Expression of Panton-Valentine Leukocidin mRNA among Staphylococcus aureus Isolates Associates with Specific Clinical Presentations

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Abstract

Panton-Valentine leukocidin (PVL; gene designation lukF/S-PV) is likely an important virulence factor for Staphylococcus aureus (S. aureus), as qualitative expression of the protein correlates with severity for specific clinical presentations, including skin and soft tissue infections (SSTIs). Development of genetic approaches for risk-assessment of patients with S. aureus infections may prove clinically useful, and whether lukF/S-PV gene expression correlates with specific clinical presentations for S. aureus has been largely unexplored. In the present study, we quantified lukS-PV mRNA among 96 S. aureus isolates to determine whether expression levels correlated with specific clinical presentations in adults and children. Expression level of lukS-PV mRNA among isolates from skin and soft tissue infections (SSTIs) was significantly greater than among isolates from blood stream infection (BSIs), and expression level of lukS-PV mRNA among BSI isolates from children was significantly greater than for BSI isolates among adults. Moreover, expression level of lukS-PV mRNA among community-acquired (CA) isolates was significantly greater than for hospital-acquired (HA) isolates. These data justify additional studies to determine the potential clinical utility for lukS-PV mRNA quantification as a predictive tool for severity of S. aureus infection.

Introduction

S. aureus causes a range of clinical infections and presentations, from mild superficial skin infection to life-threatening disease[1]. S. aureus infections, especially those caused by methicillin-resistant S. aureus (MRSA), are common in hospitals and other healthcare facilities. Highly virulent community-associated MRSA (CA-MRSA) strains emerged in the mid-1990s and are responsible for severe infections in individuals lacking health care-associated risk factors[1,2]. The S. aureus virulence factor known as Panton-Valentine leukocidin (PVL; gene designation lukF/S-PV) has been implicated in life-threatening presentations caused by CA-MRSA, including severe skin and soft tissue infections (SSTIs) and necrotizing pneumonia[3–5], possibly through induction of cytotoxicity for neutrophils[6]. However, contrasting reports have not found correlations between PVL expression and S. aureus pathogenesis[7–9]. A recent meta-analysis revealed that PVL genes are consistently associated with SSTIs, but are expressed only rarely in isolates from more invasive infections[10]. Therefore, the role of PVL in S. aureus infections remains somewhat unclear. Enhanced production of PVL in S. aureus clinical isolates was associated with less favorable outcomes in a murine skin infection model, where the quantity of PVL production among MRSA and MSSA isolates, while highly variable, remained associated with virulence[11]. Despite strain-to-strain variation, PVL is produced by all S. aureus isolates carrying lukF/S-PV [12], but whether quantitative expression of lukF/S-PV correlates with specific clinical scenarios is unknown. Therefore, the present study was designed to determine whether levels for lukF/S-PV...
expression, quantified as *lukS-PV* mRNA, correlate with specific clinical presentations for *S. aureus* infections.

Table 1. Genotypes of *S. aureus* clinical isolates.

| CC (No.) | ST(No) | MRSA (No.) | MSSA (No.) | SSTI (No.) | BSI (No.) | Adults (No.) | Children (No.) | HA isolates (No.) | CA isolates (No.) | Mean value and standard deviation |
|----------|--------|------------|------------|------------|-----------|--------------|----------------|------------------|-----------------|-------------------------------|
| 88(21)   | 88(19) | 9          | 10         | 9          | 8         | 13           | 6              | 13               | 4               | 0.933 ± 1.536±1.970           |
|          | 1219(2)| 2          | 1          | 1          | 1         | 1            | 2              |                  |                 |                               |
| 8(12)    | 239(8) | 3          | 5          | 6          | 1         | 7            | 1              | 7                | 7               | 0.052 ± 1.514±3.212           |
|          | 630(4) | 3          | 1          | 4          | 4         | 3            | 1              |                  |                 |                               |
| 59(9)    | 2207(2)| 2          | 1          | 1          | 2         | 2            | 2              | 2                | 2               | 1.025 ± 1.211±1.322           |
|          | 59(7)  | 4          | 3          | 6          | 5         | 2            | 2              | 2                | 3               |                               |
| 5(25)    | 5(3)   | 1          | 2          | 3          | 2         | 1            | 3              |                  |                 | 1.680 ± 2.210±3.050           |
|          | 6(3)   | 2          | 2          | 1          | 2         | 2            | 1              |                  |                 |                               |
|          | 15(3)  | 1          | 2          | 2          | 3         | 3            |                |                  |                 |                               |
|          | 25(3)  | 2          | 1          | 2          | 1         | 1            | 2              |                  |                 |                               |
|          | 72(1)  | 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 774(1) | 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 199(1) | 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 1018(2)| 2          | 2          | 1          | 1         | 2            |                |                  |                 |                               |
|          | 1995(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2104(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2114(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2204(2)| 2          | 1          | 2          |           | 2            |                |                  |                 |                               |
|          | 2212(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2201(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2206(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
| 121(7)   | 2213(1)| 1          | 1          | 1          |           | 1            |                |                  |                 | NC ± NC                      |
|          | 2209(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 120(2) | 2          | 2          | 1          |           | 1            |                |                  |                 | 2                             |
|          | 1301(3)| 1          | 2          | 1          | 1          | 3            |                |                  |                 | 2                             |
| 7(6)     | 7(3)   | 2          | 1          | 3          |           | 3            |                | 2                | 1               | NC ± NC                      |
|          | 943(3) | 1          | 2          | 2          |           | 1            | 3              |                  |                 |                               |
| 1(5)     | 188(5) | 5          | 5          | 5          |           | 4            | 1              |                  |                 |                               |
| 39B(4)   | 398(3) | 2          | 1          | 2          |           | 1            | 3              | 2                | 1               | NC ± NC                      |
|          | 2199(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
| 182(2)   | 944(2) | 2          | 2          | 2          |           | 1            |                |                  |                 | NC ± NC                      |
| Singletons(5) | 1349(1) | 1      | 1          | 1          |           | 1            |                |                  |                 | NC ± NC                      |
|          | 1357(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 1732(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2203(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |
|          | 2202(1)| 1          | 1          | 1          |           | 1            |                |                  |                 |                               |

Expressed as CC, clonal complex.

b NC, not count.

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Materials and Methods

Characterization of S. aureus isolates

96 isolates positive for lukS/F-PV were recovered from 67 adult and 29 pediatric patients with S. aureus infections between January 2003 to December 2010 at First Affiliated Hospital of Wenzhou Medical College, Lishui Central Hospital, the Second Affiliated Hospital of Nanchang University, and Jiangxi Provincial Children’s Hospital. Of the 96 isolates, 54 were associated with bloodstream infections (BSIs), 33 associated with SSTIs, and 9 associated with other infections. Community-acquired (CA) isolates were defined by their isolation within 48 hours after hospital admission, with hosts having no risk factors for nosocomial acquisition and no hospitalizations or nursing home residence within a year before hospital admission. Hospital-acquired (HA) isolates were defined by their isolation more than 48 hours after hospital admission, and from hosts harboring no infections before hospital admission. 58 and 28 isolates were classified as HA and CA, respectively, based on medical records. The remaining 10 isolates could not be classified. All isolates were confirmed as S. aureus using a Staph SP Agglutination kit (bioMérieux, Marcy l’Etoile, France), Gram stain, coagulase agglutination test, and a Vitek-60 microbiology analyser (bioMérieux, Marcy l’ Etoile, France). MRSA was confirmed using PCR for detection of meca[13]. The Ethics Committee of the first Affiliated Hospital of Wenzhou Medical College exempted this study from review because the present study focused on bacteria.

DNA extraction

S. aureus isolates were cultured on blood agar (Oxoid, United Kingdom) overnight at 35°C. Three to four bacterial colonies were suspended in 150 μl sterile distilled water with lysostaphin (1 mg/ml) (Sangon, China) and incubated at 37 °C without shaking for an hour. DNA was extracted following the instructions of the Genomic DNA Extraction kit (Sangon, China). The DNA was stored at -20°C and prepared for PCR detection.

Identification of MRSA isolates and PVL detection

A multiplex PCR protocol described previously was used for simultaneous amplification of meca, 16S rRNA, and lukS/F genes[13]. MRSA N315 and lukS-PV-positive MRSA isolates identified in our previous study were used as positive control strains[14]. The S. aureus isolates positive for lukS/PV as determined by the multiplex PCR mentioned above were re-detected using a simplex PCR with specific primers of the multiplex PCR above for lukS-PV. The expected DNA products were purified using PCR Production Purification kit (Sangon, China) sequenced by Shanghai Sangon Biotech Ltd.. DNA sequences were assessed by performing a similarity search by BLAST program (Blastn) available in NCBI (http://blast.ncbi.nlm.nih.gov/Blast). The gene gyrb was selected to be the endogenous control gene. The primers for detection of gyrb mRNA were designed using Primer Premier 5.0 software. The specificity of the primers was assessed by performing a similarity search by BLAST program (Blastn) available in NCBI (http://blast.ncbi.nlm.nih.gov/Blast). The gene gyrb was selected to be the endogenous control gene. The primers for detection of gyrb mRNA were designed using Primer Premier 5.0 software. The specificity of the primers was assessed by performing a similarity search by BLAST program (Blastn) available in NCBI (http://blast.ncbi.nlm.nih.gov/Blast). The gene gyrb was selected to be the endogenous control gene. The primers for detection of gyrb mRNA were designed using Primer Premier 5.0 software. The specificity of the primers was assessed by performing a similarity search by BLAST program (Blastn) available in NCBI (http://blast.ncbi.nlm.nih.gov/Blast).

RNA extraction and RT-PCR

All solutions used for RNA extraction were stored at 4°C, and centrifugation steps were performed at 4°C to limit RNase activity. S. aureus isolates were cultured in brain-heart infusion (BHI) (Oxoid, United Kingdom) broth overnight at 37°C in a shaking incubator at 220 rpm. A 20 ul aliquot of the overnight culture was added to 5 ml BHI broth and incubated at 37°C until an OD600 of 0.8 was reached. RNA was extracted from the cultures in the exponential growth phase (OD600 0.8) using PureLink™ RNA Mini Kit (Invitrogen, USA) according to the manufacturer’s instructions. RNA concentration and purity were assessed using a DU 800 Nucleic Acid Spectrometer (Beckman Coulter, USA). The extracts were adjusted to perform rigorous DNase treatments using Deoxyribonuclease I (Invitrogen, USA) in accordance with the manufacturer’s instructions. RNA was reverse transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). The cDNA was stored at -20°C and prepared for reverse transcription (RT) PCR detection. Primers for the detection of lukS mRNA were designed using Primer Premier 5.0 software. The specificity of the primers was assessed by performing a similarity search by BLAST program (Blastn) available in NCBI (http://blast.ncbi.nlm.nih.gov/Blast). The gene gyrb was selected to be the endogenous control gene. The primers for detection of gyrb mRNA were designed previously[15]. The reaction mixture (final volume of 20μl) consisted of 10μl Platinum® SYBR® Green qPCR SuperMix-UDG, 0.4μl primers (200nM final concentration), 0.4μl ROX Reference Dye, 2μl cDNA, and DEPC water for a final volume of 20μl. The thermocycling program consisted of one hold at 50°C for 2 min and one hold at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Melting curve data were performed at 60°C to 95°C as per guidelines for the ABI 7000 quantitative PCR instrument. Each sample was tested on three separate occasions, and mean values of repeated results were used for further analysis with ABI 7000 software.

Multilocus sequence typing (MLST)

MLST analysis by PCR amplification and sequencing of the seven housekeeping genes (arc, aroE, glpF, gmk, pta, tpi, and yqiL) of all tested isolates was performed as described previously [16]. The sequences were compared with the existing sequences available on the MLST website for S. aureus (http://saureus.mlst.net), and STs were assigned according to the allelic profiles. MLST clonal complexes (CCs) were determined using the program eBURST (based upon related sequence types) available on the MLST website for S. aureus (http://saureus.mlst.net).

Analysis of data

The data were analyzed using SPSS 17.0 statistic software. Given unequal distribution of lukS mRNA, the comparison of two groups was analyzed using median lukS mRNA values and the Wilcoxon rank-sum test. Statistical significance was defined by a p-value < 0.05.
The PVL Expression among *Staphylococcus aureus*

**Table 2. Expression levels of lukSmRNA among *S. aureus* clinical isolates.**

| Group              | No. of isolates | Minimum | Median | Maximum | Mean value and standard deviation |
|--------------------|----------------|---------|--------|---------|----------------------------------|
| SSTIs isolates     | 33             | 0.000337| 1.500  | 14.470  | 2.789 ± 3.468                    |
| BSI isolates       | 54             | 0.00001 | 0.818  | 3.042   | 0.941 ± 0.899                    |
| Isolates from children | 29          | 0.00003 | 1.292  | 5.296   | 1.565 ± 1.152                    |
| Isolates from adults | 67          | 0.00001 | 0.540  | 14.470  | 1.552 ± 2.706                    |
| BSI isolates from children | 22     | 0.00003 | 1.495  | 3.042   | 1.726 ± 0.792                    |
| BSI isolates from adults | 32         | 0.00001 | 0.191  | 1.912   | 0.400 ± 0.469                    |
| CA isolates        | 28             | 0.00037 | 1.034  | 14.470  | 2.858 ± 3.823                    |
| HA isolates        | 58             | 0.00001 | 0.536  | 10.891  | 1.292 ± 2.119                    |

*p <0.05*

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**Results**

**Characterization of *S. aureus* isolates**

Among 96 PVL-positive *S. aureus* isolates, 52 (54.2%) were classified as MRSA. The genotypic profiles are listed in Table 1. A total of 36 STs were identified, with ST88 accounting for 19.8% (19/96), followed by ST239 (8.3%, 8/96), ST59 (7.3%, 7/96), ST188 (5.2%, 5/96) and ST630 (4.2%, 4/96). The remaining STs accounted for ≤ 3 isolates each. Major STs representing MRSA and MSSA isolates, respectively, were as follows: ST88, 9 and 10; ST239, 3 and 5; ST59, 4 and 3; ST188, 5 and 0; ST630, 3 and 1. 36 STs belonged to 10 CCs. CC5, CC88, CC8 and CC59 accounted for 26.0%(25/96). 21.9% (21/96), 12.5% (12/96) and 9.4% (9/96), respectively.

**The expression levels of lukS-PV mRNA associate with specific clinical scenarios**

Variation of *lukS-PV* mRNA expression levels between the MLST CCs of tested *S. aureus* isolates was identified. However, significant differences were not found between the MLST CCs (p>0.05). The expression levels for *lukS-PV* mRNA are provided in Table 2. Among the 33 SSTI isolates, 12 (36.4%) were MRSA, and among the 54 BSI isolates, 33 (61.1%) were MRSA. *lukS-PV* mRNA expression among SSTI isolates was 2.964-fold higher than for BSI isolates. There was significant difference between the expression levels of *lukS-PV* mRNA among SSTI and BSI isolates (p <0.05).

41 (61.2%) of 67 isolates from adult patients were MRSA, whereas 11 (37.9%) of 29 tested isolates from children were MRSA. Compared with the isolates from adult patients, the expression level of *lukS-PV* mRNA among isolates from children was significantly higher (p <0.05). More specifically, the expression level of *lukS-PV* mRNA among BSI isolates from children was significantly higher than that among BSI isolates from adults (p < 0.01).

The frequencies of MRSA among 28 CA and 58 HA isolates were 46.4% (13/28) and 58.6% (34/58). The expression level of *lukS-PV* mRNA among CA isolates was 2.212-fold higher than that among HA isolates. There was significant difference between the expression levels of *lukS-PV* mRNA among CA and HA isolates (p <0.05)

**Discussion**

PVL is a pore-forming, bi-component toxin secreted by *S. aureus* isolates epidemiologically associated with severe infections, including necrotizing pneumonia and SSTIs[1]. PVL lyses polymorphonuclear leukocytes (PMN) of human and rabbit origin, but not those of mice and monkey origin[6]. Higher PVL concentrations cause PMN lysis, and lysed PMNs release inflammatory factors to damage tissues, while lower concentrations of PVL mediate a novel pathway of PMN apoptosis by directly binding to mitochondrial membranes[17]. The importance of PVL in pathogenesis for *S. aureus* SSTIs and necrotizing pneumonia, and MRSA infections, is controversial[5,9,18]. Conflicting data may relate to the amount of PVL produced by individual strains. One study demonstrated that strains with more PVL production produced larger skin lesions and higher bacterial burdens within the lesions in a murine skin infection model[11]. It is possible that the polymorphisms of DNA sequences in PVL genes may result in changes in the function of the PVL protein, which may help to explain the conflicting results seen in animal studies[1]. Regardless, it may be of interest to development quantitative assays for PVL gene expression to determine their clinical utility in predicting disease severity and treatment response.

One previous study reported that *S. aureus* isolates associated with severe infections were confined to a relatively small number of important clones[19]. PVL-positive *S. aureus* isolates in our study showed considerable genetic heterogeneity, with 36 genotypes. We found that the PVL was expressed in all isolates carrying Panton-Valentine leukocidin genes detected by qRT-PCR (data not shown), suggesting that PVL genes are always transcribed when they are present in clinical isolates. One study demonstrated that expression levels of PVL genes varied from strain to strain, with more than 10-fold variance[20]. Another study reported that PVL production in MRSA was variable [21]. A wide range for *lukS-PV* transcript expression was observed among clinical isolates in the present study. PVL production in MRSA was found to be associated with MLST CC [21]. In contrast, significant differences were not found between MLST CCs (p>0.05) in the present study.

*S. aureus* clinical isolates carrying PVL genes are often associated with SSTIs requiring incision and drainage[22–24], and the production of PVL correlated with SSTI severity in the aforementioned *S. aureus* animal model[11]. In our study, the expression level of *lukS* mRNA among SSTI isolates was significantly higher than that among BSI isolates, potentially indicating that quantitative PVL gene expression plays a more important role in *S. aureus* SSTIs than for other clinical presentations. However, a multinational trial found that PVL was not the primary determinant of outcome in patients with MRSA complicated skin and skin structure infections[8].
Boakes et al. reported that there is no statistical relationship between PVL production and the most severe clinical presentations of PVL-MRSA infection[21]. Given the retrospective nature of our study, we did not have sufficient data extracted from the medical record to determine SSI disease severity, but prospective studies are now justified to determine whether lukS mRNA levels correlate with severity of S. aureus SSI infections.

Children with invasive PVL-positive S. aureus infections incur significantly longer hospital stays than pediatric patients with PVL-negative invasive infections, and PVL is thought to play an important role in pathogenesis of S. aureus infection among children [25,26]. One study also reported a correlation between severity of S. aureus infections and the presence of PVL genes among adult patients, but quantitative PVL expression, at either gene or protein levels, was not determined [8]. Our study found that expression level of lukS-PV mRNA among BSI isolates from children was significantly greater than for BSI isolates from adult patients, but further prospective studies are required to determine whether quantitative lukS expression correlates with disease severity in this scenario. Carriage of PVL genes has been closely associated with infections caused by CA-MRSA, including SSTIs, necrotizing pneumonia, and severe sepsis in numerous epidemiological studies [1,27,28]. In our study, the expression level of lukS-PV mRNA for CA isolates was significantly greater than for HA isolates.

In conclusion, we found that quantitative expression of lukS-PV mRNA is greater for S. aureus SSTIs versus BSIs, for S. aureus isolates from children versus adults (in particular for BSIs), and for CA isolates versus HA isolates. These data justify additional prospective studies to determine whether quantitative lukS-PV mRNA expression may prove useful for predicting severity of S. aureus infection and/or responses to treatment.

Author Contributions
Conceived and designed the experiments: LXW FYF. Performed the experiments: YLY XYX. Analyzed the data: XYH YPL ZQW LZH. Contributed reagents/materials/analysis tools: YLY XYX YPS DPL. Wrote the manuscript: FYF YL CP.

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