Original Article

Toxin production and drug resistance profiles of pediatric methicillin-resistant Staphylococcus aureus isolates in Tehran

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Abstract

Introduction: Staphylococcus aureus is known to be a major cause of skin and soft tissue infections, pneumonia and invasive diseases. In this study, attempts were made to examine the prevalence of tss-t, eta, etb, and luk-PV genes among methicillin-resistant S. aureus (MRSA) isolated from children in Tehran.

Methodology: In the present cross-sectional study, a total of 100 MRSA were isolated from children who were referred to a pediatric hospital during 11-month period of September 2014 to August 2015. Isolates were identified using biochemical tests and then, using PCR, the isolates were tested for the presence of mecA, tss-t, eta, etb, and luk-PV genes. Susceptibility of isolates to cefoxitin, penicillin, erythromycin, clindamycin, gentamicin, rifampin, minocycline, co-trimoxazole, linezolid, and vancomycin were evaluated using standard methods.

Results: It was found that the MRSA isolates had the greatest resistance to clindamycin (72%) and erythromycin (59%), while the lowest rates of resistance were observed to be related to minocycline (6%) and rifampin (12%). All of isolates were sensitive to vancomycin and linezolid. The mecA gene was detected in all the isolates. Moreover, luk-PV and tss-t were detected in 18% and 17% of the isolates, respectively. None of the isolates harbored eta and etb genes.

Conclusions: Our data provide specifications about the toxin production status of S. aureus isolates from pediatric children. The current study showed increased resistance to different antibiotics in S. aureus isolates. Therefore, to prevent multi-resistance to other antibiotic classes, it is essential to withhold prescriptions and stop unessential use of available antibiotics.

Key words: Methicillin-resistant Staphylococcus aureus; toxic shock syndrome toxin-1; Panton-Valentine leukocidin; staphylococcal exfoliative toxin; pediatrics.


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Introduction

Although Staphylococcus aureus normally colonizes the nares and skin in up to 80% of the population, it poses as one of the most important bacterial pathogens that cause a wide variety of diseases in humans. It has long been considered as not only the leading cause of community-acquired skin and soft tissue infections, but also one of the most important organisms causing life-threatening nosocomial infections, including necrotizing pneumonia, bacteremia, and various surgical wound infections [1,2]. Meanwhile, neonates and young children are more susceptible to serious complications of S. aureus, particularly skin infections [3].

Pathogenicity of S. aureus is mainly related to the repertoire virulence factors including toxins, invasive capacity, adhesins, exoenzymes, and immunomodulating proteins it produces as well as a variety of resistance mechanisms to many existing antibiotics. The most important virulence factors with this pathogen are numerous exotoxins such as hemolysins (Hla, Hlb, Hld, and Hlg), Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs), and exfoliative toxin A and B (ETA and ETB) [4,5]. However, it should be
pointed out that most of these factors are not always expressed and their expression occurs in specified strains and under certain conditions. For instance, *S. aureus* strains that produce PVL are usually associated with skin and soft-tissue infections [6].

Pervasive resistance among clinical isolates of *S. aureus* strictly complicates management of its infections. In the recent decades, the global concerns about increasing methicillin-resistant *S. aureus* (MRSA) infections in both community and nosocomial settings have attracted significant attentions due to their therapeutic importance. MRSA strains are widespread in the hospital environment, and have recently caused a worldwide epidemic of community-acquired MRSA (CA-MRSA) infections [7,8]. In addition, several authors have reported that MRSA strains have become not only resistant to beta-lactam antibiotics, but they have also acquired additional resistance to non-beta-lactam antibiotics, especially aminoglycosides [9]. Moreover, the presence of resistance gene to methicillin may affect toxin production [6].

Given that the severity and rapid expansion of MRSA strains in the pediatric population is considered as an important health issue, the current study was conducted to characterize the virulence properties and factors of pediatric clinical isolates of MRSA in Tehran, Iran.

**Methodology**

**Bacterial isolation and identification**

The present cross-sectional study was carried out on children who had any obvious staphylococcal infection, and also those that were asymptomatic carriers referred to Children's Medical Center Hospital, from September 2014 to August 2015 in Tehran. In this study, asymptomatic carriers are defined as those subjects who had an *S. aureus*-positive nasal culture on the day of admission. Standard conventional biochemical tests for identification of the isolates were performed on colonies from primary cultures. For this purpose, all suspected colonies of *S. aureus* were examined using Gram staining and further identified using catalase, haemolysis, oxidase, coagulase, DNase, and mannitol fermentation tests. The isolates were stored at -70 °C in trypticase soy broth containing 10% glycerol for later analysis.

**Phenotypic identification of MRSA**

Methicillin resistance of *S. aureus* isolates was screened using disk diffusion test with a cefoxitin disk (30 µg/disk) (Mast Co., Germany) on Mueller-Hinton agar (Merck Co., Germany). According to the Centers for Disease Control and Prevention (CDC), the definition of CA-MRSA strains is any MRSA strain isolated from a patient within 48 to 72 hours of hospital admission, as long as the patient lacks health care-associated MRSA risk factors like surgery, haemodialysis, or hospitalization during the previous year. Accordingly, hospital-acquired MRSA (HA-MRSA) is defined as a MRSA strain isolated from a patient with established risk factors for MRSA infections, including recent surgery, recent hospitalization, residence in a long-term-care facility, or injecting-drug use [10,11].

**Antimicrobial susceptibility test**

The antibiotic susceptibility test was performed using the standardized Kirby-Bauer disc-diffusion technique on Mueller-Hinton agar (Merck Co., Darmstadt, Germany). Commercially available disks of the antimicrobial drugs (Mast Co., Darmstadt, Germany), that have been used most frequently for the treatment of *S. aureus* infections, were selected and tested as recommended by the Clinical and Laboratory Standards Institute (CLSI) [12]. Accordingly, susceptibility of the isolates to following antibiotics: penicillin G (10 units/disk), trimethoprim/sulfamethoxazole (1.25/23.75 µg/disk), clindamycin (10 µg/disk), erythromycin (15 µg/disk), gentamicin (10 µg/disk), rifampin (5 µg/disk), minocycline (30 µg/disk), linezolid (30 µg/disk) and vancomycin (30 µg/disk) were examined. The susceptibility to vancomycin was confirmed through determining of minimum inhibitory concentration (MIC) using E-test (Liofilchem Co., Roseto, Italy). *S. aureus* ATCC 25923 was used as a quality control reference strain. In this study, a multi-drug resistant (MDR) *S. aureus* strain was considered as a single isolate resistant (intermediate or complete) to three or more unique antimicrobial classes [13].

**Preparation of whole-cell DNA for PCR**

The isolates were recovered from storage via subculture on blood agar plates and incubated overnight at 37°C. Bacterial cells were suspended in 250 µl of phosphate-buffered saline (PBS) and then DNA extraction was done using whole genome DNA extraction kit (Cinnaclo Co., Tehran, Iran), according to the manufacturer's instructions. Evaluation of concentration and purity of extracted DNA was measured using Nano drop (DeNovix Inc., Wilmington, USA). Eluted DNA was stored at -20°C for later analysis.
Molecular assay for detection of mecA gene

The presence of the mecA gene encoding penicillin-binding protein 2a (PBPs2a) of S. aureus isolates were confirmed using a PCR assay, as previously described by Seni J. et al. [14]. Briefly, amplification of the mecA gene was performed using the specific primers (forward primer 5’-GTAGAAATGACTGAACGTCCGAT3A-3’ and Reverse primer 5’-CCAATTCACATTGTTCGCTCTAA-3) yielding a PCR product of 307 bp. PCR reaction was performed in a total volume of 25 μL containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 1 μL DNA template (3 μg/μL), 10 pmol of each primer, and 5 U Taq polymerase (Cinnelon Co., Iran). Amplification of mecA gene was performed via the following program: initial denaturation at 94 °C for 2 min and 35 cycles of 1 min at 94 °C, 1 min at 55°C and 1 min at 72 °C. The final extension was 5 min at 72 °C. The PCR products were analysed using agarose gel electrophoresis on 1.8% NuSieve gel DNA extraction kit (Roche Co., Mannheim, Germany) and then were sequenced using Bioneer sequencing methods (Bioneer Inc., Daejeon, South Korea).

Molecular assay for detection of toxin genes

The presence of the virulence genes encoding toxins, including PVL (luk-PV), TSST-1 (tsst-1), and exfoliative toxin A and B (eta and etb) were detected via separate monoplex PCR reactions, using specific primers, as shown in Table 1. PCR reactions were carried out in a final volume of 25 μL containing 1X PCR reaction buffer, 10 pmol of each primer, 1 μL DNA template (3 μg/μL), 1.5 mM MgCl2, 0.2 mM dNTP mixture, and 5 U of Taq DNA polymerase (Cinnelon, Tehran, Iran). The thermal cycling conditions included an initial denaturation step at 94 °C for 5 minutes followed by 30 cycles of amplification comprising of three steps: 2 minutes’ denaturation at 94 °C; one minute annealing at 55 °C for luk-PV; 46 °C for tsst-1, 44 °C for eta and 55°C for etb; and then 1 minute extension at 72°C. The final extension step was at 72°C for 5 minutes. The PCR products were analysed using Tris/Borate/EDTA (TBE) buffered agarose gel (1.5%) electrophoresis at 120 V for 45 minutes. The expected amplicon fragments were purified and sequenced as mentioned above.

Table 1. Primers sequences used in this study for the detection of genes encoding toxins in clinical isolates of methicillin-resistant S. aureus.

<table>
<thead>
<tr>
<th>PCR target</th>
<th>Sequences(5’-3’)</th>
<th>References</th>
<th>Products size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>luk-PV</td>
<td></td>
<td>Lina G et al. (32)</td>
<td>433</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>CTCAGCCGATGTCGACTCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>ATACCTGAGGCTCGCCACTG</td>
<td></td>
</tr>
<tr>
<td>tsst-1</td>
<td></td>
<td>Designated in the present study</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>TTAGCTAAGCCCTTTTGGTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TAAAAGTATTCTATGGAGTAGG</td>
<td></td>
</tr>
<tr>
<td>eta</td>
<td></td>
<td>Designated in the present study</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GAGAGGTATGAGTCAAGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GATGCTCTCTACTAAAGATG</td>
<td></td>
</tr>
<tr>
<td>etb</td>
<td></td>
<td>Designated in the present study</td>
<td>339</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>AAAAGGGAATACACGGGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TCAGTCCCCAAAGTGTCTC</td>
<td></td>
</tr>
</tbody>
</table>


Statistical analysis

SPSS version 22.0 (IBM Corp., Armonk, USA) was applied for statistical analysis. Chi-square test was used for the comparison between the prevalence of the toxins according to infection site. A p value <0.05 was considered to be statistically significant.

Results

Patients and bacterial identification

In the present study, 343 clinical isolates of S. aureus were obtained from children during an 11-month period. The patient’s age range was from infancy (1 day) to 15 years old. The median age of the patients was 44±5 months.

Among 343 S. aureus isolates, 100 (22.5%) were detected as MRSA strains by a phenotypic method. The distributions of mecA gene among MRSA isolates were 100% and its sequence is available on NCBI (GenBank accession number KU380333). Of these, 47 isolates (47%) were collected from outpatient or those referred to emergency department and classified as CA-MRSA; with the remainder from different wards, including pediatric intensive care unit (PICU) (10%), respiratory (10%), surgical (10%), infection (8%), gastroenterology (5%), cardiology (5%), and other wards (5%), which were considered as HA-MRSA. In total, 54% of all MRSA isolates were obtained from nasal of asymptomatic carriers, and the remaining 46% were recovered from clinical samples of infected children, including wound (13%), blood (11%), sputum (7%), abscess (4%), ear discharges (3%), urine (2%), eye discharges (2%), catheter (2%), and synovial fluid (2%).
Antibiotic resistance profile

The antibiotics susceptibility results showed that the most active antibiotic against tested MRSA isolates were linezolid and vancomycin (100% as the susceptibility rate), followed by minocycline (94%), rifampin (88%), gentamicin (78%), sulfamethoxazole/trimethoprim (74%). High resistance rates were detected for penicillin (100%), clindamycin (72%), and erythromycin (59%). There was no significant difference in the antibiotic resistance of the strains according to their origin (Figure 1). In the present study, 73% of isolates were classified as MDR S. aureus strains. Moreover, our results indicated that 69.5% of clindamycin, 74.5% of erythromycin and 73% of sulfamethoxazole/trimethoprim resistant strains were HA-MRSA isolates.

Toxin profiles

The MRSA isolates were also tested for the presence of PVL (luk-PV), TSST-1 (tsst-1), and exfoliative toxin A and B (eta and etb). The luk-PV gene (18%) (GenBank accession numbers KU664618 and KU664619) was the most prevalent virulence gene among the MRSA isolates, followed by tsst-1 gene (17%) (GenBank accession number KU380334).

None of the MRSA isolates carried eta and etb genes. The percentage of isolates harboring each of the virulence genes is shown in Table 2. The relationships between antibiotic resistance and virulence genes are indicated in Table 3.

Discussion

In the present study, we described the drug resistance profiles and prevalence of luk-PV, tsst-1, eta and etb virulence genes among MRSA isolated from both carrier and infected children in Tehran, Iran. MRSA is one of the most common causes of community- and hospital-acquired localized and systemic infections. Approximately, all clinical isolates of MRSA produce a range of more than 30 various extracellular proteins and exoenzymes, most of which

Table 2. Distribution of luk-PV and tsst-1 genes among methicillin-resistant S. aureus isolates according to the origin of samples.

<table>
<thead>
<tr>
<th>Origin (number)</th>
<th>% of isolates harboring luk-PV (number)</th>
<th>% of isolates harboring tsst-1 (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swab (n = 54)</td>
<td>11.1% (n = 6)</td>
<td>11.1% (n = 6)</td>
</tr>
<tr>
<td>Wound (n = 13)</td>
<td>30.7% (n = 4)</td>
<td>7.7% (n = 1)</td>
</tr>
<tr>
<td>Blood (n = 11)</td>
<td>36.3% (n = 4)</td>
<td>18.1% (n = 2)</td>
</tr>
<tr>
<td>Sputum (n = 7)</td>
<td>28.6% (n = 2)</td>
<td>28.6% (n = 2)</td>
</tr>
<tr>
<td>Abscess (n = 4)</td>
<td>0% (n = 0)</td>
<td>25% (n = 1)</td>
</tr>
<tr>
<td>Ear (n = 3)</td>
<td>0% (n = 0)</td>
<td>33.3% (n = 1)</td>
</tr>
<tr>
<td>Urine (n = 2)</td>
<td>0% (n = 0)</td>
<td>100% (n = 2)</td>
</tr>
<tr>
<td>Eye (n = 2)</td>
<td>0% (n = 0)</td>
<td>50% (n = 1)</td>
</tr>
<tr>
<td>Catheter (n = 2)</td>
<td>50% (n = 1)</td>
<td>50% (n = 1)</td>
</tr>
<tr>
<td>Synovial fluid (n = 2)</td>
<td>0% (n = 0)</td>
<td>50% (n = 1)</td>
</tr>
</tbody>
</table>

luk-PV: Panton-Valentine leucocidin gene; tsst-1: toxic shock syndrome toxin gene; A p-value < 0.05 was considered statistically significant. Only the relationship between the urine isolates and presence of the tsst-1 was significant (p value = 0.02).

Table 3. Characterization of methicillin-resistant S. aureus isolates through the determination of antibiotic resistance profiles and prevalence of virulence factors.

<table>
<thead>
<tr>
<th>Resistance pattern</th>
<th>Total number (%)</th>
<th>luk-PV- positive isolates number (%)</th>
<th>tsst-1 -positive isolates number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant to 7-8 antibiotics</td>
<td>9 (9%)</td>
<td>1 (5.5%)</td>
<td>3 (17.6%)</td>
</tr>
<tr>
<td>Resistant to 5-6 antibiotics</td>
<td>28 (28%)</td>
<td>3 (16.7%)</td>
<td>5 (29.5%)</td>
</tr>
<tr>
<td>Resistant to 3-4 antibiotics</td>
<td>36 (36%)</td>
<td>8 (44.4%)</td>
<td>3 (17.6%)</td>
</tr>
<tr>
<td>Resistant to 2 antibiotics</td>
<td>27 (27%)</td>
<td>6 (33.3%)</td>
<td>6 (35.3%)</td>
</tr>
</tbody>
</table>

contribute to staphylococcal pathogenesis. Of note, a vast majority of these factors play a direct role in pediatric morbidity and mortality [15]. Although our study revealed a relatively low incidence (22.5%) of MRSA among infants and children in the community and hospital setting, overall a higher incidence of MRSA infections have previously been reported in Iran [16-18]. However, lower distribution of MRSA (5.1%) was observed in the study reported by Mashouf et al. [19]. The observed differences can be explained by the fact that the other studies were conducted on all age groups or healthy children, while in the present study, we focused on pediatric patients.

Since a large proportion of patients (47%) enrolled in the study were outpatients, there is reason to assume about half of the MRSA isolates were considered as CA-MRSA. CA-MRSA can have the following sources: patients who were discharged from hospital and carry MRSA, employees of nursing homes with MRSA, outpatients that were colonized by MRSA, and MRSA arising de novo in the community [20]. The first three sources comprise MRSA strains that spread from health care settings, which are considered to indicate a limited number of diverse genotypes, disseminate clonally, and express resistance to multiple antimicrobial agents [21]. In contrast, de novo MRSA isolates are assumed to evolve from methicillin-susceptible S. aureus (MSSA) through acquisition of Staphylococcal Cassette Chromosome mec (SCCmec) elements containing a mec gene [22]. In the present survey, considerable prevalence of nasal MRSA carriage has been found among staphylococcal carriers. Epidemiological investigation on pediatric nasal carriage have evaluated small numbers of patients, but it can often propose that nasal carriage may be common in children [23,24]. Although the vast majority of pediatric nasal carriage of CA-MRSA are self-limited within a one-year period, some of them are at the risk of recurrent skin and soft tissue infections and therefore decolonization of these cases is recommended.

Antibiotic resistance among pathogenic bacteria became an important public health problem in Iran, similar to that in the other parts of the world. Limitation to the use of beta lactam agents in MRSA infections required use of other types of antimicrobial agents for the treatment of these infections, so survey of susceptibilities of MRSA isolates for antibiotics other than beta lactams became very vital. Inexpensive oral antibiotics commonly suggested for the treatment of MRSA infections include tetracyclines, erythromycin, clindamycin, trimethoprim/sulfamethoxazole, and rifampin [8]. In the present survey, linezolid, vancomycin, minocycline, rifampin, gentamicin, and trimethoprim/sulfamethoxazole showed a good antibacterial activity against the tested MRSA isolates compared with the other antimicrobial agents mentioned. Vancomycin, for the moment, remains the first-line intravenous agent for severe MRSA infections, both CA-MRSA and HA-MRSA, and in the present study, we observed no resistance to vancomycin. The same results have been obtained in the studies reported by Mazloomi et al. and Ghasemian et al., individually [25,26]. In addition, a vast majority of the MRSA isolates (73%) showed a MDR pattern. In accordance with our study, high rates of MDR S. aureus isolates were identified in the studies conducted by Koosha et al. and Rezaei et al. in Iran [27,28]. In developing countries, unreasonable administration of antimicrobial agents, especially beta-lactam antibiotics, could be responsible for the development of the MDR bacteria. In light of these findings, Iranian physicians and clinicians are more concerned than ever with the increasing spread of clinical isolates of MDR S. aureus.

In the present study, it was found that less than one-fifth of MRSA isolates, obtained from pediatric patients, harbored luk-PV (18%) and tsst-1 (17%) virulence genes, whereas no isolate had eta or etb. However, high detection frequencies of eta (11.3%) and etb (9%) were reported in Sabouni study [29] and almost the same incidence was reported in other studies [6,30,31]. This may be sensible because none of the MRSA strains in the current survey was isolated from children with skin diseases including staphylococcal scalded skin syndrome (SSSS), impetigo, or blisters. Generally, PVL has been associated with S. aureus soft tissue infections and severe necrotizing pneumonia, with the mortality rate for the latter being reported to be about 75% [32]. In an investigation by Lina et al., it was indicated that 50%-93% of S. aureus isolates responsible for cellulitis, furunculosis, or cutaneous abscess and 85% of those responsible for community-acquired pneumonia harbored the luk-PV gene, as compared with none of those causing diseases, such as urinary tract infection, infective endocarditis, enterocolitis, nosocomial pneumonia, or toxic-shock syndrome [33]. In the present study, luk-PV positive isolates were strongly associated with blood, respiratory secretion and wound cultures. The luk-PV gene was found in 5% of blood and 3% of pharynx samples. Overall, the prevalence of the luk-PV gene has been estimated to be 2-35% among MRSA isolates in various types of nosocomial infections [34,35]. In the study by Dormanesh et al., high luk-PV gene distribution (63.5%) among MRSA isolated from
pediatric nosocomial infections was found [36]. Studies carried out in different countries have shown the same distribution, for instance, Esan et al. in Nigeria and Shallcross et al. in the United Kingdom reported that 16% and 11.3% of MRSA isolates harbored luk-PV gene, respectively. Although the association between luk-PV gene and CA-MRSA strains is a subject of debate, to date, PVL has emerged as a significant virulence factor of CA-MRSA isolates making it a putative marker of these strains [37]. In a study conducted by D’Souza et al. in Mumbai between 2006 and 2009, it was found that none of the HA-MRSA isolates carried the luk-PV gene [38]. In contrast, in the present study, only 25% of luk-PV positive strains were isolated from outpatients and others were isolated from patients who were hospitalized for more than 48 hours. Thus, the association of CA-MRSA isolates and luk-PV gene could not be verified in the current study. The present study also showed that the prevalence of tsst-1 genes is high in eye and urine samples.

Conclusion
To the extent of our knowledge, the present study is one of the few studies that demonstrates the prevalence of virulent determinants of MRSA isolated from pediatric patients. In the current study, MRSA isolates were typically associated with both hospital and community acquired infections. In addition, the results show that the virulence gene content of MRSA isolated from pediatric patients varies widely. Therefore, efficient and accurate epidemiological typing methods such as pulsed-field gel electrophoresis (PFGE) and spa typing are suggested for surveillance and limiting the occurrence and spread of epidemic clones within and between hospitals and to community settings. Moreover, to prevent the spread of MRSA strains in health care settings, the decision was made to implement MRSA measures derived from those in use in many hospitals. The measures involved the frequent usage of nasal mupirocin and chlorhexidine showers for patients who are at risk for MRSA transmission or infection.

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Authors’ contributions
GE and ZG conceived and designed the experiment. MEB and HH performed the experiments BN and RS analysed the data. HH and HA wrote the paper. GE and ZG edited the paper. NNF encouraged the research.

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**Conflict of interests:** No conflict of interests is declared.