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Identification of Clinical Markers That Predict the Outcomes of Methicillin-Resistant Staphylococcus Aureus Infections and Identification of Synergistic Antibiotic Combinations for the Treatment of These Infections

Virginia Long
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

IDENTIFICATION OF CLINICAL MARKERS THAT PREDICT THE OUTCOMES OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS INFECTIONS AND IDENTIFICATION OF SYNERGISTIC ANTIBIOTIC COMBINATIONS FOR THE TREATMENT OF THESE INFECTIONS

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VIRGINIA AGNES LONG

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LIST OF ABBREVIATIONS

µg	microgram
ABCs	active bacterial core surveillance
ANC	absolute neutrophil count
CDC	Center for Disease Control
cef	cefazolin
CLSI	Clinical and Laboratory Standards Institute
CNS	central nervous system
CVC	central venous catheter
F	female
FIC	fractional inhibitory concentration
g	gram
gent	gentamicin
hVISA	heteroresistant vancomycin intermediate *Staphylococcus aureus*
ICU	intensive care unit
IDC	infectious disease consultant
IV	intravenous
kg	kilogram
LG	log-phase growth
LUMC	Loyola University Medical Center
M	male
MIC	minimum inhibitory concentration
mL	milliliter
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus* aureus
N	no
N/A	not applicable
NA	no antibiotic
S	suspension growth
vanc	vancomycin
Y	yes

CHAPTER ONE

INTRODUCTION

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a leading cause of hospital-acquired infection, and can lead to extended hospital stays and increased health care costs. Patients typically acquire this infection from the hands of hospital workers or their own resident flora. MRSA can cause severe problems including bloodstream infections, pneumonia and surgical site infections. MRSA is treated with antibiotic therapy and vancomycin is the primary antibiotic of choice. In addition, MRSA infections cause many patients to experience prolonged or recurrent infections.

Physicians are at a disadvantage when treating MRSA infections because the clinical outcome if often unpredictable. If the chosen antimicrobial regimen is suboptimal, patients may fail to clear their infection completely, resulting in prolonged bacteremia and/or recurrent infections with the same organism. If clinical markers could be identified that predicted the outcomes of MRSA infections, physicians could identify patients at risk for infection recurrence or prolonged MRSA bacteremia. This foreknowledge would allow physicians to modify their treatment strategies by using more efficacious therapies including combination therapy with multiple antibiotics. With this in mind I asked the question, can I predict the outcomes of MRSA infections using clinical markers?

The inability to predict MRSA infection outcome is only the first part of the current problem. The second problem addressed was the treatment of MRSA infections. Although,

vancomycin is the mainstay for treatment of MRSA infections, failures of vancomycin monotherapy are common. Current guidelines recommend vancomycin alone for treatment of most serious infections. In order to decrease vancomycin failures, physicians will often add an additional antibiotic. Combination therapy is used by many Loyola physicians to treat MRSA infections; however testing for antibiotic synergy is not performed in Loyola's clinical microbiology laboratory. My second aim was to determine whether the antibiotic combinations most commonly used at Loyola, and those suggested in recent publications, demonstrate synergy *in vitro*. I addressed this problem using timed kill-curves. The antibiotics tested were chosen based on previous chart reviews that indicated the most commonly used combination therapies for MRSA treatment at Loyola, and by review of recent publications. These combinations were vancomycin +rifampin, vancomycin +gentamicin, and vancomycin + cefazolin.

These studies will help to identify clinical markers that can predict MRSA infection outcome and confirm which combination therapy is a better method of treatment for MRSA. In addition, understanding the synergistic relationships between antibiotics used for treatment, and which combinations are most effective against MRSA will help physicians to better treat their MRSA patients.

Hypothesis

Through patient chart analysis, clinical markers will be identified that predict outcomes of MRSA infections, specifically recurrent infection and prolonged bacteremia. The antibiotic combinations commonly used at Loyola University Medical Center (LUMC) will demonstrate synergy when tested against six strains of MRSA *in vitro*.

Specific Aims

Aim 1. To determine if clinical markers can be identified that predict the outcomes of MRSA infections.

Rationale. Physicians are unable to predict the outcomes of MRSA infections, and thus cannot always treat their MRSA patients optimally. Identifying clinical markers associated with recurrent infections would help physicians identify patients at risk for recurrent infections and allow more aggressive treatment. Chart review was a good way to approach this goal because it enabled me to look at a large number of patients and identify differences in demographics (i.e. age, gender), histories (i.e. preexisting conditions), and hospital experiences (i.e. length of stay, treatment method). In addition, it allowed me to collect data from patients both living and deceased, providing information that could predict mortality. This study could give physicians more facts to consider when deciding on treatments for MRSA patients with MRSA infection. In addition, more effective treatments would shorten hospital stays for patients and subsequently lower hospital costs.

Aim 2. To determine if combination therapies commonly used at LUMC and suggested in publications demonstrate synergistic activity against MRSA.

Rationale. Vancomycin is the most frequently used antibiotic for the treatment of MRSA, but is suboptimal for its ability to rapidly kill *S. aureus*. In order to improve the efficacy of vancomycin, physicians often add an additional antibiotic.

Combination therapy is used by many Loyola physicians to treat MRSA infections; however testing for antibiotic synergy is not performed in Loyola's clinical microbiology laboratory to evaluate the efficacy of combination therapy. While studies have been done on the synergy of different antibiotics to treat multiple different infections, the resulting data are contradictory. My chart review has given me insight into the most common combination therapies currently being used at Loyola to treat MRSA. Both the conflicting literature and the differing combinations used by physicians at Loyola and suggested in publications have prompted me to study combination therapies used at Loyola and determine which of these combinations demonstrate synergy. Understanding which combination therapies actually demonstrate synergy in a lab, may ultimately support clinical decisions, but may also identify better therapies for the treatment of MRSA infections. I will use timed kill-curves to test the synergy of vancomycin + gentamicin, vancomycin + rifampin, and vancomycin + cefazolin *in vitro* against six strains of MRSA.

CHAPTER TWO

REVIEW OF RELATED LITERATURE

About Methicillin-Resistant Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a leading cause of bacterial human infections. It is a global threat that is endemic to both hospitals and communities (8). *S. aureus* infections acquired in health care environments are spread from direct contact with an infected wound, the hands of healthcare workers or from contaminated environmental surfaces(6). Many patients, however, can acquire *S. aureus* from their own flora. Studies performed by the CDC and independent scientists show that approximately 30% of people asymptomatically carry *S. aureus* in their nose, and 2 in 100 people carry methicillin-resistant *Staphylococcus aureus* (MRSA) (6, 34). In addition, *S. aureus* colonization seems to influence the epidemiology and pathogenesis of infection. In healthy individuals the rate of carriage can be classified into three patterns: persistent, intermittent, and almost never (34). The most common pattern is intermittent and 60% of people display this pattern. The remaining 40% are split between persistent (20%) and almost never (20%) (34). The presence of *S. aureus* on the skin and anterior nares increases the risk of infection for people undergoing dialysis or surgery (34).

Clinical manifestations of *S. aureus* infection can range from minor skin infections to life-threatening bloodstream infections (8). An additional danger of S. aureus is its ability to develop resistance to antibiotics. The first drug crisis occurred when *S. aureus* developed resistance to penicillin in the 1950s. To combat this development, pharmaceutical companies developed methicillin, a semi-synthetic penicillin resistant to hydrolysis by β-lactamase.

Oxacillin and nafcillin, two less toxic semi synthetic penicillins, have replaced methicillin for treating penicillin-resistant strains of *S. aureus*. In the laboratory, oxacillin is used to detect methicillin resistant strains. Oxacillin-resistance equates with methicillin-resistance and indicates MRSA. In addition, the demographics of MRSA are changing. A recent study found that although nasal colonization with *S. aureus* has decreased in the U.S. population, nasal colonization with MRSA has simultaneously increased (17). Also, while MRSA is a major public health problem typically associated with health care, it is no longer restricted to health care institutions or settings (34), and community-acquired MRSA infection has emerged as an increasing concern.

Treatment of MRSA

Vancomycin is the mainstay for treatment of infections caused by MRSA. Current guidelines recommend vancomycin monotherapy for treatment of most serious infections, including bacteremia and endocarditis (37). Exceptions include prosthetic valve endocarditis and in some instances osteomyelitis and CNS infections where combinations of antibiotics are suggested (37). However, there is a high rate of failure associated with vancomycin monotherapy. A recent study comparing daptomycin and vancomycin effectiveness for treating MRSA showed that almost 60% of MRSA patients experienced vancomycin treatment failure (42). Daptomycin was associated with a better outcome than vancomycin for the treatment of bloodstream infections caused by MRSA with higher vancomycin minimum inhibitory concentrations (MIC). Another study looked at treatment outcomes for MRSA infections with reduced vancomycin susceptibility, and reported that 76% of the study patients experienced glycopeptide therapy failure (27). The cause of vancomycin failure is currently unknown, but some studies have correlated higher vancomycin MICs (≥2) with increased mortality rate and

complicated bacteremia (1, 22, 25, 41, 65). However, there are conflicting studies that show that higher vancomycin MICs are not related to the outcome of MRSA infections (21, 53). The importance of MIC is still under investigation.

Regardless of conflicting data, MIC determination remains a prevalent method of choosing which therapy to use when treating MRSA infections. Hageman, et al. surveyed over 400 infectious disease consultants (IDCs) and determined the importance of the vancomycin MIC when confronted with a case of persistent MRSA bacteremia. While 54% of IDCs reported that they always or usually use the vancomycin MIC to guide therapy for MRSA bacteremia, 29% responded with occasionally or never (19). Unfortunately, even with the MIC guideline, vancomycin failure is common. This high prevalence has led to multiple studies looking at alternative dosing regimens and therapies.

Research has focused on the importance of vancomycin trough levels when using vancomycin monotherapy. Many experts recommend dosing to achieve a higher trough level of 15-20mg/L thus optimizing the pharmacokinetics of vancomycin and increasing its absorption into the body (35). Achieving these levels is considered particularly important in patients whose isolates have vancomycin MIC ≥ 2. In addition, the high dosage of vancomycin needed to obtain the desired trough levels may increase the potential for nephrotoxicty and could prove to be unsafe (48, 54). One study questioned the need for the 15-20mg/L trough levels, especially when the MIC ≤1 mg/L (48). In response to the toxicity problem investigators are suggesting alternate therapies. For example, as a result of one study the author recommended the use of an alternate therapy if a patient on vancomycin has not had a clinical or microbiological response to vancomycin, even after removal of the foci of the infection, regardless of vancomycin MIC (37). Other studies are suggesting new therapies that do not include

vancomycin. Another recent publication recommended that daptomycin IV be considered for patients with MRSA bacteremia, right-sided endocarditis, and complicated skin and skin structure infections (52). Yet, vancomycin remains the most frequently used antibiotic in the treatment of MRSA.

Predicting the Outcomes of MRSA Infections

Physicians are at a disadvantage when treating MRSA because they cannot reliably predict the outcome of MRSA infections. The high rate of vancomycin failure can lead to prolonged and recurrent infections. If physicians had a guideline for identifying patients at risk for these complications, they could treat patients more aggressively (60). Few studies have been done to predict these risk factors. Han et al focused on whether or not reduced vancomycin susceptibility affected the outcomes of *S. aureus* bacteremia. The study showed that reduced vancomycin susceptibility (defined as MIC >1) was associated with greater 30-day in-hospital mortality in patients with bacteremia due to MSSA, but not in patients with MRSA. In addition, reduced vancomycin susceptibility was associated with a decreased length of stay in hospital acquired, but not community acquired, *S. aureus* bacteremia. Finally, there was no effect on total hospital costs accrued after the first positive blood culture date (21). This study was limited and only focused on one possible predictor of MRSA infection outcome. Other studies have followed similar patterns, focusing on a few markers or specific patient populations.

It is known that dialysis patients are at a higher risk for MRSA infections due to frequent visits to dialysis units and the need to access the bloodstream. Nguyen et al. aimed to identify trends in invasive MRSA infections among dialysis patients over six years. The study found that 70% of infected dialysis patients from 2009-2011were hospitalized in the year prior to infection, and 60.4% of infected hemodialysis patients were dialyzed through a central venous catheter

(CVC). Despite these numbers, the overall amount of invasive MRSA infections among dialysis patients has decreased, and this is possibly due to increased efforts to control MRSA in hospitals and dialysis units (44). While important, this study was narrow because it focused on a specific population and markers for infection risk within that population (i.e. prior hospitalization and CVC). Some studies have approached the prediction of MRSA infection outcomes on a broader scale.

Klevens et al. aimed to describe the incidence and distribution of invasive MRSA infections in nine US communities during July 2004 – December 2005. This was accomplished using the Active Bacterial Core surveillance (ABCs)/Emerging Infections Program Network. The study separated MRSA infections into two groups: health-care associated or community associated. They found that most MRSA infections were health care associated. In addition, this study looked at demographic markers in relation to the incidence of MRSA infection. The results showed that incidence rates of invasive MRSA infection, regardless of whether the infection was hospital or community associated, were highest among patients 65 years and older, blacks, and males (33). This study is important in that it gives insight into patients at risk for acquiring invasive MRSA infections however; it does not determine ways to predict the outcomes of those infections.

In 2011, Moore et al. conducted a study of the factors involved in the etiology and treatment MRSA bloodstream infections in order to characterize patients at risk for vancomycin failure. This was a retrospective cohort study of 200 patients collected between July 2005 – October 2007, and vancomycin failure was defined as mortality, microbiologic failure and/or recurrence within 30 days. The analysis found that vancomycin treatment failure was associated with specific comorbidities (i.e. cardiovascular disease, acute renal failure, and

immunosuppression), definitive trough levels, the source of infection, and strain type (41). Interestingly, success of vancomycin therapy was more likely in patients with a history of intravenous drug abuse, and early combined therapy with an aminoglycoside or rifampin. Moore's study did provide physicians with clinical and biological factors to identify MRSA patients at risk for vancomycin failure.

Poor outcomes of *S. aureus* infection can range from prolonged bacteremia to mortality. Predicting factors that indicate the risk for a specific outcome is valuable, and Khatib et al. focused on characterizing patients with persistent *S. aureus* bacteremia. The authors defined persistence as bacteremia lasting ≥3 days, and found that persistent bacteremia is associated with poor outcome of *S. aureus* infection regardless of the oxacillin susceptibility of the strain (31). The results identified endovascular sources of infection, cardiovascular prosthesis, metastatic infection, vancomycin treatment, and diabetes as risk factors for persistent *S. aureus* bacteremia. This study is a valuable resource for understanding which patients are at risk for persistent bacteremia.

In another study focusing on determining risk factors of MRSA relapse, Welsh et al. performed a retrospective analysis of patients who had experienced MRSA relapse specifically after vancomycin therapy. The study included 113 patients, 12 of whom had recurrent MRSA bacteremia. The recurrent infection was considered a relapse if the subsequent strain was determined to be identical to the previous infecting strain using Diversilab typing. The results identified the presence of the *arg* type II and SCCmec type II genes, hVISA and persistent bacteremia as being associated with a relapse of MRSA bacteremia (71). This study helped to identify factors associated with relapse after vancomycin treatment of MRSA infection.

These studies all contribute pieces to the puzzle of trying to understand how to predict different outcomes of MRSA infections, including recurrence, prolonged infection, and mortality.

Combination Therapies for the Treatment of MRSA

As mentioned previously, one of the difficulties in treating MRSA is the paucity of effective antibiotics, and the frequency of vancomycin therapy failure. One possible solution to this problem is combination therapy, using two or more antibiotics to attack bacteria using multiple mechanisms. Many LUMC physicians have used various combination therapies to combat MRSA, but the microbiology laboratory at LUMC does not test these therapies to confirm their synergistic relationships.

Research reports are not always consistent when reporting which combinations are effective against *S. aureus*. For example, clinical case reports have shown that daptomycin and rifampicin in combination can successfully treat MRSA (2, 29), and the addition of linezolid to daptomycin and rifampin can further increase the efficacy of the combination (29). In contrast, lab studies failed to demonstrate synergy between daptomycin and rifampin in rifampin-resistant MRSA isolates (30). The conflicting results of these studies indicate that further tests are needed to confirm the role of combination therapy with daptomycin and rifampin.

Multiple antibiotics are used in various combinations to treat MRSA, such as daptomycin, rifampin, linezolid, vancomycin and β-lactams (2, 13, 29). A survey of over 400 IDCs revealed that in the case of persistent MRSA bacteremia with vancomycin MIC 2µg/mL, 72% of the IDCs would continue vancomycin but add an additional drug, typically rifampin or gentamicin (19). While the percentage of IDCs using combination therapy for MRSA with vancomycin MIC 4µg/mL reduced to 29%, the combinations remained vancomycin with rifampin or gentamicin.

There are multiple methods of synergy testing: disk diffusion, checkerboard, E-test and time-kill curves. Two of these methods, checkerboard and time-kill curves, are most commonly used. However, these methods do not always produce identical results. For instance the time-kill method will demonstrate synergy between two antibiotics, whereas use of the checkerboard technique will demonstrate antagonism between those same antibiotics (3, 4, 26). In addition, the results produced by the time-kill method more accurately predict the action of two antibiotics in vivo (4, 7, 14). Due to the frequent use of vancomycin alone and in combination for MRSA treatment, I chose to focus on combination therapies involving vancomycin for synergy tests.

Combination of Vancomycin & Gentamicin

One of the most commonly used antibiotic combinations used for the treatment of MRSA is vancomycin + gentamicin. Multiple studies have found synergy with this combination against staphylococcal species. Watanakunakorn et al. used time-kill curves to demonstrate enhanced activity of 10ug of vancomycin and 1ug of gentamicin against 7/10 strains of MRSA (70). A similar study found synergy in 35 isolates of *S. aureus*, 29 of which occurred with a gentamicin concentration of 5µg/mL (68). Other studies have been done using more realistic dosage regimens.

Houlihan, et al. looked at the pharmacodynamics of vancomycin used in combination against MRSA in an *in vitro* model of infected fibrin-platelet clots. The results indicated that the combination was most active when gentamicin was added to a high dose (2g) of vancomycin (26). The average vancomycin dose for a MRSA bacteremia patient at LUMC is approximately 1g, though a number of patients are placed on 2g doses. The combination dosage shown to be the most active in this study may not be ideal for all patients.

Dosage method (1 mg/kg x3 vs. 5mg/kg x1) was again shown to be important to the vancomycin + gentamicin combination in a study by Tsuji et al (62). The results of the study showed that three doses of 1 mg/kg of gentamicin did not improve vancomycin activity against MRSA (62). However, the addition of a single 5 mg/kg dose of gentamicin to vancomycin resulted in noticeable enhancement at 4 hours, and a 99.9% kill at 32 hours against MRSA (62). Overall, this study indicates that a single high dose of gentamicin in combination with vancomycin may be enough to maximize synergistic activity against MRSA while simultaneously reducing toxicity.

In contrast to the Tsuji study, Cosgrove et al. demonstrated that an initial low-dose of gentamicin as a part of *S. aureus* bacteremia treatment should not be used routinely due to nephrotoxicty. Among 53 patients treated with vancomycin and low-dose gentamicin, 19% experienced renal problems (12). They recommended against the use of this combination. Additional studies also reported the high rate of toxicity associated with this combination (16, 50). Rehm et al. found that daptomycin was an effective alternative for the vancomycin + gentamicin combination for MRSA bacteremia. However, the combination was more successful in patients who had not undergone pervious vancomycin therapy (50).

Given the likelihood of nephrotoxicty posed by the use of the vancomycin/gentamicin combination, and the frequent use of the combination for treatment of MRSA, more work should be done to support the use of this combination for MRSA treatment.

Combination of Vancomycin & Rifampin

The most commonly used combination therapy for the treatment of MRSA at LUMC is vancomycin + rifampin. This combination has been widely researched, but the data are conflicting. There are multiple studies that support the use of this combination for *S. aureus*

treatment (18, 39, 46, 64). Tuazon et al. tested 20 strains of *S. aureus* for synergy between vancomycin and rifampin. Of those 20 strains, 14 showed indifference to the combination, 5 showed a synergistic effect, and 1 showed an additive effect. This study supported the rifampin/vancomycin combination as a possible method of treatment for serious *S. aureus* infections (64).

Animal models have also been used to show the value of rifampin and vancomycin used in combination. The combination was tested in rabbit models as a method of treating *S. aureus* osteomyelitis, and it sterilized up to 90% of the infected bones in treated animals after 28 days of treatment (46). In another study, the combination proved effective in reducing bacterial counts in rat models, and was determined to be an effective treatment for a foreign body infection due to MRSA (39). However, this study also showed the combination of vancomycin and rifampin to be antagonistic against MRSA *in vitro* using the time-kill method. Studies like the one performed by Lucet et al. in which the results of antibiotic combinations are method dependent, and in which *in vitro* results do not correlate with *in vivo* results are not uncommon (39, 49).

Bayer et al. looked at the difference between the time-kill and checkerboard method for determining synergy of the vancomycin/rifampin combination against MRSA and MSSA. With respect to MRSA, the time-kill method showed that 6/26 strains demonstrated antagonism, 5/26 strains demonstrated synergy, and 15/26 strains demonstrated indifference (4). In contrast, the checkerboard method produced antagonistic results for all 26 strains of MRSA. In a similar experiment, Varaldo et al. used both time-kill and checkerboard to test the interaction of vancomycin and rifampin against MRSA and MSSA. The time-kill method produced 1 of 4 strains showing synergy and 3 of 4 showing indifference. The checkerboard method showed synergy for

1/10 strains, and indifference for 9/10 strains (66). Finally, Bayer et al. tested the combination for treatment of aortic valve endocarditis caused by MRSA both *in vitro* and *in vivo* using synergy tests and rabbit models. The time kill method resulted in synergy, but the checkerboard method showed antagonism. As seen in other studies, the time-kill was more accurate and no evidence of antagonism was observed when the combination was used in rabbit models (3). This difference in methodology is well-documented and typically the time-kill method is a more accurate predictor of the *in vivo* activity of the combination (3, 7, 14) with some exceptions (39).

Although multiple studies suggest the vancomycin + rifampin combination could be used to treat MSSA and MRSA, a comparable amount of studies suggest the opposite, demonstrating antagonism or indifference. Hackbarth et al. showed that the addition of rifampin to vancomycin markedly reduced the killing rate in *S. aureus*, discouraging the use of this combination (18). In a study comparing the vancomycin + rifampin combination to vancomycin monotherapy, 42 patients with MRSA endocarditis received either vancomycin monotherapy or the combination (61). The results showed that the combination did not affect cure rates, but instead increased the duration of the bacteremia, thus contradicting reports that say the combination is effective against MRSA. A study by Watanakunakorn et al. produced similar results *in vitro* using the time-kill method. Antagonism was demonstrated for 43 of 50 strains of both MSSA (30 total) and MRSA (20 total), and synergy was only seen in one strain (69). The study concluded that the combination cannot be accepted as superior to vancomycin monotherapy for the treatment of serious *S. aureus* infections. Indifference was also a common result in three other separate studies (63, 67, 75). Walsh et al. evaluated 20 strains of MRSA using checkerboard and time-kill. The checkerboard method showed neither synergy nor

antagonism for all 20 strains, and the time kill method showed indifference at 6 hours, but occasionally showed synergy at 24 and 48 hours (67).

To summarize, there is an abundance of research on the vancomycin + rifampin combination however, this body of knowledge is contradictory. Multiple studies that used different methods of determining synergy showed conflicting results within the study (3, 4, 66, 67). However, this combination is still used frequently despite the fact that there is little scientific evidence that this combination is effective.

Combination of Vancomycin & Cefazolin

The combination of vancomycin and cefazolin is not typically used to treat MRSA infections. While there is a large amount of research done on vancomycin combined with β-lactam drugs in general, there is less research on this specific combination for MRSA treatment. β-lactams have been used in combination with, or in place of, vancomycin for the treatment of MSSA. Multiple cohort studies have reported poor outcomes when vancomycin is used to treat MSSA. The authors have suggested that antistaphylococcal penicillin (i.e. nafcillin) or a first-generation cephalosporin (i.e. cefazolin) should be used in place of vancomycin (9, 10, 32, 40, 56, 59). These studies indicate that nafcillin or cefazolin should be used for the treatment of MSSA in place of vancomycin (40). Unfortunately, MRSA is innately resistant to these antibiotics, and thus vancomycin is the mainstay of treatment.

The value of antistaphylococcal penicillin and cephalosporins for the treatment of MSSA is well documented. Although MRSA is resistant to these antibiotics when used alone, it is possible that their value could be extended to MRSA treatment if used in combination with vancomycin. Climo et al. looked at the combination of vancomycin and oxacillin for the treatment of MRSA, and found that the combination was more likely to demonstrate synergism

against MRSA strains with higher vancomycin MICs. This same study showed no vancomycin/oxacillin synergy against 22 MRSA strains with vancomycin MICs of ≤2 (11). This "seesaw effect" was demonstrated in another study by Werth et al., which concluded that ceftaroline, consistent with traditional β-lactams such as cefazolin, demonstrates increased activity against strains that are less susceptible to vancomycin (72). Another study demonstrated the importance of MIC in that vancomycin combined with oxacillin was effective against MRSA, but only when the vancomycin and oxacillin concentrations were at sub-MIC levels (13). These studies suggest that MRSA strains with high vancomycin MICs may be effectively treated with a combination of vancomycin and a β-lactam.

Cefazolin has been suggested as another effective treatment option for MSSA (56, 59, 72), and thus the vancomycin/cefazolin combination is worth exploring. A study looking at the synergistic effects of double or triple combinations of β-lactams and vancomycin showed that the vancomycin/cefazolin combination demonstrated synergy for 50% of the MRSA strains tested (51). In addition, the addition of imipenem to the vancomycin/cefazolin combination demonstrated synergy against 69% (22 isolates) of MRSA strains, and was indifferent with 31% (10 isolates) of the MRSA strains (51). However, the vancomycin/cefazolin combination has been shown to be synergistic against staphylococcal species without the addition of other β-lactams (20, 57, 58).

Simon et al. demonstrated synergy of vancomycin and cefazolin in 10 strains of *S. aureus* using the checkerboard technique. The level of synergism was determined by fractional inhibitory concentration (FIC) index (strong synergism <0.5; weak synergism; ≤0.75; indifference 1-2; and antagonism >2). Two of 10 strains showed strong synergism with an FIC index 0.5 and 8 of 10 strains showed weak synergism with an FIC index ≤0.75 (58). Although this study did not

include MRSA, the effectiveness of the combination against MSSA is a justification for evaluating these two drugs against MRSA. The *in vitro* pharmacodynamics of vancomycin and cefazolin against MRSA were studied by Hagihara et al (20). Time-kill studies demonstrated that combination therapy significantly reduced the bacterial concentration of MRSA when compared to vancomycin alone after 12 and 72 hours of incubation. In addition to being synergistic against *S. aureus*, the vancomycin/cefazolin combination demonstrated synergy against other staphylococcal species, specifically *S. epidermidis* (57). Siebert et al. used the checkerboard method to demonstrate that the combination was synergistic in 39 of 50 cases of methicillin-resistant *S. epidermidis* (57). Although not supporting the use of vancomycin + cefazolin against MRSA, it is important to show that the combination works against multiple staphylococcal species.

Conclusion

MRSA is a severe public health problem, but limited data are available to help physicians identify patients at risk for poor infection outcomes. The current published studies have helped to establish a guideline for physicians to follow when treating MRSA infections (31, 33, 34, 41, 44), but more work is needed to expand this guideline and confirm currently suggested risk factors. Comparing patients with recurrent infection or prolonged bacteremia to patients with neither of these outcomes could provide physicians with additional information about risk factors that can identify patients at risk for poor MRSA infection outcomes.

The high rate of vancomycin treatment failure for MRSA (27, 42) is a concern and multiple studies have suggested alternate therapies for MRSA treatment (35, 36, 48, 52, 54). One approach is combination therapy with vancomycin and different antibiotics. However, these combinations are typically not tested in hospital labs, and available research is conflicting.

The vancomycin/gentamicin combination has demonstrated synergy (68, 70), but multiple studies have also shown a high incidence of nephrotoxicity associated with this combination (12, 50). The studies of the vancomycin/rifampin combination demonstrated that this combination can be synergistic (16, 54), antagonistic (10, 11, 18), or indifferent (4, 64, 75). Finally, the vancomycin/cefazolin combination has not been as thoroughly studied, but some studies have shown synergy against staphylococcal species (20, 57, 58). The disparate results between studies and lack of consistent support by clinicians for these combinations indicates that further research is needed to understand the value of these antibiotic combinations in MRSA treatment.

CHAPTER THREE

MATERIALS AND METHODS

Patient Chart Review Data Collection

The Sunquest Laboratory Information System (Sunquest information System, Tucson, AZ) was used to identify patients with blood cultures positive for *S. aureus* from January 2010 – May 2013. I separated out the MRSA patients from the MSSA patients, and focused on the MRSA patients for the remainder of the study. Using the Sunquest positive culture list I was able to identify patients with recurrence of the infection and patients with prolonged bacteremia. A recurrent infection was defined as a MRSA infection that occurred at least one month after the first MRSA infection had cleared. Prolonged bacteremia was defined as having blood cultures positive for MRSA for three days or more. In addition to infection outcomes, I was able to find the age, sex, and vancomycin MIC for each patient. Once I identified the MRSA patients to be included in my study, I used the LUMC EpiCare electronic medical records system (EPIC, Verona, WI), to review each patient chart. A total of 163 charts of patients with MRSA bacteremia were reviewed.

Each chart was searched for multiple clinical and demographic markers that could be potential predictors of MRSA infection outcome. The following markers were looked at: name, age, gender, source of infection, presence of other infections, initial vancomycin trough level, clearance vancomycin trough level, initial vancomycin dose, clearance vancomycin dose, absolute neutrophil count (ANC) level, days positive (number of days having positive blood

cultures), hospital length of stay, admission to ICU in 48 hours (yes/no), length of stay in ICU, mortality within 30 days (yes/no), cause of death (If "yes" to mortality within 30 days), injection drug use, co-morbidities (i.e. diabetes or chronic lung disease), long term intravenous access (i.e. chemotherapy or hemodialysis), recent hospitalization, and residence in a long-term care facility. A "clearance" dose or trough level was defined as the vancomycin dose or level the patient was on at the time the blood cultures became negative.

Following the chart review I separated the patients three ways; those with recurrent vs. those with non-recurrent infection (**Table 1a-b**) those with blood cultures positive for three or more days vs. those with blood cultures positive one to two days (**Table 2a-b**), and clinical response observed based on vancomycin MIC levels, i.e. vancomycin MIC of 2 vs. vancomycin MIC of 1 (**Table 3a-b**). The MIC study included 18 patients, of the 163, previously confirmed to have MICs of either 1 by microscan (12 patients), or 2 by both microscan and E-test (bioMérieux, Durham, NC) (6 patients). The other two parameters used the 163 patients previously identified through Sunquest, from January 1, 2010 – May 15, 2013. These two studies did not look at MIC because MICs of 2 or higher that appeared in the patient medical record had not been confirmed with E-test.

Statistical Analyses for Chart Reviews:

The two-tailed student T-tests and Fisher Exact tests were used to analyze the data collected from the EPIC records. The two-tailed student T-test is used to determine if two sets of data are statistically different, and tests the means of the two sets of data. The Fisher exact test is typically used when sample sizes are small. With the Fisher Exact test the significance of the deviation from the null hypothesis (i.e. the p value) can be calculated exactly rather than relying on estimation. The two-tailed student T-tests were used to find significance between the

different groups in each parameter. The Fisher exact test was used to determine significance of sub-categories of each marker, and markers that were not present in both groups. The Fisher exact test was chosen because I had to compare bins, rather than continuous data, as well as small numbers, including zeros, when analyzing the sub-categories of a marker. The MYSTAT (Systat Products, San Jose, CA) statistic computer software was used to perform the analyses. The results are shown in Tables 1-3.

Timed Kill-Curves

Bacteria Strains: The six isolates used were chosen from 180 *S. aureus* isolates, collected over 3 years by the clinical microbiology laboratory at LUMC and stored in a -80°C freezer. The strains chosen for further study were MRSA blood isolates, with MICs of 2 confirmed by both Microscan and E-test. Strains with MIC of 2 were used because those strains typically cause infections that are more difficult to clear. The six strains are referred to as Strains 1-6.

Antibiotics: The antibiotics and concentrations used were vancomycin (10µg/mL), gentamicin (5µg/mL), rifampin (1µg/mL), and cefazolin (30µg/mL). All antibiotics were obtained from SIGMA-ALDRICH (St. Louis, MO). The combinations used were vancomycin + gentamicin, vancomycin + rifampin, and vancomycin + cefazolin. The first two combinations were identified through patient chart review as being commonly used at Loyola to treat MRSA infections. The third combination was suggested in a recent publication for empiric use before clinicians know the methicillin-resistance of a *S. aureus* strain (39). The stock solution for vancomycin was made by dissolving 100mg of powder into 10mL of sterilized, de-ionized water. The gentamicin stock solution was made by dissolving 50mg of powder into 10 mL of sterilized, de-ionized water. The rifampin stock solution was made by dissolving 10mg of powder into 5mL of dimethyl sulfoxide

(DMSO). The cefazolin stock solution was made by dissolving 100mg of powder into 10 mL of sterilized, de-ionized water.

Suspension versus log-growth method: Timed kill-curve experiments typically use the log-phase growth method, but the stationary method is most commonly used in the clinical laboratory. In order to discern if there was a difference between the methods, two kill-curves were performed simultaneously using one of the strains and vancomycin. In the suspension method, colonies from a blood agar plate were inoculated into saline and adjusted to a turbidity equivalent to a 0.5 McFarland Turbidity standard (10^8cfu/mL). Then 5µL of the 0.5 McFarland saline solution were inoculated into 5mL of Mueller-Hinton Broth (MHB) (SIGMA) with or without antibiotic to achieve a 10^5cfu/mL concentration. This method ensured that the bacteria were in stationary phase at the time zero time point.

In contrast, with the log-phase growth method required that colonies be inoculated into MHB, not saline, and adjusted to a 0.5 McFarland Turbidity standard. The MHB was then incubated at 37°C for 1.5-2 hours, until the tubes were cloudy indicating the bacteria were in log-phase growth. The broth culture was again adjusted to the turbidity equivalent 0.5 McFarland Turbidity standard using MHB, and 5µL of the bacteria were inoculated into 5mL of MHB without or without antibiotic to achieve a 10^5 cfu/mL starting concentration. The bacteria were in log-phase growth at the zero time point.

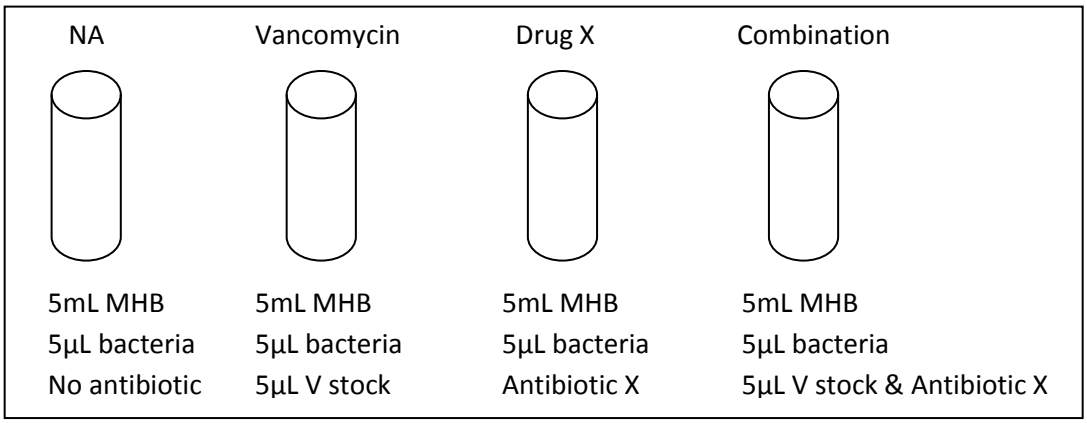
These two methods were tested to observe the difference between the kill-curves of the vancomycin only tubes. Since vancomycin works to impair cell wall growth, it is more active against organisms in log-phase growth rather than stationary phase. The log-phase growth method was used for the remaining experiments due to the noticeable difference in vancomycin

kill rate between the two methods (**Figure 2**). The timed kill-curve method is described in full detail in the following section.

Kill-Curve Experiments: Each experiment used 4 tubes, each with 5mL of MHB, a designated amount of antibiotic, and bacteria. The four tubes were the control tube which received no antibiotic, the vancomycin tube, the compliment drug alone (gentamicin, rifampin or cefazolin), and the combination tube that contained vancomycin and the compliment drug in combination (**Figure 1**). In each experiment the tubes containing vancomycin received 5µL of the stock solution, the tubes containing gentamicin received 5µL of the gentamicin stock solution, the tubes containing rifampin received 2.5µL of the rifampin stock solution, and the tubes containing cefazolin received 15µL of the cefazolin stock solution.

Each frozen bacteria strain was inoculated onto a blood agar plate (trypticase soy agar 5% sheep blood, BBL Microbiology Systems, Cockeysville, MD) and incubated for 18-24 hours at 37°C. The strain was then subbed onto a new blood agar plate and incubated for 18-24 hours at 37°C. On the day of the experiment colonies from the second plate were transferred to MHB and adjusted to the turbidity of a 0.5 McFarland standard. The broth solution was incubated at 37°C for 1.5-2 hours to achieve log-phase growth identified by a cloudy appearance. The broth was then diluted with MHB again to adjust the turbidity to the equivalent of a 0.5 McFarland standard, and 5µL of the diluted solution was added each tube of the experiment for a 10^5cfu/mL starting concentration. This process was repeated for each strain during each experiment.

Figure 1. Tube composition for each time-kill experiment. The No Antibiotic (NA) tube served as the control for each experiment, and as a growth curve for each strain. Drug X was gentamicin, rifampin, or cefazolin. Each experiment used only one of these drugs at a time, and 5µLof gentamicin stock, 2.5µL of rifampin stock, and 15µL of cefazolin stock were used to achieve the desired concentrations in each tube. The same concentration of drug used in each individual tube was used in the combination tube.



Once the tubes were inoculated with the bacteria and respective antibiotics, 1µL was taken from each tube using a 1µL loop and inoculated onto a blood plate that was incubated for 24 hours at 37°C. This was repeated twice for each tube, resulting in two blood plates per tube. This first plating was the 0 time point. The remaining time points, 4, 8, 12, and 24 hours, were handled differently for the control tube versus the antibiotic tubes. For the control tube, 10µL was taken from the tube and inoculated into 1mL of saline. Then 10 µL was taken from the first saline tube and inoculated into another 1mL of saline, resulting in two 100-fold dilutions. Finally, 1 µL was plated from each saline dilution. Each plate was done in duplicate, resulting in 4 plates per time point. In contrast, with the expectation that the antibiotics would decrease the bacterial concentration, 1µL and 100µL were plated from each antibiotic tube at the 4, 8, 12, and 24 hour time points. Each amount was plated twice, resulting in 4 plates per tube per time point. All plates were incubated at 37°C for 24-48 hours. This process was repeated with each strain for the vancomycin + gentamicin and vancomycin + rifampin combinations.

There were exceptions to this procedure when a strain was resistant to the compliment antibiotic. For example, two strains had MICs indicating rifampin resistance. The procedure for the rifampin alone tube changed to accommodate the supposed increasing bacteria concentration. The time 0 procedure was not changed. At the 4 hour time point 10µL was inoculated into 1mL of saline producing a 100-fold dilution, and 1µL was plated from the saline dilution. In addition, 1µL was plated directly from the tube. Each plate was done in duplicate resulting in 4 plates. This was repeated at the 8 hour time point, unless the tube was visibly cloudy, in which case the same 100-fold dilutions in saline were plated as for the control tube. If the tube was not cloudy by the 12-hour time point, the original 1µL and 100µL plates were done for the 12 and 24 hour time points. This procedure was followed for every strain in the vancomycin + cefazolin experiments, as MRSA is known to be innately resistant to cefazolin.

Colonies on each plate were counted after 24-48 hours of incubation. Since each plate was done in duplicate to provide technical replicates, the average of the two plates was calculated and recorded. Results are shown graphically in Figures 3-20. The following conditions were used for defining synergy, indifference, and antagonism (18, 51). A combination was considered synergistic when at least a 2 $\log_{10}$ decline in CFU/mL was achieved at 24 hours by the drug combination compared to the most active single drug. Indifference of a combination was defined as a <2 $\log_{10}$ change in CFU/mL compared to the individual drugs at 24 hours. A combination was considered antagonistic when a 2 $\log_{10}$ increase in CFU/mL was achieved by the drug combination compared to both of the drugs individually at 24 hours.

CHAPTER FOUR

EXPERIMENTAL RESULTS

Patient Chart Analyses

Recurrent versus Non-Recurrent Analysis

A common problem of MRSA infections is the recurrence of these infections. Physicians are currently unable to predict which patients are at risk for recurrent infections. I hypothesize that a review of patient chats and subsequent analysis will identify clinical markers that predict which patients are at risk for recurrent infections. I reviewed the charts of 163 patients (15 recurrent and 148 non-recurrent) with blood cultures positive for MRSA, and analyzed the results with student T-tests and Fisher Exact tests.

The results of the analysis in patients with MRSA recurrence are shown in Table 1a-b. There is a significant difference between the average age of patients who recur (42±10.23) versus patients who do not recur (57±3.07) ($p = 0.005$). My chart analysis indicates that younger patients, ranging from ages 32 – 52, are at higher risk for recurrent MRSA infections. In addition, there is a significant difference between the treatments used for recurrent patients compared to non-recurrent patients. The results showed that 93% of the recurrent patients were treated with vancomycin monotherapy, whereas only 62% of the non-recurrent patients were treated with vancomycin monotherapy ($p = 0.009$). Also, none of the recurrent patients received vancomycin combination therapy; where as 30% of the non-recurrent patients were treated with combination therapy ($p = 0.012$). No other markers reached statistical significance.

Table 1a. Demographic and Clinical Characteristics in Patients With and Without Recurrent MRSA Bacteremia

Marker	Non-Recurrent 148 Patients	Recurrent 15 Patients	P value
Age	Mean: 57± 3.08	Mean: 42 ± 10.23	0.005
Gender	67F (45%) 81 M (55%)	7 F (47%) 8 M (53%)	0.918
Source of Infection			
Skin/soft tissue	28 (19%)	4 (26.7%)	0.497*
Device related	50 (33.8%)	4 (26.7%)	0.775*
Genitourinary tract-related	6 (4%)	0	1.000*
Osteomyelitis	4 (2.7%)	0	1.000*
Lung: pneumonia, bronchitis	10 (6.8%)	1 (6.7%)	1.000*
Infection of head and neck	2 (1%)	0	1.000*
Infection of any solid organ	3 (2%)	1 (6.7%)	0.323*
Abscess of abdominal or digestive tract	1 (0.7%)	0	1.000*
Unknown, other not specified.	44 (30%)	5 (33.2%)	0.773*
Other Infections	Y 67 (45%) N 81 (55%)	Y 6 (40%) N 9 (60%)	0.698
Initial Vancomycin Trough	Mean:13.6	Mean:14.5	0.705
Clearance Vancomycin Trough	Mean:14.5	Mean:15.4	0.914
Initial Vancomycin Dose	Mean: 1,004mg	Mean: 1,124mg	0.150
Clearance Vancomycin Dose	Mean: 1,078mg	Mean: 1,161mg	0.589
ANC Level	Mean: 12.7	Mean: 17.5	0.112
Days Positive	Mean: 1.7	Mean: 1.6	0.703
Treatment			
Vancomycin Monotherapy	87 (62%)	14 (93%)	0.009*
Vancomycin in Combination	43 (30%)	0 (0%)	0.012*
No Vancomycin	11 (8%)	1 (7%)	1.000*
Hospital Length of Stay	Mean: 20.3	Mean: 43.2	0.159
Admitted to ICU in 48 hr	Y 29 (20%) N 119 (80%)	Y 1 (7%) N 14 (93%)	0.221
Length of stay in ICU	Mean: 21.2	Mean:211 (1 person)	Insufficient data
Mortality 30 Days	Y 20 (14%) N 128 (86%)	Y 0 (0%) N 15 (100%)	0.220*
Cause of Death	MRSA: 7 (35%) Non-MRSA: 13 (65%)	Not applicable	Insufficient data

* Indicates the p-value was determined using the Fisher Exact Test. All other p-values were determined using a two-tailed student T-test. Significance was defined as a p-value ≤0.05, and was seen in the analysis of age and treatment type.

Table 1b. Demographic and Clinical Characteristics in Patients With and Without Recurrent MRSA Bacteremia

Marker	Non-Recurrent 148 Patients	Recurrent 15 Patients	P value
Comorbidities			
Hypertension/heart disease	24 (16.2%)	1 (6.7%)	0.471*
Diabetes	10 (6.8%)	1 (6.7%)	1.000*
Chronic kidney disease	4 (2.8%)	1 (6.7%)	0.387*
Liver disease	2 (1.4%)	0	1.000*
Malignancy: (hematologic)	3 (2%)	0	1.000*
Other cancers	8 (5.4%)	0	1.000*
Other immunosuppressive conditions	6 (4%)	2 (13.3%)	0.160*
None	32 (21.6%)	5 (33.2%)	0.334*
2 comorbidities	40 (27%)	4 (26.7%)	1.000*
>2 comorbidities	19 (12.8%)	1 (6.7%)	0.697*
Long Term Care Facility?	Y 18 (12%) N 130 (88%)	Y 1 (7%) N 14 (93%)	0.530
Injection Drug Use	Y 3 (2%) N 145 (98%)	Y 0 (0%) N 15 (100%)	1.000*
Recent Hospitalization	Y 61 (41%) N 7 (59%)	Y 6 (40%) N 9 (60%)	0.163
Long Term IV	Y 37 (25%) N 111(75%)	Y 3 (20%) N 12 (80%)	0.670

* Indicates the p-value was determined using the Fisher Exact Test. All other p-values were determined using a two-tailed student T-test. Significance was defined as a p-value ≤ 0.05, and was seen in the analysis of age and treatment type.

These results show that younger patients (32-52) may be at a higher risk for current MRSA infections and should be treated more aggressively to avoid this outcome. In addition, treatment with vancomycin monotherapy may increase the risk of recurrent infection. Finally, treatment with combination therapy may reduce the risk of recurrent infection. Combination therapy should be used in place of vancomycin monotherapy, especially in younger patients.

Prolonged Versus not Prolonged Analysis

A complication of MRSA infections is a prolonged infection. Physicians are currently unable to predict which patients are at risk for prolonged infections. I hypothesize that a review of patient chats and subsequent analysis will identify clinical markers that predict which patients

are at risk for prolonged infections. I reviewed the charts of 163 patients (28 prolonged, and 135 non-prolonged) with blood cultures positive for MRSA, and analyzed the results with student T-tests and Fisher Exact tests.

Comparisons of demographic and clinical characteristics in patients with and without prolonged bacteremia are shown in Table 2a-b. There is a significant difference between the percentage of device-related infections in patients with prolonged bacteremia (54%) versus not prolonged bacteremia (29%) ($p = 0.015$). Looking just at long term intravenous access (i.e. for hemodialysis or chemotherapy) the percentage of patients was 54% in the prolonged bacteremia group, while only 19% in the not prolonged bacteremia group had such a device ($p > 0.001$).

Table 2a. Demographic and Clinical Characteristics in Patients With and Without Prolonged MRSA Bacteremia

Marker	1-2 Days Positive 135 Patients	3^+ Days Positive 28 Patients	p-value
Age	Mean:53.9 ± 3.47	Mean:59 ± 8.25	0.237
Gender	74 M (55%) 61F (45%)	15 M (54%) 13 F (46%)	0.905
Source of Infection			
Skin/soft tissue	27 (20%)	5 (18%)	1.000*
Device related	39 (29%)	15 (54%)	0.015*
Genitourinary tract-related	6 (4%)	0	0.591*
Osteomyelitis	4 (3%)	0	1.000*
Lung: pneumonia, bronchitis	10 (7%)	1 (3.5%)	0.691*
Infection of head and neck	1 (1%)	1 (3.5%)	0.315*
Infection of any solid organ	4(3%)	0	1.000*
Abscess of abdominal/digestive tract	1 (1%)	0	1.000*
Unknown, other not specified	43 (32%)	6 (21%)	0.366*
Other Infections	74 N (55%) 61 Y (45%)	16 N (57%) 12 Y (43%)	0.823
Initial Vancomycin Trough	Mean: 13.8	Mean: 13.4	0.826
Clearance Vancomycin Trough	Mean: 16.2	Mean: 16.3	0.975

* Indicates the p-value was determined using the Fisher Exact Test. All other p-values were determined using a two-tailed student T-test. Significance was defined as a p-value ≤0.05, and was seen in the analysis of source of infection, overall treatment type, and presence of a long-term IV.

Table 2b. Demographic and Clinical Characteristics in Patients With and Without Prolonged MRSA Bacteremia

Marker	1-2 Days Positive 135 Patients	3^{+} Days Positive 28 Patients	p-value
Initial Vancomycin Dose	Mean: 1,006mg	Mean: 1,063mg	0.392
Clearance Vancomycin Dose	Mean: 1,107mg	Mean: 980mg	0.372
ANC Level	Mean: 12.1	Mean:16.3	0.052
Recurrent	Y 13 (10%) N 122 (90%)	Y 2 (7%) N 26 (93%)	0.681
Treatment			
Vancomycin Monotherapy	87 (68%)	14 (50%)	0.083*
Vancomycin in Combination	33 (26%)	10 (36%)	0.350*
No Vancomycin	8 (6%)	4 (14%)	0.099*
Hospital Length of Stay	Mean: 22.9 days	Mean: 19.6 days	0.387
Admitted to ICU in 48 Hours	Y 24 (18%) N 111 (82%)	Y 6 (21%) N 22 (79%)	0.652
Length of stay in ICU	Mean: 29.6	Mean: 21.3	0.492
Mortality 30 Days	Y 14 (10%) N 121 (90%)	Y 6 (21%) N 22 (79%)	0.193
Cause of Death	MRSA: 4 (29%) Non-MRSA: 10 (71%)	3 MRSA, 3 non-MRSA	0.384
Long Term Care Facility?	Y 15 (11%) N 120 (89%)	Y 4 (14%) N 24 (86%)	0.636
Injection Drug Use	Y 3 (2%) N 132 (98%)	Y (0%) N 28 (100%)	1.000*
Recent Hospitalization	Y 56 (41%) N 79 (59%)	Y 14 (50%) N 14 (50%)	0.410
Long Term IV	Y 25 (19%) N 110 (81%)	Y 15 (54%) N 13 (46%)	<0.001
Comorbidities			
Hypertension/heart disease	22 (16.3%)	3 (10.7%)	0.574*
Diabetes	9 (7%)	2 (7%)	1.000*
Chronic kidney disease	4 (3%)	1 (3.6%)	1.000*
Liver disease	2 (1.5%)	0	1.000*
Malignancy: (hematologic)	3 (2.2%)	0	1.000*
Other cancers	7 (5%)	1 (3.6%)	1.000*
Other immunosuppressive conditions	7 (5%)	1 (3.6%)	1.000*
None	32 (24%)	5 (17.9%)	0.624*
2 comorbidities	34 (25%)	10 (35.7%)	0.252*
>2 comorbidities	15 (11%)	5 (17.9%)	0.344*

* Indicates the p-value was determined using the Fisher Exact Test. All other p-values were determined using a two-tailed student T-test. Significance was defined as a p-value ≤0.05, and was seen in the analysis of source of infection, overall treatment type, and presence of a long-term IV.

These results show that having a device-related infection may increase the risk of prolonged bacteremia. In addition, the presence of a long-term intravenous access (such as for hemodialysis or chemotherapy) may also increase the risk of prolonged bacteremia. Patients with these infection source types could be treated more aggressively, as well as patients undergoing hemodialysis or chemotherapy.

Vancomycin MIC 1 versus Vancomycin MIC 2 Analysis

Multiple studies have been done to determine the influence of vancomycin MIC on the outcomes of MRSA infections, particularly the mortality of MRSA infections. I hypothesize that a review of patient chats and subsequent analysis will show that the vancomycin MIC influences infection outcome. I reviewed the charts of 18 patients (6 MIC 2 and 12 MIC 1) with blood cultures positive for MRSA, and analyzed the results with student T-tests and Fisher Exact tests.

The comparisons of the demographic and clinical characteristics in patients infected with MRSA strains having vancomycin MIC of 1 or 2 are shown in Table 3a-b. The only characteristic which reached statistical significance in this comparison was the presence of two comorbidities. Patients with MRSA strains having vancomycin MICs of 2 were more likely to have two comorbidities (66.6%) had two comorbidities than patients with MRSA strains having vancomycin MICs of 1 (9%) ($p = 0.022$). None of the other markers reached significance.

Table 3a. Demographic and Clinical Characteristics in Patients Infected With MRSA Strains Having Vancomycin MIC of 1 or 2

Marker	MIC 1 12 Patients	MIC 2 6 Patients	p-value
Age	Mean: 56 ± 13.79	Mean: 64.8 ± 10.87	0.430
Gender	7 F (58%) 5 M (42%)	4 F (67%) 2 M (33%)	0.751
Source of Infection			
Skin/soft tissue	3 (25%)	1 (17%)	1.000*
Device related	5 (42%)	3 (50%)	1.000*
Genitourinary tract-related	0 (0%)	0 (0%)	N/A
Osteomyelitis	1 (8%)	0 (0%)	1.000*
Lung: pneumonia, bronchitis	0 (0%)	0 (0%)	N/A
Infection of head and neck	0 (0%)	0 (0%)	N/A
Infection of any solid organ	1 (8%)	0 (0%)	1.000*
Abscess of abdominal or digestive tract	0 (0%)	0 (0%)	N/A
Unknown, other not specified.	2 (17%)	2 (33%)	0.569*
Other Infections	5 Y (42%) 7 N (58%)	2 Y (33%) 4 N (67%)	0.751
Initial Vancomycin Trough	Mean: 14.3	Mean: 12.6	0.590
Clearance Vancomycin Trough	Mean: 17.9	Mean: 12.9	0.445
Initial Vancomycin Dose	Mean: 1000mg	Mean: 875mg	0.241
Clearance Vancomycin Dose	Mean: 979mg	Mean: 875mg	0.447
ANC Level	Mean: 10.5	Mean: 6.9	0.337
Days Positive	Mean: 2.1	Mean: 1.5	0.462
Marker	MIC 1 12 Patients	MIC 2 6 Patients	p-value
Treatment			
Vancomycin Monotherapy	9 (75%)	3 (60%)	0.600*
Vancomycin in Combination	3 (25%)	1 (20%)	1.000*
No Vancomycin	0 (0%)	1 (20%)	0.294*
Hospital Length of Stay	Mean: 13 days	Mean: 14.7 days	0.718
Admitted to ICU in 48 Hours	Y 2 (17%) N 10 (83%)	Y 2 (33%) N 4 (67%)	0.453
Length of stay in ICU	Mean: 20 days	Mean: 3.5 days	0.061
Mortality 30 Days	Y 1 (8%) N 11 (92%)	Y 1 (17%) N 5 (83%)	0.621
Cause of Death	MRSA: 1 Non-MRSA: 0	MRSA: 0 Non-MRSA: 1	1.000*
Recurrence	Y: 1 (8%) N: 11 (92%)	Y: 1 (20%) N: 5 (80%)	0.621

* Indicates the p-value was determined using the Fisher Exact Test. All other p-values were determined using a two-tailed student T-test. Significance was defined as a p value ≤ 0.05. Significance was only seen in the 2 comorbidities, sub category of the comorbidities marker.

Table 3b. Demographic and Clinical Characteristics in Patients Infected With MRSA Strains Having Vancomycin MIC of 1 or 2

Marker	MIC 1 12 Patients	MIC 2 6 Patients	p-value
Long Term Care Facility?	Y 3 (25%) N 9 (75%)	Y 0 (0%) N 6 (100%)	0.515*
Comorbidities			
Hypertension/heart disease	3 (25%)	1 (16.7%)	1.000*
Diabetes	0 (0%)	0 (0%)	N/A
Chronic kidney disease	0 (0%)	0 (0%)	N/A
Liver disease	0 (0%)	0 (0%)	N/A
Malignancy: (hematologic)	0 (0%)	0 (0%)	N/A
Other cancers	0 (0%)	0 (0%)	N/A
Other immunosuppressive conditions	0 (0%)	0 (0%)	N/A
None	4 (33%)	1 (16.7%)	0.615*
2 comorbidities	1 (9%)	4 (66.6%)	0.022*
>2 comorbidities	4 (33%)	0 (0%)	0.245*
Injection Drug Use	Y 0 (0%) N 12 (100%)	Y 0 (0%) N 6 (100%)	N/A
Recent Hospitalization	Y 5 (42%) N 7 (58%)	Y 1 (17%) N 5 (83%)	0.317
Long Term Intravenous Access	Y 2 (17%) N 10 (83%)	Y 2(33%) N 4 (67%)	0.453

* Indicates the p-value was determined using the Fisher Exact Test. All other p-values were determined using a two-tailed student T-test. Significance was defined as a p value ≤ 0.05. Significance was only seen in the 2 comorbidities, sub category of the comorbidities marker.

These results show that vancomycin MIC is not related to MRSA outcome. In addition, patients with two comorbidities may be more likely to be infected with a MRSA strain having a vancomycin MIC of 2.

Timed Kill-Curves

Suspension vs. Log-Growth Methods

The purpose of this experiment was to address whether or not the method of bacterial preparation influenced the killing activity of vancomycin alone in a timed kill-curve experiment. Since vancomycin works to impair cell wall growth, it is more active when the bacteria are in log-phase growth compared to stationary phase. Timed kill-curve experiment protocols typically call

for the log-phase growth method. However, the stationary method is most commonly used for bacterial preparation in the clinical laboratory because it is a better predictor of what is happening in the patient.

In order to discern if there was a difference between the methods, two kill-curves were performed simultaneously using Strain 2 and vancomycin. The simultaneous experiments ensured the experimental conditions were the same. For example, the time it took to take samples at each time point was consistent between the two experiments. Using the same strain and taking colonies for each experiment from the same culture plate minimized bacterial variability between the experiments. The results of the simultaneous experiments, seen in Figure 2, show that there is a difference in the vancomycin activity between the two methods.

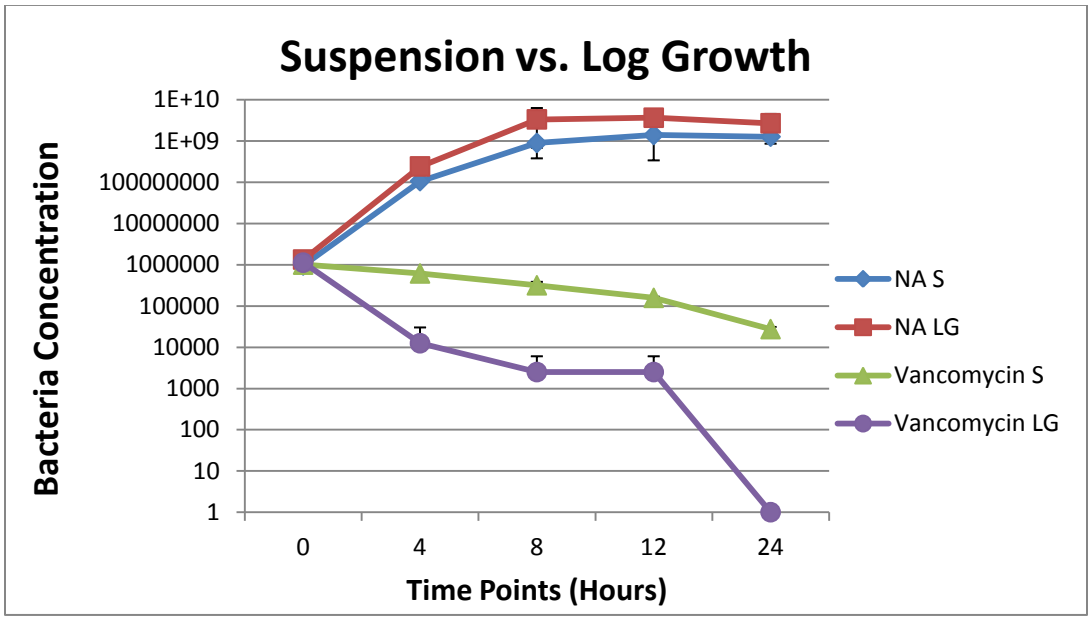


Figure 2. Comparison of the Suspension and Log Growth Vancomycin and Growth Curves. The No Antibiotic (NA) curves are the control of each experiment. The two NA curves indicate the consistent growth curve of the strain used in the two experiments. The two vancomycin curves indicate that vancomycin was more active when the bacteria were in log-phase growth at time zero, than when the bacteria were in stationary phase at time zero. Note: Log-growth (LG) and Suspension (S).

These experiments, done in parallel, showed that the activity of vancomycin was affected by the method used to prepare the bacteria for the experiment. The vancomycin curve in the log-growth method showed a more rapid decline in bacterial concentration with complete killing at 24 hours in comparison to the slow decline of the vancomycin curve with the suspension method. These results indicated that the log-growth method of bacteria preparation resulted in a better resolution in 24 hours, and thus the log-phase growth method was used for the remainder of the time kill experiments.

Kill Curves: Antibiotic Combinations

The vancomycin/gentamicin and vancomycin/rifampin combinations were chosen after they were shown to be two of the most common combination therapies used for the treatment of MRSA at LUMC. This was determined using the chart review in Aim 1. The vancomycin/cefazolin combination was chosen due to recent publications mentioned earlier, that suggested the use of this combination to treat *S. aureus* bacteremia. The experiments were done to address the question of whether or not the antibiotics demonstrated synergy *in vitro*. *In vitro* synergy was a traditional standard used to support the use of a combination in the clinic.

The timed-kill curve technique using the log-phase growth method of bacterial preparation was used to determine the synergy of vancomycin and gentamicin in combination against MRSA strains having vancomycin MICs of 2. The MICs of strain for each drug are shown in Table 4. The timed-kill curve technique allowed for observation of the killing activity of both drugs individually compared to the combination over 24 hours. The data obtained from these experiments demonstrated the effectiveness of the combination.

Table 4. Antibiotic MICs for Strains 1-6				
	Vancomycin MIC	Gentamicin MIC	Rifampin MIC	Cefazolin MIC
Strain 1	2	<= 1	<= 1	> 16
Strain 2	2	4	> 2	> 16
Strain 3	2	>8	<= 1	> 16
Strain 4	2	<= 1	<= 1	<= 4
Strain 5	2	<= 1	> 2	> 16
Strain 6	2	<= 1	<= 1	<= 4

Note: MRSA is typically innately resistant to cefazolin, but strains 4 and 6 demonstrate an intermediate MIC. The CLSI recently lowered the susceptibility break point to 2, and 4 is now considered intermediate.
Note: Strain 3 is resistant to gentamicin and strain 5 is resistant to rifampin.

Vancomycin + Gentamicin

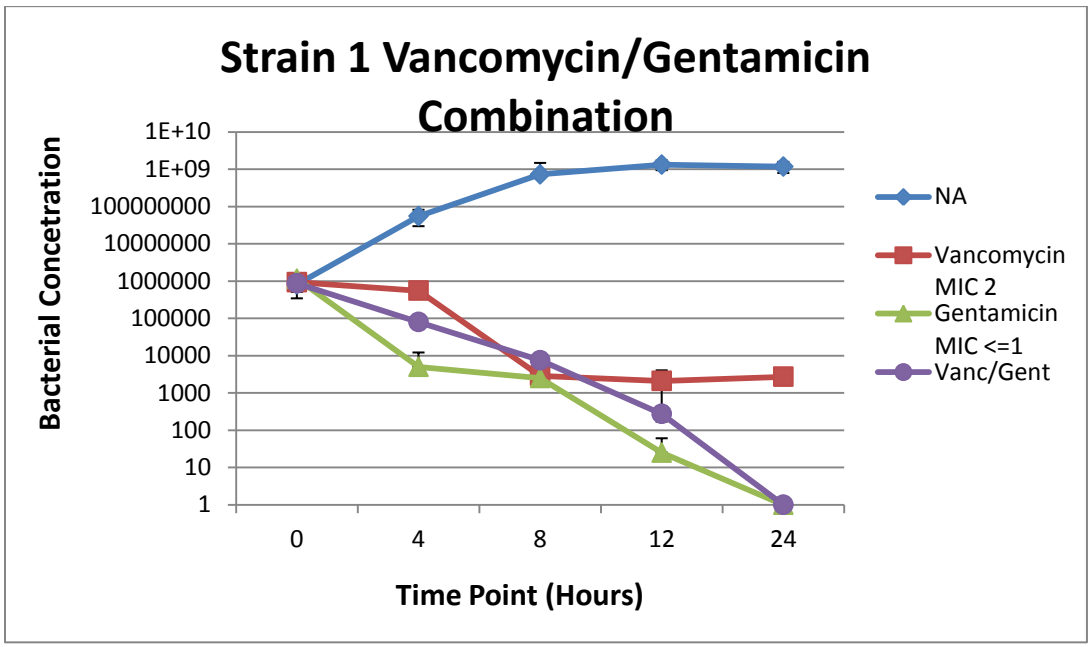


Figure 3. Results of the Strain 1 Vancomycin + Gentamicin Combination Kill-Curve Experiment. The vancomycin + gentamicin combination showed the same activity as gentamicin alone, but was more active than the vancomycin alone. Antibiotic synergy was not demonstrated.

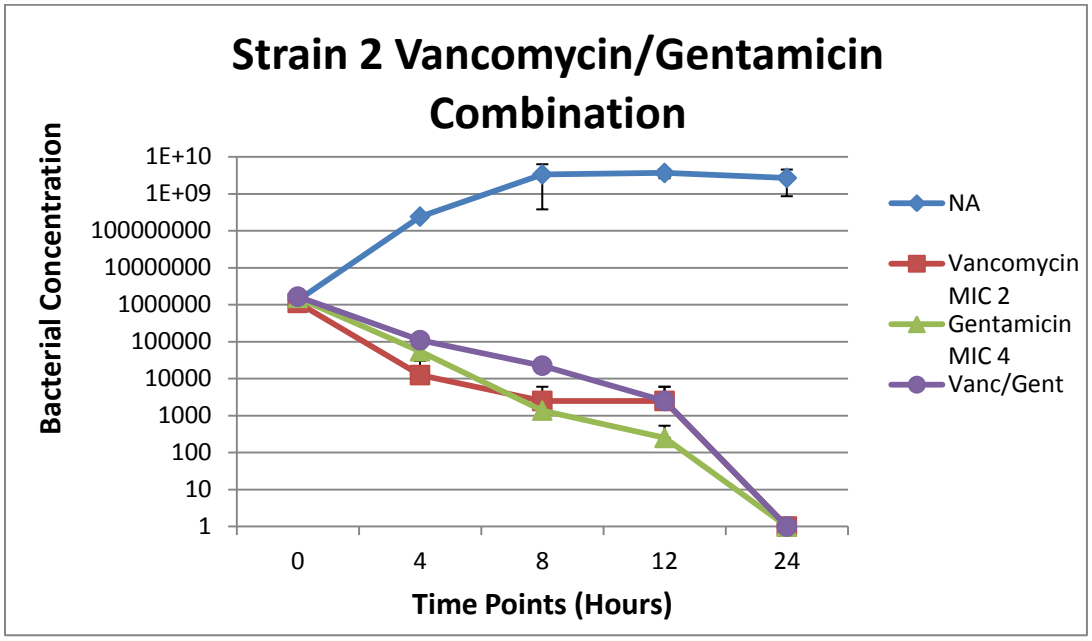


Figure 4. Results of the Strain 2 Vancomycin + Gentamicin Combination Kill-Curve Experiment. The vancomycin + gentamicin combination showed the same activity as both antibiotics individually. Antibiotic synergy was not demonstrated.

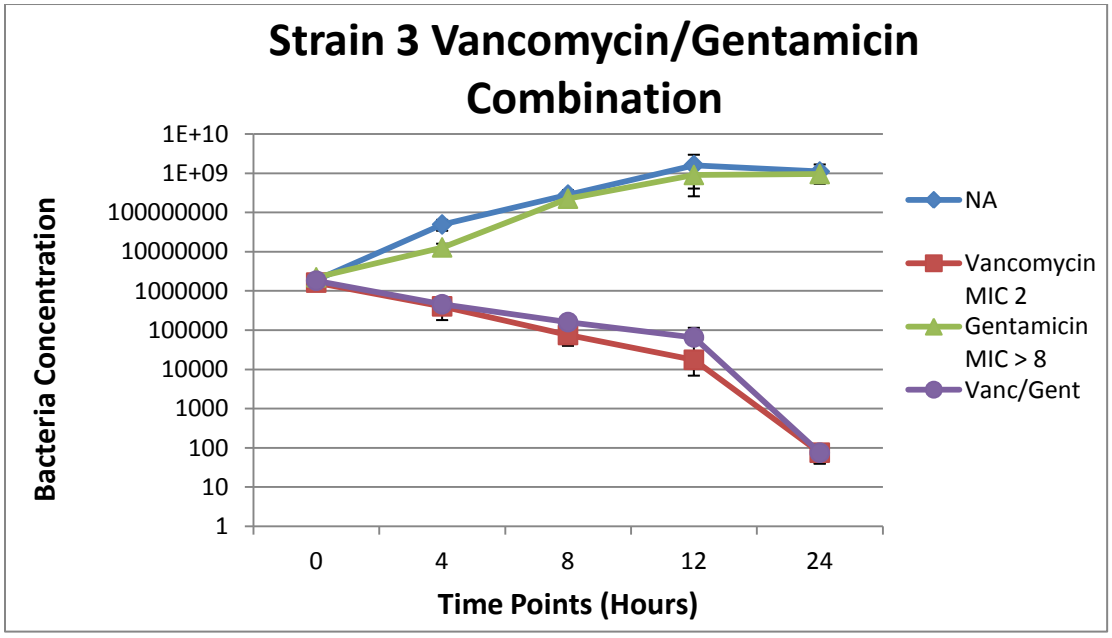


Figure 5. Results of the Strain 3 Vancomycin + Gentamicin Combination Kill-Curve Experiment. The strain was resistant to gentamicin, indicated by the MIC > 8. The vancomycin + gentamicin combination showed the same activity as vancomycin. Antibiotic synergy was not demonstrated.

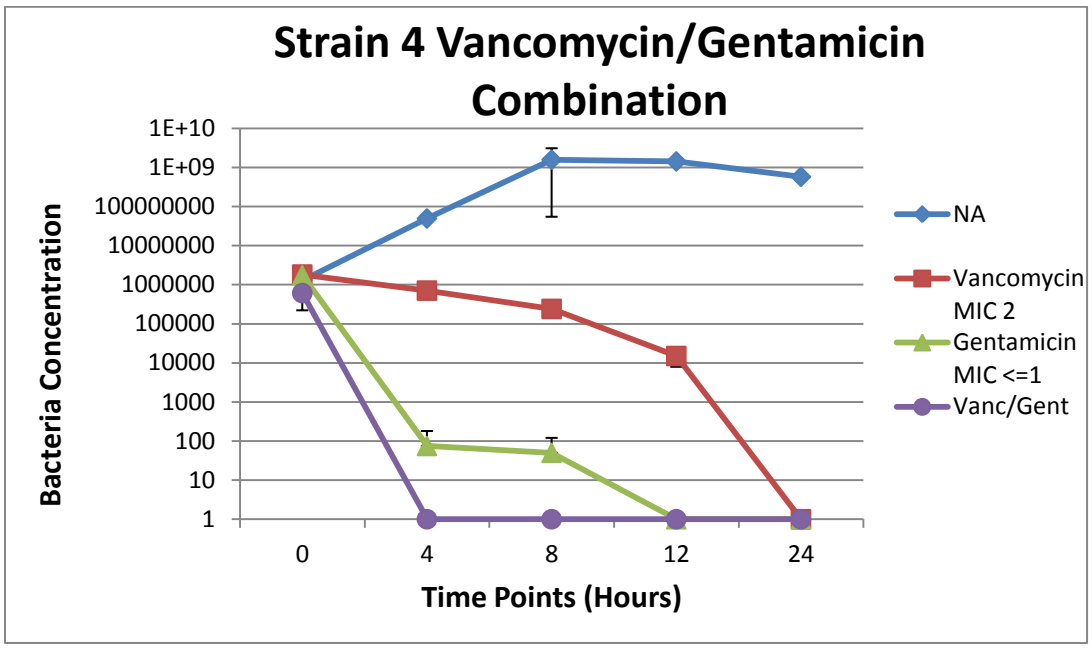


Figure 6. Results of the Strain 4 Vancomycin + Gentamicin Combination Kill-Curve Experiment. The vancomycin + gentamicin combination showed increased killing activity compared to both drugs individually. Antibiotic synergy was not demonstrated.

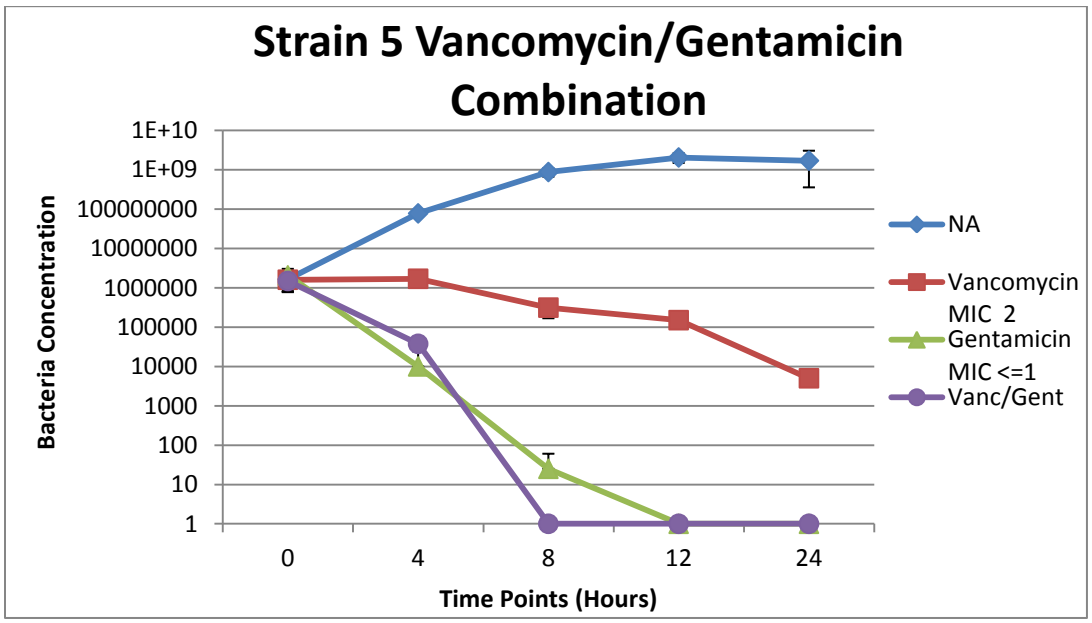


Figure 7. Results of the Strain 5 Vancomycin + Gentamicin Combination Kill-Curve Experiment. The vancomycin + gentamicin combination showed similar activity as gentamicin alone, but was more active than the vancomycin alone. Antibiotic synergy was not demonstrated.

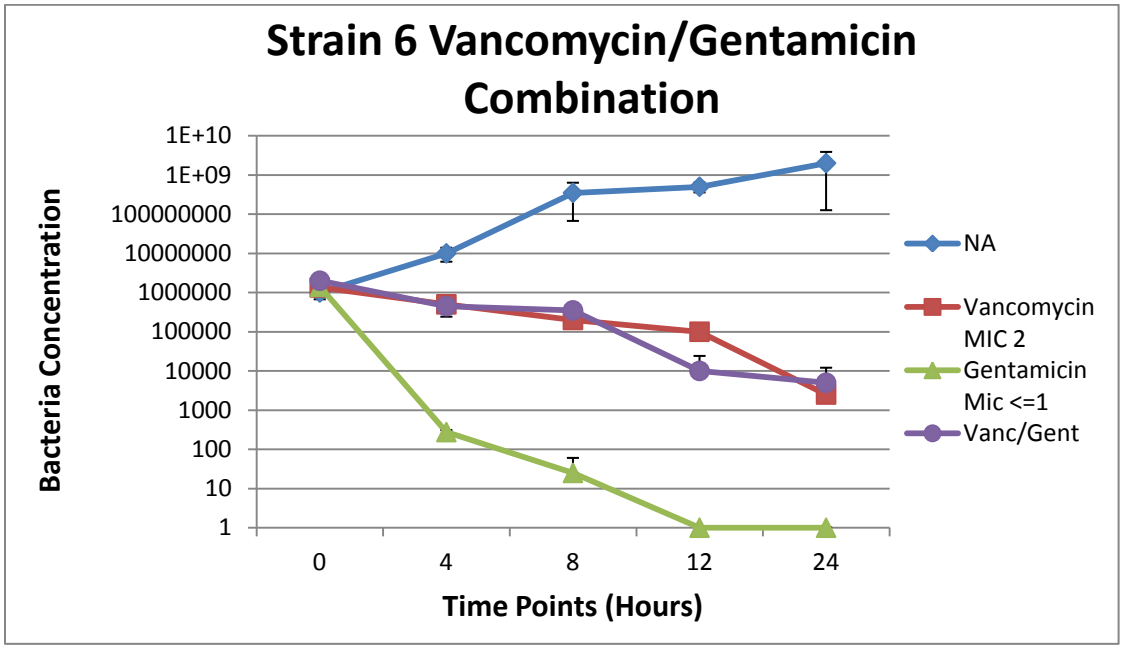


Figure 8. Results of the Strain 6 Vancomycin + Gentamicin Combination Kill-Curve Experiment. The vancomycin + gentamicin combination showed similar activity as vancomycin alone. Gentamicin was more active than both vancomycin alone and the combination. Antibiotic synergy was not demonstrated.

The results of the timed kill-curve experiments for the vancomycin + gentamicin combination showed an indifferent relationship (neither synergy nor antagonism was demonstrated) for each strain tested. Although synergy was never demonstrated, the *in* vitro killing of the combination was more active than vancomycin alone for three of the strains (1, 4, and 5). These results support the use of the vancomycin + gentamicin combination for the treatment of MRSA. However, the toxicity associated with this combination should not be overlooked.

Most of the strains showed that gentamicin alone demonstrated better or similar killing activity to the combination. Strain 3 demonstrated gentamicin resistance, and the combination curve paralleled the vancomycin alone curve indicating gentamicin was not active in the combination. It is possible that gentamicin alone could be an effective therapy for MRSA; however this method of treatment has not been tested. This is most likely because of the

toxicity associated with gentamicin, and the fact that it is typically used against gram (-) bacteria. In addition, there are less toxic antibiotics that can be used for gram (+) bacteria including *S. aureus*.

Vancomycin + Rifampin

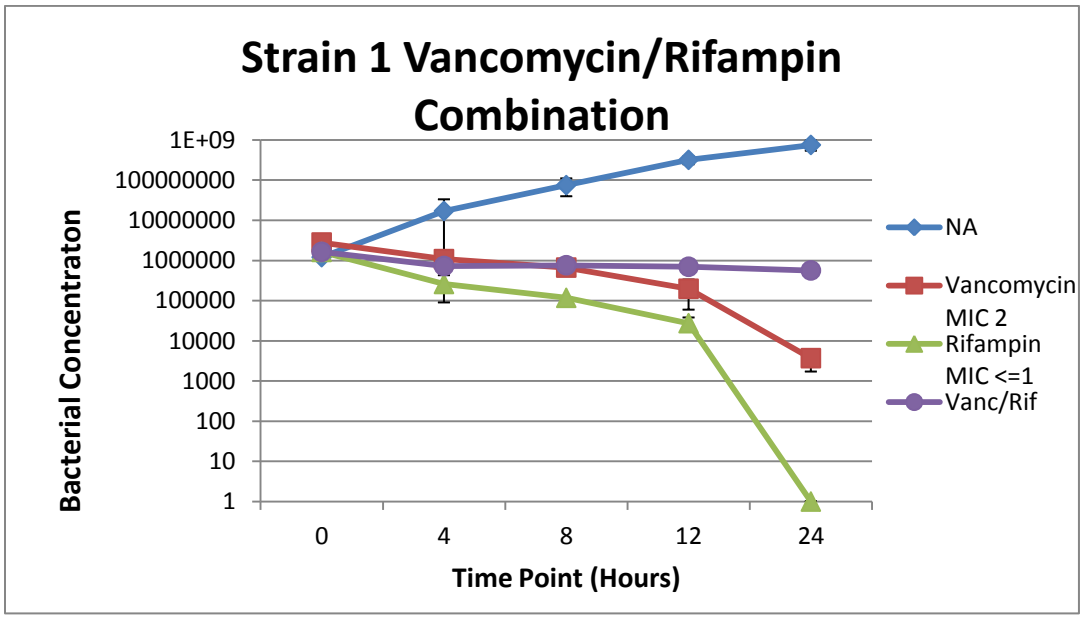


Figure 9. Results of the Strain 1 Vancomycin + Rifampin Combination Kill-Curve Experiment. The vancomycin + rifampin was antagonistic showing a 2 $\log_{10}$ decrease in killing activity compared to both antibiotics alone.

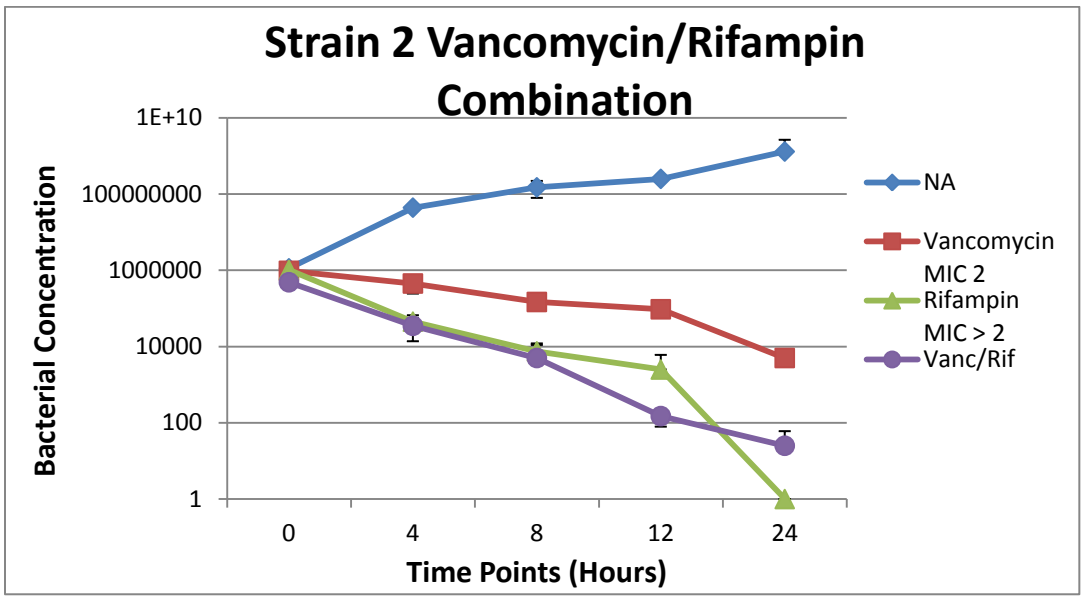


Figure 10. Results of the Strain 2 Vancomycin + Rifampin Combination Kill-Curve Experiment. The vancomycin + rifampin combination showed the same activity as rifampin alone. The combination was more active than vancomycin alone. Antibiotic synergy was not observed.

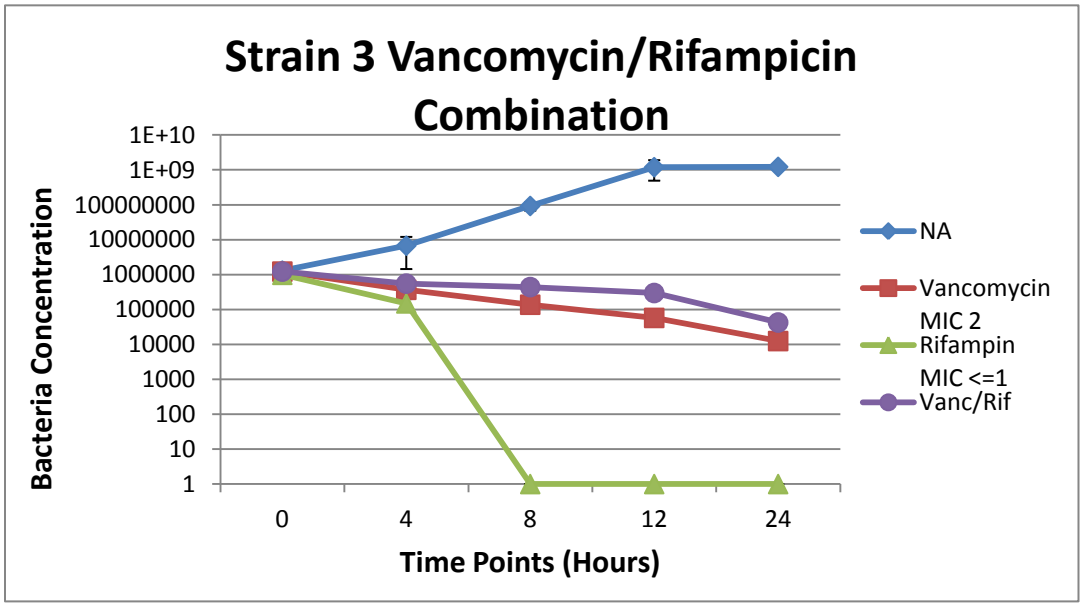


Figure 11. Results of the Strain 3 Vancomycin + Rifampin Combination Kill-Curve Experiment. The vancomycin + rifampin combination showed worse killing activity than both dugs individually, but it did not reach antagonism. The rifampin alone showed better activity than vancomycin alone and the combination. Antibiotic synergy was not demonstrated.

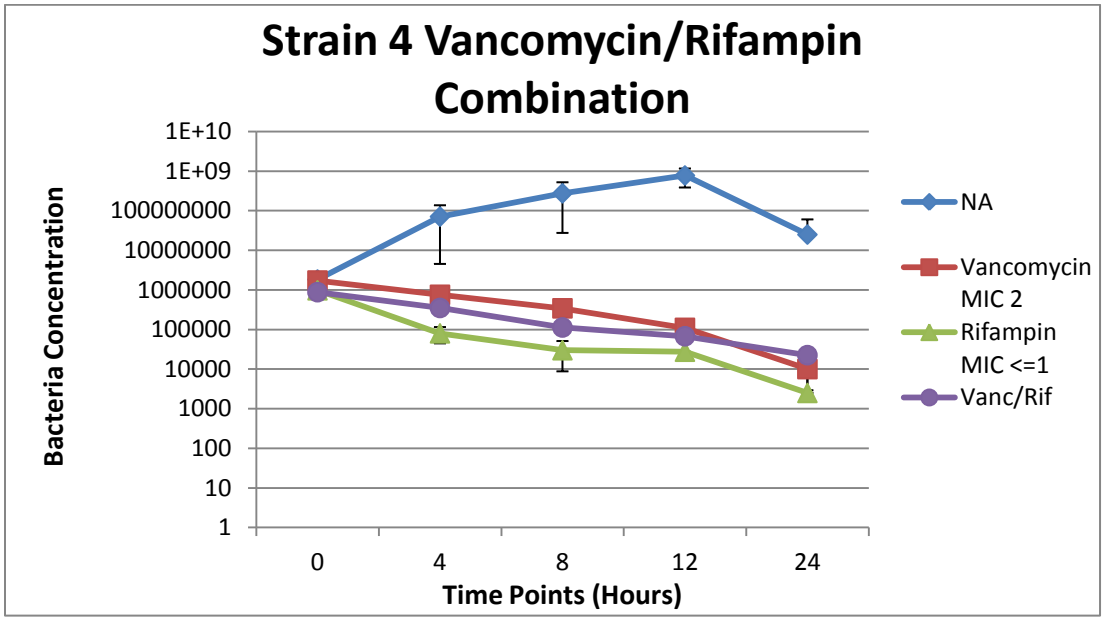


Figure 12. Results of the Strain 4 Vancomycin + Rifampin Combination Kill-Curve Experiment. The vancomycin + rifampin combination showed slightly worse killing activity than both dugs individually by 24 hours, but it did not reach antagonism. The rifampin alone showed activity similar to vancomycin alone and the combination. Antibiotic synergy was not demonstrated.

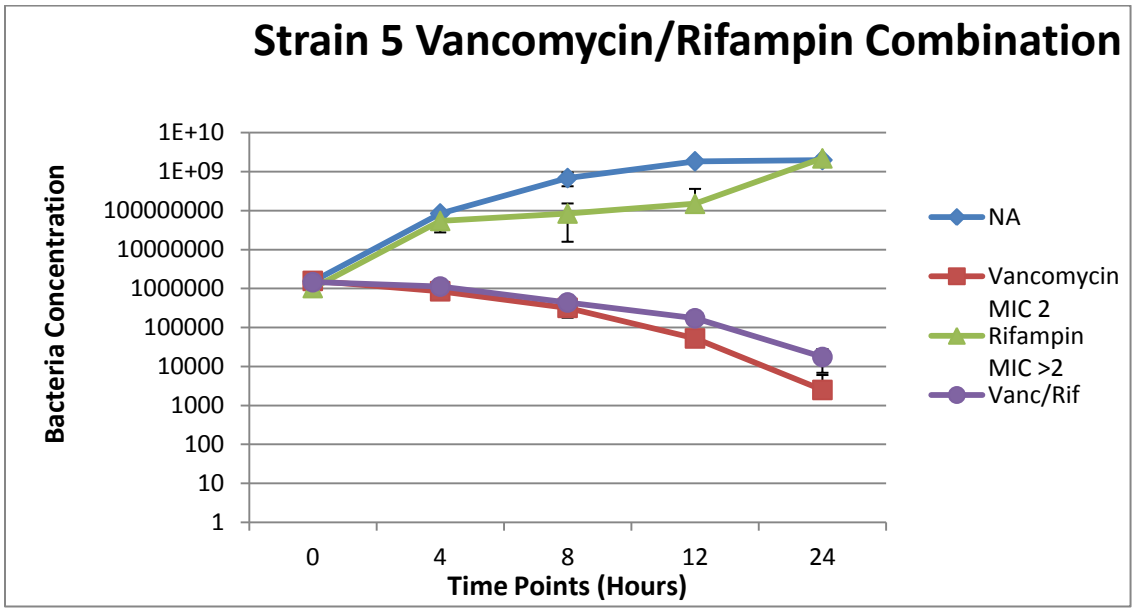


Figure 13. Results of the Strain 5 Vancomycin + Rifampin Combination Kill-Curve Experiment. The strain was resistant to rifampin, as shown by the rifampin alone curve. The vancomycin + rifampin combination showed slightly worse killing activity than vancomycin alone, but it did not reach antagonism. Antibiotic synergy was not demonstrated.

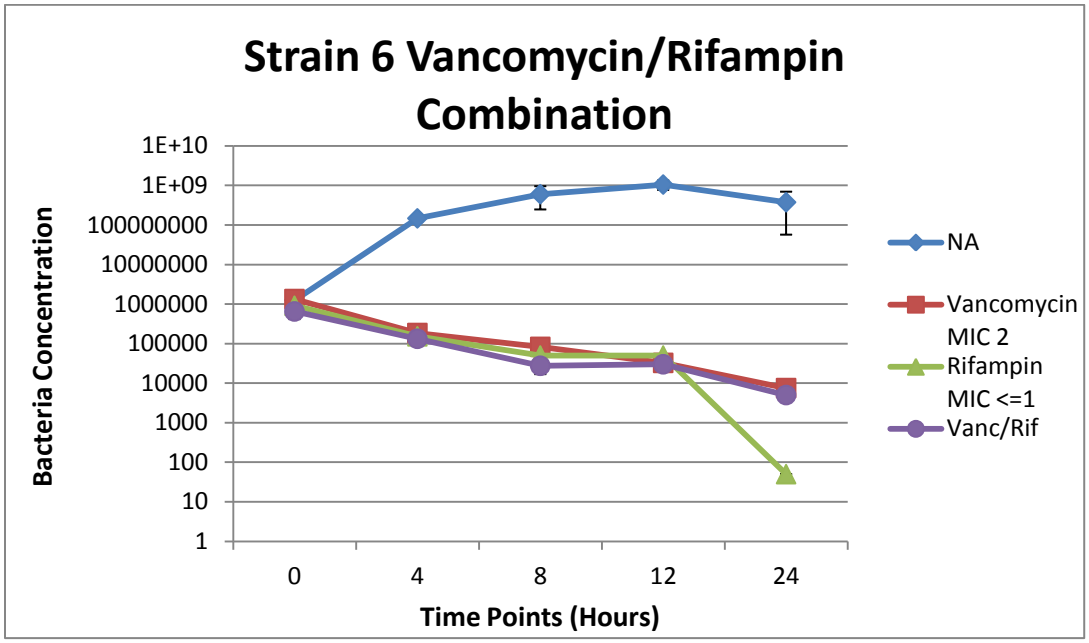


Figure 14. Results of the Strain 6 Vancomycin + Rifampin Combination Kill-Curve Experiment. The vancomycin + rifampin combination showed similar activity to vancomycin and rifampin alone. Antibiotic synergy was not demonstrated.

The results of the timed kill-curve experiments for the vancomycin + rifampin combination showed an indifferent relationship (neither synergy nor antagonism was demonstrated) for four of the strains tested and an antagonistic relationship with one strain. Only one strain showed the combination to be more effective than vancomycin alone, but synergy was not demonstrated. Although only one strain reached antagonism, the *in vitro* killing of the combination was worse than both antibiotics alone in four strains. These results do not support the use of the vancomycin + rifampin combination for the treatment of MRSA.

Vancomycin + Cefazolin

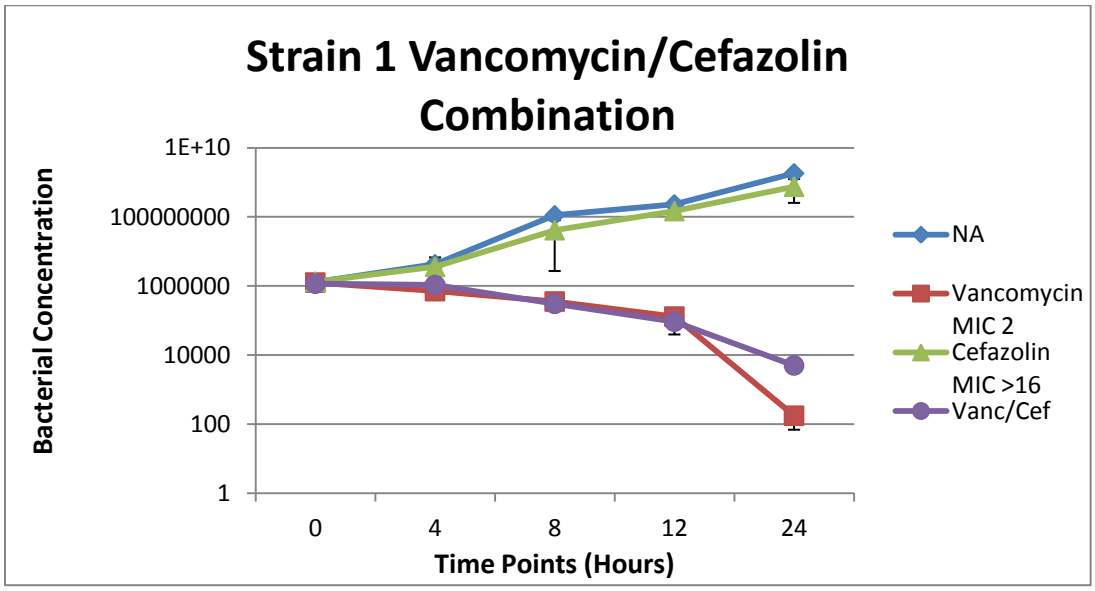


Figure 15. Results of the Strain 1 Vancomycin + Cefazolin Combination Kill-Curve Experiment. The strain was resistant to cefazolin as shown by the cefazolin curve. The vancomycin + cefazolin combination showed similar killing activity to vancomycin alone. Antibiotic synergy was not demonstrated.

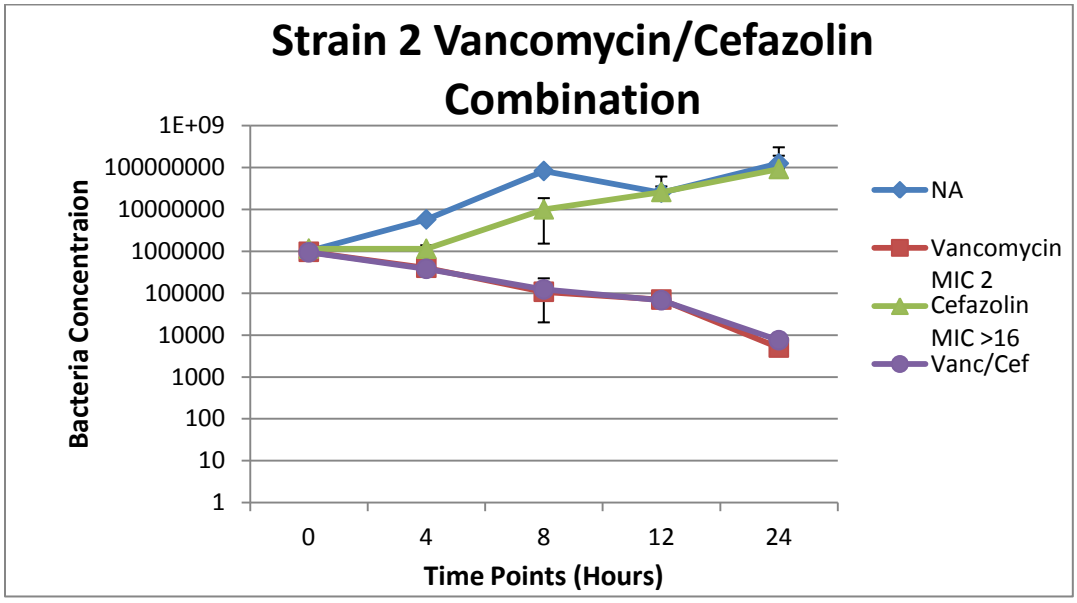


Figure 16. Results of the Strain 2 Vancomycin + Cefazolin Combination Kill-Curve Experiment. The strain was resistant to cefazolin as shown by the cefazolin curve. The vancomycin + cefazolin combination showed similar killing activity to vancomycin alone. Antibiotic synergy was not demonstrated.

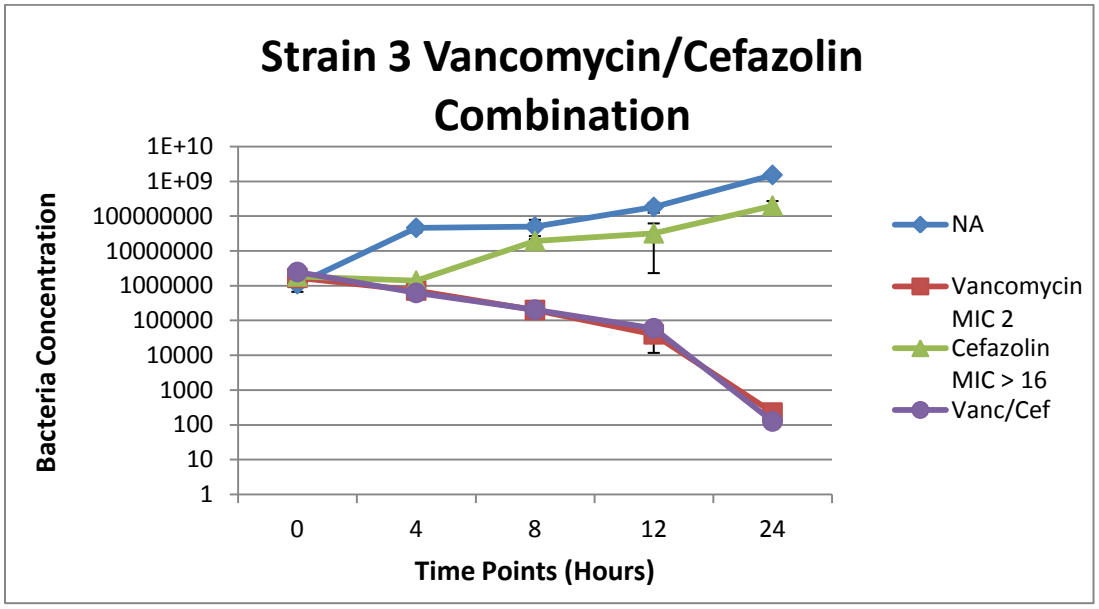


Figure 17. Results of the Strain 3 Vancomycin + Cefazolin Combination Kill-Curve Experiment. The strain was resistant to cefazolin as shown by the cefazolin curve. The vancomycin + cefazolin combination showed similar killing activity to vancomycin alone. Antibiotic synergy was not demonstrated.

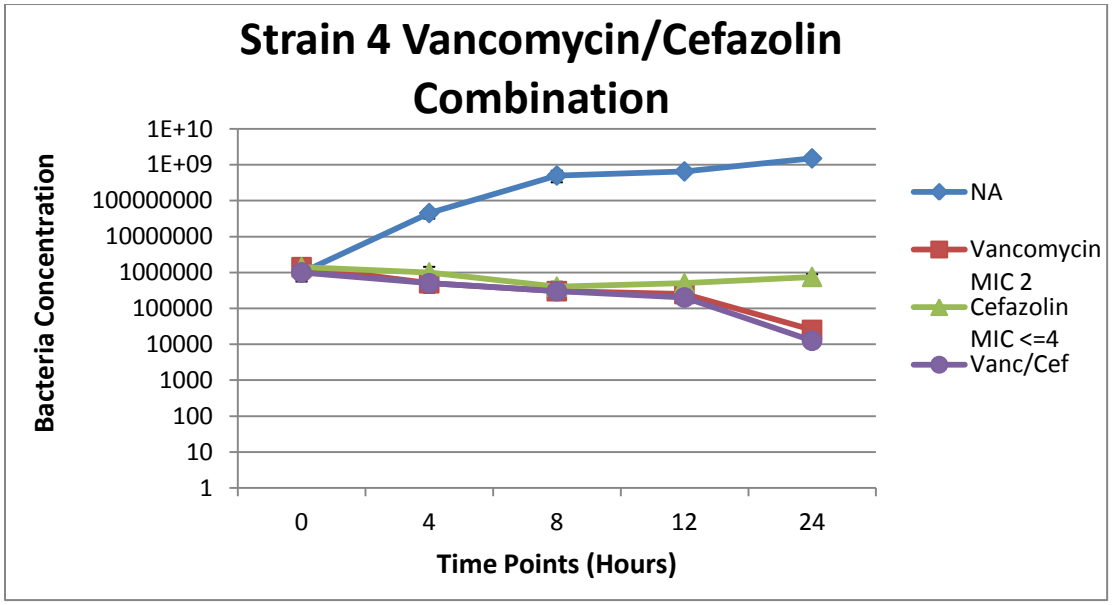


Figure 18. Results of the Strain 4 Vancomycin + Cefazolin Combination Kill-Curve Experiment. The strain was resistant to cefazolin as shown by the cefazolin MIC. The 30µg/mL concentration could have overwhelmed the bacteria, causing a delay in the resistance seen in the cefazolin curve. The vancomycin + cefazolin combination showed similar killing activity to vancomycin alone. Antibiotic synergy was not demonstrated.

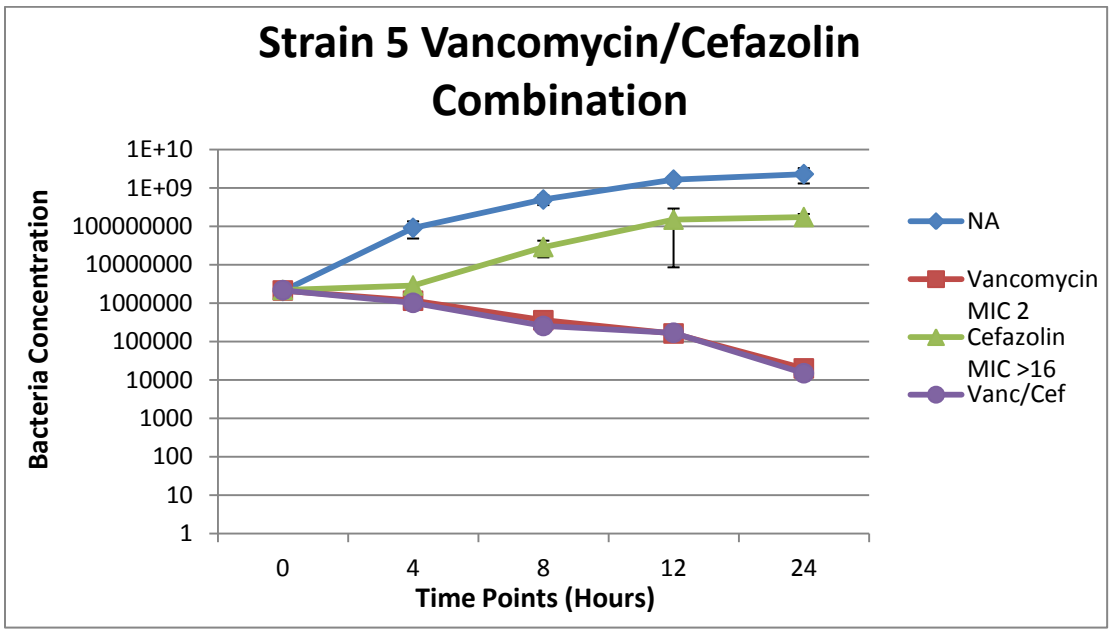


Figure 19. Results of the Strain 5 Vancomycin + Cefazolin Combination Kill-Curve Experiment. The strain was resistant to cefazolin as shown by the cefazolin curve. The vancomycin + cefazolin combination showed similar killing activity to vancomycin alone. Antibiotic synergy was not demonstrated.

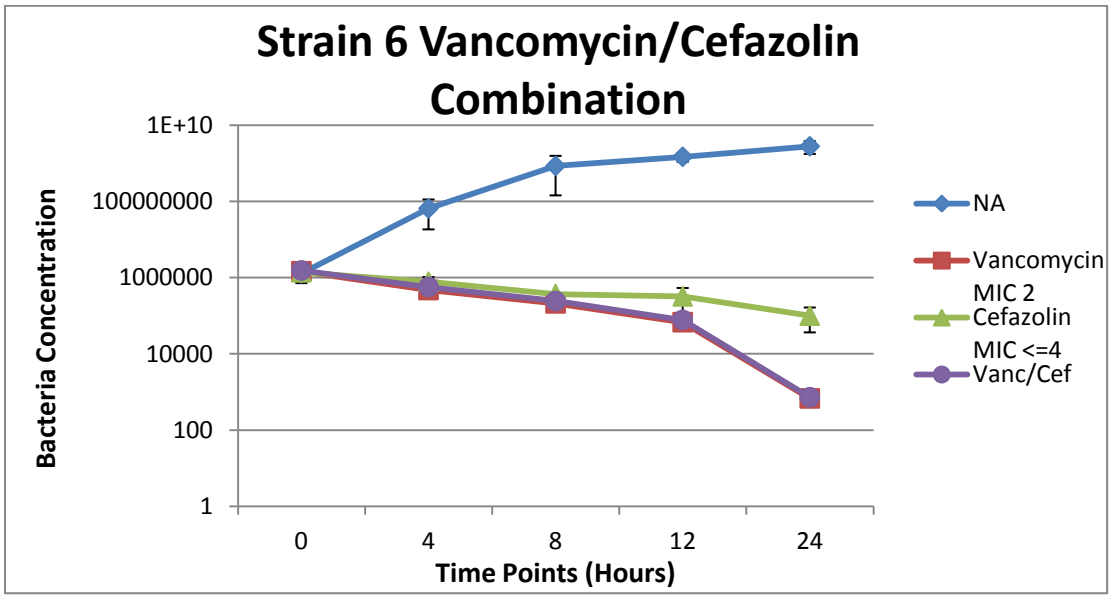


Figure 20. Results of the Strain 6 Vancomycin + Cefazolin Combination Kill-Curve Experiment. The strain was considered resistant to cefazolin by Microscan, but it did not act resistant. The vancomycin + cefazolin combination showed similar killing activity to vancomycin alone. Antibiotic synergy was not demonstrated.

The results of the timed kill-curve experiments for the vancomycin + cefazolin combination showed that the combination paralleled the *in vitro* killing activity of vancomycin alone. These results indicate that the cefazolin did not play a role in the combination, and only the vancomycin was active against the bacteria. This was seen with all of the strains, even when the strain had an intermediate MIC of <= 4. All six strains demonstrated indifference. Neither antibiotic synergy nor antagonism was achieved. These results do not support the use of the vancomycin + cefazolin combination for the treatment of MRSA.

CHAPTER FIVE

DISCUSSION

MRSA is an important public health concern, and MRSA infections are no longer limited to health care institutions (34). This study was unique in that it aimed to identify predictors of both recurrent and prolonged bacteremia as individual outcomes of MRSA, instead of combining them into a general category of vancomycin treatment failure (21, 32, 38, 41). Few studies have done this type of analysis and those that have focused on either one outcome or the other (10, 15, 24, 31, 43, 47, 71, 74). One recent study by Wong et al. examined organism characteristics of MRSA isolates from patients with persistent or recurrent bacteremia but did not look at clinical predictors for recurrent or prolonged bacteremia (73). In the present study, I examined patient records and laboratory data, to determine if clinical or microbiologic characteristics could be identified that would predict patient populations at increased risk for prolonged or recurrent MRSA bacteremia. I used the same patient population to determine risk factors for both outcomes. In addition, I tested combinations antibiotics commonly used at LUMC for treating MRSA bacteremia to determine the *in* vitro effects of combination therapy on select MRSA isolates recovered from patients with bacteremia.

My first aim was to determine if clinical or microbiologic markers could be identified that predict less favorable outcomes of MRSA infections. These outcomes were defined as recurrent infection or prolonged bacteremia (≥3 days of positive blood cultures). To answer this question I performed a retrospective analysis of 163 patients to determine if there were any demographic or clinical factors that could predict patients at a greater risk for having for having

recurrent or prolonged bacteremia. In addition, I evaluated the vancomycin susceptibility of MRSA isolates to determine if vancomycin MIC 1 vs. MIC 2 could be used to predict patients at risk for negative infection outcomes.

My chart review indicated that 9.2% of patients experienced recurrent infection. The current study did not differentiate between relapse and reinfection when defining recurrence, and both sub-classifications are included in the recurrent category. I found that the younger patients (32-52) are more likely to experience recurrent infections (p=0.005). The average age of recurrent patients was 42 ± 10.23 years, and the average age of non-recurrent patients was 57 ± 3.07 years (**Table 1a-b**). These results were not expected. One would have expected older age to be associated with recurrent infection given the weaker immune system and increased amount of comorbidities seen in older patients. A study of *S. aureus* bacteremia done by Hill et al. indicated that age greater than 60 was a risk factor for a poor outcome (defined as 30-day mortality) (24). In addition, Klevens et al. reported that the incidence of invasive MRSA infections was highest among persons 65 years and older (33). However, neither study associated age with the likelihood of recurrent infections. I did find that patients who did not have recurrent infections were more likely to be between 54-60 years old, and 20% of patients in this group experienced 30-day mortality, which is similar to the age identified to put people at risk for MRSA infections and 30-day mortality (24, 33).

One theory possibly explaining a younger age being associated with recurrence is that many activities identified as predisposing people to community-associated MRSA infections are more likely to have younger participants, especially in the 32-52 age range. Some of these activities include contact sports such as wrestling, fencing and football, and military recruitment (5, 6, 23, 45, 55). These activities provide ample opportunities for people to be in close contact

with not only one another, but also with mats, masks, towels, and other surfaces exposed to multiple people. As a result, those participants would be more likely to be exposed to MRSA multiple times, possibly resulting in multiple MRSA infections.

A second theory must address the limitations of this study. I only included patients who were treated at LUMC. It is feasible that patients grouped as non-recurrent could have experienced recurrent MRSA infections, but received treatment at another medical facility. In these cases their recurrence would not have been documented by LUMC, and they would have been categorized as non-recurrent. It is possible there were more recurrent patients in this study than were documented. In addition, I only included patients with a bloodstream infection indicated by blood cultures positive for MRSA. It is possible that patients with non-bloodstream infectious could have also recurred, but I did not document those cases. Had more patients been included in the recurrent group, the age ranges could have been different.

I also found that treatment type is associated with recurrent infection. The results showed that 93% of recurrent patients received vancomycin monotherapy, compared to only 62% of non-recurrent patients (p=0.009). In addition, 30% of non-recurrent patients received a combination therapy with vancomycin, compared to 0% of recurrent patients (p=0.012) (**Table 1a-b**). These results indicate that that treatment with vancomycin monotherapy may increase the risk of recurrent infection. These results are not surprising. High rates of vancomycin failure in MRSA treatment are well documented (27, 41, 42), and these results are supported by findings that treatment type is associated with relapse of *S. aureus* bacteremia (MRSA and MSSA) (10, 15, 71). Chang et al. found that recurrence, primarily relapse, occurred in 9.4% of *S. aureus* bacteremias, similar to the 9.2% seen in the present study, following treatment and was significantly associated with vancomycin therapy (10). Similar reports showed that patients who

relapsed with either MSSA or MRSA were more likely to have received vancomycin therapy (15, 71). These results suggest that vancomycin should not be the mainstay of therapy for *S. aureus* bacteremias regardless of oxacillin susceptibility.

Studies have suggested alternate therapies (37, 52), and these alternate therapies include combining vancomycin with another antibiotic, such as rifampin or gentamicin. Regarding combination therapy, my results show that this method of treatment may decrease the risk of recurrent infection. None of the recurrent patients received combination therapy, compared to 30% of non-recurrent patients (p=0.012). Moore et al. also showed that early combined therapy with an aminoglycoside or rifampin is associated with vancomycin treatment success (where failure was defined as recurrence, 30-day mortality and/or microbiologic failure) (41). Many other studies show that combination therapies can be successful against MRSA (3, 11, 39, 46, 62), so this finding is not unexpected.

The remainder of the markers analyzed did not reach statistical significance. This contradicts some of the findings by similar studies (10, 15, 24, 41, 71) that identify specific comorbidities (i.e. acute renal failure) and sources of infection, vancomycin trough levels, strain type, persistent bacteremia and presence of indwelling foreign bodies as risk factors predisposing patients to vancomycin failure. Similar statistics (i.e. t-test and fisher exact test) were used in this and other studies (41, 71). The difference in findings could be attributed to the different patient populations included in each study, and the specification of MRSA versus MSSA. In addition, Moore et al combined recurrence, 30-day mortality, etc. into one category (therapy failure); while my study separated the outcomes that are considered vancomycin failure and looked to identify markers that could more specifically predict each outcome (41).

This detailed analysis led to smaller numbers of patients in each marker category and could have resulted in different findings.

I also identified markers that may predict patients at risk for prolonged bacteremia. In the current study 17% experienced this outcome. Patients with device related infections may be at a greater risk for prolonged MRSA bacteremia. The percentage of patients experiencing prolonged bacteremia with device related infections (54%) was significantly higher (p=0.015) than the non-prolonged bacteremia group (29%). Similarly, the percentage of patients with long-term intravenous access (such as for hemodialysis or chemotherapy) was significantly higher ($p < 0.001$) in the prolonged group (54%), than in the non-prolonged group (19%) (**Table 2a-b**).

These results are not surprising, and are supported by multiple studies that indicate device related infections are associated with prolonged bacteremia (31, 43, 47, 74). The "device-related" category used in the current study includes all devices, ranging from pacer wires and prosthetics to PICC lines and catheters. Khatib et al. identified cardiovascular prosthesis and vancomycin treatment as risk factors for persistent *S. aureus* bacteremia (≥3 days) regardless of oxacillin susceptibility (31). The ability of biofilms to form on medical devices is well understood in hospitals. Additionally, some devices are easily replaced, such as catheters, while others require complex extraction or surgical procedures to replace such as pacer wires and prosthetic joints. This problem also explains the result that identifies patients with long-term intravenous access as being at risk for prolonged MRSA bacteremia. Some studies have mentioned the importance of removing devices responsible for infection, and indicate persistence is associated with delayed device removal (28, 43, 47, 74).The ability to identify and remove a device source affects the length of bacteremia.

Other studies have identified endovascular sources of infection, metastatic infections, multiple infection sites, treatment type and comorbidities as factors associated with persistent *S. aureus* infection (MRSA and MSSA) (31, 43, 47, 74). None of these other factors were significantly associated with prolonged bacteremia in the present study. This could be due to the smaller sample size, 28 prolonged bacteremias out of 163 patients, seen in this study. The small sample size split among twenty possible source and comorbidity categories (**Table 2a-b**), results in much smaller numbers in each category, which could reduce the incidence of significance. However, other markers in the current study reported as insignificant (p values >0.05), such as vancomycin trough level and treatment type, were also reported insignificant by similar studies (47, 74).

My last chart analysis looked at the differences between patients whose MRSA strains had a vancomycin MIC of 1 versus patients whose MRSA strain had a vancomycin MIC of 2. My results showed that the vancomycin MIC of the MRSA strain was not associated with recurrence (p=0.621) or prolonged bacteremia (p=0.462) (**Table 3a-b**). The only characteristic that reached significance was the presence of two comorbidities (p=0.22). The results showed that 66.6% of patients with MRSA strains having vancomycin MICs of 2 had two comorbidities, compared to only 9% of patients with MRSA strains having vancomycin MICs of 1.

This analysis was limited in that the MIC 2 group only contained six isolates. Those isolates were chosen from 180 isolates because they were MRSA blood isolates that had confirmed MICs of 2 by both MicroScan and Etest. To account for this small number, only 12 randomized patients of the 163 analyzed, are included in the MIC 1 comparison group. The 12 randomized patients were chosen from the list of 157 remaining patients by selection of every 13th patient. These data are supported by other studies stating MIC is unrelated to MRSA

infection outcome (21, 47, 53, 71). Other authors have shown that vancomycin MIC is related to *S. aureus* (MRSA and MSSA) infection outcome (38, 43, 73, 74). Finally, the significance of having 2 comorbidities could have been due to coincidence as a result of the small sample population. The vancomycin MIC of a MRSA strain infecting an individual should not be dependent upon the presence of comorbidities. The issue of MRSA vancomycin MIC importance should be further studied with larger populations.

The results of the first aim suggest further research is necessary to elaborate on the current findings. For example, Welsh et al. found prolonged bacteremia to be associated with MRSA infection relapse (71). However after looking at a group of patients, some of which had either recurrent MRSA infection or prolonged bacteremia, I did not find any correlation between the two outcomes. This lack of association was also found in a similar study that looked at the recurrent and prolonged bacteremia outcomes using the same patient population (73). The differences in these results suggest that future studies regarding predictors of MRSA outcomes should not only look at the outcomes of MRSA infections individually, but use the same patient populations in those studies. The variability between patients is clear and may contribute to the lack of consistency between studies of this nature. Using different patient populations to study different MRSA outcomes could play a role in the current contradicting studies.

An important note of the current study is that recurrence included both relapse and reinfection. A relapse infection occurs when a patient's infection is caused by the same strain of MRSA as the previous infection. Reinfection occurs when the patient is exposed to MRSA again however; it does not have to be the same strain as the previous infection. Relapse is a concern for patients who are in a hospital or medical setting for an extended period of time, or for patients who are in medical settings often, such as dialysis patients. Therefore, a study focusing

on the predictors of relapse infections would be beneficial to clinicians. Such a study would require access to the MRSA strains of multiple infections for each patient included in the study. In addition, strain typing would be necessary to determine if the MRSA strains from different infections were identical. The study could utilize chart review to analyze the difference characteristics seen in relapse versus non-relapse patients.

The results of the current study also suggest that future studies should include larger numbers of patients. For example, the comparison of the clinical and demographic characteristics between patients with and without prolonged bacteremia did not reveal ANC level to be significant. However, the p value was 0.052 which is approaching statistical significance. Had a larger patient population been used, it is possible that this characteristic could have demonstrated significance. In addition, the importance of the vancomycin MIC in terms of predicting negative MRSA outcomes should also be studied with a larger patient population, as the current study was limited to 18 patients. In addition, while the current study did not specifically address the 30-day mortality outcome, it is a concern and should be studied. This characteristic did not demonstrate significance in this study, but a study focusing on 30-day mortality as a negative outcome of MRSA infection using chart review could provide different results.

The second aim of my study was to determine if combination therapies commonly used at LUMC and suggested in publications demonstrate synergistic activity against MRSA. To answer this question I performed time-kill curve experiments on six strains of MRSA with vancomycin MICs of 2 confirmed by both microscan and Etest. I performed simultaneous experiments to determine whether there was a difference between the log growth method of bacterial preparation and the clinically used suspension method of bacterial preparation. Once

the method of bacterial preparation was decided, I tested three antibiotic combinations: vancomycin + gentamicin, vancomycin + rifampin, and vancomycin + cefazolin.

Since vancomycin affects cell-wall maintenance and growth it is more effective when cells are actively dividing. Therefore, the log-growth method of bacterial preparation is typically used in time-kill studies (3, 4, 64, 68, 69). In addition, the time-kill method of synergy testing is usually a more accurate predictor of a combination's activity *in vivo* (3, 7, 14). However, clinical laboratories susceptibility testing procedure calls for preparation using the suspension method. The bacteria are in stationary phase at the start of the test. My results (**Figure 2**) indicate that the method of bacterial preparation does affect the results of a time-kill experiment. The vancomycin curve showed a much steeper decline when the log-growth method was used compared to the suspension method. This supports a study done by Lamp et al. that shows that vancomycin produces higher kill rates against exponentially growing organisms (36). The log-growth method is used in the remainder of the time-kill experiments discussed.

In the vancomycin + gentamicin experiments, the combination does not demonstrate synergy against any of the six strains tested (**Figures 3-8**). Indifference is seen for all strains tested. However, the combination does show enhanced killing when compared to vancomycin alone in three of the six strains. Interestingly, one strain (strain 3) was resistant to gentamicin, and the combination kill rate was similar to that of vancomycin alone. This is logical in that the gentamicin resistance most likely results in the antibiotic having a diminished role in the combination. However, this result contradicts a study that uses the same antibiotic concentrations and demonstrates synergy regardless of gentamicin resistance (70). The reason for this contradiction probably lies in the variability of bacteria. Although synergy is not seen in

my studies, each strain does demonstrate different reactions to the same antibiotic combinations (i.e. vancomycin + gentamicin and vancomycin + rifampin).

While these results contradict studies that have shown *in vitro* synergy of vancomycin and gentamicin (26, 68, 70) they do support the use of this combination for the treatment of MRSA due to the enhanced killing rates (62). Although this combination is a promising alternative to vancomycin monotherapy for the treatment of MRSA, the dangers associated with it should not be overlooked. Multiple studies show a high rate of nephrotoxicity (16-26.3% of patients) with this combination (12, 16, 50). Current publications on this subject are contradictory. Tsuji et al. suggests one high dose of gentamicin in combination with vancomycin is enough to both achieve antibiotic synergy and decrease nephrotoxicity (62), but Cosgrove et al. states that an initial lose dose of gentamicin (1mg/kg every 8 hrs. for 4 days) with vancomycin is enough to cause nephrotoxicity (12). More research should be done *in vitro* and *in vivo* to clarify which doses result in the least nephrotoxicity while maintaining the most synergy.

The experiments testing the vancomycin + rifampin combination do not demonstrate synergy (**Figures 9-14**). The combination shows indifference against five of the six strains tested, and antagonism in one strain. The combination demonstrates enhanced killing when compared to vancomycin alone in only one strain. In addition, the combination shows diminished killing compared to both antibiotics individually in four of the six strains, including the antagonistic strain. Finally, the combination has a similar kill rate as vancomycin alone in one strain. These results are supported by studies that do not recommend the use of vancomycin + rifampin for the treatment of MRSA infections (61, 67, 75). However, there other reports that demonstrate synergy with this combination (4, 39, 46, 64). A possible reason for this discrepancy is the fact that I defined the combination as indifferent or antagonistic at 24 hours. In one study Bayer et

al. noted that synergy was more commonly seen at 48 hours than at 24 (4). It is possible that an extending the incubation time might have resulted in synergy, such as in the strain in which the combination kill rate paralleled that of vancomycin alone.

One discrepancy of these experiments is that both strains 2 and 5 were initially reported to have rifampin MICs >2 which would be considered resistant, but only strain 5 demonstrated resistance in the time-kill experiments. The experiment was done in duplicate for strain 2, and in both experiments the strain demonstrated susceptibility to rifampin. The definitive answer to this inconsistency is not known.

The vancomycin + cefazolin combination demonstrates indifference with all six strains tested (**Figures 15-20**). Interestingly, the rate of killing seen in the combination parallels that of vancomycin for each strain, whereas the strains show different reactions to the previous combinations used (i.e. vancomycin + gentamicin, and vancomycin + rifampin). These results are supported by the Climo et al. study which showed that the combination of vancomycin and a β-lactam is less likely to be synergistic against MRSA with a vancomycin MIC ≤2 (11). In their study 22 MRSA strains with MICs ≤2 showed a lack of synergy (11). This "seesaw effect" was demonstrated by Werth et al. as well, when their study showed ceftaroline, similar to a traditional β-lactam like cefazolin, was more effective against *S. aureus* strains with lower vancomycin susceptibilities (72). The six strains used in my study had MICs of 2, so the lack of synergy demonstrated by the vancomycin + cefazolin combination is not unusual.

However, these results do contradict studies reporting that β-lactam drugs, including cefazolin, work well in combination with vancomycin against *S. aureus* infections (MRSA and MSSA) (20, 51, 58). The concentrations used in the present study were similar to those used in the aforementioned studies (10µg/mL of vancomycin and 30 µg/mL of cefazolin). It is possible

that my results differ because the last time point is 24 hours versus 48 hours (20), so 24 hours may be too early to determine synergy with this combination. Another possibility is the well-documented discrepancy between *in vitro* methods of synergy testing (3, 4, 26). Simon et al. demonstrates vancomycin + cefazolin synergy against 10 strains of *S. aureus* but uses the checkerboard method, whereas I use the time-kill method.

The limitations to the current study were the small sample size, the amount of drug concentrations that could be tested, and the fact that it is difficult to predict *in vivo* activity of combinations using *in vitro* methods. In addition, synergy, antagonism and indifference were identified at 24 hours. It is possible that identifying the combination activity at 48 hours could have provided different results. In addition, if more than six samples had been used more of the samples could have demonstrated synergy. Finally, because only one concentration was tested for each drug, other concentrations could have resulted in different drug interactions.

These results suggest more research is needed to confirm the efficacy of these combinations for the treatment of MRSA. Many of the current studies provide conflicting results, but the definitive reasoning behind this is not known. It is possible that that difference between *in vitro* methods contributes to the conflicting body of knowledge. Establishing a standard protocol that suggests one specific method for synergy testing could start to reconcile the differences between studies of this nature. Since the time-kill curve method has been shown to most accurately predict in vivo results (3, 7, 14) this method may be the best option for a standard protocol. In addition, future studies should include both *in vitro* and *in vivo* methods in order to confirm the results seen *in vitro*.

In conclusion, this study provided guidelines for the treatment of MRSA and how to predict infection outcomes. Age and method of treatment were identified as markers that may

predict recurrent MRSA infections (**Table 1a-b**), and these results supported similar studies (10, 15, 41, 71). A device-related source of infection, presence of a long-term IV, and method of treatment were identified as markers that may identify patients at risk for prolonged bacteremia (**Table 2a-b**). These results were supported by multiple studies (31, 43, 47, 74). I also found that vancomycin MIC is not associated with the outcome of MRSA infections, which is supported by multiple studies (21, 47, 53, 71).

The second part of my study shows that combining gentamicin, rifampin or cefazolin with vancomycin against six strains of MRSA with vancomycin MIC of 2 is not synergistic *in vitro*. The contradicting results found in my study compared to other studies (26, 39, 58) highlight the need for continued research of combination therapies. However, my results did show enhanced killing with vancomycin +gentamicin in three strains, and vancomycin + rifampin in one strain. Killing with vancomycin + rifampin was worse than both antibiotics alone in four strains, and achieved antagonism in one of those strains. These results, although not demonstrating synergy may support the use vancomycin + gentamicin, but, although only showing antagonism once, do not support the use of vancomycin + rifampin.

Future directions for Aim 1 should focus on defining predictors of individual MRSA outcomes, including 30-day mortality which was not looked at in the current study, and using the same patient populations in those studies. In addition, a study focusing on the predictors of relapse infections would be beneficial to clinicians. Finally, the results of the current study also suggest that future studies should include larger numbers of patients to increase the likelihood of significance. Future directions for Aim 2 include testing the combinations for synergy using different drug concentrations, and extending the observation time to 48 hours. In addition, establishing a standard protocol that suggests one specific method for synergy testing could

start to reconcile the differences between studies of this nature. Finally, future studies should include both *in vitro* and *in vivo* methods in order to confirm the results seen *in vitro*.

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VITA

Virginia Long was born on August 9, 1990 in Silver Spring, MD to Kenneth and Nancy Long. For her undergraduate career, she attended Washington College in Chestertown, Maryland, where she earned her Bachelor of Science in Biology, with a minor in Chemistry, *magna cum laude*, in 2012.

In August 2012, she began her graduate studies at Loyola in the program of Infectious Disease and Immunology. She joined Dr. Paul Schreckenberger's lab where she studied clinical aspects of MRSA infections and combination therapies used to treat MRSA infections at Loyola Medical Center. During her time as a graduate student Virginia actively volunteered at the local food pantry in her community.