Accessory gene regulator (agr) dysfunction was unusual in *Staphylococcus aureus* isolated from Chinese children

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**Abstract**

**Background:** *Staphylococcus aureus* (*S. aureus*) with accessory gene regulator (*agr*) dysfunction occurs in health care settings. This study evaluated the prevalence and the molecular and drug resistance characteristics of *S. aureus* with dysfunctional *agr* in a pediatric population in Beijing, China.

**Results:** A total of 269 nonduplicate *S. aureus* clinical isolates were isolated from Beijing Children’s Hospital, including 211 methicillin-resistant *S. aureus* (MRSA) from September 2010–2017 and 58 methicillin-sensitive *S. aureus* (MSSA) from February 2016–2017. Only 8 MRSA and 2 MSSA isolates were identified as *agr* dysfunction, and the overall prevalence rate was 3.7%. For MRSA isolates, ST59-SCC mec IV and ST239-SCC mec III were the most common clones, and the prevalence rate of *agr* dysfunction in ST239-SCC mec III isolates (17.39%) was significantly higher than in ST59-SCC mec IV (1.69%) and other genotype strains (*P* = 0.006). Among the *agr* dysfunctional isolates, only one MRSA ST59 isolate and one MSSA ST22 isolate harbored *pvl*. No significant difference was detected between *agr* dysfunctional and *agr* functional isolates regarding the biofilm formation ability (*P* = 0.4972); however, 9/10 *agr* dysfunctional isolates could effectuate strong biofilm formation and multidrug resistance. Among MRSA, the non-susceptibility rates to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole were significantly higher in *agr* dysfunctional isolates than in isolates with functional *agr* (*P* < 0.05). Two isolates belonging to ST239 had no mutations in *agr* locus, but a synonymous mutation was found in *agrA* in another ST239 isolate. The inactivating mutations were detected in other seven *agr* dysfunctional isolates. The variants were characterized by non-synonymous changes (n = 5) and frameshift mutations (insertions, n = 2), which mainly occurred in *agrC* and *agrA*.

**Conclusions:** The results showed that *agr* dysfunctional *S. aureus* was not common in Chinese children, and ST59-SCC mec IV was associated with lower prevalence of *agr* dysfunction as compared to ST239-SCC mec III isolates. The *agr* dysfunctional isolates were healthcare-associated, multidrug resistant and form strong biofilm, which suggested that *agr* dysfunction might offer potential advantages for *S. aureus* to survive in a medical environment.

**Keywords:** *Staphylococcus aureus*, Accessory gene regulator, Children, China
Background

Staphylococcus aureus (S. aureus) continues to be a leading cause of both community- and healthcare-associated infections, including skin and soft tissue infections, bacteremia, pneumonia, osteomyelitis, and endocarditis. Virulence factors play a major role in the pathogenesis of S. aureus, such as Panton-Valentine leukocidin (PVL). PVL is a pore-forming exotoxin composed of LukS-PV and LukF-PV. The PVL-producing strains have been associated with the onset of skin and soft tissue infections (SSTIs) and can also cause severe invasive infections (necrotizing pneumonia, etc) [1]. In addition, S. aureus can form biofilms not only in biological samples and surfaces of medical devices but also in tissues [2]. Biofilm can protect S. aureus from the damage of antibiotics and the host immune system [3]. Subsequently, the successful eradication of S. aureus infections is difficult, rendering biofilm as a vital factor in chronic infections.

The accessory gene regulator (agr)-mediated quorum sensing plays a major role in staphylococcal pathogenesis, which can downregulate the expression of cell surface-associated proteins (microbial surface components recognizing adhesive matrix molecules [MSCRAMMs]) and upregulate the expression of extracellular toxin (hemolysins, enterotoxins, extracellular proteases, etc.) [4]. The regulation of virulence factors by agr is important for disease progression. Several studies demonstrated that genetically engineered agr-knockout strains had attenuated virulence in animal models of skin and soft tissue infections, pneumonia, infective endocarditis, arthritis, and osteomyelitis [5–10].

However, recent evidence indicated that agr dysfunction exists extensively in healthcare settings (13–82%) [11–14], which might be attributed to the agr dysfunction that confers a potential advantage for S. aureus in the current medical environment [15]. For example, agr dysfunction has been linked to attenuated vancomycin activity; both laboratory-derived and clinical vancomycin-resistant S. aureus (VISA) and clinical VISA isolates developed during vancomycin therapy often exhibit as agr dysfunction [16]. In addition, infections, such as bacteremia and bone and joint infection, caused by strains with dysfunctional agr often manifest as a chronic course and result in adverse outcomes [17–19]. Thus, understanding the prevalence of agr dysfunction in a specific area is essential.

Herein, we conducted a molecular epidemiology study in Beijing Children’s Hospital in China. The primary objectives of the present study were as follows: (1) to detect the prevalence of agr dysfunction in MRSA and MSSA isolates; (2) to explore the molecular characteristics, pvl carriage rate, biofilm formation ability, and antibiotic susceptibility of S. aureus isolated from pediatric patients; these indicators were compared mainly based on agr functionality.

Methods

Bacterial isolates

This study was approved by the Ethics Committee of Beijing Children’s Hospital affiliated to the Capital Medical University (No. 2016–93, 23/06/2016), and obtained clearance from the Institutional Biosafety Committee (IBSC) ([2017] No.43). S. aureus strains were collected and identified as follows. If the clinical samples (blood, pleural effusion, and joint effusion, etc.) were obtained from sterile specimens (blood, pleural effusion, bone marrow, cerebrospinal fluid, joint effusion, seroperitoneum, etc), the bacterial growth in the culture medium could be directly identified by VITEK® MS system (BioMérieux, France). If the clinical samples were obtained from the non-sterile specimens (respiratory tract, skin, etc.), several suspected colonies were selected according to the morphological characteristics and identified by VITEK® MS system. The coagulase test and detection of nuc gene were employed to identify S. aureus as described previously by Kateete et al. [20] and Petersson et al. [21], respectively; these isolates were further confirmed to be S. aureus. The MRSA isolates were screened by cefoxitin disc (30 mg, Oxoid) diffusion test, while the polymerase chain reaction (PCR) was employed for the detection of the mecA gene [22]. In the case of strains isolated from the same patient, if the genotyping studies revealed identical genotype, only one of them was included in the study, which ensured that all strains involved in the current study were non-repetitive. All the strains were preserved at 80 °C in a bacterial cryopreservation reagent comprised of 2.5% TSB (w/v), 16.7% glycerol (v/v), and 66.7% sterile horse serum (v/v).

The clinical data of children, including age, sex, medical history, isolation site, infection sites, and medication use, were collected. S. aureus infections were categorized as healthcare-associated (HA) or community-associated (CA) according to the epidemiology definitions established previously [23].

Delta-hemolysin expression

RNAIII is the major effector molecule of agr system, and also encodes the gene for delta-hemolysin (hld) [24]. Thus, δ-hemolysin production was used to assess the function of agr operon. The expression of δ-hemolysin was determined using S. aureus RN4220, which produced only β-hemolysin without the interference of α- or δ- hemolysins. Furthermore, β-hemolysin and δ-hemolysin have synergetic effects, while β-hemolysin inhibits lysis by α-hemolysin [25]. Therefore, the presence of enhanced hemolysis within the β-hemolysin zone of RN4220 indicates the production of δ-hemolysin by the test strains.
**agr sequencing**

The *agr* locus of non-hemolytic isolates was amplified using the method reported by Robinson et al. [26]. In addition, *agr*-w1f (5′-CCATTGcCCTATATGTTTC-3′) was used for sequencing the PCR product amplified by *agrX1F* (5′-TCGT

**Molecular genotyping analysis**

Multilocus sequence typing (MLST) was performed as described by Enright et al. [27]. The allelic profiles (allele numbers) and ST types were determined based on the MLST database (http://saureus.mlst.net/). The staphylococcal protein A (*spa*) gene repeat region was amplified and sequenced as described previously [28], and the sequencing data were submitted to the *S. aureus spa* database (http://spaserver.ridom.de) to determine the *spa* type. *Agr* typing was assigned by multiplex PCR according to the method described by Gilot et al. [29]. COL (*agr* I), N315 (*agr* II), TY114 (*agr* III), and A920210 were used as positive controls.

The staphylococcal cassette chromosome *mec* (SCC*mec*) types of MRSA isolates were determined using a multiplex PCR as described previously [30]. The reference strains used for SCC*mec* typing included NCTC10442 (SCC*mec* I), N315 (SCC*mec* II), 85/2082 (SCC*mec* III), JCSC4744 (SCC*mec* IV), and IMVS 67 (SCC*mec* V).

**Detection of pvl gene**

The detection of *pvl* was carried out using primers and conditions as described by Jarraud et al. [31]. ATCC25923 was used as a positive control.

**Biofilm formation assays**

Tissue culture plate method (TCP) was used to assess the biofilm forming ability of the nonhemolytic strains, as described in our previous study [32]. Briefly, overnight cultures in tryptic soy broth (TSB) (OXOID, USA) containing 0.25% glucose were adjusted to $10^6$ CFU/mL. Then, 0.2 mL cell suspension was inoculated into each well of 96-well flat-bottom plates (Corning Costar #3599, USA) at 37 °C for 48 h. Subsequently, the wells were washed two times with normal saline, fixed by methanol for 15 min, stained with 0.1% crystal violet for 5 min, rinsed, and air-dried. The stained biofilm was solubilized with 33% glacial acetic acid, and the optical density (OD) was measured at 590 nm using on a CLARIOstar Microplate reader (BMG LABTECH, Germany). Each isolate was tested in triplicates. The negative control wells contained only the broth. The cut-off OD value (ODc) was defined as an average OD of negative control with three times of standard deviation. The biofilm formation ability was classified as negative (OD ≥ ODc), weak (ODc < OD ≤ 2ODc, WBF), moderate (2ODc < OD ≤ ODc, MBF), and strong (4ODc < OD, SBF).

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing to 12 antimicrobial agents (penicillin G, oxacillin, erythromycin, clindamycin, tetracycline, gentamicin, chloramphenicol, ciprofloxacin, rifampin, linezolid, vancomycin, moxifloxacin; National Institutes for Food and Drug Control, China) were performed by agar dilution method as described by Wiegand et al. [33] with slight modifications. Mueller-Hinton Agar (MHA; OXOID CM0337B, UK) medium without cation-adjustments were prepared and autoclaved according to the manufacturer’s instructions. The antibacterial drugs were diluted for usage according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [34]. Appropriate MHA medium was cooled to about 50 °C and poured to the 15 × 100 mm petri dish to produce the required depth of 3–4 mm. The final antibiotic concentration for each drug ranged from 0.032–256 mg/L and three control agar plates were without any antibiotic. The inoculation was carried out using a multipoint inoculator. Bacterial suspension, at a density of $1 \times 10^7$ CFU/mL, was inoculated to the agar plates starting from the lowest concentration. Then, the inoculum spots were dried at room temperature before inverting the plates and incubated at 35 °C for 16–20 h before obtaining the minimal inhibitory concentration (MIC). In addition, the E-test method was used to determine the MIC of all isolates to sulphasethoxazole/trimetoprim (SXT) (BioMérieux, France), MHA plates were inoculated by streaking the standardized inoculums (0.5 McFarland, about $1.5 \times 10^8$ CFU/mL), with a sterile swab. The SXT E-test strips (BioMérieux) were placed on the plates, followed by incubation at 35 °C for 16–20 h.

The MIC reading for both E-test and agar dilution method was conducted independently by a senior experimenter, with the result confirmed by a second reader. The results of MIC were interpreted according to the CLSI breakpoints for *Staphylococcus* spp. [34]. *S. aureus* ATCC29213 was used as quality control. Multi-drug resistance (MDR) was defined as isolates resistant to ≥3 classes of non-β-lactam antimicrobials for MRSA, and resistant to ≥3 classes of antibiotics including β-lactam antibiotics for MSSA.
Statistical analysis
SAS JMP Statistical Discovery v11.0 was used for statistical analysis. Chi-squared ($\chi^2$) test or Fisher’s exact test was used to analyze the categorical variables, and Wilcoxon rank sum test was used to compare the biofilm formation ability between the two groups. $P < 0.05$ was considered as statistically significant.

Results
Clinical characteristics
A total of 269 non-duplicate S. aureus clinical isolates were collected from Beijing Children’s Hospital, including 211 MRSA from September 2010–2017 and 58 MSSA from February 2016–2017. These strains were isolated from several clinical sources, including respiratory tract (6 from throat swab, 69 from sputum, and 37 from bronchial alveolar lavage fluid), skin and soft tissue (38 from pus, 20 from secretions of omphalitis, 10 from skin secretions, 17 from wound surface, 6 from eye secretions, and 2 from ear secretions), sterile sites (48 from skin secretions, 17 from wound surface, 6 from eye secretions), and invasive infections. All children included in this study were treated with antibiotics. Vancomycin and linezolid were used in 4.54% (66/269) and 16.73% (45/269) patients, respectively. The median hospital stay was 15 (interquartile range, IQR: 10–24) Days.

Molecular typing and virulence characteristics
Among the 269 isolates, only 3.79% (8/211) MRSA and 3.45% (2/58) MSSA isolates were identified with $agr$ dysfunction (no apparent hemolytic activity as shown in Fig. 1), and the overall prevalence rate was 3.71% (10/269).

The molecular characteristics of MRSA and MSSA stratified by $agr$ functionality were shown in Table 2 and Table 3, respectively. ST59-SCCmec IV-t437/t441-agr I (49.29%, 104/211) and ST239-SCCmec III-t030/t037-agr I (9.00%, 19/211) were the most common MRSA clones. The prevalence rate of $agr$ dysfunction in ST239-SCCmec III isolates (17.39%, 4/23) was significantly higher than in ST59-SCCmec IV (1.69%, 2/118) and other genotype strains (2.86%, 2/70) ($P = 0.006$ (Table 1). For MSSA isolates, the top 4 genotypes were ST22-t309-agr I (15.52%, 9/58), ST398-t571-agr I

Table 1: Characteristics of patients for samples

| Patient characteristics | Total | Dysfunctional $agr$ (N = 10) | Functional $agr$ (N = 159) | $P$-value |
|-------------------------|-------|-----------------------------|-----------------------------|-----------|
| Male sex, N (%)         | 162 (60.22) | 5 (50.00) | 157 (60.62) | 0.5253    |
| Age (months), median (IQR$^2$) | 11.9 (1.27–55.14) | 33.27 (13.15–108.38) | 11.33 (1.17–54.90) | 0.0797    |
| Age distribution        | 0.1581 |
| ≤ 28 days               | 59 (21.93) | 0 | 59 (22.78) |          |
| 29 days–3 years         | 137 (50.93) | 7 (70.00) | 130 (50.19) |          |
| 4–6 years               | 28 (10.41) | 0 | 28 (10.81) |          |
| 7–15 years              | 45 (16.73) | 3 (30.00) | 42 (16.22) |          |
| Origin, N (%)           | 0.0102 |
| CA                      | 135 (50.19) | 1 (10.00) | 134 (51.74) |          |
| HA                      | 134 (49.81) | 9 (90.00) | 125 (48.26) |          |
| Disease                 | 0.2900 |
| Invasive infection$^6$, N (%) | 80 (29.74) | 1 (10.00) | 79 (90.00) |          |
| Non-invasive infection$^5$, N(%) | 189 (70.26) | 79 (30.50) | 180 (69.50) |          |
| Vancomycin treatment, N (%) | 66 (24.54) | 2 (20.00) | 64 (24.71) | 1.0000    |
| Linezolid treatment, N (%) | 45 (16.73) | 2 (20.00) | 43 (16.60) | 0.6758    |
| Hospitalization-Median (IQR) | 15 (10–24) | 21 (10.25–30.25) | 14 (10–23) | 0.4000    |

$^a$IQR interquartile range
$^b$SSTI including skin and soft tissue infection, BJI bloodstream infection, CNSI central nervous system infection, IE infective endocarditis, BJI bone and joint infection, SP severe pneumonia, AI intra-abdominal infection. Details were as follows: SSTI (1 case), AI (1 case), CNSI (1 case), BJI (3 cases), SP (3 cases), BJI (1 case), BJI (1 case), BJI (6 cases), BJI + CNSI (2 cases), SP + SSTI (1 case), SP + SSTI (1 case), SP + BJI (1 case), BJI + SSTI + CNSI (1 case), BJI + CNSI + IE (1 case), BJI + SP + CNSI (1 case), BJI + SP + SSTI (3 cases), BJI + BJI + SSTI (7 cases), BJI + BJI + SSTI + CNSI (1 cases), BJI + SP + 6 + SSTI + CNSI (1 case)

$^c$Including SSTI (83 cases), pneumonia (103 cases), pneumonia+SSTI (2 cases), urinary system infection (1 case)
The detection rate of \( pvl \) was similar in MRSA (35.55%, 75/211) and MSSA (29.31%, 17/58) isolates (\( P = 0.4359 \)). Among MRSA, the \( pvl \) prevalence rate of ST239-SCCmec III isolates (4.35%, 1/23) was significantly lower than that of ST59-SCCmec IV (37.3%, 44/118) and other strains (42.86%, 30/70) (\( P = 0.0031 \)). All the ST22 isolates were \( pvl \)-positive. Among the ten agr dysfunctional isolates, only one MRSA belonging to the ST59-SCCmec IV-t437-\( agr \) I and one MSSA belonging to ST22-t310-\( agr \) I harbored \( pvl \).

**Biofilm formation**

Table 4 shows that 88.63% (187/211) of MRSA and 56.90% (33/58) of MSSA isolates were strong biofilm formers. However, the biofilm formation ability of MRSA was significantly higher than that of MSSA isolates (\( P < 0.0001 \)) (Fig. 2a). Interestingly, no significant difference was detected between \( agr \) dysfunction and \( agr \) functional isolates regarding the biofilm formation ability (\( P = 0.4972 \)) (Fig. 2b); nevertheless, all the eight \( agr \) dysfunctional MRSA isolates and one MSSA isolate showed strong biofilm formation. Any association between \( pvl \) and biofilm formation in both MRSA and MSSA isolates was not observed (\( P = 0.4004 \) and \( P = 0.0509 \), respectively) (Figs. 2c and d).

**Antimicrobial resistance**

The results of antimicrobial susceptibility test are shown in Table 5. In this study, the \( S. aureus \) isolates were sensitive to vancomycin and linezolid, but highly resistant to penicillin (97.03%, 261/269) and erythromycin (98.89%, 266/269). Approximately, 80.09% (169/211) of MRSA and 74.14% (43/58) of MSSA isolates were MDR strains. The non-susceptibility rate of MRSA to oxacillin, clindamycin, tetracycline, and rifampin was significantly higher than that of MSSA isolates (\( P < 0.05 \)). In addition, the non-susceptibility rates to ciprofloxacin, gentamicin, and SXT among MRSA isolates were significantly higher in \( agr \) dysfunctional isolates than in those with functional \( agr \).

**Discussions**

Recent evidence indicated that \( agr \) dysfunction was common among \( S. aureus \) clinical isolates, especially MRSA [11–14, 17, 18]. However, the dysfunction was unusual in pediatric populations in China according to the current study. Moreover, the data showed that infections caused by \( agr \)-defective strains were always HA. Similarly, the study by Shopsin et al. [35] showed that the carriage of an \( agr \)-defective strain was associated with hospitalization. Butterfield et al. [13] also indicated that \( agr \) dysfunction was closely associated with prior administration of \( \beta \)-lactam and fluoroquinolone. Therefore, the high antibiotic selection pressure in the medical environment might lead to the emergence of \( agr \) dysfunction, which might be due to the \( agr \)-controlled virulence that is energy-consuming and needs to be balanced with the expression of antibiotic resistance in a healthcare environment filled with antibiotics [36].

In the current study, MRSA strains showed strong homology, and ST59-SCCmec IV and ST239-SCCmec III were the most prevalent clones. This result was consistent with that of a previous study conducted by Qiao et al. in Chinese children [37]. These results showed that \( agr \) dysfunction was more common in ST239-SCCmec III than in ST59-SCCmec IV isolates, the phenomenon was consistent with a previous study, which indicated that SCCmec IV/IVa MRSA (3%, known as CA-MRSA...
clone) was associated with lower rates of agr dysfunction as compared to SCCmec I-III MRSA (43%, known as HA-MRSA clone) [38]. Thus, the low prevalence of agr dysfunction in this study might be attributed to more than half of the MRSA isolates belonging to ST59 harboring the SCCmec IV.

In addition, infections caused by PVL-producing ST22-t309-agr I clone should be under intensive research.

Table 2 Genotypic characteristics of MRSA isolates stratified by agr functionality

| Genotype | No. of isolates (%) | agr Dysfunction | Function | pvl (%) | N (%) |
|----------|---------------------|----------------|----------|---------|------|
| Total    | 211                 | 8 (3.79)       | 203 (96.21) | 75 (35.50) |
| Origins  |                     |                |           |         |
| Community-associated | 104 (49.29) | 0 | 104 (100.00) | 44 (42.30) |
| Hospital-associated | 107 (50.71) | 8 (7.47) | 99 (92.52) | 31 (28.97) |
| MLST     |                     |                |           |         |
| 1        | 8 (3.79)            | 0              | 8 (100.00) | 2 (25.00) |
| 22       | 7 (3.32)            | 0              | 7 (100.00) | 0 (0.00) |
| 59       | 128 (60.56)         | 2 (1.56)       | 126 (98.44) | 49 (38.28) |
| 88       | 8 (3.79)            | 0              | 8 (100.00) | 5 (62.50) |
| 239      | 27 (12.80)          | 4 (14.81)      | 23 (85.19) | 2 (7.41) |
| Others   | 33 (15.64)          | 2 (6.06)       | 31 (93.94) | 10 (30.30) |
| SCCmec   |                     |                |           |         |
| I        | 1 (0.47)            | 0              | 1 (100.00) | 0 (0.00) |
| II       | 26 (12.32)          | 4 (15.38)      | 22 (84.62) | 2 (7.69) |
| IV       | 145 (68.72)         | 3 (2.07)       | 142 (97.93) | 51 (35.17) |
| V        | 32 (15.17)          | 1 (3.13)       | 31 (96.87) | 19 (59.38) |
| NF*      | 7 (3.32)            | 0              | 7 (100.00) | 3 (42.86) |
| spa type |                     |                |           |         |
| t030     | 16 (7.58)           | 0              | 16 (100.00) | 0 (0.00) |
| t037     | 7 (3.32)            | 3 (42.86)      | 4 (57.14) | 1 (14.29) |
| t309     | 7 (3.32)            | 0              | 7 (100.00) | 0 (0.00) |
| t437     | 107 (50.71)         | 2 (1.87)       | 105 (98.13) | 47 (43.92) |
| t441     | 14 (6.64)           | 0              | 14 (100.00) | 7 (50.00) |
| Others   | 60 (28.43)          | 3 (5.00)       | 57 (95.00) | 13 (21.67) |
| agr type |                     |                |           |         |
| I        | 185 (87.68)         | 7 (3.78)       | 178 (96.22) | 67 (36.22) |
| II       | 6 (2.84)            | 1 (16.67)      | 5 (83.33) | 1 (16.67) |
| III      | 17 (8.06)           | 0              | 17 (100.00) | 7 (41.18) |
| IV       | 3 (1.42)            | 0              | 3 (100.00) | 0 (0.00) |
| MLST-SCCmec type |         |                |           |         |
| ST1-SCCmec IV | 6 (2.84)    | 0              | 6 (100.00) | 0 (0.00) |
| ST22-SCCmec V | 7 (3.32)     | 0              | 7 (100.00) | 0 (0.00) |
| ST59-SCCmec IV | 118 (55.92) | 2 (1.69)       | 116 (98.31) | 44 (37.29) |
| ST239-SCCmec III | 23 (10.90) | 4 (17.39)      | 19 (82.61) | 1 (4.35) |
| Others   | 57 (26.43)          | 2 (3.51)       | 55 (96.49) | 23 (40.35) |
| MLST-SCCmec-spa-agr type |         |                |           |         |
| ST59-SCCmec IV-t037-agr I | 92 (43.60) | 1 (1.09)       | 91 (98.91) | 36 (39.13) |
| ST59-SCCmec IV-t441-agr I | 12 (5.69)   | 0              | 12 (100.00) | 6 (50.00) |
| ST239-SCCmec III-t039-agr I | 12 (5.69)   | 0              | 12 (100.00) | 0 (0.00) |
| ST239-SCCmec III-t037-agr I | 7 (3.32)    | 3 (42.86)      | 4 (57.14) | 0 (0.00) |
| ST22-SCCmec V-t437-agr I | 7 (3.32)    | 0              | 7 (100.00) | 0 (0.00) |
| Others   | 81 (38.39)          | 4 (4.94)       | 77 (95.06) | 26 (32.10) |

*The other MLSTs were ST5 (2 isolates), ST6 (1 isolate), ST79 (1 isolate), ST130 (1 isolate), ST172 (2 isolates), ST179 (1 isolate), ST120 (1 isolate), ST121 (1 isolate), ST375 (1 isolate), ST509 (1 isolate), ST358 (1 isolate), ST630 (1 isolate), ST896 (1 isolate), ST950 (1 isolate), ST965 (2 isolates), ST1224 (1 isolate), ST1296 (1 isolate), ST1777 (1 isolate), ST1821 (1 isolate)

*Not determined

*The other spa types were t008 (1 isolate), t011 (1 isolate), t021 (1 isolate), t034 (2 isolates), t062 (2 isolates), t078 (2 isolates), t114 (6 isolates), t127 (3 isolates), t138 (1 isolate), t163 (1 isolate), t172 (4 isolate), t186 (1 isolate), t267 (1 isolate), t318 (1 isolate), t459 (2 isolate), t664 (1 isolate), t695 (2 isolates), t895 (1 isolate), t1977 (1 isolate), t2270 (1 isolate), t2310 (1 isolate), t2755 (2 isolate), t4001 (1 isolate), t515 (2 isolates), t5523 (1 isolate), t5590 (1 isolate), t4431 (1 isolate), t4549 (2 isolates), t617 (1 isolate), t7637 (1 isolate), t8660 (2 isolates), t8723 (1 isolate), t10555 (1 isolate), t12946 (1 isolate), t16365 (1 isolate)
The current literature demonstrated that ST22-MRSA isolates mainly carry SCCmec IV \[39, 40\]. However, in this study, ST22-t309-agrI-MRSA strains were classified as SCCmec V. Moreover, ST22-t309-agrI present in MSSA isolates indicated that ST22-t309-MRSA probably arose from ST22-t309-MSSA.

Furthermore, *S. aureus* can lead to chronic infections by forming the biofilm on the surface of medical implants \[41\]. Herein, a majority of the *S. aureus* isolates were strong biofilm formers, especially MRSA, which should be brought to the attention of Chinese pediatricians. In addition, *agr* dysfunction has been linked to increased biofilm formation and enhanced colonization ability previously \[11\]; however, this phenomenon was inconsistent with that in the current study and could be attributed to the small sample size of *agr* dysfunctional isolates.

Both PVL and biofilm are major virulence factors of *S. aureus*. Previous studies found that some secreted virulence factors are closely related to biofilm formation, such as phenol-soluble modulin α (PSMα) \[42\]. Thus, we tried to explore the correlation between PVL and

### Table 3

Genotypic characteristics of MSSA isolates stratified by *agr* functionality

| Genotype | No. of isolates (%) | No. (%) of isolates | pvl positive [No. (%)] |
|----------|---------------------|---------------------|------------------------|
|          |                     | dysfunctional *agr* | functional *agr*       |
| Total    | 58                  | 2 (3.45)            | 56 (96.55)             | 17 (29.31)             |
| Community-associated | 31 (53.45) | 1 (3.23)            | 30 (96.77)             | 12 (38.71)             |
| Hospital-associated  | 27 (46.55) | 1 (3.70)            | 26 (96.30)             | 5 (18.52)              |
| **MLST** |                     |                     |                        |                        |
| 5        | 5 (8.62)            | 0                   | 5 (100.00)             | 0                      |
| 7        | 5 (8.62)            | 0                   | 5 (100.00)             | 1 (20.00)              |
| 22       | 12 (20.69)          | 1 (8.33)            | 11 (91.67)             | 12 (100.00)            |
| 25       | 6 (10.34)           | 0                   | 6 (100.00)             | 1 (16.67)              |
| 398      | 10 (17.24)          | 1 (10.00)           | 9 (90.00)              | 1 (10.00)              |
| Others   | 20 (34.48)          | 0                   | 20 (100.00)            | 2 (10.00)              |
| **spa type** |                     |                     |                        |                        |
| t002     | 4 (6.89)            | 0                   | 4 (100.00)             | 0                      |
| t189     | 4 (6.89)            | 0                   | 4 (100.00)             | 0                      |
| t309     | 11 (18.97)          | 0                   | 11 (100.00)            | 11 (100.0)             |
| t571     | 6 (10.34)           | 1 (16.67)           | 5 (83.33)              | 0                      |
| t701     | 4 (6.90)            | 0                   | 4 (100.00)             | 0                      |
| Others   | 29 (50.00)          | 1 (3.45)            | 28 (96.55)             | 6 (20.69)              |
| **agr type** |                     |                     |                        |                        |
| I        | 46 (79.31)          | 2 (4.35)            | 44 (95.65)             | 16 (34.78)             |
| II       | 9 (15.52)           | 0                   | 9 (100.00)             | 0                      |
| III      | 1 (1.72)            | 0                   | 1 (100.00)             | 0                      |
| IV       | 1 (1.72)            | 0                   | 1 (100.00)             | 0                      |
| NT       | 1 (1.72)            | 0                   | 1 (100.00)             | 1 (100.00)             |
| **MLST-spa-agr type** |                 |                     |                        |                        |
| ST5-t002-agrII | 4 (6.90) | 0                   | 4 (100.00)             | 0                      |
| ST6-t189-agrI | 4 (6.90) | 0                   | 4 (100.00)             | 0                      |
| ST22-t309-agrI | 9 (15.52) | 0                   | 9 (100.00)             | 9 (100.00)             |
| ST188-t189-agrI | 4 (6.90) | 0                   | 4 (100.00)             | 0                      |
| ST398-t571-agrI | 6 (10.34) | 1 (16.67)           | 5 (83.33)              | 0                      |
| Others   | 31 (53.45)          | 1 (3.23)            | 30 (96.77)             | 8 (25.81)              |

*The other MLSTs were ST1 (1 isolate), ST6 (4 isolates), ST8 (1 isolate), ST15 (3 isolates), ST25 (6 isolates), ST59 (4 isolates), ST121 (1 isolate), ST188 (4 isolates), ST950 (1 isolate), ST1281 (1 isolate)

*The other spa types were t034 (3 isolates), t078 (2 isolates), t081 (1 isolate), t084 (3 isolates), t091 (2 isolates), t127 (1 isolate), t163 (1 isolate), t164 (1 isolate), t167 (1 isolate), t310 (1 isolate), t437 (2 isolates), t660 (1 isolate), t796 (2 isolates), t1062 (1 isolate), t1818 (1 isolate), t2092 (1 isolate), t4377 (1 isolate)

*Not determined
biofilm formation. However, no such association was found in both MRSA and MSSA isolates. A previous meta-analysis demonstrated that PVL strains are rare in colonizing isolates as compared to isolates causing skin and soft-tissue infections [43]. These results indicated that PVL was primarily associated with disease rather than biofilm formation and colonization.

Intriguingly, we found that agr dysfunctional isolates were more resistant than isolates with functional agr. However, at present, agr dysfunction is the result or cause of drug resistance remains unclear, while methicillin resistance might lead to agr dysfunction. Rudkin et al. [44] found that the expression of mecA (major

| agr functionality | No. of isolates | WBF | MBF | SBF |
|-------------------|----------------|-----|-----|-----|
| MRSA              | 211            | 1 (0.47) | 23 (10.90) | 187 (88.63) |
| Functional agr    | 203            | 1 (0.49) | 23 (11.33) | 179 (88.18) |
| Dysfunctional agr  | 8              | 0     | 0     | 8 (100.00) |
| MSSA              | 58             | 2 (3.45) | 23 (39.66) | 33 (56.90) |
| Functional agr    | 56             | 2 (3.57) | 22 (39.29) | 32 (57.14) |
| Dysfunctional agr  | 2              | 0     | 1 (50.00) | 1 (50.00) |
| Total             | 269            | 3 (1.11) | 48 (17.84) | 218 (81.04) |

WBF weak biofilm formation, MBF moderate biofilm formation, SBF strong biofilm formation

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resistance determinant of MRSA) could subtly affect the peptidoglycan structure or its interaction with other cell wall-associated proteins and prevented the detection of autoinducing peptide (AIP), thereby resulting in an unresponsive agr system and the subsequent low-level toxicity. When the type II SCCmec element or only mecA was deleted from an isolate with dysfunctional agr, the agr activity was restored. However, Tsuji et al. [45] suggested that agr dysfunction might directly influence the acquisition of intermediate resistance to vancomycin after subtherapeutic exposure.

Nonetheless, all S. aureus strains, including the 10 agr dysfunctional isolates, were sensitive to vancomycin. Kim et al. [46] showed that 2/12 initial agr-functional isolates acquired agr dysfunction during vancomycin therapy for persistent bacteremia, but were still sensitive to vancomycin. In addition, all the 4 strains developed from vancomycin-susceptible S. aureus (VSSA) to heterogeneous VISA (hVISA) were initially agr dysfunctional strains. Therefore, we speculated that agr dysfunctional isolates can more easily adapt to glycopeptide selection pressure than agr functional isolates. Taken together, further studies assessing the correlation between agr dysfunction and antibiotic resistance, especially vancomycin resistance, are imperative.

The agr quorum sensing system has become a new target for developing new antibiotics. Hitherto, many natural and synthetic compounds have been found to interfere with the functions of agr [47]. However, the prevalence of agr dysfunction among S. aureus, and the potential correlation between agr dysfunction and antibiotic resistance indicated that isolates could withstand

### Table 5 Non-susceptibility rates of S. aureus in pediatric population in China [N (%)]

|     | PEN | OXA | ERY | CLI | TET | GEN | CHL | RIF | CIP | SXT | MDR |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| MRSA |     |     |     |     |     |     |     |     |     |     |     |
| No. of isolates | 211 | 207 | 176 | 208 | 181 | 103 | 38  | 121 | 22  | 65  | 2  |
| aP-value | 0.0688 | <0.0001 | 1.0000 | <0.0001 | <0.0001 | 0.7033 | 1.0000 | 0.0057 | 0.0990 | 0.5883 | 0.3648 |
| MDR | 2 | 1.0000 | <0.0001 | 1.0000 | <0.0001 | <0.0001 | 0.7033 | 1.0000 | 0.0057 | 0.0990 | 0.5883 |

All isolates were susceptible to vancomycin and linezolid, and hence, not listed in the table.

Comparison between MRSA and MSSA isolates

Comparison between agr dysfunctional and agr functional isolates among MRSA

*P*-value 0.0688 < 0.0001 1.0000 < 0.0001 < 0.0001 0.7033 1.0000 0.0057 0.0990 0.5883 0.3648

### Table 6 agr mutations for non-hemolytic isolates

| Strain | MLST | agr mutations | Predicted results |
|--------|------|---------------|------------------|
| R85⁵   | 59   | agrC 62–63 ins t | Frameshift-truncated AgrC |
| R90⁵   | 59   | agrA g443a     | Asp>Gly at aa 148 in AgrA |
| 11-3⁴  | 239  | agrD a48g; agrC t884c | No aa change in agrD; Ile > Thr at aa 295 in AgrC |
| 11-6⁴  | 239  | agrC t884c     | None |
| 11-15⁵ | 9    | agrB g519a; Many mutations in agrC and agrA | No aa change in AgrB; Many aa changes in AgrC and AgrA |
| 12-31⁵ | 239  | None          | None |
| 12-98⁶ | 1296 | agrD a7g; agrC a704c + a875g | Thr > Ala at aa 3 in AgrD; Tyr > Ser at aa 235 + Asn > Ser at aa 292 in AgrC |
| SA2017112⁶ | 239 | agrE t651c | No aa change in AgrA |
| S14⁴  | 308  | agrC c415t; agrA c263t | Pro>Ser at aa 139 in AgrC; Thr > Met at aa 88 in AgrA |
| S78⁴  | 22   | agrC 434–435 ins t | Frameshift-truncated AgrC |

*Compared to SA957 genome (NC_022442.1); *Compared to NCTC8325 genome (CP000253.1); *Compared to N315 genome (NC_002745.2); *Compared to ST398 genome (NC_017333.1); *Compared to H-EMRSA-15 genome (CP007659.1); ins, insertion; del, deletion; aa, amino acid; --, not applicable.
drug interference with agr functionality emerge rapidly. Strikingly, the promotion effects of agr dysfunction on biofilm formation may pose a great threat for patients using indwelling devices. Thus, additional studies are needed to explore the feasibility of agr system as a new target for antimicrobial agents.

Herein, we found that the agr sequence was not only associated with the agr group but also to the MLST types. Thus, the agr group and MLST types were considered while detecting the occurrence of mutation in the current agr dysfunctional isolates. Furthermore, agr dysfunctional isolates detected mutations that mainly occurred in agrC and agrA, leading to the inactivation of the auto-activation circuit and RNAIII expression [48]. However, no mutation or synonymous mutation or no mutations were also detected in our study, which suggested the involvement of additional mechanisms in agr dysfunction. Interestingly, mecA expression [49, 50] and abnormal expression of regulators of agr (sarA) can also be the cause of agr dysfunction [11].

Nevertheless, the current study had limitations. First, the sample size was relatively small. Second, this was a single-center study, and all the isolates were collected from one hospital. However, the hospital serves the whole of China, and > 60% of the hospitalized children come from all over the country. Therefore, the present study is still representative in China. Third, some MRSA strains used in this study have been sub-cultured 2 or 3 times since first collected, and multiple subcultures and long-term cryopreservation might have affected the characteristics of the strain.

Conclusions
In summary, agr dysfunction was not common in pediatric populations in China, and ST59-SCCmec IV was associated with a low prevalence of agr dysfunction as compared to the ST239-SCCmec III isolates. Agr dysfunctional isolates were always healthcare-associated and multidrug resistant. Except for the gene mutations, other mechanisms might also be involved in agr dysfunction. The agr dysfunction might be a major adaption mechanism of S. aureus to antibiotic selection pressure. Thus, an in-depth understanding of agr dysfunction is an urgent requirement for the development of new antibiotics that target agr expression.

Abbreviations
agr, accessory gene regulator; AI: Intra-abdominal infection; BII: Bone and joint infection; BSI: Bloodstream infection; CA: Community-associated; CHL: Chloramphenicol; CIP: Ciprofloxacin; CLI: Clindamycin; CLSI: Clinical and Laboratory Standards Institute; CNSI: Central nervous system infection; ERY: Erythromycin; GEN: Gentamicin; HA: Healthcare-associated; hVISA: heterogeneous VISA; IE: Infective endocarditis; LA-MRSA: Livestock-associated MRSA; LNZ: Linezolid; MDR: Multidrug resistance; MLST: Multilocus sequence typing; MRSA: Methicillin-resistant S. aureus; MRSA: Methicillin-resistant S. aureus; MSCRAMMs: Microbial surface components recognizing adhesive matrix molecules; OD: Optical density; ODc: Cut-off OD value; OXA: Oxacillin; PCR: Polymerase chain reaction; PEN: Penicillin G; pvL: pantost-Valentine leukocidin; RF: Rifampicin; S. aureus: Staphylococcus aureus; SCCmec: Staphylococcal cassette chromosome mec; SP: Severe pneumonia; spa: staphylococcal protein A; SSSI: Skin and soft tissue infection; SXT: Sulphamethoxazole-trimethoprim; TCP: Tissue culture plate method; TET: Tetracycline; TSB: Tryptic soy broth; VAN: Vancomycin; VISA: Vancomycin intermediate S. aureus; VSSA: Vancomycin-susceptible S. aureus.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors’ contributions
XY detected the biofilm formation ability, performed MLST, SCCmec and spa typing, analyzed the data, and drafted the manuscript. FD analyzed the data and drafted the article. SQ designed the study and revised the article. KY statistically analyzed the data and revised the article. LW and YL performed MLST and spa typing. WS analyzed the data and drafted the article. JZ, WZ, HX, and HZ collected and identified the clinical strains of S. aureus and analyzed the data. All authors had read and approved the final manuscript.

Ethics approval and consent to participate
This study mainly used the bacterial isolates from the biological specimens obtained during the clinical diagnosis and management of patients without threat to the subjects’ rights and health. The applications for the exemption of written informed content and ethical review were approved by the Ethics Committee of Beijing Children’s Hospital Affiliated to Capital Medical University according to national regulations (No. 2016–93, 23/06/2016). Thus, only verbal consent was obtained from the patient’s legal guardian. This study also obtained clearance from the Institutional Biosafety Committee (IBSC) (2017 No.43).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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