Detection of Panton-Valentine Leukocidin (PVL) Genes within CA-MRSA Carriers of the Oral Roberts University Community

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Abstract: Over the years, the misuse of antibiotics has become a significant factor in the emergence of resistant bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA) is *staphylococci* that are resistant to Beta-lactam drugs. There are two types of MRSA infections: community-acquired MRSA (CA-MRSA) and hospital-acquired MRSA (HA-MRSA). They can be differentiated by the presence of the Panton-Valentine Leukocidin (PVL) gene in the CA-MRSA strain. Studies have shown that CA-MRSA is more virulent than HA-MRSA. The aim of this study is to determine the population of students at Oral Roberts University who are carriers of CA-MRSA. This was done by obtaining nasal swabs from 50 students and performing coagulase and antibiotic susceptibility tests to identify the samples that contained MRSA. Polymerase chain reaction (PCR) was then used to detect the presence of PVL genes in the positive samples using *lukS-PV*, *hlg*-1 and Sa16 primers. Results showed that 18.0% of the student nasal samples contained MRSA. Of those samples 6.0% were CA-MRSA.

Introduction

Staphylococcus aureus (*S. aureus*) is a common skin bacterium that only becomes a problem when it enters the skin through a cut, open wound, or breathing tube. These bacteria occur harmlessly in the nasal passages of roughly 30% of the U.S. population, and 20% of the human population are long-term carriers (Staywell et al. 2001). *Streptococcus aureus* causes several diseases from minor skin infections (boils, pimples, abscesses, scalded skin syndrome) to life-threatening diseases (pneumonia, meningitis, toxic shock syndrome). A recent study by Graffunder and Venezia (2002) has shown that *S. aureus* is one of the most common causes of nosocomial infections, often causing postsurgical wound infections. Over the years, *S. aureus* has developed multiple antibiotic resistance mechanisms.

The first methicillin resistant strain of *S. aureus* was isolated from a hospital in the UK in 1942 (Jevons et al.1961). By 1960, 80% of all *S. aureus* isolates were resistant to Beta-lactam antibiotics. These strains have gained methicillin resistance through the acquisition of the *mecA* gene (Deurenberg et al. 2006). The *mecA* gene is found on a genetic element known as the staphylococcal chromosomal cassette (SCC*mec*) and encodes low-affinity penicillin-binding protein (PBP-2a) (de Lencastre et al. 1994). This attribute causes the bacteria to be resistant to methicillin. Methicillin is a Beta-lactam antibiotic, structurally analogous to penicillin, oxacillin, and vancomycin. Under normal circumstances, the β-lactam nucleus binds to PBP-2a in the cell wall and inhibits the synthesis of peptidoglycan, thereby causing bacterial cell

death (Yocum et al. 1980). However, when the *mecA* gene is expressed, the β-lactam nucleus does not bind to PBP-2a; hence, the cell continues to synthesize peptidoglycan and is able to replicate and survive even in the presence of Beta-lactam antibiotics. *Staphylococcus aureus* that are resistant to methicillin are classified as Methicillin-resistant *Staphylococcus aureus* (MRSA).

MRSA constitutes 30% - 60% of *Staphylococcal* infections in the USA, Japan, and Europe (Davis et al. 2007). According to the Center for Disease Control, 0.8%-2% of the U.S. population is MRSA carriers; 94,000 people are infected each year, and 19,000 people die as a result (CDC Control and Prevention 2011). MRSA has consequently become a notorious life-threatening pathogen.

MRSA is commonly transmitted through physical contact. Skin-to-skin contact, cuts in the skin, sharing of personal hygiene items (towels, razors), and contact with contaminated items (door handles and athletic equipment) helps spreading this contagious disease. MRSA causes infections such as surgical wound infection, urinary tract infection, pneumonia, and skin infection. The most common signs of MRSA are cellulitis, abscess, folliculitis, and impetigo.

There are two strains of MRSA: hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA). In recent years, HA-MRSA (85% of MRSA) has become more abundant in hospitals and other healthcare facilities, representing a growing public health problem in the United States (Groom et al. 2008). However, in 2010, encouraging results from a CDC study stated that the invasive MRSA infections in healthcare facilities declined by 28% from 2005 through 2008 (Kallen et al. 2010). On the other hand, it has been discovered that people who have not recently been in the hospital or have had no medical procedure such as dialysis or surgery within the past year are also vulnerable to MRSA infections. This type of MRSA is often known as community-acquired MRSA. Outbreaks of CA-MRSA have been seen among athletes, prisoners, military recruits, daycare attendees, school students, and other groups of people who live in a close quarters and share contaminated items.

Though both CA-MRSA and HA-MRSA cause similar infections, both strains differ in the virulence factors they express. Characteristically HA- MRSA s trains elicit staphylococcal enterotoxin A (SEA), while the CA-MRSA produces Panton Valentine Leukocidin (PVL), which is a toxin that belongs to the synergohymenotropic toxin family (Tristan et al. 2007, Dufour et al. 2002). It consists of LukS-PV, LukF-PV, and γ-hemolysin proteins. The association of PVL with five major pandemic clones of CA-MRSA led to the hypothesis that PVL is the key virulence factor for CA-MRSA (Labandeira-Rey et al. 2007, Shallcross et al. 2012, Wannet et al. 2005). The exact mechanism by which PVL toxins harm host cells is not clear. A study by Spann et al (2013) showed that PVL toxin binds to the complement receptors C5aR and C5L2, inhibiting the immune cell activation. Another study indicates that PVL binds to the ganglioside GM1 in cells and causes its cytotoxic effects (Nishiyama et al 2012). PVL has been shown to be a strong inducer of IL-1and inflammasome activation in primary human alveolar macrophages that in turn can trigger the release of chemotactic factors leading to massive neutrophil infiltration of the lung (Perret et al. 2012). Hence, PVL toxin is considered to be responsible for the high mortality rates associated with infection by CA-MRSA (Tristan et al. 2007). Gamma hemolysins (Hlg) are reported to lyse human and other mammalian erythrocytes (Kaneko et al. 2004), PMNs (Joubert et al. 2006, Malachowa et al. 2011), and to enhance the survival of *S. aureus* in human blood (Malachowa et al. 2011). Karauzum et al (2013) have recently shown that Hlg(B) can form heterologous oligomers with LukS-PV. A report has shown that a combination of Hlg(A) and LukF-PV is hemolytic towards rabbit red blood cells (Prevost et al. 1995). Intravitreal injection of rabbits with six different combinations of PVL and Hlg and comparing these combinations based on ability to induce inflammation and necrosis showed various degrees of symptoms with the following order of severity: Hlg(A) +

LukF-PV > Hlg(AB) ≥ LukS-PV + Hlg(B) ≥ PVL > Hlg(CB), suggesting that a variety of new toxins with distinct potencies can be generated by these cross combinations (Siqueira et al. 1997). The above study has clearly demonstrated that the individual virulence factors PVL and Hlg can play a role in clinical infections.

In this study, MRSA strains obtained from the Oral Roberts University student population were tested for the presence of Panton Valentine Leukocidin (*pvl*) and γ-hemolysin gene (*hlg*).

Methods

Study design and collection. Nasal swabs were obtained from fifty student volunteers who were recruited for this study. All samples were collected in the microbiology lab in a single day. Briefly, prepackaged sterile cotton swabs (Puritan Medical Products, Gilford, ME) were removed from the packing and soaked briefly 20 sec in sterile water. The moistened swab tip was then inserted into the volunteer student's right nostril and rotated gently for 5 seconds. The same procedure was done on the right nostril. The sample swabs were immediately placed into Tryptic Soy Broth (TSB) and incubated for 18 hours at 37 ºC. Each sample was then streaked onto Mannitol Salt Agar (MSA, Carolina Biological Supply Co, Burlington, NC) plates and incubated for 18 hours at 37 ºC to select for members of the genus *Staphylococcus*. Two isolated colonies of *Staphylococcus* were taken from each positive MSA plate, streaked onto a TSB slant, and incubated for 18 hours at 37 ºC.

Coagulase test. The Staphylo Monotec Test Kit (Sigma Aldrich, St. Louis, MO) was used to confirm *Staphylococcus aureus* identity. As per the manufacturer instructions, a drop of control reagent was placed on an analysis card, and a small amount of the sample (*Staphylococcus*) was mixed with the reagent. A test reagent was then added to the mixture, and mixed for less than 20 seconds to observe agglutination on the analysis card. A coagulase positive culture of *Staphylococcus aureus* (Presque Isle Cultures, Erie, PA, catalog number 4651) was used as a positive control.

Antibiotic resistance test. The use of methicillin has been discontinued. Oxacillin is in the same class of drugs as methicillin and was chosen as the agent of choice for testing *staphylococci* in the early 1990s. The acronym MRSA is still used to describe these isolates because of its historic role. Sensitivity to oxacillin (1ug), and vancomycin (30 ug) (Beckton Dickenson and Company, San Diego, CA) was performed by disk diffusion method. Coagulase-positive cultures were regrown in TSB broth for 18 hours at 37 ºC. Approximately 1 mL of culture was spread on Muller Hinton agar plate (Carolina Biological Supply Co, Burlington, NC) and allowed to dry at room temperature. The antibiotic discs were then placed on the agar plate and incubated for 18 hours at 37 ºC. The zone of inhibition was measured using a ruler to determine the sensitivity of the bacteria according to the manufacturer's instructions.

Genomic DNA extraction. The extraction of genomic DNA was performed on resistant strains using the PureLink Genomic DNA Kit (Life Technologies, Grand Island, NY). TSB cell culture (1 ml) was taken from each sample and placed in a 1.5-ml micro centrifuge tube and centrifuged. The supernatant was discarded and the cell pellet was suspended in 180 µl of lysozyme solution (20 mg/ml). The solution was incubated for 30 minutes at 37 ºC, treated with 20 µl Proteinase K and 200 µl PureLink genomic lysis/binding buffer. The solution was again incubated for 30 minutes at 55 ºC, and the DNA was extracted by running the lysate through a PureLink spin column. The bound DNA in the column was eluted with Millipore water.

PVL and γ-hemolysin gene analysis. Extracted genomic DNA was used as a template for amplification. Oligonucleotide primers of *pvl* and *hlg* genes were used to obtain amplification of *lukS-PV* and *hlg,* respectively. The primer set sequences for the genes were as follows:

For *lukS-PV:* 5' ATCATTAGGTAAAATGTCTGGACATGAT CCA 3' and

5' GCATCAASTGTATTGGATAGCAAAAGC 3'. The expected amplicon size is 533 base pairs.

For *hlg*: 5' GCCAATCCGTTATTAGAAAATGC 3' and 5' CCATAGACGTAGCAACGGAT 3'

The expected amplicon size is 937 base pairs.

The 16S rRNA sequence for *S. aureus*, Sa16, was used as an internal control. The primer set for Sa16 was: 5'GAAAGCCACGGCTAACTACG 3' and 5'CATTTCACCGCTACACATGG 3'. The expected amplicon size is 203 bp.

PCR primers were designed using the freeware Primer 3 (Rozen et al. 2000). In brief, the PCR protocol consisted of an initial denaturation at 94 ºC for 2 min; 30 cycles of 30 s denaturation at 94 ºC, 30 s of annealing at 55 ºC, and 1 min of extension at 72 ºC. The PCR products were resolved by electrophoresis through a 1.5% agarose gel.

Results

MSA plate: *Staphylococcus* strains were recovered from 29 out of 50 (58.0%) were staphylococcus positive as shown by the production of acid on the selective MSA agar (Fig 1)

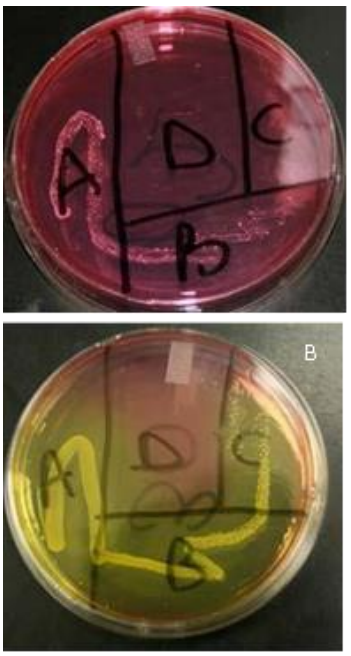

Figure 1. MSA plate after 18 hours at 37°C inoculated with nasal swabs. (A. Upper) MSA plate showing growth of non-*Staphylococci*; as indicated by the absence of yellow colonies. (B. Lower) MSA plate showing growth of *Staphylococci,* indicated by the presence of yellow colonies.

Coagulase test: Coagulase test is used to differentiate the potentially pathogenic species, *Staphylococcus aureus* from other nonpathogenic *Staphylococcus* isolates, which are usually coagulase-negative. Coagulase test was positive for the 13 out of 29 S. aureus culture (44.0%) samples tested (Fig. 2).

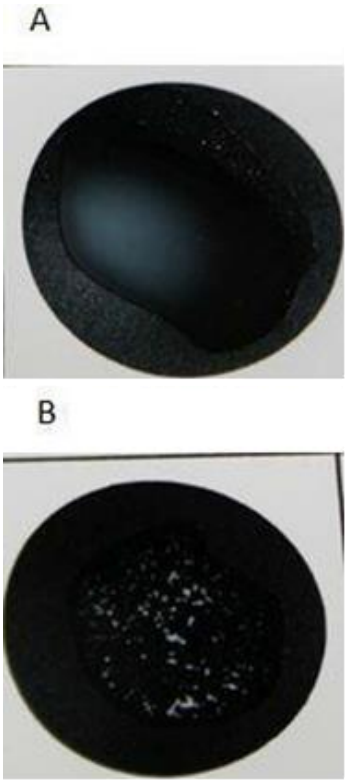

Figure 2. Coagulase test to identify pathogenic *Staphylococcus aureus.* (A) No agglutination observed on the analysis card, indicating a negative coagulase test (B) Positive coagulase test showing agglutination on the analysis card. Isolate is *Staphylococcus aureus* and likely pathogenic.

Antibiotic resistance: The analysis of oxacillin and vancomycin zone of inhibition was compared to the standard values provided by the manufacturer to determine the susceptibility or resistance to the antibiotics. Out of 50 samples, 9 samples (18%) were resistant to oxacillin and vancomycin antibiotics; these were considered to be MRSA. The use of methicillin has been discontinued. Oxacillin, which is in the same class of drugs as methicillin, is chosen as the agent of choice for testing *Staphylococci* (NCCLS). The acronym MRSA is still used to describe these isolates because of its historic role. The diameter of the zone of inhibition

measured from these samples was within the range that was considered to be resistant as per the manufacturer (Fig. 3).

Figure 3. Result of antibiotic susceptible test. (Upper left half) The sensitivity of the isolate to oxacillin with zone of inhibition of 19 mm. (Lower bottom half) The sensitivity of the isolate to vancomycin with zone of inhibition of 14 mm.

PVL(*pvl*) and γ-hemolysin (*hlg*) gene analysis: PCR for *pvl*, *hlg* and *Sa16* genes was carried out in separate tubes. The genomic DNA isolated from the bacterial strains was subjected to PCR to amplify genes for *pvl* and *hlg* genes. Of the 9 samples tested, 8 samples (88%) showed an amplicon size of ~ 937 bp as expected for the *hlg* gene; 2 out of 9 samples (22%) showed fragments for both *pvl* (amplicon size 533) and *hlg* genes, and 1 out of 9 samples (11%) showed fragment only for *pvl* gene (Fig. 4A). This indicated that 6.0% (*pvl* positive) of the samples are CA-MRSA. All PCR samples using the Sa16 primers as an internal control yielded a fragment of expected size, 203 bp, except for sample 38 (Fig. 4B)

Discussion

MRSA is no longer limited to the hospitalized, but also occurs among otherwise healthy communities. Such community-acquired MRSA is an emerging pathogen that primarily causes skin and soft tissue infections. A marked increase in CA-MRSA infection among people living in a closed area is demonstrated in the present study. Carriers of CA-MRSA in the Oral Roberts University student community were identified by detecting the presence of PVL genes among 50 isolates of bacteria randomly collected from students.

In this study, it was found that more than half of the samples (58.0%) contained *Staphylococcus*. These samples produced acid by-product that reacted with the phenol in the MSA plate to convert the red color of the plate to yellow. The frequency of *Staphylococcus* in our samples is consistent with the known prevalence of the bacteria in humans.

There are over 30 different types of *staphylococci*, but *Staphylococcus aureus* causes most staph infections. *Staphylococcus aureus* is part of the human flora, and its ability to clot plasma is the most widely accepted measure for identification. Coagulase test is often used to identify *Staphylococcus aureus* from other *Staphylococcus* species (Ryan 2004). From this study, it was determined that 26.0% of the student samples were positive for coagulase and were thus positive for *Staphylococcus aureus*. Based on previous research, 11-32% of healthy adults are carriers (Tolan 2011). Therefore, our results were consistent with previous estimates of the incidence of *Staphylococcus aureus* in the general population.

The increasing use of antibiotics is a factor in the occurrence of MRSA. Over time, *Staphylococcus aureus* has developed mechanisms that impart it resistance to multiple antibiotics. Oxacillin and vancomycin (β-lactams) were used during the study to determine percentage of samples that were MRSA. In this study we report that 18.0% (9/50) of the samples were resistant to the antibiotics. MRSA has been identified to be one of the leading causes of nosocomial infections. MRSA was originally isolated from patients in health care facilities; however, it has become increasingly common among healthy people who have not been living in the hospital. This change in disease distribution is illustrated in this study, which concludes that 6.0% of the students are carriers of community-acquired MRSA. CA-MRSA can be differentiated from HA-MRSA by the presence of *pvl* and/or *hgl*

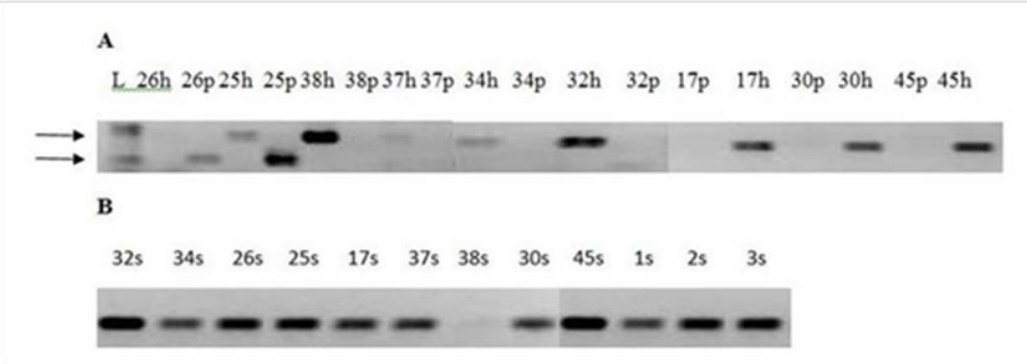

Figure 4. PCR for the identification of *hlg* and *pvl* genes. (A) Amplicon for *pvl* seen in three samples, 26, 25, and 32. All samples showed the presence of *hlg* amplicon, except 26. (h = *hlg*, p = *pvl* , L = DNA ladder: top arrow 1000 bp, bottom arrow 500bp,). (B) PCR results showing PCR products for Sa16 primers as an internal control

genes. Vandenesch et al (2003) described CA-MRSA clones in three continents that were tested positive for *pvl* gene specific sequences. The clones from Oceania showed the presence of both *pvl* and *hlg* sequences. During this research, PCR was used to amplify the PVL genes to validate the study by determining the prevalence of CA-MRSA within the population. The products observed in 6.0% of the samples indicated that those samples contained PVL-producing isolates. In a study of 123 uninfected children, 59.0% carried *Staphylococcus aureus*, and 2% were carriers of CA-MRSA (Sdougkos 2008). Our study corroborates this prevalence of CA-MRSA among healthy people.

Conclusion

MRSA is a major pathogen and threat to lives worldwide. Therefore, strategies must be taken to prevent this infection. Incision and Drainage (I&D) and Oral Antimicrobial Therapy are the two clinical approaches that are most useful in treating MRSA. As the name suggests, Incision and Drainage is a minor surgical procedure to release (drain) the pus under the skin (abscess) by using a sterile instrument such as a sharp needle to puncture the skin. In the case of Oral Antimicrobial Therapy, cultures of bacteria are taken from the infected person, and several antibiotics are used to test for the susceptibility of the bacteria. Every strain of bacteria is susceptible to a specific antibiotic; hence, it is important to identify the antibiotics before prescribing to the infected person. Even though these clinical approaches can be helpful, it is advisable to take strategies such as keeping personal items uncontaminated, covering open wounds, and washing hands in order to prevent the transmission of MRSA.

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