PREVALENCE OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS ON ENVIRONMENTAL SOURCES IN THE CLARKSVILLE, TN AND FT. CAMPBELL, KY AREAS

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PREVALENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ON ENVIRONMENTAL SOURCES IN THE CLARKSVILLE, TN AND FT. CAMPBELL, KY AREAS

A Thesis Presented in Partial Fulfillment of the

Requirements for a Master of Science Degree in Biology

Austin Peay State University

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Jessica Anderson

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been an increasing and evolving problem since it was first reported nearly a half century ago. The increasing resistance of hospital-associated MRSA infections (HA-MRSA) to antibiotics and the emergence of community-associated MRSA (CA-MRSA) infections has necessitated the need for more research into the prevalence, prevention, and treatment of MRSA strains. In this study, the prevalence of MRSA isolates among environmental sites frequently contacted by human skin was evaluated. The sample sites included military fitness facilities, university library and science complex facilities, and local fast food restaurants in the Clarksville, TN and Ft. Campbell, KY areas. All of the sample sites were found colonized with MRSA. A total of 464 isolates of *S. aureus* were obtained and 169 (36.4%) of these were resistant to methicillin. Interestingly, when the MRSA isolates were analyzed by conventional PCR for the *mecA* gene, only 49 (29%) were positive. To assess the virulence of the MRSA isolates, colonies were screened for the presence of the Panton-Valentine Leukocidin (PVL) genes. These genes encode a toxin that has been associated with severe skin and soft tissue lesions and more rarely, necrotizing pneumonia. This leukocidin is closely associated with CA-MRSA infections, and has been shown to be a useful marker for these types of infections. Real-time PCR was used to confirm the presence of PVL genes in a single isolate. These data suggest that these environments may play a role as reservoirs of MRSA, but currently are not a source of the highly-virulent PVL positive MRSA strains indicative of CA-MRSA.

Table of Contents

CHAPTER I

Introduction

Staphylococcus aureus is a ubiquitous, opportunistic pathogen that can cause a variety of diseases including skin and soft tissue abscesses, furuncles, pneumonia, and sepsis (Baggett et al., 2004; Cosgrove et al., 2003; Diep, Sensabaugh, Somboona, Carleton, & Perdreau-Remington, 2004; Francis et al., 2005). It is carried intermittently or continuously in a small portion of the human population within the anterior nares of the nasal passages, although it may also be found on the skin and other areas of the body though not consistently (Kluytmans, van Belkum, & Verbrugh, 1997; VandenBergh et al., 1999). When infections do occur, they can often be effectively treated with β-lactam antibiotics. These antibiotics bind to specific proteins in the bacterial membrane called penicillin-binding proteins (PBP) (Georgopapadakou & Liu, 1980). The β-lactam antibiotics are structural analogs of the peptide D-alanyl-alanine, used in peptidoglycan synthesis, and competitively inhibit its binding to the penicillin-binding proteins (Tipper & Strominger, 1965). This binding of the antibiotic to the PBP inhibits peptidoglycan cross-linking in the cell wall (Izaki, Matsuhashi, & Strominger, 1966). Peptidoglycan is a vital structural component of the bacterial cell wall that provides shape and support. Inhibiting the peptidoglycan cross-linking during cell growth decreases cell wall integrity until osmotic pressure causes lysis to occur, inducing cell death (Wise & Park, 1965).

Concern in treating *S. aureus* began with the occurrence of infections that exhibited resistance to β-lactam antibiotics, particularly after the introduction of the penicillin-like antibiotic methicillin in 1959. *S. aureus* exhibiting resistance to methicillin, under its trade name Celbenin, was first reported in 1961 in the United Kingdom (Barber, 1961). This new methicillin-resistant *Staphylococcus aureus* (MRSA)

has since evolved into a major concern for the medical community, becoming an increasing cause of nosocomial infections. These initial infections were seen in hospitalized patients or with people undergoing chronic medical treatment like dialysis or catheterization. Hospital-associated cases of MRSA (HA-MRSA) were first reported in the United States in 1968 and have increased at an alarming rate, from 2.4% of infections in hospitals in 1975 to 29% in 1991 (Barrett, McGehee, & Finland, 1968; Panlilio et al., 1992). By 1995, MRSA infections accounted for 0.20% of hospitalizations and had increased to 0.21% by the year 2000 although overall *S. aureus* hospitalization rates remained constant (Kuehnert et al., 2005). In 2003, MRSA accounted for 57.1% of *S. aureus* infections in hospitals (Surveillance, 2003). This is a cause for concern because while the overall number of *S. aureus* hospitalizations is constant, the number of drug resistant hospitalizations is increasing with MRSA becoming the most common nosocomial pathogen in hospitals in the United States (Diekema et al., 2004), resulting in higher mortality rates as has been shown with cases of bacteremia (Cosgrove et al., 2003). Newer antibiotics, such as vancomycin can often effectively treat these infections (Maple, Hamilton-Miller, & Brumfitt, 1989); however, there has been an emergence of strains exhibiting resistance to these antibiotics. The first case of vancomycin-resistance was reported in the United States in 1997, followed by fifteen more cases being reported to date according to the Centers for Disease Control and Prevention (Tenover et al., 2004).

Initially, MRSA infections were confined to the hospital setting in the United States, but in the early 1980's cases of community-associated MRSA (CA-MRSA) infections were reported (Saravolatz, Markowitz, Arking, Pohlod, & Fisher, 1982).

Community-associated MRSA infections are differentiated from hospital-associated infections by acquiring the infection without coming into contact with a healthcare facility or undergoing chronic medical treatment. CA-MRSA infections are easily spread by skin contact, so outbreaks commonly occur in close living facilities, such as prisons and military facilities, and with intravenous drug users and athletic teams (Campbell et al., 2004; Charlebois et al., 2002; Diep, Sensabaugh, Somboona, Carleton, & Perdreau-Remington, 2004; Huang et al., 2006; Moran, Amii, Abrahamian, & Talan, 2005; Wannet et al., 2004). Infections usually include skin and soft tissue infections such as abscesses and furuncles and more recently, serious cases of necrotizing pneumonia, particularly in otherwise healthy individuals and children (Baggett et al., 2004; Francis et al., 2005).

β-lactam resistance in *S. aureus* is heterogeneous meaning that depending on growth conditions such as differing temperatures, cultures will have subpopulations with varying levels of resistance (Hartman & Tomasz, 1986). The majority of subpopulations have a minimum inhibitory concentration (MIC) of 5μg/ml of antibiotic or less (Hartman & Tomasz, 1986) and are termed borderline. Also present is a small subset that has a very high level of resistance, possessing an MIC of 600 to 1000μg/ml of antibiotic or higher (Hartman & Tomasz, 1986).

High-level β-lactam resistance is conferred by the *mecA* gene, which is located on a mobile genetic element, the *Staphylococcal* cassette chromosome *mec* (SCC*mec*) (Katayama, Ito, & Hiramatsu, 2000). There are four types of SCC*mec*, I-IV, and community-associated MRSA strains usually have type SCC*mec* IV, which is a highly mobile, smaller cassette than the other three and only confers resistance to β-lactam antibiotics (Diep et al., 2004; Francis et al., 2005;Ma et al., 2002). The *mecA* gene

encodes an inducible altered penicillin binding protein, PBP2a, which reduces the bacterium's affinity for β-lactam antibiotics (Chambers, Hartman, & Tomasz, 1985; Hartman & Tomasz, 1984). Because most CA-MRSA strains have type SCC*mec* IV, they have greater susceptibility to non β-lactam antibiotics than do HA-MRSA strains, which commonly demonstrate multiple drug resistance due to intense selection pressure in the hospital setting (Kilic, Li, Stratton, & Tang, 2006).

Most virulent strains of CA-MRSA that cause community-associated infections have SCC*mec* type IV and also secrete the virulence factor Panton-Valentine Leukocidin (PVL) (Moroney, Heller, Arbuckle, Talavera, & Widen, 2007). The PVL consists of two polypeptide components, LukF and LukS which are encoded by genes *lukF* and *lukS*, respectively. The leukocidin belongs to the group of pore forming, synergohymenotropic toxins (Genestier et al., 2005). These toxins form pores in the membrane of host defense cells, particularly neutrophils, macrophages, and monocytes, by the synergistic action of the LukS and LukF components, leading to apoptosis (Genestier et al., 2005). *In vitro*, the toxin has been shown to induce apoptosis, indicated by rounding of cells and nuclei and marked condensation of chromatin, in neutrophils, specifically targeting mitochondrial membranes (Genestier et al., 2005). PVL is particularly associated with increased virulence in CA-MRSA strains often resulting in severe pyogenic skin lesions or an aggressive necrotizing pneumonia (Francis et al., 2005; Gauduchon et al., 2004; Lina et al., 1999) and is associated with a lower survival rate for patients with pulmonary disease (Lopez-Aguilar et al., 2007). These diseases are a significant concern because they occur in individuals exhibiting no risk factors such as chronic health problems like diabetes, intravenous drug use, and other conditions commonly associated with *S. aureus*

(Charlebois et al., 2002; Gorak, Yamada, & Brown, 1999). An additional concern regarding CA- MRSA strains is they have begun appearing in healthcare facilities where they cause nosocomial disease (Diep et al., 2004). These strains can be effectively treated with antibiotics other than β-lactams; however, multidrug resistance is likely to develop in the future (Diep et al., 2004). Horizontal transfer of genetic information is a common occurrence between bacteria so it is anticipated that CA-MRSA strains will acquire multidrug resistance after contact with the highly resistant strains often found in healthcare facilities. Alternatively, CA-MRSA strains may transfer the genetic information for PVL to the already multidrug-resistant HA-MRSA strains.

The scarce number of studies done to determine the presence of MRSA on environmental surfaces indicates these have not been considered a major avenue of distribution of PVL positive CA-MRSA. The role of environmental surfaces in the persistence of MRSA in the community needs to be determined. Some studies have been done during the course of outbreaks including sampling of possible environmental sources of infection, such as saunas (Baggett et al., 2004); however the role these sources play in transmission of the bacterium has only been assessed during an ongoing outbreak and not as sources of possible transmission throughout the population on a daily basis. Assessing the prevalence of PVL positive CA-MRSA strains in high traffic, public areas with diverse populations will give insight into the possible role that environmental sources may play in assisting the spread of the organism throughout the community.

In this study, surfaces were sampled from eight different locations in the Clarksville, TN and Ft. Campbell, KY areas. These locations included two sites on the campus of Austin Peay State University (APSU), five fitness facilities on Ft. Campbell,

and a combination of several fast food restaurants within Clarksville, TN. The surfaces selected for this study were those that are touched frequently by a variety of individuals but do not require immediate hand washing or are not thoroughly cleaned on a regular basis. Each surface was initially tested for the presence of *S. aureus*. The *S. aureus* isolates were subsequently assessed for resistance to β-lactam antibiotics and if found to be resistant, they were then tested for the PVL genes. Additionally the isolates were tested for resistance to vancomycin, which is now commonly used to treat MRSA infections. While vancomycin is usually successful in treating these infections, instances of isolates exhibiting limited or complete resistance to vancomycin have been reported (Tenover et al., 2004). The knowledge gained from determining the presence of MRSA and PVL-positive MRSA, particularly in areas with diverse, dense populations such as at military bases and universities will be useful in determining possible challenges for facility maintenance to assist in limiting transmission. Supervisors of areas with high concentrations of MRSA, particularly MRSA positive for the PVL virulence factor, should be encouraged to reexamine existing cleaning procedures and the types of cleaning agents used to limit the spread of MRSA within the facilities and to control outbreaks of infection among the patrons who use the facilities.

CHAPTER II

Methods and Materials

MRSA Isolate Collection

Positive control MRSA isolates containing the PVL genes were obtained from three different sources. Bacterial cultures of PVL-positive MRSA isolates (Ryan McDonald HEO of the Saskatchewan Disease Control Laboratory and Dr. Binh An Diep of the Department of Medicine at the University of California, San Francisco) and purified PVL-positive MRSA DNA (Dr. Michael Dunne, Jr. of the Washington University School of Medicine, Barnes-Jewish Hospital) were used as positive controls for DNA isolation and PCR. Environmental samples were obtained from eight local areas to include the Felix G. Woodward Library and Sundquist Science Complex at APSU, the Estep, Gertsch, Fratellenico, Lozada, and Olive physical fitness facilities at Ft. Campbell, and several fast food facilities in the Clarksville, TN area. In the APSU Felix G. Woodward Library, one hundred and eight surfaces were sampled focusing on computer keyboards and computer mice. In the Sundquist Science Complex, one hundred and twenty-four surfaces were sampled including water fountains, door handles, computer keyboards and mice, elevator buttons, light switches, and vending machine buttons. On Ft. Campbell, one hundred samples were collected from four of the fitness facilities and from the Estep fitness facility; one hundred and four samples were taken. Surfaces sampled in the gyms included weight bars, cardiovascular equipment handles, exercise mats, equipment spray bottles, and water fountains. The types of cleaners used at the fitness facilities were recorded. The Estep fitness facility uses Rejuvenal®, a hospital

grade disinfectant and the other fitness facilities all use Evirox® H_2Orange2 Concentrate 117, a hydrogen peroxide based multi-purpose cleaner. Eighty-four samples were taken from fast food restaurants from the Clarksville, TN area focusing on areas including the ordering and condiment counters, bathroom door surfaces and sinks, food trays, and tables.

All surfaces were sampled using cotton swabs slightly moistened with sterile, distilled water. Each swab was then streaked immediately onto an Oxoid® mannitol-salt agar plate at the site. Mannitol-salt agar selects for *Staphylococcus spp.* because it has a high NaCl content, 7.5%, which is inhibitory to most bacteria. The ability of *S. aureus* to ferment mannitol differentiates it from non pathogenic *Staphylococcus spp.* on mannitol-salt agar because the resulting acid production induces the pH indicator phenol red to turn yellow. The presence of *S. aureus* was determined by growth on mannitol-salt agar after 24 h incubation at 37°C accompanied by a yellowing of the colony and surrounding media. The *S. aureus* isolates were then streaked onto BBL® oxacillin screen plates containing 6μg/ml oxacillin and 4% NaCl to test for methicillin resistance. Oxacillin is a penicillin-like antibiotic similar to methicillin that is used in commercial media to detect methicillin resistance because it is more stable, enabling long-term storage of the media. Both antibiotics exhibit resistance to β-lactamase by inhibiting its binding through steric hindrance with their side chains. Methicillin is no longer clinically used in the United States and oxacillin is an acceptable antibiotic to use for screening for beta-lactam resistance in accordance with the National Committee for Clinical Laboratory Standards guidelines (Skulnick et al., 1992). Isolates were considered positive for methicillin resistance if any growth was observed after 24 h incubation at 37°C. The isolates were

then grown in brain heart infusion medium and stored at -80°C in a mixture of 30% glycerol and brain heart infusion medium.

Isolation of DNA

The MRSA isolates were grown on brain heart infusion agar plates and two to three colonies were picked for cell lysis. The colonies were suspended in 100µL of sterile, de-ionized water and then centrifuged for 15 minutes at 14000 rpm in an Edvotek microcentrifuge. The water was then discarded from the bacterial cell pellet. Cells were suspended in 100µL of lysis buffer consisting of 20mM Tris-HCl [pH 8.3] , 50 mM KCl, 1.5 mM $MgCl_2$, 0.50% [vol/vol] Tween 20, 0.45% [vol/vol] Nonidet P-40, 0.01% [wt/vol] gelatin, and 60 µg/ml of proteinase K and then lysed by heating for one hour at 55°C followed by continued incubation for five minutes at 95°C (Brakstad, Aasbakk, & Maeland, 1992). The lysates were then stored at -80°C.

PCR Detection of *mecA* gene

Molecular detection of the *mecA* gene was obtained by endpoint PCR in an Applied Biosystems 2720 96-well thermal cycler. The *mecA* primers used in the assay were made available from a previous project at Austin Peay State University and the sequences were as follows: forward primer 5' GTA GAA ATG ACT GAA CGT CCG ATA A 3'; reverse primer 5' CCA ATT CCA CAT TGT TTC GGT CTA A 3'. Each PCR reaction had a total volume of 25µl and consisted of: 5µl of Green GoTaq® Flexi Buffer (5X), 4µl of MgCl (25mM), 0.5µl of deoxyribonucleotides (10µM each), 2µl of forward and reverse primer mix (10µM each), 0.125µl of GoTaq® DNA 5µg/µl, 6.375µl of molecular grade water. The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, then 38 cycles of

the following steps : denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 45 seconds, with a final extension at 72°C for 5 minutes. The resulting amplicon was 309 base pairs (bp) in size, which was visualized by electrophoresis on a 1.5% agarose gel.

PCR Detection of Panton-Valentine Leukocidin genes

The presence of PVL genes was determined using a TaqMan® real time PCR assay. The PCR assays were performed on an Applied Biosystems standard 7500 system. The probe and primer sequences picked have been successfully used for a triplex real time assay for the PVL genes (McDonald et al., 2005); however the reporter dye on the probe was changed to FAM due to the dye calibration standards available for our ABI 7500 system. The probe sequence was 5' FAM ATT TGT AAA CAG AAA TTA CAC AGT TAA ATA TGA TAMRA 3'. The forward primer sequence was 5' ACA CAC TAT GGC AAT AGT TAT TT 3' and the reverse primer sequence was 5' AAA GCA ATG CAA TTG ATG TA 3'. The size of the PVL-specific amplicon was 175 bp. Each PCR reaction had a total volume of 25µl and consisted of: 12.5µl of TaqMan® Universal PCR Master Mix (2X), 2µl of forward and reverse primer mix (10µM each), 0.25µl of probe (10µM), and 3.25µl of molecular grade water. The cycling parameters were as follows: 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles with the following steps: denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute. Prior to amplification of the PVL genes, the samples and positive controls were also amplified with an ABI TaqMan® Exogenous Internal Positive Control kit (IPC). This contains a set of pre-designed primers and probe with accompanying DNA that produces a small amplicon. This kit is used to ensure that negative amplification results were the result of the absence of the

PVL encoding genes and not the result of an agent in the reaction mix. The IPC reactions were performed with the same cycling parameters as the PVL reaction. Positive controls containing PVL genes including both pure DNA and bacterial cultures that underwent the same DNA extraction process as the environmental samples were run to ensure efficacy of primers. PVL genes were considered present if logarithmic amplification occurred before forty cycles. End-point detection of PVL-positive isolates was then visualized by electrophoresis on a 12% polyacrylamide gel.

Agarose and Polyacrylamide Gel Electrophoresis of *mecA* and PVL genes

In order to visualize the amplified segment of the *mecA* gene, the samples were electrophoresed through a 1.5 % agarose gel in Tris-Acetate-EDTA (TAE) buffer containing 10μg/μl ethidium bromide. Seven microliters of the PCR reaction were loaded into the gel submerged in TAE buffer and electrophoresed at 80V for approximately one hour. The gel was then placed over ultraviolet light and the image was recorded using Kodak Gel Logic 100 imaging software.

A 12% polyacrylamide gel in a vertical electrophoresis apparatus was used to visualize the PVL positive isolates. A polyacrylamide gel was used in order to get better resolution of the small DNA fragment produced by the PCR assay. The polyacrylamide gel was prepared with and electrophoresed in Tris-Borate-EDTA (TBE) buffer. Electrophoresis was performed at 60V for approximately one hour and the gel was then placed in a small tray and covered with 10μg/ml of ethidium bromide prepared in TBE. The gel was incubated for approximately fifteen minutes with constant shaking. The buffer was then removed and the gel was washed with constant shaking in distilled water

for an additional ten minutes. The gel was then placed over ultraviolet light and the image was recorded with Kodak Gel Logic 100 imaging software.

Vancomycin Resistance Screening

Vancomycin is a commonly used antibiotic that is effective in treating MRSA infections however there have been reports of MRSA infections showing limited to complete resistance to vancomycin (27). In order to test for MRSA isolates that also exhibited vancomycin resistance, all MRSA isolates were plated onto BBL® vancomycin screen agar containing 6µg/ml vancomycin. Isolates were considered positive for vancomycin resistance if any growth was observed after 24 h incubation at 37°C.

The entire process of sample analysis is summarized in Figure 1.

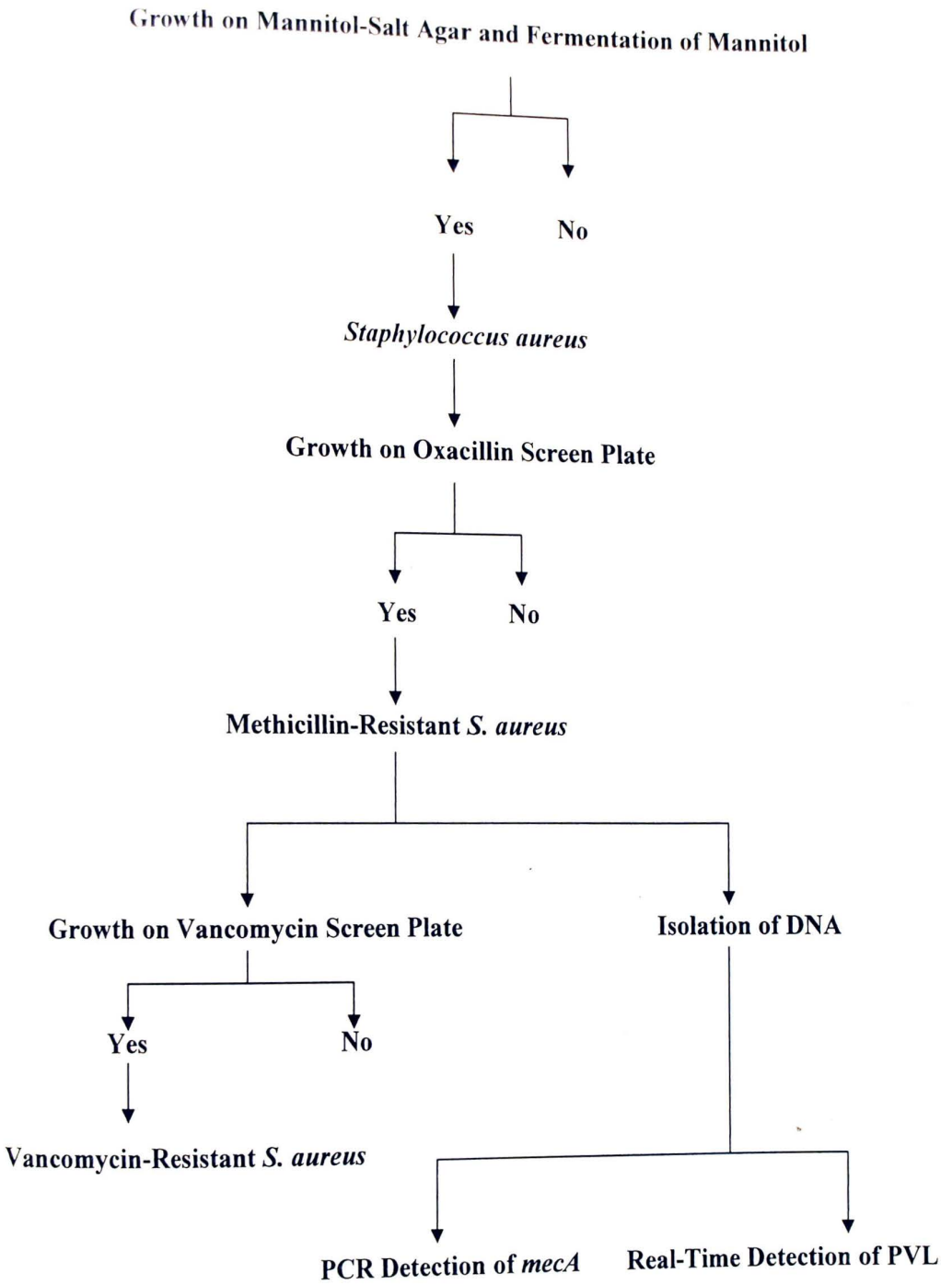

Figure 1. Flow chart depicting the sample analysis process.

CHAPTER III

Results

MRSA Isolate Collection

A total number of 822 environmental samples were collected and *S. aureus* was identified by growth and mannitol fermentation on mannitol salt agar from 464 (56.4%) of the samples. Of these, 169 (36.4%) were confirmed positive for methicillin resistance by growth on oxacillin screen agar. The culture results for the total number of samples from each site are summarized in Table 1 and the results from each type of surface at each site are listed in Table 2. Figure 2 shows a representative *S. aureus* culture on mannitol-salt agar and Figure 3 shows a representative oxacillin screen plate inoculated with methicillin-resistant and methicillin-susceptible *S. aureus* isolates.

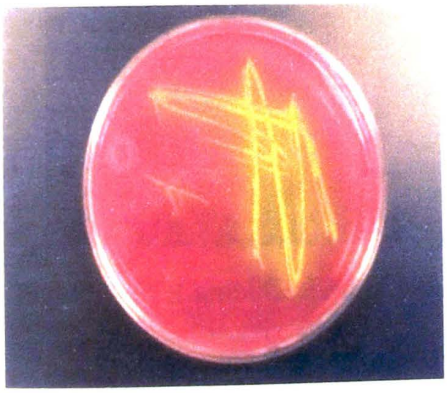

Figure 2. *S. aureus* cultured on mannitol salt agar. Growth indicates *Staphylococcus spp.* Yellow color indicates *S. aureus*.

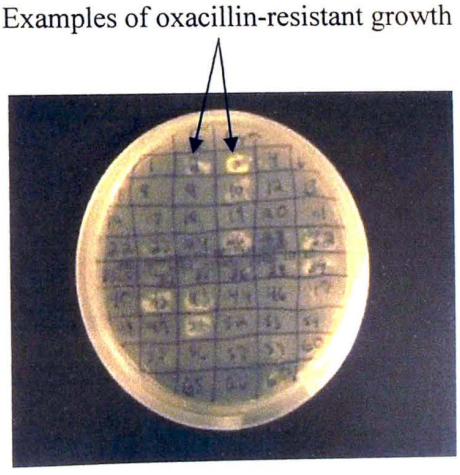

Figure 3. *S. aureus* isolates on oxacillin screen agar 6µg/ml. Growth indicates resistance to beta-lactam antibiotics.

PCR Detection of *mecA* Gene

All 169 methicillin-resistant isolates were screened for the *mecA* gene by conventional endpoint PCR. A 309 bp band corresponding to the endpoint PCR product of the *mecA* gene was visualized by agarose gel electrophoresis for 49 (29%) of the 169 isolates. The distribution of samples positive for the *mecA* gene from each site is summarized in Table 1. Figure 4 shows a representative agarose gel with a *mecA* positive control in Lane 1, *mecA* no template control in Lane 2 and *mecA* positive and negative MRSA isolates from our study in the remaining lanes.

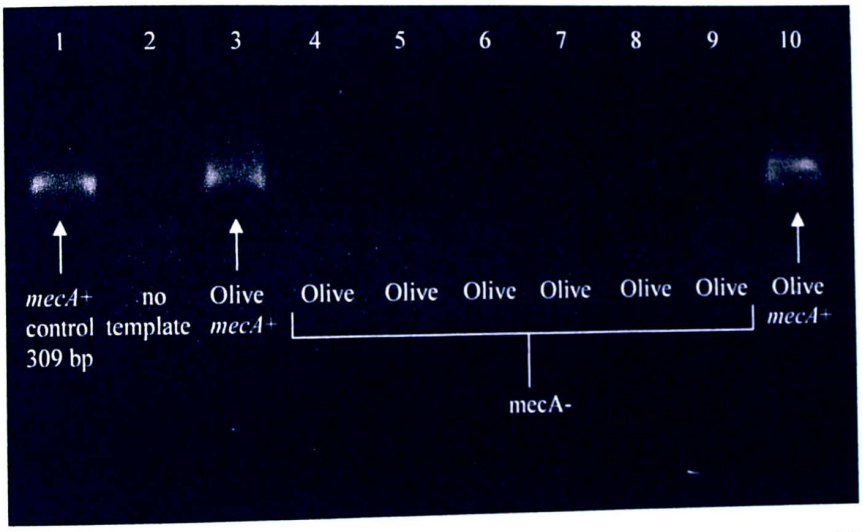

Figure 4. PCR amplification of *mecA* gene from MRSA isolates from the Olive fitness facility on Ft. Campbell, KY.

Real Time PCR Detection of Panton-Valentine Leukocidin Genes

All MRSA isolates were also screened for the presence of the PVL genes by a real-time PCR assay. Logarithmic amplification was recorded for one isolate, taken from

Gertsch physical fitness facility, with threshold crossover value (Ct) at 33.45 cycles. The Ct values for the purified DNA and lysate positive controls were 25.87, 30.33, and 34.96 cycles respectively. Figure 5 shows the amplification plot generated by the real time PCR run with the threshold value at 0.1. The amplified product was also visualized on a 12% polyacrylamide gel. Figure 6 shows the 12% polyacrylamide gel with the Gertsch fitness facility PVL-positive environmental isolate.

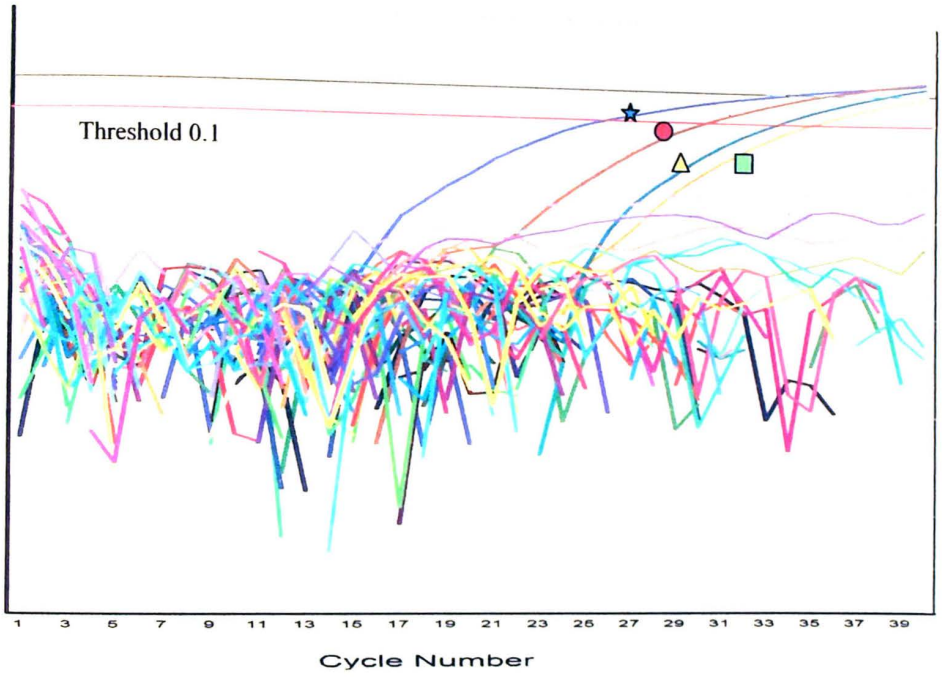

Figure 5. Amplification plot from real-time PCR. Symbols correspond as follows: ☆purified DNA PVL+ control (Ct 25.87), ● - bacterial lysate PVL+ control (Ct 30.33),△- Gertsch PVL+ envonmental sample (Ct 33.45), □-bacterial lysate PVL+ control (Ct 34.96).

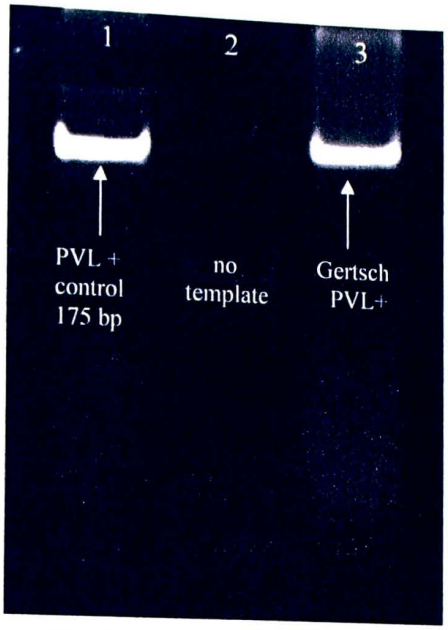

Figure 6. PCR amplification of PVL genes from PVL+ Gertsch fitness facility. Lane 1, PVL-positive isolate, Lane 2, no template control, Lane 3, PVL-positive isolate from Gertsch fitness facility.

Vancomycin Resistance Screening

Methicillin-resistant isolates were screened for vancomycin resistance by growth on vancomycin screen agar after twenty hours incubation at 37°C. Fourteen (8.3%) of the 169 MRSA isolates were determined resistant to vancomycin. Seven of these were from fast food restaurants, one from the Gertsch fitness facility, three from the Olive fitness facility, one from the Lozada fitness facility, and two from the APSU Felix G. Woodward Library (Table 1). The single PVL-positive isolate from the Gertsch physical fitness facility was not vancomycin resistant. Figure 7 shows a representative vancomycin screen plate with vancomycin-resistant and susceptible MRSA isolates.

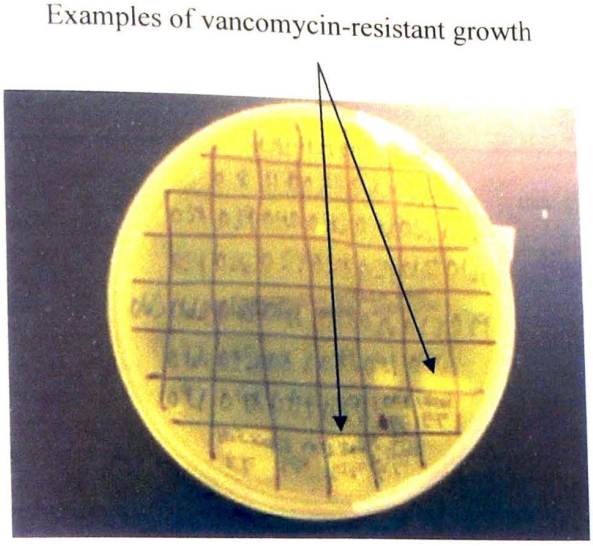

Figure 7. Vancomycin-resistant and vancomycin-susceptible MRSA isolates on vancomycin screen agar 6µg/ml.

Table 1. Environmental isolate distribution from sites in Clarksville, TN and Ft. Campbell, KY.

Site	# samples	*S. aureus*[1]	MRSA[2]	*mecA*[3]	PVL[4]	Van[5]
Felix G. Woodward Library	108	94	28	18	0	2
Sundquist Science Complex	124	61	14	6	0	0
Estep Fitness Facility	104	23	3	1	0	0
Gertsch Fitness Facility	100	72	28	11	1*	1
Fratellenico Fitness Facility	100	71	17	3	0	0
Olive Fitness Facility	100	65	38	6	0	3
Lozada Fitness Facility	100	57	31	4	0	1
Fast Food	86	21	10	0	0	7
Total	**822**	**464**	**169**	**49**	**1**	**14**

[1] Isolates confirmed positive for *S. aureus* on mannitol salt agar

[2] Isolates confirmed positive for methicillin resistance on oxacillin screen agar

[3] MRSA isolates confirmed positive for *mecA* gene by PCR

[4] MRSA isolates confirmed positive for PVL genes by real time PCR

[5]MRSA isolates confirmed positive for vancomycin resistance

* PVL positive isolate was not also vancomycin resistant

Table 2. Surface distribution of environmental isolates obtained from sites in Clarksville, TN and Ft. Campbell, KY

Site	Surface	# samples	*S. aureus*[1]	MRSA[2]	*mecA*[3]	PVL[4]	Van[5]
Felix G. Woodward Library	Keyboard	50	46	15	9	0	1
	Mouse	50	43	13	9	0	1
	Elevator button	7	5	0	0	0	0
	Copier button	1	0	0	0	0	0
Sundquist Science Complex	Keyboard	28	15	3	1	0	0
	Mouse	28	11	1	1	0	0
	Vending machine	26	11	3	2	0	0
	Elevator button	16	11	3	1	0	0
	Bathroom door	14	6	2	0	0	0
	Water fountain	8	6	1	0	0	0
	Light switch	2	0	0	0	0	0
	Telephone	2	1	1	1	0	0
Estep Fitness Facility	Weight bar	37	7	1	1	0	0
	Weight adjuster	24	7	1	0	0	0
	Weight machine handle	20	3	0	0	0	0
	Cardiovascular equipment	7	1	0	0	0	0
	Mat	5	1	1	0	0	0
	Water fountain	4	1	0	0	0	0
	Ball	4	3	0	0	0	0
	Spray bottle	3	0	0	0	0	0
Gertsch Fitness Facility	Weight bar	47	34	10	6	1*	0
	Weight machine handles	26	18	10	3	0	0
	Weight adjuster	14	9	5	1	0	0
	Ball	7	6	2	0	0	1
	Spray bottle	3	2	0	0	0	0
	Mat	2	2	0	0	0	0
	Water fountain	1	1	1	1	0	0

Table 2. (continued)

Site	Surface	# samples	*S. aureus*[1]	MRSA[2]	*mecA*[3]	PVL[4]	Van[5]
Fratellenico Fitness Facility	Weight bar	57	39	11	2	0	0
	Weight machine handles	16	14	1	0	0	0
	Weight adjuster	11	8	3	1	0	0
	Cardiovascular equipment	8	4	2	0	0	0
	Mat	7	6	0	0	0	0
	Pull up bar	1	0	0	0	0	0
Olive Fitness Facility	Weight bar	46	28	13	1	0	0
	Cardiovascular equipment	13	7	6	1	0	1
	Weight machine handles	12	9	8	1	0	1
	Ball	8	7	3	1	0	0
	Weight adjuster	7	5	4	1	0	0
	Paper towel dispenser	5	3	1	1	0	1
	Spray bottle	4	2	2	0	0	0
	Scale	2	2	1	0	0	0
	Mat	1	1	0	0	0	0
	Water fountain	1	0	0	0	0	0
	Rope	1	1	0	0	0	0
Lozada Fitness Facility	Weight bar	41	19	11	2	0	0
	Weight machine handles	22	16	7	0	0	0
	Cardiovascular equipment	13	10	4	0	0	0
	Ball	12	10	8	2	0	1
	Spray bottle	3	0	0	0	0	0
	Weight adjuster	2	0	0	0	0	0
	Water fountain	2	0	0	0	0	0
	Hand grip	2	0	0	0	0	0
	Paper towel dispenser	1	0	0	0	0	0
	Mat	1	1	1	0	0	0
	Scale	1	1	0	0	0	0

Table 2. (continued)

Site	Surface	# samples	*S. aureus*[1]	MRSA[2]	*mecA*[3]	PVL[4]	Van[5]
Fast Food	Ordering counter	13	2	2	0	0	0
	Bathroom door	13	4	1	0	0	1
	Sink	13	3	2	0	0	1
	Condiment counter	12	5	1	0	0	1
	Tray	12	1	0	0	0	0
	Front door	12	4	2	0	0	2
	Table	11	2	2	0	0	2

[1]Isolates confirmed positive for *S. aureus* on mannitol salt agar

[2] Isolates confirmed positive formethicillin resistance on oxacillin screen agar

[3] MRSA isolates confirmed positive for *mecA* gene by PCR

[4] MRSA isolates confirmed positive for PVL genes by real time PCR

[5] MRSA isolates confirmed positive for vancomycin resistance

* PVL positive isolate was not also vancomycin resistant

Statistical analysis

A Chi-Square test for independence was done to determine if the distribution of MRSA isolates and methicillin-susceptible *S. aureus* (MSSA) isolates among the eight sites was equal or if they were site dependent. The Chi-Square value was 53.83, which was much higher than the table value of 14.1 at an alpha level of 0.05. This indicates that the number of MRSA isolates at each site was site dependent and due to site-specific factors and not merely natural prevalence. These factors include cleaning frequency, types of cleaners used, and the number of people using the facility. The Chi-Square test is summarized in Table 3.

Table 3. Chi-Squared Test for independence of MRSA and MSSA isolates obtained from environmental sources at sites in Clarksville, TN and Ft. Campbell, KY

	Felix G. Woodward Library	Sundquist Science Complex	Estep	Gertsch	Fratellenico	Olive	Lozada	Fast Food
MRSA								
Obs.	28	14	3	27	38	17	31	10
Exp.	34	22	8	26	24	25	21	8
MSSA								
Obs.	66	47	20	45	27	53	26	11
Exp.	60	39	15	46	41	45	12	13

Degrees of Freedom	7
Chi-Square Table value	14.1
Chi-Square calculated	52.667

CHAPTER IV

Discussion

Environmental sources have not been considered a significant source of infection for MRSA strains. In this study, MRSA was found on a wide variety of environmental sources, especially on surfaces that are not widely recognized by the public as reservoirs of pathogenic bacteria, such as computer keyboards, and are not disinfected with the same vigor as more commonly cleaned surfaces like those found in bathrooms. The surfaces sampled in this study can be excellent sources of MRSA because they are infrequently cleaned, as in the case with keyboards, or as in the case with the gym equipment, a person has close physical contact with the surface and does not generally cleanse the skin after each instance of contact.

The difference between the percentage of *S. aureus* isolates found positive for methicillin resistance by growth on oxacillin screen agar (36.4%) and the percentage of those confirmed to have the *mecA* gene by PCR (10.6%) was substantial. Examination of the plates yielded the observation that positive growth often occurred in clusters as shown in Figure 3, but none of the isolates invaded the section of any other, except in rare cases of very vigorous growth. The presence of clusters does raise doubts on the validity of the results because a more random distribution was expected however, since oxacillin screen agar plates have been shown to give false positive results of up to only 1% with *S. aureus*, the results from the plates were deemed sufficient for screening purposes (Sakoulas et al., 2001). It is possible that alternate methods of methicillin resistance are present other than the mechanism of the *mecA* gene.

The large difference between the number of total methicillin-resistant strains and those containing the *mecA* gene could also be explained by the fact that most strains demonstrate heterogeneous methicillin resistance which can be low or borderline and can occur with or without the presence of the *mecA* gene. Three mechanisms in particular have been shown to provide borderline resistance, even without the presence of the *mecA* gene. First, MRSA strains can have altered PBP genes with point mutations that result in slower β-lactam binding and faster β-lactam release (Hackbarth, Kocagoz, Kocagoz, & Chambers, 1995; Tomasz et al., 1989). Second, the over production of PBP4 has been shown to produce resistance because more PBP's are available than can be bound by the antibiotic, allowing continued peptidoglycan cross-linking and cell wall synthesis even in the presence of antibiotic (Henze & Berger-Bachi, 1996). Third, since β-lactamase, an enzyme that inactivates β-lactam antibiotics is extracellular, it is also plausible that one or more of the MRSA strains is hyper-producing and secreting such an enzyme (de Lencastre, Sa Figueiredo, Urban, Rahal, & Tomasz, 1991) There has been evidence that sometimes the *mecA* gene can be "lost" due to potential instability; meaning that it could have grown on the oxacillin plate and then not been amplified during PCR, but this is associated with long term storage and is not considered a cause in this study (Sakoulas et al., 2001). The difference is most likely due to non-*mecA* methods producing lower level resistance, but the exact mechanism of resistance cannot be determined without further molecular analysis.

The large difference between the oxacillin screen results and the PCR amplification also emphasizes the need for additional analysis of the vancomycin resistance results. Of the 169 isolates plated on vancomycin screen agar, 14 (8.3%)

showed resistance. These also occurred in clusters of growth on the plate as seen in Figure 7 indicating that the actual number is possibly lower. Four isolates were positive for both the *mecA* gene, and vancomycin resistance however PCR amplification of the gene encoding vancomycin resistance, *vanA*, would be necessary to determine which isolates actually exhibit vancomycin resistance and if there are any false positives. Further analysis would also need to be done to determine if these isolates exhibit other forms of vancomycin resistance, other than the presence of the *vanA* gene, including a thickening of the cell wall (Cui et al., 2003).

The real time PCR results showed that one sample was positive for the presence of the PVL genes. Panton-Valentine Leukocidin genes are often found in CA-MRSA strains and are used as a marker for virulent forms of these strains (Kilic et al., 2006). In a study in middle Tennessee of 1,315 MRSA isolates clinical isolates, 64.4% contained the SCC*mec* type IV which is indicative of CA-MRSA strains, and 93.6% of those were positive for the PVL genes (Kilic et al., 2006). Additional studies have also shown the high PVL occurrence among clinical isolates containing SCC*mec* type IV (Moroney et al., 2007). Since only one of the methicillin-resistant *S. aureus* isolates identified in our study was positive for PVL-producing genes, this indicates that the expectation for clinically relevant CA-MRSA infections from these facilities should be relatively low.

Further analysis to determine the mechanisms of resistance for the isolates that do not contain the *mecA* gene as well as the minimum inhibitory concentrations of β-lactam antibiotics needed to kill these possibly borderline bacteria would be helpful in determining how much of an antibiotic resistance concern these isolates are if they were to cause infection.

The presence of MRSA in these environments is clinically relevant in that MRSA isolates exhibiting strong methicillin-resistance conferred by the *mecA* gene are found on a variety of surfaces that do not receive the same sterilization efforts as other environments. However, the occurrence of only a single PVL positive isolate is important because although these surfaces harbor MRSA, these isolates will not cause the highly virulent CA-MRSA infections, particularly skin and soft tissue, commonly seen with PVL-positive infections. It would be necessary to do further analysis testing multiple resistance to determine if these isolates would cause infections difficult to treat with the current antibiotic arsenal.

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