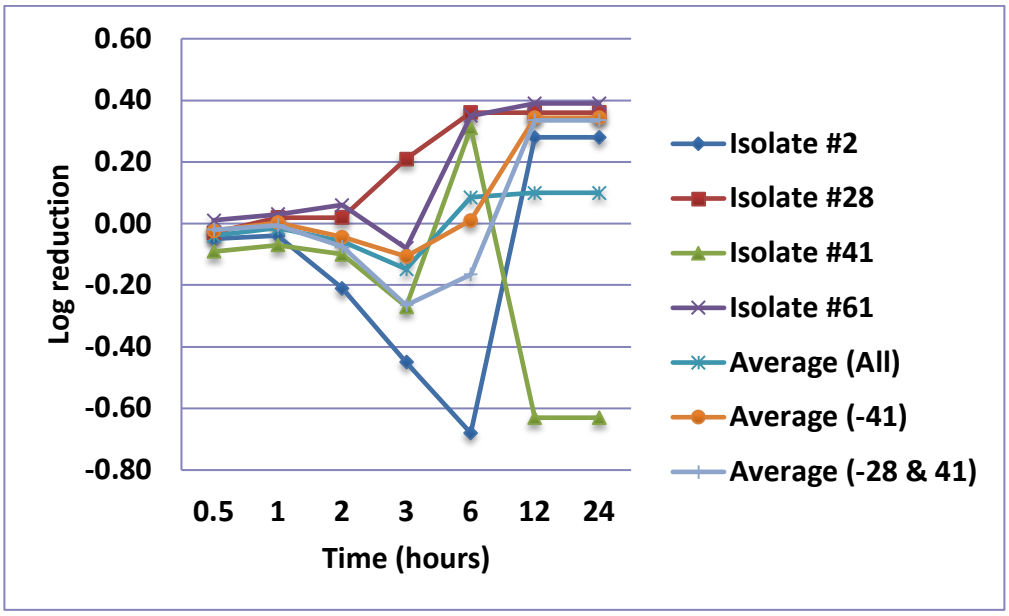


Figure 3.7.57: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the MIC drug concentration of vancomycin.

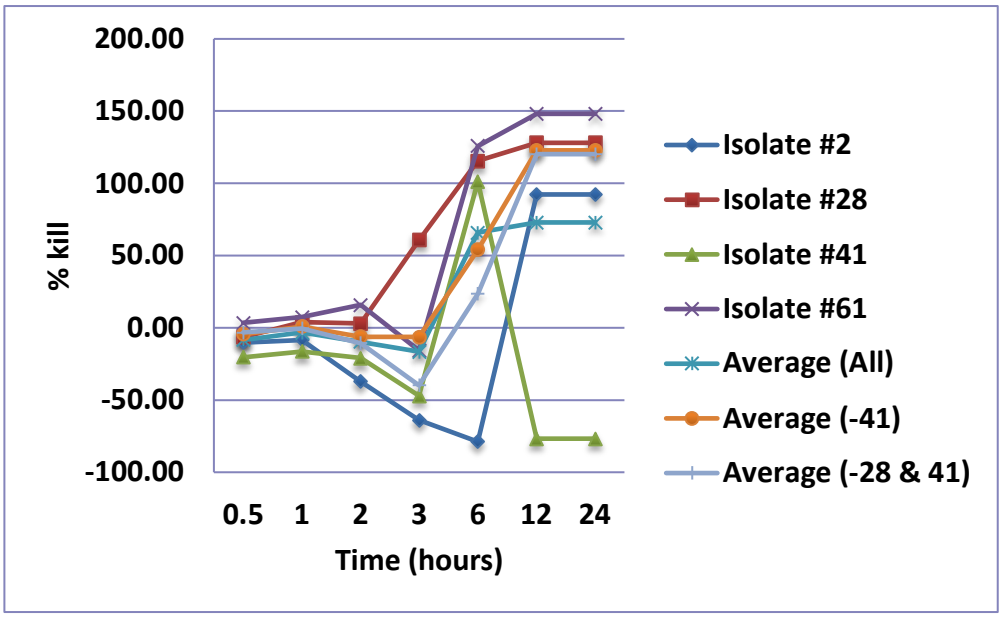


Figure 3.7.58: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the MIC drug concentration of vancomycin.

Exposure of 10^7 CFU/ml to the vancomycin MIC drug concentration showed a 0.65-0.7 $\log_{10}$ reduction for strains 2 (80% kill) following 6 hours of drug exposure but thereafter growth occurred. For strain 41 approximately 80% of cells were killed following 12 and 24 hours of drug exposure.

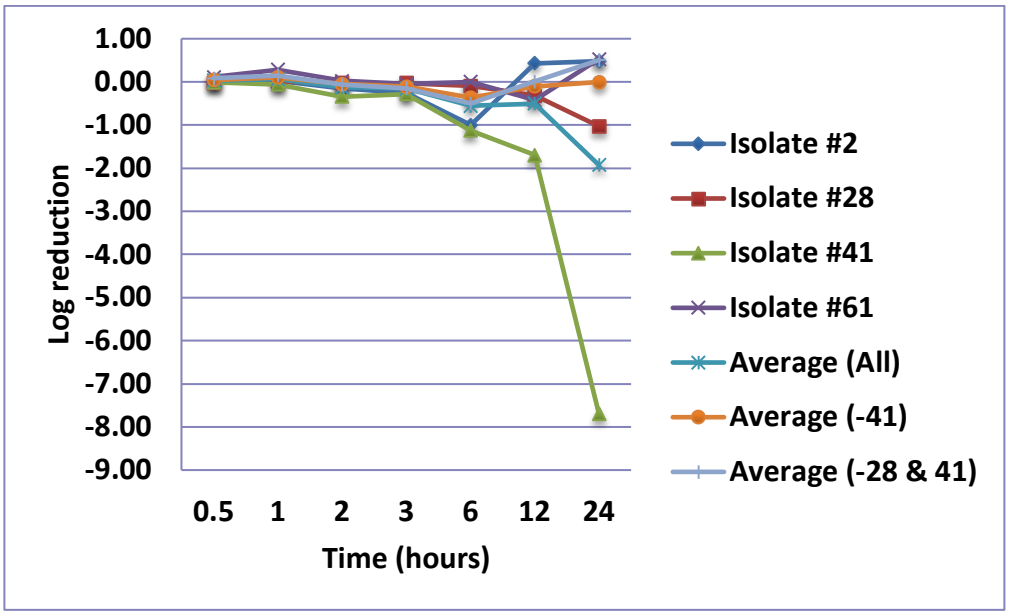


Figure 3.7.59: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the MPC drug concentration of vancomycin.

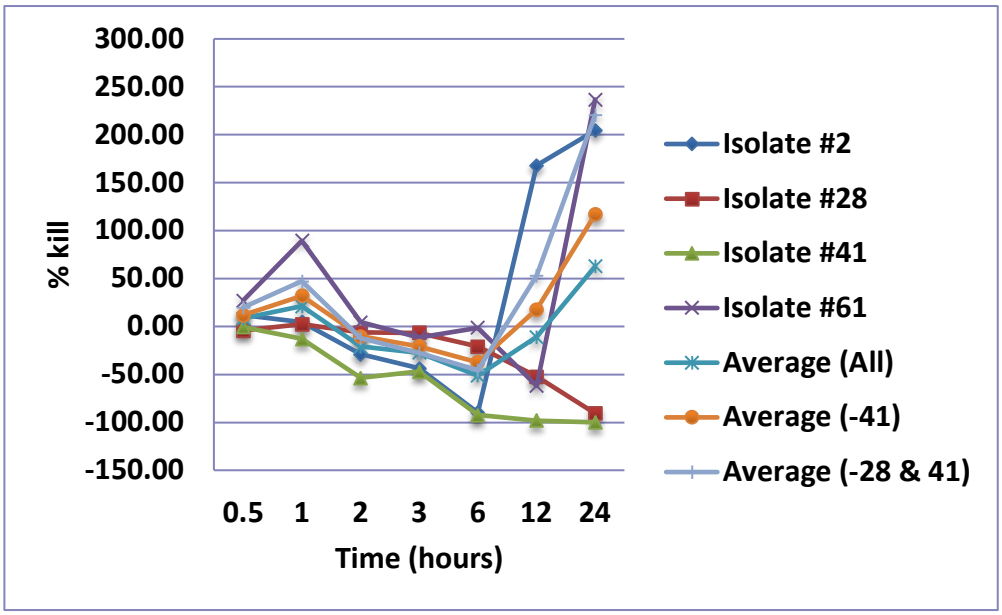


Figure 3.7.60: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the MPC drug concentration of vancomycin.

Exposure of 10^7 CFU/ml to the vancomycin MPC drug concentration showed killing of strain 2 to 6 hours of drug exposure but growth thereafter. Strain 28 had a 1 $\log_{10}$ reduction following 24 hours of drug exposure which translated to 90% kill. For strain 41, a 7.9 $\log_{10}$ reduction was seen following 24 hours of drug exposure corresponding to 100% kill.

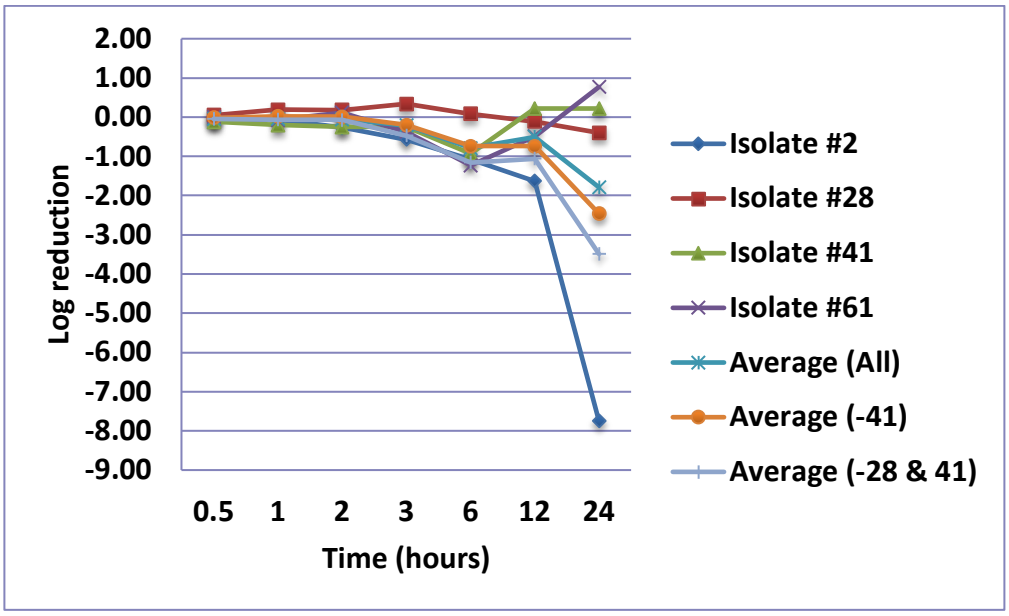


Figure 3.7.61: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

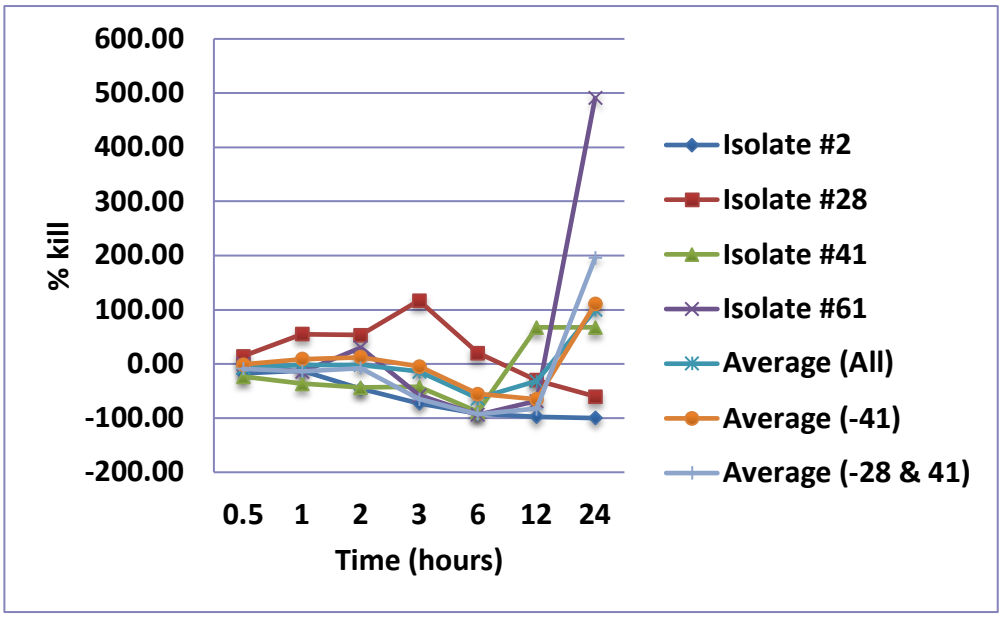


Figure 3.7.62: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

Exposure of 10^7 CFU/ml to the vancomycin $Tiss_{max}$ drug concentration resulted in reduction in viable cells for all strains, however, growth occurred for strain 61 following 6 hours of drug exposure. For strains 28 and 2, 80-100% kill occurred following 24 hours of drug exposure.

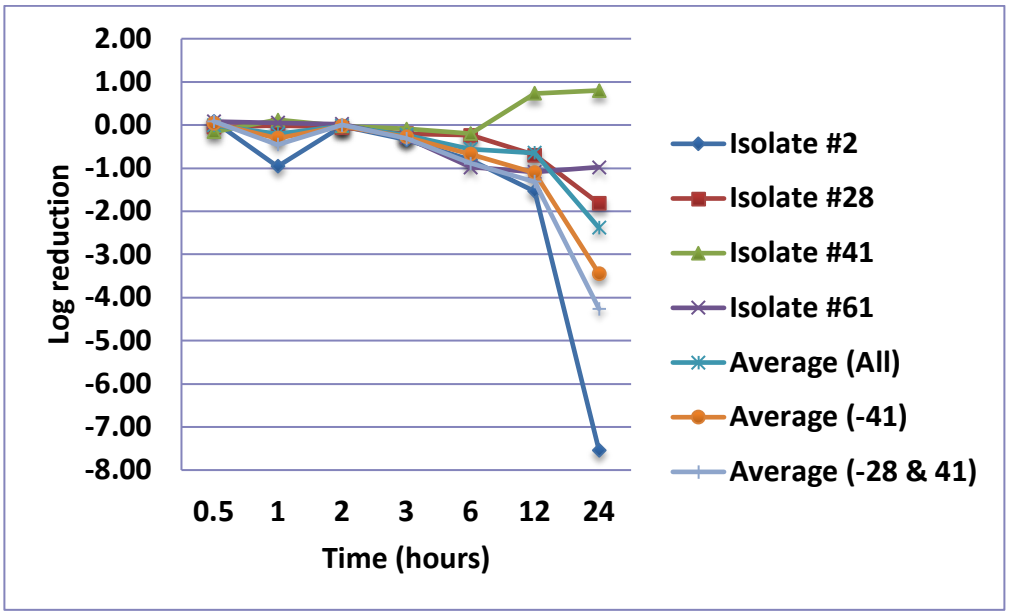


Figure 3.7.63: Log reduction of MRSA strain at 10^7 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

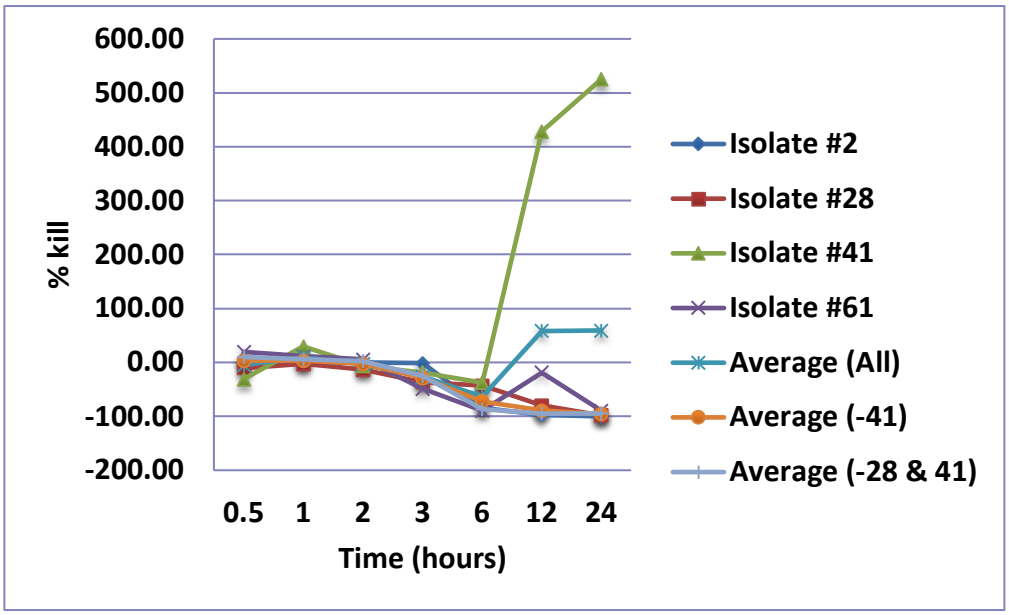


Figure 3.7.64: Percent kill of MRSA strain at 10^7 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

Exposure of 10^7 CFU/ml to the vancomycin C_{max} drug concentration resulted in 100% kill for strains 2, 28 and 61 following 24 hours of drug exposure. Growth occurred with strain 41 between 6-12 hours of drug exposure.

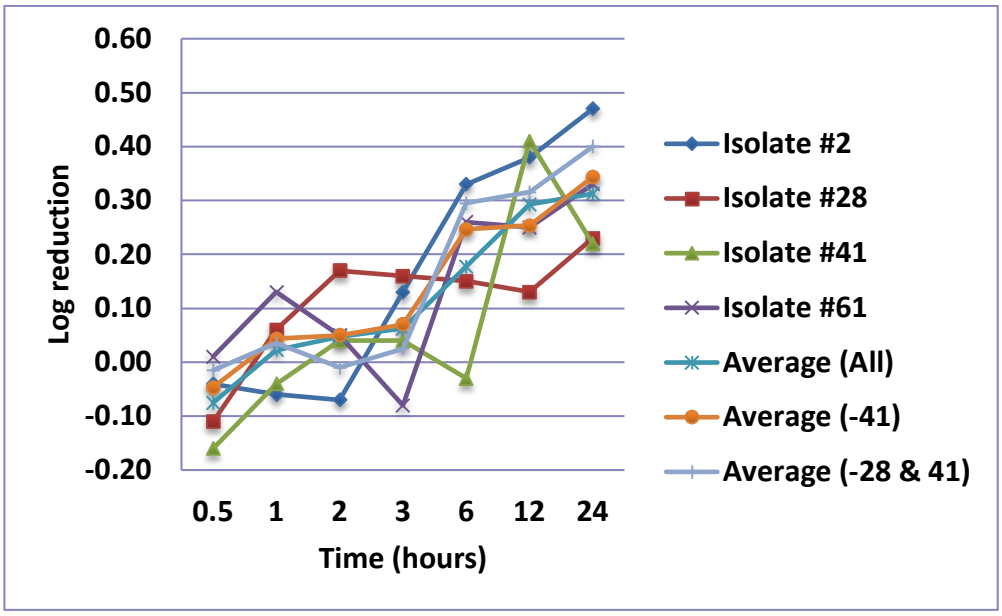


Figure 3.7.65: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the MIC drug concentration of vancomycin.

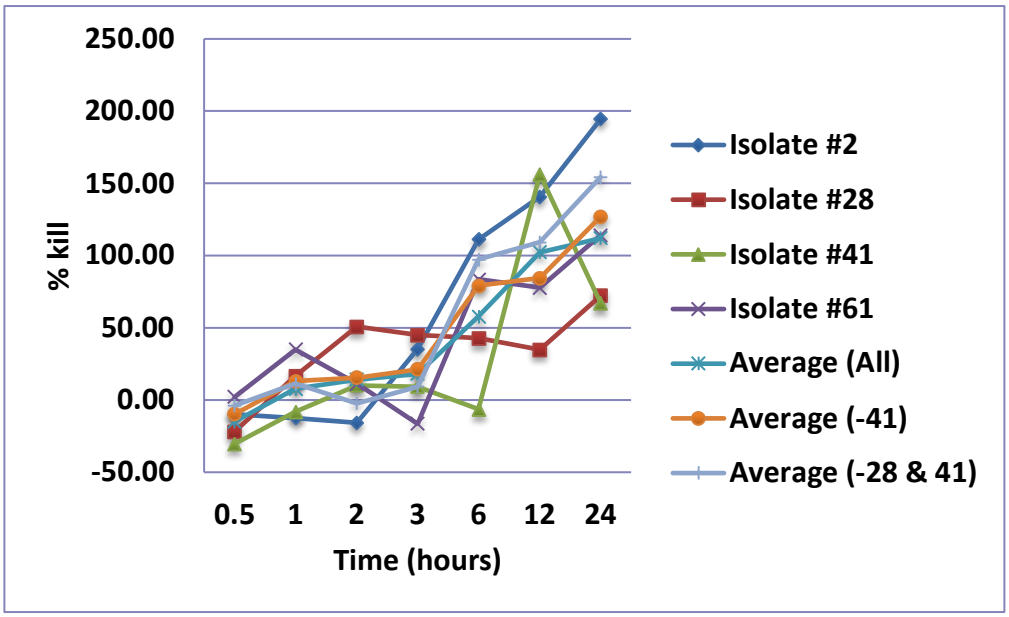


Figure 3.7.66: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the MIC drug concentration of vancomycin.

Exposure of 10^8 CFU/ml to the vancomycin MIC drug concentration showed some initial reduction in viable cells following 30 minutes of drug exposure but growth thereafter.

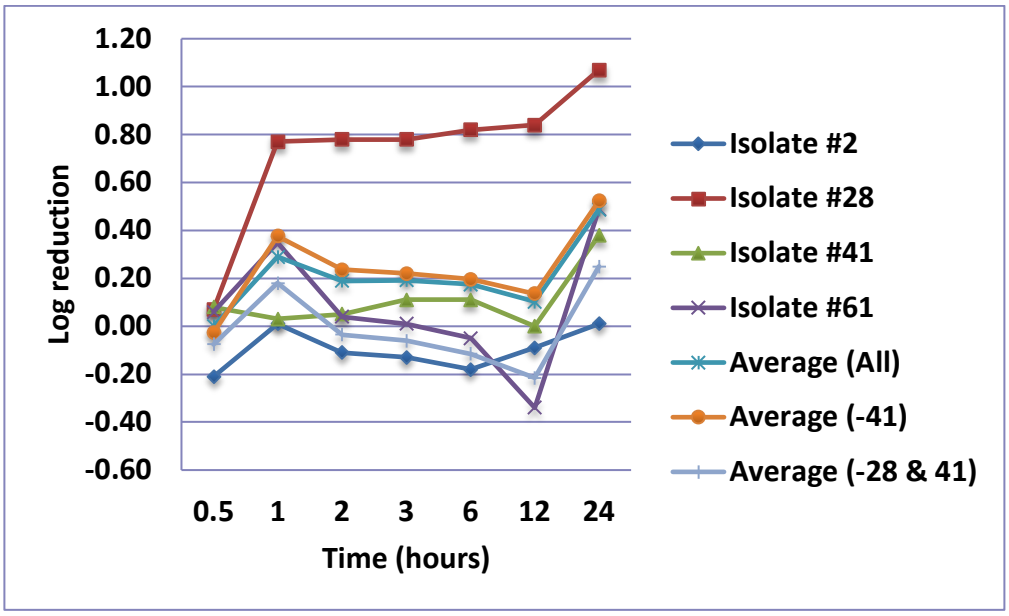


Figure 3.7.67: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the MPC drug concentration of vancomycin.

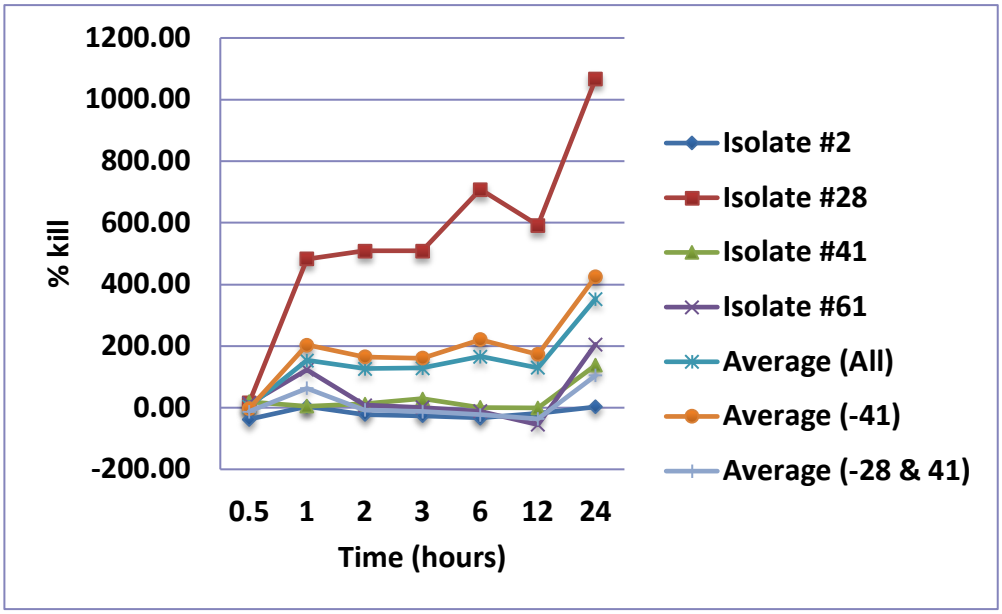


Figure 3.7.68: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the MPC drug concentration of vancomycin.

Exposure of 10^8 CFU/ml to the vancomycin MPC drug concentration failed to result in substantial killing of any strain over the 24 hour time intervals investigated. Following 12 hours of drug exposure, 55% (0.34 $\log_{10}$) of cells of strain 61 were killed by vancomycin, however, regrowth occurred between 12 and 24 hours.

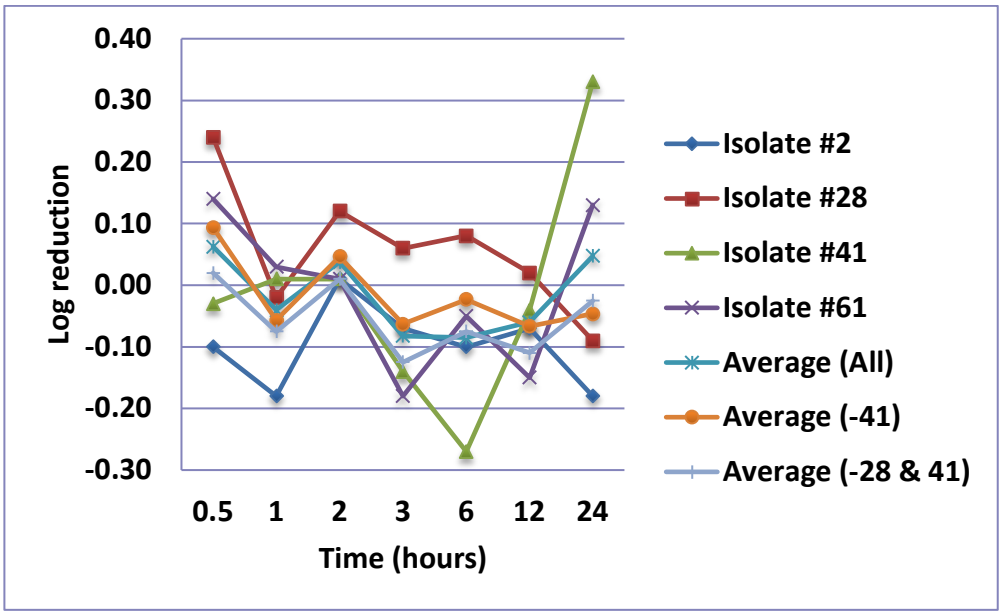


Figure 3.7.69: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

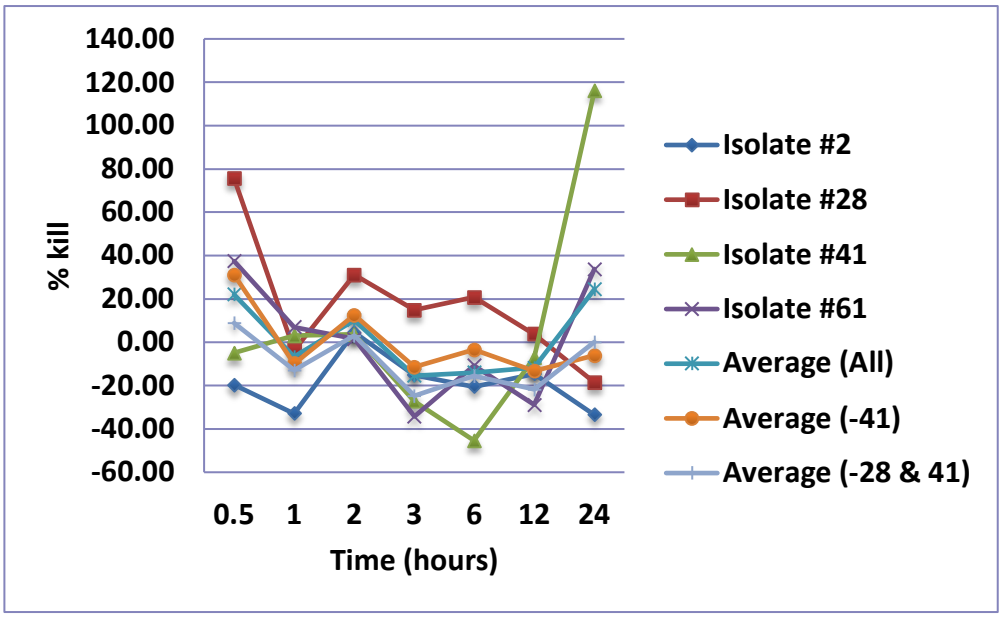


Figure 3.7.70: Percent kill of MRSA strain at 10^8 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

Exposure of 10^8 CFU/ml to the vancomycin $Tiss_{max}$ drug concentration yielded no more than 45% kill for any strain and following 24 hours of drug exposure, 20-35% kill for strains 2 and 28.

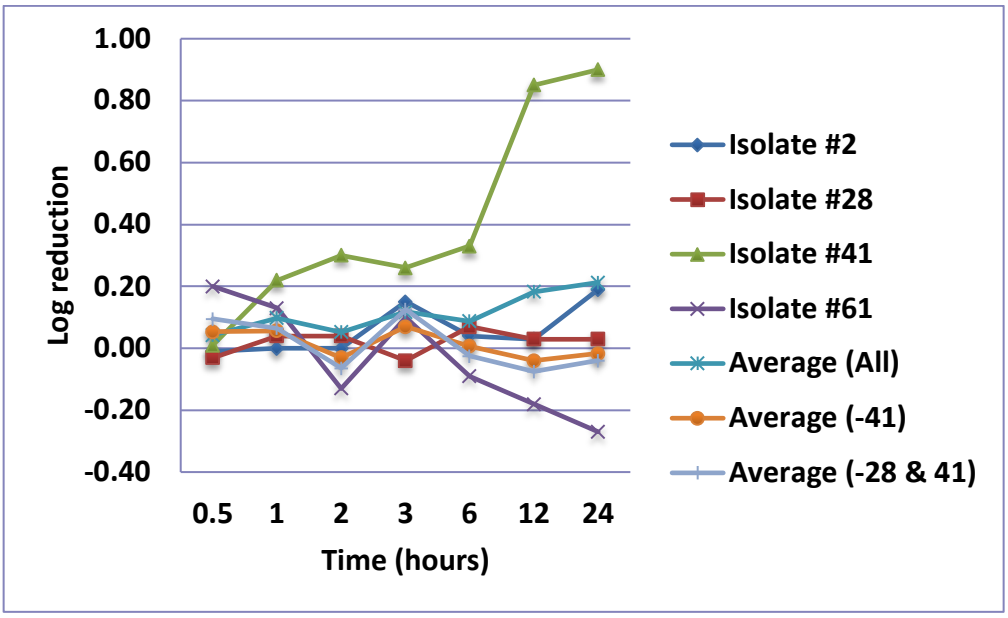


Figure 3.7.71: Log reduction of MRSA strain at 10^8 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

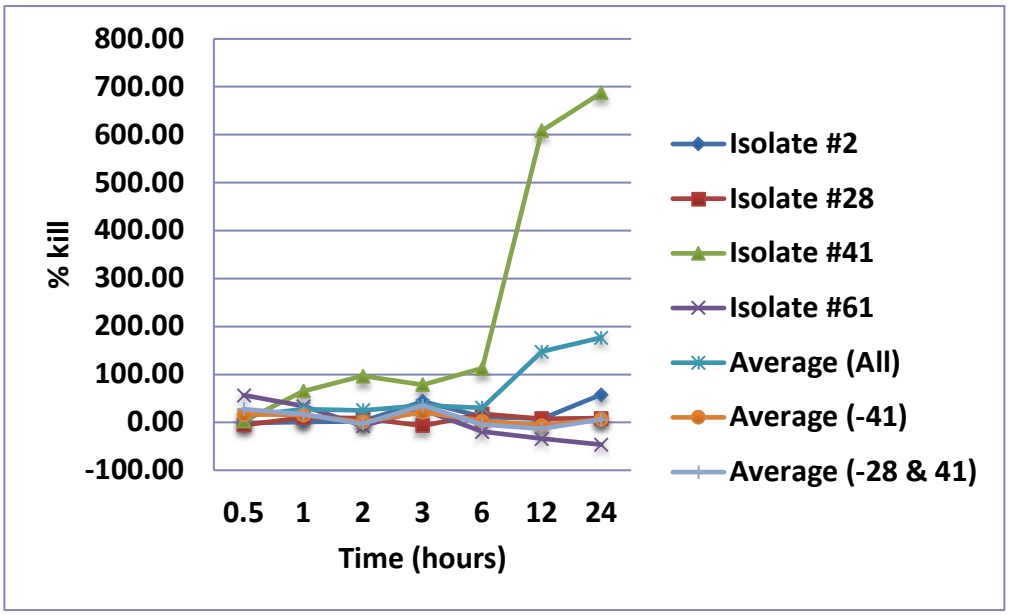


Figure 3.7.72: Percent kill of MRSA strain at 10 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

Exposure of 10^8 CFU/ml to the vancomycin C_{max} drug concentration yielded a 0.25 $\log_{10}$ reduction (50% kill) for strain 61 following 24 hours of drug exposure. Reduction was minimal or growth occurred for the other strains.

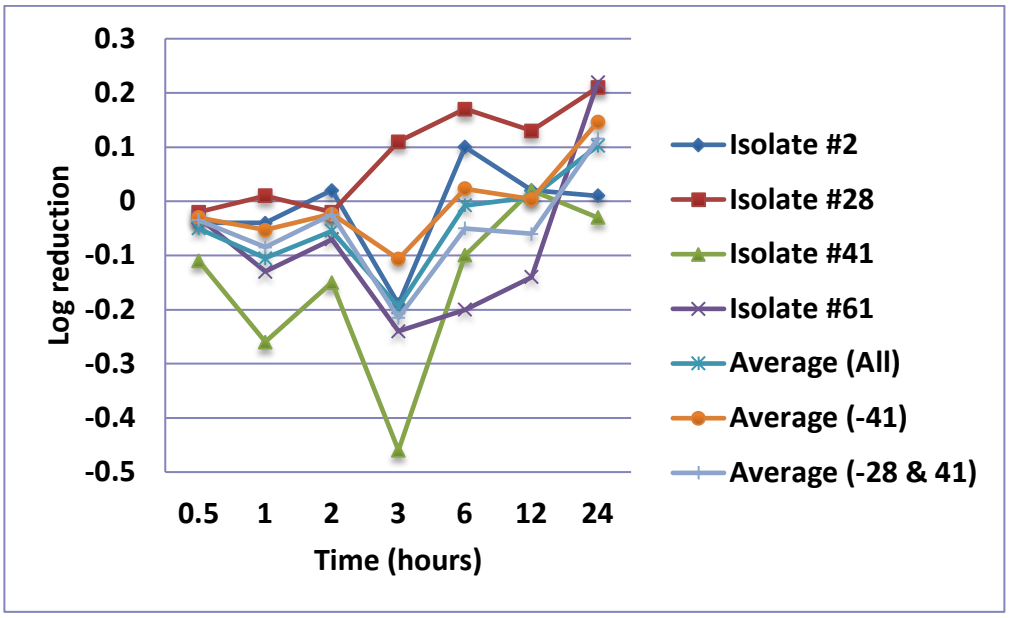


Figure 3.7.73: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the MIC drug concentration of vancomycin.

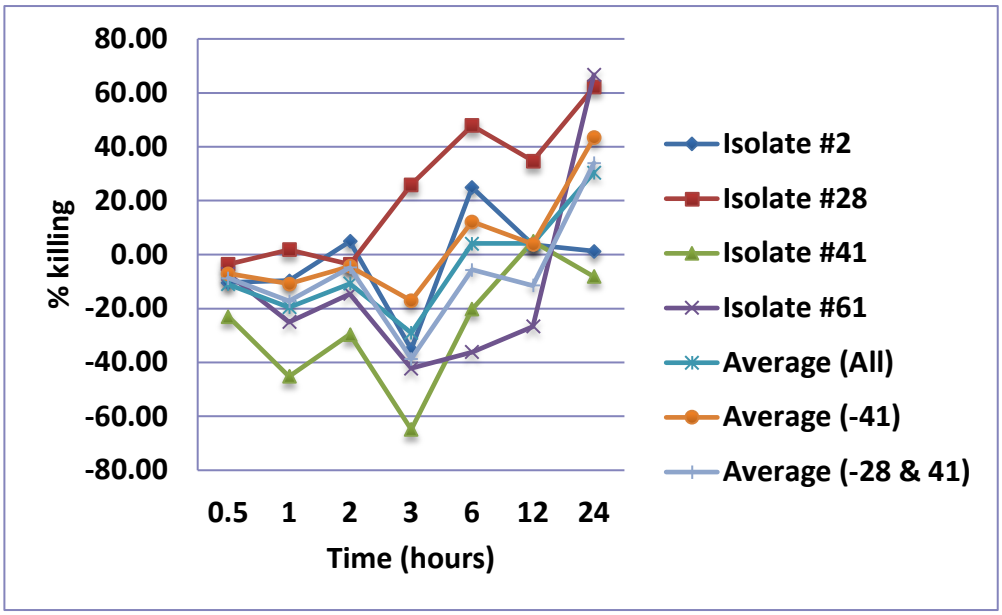


Figure 3.7.74: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the MIC drug concentration of vancomycin.

Exposure of 10^9 CFU/ml to the vancomycin MIC drug concentration yielded approximately 65% killing of strain 41 after which growth occurred. Substantial killing did not occur with the other strains tested.

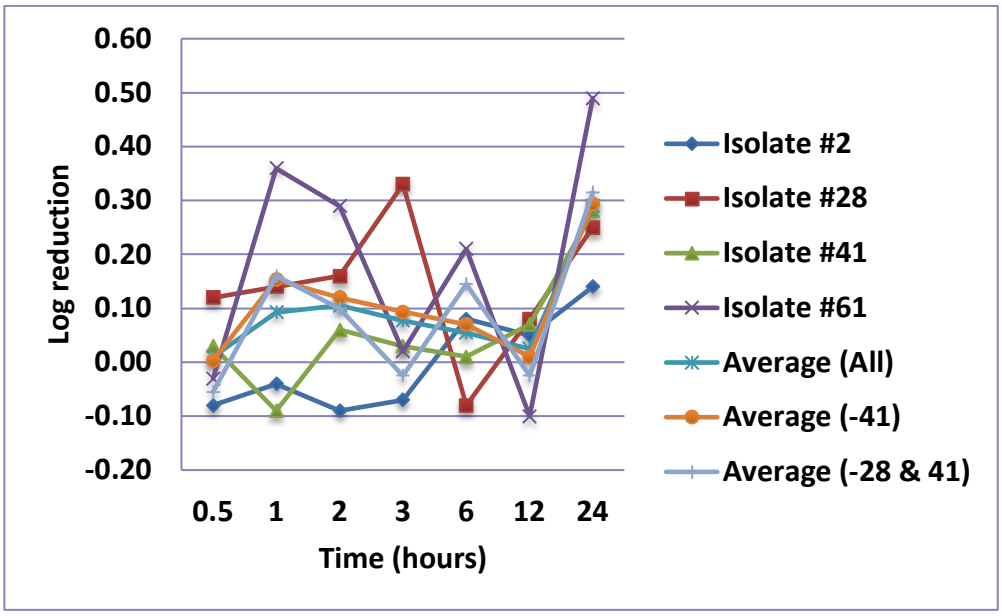


Figure 3.7.75: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the MPC drug concentration of vancomycin.

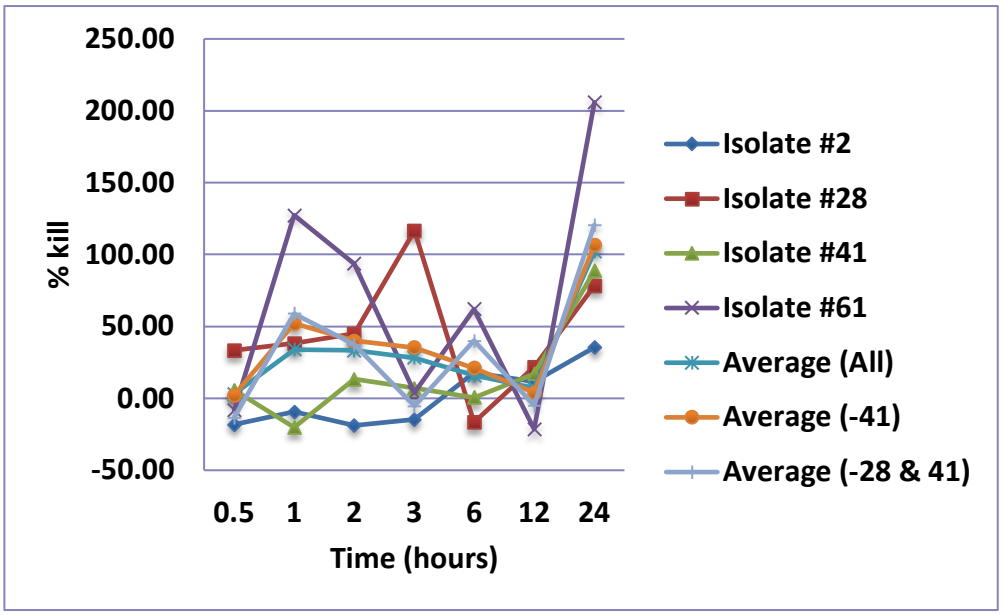


Figure 3.7.76: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the MPC drug concentration of vancomycin.

Exposure of 10^9 CFU/ml to the vancomycin MPC drug concentration did not yield substantial killing for any strain tested. Less than 30% killing was seen for strains 2, 28 and 41 at any time point and growth occurred between 6 and 24 or 12 and 24 hours of drug exposure depending on strains.

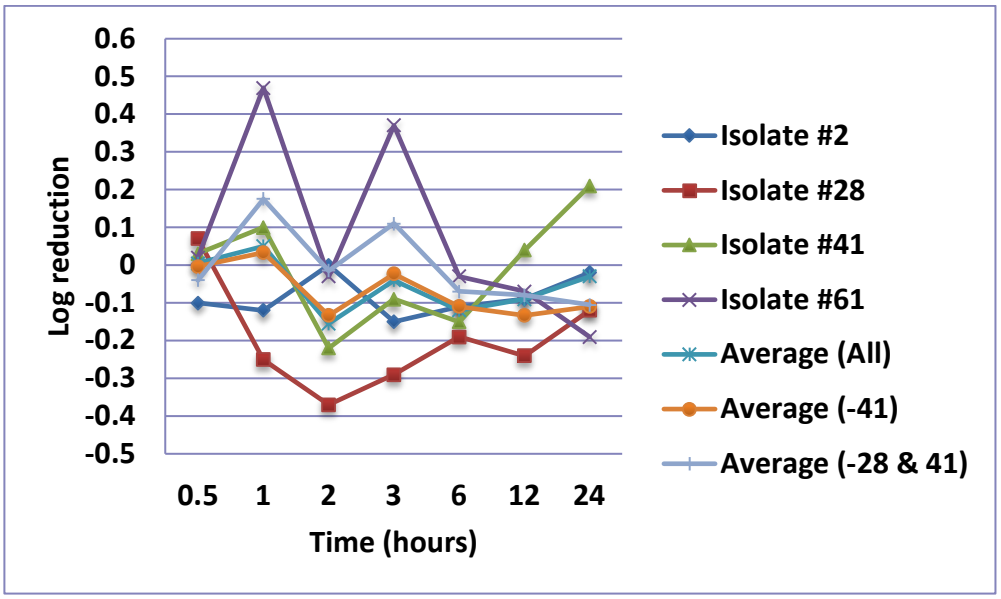


Figure 3.7.77: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

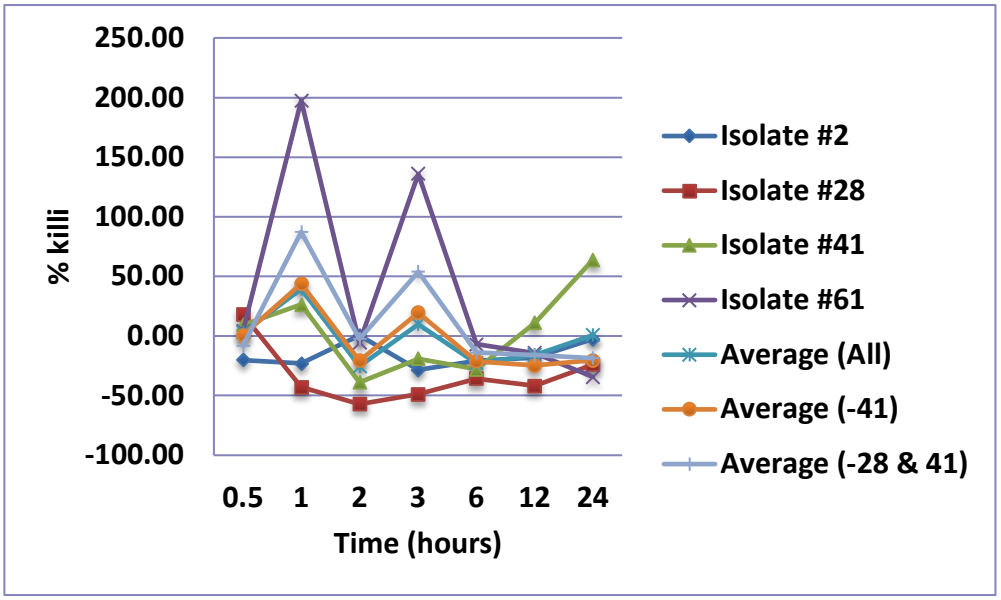


Figure 3.7.78: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of vancomycin.

Exposure of 10^9 CFU/ml to the vancomycin $Tiss_{max}$ drug concentration did not result in substantial killing of any strain. Approximately 55% of viable cells were killed for strain 28 following 2 hours of drug exposure but growth occurred thereafter.

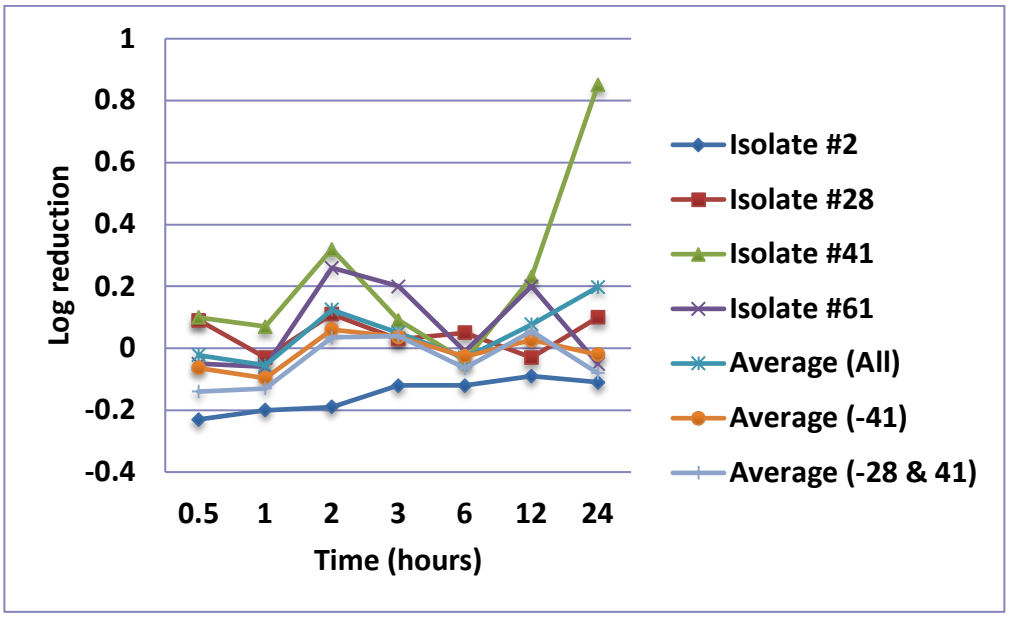


Figure 3.7.79: Log reduction of MRSA strain at 10^9 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

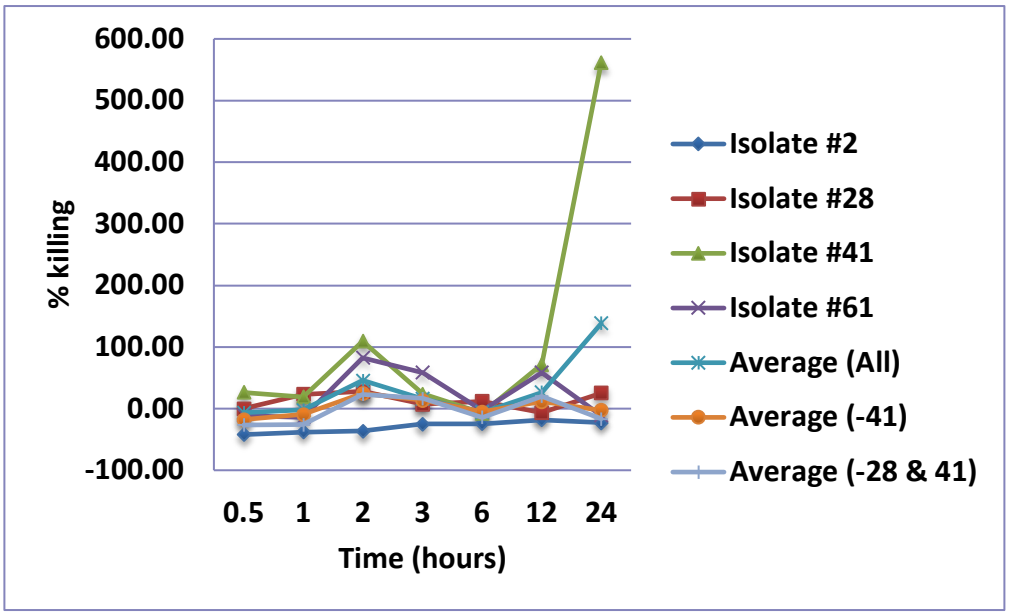


Figure 3.7.80: Percent kill of MRSA strain at 10^9 CFU/ml inocula using the C_{max} drug concentration of vancomycin.

Exposure of 10^9 CFU/ml to the vancomycin C_{max} drug concentration did not result in substantial killing for any strain tested.

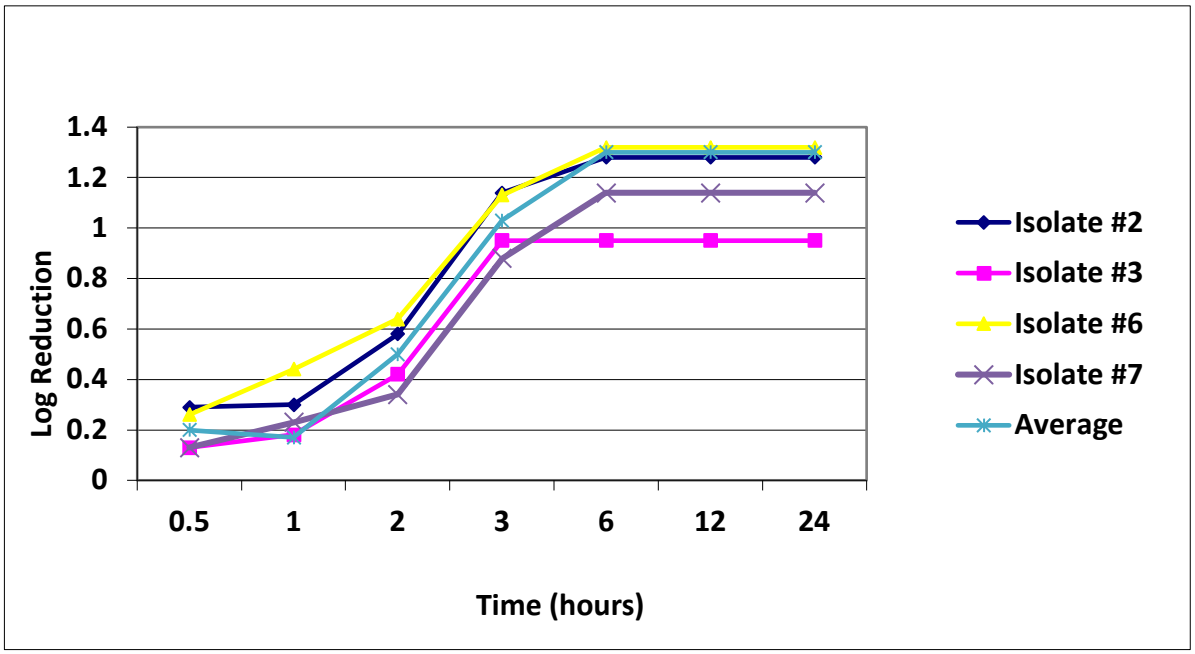


Figure 3.7.81: Log reduction of MRSA strain at 10^5 CFU/ml inocula without drug.

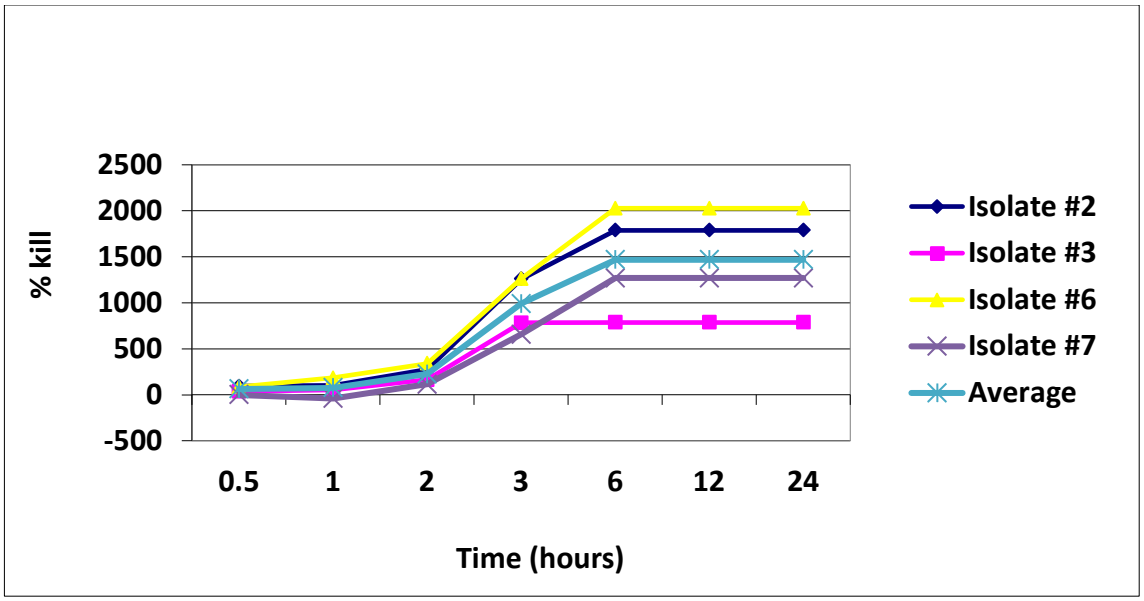


Figure 3.7.82: Percent kill of MRSA strain at 10^5 CFU/ml inocula without drug.

Four independent strains were included in assays without drug. In these experiments, 10^5 CFU/ml were used. Growth for all strains occurred at 30 minutes and at each time point thereafter.

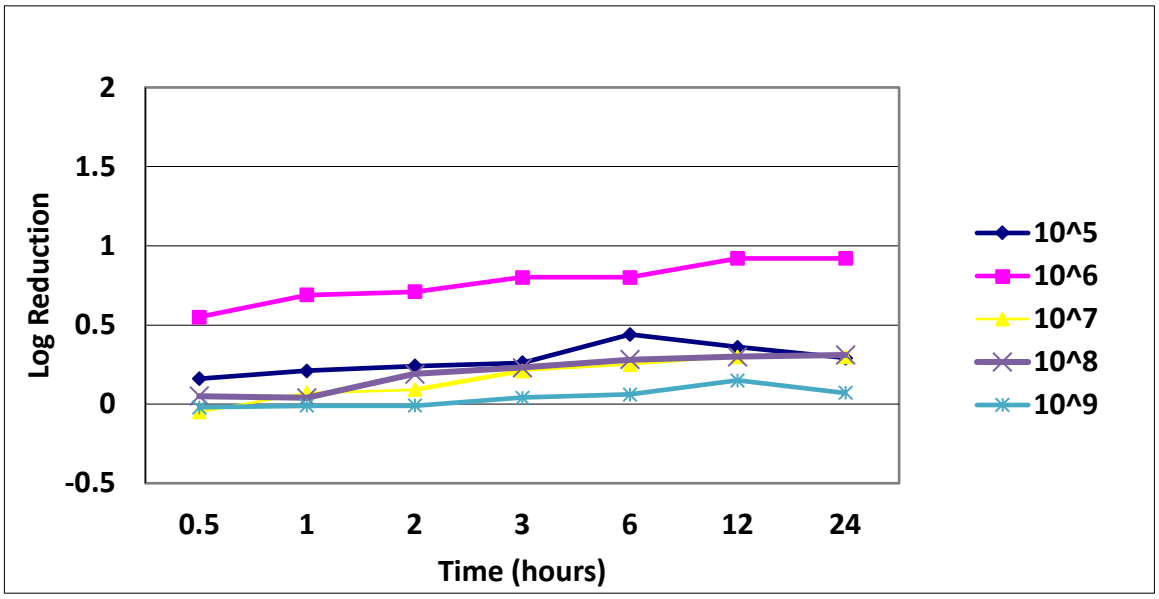


Figure 3.7.83: Log reduction of MSSA strain at 10^5 -10^9 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

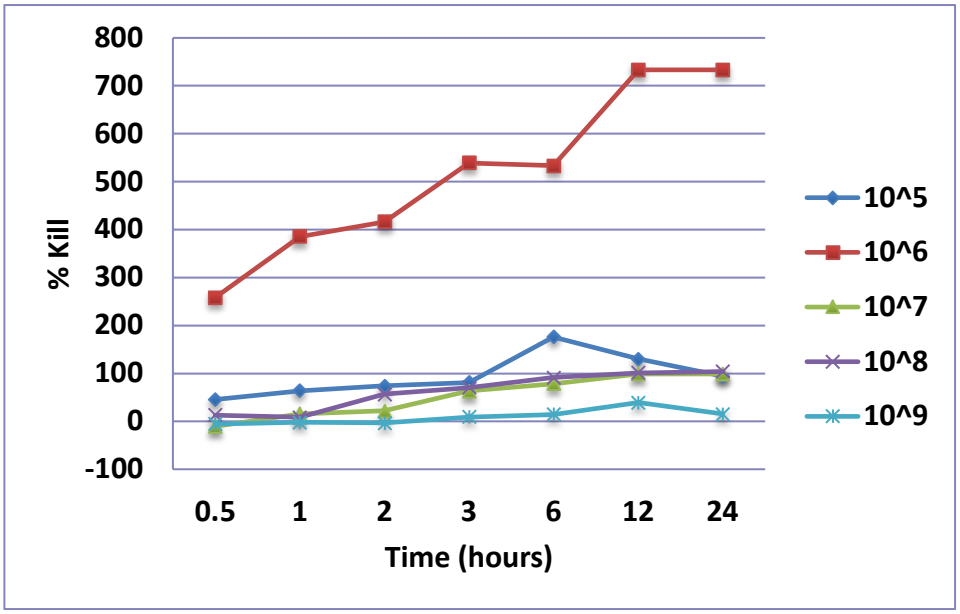


Figure 3.7.84: Percent kill of MSSA strain at 10^5 -10^9 CFU/ml inocula using the MIC/MPC drug concentration of linezolid.

Exposure of 10^5-10^9 CFU/ml to the MIC and MPC linezolid drug concentrations are shown above. Killing as measured by reduction in viable cells did not occur in the presence of linezolid for any of the bacterial densities investigated for the MSSA strains.

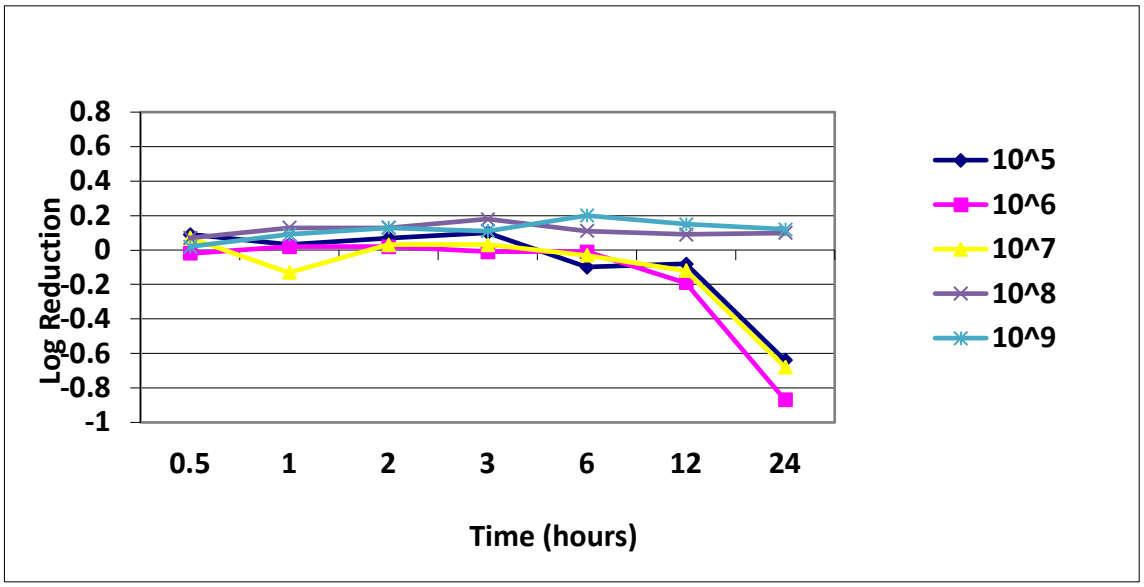


Figure 3.7.85: Log reduction of MSSA strain at 10^5 -10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

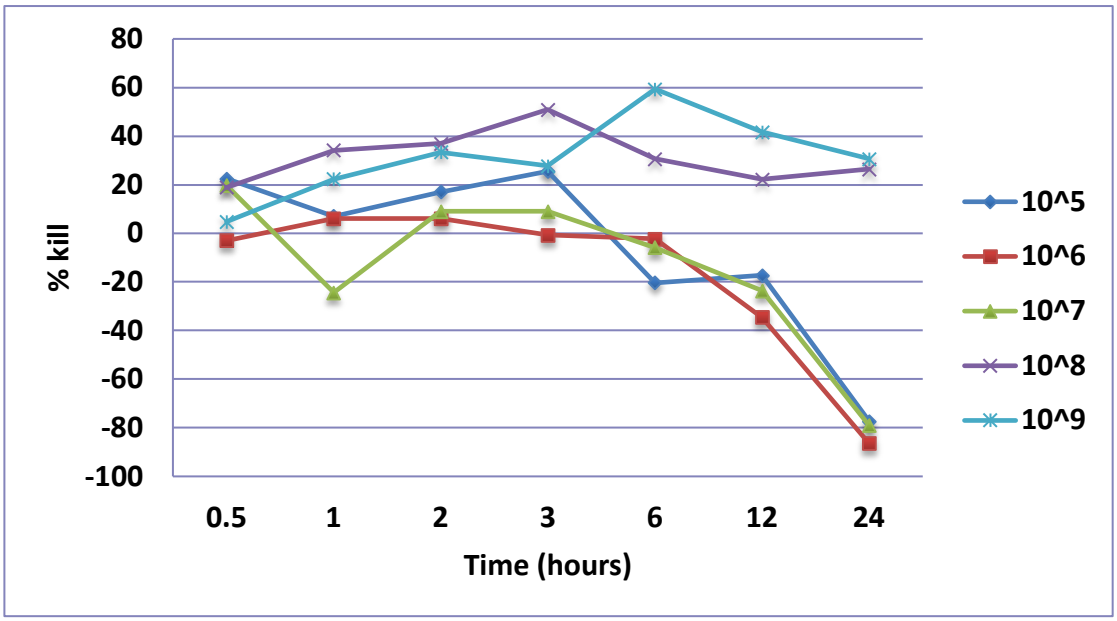


Figure 3.7.86: Percent kill of MSSA strain at 10^5 -10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of linezolid.

Exposure of 10^5-10^9 CFU/ml to the linezolid $Tiss_{max}$ drug concentration is shown above. For the 10^5 CFU/ml and 10^6 CFU/ml, a 0.6-0.9 log_{10} reduction (75-85% kill) was seen following 24 hours of drug exposure. Killing did not occur for the other densities tested.

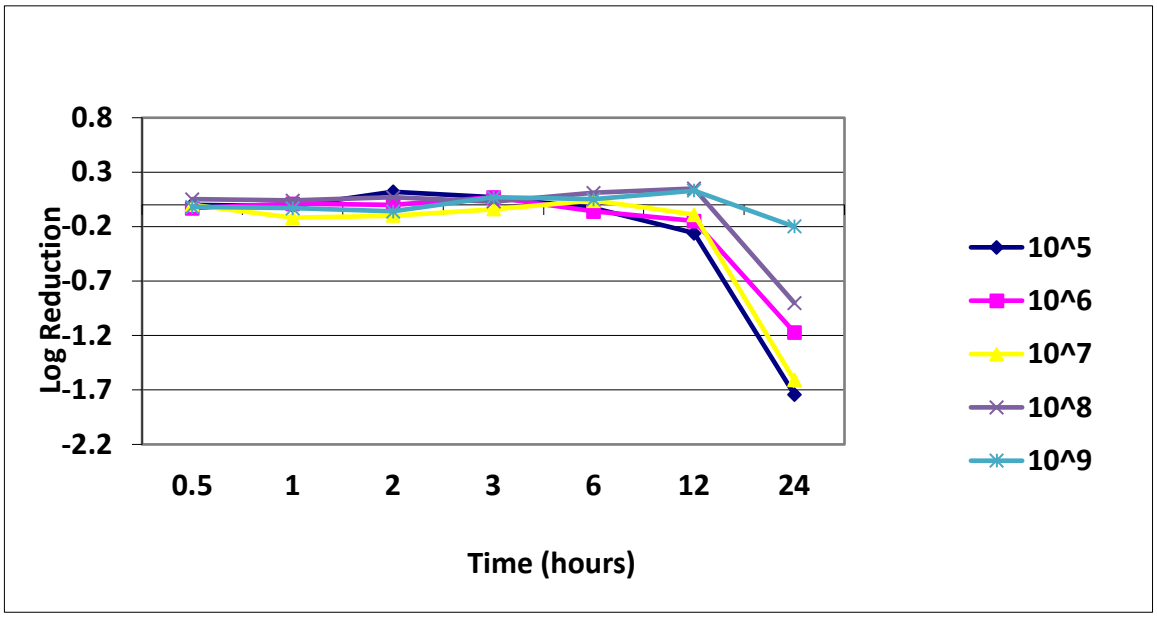


Figure 3.7.87: Log reduction of MSSA strain at 10^5 -10^9 CFU/ml inocula using the C_{max} drug concentration of linezolid.

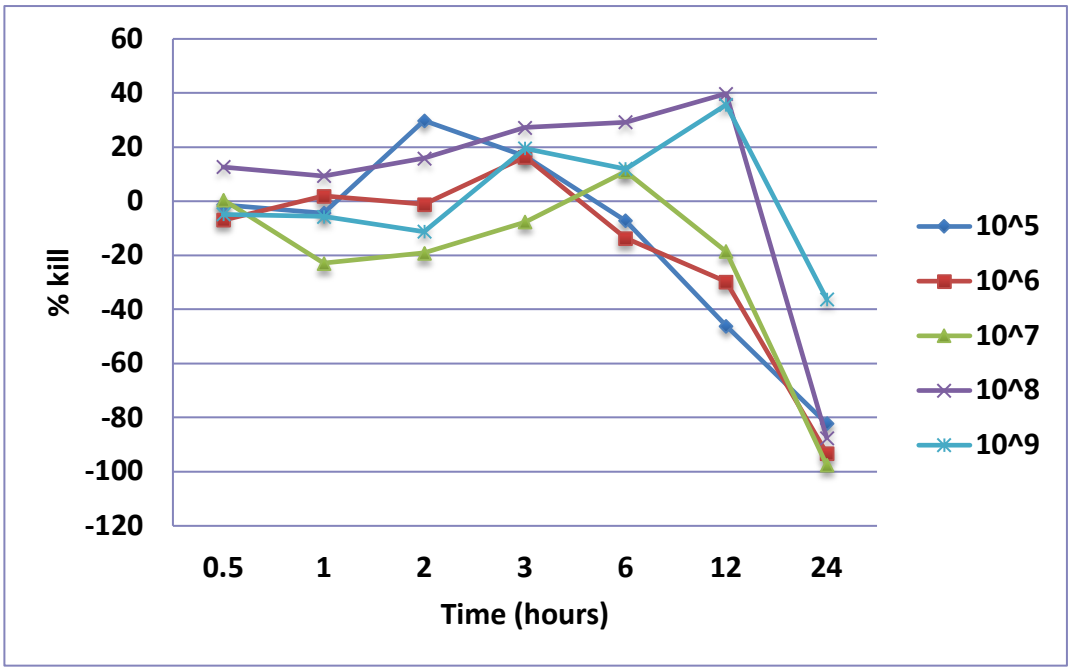


Figure 3.7.88: Percent kill of MSSA strain at 10^5 -10^9 CFU/ml inocula using the C_{max} drug concentration of linezolid.

Exposure of 10^5-10^9 CFU/ml to the linezolid C_{max} drug concentration is shown above. A 0.2-1.7 $\log_{10}$ reduction was seen for the various densities tested corresponding to 35-100% kill.

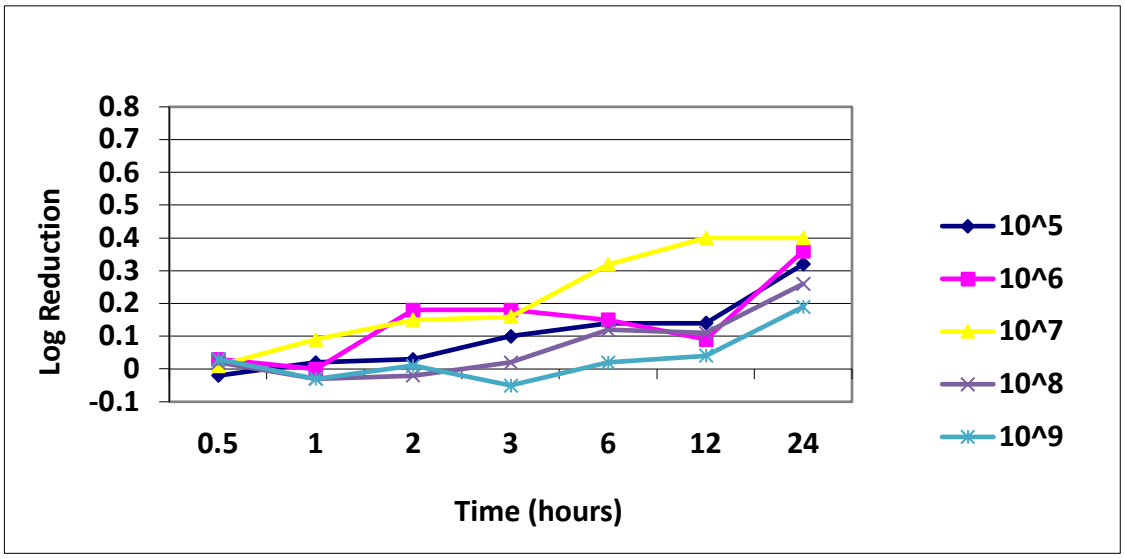


Figure 3.7.89: Log reduction of MSSA strain at 10^5 -10^9 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

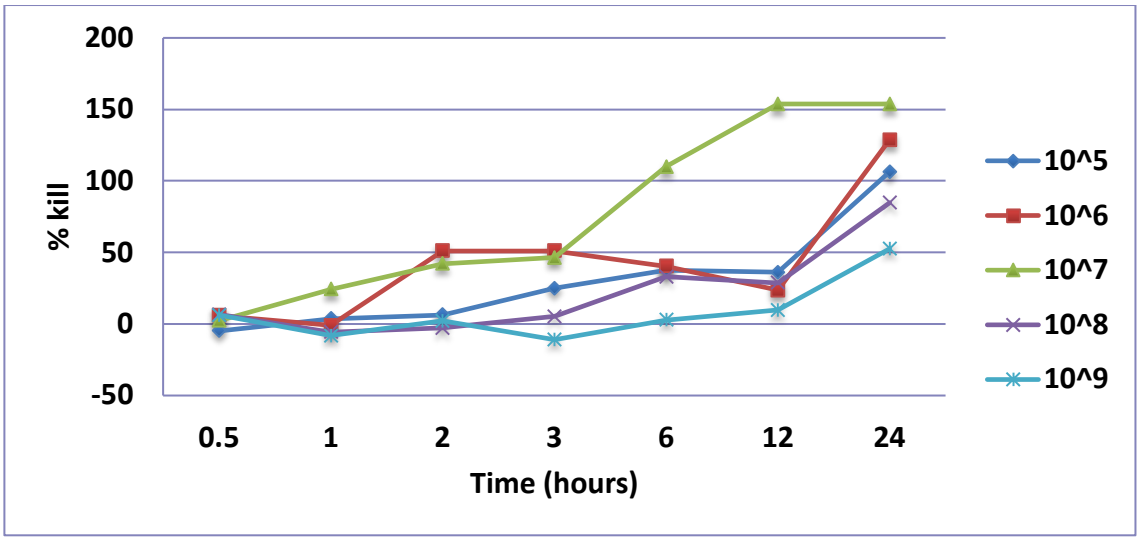


Figure 3.7.90: Percent kill of MSSA strain at 10^5 -10^9 CFU/ml inocula using the MIC/MPC drug concentration of tedizolid.

Exposure of 10^5-10^9 CFU/ml to the tedizolid MIC and MPC drug concentration failed to result in substantial killing of any density tested.

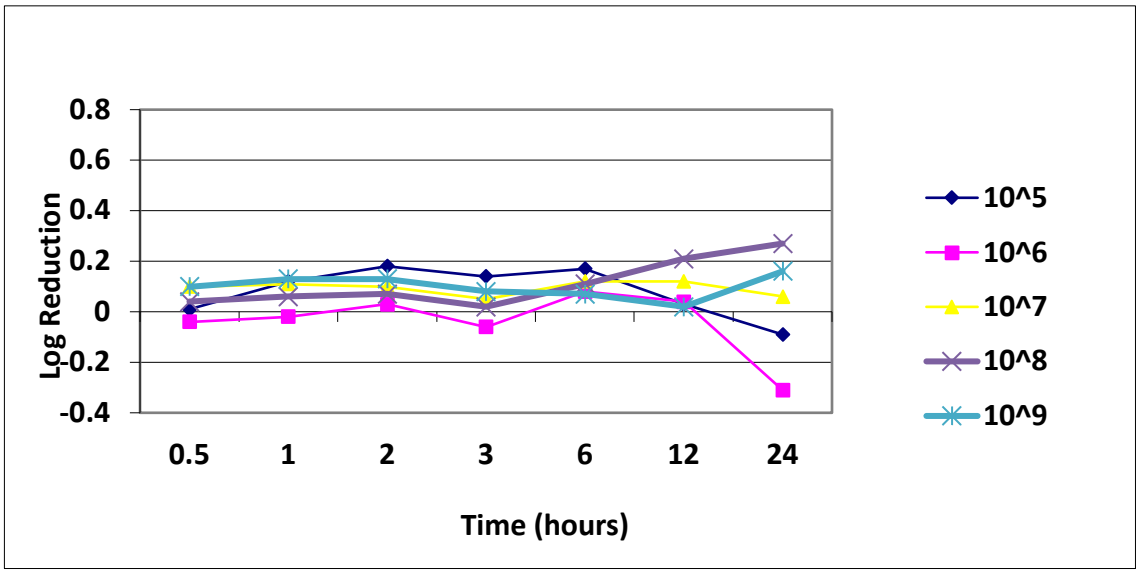


Figure 3.7.91: Log reduction of MSSA strain at 10^5 -10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

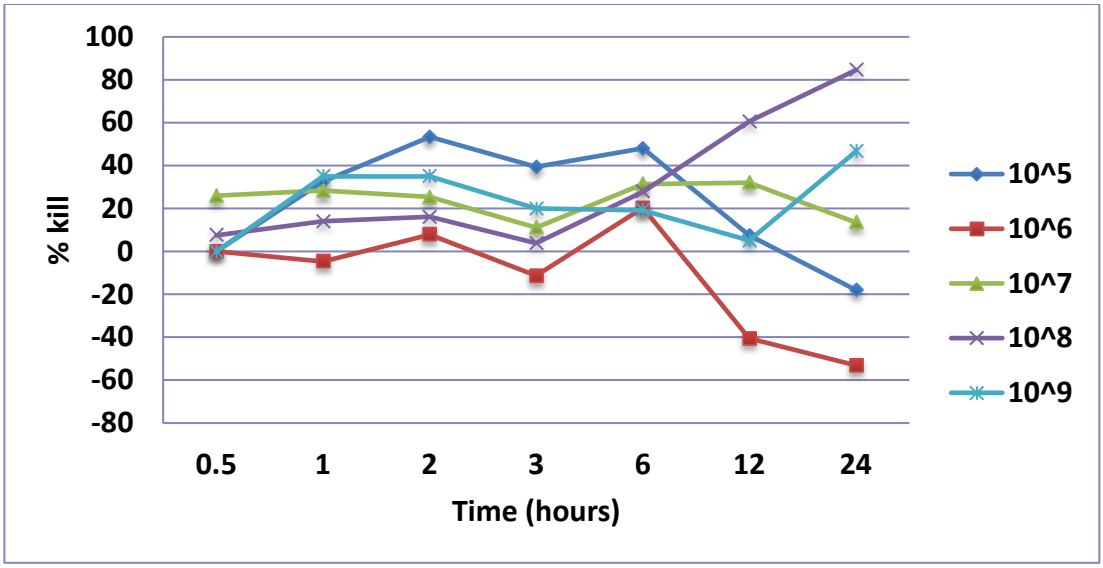


Figure 3.7.92: Percent kill of MSSA strain at 10^5 -10^9 CFU/ml inocula using the $Tiss_{max}$ drug concentration of tedizolid.

Exposure of 10^5-10^9 CFU/ml to the tedizolid $Tiss_{max}$ drug concentration resulted in 20-50% kill for 10^5 and 10^6 densities tested following 24 hours of drug exposure. Growth occurred with the other densities tested.

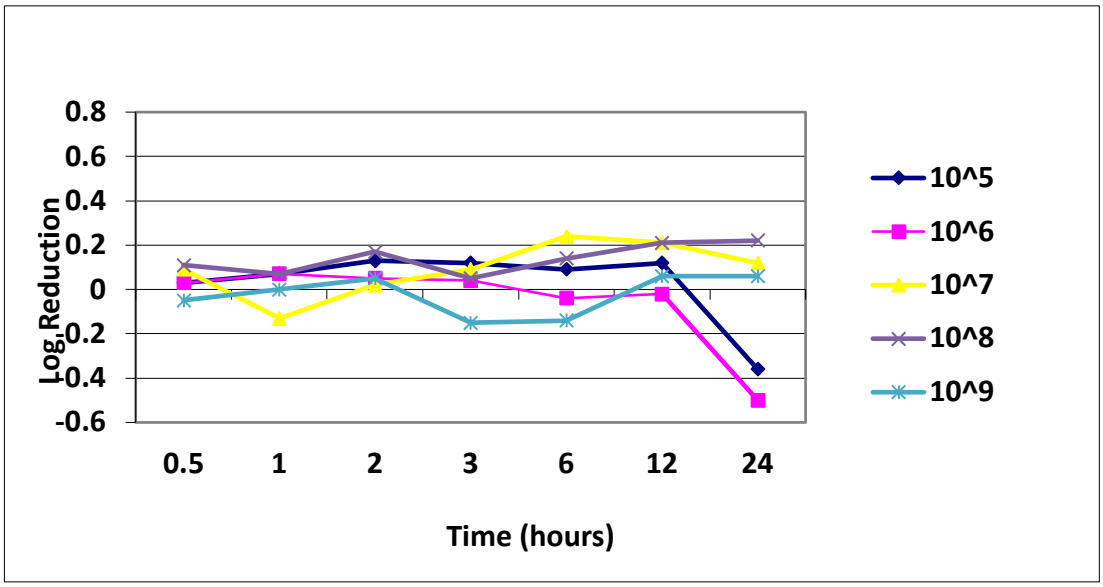


Figure 3.7.93: Log reduction of MSSA strain at 10^5 -10^9 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

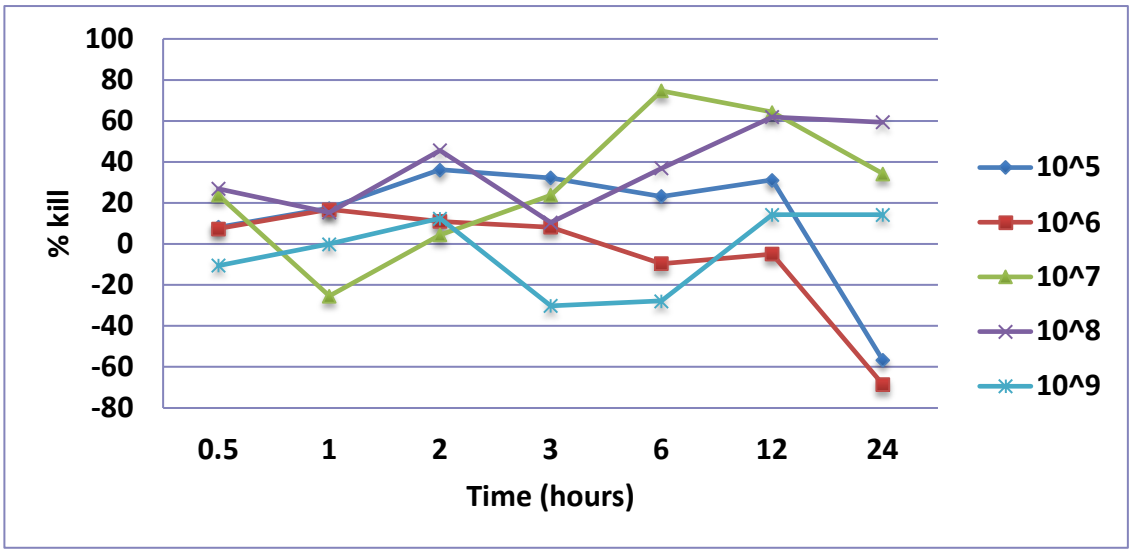


Figure 3.7.94: Percent kill of MSSA strain at 10^5 -10^9 CFU/ml inocula using the C_{max} drug concentration of tedizolid.

Exposure of 10^5-10^9 CFU/ml of the tedizolid C_{max} drug concentration resulted in 60-70% kill of the 10^5-10^6 CFU/ml densities following 24 hours of drug exposure. Growth occurred at the other densities tested.

4.0 DISCUSSION

The MIC is the standard measurement and most commonly used parameter in clinical microbiology to determine the susceptibility of microorganisms to antimicrobial agents and to confirm unusual resistance. It is also used as a research tool to determine *in-vitro* activity of new antimicrobial agents, compare the *in vitro* activity of different antimicrobial agents, assess the *in vitro* activity of antibiotic combinations, and to set antibiotic dosage recommendations by various committees.

MIC testing is known to be efficient, as many isolates may be tested simultaneously. This type of testing also allows for results to be available within 24 hours, which is important for patients with serious bacterial infections. Moreover, it is cost-effective, easy to determine experimentally, and a reproducible (within one doubling dilution) technique.

Nevertheless, MIC testing has a number of disadvantages. This procedure tests organisms at an inoculum of ~10^5 CFU/ml, whereas the common bacterial burdens during infection reported may be as high as10^{10} CFU/ml [Gould & MacKenzie, 2002] and resistant subpopulations are believed to be present in such large bacterial inoculum [Allen, 2003]. According to the principle of spontaneous mutation frequency at one mutation for every 10^6-10^8 cells, an inoculum exceeding 10^6 cells will probably contain bacterial cells that have developed resistance (point mutations) and consequently reduced susceptibility to the antibiotic [Zhao & Drlica, 2001]. Therefore, testing based on an inoculum of 10^5 CFU/ml may not be representative of the true susceptibility of the bacterial population when there is >10^5 CFU/ml at the infection site [Blondeau, 2003]. In other words, the MIC may not predict the resistance development, since MIC testing utilizes a lower inoculum in which resistant subpopulations are most likely to be absent. The MIC may therefore not accurately represent a drug concentration that will inhibit all the cells in a bacteria population that is present during infection.

Furthermore, MIC indicates a threshold concentration i.e., no quantitative distinction between all concentrations above the MIC or below the MIC. This static approach does not

also reflect the *in vivo* interaction of exposing bacteria to continuously changing antibiotic concentrations. It also fails to provide a description of pharmacodynamic scenario between the drug and micro-organisms in the target organ as well as the bactericidal activity and its persistent activity or PAE [Mueller*, et al*, 2004]. Regardless of these shortcomings, MIC testing remains the standard *in vitro* measurement of drugs' potency to determine their therapeutic potentials [National Institute of Allergy and Infectious Diseases, 2017].

In our study, MIC values for the MRSA strains tested against the various antimicrobial agents were consistent with MIC values reported by others [Allen*, et al*, 2004; Blondeau & Borsos, 2007; Blondeau*, et al*, 2007; Blondeau*, et al*, 2006; Blondeau & Metzler, 2005; Blondeau*, et al*, 2001; Hansen & Blondeau, 2005; Hedlin & Blondeau, 2004; Hesje*, et al*, 2015; Metzler*, et al*, 2004; Sievert*, et al*, 2008] and within susceptibility or resistant designated categories as recommended by the Clinical and Laboratory Standards Institute. As such, there was nothing unusual from a susceptibility or resistance perspective with the collection of MRSA strains included for investigation in our study.

MPC is a new concept aimed to limit the prevalence of antibiotic resistance by preventing the selection of resistant mutants. It determines the antibiotic concentration that inhibits not only susceptible cells but also the growth of first-step resistant phenotypes in a high-density bacterial population. The innovativeness of this approach is the utilisation of bacterial populations which are equal to or exceed 10^9 CFU [Lu*, et al*, 2003; Zhao & Drlica, 2002; Zhao*, et al*, 2003].

This approach may help to develop new dosing strategies that minimize the development of resistance. It can also be applied to evaluate and compare potency of antibiotics against single step resistant mutants, which may aid in identifying agents that are least likely to select for resistance [Zhao & Drlica, 2002]. However, there are no animal or human trials available to test this hypothesis; thus, more investigation is required. Also, the concept behind this method "the MSW' has some limitations as it excludes cases of natural resistance and the enzymatic inactivation of a compound grants resistance to both enzyme-producing and non-producing cells.

MPC has some drawbacks. It is a lengthy procedure requiring 4 days and only a few

isolates can be tested in one assay, and it is not as cost effective as MIC testing. Therefore, testing in its current format cannot be performed easily by many laboratories. A liquid based microbroth MPC assay has been investigated and further development work is needed [Hesje & Blondeau, 2008].

Our study also represents the first report of MPC values for the drug tedizolid, the newest oxazolidinone, tested against MRSA strains and also the first report of MPC values for linezolid against MRSA blood culture isolates. In a summary (abstract only) of MRSA strains tested against linezolid, Zhao *et al* (2010) reported a narrow mutant selection window for linezolid, a finding substantiated in our research [Zhao*, et al*, 2010]. The width of the MSW is determined by the fold difference between the measured MIC and MPC for the individual strain tested. Data from multiple strains can be considered by comparing the MIC_{90} and MPC_{90} values. In our work the MIC_{90} for linezolid was 2 µg/ml and the MPC_{90} was identical meaning the MSW was narrow. The MPCs of linezolid against *Mycobacterium tuberculosis* were previously tested by Rodriguez *et al* [Rodríguez*, et al*, 2004] and MRSA [Zhao*, et al*, 2010] and showed great ability to restrict the selection of resistant mutants of MRSA. Similarly for tedizolid, the majority of MRSA strains (88% and 100% respectively) had both MIC and MPC values at or below the susceptibility break points which narrows the MSW and keeps the serum drug concentration in excess of the selection window over the duration of the dose, and consequently reduces the likelihood for resistance selection to occur for those drugs.

A major finding in our study was the high MPC values for vancomycin with some MRSA strains. While all strains of MRSA were inhibited by an MIC $\leq$1 µg/ml, >50% of the strains were non-susceptible (8 µg/ml) and 40% of strains were resistant ($\geq$16 µg/ml) by MPC testing. Such values have not been previously reported and are likely a major concern for clinical medicine as the number of such strains increase in prevalence in infected patients for whom vancomycin is used for therapy. Our data shows the survival of MRSA bacterial cells in high or concentrations of vancomycin and such concentrations cannot be safely achieved in treated patients with known dosing strategies.

We attempted to determine the mechanism(s) responsible for the survival of MRSA bacterial cells in high vancomycin drug concentrations. To do this we investigated cell wall thickness by electron microscopy, we tested for cell wall markers, and examined PFGE profiles after serial passage in increasing vancomycin drug concentrations. Serial passage experiments were performed to try and induce/generate a stably resistant clone from which more extensive investigations could be performed as to mechanism of resistance. Unfortunately we were unable to generate such a clone (serial passage failed to induce a stably resistant bacterial cell) and others [Kosowska-Shick*, et al*, 2009] have reported similar difficulties but not with strains showing high MPC values to vancomycin.

PFGE on select strains with the high MPC values to vancomycin were compared to the profiles of the wild type (parental) strains. No single dominant clone was associated with the high MPC values and profiles from the parental strain were identical to the matched isolate recovered from the drug containing MPC plates.

Our EM investigations did provide some interesting and previously unrecognized observations. First, cell wall thickening was seen for 50% of bacterial cells from a limited sampling of cells removed from agar plates containing 32 µg/ml of vancomycin. Second, from MRSA inoculated agar plates containing 32 µg/ml of vancomycin and from MRSA inoculated MH broth containing 32 µg/ml of vancomycin, apparent dividing bacterial cells were seen on EM micrographs (organisms continued to replicate in the presence of the vancomycin).

Septum formation, indicating cell division, was originally seen on micrographs initially generated to investigate if cell wall thickening could be seen with MRSA cells sampled from agar plates containing high vancomycin drug concentrations and as such an explanation for cell survival in the presence of the drug. With this unexpected observation, a series of experiments were designed to explore this observation further. First, MRSA was inoculated to agar plates containing 32 µg/ml of vancomycin, incubated overnight and then sub-cultured to fresh plates containing 32 µg/ml of vancomycin and incubated overnight. From these inoculated drug-containing plates, bacterial colonies were removed and placed immediately into fixative for electron microscopy. Cells showing septum formation were seen. To

investigate if the cells showing septum formation might have been the result of poor drug exposure on agar plates, the same experiments were repeated in broth media. Again, bacterial cells were sampled from MHB containing 32 μg/ml of vancomycin and immediately placed in fixative for electron microscopy. Once again septum formation was seen and confirmed the observations seen for cells taken from drug-containing agar plates. This eliminated the possibility that replication was occurring due to lack of contact with drug on the plates. To the best of our knowledge, we are unaware of such an observation being previously reported. Surewaard *et al* reported replication of MRSA in Kupffler cells in the liver in the presence of vancomycin, however, vancomycin does not penetrate the Kupffler cells so their observations are different than ours and replication was acknowledged as occurring in the absence of the drug [Surewaard*, et al*, 2016]. In that study, bacterial cell replication was demonstrated by electron microscopy showing septum formation, images similar to those reported here. As such, our observation of septum formation, indicating bacterial replication is consistent with the report of Surewaard *et al*. The observation of MRSA replication in the presence of high vancomycin drug concentrations is disturbing and may have important clinical implications for patients with MRSA infection and requiring vancomycin therapy.

The thickness of the cell wall in vancomycin-tolerant (VT) MRSA isolates has not been evaluated. However, the study of Cazares-Domínguez *et al* indicated vancomycin induced cell wall thickening in VT- MRSA isolates tested. In their study, a total of 88.88% of these isolates were associated with *agr* type II and *SCCmec* group II and showed adaptive resistance (lowered susceptibility) to vancomycin. This type of resistance was interpreted to be inducible in the presence of the drug [Cazares-Dominguez*, et al*, 2015]. In multiple studies [Moise-Broder*, et al*, 2004; Rose*, et al*, 2012; Sakoulas*, et al*, 2003; Sakoulas*, et al*, 2002], *agr* type II polymorphism was identified to be strongly associated with vancomycin failures against *S. aureus* and MRSA bacteremia. Additionally, Sakoulas *et al* suggested that the loss of *agr* function confer MRSA survival and resistance to a vancomycin effect [Sakoulas*, et al*, 2004] accessory gene regulator (*agr*) is known to regulate various metabolic pathways and the expression of multiple virulence factors [French, 2006].

Furthermore, the *ClpP* role in the growth of Gram-positive bacteria under stress conditions has been demonstrated in multiple studies [Frees & Ingmer, 1999; Gaillot*, et al*, 2000; Msadek*, et al*, 1998; Thomsen*, et al*, 2002]. Frees *et al* suggested the presence of *S. aureus ClpP* contribute in the degradation of stress-damaged proteins[Frees*, et al*, 2003], which was also observed in other Gram-positive bacteria, such as *Bacillus subtilis, Listeria monocytogenes* and *Lactococcus lactis* [Frees & Ingmer, 1999; Gaillot*, et al*, 2000; Msadek*, et al*, 1998].

Another study was conducted by Springer *et al* to determine the effect of *ClpP* and *ClpC* deletion on *S. aureus* persister cells following antimicrobial therapy. That study showed that the removal of *ClpP* resulted in a significant decrease of persisters after the administration of erythromycin and oxacillin [Springer*, et al*, 2016]. Persister revival assays (isolating persister cells by rapidly killing normally growing cells using lytic solutions) demonstrated a significant delay in resumption of persisters growth, implying that the surviving organisms are not caused by spontaneous resistance and suggesting that *ClpP* plays a role in persisters formation, which was also affected by the antibiotic class and the growth phase of the bacterial cells. In addition, Donegan *et al* examined the proteolytic regulation of toxin-antitoxin systems by the *ClpP* in *S. aureus*, and concluded that *ClpP* has an essential role in the degradation of *S. aureus* antitoxins [Donegan*, et al*, 2010].

These aforementioned studies were performed on organisms with reduced susceptibility to vancomycin, as determined by MIC measurements. These differ from our investigations as our MRSA strains were fully susceptible to vancomycin by MIC testing (MICs $\leq$1 µg/ml; breakpoint for resistance is $\geq$8 µg/ml) yet survived on agar plates containing $\geq$32 µg/ml of vancomycin. As well, and as previously stated, organisms surviving on agar plates containing $\geq$32 µg/ml of vancomycin showed wildtype susceptibilities when retested by MIC testing. Again, this is different than previously performed investigations where strains with elevated MIC values were used. These observations from our strains suggest a tolerance or persistence effect for which the mechanism remains unknown.

In our experiments, cell wall markers including *agr* 1-4, *ClpP* and SCC 1-5 did not identify a common factor that could be associated with the survival of MRSA bacterial cells in

high vancomycin drug concentrations. This has also been previously reported [Cazares-Dominguez*, et al*, 2015]. Additionally, we performed PCR analysis for the *pvl* gene and confirmed its presence in a number of our MRSA strains. This rules out the possible association between *pvl* presence and the high MPC values observed.

In light of our observations of MRSA strains with high vancomycin MPC values, we were interested in determining if the visual endpoint meant that no viable organisms were present on the drug-containing plates. This differentiation might be important when comparing bactericidal (kill bacteria) versus bacteriostatic agents (inhibit bacterial growth). From our experiments, we found that visual endpoints did not reflect the absence of viable organisms. A negative MPC plates (no visual growth), showed growth when sub-cultured to drug-free plates, including bacteriostatic and bactericidal agents. For MIC testing (performed at 10^5 - 10^9 CFU/ml), results varied although most of negative wells showed growth when sub-cultured to drug-free plates (vancomycin at 10^5 was the only exception).

Previously published MPC reports summarized MPC data based on the visual endpoint (discernable colonies present or absent) and by definition, the MPC is the drug concentration blocking 100% of visible growth. For routine susceptibility testing by microbroth dilution, the visual endpoint is for inhibition of growth and not 100% killing of viable cells. Neither MIC nor MPC testing are measurements of kill but rather inhibition of growth. Therefore, these observations add to the growing body of evidence related to MPC testing.

Time-kill curves have contributed to providing comprehensive details about the time course of an antibacterial effect [Mueller*, et al*, 2004]. This approach has been used to determine the kinetics of bacterial killing *in-vitro* and determine if the agent is bactericidal or bacteriostatic. Bacterial killing may be time-dependent or concentration-dependant. Concentration-dependent bacterial killing occurs when the rate of killing increased proportionally with antibacterial concentrations (e.g., for aminoglycosides and fluoroquinolones). For antimicrobials that demonstrate time-dependent killing does not increased with higher concentrations (e.g., for oxazolidinones and ß-lactams) but rather is affected by the amount of time concentrations exceeding the MIC during the dosing intervals.

Distinction between bacteriostatic and bactericidal in clinical practice is not absolute

and can result in inaccurate assumptions regarding antibacterial therapy. *In vitro* definitions of bacteriostatic and bactericidal agents are applied to specific drug-bug combinations under strict laboratory conditions. But, it is inconsistent for a specific agent against all pathogens. Therefore, this classification is considered arbitrary in clinical situations [Pankey & Sabath, 2004].

Moreover, some antibacterial agents that are classified as bacteriostatic may demonstrate bactericidal activity against some bacteria *in vitro*, and vice versa. For example, quinupristin-dalfopristin is considered to be bactericidal against most strains of streptococci and staphylococci, but it is considered bacteriostatic against *Enterococcus faecium.* Blondeau *et al.* demonstrated killing of *S. pneumoniae* isolates by gemifloxacin – a fluoroquinolone – and with macrolide agents, which are considered to be bactericidal and bacteriostatic respectively [Blondeau*, et al*, 2015]. Furthermore, drugs considered to be bactericidal may also act as bacteriostatic at low concentrations. Similarly, bacteriostatic agents at high concentrations can exhibit bactericidal activity against some susceptible organisms [Pankey & Sabath, 2004].

In terms of pharmacodynamic and pharmacokinetic assessment, this approach has some drawbacks in regard to protein binding and tissue distribution. Protein binding should be considered because most drugs bind to plasma proteins, which are not identified by serum concentrations. It should be noted that only unbound drug will display a pharmacological effect. Another parameter to consider is tissue distribution, which is important due to the fact that most infections occur in tissues rather than in plasma [Mueller*, et al*, 2004].

Kill studies were previously published for staphylococci with vancomycin [Herrera*, et al*, 2013], linezolid [Tsuji*, et al*, 2012] and tedizolid [Nunart*, et al*, 2017]. These studies are different than ours as they used drug concentrations that are multiples of the MIC drug concentration (one-half, 2X, 4X, 8X and 16X the MIC) and testing lower densities of bacteria, approximately 10^5-10^6 CFU/ml. In our experiments we used bacterial densities ranging between 10^5-10^9 CFU/ml and drug concentrations of the measured MIC, MPC and published values for the $Tiss_{max}$ and C_{max}. Our kill studies are consistent with previous studies demonstrating tedizolid and linezolid as bacteriostatic agents against *S. aureus*. However, for

some MRSA strains better killing was seen in the presence of linezolid and tedizolid than others and some strains showed no killing following drug exposure (at 24hr). Therefore, current data is not absolutely clear for linezolid and tedizolid and the designation of bacteriostatic versus bactericidal might be more related to the strains tested and drug concentrations used. For example, with linezolid and strain number #2, a 1.3 $\log_{10}$ reduction translated to ~99% killing following 24 hours of drug exposure at the maximum tissue drug concentration.

Similarly for vancomycin, our results were consistent with previously published studies demonstrating its bactericidal activity against *S. aureus* at lower bacterial inoculum $\leq 10^6$ CFU/ml. However, we observed that at higher bacterial inoculum ($\geq 10^7$ CFU/ml) the drug tended to act as a bacteriostatic agent. Further kill experiments on a larger number of MRSA strains might help to resolve this observation.

One consideration as an explanation for the high vancomycin MPC values with some MRSA strains is a tolerance or persistence effect. Conlon *et al* indicated that treatment failure following *S. aureus* infections in some cases was due to antibiotic tolerant cells called persisters [Conlon*, et al*, 2013]. Tomasz *et al* defined the antibiotic tolerance as "the ability of bacteria to survive, but not grow, in the presence of drug" and assumed tolerance to be the precursor phenotype to resistance [Tomasz*, et al*, 1970].

Tolerance may be a reversible phenotypic response, which can be easily produced *in vitro* by limiting the supply of essential nutrients as well as *in vivo* in animal models [Handwerger & Tomasz, 1985; Tuomanen*, et al*, 1986]. Phenotypic tolerance may provide an explanation for the persisters subpopulation, which comprise only 0.1-1% of a total bacterial population [Dawson*, et al*, 2011]. This small population consists of cells that are replicating at a slower rate than the majority of the population. Persisters are not truly resistant and usually are susceptible during repeat testing. Persistence can be defined as the ability of a subpopulation to survive high concentrations of an antibiotic which is a possible explanation of our observations but remains unproven.

The tolerance phenomenon is known to occur in staphylococci, and vancomycin tolerance in *S. aureus* has been reported in several studies [May*, et al*, 1998; Perry*, et al*,

1999; Voorn, *et al*, 1994]. In a study including nine hospitals, vancomycin tolerance was identified in 20% of MRSA isolates, while some institutions showed a higher prevalence at 43% [Gemmell, *et al*, 2006].

Vancomycin tolerance has proven to be more common in MRSA than in MSSA strains, and especially from endocarditis cases [May, *et al*, 1998]. The tolerance mechanism in *S. aureus* is unclear and the clinical properties of these strains are still unknown. However, it was demonstrated to be linked to autolysis deficiency [Handwerger & Tomasz, 1985].

It has been reported in a number of studies that the infections (endocarditis and bacteremia) caused by VT-MRSA are more difficult to treat due to the poor responses to vancomycin therapy, therefore additional agents to achieve bactericidal effects are necessary [Faville, *et al*, 1978; Gopal, *et al*, 1976; May, *et al*, 1998; Reis, *et al*, 1995; Sorrell, *et al*, 1982].

Tolerance to antibiotics allows bacterial regrowth after antibiotic removal, without altering the MIC. This phenomenon may reduce the drug susceptibility and thus increase the difficulty of treating infections that require bactericidal action e.g., endocarditis. Tolerance, therefore, should be evaluated to assess the potential of clinical failure during treatment [Jones, 2006]. Liu and Tomasz indicated that tolerance allows for survivors in the presence of the drug, which may speed up the development of resistance[Liu & Tomasz, 1985].

Vancomycin tolerance is not only limited to *S. aureus*. Novak *et al* described vancomycin tolerance in *S. pneumoniae* caused by *vncS* mutation (the operon encoding histidine kinase) [Novak, *et al*, 1999]. Due to the *vncS* mutation, cells remain viable but cease replication without altering MIC, suggesting that the drugs still have access to their targets. However, such tolerant strains are difficult to detect by routine antimicrobial susceptibility testing [Novak, *et al*, 1999].

It remains unclear from our investigations whether the observations of high vancomycin MPC values are explained by tolerance or persistence. In a series of experiments we conducted, MRSA colonies from agar plates containing $\geq$32 µg/ml of vancomycin were retested to determine the MIC and in all instances the MIC was identical to the MIC of the parental strain. Our experiments neither proved nor disproved the exact mechanism however, and further measurements are required. Regardless of the mechanism, this

observation is likely clinically important, especially in patients with higher bacterial burdens and could be an important risk factor for vancomycin therapeutic failure.

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In the thesis, density refers to CFU/ml [Blondeau, *et al*, 2015] whereas inocula/inoculum refers to the number of CFUs inoculated to agar plates or broth. The terms density and inoculal/inoculum are often used interchangeably in the peer reviewed literature [Blondeau & Shebelski, 2016].

Future Considerations:

- More extensive search for molecular markers that might provide an explanation for high vancomycin MPC values
- Investigate more strains for killing by the 3 drugs over a range of bacterial densities

- More measurements of bacterial replication in the presence of vancomycin
- Further investigations to define visual versus actual endpoints for MPC testing with more types of bacteria and drugs

4.1 Conclusions

The conclusions from this thesis are as follows:

1. A majority of MRSA blood culture isolates had MPC values to vancomycin ≥16 μg/ml despite the fact that MIC values ranged from 0.5-1 μg/ml.
2. There was no single variable identified from patients that could explain high vancomycin MPC values.
3. MRSA strains with high vancomycin MPC values had thicker cell walls as seen by electron microscopy.
4. For both drug-containing agar media and drug-containing liquid media, MRSA strains showed signs of continued replication in the presence of vancomycin.
5. The presence or absence of select cell wall accessory genes were not responsible for high vancomycin MPC values.
6. Linezolid and tedizolid gave inconsistent kill results with considerable strain variability and appear to be bacteriostatic.

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APPENDIX A

Solutions and Buffers

10X TE Buffer

Dissolve 12.1 g of Tris Base and 3.72 g of EDTA in 750 ml of distilled water. Bring to pH 8.0 using HCl. Adjust volume to 1 L and autoclave.

10X TBE

Dissolve 90.8 g of Tris Base, 15.4 g of Boric acid, and 0.37 g of disodium EDTA in 1000 ml of distilled water and autoclave.

0.5 M EDTA

Add 93.05 g of EDTA (disodium salt) to 400 ml distilled water. Add NaOH pellets one at a time until the EDTA is completely dissolved. Bring the pH to 8.0 by adding 5 M NaOH. Adjust volume to 500 ml and autoclave.

0.1M Cacodylate Buffer

Combine 16 g of sodium cacodylate (Mol. Wt. 160.0) with 920 ml of distilled water. pH to 7.2 with 0.1N HCL. Adjust to 1000 ml with distilled water.

1% Pulsed Field Agarose

Add 1.0 g pulsed field agarose to 100 ml 0.5X TBE. Boil until agarose is dissolved and cool to 50°C before pouring the gel. Allow gel to solidify for a minimum of 30 min before use.

1% Agarose Gel for PCR

Add 0.35 g to 35 ml of TBE Buffer containing ethidium bromide (*ETBr*). Microwave on high until the agarose is completely dissolved. Pour gel and allow it to solidify for ~30 min.

2% Glutaraldehyde

Add 10 ml of 25% Glutaraldehyde EM grade to 115 ml of 0.1M cacodylate buffer.

Cell Suspension Buffer

Combine 100 µl of 1M Tris-HCl (pH 7.2), 200 µl of 1M NaCl, and 1.0 ml of 0.5M EDTA (pH 8.0). Dilute to 10 ml with sterile distilled water.

***ETBr* Buffer**

Add 50 µl of *ETBr* at 10 mg/ml to 1 L of TBE Buffer for a final *ETBr* concentration of 500 µg/ml.

***ETBr* Solution For Staining a Pulsed Field Gel**

Combine 40 ul stock ethidium bromide with 400 ml of distilled of distilled water.

Low Melting Point Agarose

Dissolve 1 g into 100 ml of TE Buffer at pH 8.0. Boil for 1-1.5 min and cool to 50-65°C in water bath.

Lysis Buffer

Combine 100 ul of 1M Tris-HCl (pH 7.2), 500 µl of 1M NaCl, and 1.0 ml of 0.5M EDTA (pH 8.0). Weigh out 20 mg of deoxycholate and 50 mg of N-Lauroylsarcosine and add to above mixture. Dilute to 10 ml with sterile distilled water.

PFGE Running Buffer

Dilute 300 ml of 10X TBE in 2700 ml of distilled water for a concentration of 1X TBE.

Proteinase K Buffer

Combine 50 ml of 0.5M EDTA (pH 8.0) and 10 ml of 10% N-lauroylsarcosine. Adjust pH to 9.0, then dilute to 100 ml with sterile distilled water.

Proteinase K/Proteinase K Buffer

Add 25 µl of 20 mg/ml of Proteinase K to 10 ml of Proteinase K Buffer.

Skim Milk

Dissolve 200 g of powdered skim milk into 1000 ml of distilled water and autoclave.

TE Buffer

Add 5 ml of 1M Tris-HCl pH 8.0 and 8 ml of 0.25M EDTA pH 8.0 into 494 ml of distilled water.

Tracking Dye

Add 60 g of sucrose into 100 ml sterile distilled water to make a 60% sucrose mixture. Add 0.25 g of xylene cyanol into 100 ml sterile distilled water. Add 10mM Tris at pH 8.0. Combine the 60% sucrose, the 0.25% xylene cyanol, and the Tris.

TSA

Add 40 g to 1 L distilled water. Autoclave.

Wash Buffer

Combine 10 ml of 1M Tris- HCl (pH 7.6) and 20 ml of 0.5M EDTA (pH 8.0). Dilute to 100 ml with sterile distilled water.

APPENDIX B

PFGE Conditions

Initial Time:	5.3 seconds
Final Time:	34.9 seconds
Included Angle:	120
Voltage:	200V, 6V/cm
Run Time:	18 hr
Variant Speed Pump:	55

APPENDIX C

MIC Breakpoints for Control Strains

ATCC *S. aureus* Strain 29213

Amikacin	≤16 µg/ml
Azithromycin	≤2 µg/ml
Ciprofloxacin	≤1 µg/ml
Gatifloxacin	≤0.5 µg/ml
Gentamicin	≤4 µg/ml
Linezolid	≤4 µg/ml
Moxifloxacin	≤0.5 µg/ml
Tedizolid	≤0.25 µg/ml
Tigecycline	≤0.25 µg/ml
TMP/SMX	≤2/38 µg/ml
Tobramycin	≤4 µg/ml
Vancomycin	≤2.0 µg/ml

ATCC *S. pyogenes* Strain 19615

Azithromycin	≤0.5 µg/ml
Moxifloxacin	≤1* µg/ml
Penicillin	≤0.12 µg/ml
Vancomycin	≤1 µg/ml

*Based on *S. pneumoniae* ATCC 49619.

APPENDIX D

Suppliers

Media

Mueller Hinton Broth (MHB)	Fisher Scientific, Toronto, ON
Todd Hewitt Broth (THB)	Becton, Dickinson and Co., Sparks, MD
Tryptic Soy Agar (TSA)	Fisher Scientific, Toronto, ON
5% Sheep Blood	Oxoid, Nepean, ON

Reagents, Chemicals, and Enzymes

25% Glutaraldehyde (EM Grade)	Marivac, St. Laurent, QU
95% Alcohol	Commercial Alcohols Inc., Brampton, ON
1% Agarose	Invitrogen, Corisbad, CA
Buffer A	New England BioLabs, Mississauga, ON
EDTA	Sigma-Aldrich Co., St. Louis, MO
Ethidium Bromide	BioRad Loaboratories, Hercules, CA
Hydrochloric Acid (HCl)	BDH Inc., Toronto, ON
InstaGene Matrix	BioRad Laboratories, Hercules, CA
Lambda Ladder	New England BioLabs, Mississauga, ON
Low Melting Point Agarose	BioRad Laboratories, Hercules, CA
Lysostaphin	Sigma Chemical Co., St. Louis, MO
PCR Ladder	Invitrogen, Carisbad, CA
PCR Primers	Sigma-Genosys, Oakville, ON
PuReTaq Ready-To-Go PC Beads	Amersham/Pharmacia, Piscataway, NJ
Proteinase K	Sigma Chemical Co., St. Louis, MO
Pulsed Field Certified Agarose	Sigma Chemical Co., St. Louis, MO
Saline	Baxter, Deerfield, IL
Skim Milk	Becton, Dickinson and Co., Sparks, MD
SmaI	New England BioLabs, Mississauga, ON
Sodium Cacodylate	Ted Pella, Inc., Millville, NJ
Sodium Chloride (NaCl)	BDH Inc., Toronto, ON
Tris-HCl	Sigma Chemical Co., St. Louis, MO

Disposable Labwares

Pipette Tips	VWR International, Mississauga, ON
Corning Cryovials	Corning Inc., Corning, NY
Cuvettes	Fisher Scientific, Toronto, ON
Glass Tubes	Fisher Scientific, Toronto, ON
Latex Gloves	Fisher Scientific, Toronto, ON

McFarland Tubes	Fisher Scientific, Toronto, ON
Microcentrifuge Tube	Fisher Scientific, Toronto, ON
Microtitre Plates	Sarstedt, Newton, NC
Pasteur Pipettes	Fisher Scientific, Toronto, ON
Sterile Plastic Petri Plates	Fisher Scientific, Toronto, ON
Swabs	Fisher Scientific, Toronto, ON
Wooden Applicator Sticks	Puritan, Guilford, Maine

APPENDIX E

MIC Protocol – Microbroth Dilution

Day One
- Subculture microorganisms onto the appropriate agar plates and incubate overnight.
- Label.

Day Two
Microbroth Dilution:
- Make a 0.5 McFarland standard using the colourometer.
- Take 50 µl of the 0.5 McFarland and add it to 5 ml of the appropriate broth (1/100 dilution).
- Vortex the bacterial suspension and continue with step #1 in the Test Dilution.

Drug Dilution:
- In each panel add 100 µl of the appropriate broth into wells 2-12 (reverse pipette).
- Add 200 µl of drug into well #1 (reverse pipette).
- Take 100 µl of drug from well #1 and add it to well #2 (normal pipette), mix 3 times, aspirate 100 µl of that suspension into the following well (#3).
- Continue doing this until you reach well #11.
- When you are finished mixing the contents of well #11 DO NOT TRANSFER them to well #12. DISPOSE OF THEM because well #12 is the growth control (contains broth and microorganism, no drug).
- This result is a serial dilution (example 512, 256, 128……).

Test Dilution:
- Take 100 µl of the diluted test microorganism and add it to wells 1-12 of the appropriate row.
- Streak out a purity plate.
- Incubate overnight in the appropriate incubator.
- The following day record your results (MIC=the first well showing no growth).

Example of Math for Microbroth Dilution:

0.5 McFarland (50 µl) + 5 ml broth = 1/100 dilution

↓ ↓

1-2 x 10^8 CFU/ml 1-2 x 10^6 CFU/ml

Microbroth Panel:

100 µl of diluted microorganism + 100 µl of broth in the wells = 200 µl /well (1/2 dilution) therefore the 0.5 McFarland is diluted to total of 1/2 with a resulting cfu equaling 0.5-1 x 10^6 CFU/ml or 0.5 – 1 x 10^5 CFU/ml.

APPENDIX F

MPC Protocol

Day One:

- Subculture microorganisms onto 6½ agar plates and incubate overnight in a 35°C CO_2 incubator.
- Pour plates that you will need for the week.
- Each plate contains approximately 20 ml of agar.
- We usually pour 7 drug dilutions

eg. moxifloxacin frozen at 1756 µg/ml

c1v1 = c2v2

1756µg/ml (x) = 4 µg/ml(100 ml)

x = 227.8 ul

c1 = 1756µg/ml = known drug stock

v1 = x = the amount of drug we need to add

c2 = 4µg/ml = the drug concentration we want in the plate

v2 = 100 ml = the amount of agar made

Day Two:

- Transfer microorganisms to 500 ml of Todd Hewitt Broth and incubate overnight in a 35°C CO_2 incubator.
- Label supplies needed for the next day.

Day Three:

- Remove the bottles containing the bacterial suspension from the incubator and mix briefly.
- Pipette 2 ml of the bacterial suspension into a cuvette and take a spectrophotometer read at 600nm (must read at 0.300 or greater).
- Balance the remaining bacterial suspension between 2 500ml centrifuge bottles.
- Centrifuge conditions: 600rpm, 20 minutes, 4°C.
- When the centrifuge has fully come down, remove the bottles and pour off the supernatant being careful not to disturb the pellet.
- Resuspend the pellet in 1.5 ml of fresh Todd Hewitt broth.
- Pipette 200 µl of the bacterial suspension to the appropriately labeled drug plates.
- Spread the inoculum on the plate with a sterile loop.
- Streak out a purity plate (blood agar plate).
- Incubate the plates overnight in a 35°C CO_2 incubator.

Day Four:

- Remove the plates from the incubator and record your results (growth, no growth or unsure).
- DO NOT GUESS if it is growth or not, if you are unsure, question mark it.
- Return the plates to the incubator for another 24 hours.

Day Five:

- Remove the plates from the incubator and once again record your results.
- This time you can streak out any plates that you are unsure of onto new drug plates.

Example: Moxi 2 µg/ml has growth (visible colonies) – record as +.

Moxi 4 µg/ml has a smear (no visible colonies) – take a swab of this plate and replate it onto a new moxi 4 µg/ml plate.

- Incubate the streaked drug plates overnight in a 35°C CO_2 incubator.

Day Six:

- Record your results.

APPENDIX G

Kill Curve Protocol – 3 hour

Time Condensed and only Dealing with 10^5 Dilution

Day One

- Inoculate the microorganism onto the appropriate number of plates and incubate overnight.
- Label all the necessary supplies.
- Figure out the math for the next day.

Day Two

- Transfer all the growth from the plate into 10 ml of appropriate broth and incubate for 2 hours.
- Pipette 700 µl of bacterial suspension from the tube labelled 10^9 into the tube labelled 10^8, vortex.
- Pipette 700 µl of bacterial suspension from the tube labelled 10^8 into the tube labelled 10^7, vortex.
- Pipette 700 µl of bacterial suspension from the tube labelled 10^7 into the tube labelled 10^6, vortex.
- Pipette 700 µl of bacterial suspension from the tube labelled 10^6 into the tube labelled 10^5, vortex and discard 700 µl from the 10^5 tube.
- Add the appropriate amount of antimicrobial agents to the tube containing 10^5 bacterial suspension.
- Working with the appropriate labelled rack of tubes (that contain 900 µl of MHB) start by Pipetting 100 µl from the main 10^5 tube into your first set of tubes that you are going to serially dilute down and plate.
- Serially dilute down your tubes of broth by removing 100 µl of the bacterial suspension from the first tube and placing it into the second tube, vortex, remove 100 µl of the bacterial suspension from the second tube and plate it into the third tube, vortex and so forth until all the tubes are diluted then set this rack of diluted tubes aside.
- Pull the corresponding labelled blood agar plates and Pipette 100 µl onto each, then set aside.
- Dispose of the tubes that were not plated and pull forward your next set of tubes to be diluted at the next time interval.
- You are now ready to start spreading the bacterial suspension on the freshly inoculated plates.
- Sterilize a yellow cell spreader; you can use the same spreader on your stack of three plates labelled with the same concentration and time interval.
- Place the inoculated blood agar plates into the appropriate incubator overnight.
- This was done at time intervals of 0 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 1 hr, 2 hrs and then finishing at 3 hrs.

Day Three

- Remove plates inoculated on the previous date and begin counting your colonies (double digits is ideal, single digits are countable but are less reliable, once you get into the higher hundreds it also becomes unreliable) and record your results.

APPENDIX H

Kill Curve Protocol – 24 hour (10^6-10^9 CFU/ml)

Day One

- Inoculate the microorganism onto the appropriate number of plates and incubate overnight.
- Label all the necessary supplies.
- Figure out the math for the next day.

Day Two

- Transfer all the growth from the plate into 5 ml of appropriate broth and incubate for 2 hours.
- Vortex the bacterial suspension and pour the contents into a petri dish.
- Pipette 3 ml into a cuvette and perform a spec read at 600 nm; when done do not discard the bacterial suspension, pour it into a new tube labelled 10^9.
- Pipette the remaining bacterial suspension into the new tube labelled 10^9 remembering the volume that you Pipetted (nb. for the math).
- Pipette 700 µl of bacterial suspension from the tube labelled 10^9 into the tube labelled 10^8, vortex.
- Pipette 700 µl of bacterial suspension from the tube labelled 10^8 into the tube labelled 10^7, vortex.
- Pipette 700 µl of bacterial suspension from the tube labelled 10^7 into the tube labelled 10^6, vortex.
- Working with the appropriate labelled rack of tubes (that contain 900 µl of MHB) start at 10^9; Pipette 100 µl from the main 10^9 tube into your first set of tubes that you are going to serially dilute down and plate; set this rack aside when done and do the same for the corresponding racks for 10^8, 10^7, and 10^6.
- After adding the initial 100 µl to the first tube in each rack take the rack containing the main 10^9, 10^8, 10^7 and 10^6 and place it in the appropriate incubator and start your timer.
- Starting with the 10^9 rack, serially dilute down your tubes of broth by removing 100 µl of the bacterial suspension from the first tube and placing it into the second tube, vortex, remove 100 µl of the bacterial suspension from the second tube and plate it into the third tube, vortex and so forth until all the tubes are diluted then set this rack of diluted tubes aside.
- Pull the corresponding labelled blood agar plates and Pipette 100 µl onto each, then set aside.
- Do the exact same procedure for the 10^8, 10^7 and 10^6 rack of tubes.
- Starting with the 10^6 rack of diluted tubes, pull the corresponding labelled blood agar plates and Pipette 100 µl onto each then set aside.
- Dispose of the tubes that were not plated and pull forward your next set of tubes to be diluted at the next time interval.
- Do the exact same procedure for the 10^8, 10^7 and 10^6 racks of tubes.
- You are now ready to start spreading the bacterial suspension on the freshly inoculated plates.
- Sterilize a yellow cell spreader; you can use the same spreader on your stack of three plates labelled with the same concentration and time interval.
- Place the inoculated blood agar plates into the appropriate incubator overnight.
- This was done at time intervals of 0 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr and finishing at 24 hours.

Day Three

- Remove the main rack of tubes (10^9, 10^8, 10^7 and 10^6) from the incubator and set up the 24 hour results (same procedure as the previous day when you simply remove 100 µl from the main tube and place it into the corresponding rack of tubes needed to be serially diluted down and then plate 100 µl in triplicate).
- Remove plates inoculated on the previous date and begin counting your colonies (double digits is ideal, single digits are countable but are less reliable, once you get into the higher hundreds it also becomes unreliable) and record your results.