In Vitro Production of Panton-Valentine Leukocidin among Strains of Methicillin-Resistant \textit{Staphylococcus aureus} Causing Diverse Infections

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\textbf{Background.} Community-acquired methicillin-resistant \textit{Staphylococcus aureus} strains have recently been associated with severe necrotizing infections. Greater than 75\% of these strains carry the genes for Panton-Valentine leukocidin (PVL), suggesting that this toxin may mediate these severe infections. However, to date, studies have not provided evidence of toxin production.

\textbf{Methods.} Twenty-nine community-acquired methicillin-resistant \textit{Staphylococcus aureus} and 2 community-acquired methicillin-susceptible \textit{S. aureus} strains were collected from patients with infections of varying severity. Strains were analyzed for the presence of \textit{lukF-PV} and SCCmecA type. PVL production in \textit{lukF-PV} gene–positive strains was measured by ELISA, and the amount produced was analyzed relative to severity of infection.

\textbf{Results.} Only 2 of the 31 strains tested, 1 methicillin-resistant \textit{Staphylococcus aureus} abscess isolate and 1 nasal carriage methicillin-susceptible \textit{S. aureus} isolate, were \textit{lukF-PV} negative. All methicillin-resistant \textit{Staphylococcus aureus} strains were SCCmec type IV. PVL was produced by all strains harboring \textit{lukF-PV}, although a marked strain-to-strain variation was observed. Twenty-six (90\%) of 29 strains produced 50–350 ng/mL of PVL; the remaining strains produced PVL in excess of 500 ng/mL. The quantity of PVL produced in vitro did not correlate with severity of infection.

\textbf{Conclusions.} Although PVL likely plays an important role in the pathogenesis of these infections, its mere presence is not solely responsible for the increased severity. Factors that up-regulate toxin synthesis in vivo could contribute to more-severe disease and worse outcomes in patients with community-acquired methicillin-resistant \textit{Staphylococcus aureus} infection.

\textit{Staphylococcus aureus} has re-emerged as a major worldwide threat to human health. Epidemics of \textit{S. aureus} infection occurred in the 1950s, when strains acquired resistance to penicillin, and again in the 1980s, when strains acquired staphylococcal toxic shock syndrome toxin 1 [1]. Nosocomial infections due to methicillin-resistant \textit{S. aureus} (MRSA) were first reported in the 1970s [2], and the number of these infections has steadily increased over the past 3 decades [3, 4].

True community-acquired (CA) MRSA infections have only recently emerged [5–12]. PFGE of the strains causing these infections demonstrated an identical pattern, despite their association with diverse infections from widely disparate geographical areas [12–15]. Subsequently, investigators demonstrated at least 4 different types of methicillin-resistance (mecA) genes [16–18]. Types I, II, and III were associated with strains causing hospital-acquired infections. A novel cassette, type IV, distinguished CA-MRSA strains [18]. The type IV mecA gene cassette is relatively small (23 kD) [19], and its ease of transfer to permissive strains likely accounts for the dramatic increase in the number of CA-MRSA infections.

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CA-MRSA infection has been associated with severe disease, including fatal infections in children [5], necrotizing fasciitis [20], and fulminant necrotizing hemorrhagic pneumonia [21]. CA-MRSA strains associated with these infections all carried the genes for Panton-Valentine leukocidin (PVL)—an extracellular, 2-component toxin that forms lytic pores on the surface of leukocytes and other cell types [22]. PVL has previously been associated with severe methicillin-susceptible \textit{S. aureus} (MSSA) strains in France [14, 23, 24]; however, in the United States, PVL has been largely associated with MRSA strains—most predominantly with CA-MRSA strains [11, 20, 21, 25–27]—although some PVL gene–positive MSSA strains have been reported [15, 25, 27, 28].

Because the genes for PVL have been found in strains causing fulminant CA-MRSA infection, the assumption has been that PVL plays a major role in pathogenesis. Yet many minor infections have also been caused by PVL gene–positive strains of CA-MRSA [29, 30]. Furthermore, the mere presence of a toxin gene does not mean that the protein will be expressed and, if it is, toxin levels could vary widely from strain to strain.

Thus, in the current study, we sought to determine whether PVL is indeed expressed in PVL gene–positive strains of CA-MRSA and whether the quantity of toxin produced correlates with the severity of the clinical infection. Strains of \textit{S. aureus} isolated from patients with a wide variety of clinical infections were screened for the presence of type IV \textit{mecA} and for the PVL genes. A PVL-specific ELISA was developed and used to quantify the amount of extracellular PVL produced by these strains. Results demonstrate the production of PVL by all PVL gene–positive MRSA strains. In addition, the quantity of PVL produced in vitro varied up to 10-fold among CA-MRSA strains; however, this variation was not correlated with the severity of infection.

**MATERIALS AND METHODS**

**\textit{S. aureus} Strains**

MRSA strains were isolated from patients with a variety of infections, including patients with necrotizing fasciitis, bacteremia, pneumonia, abscesses, and superficial wound infections. Two MSSA strains associated with nasal colonization were also studied. Some strains have been characterized in previous reports (table 1) [20, 21, 29, 31]. For other strains, SCC\textit{mecA} typing was performed by the State of Idaho Bureau of Laboratories (Boise, ID). Two control strains from the American Type Culture Collection (ATCC) were also included. Strain ATCC 49775 is positive for the PVL genes (herein referred to as \textit{lukF-PV} and \textit{lukS-PV}) and has been used for the purification of the 2 PVL proteins, LukF-PV and LukS-PV [30, 32]. Strain ATCC 31889 is PVL gene negative [22]. Both strains produce other homologous bicomponent toxins, such as \(\gamma\)-hemolysin [22, 30].

Routine culture of all \textit{S. aureus} strains was performed using Mueller-Hinton II broth, and no difference in growth rates among strains was observed. For analysis of toxin production, washed \textit{S. aureus} strains from overnight cultures were diluted to 1–3 \(\times 10^5\) CFU/mL in fresh culture media and grown at 37°C in 5% CO\(_2\), with shaking (200 rpm) for 20 h, at which time all culture specimens had reached a plateau of 1–4 \(\times 10^7\) CFU/mL. Bacterial culture specimens were rendered free of bacteria by centrifugation and filter sterilization and then frozen at –70°C until they were tested for the presence of Luk-F-PV by ELISA, as described below.

**PCR-Based Detection of PVL Genes**

Freshly isolated colonies (diameter, 1.5 mm) of \textit{S. aureus} were collected and transferred to microtubes containing 50 \(\mu\)L of lysis solution (50 mmol/L NaCl, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl; pH, 8.0). After vigorous vortexing, tubes were heated to 100°C for 5 min and clarified by centrifugation; the recovered supernatant was kept on ice or frozen until use. Standard PCR was performed on 1 \(\mu\)L cleared supernatant using 2 primers designed to amplify the 3’ end of \textit{lukS-PV} (primer #127, 5’-ATCATTCATAGTTAAGATCTGGAATGATGAC-3’) and the 5’ end of \textit{lukS-PV} (primer #128, 5’-CATCAASTGTTATGGATAGCAAAGC-3’) [20, 26]. The PCR program included 5 min of denaturing at 95°C, 30 cycles of 30 s at 95°C, 30 s at 53°C, and 2 min at 72°C, followed by a final 10 min extension step at 72°C. PCR products were visualized on a 1% Tris-acetate/EDTA (TAE) gel. This strategy amplified a portion of the bicistronic genes \textit{lukS-PV} and \textit{lukF-PV}, resulting in a PCR product of 432 bp.

**Quantitation of In Vitro PVL Production**

**Production of recombinant LukF-PV.** Chromosomal DNA was isolated from the PVL–positive \textit{S. aureus} strain ATCC 49775 by a guanidium thiocyanate–based method [33]. PCR amplification of the \textit{lukF-PV} used the following primers: forward 5’-CTCTTCGAGGCTCAACATATC-CTG-3’ and reverse 5’-ATTGGATCCCTAGCTCATAGGATTTTTTTCC-3’. The following conditions were used: 4 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and a final 5 min at 72°C. The primers amplified a portion of \textit{lukF-PV} encoding for the mature protein (i.e., minus the leader sequence). The resulting PCR product was TA-TOPO cloned into pCR2.1 (Invitrogen), creating plasmid pDB989. Sequencing of this plasmid using M13 forward and reverse primers verified that no mutations were present within \textit{lukF-PV}. The Luk-F-PV gene was subsequently cloned into the pET14b expression vector (Novagen) as an \textit{Xhol}-to-\textit{BamH}I fragment; however, because of the close proximity of the \textit{Xhol} site to the \textit{BamH}I restriction site in the pET14b expression vector, a 3-part ligation was performed. For this, 2 separate 5-\(\mu\)g samples were heated to 100°C with 10 mmoL/L Tris-HCl, 1 mmol/L EDTA, and 10 mmoL/L Tris-HCl; pH, 8.0). After vigorous vortexing, tubes were heated to 100°C for 5 min and clarified by centrifugation; the recovered supernatant was kept on ice or frozen until use. Standard PCR was performed on 1 \(\mu\)L cleared supernatant using 2 primers designed to amplify the 3’ end of \textit{lukS-PV} (primer #127, 5’-ATCATTCATAGTTAAGATCTGGAATGATGAC-3’) and the 5’ end of \textit{lukS-PV} (primer #128, 5’-CATCAASTGTTATGGATAGCAAAGC-3’) [20, 26]. The PCR program included 5 min of denaturing at 95°C, 30 cycles of 30 s at 95°C, 30 s at 53°C, and 2 min at 72°C, followed by a final 10 min extension step at 72°C. PCR products were visualized on a 1% Tris-acetate/EDTA (TAE) gel. This strategy amplified a portion of the bicistronic genes \textit{lukS-PV} and \textit{lukF-PV}, resulting in a PCR product of 432 bp.

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<table>
<thead>
<tr>
<th>Infection type, strain, Type of strain</th>
<th>Clinical notes</th>
<th>Result of gene probe for PVL</th>
<th>SCCmec type</th>
<th>Reference or institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotizing fasciitis</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[20]</td>
</tr>
<tr>
<td>05–001/M0839 MRSA Necrotizing fasciitis</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[20]</td>
</tr>
<tr>
<td>05–002/M2242 MRSA Necrotizing fasciitis</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[20]</td>
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<tr>
<td>05–003/M2794 MRSA Necrotizing fasciitis</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[20]</td>
</tr>
<tr>
<td>05–004/M1588 MRSA Necrotizing fasciitis</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[20]</td>
</tr>
<tr>
<td>BAC02870 MRSA Suspected spider bite on right leg; infection presented like streptococcal myonecrosis</td>
<td>+</td>
<td>IV</td>
<td>ID</td>
<td></td>
</tr>
<tr>
<td>Bacteremia</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[29]</td>
</tr>
<tr>
<td>3801 MRSA Erythromycin, tetracycline, and ciprofloxacin resistant</td>
<td>+</td>
<td>IV</td>
<td>ID</td>
<td></td>
</tr>
<tr>
<td>BAC02693 MRSA Endocarditis</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>ID</td>
</tr>
<tr>
<td>BAC02840 MRSA Female prison inmate with FUO and a suspected spider bite under her right arm; developed sepsis, hypotension, and renal failure; survived +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[21]</td>
</tr>
<tr>
<td>04-005 MRSA Thoracentesis isolate from a 17-year-old male patient with empyema and pneumonia</td>
<td>+</td>
<td>IV</td>
<td>MT-B</td>
<td></td>
</tr>
<tr>
<td>05–007 MRSA 31-year-old female smoker with acute influenza A virus infection and multiple bilateral cavitary lung lesions; refractory to azithromycin</td>
<td>+</td>
<td>IV</td>
<td>[21]</td>
<td></td>
</tr>
<tr>
<td>05–008 MRSA 52-year-old male smoker with subcutaneous abscesses on arms and back, cavitary lung lesions with nodular opacities in right middle lobe and bilateral lobes; fatal +</td>
<td>IV</td>
<td>[21]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05–009 MRSA 20-year-old woman with acute influenza A virus infection and pneumonia; developed necrosis of lower extremity and digits requiring below-the-knee amputation +</td>
<td>IV</td>
<td>[21]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05–010 MRSA 33-year-old woman with fever, sore throat, rhinorrhea, and pneumonia</td>
<td>+</td>
<td>IV</td>
<td>[21]</td>
<td></td>
</tr>
<tr>
<td>BAC02690 MRSA Sputum isolate; fatal +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAC02781 MRSA Fatal case associated with influenza A virus infection +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
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<tr>
<td>Abscess</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>[29]</td>
</tr>
<tr>
<td>2349 MRSA Uncomplicated abscess isolate</td>
<td>+</td>
<td>IV</td>
<td>[29]</td>
<td></td>
</tr>
<tr>
<td>1560 MRSA Wound/abscess isolate from female prison inmate; erythromycin resistant +</td>
<td>IV</td>
<td>[29]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2241 MRSA Isolate from a male with neck abscess +</td>
<td>IV</td>
<td>[29]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1681 MRSA Abscess isolate; erythromycin resistant +</td>
<td>IV</td>
<td>[29]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1740 MRSA Abscess isolate; erythromycin resistant –</td>
<td>IV</td>
<td>[29]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPR-S1-3757 MRSA Multidrug-resistant USA 300 strain for which the genome has been sequenced; originally isolated from an HIV-infected patient with a partially drained abscess/wound on left wrist +</td>
<td>IV</td>
<td>[31]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04–008 MRSA 48-year-old man with redness on his buttocks following possible insect bite; abscess required deep-tissue incision and drainage +</td>
<td>ND</td>
<td>MT-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAC02703 MRSA Severe soft-tissue infection of buttocks +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAC02875 MRSA Wound/abscess isolate from 14-year-old girl +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAC02880 MRSA Arm abscess isolate from a 17-year-old female patient +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial wound</td>
<td></td>
<td>+</td>
<td>IV</td>
<td>ID</td>
</tr>
<tr>
<td>BAC02873 MRSA Superficial arm wound, self-inflicted in a 17-year-old female patient +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAC02874 MRSA Superficial arm wound, self-inflicted in a 17-year-old female patient +</td>
<td>IV</td>
<td>ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04–012 MRSA Superficial abdominal wound from insect bite; patient developed toxic shock syndrome +</td>
<td>ND</td>
<td>MT-M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04–021 MRSA Superficial wound isolate +</td>
<td>ND</td>
<td>ID-VA</td>
<td></td>
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</tbody>
</table>
pET14b were cut with Psfl and BamHI or with Psfl and XhoI, and the resultant fragments (1260 bp and 3406 bp, respectively) were purified. Ligation of these 2 fragments plus the lukF-PV XhoI-to-BamHI fragment resulted in plasmid pDBS112, in which LukF-PV carries a C-terminal 6x-histidine tag. pDBS112 was transformed into strain BL21(DE3) (Novagen).

A starter culture sample of the LukF-PV expression strain was grown overnight at 37°C in 2 mL of Luria Bertani broth supplemented with 50 μg/mL of carbenicillin and 1% glucose. After 18 h, bacteria were recovered by centrifugation, added to 600 mL of fresh Luria Bertani broth supplemented with 50 μg/mL of carbenicillin and 1% glucose, and incubated at 37°C with shaking (225 rpm) until mid-log phase (OD600nm = 0.5) growth was achieved. Expression of PVL was induced with 0.4 mmol/L IPTG and grown at 37°C (225 rpm for 8 h). Bacteria were lysed using a commercial reagent (Bug Buster; Novagen), and LukF-PV was purified by nickel chromatography using standard techniques. The recombinant LukF-PV was dialyzed against 20 mmol/L potassium phosphate, 150 mmol/L NaCl, and 20 mmol/L imidazole (pH, 8.0) and was treated with human thrombin (4 U of thrombin per 1 mg of LukF-PV; Sigma) to remove the 6x-histidine tag. The cleaved 6x-histidine tag and the residual thrombin were removed by sequential nickel chromatography and p-aminobenzamidine agarose beads (Sigma), respectively. Recombinant LukF-PV was dialyzed against PBS, aliquotted, and frozen at −70°C.

**Development of an ELISA for quantitation of PVL.**

PVL and other members of the *S. aureus* 2-component pore-forming family of toxins share remarkable amino acid sequence homology and, thus, antibodies are often cross-reactive. In a previous report [35], we described the development of a LukF-PV–specific antibody that showed no cross-reactivity with other members of this family by Western blotting. Specifically, an antibody directed against a 27–amino acid peptide, RYT-NF-WNWGNNYK-DN-RATHTSI (corresponding to amino acids 275–301 of mature LukF-PV), was commercially prepared in rabbits [35]. This region was selected for anti–PVL-specific antibody production, because it is the most divergent from the F-components of the other homologous bicomponent toxins. The LukF-PV peptide–specific IgG was purified from the hyperimmune rabbit serum samples using immobilized protein A agarose (Pierce). This LukF-PV peptide antibody was used as the capture antibody in the ELISA.

In addition to this LukF-PV peptide–specific antibody, an anti-LukF-PV whole-toxin antibody was commercially prepared by repeated immunization of rabbits with recombinant LukF-PV [35]. The LukF-PV whole-toxin antibody recognized LukF-PV as well as the F-components of other homologous bicomponent *S. aureus* toxins. For use as a secondary antibody in the ELISA, the LukF-PV whole-toxin IgG was biotinylated using a commercial kit (EZ-Link NHS-biotin; Pierce).

For the ELISA, a 96-well microtiter plate was coated overnight at 4°C with 5 μg/mL of the LukF-PV peptide IgG in 100 mmol/L carbonate/bicarbonate buffer (pH, 9.6). After washing, the wells were blocked with 3% bovine serum albumin in PBS. Proteins in the 20-h bacterial culture supernatants were concentrated 5-fold by ethanol precipitation and resuspended in 0.05% sodium dodecyl sulfate in Dulbecco’s PBS. Un inoculated bacterial media were similarly treated and used both as a negative control and as a diluent for recombinant LukF-PV in preparing the standard curve. Concentrated bacterial culture supernatant samples and dilutions of recombinant LukF-PV (100 μL) were treated with 1 μL of a 11.2 mg/mL solution of purified normal rabbit IgG (Sigma) for 60 min at 4°C to block nonspecific binding of antibodies by protein A and/or Sbi [36]. Samples were added to the plate in duplicate. After 2 h, the plate was washed, and biotinylated rabbit anti–LukF-PV whole-toxin IgG was added (diluted to 2 μg/mL in PBS/bovine serum albumin) for another 2 h. After washing, the plate was developed with streptavidin–horseradish peroxidase conjugate (Zymed) diluted 1:1000 in PBS and the colorimetric substrate.
2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Zymed). After 20 min, absorbance was read at 405 nm. The assay was linear over the range of 156–2500 ng/mL, with a lower limit of detection of 36 ng/mL. Samples that were positive by ELISA were analyzed by Western blotting with the LukF-PV peptide antibody to verify the presence of a single band of molecular weight that was equal to that of recombinant LukF-PV.

RESULTS

Twenty-nine CA-MRSA and 2 MSSA strains, isolated from patients with infections of varying severity, were analyzed for the presence of lukF-PV. Only 2 of the 31 strains tested, MRSA 1740 (an abscess isolate) and MSSA SNIF 121 (a nasal carriage isolate), were lukF-PV negative (table 1). Twenty-six of the 29 MRSA strains were analyzed for the type of SCCmec cassette that was present, and all (100%) were type IV.

Extracellular production of PVL by the 29 lukF-PV–positive strains was measured by ELISA, as described in Materials and Methods. The specificity of the PVL peptide antibody was again confirmed in the present study using 2 well-characterized strains from ATCC. First, the PVL gene–negative strain ATCC 31889 yielded no measurable signal in the ELISA, despite its ability to produce the homologous toxin γ-hemolysin [22]. In contrast, the PLV gene–positive strain ATCC 49775 had a strongly positive ELISA result, producing 773 ng/mL of PVL.

The quantity of PVL produced was variable among strains (figure 1). Twenty-six (90%) of 29 strains produced PVL within the range of 50–350 ng/mL (figure 1). However, 3 strains produced PVL in excess of 500 ng/mL (figure 1), and 1 of these strains, isolate 2241, produced nearly 800 ng/mL of PVL.

The greatest strain-to-strain variability in the quantity of PVL produced was observed among isolates obtained from patients with abscesses and from patients with pneumonia (figure 2). For example, abscess-associated strains 2241 and 2349 produced 3–4-fold greater amounts of PVL than the other strains in this category (figure 2A). Similarly, a 10-fold difference was observed between the high and low PVL–producing pneumonia strains (figure 2B). Strains associated with bacteremia or strains isolated from focal wounds also showed variability in the amount of PVL produced, but to a lesser extent (figure 2C, D). Interestingly, strains isolated from patients with necrotizing fasciitis showed little to no strain-to-strain variability in the amount of PVL produced (figure 2E).

Production of high levels of PVL was not associated with increased severity of infection. In fact, the strains that produced the highest levels of PVL (strains 2349 and 2241, producing 531 ng/mL and 796 ng/mL of PVL, respectively) were from patients with uncomplicated abscesses, whereas invasive CA-MRSA strains causing lethal necrotizing pneumonia (strains 05-008, BAC02690, and BAC02781) or bacteremia (strains 3801, BAC02693, and BAC02840) produced intermediate quantities of the toxin; strains causing pneumonia produced a mean PVL level (± SD) of 37 ± 39 ng/mL, and strains causing bacteremia produced a mean PVL level (± SD) of 245 ± 88 ng/mL. Among pneumonia isolates, the strain that produced the highest PVL level (strain 04-005; 504 ng/mL) was from a 17-year-old male patient with pneumonia and empyema who survived without complications, whereas the strain that produced the lowest PVL level (strain 05-009; 72 ng/mL) was from a 20-year-old woman who developed cavitary pneumonia, disseminated intravascular coagulopathy, and lower extremity necrosis requiring below-the-knee amputation [21].

DISCUSSION

Severe infections due to CA-MRSA have recently emerged, and studies have shown a high prevalence of the genes for PVL in strains associated with these infections. This observation led many researchers to conclude that PVL was the most likely cause of the increased severity. However, the genes for PVL have also been found in strains causing very minor infections, such as superficial abscesses and furunculosis [30]. Acquisition of PVL genes occurs via mobile genetic elements; however, PVL is not part of the methicillin-resistance (SCCmec) cassette. Thus, the current epidemic of CA-MRSA infection is historically significant, because strains have not only acquired methicillin resistance genes, but have also acquired additional virulence genes, albeit by independent genetic mechanisms.

In the current study, in every strain containing lukF-PV, the PVL toxin was expressed, although strain-to-strain variation was observed. Strains associated with severe infections, such as...
necrotizing fasciitis and necrotizing pneumonia, did not produce greater quantities of PVL toxin than those associated with more minor infections. Thus, it appears that PVL may be necessary but not sufficient to cause these severe infections, implying that other bacterial and/or host factors contribute to poor outcomes.

In a recent report of necrotizing fasciitis caused by CA-MRSA, contributing host factors included comorbid conditions, such as chronic hepatitis C, diabetes, and injection drug use, although 29% of patients had no coexisting conditions or risk factors [20]. Other studies identified age <2 years and black race as factors associated with increased prevalence of infection with CA-MRSA strains, but no other host factors that could be related to severity of infection were identified [37]. Indeed, most of these severe infections have occurred in healthy hosts, suggesting that general immunosuppression is not necessary. Concomitant influenza virus infection may predispose to necrotizing pneumonia [38, 39], although a molecular mechanism has not been elucidated. Still, influenza virus infection could alter barrier function of the respiratory epithelium and
provide a portal of entry for CA-MRSA–bearing PVL toxin genes.

Other toxins produced by PVL gene–positive CA-MRSA strains have not been implicated, although many of these strains harbor genes for multiple enterotoxins [11]. In 1 study involving patients with necrotizing fasciitis, strains were gene–positive for PVL, LukD, and LukE, but no other toxin genes, including the enterotoxins and exfoliative toxins, were found [20]. Notably, toxic shock syndrome toxin 1 was absent from PVL gene–positive strains [11, 20]. Together, these findings suggest that PVL is the principal mediator of these invasive infections. Some experimental data support this notion. Specifically, wild-type CA-MRSA strains expressing PVL genes but not their PVL gene–deficient isogenic counterparts caused pneumonia in a murine model of acute lung infection [40]. Pneumonia was also induced by direct instillation of PVL into the lungs of experimental animals [40].

Conversely, a recent study using PVL knockout strains of CA-MRSA in a murine soft-tissue infection model showed that isogenic mutant strains of CA-MRSA lacking PVL genes were as lethal as wild-type strains [41]. The authors concluded that virulence factors other than PVL production were responsible for the increased severity of CA-MRSA infections [41].

Clearly, most strains of S. aureus contain genes for important surface adhesins [42] that contribute to the pathogenesis of a variety of staphylococcal infections. In the series by Miller et al. [20], the authors showed that all PVL gene–positive CA-MRSA strains associated with necrotizing fasciitis possessed genes coding for multiple adhesins, such as fibronectin-binding protein A and cell wall–associated and extracellular fibrinogen-binding proteins. Thus, concomitant expression of surface adhesins and production of PVL may be required for development of necrotizing soft-tissue infections.

Several known factors affect S. aureus exotoxin production. For example, maximal toxic shock syndrome toxin 1 production occurs with 0.3–1.5 mmol/L Mg++ at a temperature of 37°C–40°C, and with regulation of CO₂ and O₂ levels, such that an anaerobic environment is not present [43]. Similarly, production of α-hemolysin requires both CO₂ and O₂ and is repressed by high osmolarity [44, 45]. However, the factors that contribute to optimal PVL production in vitro, as well as those that induce its expression in vivo, have not been established.

Empirical use of β-lactam antibiotics for treatment of S. aureus infection has obvious consequences if the organism targeted is resistant. Furthermore, studies from our laboratory have shown that β-lactam antibiotics (specifically, nafcillin) up-regulate and prolong transcription and translation of the genes for α-hemolysin and PVL in MRSA strains [35]. Up-regulation of toxin gene expression (i.e., α-hemolysin and toxic shock syndrome toxin 1) by β-lactam antibiotics also occurred in MSSA strains [35] but may not be clinically relevant, because these agents ultimately reduce the bacterial burden. However, for MRSA strains, inadvertent use of β-lactam antibiotics to treat mild infections caused by CA-MRSA strains bearing these toxin genes, and particularly the PVL gene, could contribute to more aggressive infection by increasing and extending toxin production while continued bacterial proliferation proceeds unchecked.

Pre-existing antibody to PVL could affect the outcome of patients infected with S. aureus by preventing PVL-mediated cytotoxicity, although data regarding anti-PVL titers in humans is lacking. We and other researchers have ascertained that some batches of commercial intravenous immunoglobulin contain neutralizing antibody against PVL (authors’ unpublished data) [46], suggesting that such treatment may be an effective adjuvant for patients infected with PVL-producing strains.

Inappropriate management of CA-MRSA infections could also contribute to more-severe infections and worse outcomes. In 1 study involving 14 patients with necrotizing fasciitis and/or myositis caused by CA-MRSA, 10 patients (71%) received empirical treatment with an antimicrobial agent that was active against their infecting strain at hospital admission [20]; other patients received a variety of antimicrobials, including β-lactam antibiotics. All patients underwent timely surgical debridement, and all survived, although serious complications were common [20]. In contrast, in a much larger study of CA-MRSA infections (1647 cases), 73% of patients initially received antimicrobials to which the CA-MRSA strain was resistant, and 58% received a β-lactam antibiotic [37]. Severe necrotizing infections were not described, although 23% of patients required hospitalization; one-half of these patients were hospitalized in the intensive care unit [37]. Lastly, a recent report of CA-MRSA pneumonia reported a mortality rate of 60%, and 80% of patients had initially received ceftriaxone treatment [38].

In summary, molecular epidemiologic studies and some experimental data support a role for PVL in the pathogenesis of CA-MRSA infection. In vitro, PVL production by CA-MRSA strains is highly variable, suggesting important differences in transcriptional and/or translational control of gene expression. Up-regulation of PVL gene expression by β-lactam antibiotics in vivo may contribute to more-severe infections and worse outcomes. An understanding of the additional environmental and host factors that influence PVL gene expression in vivo is sorely needed.

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