1-1-2016

Impact Of Cefazolin Co-Administration With Vancomycin To Reduce Development Of Vancomycin Intermediate Staphylococcus Aureus

Nivedita Birbahadur Singh
Wayne State University,

IMPACT of CEFAZOLIN CO-ADMINISTRATION WITH VANCOMYCIN to REDUCE DEVELOPMENT of VANCOMYCIN INTERMEDIATE *STAPHYLOCOCCUS AUREUS*

by

NIVEDITA B SINGH

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER of SCIENCE

2016

MAJOR: PHARMACEUTICAL SCIENCES

Approved By:

Advisor Date

DEDICATION

I would like to dedicate my work to my loving husband, Sushil, for your unconditional love, support and encouragement. I would also dedicate to my in-laws for believing in my dreams. Finally, this thesis is dedicated to my mom and dad, my inspiration and role models in life.

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor Dr. Michael J. Rybak, Pharm.D., M.P.H. for his constant support and guidance throughout the research project for my M.S. program at Wayne State University. He was always available when I needed his help in tackling problems during the research.

I am grateful to Ms. Juwon Yim, Pharm.D. for her help during the project. I thank her for sparing the time in teaching me the techniques during the project and guiding and assisting me in various aspects of my work.

I also place on record, my sense of gratitude to the “Anti-infective Laboratory’ (ARL) team who directly or indirectly have lent a helping hand towards accomplishing my goal.

I wish to thank my committee members Dr. Steven Firestine and Dr. Glenn Kaatz for taking the time out of their busy schedule and being a part of the committee. Their views and encouragement mean a lot to me.

I would take this opportunity to thank Dr. George Sakoulas from the University of California, San Diego for his valuable inputs during the project.

I would also thank Dr. George Corcoran, our departmental chair and all the faculty members of the pharmaceutical sciences department at Wayne State University for providing the facilities and knowledge during the M.S. program that will be very useful in the future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

1.1. *Staphylococcus aureus*:

Staphylococcus aureus (S. aureus) are Gram positive, non-spore forming, non-motile, facultative anaerobic bacteria. They are spherical in shape with sizes ranging from 0.5 to 1µm in diameter. On Gram stain they appear in clusters and are positive for coagulase and catalase production. These properties are useful in the identification of this organism.(*1*)

S. aureus infections has been recognized as a major contributor to morbidity and mortality. The organism was first identified as a pathogenic bacterium in 1880 by Alexander Ogston of Aberdeen, Scotland. Ogston proved that these bacteria played an important role in wound infection and subsequent septicemia. He referred to the organism as "*micrococci*" in his early work and later named them "Staphylococci", from the Greek word staphyle, meaning bunch of grapes and kokkos meaning berry.(*2, 3*) The official name '*Staphylococcus aureus*" is credited to Dr.Friedrich Julius Rosenbach, who isolated the genus and named it "aureus" because of the golden pigment observed on the colonies. (*4*)

S. aureus is an opportunistic pathogen commonly found on skin and in the upper respiratory tracts of healthy individuals without any symptoms. When provided with optimum opportunity this pathogen can cause a wide range of infections including minor skin infections to fatal osteomyelitis, toxic shock syndrome, infective endocarditis and septicemia. (*5*) An estimated 30% of the population are asymptomatic carriers. Its ability to survive on dry skin and inanimate surfaces further helps spread of infection.(*6,*

7) Before the discovery of antibiotics, *S. aureus* was responsible for 82% mortality.(*8*) It remains one of the major cause of hospital and community acquired infections.(*9*)

The biggest challenge with the treatment of *S. aureus* infections is its ability to evolve and develop resistance not only to the immune system but also to various antibiotics, either through mutation and/or DNA transfer. Antibiotic resistance is linked to antibiotic overuse and misuse including poor adherence to dosage regimens, self-medication, inadequate dosing, failure to complete treatment courses, substandard antimicrobials etc.(*10*)

Within a short time following the introduction of penicillin into clinical practice there were reports of *S. aureus* strains already resistant to it. The incidence of infections due to penicillin resistant strains increased rapidly from 14.1% to 59% over a period of just two years (1946 to 1948). (*11*) Currently approximately 90% of *S. aureus* isolates are resistant to penicillin. (*12*) A similar pattern followed for other classes of antibiotics including semisynthetic penicillins (e.g. methicillin), macrolides (e.g. erythromycin), aminoglycosides (e.g. gentamicin) and fluoroquinolones (e.g. ciprofloxacin).(*13*) Table 1 provides an overview of the various resistance mechanisms. In addition to antibiotic resistance, *S. aureus* has developed multiple pathways to combat host defense mechanisms including neutrophils - the main line of defense for the human immune system. Neutrophils are recruited by chemotaxis through the release of various agents (e.g. cytokines, IL-7) by the host cell in response to a pathogen. Neutrophils phagocytize the pathogen and kill it by various mechanisms including formation of superoxides. (*14*) *S. aureus* can limit chemotaxis function to prevent neutrophil recruitment. They are also capable of limiting superoxide function preventing digestion

and killing of the organism. In addition to these mechanisms, *S. aureus* also prevent host cells from sequestering essential nutrients such as iron, manganese, copper and zinc, which are needed for the proper functioning of the host defense machinery. In addition to this they also induce apoptosis in macrophages. The risk of acquiring *S. aureus* infections in immunocompromised patients increases multiple fold. (*15, 16*)

Table 1 Mechanism of resistance in Staphylococcus aureus

Antibiotic	**Notes**	**Strategy of resistance**
β -lactams(*17*)	Producing β-lactamase	Drug inactivation
	mecA gene responsible for synthesis of a modified Penicillin Binding Protein PBP2a	Target modification: Decreased affinity of drug for PBP
Macrolide	Erm gene responsible for structural changes to rRNA	Target modification: Prevent macrolide binding to rRNA(*18*)
Aminoglycosides	Modification of drug by cellular enzymes such as aminoglycoside acetyltransferases or aminoglycoside phosphotransferases	Drug structure modification: Reduce affinity to ribosome binding.(*19*)
Quinolone(*20*)	Mutations in the *grlA* and *gyrA* genes	Target modification: Mutations in the quinolone-resistance determining region (QRDR) reducing drug affinity at the DNA-DNA gyrase complex
	membrane-associated multidrug efflux protein (NorA) over expression	Increased drug efflux
Glycopeptides(*17, 21*)	Synthesis of excess amounts of D-alanine-D-alanine	Increased wall thickness, preventing drug to reach the division septum the site of action.
	Acquiring *VanA* gene that replaces D-Ala-D-Ala with D-Ala-D-Lac	Target modification: Reduced drug affinity to di-peptide target.

1.2. Treatment of *Staphylococcus aureus* Infection.

For treatment of *S. aureus* infection, the choice of drug is very important. The first line of treatment for staphylococcal infections are β-lactam antibiotics such as nafcillin or oxacillin. For patients with non-anaphylactic type penicillin allergies cefazolin, a first generation cephalosporin, may be an alternative. Organisms resistant to methicillin and its analogues are referred to as methicillin resistant *S. aureus* (MRSA). MRSA is a serious problem for hospitals around the world. Serious MRSA infections are associated with high treatment failure and mortality. According to the Centers for Disease Control and Prevention, the invasive MRSA incidence rate in 2014 was 22.72 per 100,000 individuals, while the mortality rate of MRSA infection in 2014 was 2.88 per 100,000 individuals.(*22*)

For patients with anaphylactic type penicillin allergy, or if the organism is resistant to β-lactam antibiotics (MRSA), vancomycin is considered the drug of choice. Unfortunately, low-level vancomycin resistance is an emerging problem in hospital settings. For patients who do not respond to vancomycin therapy alternatives such as daptomycin is the last resort of treatment.(*23*)

1.2.1. β-lactams

1.2.1.1. Mechanism of action:

β-lactam antibiotics interfere with the bacterial cell wall synthesis. They prevent the crosslinking of the peptidoglycan layer of the cell wall. This layer is a highly cross-linked latticework structure that provides mechanical stability to the cell wall. Thus β-lactams weaken the cell wall of the organism.

As shown in Figure 1, the bacterial cell wall is composed of repeating units of N-acetylglucosamine (NAG) [shown in blue] and N-acetylmuramic acid (NAM) [shown in green]. Short peptide chains are attached to the NAM subunits with D-Ala-D-Ala at the distal end. Penicillin binding proteins (PBP), also known as transpeptidase or transamidase enzymes, bind to these peptide side chains and form a cross-link with the expulsion of a D-Ala in the process as shown in step 2. The PBP dissociates from the cell wall once the cross-link is formed as shown in step 3, and then is ready for reuse in the crosslinking process. Thus a normal cell wall is formed.

In the presence of a β-lactam, the drug mimics the D-Ala-D-Ala residue of the peptidoglycan and binds irreversibly to the active site of the PBP forming a stable complex, as shown in step 5. The PBP is therefore unavailable for the formation of cross-linking in the peptidoglycan layer. This disrupts the cell wall synthesis.

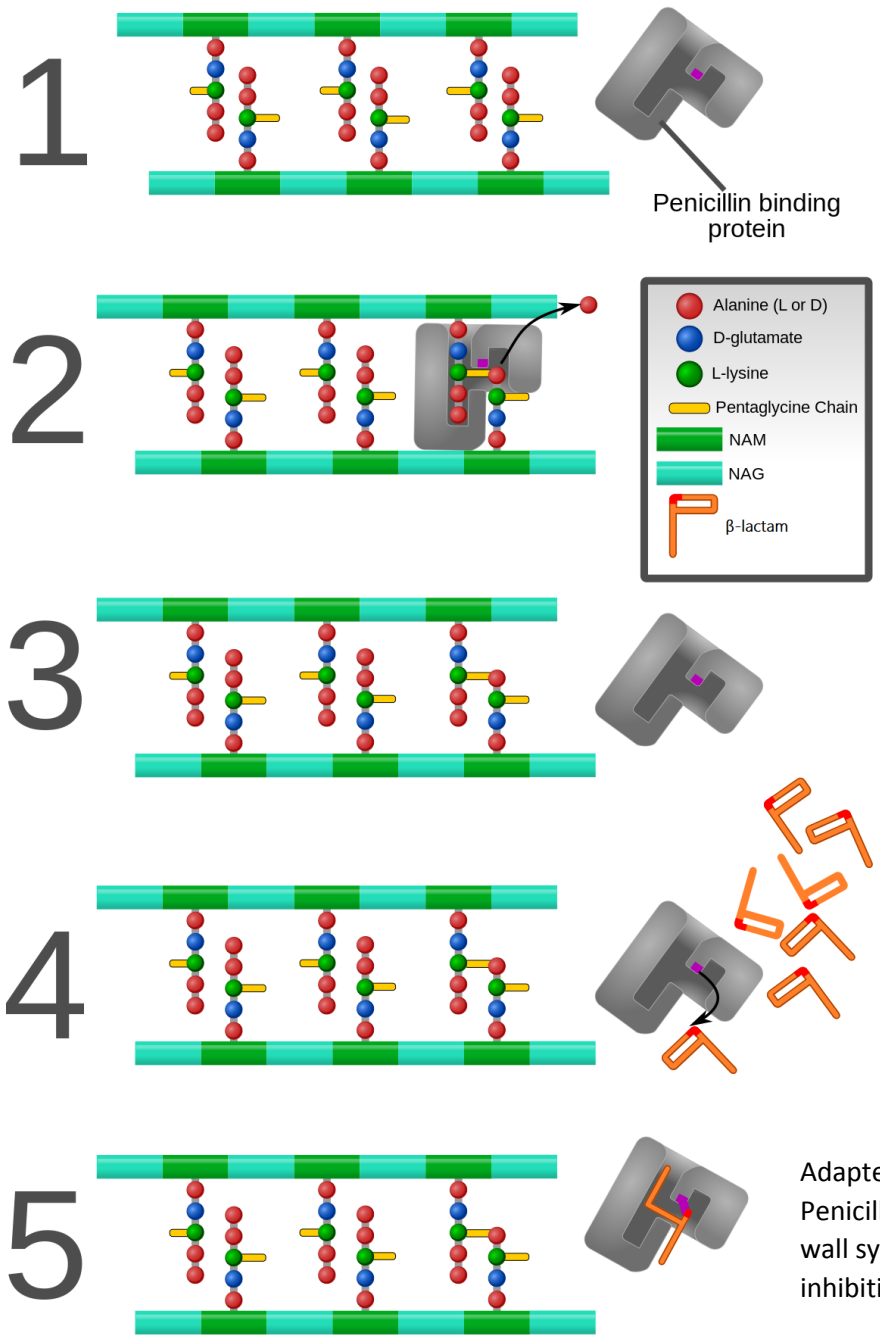


Figure 1 Mechanism of Action of β-lactam antibiotics

(1) The bacterial cell-wall before cross-linking. (2) Formation of cross-link with the expulsion of one D-Alanine from one peptide side chain. (3) The PBP dissociates from the wall once the cross-link has been formed. (4) β-lactam enters the active site of the PBP (5) The β-lactam irreversibly binds to PBP and permanently blocks the active site.

1.2.1.2. Resistance to β-lactams

S. aureus resistance to β-lactams occurs by two main mechanisms. The organism produces an extracellular enzyme called β-lactamase, which cleaves the β-lactam ring through hydrolysis and inactivates the drug. The *blaZ* gene is believed to be the gene responsible for the production of β-lactamase.(*24*)

The second mechanism, which is of greater concern, is the acquisition of the *mecA* gene which encodes a modified PBP named PBP2a. This PBP has low affinity to β-lactam antibiotics but can efficiently participate in cell wall synthesis.(*17*) This is the main mechanism by which an organism becomes resistant to methicillin and its analogues (MRSA).

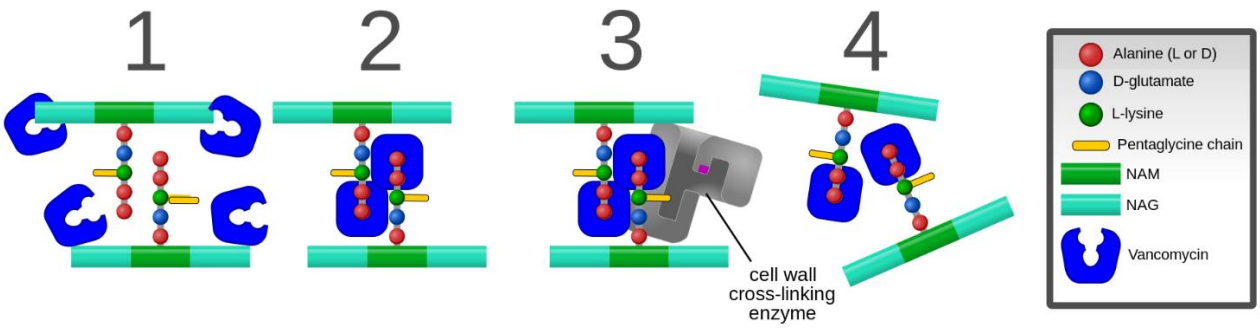
1.2.2. Vancomycin

1.2.2.1. Mechanism of action:

Vancomycin(VAN) interferes with cell wall synthesis by inhibiting the transpeptidation reaction. It renders the cell wall unstable and the organism becomes prone to lysis. However, the mechanism of inhibiting transpeptidation is entirely different from that employed by β-lactams. Instead of binding to the PBP VAN binds to the substrate of the transpeptidation reaction (i.e. C-terminal D -ala-D -ala end of the peptide). VAN forms a stable non-covalent complex involving a number of hydrogen bonds between VAN and D -ala-D -ala residue in the precursor, making it unavailable for cell wall synthesis. The result is inhibition of cell wall synthesis and lysis of the organism.(*25*)

VAN is a large and bulky glycopeptide, therefore it cannot penetrate to the cytoplasm. The interaction with the target i.e. D-ala-D-ala terminus of the precursor peptide takes place only on the peptides that have been translocated to the outer surface of the cytoplasmic membrane and where new cell wall is being synthesized. Therefore, for VAN to be effective it should interact with the lipid linked precursors at the site of cell wall synthesis, (i.e. at the division septum for *S. aureus)*. (*26, 27*)

The mechanism of action is shown in Figure 2. In step 1 the peptidoglycan precursors have been synthesized but not yet cross-linked. VAN binds to the D-ala-D-ala residue of the peptidoglycan as shown in step 2 forming stable non-covalent bonds. Therefore, the peptide chains are not available to the PBP for cross-linking as shown in step 3. Thus the cell wall falls apart (step 4)



Adapted from Mcstrother, Action of vancomycin and resistance via alteration of peptide side-chains.

Figure 2 Vancomycin mechanism of action

(1) Lipid linked precursor at the site of cell wall synthesis before cross-linking.
(2) VAN binds to the two D-ala residues on the end of the peptide chains.
(3) VAN bound to the peptide chains prevents them from interacting properly with the cell wall cross-linking enzyme (PBP).
(4) Cell wall falls apart

1.2.2.2. Resistance to vancomycin:

There are a number of methods and terminologies used to define vancomycin resistance. With respect to the VAN minimum inhibitory concentrations (MIC) the organism is said to be susceptible if its MIC is ≤ 2 mg/l (VSSA). If the VAN MIC is ≥16 mg/l they are called VAN resistant *S. aureus* (VRSA). *S. aureus* becomes resistant to VAN by acquisition of the *vanA* gene from enterococci. The organism synthesizes a modified peptidoglycan precursor D-ala-D-lac instead of D-ala-D-ala. This reduces the affinity of VAN to the dipeptide substantially while retaining the activity of PBP. Therefore the crosslinking can occur without any hindrance from the drug.(*28*) This is shown in Figure 3.

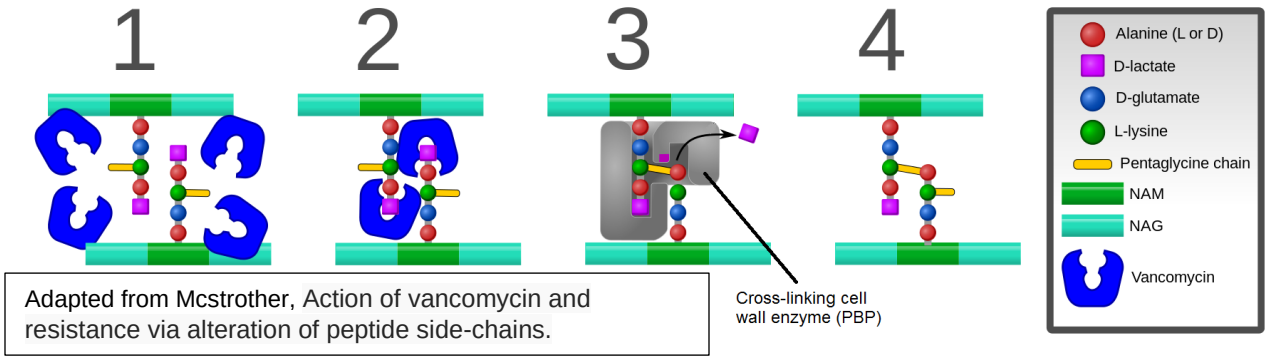


Figure 3 Vancomycin Resistant S. aureus.

(1) Lipid linked precursor at the site of cell wall synthesis before cross-linking.
(2) VAN cannot bind to the D-ala-D-lac side chain.
(3) Cell wall cross-linking enzyme (PBP) successfully cross-links the peptide chain.
(4) Stable cell wall is formed.

It is not very common for an organism to become VRSA. More common and concerning is the development of low-level VAN resistance (LLVR). There are two types of LLVR including VAN intermediate *S. aureus* (VISA), defined as VAN MIC values between 4 and 16 mg/l and heterogeneous VAN intermediate *S. aureus* (hVISA), which have MIC values in the susceptible range but contain a sub-population that are in the intermediate range. Since hVISA have MIC values in the normal range, they can only be identified by population analysis.(*29, 30*) LLVR has been documented to have a major impact on clinical outcomes. Higher treatment failure and persistence of infection has been associated with both VISA and hVISA as compared to VAN susceptible *S. aureus*. (*31*)

There are multiple pathways and genes by which *S. aureus* can gain intermediate resistance to VAN.(*32*) A link has been found between the Accessory Gene Regulator (*agr*) locus and reduced VAN susceptibility. Agr is a global regulator gene affecting the production of various *S. aureus* virulence factors. Organisms with a dysfunctional *agr* II locus are more prone to becoming resistant to VAN.(*33*). Mutations in *vraAR, graSR* or *walKR* operons, singly or in combination, lead to modification of their respective regulons. This results in increased thickness of the cell wall due to up regulation of cell wall synthesis or decreased autolysis or both. There is also an increase in capsule expression. The upregulation of the *dlt* operon leads to an increase in alanylation of teichoic acid, giving a net positive charge to the cell wall. All of these changes interfere with the interaction of drugs with the target.(*34*)

The increase in cell wall thickness and increase in D-ala-D-ala containing muropeptides (false binding sites) in the cell wall leads to a reduction in the amount of

drug reaching the division septum (site of cell wall synthesis) and therefore a reduction in activity of the drug. This is shown in Figure 4.

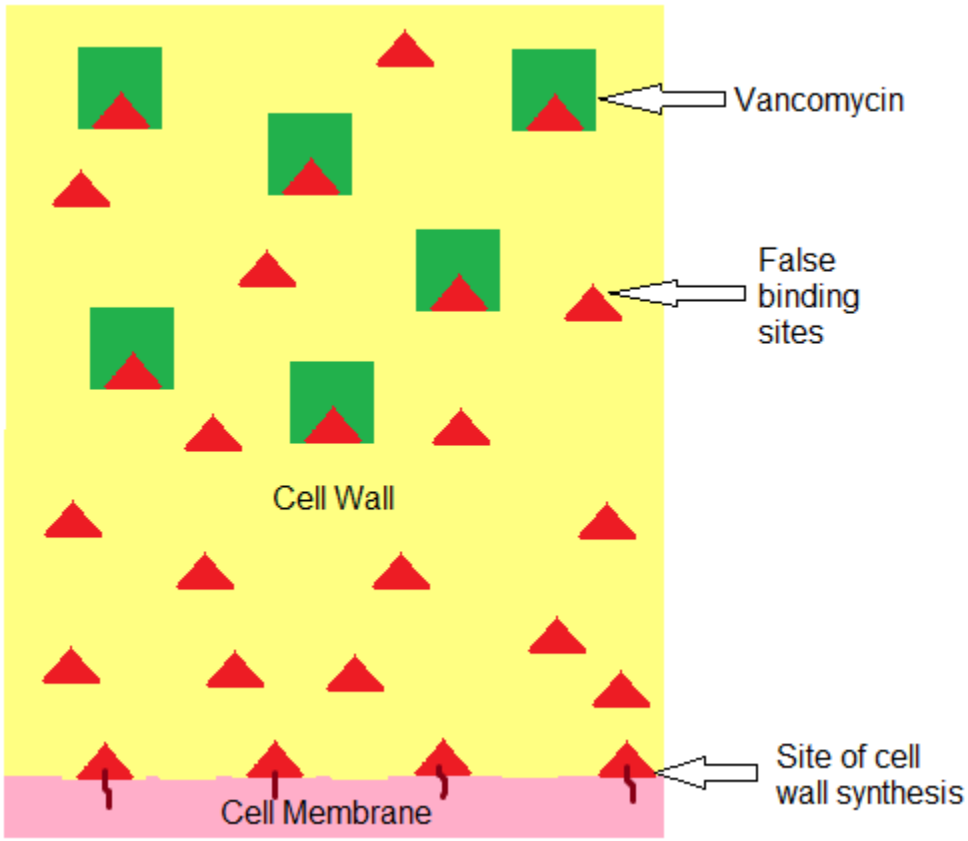


Figure 4 Reduced vancomycin activity in VISA

VAN gets trapped in outer layers by binding to muropeptides. Therefore, the drug is unable to reach the site of cell wall synthesis.

1.2.3. Daptomycin

1.2.3.1. Mechanism of action:

For patients who do not respond to VAN therapy, daptomycin (DAP) is used as a last resort. It is a cyclic lipopeptide with a calcium dependent mechanism. The exact mechanism by which DAP causes cell death is not fully understood.

The current hypothesis is that DAP exerts its effect on the target cell by primarily disrupting membrane functions. DAP is anionic in charge by itself. When it comes in contact with calcium in blood it becomes cationic. This facilitates its penetration into cell membranes. The functional integrity of the membrane is disrupted and the membrane depolarizes, triggering the leakage of potassium ions and ultimately cell death.(*35, 36*)

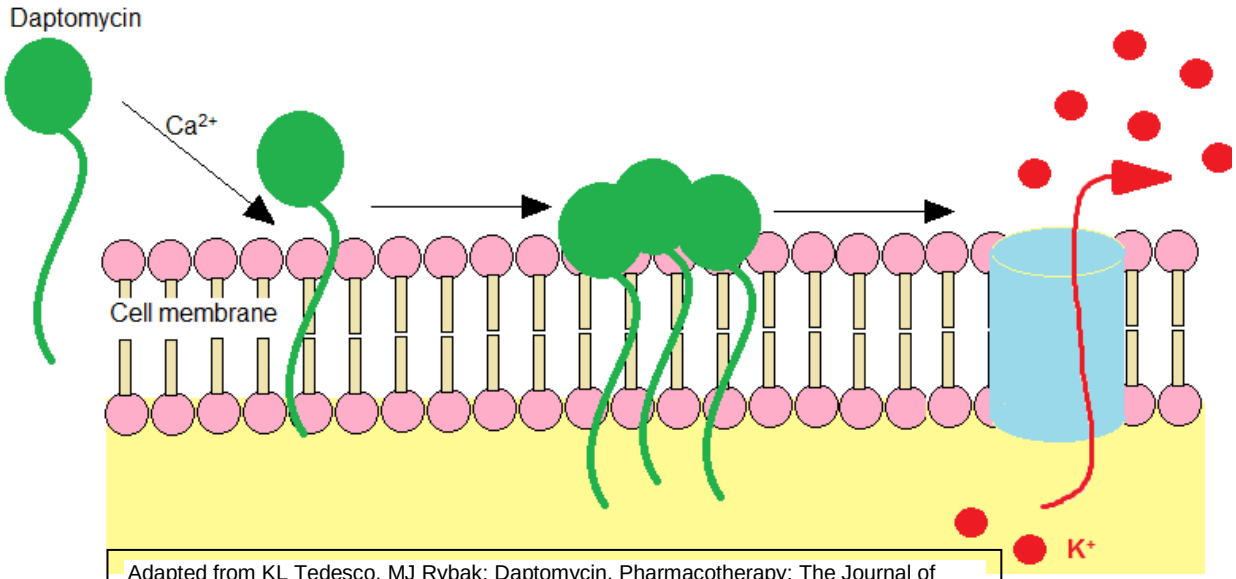


Figure 5 Mechanism of action of Daptomycin(36)

1.2.3.2. Resistance to Daptomycin

The mechanism of resistance to DAP is not completely understood. When an organism becomes resistant to DAP changes in both cell wall and membrane take place. The resultant changes to the membrane reduces depolarization secondary to DAP binding, which results in a loss of its antibacterial effect.

In addition, changes in the cell wall including increased thickness and an increased positive charge of the cell membrane due to over expression of the *dlt* operon contribute to DAP resistance.(*37*) Figure 6 shows DAP molecules repelled from the cell wall that is positively charged.

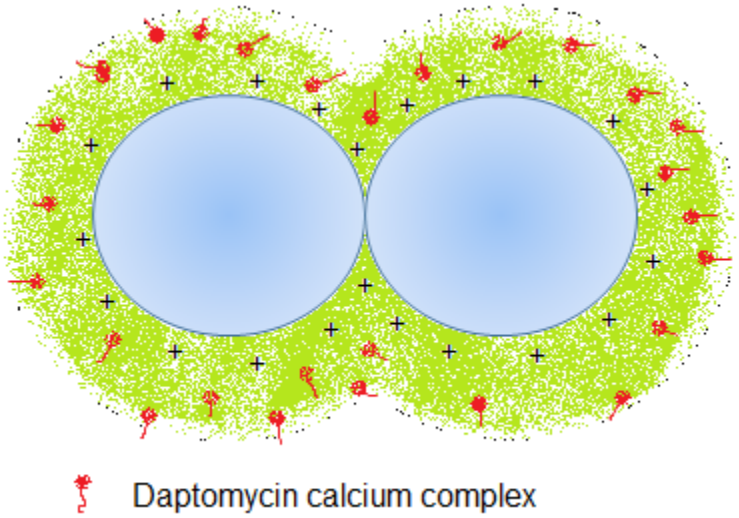


Figure 6 Positive charged daptomycin-calcium complex repelled by the positive charged cell wall.

DAP is often used as second line therapy and often after VAN has failed. DAP non-susceptibility is a rising concern in health care. Many studies have shown a positive correlation between prior low-level VAN resistance and DAP resistance.(*38-40*) The changes that occur in a cell wall as it becomes resistant to VAN, like a thickened cell wall, enhanced cell wall turnover, etc. interfere with the antimicrobial action of DAP. (*40*) It has been demonstrated that there is a correlation between cell wall thickness and the DAP MIC. In fact, a positive correlation between VAN and DAP MIC has also been reported. Consequently, as an organism develops intermediate resistant to VAN, it is more prone to DAP treatment failure. (*41, 42*)

It is interesting to note that VAN resistance acquired through acquisition of *Van-A* does not affect DAP susceptibility. (*43*)

Regardless, alternatives to VAN for the treatment of *S.* aureus are needed.

1.3. Synergy between vancomycin and β-lactam.

A β-lactam in combination with VAN has been demonstrated to be synergistic. Many *in vitro* studies demonstrate synergistic activity of the combination against most *S. aureus* strains. (*44-47*) Animal models have also corroborated the data.(*48, 49*)
The exact mechanism of synergy is not known but there are many hypotheses for the synergy.

One of the theories is that the synergy is due to substrate specificity of PBP 2a. In the presence of β-lactams all the cross-linking in the cell wall is carried out by PBP 2a. PBP 2a is able to carry out the cross-linking reaction only in monomeric disaccharide pentapeptides. VAN competes for these monomeric disaccharide pentapeptides. They therefore interfere with the cell wall synthesis, reducing β-lactam

resistance. This would lead to better activity with the combination.(*47, 48*) The reduction in β-lactam resistance with vancomycin use is also described as the *seesaw* effect. (*42*)

Another mechanism, though less common, is that the stress caused by the addition of VAN to a β-lactam can cause deletion of *mecA* in some strains. This will result in disruption of production of PBP2a, which is the product of *mecA,* leading to the organism becoming susceptible to β-lactam antibiotics. (*50, 51*)

A newer concept for synergy is due to PrsA, an auxiliary factor in oxacillin resistance. It is a chaperone protein responsible for posttranslocational folding at the outer surface of the cytoplasmic membrane. PrsA is responsible for folding and stabilization of PBPs. Any disruption in PrsA will lead to disruption in PBP 2a, which will decrease β-lactam resistance.(*52*) The working hypothesis is that VAN pressure disrupts PrsA, which can lead to a decrease in oxacillin MIC.

1.4. Cefazolin (β-lactam of choice)

Cefazolin (CFZ) is a first generation cephalosporin antibiotic. It has been documented that CFZ shows better synergy than most other β-lactam antibiotics with VAN.(*45*) CFZ has a non-selective PBP profile and therefore binds to all PBPs including PBP1, an important protein in cell division and septum formation. (*53*) Thus it will interfere with cell wall repair. In addition, CFZ is a safer drug as compared to other β-lactams such as nafcillin and oxacillin, having markedly fewer side effects.(*54*) Lastly, the drug has a comparatively long half-life of almost 1.8 hours. The dosing interval is 8 hours making it a convenient drug to use in clinical practice.

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

Development of resistance in *S. aureus* has been a big concern in the treatment of infections caused by the organism. The line of therapy used today has a disadvantage of slowly developing cross-resistance for antibiotics such as DAP secondary to prior VAN exposure. Therefore, alternative therapy is needed to prevent the emergence of VAN resistance and cross-resistance to other antibiotics. The primary aim of this experiment is first: to prove that sub-optimal VAN exposure in S. *aureus* will lead to development of VISA. Secondly, VAN in combination with CFZ will prevent the development of VISA, regardless of sub-therapeutic VAN exposure. In addition to this we will evaluate if the combination of VAN plus CFZ is synergistic against various phenotypes of MRSA.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains

Two *S. aureus* strains were selected for the studies for the development and prevention of resistance to VAN in presence of CFZ. The strains chosen were RN9120, which is a MSSA with dysfunctional agr- II (knockout) and JH1, which is a well described clinical MRSA strain. Both of these strains have been extensively studied in the literature and have a proclivity to gain resistance to VAN and become VISA upon VAN exposure.(*33, 55-58*).

An additional 10 MRSA isolates of different drug resistant phenotypes including MRSA, hVISA, VISA and linezolid-resistant *S. aureus* (LZD-R) were selected for time kill studies to demonstrate synergy with the combination of VAN and CFZ in different phenotypes of *S. aureus*. The organisms selected are given in Table 2.

Table 2 Microorganisms selected for time kill studies

MRSA	hVISA	VISA	LZD-R
494	6911	3219	6499
8845	6837	2325	6256
3651			
6387			

Kocuria rhizophila ATCC 9341 was the indicator organism used for bioassays to verify VAN concentrations and 24 hour exposures in the *in-vitro* pharmacokinetic/pharmacodynamic (PK/PD) models.

3.1.2 Media

Mueller Hinton Broth (MHB, Difco, Detroit, MI), was used for susceptibility testing, *in-vitro* one-compartment PK/PD models and time kill assays. Tryptic soy agar (TSA, Difco, Detroit, MI) was used for *S. aureus* culturing, growth and colony enumeration. Brain heart infusion agar (BHIA, Difco, Detroit, MI), a highly nutritious media, supplement with VAN was used for resistance screening and population analysis. Antibiotic assay medium 1 agar (AA1) was used for determination of VAN concentrations *from in-vitro* PK/PD models.

3.1.3 Antimicrobials

VAN, the primary drug for the studies, was purchased commercially from Pfizer, New York City, NY. The β-lactams; CFZ was purchased commercially from Sandoz, Princeton, NJ and oxacillin (OXA) was purchased from Sigma Aldrich, St Louis, MO. Teicoplanin (TEI) a glycopeptide used for susceptibility testing was purchased commercially from Sanofi Aventis, Bridgewater, NJ. DAP a lipopeptide used for susceptibility testing was purchased commercially from Merck Pharmaceuticals, Lexington, MA.

3.2 Methods

Susceptibility testing was carried out for each antibiotic alone and in combination. Minimum inhibitory concentration (MIC) for all *S. aureus* isolates was determined using

broth dilution method or Epsilometer test (Etest). Methods used to generate a VISA from a VAN susceptible organism included a gradient plate method and serial passage. A one compartment *in-vitro* PK/PD model was used to simulate human PK of each antimicrobial agent including VAN and CFZ. The pharmacokinetics for VAN were determined by bioassay.

The organisms obtained at the end of the PK/PD models were tested for changes in MIC to the various antibiotics. If a change in MIC was observed, population analysis (PAP) was completed to determine if a shift in population occurred. In addition to this passage, studies using antibiotic free media were done to study if the mutant obtained was stable.

In the second part of this investigation, time kill studies were carried out using VAN alone and in combination with CFZ. The experiments evaluated the bactericidal nature of the antibiotics and provided data on whether the combination met the definition of synergy.

3.2.1 Susceptibility testing

Susceptibility testing for initial profiling of the organisms was completed by Etest. For all other susceptibility testing the microbroth dilution method was used.

Etest: In the E-test method of antimicrobial susceptibility testing antibiotic containing strips (Estrips) are placed onto Mueller-Hinton agar(MHA) plates inoculated with known bacterial density. For our study the bacterial suspension of 0.5 McFarland for *S. aureus* was used containing about 10^8 CFU/ml. The sample is incubated at 35°C for 24 hr. During the incubation period, the antimicrobial agents diffuse from their respective antibiotic impregnated strips into the surrounding agar and producing an elliptical zone

of growth inhibition. The MIC is determined at the point on the scale where the ellipse of organism growth inhibition intercepts the strip.

For the oxacillin(OXA) Etest MHA agar was supplemented with 2% sodium chloride as suggested by clinical laboratories standard institute (CLSI) guidelines. VAN and the OXA Etest was performed for RN9120 and JH1.

Microbroth dilution method: Microbroth dilution (MBD) method for susceptibility testing is the gold standard for determining *S. aureus* resistance to VAN according to Clinical Laboratory and Standards Institute (CLSI) guidelines. The disc diffusion method is unable to differentiate between VSSA, VISA and VRSA isolates. The bacterial concentration used for MBD was about 10^6 CFU/ml. All studies were done in duplicate to ensure reproducibility of results. MHB was used for all other drugs except oxacillin. 2% NaCl supplemented MHB was used for OXA MBD susceptibility testing. Evaluation of MIC was done after incubation for 24 hr for VAN and OXA at 35±2°C. For all other drugs in question the samples were read after an incubation period of 16-20 hr.

Thus this method was used to determine the initial MIC of the organism to different drugs under consideration such as VAN, CFZ, OXA, DAP and TEI. In addition, MBD was used to evaluate the change in MIC as a consequence to exposure to suboptimal exposure of VAN.

3.2.2 Gradient plate serial passage

The Gradient plate method was evaluated to induce resistance using JH1. The method involves consecutively pouring two layers of agar onto a Petri dish. For the bottom layer 25ml of plain BHIA poured over a propped up plate to form a wedge. When the agar solidifies the Petri dish is placed in a horizontal position and another 25ml of

BHIA containing VAN at different concentrations being tested is poured over the wedge. The representation of plate is shown in Figure 7. A 0.5 McFarland suspension was made for the *S. aureus* being tested. The organism was streaked on the plate with a swab from the area of lowest to highest antibiotic concentration. The plates were incubated for 48 hours at 35±2◦C. The colonies growing towards the highest antibiotic concentration were taken and tested for MIC. They were also transferred and streaked on plate with higher antibiotic concentration and the process was repeated. The antibiotic used for the study was VAN and the concentration used in the plates were 2 µg/ml, 8 µg/ml, 16 µg/ml and 24 µg/ml. The MIC testing was done to see if any change in susceptibility to VAN occurred on VAN exposure.

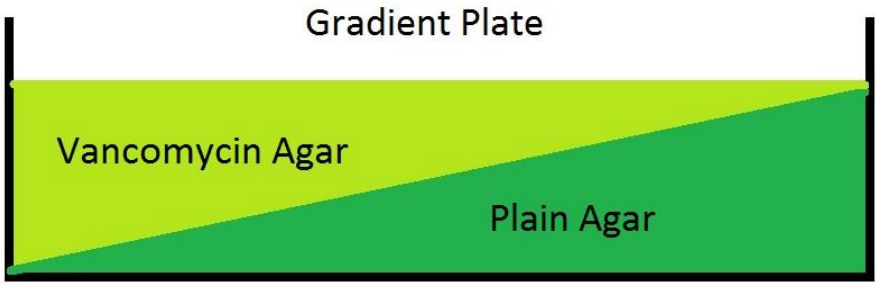


Figure 7 Gradient Vancomycin agar plate

3.2.3 Broth serial passage

A *S. aureus* suspension containing~10^6 CFU/ml of the organisms being tested in BHI containing 25µg/ml VAN was made. The culture was incubated in a shaker incubator at 35±2◦C till they showed visual turbidity. Cultures showing visual turbidity were centrifuged and the pellet was re-suspended in saline to get a 0.5 McFarland suspension. 1:100 dilution of the bacterial suspension was done fresh BHI containing

elevated concentration of VAN to get a 10^6 CFU/ml and returned to shaker incubator. Samples showing visual growth were used to inoculate the next day's passage, and the concentration of VAN shall be increased stepwise WITH 0.25µg/ml increments in concentration of VAN was made till growth was observed in 2µg/ml broth. Later 0.5µg/ml in VAN concentration increment was made till growth in 8µg/ml is observed. The experiment was performed in duplicate. The MIC was checked every 7 days or on from the growth in 4µg/ml VAN broth and every 2µg/ml increment higher.

The experiment was also carried out for VAN in presence on β-lactam at ½ MIC and ¼ MIC.

3.2.4 *In vitro* one-compartment PK/PD Models

In vitro one-compartment PK/PD model of 250-ml capacity having inflow and outflow ports was used. The apparatus was prefilled with medium, and VAN was administered as boluses over the period of the run. For models with VAN in presence of CFZ, VAN was administered as a bolus while CFZ administered as a bolus for MRSA or as an infusion for MSSA.

Bacterial lawns from an overnight growth on TSA were suspended and added to each model to obtain a starting inoculum of $\sim 10^6$CFU/ml. Fresh medium was continuously supplied and removed from the compartment, along with the drug, via peristaltic pump (Masterflex; Cole-Parmer Instrument Company, Chicago, IL) at an appropriate rate to simulate the average human antibiotic exposure concentrations, clearances and half-lives of the antimicrobials. The antimicrobial regimens evaluated for preliminary screening for selection of dosage most likely to produce VISA for the studies was as follows:

VAN 62.5 mg q 12, VAN 125 mg q 12, VAN 200 mg q 12,

VAN 250 mg q 12, VAN 500 mg q 12 for 120 hr.

Samples from each model were collected at 0, 4, 8, 24, 32, 48, 56, 72, 96, 102 and 120h in duplicate and diluted in 0.9% saline. Colony counts were determined by spiral plating appropriate dilutions using an automatic spiral plater to enumerate the CFU per milliliter. Colonies were counted using a laser colony counter. In addition, samples were plated on 3x MIC VAN BHIA plate for resistance determination. If growth was observed on the resistance plates the colonies were tested for MIC change.

In the second part of this investigation, a VAN exposure and recycling one compartment *in-vitro* PK/PD model was used in the selected dosage regimen chosen from preliminary run, for 72 hr. This procedure was incorporated to increase the rate and reproducibility of VISA production to test our primary hypothesis. Samples from each model were collected at 0, 4, 8, 24, 32, 48, 56, 72 hours in duplicate. The samples were diluted, plated and incubated for 24hr on TSA before being counted. In addition, all samples were plated on BHIA drug plate with VAN concentration 3x MIC to check for resistance development as in previous runs. The drug plates were incubated for 48 hr. Colonies growing on 3xMIC VAN plate (If no organisms present, colonies from TSA plate) at T72h was re-exposed to the same regimen for additional 72-144hr to develop VISA

To evaluate weather β-lactams could prevent the emergence of VISA Regimens for MSSA PK/PD models included VAN at the selected dose q 12h given as a bolus+ CFZ at an increment of the MIC concentration as an infusion. If no colonies were observed in these experiments, the concentration of CFZ is lowered until some colonies

survived. The model sampling and plating procedure was identical for VAN alone. The colonies obtained at T72 was re-exposed to the same regimen for additional 72-144hr and studied for change in resistance.

Regimens for the prevention of resistance in MRSA, included VAN at the selected dose q 12h + CFZ at a clinical therapeutic dosage regimen of 1g q 8 (since CFZ has no activity against MRSA). If no colonies were observed, the concentration of CFZ was lowered and dosing interval increased, until some colonies survive. The model sampling and plating procedure was identical to VAN alone. The colonies obtained at T72 was re-exposed to the same regimen for additional 72-144hr and studied for change in resistance.

Resistance: The emergence of resistance was evaluated by plating 120-µl samples from the model on plates supplemented with VAN at 3X the MIC. The plates were examined for growth after 48 h of incubation at 35°C. Resistant colonies grown on screening plates were evaluated by broth microdilution methods to determine the MIC. If resistance was detected, the organism at the end of the model was characterized for its change in MIC to other drugs.

Pharmacokinetic analysis: Pharmacokinetic samples for model run with VAN alone at selected concentration was performed by bioassay. The organism selected was *Kocuria rhizophila* (formerly *Micrococcus luteus*) strain ATCC 9341.

Wells were bored on agar plate with capacity of 150 µl for standard concentrations or pharmacokinetic samples. Each standard was tested in duplicate by placing in wells on agar plates (antibiotic medium no. 1; Difco, Detroit, MI) and inoculated with a 0.5 McFarland standard suspension of the test organism. The samples

were incubated for 24 hours before reading the zone of inhibition for standard and samples. The half-life and peak concentrations were determined using the PK Analyst software (version 1.10; MicroMath Scientific Software, Salt Lake City, UT).

3.2.5 Population Analysis Profile:

The VAN PAP/AUC was measured for isolates obtained at the end of 216 hours or at the end of last run where any organism survived, along with the parent strain. A 2 McFarland suspension for the organism being tested containing ~10^9 CFU/ml was prepared. Serial dilutions of the test organism were plated using automatic spiral plater (WASP, DW Scientific, West Yorkshire, England) onto decreasing concentrations of VAN BHI.

The VAN agar concentrations used were 0, 0.125, 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, 6, 8 and 16 mg/liter for MSSA and 0, 0.50, 1, 1.5, 2, 3, 4, 5, 6 and 8mg/liter for MRSA.

Colony growth at 48 h was measured using laser colony counter (ProtoCOL, Synoptics limited, Frederick, MD) and graphed as $\log_{10}$ CFU/ml versus the VAN concentration. Parent Strain was included in the run. The area under the curve was calculated for each sample and compared to the mean parent AUC for each group of PAP.

3.2.6 Serial Passage:

Serial passage in a drug free medium was carried out to check if the mutation was stable. The organism is streaked on a drug free TSA plate and incubated at 35±2°C for 24 hr. This is first passage. The organism obtained on the plate was re-streaked onto TSA plate and incubated again at 35±2°C. This was the second passage. The

process was repeated once more and therefore three passages were completed. The organism obtained after each passage was analyzed for change in MIC. If the MIC remains unchanged the mutation was considered stable.

3.2.7 Time Kill Studies:

The time kill experiments were performed in duplicate to ensure reproducibility of the results. The 2.0ml flat bottom well trays were used for the study. The medium used was MHB 25. Each well was inoculated to get an initial inoculum of 10^6 CFU/ml. The antimicrobials being tested i.e. VAN and CFZ was tested at a concentration of 0.5x MIC of each microorganism being tested. If the MIC was greater than the maximum biologic concentration a standard dose would achieve in serum (C_{max}), the C_{max} value was used. Since all *S. aureus* tested were MRSA and had a MIC greater than C_{max}, the C_{max} value for CFZ was used.

Both of the drugs were tested alone and in combination were tested. 0.1ml aliquot was withdrawn from each well at 0, 4, 8 and 24hr intervals. They were serially diluted with saline and plated onto TSA plates for colony enumeration using automatic spiral plater (WASP, DW Scientific, West Yorkshire, England). The samples were incubated at 35±2°C before being read using a laser colony counter (ProtoCOL, Synoptics Limited, Frederick, MD)

The time kill curves were generated by plotting mean colony counts (log_{10} CFU/ml) verses time. The effect of 24hr antibiotic of single agent verses combination was compared. If there was a greater than 100-fold increase in killing with the combination, in other words greater 2 log_{10}CFU/ml difference between the more active

agent and combination, the combination was considered to be synergistic. Figure 8 represents the definition of synergy in a time kill study.

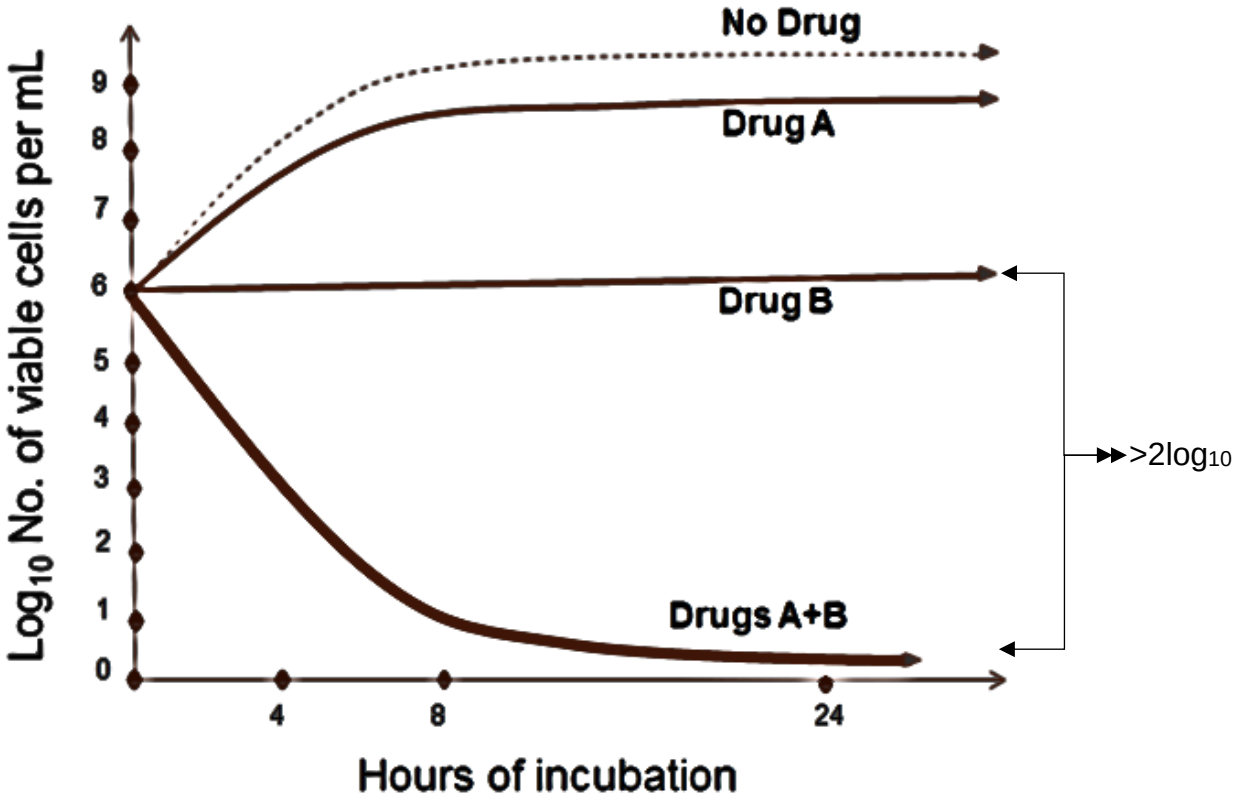


Figure 8 Definition of synergy

The number of CFU/ml is represented on the Y axis in a log scale. The hours of incubation are on the X-axis. The dotted line represents the growth curve in the absence of an antibiotic. The lines labelled Drug A and Drug B are the growth curves in the presence of an antibiotic A and antibiotic B respectively. The line labelled Drugs A+B represents when the combination of antibiotic A and B was administered. If the difference between the most active single drug, it this case antibiotic B, and the combination growth curve is greater than 2 log units; the combination is synergistic.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Susceptibility testing

RN9120 a MSSA with dysfunctional agr II locus and JH1 an MRSA both have a proclivity to gain resistance and become a VISA following VAN exposure. This made them suitable candidates to study induction of resistance and prevention of resistance development. VAN susceptibility testing for these two strains of S. aureus, RN9120 and JH1, was performed by E-test against VAN. The results are given in Table 3

Table 3 Etest results against vancomycin

Organism	**RN9120**	**JH1**
Vancomycin MIC (mg/l)	1.5	1.5
Oxacillin MIC mg/l	0.38	48

Susceptibility testing for all other *S. aureus* strains (one MSSA and 11 MRSA strains) being used for the study was performed using broth microdilution. The MIC values for VAN, CFZ and the combination of the two drugs is given in table 4.

Table 4 Vancomycin and Cefazolin MIC alone and in combination against S. aureus

Organism	VAN	CFZ	VAN MIC+ CFZ	VAN MIC Fold reduction from baseline
RN9120	1	0.25	<0.0078	>128
JH1	1	16	0.125	8
494	1	>32	0.25	4
8845	0.5	>32	0.125	4
3651	1	32	0.125	8
6387	2	>32	0.5	4
6911	2	>32	0.5	4
6837	2	>32	0.5	4
2319	8	>32	1	8
2325	8	>32	2	4
6499	1	>32	0.125	8
6256	1	>32	0.125	8

The MIC range for VAN was 0.5-8 mg/l for the organisms tested. The MSSA isolate, RN9120, had a CFZ MIC of 0.25mg/l. The MRSA isolates had a CFZ MIC in the range of 16 to >32mg/l.

Of note, Etest MIC for both organisms reads slightly higher that that obtained by broth microdilution technique as shown in table 3 and table 4 respectively. It has been described in the literature that Etests tend to give a higher value than microbroth dilution. (*59*) One of the reasons for this is that a broth microdilution can read results only in 2-fold values and not in between. Whereas, Etest is capable of obtaining readings between the 2 values.

The combination of VAN and CFZ produced an MIC was in the range of <0.0078 to 2. A reduction in MIC was observed anywhere between 2-fold to 5-fold. Maximum reduction with combination use was observed with MRSA.

Combination MIC of VAN and CFZ caused reduction in MIC for all organisms tested as compared to either agent alone. The maximum reduction was obtained with the combination on a MSSA. This can be due to the fact that the MSSA isolate under consideration is susceptible to both the study drugs and therefore combination results in effects more pronounced than in organisms not susceptible to one of the drug being tested. Over all there was a decrease in MIC to all the organisms being tested to a combination of VAN and CFZ. The results are described in table 4.

RN9120 and JH1 were also tested for MIC against DAP, teicoplanin and oxacillin by broth microdilution method. The MIC to these agents is given in Table 5.

Table 5 MIC (mg/l) of JH1 and RN9120 against daptomycin, teicoplanin and oxacillin.

	Daptomycin	Teicoplanin	Oxacillin
RN9120	0.125	0.125	0.25
JH1	0.0625	0.25	>32

4.2. Gradient plate serial passage

Various techniques were tried to induce resistance in *S. aureus.* The gradient plate technique has been described to induce resistance in *Streptococcus pyogenes* strains to β-lactams. (*60*) The technique was evaluated against JH1 a VSSA. The impact of VAN exposure on the VAN MIC by this method was evaluated. The results are summarized in Table 6

Table 6 Vancomycin susceptibility change in gradient plate method to JH1

Gradient plate highest vancomycin concentration (mg/l)	VAN MIC (mg/l)	ΔMIC to VAN (mg/l)
0	1	Not applicable
8	1	0
16	1	0
24	1	0

As shown in Table 6, no change in VAN susceptibility could be obtained by this method. Resistance could not be developed even after 3 passages on consecutively higher VAN containing gradient plates.

4.3. Broth serial passage

Serial passage studies have been shown to induce resistance in *S. aureus, S.epidermidis, E.faecalis, E.faecium* and others to DAP in literature.(*61*) The technique when tested on *S. aureus* RN9120 for the induction of resistance in duplicate. The organism was inoculated in broth with VAN concentration of 0.25mg/l which is ¼ times its MIC. After incubation at 37°C the until the organism showed visual turbidity. The organism was then transferred to fresh BHI with elevated concentration of VAN. The organism's MIC was checked at every 2 mg/l increment and every 7 days. The change in MIC as respect to time is given in Table 7.

Table 7 Change in MIC by serial passage in presence of increasing vancomycin exposure.

Vancomycin MIC (mg/l)	1	2	4
No. of days	0	10 days	19-27 days

It took 10 days to increase the MIC from 1 to 2 mg/l to vancomycin. A VISA with MIC of 4 mg/l to VAN was obtained in 19-27 days of serial passage. The process took

a very long time and showed a high variation in the two sample with reference to the time of emergence of a VISA.

Effect of VAN exposure in presence of CFZ at ½ MIC and ¼ MIC (i.e. 0.125mg/l and 0.0625mg/l) was evaluated in a similar fashion. The concentration of CFZ was kept constant and the VAN concentration was increased in a fashion similar to the study with VAN alone. When CFZ was added to the method no growth was observed at VAN concentration of 0.75mg/l with CFZ at ½ MIC (0.125mg/l) and 1mg/l for CFZ at MIC (0.0625mg/l). The explanation can be that the combination is bactericidal at this concentration in static medium. Also it can be accounted to CFZ preventing the organism from developing resistance and therefore the organism is unable to survive as the concentration of MIC increases.

The study takes a very long time to induce a VISA and is cumbersome with preparation of new media with increased concentration of drug at every step, thus increasing the chances of contamination. Also the variation in the 2 samples at which VISA emerged is high. Therefore, this method was not used for further studies.

4.4. *In vitro* one-compartment PK/PD Models

4.4.1. Screening for dosage for vancomycin to induce resistance in VSSA.

The PK/PD model was used to select a dosage regimen most likely to induce a VISA. The regimens evaluated for preliminary screening for selection of dosage for VAN in the studies to induce low level VAN resistance in *S. aureus* are represented in Figure 9. The plot represents the number of colonies growing in log scale, represented on Y axis as $\log_{10}$ CFU/ml verses time on the X axis in hours.

S. aureus RN9120 (Van MIC 1) *VS vancomycin at different concentration.*

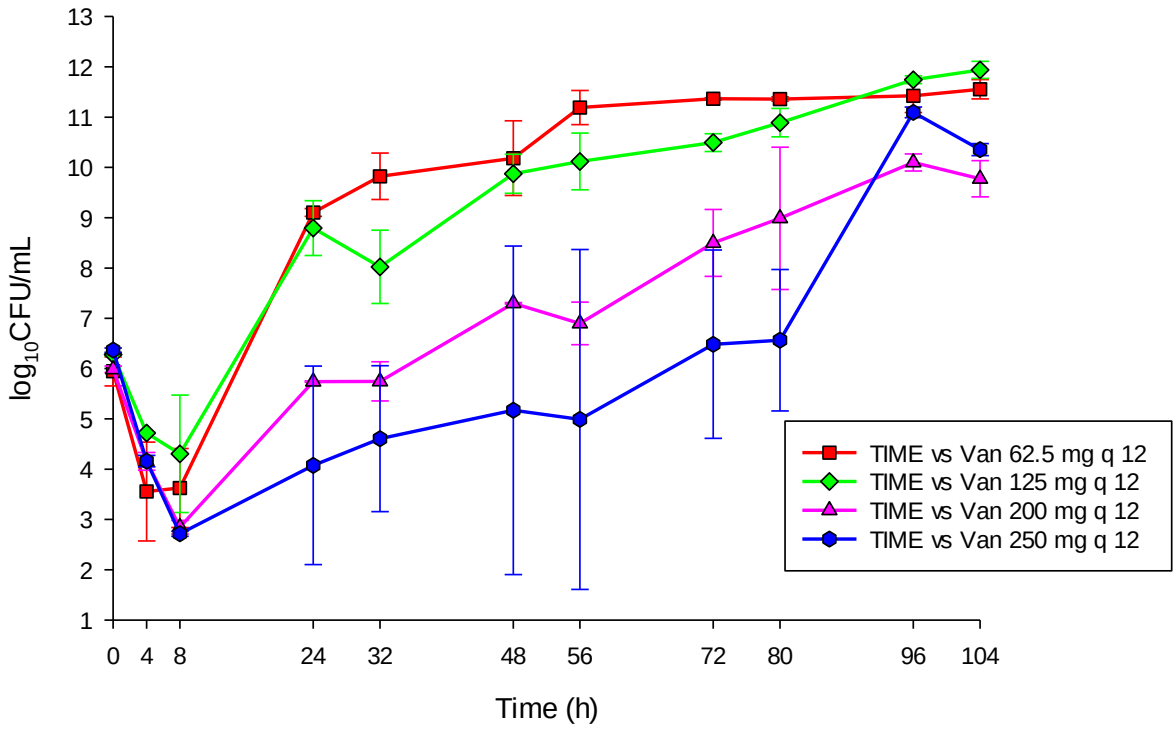


Figure 9 Preliminary study for selection of vancomycin dosage to induce resistance to vancomycin in VSSA.

The results of resistance determination on colonies growing on 3x MIC VAN BHIA plates is given in Table 8.

Table 8 MIC of S. aureus at the end of 120 hour from resistance plates

Simulated regimen of vancomycin	MIC at the end of run at T 120 on resistance plate	
	Model A	Model B
62.5 mg q 12	2	2
125 mg q 12	2	2
200 mg q 12	2	2
250 mg q 12	2	2

All the models were able to increase the MIC of the organism from 1 to 2 mg/l for vancomycin within 120 hours. Tsuji and colleagues had worked on RN9120 earlier and successfully converted it to a VISA. (*58*) Also Vidaillac and colleagues worked on JH1 and transformed it into a VISA with VAN exposure.(*32*) In both studies the organism

mutated to become a VISA when its growth curve was static. In other words, the growth curve was in the mutation selection window. As shown in figure 9 only VAN 200mg q 12 regimen curve met the requirement of a potential dosage regimen to induce mutation to generate a VISA. Thus, the dosage regimen of 200mg q 12 was selected for further studies. The strategy of recycling was implemented to speed the process of generating resistance.

4.4.2. Generation of VISA from VSSA

Selected *S. aureus* i.e. RN9120 (MSSA) and JH1(MRSA) were exposed to VAN 200 mg q 12 for 72 hours based on the data obtained from preliminary studies. The organism obtained at the end of first run were recycled for additional exposure of 72-144 hr. VAN 200mg q 12 exposure resulted in a VISA (MIC= 4mg/l) in both the *S. aureus* strains under consideration within 144 hour of exposure. In addition to this JH1 was exposed to higher concentration of VAN (i.e. 500 mg q 12 for 72 hours) and recycled for an additional 144 hour.

The graphs obtained from the study are given in figure 10 and 11 for RN9120 and JH1 respectively. The time point for change in MIC is given in Table 9 and 10 for RN9120 and JH1 respectively.

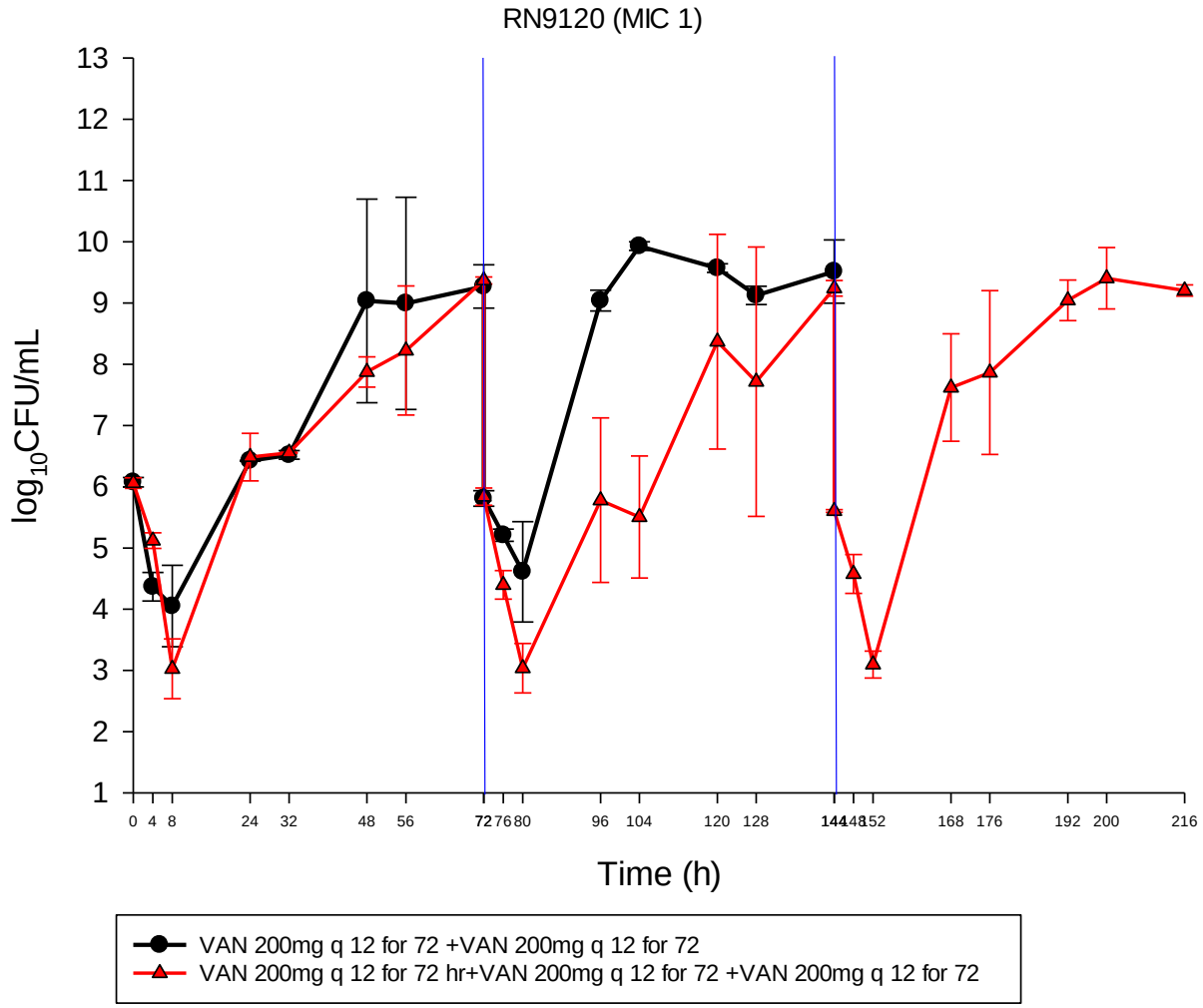


Figure 10 RN9120 VS Vancomycin 200mg q 12

Table 9 Change in MIC in PK/PD model at van 200mg q 12 exposure

Organism	RN9120 Parent	RN9120(1) Mutant 1	RN9120(2) Mutant 2
Vancomycin MIC	1 mg/l	2 mg/l	4 mg/l
No. of hours	0	48-56 hours	120-128 hours

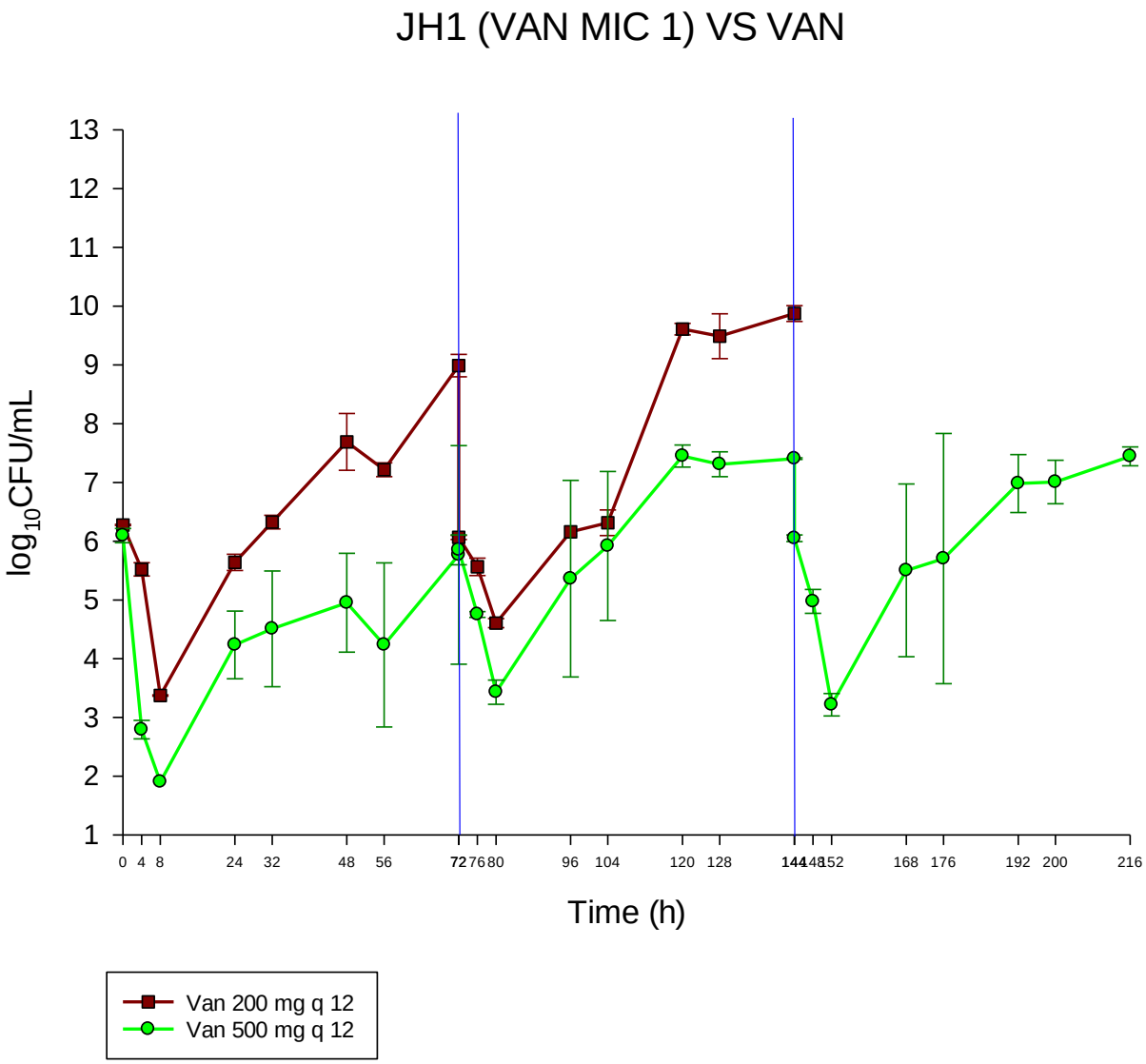


Figure 11 JH1 Vs Vancomycin 200mg q 12 and 500mg q 12

Table 10 Change in MIC in PK/PD model at van 200mg q 12 and vancomycin 500mg q 12 exposure

JH1 vs Vancomycin 200mg q 12			
Organism	JH1 Parent	JH1-1 Mutant 1	JH1-2 Mutant 2
Vancomycin MIC	1 mg/l	2 mg/l	4 mg/l
No. of hours	0 hour	48-56 hours	120-128 hours
JH1 vs vancomycin 500mg q 12			
Organism	JH1 Parent	JH1-1 Mutant 1	JH1-2 Mutant 2
Vancomycin MIC	1 mg/l	2 mg/l	4 mg/l
No. of hours	0 hour	>216 hours	>216 hours

In both the organisms under study RN9120 and JH1 the MIC increased to vancomycin from 1 mg/l to 2 mg/l within the first 72hours of the cycle. In other words, first mutant of each namely RN9120(1) and JH1-1 having MIC of 2 mg/l to VAN was generated. When the organism was recycled for the next run The MIC to both the organisms increased to 4mg/l to VAN within 144 hours. Thus, the second mutant RN9120(2) and JH1-2 having VAN MIC 4mg/l was generated. Even on further exposure till 216 hours the mutant RN9120(2) remained stable.

The effect of exposing the organism at higher concentration to VAN for JH1 when performed, no change in MIC was obtained. Therefore, it was concluded that the organisms under study gain resistance to VAN and become a VISA when exposed to VAN 200mg q 12 within 144 hours of suboptimal drug exposure.

4.4.3. Cefazolin in prevention of resistance in event of suboptimal VAN exposure.

In order to prove our hypothesis that CFZ is capable of preventing emergence of VISA in the event of suboptimal dosing CFZ was added to the regimen of VAN 200mg q 12 either as infusion or as a bolus.

After the observation that the van 200mg q 12 regimen was capable of producing a VISA from a VSSA. CFZ was added to the VAN regimen for both RN9120 and JH1 to study its effect on prevention of resistance. For RN9120 CFZ was administered as a low dose continuous infusion since the strain was MSSA and required a CFZ dose that produced the desired organism effect but not enough concentration to produce bactericidal killing on its own. This PK/PD model was evaluated at VAN regimen of 200mg q 12 with CFZ continuous infusion of CFZ at MIC the concentration of 0.25 mg/l. The regimen produced a rapid bactericidal combination resulting in organisms below

detection within the first 24 hours with no colonies detected at T 24h and onwards. Therefore, we adjusted the subsequent PK/PD model run with VAN 200mg q 12 and CFZ concentration was reduced to half (½ MIC) at 0.125mg/l. The graphs for these models are depicted in Figure 12 and the MIC change in Table 11.

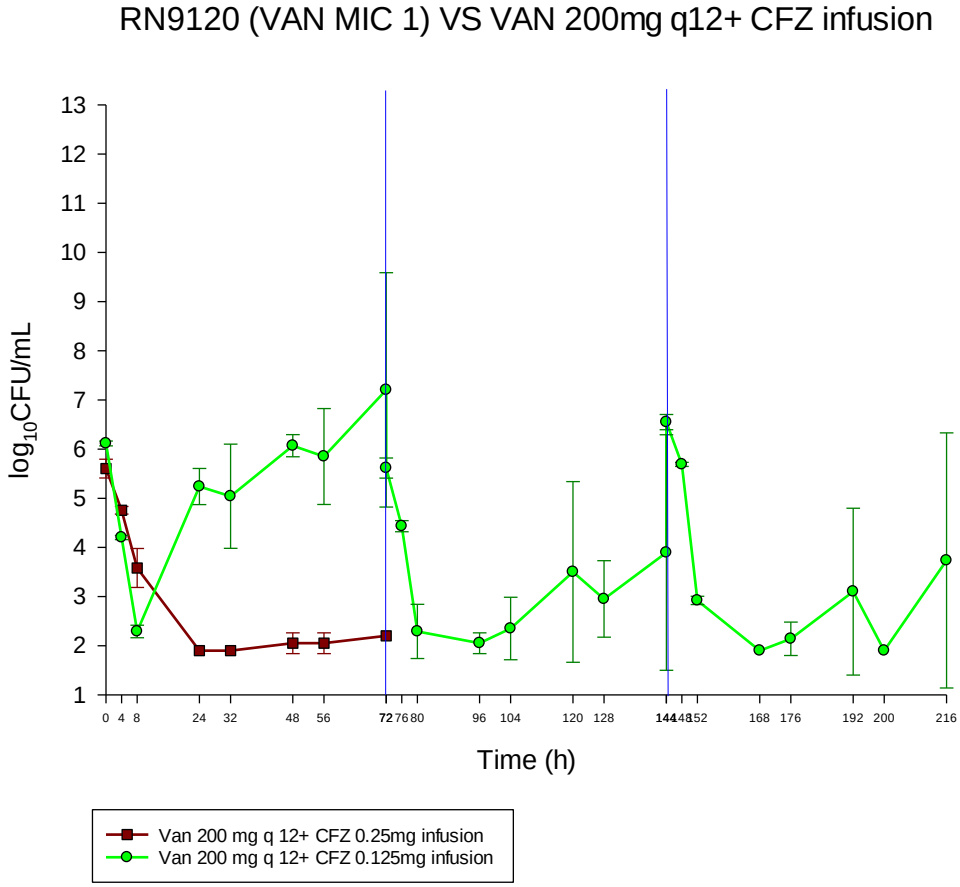


Figure 12 RN9120 with Vancomycin 200mg q 12+ Cefazolin infusion.

Table 11 Change in MIC in PK/PD model at van 200mg q 12 and cefazolin infusion exposure

RN9120 vs Vancomycin 200mg q 12+ CFZ 0.25 mg/l infusion			
Vancomycin MIC	1 mg/l	2 mg/l	4 mg/l
No. of hours	0 hour	>216 hours	>216 hours
RN9120 vs Vancomycin 200mg q 12+ CFZ 0.125 mg/l infusion			
Vancomycin MIC	1 mg/l	2 mg/l	4 mg/l
No. of hours	0 hour	72 hours	>216 hours

Since JH1 was a MRSA isolate and therefore resistant to CFZ (MIC=16mg/l) a therapeutic dose of CFZ i.e. 1g q 8h, was administered with VAN regimen of 200mg q 12h. The PK/PD model resulted in organism colony dropping below detection limits within 8 hours and no organisms surviving at the end of first run of 72 hours. Therefore, the CFZ and dosing interval was increased until organisms survived at end of first model run to be recycled. The regimens that were attempted until surviving colonies were obtained at the end of 72 hours were as follows:

- VAN 200 mg q 12h + CFZ 1 g q 8h
- VAN 200 mg q 12h + CFZ 500 mg q 12h
- VAN 200 mg q 12h + CFZ 250 mg q 24h
- VAN 200 mg q 12h + CFZ 125 mg q 24h

On exposure to VAN 200 mg q 12h + CFZ 125 mg q 24h some organisms survived by T72. However, no growth was observed on VAN resistance plates. The colonies obtained at T 72h were recycled. The graph of JH1 exposed to VAN and CFZ regimen is depicted in Figure 13. Change in MIC is given in Table 12.

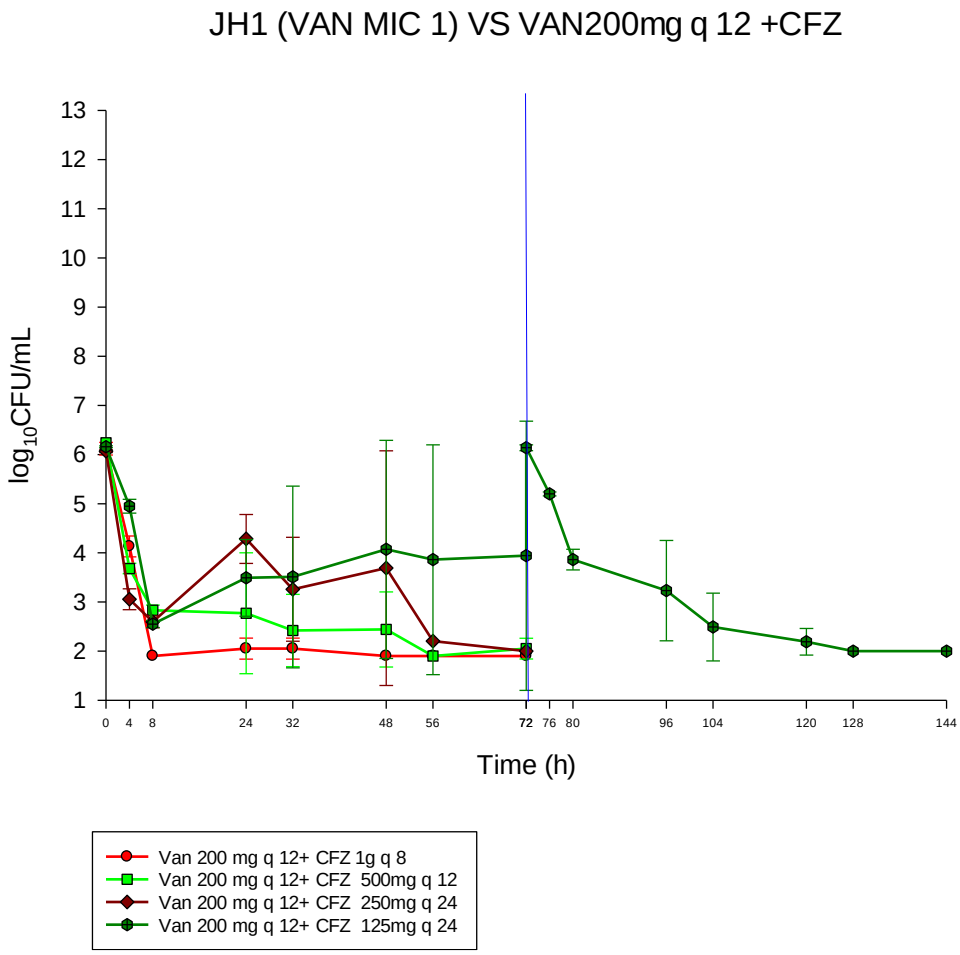


Figure 13 JH1 with Vancomycin 200mg q 12+ Cefazolin

Table 12 Change in MIC to vancomycin for treatment with vancomycin in presence of cefazolin.

JH1 vs Vancomycin 200mg q 12+ CFZ 125 mg q 24			
Vancomycin MIC	1 mg/l	2 mg/l	4 mg/l
No. of hours	0 hour	>216 hours	>216 hours

For both the organisms RN9120 and JH1 addition of CFZ to the VAN 200mg q 12 regimen prevented the organism to become a visa. This is described in table 11 and 12. The MIC of the organism increased to 2 mg/l to VAN in case of RN9120 but did not increase further. Therefore, CFZ in presence of VAN did have a beneficial effect in prevention of resistance development.

4.4.4. Pharmacokinetic analysis for model with VAN 200mg q 12h

The pharmacokinetic analysis VAN was performed by bioassay. The zone of inhibition for standards is given in table 13. The mean zone of inhibition vs concentration was plotted as given in Figure 14.

Table 13 Zone of inhibition for standard concentrations

Concentration (mg/l)	**Zone of Inhibition**		**Mean Zone of Inhibition**
	A	**B**	
4	15.2	15.4	15.3
3	13.9	14.1	14
2	12.8	12.6	12.7
1	9.1	9.8	9.45

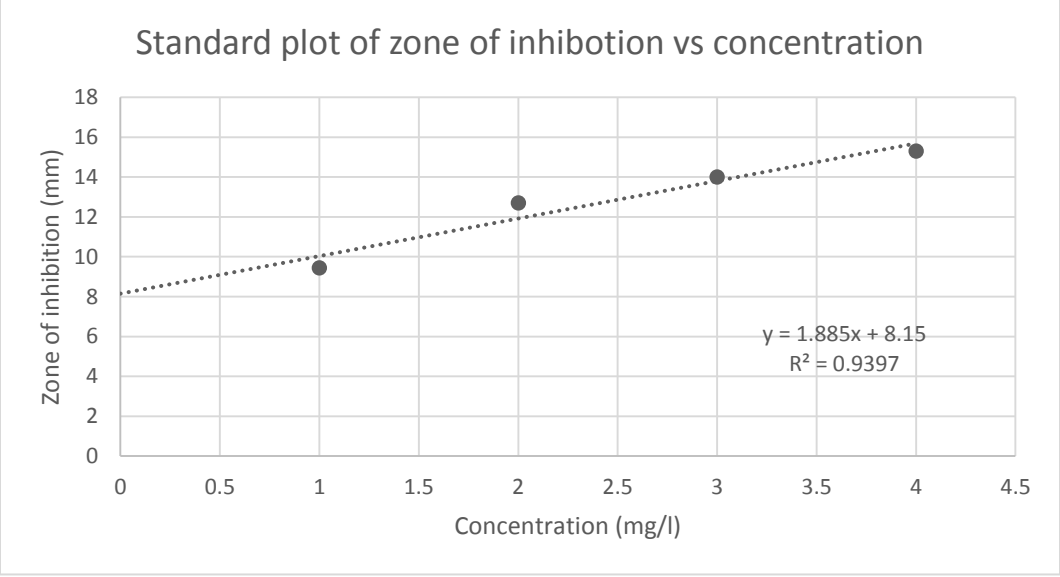


Figure 14 Standard plot of zone of inhibition vs concentration of standards.

Thus using the equation of the standard plot the concentration of samples was determined along with Half-life and peak concentration as shown in table 14.

Table 14 Pharmacokinetic analysis for Van 200mg q 12

Time (h)	Mean zone of inhibition		Concentration (mg/l)		C_{max}	$T_{1/2}$
	A	B	A	B		
0	15.2	15.1	3.74	3.69		
2	13.9	13.45	3.05	2.81		
4	12.6	12.4	2.36	2.25	3.715±0.092	5.81±0.071
8	10.85	10.85	1.43	1.43		
C_{max}	3.78 mg/l		3.65 mg/l			
$T_{1/2}$	5.86 h		5.76 h			

The pharmacokinetic study revealed that with this regimen the peak concentration achieved was 3.7 mg/l and the half-life was 5.81. The data is given in detail in table 14.

Furthermore, analysis of the organisms obtained at the end of the cycle after exposure to VAN alone and exposure to VAN and CFZ was done to study difference in the organisms obtained.

4.4.5. Population Analysis Profile

The population analysis profile demonstrated a shift in population and an increase in Area-under-curve (AUC) with exposure to VAN 200mg q 12h as compared to parent. In addition, CFZ prevented the population shift upon exposure to VAN. The population analysis profile for RN9120 and JH1 are depicted in Figure 15 and 16 respectively. The change in area under curve is shown in table 15.

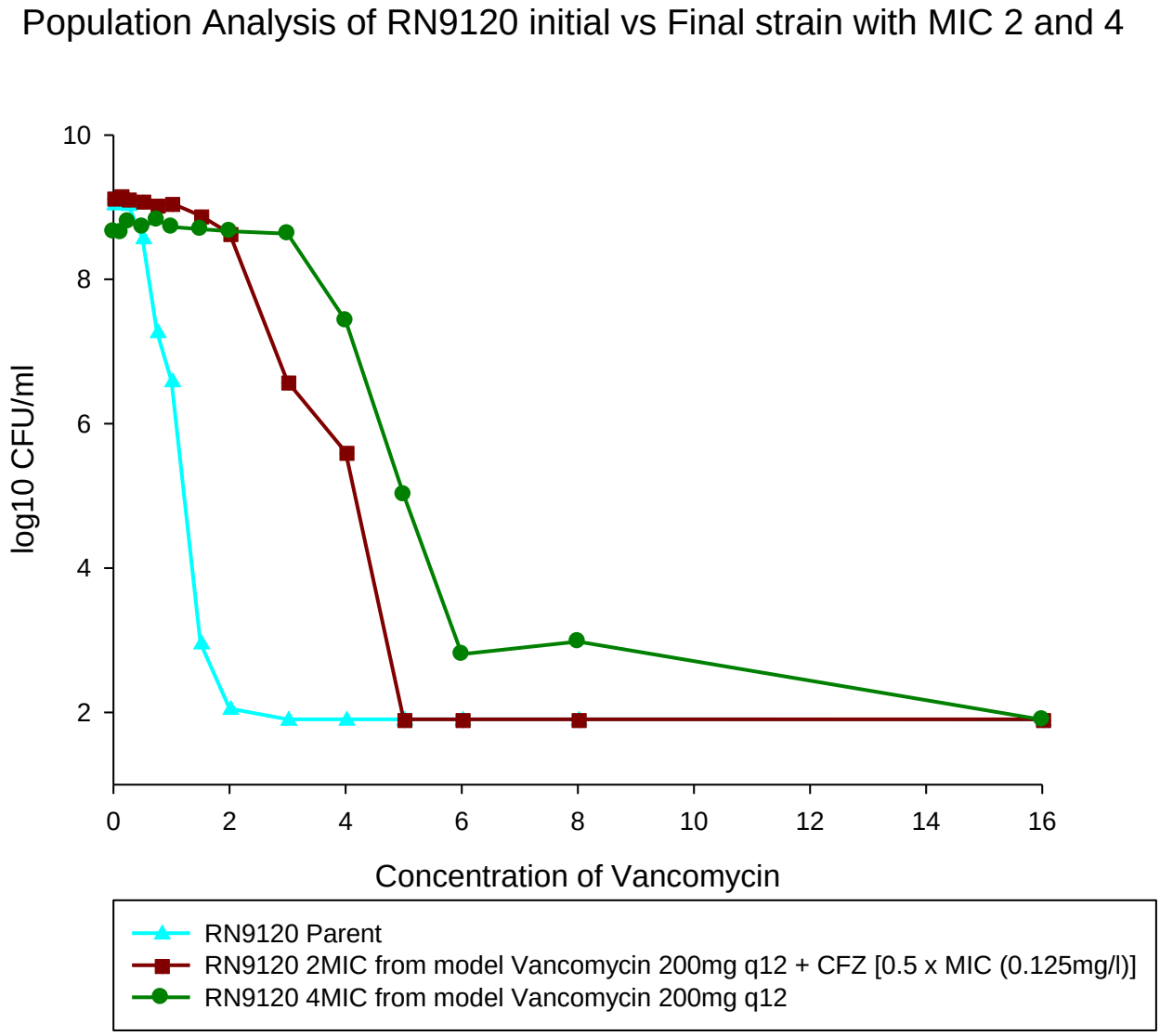


Figure 15 Population analysis profile for RN9120 for parent, exposed to van 200mg q 12 alone and exposed to van 200mg q 12 in presence of cefazolin.

PAP of JH1 parent(MIC 1) vs JH1 Mutant (MIC 4) and JH1 with VAN+CFZ exposure(MIC 1)

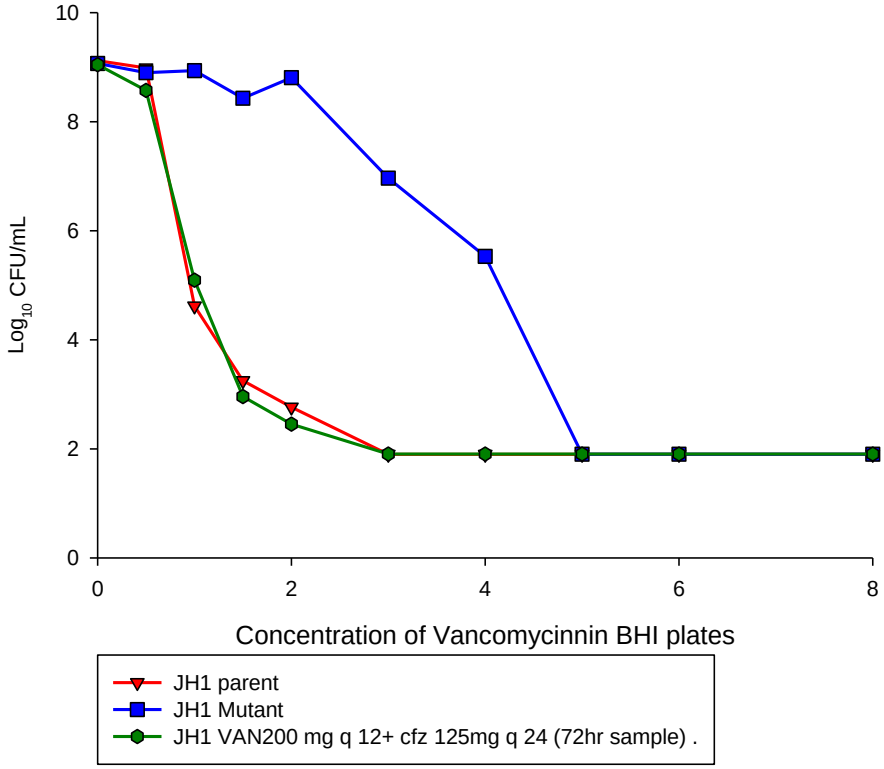


Figure 16 Population analysis profile for JH1 for parent, exposed to van 200mg q 12 alone and exposed to van 200mg q 12 in presence of cefazolin.

Table 15 Change in MIC and AUC with exposure to vancomycin alone and vancomycin in presence of cefazolin

RN9120			
	Parent	**Vancomycin 200mg q 12**	**Vancomycin 200mg q 12+ cefazolin**
MIC to vancomycin	1 mg/l	4 mg/l	2 mg/l
AUC	38.5305	69.5729	56.3428
JH1			
MIC to vancomycin	1 mg/l	4 mg/l	1 mg/l
AUC	23.2313	41.1541	22.8814

The population analysis profile of parent (initial strain) vs the organisms obtained at the end of the cycle with only VAN exposure vs exposure to VAN in presence of cefazolin showed a distinct difference in population and area under the curves. CFZ

when added to VAN regimen was able to reduce the shift of population to higher MIC and reduce the area under curve for both the organisms. Figure 15 and 16 show the shift in population for RN9120 and JH1 respectively.

CFZ addition resulted in no change in MIC for MRSA JH1 while there was some increase in MIC to MRSA. The plausible explanation to this phenomenon would be that the CFZ concentration in the MSSA model used was very low as compared to that used in MRSA. Thus the amount of CFZ present was not sufficient to entirely prevent the resistance development. Though as shown in figure 12 at higher concentration CFZ addition to VAN regimen did not allow any organism to grow. This can also be that the organism is unable to mutate to gain resistance and therefore is annihilated at that concentration.

4.4.6. Serial Passage

The VISA obtained from exposure of RN9120 and JH1 to VAN namely RN9120(2) and JH1-2 when tested for stability of mutation by serial passage, the mutation was stable to serial passage onto antibiotic free media. There was no change in MIC after serial passage for both RN9120(2) and JH1-2 (i.e. second mutant with MIC 4), even after 3 passages. The data is presented in table 16

Table 16 MIC on serial passage for second mutant of JH1 and RN9120

	MIC from resistance plate	MIC from Passage 1	MIC from Passage 2	MIC from Passage 3
RN9120(2)	4 mg/l	4 mg/l	4 mg/l	4 mg/l
JH1-2	4 mg/l	4 mg/l	4 mg/l	4 mg/l

4.5. Change in MIC to other antimicrobials.

The MIC of parent with VAN MIC 1 mg/l vs second mutant with VAN MIC 4 mg/l was compared for various glycopeptide and lipopeptide and β-lactam to check for cross resistance. The agents used in the study include VAN, DAP, Teicoplanin, CFZ and oxacillin. The MIC are given in table 17.

Table 17 MIC of parent and mutant.

	VAN (mg/l)	**DAP (mg/l)**	**TEI (mg/l)**	**CFZ (mg/l)**	**OXA (mg/l)**
RN9120	1	0.125	0.125	0.25	0.25
RN9120(2)	4	1	4	0.25	0.25
JH1	1	0.0625	0.25	16	>32
JH1-2	4	0.5	8	1	2

An interesting pattern observed was that as the VAN MIC increased the MIC to other glycopeptide and lipopeptide also increased in case of both organisms. This proves the potential of development of cross resistance by prior VAN exposure. In addition to this the MIC for beta lactams in case of MRSA decreased as the organism became more resistant to VAN. Thus demonstrating the seesaw effect in accordance to the literature.(*42*) The MIC of beta lactam in MSSA remained unchanged. The tentative explanation is that VAN interferes with the production of folding of PBP2A present only in MRSA, either by deleting the *mecA* gene or by disrupting PrsA, a protein responsible for folding in the region of PBP 2A. This increases the susceptibility of the organism to β-lactams.

4.6. Time kill studies

The green cross is the Growth control, blue closed circle is VAN alone, pink closed square is CFZ alone and red open square is the combination drug treatment.

Figure 17-21 represent time kill for MRSA. Figure 22 and Figure 23 denote hVISA while Figure 24 and 25 are plots of time kill curve of VISA. Linezolid (LNZ) resistant strain time kill curves is shown in figure 26 and 27.

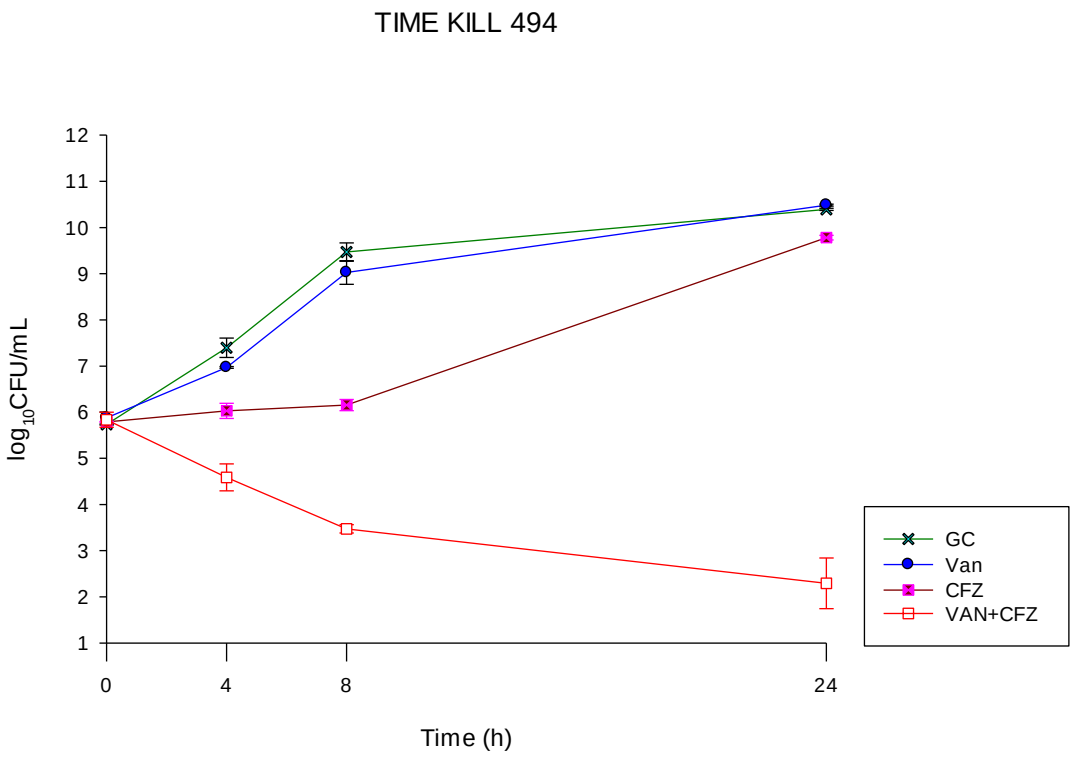


Figure 17 MRSA 494 Time kill plot

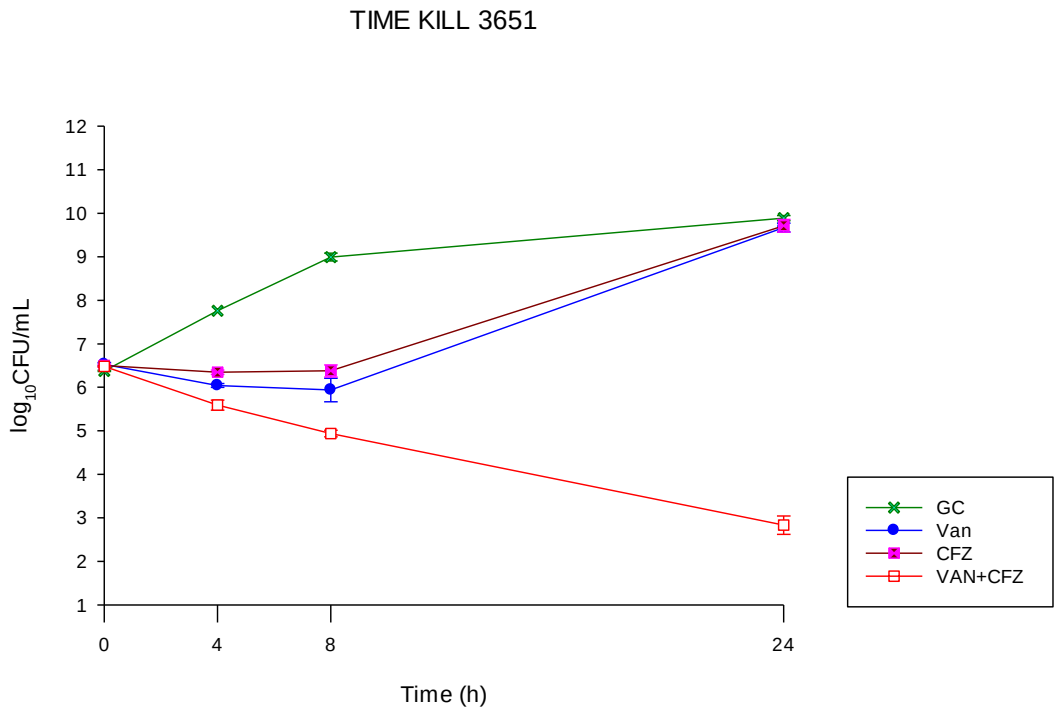


Figure 18 MRSA 3651 Time kill plot

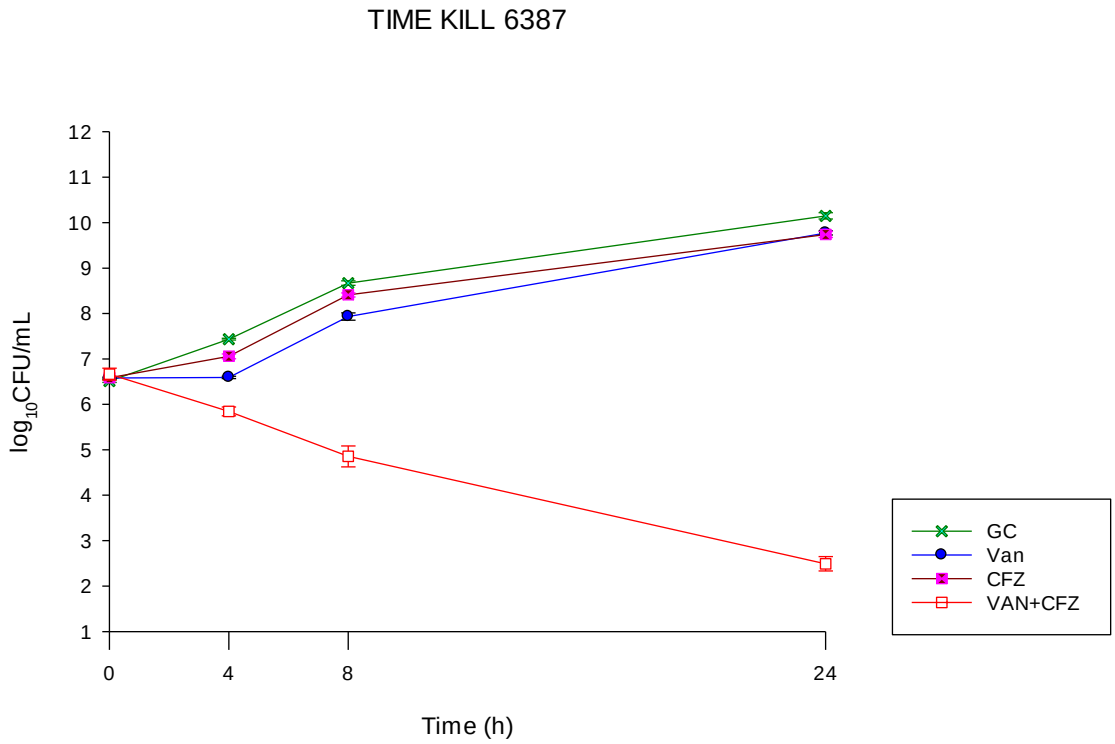


Figure 19 MRSA 6387 Time kill plot

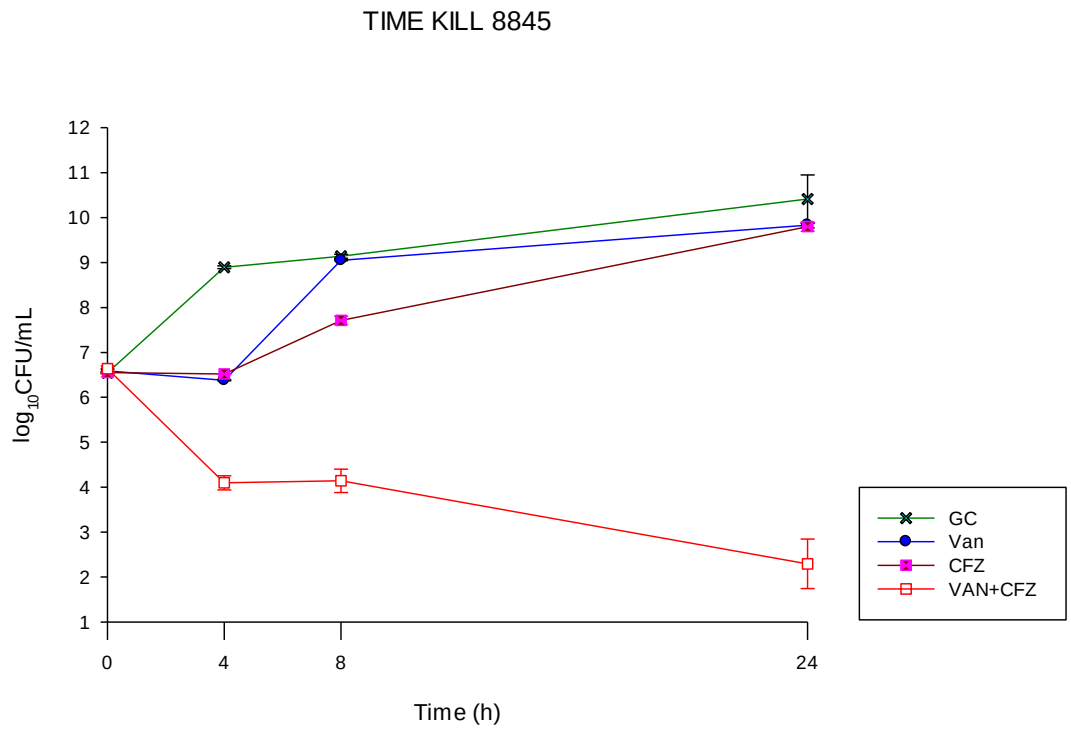


Figure 20 MRSA 8845 Time kill plot

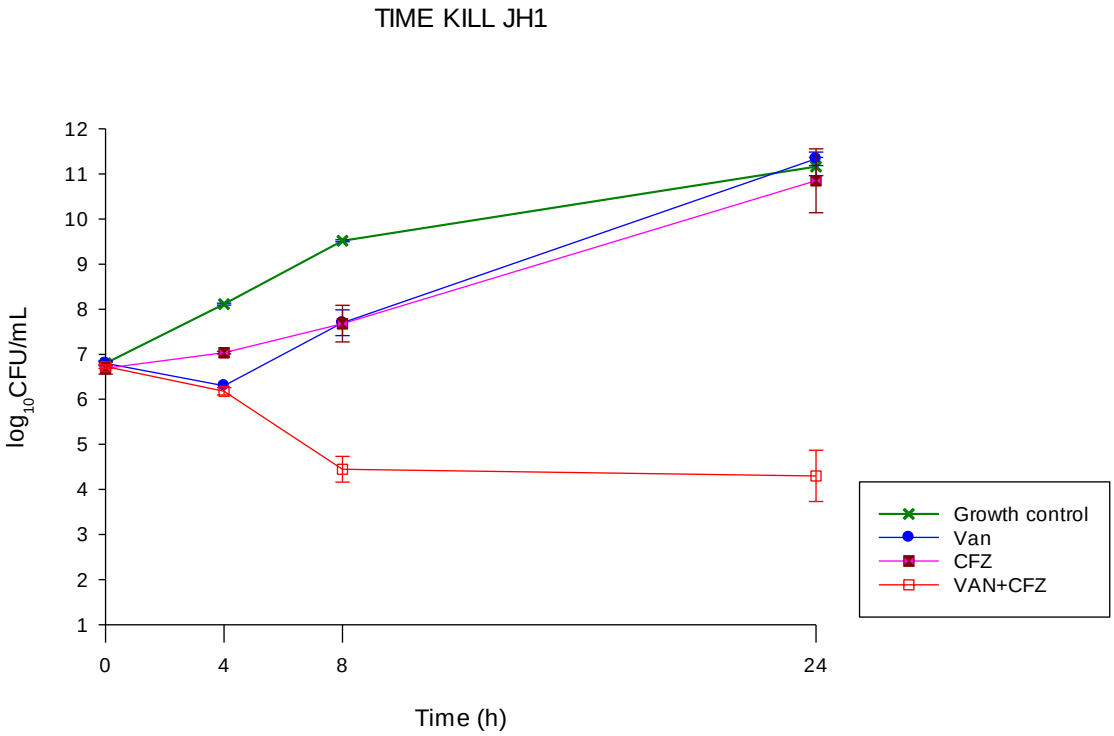


Figure 21 MRSA JH1 Time kill plot

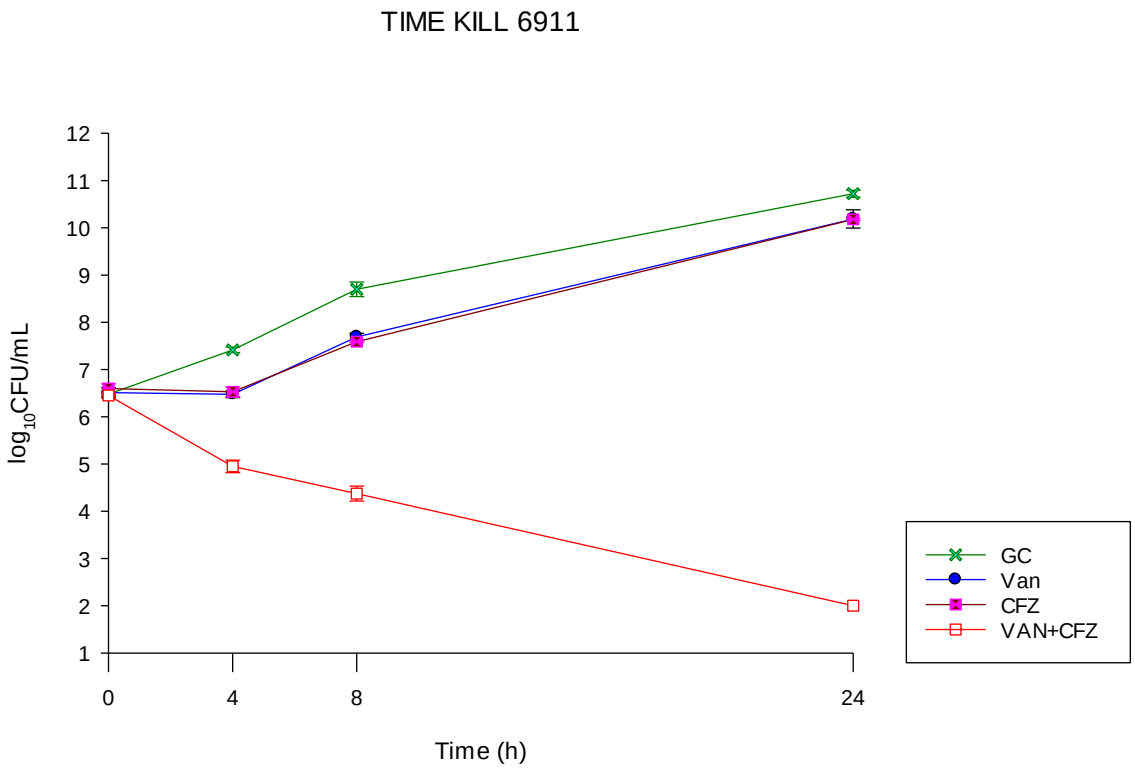


Figure 22 hVISA 6911 Time kill plot

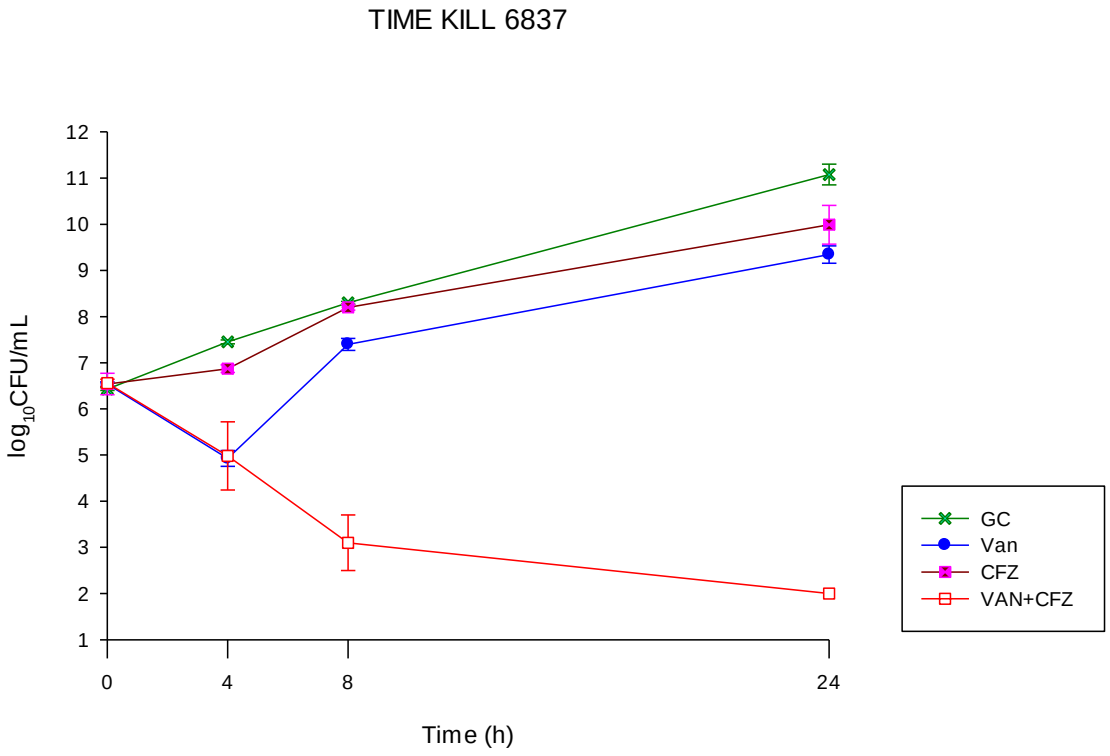


Figure 23 hVISA 6837 Time kill plot

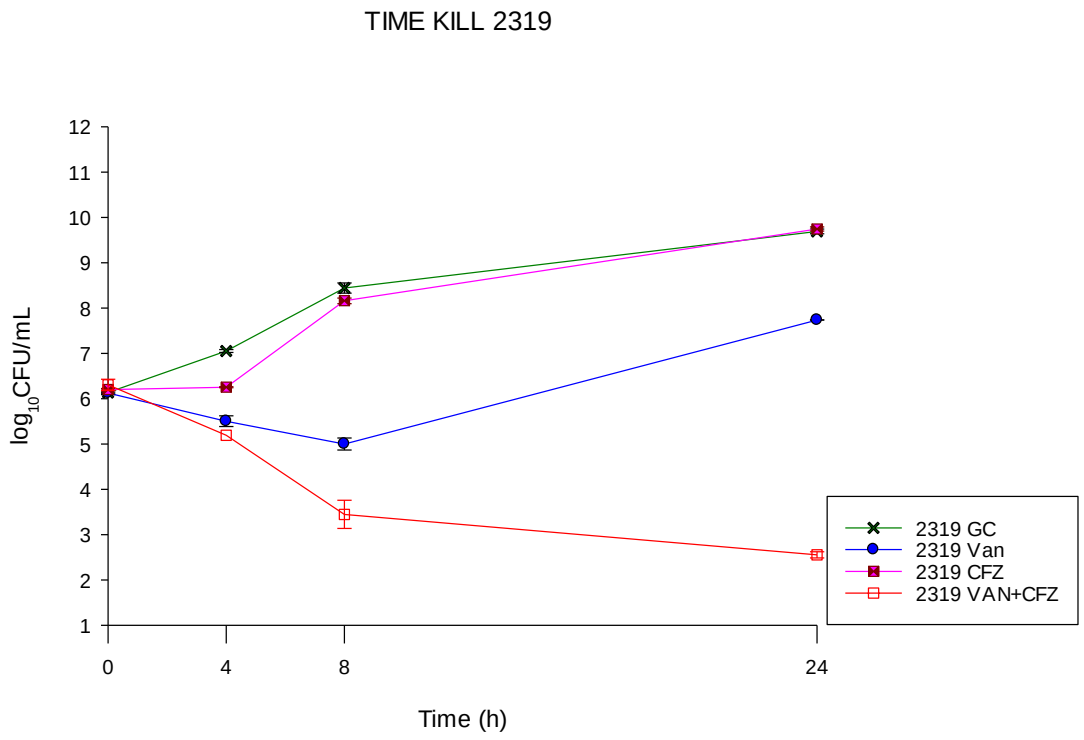


Figure 24 VISA 2319 Time kill plot

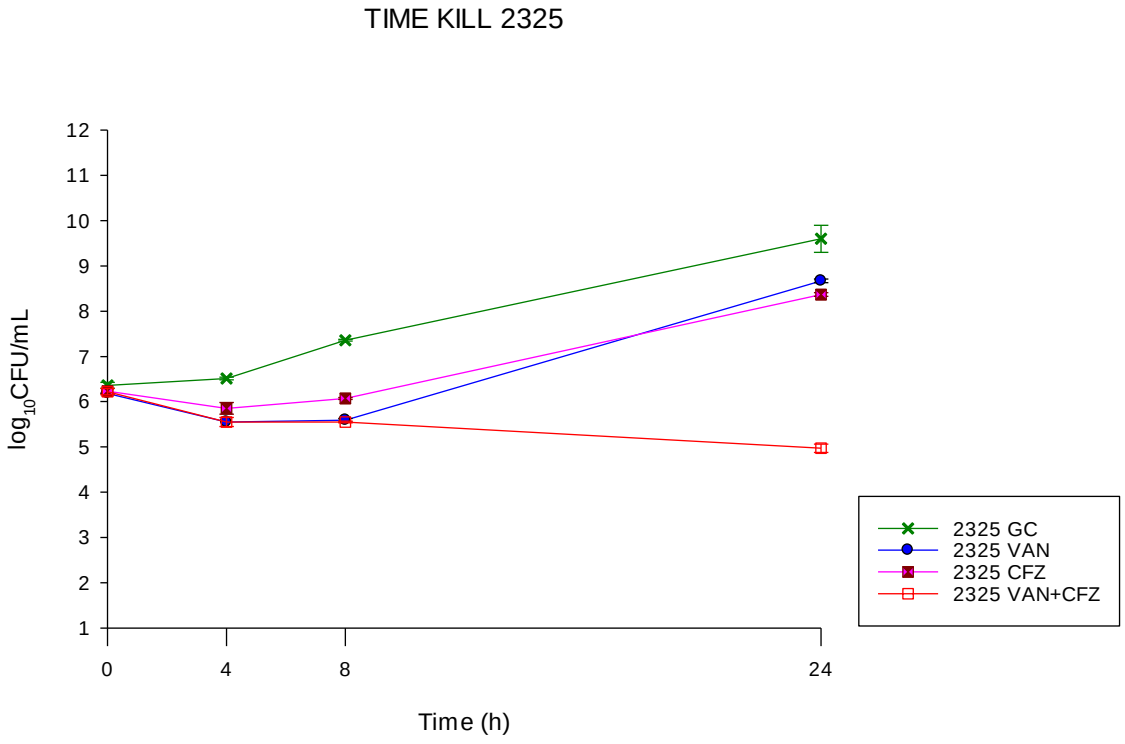


Figure 25 VISA 2325 Time kill plot

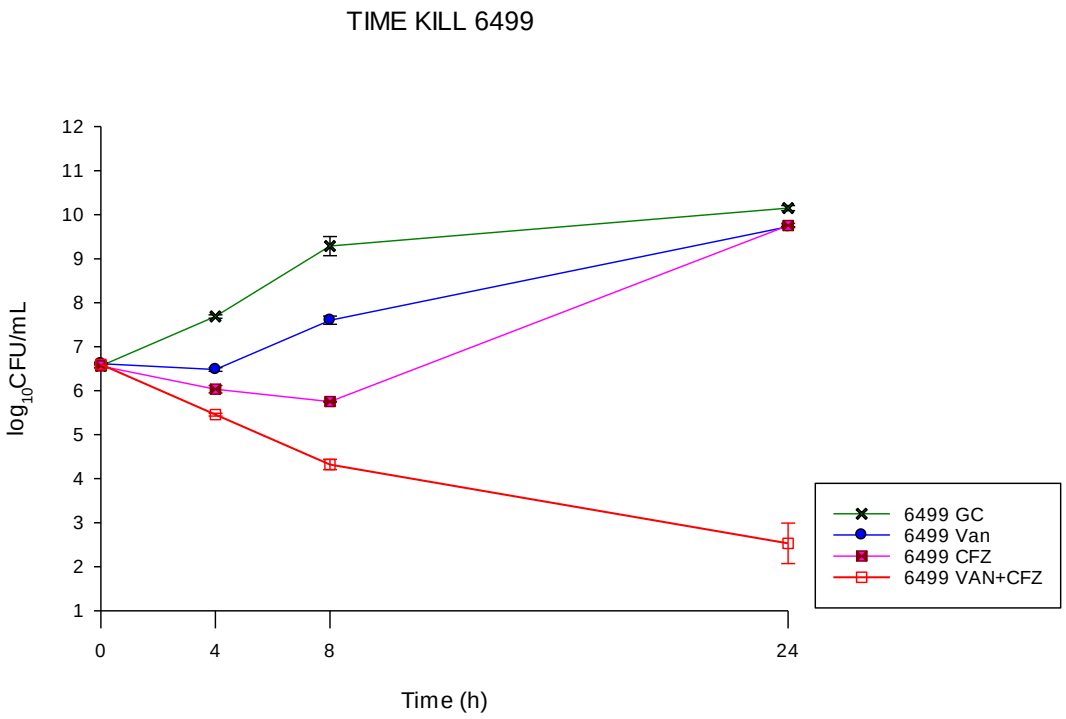


Figure 26 LZD-R 6499 Time kill plot

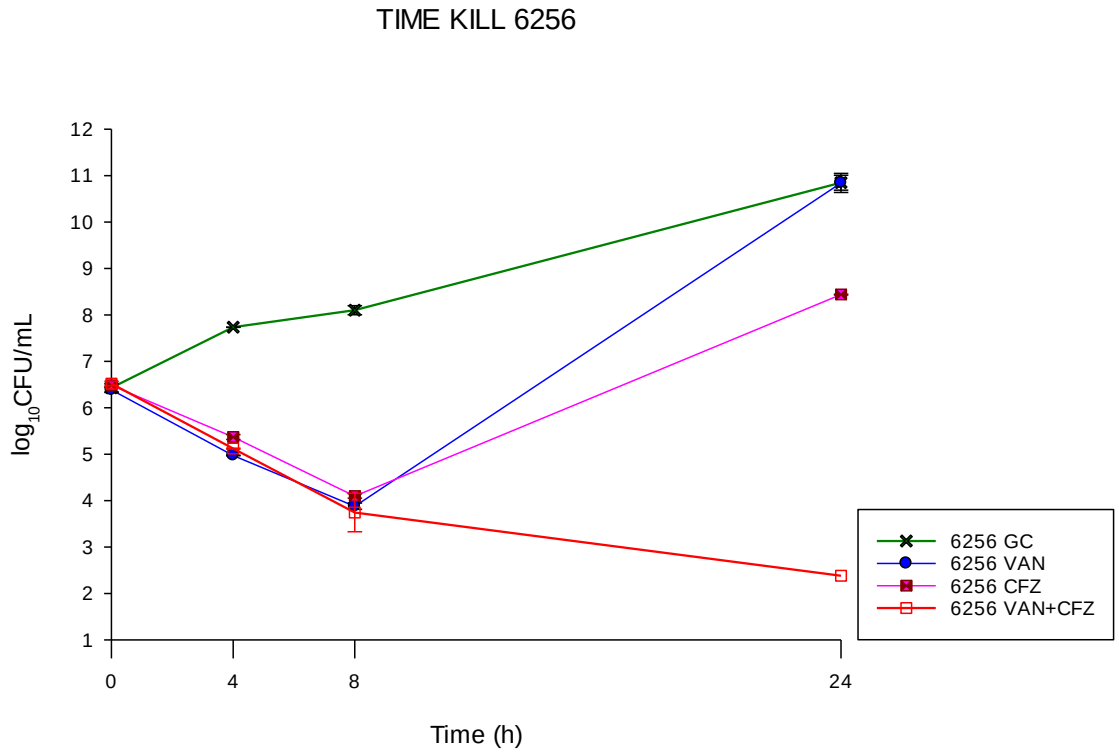


Figure 27 LZD-R 6256 Time kill plot

The time kill studies on various phenotypes of MRSA shown above, corroborate our data of the advantage of using CFZ in combination to VAN. In all 11 strains studied for time kill study, the addition of CFZ to VAN resulted in synergy for all strains tested regardless of varying susceptibility patterns to VAN or LNZ. The strain 2325 was the only strain which showed less pronounced effect. This may be strain specific. The organism was a VISA with MIC to VAN 8. The synergy was seen even on this strain though bactericidal activity of the combination was lower.

DISCUSSION:

In this study we validated that suboptimal VAN dosing in *S. aureus* leads to VISA for both MSSA (RN9120) and MRSA (JH1).

Previous experiments by Celine and colleagues have shown that over a period of 30 and 60 days of VAN dosing of 500 mg q 12h and 1g q 12 h against JH1 results in a VISA. Several mutations were reported namely yvqF (H164R), rpoB (D471Y, A473S, A477S, E478D), rpoC (E854A) and SA1129 (D296Y) associated with VISA. In addition to this phenotypic changes associated with VISA was observed, specifically reduced susceptibility to VAN and thickening of cell wall. (*56*)

In addition to MRSA, VAN reduced susceptibility had also been reported for MSSA in clinical strains.(*62*) B. Tsuji and his colleagues proved that MSSA with dysfunctional AGR group have a proclivity towards developing resistance to VAN on suboptimal exposure.(*58*)

Using our method of recycling at suboptimal VAN concentration we were able to generate VISA faster and more reproducibly as compared to other methods including

gradient plate method and broth serial passage methods. In both MSSA and MRSA we were able to generate a VISA within 144 h.

Previous studies have evaluated combination of VAN and a variety of beta-lactam for synergy. VAN and Nafcillin have been shown to be synergistic in more than 90% strains in time kill studies with overall increase in magnitude of activity in PK/PD models. (*47*)

Similarly, many previous studies have reported synergy with VAN with beta-lactams such as cefepime, cefpirome, carbapenam, cefotaxime and many others. (*63, 64*) CFZ in combination to VAN had been evaluated for synergy against S. epidermidis and had been found effective in about 40% of the cases.(*65*) There is very little information about the combination of VAN and CFZ against *S. aureus*. Rochon and his colleagues had tested 26 different beta-lactams in combination with VAN against 32 MRSA. They found in their studies that CFZ was one of the most effective beta-lactams in combination with VAN. (*45*) In addition to this CFZ had fewer side effects as compared to drugs like nafcillin.(*54*) These advantages of CFZ paved the way for use of CFZ in our study.

Combination MIC of VAN and CFZ revealed in accordance to the studies performed by Rochon and his colleagues, that the combination caused reduction in MIC for all organisms tested as compared to either drug alone.(*45*)

Some studies have evaluated the pharmacodynamic of VAN in combination of beta-lactam in PK/PD models. The combination has been reported to kill faster than single agent in MRSA, VISA and hVISA.(*46*)

The studies done by previous researchers concentrate on the combination of VAN and CFZ being synergistic. The combination demonstrated to have improved bactericidal activity against an MRSA and hVISA strain in an in vitro studies. However, there is little to no data examining the impact of beta-lactam exposure including CFZ on the prevention of generation of VISA. In fact, CFZ even at suboptimal doses of 125mg q 24 for JH1 and 0.125mg/l infusion for RN9120 was effective in preventing the generation of a VISA in both phenotypes.

Clinical investigations have demonstrated that suboptimal VAN exposure can lead to VISA and treatment failure.(*66*) The acquisition of VISA is important since these strains are often cross resistant to DAP which is one of the primary alternatives to VAN therapy especially in the face of VAN failure. (*67*) In our study we found that increase in VAN resistance resulted in increase in MIC to other glycopeptide teicoplanin and lipopeptide DAP. In addition, our data would suggest that application of a beta-lactam like CFZ and VAN as initial therapy may prevent the emergence of VISA on VAN therapy. This has potential important clinical implications since the VAN failure often leads to subsequent failure with alternative (salvage) antibiotics such as DAP. The prevention of resistance by using a combination of VAN and CFZ will be beneficial to patient.

In addition, the choice of beta-lactam is important as safety, the ease of administration, cost are important factors in selecting antibiotic therapy. While there is data to suggest that alternative beta-lactams such as nafcillin may demonstrate synergy against S. aureus including MRSA, CFZ has been shown to be a safer alternative to nafcillin and can be more easily administered (q 4-6 hours for nafcillin versus q 8-12

hours for CFZ). (*54, 68*) Berti and his colleagues in a study showed that beta-lactam antibiotics targeting PBP1 had enhanced DAP activity against MRSA.(*53*) PBP1 is a membrane protein required with cell wall synthesis. CFZ had affinity to PBP1 making it a potential antibiotic for synergy. In addition to this low cost of therapy made CFZ an ideal candidate for the study.

In the second part of our study synergy was observed in various phenotypes of MRSA including VISA, hVISA and LNZ-R strains. This information is useful clinically and makes it a potential candidate for treatment of difficult MRSA.

Limitations of the research:

Only one organism of each phenotype was used for the study. To verify that the combination of VAN and CFZ is effective in preventing resistance additional organisms from MSSA and MRSA should be explored.

Also VAN and CFZ combination was studied for a relatively short period of time. In clinical setting a patient may receive the antibiotics for a longer duration of time and therefore study should be carried out for a longer period of time to ensure it prevents resistance.

Only one beta-lactam (CFZ) was explored in our study. Other beta-lactams like Nafcillin, cefepine, ertamenam, meropenem need to be explored with VAN to study their potential in prevention of resistance.

CHAPTER 5

CONCLUSION

From the studies it can be concluded that sub-therapeutic exposure to VAN induces resistance in both MSSA (RN9120) and MRSA(JH1) isolates and the addition of CFZ to the regimen prevented of emergence of VISA for both MSSA and MRSA.

A positive correlation was established between the MIC changes for VAN and other glycopeptides/lipopeptides. Thus indicating a potential of developing cross-resistance to other drugs as the organism develops resistance to VAN.

A negative correlation was observed between VAN MIC and β-lactam (CFZ) in case of MRSA indicating VAN may interfere with the organism's mechanism to gain β-lactam resistance. In other words, it interferes with PBP2A by some mechanism making the organism more susceptible to β-lactam.

The time kill study corroborates the data of advantage of using VAN in combination to CFZ.

Additional studies on a wider range of isolates, more antibiotic combinations along with experiments on animal models will further validate the utility of this antibiotic combination for clinical use.

REFERENCES

1. A. Rohilla, *Handbook of Bacteriology*. (Oxford Book Co., Jaipur, IND, 2010).
2. A. Lyell, Alexander Ogston, micrococci, and Joseph Lister. *Journal of the American Academy of Dermatology* **20**, 302-310 (1989).
3. A. Ogston, Micrococcus Poisoning. *Journal of Anatomy and Physiology* **17**, 24-58 (1882).
4. F. J. Rosenbach, Mikro-Qrganismen bei den Wund-Infections-Krankheiten des Menschen. *Wiesbaden J.F. Bergmann*, 18 (1884).
5. P. D. Stapleton, P. W. Taylor, Methicillin resistance in Staphylococcus aureus: mechanisms and modulation. *Science progress* **85**, 57-72 (2002).
6. C. Solberg, A Study of carriers of Staphylococcus Aureus: with special reguard to quantitative bacterial estimations. *Acta Medica Scandinavica, Supplement* **436**, 1-98 (1965).
7. R. J. Gorwitz *et al.*, Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004. *J Infect Dis* **197**, 1226-1234 (2008).
8. D. Skinner, C. S. Keefer, Significance of bacteremia caused by staphylococcus aureus: A study of one hundred and twenty-two cases and a review of the literature concerned with experimental infection in animals. *Archives of Internal Medicine* **68**, 851-875 (1941).
9. M. J. Richards, J. R. Edwards, D. H. Culver, R. P. Gaynes, Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Critical care medicine* **27**, 887-892.

10. WHO GLOBAL STRATEGY FOR CONTAINMENT OF ANTIMICROBIAL RESISTANCE • WHO/CDS/CSR/DRS/2001.2. 11-23.

11. G. B. Forbes, Infection with Penicillin-resistant Staphylococci in Hospital and General Practice. *British Medical Journal* **2**, 569-571 (1949).

12. C. J. Henwood *et al.*, Susceptibility of Gram-positive cocci from 25 UK hospitals to antimicrobial agents including linezolid. *Journal of Antimicrobial Chemotherapy* **46**, 931-940 (2000).

13. G. C. Schito, The importance of the development of antibiotic resistance in Staphylococcus aureus. *Clinical Microbiology and Infection* **12, Supplement 1**, 3-8 (2006).

14. A. W. Segal, How Neutrophils Kill Microbes. *Annual review of immunology* **23**, 197-223 (2005).

15. I. A. Myles, S. K. Datta, Staphylococcus aureus: an introduction. *Seminars in Immunopathology* **34**, 181-184 (2012).

16. A. Du Toit, Immune evasion: Staphylococcus slips through the net. *Nat Rev Immunol* **14**, 4-5 (2014).

17. F. D. Lowy, Antimicrobial resistance: the example of Staphylococcus aureus. *Journal of Clinical Investigation* **111**, 1265-1273 (2003).

18. J. C. Pechère, Macrolide resistance mechanisms in Gram-positive cocci. *International Journal of Antimicrobial Agents* **18, Supplement 1**, 25-28 (2001).

19. R. Kelmani Chandrakanth, S. Raju, S. A. Patil, Aminoglycoside-Resistance Mechanisms in Multidrug-Resistant Staphylococcus aureus Clinical Isolates. *Current Microbiology* **56**, 558-562 (2008).

20. M. Tanaka, T. Wang, Y. Onodera, Y. Uchida, K. Sato, Mechanism of quinolone resistance in Staphylococcus aureus. *Journal of Infection and Chemotherapy* **6**, 131-139 (2000).

21. D. Hughes, Exploiting genomics, genetics and chemistry to combat antibiotic resistance. *Nat Rev Genet* **4**, 432-441 (2003).

22. Active Bacterial Core Surveillance (ABCs) Report Emerging Infections Program Network Methicillin-Resistant Staphylococcus aureus, 2014 (2016).

23. M. Larry M. Baddour, FAHA, Chair; Walter R. Wilson, MD; Arnold S. Bayer, MD; Vance G. Fowler, Jr, MD, MHS; Imad M. Tleyjeh, MD, MSc; Michael J. Rybak, PharmD, MPH; Bruno Barsic, MD, PhD; Peter B. Lockhart, DDS; Michael H. Gewitz, MD, FAHA; Matthew E. Levison, MD; Ann F. Bolger, MD, FAHA; James M. Steckelberg, MD; Robert S. Baltimore, MD; Anne M. Fink, PhD, RN; Patrick O'Gara, MD, FAHA; Kathryn A. Taubert, PhD, FAHA, Infective Endocarditis in Adults: Diagnosis, Antimicrobial Therapy, and Management of Complications. *American Heart Association, Inc.*, 16-20 (2015).

24. J. E. Olsen, H. Christensen, F. M. Aarestrup, Diversity and evolution of blaZ from Staphylococcus aureus and coagulase-negative staphylococci. *Journal of Antimicrobial Chemotherapy* **57**, 450-460 (2006).

25. O. ld, *Blackwell Handbooks of Behavioral Neuroscience : Antibiotics and Antibiotic Resistance (1)*. (Wiley, Hoboken, US, 2011).

26. J. Pootoolal, J. Neu, G. D. Wright, GLYCOPEPTIDE ANTIBIOTIC RESISTANCE. *Annual Review of Pharmacology and Toxicology* **42**, 381-408 (2002).

27. P. M. Pereira, S. R. Filipe, A. Tomasz, M. G. Pinho, Fluorescence Ratio Imaging Microscopy Shows Decreased Access of Vancomycin to Cell Wall Synthetic Sites in Vancomycin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **51**, 3627-3633 (2007).

28. B. Perichon, P. Courvalin, VanA-type vancomycin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother* **53**, 4580-4587 (2009).

29. B. P. Howden, J. K. Davies, P. D. Johnson, T. P. Stinear, M. L. Grayson, Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clinical microbiology reviews* **23**, 99-139 (2010).

30. K. Hiramatsu *et al.*, Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* **40**, 135-136 (1997).

31. A. M. Casapao *et al.*, Clinical Outcomes in Patients with Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Bloodstream Infection. *Antimicrobial Agents and Chemotherapy* **57**, 4252-4259 (2013).

32. C. Vidaillac *et al.*, Alternative mutational pathways to intermediate resistance to vancomycin in methicillin-resistant Staphylococcus aureus. *J Infect Dis* **208**, 67-74 (2013).

33. G. Sakoulas *et al.*, Accessory Gene Regulator (agr) Locus in Geographically Diverse Staphylococcus aureus Isolates with Reduced Susceptibility to Vancomycin. *Antimicrobial Agents and Chemotherapy* **46**, 1492-1502 (2002).

34. B. P. Howden, J. K. Davies, P. D. R. Johnson, T. P. Stinear, M. L. Grayson, Reduced Vancomycin Susceptibility in Staphylococcus aureus, Including Vancomycin-Intermediate and Heterogeneous Vancomycin-Intermediate Strains: Resistance Mechanisms, Laboratory Detection, and Clinical Implications. *Clinical Microbiology Reviews* **23**, 99-139 (2010).

35. J. A. Silverman, N. G. Perlmutter, H. M. Shapiro, Correlation of Daptomycin Bactericidal Activity and Membrane Depolarization in Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **47**, 2538-2544 (2003).

36. K. L. Tedesco, M. J. Rybak, Daptomycin. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* **24**, 41-57 (2004).

37. A. S. Bayer, T. Schneider, H.-G. Sahl, Mechanisms of daptomycin resistance in Staphylococcus aureus: role of the cell membrane and cell wall. *Annals of the New York Academy of Sciences* **1277**, 139-158 (2013).

38. R. A. Keel, C. A. Sutherland, J. Aslanzadeh, D. P. Nicolau, J. L. Kuti, Correlation between vancomycin and daptomycin MIC values for methicillin-susceptible and methicillin-resistant Staphylococcus aureus by 3 testing methodologies. *Diagnostic Microbiology and Infectious Disease* **68**, 326-329 (2010).

39. G. Sakoulas, J. Alder, C. Thauvin-Eliopoulos, R. C. Moellering, G. M. Eliopoulos, Induction of Daptomycin Heterogeneous Susceptibility in Staphylococcus aureus by Exposure to Vancomycin. *Antimicrobial Agents and Chemotherapy* **50**, 1581-1585 (2006).

40. J. B. Patel, L. A. Jevitt, J. Hageman, L. C. McDonald, F. C. Tenover, An Association between Reduced Susceptibility to Daptomycin and Reduced

Susceptibility to Vancomycin in Staphylococcus aureus. *Clinical Infectious Diseases* **42**, 1652-1653 (2006).

41. L. Cui, E. Tominaga, H.-m. Neoh, K. Hiramatsu, Correlation between Reduced Daptomycin Susceptibility and Vancomycin Resistance in Vancomycin-Intermediate Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **50**, 1079-1082 (2006).
42. K. E. Barber, C. E. Ireland, N. Bukavyn, M. J. Rybak, Observation of “Seesaw Effect” with Vancomycin, Teicoplanin, Daptomycin and Ceftaroline in 150 Unique MRSA Strains. *Infectious Diseases and Therapy* **3**, 35-43 (2014).
43. L. M. W. Fred C. Tenover, Peter C. Appelbaum, Linda K. McDougal, Jasmine Chaitram, Sigrid McAllister, Nancye Clark, George Killgore, Caroline M. O'Hara, Laura Jevitt, Jean B. Patel and Bülent Bozdogan, Vancomycin-resistant Staphylococcus aureus isolate from a patient in Pennsylvania. *Antimicrobial Agents Chemotherapy* **48**, 275-280 (2004).
44. J. G. Barr, E. T. M. Smyth, G. M. Hogg, In vitro antimicrobial activity of imipenem in combination with vancomycin or teicoplanin againstStaphylococcus aureus andStaphylococcus epidermidis. *European Journal of Clinical Microbiology and Infectious Diseases* **9**, 804-809 (1990).
45. S. Rochon-Edouard, M. Pestel-Caron, J.-F. Lemeland, F. Caron, In Vitro Synergistic Effects of Double and Triple Combinations of β-Lactams, Vancomycin, and Netilmicin against Methicillin-Resistant Staphylococcus aureus Strains. *Antimicrobial Agents and Chemotherapy* **44**, 3055-3060 (2000).

46. M. Hagihara, D. E. Wiskirchen, J. L. Kuti, D. P. Nicolau, In Vitro Pharmacodynamics of Vancomycin and Cefazolin Alone and in Combination against Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **56**, 202-207 (2012).

47. S. N. Leonard, Synergy between Vancomycin and Nafcillin against Staphylococcus aureus in an In Vitro Pharmacokinetic/Pharmacodynamic Model. *PLoS ONE* **7**, e42103 (2012).

48. M. W. Climo, R. L. Patron, G. L. Archer, Combinations of Vancomycin and β-Lactams Are Synergistic against Staphylococci with Reduced Susceptibilities to Vancomycin. *Antimicrobial Agents and Chemotherapy* **43**, 1747-1753 (1999).

49. P. M. Fox, R. J. Lampen, K. S. Stumpf, G. L. Archer, M. W. Climo, Successful Therapy of Experimental Endocarditis Caused by Vancomycin-Resistant Staphylococcus aureus with a Combination of Vancomycin and β-Lactam Antibiotics. *Antimicrobial Agents and Chemotherapy* **50**, 2951-2956 (2006).

50. R. P. Adhikari *et al.*, Vancomycin-induced deletion of the methicillin resistance gene mecA in Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy* **54**, 360-363 (2004).

51. K. Sieradzki, S. W. Wu, A. Tomasz, Inactivation of the methicillin resistance gene mecA in vancomycin-resistant Staphylococcus aureus. *Microb Drug Resist* **5**, 253-257 (1999).

52. A. Jousselin *et al.*, The Posttranslocational Chaperone Lipoprotein PrsA Is Involved in both Glycopeptide and Oxacillin Resistance in Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **56**, 3629-3640 (2012).

53. A. D. Berti, G. Sakoulas, V. Nizet, R. Tewhey, W. E. Rose, β-Lactam Antibiotics Targeting PBP1 Selectively Enhance Daptomycin Activity against Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **57**, 5005-5012 (2013).

54. S. Lee *et al.*, Is Cefazolin Inferior to Nafcillin for Treatment of Methicillin-Susceptible Staphylococcus aureus Bacteremia? *Antimicrobial Agents and Chemotherapy* **55**, 5122-5126 (2011).

55. G. Sakoulas *et al.*, Reduced Susceptibility of Staphylococcus aureus to Vancomycin and Platelet Microbicidal Protein Correlates with Defective Autolysis and Loss of Accessory Gene Regulator (agr) Function. *Antimicrobial Agents and Chemotherapy* **49**, 2687-2692 (2005).

56. C. Vidaillac *et al.*, Alternative Mutational Pathways to Intermediate Resistance to Vancomycin in Methicillin-Resistant Staphylococcus aureus. *The Journal of Infectious Diseases* **208**, 67-74 (2013).

57. F. McAleese *et al.*, Overexpression of Genes of the Cell Wall Stimulon in Clinical Isolates of Staphylococcus aureus Exhibiting Vancomycin-Intermediate- S. aureus-Type Resistance to Vancomycin. *Journal of Bacteriology* **188**, 1120-1133 (2006).

58. B. T. Tsuji, M. J. Rybak, K. L. Lau, G. Sakoulas, Evaluation of accessory gene regulator (agr) group and function in the proclivity towards vancomycin intermediate resistance in Staphylococcus aureus. *Antimicrobial agents and chemotherapy* **51**, 1089-1091 (2007).

59. H. S. Sader, P. R. Rhomberg, R. N. Jones, Nine-Hospital Study Comparing Broth Microdilution and Etest Method Results for Vancomycin and Daptomycin against Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **53**, 3162-3165 (2009).

60. H. Carsenti-Etesse *et al.*, Gradient plate method to induce Streptococcus pyogenes resistance. *Journal of Antimicrobial Chemotherapy* **44**, 439-443 (1999).

61. J. A. Silverman, N. Oliver, T. Andrew, T. Li, Resistance Studies with Daptomycin. *Antimicrobial Agents and Chemotherapy* **45**, 1799-1802 (2001).

62. J. Hu *et al.*, Reduced Vancomycin Susceptibility Found in Methicillin-Resistant and Methicillin-Sensitive Staphylococcus aureus Clinical Isolates in Northeast China. *PLoS ONE* **8**, e73300 (2013).

63. S. Deresinski, Vancomycin in Combination with Other Antibiotics for the Treatment of Serious Methicillin-Resistant Staphylococcus aureus Infections. *Clinical Infectious Diseases* **49**, 1072-1079 (2009).

64. C. L. Sy *et al.*, Synergy of beta-Lactams with Vancomycin against Methicillin-Resistant Staphylococcus aureus: Correlation of Disk Diffusion and Checkerboard Methods. *J Clin Microbiol* **54**, 565-568 (2016).

65. W. T. Siebert, N. Moreland, T. W. Williams, Synergy of Vancomycin plus Cefazolin or Cephalothin against Methicillin-Resistant Staphylococcus epidermidis. *Journal of Infectious Diseases* **139**, 452-457 (1979).

66. M. Rybak *et al.*, Therapeutic monitoring of vancomycin in adult patients: a consensus review of the American Society of Health-System Pharmacists, the

Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists. *Am J Health Syst Pharm* **66**, 82-98 (2009).

67. C. J. Chen, Y. C. Huang, C. H. Chiu, Multiple pathways of cross-resistance to glycopeptides and daptomycin in persistent MRSA bacteraemia. *J Antimicrob Chemother* **70**, 2965-2972 (2015).

68. K. G. Blumenthal, I. Youngster, E. S. Shenoy, A. Banerji, S. B. Nelson, Tolerability of Cefazolin after Immune-Mediated Hypersensitivity Reactions to Nafcillin in the Outpatient Setting. *Antimicrobial Agents and Chemotherapy* **58**, 3137-3143 (2014).

ABSTRACT

IMPACT OF CEFAZOLIN CO-ADMINISTRATION WITH VANCOMYCIN TO REDUCE DEVELOPMENT OF VANCOMYCIN INTERMEDIATE *STAPHYLOCOCCUS AUREUS*

by

NIVEDITA B SINGH

August 2016

Advisor: Dr. Michael J. Rybak

Major: Pharmaceutical Sciences

Degree: Master of Science

Objective: Development of resistance in *S. aureus* has been a big concern in the treatment of infections caused by the organism. The line of therapy used today has a disadvantage of slowly developing cross-resistance for antibiotics such as DAP secondary to prior VAN exposure. Therefore, alternative therapy is needed to prevent the emergence of VAN resistance and cross-resistance to other antibiotics. The primary aim of this experiment is first: to prove that sub-optimal VAN exposure in S. *aureus* will lead to development of VISA. Secondly, VAN in combination with cefazolin (CFZ) will prevent the development of VISA, regardless of sub-therapeutic vancomycin exposure. In addition to this we will evaluate if the combination of vancomycin and CFZ is synergistic against various phenotypes of MRSA.

Methods: Two strains of *S. aureus*, one MSSA (RN9120) and one MRSA (JH1) having a proclivity to gain resistance to vancomycin was used to induce resistance. The organisms were exposed to subtherapeautic VAN concentrations in a 1-compartment pharmacokinetic/pharmacodynamic (PK/PD) model, simulating human PK of VAN 200 mg q 12h over 72 h to induce resistance. At 72h, organisms recovered from the model

were re-exposed to the same VAN regimen for an additional 72-144 h exposure to generate VISA. The same experiment was repeated with continuous infusion of CFZ for MSSA and bolus administration for MRSA. Changes in MIC were evaluated at the end of each 72 h exposure. A population analysis profile (PAP) was performed to evaluate for shifts in population susceptibility. All PK/PD models were completed in duplicate to ensure reproducibility.

In addition to this Time kill experiments were carried out on 10 isolates of MRSA with various phenotypes including MRSA, VISA, hVISA and LNZR strains.

Results: VAN MIC of RN 9120 and JH1 increased to 4 mg/L as soon as 144h under sub-therapeutic VAN exposure. When CFZ was concomitantly administered, VAN MIC increased to 2 mg/L at 72h. However, no further increase in MIC was noted up to 216h of sub-therapeutic VAN administration. The MIC for MRSA remained unchanged when combination of VAN and CFZ was evaluated. PAP revealed a shift in the overall population towards non-susceptibility with VAN alone. CFZ when added to VAN caused a lesser shift in MSSA and no shift in MRSA.

Time kill studies showed synergy in all 10 MRSA under study.

Conclusion: The addition of low concentration of CFZ appears to prevent emergence of VISA under sub-therapeutic exposure to VAN. The time kill study corroborates the data of advantage of using VAN in combination to CFZ. Additional studies on a wider range of isolates, more antibiotic combinations along with experiments on animal models will further validate the utility of this antibiotic combination for clinical use.

AUTOBIOGRAPHICAL STATEMENT

NIVEDITA SINGH

- **EDUCATIONAL BACKGROUND**
  - **M. Pharm in quality assurance with project in pharmaceutics** **2003-2006**
    C.U. Shah College of pharmacy
    Mumbai, India

  - **Bachelor in pharmacy** **2001-2003**
    C.U. Shah College of pharmacy
    Mumbai, India

- **INDUSTRIAL EXPERIENCE**
  - **Research Associate (Formulation development)** **2006-2009**
    Unichem laboratories Ltd
    Mumbai, India

  - Underwent 4 weeks In-plant training (internship) **May 2004-june 2004**
    Alkem Laboratories Limited
    Mumbai, India

  - Underwent 4 weeks In-plant training (internship) **May 2002-june 2002**
    Sanjivini Parenteral Limited
    Mumbai, India

- **Publications and presentations:**
  - **Singh NB**, Yim J, Sakoulas G, Rybak MJ. Beta-Lactams (Bl) Plus Vancomycin (Van) Prevent Emergence of Van Resistance in Staphylococcus aureus (S. aureus); Poster presentation at ASM Microbe 2016
  - Yim J, Smith JR , Hallesy JA , **Singh N** , Arias CA , Rybak MJ. Synergy between Beta-Lactams (Bls) and Daptomycin (Dap) Against Vancomycin-resistant Enterococci (Vre) in an In Vitro Pharmacokinetic/Pharmacodynamic (Pk/Pd) Biofilm Model: Does This Apply to All Vre? Poster presentation at ASM Microbe 2016
  - Yim J , Smith JR , **Singh N** , Hallesy JA , C Garcia de la Mària , Bayer A , Mishra NN , Miro JM , Arias CA , Truc TT , Sullam P , Rybak MJ. Combination of Daptomycin (Dap) and Ceftaroline (Cpt) or Gentamicin (Gen) Against Streptococcus mitis in an In Vitro Model of Simulated Endocardial Vegetations (Sevs). Poster presentation at ASM Microbe 2016
  - Smith, J. R., Arya, A., Yim, J., Barber, K. E., Hallesy, J., **Singh, N. B.**, & Rybak, M. J. (2016). Daptomycin in Combination with Ceftolozane-Tazobactam or Cefazolin against Daptomycin-Susceptible and -Nonsusceptible Staphylococcus aureus in an In Vitro, Hollow-Fiber Model. Antimicrob Agents Chemother, 60(7), 3970-3975. doi:10.1128/AAC.01666-15
  - Hall Snyder, A. D., Werth, B. J., Nonejuie, P., McRoberts, J. P., Pogliano, J., Sakoulas, G., Yim, J., Singh, N. and Rybak, M. J. (2016) 'Fosfomycin enhances the activity of daptomycin against vancomycin-resistant Enterococcus in an in vitro pharmacokinetic/pharmacodynamic model', Antimicrobial Agents and Chemotherapy. AAC.00687-16; Accepted manuscript posted online 18 July 2016, doi:10.1128/AAC.00687-16