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Biochemical and Molecular Identification of Type V MRSA among Students at a Small Southern University

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Abstract

Staphylococcus aureus is a commensal organism of healthy individuals associated with the skin and mucosal membranes. Methicillin-resistant *S. aureus* (MRSA) has become a serious health concern due to its resistance to antibiotics. There are eight types of MRSA, with type V MRSA having its origins in India; few type V have been reported in the United States. This study was initiated to determine if type V is present in south Texas and to find out if the transmission of this uncommon type amongst students at Texas A&M University-Kingsville is possible. Through biochemical testing, seventy-eight out of 200 domestic students (39) and international students (39) were identified as carriers for *S. aureus*. Of those 78 students, 19 (25%) were positive for MRSA. Six (32%) of the 19 students were domestic and 13 (68%) were international. Polymerase chain reaction methodology was used to identify one type I, five type IV, and 10 type V MRSA isolates. Four isolates were determined to be “non-typable”. Two (33%) of the six domestic student MRSA isolates and eight (62%) of the 13 international student isolates were determined to be type V MRSA. A Pearson’s Chi-square test found a significant difference between the number of type V strain positive domestic students and international students (X2=4.08, df=1, p=0.043). There was no significant difference between the number of domestic and international students as carriers of *S. aureus* or MRSA. Additionally, health-care associated MRSA type I was isolated from a campus computer along with two type V community-associated MRSA strains, which suggests that indirect transmission may play a role in the spread of these pathogens. These results indicate that although type V MRSA is considered rare in the United States, it is probably more prevalent than suspected.

Keywords: *Staphylococcus aureus*; MRSA; Type V

Introduction

Staphylococcus aureus remains one of the top public health concerns due to its ability to acquire resistance to antibiotics. Although these bacteria are part of the normal microbiota of various animals (e.g. humans, cats, pigs), they can potentially invade tissues and cause infection in both animals and humans [1,2]. These types of infections occur endemically in the United States and have been on the rise in the past decade [3,4]. The severity of a skin and soft tissue infection depends on which tissue layer has been colonized by the pathogen. Mild infections occur on the epidermal tissue layer while more serious infections are present in the subcutaneous or muscle tissue [5]. In a North American study conducted from 1998-2004, *S. aureus* was the most commonly isolated organism from patients diagnosed with skin and soft tissue infections [6]. Lack of sanitation and misuse of antibiotics in both health care and community settings has led to an increase of antibiotic resistance in *S. aureus.* In many countries, distribution of antibiotics is highly unregulated, allowing them to be sold without a prescription [7]. According to an article published by the Canadian Medical Association, only 10% to 20% of patients who experience signs and symptoms of a bacterial infection need to be prescribed antibiotics [8].

β-lactam antibiotics are commonly prescribed when treating patients with soft tissue infections and usually eliminate *S. aureus* and other Gram-positive pathogens. Increase of antibiotic resistance in *S. aureus* has been linked to the acquisition of a set of genes that allow it to continue with cell wall synthesis during exposure to β-lactam treatments [9]. Such strains are referred to as methicillin resistant *S. aureus* (MRSA) and have traditionally been classified based on where the patient acquires them. Healthcare-associated MRSA (HA-MRSA) has been associated with medical centers as well as hospitals, and are typically characterized by the patient having signs and symptoms of the infection 48 hours after being in a medical facility and tend to be resistant to multiple drugs [10,11]. Community-acquired MRSA (CA-MRSA) are the antibiotic-resistant strains that tend to be more virulent and are associated with physical contact such as participation in sports, sharing of toiletry items and improper care of lesions during contact. CA-MRSA are also associated with more virulence factors such as the production of toxins, stronger adherence factors, and membrane surface proteins that resist and target cells of the immune system [12,13]. At the genomic level, these strains are classified based on the SCC*mec* cassette I-VIII with types I-III being HA-MRSA and the others being classified as CA-MRSA. More recently, community acquired strains have been found in health care facilities and vice versa, suggesting that the classification of MRSA based on location may soon be unnecessary [14-16]. Types-IV and -V MRSA have small SCCmec cassettes compared to the other MRSA types and it has been theorized that this allows them to grow more rapidly resulting in quicker colonization and ensuing infection. However, Lee et al. [17] compared type I and type IV MRSA under different conditions and suggested that it may be due to the over-expression of PBP 2a by type I leading to the organism needing to compensate in its growth rate, rather than the large size of the cassette when compared to type IV. The smaller cassette may allow for easier insertion into the genome

making type IV one of the most prevalent MRSA being distributed worldwide [18,19]. Molecular epidemiological studies have determined that type IV MRSA strains are the most common cause of CA-MRSA infections in the United States, whereas type II MRSA strains are the mostly responsible for HA-MRSA infections [20,21].

Type V has emerged in over 11 different countries throughout Asia and has been reported in other continents in people as well as swine [22,23]. In contrast to types I-IV and VI, type V MRSA contains a single ccrC fragment, without the presence of *ccrA* and *ccrB*. *ccrC* is a non- conserved region of the SCC*mec*, which results in a rare form of the cassette [24]. Type V MRSA has only been recorded in the United States in Iowa among a group of employees at a livestock sorting facility. The same type V MRSA strain was also isolated from the swine in the facility which begs the question of where the MRSA originated [25].

Some researchers suggest that type V MRSA is largely limited to Asia and will remain rare in other countries [3]. However, more research involved with tracking the emergence of type V MRSA in countries where it is considered uncommon must be carried out in order to come to this conclusion. The high rate of immigration to the United States from various countries in Asia raises the question as to whether or not type V MRSA will continue to remain an uncommon type among the population. Genetic differences present between MRSA strains originating from different geographic locations allow for the categorization into different types, some possessing more antibiotic resistance and virulence factors than others. This is why it is important to track the emergence of MRSA strains in countries where they are uncommon among the population so more can be learned regarding transmission and control. In this study, domestic and international students were tested for the presence of MRSA and more specifically, type V MRSA, to determine if it is present and if the international students were the predominant carriers of this strain which is rare to the United States.

Materials and Methods

Sample collection and bacterial cultures

Collections took place at the Biological & Health Sciences building, Student Union building, and Engineering complex at Texas A&M University-Kingsville. Collection of samples was approved by the TAMUK Internal Review Board proposal #2013-073. The students were given a survey in order to determine their age group, travel history to Asia, family, spouse, and roommate travel history to Asia, and if they had ever been diagnosed with MRSA. The international students were to list where they were from if not originally from Asia.

Two hundred students voluntarily swabbed their nasal and pharyngeal passages using a Becton Dickenson Culture Swab^{TM} (Sparks, MD) resulting in a total of 400 samples that were plated on mannitol salt agar (MSA) and incubated for 24 hours at 37°C. Putative Staphylococcus aureus isolates were plated on Mueller-Hinton agar and tested for oxacillin resistance using the disc diffusion method [26]. Oxacillin was substituted for this study due to its similar mode of action as methicillin, availability, and lower cost. Strains that grew up to the 1 μg oxacillin disc (Becton Dickenson BBL^{TM} sensi-discs) were cultured in Mueller Hinton broth containing 4 μg/ml oxacillin to confirm antibiotic resistance. Bacteria that grew in the broth after 24 hours of incubation were considered MRSA and further characterized while those that could not grow in the presence of the oxacillin were considered methicillin sensitive and not characterized further. Putative MRSA strains were also tested for antibiotic sensitivity against a panel of antibiotics chosen because they are recommended by the Infectious Diseases Society of America (IDSA) for treatment of CA-MRSA especially for skin and soft tissue infections, as well as pneumonia [27]. These antibiotics included vancomycin (30 μg), clindamycin (5 μg), gentamycin (10 μg), doxycycline (30 μg), trimethoprim (5 μg) and sulfamethoxazole with trimethoprim (23.75 μg with 1.25 μg, respectively).

In addition to the testing of students, thirty-four computer keyboard and mouse surfaces located in two computer laboratories were tested for the presence of *S. aureus* and MRSA. Sterile culture swabs were moistened in sterile phosphate buffer saline (PBS) prior to collection, which aided in picking up bacteria from dry surfaces. Specifically, the main keys where fingertips are placed during typing such as caps lock, shift, enter, spacebar, and delete button were swabbed and samples were characterized enzymatically and at the genomic level.

Four strains of type V MRSA were generously donated from the St. James' National MRSA Reference Laboratory and have been previously characterized [14]. All four strains contain ccrC and mecA in their SCCmec complex and are positive for lukS. These strains served as molecular controls for type V strains for this study.

Biochemical characterization

In order to confirm the MSA isolated organisms were *S. aureus*, all isolates were tested for catalase and coagulase activity which are characteristic of *S. aureus* and distinguishes them from other species of *Staphylococcus* as well as streptococcal species.

Catalase is an enzyme that converts H_2O_2, a potentially toxic compound, to water and oxygen. *S. aureus* is a facultative anaerobe that produces this enzyme in order to detoxify oxygen radicals that could destroy it [28]. Catalase activity was detected by the addition of 20 μl of hydrogen peroxide with 20 μl of overnight broth culture. A positive reaction was indicated by the appearance of bubbles in solution.

Coagulase allows *S. aureus* to encase itself in a protective layer of fibrin that prevents recognition by phagocytic cells and opsonization by IgG [29]. To test for coagulase activity, 500 μl of Becton Dickenson BBL^{TM} Rabbit Plasma (Franklin Lakes, NJ) was combined with 50 μl of overnight broth culture and incubated at 37°C from four to 24 hours. Samples were considered positive for coagulase if a thick, white clot or a loose clot was formed in the tube. Isolates those were negative for either test was eliminated from the study.

Minimal inhibitory concentration determination (MIC)

The MIC of resistant samples were tested in 24 well plate dishes with each well containing 500 μl of LB broth with 2 fold concentrations of oxacillin ranging from 4 μg/ml to 128 μg/ml. 10 μl of overnight culture were inoculated into each concentration of oxacillin and the growth was observed after 24 hours at 37°C. The MIC was determined by the lowest dilution of antibiotic that inhibited the growth of the bacteria.

DNA isolation

Genomic DNA isolation was performed on all resistant strains including control strains sent from the St. James' National MRSA

Reference Laboratory. The genomic DNA was isolated from three mL of overnight culture using a commercially available E.Z.N.A bacterial DNA isolation kit from Omega Bio-Tek (Norcross, GA). Bacterial cells were collected by centrifugation at 2300 g for five minutes in microfuge tubes (Eppendorf 5415 R). The supernatant was removed and the cells were lysed and treated with proteinase K and RNase to eliminate interfering proteins and RNA. The resulting mixture was applied to a HiBind® DNA mini column and centrifuged at 9300 g for two minutes to eliminate enzymes and any remaining cellular material. The DNA-bound column was washed to remove cellular material and the DNA was eluted using the elution buffer. Genomic DNA was quantified spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany) and the quality of DNA was evaluated by visualization in an agarose gel containing ethidium bromide (2 µg/ml) and by PCR amplification.

Polymerase Chain Reaction (PCR)

Primers used in this study were described previously by other authors. To check quality control of the DNA isolation procedure, primers 2491F and 3554R [30,31] which are specific for the RNA polymerase subunit B gene, were used at an annealing temperature of 52°C resulting in an amplified 1,081bp *rpoB* product. The initial detection of the *mecA* cassette was carried out using the primer pair of *ccrB-R* and *ccrB-F* at the annealing temperature of 50°C [19,28] for types l-lV. The preliminary identification of type V MRSA was performed using the *mecA*-IS431 specific primer pair 5R*mecA* and 5R431 at the annealing temperature 55°C [32]. DNA amplification was performed in a final volume of 20µl containing 0.4 µm of forward and reverse primers, 12 µl of 2X GoTaq (Promega, Madison, WI), 100 ng of DNA, and 2 µl of H_20. Samples were amplified in a thermal cycler (BIO-RAD T100^{TM}, Hercules, CA) after an initial denaturation of 5 minutes at 95°C followed by 30 cycles of the following: denaturation at 95°C for 30 seconds, specific annealing temperature for 30 seconds, elongation at 72°C for 1 minute, with a final extension of 5 minutes at 72°C. Products were then separated via electrophoresis through a 2% agarose gel and visualized using 2 µg/mL of ethidium bromide. Expected sizes for the amplified *ccrB* product are 697 base pair (bp) while the amplified mecA-IS431 product is approximately 329 bp.

Additionally, multiplex PCR was performed as described by Boye et al. [32] that allowed for the discrimination between types 1-IV and confirmed type V. The primer pairs used were: alpha/beta-forward and reverse, 1272-forward and reverse, 5RmecA/5R431-forward and reverse, and ccrC-F/R -forward and reverse. As described in Boye et al. [32], amplification of type I MRSA DNA yields a single 415 bp band. Types-II and -IV DNA amplification results in a 937 bp fragment and type IV amplification products will include an additional 415 bp band. The amplification of types-III and -V will yield a 518 bp fragment and type V will have an additional 359 bp band. Finally, all isolates were tested for the presence of *lukS* using the primer pair *luk*S-R and *luk*S-F at the annealing temperature 60°C with an expected 200 bp band [33].

Statistical analysis

The group differences among domestic and international students were determined using independent samples Chi square test for categorical variables. Pearson's correlation was computed to find correlation between prevalence of *Staphylococcus aureus* carriage, MRSA carriage and presence of type V MRSA between student groups. A P value less than 0.05 was considered as the level of statistical significance. Statistical analyses were carried out using a chi- square test statistical analysis calculator [34].

Results

Sample collection

The four hundred nasal and pharyngeal samples collected from 200 students were inoculated on MSA as a preliminary selection for *S. aureus*, a haloduric bacterium which can tolerate higher salt concentrations than most human microbiota and will ferment mannitol. Ninety-nine (25%) of the 400 samples grew on the media and tested positive for mannitol fermentation as indicated by a change from red to yellow around the mannitol fermenting colonies. Fifty-two *S. aureus* isolates were obtained from domestic students and 47 isolates from international students. The 99 samples represent 78 students due to redundancy of sampling (nasal and pharyngeal passages); therefore 39% (78 of 200) of all of the students tested were carriers of *S. aureus*. These numbers represent 39 students from both groups indicating that neither group has a higher tendency to carry S. aureus (Table 1).

Source	Domestic Students *S. aureus* (100)	International Students *S. aureus* (100)	*S. aureus* (200)
Nasal	26 (26%)	28 (28%)	54 (27%)
Pharyngeal	26 (26%)	19 (19%)	45 (23%)
Total (200)	52 (26%)	47 (23%)	99 (25%)

Table 1: Detection of 99 *S. aureus* isolates at different anatomical sites between domestic and international students. Percentage at each site per student type was determined per 100 samples. Percentage per site or per type of student was determined per 200 but the final totals for *S. aureus* isolated regardless of site or type of student was determined per 400 samples (Two sites per 100 domestic and international students).

Nasal and pharyngeal passages of each participant were tested in order to determine which collection site would be more accurate for determining carrier status. Fifty four of the 99 S. aureus isolates were from the nasal passage and 45 of the S. aureus isolates were from the pharyngeal passage (Table 2). The positive samples represent students who had *S. aureus* present in both of the nasal and pharyngeal passages, the nasal passage or the pharyngeal passage. Had we chosen to collect from only the nasal passage, 22 participants would not have been identified as *S. aureus* carriers. Thirty-four strains would have been missed if we had only collected from the nasal passage. The presence of *S. aureus* in the nasal passage is not insignificant. Wertheim, et al. [35] found as part of a pre-operative procedure involving patients' nasal passages being swabbed, that nosocomial bacteremia was three times more frequent in S aureus carriers than in non-carriers. Therefore the nasal carrier status may be predictive of future complication from MRSA. Statistically, neither site was more accurate than the other so it was determined that both sites should be sampled in order to detect as many carriers as possible thus increasing accuracy of the collection [X^2=4.791, df=2, p=0.09].

Enzymatic activity

Enterococcus species are mannitol fermenting, Gram-positive cocci that are catalase negative. These potential pathogens normally inhabit

the intestinal tract of humans and animals however; they may also colonize the human oral cavity, vagina, and skin of healthy individuals [36]. Two enzymatic assays, catalase and coagulase, were performed on all putative isolates to eliminate Enterococci and other Staphylococci species from this study. When broth cultures of the samples were combined with hydrogen peroxide, all 99 samples were catalase positive as indicated by the production of bubbles. Additionally, the isolates were tested for coagulase activity using the direct tube method with rabbit plasma. The isolates were grown overnight in MH broth containing oxacillin and added to reconstituted rabbit plasma to test for coagulase. Assays were observed starting at four hours and every isolate was able to convert the fibrinogen to soft or firm fibrin clots within six hours of the intiation of the incubation period (data not shown). Not only does the catalase test results distinguish *S. aureus* from Enterococci species, the coagulase test results also separates *S. aureus* from the other Staphylococcal species (coagulase negative or CoNS) which happen to be mannitol positive, such as *S. haemolyticus*.

Source	Domestic Students *S. aureus*	International Students *S. aureus*	Total
Nasal	13	21	34
Pharyngeal	11	11	22
Both	15	7	22
Totals	39	39	78

Table 2: Presence of *S. aureus* in the pharyngeal or nasal passages of tested students. Detection was indicated as "nasal", "pharyngeal" or "both" to demonstrate that testing of only one passage could result in missing the accurate carrier status of the student. Testing only the nasal passage would have resulted in missing 22 isolates. Testing only the pharyngeal passage would have resulted in missing 34 isolates. Only 22 students were positive in both passageways.

Antibiotic resistance

Twenty-seven of the 99 *S. aureus* isolates were tentatively labeled as resistant to oxacillin based on the disc-diffusion assay. Because disc diffusion does not reflect a precise amount of antibiotic in the medium (different volumes of media affect the concentration around the disc), all putative MRSA samples were cultured in broth supplemented with 4 μg/ml OX, which is the laboratory standard for MRSA determination (Laboratory Corporation of America), in order to confirm that they were MRSA. Twenty-three of the twenty-seven isolates grew in oxacillin- containing broth indicating the samples were MRSA based on these standards (Table 3). The 23 confirmed MRSA strains represent 19 students because of the redundancy in sampling. From this point forward, the duplicate samples from the students will not be included in the result and discussion because they were characterized as the same strain (comparing mecA type, MIC and antibiotic resistance). Results will be presented as "student" rather than "sample". Interestingly, one student had two different MRSA strains isolated from the nasal and pharyngeal passages as indicated by phenotypic as well as genotypic differences. Therefore, these particular isolates will be included as individual isolates in the testing however, they both will be considered as one student (Table 4).

S. aureus isolates picked from MSA plates were tested for antibiotic resistance to oxacillin using antibiotic disk diffusion method on MH agar plates as described above. Strains that tested positive for oxacillin resistance in this manner, were further characterized using antibiotic discs containing vancomycin (30 μg), clindamycin (5 μg), doxycycline (30 μg), and sulfamethoxazole with trimethoprim (23.75 μg with 1.25 μg, respectively). The MRSA isolates were placed into four groups based on their antibiotic resistance patterns (Table 5). Group A contained 10 of the 20 MRSA which were sensitive to the all of the antibiotics tested other than oxacillin. Group B contained eight strains that were resistant to sulfamethoxazole-trimethoprim as well as oxacillin. The sole MRSA strain placed in Group C was classified as such due to its resistance to doxycycline. The final MRSA was the sole member of Group D due to its resistance to clindamycin and sulfamethoxazole-trimethoprim. All MRSA tested were sensitive to vancomycin, a glycopeptide that interferes with cell wall synthesis, but by a different mechanism than oxacillin. Not surprising, when comparing MRSA strains grouped by antibiotic resistance patterns, it is apparent that there are several different strains of MRSA present on the campus and there doesn't appear to be a common association based on the source being an international or domestic student. These antibiotics were chosen for testing because they are the commonly recommended drugs to prescribe for MRSA infections [26]. Fifty percent of the MRSA strains isolated were only resistant to oxacillin in this study (Group A) which is typical of type IV MRSA, a CA-MRSA that is considered a non-multidrug resistant strain. However, results described below indicate that types I, V and some strains that cannot be typed are in Group A as well, so this non-multidrug resistant classification is apparently not reserved for type IV.

Test	*S. aureus*	Total
MSA	99 (25%)	400
OX-Disk	27 (27%)	99
OX-Broth	23 (23%)	99

Table 3: Results for initial characterization of isolates as *S. aureus* on MSA and oxacillin-resistance screened by disc diffusion and confirmed by growth in MH supplemented with 4 μg/ml of oxacillin. Percentage positive for MSA was determined as positive out of 400 samples (two sites on 200 students) while percentage positive for resistance was determined by positive out of 99 *S. aureus* isolates.

Students	Domestic *(100)*	International (100)	Totals (200)
S. aureus samples	52 (26%)	47 (24%)	99 (25%)
S. aureus students	39 (39%)	39 (39%)	78 (39%)
MRSA samples	8 (15%)	15 (32%)	23 (23%)
MRSA students	6 (15%)	13 (33%)	19 (24%)
Unique strains	7	13	20
Type I	1	0	1
Type IV	4	1	5
Type V	2	8	10
ND	0	4	4

Table 4: MSA and antibiotic testing results distinguishing sample numbers compared to student numbers. *S. aureus* samples were

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calculated based on 200 test samples from each group (international and domestic). *S. aureus* positive students was determined based on 100 test subjects per group for a total of 200 (100 per group). MRSA sample percentage was calculated based on *S. aureus* positive samples and MRSA positive students was calculated as a percentage of *S. aureus* positive students. It should be noted that one domestic student had two distinct MRSA isolates which resulted in more unique strains than students.

Group	VA	C	DX	ST	Totals
A	S	S	S	S	10
B	S	S	S	R	8
C	S	S	R	S	1
D	S	R	S	R	1

Table 5: Grouping of MRSA strains based on antibiotic resistant patterns to commonly used antibiotics for treatment of MRSA infections. By definition, all of the isolates are resistant to oxacillin. Additionally, all strains tested were susceptible to gentamicin. Abbreviations: VA- Vancomycin, C-Clindamycin, DX-doxycyclin, and ST-sulfamethoxazole/ trimethoprim.

Molecular analysis

Genomic DNA was isolated from MRSA isolates and screened for the presence of genes associated with the staphylococcal chromosome, SCCmec, and the virulence associated lukS which encodes for the Panton-Valentine leukocidin. Prior to amplification of target genes, PCR was performed to amplify the predicted 1,081 bp rpoB fragment which served as a quality control for DNA isolation (Figure 1, Panel A). Because every bacterium has the RNA polymerase subunit B gene, there should be no negative strain; therefore, a negative PCR result indicated that the sample needed to be re-grown and the DNA re-isolated for repeat testing to ensure the DNA was amplifiable for the other target genes so there would be no false-negatives.

Screening of genomic DNA from the 20 MRSA isolates involved the amplification of mecA using ccrB specific primers which can amplify types I-IV. The presence of a 697 bp band following PCR indicated that mecA was present but did not indicate which of the four mentioned types of cassette. Six of the 20 samples were positive for the ccrB PCR product which narrowed the typing to types I-IV (Figure 1, Panel C). MRSA isolates that were negative for the ccrB product were further screened using primers which are specific for type V strains. Amplification of ten of the 20 samples resulted in a 359 bp type V associated band as shown in Figure 1, Panel B indicating that these strains were type V MRSA isolates. There were four samples that could not be amplified with either set of primers and following multiplex PCR described below, were considered "non-typable", but were still included in the study due to their oxacillin resistance.

The six MRSA isolates which were positive by PCR using the ccrB specific primers and the 10 isolates that were tentatively identified as type V were further typed using four primer pairs that allowed for the identification of specific SCCmec types. As described by Boye, et. al. [32], analysis of the multiplex products allows for classification of types 1-V. An agarose gel showing the PCR amplification results of representative type 1, IV and V isolates is shown in Figure 2. Using this methodology, it was possible to identify one type I MRSA, six type IV MRSA and four MRSA strains that could not be typed using this method. Although four of the strains could not be typed, they are phenotypically MRSA and rpoB was amplified using the genomic DNA indicating the failure to type was not an issue of the quality of DNA. The samples may not be able to be typed due to mutations that could disrupt primer annealing, association with a MRSA type that our primers do not recognize, or they could contain a different mechanism or type not yet recognized.

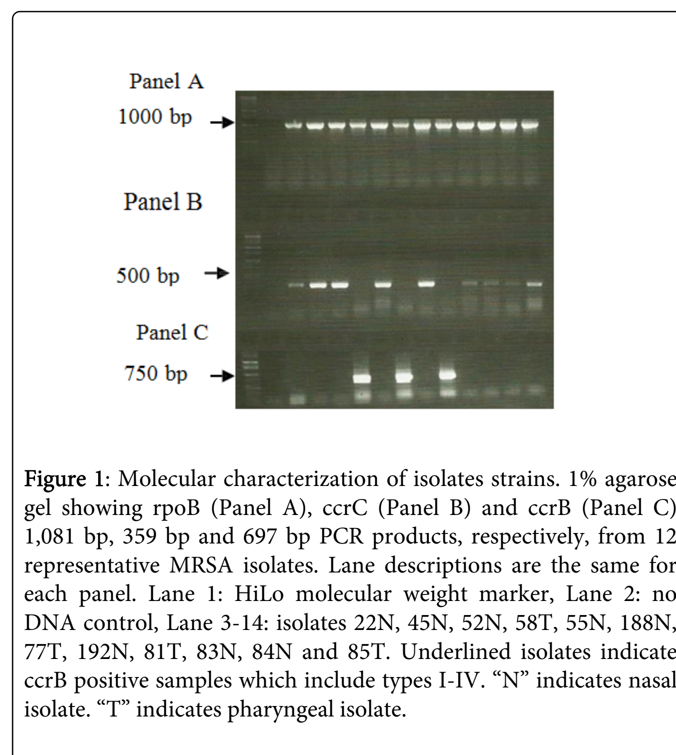

Figure 1: Molecular characterization of isolates strains. 1% agarose gel showing rpoB (Panel A), ccrC (Panel B) and ccrB (Panel C) 1,081 bp, 359 bp and 697 bp PCR products, respectively, from 12 representative MRSA isolates. Lane descriptions are the same for each panel. Lane 1: HiLo molecular weight marker, Lane 2: no DNA control, Lane 3-14: isolates 22N, 45N, 52N, 58T, 55N, 188N, 77T, 192N, 81T, 83N, 84N and 85T. Underlined isolates indicate ccrB positive samples which include types I-IV. "N" indicates nasal isolate. "T" indicates pharyngeal isolate.

Nineteen of the 78 *S. aureus* carriers were colonized with MRSA (Table 4). Six were domestic students and 13 were international students. Two of the six domestic students (30%) were positive for type V MRSA, one isolated from the nasal passage and one from the pharyngeal passage. Four of the domestic students (67%) had types IV MRSA strains present which were isolated for the pharyngeal passage. Although it does not appear to be significant where the MRSA is located, it was interesting because one of the domestic students whose pharyngeal isolate was determined to be a type -IV MRSA, also carried a type I MRSA in their nasal passage. Type I strains are classified as being HA-acquired and this student did not have any of the typical associations with HA-MRSA such as admission to hospital or clinical setting. Eight of the 13 international students (61.5%) were positive for type V MRSA, one isolate was determined to be a type IV, while the four remaining MRSA strains could not be typed using Boye's methodology [32].

Two of the 39 *S. aureus* positive domestic students tested positive for type V MRSA, while eight of the 39 *S. aureus* positive international students tested positive for type V MRSA (5% and 20.5%, respectively). There was no significant difference between the number of domestic students and international students as carriers of *S. aureus* or MRSA. Of the seven MRSA strains isolated from domestic students, two were type V strains compared to eight type V strains identified in 13 MRSA isolates from international students (28.5% and 61.5%, respectively). A Pearson's Chi-square test found a significant

difference between the number of MRSA positive domestic students testing positive for type V strains and the number of MRSA positive international students testing positive for type V strains (X2= 4.08, df= 1, p=0.043). This is not surprising due to the reported origin of type V being in India and only one report of type V in the United States [25]. It is interesting that the four non-typable MRSA strains are also from international students from India. It may be that these strains represent yet another new form of MRSA that has evolved and has not been recognized throughout the world.

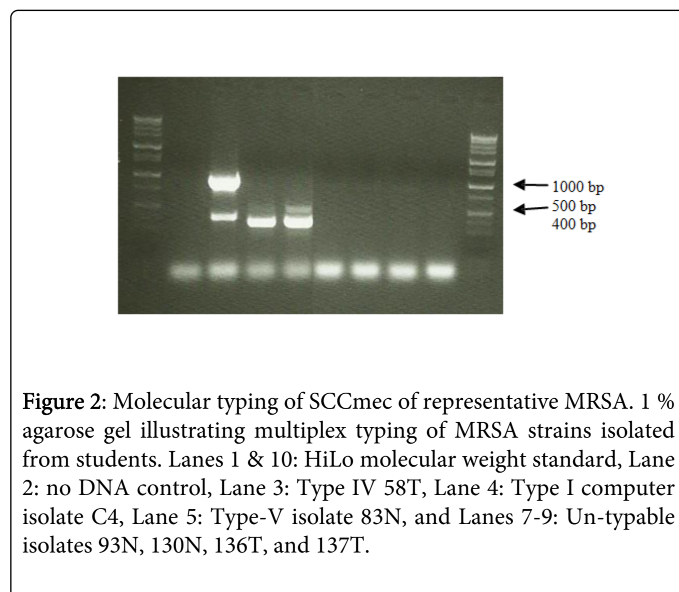

Figure 2: Molecular typing of SCCmec of representative MRSA. 1 % agarose gel illustrating multiplex typing of MRSA strains isolated from students. Lanes 1 & 10: HiLo molecular weight standard, Lane 2: no DNA control, Lane 3: Type IV 58T, Lane 4: Type I computer isolate C4, Lane 5: Type-V isolate 83N, and Lanes 7-9: Un-typable isolates 93N, 130N, 136T, and 137T.

All 20 MRSA isolates were tested by PCR for the lukS which encodes for the Panton-Valentine (PVL) leukocidin. None of the isolates from this study tested positive for this gene although type V HA-MRSA strains which have this phage encoded gene have been reported [14]. Interestingly, PVL is commonly associated with type IV CA-MRSA but not with type IV HA- MRSA [37,38], yet the five community-associated type IV isolates in this study did not have it. Review of the student surveys which specifically asked about hospitalization and prior MRSA infections, reveal that all but two students answered negatively. The two students who had been diagnosed previously with MRSA infections did not test positive for MRSA in this study. Together, all of these results suggest that these isolates are most likely CA-MRSA.

The swine associated type V isolates from the United States are also characterized as PVL-negative [25]. Furthermore, HC Lewis et al. [22] reported on the isolation of 21 type V human isolates in Denmark. Nineteen of the isolates are PVL negative and appear to be swine associated. The remaining two isolates are from people who have ties to China and the associated MRSA isolates are PVL positive. As controls in this present study, four type V MRSA strains had been generously donated to the laboratory and were used for confirmation of type V mecA, as well as positive controls for lukS [14]. PCR amplification of these samples resulted in the expected 151 bp lukS fragment indicating that the negative results from this study's isolates were not due to a technical problem, but because the isolates do not have lukS due to differences in strains.

Minimal inhibitory concentration

Although clinical laboratory standards and Center for Disease Control (CDC) categorize *S. aureus* as methicillin-resistant if the organism grows in the presence of 4 or 6 µg/ml OX, respectively, there are differences amongst MRSA strains regarding how resistant they are. The MICs of the isolated strains were measured to determine if there was a correlation between MIC and MRSA association (CA-MRSA compared to HA-MRSA) or type of mecA cassette in the strain. Strains that were characterized as type V had MICs of 32 µg/ml OX to as high as 128 µg/ml OX (data not shown). One type IV MRSA sample had an MIC of 16 µg/mL. One type IV MRSA and one type V MRSA had MICs of 32 µg/mL. Four type V MRSA samples and three un-typable MRSA strains had MICs of 64 µg/mL. The type I MRSA, two type IV MRSA, four type

V MRSA, and one un-typable sample were capable of growing in media supplemented with oxacillin as high as 128 µg/mL. Because 11 isolates grew in 128 µg/ml, additional concentrations of oxacillin (160 µg/ml, 192 µg/ml and 224 µg/ml) were included. The two highest MIC determined were for a type V MRSA which had an MIC of 160 µg/ml and a type IV which had an MIC of 192 µg/ml. All of the strains that were isolated during this study had MICs that were well above CDC's defining limit of 6 µg/ml OX. These results show how variable the strains are in the ability to survive in the presence of different concentrations of antibiotic and that there is no association between type and MIC. These high MICs could be significant if these MRSA carriers were to acquire an endogenous infection. Recommended intravascular (IV) treatment for mild to moderate infections in adults is 250-500 mg every 4-6 hours or one gram by IV every 4 hours for severe infections [39]. These prescribed IV doses theoretically yield physiological concentrations that would not kill several of these MRSA isolates.

Fomite transmission

Thirty-four keyboards were sampled from two computer laboratories and treated identically as the student samples. The twenty samples taken from computer laboratory A had *S. aureus* present on 14 (70%) keyboards, and one was identified as MRSA (7%) and determined to contain the type V cassette. Fourteen keyboards from computer laboratory B were sampled and all contained *S. aureus* with three of the 14 being confirmed as MRSA (21%). Two of those 14 strains were identified as type V MRSA (14%) and one was identified as type I MRSA (Figure 2). Testing for patterns of antibiotic resistance showed that the three type V MRSA samples and the type I MRSA were only resistant to oxacillin and were sensitive to the other antibiotics which were tested, indicating a narrow range of resistance to antibiotics similar to the student isolates (Group A, Table 5). Similar to the MRSA isolated from the students, the MIC of the four computer isolates ranged from 32 µg/ml to 128 µg/ml without apparent association with type of MRSA.

Discussion

The goal of this study was to determine if type V MRSA was present in this United States university community due to a significant international student enrollment. According to the 2013 university statistical profile, the total number of students at Texas A&M University-Kingsville was 7,730. The report on student demographics stated that there were 809 international students (10.5%) with the top five countries of origin being India, Mexico, Nigeria, China, and

Taiwan, in descending order [36]. One hundred international students and 100 domestic students were tested for this study. Eighty-seven of the international students were recent arrivals to the United States. Although the sampling of international students was random, it is not surprising that 80 of the international students who were included in this study were from India due to the fact that India is the number one country of origin for the international students at this university. The other 20 students were from Mexico, Nigeria, Thailand, Australia, and Germany. MRSA strains I-IV have been documented in most countries; however, type V has most frequently been detected in Asia and some researchers suggest that it will remain mostly on that continent [3]. More recently type V MRSA has emerged in various countries worldwide [21], yet few cases have been reported in the United States. The one human case that has been reported was associated with swine on a Northern Idaho farm where type V MRSA was also identified on the animals [25]. Other than this report, type V MRSA does not appear to be prevalent in the human or animal associated cases in the United States. This present study involved the isolation of type V MRSA from both international and domestic students and suggests that like MRSA types I-IV, type V will most likely become common place even in the United States.

Each participant completed a short survey to document their country of origin, MRSA status (if known), association with anyone with MRSA and travel history. It should be noted, that at the time of the sampling none of the students had an active infection, abscess or illness. Therefore, detection of MRSA on these students would only classify them as carriers and does not suggest that these students are ill. According to the surveys, two students who participated in this study had been previously diagnosed with MRSA infections, but none of the students were on antibiotic therapy at the time of testing. MRSA was not detected on either one of these two students suggesting that they were not carriers but suffered from an infection at one time and the treatment they received most likely eliminated the pathogen.

The identification of two type V MRSA strains from domestic students and eight type V MRSA strains from international students answers the question whether type V MRSA is present in the United States. Based on these data, the presence of type V MRSA associated with the international student body at Texas A&M University-Kingsville is significantly higher than in the domestic students. Unfortunately at the time of the survey of the university students, there was no question regarding the students' association with swine. Due to the lone case of type V MRSA in United States being associated with swine and many cases throughout Europe and Canada being associated with swine, it would have been beneficial to have this information to determine if swine association was responsible for the type V carriage.

Although a small number of domestic students had type V MRSA isolated from the nasal and pharyngeal passage, it is not enough to suggest that type V MRSA is spreading throughout the population. It does suggest that type V MRSA may not be as rare in the United States as originally thought. Many *S. aureus* isolates in the United States are tested for patterns of antibiotic resistance in order to treat the infection. Doctors do not request a molecular diagnosis of the sample; therefore, many type V MRSA strains may be overlooked.

In addition to person-to-person transmission, indirect contact transmission can serve as a source for type V MRSA as well as any other type. Past studies confirmed survival of CA-MRSA on fomite surfaces over long periods of time. One study reported the transfer of MRSA from fomite surface to pig skin after prolonged contact [41]. It was reported that CA-MRSA strains, like type V, survive longer on non-porous surfaces and is more likely to transfer through indirect contact transmission rather than direct contact transmission. According to the student survey, the two domestic students who carried type V MRSA never traveled to Asia or roomed with anyone that had ties to Asia. It is possible that these students picked up the type V strains through indirect transmission as well as person-to-person contact. Two computer laboratories on campus that are frequently used by both domestic and international students housed computer keyboards that tested positive for one type I and three type V MRSA strains. These surfaces serve as potential vehicles for MRSA increasing the likeliness of transmission to students. Published research articles report that MRSA can survive on surfaces for long periods of time [41,42] and thus the surfaces can contribute to the transmission of the pathogen. Therefore, students who did not report any association with MRSA, hospitalization or travel to countries in Asia yet had type V or other MRSA types isolated from them, may have acquired them from a surface such as a computer. These results reinforce the need for extra rigor regarding hygiene when using equipment that is shared by many people regardless of their origins.

Two isolates from the study were classified as HA-MRSA, a type I from a keyboard and from a student. The type I MRSA isolate was present in the nasal passage of a domestic student who had none of the typical HA-MRSA associations such as hospital admission. It is not uncommon for HA-MRSA strains to be isolated from the community and CA-MRSA strains to be isolated from clinical settings, yet researchers still use these classifications. A study conducted in a hospital in Ireland isolated type V MRSA, a CA-MRSA, from a neonatal intensive care unit [14]. Evidence from our data and others' data suggests that the categorization of MRSA types based on location is outdated and no longer applicable and should rely more on genetic composition.

PVL has been associated with CA-MRSA strains which have been differentiated from HA-MRSA by non-multidrug resistance and the presence of the type IV cassette [38]. The type V MRSA strains that have been isolated in swine and one swine worker in Northern Idaho have been identified as ST 398, which has also been characterized in this study, as well as in other countries as PVL negative [25]. A study carried out in Denmark [22] found that 21 out of 23 PVL negative type V ST 398 strains were isolated from people who had contact with swine. The two PVL positive type V strains isolated in this same study were isolated from individuals from China with no known association with swine. Chen et al. [43] characterized MRSA isolates containing the type IV and type V cassettes based on differences such as health-care association as well as the presence of PVL. Interestingly, in their study only 3.4% of type IV and type V HA-MRSA were PVL positive, compared to 35% of the CA-MRSA type IV and type V which were positive for PVL. These results are in agreement with previously published findings that demonstrate a strong association between PVL with CA-MRSA [37,44,45], however they contrast with studies associating PVL specifically with type IV cassette. The present university study resulted in the isolation of 24 MRSA strains; 20 of these strains were from students and four were from computers. All of these strains were tested for the presence of PVL and all were PVL-negative, although the type V PVL controls were positive in this study. Although nothing is known about the origin of the computer isolated strains, the strains isolated from the students should be characterized as CA-MRSA due to the lack of traditional HA-MRSA associations and type of cassette present. The results from Chen et al. [43] support the lack of PVL in CA-MRSA type IV and type V. Additionally, the

type V isolates associated with swine in the U.S., Canada and Denmark are PVL-negative [25,22,46]. This study and others demonstrate that previously determined characteristics and traits of these pathogens are not permanent due to the rapid evolution of bacteria. As MRSA acquires new genes it often loses other genes, thus changing the very characteristics by which they are defined. Obviously, the presence of a traditionally defined HA-MRSA type I isolate from a student who had no history of MRSA or hospital associations leads to the question of where and how the student acquired this strain, as well as the aforementioned strains. The presence of type I and type V MRSA on the computers, as well as previous studies demonstrating the stability of *S. aureus* on inanimate objects, suggest that the transmission of these pathogens may be indirect. Thus, the HA-MRSA and CA-MRSA labeling becomes confusing in the face of no medical association or other defining associations.

This study is not intended to point out specific ethnicities and their association with MRSA. The aim was to identify a type of MRSA uncommon to the United States and to determine if type V was more common in the United States than previously suspected. Continental or localized transfer of MRSA strains is a public health concern due to the threat to humans, regardless of their geographic origin. Previous studies have shown that animals, especially swine, are carriers of type V MRSA and most likely play a very important role in the transmission of this pathogen. This study has its limitations with the small size being one of them. However, the goal was not to determine the prevalence of type V MRSA in the United States, but to establish that these strains are present and may play an important role in health care for humans as well as animals.

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