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A GENOME WIDE APPROACH TO IDENTIFY FACTORS ASSOCIATED WITH VANCOMYCIN NON-SUSCEPTIBILITY IN *STAPHYLOCOCCUS AUREUS*

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by

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Dedication

I dedicate this dissertation to my parents and my sister for their hard work, love, humor and support.

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Vancomycin (VAN) represents the standard of care in the treatment of Methicillin-Resistant *S. aureus* (MRSA) infections and widespread use has driven antimicrobial resistance. While fully resistant *S. aureus* strains have been identified, vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate susceptible *S.aureus* (hVISA) strains which contain resistant subpopulations despite appearing susceptible, are more common. These strains are associated with poorer outcomes including persistent infections or prolonged bacteremia. Because hVISA can be challenging to identify, prevalence is unclear and varies significantly as currently reported. Identifying the prevalence of hVISA is vital to understanding the clinical impact of these infections. Further, whole genome sequencing (WGS) offers a tool to identify factors such as multi locus sequence types (MLSTs) or single nucleotide polymorphisms (SNPs) that may aid in identifying or predicting the presence of hVISA.

This study therefore utilized a stepwise phenotypic approach to elucidate the prevalence of hVISA among a collection of clinical isolates derived from hospitals in the states of Texas and California and employed WGS to identify MLSTs and SNPs associated with hVISA. VAN E-tests, VAN impregnated BHI agar and population analysis profile-area under the curve tests were used to identify the prevalence of hVISA in a collection of 320 clinical isolates. Additionally, a subset of these isolates underwent WGS to identify MLSTs while their genomes were compared to the reference (N315) to identify SNPs. The overall prevalence of hVISA in this study was 3% and was most common among isolates with an MIC of 2µg/mL. The predominant MLST among hVISA isolates was ST5, contributing to 70% of the isolates. Further, by comparing hVISA to VSSA isolates matched by year, location, MIC and MLST, we identified SNPs in four candidate genes that were exclusive to hVISA. Finally, a phylogenetic analysis demonstrated the heterogeneity of isolates displaying the hVISA phenotype. While this study highlights the complex and multifactorial nature of hVISA, it does provide insight into its prevalence and factors that may provide utility while identifying these infections. These tools may aid in identifying patients suffering from hVISA infections and allow clinicians to make more rapid and informed decisions during treatment.

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CHAPTER 1

INTRODUCTION TO VANCOMYCIN-INTERMEDIATE *STAPHYLOCOCCUS AUREUS*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of infectious diseases in the United States and elsewhere in the world. Vancomycin serves as the main therapeutic agent for infections caused by MRSA strains. However, its increased use has led to the emergence of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) strains[1]. Infections with vancomycin-nonsusceptible isolates are associated with prolonged bacteremia, longer hospital stays, and greater rates of clinical treatment failure than infections with vancomycin-susceptible *S. aureus* (VSSA).[2,3]

Classification of vancomycin susceptibility

There are 3 main classifications of vancomycin non-susceptible *Staphylococcus aureus.* The concentration of vancomycin to inhibit *S. aureus* [e.g., minimum inhibition concentration (MIC)] is typically ≤ 2 μg/mL. By contrast, *S. aureus* isolates with vancomycin MICs of 4-8 μg/mL are classified as vancomycin-intermediate susceptible *S. aureus* (VISA), and isolates with MICs $\geq$16μg/mL are classified as vancomycin resistant *S. aureus* (VRSA). Heterogeneous vancomycin resistant *S. aureus* (hVISA) strains commonly display susceptible MICs (1-2 μg/mL) but contain resistant sub-populations.[4,5,6] Table 1.1 describes the current breakpoints for vancomycin in *S. aureus.*

Table 1.1 Vancomycin breakpoints for *S. aureus* according to the Clinical and Laboratory Standards Institute

Classification	Minimum inhibitory concentration
Susceptible	≤ 2μg/mL
Intermediate resistance	4-8μg/mL
Resistant	≥16μg/mL

Discovery of VISA

The first report of *S. aureus* exhibiting VISA was reported in Japan after a 4 month old male patient received vancomycin to treat a surgical wound infection in 1996. This strain has been referenced to as Mu50. The patient failed therapy with vancomycin and the infection did not resolve until treatment with arbekacin and ampicillin/sulbactam. Broth microdilution later revealed an MIC of 8μg/mL.[7] Further, Hiramatsu et al. also identified another *S. aureus* isolate (Mu3) in 1996 with an MIC of 3μg/mL from the sputum of an 86 year old pneumonia patient that had failed therapy with vancomycin.[5] Further studies have since reported Mu3 MICs of 2μg/mL.[7,8]

Prevalence of VISA/hVISA

VISA was first reported in 1997; however, studies have suggested that VISA and hVISA emerged earlier. In a retrospective study of 750 isolates collected from 31 Japanese hospitals in 1990 (one year before the approval of IV vancomycin in Japan), 38 strains (5.1%) were hVISA as determined by population analysis.[9] Another retrospective study assessed the MICs of 1,445

isolates from a French hospital from 1983 to 2002. In this study MICs were determined via Etest and ranged from 0.5-6μg/mL.[10] Additionally, Rybak et al. studied 50 isolates collected from patients during a clinical trial spanning from 1987-1992. Among these isolates, 7 had MICs ≥3μg/mL ranging from 3-8μg/mL.[11]

While these studies demonstrate the occurrence of hVISA/VISA well before it was reported by Hiramatsu in 1997, evidence also suggests that the prevalence of hVISA has risen over time. In the United States, Rybak et al. screened ~1500 MRSA isolates to detect hVISA from three hospitals in the Detroit metropolitan area over a 22 year period. Isolates were screened using the Macro Etest method (MET) and confirmed using the population-area under the concentration-time curve (PAP-AUC) method (these methods were discussed in more detail below). The rate of hVISA increased from 2.2% in 1986-1993 to 7.6% in 1994-2002 and to 8.3% in 2003-2007. The rate of VISA fluctuated from 0.4% from 1986-1993 to 2.3% from 1994-2002 and was 0.3% from 2003-2007.[12] Another study in the Detroit area screened 202 *S. aureus* isolates from the Detroit Medical center collected from 2002 through 2013 and identified 38 (19%) hVISA isolates via PAP-AUC.[13] The reported prevalence seems to vary greatly between studies. Richter et al. reported substantially lower frequencies. A study evaluated 4,210 *S. aureus* isolates collected from 43 US centers in 2009. Isolates were screened using Etests and hVISA was confirmed by PAP-AUC. This study identified 11 (0.3%) hVISA isolates and no VISA isolates. Further, 56 isolates had an MIC of 2μg/mL of which 6 (10.7%) were hVISA.[14] In a subsequent study by Richter et al., 2,093 isolates collected from 42 centers across the US in 2011 were studied using the same methods. Of these isolates, 25 (1.2%) were hVISA. Among 22 isolates with an MIC of 2μg/mL, 50% were hVISA.[14] While the prevalence of hVISA was low, these studies demonstrated a 4-fold increase in the frequency of hVISA from 2009 to 2011.

VISA/hVISA has been detected in countries across the globe including Asia, Europe, Australia, North and South America, and Africa. Table 1.2 describes the hVISA/VISA prevalence by country. A recent systematic review included 91 studies from Asia, America, Europe and Australia and grouped studies into three periods; before 2006, 2006-2009, and 2010-2014. The global prevalence of hVISA isolates increased steadily from 4.7% before 2006 to 5.4% in 2006–2009, then to 7.0% in 2010–2014. The prevalence of VISA was 2.1% before 2006, 2.6% in 2006–2009, and 7.9% in 2010–2014.[16] Moreover, the rate of hVISA is increasing more rapidly in some geographic regions. In a study conducted in Turkey for example, the rate of hVISA was reported as 1.6% in 1998 and increased to 32% in 2001, while a study in Australia reported rates as high as 48%.[17,18] Such wide variation in hVISA/VISA prevalence may be partially explained by geographic location, variation in testing methods, and change in breakpoints.

Table 1.2 Prevalence of vancomycin intermediate resistance among MRSA isolates

Country	Time frame	Source	Methods	hVISA (%)	VISA (%)	Reference
Japan	2008/01–2011/05	Blood samples	MET	55/830 (6.5)	8/830 (1.0)	Hanaki et al, 2014
Korea	2008/08–2010/09	Blood samples	Etest, PAP-AUC	101/268 (37.7)		Park et al, 2012
India	2009–2010	Pharyngitis throat swabs	Van supplanted agar, BMD		10/63 (15.9)	Gowrishankar et al, 2013
Lebanon	2006/02–2013/03	All clinical samples	Agar dilution		5/113 (3.8)	El Ayoubi et al, 2014
India	2009/09–2012/04	All clinical samples	Etest		545/1214 (44.9)	Dubey et al, 2013
India	2010/09–2013/03	All clinical samples	Etest,MET,PAP-AUC	4/58 (6.9)	2/58(3.4)	Chaudhari et al, 2015
Thailand	2010/11–2011/11	All clinical samples	Agar dilution, PAP-AUC	2/68 (2.9)		Panomket et al, 2014

Table 1.2 (continued) Prevalence of vancomycin intermediate resistance among MRSA isolates

Country	Time frame	Source	Methods	hVISA (%)	VISA (%)	Reference
China	2011/06–2012/05	Sterile body fluids	PAP-AUC	17/77 (22.1)		Liu et al, 2014
Pakistan	2012	All clinical samples	Etest	6/347 (1.7)		Kaleem et al, 2012
India	2013	All clinical samples	Agar dilution, MET, PAP-AUC	8/130		Chaudhary et al, 2013
Turkey	2009–2010	Blood samples	MET, PAP-AUC	24/175 (13.7)		Sancak et al, 2013
United States	2002/01–2013/06	All clinical samples	PAP-AUC	38/202 (18.8)	3/202 (1.5)	Casapao et al, 2014
United States	2011	All clinical samples	PAP-AUC	25/2093 (1.2)		Richter et al, 2014

MET: Macromethod Etest, Van: vancomycin, PAP-AUC: population analysis profile-area under the curve
Note: Table was adopted and modified from Zang et al. PLOS one. 2015 Aug.

Laboratory detection of VISA and hVISA

The current breakpoints for *S. aureus* to determine susceptibility to vancomycin include: susceptible < 2 ug/mL, intermediately susceptible 4-8 ug/mL, and resistant > 16 ug/mL. VISA is defined by an MIC of 4-8µg/mL while hVISA strains commonly display elevated MICs (1-2 µg/mL) but are within the susceptible range[4,5,6]

VISA has been identified using conventional susceptibility testing methods such as agar dilution, broth microdilution, and Etests. The agar dilution method utilizes agar plates imbedded with vancomycin at varying concentrations. A 0.5 McFarland inoculum is prepared, diluted and plated. The MIC is determined by the concentration of vancomycin that inhibits visible growth after 16-24hrs.[4] The broth microdilution technique utilizes a similar approach where vancomycin is added to the first well of a 96-well microtiter plate and serially diluted such that each well contains a two-fold dilution from the previous well. A 0.5 McFarland inoculum is then prepared, diluted and added to wells of a microtiter plate such that the final concentration is 5 X 10^5 CFU/mL. After a 16-20hr incubation time, MICs are determined by identifying the concentration which inhibits visible growth.[4] The Etest utilizes a strip containing a drug concentration gradient. After forming a 0.5 McFarland standard inoculum, a Mueller Hinton Agar (MHA) plate is streaked and the strip is placed. The point where the inhibition ellipse intersects the test strip indicates the MIC[30]. Though the above mentioned methodologies are common, discrepancies between tests have been reported. While broth and agar dilution MICs tend to be fairly well correlated, Etest MICs tend to be higher and a one 2-fold dilution increase in MIC versus broth microdilution is common.[5,31,32]

Detection of hVISA presents additional problems in that while isolates may appear susceptible based on traditional microbroth methods, resistant sub-populations at undetectable frequencies are present.[5] To combat this issue, tests may utilize higher inoculums, prolonged growth time, and more nutritious media to detect these sub populations.[5] The MET for example utilizes a McFarland 2.0 standard instead of 0.5 and assesses growth at 8µg/mL.[33] The Etest glycopeptide resistance determination (GRD) utilizes a double sided strip containing vancomycin and teicoplanin. Isolates at a 0.5 McFarland standard concentration are plated onto a MHA plate containing 5% sheep blood. The plate is read at 24 and 48 hours after incubation at 35^0C and an MIC ≥8µg/mL for either drug indicates a positive test.[33] Further, BHI screen agar plates utilize BHI agar infused with casein and vancomycin (4µg/mL). A series of four 10µL droplets at 0.5 McFarland standard concentration is plated and growth is monitored after 24 and 48 hours of incubation at 35^0C. Growth of more than one colony indicates vancomycin resistance.[33] These methods are often used as a means to screen for hVISA with the phenotype being conformed using population analysis profiles (PAP). Using the PAP-AUC method, cultures are diluted to 10^{-3} and 10^{-6}, and plated on agar containing various concentrations of vancomycin. After 48 hours of incubation, viable colonies are plotted against vancomycin concentration. An area under the curve (AUC) is then calculated and compared to the AUC of Mu3 grown under the same conditions. The ratio of the test strain AUC divided by the Mu3 AUC is calculated with a ratio ≥0.9 being classified as hVISA.[34] Table 1.3 compares the sensitivity and specificity of the above mentioned screening methods.

Table 1.3 Sensitivity and specificity of screening methods to detect hVISA using PAP-AUC as reference

Test	Sensitivity %	Specificity %
MET	57	96
GRD	57	97
BHI agar screen	91	94

Satola et al. J Clin Microbiol 2011 Jan 49(1) 177-183

Note: Sensitivity and specificity based on MIC of 2µg/mL

Mechanisms of vancomycin resistance

Vancomycin exerts activity through binding of d-ala-d-ala residues on peptidoglycan precursors, preventing crosslinking by penicillin binding proteins and subsequent cell wall formation.[5] The van A gene confers complete resistance to vancomycin in *S. aureus* and Enterococcus by altering the binding site to d-alanine-d-lactate residues.[5] In the late 1990s, Sieradzki et al. noted reduced vancomycin susceptibility in *S. aureus* mutants lacking d-alanine-d-lactate and suggested that other structural cell wall changes could be responsible for vancomycin resistance.[35,36] Since the primary site of cell wall synthesis occurs at the septum during cell division, vancomycin must diffuse to this area to exert its activity.[5] Therefore, mutations reducing the ability of vancomycin to reach the septum such as, increased cell wall formation and increased free d-alanine-d-alanine residues, are of particular interest when studying VISA.

Diffusion of vancomycin can be inhibited in multiple ways. Increased cell wall diameter resulting from increased biogenesis or reduced autolysis can increase the distance to the active site. Additionally, cells exhibiting this characteristic display a greater ability to bind vancomycin due to an increased number of free d-ala-d-ala residues. Reduced cross linking is also commonly observed in VISA isolates and d-ala-d-ala residues that are not linked to the existing peptidoglycan layer are once again free to bind vancomycin. As a result, several genes are implicated in VISA including genes regulating cell wall formation, cell wall autolysis, penicillin binding protein function (crosslinking), and peptidoglycan precursor formation and therefore serve as targets to identify VISA.

The number of genes and mutations that can lead to structural changes and reduced vancomycin susceptibility are vast; however, a relatively small proportion has been experimentally validated. The following summary will focus on mutations conferring the VISA phenotype that have been experimentally validated. These loosely fall into four categories of gene function including: (1) genes which govern cell wall synthesis and remodeling/autolysis, (2) metabolic genes, (3) genes regulating transcription, (4) genes governing post translational modification of protein. Additionally these processes are regulated by multiple genes simultaneously and operons can modulate several pathways. Please see the summary of genes and variants listed in Table 1.4.

Cell wall synthesis and remodeling: Deletion in the gene Sle1, the hydrolase of N-acetylmuramyl-L-alanine amidase involved in peptidoglycan biosynthesis was observed

in the VISA strain Mu3 compared to the vancomycin susceptible (VSSA) strain N315ΔIP. Introduction of the loss of function mutation (Δ67AA) led to reduced autolysis and constitutive cell wall thickening.[1,37] Other genes involved in cell wall synthesis include gtaB, tagO, and msrR which is necessary for the attachment of wall teichoic acid (WTA) to peptidoglycan. Since WTA prevents the binding of autolysin to assembled cell walls, the mutation in msrR gene (E164K) is thought to increase vancomycin resistance through reduced cell wall autolysis.[1]

Cell metabolism: While alteration of cell wall remodeling (decreased autolysis) is implicated in cell wall size, alteration of cell metabolism is necessary to compensate for larger cell wall diameters. Perhaps the most noteworthy mutations occur in the cmk gene. Mutations in the cmk gene (A20G) have been shown to convert hVISA isolates to VISA. The cmk is thought to increase the formation of the peptidoglycan precursor uridine diphosphate N-acetylglucosamine, leading to greater cell wall formation.[1,37]

Transcriptional regulation: Several regulatory systems modulate downstream genes involved in cell wall formation including the vraTSR, graSR and walKR operons as well as the rpoB gene. Kuroda et al. noted the upregulation of the vraTSR operon in Mu50 VISA strain. The vraTSR operon is thought to regulate the cell wall stress stimulon and increase cell wall biosynthesis.[39,40,41] The graSR and walKR systems are both thought to increase vancomycin resistance through modulation of genes that control autolysis.

Finally rpoB codes for the β subunit of RNA polymerase and the H481Y mutation has been observed in VISA.[1]

Post translational modification: The protein phosphorylation processes, particularly by Stk1/Stp1, a two-part global regulatory system, has been described to have important roles in *S. aureus*. Mutations in stp1 and clpP have been described to have on vancomycin resistance through downstream elements through the phosphorylation of downstream elements including vraR, graR, sarA, mgrA, sarZ, spoVG, luxS, and purA.

Table 1.4 Experimentally verified mutations associated with vancomycin intermediately resistant *S. aureus*

Target	Functional category	Mutation Sites	Vancomycin MIC (μg/mL) changes
VraTSR	Transcriptional regulation	VraS-S329L	1 → 2
		VraT-Y220C	3 → 1.5
		VraS-234Δ	1 → 3
		VraS-L114S+D242G	1.5 → 4
GraSR	Transcriptional regulation	GraS-T136I	2 → 6
		GraR-N197S	2 → 4
		GraS-T136I	1.5 → 2
WalKR	Transcriptional regulation	WalK-G223D	1.5 → 3
		WalR-K208R	1.5 → 4 or 4 → 1.5
		WalK-ΔQ371	1.5 → 3
		WalK-G223D	2 → 4
ClpP	Post translational modification	ClpP-ΔN	1.5 → 2
Stp1	Post translational modification	Stp1 deletion	1.5 → 3
		Stp1-E18D19 duplication	6–8 → 3

Table 1.4 (continued) Experimentally verified mutations associated with vancomycin intermediately resistant S. aureus

Target	Functional category	Mutation Sites	Vancomycin MIC (μg/mL) changes
Cmk	Cell metabolism	Cmk-A20G, CmK-T(-13)A	2 → 8, 3 → 8
		Cmk-A20G, CmK-T(-13)A	8 → 2, 8 → 3
VraS+GraR	Transcriptional regulation	VraS-I5N+GraR-N197S	4 → 6
GraS+WalK	Transcriptional regulation	GraS-T136I+ WalK-G223D	1.5 → 4
GraR+RpoB	Transcriptional regulation	GraR-N197S+RpoB-H481Y	2 → 6
WalK+ClpP	Transcriptional regulation Post translational modification	WalK-ΔQ371+ ClpP-ΔN	1.5 → 4
VraS+Stp1+YjbH	Transcriptional regulation Post translational modification	VraS-G45R+Stp1-Q12ΔC+YjbH-K23ΔC	2 → 4
VraS+GraR+RpoB+Fdh2+Sle1+MsrR	Cell wall synthesis and remodeling Transcriptional regulation Post translational modification	VraS-S329L+GraR-N197S+RpoB-H481Y+Fdh2-A297V+Sle1-Δ67aa+MsrR-E164K	1 → 12

Note: Table was adapted from Hu et al. Front. Microbiol 7:1601, 2016.

Outcomes associated with hVISA

While the effect on mortality is controversial, hVISA has been associated with poorer outcomes in patients. In a meta-analysis comprised of eight studies, Van Hal and Paterson noted an increased likelihood of treatment failure in patients with hVISA.[3] See figure 1.1

Figure 1.1 Association of hVISA and treatment failure

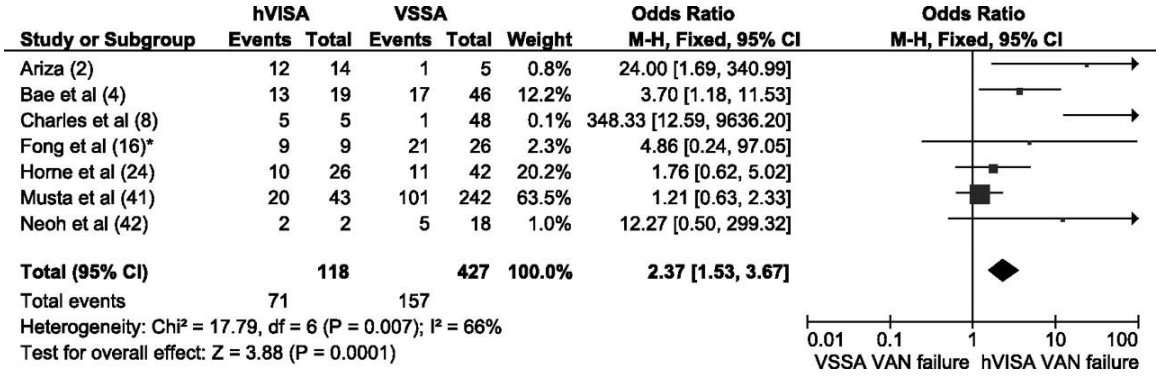


Figure adopted from Van Hal and Paterson. Antimicrob. Agents. Chemother. Dec 2010, 55 (1) 405-410.

Further, Casapao et al suggested a substantial increase in the odds of treatment failure (OR= 11.138) among patients experiencing blood stream infections due to hVISA compared to VSSA.[2]

The application of genome-wide association studies to identify variants associated with vancomycin non-susceptibility

Genome wide association studies (GWAS) are a hypothesis free method that can test hundreds of thousands of genetic variants across a genome to identify alleles that are associated with a specific phenotype.[42] This method is unique in that it can evaluate the full range of genetic variants that are found in a given phenotype, for example vancomycin intermediate susceptibility, and does not rely on targeting candidate genes. By comparing genetic variants found in VISA strains to susceptible isolates, GWAS has the potential to identify new single nucleotide polymorphisms (SNPs) that are associated with VISA.

Studies have demonstrated links between single SNPs and vancomycin resistance, however these SNPs are often identified in a laboratory environment from induced VISA strains and may differ from mutations observed *in vivo*. Other studies have compared genomes prior to and after vancomycin exposure or periodically throughout the course of treatment.[43]

In 2007, Mwangi et al. conducted a study to track genetic changes that lead to vancomycin resistance.[43] This study involved the serial collection of MRSA isolates from the bloodstream of a patient with endocarditis. The patient was treated with vancomycin, rifampin, and imipenem. Samples were taken at nine time points before being sequenced. A total of 35 mutations were identified over the course of the study and included mutations in the operon encompassing the vraR gene. Sequencing also identified

mutations in the agrC gene, and yycH gene. Mutations in these genes have been associated with increased vancomycin MICs and the VISA phenotype. Subsequently, the MIC increased from an MIC of 1μg/mL (susceptible) to 8μg/mL (intermediate) over the course of treatment before the patient ultimately experienced treatment failure. This study identified the multifactorial and sequential nature of VISA development and suggests that mutations differ based on exposure. Further, it provides support for the use of whole genome sequencing studies to identify antimicrobial resistance markers in patients. While this study is unique in following the development of resistance *in vivo*, it is limited in that it followed only one patient.

GWAS offer a means to study a multitude of samples in order to identify mutations associated with a particular phenotype such as vancomycin resistance. A study conducted in 2014 sought to identify genetic variants leading to vancomycin intermediate susceptibility through GWAS. This study evaluated 75 strains of which 26 were typed as VISA by Etest and a total of 33 strains were typed as hVISA by PAP-AUC. This study revealed a strong association between mutations in the rpoB H481 locus and increased MICs. Mutations at this site were not found in all VISA strains. No other candidate genes or variants reached the significant threshold in this study.[44]

Another study conducted in 2015 analyzed a subset of 24 isolates with known vancomycin resistance. While the initial results of this GWAS did not identify any statistically significant SNPs, mutations in the rpoB gene demonstrated the lowest p-value. Additionally, the study identified multiple SNPs in the walKR operon. The researchers then conducted a modified GWAS in which protein coding sequences

containing SNPs across a given MIC range were compared. In this analysis, the walR and walk genes were highly associated with vancomycin MIC.[45]

While these studies demonstrate the potential of GWAS to identify SNPs associated with vancomycin intermediate resistance and may serve as a benchmark for future studies, they also highlight the need for studies with greater sample sizes to detect less common genetic variants. Mutations in genes such as clp, stpP1 and Clk for example have been experimentally shown to affect vancomycin MIC when mutated, but were not detected by these studies**.**

CHAPTER 2
INTRODUCTION OF SPECIFIC AIMS

Aim 1: Describe the epidemiology of hVISA and VISA among a collection of clinical isolates

Sub aim a. Identify the overall prevalence of hVISA/ VISA among a diverse collection of clinical isolates

Sub aim b. Determine the association of MIC and prevalence of hVISA among a collection of clinical isolates

Aim 2: Characterize the genetic differences between vancomycin susceptible and non-susceptible clinical isolates.

Sub aim 2a. Describe the molecular epidemiology of hVISA/VISA among a collection of clinical isolates

Sub aim 2b. Identify SNPs in candidate genes associated with hVISA/VISA among a diverse collection of isolates

Sub aim 2c: Identify SNPs associated with evolutionary progression of hVISA to VISA among individual colonies of an hVISA strain

CHAPTER 3

SPECIFIC AIM 1: DESCRIBE THE EPIDEMIOLOGY OF HVISA AND VISA AMONG A COLLECTION OF CLINICAL ISOLATES

Introduction

Infections due to hVISA have been associated with poorer outcomes including persistent infection and prolonged bacteremia.[2] Reported prevalence varies considerably based on location and the variety of testing methods further complicates epidemiological studies. Therefore this study aimed to employ a series of phenotypic tests including the standard PAP-AUC in order to gain further insight into the prevalence of hVISA in the United States. The results of this study are important in understanding the clinical impact of hVISA.

Materials and methods

Isolates

Isolates were collected from multiple sources including a previous study of purulent skin infections in the South Texas area, the Network of Antimicrobial Resistance in *S. Aureus* as well as clinical specimen repositories from Seton hospital, TX, San Leandro Hospital, CA and Highland Hospital, CA.

Isolates were grown blood agar plates (Hardy Diagnostics, Santa Maria, CA) and incubated for 24 hours at 36^0 C. Isolates were then streaked for isolation on additional

blood agar plates which were subsequently incubated for 24hours. Morphology was assessed and agglutination tests were performed on individual colonies to assess purity. After the second incubation, isolates were sub cultured into Brain heart infusion broth (Hardy Diagnostics, Santa Maria, CA) and grown in a shaking incubator overnight (18-24hrs) at 36^0C.

BHI agar plates

Plates used for the BHI agar screen and PAP-AUC were hand poured at a concentration of 49.7 grams per liter of filtered water. After being dissolved in water, the mixture was autoclaved for 15 minutes. The mixture was then cooled to 55^0C before vancomycin was added. Vancomycin (Sigma Aldrich, St. Louis, MO) was added to form concentrations of 0.5, 1, 2, 2.5 and 4ug/mL for PAP-AUC plates and 3µg/mL for BHI agar screen plates. Approximately 18-23 mL of molten agar was then poured into 100mm sterile polystyrene petri dishes (Fisher, Pittsburg, PA). Plates were allowed to cool before storing at 40ºC. For each batch, remaining agar as well as a sample plate was incubated overnight to ensure that agar was free of contaminants.

Etest

Etests were performed on Mueller Hinton Agar (Hardy Diagnostics, Santa Maria, CA) using vancomycin Etest strips (bioMérieux, Durham, NC) according to the manufacturer's instructions. Specifically, inoculum was adjusted to 0.5 McFarland standard concentration and streaked onto agar plates using sterile cotton swabs. A single

Etest strip was then placed in the center and the plate was incubated at 36^0 C for 16-20hrs. MICs were determined by observing the intersection between the test strip and the area of inhibited growth. Isolates were then classified into the following breakpoints by MIC; susceptible (MIC ≤ 2μg/mL), intermediate (2μg/mL <MIC < 4μg/mL) or resistant (MIC >4 μg/mL).

BHI agar screen

Isolates with MICs falling within the susceptible range (≤2 μg/mL) were screened for hVISA using vancomycin impregnated agar plates. Overnight cultures were adjusted to 0.5 McFarland and four 10μL droplets were placed on the agar. A positive test was defined as growth within any of the four droplets after 48 hours of incubation at 36^0C.

PAP-AUC

Isolates which grew on vancomycin impregnated BHI agar were selected for testing using the population analysis area under the curve. Isolates were sub cultured into Tryptic soy broth (Hardy Diagnostics, Santa Maria, CA) and grown in a shaking incubator overnight (18-24hrs). Isolates were vortexed and diluted to a concentration equal to that of a latex 0.5 McFarland Standard (Hardy Diagnostics, Santa Maria, CA).

Four inoculums were plated for each isolate and included cell densities of 1.0 X 10^8, 1.0 X 10^7, 1.0 X 10^6 and 1.0 X 10^5 CFU/mL. The 1.0 X 10^8 inoculum was formed by vortexing the overnight culture and diluting the isolates to a concentration equal to that of

a latex 0.5 McFarland Standard. Serial 10-fold dilutions using 1x Sterile Phosphate buffered saline (Thermo Fisher, Waltham, MA) were used to form the remaining dilutions.

For each isolate, all four inoculums were spiral plated (Spiral Biotech, Norwood, MA) on agar plates containing each vancomycin concentration. Prior to spiral plating, the spiral plate stylus was cleaned in 5% sodium hypochlorite once and twice in filtered water. For each plate, 100uL of inoculum was aspirated and spiral plated. Inoculums were plated in increasing concentrations, beginning with 1.0×10^5 CFU/mL to reduce the effects of microbial carryover. Further, the stylus was cleaned in between concentrations and in between isolates. Plates were allowed to dry briefly at room temperature before being placed into the incubator. Isolates were then incubated at 36^0C for 48 hours before being analyzed.

Bacterial counts were enumerated using the Interscience scan 300 (Woburn, MA). Parameters were adjusted to account for the volume plated as described in Table 3.1. The phenotype identification workflow is outlined in figure 3.1.

Figure 3.1 HVISA phenotype determination workflow

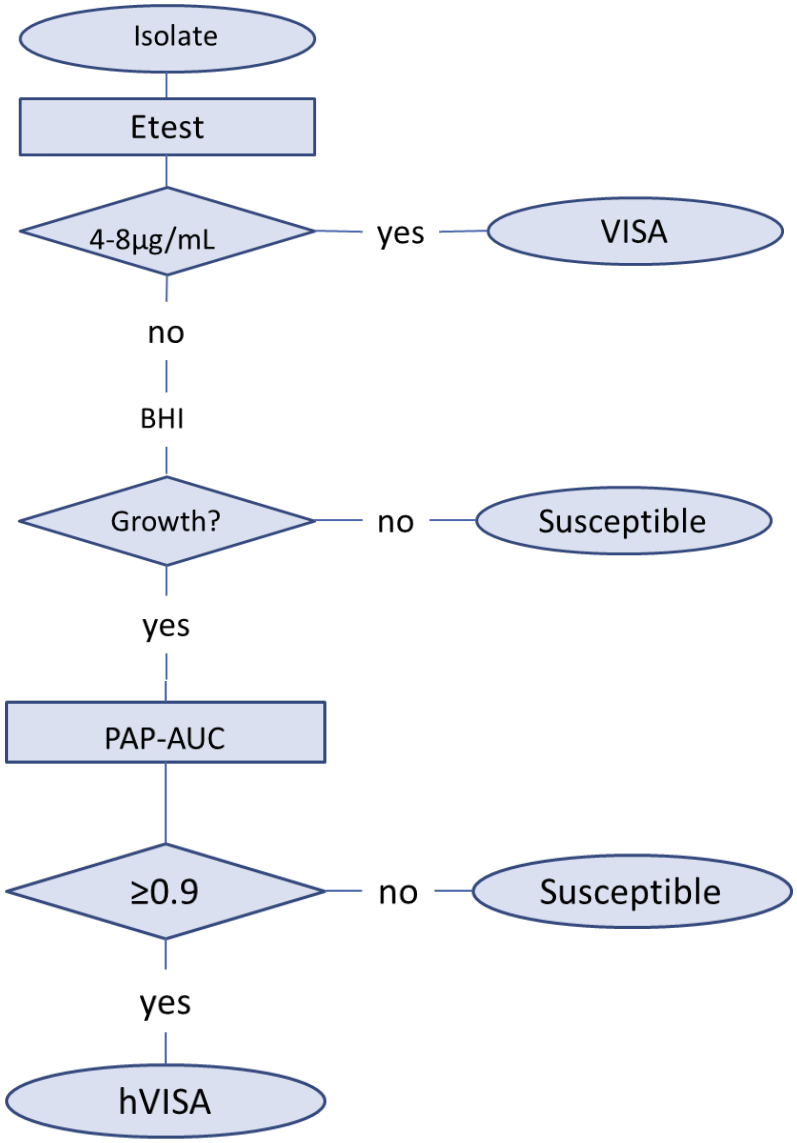
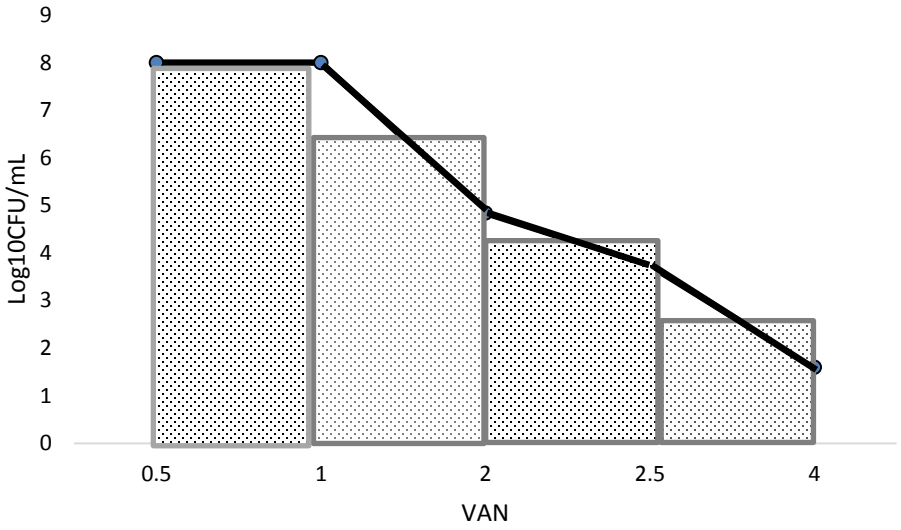


Table 3.1 Volume of inoculum deposited during spiral plating of PAP-AUC plates

Segment Pair	Volume deposited (µL)
3c	0.58
3b	2.36
3a	5.04
4c	9.14
4b	15.06
4a	25
Total	100

The above parameters, dilution factor and visible colonies were used to calculate the bacterial growth expressed in CFU/mL. After recording, the log CFUs/mL were plotted against vancomycin concentration to determine the area under the curve. The area under the curve was calculated using the trapezoidal rule described in Figure 3.2.

Figure 3.2 Trapezoidal rule formula used to calculate AUC



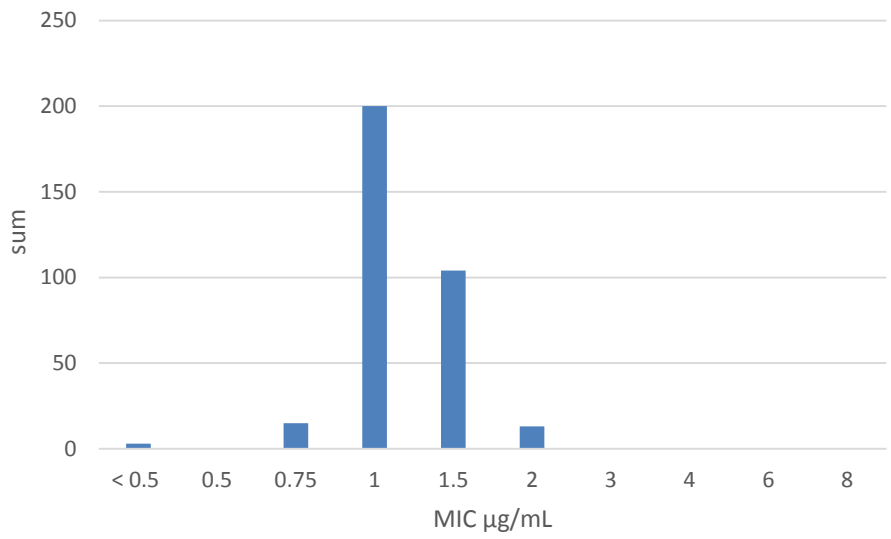
$AUC = \Sigma[(Log_{10}\ CFU/mL_{0.5} + Log_{10}\ CFU/mL_{1})/2]\ W_{0.5\text{-}1} + [(Log_{10}\ CFU/mL_{1} + Log_{10}\ CFU/mL_{2})/2]\ W_{1\text{-}2} + [(Log_{10}\ CFU/mL_{2} + Log_{10}\ CFU/mL_{2.5})/2]\ W_{2\text{-}2.5} + [(Log_{10}\ CFU/mL_{2.5} + Log_{10}\ CFU/mL_{4})/2]\ W_{2.5\text{-}4}$

The Mu3 reference strain (ATCC 700698) was spiral plated along with each batch of isolates. After calculating the AUC for each isolate, the AUC was compared to that of the reference strain. A ratio of the sample AUC to the reference AUC of 0.9 or greater indicated a positive result for hVISA.

Results

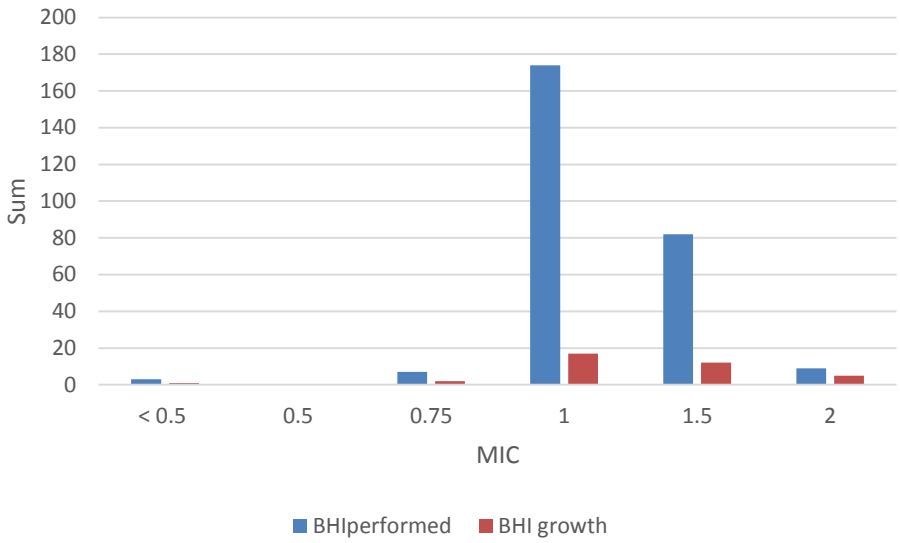
MICs were performed on a total of 335 isolates. Of these, 3 had an MIC below 0.5µg/mL (0.89%), 0 had an MIC of 0.5 µg/mL (0%), 15 had an MIC of 0.75 µg/mL (4.48%), 200 had an MIC of 1 µg/mL (59%), 104 had an MIC of 1.5 µg/mL (31%) and 13 had an MIC of 2ug/mL (3.89%). See Figure 3.3

Figure 3.3 Distribution of MIC among clinical isolates



BHI agar screens were performed on 275 of the 335 isolates to identify hVISA. A total of 37 isolates grew on vancomycin impregnated BHI agar of which 1 had an MIC less than 0.5 µg/mL (33%), 2 had an MIC of 0.75 (13%), 17 had an MIC of 1 (8.5%) µg/mL, 12 had an MIC or 1.5 µg/mL (11.5%) and 5 had an MIC of 2 µg/mL (38%). See figure 3.4.

Figure 3.4 Growth of isolates on vancomycin impregnated BHIA



The population analysis profile area under the curve was performed on the 37 isolates that grew on BHI agar. In total, nine isolates were positive. Of these isolates, one had an MIC of 1 µg/mL (11%), four had an MIC of 1.5 µg/mL (44%) and four had an MIC of 2 µg/mL (44%). Overall, isolates across the entire range of the susceptible break point displayed resistant populations as evidenced by growth on BHI agar containing 3 µg/mL vancomycin, growth on vancomycin impregnated agar was more commonly observed among isolates with higher MICs ranging from 1.5-2 µg/mL. Further, the frequency of hVISA isolates as identified by PAP-AUC was highest among isolates with an MIC of 2 µg/mL. See figure 3.5 for population analysis curves.

Figure 3.5 Population analysis profiles of isolates undergoing PAP-AUC

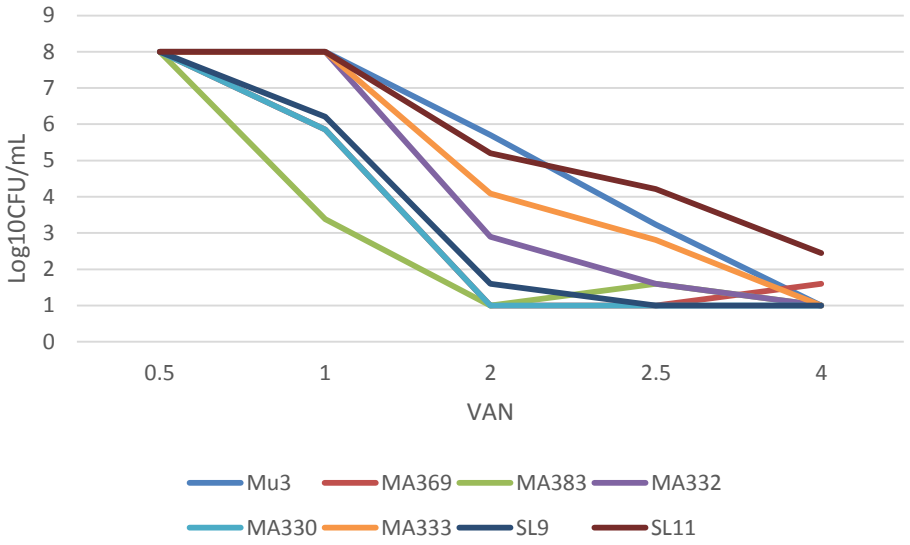


Figure 3.5 (continued) Population analysis profiles of isolates undergoing PAP-AUC

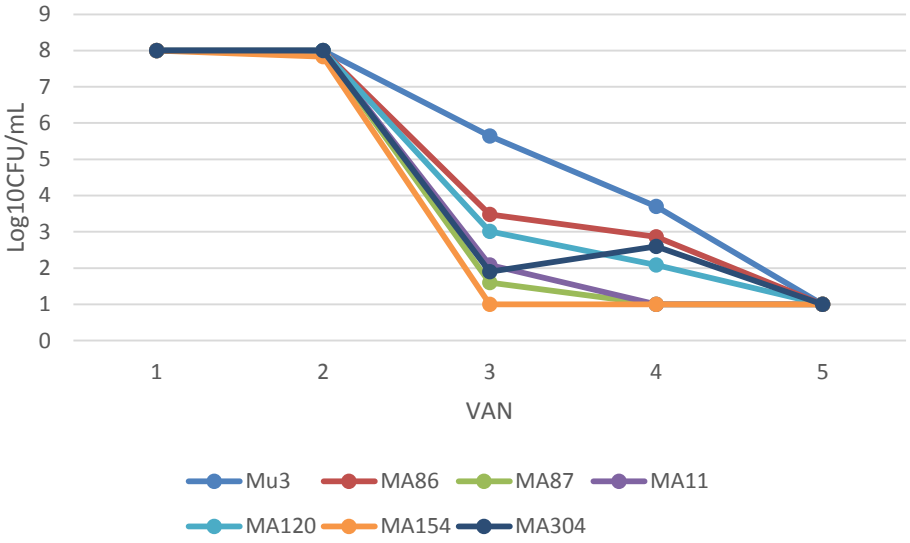


Figure 3.5 (continued) Population analysis profiles of isolates undergoing PAP-AUC

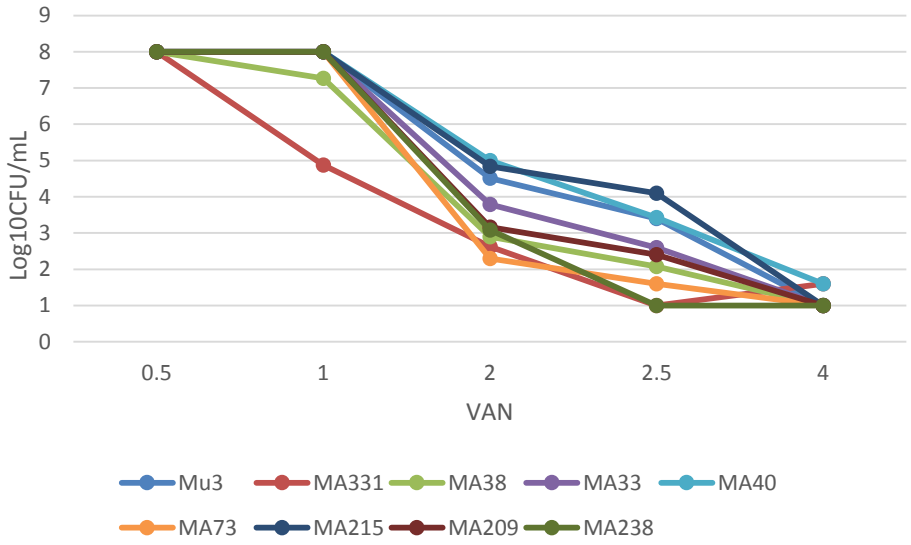


Figure 3.5 (continued) Population analysis profiles of isolates undergoing PAP-AUC

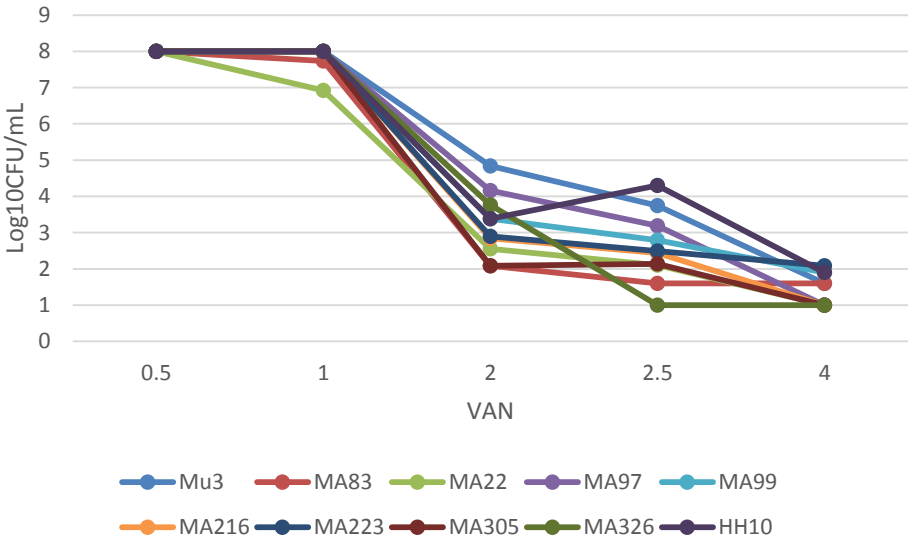


Figure 3.5 (continued) Population analysis profiles of isolates undergoing PAP-AUC

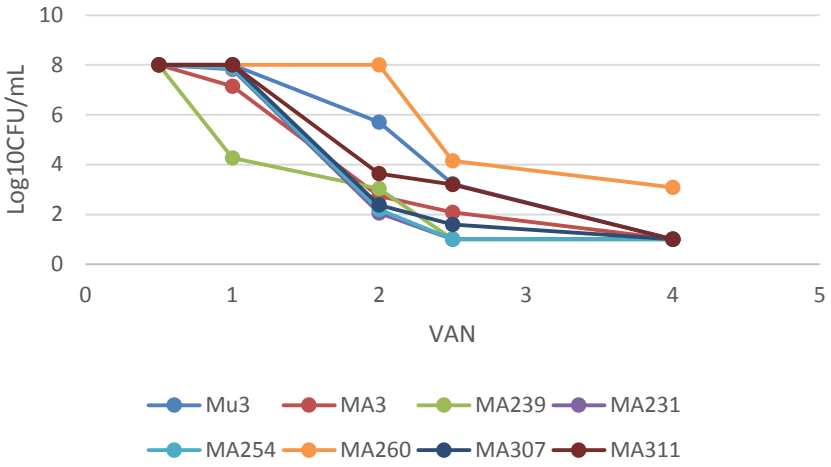
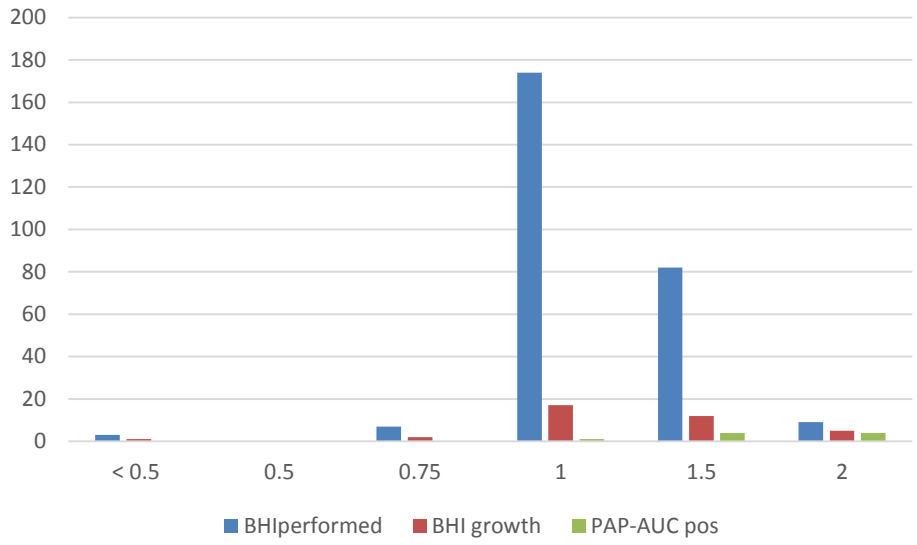


Table 3.2 AUC ratios of isolates in population analysis

Isolate	AUC	Reference	Ratio
MA369	9.34	16.26	0.57
MA383	7.63	16.26	0.47
MA332	12.54	16.26	0.77
MA330	8.89	16.26	0.55
MA333	13.82	16.26	0.85
SL9	9.61	16.26	0.59
SL11	17.95	16.26	1.10
MA304	12.64	17.24	0.73
MA120	13.09	17.24	0.76
MA154	10.38	17.24	0.60
MA11	11.31	17.24	0.66
MA86	14.22	17.24	0.82
MA87	10.95	17.24	0.64
MA331	9.82	13.74	0.71
MA38	12.46	13.74	0.91
MA33	14.72	13.74	1.07
MA40	16.39	13.74	1.19
MA73	10.78	13.74	0.78
MA215	16.48	13.74	1.20
MA209	13.52	13.74	0.98
MA238	12.06	13.74	0.88
MA83	12.16	16.58	0.73
MA22	11.96	16.58	0.72
MA97	15.06	16.58	0.91
MA99	14.75	16.58	0.89
MA216	14.29	16.58	0.86
MA223	14.23	16.58	0.86
MA305	12.45	16.58	0.75
MA326	12.57	16.58	0.76
HH10	16.27	16.58	0.98
MA3	12.25	16.26	0.75
MA239	9.23	16.26	0.57
MA231	11.17	16.26	0.69
MA254	11.30	16.26	0.69
MA260	20.46	16.26	1.26
MA307	12.14	16.26	0.75
MA311	14.69	16.26	0.90

Ratio values of ≥0.9 indicate positive test

Figure 3.6 Distribution of hVISA as determined by BHIA and PAP-AUC



Discussion and Limitations

The prevalence of hVISA among *S. aureus infections* remains unclear largely due to the wide range of reported frequency among studies and multiple techniques used to identify the phenotype. Additionally, while the prevalence of hVISA seems to vary by geography, studies have reported differences in prevalence even within the same country. In a pair of studies conducted in the US for example, Casapao et al reported a prevalence of 18.8% when utilizing the PAP-AUC while Reichter et al reported a lower prevalence of 1.2% despite using the same test. The wide variation in reported prevalence warrants further clarification to elucidate the impact of hVISA related infections.

In this study the PAP-AUC was utilized to confirm the hVISA phenotype among candidate isolates identified by screening with vancomycin impregnated BHI agar. Among the 275 isolates screened, 37 were subjected to PAP-AUC and 10 had a growth ratio of 0.9 or greater when compared to the reference. While some studies reported substantially higher frequencies (eg Park et al, Korea), the prevalence reported in this study (3.27%) fell within the range of US studies conducted by Casapao and Reichter. Further, this study demonstrated a notable difference in frequency of hVISA based on MIC and suggests that hVISA is more common in isolates with an MIC of 2µg/mL. While only nine isolates displayed an MIC of 2µg/mL, four of these isolates were hVISA. Therefore while isolates with an MIC of 2, accounted for only 3.27% of the isolates used in the study, isolates with an MIC of 2 accounted for nearly half (44%) of all hVISA isolates identified. Although hVISA was most common in isolates with an MIC of 2, it should be noted that one hVISA isolate was identified by PAP-AUC among

those with an MIC of one and several isolates with MICs of one or below grew on vancomycin impregnated agar, suggesting that isolates with low MICs may still contain resistant sub populations.

This study was limited in by multitude of tests used to identify the hVISA phenotype. The hVISA phenotype was identified using Etests, BHI agar screens and PAP-AUCs in a stepwise fashion opening up the possibility for inconsistencies in cell viability, growth conditions and inoculums between tests. Further, since the BHI agar was used to screen for potential hVISA isolates, the PAP-AUC was not performed on all isolates and any false negatives occurring during the BHI agar test would lead to an isolate being classified as VSSA. Finally PAU-AUC tests were conducted in batches, so individual isolates may have been exposed to slight variation in agar or other growth conditions (humidity, growth temperature etc.)

To address these limitations, Etests and BHI agar screens were performed together to ensure no variation in isolates between tests. Although the PAP-AUC was conducted at a later date, isolates were grown from fresh frozen stock before each test. While the use of a screening method has the potential to miss classify isolates due to false negatives, this study employed a highly sensitive modified BHI agar test to maximize the number of isolates subjected to PAP-AUC. Multiple studies have utilized a similar agar dilution method to identify hVISA as well as VISA isolates while adjusting vancomycin concentration. A study conducted by Satola et al demonstrated a sensitivity of 91% when using a BHI agar screen.[33] In their study, BHI agar was impregnated with 4μg/mL vancomycin and a positive result was defined as growth of at least two colonies in one of

four 10μL droplets. To minimize the potential for false negatives, this study used a vancomycin concentration of 3μg/mL and all isolates displaying growth were selected for the PAP-AUC regardless of the number of colonies present. Though false negatives were likely reduced by employing these modifications, false positives likely increased as well. To further ensure that hVISA isolates were being identified, BHI screens were performed on 27 hVISA and VISA isolates. In all cases, growth was observed. Lastly, while the PAP-AUC was performed in batches, incubation time and temperature were held constant. Further, agar plates were poured before each run to ensure that drug concentrations and nutrients were not altered by prolonged storage. Finally the reference strain Mu3 was used in conjunction with each batch to ensure that the isolates and reference were subjected to the same conditions.

CHAPTER 4

SPECIFIC AIM 2: CHARACTERIZE THE GENETIC DIFFERENCES BETWEEN VANCOMYCIN SUSCEPTIBLE AND NON-SUSCEPTIBLE ISOLATES

Sub aim 2a. Describe the molecular epidemiology of hVISA/VISA among a collection of clinical isolates

Sub aim 2b. Identify SNPs in candidate genes associated with hVISA/VISA among a diverse collection of isolates

Sub aim 2c: Identify SNPs associated with evolutionary progression of hVISA to VISA among individual colonies of an hVISA strain

Introduction

Due to the low frequency of resistant populations, and the propensity for colonies to exhibit reduced growth rates, hVISA is undetectable by conventional susceptibility testing methods such as broth microdilution or E-test. Further, while methods such as the macromethod E-test and teicoplannin disk diffusion test have been used to identify hVISA, studies have shown discrepancies of the prevalence of hVISA based on testing method. Further, the gold standard PAP-AUC requires growth on a larger number of agar plates, and a prolonged incubation. The cost, labor, and delayed results associated with this method severely limit its utility as a diagnostic tool in the clinical setting. Further, studies have shown poorer outcomes in patients suffering from hVISA infections.[2,3] The difficulty identifying these infections combined with their clinical implications, highlight the importance of developing novel methods to predict hVISA. The development of rapid diagnostic platforms has allowed for the use of molecular markers to identify pathogens in a given infection. The use of GWAS offers the potential to identify markers that suggest antimicrobial resistance. Such information, combined with the speed of modern diagnostic platforms can lead to rapidly tailored therapy, reduced hospital length of stay and improved clinical outcomes. Additionally WGS can have a profound impact on the surveillance of hVISA by tracking the multi locus sequence types of these infections. These factors may in time aid clinicians in identifying and monitoring the spread of hVISA infections. This study therefore aimed to compare the MLSTs of VSSA and hVISA isolates. Genome wide association was used to identify SNPs associated with hVISA among a group of VSSA and hVISA isolates while a comparative genomics

approach was employed on a subset of matched isolates as well as hVISA colonies with and without vancomycin exposure to identify SNPs unique to hVISA.

Materials/Methods

DNA extraction

Samples were incubated in lysis buffer for 30 min at 37^0C then subjected to mechanical bead beating (6 m/s for 2 minutes) using the MP fast prep homogenizer (MP biomedicals Salon, OH) and 0.7mm garnet beads. DNA was then be column purified using the Qiagen DNeasy Powerlyzer Power soil kit (Germantown, MD). DNA concentration was quantified using the Qubit 2.0 fluorometer (Thermofisher Waltham, MA) and quality was assessed using the Nanodrop 1000 (Thermofisher Waltham, MA).

Whole genome sequencing and variant calling

Sequencing was conducted at the Genome Sequencing Center at UT Health San Antonio. Sequencing libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina Inc., San Diego, CA) per manufacturer's instructions and sequenced on the Illumina Nextseq (Illumina Inc., San Diego, CA) with 2x100-base paired end reads. Data were analyzed using CLC Genomics Workbench (Qiagen, Redwood City, CA). Poor quality reads (≥ 2 ambiguous bases or phred quality score less than 20) were filtered out. Paired-end reads were assembled into contigs using Spades v3.9.0, and contigs annotated using Prokka v1.12. Reads were mapped to the N315 genome. SNPs were detected using the fixed ploidy variant detection tool and the structural variant tool were used to detect insertions and deletions (Indels). A SNP or indel were considered valid if that position contains at least 15-high quality reads and ≥ 90% support and alternate allele from the reference.

Phylogenetic analysis

Whole genome-mapped sequences and concatenated core genome sequences were used for phylogenetic analyses. Phylogeny were inferred by maximum likelihood using RAxML using a general time-reversible nucleotide substitution model with 500 bootstrap support. Individual strains were partitioned into clusters based on multiple runs of the estimation algorithm. A subset of isolates were selected for Multilocus sequence typing (MLST). Isolates were prioritized based on MIC and growth on BHIA. The characteristics of isolates undergoing MLST are summarized in Table 4.1. MLST was derived by mapping reads against the *S. aureus* MLST scheme.

Table 4.1 Characteristics of WGS clinical isolates

Characteristic	N=103
Year	
2007	17
2008	38
2009	23
2018	25
Location	
Texas	78
California	25
MIC	
≤1	36
1.5	87
2	9
BHIA	
pos	37
neg	66
Phenotype	
VSSA	93
hVISA	10

Identification of Variants in Candidate Genes Associated with hVISA/VISA

We employed a targeted approach to evaluate the presence of experimentally validated SNPs described in table 1.4 and a matrix of the presence and absence of genes and SNPs among the isolates was constructed.

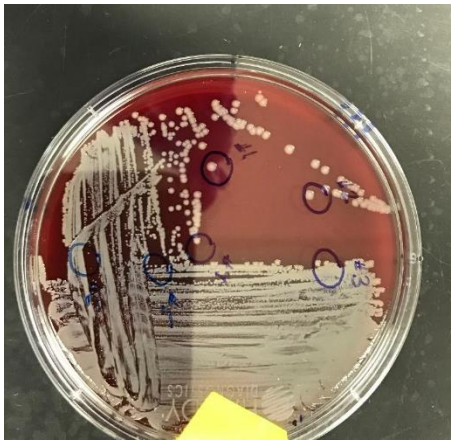
Genome wide association analysis was conducted to examine potentially novel or validate these prior experimentally validated SNPs. GWAS were conducted to identify genetic variants that may contribute to vancomycin non-susceptibility. The GWAS was used to identify SNPs that are associated with one of two phenotypes: vancomycin susceptible (VSSA) or vancomycin non susceptible (hVISA, VISA). All isolates used in this GWAS were ST5 and derived from one of four sources, clinical isolates described in Aim 1 (n=44), the Network on Antimicrobial Resistance in *S. aureus* (n=10), a previous study conducted by Alam et al (n=41) and the American Type Culture Collection (n=1).[44] These analyses were conducted using the CLC Genomics Workbench and Microbial Module and PLINK v1.9 software.[51]

A frequency cutoff for the occurrence of a polymorphism across the population of >90% and a minor allele frequency of >5% were applied. Significance levels were corrected for multiple tests using the Bonferroni's correction factor. The impact of the population structure in the reduction of false positive associations were estimated using the genomic inflation factor.

Evolutionary studies of hVISA to VISA

To better understand the heterogeneity of hVISA and the effect of colony selection on response to vancomycin we selected individual colonies from the Mu3 (ATCC 700698, Manassas, VA) isolate and exposed them to vancomycin. Mu3 was grown blood agar plates (Hardy Diagnostics, Santa Maria, CA) and incubated for 24 hours at 360^0C. It was then streaked for isolation on an additional blood agar plate which was subsequently incubated for 24 hours. Colony morphology was assessed and a plate streak was transferred to a tube containing BHI broth. Additionally six colonies were selected and transferred into individual tubes containing BHI. Tubes were placed in a shaker and incubated at 36^0C for 48 hours. After 48hours, tubes were removed and used to perform Etests as described in previously. Further, aliquots were transferred into a fresh tube of BHI to reach a McFarland standard of 0.5. Vancomycin was added into each tube at a concentration of 1μg/mL before incubation for 48hours at 36^0C. After 48 hours, tubes were removed and Etests were performed. Aliquots were transferred in a stepwise fashion to fresh tubes containing BHI and vancomycin at concentrations of 1.5,2, 2.5,3,3.5,4,4.5,5.5,6,7,7.5 and 8μg/mL. After exposure to each successive vancomycin concentration, Etests were performed to track the development of resistance. Further, aliquots were removed, pelleted and stored for DNA extraction.

Figure 4.1 Morphology of Mu3 colonies selected prior to vancomycin exposure



Results Sub aim 2a. Describe the molecular epidemiology of hVISA/VISA among a collection of clinical isolates

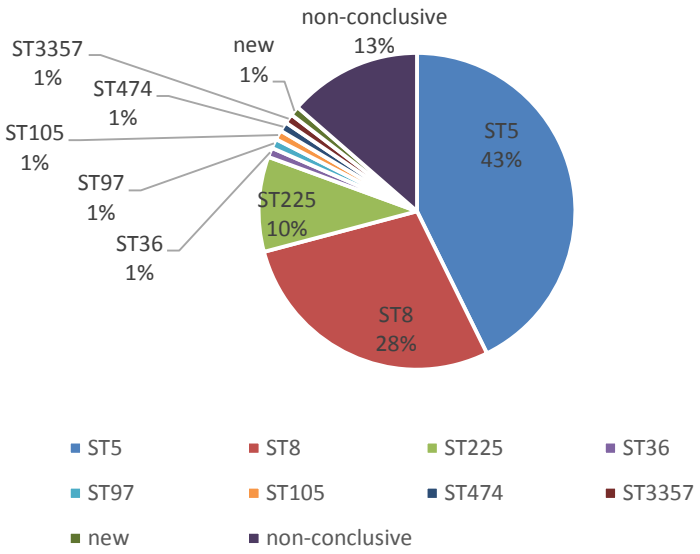
Multi locus sequence typing was performed on 103 clinical isolates. Overall, a total of eight distinct MLSTs were identified including ST5 (n=44, 43%) ST8 (n=29, 28%), ST225 (n=10, 10%), ST36 (n=14, 14%), ST97 (n=1, 1%), ST105 (n=1, 1%), ST474 (n=1, 1%) and ST3357 (n=1, 1%). Isolates were unidentified in a total of 14 (13%) isolates and labeled non-conclusive. Additionally one isolate (1%) displayed a potentially new MLST.

A total of 93 vancomycin susceptible isolates were classified by MLST. All eight MLSTs were observed among VSSA. ST5 was the most common sequence type (n=37, 47%) followed by ST5 (n=26, 33%). A single isolate of ST225 (1%), ST36 (1%) ST97 (1%) ST105 (1%) and ST3357 (1%) were observed. A total of 14 isolates (15%) were non-conclusive and one isolate (1%) displayed a potentially new sequence type.

MLSTs were identified in 10 isolates, of these, 7 isolates were ST5 (70%) and the remaining 3 (30%) were ST8. There were no unidentified or new MLSTs in this group.

Figure 4.2 A. Overal distribution of MLST types among clinical isolates(n=103). B. Distribution of MLST among VSSA isolates(n=93). C. Distribution of MLST among hVISA(n=10).

4.2A



4.2B

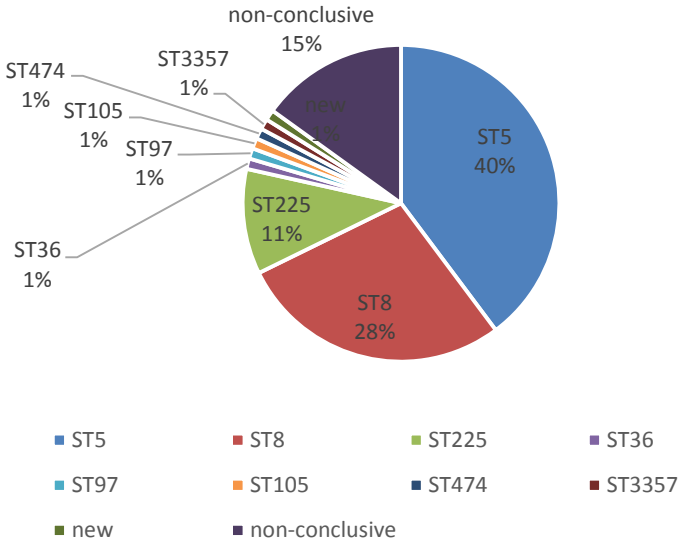
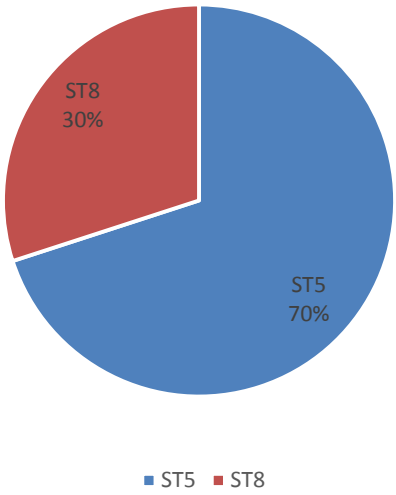


Figure 4.2 (continued) A. Overal distribution of MLST types among clinical isolates(n=103). B. Distribution of MLST among VSSA isolates(n=93). C. Distribution of MLST among hVISA(n=10).

4.2C



In addition to phenotype, isolates were grouped by MIC. Of the 103 isolates, 36 (35%) displayed an MIC of 1µg/mL or less. The most common sequence type in this group was ST and accounted for 15 (41%) of isolates. ST8 accounted for 9 (25%) isolates while ST225 accounted for 2 (5%) isolates. MLSTs 36, 105 and 3357 were each observed in one isolate (3%). One potentially new MLST (3%) was observed and 6 (17%) of isolates were unidentified.

Of the 103 isolates, 58 (56%) displayed an MIC of 1.5µg/mL. The most common sequence types in this group were ST5 (n=26) and ST8 (n=16) and accounted for 45% and 27% of isolates respectively. ST225 accounted for 7 isolates (12%) while ST97 and ST474 accounted for one isolate (2%) each. In this group, 7 isolates displayed inconclusive MLSTs and no new sequence types were identified.

A total of nine isolates (9%) displayed an MIC of 2µg/mL. Three MLSTs were observed in this group. ST8 was the most common sequence type observed and accounted for 4 isolates (45%), followed by ST5 (n=3, 33%) and ST225 (n=1, 11%). One isolate was inconclusive in this group.

Figure 4.3A Distribution of MLST among isolates with an MIC ≤ 1µg/mL (n=36) B. 1.5µg/mL (n=58) and C. 2µg/mL (n=9)

4.3A

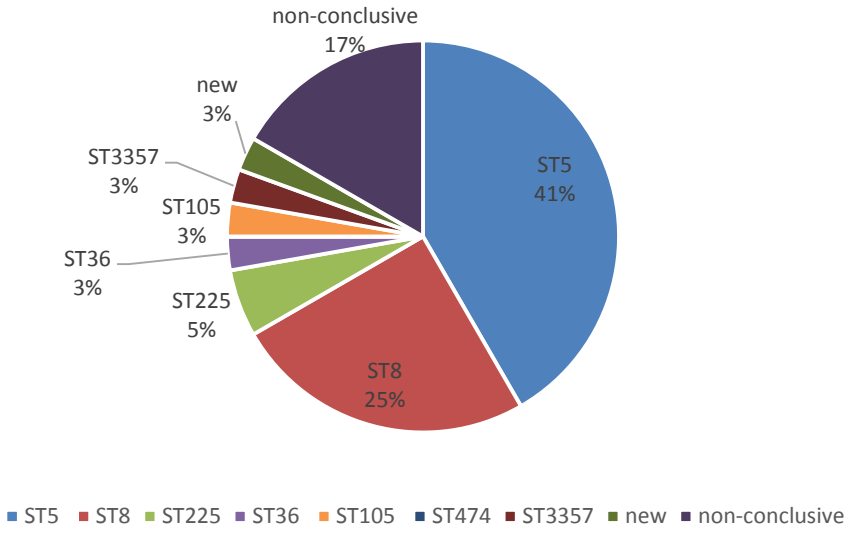
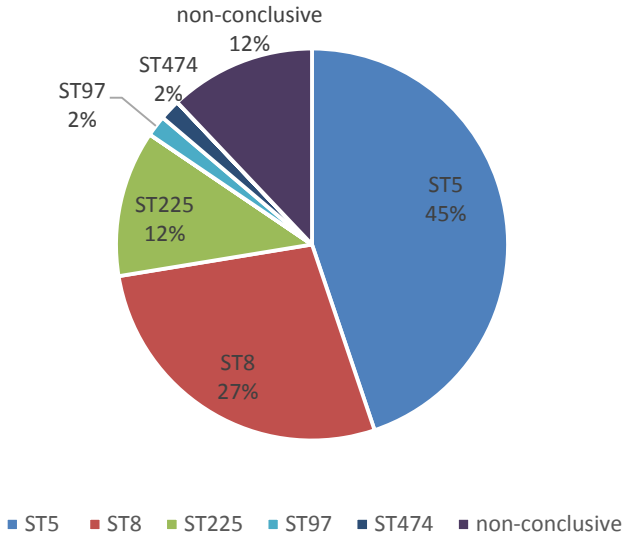
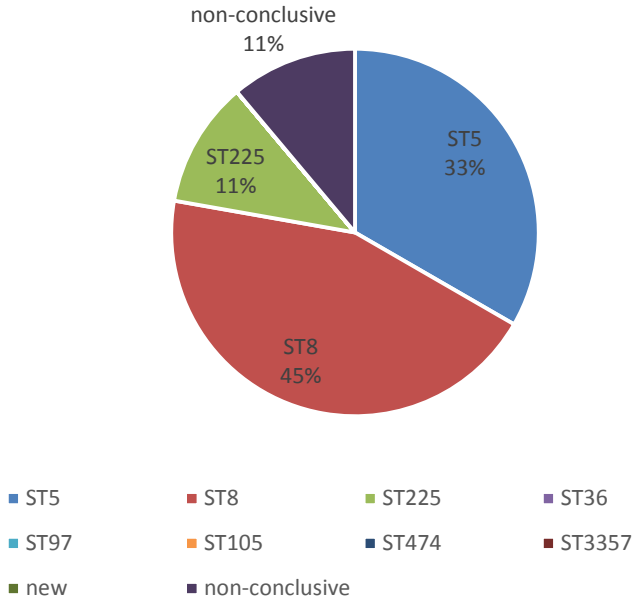


Figure 4.3 (continued) A. Distribution of MLST among isolates with an MIC ≤ 1µg/mL (n=36) B. 1.5µg/mL (n=58) and C. 2µg/mL (n=9)

4.3B



4.3C



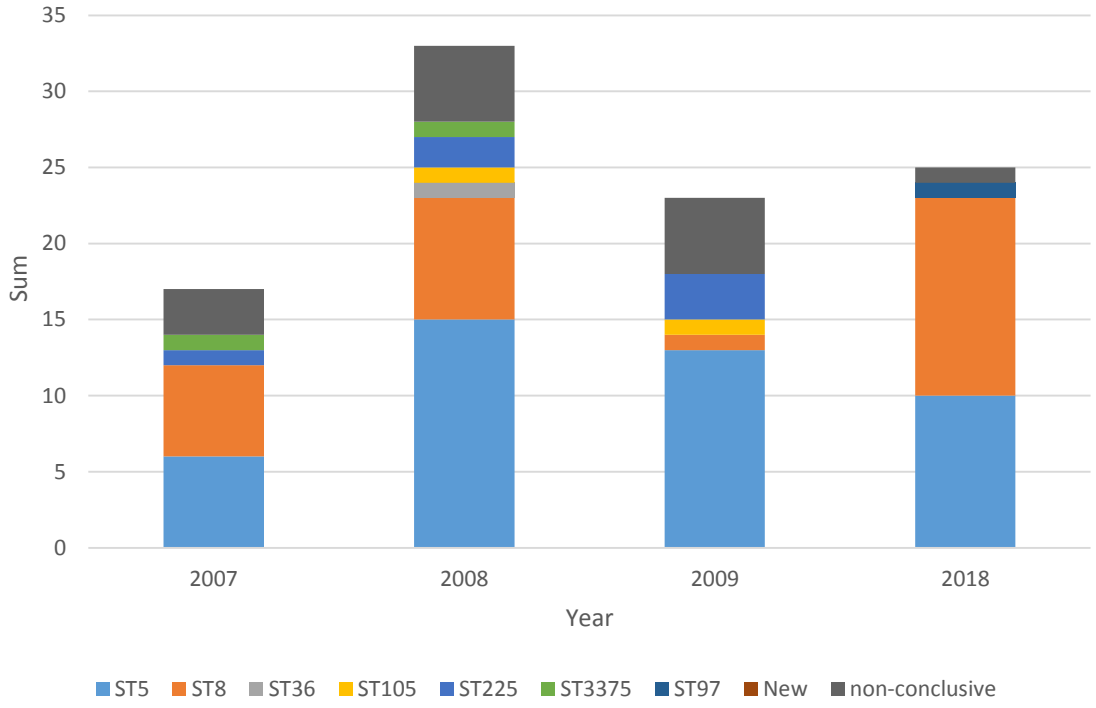
Isolates were also divided into four groups based on year (2007, 2008, 2009, 2018) of collection. Seventeen of the 103 isolates were collected in 2007 (17%). Of these, 6 isolates were ST5 (35%), 6 were ST8 (35%), one was ST225 (6%) and one was ST3375 (6%). Three isolates were inconclusive (18%) and no new MLSTs were identified.

Of the 103 isolates, 38 were collected in 2008 (37%). Of these, 15 isolates were ST5 (39%), 9 were ST8 (35%) and 6 were ST225 (16%). One ST36 (3%) and one ST474 (3%) were also observed. One isolate (3%) displayed a potentially new MLST and 5 isolates were inconclusive.

Of the 103 isolates, 38 were collected in 2009 (37%). In this group ST5 was the predominant sequence type and accounted for 13 isolates (57%). ST225 accounted for 3 isolates (13%) while ST8 (4%) and ST105 (4%) accounted for a single isolate each. There were no new sequence types observed and 5 (22%) were inconclusive.

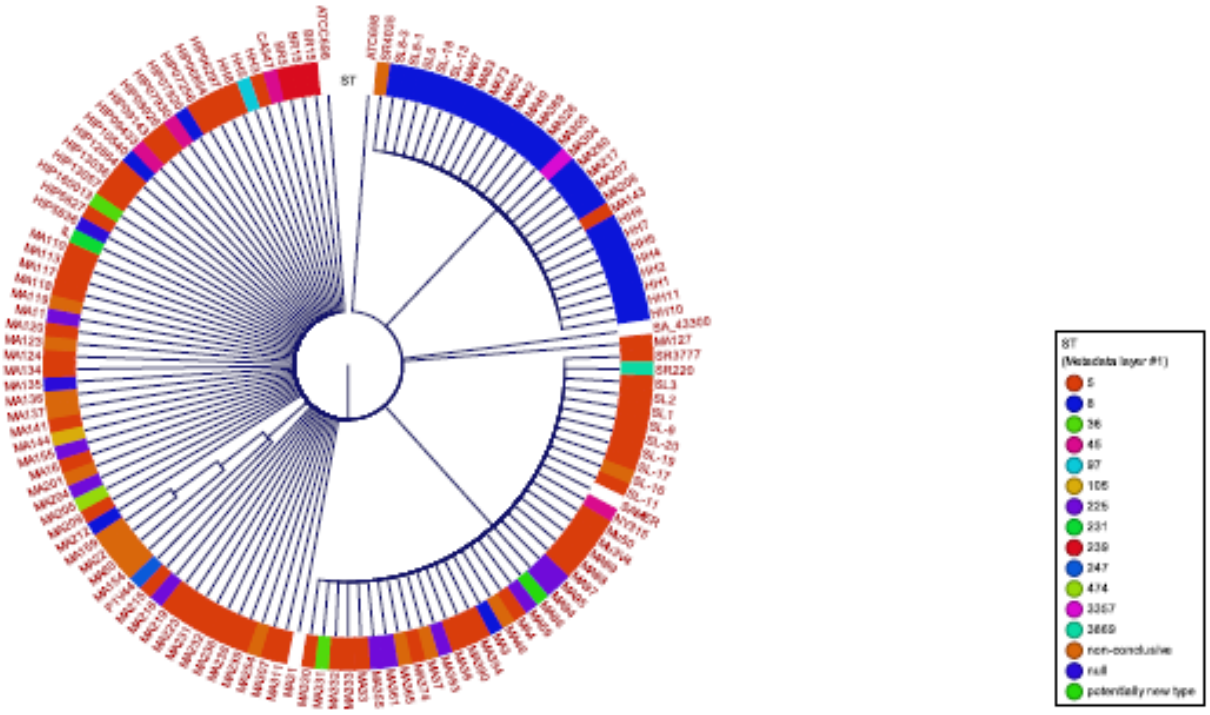
The remaining 25 (24%) isolates were collected in 2018. The majority of these isolates (n=13, 52%) were ST8. ST5 accounted for 10 isolates (40%) and ST97 accounted for one isolate (4%). One isolate was inconclusive (4%) and no new MLSTs were identified.

Figure 4.4 Distribution of MLST by year



Lastly, phylogenetic analysis demonstrated similarities among certain MLSTs especially ST8. These results suggest that ST8 isolates may harbor a similar a similar set of SNPs.

Figure 4.5 Phylogenetic tree demonstrating relationships based on SNPs.



Outer ring represents MLST

Results: Sub aim 2b. Identify SNPs in candidate genes associated with hVISA/VISA among a diverse collection of isolates

SNPs were manually identified in 23 candidate genes shown to be associated with hVISA including walR, walK, rpoB, graR, graS, clpP, stP, vraS , cmK, vraT, yvqF, clP, cmK, yjbH, cle, msrR, sarA, agrA, ccpA, prsA, vraR, vraF, and vraG. VSSA and hVISA isolates were matched by year, location, MIC and MLST to identify SNPs that were unique to hVISA. Growth on vancomycin impregnated agar was also considered, and when possible, VSSA isolates which did not grow on BHIA were prioritized to minimize the influence of resistant sub populations that may have been present in VSSA. Both MA98, and MA212 served as a match for multiple isolates. In the event that multiple VSSA isolates served as a suitable match for hVISA, a single isolate was chosen at random. A list of paired isolates and matching parameters is described in Table 4.2.

Table 4.2 Characteristics of matched isolate pairs used in comparative analysis

Pair	Isolate	Phenotype	year	location	MIC	BHIA	MLST
1	MA33	hVISA	2007	TX	1.5	pos	5
	MA4	VSSA	2007	TX	1.5	neg	5
2	MA38	hVISA	2007	TX	1	pos	5
	MA307	VSSA	2007	TX	1	pos	5
3	MA40	hVISA	2007	TX	2	pos	8
	MA212	VSSA	2008	TX	2	neg	8
4	MA97	hVISA	2008	TX	1.5	pos	5
	MA98	VSSA	2008	TX	1.5	neg	5
5	MA209	hVISA	2008	TX	1.5	pos	5
	MA98	VSSA	2008	TX	1.5	neg	5
6	MA215	hVISA	2008	TX	2	pos	5
	MA98	VSSA	2008	TX	1.5	neg	5
7	MA260	hVISA	2008	TX	2	pos	8
	MA212	VSSA	2008	TX	2	neg	8
8	MA311	hVISA	2007	TX	1	pos	5
	MA307	VSSA	2007	TX	1	pos	5
9	SL-11	hVISA	2018	CA	1.5	pos	5
	SL1	VSSA	2018	CA	1.5	neg	5
10	HH10	hVISA	2018	CA	2	pos	8
	HH7	VSSA	2018	CA	1.5	neg	8

Among the 7 VSSA isolates, SNPs were identified in 17 of the 21 candidate genes. A total of 149 synonymous SNPs were identified and were most commonly observed in the walK (2 isolates, 18 SNPs), rpoB (2 isolates, 12 SNPs), graS (2 isolates, 12 SNPs), vraS (2 isolates, 12 SNPs), slE (2 isolates, 16 SNPs), vraF (2 isolates, 17 SNPs) and vraG (2 isolates, 16 SNPs) genes. No synonymous SNPs were identified in walR, clpP, cmK or sarA genes. See Table 4.3. Further, a total of 34 non-synonymous SNPs were observed among these isolates. The largest number of non-synonymous SNPs were identified in the vraF (one isolate, 6 SNPs) and vraG (5 isolates, 8 SNPs) genes however multiple isolates displayed SNPs in the walk (2 isolates, 2 SNPs), graR (2 isolates, 4 SNPs), graS (2 isolates, 6 SNPs), stP (2 isolates, 2 SNPs), prsA (2 isolates, 2 SNPs) and vraR (2 isolates, 2 SNPs) genes. Non-synonymous SNPs identified in VSSA isolates are summarized in Table 4.4**.**

Table 4.3 Summary of synonymous and non-synonymous SNPs identified in VSSA and hVISA

	VSSA n=7				hVISA n=10			
Gene	No. isolates	No. syn	No. isolates	No. non-syn	No. isolates	No. syn	No. isolates	No. Non-syn
walR	0	0	0	0	0	0	0	0
walK	2	18	2	2	3	27	1	1
rpoB	2	12	0	0	4	20	1	1
graR	2	6	2	4	3	9	3	6
graS	2	12	2	6	3	18	3	9
clpP	0	0	0	0	0	0	0	0
stP	2	4	2	2	3	6	3	4
vraS	2	12	0	0	3	18	0	0
cmK	0	0	1	1	0	0	0	0
vraT	1	5	0	0	3	15	1	1
yvqF	1	5	0	0	3	15	1	1
yjbH	2	4	0	0	3	6	0	0
slE	2	16	0	0	3	24	0	0
msrR	2	8	0	0	3	12	0	0
sarA	0	0	0	0	0	0	0	0
agrA	2	4	1	1	3	6	0	0
ccpA	2	2	0	0	3	3	0	0
prsA	2	6	2	2	3	9	1	1
vraR	2	2	2	2	3	3	3	3
vraF	2	17	1	6	3	25	1	1
vraG	2	16	5	8	3	36	5	12

Table 4.4 Non-synonymous SNPs and amino acid changes identified in VSSA

Isolates	walR (SA0017)	walk (SA0018)	rpoB (SA0500)	graR (SA0614)	graS (SA0615)	clpP (SA0723)	stP (SA1062)	Vras (SA1701)
MA4	0	Thr595Lys	0	0	0	0	0	0
MA16	0	Thr595Lys	0	0	0	0	0	0
MA98	0	0	0	0	0	0	0	0
MA212	0	0	0	Asp148His, Asp148Glu	Leu26Phe, Ile59Leu Thr224Ile	0	Glu68Gln	0
MA307	0	0	0	0	0	0	0	0
SL1	0	0	0	0	0	0	0	0
HH7	0	0	0	Asp148His, Asp148Glu	Leu26Phe Ile59Leu Thr224Ile	0	Glu68Gln	0

Table 4.4 (continued) Non-synonymous SNPs and amino acid changes identified in VSSA

Isolates	cmK (SA1309)	vraT/yvqF (SA1702)	clP (SA0723)	yjbH (SA0860)	sle (SA0423)	msrR (SA1195)
MA4	0	0	0	0	0	0
MA16	0	0	0	0	0	0
MA98	0	0	0	0	0	0
MA212	Ala116Asp	0	0	0	0	0
MA307	0	0	0	0	0	0
SL1	0	0	0	0	0	0
HH7	0	0	0	0	0	0

Table 4.4 (continued) Non-synonymous SNPs and amino acid changes identified in VSSA

Isolates	sarA (SA0573)	agrA (SA1844)	ccpA (SA1557)	prsA (SA1659)	vraR (SA1700)	vraF (SA0616)	vraG (SA0617)
MA4	0	0	0	Pro269Ser	0	0	0
MA16	0	0	0	Pro269Ser	0	0	Thr499Ile
MA98	0	0	0	0	0	0	Thr217Ile
MA212	0	Ala127fs (frame shift)	0	0	Glu59Asp	Thr274Lys Ile230Thr Gly227Ala His184Arg Lys135Asn Ala136Val	Pro246Leu Val8Ile
MA307	0	0	0	0	0	0	0
SL1	0	0	0	0	0	0	Thr217Ile
HH7	0	0	0	0	Glu59Asp	0	Ile235Thr Val489Ala Lys498Glu

Among the 10 hVISA isolates, SNPs were identified in 17 of the 21 candidate genes. A total of 254 synonymous SNPs were identified and were most commonly observed in the walK (3 isolates, 27 SNPs), rpoB (4 isolates, 20 SNPs), graS (3 isolates, 18 SNPs), vraS (3 isolates, 18 SNPs), slE (3 isolates, 24 SNPs), vraF (3 isolates, 25 SNPs) and vraG (3 isolates, 36 SNPs) genes. No synonymous SNPs were identified in walR, clpP, cmK or sarA genes. In addition, a total of 40 non-synonymous SNPs were observed among hVISA isolates. The largest number of non-synonymous SNPs was identified in the vraG (5 isolates, 12 SNPs) gene though multiple isolates displayed SNPs in the graR (3 isolates, 6 SNPs), graS (3 isolates, 9 SNPs), stP (3 isolates, 4 SNPs), and vraR (3 isolates, 3 SNPs) genes. A summary of synonymous and non-synonymous SNPs is provided in Table 4.3.

This comparison identified 4 non-synonymous SNPs within candidate genes that were unique to hVISA including SNPs within rpoB (His481Tyr), stP (Gly195Arg), vraF (Gly26Ser) and vraG (Ala580Glu). A full list of non-synonymous SNPs and amino acid changes among VSSA can be observed in Table 4.4 and a full list of SNPs and amino acid changes in hVISA can be observed in Table 4.5. A comparison of amino acid changes between VSSA and hVISA is described in Figure 4.6.

Table 4.5 Non-synonymous SNPs and amino acid changes identified in hVISA

Isolates	walR (SA0017)	walk (SA0018)	rpoB (SA0500)	graR (SA0614)	graS (SA0615)	clpP (SA0723)	Stp (SA1062)	Vras (SA1701)
MA33	0	0	0	0	0	0	0	0
MA38	0	0	0	0	0	0	0	0
MA40	0	0	His481Tyr	Asp148His Asp148Glu	Leu26Phe Ile59Leu Thr224Ile	0	Glu68Gln	0
MA97	0	0	0	0	0	0	0	0
MA209	0	0	0	0	0	0	0	0
MA215	0	Thr595Lys	0	0	0	0	0	0
MA260	0	0	0	Asp148His Asp148Glu	Leu26Phe Ile59Leu Thr224Ile	0	Glu68Gln Gly195Arg	0
MA311	0	0	0	0	0	0	0	0
SL-11	0	0	0	0	0	0	0	0
HH10	0	0	0	Asp148His Asp148Glu	Leu26Phe Ile59Leu, Thr224Ile	0	Glu68Gln	0

Table 4.5 (continued). Non-synonymous SNPs and amino acid changes identified in hVISA

Isolates	cmK (SA1309)	vraT (SA1702)	yvqF (1702)	clP (SA0723)	cmK (SA1309)	yjbH (SA0860)	Sle (SA0423)	msrR (SA1195)
MA33	0	0	0	0	0	0	0	0
MA38	0	0	0	0	0	0	0	0
MA40	0	0	0	0	0	0	0	0
MA97	0	0	0	0	0	0	0	0
MA209	0	0	0	0	0	0	0	0
MA215	0	0	0	0	0	0	0	0
MA260	0	0	0	0	0	0	0	0
MA311	0	Pro99Thr	Pro99Thr	0	0	0	0	0
SL-11	0	0	0	0	0	0	0	0
HH10	0	0	0	0	0	0	0	0

Table 4.5 (continued). Non-synonymous SNPs and amino acid changes identified in hVISA

	sarA (SA0573)	agrA (SA1844)	stp1 (SA1062)	ccpA (SA1557)	prsA (SA1659)	vraR (SA1700)	vraF (SA0616)	vraG (SA0617)
MA33	0	0	0	0	0	0	0	0
MA38	0	0	0	0	0	0	0	
MA40	0	0	Glu68Gln	0	0	Glu59Asp	Gly26Ser	Ile235Thr, Val489Ala, Lys498Glu
MA97	0	0	0	0	0	0	0	0
MA209	0	0	0	0	0	0	0	0
MA215	0	0	0	0	Pro269Ser	0	0	0
MA260	0	0	Glu68Gln, Gly195Arg	0	0	Glu59Asp	0	Ile235Thr, Val489Ala, Lys498Glu, Ala580Glu
MA311	0	0	0	0	0	0	0	Thr217Ile
SL-11	0	0	0	0	0	0	0	Thr217Ile
HH10	0	0	Glu68Gln	0	0	Glu59Asp	0	Ile235Thr, Val489Ala, Lys498Glu

Figure 4.6 Comparison of SNPs identified in candidate genes among VSSA and hVISA

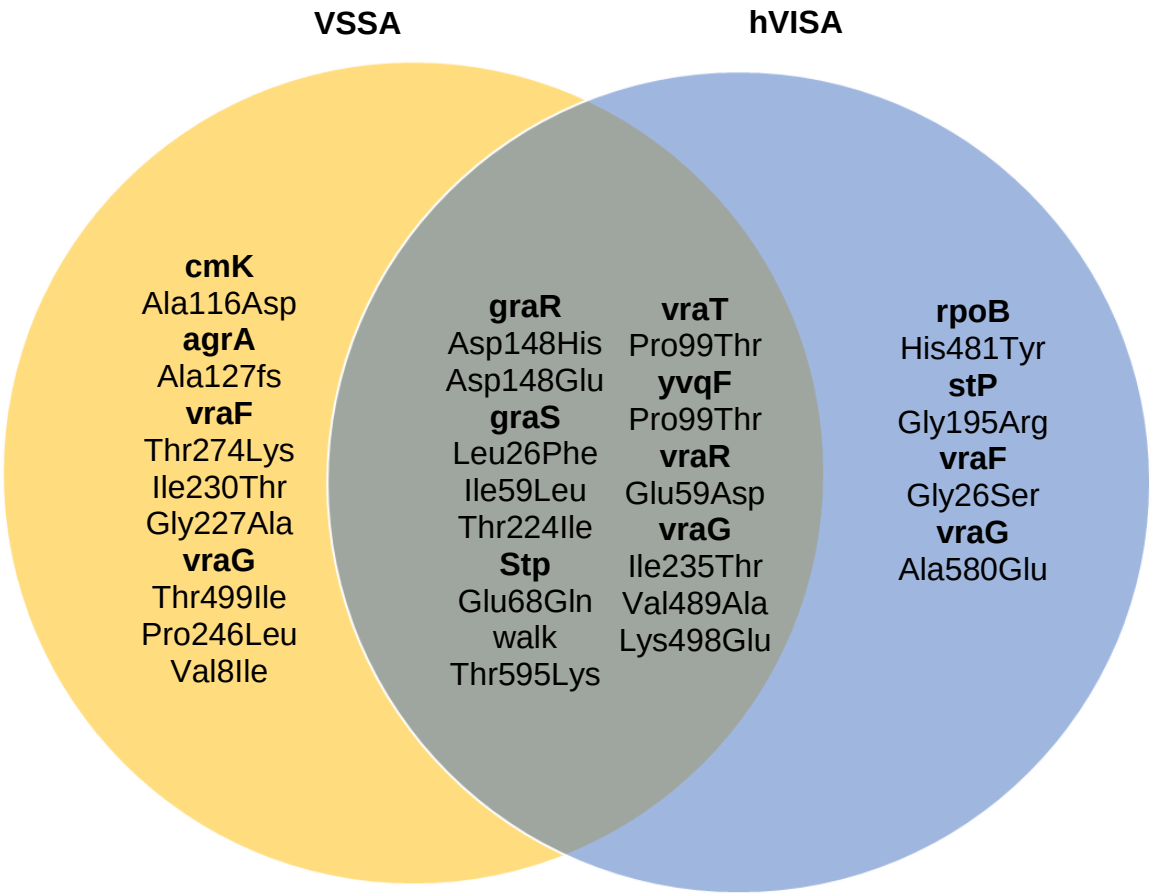
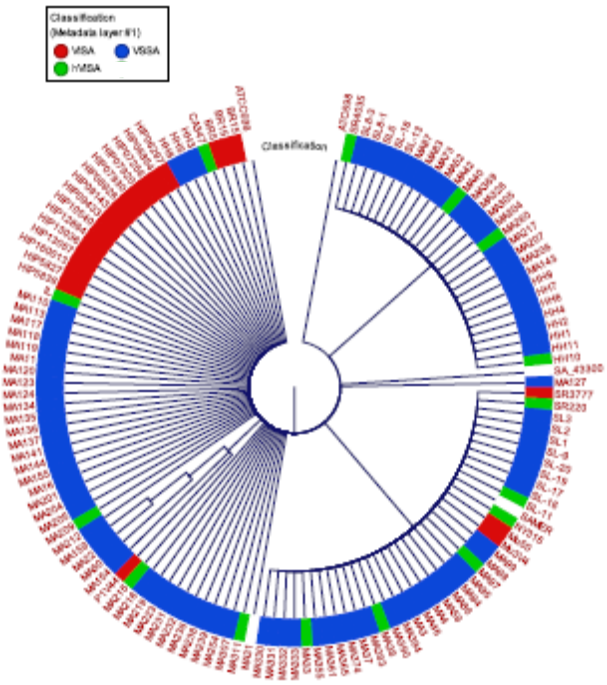


Figure 4.7 Location of common SNPs differeing between VSSA and hVISA as identified by GWAS.

After identifying SNPs unique to hVISA in clinical isolates we conducted a genome wide association study using clinical isolates described in aim one along with known reference strains (NARSA) and additional sequences deposited on NCBI.[44] The GWAS compared VSSA to a composite phenotype of hVISA and VISA and was performed exclusively among ST5 isolates, however no statistically significant SNPs were observed ($p = 8.92 \times 10^{-6}$, Bonferroni corrected $p = 0.08$)

Further, phylogenetic analysis demonstrated similarities among VISA isolates as they generally resided in a single clad. See figure 4.8. HVISA isolates demonstrated marked heterogeneity however, and were distributed widely among clads. These results highlight the variety and number of genes that may be implicated in the development of hVISA.

Figure 4.8 SNP tree demonstrating relationships between clinical isolates.



Outer ring color coded based on phenotype

Results Sub aim 2c

HVISA is noteworthy in the heterogeneous nature of its resistant populations. This study therefore sought to characterize the differences between individual colonies especially in the context vancomycin exposure. All colonies displayed an MIC of 1.5µg/mL which was consistent with the parent Mu3 isolate. Predictably, all six colonies as well as Mu3 developed resistance when exposed vancomycin in a stepwise fashion. Interestingly however, while all colonies increased in MIC, colony 3 achieved the highest MIC (12µg/mL) after exposure to 8µg/mL. Further the Mu3 isolate and colony 4 did not display a viable inoculum sufficient to perform MICs after exposure to 6.5µg/mL and 6µg/mL vancomycin. These results highlight the heterogeneous nature of Mu3. Further, SNP based phylogenetic analysis demonstrated colonies clustering together, in some cases a single colony accounted for an entire clad regardless of vancomycin exposure. See Figure 4.10. This suggests that individual colonies may differ inherently in terms of SNPs even before being subjected to vancomycin. Finally SNPs in candidate genes were studied prior to and after exposure to vancomycin. While multiple SNPs were identified, these did not occur within candidate genes.

Figure 4.9 Effect of vancomycin exposure on Mu3 colony MICs

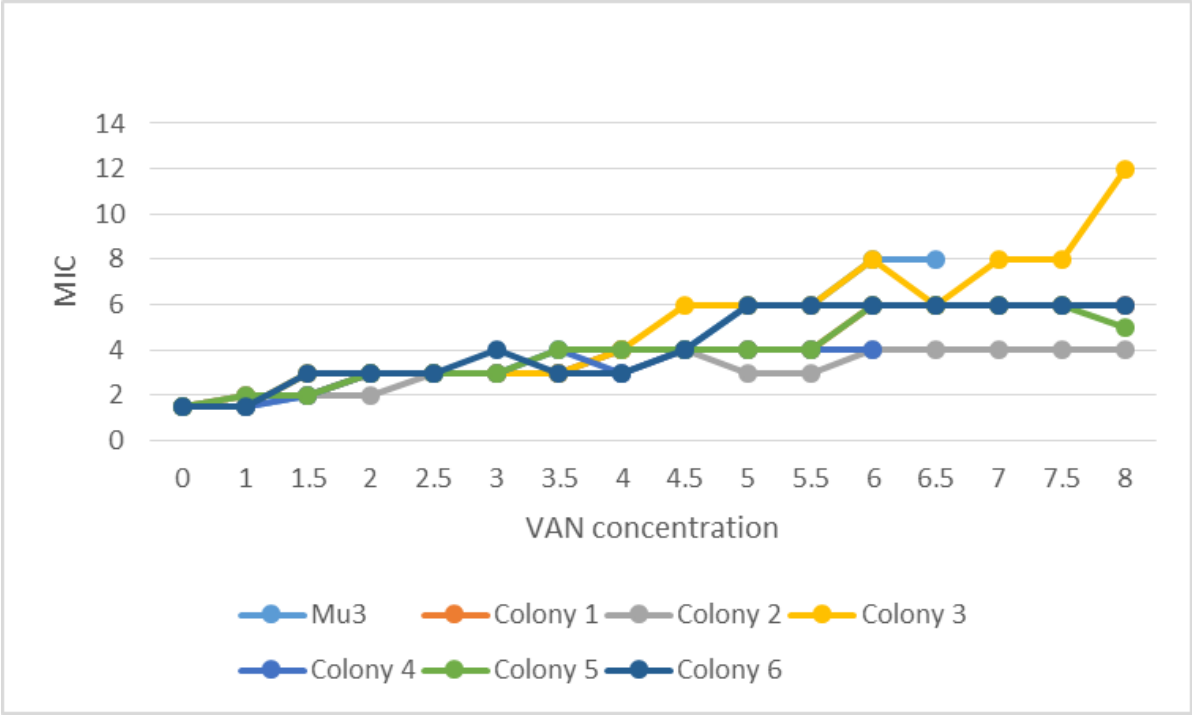
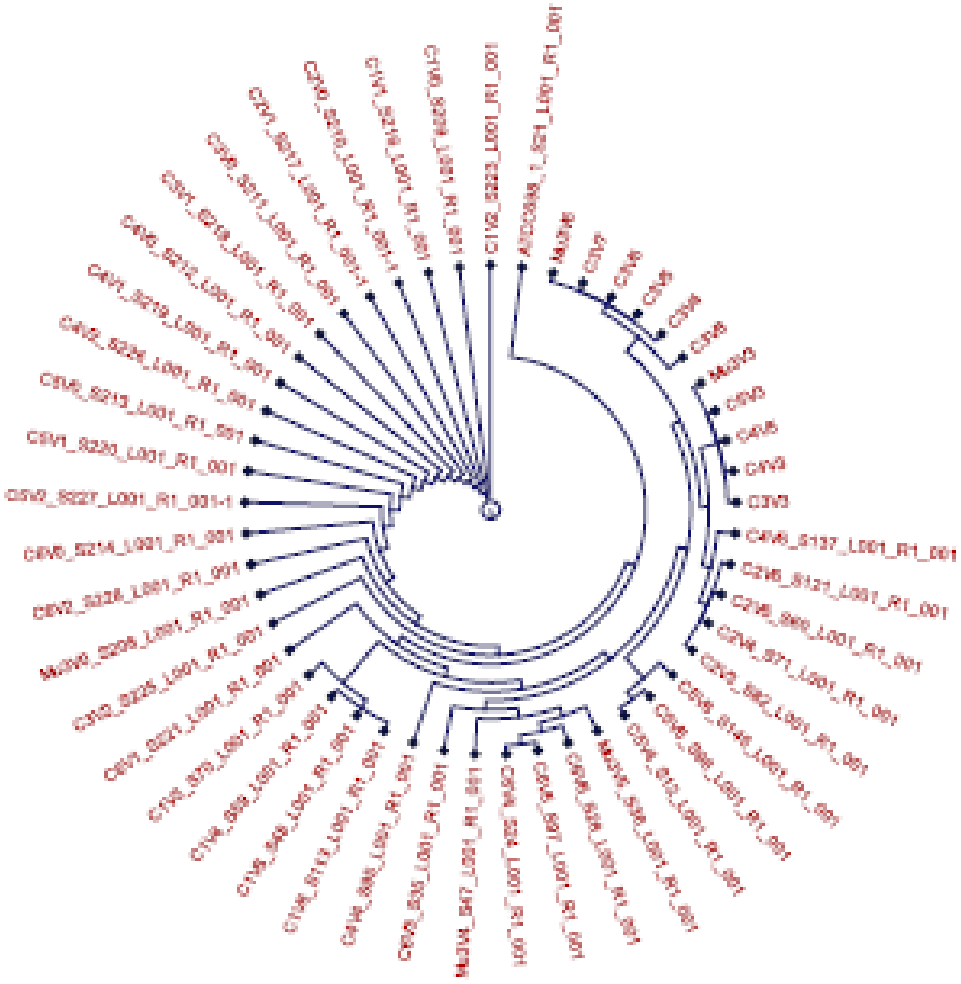


Figure 4.10 Cladogram displaying relationships of Mu3 colonies prior to and post vancomycin exposure.



Discussion

MLST

This study suggests ST5 as the most common MLST among clinical isolates followed by ST8. These findings are consistent with most epidemiological studies conducted in the United States although MLST seems to vary substantially based on country.[52] A previous study has demonstrated variation in the proportion of hVISA isolates based on MLST.[53] Such information has potential in predicting the presence of hVISA or for use in surveillance studies. This study therefore aimed to describe differences in phenotype based on MLST. In this study we noted that similar to VSSA, ST5 was the most common MLST among hVISA isolates. However, unlike with VSSA, ST5 made up the majority of the hVISA isolates. It is important to note that the majority of isolates in this cohort were VSSA and therefore, these isolates drove the overall frequencies of observed MLSTs. Additionally this study observed several MLSTs that occurred in single isolates only, these sequence types were only observed in VSSA isolates and their absence among hVISA may have allowed for the distribution to more easily become skewed towards a single MLST.

This study observed a trend regarding MLST and MIC as ST8 became more prevalent with increasing MICs. Previously, hVISA was shown to be more common in isolates with elevated MICs. This study showed a similar phenomenon. Interestingly among ST8 isolates with an MIC of 2µg/mL, 75% were hVISA. While it's apparent that resistant sub-populations can be observed across MIC and MLST, these observations may aid in

predicting the presence of hVISA within infections. Finally, we reported the frequency of MLSTs by year. As in the overall distribution, ST5 was the predominant MLST for most years except 2018, in which ST8 made up the majority. While these data may suggest a change in the frequency of MLSTs, further studies in the years between 2010 and 2017 must be conducted to confirm any such trend. Further, many of the isolates collected in 2018 were from California where as Texas isolates constituted the majority of isolates collected previously. Therefore additional multicenter studies with similar distributions of isolates among locations would be needed to confirm this finding.

Overall this study is limited as a comprehensive epidemiological study of MLST among MRSA in that a subset of the entire cohort was selected and a gap in time of collection compromises the ability to observe temporal trends. This study's greatest utility however may lie in the ability to describe MLST frequencies in isolates with increased likelihoods of hVISA, that is, among isolates with increased MICs or those displaying growth on vancomycin impregnated BHIA. Additionally, SNP based phylogenetic analysis showed similarity of isolates among certain MLSTs especially ST8 suggesting that certain MLSTs may harbor similar SNPs. Finally, this study adds to a limited number of studies that have selectively focused on the distribution of MLST among hVISA.

While factors such as MIC and MLST may provide insight into scenarios when hVISA might be present, they alone cannot predict all hVISA infections. Further, the time, labor and cost of conducting PAP-AUCs severely limit their utility in the clinic.

Therefore this study aimed to identify genetic factors that could potentially be used in conjunction with rapid diagnostic platforms or mobile sequencers to identify hVISA.

A comparative analysis of matched VSSA/hVISA pairs resulted in the identification of four SNPs that were unique to hVISA. While the majority of these SNPs are not well studied, they were identified in genes that have been shown to have profound effects on vancomycin resistance. StP has profound effects on post translational modification and alterations have been associated with altered muropeptide composition during stationary phase.[54] VraF and vraG mutations have been shown to alter cell wall charge and therefore have an effect on susceptibility to vancomycin, daptomycin and polymixins.[55] Among the SNPs identified in this study, the rpoB his481tyr is by far the most studied. This mutation was identified through GWAS and other studies have cited its presence in the conversion from hVISA to VISA.[44,] While commonly studied in the context of rifampin, rpoB mutations have also been shown to be a factor in dual vancomycin/daptomycin resistance.[55] Though unique SNPs were identified in hVISA, this study may have been limited by sample size, forcing a single isolate (MA98) to serve as a match to multiple hVISA isolates. Further if more than one isolate served as a match, the isolate was chosen at random which may have led to unidentified SNPs had other isolates been incorporated into the analysis. Fortunately, most isolates matched on all characteristics with a few matching on all but one characteristic. Further while additional SNPs unique to hVISA were noted within each pair, the four reported SNPs described in figure 4.6 did not occur in any VSSA isolates. Therefore each SNP identified in hVISA was compared to SNPs identified in a composite of seven VSSA isolates. Though the comparative analysis

identified unique SNPs in hVISA isolates, these SNPs did not reach statistical significance in GWAS. While our GWAS contained additional reference isolates, previously submitted sequences, and was unique to ST5 isolates, the sample size may have still been too small to identify variants. A similar study conducted by Alam et al noted similar issues when utilizing 75 isolates.[44] While our study incorporated ~90 isolates, a larger sample would likely aid in identifying SNPs that reached statistical significance. Since this GWAS may have been limited by the use of multiple isolate were sources MIC testing and WGS was performed among a subset of previously sequenced reference isolates. In all three cases we noted similar MICs and were able to identify identical SNPs in candidate genes.

While the comparative analysis garners strength from the fact that it approximates an *in vivo* study by utilizing samples derived from actual patient infections, this format introduces other limitations since patient demographics were unknown. In essence, patients developing hVISA infections, did so in a non-controlled environment, limiting our ability to characterize the effect of outside factors on phenotype.

Performing a controlled *in vitro* experiment in which colonies were selected and exposed to vancomycin in a controlled manor allowed us to study inherent differences among colonies as well as study the development of resistance as it applies only to the exposure to vancomycin. The clustering of individual colonies combined with the different MICs achieved through vancomycin exposure, highlight differences between colonies. Interestingly, these differences were observed even though only six colonies were studied, while resistant subpopulations are thought to occur at frequencies at or

below 10^{-5}. While all colonies displayed an initial MIC of 1.5µg/mL, these results introduce the possibility that individual colonies may display different MICs especially after drug exposure and thereby influence treatment.

CHAPTER 5

SUMMARY

Purpose

MRSA infections pose a huge burden on healthcare. This problem is complicated by the development of antimicrobial resistance. While vancomycin currently reflects the standard of care, its effectiveness may be reduced in patients suffering from hVISA infections. These infections pose additional challenges by going undetected by conventional susceptibility tests due to the low frequency of resistant populations. Current methods used to identify hVISA are unpractical as diagnostic tools, necessitating the development of alternative methods to identify hIVSA in MRSA infections.

Study

This study employed a series of experiments to identify factors associated with vancomycin non-susceptibility in effort to more reliably identify hVISA among MRSA infections. We used a stepwise approach including a susceptibility test, screening method and confirmatory test to identify and report the prevalence of hVISA in a collection of clinical isolates. This study reported a prevalence of 3% and suggests that hVISA is more common among isolates with MICs of 1.5µg/mL and 2µg/mL. We also employed WGS to characterize multi locus sequence types in VSSA compared to hVISA as well as to identify SNPs that are associated with vancomycin non-susceptibility in MRSA. We identified ST5 and ST8 as the predominant sequence types among hVISA isolates and

using a comparative genomics approach, identified SNPs in four candidate genes that were exclusive to hVISA.

Innovation

This study is unique in several ways. While epidemiological studies have reported MLSTs, most center on MRSA without consideration of hVISA. While we analyzed a subset of clinical isolates, these isolates focused on susceptible isolates with elevated MICs, and resistant sub populations.

We also employed a multi-pronged approach to identify SNPs associated with hVISA. The comparative analysis employed a focused approach by targeting SNPs found within specific genes known to be associated with vancomycin intermediate susceptibility. Additionally the GWAS conducted in this study represents that largest GWAS comparing VSSA and hVISA while also focusing on ST5 isolates. Finally, while evolutionary studies have tracked the development of resistance in MRSA, this study represents the only work characterizing response of individual colonies to vancomycin within a single isolate.

Impact

By incorporating a large collection of isolates and a range of susceptibility and confirmatory tests, this study provides a robust understanding of the prevalence of hVISA, especially in the state of Texas. Further, identification of SNPs unique to hVISA may provide targets for rapid diagnostic platforms. Rapid identification of hVISA could

allow clinicians to quickly employ targeted therapy as opposed waiting on culture and sensitivities or monitoring patient response prior to switching agents. Quickly employing appropriate antibiotics would subsequently improve patient outcomes and reduce health care costs. Further, SNPs in certain genes have been associated with cross resistance or in other cases increased susceptibility among agents. Therefore, identifying mutations associated with hVISA may offer additional information regarding the utility of other agents other than vancomycin in a given infection. Finally, identifying resistant populations may be of importance during pharmacokinetic studies when optimizing doing based on AUC:MIC ratios.

Future directions

Future directions would include improving upon limitations of this study. Maintaining a collection of new MRSA infections could lead to more robust and current epidemiological data. Additionally while unique SNPs were identified in candidate genes, functional studies must be performed to determine their impact.

References

1. Hu Q, Peng H et al. Molecular Events for Promotion of Vancomycin Resistance in Vancomycin Intermediate Staphylococcus aureus. Front Microbiol. 2016 Oct; 1601 (7):1-18
2. Casapao AM, Leonard SN et al. Clinical Outcomes in Patients with Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* Bloodstream Infection. Antimicrob Agents Chemother. 2013 Sep; 57(9): 4252–4259
3. Sebastiaan J. van Hal, David L. Paterson. Systematic Review and Meta-Analysis of the Significance of Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Isolates. Antimicrob. Agents. Chemother. 2001;55:405-410
4. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Fourth Informational Supplement M100-S24.CLSI,Wayne,PA,USA,2014.
5. Howden P, Davies J et al. Reduced Vancomycin Susceptibility in Staphylococcus aureus, Including Vancomycin-Intermediate and Heterogeneous Vancomycin-Intermediate Strains: Resistance Mechanisms, Laboratory Detection, and Clinical Implications. Clin Microbiol Rev. 2010 Jan; 23(1): 99–139.
6. Leonard S, Rossi K et al. Evaluation of the Etest GRD for the detection of Staphylococcus aureus with reduced susceptibility to glycopeptides. J Antimicrob Chemother 2009 Jan: 63 (3): 489-492.
7. Hiramatsu K, Hanaki H et al. Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother. 1997 Jul;40(1):135-6.
8. Hiramatsu K, Aritaka N et al. Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. Lancet Infect Dis. 1997 Dec; 350 (9092): 1670-1673
9. Yamakawa J, Aminaka M et al. Heterogeneously vancomycin-intermediate Staphylococcus aureus (hVISA) emerged before the clinical introduction of vancomycin in Japan: a retrospective study. J Infect Chemother. 2001 Jun
10. Robert J, Bismuth RJ et al. Decreased susceptibility to glycopeptides in methicillin-resistant Staphylococcus aureus: a 20 year study in a large French teaching hospital, 1983-2002. Antimicrob Chemother. 2006 Mar;57(3):506-10. Epub 2006 Jan 12.
11. Rybak M, Raymond C et al. Clinical isolates of Staphylococcus aureus from 1987 and 1989 demonstrating heterogeneous resistance to vancomycin and teicoplanin. 2005 Feb 51(2): 119-125
12. Rybak M, Leonard S et al. Characterization of Vancomycin-Heteroresistant Staphylococcus aureus from the Metropolitan Area of Detroit, Michigan, over a 22-Year Period (1986 to 2007) J. Clin. Microbiol. Sep 2008; 46 (9) 2950-2954
13. Casapao AM, Davis SL et al. Evaluation of vancomycin population susceptibility analysis profile as a predictor of outcomes for patients with infective endocarditis due to methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. 2014;58(8):4636–41.

14. Richter SS, Satola SW et al. Detection of Staphylococcus aureus isolates with heterogeneous intermediate-level resistance to vancomycin in the United States. J. Clin. Microbiol. 2011;49 (12):4203–7.
15. Richter SS, Diekema DJ et al. Activities of Vancomycin, Ceftaroline, and Mupirocin against Staphylococcus aureus Isolates Collected in a 2011 National Surveillance Study in the United States. Antimicrob Agents Chemother. 2014 Feb; 58(2): 740–745.
16. Zhang S, Sun X et al. Systematic Review and Meta-Analysis of the Epidemiology of Vancomycin-Intermediate and Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Isolates. PLOS one. 2015 Aug.
17. Sancak B, Ercis SJ et al. Methicillin-resistant Staphylococcus aureus heterogeneously resistant to vancomycin in a Turkish university hospital. Antimicrob Chemother. 2005 Sep;56(3):519-23
18. Horne KC, Howden BP et al. Prospective Comparison of the Clinical Impacts of Heterogeneous Vancomycin-Intermediate Methicillin-Resistant Staphylococcus aureus (MRSA) and Vancomycin-Susceptible MRSA Antimicrob Agents Chemother. 2009 Aug; 53(8): 3447–3452.
19. Hanaki H, Cui L et al. Antibiotic susceptibility survey of blood-borne MRSA isolates in Japan from 2008 through 2011. J. Infect. Chemother.2014;20(9):527–34.
20. Park KH, Kim ES et al. Comparison of the clinical features, bacterial genotypes and outcomes of patients with bacteraemia due to heteroresistant vancomycin-intermediate Staphylococcus aureus and vancomycin-susceptible S. aureus. J Antimicrob Chemother. 2012;67(8):1843–9
21. Gowrishankar S, Thenmozhi R et al. Emergence of methicillin-resistant, vancomycin-intermediate Staphylococcus aureus among patients associated with group A Streptococcal pharyngitis infection in southern India. Infect Genet Evo. 2013;14:383–9.
22. Ayoubi EL, Hamze M et al. Glycopeptide intermediate Staphylococcus aureus and prevalence of the luk-PV gene in clinical isolates, in Northern Lebanon. Med Mal Infect. 2014;44(5):223–8.
23. Dubey D, Rath S et al. Surveillance of infection status of drug resistant Staphylococcus aureus in an Indian teaching hospital. Asian Pac J Trop Biomed. 2013;3(2):133–42.
24. Chaudhari S, Tandel M et al. Heterogeneous vancomycin-intermediate among methicillin resistant Staphylococcus aureus. Med J Armed Forces India. 2014 Jun:2015(71) 15-18
25. Panomket P, Thirat Set al. Methicillin-resistant Staphylococcus aureus with reduced susceptibility to vancomycin in Sanprasitthiprasong Hospital. J Med Assoc Thai. 2014;97
26. Liu C, Chambers HF et al. Staphylococcus aureus with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. Antimicrob Agents Chemother. 2014;47(10):: 3040–5
27. Kaleem F, Usman J, Amanat S. Current status of Glycopeptide intermediate and heterogenous Glycopeptide intermediate Staphylococcus aureus and their prevailing susceptibility pattern at two tertiary care hospitals of Pakistan. Int J Infect Dis. 2012;16:e420–e1
28. Chaudhary M, Payasi A. Prevalence of heterogeneous glycopeptide intermediate resistance in Methicillin-Resistant Staphylococcus aureus. Am J Infect Dis. 2013;9(3):63–70.

29. Sancak B, Ercis S et al. Methicillin-resistant Staphylococcus-aureus heterogeneously resistant to vancomycin in a Turkish university hospital. J antimicrob chemother. 2005; 56(3):519–23.
30. Etest [package insert] 69280 Marcy-l'Etoile – France. bioMérieux SA. 2012
31. Prakash V, Lewis J et al. Vancomycin MICs for methicillin-resistant Staphylococcus aureus isolates differ based upon the susceptibility test method used. Antimicrob. Agents Chemother. 2008 Oct: 52 (12):4528
32. Sader H. S, Rhomberg P et al, Nine-hospital study comparing broth microdilution and Etest method results for vancomycin and daptomycin against methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother.2009 Jul 53(7):3162-3165.
33. Satola S, Farley F et al. Comparison of Detection Methods for Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus,* with the Population Analysis Profile Method as the Reference Method. J. Clin. Microbiol. 2011 Jan 49(1) 177-183
34. Wootton M, Howe R et al. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. J Antimicrob Chemother. 2001 Apr;47(4):399-403
35. Sieradzki K, Tomasz A. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of Staphylococcus aureus. J Bacteriol. 1997 Apr; 179(8): 2557–2566.
36. Sieradzki K, Pinho M et al. Inactivated pbp4 in Highly Glycopeptide-resistant Laboratory Mutants of Staphylococcus aureus. J. Biol. Chem.1999 Jul ; 274, 18942-18946.
37. Katayamaa Y, Sekinea M et al. Complete Reconstitution of the Vancomycin-Intermediate Staphylococcus aureus Phenotype of Strain Mu50 in Vancomycin-Susceptible S. aureus.
38. Matsuo M, Cui L et al. Comprehensive identification of mutations responsible for heterogeneous vancomycin-intermediate Staphylococcus aureus (hVISA)-to-VISA conversion in laboratory-generated VISA strains derived from hVISA clinical strain Mu3. Antimicrob Agents Chemother. 2013 Dec;57(12):5843-53.
39. Kuroda M1, Kuwahara-Arai K et al. Identification of the up- and down-regulated genes in vancomycin-resistant Staphylococcus aureus strains Mu3 and Mu50 by cDNA differential hybridization method. Biochem Biophys Res Commun. 2000 Mar;269(2):485-90.
40. McCallum N, Meier PS et al. Mutational analyses of open reading frames within the vraSR operon and their roles in the cell wall stress response of Staphylococcus aureus. Antimicrob. Agents Chemother. 2011 Apr; 55(4):1391-402
41. Boyle-Vavra S, Yin S et al. VraT/YvqF is required for methicillin resistance and activation of the VraSR regulon in Staphylococcus aureus. Antimicrob Agents Chemother. 2013 Jan; 57(1): 83–95.
42. Power R, Parkhill J et al. Microbial genome-wide association studies: lessons from human GWAS. Nat Rev Genet. 2017; 18:41-50.
43. Mwangi MM, Wu SW et al. Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. Proc Natl Acad Sci U S A. 2007; 104 (22): 9451–9456.
44. Alam T, Petit R et al. Dissecting Vancomycin-Intermediate Resistance in Staphylococcus aureus Using Genome-Wide Association. Genome Biol Evol. 2014; 6(5): 1174–1185.
45. Baines SL, Holt KE et al. Convergent Adaptation in the Dominant Global Hospital Clone ST239 of Methicillin-Resistant Staphylococcus aureus. 2015 mBio; 6 (2 e00080-15)

46. Gardete S, Aires-De Sousa M et al. Identification of the first vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) isolate from a hospital in Portugal. Microb Drug Resist 2008; 14:1-6
47. Ok HS, Lee HS, Park MJ, et al. Predictors and clinical outcomes of persistent methicillin-resistant Staphylococcus aureus bacteremia: a prospective observational study. Korean J Intern Med. 2013;28(6):678-686.
48. Dhand A, Sakoulas G et al. Reduced vancomycin susceptibility among clinical Staphylococcus aureus isolates ('the MIC Creep'): implications for therapy. F1000 Medicine Reports 2012 Feb:4(4) 1-11
49. Wootton M, Howe R et al. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. J Antimicrob Chemother. 2001 Apr;47(4):399-403
50. Cheng L, Connor TR et al. Hierachial and spatially explicit clustering of DNA sequences with BAPS software. Mol Biol Evol 2013; 30:1224-28
51. Purcell S, Neale B et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007; 81: 559-575
52. Park K-H, Greenwood-Quaintance et al. Molecular epidemiology of Staphylococcus aureus bacteremia in a single large Minnesota medical center in 2015 as assessed using MLST, core genome MLST and spa typing. PLoS One. 2017; 12(6)
53. Park K-H, Kim ES, et al. Comparison of the clinical features, bacterial genotypes and outcomes of patients with bacteraemia due to heteroresistant vancomycin-intermediate Staphylococcus aureus and vancomycin-susceptible S. aureus. J. Antimicrob. Chemother.2012; 67(8): 1843-1849
54. Jarick M, Bertsche U et al. The serine/threonine kinase Stk and the phosphatase Stp regulate cell wall synthesis in Staphylococcus aureus. Sci Rep. 2018; 8: 13693.
55. Yang S-J, Bayer AS et al. The Staphylococcus aureus Two-Component Regulatory System, GraRS, Senses and Confers Resistance to Selected Cationic Antimicrobial Peptides. Infect Immun. 2012 Jan; 80(1): 74–81.
56. Cui L, Isii L et al. An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in Staphylococcus aureus. Antimicrob Agents Chemother. 2010 Dec;54(12):5222-33.