Abstract

One issue associated with *Staphylococcus aureus* is the development of drug resistance. A recently emerged strain of MRSA, ST398, has been identified as livestock-associated and transmission has been found between animal and human. In the U.S., ST398 has been identified in livestock workers in Iowa and North Carolina. The development of virulence factors in *S. aureus* is also an issue. The virulence factor Panton-Valentine leukocidin increases the pathogenicity of a specific *S. aureus* infection. The goal of this study is to evaluate the prevalence of *pvl* in *S. aureus* isolates collected from a community of North Carolina livestock workers. PCR was utilized to identify *pvl* carriage. All *S. aureus* isolates examined have been negative for *pvl*. In order to achieve a better understanding of the isolates, more research can be done looking at the prevalence of other virulence factors.

Introduction

*Staphylococcus aureus* is a gram-positive bacterium that has the ability to cause infection and disease in humans. Human infections caused by *S. aureus* can vary from soft tissue infections to more invasive infections including endocarditis, pneumonia, and tissue necrosis (Gorwitz et al., 2008; Lina et al., 1999). *S. aureus* also has the ability to colonize specific body sites including the nose, hand, forearm, and abdomen. The anterior nares are the body site associated with the highest percentage of *S. aureus* colonization and are the principal site of multiplication for *S. aureus* (Williams, 1963). Nasal carriage of *S. aureus* and colonization of body sites by *S. aureus* are considered risk factors for the development of human infection (Kluytmans et al., 1997).
Panton-Valentine Leukocidin

Genes encoding various virulence factors and antibiotic resistance in *S. aureus* are located on the single circular chromosome as well as on extrachromosomal DNA such as plasmids (Lowy, 1998). One common virulence factor is Panton-Valentine leukocidin (PVL), a cytotoxic extracellular protein associated with necrotizing pneumonia, a type of human infection (Genestier et al., 2005). This virulence factor is encoded by a prophage and is integrated into the *S. aureus* circular chromosome (Boakes et al., 2011). PVL affects the pathogenicity of a specific *S. aureus* infection as it forms pores in host defense cells, especially neutrophils, which damage these cells irreversibly and provides nutrients to the specific *S. aureus* infection (Löffler et al., 2010). Specifically, PVL is a synergohymenotropic toxin, a toxin that works via the association of two distinct secreted polypeptides, LukS-PV and LukF-PV (Lina et al., 1999; Miles et al., 2002). The two polypeptides, LukS-PV and LukF-PV, are encoded by two genes on the integrated prophage. Less than 2% of current *S. aureus* strains produce the virulence factor PVL (Dyer, 2007).

Livestock-Associated ST398 MRSA

Another issue associated with *S. aureus* is the development and carriage in the community of drug-resistant *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA) (Holden et al., 2004). A recently emerged strain of MRSA, ST398, was first described and examined in the Netherlands. It was found that a relatively high proportion of pig farmers and the pigs themselves were positive for livestock-associated ST398 MRSA and regional pig farmers in the Netherlands were found to have an incidence of ST398 MRSA at a rate >760 times higher than the general population (Wulf et al., 2008; Voss et al., 2005). In addition, transmission of ST398 MRSA was found to be possible between animal and human as well as
between different humans (Voss et al., 2005). ST398 MRSA isolates from pigs were all found to be negative for \textit{pvl}. However, some ST398 MRSA isolates from farmers were found to be positive for \textit{pvl}, suggesting that this specific strain has the ability to acquire the PVL virulence factor (Wulf et al., 2008). In the United States, ST398 has been identified in pig farm workers in Iowa and North Carolina (Smith et al., 2009; Rinsky et al., 2013). However, no studies in the United States have examined whether ST398 or other livestock-associated strains carry PVL. Without this information, it is difficult to determine whether these strains pose a potential health risk to workers, their household members, and their communities.

The goal of this study is to evaluate the prevalence of \textit{pvl} in \textit{S. aureus} isolates collected from North Carolina livestock workers and their corresponding household members. Data so far is limited with regard to the virulence of livestock-associated strains of \textit{S. aureus}, and because these strains can develop high levels of antibiotic-resistance, more information concerning the pathogenicity of livestock-associated strains of \textit{S. aureus} is required.

\section*{Methods}

\subsection*{Data Collection}

\textit{S. aureus} isolates collected for a four month epidemiology study of North Carolina livestock workers were tested for Panton-Valentine leukocidin gene positivity. A total of 722 \textit{S. aureus} isolates were identified and archived from 183 North Carolina participants, forming the collection of \textit{S. aureus} isolates to be examined in this study. The 183 participants from this study were recruited by members from the Rural Empowerment Association for Community Help (REACH). To be eligible for participation, participants had to be residents of North Carolina, work at an industrial hog operation, and be at least 18 years of age. From these eligible livestock workers, up to two members above 7 years of age from their individual households
were also able to be participants. REACH members utilized a snowball sampling method to recruit all participants of this study. Participants provided samples with BD BBL CultureSwabs every two weeks from both nares, resulting in a baseline sample and up to eight chronological follow-up samples. Isolates were grown from these nasal samples on both Baird Parker and BBL CHROMagar media plates and stored cryogenically at -80°C.

**Detection of Panton-Valentine Leukocidin Gene**

For *pvl* testing, isolates were collected from freezer storage and grown at 37°C on 5% Sheep Blood Agar (Thermo Scientific Remel, Lenexa, KS) for a period of 24 hours. A sterile 10µL loop was used to transfer *S. aureus* culture to microcentrifuge tubes containing 100µL of DNA Extraction Buffer (Reischl et al., 2000). The microcentrifuge tubes were vortexed and then heated on a heating block set to 100°C for 10 minutes. After letting the microcentrifuge tubes cool for 2 minutes, they were again vortexed and then placed in a microcentrifuge.

Centrifugation occurred for 5 minutes at 13,000 rpm. 2µL of the completed DNA extract for each *S. aureus* isolate were pipetted into PCR tubes containing 23µL of PCR master mix. One aliquot of PCR master mix was composed of 10µL 2.5x 5’ MasterMix, 5pmol *pvl* forward primer, 5pmol *pvl* reverse primer, and 12.5nmol MgCl₂. The two primers utilized to amplify the *pvl* were *luk-PV*-1 (forward) and *luk-PV*-2 (reverse). The primer sequences for *luk-PV*-1 and *luk-PV*-2 can be seen below in Table I.

**Table I. Primer sequences used for *pvl***

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Direction</th>
<th>Sequence (5’--3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pvl</em></td>
<td>F (luk-PV-1)</td>
<td>ATC ATT AGG TAA ATG GAC ATG ATC CA</td>
</tr>
<tr>
<td></td>
<td>R (luk-PV-2)</td>
<td>GCA TCA AST GTA TGG ATA GCA AAA GC</td>
</tr>
</tbody>
</table>

Source: (Lina et al., 1999)

Polymerase chain reaction amplification was run using a BioRad C1000 Touch Thermal Cycler. The program used started at 95°C for 5 minutes and then went to 30 cycles at 94°C for
30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 1 minute (extension) (Lina et al., 1999). PCR products were separated via gel electrophoresis utilizing ethidium bromide staining on 1.5% agarose gel for 45 minutes at 120V. A clinical isolate (provided courtesy of Dr. Jill Stewart, UNC Gillings School of Global Public Health) was used as a positive control for presence of \( pvl \). The positive and negative controls were run through the same extraction protocol used for the test \( S. aureus \) isolates. Agarose gels were visualized using ultraviolet light.

**Results**

All \( S. aureus \) isolates examined have been negative for the presence of the Panton-Valentine leukocidin gene. Figure 1, below, shows a visualized agarose gel utilized to detect the presence of \( pvl \).

**Figure 1. Detection of Panton-Valentine leukocidin gene via polymerase chain reaction amplification.** Gene product was visualized with ethidium bromide staining on 1.5% agarose gel. DNA ladder was loaded in lane 1 as a means of molecular weight identification (far left). Lanes 2-17 were loaded with PCR product for 16 different \( S. aureus \) isolates. Lane 18 was loaded with positive \( pvl \) control PCR product from \( S. aureus \) isolate CA 5003. Lane 19 (far right) was loaded with negative \( pvl \) control PCR product.
In addition to the above results, all of the collected isolates from the 183 participants were identified and characterized as *S. aureus*, methicillin-resistant *S. aureus* (MRSA), or multidrug-resistant *S. aureus* (MDRSA) by a graduate student. MRSA are *S. aureus* isolates positive for the mecA gene. MDRSA are *S. aureus* isolates resistant to three or more different classes of antibiotics. This data can be seen presented below in Table II.

**Table II. Occurrence of nasal carriage of *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), and multidrug-resistant *S. aureus* (MDRSA) from 183 North Carolina livestock worker, adult household member, and minor household member (age 7-17) participants.**

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Carriage states, N (%)</th>
<th>Persistent</th>
<th>Intermittent</th>
<th>Non-carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Workers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>87 (84.5)</td>
<td>28 (27.2)</td>
<td>59 (57.3)</td>
<td>16 (15.5)</td>
</tr>
<tr>
<td>MRSA</td>
<td>9 (8.7)</td>
<td>1 (1.0)</td>
<td>8 (7.8)</td>
<td>94 (91.3)</td>
</tr>
<tr>
<td>MDRSA</td>
<td>45 (43.7)</td>
<td>12 (11.7)</td>
<td>33 (32.0)</td>
<td>58 (56.3)</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>14 (53.8)</td>
<td>6 (23.1)</td>
<td>8 (30.8)</td>
<td>12 (46.2)</td>
</tr>
<tr>
<td>MRSA</td>
<td>1 (3.8)</td>
<td>0</td>
<td>1 (3.8)</td>
<td>25 (96.2)</td>
</tr>
<tr>
<td>MDRSA</td>
<td>7 (26.9)</td>
<td>2 (7.7)</td>
<td>5 (19.2)</td>
<td>19 (73.1)</td>
</tr>
<tr>
<td><strong>Minors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>41 (75.9)</td>
<td>19 (35.2)</td>
<td>22 (40.7)</td>
<td>13 (24.1)</td>
</tr>
<tr>
<td>MRSA</td>
<td>3 (5.6)</td>
<td>0</td>
<td>3 (5.6)</td>
<td>51 (94.4)</td>
</tr>
<tr>
<td>MDRSA</td>
<td>12 (22.2)</td>
<td>3 (5.6)</td>
<td>9 (16.7)</td>
<td>42 (77.8)</td>
</tr>
</tbody>
</table>

Participants from the study were divided into three groups, livestock workers (103 participants), adult household members (26 participants), and minor household members (54 participants). For each of the participant groups, the number of cases that a participant ever tested positive throughout the four-month study for any of the three classifications (*S. aureus*, MRSA, or MDRSA) was determined. Carriage states persistent, intermittent, and non-carrier were also evaluated for each of the three classifications. The persistent carriage state was defined as being positive for the outcome of interest for either all (9/9) or all but one (8/9) of the sample nasal swabs. The intermittent carriage state was defined as being positive for one of the
classifications for at least one (1/9) but less than all but one (<8/9) of the sample nasal swabs. The non-carrier carriage state was defined as being negative for one of the classifications for all of the sample nasal swabs (Nadimpalli et al., 2014).

Discussion

The overall goal of this study was to evaluate the prevalence of the Panton-Valentine leukocidin gene in *S. aureus* isolates collected from livestock workers with occupations in industrial hog operations in North Carolina as well as their corresponding household members. As seen in the Results section, all isolates analyzed with polymerase chain reaction amplification have been negative for *pvl*.

One important consideration when evaluating current findings is the snowball sampling method utilized in this study to enroll participants. In this specific method of sampling, a participant was enrolled initially and additional participants resulted from communication from this initial participant. As more participants enrolled by word of mouth, the sample size became larger and larger. This method was utilized in order to ensure this study remained within the community of livestock workers and their household members with no risk to their careers or livelihood. Because this snowball sampling method was utilized, it cannot be said that acquired data represents the state of North Carolina or any specific community as a whole. Although biases do exist with this sampling method and the extent to which this data can be utilized is somewhat restricted, the collected data is still valuable to investigate the disease ecology of *S. aureus* among livestock workers in the United States.

Global and Regional Prevalence of Panton Valentine-Leukocidin Gene

This absence of *pvl* in this collection of *S. aureus* isolates is a positive sign for public health. It is also important to note that this absence of *pvl* is not abnormal, as less than 2% of *S.
*aureus* bacteria in general produce PVL (Dyer, 2007). Although minimal research documents the prevalence of *pvl*, especially with regard to different regions of the world, in the Netherlands ST398 MRSA has been found in 20% of pig farmers and 39% of slaughterhouse pigs (Wulf et al., 2008). As mentioned briefly in the Introduction, tested isolates from slaughterhouse pigs were all found to be negative for *pvl*, however, some tested isolates from pig farmers were found to be positive for *pvl*. This research indicates that strain ST 398 MRSA can acquire the PVL virulence factor (Wulf et al., 2008). With regard to North Carolina, ST398 has been found to be present in humans in North Carolina (Rinsky et al., 2013). However, no *pvl* positive *S. aureus* isolate, ST398 or other, has been found among livestock workers in North Carolina. Presence of *pvl* would warrant cause for concern, as PVL has been found to be associated with necrotic infections as well as increased virulence of *S. aureus* in general (Lina et al., 1999). From the public health perspective, presence of *pvl* in this North Carolina community and environment would reinforce the idea that steps need to be taken towards limiting the spread and development of drug-resistant *S. aureus*, especially with regard to antibiotic use in livestock.

**Other Virulence Factors**

Although all *S. aureus* isolates examined so far have been negative for *pvl*, this data alone is not enough to classify the *S. aureus* isolates found in this North Carolina community. PVL is just one of many *S. aureus* virulence factors which all have the possibility to affect pathogenicity and virulence of this specific microbe. In order to achieve a better understanding of the *S. aureus* isolates collected from these North Carolina livestock workers and their corresponding household members, more research can be done looking at the prevalence of other virulence factors other than PVL such as surface proteins which allow for attachment and colonization and hemolysins which damage cell membranes.
Prevalence of *S. aureus*, MRSA, and MDRSA and Conclusions

Despite having witnessed no positive *pvl* *S. aureus* isolates, presence of methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus* (MDRSA) isolates collected from livestock members and their household members in this study suggests that there may be cause for concern. Livestock worker and household member participants from this study were found to have a high prevalence of *S. aureus* (84.5% for livestock workers, 53.8% for adult household members, and 75.9% for minor household members) and MDRSA (43.7% for livestock workers, 26.9% for adult household members, and 22.2% for minor household members). There were also a relatively large percentage of persistent carrier state participants for *S. aureus* in general. These results suggest that multi-drug resistant strains of *S. aureus* are circulating and persisting in livestock workers that could become a public health problem if the strains also acquire virulence factors.
References


