Antimicrobial resistance and prevalence of CvfB, SEK and SEQ genes among Staphylococcus aureus isolates from paediatric patients with bloodstream infections

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Abstract. Staphylococcus aureus (S. aureus) is one of the most frequently isolated pathogens in neonatal cases of early and late-onset sepsis. Drug resistance profiles and carriage of toxin genes may affect the treatment and outcome of an infection. The present study aimed to determine the antimicrobial resistance patterns and frequencies of the toxin-associated genes conserved virulence factor B (CvfB), staphylococcal enterotoxin Q (SEQ) and staphylococcal enterotoxin K (SEK) among S. aureus isolates recovered from paediatric patients with bloodstream infections (BSIs) in Guangzhou (China). Of the 53 isolates, 43.4% were methicillin-resistant S. aureus (MRSA), and resistance rates to penicillin, erythromycin, clindamycin, trimethoprim/sulfamethoxazole, tetracycline, and ciprofloxacin of 92.5, 66.0, 62.3, 13.2, 20.8 and 1.9% were recorded, respectively. However, no resistance to nitrofurantoin, dalfopristin/quinupristin, rifampicin, gentamicin, linezolid or vancomycin was detected. Resistance to erythromycin, clindamycin and tetracycline in the MRSA group was significantly higher than that in the methicillin-susceptible S. aureus (MSSA) group. No significant differences in antimicrobial resistance patterns were noted between two age groups (≤1 year and >1 year). The proportion of S. aureus isolates positive for CvfB, SEQ and SEK was 100, 34.0 and 35.8%, respectively, with 24.5% (13/53) of strains carrying all three genes. Compared with those in MSSA isolates, the rates of SEK, SEQ and SEK + SEQ carriage among MRSA isolates were significantly higher. Correlations were identified between the carriage of SEQ, SEK and SEQ + SEK genes and MRSA (contingency coefficient 0.500, 0.416, 0.546, respectively; P<0.01). In conclusion, MRSA isolated from the blood of paediatric patients with BSIs not only exhibited higher rates of antimicrobial resistance than MSSA from the same source, but also more frequently harboured SEK and SEQ genes. The combination of the two aspects influenced the dissemination of MRSA among children. The present study clarified the characteristics of BSI-associated S. aureus and enhanced the current understanding of the pathogenicity and treatment of MRSA.

Introduction

Staphylococcus aureus (S. aureus) is a major causative agent of various infectious diseases worldwide, ranging from furuncles to endocarditis, toxic shock syndrome and sepsis (1,2). Of the pathogens most frequently isolated from patients with bloodstream infections (BSIs), S. aureus, which is common among neonates and elderly patients (3,4), ranked third (11.4%) in 2012 in China (5). Furthermore, methicillin-resistant S. aureus (MRSA) strains accounted for 44.6 and 42.2% of BSIs in China in 2014 and 2015, respectively (6,7). Invasive MRSA infection has been identified as an independent risk factor for poor prognosis and is associated with a significant increase in the duration of hospitalization (8,9). Although MRSA-ST239-SCCmec III represents the most common clone in China, others are also in circulation, whose antimicrobial resistance profiles differ (10). Thus, the provision of data concerning antibiotic resistance is important to enable clinicians to choose appropriate treatments.

S. aureus produces a wide variety of virulence factors, including pan-valentine leucocidin (PVL), toxic shock...
syndrome toxin-1, and staphylococcal enterotoxins (SEs), which facilitate bacterial colonization of host tissues and evasion of host immune responses, resulting in disease. MRSA appears to harbour more virulence genes than other types. In a study by Yu et al (1), the frequencies of virulence genes, including the PVL and SEs genes, in S. aureus isolated from BSIs in the MRSA group were higher than those in the methicillin-susceptible S. aureus (MSSA) group. The course of an infection may be affected by the genes encoding these virulence factors; given their diversity and great variability (11), toxin gene profiling is of importance.

Conserved virulence factor B (CvfB) is a novel virulence-associated protein that acts via activation of the accessory gene regulator (AGR) locus, or through an AGR-independent pathway, to control exoprotein production. Deletion of CvfB results in decreased production of hemolysin, DNase and protease, and reduced virulence (12,13). SEs are pyrogenic toxin superantigens that are thought to be a leading cause of foodborne illness and toxic shock (14). Staphylococcal enterotoxin Q (SEQ) and staphylococcal enterotoxin K (SEK) are newly described SEs with demonstrable superantigenic activity that may cause severe gastroenteritis, nausea and vomiting (15-17). To the best of our knowledge, carriage of CvfB by a clinical strain of S. aureus and the prevalence of SEQ and SEK among S. aureus isolates from paediatric patients with BSIs in China have not yet been reported. Therefore, the present study examined the prevalence of these three genes among isolates of this bacterium from Chinese paediatric patients with BSIs.

Materials and methods

Bacterial strains. Between 2012 and 2015, a total of 53 clinical S. aureus isolates were recovered from blood cultures derived from paediatric patients with BSIs attending Guangzhou Women and Children's Medical Center (Guangzhou, China). To avoid duplication, strains consecutively isolated from the same patient were excluded. All the isolates were stored at -70˚C. The present study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center (Guangzhou, China). Informed consent was obtained from the guardians of all participants.

Antibiotic susceptibility tests. S. aureus isolates were manually identified by routine microbiological methods prior to final confirmation and assessment of susceptibility to 12 antibiotics [penicillin, erythromycin, clindamycin, trimethoprim/sulfamethoxazole (SXT), tetracycline, ciprofloxacin, nitrofurantoin, rifampicin, dalfopristin/quinupristin, gentamicin, linezolid and vancomycin] by broth microdilution using an automated VITEK2 compact system (bioMérieux, Marcy l’Etoile, France). Antimicrobial susceptibility testing performance standards and minimum inhibitory concentration (MIC) interpretive criteria followed the Clinical and Laboratory Standards Institute guidelines. MRSA was determined based on cefoxitin, a surrogate for oxacillin, for which the MIC value was ≥8 µg/ml (18). S. aureus ATCC 29213 (American Type Culture Collection, Manassas, VA, USA) was used as a reference strain.

Preparation of staphylococcal DNA. The boiling method (19,20) was employed to extract crude DNA for use in PCR. S. aureus was cultured at 37˚C in lysogeny broth (Oxoid; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 16 h, 1 ml of which was then centrifuged for 10 min at 4˚C at 13,839 x g to collect the cells, which were subsequently resuspended in 100 µl Tris-EDTA buffer. This suspension was incubated in boiling water for 10 min prior to being cooled on ice for 5 min. Following centrifugation at 4˚C at 13,839 x g for 5 min, supernatants containing DNA were recovered.

Primer design and polymerase chain reaction (PCR) amplification

Primer design. Primer pairs specific for the CvfB, SEQ and SEK genes were designed based on the S. aureus TW20 genome (GenBank accession no. FN433596.1). Primer sequences, predicted PCR product sizes, locations within the genome and GenBank accession numbers are presented in Table I. Primers were synthesized by the Beijing Genomics Institute (Shenzhen, China).

PCR amplification. All PCRs were singleplex and performed using a Takara taq™ Package (ro01a) (Takara Bio, Inc., Otsu, Japan), according to the manufacturer’s instructions. Each reaction had a final volume of 50 µl, comprising 1 µl DNA, 4 µl deoxynucleotide triphosphates, 5 µl 10X PCR buffer, 1 µl of each primer, 0.5 µl rTaq polymerase and 37.5 µl double-distilled H₂O. For each PCR run, a negative control was included using ddH₂O in place of the DNA template. Using a Biometra personal PCR thermocycler (Biometra, Gottingen, Germany), the following cycling conditions were applied: 94˚C for 5 min, followed by 30 cycles of amplification, consisting of denaturation at 94˚C for 30 sec, annealing at 52˚C for 30 sec and extension at 72˚C for 80 sec. PCR products and marker DL1,000 (Takara Bio, Inc.) were electrophoresed using a DYY-8C instrument (Beijing Liuyi Biotechnology Co., Beijing, China) on 2% (w/v) Biowest agarose G10 gels (Gen Company Ltd., Hong Kong, China) and stained with GoldView I dye (SBS Gene Tech, Shanghai, China), prior to visualisation on an ultraviolet transilluminator GAST508-T20 (Uvitec, Cambridge, UK).

Sequence analysis. The CVFB, SEQ and SEK PCR products from one isolate were purified and subjected to Sanger sequencing using an ABI 3730x1 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) to confirm the reliability of the PCR. The PCR products were bi-directionally sequenced multiple times and the sequences were assembled using ContigExpress software (ContigExpress LLC, New York, NY, USA). The obtained DNA sequences and deduced protein sequences were searched using the Basic Local Alignment Search Tool (BLAST) (21) with the default parameters of the National Center for Biotechnology Information database (22) to assess homology with TW20 and other S. aureus strains.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 Data Editor (SPSS, Inc., Chicago, IL, USA). Categorical variables were described using frequencies.
Results

Clinical features of the patients with S. aureus BSIs. The ages of the 53 patients with S. aureus BSIs ranged from 5 days to 13 years, with a median age of 8 months. Male patients accounted for 49.1% (26/53) of the cases, while the remaining 50.9% (27/53) were female and one isolate strain was obtained from each patient. The probable primary lesion was mostly in the respiratory tract, being responsible for BSI in 39.6% (21/53) of all cases, followed by skin and soft tissue (28.3%), bone and joint (17.0%), and surgical site infection (1.9%) (Table II). The distribution of strains by clinical department was as follows: Neonatal and paediatric intensive care units (n=16), emergency department (n=14), internal medicine department (n=14) and surgery department (n=9).

Antimicrobial susceptibility profiles of S. aureus isolated from patients with BSIs. All isolates were tested for susceptibility to the 12 antibiotics mentioned above. In total, 43.4% (23/53) were MRSA, while the remaining 56.6% (30/53) were MSSA. Antibiotic susceptibility profiles for MRSA and MSSA isolates are listed in Table III. Among the 53 isolates recovered, the highest rate of resistance was recorded in association with penicillin (92.5%), followed by erythromycin (66.0%), clindamycin (62.3%), tetracycline (20.8%), SXT (13.2%) and ciprofloxacin (1.9%). None of the isolates was resistant to nitrofurantoin, dalfopristin/quinupristin, rifampicin, gentamicin, linezolid or vancomycin. All (23/23) MRSA strains and 33.3% (10/30) of the MSSA strains were multidrug resistant (≥ 3 classes). Rates of resistance to erythromycin, clindamycin and tetracycline were significantly higher in the MRSA group than in the MSSA group. Although resistance to penicillin and ciprofloxacin was more prevalent among MRSA isolates and resistance to SXT was more common among MSSA isolates, no significant differences were noted between the two groups in this respect. The antimicrobial resistance patterns between two age groups (≤1 year and >1 year) were also not significantly different.

Prevalence of CvfB, SEQ and SEK genes among S. aureus isolates from paediatric patients with BSIs. As presented in Fig. 1, the amplified CvfB, SEQ and SEK fragments were ~903, 771 and 729 bp long, respectively. BLAST searches revealed that the amplified sequences were highly similar to the corresponding genes of strain TW20, as follows: CvfB, 99.0%; SEQ, 100%; and SEK, 99.0%. High levels of similarity were also noted with sequences of other S. aureus strains, such as those for AB297388.1 (99% similarity in CvfB), KU574280.1 (99% similarity in SEK) and U93688.2 (100% similarity in SEQ).
The CvfB, SEQ and SEK sequences generated in the present study have been deposited in GenBank under the accession nos. KY705045, KY873303 and KY684176, respectively.

Of the 53 strains isolated, all carried CvfB, 34.0% (18/53) harboured SEQ and 35.8% (19/53) were positive for SEK. Furthermore, 45.3% (24/53) possessed SEK or SEQ, while 24.5% (13/53) carried both. The presence of SEK + SEQ was only identified in MRSA isolates. The number of isolates harbouring SEK, SEQ or both genes was significantly higher in the MRSA group than in the MSSA group (P<0.01). In addition, correlations were found between the virulent genes SEK, SEQ, SEK + SEQ and MRSA (contingency coefficient 0.416, 0.500, 0.546 respectively; P<0.01; Table IV).

### Discussion

In the present study, the antimicrobial resistance profiles of *S. aureus* isolates recovered from paediatric patients with BSIs in Guangzhou (China) were assessed and the prevalence of the toxin-associated genes CvfB, SEQ and SEK was estimated. In total, 43.4% were identified to be MRSA, and high rates of resistance to penicillin (92.5%), erythromycin (66.0%) and clindamycin (62.3%) were recorded. All isolates carried CvfB,
while 34.0 and 35.8% harboured SEQ and SEK, respectively; 24.5% possessed all three genes. Carriage of SEK, SEQ, and SEK + SEQ, and resistance to erythromycin, clindamycin and tetracycline, was significantly higher among MRSA isolates than in those classified as MSSA.

The emergence of antimicrobial resistance and presence of virulence genes among bacteraemia-associated *S. aureus* strains result in limited therapeutic options and challenging patient management, particularly for patients <1 year of age that involve MRSA. Among the 53 patients with *S. aureus* BSIs, the age ranged from 5 days to 13 years, with a median age of 8 months. The group aged ≤1 year accounted for 60.4% (32/53). In a nationwide, multicentre surveillance study of antimicrobial susceptibility among bacteria causing BSIs performed in 2011, *Yuan et al.* (5) reported rates of *S. aureus* resistance to penicillin, erythromycin and clindamycin of 90.2, 70.7 and 52.0%, respectively. In the present study, high rates of resistance to penicillin (92.5%), erythromycin (66.0%) and clindamycin (62.3%) were identified among *S. aureus* isolates from paediatric patients with BSIs in Guangzhou (China). These results indicated that these drugs are not effective to treat invasive *S. aureus* infections as empiric options.

In China, MRSA strains accounted for 44.6 and 42.2% of BSIs on average in 2014 and 2015, respectively (6,7). In the present study, 43.4% of isolates recovered from paediatric patients with BSIs in Guangzhou were MRSA. Vancomycin, a glycopeptide antibiotic, is the drug of choice for MRSA infections, as recommended by the Infectious Diseases Society of America (23). *The results of the present study imply that vancomycin and linezolid may be effective treatments for MRSA in paediatric BSIs, given that all isolates tested were susceptible to these antimicrobial agents. Thus, for patients who are suspected of having *S. aureus* bacteraemia and who are in a critical condition, it is better to choose vancomycin as empiric option in case of MRSA infections being present.*

*Casapao et al.* (24) reported that vancomycin treatment is more likely to fail for patients with heterogeneous vancomycin-intermediate *S. aureus* BSIs, and vancomycin monotherapy may not be adequate for severe MRSA infections (25). Linezolid or rifampicin may be used as adjunctive therapies for severe, invasive MRSA infections, as could gentamicin, which has been recommended by the American Academy of Pediatrics for use with other drugs in the treatment of endocarditis, persistent bacteraemia, meningitis and ventriculitis (26).

Table IV. Occurrence of SEK, CvFB and SEQ genes in 53 strains of *S. aureus* clinical isolates in n (%).

<table>
<thead>
<tr>
<th>Gene</th>
<th>MRSA + MSSA (n=53)</th>
<th>MRSA (n=23)</th>
<th>MSSA (n=30)</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CvFB</td>
<td>53 (100)</td>
<td>23 (100)</td>
<td>30 (100)</td>
<td>0.416</td>
<td>0.546</td>
</tr>
<tr>
<td>SEK</td>
<td>19 (35.8)</td>
<td>14 (60.9)</td>
<td>5 (16.7)</td>
<td>&lt;0.01</td>
<td>0.500</td>
</tr>
<tr>
<td>SEQ</td>
<td>18 (34.0)</td>
<td>15 (65.2)</td>
<td>3 (10.0)</td>
<td>&lt;0.01</td>
<td>0.500</td>
</tr>
<tr>
<td>SEK + SEQ</td>
<td>13 (24.5)</td>
<td>13 (56.5)</td>
<td>0 (0.0)</td>
<td>&lt;0.01</td>
<td>0.546</td>
</tr>
</tbody>
</table>

<sup>a</sup>MRSA vs. MSSA by Chi-square test (2‑sided); <sup>b</sup>Contingency coefficient values of correlations between the virulent genes SEK, SEQ, SEK + SEQ and MRSA. *S. aureus*, *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; CvFB, conserved virulence factor B; SEK, staphylococcal enterotoxin K; SEQ, staphylococcal enterotoxin Q.

In conclusion, based on the observed antimicrobial resistance rates, penicillin, erythromycin and clindamycin may not be appropriate antibiotics for the treatment of paediatric patients with BSIs, whereas vancomycin may be more effective as an empiric option. Of the isolates recovered from paediatric patients with BSIs in Guangzhou (China), 43.4% were MRSA, which demonstrated not only higher rates of antimicrobial resistance, but also more frequent carriage of SEK and SEQ genes compared with the MSSA isolates obtained. The combination of the two aspects influenced the dissemination of MRSA among children. The present study clarified the characteristics of BSIs-associated *S. aureus* and enhanced the current knowledge regarding the pathogenicity and treatment of MRSA.

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References


