Comparative Study Of The Prevalence Of Psk41 In Heterogenous Vancomycin-Intermediate Staphylococcus Aureus Isolates Against Non-Heterogenous Vancomycin-Intermediate Staphylococcus Aureus Isolates And The Prevalence By Patient Severity

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COMPARATIVE STUDY OF THE PREVALENCE OF PSK41 IN HETEROGENOUS VANCOMYCIN-INTERMEDIATE STAPHYLOCOCCUS AUREUS ISOLATES AGAINST NON-HETEROGENOUS VANCOMYCIN-INTERMEDIATE STAPHYLOCOCCUS AUREUS ISOLATES AND THE PREVALENCE BY PATIENT SEVERITY

by

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Approved By:

Advisor Date
DEDICATION

I dedicate my thesis research to my parents. Without their support, understanding and patience, this research would not have been possible.
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TABLE OF CONTENTS

DEDICATION ............................................................................................................................... II

ACKNOWLEDGMENTS ................................................................................................................... III

LIST OF FIGURES ........................................................................................................................ VII

LIST OF TABLES .......................................................................................................................... VIII

LIST OF ABBREVIATIONS .......................................................................................................... IX

CHAPTERS

CHAPTER 1: INTRODUCTION ........................................................................................................ 1

  1.1 Background .......................................................................................................................... 1

  1.2 Antibiotic resistance ............................................................................................................ 2

  1.3 *Staphylococcus* aureus ....................................................................................................... 2

  1.4 Clinical significance of *Staphylococcus* aureus .................................................................. 3

  1.5 Development of drug resistance in *S. aureus* .................................................................... 4

  1.6 Characteristics of the *S. aureus* genome ............................................................................ 5

       1.6.1 Important MGE’s of *S. aureus* .................................................................................. 6

       1.6.2 Molecular-based typing techniques ............................................................................. 7

  1.7 Vancomycin Resistance in *S. aureus* ................................................................................. 8

       1.7.1 hVISA and VISA ........................................................................................................ 8

       1.7.2 VRSA .......................................................................................................................... 9

CHAPTER 2: RATIONALE AND SPECIFIC AIMS ......................................................................... 13

  2.1 Rationale ............................................................................................................................ 13

  2.2 Specific Aims ...................................................................................................................... 14

CHAPTER 3: MATERIALS AND METHODS .................................................................................. 15
3.1 Materials

3.1.1 Samples
3.1.2 Controls

3.2 Methods

3.2.1 Bacterial Growth

3.2.2 DNA Extraction
   3.2.2.1 Total DNA Extraction
   3.2.2.2 Plasmid Purification

3.2.3 PCR Assays
   3.2.3.1 Preliminary screening for pSK41 plasmid
   3.2.3.2 Confirmation of pSK41 plasmid

3.2.4 Gel Electrophoresis

3.2.5 spa typing

3.2.6 Statistical Analysis

CHAPTER 4: RESULTS

4.1 Detection of pSK41 plasmid
   4.1.1 Preliminary screening to detect pSK41
   4.1.2 Confirmation of pSK41 with primer set II

4.2 Calculation of the prevalence of pSK41
   4.2.1 Comparison of the prevalence of pSK41 in hVISA isolates against non-hVISA isolates
   4.2.2 The prevalence of pSK41 in all hVISA isolates compared to non-hVISA isolates
4.2.3 The prevalence of pSK41 in hVISA isolates compared to matched non-hVISA isolates

4.3 Comparison of the prevalence of pSK41 in hVISA by the patient severity

4.4 Comparison of the strain types between hVISA isolates and non-hVISA isolates

4.4.1 Results of spa PCR

4.4.2 Distribution of strain types

CHAPTER 5: DISCUSSION

CHAPTER 6: CONCLUSION

CHAPTER 7: FUTURE EXPERIMENTS

REFERENCES

ABSTRACT

AUTOBIOGRAPHICAL STATEMENT
LIST OF FIGURES

Figure 1: Mechanism of the transfer of vancomycin resistance from VRE to *S. aureus* 11

Figure 2: False positive data for the ‘pre’ gene assay 23

Figure 3: BLAST Hits matching to the sequence of ‘pre’ gene 24

Figure 4: Inconsistent detection of genes with the primer set I 25

Figure 5: Inconsistent gene detection amongst duplicate runs 26

Figure 6: Consistency of results amongst various colonies from the same sample 27

Figure 7: Confirmation of pSK41-positive isolates by primer set II 28

Figure 8: Bands of *spa* gene after amplification 33
LIST OF TABLES

Table 1: Baseline demographics and clinical outcomes of hVISA and non-hVISA patients.............................................. 21

Table 2: The marker genes observed in pSK41-positive samples.............................................. 29

Table 3: The prevalence of pSK41 in various study groups.............................................. 30

Table 4: The prevalence of pSK41 in all the hVISA isolates compared to non-hVISA isolates.............................................. 31

Table 5: The prevalence of pSK41 in hVISA isolates compared to matched non-hVISA isolates.............................................. 31

Table 6: Comparison of the prevalence of pSK41 in hVISA by the patient severity.............................................. 32

Table 7: Distribution of the strain types between hVISA and non-hVISA isolates and between pSK41-positive and pSK41-negative isolates by spa typing.............................................. 34

Table 8: Summary of pSK41-positive isolates.............................................. 34
LIST OF ABBREVIATIONS

hVISA: Heterogeneous vancomycin-intermediate *S. aureus*

VISA: Vancomycin-intermediate *S. aureus*

VRSA: Vancomycin-resistant *S. aureus*

MRSA: Methicillin resistant *S. aureus*

CDC: Centers for disease control and prevention

ECDC: European centers for disease control and prevention

MGE: Mobile genetic elements

PFGE: Pulsed field gel electrophoresis

MLST: Multi-locus sequence typing

*spa*: Staphylococcal protein A

PBP: Penicillin binding proteins

MIC: Minimum inhibitory concentration

NAMA: N-acetyl muramic acid

NAGA: N-acetyl glucosamine

VRE: Vancomycin-resistant *enterococci*

*Tn1546*: Transposon 1546
$vanA$: Vancomycin-resistant gene-A

TSA: Tryptic soy agar

MHB: Muller Hinton broth

PBS: Phosphate buffered saline

TE: Tris-EDTA

PCR: Polymerase chain reaction

ICU: Intensive care unit

BMI: Body mass index

APACHE II: Acute physiology and chronic health evaluation II

BLAST: Basic local alignment search tool
CHAPTER 1

INTRODUCTION

1.1 Background:

Antibiotic resistance is a threat to public health globally. It is one of the major challenges faced by the pharmaceutical industry and medical community today [1].

According to the European Centers for Disease Control and Prevention (ECDC), every year approximately 25,000 people die from the consequences of antibiotic resistance in Europe [2]. In the United States, the Centers for Disease Control and Prevention (CDC) has estimated that antibiotic-resistant infections make more than 2 million people ill every year, and at least 23,000 people die from these infections [3]. In addition to the pharmaceutical industry and medical community, antibiotic resistance has jeopardized the financial sector as well. Studies have shown that an increase in antibiotic resistance has caused a $20 billion increase in healthcare costs in the United States, including, a $1.1 billion attributable to unnecessarily prescribed antibiotics [4, 5]. Infections caused by antibiotic resistance have increased the length of hospital stays in the United States by approximately eight days, thereby increasing the cost of hospitalization. [4].

These findings strongly indicate the effect of antibiotic resistance on the healthcare system, the pharmaceutical industry, and on the financial sector. Thus, the necessity for new antibiotics is urgent because of the resistance to many existing and previously effective drugs [1]. Currently, however, there are very few new antibiotics in the development pipeline [1]. Therefore, it is important to understand the mechanisms and transfer of antibiotic resistance in order to optimize our currently available antibiotics.

1.2 Antibiotic resistance:
**Antibiotic resistance** occurs when the targeted microorganism no longer remains susceptible to treatment and survives exposure to one or more antibiotics [6-8]. These resistant bacteria continue to grow and multiply even in the presence of therapeutic levels of antibiotics and thus severely compromise the health of patients [6, 8]. Antibiotic resistance is an increasingly serious issue in contemporary medicine worldwide and has emerged as the pre-eminent public health concern of this century [8].

Development of antibiotic resistance is a natural phenomenon [6, 8]. Antibiotic resistance can be acquired: a) through mutations (vertical transfer of resistant genes via reproduction) [8, 9], and b) via horizontal transfer of resistance (gene exchange among different bacteria) [8, 10, 11]. Several mobile genetic elements (MGE’s) play an important role in the latter kind of resistance.

1.3 *Staphylococcus aureus*:

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive, coccal bacterium. It is one of the five most common causes of nosocomial infections [12].

*S. aureus* also occurs as a commensal. Approximately 30-70% of humans are long-term carriers of *S. aureus* [13]. It is found in the anterior nares of the nasal passages and as a part of the normal skin flora [14, 15]. Thus, the presence of *S. aureus* in the human body does not necessarily indicate an infection [13].

*S. aureus* infects tissues when the skin or mucosal barrier is breached. It usually affects the skin, respiratory tract, soft tissues, bones, and joints [16, 17]. It can cause a plethora of infections, from minor skin infections to life-threatening diseases [17]. Minor skin infections include pimples, cellulitis, folliculitis, impetigo, furuncles (boils) and carbuncles (a collection of furuncles), abscess, and scalded skin syndrome [17]. The life-threatening conditions such as pneumonia,
sepsis, meningitis, bacteremia, osteomyelitis, toxic shock syndrome (TSS), and endocarditis can also arise due to *S. aureus* [16, 17].

### 1.4 Clinical significance of *Staphylococcus aureus*:

The CDC has reported that *S. aureus* causes around 80,000 infections and 11,000 deaths in the United States every year [3]. Another study has also reported that the *S. aureus* associated Skin and Soft Tissue Infections (SSTIs) have increased by 123% in USA over the period of ten years (2001-2009) [4]. This study reported an increase of 44% in the cost of hospitalization because of *S. aureus* infections as well.

Currently, many *S. aureus* bacteria isolated in the clinical settings have developed partial to complete resistance against many antibiotics. Methicillin Resistant *S. aureus* (MRSA) is the most common type of *S. aureus* that has become endemic over the past few years [10, 18-20]. According to Gould et al, the recent rate of occurrence of MRSA bacteremia is 30-40%, and if MRSA is treated with a glycopeptide antibiotic (for example, vancomycin) alone, then this rate of bacteremia is as high as it was in the pre-antibiotic era (70-80%) [21]. These statistics explains how antibiotic resistance can render an earlier effective drug ineffective.

Recently, MRSA has developed resistance against vancomycin, the drug of choice to treat MRSA infections [1, 5, 22-27]. Three types of *S. aureus* that show partial or complete resistance against vancomycin are, heterogeneous vancomycin-intermediate susceptible *Staphylococcus aureus* (hVISA), vancomycin-intermediate susceptible *Staphylococcus aureus* (VISA) and high-level vancomycin-resistant *Staphylococcus aureus* (VRSA) [25, 27-32]. This is alarming, because, these organisms are very difficult to treat with because of their non-responsiveness to vancomycin treatment. According to a surveillance study by Richter et al hVISA occurrence increased between
2009 and 2011 [33]. VRSA, although rare, has been reported in several parts of the world. The United States has had 13 cases of VRSA since 2002 [34].

Due to an alarming rate of \textit{S. aureus} infections and also, due to the continued development of resistance against currently employed antibiotics, \textit{S. aureus} proves to be clinically significant.

1.5 Development of drug resistance in \textit{S. aureus}:

Various drugs have been employed to date to treat the infections caused by \textit{S. aureus}. The first drug used was penicillin (1943). Penicillin is a \(\beta\)-lactam antibiotic that acts by preventing the cross-linking of the peptidoglycan layer, thereby preventing the formation of bacterial cell wall [35]. However, by 1950, 40\% of the patients were infected with penicillin-resistant \textit{S. aureus} [36]. Penicillin-resistant \textit{S. aureus} produces \(\beta\)-lactamase enzyme that cleaves the active component of penicillin, the \(\beta\)-lactam ring, and renders the drug inactive [37].

In response to the emergence of penicillin resistance, semisynthetic antibiotics belonging to the class of \textit{penicillinase-resistant \(\beta\)-lactam antibiotics} were introduced. Methicillin, oxacillin and flucloxacillin are a few examples of drugs in this class [38]. In 1959, the first antibiotic in this class, \textit{methicillin}, was introduced. Methicillin acts by inhibiting the penicillin-binding proteins (PBP). PBPs are involved in the synthesis of the peptidoglycan layer. By inhibiting PBPs, bacterial cell wall synthesis is hampered, and thus bacterial growth is inhibited [39]. After two years of clinical use, the first case of methicillin-resistant \textit{S. aureus} (MRSA) was reported in England [40]. Methicillin resistance is conferred by \textit{mecA} gene that synthesizes an analog of PBP, PBP 2-a, having low affinity for methicillin. As a result, PBP remains available for the synthesis of the bacterial cell wall and thus the resistance is developed against methicillin [39, 41]. Methicillin and oxacillin were still used as a first-line treatment until the 1990s, when the prevalence of MRSA became endemic in most hospital settings across the U.S. [23].
After MRSA infections had become endemic, vancomycin became the drug of choice to treat *S. aureus* infections. Vancomycin is a glycopeptide antibiotic which was introduced in 1956 [6]. In 1997, the presence of VISA, the strains of *S. aureus* with reduced susceptibility towards vancomycin, was reported for the first time [30]. The first case of VRSA, high level Vancomycin-resistant *S. aureus* was reported in 2002 in Michigan [34]. Since 2002, 13 cases of VRSA have been reported in the U.S. [42].

1.6 Characteristics of the *S. aureus* genome:

Genetics plays a key role in the development of antibiotic resistance. Therefore, in order to understand the development of antibiotic resistance in *S. aureus*, it is important to understand the characteristics of *S. aureus* genome first.

The genome of *S. aureus* consists of a core/stable component which contains all of the genes that are essential for the survival of the bacterium and an auxiliary/variable component which carries genes that are found in some strains of *S. aureus* [43-45]. DNA microarray analysis has shown that ~22% of *S. aureus* genomes consists of variable regions [16, 44, 45].

The core genome of *S. aureus* is approximately 2.8 Mb in size. It is highly conserved, and it makes up ~75% of the *S. aureus* genome. The auxiliary genome of *S. aureus* accounts for ~25% that is mainly composed MGE’s. MGE’s include plasmids, transposons, chromosomal cassettes, bacteriophages, genomic and pathogenicity islands [11, 16, 43, 44, 46].

1.6.1 Important MGE’s of *S. aureus*:

MGE’s, specifically plasmids and transposons play an important role in the transfer of vancomycin resistance.
Plasmids are extra-genomic, small circular DNA molecules capable of independent replication [45, 47]. They have the ability to integrate with genomic DNA [47]. *S. aureus* isolates, especially hospital associated isolates often carry one or more such plasmids [44]. Based upon the virulence genes carried and the size of the plasmid, *S. aureus* plasmids are classified into three types: Type I, II and III. Type I plasmids are smaller in size and are present in multiple copies. They usually carry a single virulence gene. Type II plasmids are low copy plasmids and are 15-30 kb in size. These plasmids carry several virulence genes. Type III plasmids are multi-resistant conjugative plasmids that are > 30 kb in size [11, 43, 45, 46, 48].

Transposons or transposable elements are the DNA sequences that are capable of changing their positions within a genome [49]. Transpositions occur by creating or reversing mutations [49]. Transposons are either smaller in size (and present in multiple copies) or larger in size (and usually present in a single copy) [1, 16, 43, 44]. Larger transposons are often found responsible for encoding antibiotic resistance genes in *S. aureus* [43]. Transposons transfer horizontally with the help of another MGE like plasmids [48, 50]. Transposons first separate from genomic DNA and get inserted into a vector such as a plasmid. Then this assembly gets transferred to a second organism where the transposon separates again from the vector plasmid and integrates with genomic DNA of the new organism. When the transposons get transferred, they may carry resistant determinants with them and thus transfer the drug resistance from one organism to another organism [51, 52].

Apart from the usefulness in understanding the development of drug resistance, the genomic studies are also useful for characterizing various strains of the microorganism.

1.6.2 Molecular-based typing techniques:
The genome of *S. aureus* can be used for molecular analysis of *S. aureus* to differentiate various strains of *S. aureus* [53]. Characterization of these strains aids in our understanding of the epidemiology of *S. aureus* and allow us to explore whether specific resistance traits are harbored by specific types of *S. aureus* [54].

Various molecular-based typing techniques include pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), *spa* typing, Staphylococcal cassette chromosome typing (*SCCmec* typing), and *coa* typing [53].

PFGE is considered as the ‘gold standard’ in determining different strain types of *S. aureus*. However, its disadvantage is that it is time-consuming, and inter-laboratory comparisons require homogenization of protocols [54]. MLST has been successfully applied for strain typing of *S. aureus*; however, it is more costly [54]. *spa* typing can be used to eliminate the disadvantages of PFGE and MLST [55].

*spa* typing is a single locus typing technique, meaning that, it requires the sequence analysis of only one gene, the *spa* gene which encodes *S. aureus* Protein A. Thus, this technique is more economical, faster and more reproducible than the other typing techniques. The *spa* gene has a region ‘X’ that is composed of a variable number of tandem repeats [55]. These repeats are assigned a numeric code and then the order of specific repeats is used to elucidate the particular *spa* type [55]. Ridom *spa* server, Bionumerics, and DNAGear are few examples of software programs that are designed for *spa* typing [56].

This project focuses on vancomycin resistance in *S. aureus*. The subsequent sections discuss different types of vancomycin resistance in *S. aureus*. 
1.7 Vancomycin resistance in *S. aureus*:

*S. aureus* has developed partial (hVISA, VISA) to complete resistance (VRSA) to vancomycin. Since vancomycin is a drug of choice to treat MRSA infections, the development of vancomycin resistance in *S. aureus* has become a great concern.

**Mechanism of action of vancomycin:** Vancomycin is a glycopeptide antibiotic which acts by blocking the bacterial cell wall synthesis. D-Ala-D-Ala residues are an important part of N-acetylglucosamine (NAGA) and N-acetylmuramic acid (NAMA), which form the backbone of the bacterial cell wall. Vancomycin binds with the terminal D-Ala residue and thus prevents cell wall formation, thereby preventing the bacterial growth [29].

1.7.1 hVISA and VISA:

An intermediate level of resistance against vancomycin is found in hVISA and VISA strains [30, 57]. These strains have reduced susceptibility towards vancomycin. VISA strains were first observed in Japan in 1997 [30]. They have the MICs of 4-8 μg/ml [57].

hVISA can be considered as a pre-VISA stage of vancomycin resistance. These strains are susceptible to vancomycin, but, also contain a few vancomycin-resistant subpopulations [57, 58]. This heterogeneity arises due to selective pressure resulting from vancomycin exposure. Upon continuous exposure to vancomycin, hVISA can convert into a uniform population of VISA [30, 57].

**Mechanism of intermediate resistance to vancomycin:** The reduced susceptibility towards vancomycin can occur in two different ways. Firstly, from the changes in the peptidoglycan biosynthesis and decreased cross-linking between the peptidoglycan strands. As a result of this, more D-Ala-D-Ala residues remain exposed to vancomycin and thus trap vancomycin. This bound vancomycin then obstructs the drug molecules from reaching their target
site [17, 29, 30, 57]. Little is known about the mechanisms behind these alterations in peptidoglycan biosynthesis. Another way by which vancomycin susceptibility can be reduced is by thickening of the bacterial cell wall, which reduces the ability of vancomycin to diffuse into the division septum of the cell [29, 57].

**Clinical significance of hVISA:** Patients with hVISA infections show higher rates of treatment failure with vancomycin therapy [59]. hVISA stays in the body of patients for a long time [25]. Therefore, these patients receive longer course of treatments to eliminate the bacteria and thus report longer hospital stays [59]. Because of the longer presence of hVISA, these patients have also reported having the higher rates of persistent bacteremia and congestive heart failure [58].

1.7.2 VRSA:

The strains of *S. aureus* that are completely resistant to vancomycin, are known as VRSA (MICs ≥ 16 μg/ml) [29]. Treating VRSA infections is very difficult as VRSA is resistant to almost all of the commonly employed antibiotics. Therefore, the emergence of VRSA is considered to be a big threat. Since 2002, thirteen cases of VRSA have been reported in the United States [42]. Apart from the United States, VRSA is also reported in India, Pakistan, Iran and Brazil [60-62].

**Mechanism of complete resistance to vancomycin:** In *S. aureus*, vancomycin resistance is acquired from the conjugal transfer of the *vanA* gene from Vancomycin-resistant *E. faecalis* (VRE) [27, 32, 42]. In VRSA, *vanA* gene causes an alteration of the terminal peptide to ‘D-Ala-D-Lac’ in place of D-Ala-D-Ala that has reduced affinity towards vancomycin. As a result, vancomycin cannot bind with the terminal D-Ala residue and thus allows the cell wall formation [29].
The conjugal transfer of vanA gene from VRE to *S. aureus* is the key step in the development of VRSA. The mechanism of this transfer is explained next.

Earlier studies have shown that VRSA has emerged through the transfer of a vancomycin resistance determinant gene, *vanA*, from VRE to *S. aureus* [27, 32, 42, 52, 63]. The Inc-18 like plasmid present in VRE, carries the *vanA* gene on a transposon, *Tn1546* [27, 63]. This Inc-18 like plasmid is then transferred from VRE to *S. aureus* only in the presence of pSK41 plasmid in *S. aureus* [52] (Figure 1).
Figure 1-Mechanism of the transfer of vancomycin resistance from VRE to S. aureus: The mechanism of transfer of vancomycin resistance from VRE to S. aureus is depicted in Figure 1. VRE containing the vanA gene (associated with Tn1546) on an Inc-18 like plasmid (A) acts as a donor cell and a S. aureus containing the pSK41 plasmid (B) acts as a recipient cell. The vancomycin resistance is transferred via conjugative transfer of the Inc-18 like plasmid to S. aureus. This transfer is facilitated by the presence of the pSK41 plasmid. Post conjugation, VRE with the Inc-18 like plasmid (C) and S. aureus containing pSK41, as well as the Inc18 like plasmid carrying vanA gene, are obtained. The Inc-18 like plasmid in S. aureus is lost during replication, but, it survives enough for Tn1546 carrying the vanA gene to transfer to the genome of S. aureus or pSK41.

(This figure is adapted from the mechanism of vancomycin resistance transfer as published previously [27, 52, 64])
pSK41-like plasmids are multi-resistant, low copy, conjugative plasmids of S. aureus [65]. Plasmids belonging to this family include pSK41, pGO1, and pJE1 [66]. These plasmids are known to confer resistance against aminoglycoside antibiotics like gentamycin, kanamycin, and tobramycin via aadD and aacA-aphD genes [65-67].

The pSK41 plasmid is 46.4 kb in size [65]. Genetically, it consists of a highly conserved backbone (which contains sequences for replication, conjugative transfer and maintenance), transposon-like structures, and co-integrated plasmids flanked by IS257 (that contain resistance determinants) [65]. IS257 divides the backbone of pSK41 into two regions, the tra region (which contains genes that regulate conjugative transfer of the plasmid) and region 1 (which contains genes involved in replication, partitioning and multimer resolution) [65].

Inc-18 like plasmids carried by Enterococci have an ability to transfer a transposon encoding vanA (Tn1546) to S. aureus [51]. However, this transfer appears to be rare, possibly because of robust barriers like S. aureus endonuclease, which block this transfer [44].

All thirteen VRSA cases of the United States and their associated VRE and MRSA isolates have been characterized for the presence of Inc-18 like plasmid and pSK41 [42]. Nine (four MRSA and five VRSA) isolates representing seven VRSA cases were found to contain marker genes that indicate the presence of pSK41. In two cases, pSK41 was found in VRSA as well as in associated MRSA isolates, whereas, in the remaining five cases, pSK41 was found to be present in either the actual VRSA isolate or in an associated MRSA isolate. In the case of Inc-18 like plasmid, eight associated VRE isolates carried Inc-18 like plasmid [42]. This information was used to confirm the mechanism of transfer of vanA gene from VRE to S. aureus.
CHAPTER 2

RATIONALE AND SPECIFIC AIMS

2.1 Rationale:

As mentioned earlier, vancomycin is currently the first-line treatment to treat MRSA infections. Therefore, the emergence of VRSA is a severe health care threat, as there are limited antibiotics that are available to effectively treat patients, who are infected with this pathogen [1].

In order to evaluate the potential threat of emergence of VRSA, we specifically aimed to determine the prevalence of the pSK41 plasmid, which plays an important role in the transfer of vancomycin resistance from VRE to S. aureus.

The importance of hVISA: VRSA emerges as a result of vancomycin resistance gene (vanA) transfer from VRE to S. aureus. For this transfer, it is required that the patient has a VRE and S. aureus co-colonized in his body. Thus, this co-colonization of VRE and S. aureus in a patient can be considered the first step towards the possible transfer of vancomycin resistance. A patient’s likelihood of acquiring VRE is directly associated with hospital admission. hVISA has reported longer treatment courses and thus longer hospital stays, increasing the time during which the patient is at risk of VRE infection. Therefore, hVISA persistence in the body along with frequent and longer hospitalization, increase the chances of VRE co-infection in hVISA infected patients compared to non-hVISA infected patients.

The importance of the pSK41 plasmid: The pSK41 plasmid mediates the transfer of vanA from VRE to S. aureus. In the absence of pSK41, this transfer is unlikely to take place. Thus, this plasmid is very important to evaluate the emergence of VRSA. Plus, if hVISA carries the pSK41
plasmid, the risk of the VRSA emergence is increased because of the higher chances of co-colonization of VRE and *S. aureus* in hVISA.

### 2.2 Specific Aims:

1. **A)** To determine the prevalence of pSK41 in all hVISA and non-hVISA isolates.
   
   B) To compare the prevalence of pSK41 in hVISA isolates against matched non-hVISA isolates.

2. To compare the prevalence of pSK41 by patient severity in hVISA isolates.

3. To compare the strain types between hVISA and non-hVISA isolates and between pSK41-positive and pSK41-negative isolates by *spa* typing.
CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples:

The samples were collected from patients presenting for care in Michigan, New York, Pennsylvania and Ohio. The patients included in the study were adults aged ≥ 18 years, who had blood cultures positive for MRSA with vancomycin MIC ≤ 2µg/ml, and had received vancomycin for ≥ 72 hours. All the isolates were confirmed to be hVISA by Population Analysis Profiling (PAP). In order to compare hVISA isolates to non-hVISA isolates, non-hVISA isolates were identified and matched to hVISA surveillance isolates when possible, based on age, source of infection, and year of infection, in a nested matched cohort design.

3.1.2 Controls:

A S. aureus isolate from a previous study [68], which was known to contain pSK41 and gave reproducible results, was used as a positive control in this project. A positive control was included at every step to confirm the success of that step. Molecular-grade water was used as a negative control to confirm the absence of contamination at any step.

3.2 Methods

3.2.1 Bacterial Growth:

All the organisms were first grown on tryptic soy agar (TSA) plates. After an incubation of 18-24 hours, at 37°C, one colony was selected, and further grown in Muller Hinton broth (MHB), (5ml for genomic DNA extraction and 10 ml for plasmid DNA extraction) for 18-24 hours at 37°C.
3.2.2 DNA Extraction:

3.2.2.1 Total DNA Extraction

Total DNA was extracted from all the isolates by using Wizard® SV Genomic DNA Purification Kit (Promega). This kit is used to extract the total bacterial DNA (genomic DNA, as well as accessory DNA). Bacterial cultures were grown overnight, centrifuged, and cell pellets were re-suspended in PBS. Bacterial lysates were then prepared by adding Wizard SV lysis buffer. Bacterial lysates were later transferred to Wizard SV mini-columns for vacuum purification and washed with column wash solution, four times. Total DNA was eluted using nuclease-free water. DNA was stored at -70°C until further testing was performed. This DNA was further used to determine the spa type (See Section 3.2.5).

3.2.2.2 Plasmid Purification

Plasmid DNA was extracted from all the samples using PureYield™ Plasmid Miniprep System (Promega). Bacterial cultures were grown overnight, centrifuged, and cell pellet were re-suspended in TE buffer. Bacterial lysates were prepared by adding cell lysis solution followed by a cold neutralization solution. Lysates were thoroughly mixed in between addition of these reagents. Later, the lysates were centrifuged, and further purified using a vacuum manifold. DNA was eluted using nuclease-free water and stored at -70°C until further testing was performed.

3.2.3 PCR Assays:

Polymerase Chain Reaction (PCR) was used for the amplification of target genes used to determine the presence of pSK41. Two sets of pSK41 specific primers were used [59, 69]. Primer set I [69] was used as a preliminary screen, to detect how many samples possibly carry pSK41. The actual number of samples carrying pSK41 was then confirmed by performing another PCR using primer set II [59]. Two primer sets were used because of the problem of inconsistent and non-reproducible
detection of pSK41 by Primer set I. Detection of pSK41 using Primer set II was found to be reproducible.

3.2.3.1 Preliminary Screening for pSK41 plasmid

Previously published primers were used for preliminary screening for the presence of pSK41 marker genes [69]. These primers have been designed to target the following regions of pSK41: pre, nes, traG, traK, traL, traE and traM. pSK41 was identified using these specific marker genes. 50µl of PCR reaction mixture was set up using 17µl of distilled water, 1.5µl each of 10 µM forward and reverse primers, 5µl of extracted DNA solution and 25µl of PCR Master Mix 2X (Promega). DNA amplification was carried out using T100 Thermal Cycler (Bio-Rad). PCR conditions were an initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 1 min, 52–56 °C for 1 min and 72 °C for 1 min and a final elongation at 72 °C for 5 min [69].

3.2.3.2 Confirmation of pSK41 plasmid

A second set of previously published primers was used to confirm the presence of pSK41 [59]. These primers have been designed to target the following regions of pSK41: traE, traI, repA. The PCR reagent volumes were as described earlier. Thermal cycling parameters included an initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 90 s; and a final extension at 72°C for 10 min [59].

3.2.4 Gel Electrophoresis:

The presence of amplified marker genes was determined by performing gel electrophoresis. Agarose gel (3%) was casted; ethidium bromide was added to help DNA visualization and amplified DNA mixed with loading dye was loaded onto the gel along with an appropriate DNA ladder. Gel electrophoresis was carried out at 3Volts/cm for 45 min. The bands of DNA referring to these specific genes were observed under UV light. For the last few experiments, ethidium
bromide-UV illumination was replaced by the Sybr Safe- Embi Tec PrepOne™ Sapphire gel electrophoresis system that uses LED illumination. This illumination prevents DNA damage during electrophoresis. A selection of samples was retested to confirm that this new method of detection did not affect the detection of pSK41. The results produced by LED illumination gel-electrophoresis were no different from UV illumination gel-electrophoresis.

3.2.5 *spa* typing:

Strain types of these isolates were determined by *spa* typing. Previously published primers were used to carry out PCR [70]. These primers have been specifically designed to amplify a variable region of the *spa* gene. The PCR reaction was set up, in the same way, as described above. The temperature profile included an initial denaturation for 10 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C; and a final extension at 72°C for 10 min [70]. PCR products were purified using Wizard® SV 96 PCR Clean-Up System by Promega. After that, DNA was sequenced by Sanger sequencing technique at the Applied Genomic Technology Center (AGTC) of Wayne State University. DNA sequences were then analyzed to determine the *spa* types using the Sequencher program and DNA Gear software [55].

3.2.6 Statistical Analysis:

The prevalence of pSK41 in hVISA isolates was calculated and compared to the prevalence of pSK41 in non-hVISA isolates. The prevalence of pSK41 was compared in patients with high severity (requiring ICU admission) to pSK41 prevalence in patients with low severity (not requiring ICU admission). These comparisons were used to analyze the association of pSK41 with hVISA/Non-hVISA and patient severity. All the comparative studies were done using Fisher’s exact test; except for the comparison of prevalence of pSK41 within matched pairs, which was
performed by McNemar’s test for matched data. Graphpad and SPSS were used to perform all statistical analyses.
CHAPTER 4

RESULTS

A total of 144 samples were collected from patients presenting for care of bloodstream infections or infective endocarditis in Michigan, New York, Pennsylvania and Ohio. Out of these 144 patients, 84 patients had infection due to hVISA, and 60 patients had infection due to non-hVISA. The study group chosen for this project was subdivided into a matched cohort (60 hVISA patients matched with 60 non-hVISA patients based upon age, source of infection and year of infection) and a surveillance cohort (24 hVISA patients, unmatched). Baseline demographics and clinical outcomes of all hVISA and non-hVISA patients with available clinical data are presented in Table 1.
Table 1: Baseline demographics and clinical outcomes of hVISA and non-hVISA patients.

<table>
<thead>
<tr>
<th>Feature</th>
<th>hVISA (n=73)*</th>
<th>Non-hVISA (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basic Demographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (Median, IQR)</td>
<td>24.93 (21.27-28.67)</td>
<td>25.31 (21.79-29.44)</td>
</tr>
<tr>
<td>Female Sex</td>
<td>32 (43.8%)</td>
<td>21 (35%)</td>
</tr>
<tr>
<td>APACHEII Score (Median, IQR)</td>
<td>12 (8-18)</td>
<td>10 (6.25-15)</td>
</tr>
<tr>
<td>Charlson Index (Median, IQR)</td>
<td>3 (1-4)</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td>ICU Ever</td>
<td>29/61 (39.7%)</td>
<td>23 (38.3%)</td>
</tr>
<tr>
<td>Previous Hospitalization</td>
<td>41 (56.1%)</td>
<td>33 (55%)</td>
</tr>
<tr>
<td>Nursing Home Admission</td>
<td>5/61 (8.2%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>Previous Surgery</td>
<td>4 (6.5%)</td>
<td>10 (16.6%)</td>
</tr>
<tr>
<td>Previous Antibiotics</td>
<td>33/61 (45.2%)</td>
<td>24 (40%)</td>
</tr>
<tr>
<td>Prior Staph Infection</td>
<td>9 (14.8)</td>
<td>8 (13.1)</td>
</tr>
<tr>
<td>Renal Disease</td>
<td>28/61 (45.9%)</td>
<td>26 (43.3%)</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>17 (27.9%)</td>
<td>10 (16.4)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>20/61 (32.7%)</td>
<td>16 (26.6%)</td>
</tr>
<tr>
<td>Decubitus Ulcer</td>
<td>11 (15%)</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>Prosthetic</td>
<td>4/62 (6.4%)</td>
<td>4 (6.6%)</td>
</tr>
<tr>
<td><strong>Clinical Outcomes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deaths at Discharge</td>
<td>14 (22.9%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>Status after 30 days (Deaths)</td>
<td>15 (20.5%)</td>
<td>7 (11.6%)</td>
</tr>
</tbody>
</table>

(*: Patient data for 11 hVISA infected patients could not be obtained through previous records)
Specific Aim: 1. A) To determine the prevalence of pSK41 in all hVISA and non-hVISA isolates.

4.1 Detection of pSK41:

All 144 samples were tested for the presence of marker genes for the plasmid, pSK41. Detection of pSK41 was carried out by a two-step screening. Two different assays were required due to inconsistent gene detection in the first PCR assay.

4.1.1 Preliminary screening to detect pSK41:

Preliminary screening for the detection of pSK41 was performed by using primer set I [69]. pSK41 genes targeted by this primer set were: pre, nes, traG, traK, traE, traL, and traM. Two problems were found while working with these primers: (A) False positive detection using the ‘pre’ gene primer and (B) Inconsistent and non-reproducible detection of genes, leading to questionable determinations of pSK41-positive samples.

These two problems are described further.

(A) False positive data for the ‘pre’ gene assay:

The ‘pre’ gene was one of the pSK41 marker genes from the primer set I. Upon DNA amplification, 77.7% of the samples (112/144) had bands representing the presence of the ‘pre’ gene. As it was the only gene observed in most of the samples, the specificity of this marker gene became questionable, as compared to the other reports [29]. An example of gel electrophoresis result showing false positive detection of ‘pre’ gene is shown in Figure 2.
In order to confirm the non-specificity of this 'pre' primer with pSK41, the sequences of the amplicon from study samples were compared to the other available sequences to see a possible match between the sequence of 'pre' gene and other sequences of the *S. aureus* genome. This comparison was performed using the BLAST tool by NCBI (http://blast.ncbi.nlm.nih.gov/). The results showed that 106 sequences from the *S. aureus* genome had a sequence similar to the sequence of 'pre' gene. These similar sequences were found to be present in different MGEs of *S. aureus* (like pUB110 and SCCmec), as well as a part of the genome of *S. aureus*. Because of these similarities, most of the samples showed an amplification with the 'pre' gene primer, however, the pSK41-specific 'pre' gene was not necessarily getting amplified in every sample, therefore, giving a false positive data. The previous study using these primers did not report this non-specificity issue of the 'pre' gene assay [69]. The results of BLAST are shown in Figure 3.

**Figure 2-False positive data for the 'pre' gene assay:** Five samples, #3762, #3775, #3782, #3783, and #3817, were tested for the presence of pSK41 using primer set I. Out of the five genes tested for each sample, only the 'pre' gene had amplification in all five samples. The bands observed for the 'pre' gene are highlighted in the picture.
Inconsistent and non-reproducible detection of genes:

Inconsistent detection: A detection was called as an ‘inconsistent’ detection, when, all seven marker genes did not amplify. Inconsistent detections gave amplifications for a variable number of marker genes, thus, complicating the detection of pSK41 (Figure 4). The previous study using these primers have considered the isolate to be pSK41-positive if an amplification was observed for any of the marker genes [69]. With that definition of positive detection, we got misleading positive data for our samples.

Figure 3-BLAST hits matching to the sequence of ‘pre’ gene: Distribution of 106 BLAST hits is shown in this figure. Red colored bands indicate an alignment of ≥200bp and the pink colored bands indicate an alignment of 90-200bp. One red band represents the sequence of ‘pre’ gene from pSK41. Similarly, two other bands show similar sequences from pGO1 and SCCmec Other commonly found sequences of S. aureus, having a similar sequence like the ‘pre’ gene are shown by other red bands in this figure.
Non-reproducible results: The results were called ‘non-reproducible’, when the same sample produced different results in different independent PCR runs. For example, if sample ‘A’ gave an amplification for three genes in its first PCR run, it did not amplify the same three genes in its second PCR run or in other words it did not reproduce the same result in its second run (Figure 5).
To check whether this inconsistency in detection was dependent on a particular colony chosen for an experiment, a five-colony experiment was run as well. In this experiment, five colonies of the same sample from the same agar plate were randomly chosen and simultaneously tested for the presence of pSK41. The detection of pSK41 was found to be consistent amongst all chosen colonies (Figure 6). Therefore, it was confirmed that the detection of pSK41 genes did not depend on the colony chosen.
4.1.2 Confirmation of pSK41 by another primer set:

Due to an unreliable detection of pSK41 by primer set I, the extracted plasmids from all the samples were tested by a second, previously published set of primers, primer set II [59]. These were three primers, \textit{traE}, \textit{traI}, and \textit{repA}. Detection of pSK41 was found to be reproducible and reliable with this primer set (Figure 7).
28

10 samples were found to carry the pSK41 plasmid by primer set II [59]. The marker genes observed in these pSK41-positive isolates are summarized in Table 2.

---

**Figure 7-Confirmation of pSK41-positive isolates by primer set II:** Detection of pSK41 by primer set II is shown in this figure. #3875 and #4932 showed bands for one or two genes and in #3044, #5582, #5253 and #4941 amplification of all three genes was observed.
Table 2: The marker genes observed in pSK41-positive samples

<table>
<thead>
<tr>
<th>hVISA/Non-hVISA</th>
<th>SAMPLE #</th>
<th>PRIMER SET II [59]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>traE</td>
</tr>
<tr>
<td>hVISA</td>
<td>3044</td>
<td>+</td>
</tr>
<tr>
<td>Non-hVISA</td>
<td>4941</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>5582</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>5747</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>3829</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>5992</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>5253</td>
<td>+</td>
</tr>
<tr>
<td>Non-hVISA</td>
<td>3606</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>3875</td>
<td>+</td>
</tr>
<tr>
<td>hVISA</td>
<td>4932</td>
<td>+</td>
</tr>
</tbody>
</table>

Specific Aim: 1.B) To compare the prevalence of pSK41 in hVISA isolates against matched non-hVISA isolates.

4.2 Calculation of the prevalence of pSK41

Ten samples out of 144 samples were found to carry pSK41. The prevalence of pSK41-positive isolates was calculated in overall study group, in hVISA isolates (unmatched), in hVISA isolates (matched) and in non-hVISA isolates (matched) (Table 3).
Table 3: Prevalence of pSK41 in various study groups

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Prevalence of pSK41</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVISA from a surveillance study</td>
<td>2/24 (8.3%)</td>
</tr>
<tr>
<td>hVISA from a matched study</td>
<td>6/60 (10%)</td>
</tr>
<tr>
<td>Non-hVISA from a matched study</td>
<td>2/60 (3.3%)</td>
</tr>
<tr>
<td>Overall Prevalence of pSK41</td>
<td>10/144 (6.9%)</td>
</tr>
</tbody>
</table>

4.2.1 A comparison of the prevalence of pSK41 in hVISA isolates against non-hVISA isolates:

The pSK41 plasmid was three times more commonly found in hVISA isolates (6/60, 10%) than in non-hVISA isolates (2/60, 3.33%). However, by McNemar’s test for matched data, results were not statistically significant. In the whole study group, the prevalence of pSK41 was found to be more in hVISA isolates (9.5%) as compared to non-hVISA isolates (3.3%).

4.2.2 The prevalence of pSK41 in all hVISA isolates compared to non-hVISA isolates:

To calculate the overall prevalence of pSK41 amongst hVISA and non-hVISA patients, all 144 samples were considered. This analysis did not control for heterogeneity of patient characteristics including age, source of infection and the year of infection. A comparison of the overall prevalence of pSK41 in all hVISA (n=84) and non-hVISA (n=60) is shown in Table 4. The overall prevalence of pSK41, in the whole study group, was found to be 6.9%. In hVISA isolates, this prevalence was found to be 5.5% whereas in non-hVISA, only 1.3% prevalence was reported. The association between hVISA status and the plasmid, pSK41, was not statistically significant (p = 0.20, Fischer’s exact test).
Table 4: The prevalence of pSK41 in all the hVISA isolates compared to non-hVISA isolates.

<table>
<thead>
<tr>
<th></th>
<th>pSK41-positive</th>
<th>pSK41-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVISA</td>
<td>8 (5.5%)</td>
<td>76 (52.7%)</td>
<td>84 (58.3%)</td>
</tr>
<tr>
<td>Non-hVISA</td>
<td>2 (1.3%)</td>
<td>58 (40.2%)</td>
<td>60 (41.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>10 (6.9%)</td>
<td>134 (93.1%)</td>
<td>144</td>
</tr>
</tbody>
</table>

4.2.3 The prevalence of pSK41 in hVISA isolates compared to matched non-hVISA isolates:

To determine if controlling for patient characteristics affected our results, a second analysis was performed. Only the samples from matched study were chosen to compare the prevalence of pSK41. A pair-wise comparison was performed to know if the prevalence of pSK41 was associated with hVISA/non-hVISA.

Table 5 shows the prevalence of pSK41 in hVISA isolates compared to matched non-hVISA isolates. The association between heterogeneity in vancomycin resistance and the presence of pSK41 was statistically non-significant. (p = 0.29, McNemar's test for matched data).

Table 5: The prevalence of pSK41 in hVISA compared to matched non-hVISA isolates.

<table>
<thead>
<tr>
<th></th>
<th>pSK41-positive</th>
<th>pSK41-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVISA</td>
<td>0 (0%)</td>
<td>6 (10%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>pSK41-positive</td>
<td>2 (3.3%)</td>
<td>52 (86.6%)</td>
<td>54 (90%)</td>
</tr>
<tr>
<td>pSK41-negative</td>
<td>58 (96.6%)</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Specific Aim 2: To compare the prevalence of pSK41 in hVISA isolates by the patient severity:

hVISA infected patients admitted to the ICU were compared with hVISA infected patients admitted to the general inpatient ward. Results showed that there were only two patients who were admitted to the ICU and detected with pSK41 in the hVISA group. The other four patients with pSK41 detected were not admitted to ICU. This data is shown in Table 6. The association between patient severity and the presence of the pSK41 plasmid was not statistically significant (p = 0.99, Fisher’s exact test).

Table 6: Comparison of the prevalence of pSK41 in hVISA isolates by the patient severity.

<table>
<thead>
<tr>
<th></th>
<th>ICU</th>
<th>Non-ICU</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSK41-positive</td>
<td>2 (3.2%)</td>
<td>4 (6.4%)</td>
<td>6 (9.6%)</td>
</tr>
<tr>
<td>pSK41-negative</td>
<td>15 (24.5%)</td>
<td>40 (65.5%)</td>
<td>55 (90.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>17 (27.8%)</td>
<td>44 (72.1%)</td>
<td>61</td>
</tr>
</tbody>
</table>

4.4 Specific Aim 3: To compare the strain types between hVISA isolates and non-hVISA isolates and between pSK41-positive and pSK41-negative isolates by spa typing:

4.4.1 Results for spa PCR:

Detection of the spa gene was found to be consistent. Figure 8 shows the gel picture of spa detection. The spa bands from different samples were observed at different lengths. This happens as a result of a variable size of the gene that is amplified during PCR. spa primers are designed for the amplification of a variable region, region X, within the ‘spa’ protein. Because of mutations, this region X has a variable number of tandem repeats (VNTR). VNTR give rise to spa genes of variable sizes and thus these are observed at different lengths on gel [55, 71].
4.4.2 Distribution of strain types:

Amongst 144 hVISA and non-hVISA samples, the spa types t002 (n= 36, 25%) and t008 (n=35, 24.3%) were most commonly found. Seventeen (11.8%) isolates could not be typed. Other common spa types were t004 (n=7, 4.8%), t062 (n=8, 5.5%) and t688 (n=5, 3.4%). pSK41 plasmid was found to be present in multiple spa types. (Table 7).

Figure 8-Bands of spa gene after amplification: Bands for spa genes after amplification, are shown in this figure.
Table 7: Distribution of the strain types between hVISA and non-hVISA isolates and between pSK41-positive and pSK41-negative isolates by spa typing.

<table>
<thead>
<tr>
<th>Category</th>
<th>t002</th>
<th>t008</th>
<th>t004</th>
<th>t062</th>
<th>t688</th>
<th>Other types</th>
<th>Unknown type</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVISA</td>
<td>21</td>
<td>21</td>
<td>7</td>
<td>8</td>
<td>2</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Non-hVISA</td>
<td>15</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>pSK41-positive</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pSK41-negative</td>
<td>35</td>
<td>33</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>35</td>
<td>15</td>
</tr>
</tbody>
</table>

hVISA status (hVISA/non-hVISA), spa type and patient severity of pSK41-positive isolates is summarized below (Table 8).

Table 8: Summary of pSK41-positive isolates

<table>
<thead>
<tr>
<th>Isolate #</th>
<th>hVISA/Non-hVISA</th>
<th>spa type</th>
<th>Patient severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3044</td>
<td>hVISA</td>
<td>t062</td>
<td>ICU</td>
</tr>
<tr>
<td>3606</td>
<td>Non-hVISA</td>
<td>t008</td>
<td>ICU</td>
</tr>
<tr>
<td>3875</td>
<td>hVISA</td>
<td>t004</td>
<td>Non-ICU</td>
</tr>
<tr>
<td>4932</td>
<td>hVISA</td>
<td>t004</td>
<td>Non-ICU</td>
</tr>
<tr>
<td>4941</td>
<td>Non-hVISA</td>
<td>Unknown</td>
<td>ICU</td>
</tr>
<tr>
<td>5253</td>
<td>hVISA</td>
<td>t008</td>
<td>ICU</td>
</tr>
<tr>
<td>5582</td>
<td>hVISA</td>
<td>t002</td>
<td>Non-ICU</td>
</tr>
<tr>
<td>5747</td>
<td>hVISA</td>
<td>t3234</td>
<td>Non-ICU</td>
</tr>
<tr>
<td>3829</td>
<td>hVISA</td>
<td>t062</td>
<td>Missing</td>
</tr>
<tr>
<td>5992</td>
<td>hVISA</td>
<td>Unknown</td>
<td>Missing</td>
</tr>
</tbody>
</table>
CHAPTER 5
DISCUSSION

This study included 144 samples, of which 84 were hVISA, and 60 were non-hVISA. 120 of the samples were collected from a matched study (60 hVISA samples matched to 60 non-hVISA samples), and 24 hVISA samples could not be matched. All isolates were tested for the presence of pSK41. Ten of 144 samples were found to contain pSK41. Eight of these pSK41-positive samples were hVISA, and the remaining two were non-hVISA.

In a previous study based on these patients, the odds of vancomycin treatment failure were found to be 11 times greater for patients with hVISA infection than for patients with non-hVISA infection [58]. Due to the patterns of persistent and recurrent hVISA infections, vancomycin therapy has shown greater failure rates in hVISA patients than in non-hVISA patients. Vancomycin treatment failure in hVISA patients was found to be associated with bacteremia, recurrence of MRSA-blood stream infections and change or addition of antibiotic against MRSA [58]. All patients had MRSA-blood stream infections, infective endocarditis, or bacteremia. Furthermore, the patients with hVISA infections reported longer hospital stays than the patients with non-hVISA infections [58], thus, they had a higher risk of VRE exposure.

It was also observed that few common underlying characteristics of the hVISA patients of this study were similar to those found in thirteen VRSA patients of the United States (discussed in Section 1.7.2) [58, 60] including renal disease (45.9%), diabetes (32.7%) and hemodialysis (23%) [58, 60].

Therefore, we can say that few of the hVISA patients in our study had more than one risk factors (Underlying conditions similar to known VRSA patients, persistent and frequent hospitalization, and the presence of pSK41) to develop VRSA infection.
Detection of pSK41 in hVISA and non-hVISA isolates: The pSK41 plasmid was detected by two sets of previously published primers [59, 69]. Primer set I (pre, nes, traG, traK, traL, traE and traM) [69] was used as the first marker set of pSK41. A previous study using this primer set did not report any non-specificity issues with the assay of the ‘pre’ gene [69]. However, we found that the primer sequence for the ‘pre’ gene was not uniquely associated with pSK41. The primer sequence was found to be similar to 106 other sequences belonging to the genome of S. aureus. Hence, we confirmed that the ‘pre’ gene assay cannot be used as a reliable marker for pSK41 because of these similarities with non-pSK41 sequences.

Another problem with this primer set was the inconsistent and non-reproducible detection of pSK41. We were unable to determine the reason for the inconsistent detection of pSK41 using this primer. However, this inconsistency was reported in the previous study as well [69]. The previous study considered the isolate to be pSK41-positive if it amplified any of the marker genes [69]. Based upon this consideration, we found that most of our samples (77.7%) would be falsely detected as pSK41-positive by the ‘pre’ gene assay alone. Therefore, this consideration did not work in our study.

We also addressed the problem of non-reproducibility of primer set I. We performed an experiment in which one batch of samples was independently run twice. The results from independent duplicate runs were not reproduced, which indicates that the assay lacked reproducibility of the results.

Lastly, we conducted a five-colony experiment to check if the inconsistency of plasmid detection was dependent on the particular colony chosen for the experiment. However, when five different colonies from the same sample were run simultaneously, the assay produced consistent
results. Hence, we can confirm that the presence of pSK41 does not depend on the colony chosen for the experiment.

We were able to demonstrate reproducible detection of pSK41 using the second set of primers (traE, traI, and repA) [59]. Ten samples were found to carry the pSK41 plasmid. We found that, out of the ten samples that carried pSK41, eight samples amplified all three pSK41 marker genes, and two samples showed amplification for only two marker genes. However, the amplification of any one of the three marker genes was considered to be a positive indication of the presence of pSK41 by McDougal et al. [59]. The reason for this inconsistency is not known. Also, traE and repA have been used to detect pSK41 plasmid previously [29]. Our results were consistent with their data as all the pSK41 plasmids from our study showed amplification for traE and repA genes.

**Prevalence of pSK41 among hVISA and non-hVISA isolates:** In this study, 6.9% of hVISA isolates (8/84) and 3.33% of non-hVISA isolates (2/60) were found to carry pSK41. The pSK41 plasmid was found to be a low-prevalence plasmid in both populations. The overall prevalence of pSK41 in hVISA isolates compared to non-hVISA isolates was not statistically significant (p=0.20). Controlling for age group, source of infection and year of infection via a matched analysis, we found that the results of matched analysis were not different from the overall results. Another study has reported a low (3%) prevalence of pSK41 in *S. aureus* [29]. This study looked for the presence of vancomycin resistance precursors in patients in Michigan with lower extremity wounds. Our results were consistent with Tosh et al findings.

Although the prevalence of pSK41 was not statistically significant, the presence of pSK41 cannot be overlooked because of its critical role in the transfer of vancomycin resistance. Previous studies have shown that pSK41 facilitates the transfer of vanA, a vancomycin resistance
determinant, carried by *Tn1546* in association with an Inc-18-like plasmid, from VRE to *S. aureus* [27, 72]. Out of thirteen VRSA cases reported in the U.S., eight VRE strains associated with VRSA cases showed the presence of *vanA* operon on Inc-18-like plasmid [73] and seven of the recipient MRSA were found to contain pSK41 [27]. It was also shown that pSK41 can facilitate this transfer via a helper cell or even in a cell-free filtrate [52]. However, the presence of pSK41 appears to be essential for this discussed conjugation [52]. One study has recently reported a case of VRSA in Brazil that lacked the presence of pSK41-like plasmid. Instead, this isolate carried the plasmid pBRZ01. However, as reported in earlier studies, pSK41 need not be present in the recipient MRSA cell, it can facilitate the conjugation of Inc-18-like plasmid through a helper cell as well [52, 62]. Resemblance between pSK41 and pBRZ01 has not been reported to date.

Despite being a low prevalence plasmid, pSK41 remains strongly associated with the emergence of VRSA cases. By having an infection with *S. aureus* containing pSK41 plasmid, patients may be at risk of developing a VRSA infection. Moreover, owing to reduced susceptibility towards vancomycin in hVISA, these patients receive longer treatments of vancomycin and stay in hospitals for a relatively long time, therefore, increasing the chance of VRE exposure and transfer of a *vanA* gene. Such patients have infections that are completely resistant to vancomycin and are left with very few options for treatment. Due to all these reasons, the incidence of pSK41 cannot be overlooked.

**Prevalence of pSK41 in hVISA by patient severity:** Only two of six patients infected with hVISA and carrying pSK41 were admitted to the ICU. Only the hVISA-infected patients were chosen to compare this prevalence. hVISA-infected patients do not respond effectively to vancomycin. Additionally, the presence of the multi-resistant plasmid, pSK41, was associated with
a reduction in cure rate as it confers resistance to many drugs, such as gentamycin, kanamycin, tobramycin, neomycin, and bleomycin [73]. Patients infected with pSK41-positive hVISA who are at a high level of clinical severity have very few options for treatment.

In our study, the prevalence of pSK41 in hVISA by patient severity was not statistically significant (p=0.99), which suggests that the presence of pSK41 is not necessarily associated with ICU admission. However, our data were not sufficient to generalize these results, and more work is needed. Little has been documented about the association of pSK41 with overall patient severity.

**Prevalence of pSK41 by spa type:** The most common spa types among all the isolates were t002 (25%) and t008 (24.3%). These spa types have been associated with hospital associated-MRSA and community associated-MRSA [74]. They are also the most common spa types in the U.S. [74]. Our results were consistent with these findings.

The overall distribution of pSK41-positive samples was not specifically related to any spa type. Two isolates carried pSK41, in spa types t008, t004, and t062. One pSK41-positive isolate was detected in type t002 and one in type t3234. However, these results were in slight contrast with the results of spa types of 13 VRSA cases found in the U.S, in which nine of the thirteen VRSA isolates were spa type t002 [34]. Of the nine isolates, five carried pSK41, and four isolates did not carry pSK41 [34]. We did not find a high prevalence of t002 in pSK41-positive isolates; only one isolate carrying pSK41 was spa type t002.

This association between the presence of pSK41 and the spa type has not been documented previously. We found that strains of *S. aureus* can carry this plasmid irrespective of strain type.
CHAPTER 5

CONCLUSION

From this study, it can be concluded that the multi-resistant plasmid, pSK41, is found at a low prevalence in hVISA isolates, and non-hVISA isolates recovered from patients with bloodstream infection. Even though the number was not sufficient to generate statistically significant data, 6.9% of the total isolates in the study carried pSK41. This number cannot be ignored as it might indicate future development of VRSA.

Secondly, pSK41 can be found in patients that are at high and low clinical severity. However, more work is needed to produce robust data regarding this association.

Lastly, we can also conclude that the presence of pSK41 can be found in many spa types. The pSK41 plasmid does not appear to be associated with any particular spa type.
CHAPTER 6

FUTURE EXPERIMENTS

Future directions may include a comparative study of the clinical characteristics and outcomes of pSK41-positive and pSK41-negative isolates. A risk analysis would be performed to understand which population may be at risk of carrying pSK41-positive *S. aureus* infection, and also, to understand whether patients with pSK41 are unique in their clinical outcomes.

Only thirteen cases of VRSA have been reported in the United States to date. Although pSK41 has been found at a low prevalence, its prevalence is still more than the incidence of VRSA cases in the United States. In future, the role of other possible barriers in the prevention of VRSA development might be studied.

Eight out of thirteen VRSA cases were reported in Michigan. Therefore, studying the prevalence of pSK41 in patients from Michigan and comparing it with the patients from other states in USA is needed. More knowledge as to the prevalence in Michigan, compared to other states would inform current hypotheses on the mechanism of VRSA emergence.

We also plan to study the prevalence of pSK41 by infection source, to understand if any particular infection is associated with detection of the pSK41 plasmid.
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ABSTRACT

COMPARATIVE STUDY OF THE PREVALENCE OF pSK41 IN HETEROGENOUS VANCOMYCIN-INTERMEDIATE STAPHYLOCOCCUS AUREUS ISOLATES AGAINST NON-HETEROGENOUS VANCOMYCIN-INTERMEDIATE STAPHYLOCOCCUS AUREUS ISOLATES AND THE PREVALENCE BY PATIENT SEVERITY

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Earlier studies have reported that pSK41 plays an important role in the transfer of vancomycin resistance from Vancomycin-resistant Enterococci (VRE) to S. aureus. This transfer leads to the development of Vancomycin-resistant S. aureus (VRSA). Thirteen VRSA cases have been reported in the United States since 2002. To determine and compare the prevalence of pSK41 in hVISA isolates against non-hVISA isolates and the comparison of the prevalence of pSK41 by patient severity and spa type.

Samples were collected from patients presenting for the care of bloodstream infection. Collection was done in Michigan, New York, Pennsylvania and Ohio. DNA was extracted from all the samples and checked for the presence of pSK41 using pSK41 marker genes. After confirming the final number of pSK41-positive isolates, the prevalence of pSK41 was compared between hVISA isolates and non-hVISA isolates. Secondly, the prevalence of pSK41 was compared by patient severity. Lastly, the distribution of pSK41 isolates among various spa types was studied by performing spa typing.
Out of 144 samples, 10 (6.9%) samples were found to contain pSK41. Eight pSK41-positive isolates were hVISA and two were non-hVISA. The pSK41 plasmid was found to be a low-prevalence plasmid in both hVISA, and non-hVISA isolates. There was no significant association between the presence of pSK41 and patient severity. Also, the presence was pSK41 was observed in many spa types.

The pSK41 plasmid is found at a low prevalence in hVISA isolates, and non-hVISA isolates recovered from patients with bloodstream infection. Even though a low prevalence of this plasmid was observed, 6.9% of the total isolates in the study carried pSK41. This number can’t be ignored as it might indicate future development of VRSA. Secondly, pSK41 can be found in patients that are at high and low clinical severity. However, more work is needed to produce robust data regarding this association. Lastly, we can also conclude that the presence of pSK41 can be found in many spa types. The pSK41 plasmid does not appear to be associated with any particular spa type.
AUTOBIOGRAPHICAL STATEMENT

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