Molecular Epidemiology of Panton-Valentine Leukocidin Harboring Hospital-Associated Methicillin-Resistant Staphylococcus aureus in Septicemic Children, Northeastern Iran, Bojnurd

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Abstract

Background: Methicillin-resistant Staphylococcus aureus (MRSA) is responsible for an increasing number of serious hospital- and community-acquired infections in adults and children. Sepsis caused by S. aureus is one of the major health problems associated with treatment failure in adults; however, its clinical outcomes, the rate of treatment failure, and its molecular epidemiology are poorly understood.

Objectives: The objective of this study was to evaluate the molecular epidemiology of Panton-Valentine Leukocidin (PVL) harboring MRSA strains isolated from children's blood culture in Bojnurd.

Methods: Totally, 58 S. aureus strains were isolated from blood cultures in the major teaching hospital in Bojnurd. After the primary verification of Methicillin resistance by agar screening method, the isolated MRSA strains were confirmed with the detection of the meca gene. Meca-positive strains evaluated for SCCmec, agr, and toxin profiles. Panton-valentine leucocidin- positive isolates were subjected to be evaluated for spa and sequence type (ST).

Results: Our data indicated 53.4% (31) of isolates were MRSA. Twelve (38.7%) of these isolates had PVL gene that 25% (3) of them had tsst-1 gene and 58.3% (7) had etb gene. One (3.2%), 64.5% (20), and 32.2% (10) of these isolates belonged to SCCmec I, III, and IV, respectively. Predominant ST and spa types among PVL positive isolates were ST6 and t304, respectively.

Conclusions: We had an uncommon finding because PVL was routinely found in community-acquired MRSA, but in this study we found PVL harboring hospital-associated MRSA. A notable point about these isolates is that most of them belonged to Asian endemic clones.

Keywords: Staphylococcus aureus, Methicillin, Panton-Valentine Leukocidin, Sepsis, Child

1. Background

Bacterial bloodstream infections, called bacteremia, are life-threatening infections with a high mortality rate. These infections commonly appear with other serious infections such as urinary tract infections, endocarditis, and respiratory tract infections (1). Untreated bacteremia can progress to systemic inflammatory responses, sepsis, septic shock, and multiple organ dysfunction syndromes. A large number of Gram-negative and Gram-positive bacteria can lead to bacteremia. One of the most important Gram-positive bacteria is Staphylococcus aureus (2). This bacterium is one of the major human pathogens that cause hospital- and community-acquired infections ranging from skin and soft-tissue mild infections to life-threatening diseases such as toxin-mediated diseases, pneumonia, and septicemia (3, 4).

Antimicrobial resistance has appeared soon after the first use of antibiotics to treat staphylococcal infections (5). Methicillin-resistant Staphylococcus aureus (MRSA) has been identified as a virulent bacterium in healthcare set-
ttings since 1960, named hospital-associated MRSA (HA-MRSA) (6). Since 1990 the arrival of community-acquired MRSA (CA-MRSA) outside the hospitals has been increasingly reported and led to change in the epidemiology of MRSA (7). Community-acquired MRSA and HA-MRSA differ-entiated with genetic characteristics. Small SCCmec elements such as SCCmec IV and V presented in CA-MRSA instead of large elements such as SCCmec I, II, and III that are more common in HA-MRSA (8). Moreover, they express different patterns of specific virulence factors, including toxins and enzymes.

Phage harbored Panton-Valentine Leukocidin (PVL) is one of the most important toxins (8). This is a two-component toxin encoded by phage that can be inserted into the S. aureus genome (9). This toxin was introduced as one of the CA-MRSA markers, especially in skin and soft tissue infections (10). In Asia, the most prevalent MRSA clone is ST239 that is different from the USA and European clones (11-13). In previous works performed separately on SCCmec and sequence types in Iran, researchers have shown that the common types in HA-MRSA strains are SCCmec III and ST239 and in CA-MRSA are SCCmec IV and ST22, respectively (14-17).

2. Objectives

In this study we evaluated the molecular epidemiology of PVL-harboring MRSA strains isolated from children’s blood culture in Bojnurd with multi-locus sequence typing (MLST), spa typing, agr typing, toxin profiling, and SCCmec typing methods.

3. Methods

3.1. Strains and Identification

We totally evaluated 58 strains of S. aureus isolated from blood cultures of children admitted to a university reference hospital in Bojnurd between September 2015 and April 2016. The ages of the patients were between 6 months to 12 years old. Thirty-five blood culture-positive patients (60.3%) were male and the 23 patients (39.7%) were female. All of these strains isolated from the patients (Table 1). Primary isolation of bacteria was performed using biphasic blood culture media (Pasteur Institute of Iran). Suspected positive blood cultures, sub-cultured to blood agar (Merck, Germany) and also MacConkey agar media (Merck, Germany). Staphylococcus aureus strains were characterized by laboratory microbiologic tests, including microscopic evaluation, DNase (Merck, Germany), coagulase (Razi Institute, Iran), mannitol fermentation (Merck, Germany), and catalase (Bahar Afshan, Iran). For classification of isolates to community-acquired and hospital-acquired MRSA, we used criteria set by Clinical Laboratory Standards Institute (CLSI) and also SCCmec typing and toxin profiling methods (18).

3.2. Examination for Resistance to Methicillin

All isolates were screened for resistance to methicillin using cefoxitin disc (30 µg) (MAST DISKS™, UK) on Mueller hinton agar media (Merck, Germany) completed with 4% NaCl. Minimum inhibitory concentration (MIC) was determined by E-test method (MAST DISKS™, UK).

3.3. Antimicrobial Susceptibility Test

Antibiotic susceptibility test was performed using minocycline, levofloxacin, ciprofloxacin, tetracycline, co-trimoxazole, gentamycin, clindamycin, and rifampicin antimicrobial disks using Kirby Bauer method (MAST DISKS™, UK) based on CLSI guidelines (18). All antibiotic disks used in this work were purchased from Mast, UK. Staphylococcus aureus ATCC 25923 used as the control.

3.4. DNA Extraction

The whole genome of S. aureus isolates was extracted using QIAamp® DNA blood mini kit (QIAGEN, Germany). As per kit recommendations, lysozyme (Sigma, Germany) was added at final concentration of 30 µg/mL to the lysis buffer.

3.5. PCR

PCR reaction was performed using TAKARA gradient PCR TP6000 thermal cycler (TAKARA, Japan). We used Hot Star® 2X Master Mix (QIAGEN, Germany) for PCR reactions.

(I) Detection of the mecA gene. The presence of the mecA gene was evaluated by PCR as previously described (3).

(II) Multiplex PCR for the detection of toxin genes. The presence of the PVL, enterotoxin B and toxic shock syndrome toxin genes were evaluated by PCR as previously described (19).

(III) Typing of SCCmec and agr. Agr and SCCmec typing were performed as previously described (20).

3.6. Multi-Locus Sequence Typing

Multi-locus sequence typing was carried out by PCR and sequencing of the internal fragments of yqi, tpi, glp, gmk, aro, pta, and arc genes of S. aureus isolates on PVL-positive isolates as previously described (21).

3.7. Spa Typing

Spa typing was performed by PCR amplification of X region and sequence analysis of spa gene of the PVL-positive isolates as previously described (22).
Table 1. Phenotypic and Genotypic Characteristics of PVL-Positive MRSA Isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gender/Age</th>
<th>Ward</th>
<th>Agr Type</th>
<th>SCCmec Type</th>
<th>Spa Type</th>
<th>Sequence Type</th>
<th>Virulence Genes</th>
<th>Antimicrobial Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA 1</td>
<td>M/4</td>
<td>Internal</td>
<td>I</td>
<td>III</td>
<td>t7688</td>
<td>ST97</td>
<td>etb, tsst1</td>
<td>tet, gen</td>
</tr>
<tr>
<td>MRSA 2</td>
<td>M/0</td>
<td>Internal</td>
<td>I</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>etb</td>
<td>cip, gen</td>
</tr>
<tr>
<td>MRSA 3</td>
<td>M/3</td>
<td>Surgery</td>
<td>I</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>tst1</td>
<td>lev, cli, rif</td>
</tr>
<tr>
<td>MRSA 4</td>
<td>F/4</td>
<td>Internal</td>
<td>I</td>
<td>III</td>
<td>t037</td>
<td>ST239</td>
<td>-</td>
<td>lev, cli, rif</td>
</tr>
<tr>
<td>MRSA 5</td>
<td>F/3</td>
<td>Internal</td>
<td>I</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>etb</td>
<td>tet, cli, rif</td>
</tr>
<tr>
<td>MRSA 6</td>
<td>M/7</td>
<td>Internal</td>
<td>I</td>
<td>IV</td>
<td>t267</td>
<td>ST97</td>
<td>etb</td>
<td>cli, rif</td>
</tr>
<tr>
<td>MRSA 7</td>
<td>F/3</td>
<td>Surgery</td>
<td>I</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>etb, tsst1</td>
<td>cot, cli</td>
</tr>
<tr>
<td>MRSA 8</td>
<td>F/6</td>
<td>Surgery</td>
<td>I</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>etb</td>
<td>gen, cli, rif</td>
</tr>
<tr>
<td>MRSA 9</td>
<td>M/3</td>
<td>Infectious</td>
<td>I</td>
<td>III</td>
<td>t037</td>
<td>ST239</td>
<td>etb</td>
<td>lev, cli</td>
</tr>
<tr>
<td>MRSA 10</td>
<td>M/0</td>
<td>Infectious</td>
<td>III</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>-</td>
<td>lev, cip, gen, cli, rif</td>
</tr>
<tr>
<td>MRSA 11</td>
<td>M/3</td>
<td>Internal</td>
<td>I</td>
<td>IV</td>
<td>t304</td>
<td>ST6</td>
<td>-</td>
<td>lev, cip, gen, cli, rif</td>
</tr>
<tr>
<td>MRSA 12</td>
<td>M/3</td>
<td>Surgery</td>
<td>I</td>
<td>IV</td>
<td>t309</td>
<td>ST339</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: cip, ciprofloxacin; cli, clindamycin; cot, co-trimoxazole; etb, enterotoxin B; gen, gentamycin; lev, levofloxacin; rif, rifampicin; tet, tetracycline; tsst-1, toxic shock syndrome toxin 1.

3.8. Nucleotide Sequencing

QIAquick® Gel Extraction Kit (QIAGEN- Germany) was used for PCR product purification. The PCR products were sequenced in both directions with an ABI 3730XL DNA analyzer.

4. Results

Thirty-one of 58 analyzed S. aureus isolates (53%) were MRSA. Of these 12 were PVL-positive and various antimicrobial resistance patterns were found to rifampicin, clindamycin, gentamycin, co-trimoxazole, tetracycline, ciprofloxacin, and levofloxacin in these isolates. All of these isolates were susceptible to vancomycin and minocycline. The patients’ data and complete antibiogram results of these isolates are listed in Table 1. Twenty-five percent and %75 of MRSA isolates belonged to SCCmec III and IV, respectively. Also, 91.7% and 8.3% belonged to agr I and III, respectively. Evaluation of tsst-1 and etb genes led to the finding of 3/12 (25%) tsst-1 and 7/12 (58.3%) etb gene harboring isolates. We had three PVL-positive SCCmec III isolates. Most of our PVL-positive isolates belonged to ST6 (Table 2). Spa typing revealed that the most prevalent spa type among PVL-harboring isolates was 304 (Table 2). Regarding MLST and spa typing results, we can infer that the most prevalent types in our isolates are Asian endemic types.

5. Discussion

The present study describes genetic characteristics of PVL harboring HA-MRSA strains isolated from blood cultures of children admitted to teaching hospital in Bojnurd. We found 25% (3/12) of PVL gene harboring HA-MRSA in children are less than five years old. Khorasani et al. reported that the prevalence of PVL gene harboring MRSA in burn patients in Ahvaz-Iran was 7.23% and PVL gene harboring MSSA was 33.3% (23). In another study in Shiraz-Iran, Hoseini Alfatemi et al. reported that the prevalence of PVL gene between MRSA isolates was 5.47% (24). Panton-Valentine Leukocidin is commonly found in SCCmec IV MRSA strains that are often classified as community-acquired. Momtaz and Hafezi reported 40.9% PVL gene harboring isolates in clinical samples that most of them belonged to SCCmec type V (25). A similar result was reported by Dormanesh et al. They reported 63.5% PVL-positive MRSA that most of them belonged to SCCmec type V (26).

Surprisingly in this study, we found the PVL gene in SCCmec type III MRSA often classified as HA-MRSA. This is in concordance with some previous findings that proved the sole existence of PVL is not a decisive clue for characterization of an MRSA as CA-MRSA (27-31). Havaei et al. reported that the prevalence of PVL harboring isolates in five Hospitals of Tehran was 24.2%. Of these, 61.8% was HA-MRSA (32). Rasigade et al. expressed three hypotheses about the relationship between SCCmec elements and PVL gene acquisition. First, the subset of S. aureus lineages that are prone to the stable integration of SCCmec IV (for example ST8/USA300 or ST80) is also prone to the integration of the PVL gene (33). Second, there is no direct interaction between the PVL gene and SCCmec integration. Third, the PVL-harboring MSSA lineages pre-exist.
to PVL positive MRSA (33). Based on the last hypothesis, if PVL-harboring MSSA were prone to accept any SCCmec element, they would likely integrate the most prevalent SCCmec element in the environment. In our isolates similar to other Asian countries, the most prevalent HA-MRSA clone is ST239-SCCmec III (II, 12, 34). Prevalence of PVL-harboring SCCmec III MRSA is uncommon but interestingly we found PVL-positive SCCmec III MRSA in our region.

Song et al. reported the variable presence of the PVL gene in various SCCmec types. About 10% of their SCCmec I, 7.7% of SCCmec III, and 16.3% of SCCmec IV isolates were PVL-positive (13). All of SCCmec II isolates were negative for PVL; however, in our study the presence of PVL in various SCCmec types was different and we just found PVL gene among SCCmec III and IV isolates (Tables 1 and 2). Most of our PVL-positive SCCmec III isolates belonged to major Asian endemic clone (CC8/ST239). Perhaps the reason for this is that PVL encoding phages have been recently integrated into dominant Asian MRSA clones. This would suggest the previously mentioned hypothesis, PVL phage insertion into pre-existing MRSA lineages, rather than the accepted model in which SCCmec inserted itself into PVL-positive MSSA strains (33).

In the present study, the PVL-positive isolates were evaluated for the presence of toxin genes, including tst 1 and etb. We observed tst 1 and etb genes in PVL-harboring strains and it can be an indicator of high pathogenicity of these isolates. The emergence of ST239/SCCmec III MRSA strains carrying tst 1, in addition to PVL could be an alarming sign because these strains belong to major hospital-acquired clones. Actually, toxins encoded by these genes are associated with pathogenicity of S. aureus. The genetic evaluation showed that a mainland genetic base of CA-MRSA strain does not relate to HA-MRSA, indicating that CA-MRSA does not appear from residential HA-MRSA (35). One of the most prevalent MRSA genetic backgrounds in Asia is ST239 and spa type t037 (36). The majority of our PVL-positive HA-MRSA isolates belong to ST239 and spa type t037. Only one ST239 isolate had SCCmec IV (CA-MRSA). Moreover, we didn’t have CA-MRSA strain with spa type t037. Regarding these facts and also the high-prevalence rate of spa type t037 in HA-MRSA, it is possible that there is a different genetic background of HA- and CA-MRSA in our isolates.

5.1. Conclusions

In conclusion, for the first time, we have shown PVL is not restricted to SCCmec IV CA-MRSA isolates in Bojnurd, Iran contrary to the previous hypothesis. It seems that PVL carrying phages were integrated into pre-existing endemic MRSA strains; therefore, a previously accepted hypothesis about markers of CA-MRSA strains could be speculated. Our findings and some previous studies suggest that the PVL gene could not be an appropriate marker for CA-MRSA (9, 10, 27, 37-39). Altogether, these findings are alarming with respect to the genetic background of these HA-MRSA strains (ST239/SCCmec III), since it is possible that these highly virulent strains spread to other hospitals in various geographical regions.

Footnotes

Authors’ Contribution: Reza Besharat: laboratory analysis; Majid Ghafoori: study design; Saghaf Safamanesh: laboratory analysis and English editing; Mahsa Khosrojerdi: sample collection and scientific advice; Kiarash Ghazvini: scientific advice; Sara Nojumi: sample collection; Toktam Memariani: laboratory analysis; Hosein Lashkardoost: statistical analysis; Amir Azimian: study design, laboratory analysis and write manuscript.

Conflict of Interests: All authors declare no conflicts regarding this manuscript.

Ethical Considerations: This study was approved by Ethics Committee of North Khorasan University of Medical Sciences for using patient samples with number 92P713.

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References


Table 2. The Prevalence of Various Spa and Sequence Types Among PVL-Positive MRSA Isolates

<table>
<thead>
<tr>
<th>Spa Types</th>
<th>Sequence Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>t037</td>
<td>ST239</td>
</tr>
<tr>
<td>t7688</td>
<td>ST6</td>
</tr>
<tr>
<td>t304</td>
<td>ST97</td>
</tr>
<tr>
<td>t267</td>
<td></td>
</tr>
<tr>
<td>t189</td>
<td></td>
</tr>
</tbody>
</table>

58.3 | 25 | 8.3 | 16.7 | 8.3 | 8.3 | 58.3 | 16.7

Percent


