

# The Correlation Between Biofilm-Forming Ability of Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Isolated from the Respiratory Tract and Clinical Characteristics in Children

Shumin Huang<sup>1,2</sup>, Jing He<sup>1,2</sup>, Yiting Zhang<sup>1,2</sup>, Lin Su<sup>1,2</sup>, Lin Tong<sup>1,2</sup>, Ying Sun<sup>1,2</sup>, Mingming Zhou<sup>2,3</sup>, Zhimin Chen<sup>1,2</sup>

<sup>1</sup>Department of Pulmonology, Children's Hospital, Zhejiang University School of Medicine, Hangzhou, 310052, People's Republic of China; <sup>2</sup>National Clinical Research Center for Child Health, National Children's Regional Medical Center, Hangzhou, 310052, People's Republic of China; <sup>3</sup>Department of Clinical Laboratory, Children's Hospital, Zhejiang University School of Medicine, Hangzhou, 310052, People's Republic of China

Correspondence: Zhimin Chen, Email zmchen@zju.edu.cn

**Objective:** This study aimed to investigate the biofilm-forming ability, molecular typing, and antimicrobial resistance of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains isolated from the respiratory tract of children and their correlation with clinical characteristics.

**Methods:** All CA-MRSA strains were isolated from hospitalized children, and their presentation, molecular typing, antimicrobial susceptibility, and biofilm formation were investigated. The clinical characteristics were compared between the strong and weak biofilm producer groups.

**Results:** Fifty-three CA-MRSA strains were isolated from the respiratory samples of 53 children, with nearly half of them being young infants (0–12 months). Approximately, 88.7% (47/53) of the isolates were resistant to four or more antibiotics, mainly  $\beta$ -lactam antibiotics, lincosamides, and macrolides. Twelve sequence types (STs) and 20 subtypes of staphylococcal protein A (spa) typing were identified, with ST59-t437 (39.6%, 21/53) as the predominant subtype. All strains showed the ability to form biofilms. When compared to children with weak biofilm-forming CA-MRSA strains, those with strong biofilm-forming strains had higher proportions of lower respiratory tract infections (LRTI) (88.5% vs 59.3%), obvious cough symptoms (84.6% vs 51.9%), and severe chest imaging manifestations (76.9% vs 37.0%). Furthermore, a strong biofilm-forming ability significantly increased the risk of prolonged cough in children with LRTI (44.4% vs 14.3%), and a positive correlation between the duration of cough and the extent of biofilm formation was observed. Medical history investigation revealed that the strong biofilm-forming group had a much higher percentage of macrolides intake than the weak biofilm-forming group in the last month before admission (61.5% vs 14.8%).

**Conclusion:** ST59-t437 was the most prevalent clone in CA-MRSA respiratory isolates among the hospitalized children. All CA-MRSA strains formed biofilms. The stronger the biofilm-forming ability, the more serious and prolonged were the respiratory symptoms.

**Keywords:** CA-MRSA, biofilm, respiratory infection, child, genotype

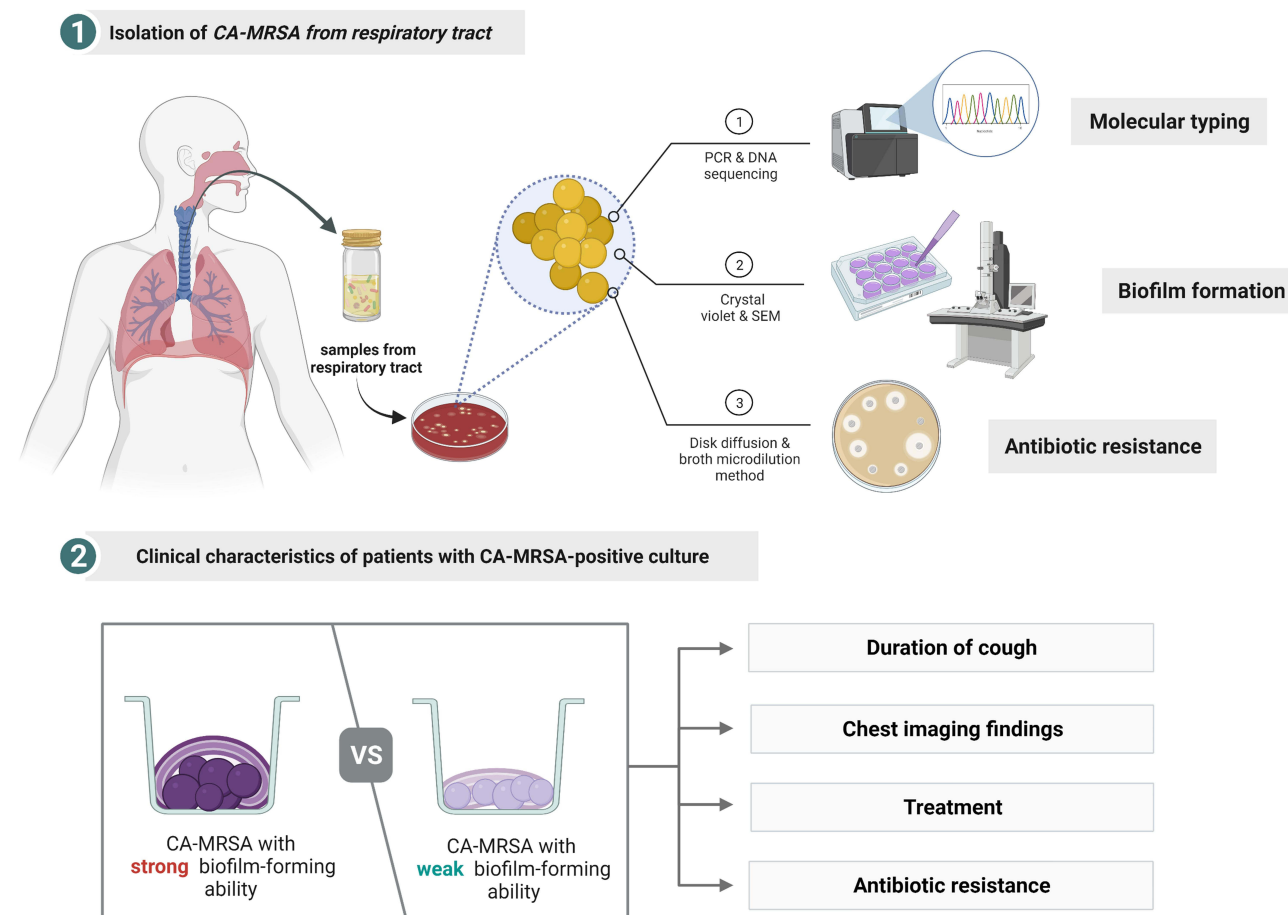
## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen and the most common multidrug-resistant bacterium that causes nosocomial infections, with high morbidity and mortality. MRSA is closely associated with health care settings.<sup>1</sup> However, since the 1990s, MRSA infections have increased among people without hospital-related risk factors worldwide.<sup>2,3</sup> Furthermore, there have been notable epidemics, such as the USA300, which spread internationally and caused many deaths.<sup>4,5</sup> All of these are associated with the emergence of new MRSA clones known as community-acquired MRSA (CA-MRSA). Compared to hospital-acquired MRSA (HA-MRSA), CA-MRSA is more clonally diverse and highly virulent

because it produces Pantone-Valentine leukocidin (PVL) exotoxin and is more likely to cause severe infections, particularly among young, healthy individuals.<sup>6–9</sup> Additionally, more CA-MRSA strains contain the staphylococcal cassette chromosome *mec* (SCC*mec*) element type IV or V, conferring resistance to  $\beta$ -lactam antibiotics.<sup>8,9</sup> It can cause a wide spectrum of clinical diseases, from the skin and soft tissue infections (SSTIs) to severe invasive infections, accounting for a large proportion of the increased disease burden observed in the last decade, particularly among children.<sup>6,7,9–11</sup>

Biofilms are bacterial aggregates embedded in a matrix composed of extracellular polysaccharides, fibrin, and other substances produced by bacteria. They are highly resistant to antibiotics and host immunity, making biofilm-associated infections a major concern in clinical practice. A biofilm can be formed not only on the surface of medical implants but also on mucous membranes. The formation of bacterial biofilms on the respiratory tract has been linked to recurrent respiratory diseases and asymptomatic colonization.<sup>12,13</sup> According to data reported in published studies, approximately 2–10% of healthy children have MRSA nasal colonization, and the figures are still on the rise.<sup>14–17</sup> Children may be an important reservoir for the community transmission of CA-MRSA.<sup>6,18</sup> However, the role of biofilms in respiratory diseases has not yet been fully explored.

In this study, we examined the molecular typing, antimicrobial resistance, and biofilm-forming ability of CA-MRSA strains isolated from the respiratory tract of children. We mainly investigated the relationship between the biofilm-forming ability and the clinical characteristics of respiratory diseases, which might provide new insights for treating patients with CA-MRSA infections. A simplified description of the study protocol is shown in Figure 1.



**Figure 1** Flow chart of the simplified process. Created with BioRender.com.

**Abbreviations:** CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; PCR, polymerase chain reaction; SEM, scanning electron microscopy.

## Materials and Methods

### Collection of Isolated Strains and Clinical Data

This retrospective study was conducted at a tertiary-care academic pediatric center. In this study, 53 non-duplicate CA-MRSA isolates were collected from inpatients at Children's Hospital, Zhejiang University School of Medicine, from April 2015 to July 2020. All strains were isolated from respiratory specimens, including sputum, bronchoalveolar lavage fluid (BALF), pharyngeal swabs, and pleural fluids. MRSA identification is mediated by the *mecA* gene detection and the cefoxitin disk diffusion methods, which will be discussed later. In addition, we defined CA-MRSA according to the United States Centers for Disease Control and Prevention criteria: (1) positive culture for MRSA within 48 h after admission; (2) no medical devices or indwelling catheters permanently placed through the skin; (3) no history of MRSA infections; and (4) no history of hospitalization or residence in nursing homes or long-term care facilities in the previous year.<sup>19</sup> Each strain was obtained independently and maintained as a stock culture at  $-80^{\circ}\text{C}$  in Luria broth (LB) supplemented with 20% glycerol until further examination.

Clinical data, including age, sex, length of hospital stay, clinical characteristics, laboratory examinations, chest imaging findings, treatment, and prognosis, were collected from patients' medical records. To investigate the relationship between the biofilm-forming ability and medication history, the information on each child's antibiotic medication in the month before admission was collected. This study was approved by the Ethics Committee of the Children's Hospital, Zhejiang University School of Medicine (approval number: EC2022-IRB-005).

### Identification and Antimicrobial Susceptibility Testing

Species confirmation was using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics GmbH, Germany). MRSA was identified using the *mecA* gene detection and disk diffusion method with a 30  $\mu\text{g}$  cefoxitin disk according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Standard *S. aureus* ATCC 25923 and ATCC 43300 strains (supplied by the Microbiology Laboratory of Children's Hospital, Zhejiang University School of Medicine) were used as the quality controls.

The antimicrobial susceptibility of 14 different antimicrobial agents was determined among all the collected CA-MRSA isolates using the commercialized microdilution method (VITEK COMPACT, BioMérieux, Marcy-l'Étoile, France), and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines M100-Ed30. The antibiotics tested were penicillin, ceftaroline fosamil, oxacillin, sulfamethoxazole/trimethoprim, gentamicin, rifampicin, levofloxacin, moxifloxacin, clindamycin, daptomycin, erythromycin, linezolid, vancomycin, teicoplanin, and tigecycline. *Staphylococcus aureus* ATCC 29213 was used as the quality control.

### DNA Extraction

The DNA was extracted by using a DNA extraction kit (Vazyme Biotech Co. Ltd., Nanjing, China) with lysozyme (20 mg/mL) according to the manufacturer's instructions, and used as the template for all the polymerase chain reaction (PCR) products.

### Molecular Typing Methods and Detection of PVL and *mecA* Genes

#### Spa Typing

Spa typing is mainly based on polymorphism of the X-region repeats of staphylococcal protein A (spa). After amplifying the X-region by PCR with primers (5'-AGACGATCCTTCGGTGAGC-3'; 5'-GCTTTTGCAATGTCATTTACTG-3'),<sup>20</sup> and subsequent sequencing, the sequences were submitted to spaTyper (<http://spatyper.fortinbras.us/>) for spa typing.

#### Multilocus Sequence Typing (MLST)

MLST was performed by sequencing the PCR products of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmK*, *pta*, *tpi*, and *yqi*). The sequence types (STs) and clonal complexes (CCs) of each strain were determined by comparison with known alleles from the MLST database (<https://pubmlst.org/organisms/staphylococcus-aureus>), which also provides the primers and conditions for PCR. The software PHYLOViZ 2.0 (<http://www.phyloviz.net>) was used to construct the dendrogram of STs and to assess the evolutionary relationship between the isolates.

## Detection of PVL and *mecA* Genes

The PVL genes (*lukS/F-PV*) and *mecA* gene were detected by PCR amplification using primers (5'-GGCCTTTCCAA TACAATATTGG-3'; 5'-CCCAATCAACTTCATAAATTG-3') and (5'-GTGAAGATATACCAAGTGATT-3'; 5'- ATG CGCTATAGATTGAAAGGAT-3') respectively, as previously described.<sup>21</sup> The reference strains ATCC-43300-*mecA* +/PVL-(MRSA), ATCC-29213-*mecA*-/PVL-(MSSA) and ATCC-25923-*mecA*-/PVL+(MSSA) were used as positive or negative controls.

## Biofilm Formation

CA-MRSA strains were inoculated onto Columbia blood agar (Bioivd, Zhengzhou, China) and incubated for 18–24 h at 35 °C in a 5% CO<sub>2</sub>-enriched atmosphere. A single clone was picked from each blood agar sample and cultured in tryptic soy broth (TSB; Oxoid, Basingstoke, UK) with shaking (200 rpm) for 16 h at 35 °C to reach the logarithmic growth phase. The cultures were inoculated into a sterile 0.9% NaCl solution to match a 0.5 McFarland turbidity of 100-fold dilution using TSB. Subsequently, 200 µL of this suspension was inoculated into triplicate in a 96-well polystyrene plate (Corning Costar, Acton, MA, USA) at 35 °C for 24 h. The blank group was treated with 200 µL of TSB. Biofilm formation was quantified by crystal violet staining as previously described.<sup>22</sup> After incubation, the supernatant was removed, and each well of the microtiter plate was washed twice with phosphate buffer (0.1 M, pH 7.0), fixed with 100% methanol for 20 min, stained with crystal violet staining solution (Beyotime, Shanghai, China) for 15 min, and washed again. To solubilize the crystal violet, 150 µL of 33% acetic acid in water was added to each well.

The optical density (OD) of each well was measured at 590 nm using an automated ELx800 universal microplate reader (Bio-Tek, USA). According to previous studies,<sup>23,24</sup> the results were categorized based on the calculated cut-off optical density (OD<sub>C</sub>) value. The OD<sub>C</sub> value was defined as the mean OD of the blank group plus three times the standard deviation (SD),<sup>25</sup> and the results of the biofilm formation assay were analyzed as follows: non-biofilm producers (OD ≤ OD<sub>C</sub>), weak producers (OD<sub>C</sub> < OD ≤ 4OD<sub>C</sub>), and strong producers (OD > 4OD<sub>C</sub>).

## Scanning Electron Microscopy (SEM)

After biofilm formation on a sterile slide after 24 h of culturing in TSB, the sample was fixed with 2.5% glutaraldehyde overnight, washed three times in phosphate buffer (0.1 M, pH 7.0) for 15 min prior to postfixing with 1% OsO<sub>4</sub> for approximately 1–2 h, and then washed again. Subsequently, the sample was dehydrated using gradient ethanol, dried in a Hitachi Model HCP-2 critical point dryer, coated with gold-palladium, and observed using a Hitachi Model SU-8010 SEM at 30 kV.

## Statistical Analysis

The results for continuous variables were presented as the means (SD) or median (IQR), and proportions were expressed as percentages. The chi-squared test, Fisher's exact test and Mann–Whitney *U*-test were used for between-group comparisons. Statistical analyses were performed using the SPSS, version 19.0 (Chicago, IL, USA). A *P* value of ≤ 0.05 was considered statistically significant.

## Results

### General Information and Clinical Characteristics

Fifty-three CA-MRSA strains were isolated from the respiratory samples of 53 children aging from 0.3–144 months (median, 11.1). However, approximately half of these children (27/53, 50.9%) were young infants (0–12 months). The male to female ratio was 1.30. Of the 53 children, 92.4% (49/53) were previously healthy, three (5.7%) had a history of pneumonia, one (1.9%) had congenital heart disease, three (5.7%) had a history of premature delivery. The clinical features and demographic data are presented in Table 1.

Regarding sample sources, 49.1% (26/53) of the CA-MRSA strains were collected from sputum samples. Of the enrolled pediatric patients, 41.5% (22/53) were from the respiratory department, followed by the neonatal department (20.8%), and the remaining were scattered in the various clinical departments in the hospital. The most common

**Table 1** General Information and Clinical Characteristics of Enrolled Children

Characteristics	CA-MRSA Isolates (n=53)
Age (month), median (range)	11.1 (0.3–144)
Gender (female vs male), n (%)	23 vs 30 (43.4% vs 56.6%)
Preterm birth, n (%)	3 (5.7%)
Medical history in the last year, n (%)	
Pneumonia	3 (5.7%)
Congenital heart disease	1 (1.9%)
None	49 (92.4%)
Clinical sources, n (%)	
Sputum	26 (49.1%)
Bronchoalveolar Lavage Fluid	8 (15.0%)
Pharyngeal swab	17 (32.1%)
Pleural effusion	2 (3.8%)
Department, n (%)	
Respiratory department	22 (41.5%)
Neonatal department	11 (20.8%)
Neurosurgery	7 (13.2%)
PICU	4 (7.5%)
Other Clinical Departments	9 (17.0%)
Categories of clinical phenotype, n (%)	
LRTI	39 (73.6%)
Tonsillitis	2 (3.8%)
Lymphadenitis	1 (1.9%)
Sepsis	3 (5.7%)
Asymptomatic colonization	8 (15.0%)
Outcomes, n (%)	
Recovery	50 (94.3%)
Death or failure to treatment	3 (5.7%)

**Abbreviations:** CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; PICU, pediatric intensive care unit; LRTI, lower respiratory tract infection.

diagnosis was lower respiratory tract infection (LRTI; 39/53, 73.6%), including 36 cases of pneumonia and 3 cases of bronchitis. Moreover, eight children (15.0%) were classified as asymptomatic colonization because they had no obvious respiratory symptoms and the isolates were recovered from pharyngeal swabs on the day before they received planned elective surgical procedures.

## Antibiotic Resistance

Antimicrobial susceptibility results are presented in Table 2. All CA-MRSA strains were resistant to penicillin and oxacillin. The rates of resistance were considerably high to erythromycin (47/53, 88.7%) and clindamycin (46/53, 86.8%) but were less than 10% to the other antibiotics. In addition, all strains were sensitive to linezolid, vancomycin, tigecycline, teicoplanin, rifampicin, and daptomycin. Overall, 88.7% (47/53) of the isolates were resistant to four or more antibiotics, mainly  $\beta$ -lactam antibiotics, lincosamides, and macrolides.

## Molecular Characteristics

Among the CA-MRSA isolates, 12 STs and 20 subtypes of *spa* were identified. In MLST, ST59 (60.4%, 32/53) was the most prevalent subtype, followed by ST22 (9.4%, 5/53), and ST398 (7.5%, 4/53). The *spa* typing analysis showed that t437 was the most common subtype (39.6%, 21/53), followed by t3592 (7.5%, 4/53), and the remaining subtypes occupied minor proportions. By integrating the results of MLST and *spa* typing, the ST59-t437 clone (39.6%, 21/53) was

**Table 2** The Antimicrobial Resistance Profiling of 53 CA-MRSA Isolates

Antimicrobials	CA-MRSA (n=53)	
	No.*	%
Ceftaroline Fosamil	53	100.0
Oxacillin	53	100.0
Penicillin	53	100.0
Erythromycin	47	88.7
Clindamycin	46	86.8
Moxifloxacin	5	9.4
Sulfamethoxazole/trimethoprim	2	3.8
Levofloxacin	2	3.8
Gentamicin	1	1.9
Linezolid	0	0
Daptomycin	0	0
Rifampicin	0	0
Tigecycline	0	0
Teicoplanin	0	0
Vancomycin	0	0

**Note:** No.\* indicates the number of isolates that were resistant to the antimicrobials.

found to be predominant among the CA-MRSA isolates. According to gene detection, the *mecA* gene was present in all CA-MRSA strains in this study, while the PVL genes were found in only 35.8% (19/53) of the strains. Furthermore, no significant difference in the presence of the PVL genes was found between the dominant clone group and other clonal typing groups. The genotypic characteristics of the CA-MRSA isolates are shown in [Figure 2](#).

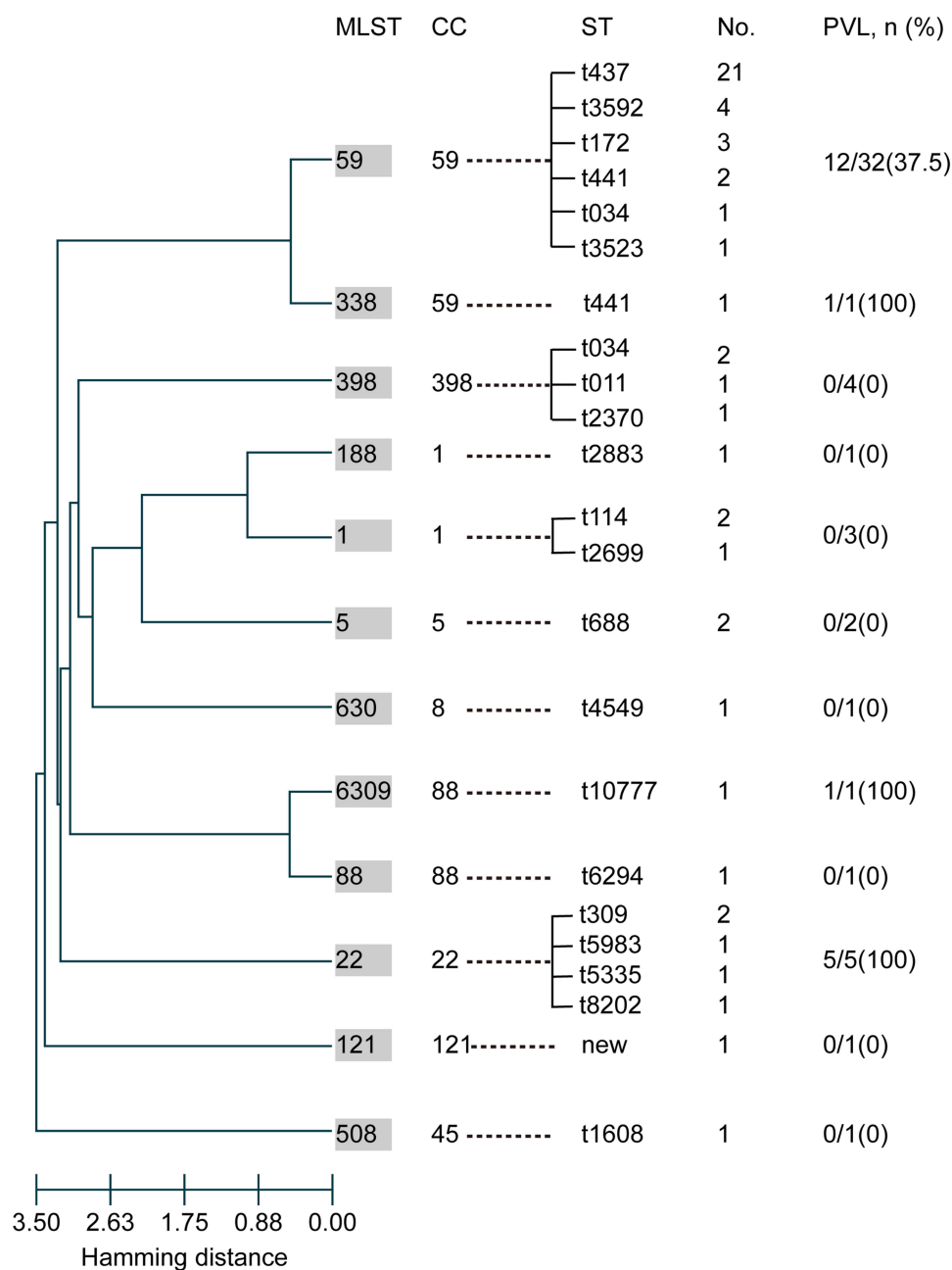
## Biofilm Assay and Its Effect on the Clinical Picture

All CA-MRSA strains were capable of forming biofilms. The ability of the CA-MRSA strains to form biofilms and representative SEM images are shown in [Figure 3](#). According to the standard described in the Methods section, 50.9% (27/53) of the isolated CA-MRSA strains belonged to the weak producer group and 49.1% (26/53) to the strong producer group. Based on this classification, the correlation between the biofilm-forming ability and the clinical characteristics of these patients was analyzed ([Table 3](#)). Compared with the weak biofilm-forming group, the patients in the strong biofilm-producing group experienced a higher proportion of lower respiratory tract infections (88.5% vs 59.3%,  $P=0.026$ ), more obvious cough symptoms (84.6% vs 51.9%,  $P=0.006$ ), and more severe chest imaging (76.9% vs 37.0%,  $P=0.002$ ). However, no significant differences in demographics, hospital stay duration, other symptoms, laboratory results, drug resistance rates, or positive rate of the PVL genes were found between the two groups.

To further explore the adverse impact of biofilm formation in children with LRTI ( $n=39$ ), a subgroup analysis was conducted. Intriguingly, as shown in [Figure 4A](#), biofilm-forming significantly increased the risk of prolonged cough (44.4% vs 14.3%,  $P=0.037$ ). Meanwhile, the line chart revealed a positive correlation between the duration of cough and the extent of biofilm formation ( $P=0.0496$ , [Figure 4B](#)). Furthermore, the strong biofilm-forming group had a significantly higher proportion of antibiotic changes owing to inefficient antimicrobial treatment than the weak producer group (50% vs 19%,  $P=0.041$ ) ([Figure 4C](#)). However, no significant differences were found between the two groups in terms of oxygen demand or systemic glucocorticoid administration.

Additionally, in order to explore the factors underlying different biofilm-forming abilities of the two groups of CA-MRSA isolates, we investigated the relationship between their biofilm-forming ability and previous medication history. The strong biofilm-forming group had a much higher percentage of macrolides intake history in the last month before admission than the weak biofilm-forming group (61.5% vs 14.8%,  $P=0.001$ , [Supplementary Table 1](#)).



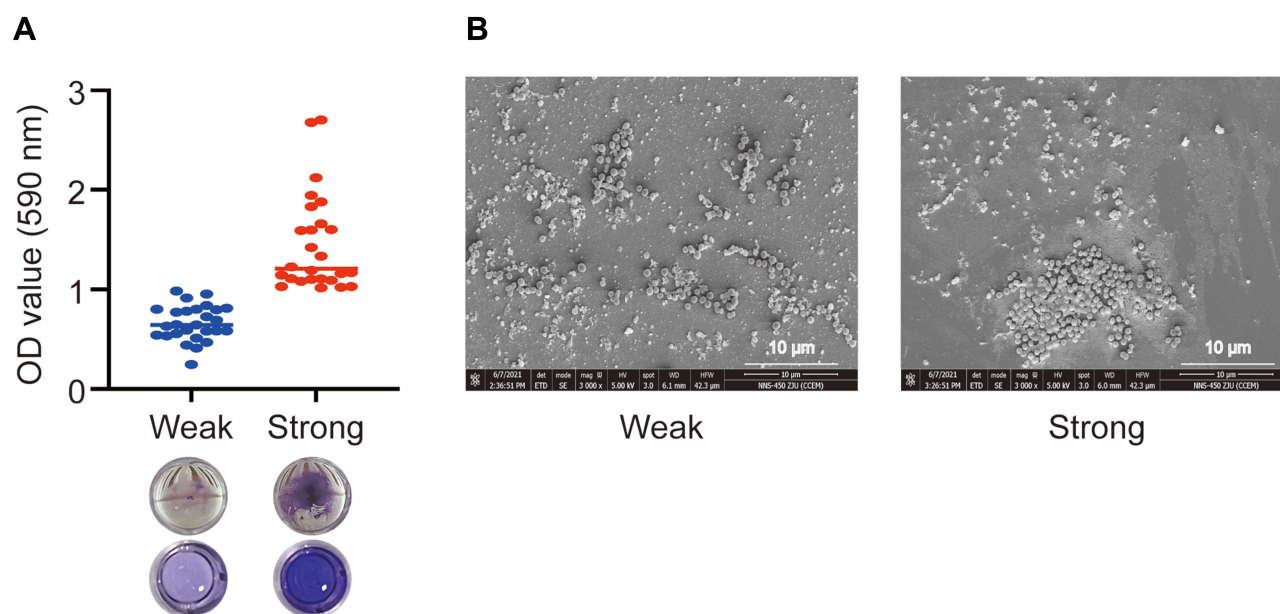


**Figure 2** Molecular characteristics of 53 CA-MRSA isolates. Evolution patterns of the 53 CA-MRSA isolates (calculated by goeBURST hierarchical clustering analysis) and the carriage of PVL genes among the different MLST groups.

**Abbreviations:** MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type; PVL, Pantone-Valentine leukocidin.

## Discussion

CA-MRSA infection remains a challenging issue worldwide. There has been an increase in the number of CA-MRSA infections among children.<sup>7,26</sup> CA-MRSA is often associated with severe invasive infections characterized by acute exacerbation and high mortality.<sup>6,27</sup> In addition, CA-MRSA can cause asymptomatic colonization of the nasopharynx in children, contributing to community transmission and recurrent infections. As a susceptible group, children play an important role in the transmission of CA-MRSA. Therefore, it is meaningful to characterize CA-MRSA respiratory strains from pediatric patients in terms of presentation, molecular characteristics, antibiotic resistance, and biofilm profiles.



**Figure 3** Biofilm formation by 53 CA-MRSA isolates. **(A)** Representative images and quantification of biofilm formation of the two CA-MRSA strain groups by crystal violet staining. **(B)** Representative scanning electron microscopy images of CA-MRSA biofilms in both groups.

In the present study, 53 CA-MRSA strains were isolated from the respiratory samples of 53 children, of which half were young infants (0–12 months) (27/53, 50.9%), including 11 neonates. Although our data did not directly reflect the incidence rate of CA-MRSA respiratory infection in this age group, it did indicate that the neonatal group was at a greater risk for CA-MRSA infection. Neonates are classified as a putative high-risk group in the USA, as reported in a previous study.<sup>6</sup> Furthermore, several countries have reported outbreaks of CA-MRSA in the neonatal group, and some evidence has revealed that multiple sources of transmission may be involved, such as family members<sup>28–30</sup> and health care workers who have asymptomatic MRSA

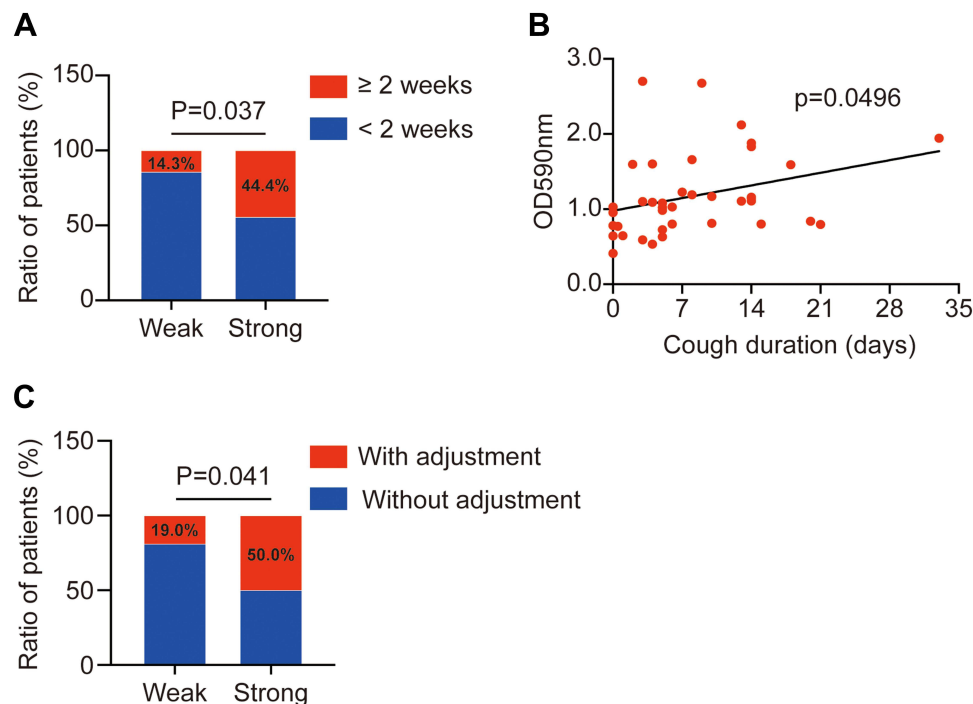
**Table 3** Clinical Characteristics Between CA-MRSA Groups with Different Biofilm-Forming Capacity

Characteristic	CA-MRSA Isolates (n=53)		P value
	Weak Producer (n=27)	Strong Producer (n=26)	
No. of patients (male/female)	15/12	15/11	0.548
Age (month), median (IQR)	23.0 (71.0)	5.1 (61.8)	0.722
Hospital stay length (days), median (IQR)	5.5 (7.0)	7.0 (5.0)	0.180
% Patients with LRTI	59.3 (16/27)	88.5 (23/26)	0.026
% Cough	51.9 (14/27)	84.6 (22/26)	0.006
% Fever	37.0 (10/27)	46.2 (12/26)	0.347
% Shortness of breath	18.5 (5/27)	26.9 (7/26)	0.344
WBC on admission, median (IQR)	8.4 (5.0)	8.7 (4.5)	0.943
CRP on admission, median (IQR)	1.2 (4.5)	2.0 (11.5)	0.202
% Patients with increased PCT	33.3 (9/27)	34.6 (9/26)	0.576
% Patients with increased IL-6	25.9 (7/27)	26.9 (7/26)	0.590
% Patients with increased IL-10	14.8 (4/27)	23.1 (6/26)	0.339
% Patients with Chest Imaging Findings <sup>a</sup>	37.0 (10/27)	76.9 (20/26)	0.002
% Antibiotic resistance <sup>b</sup> , n (%)	81.5 (22/27)	96.2 (25/26)	0.104
% Isolates with PVL	37.0 (10/27)	34.6 (9/26)	0.541

**Notes:** <sup>a</sup>Chest imaging findings include definite pneumonia, atelectasis, pleural effusion, and lung necrosis; <sup>b</sup>antibiotic resistance means that related isolates were resistant to more than 4 classes of antibiotics.

**Abbreviations:** No, number; LRTI, lower respiratory tract infection; WBC, white blood cell; CRP, C-reactive protein; IQR, interquartile range; PCT, procalcitonin; PVL, Panton-Valentine leukocidin.





**Figure 4** The impacts of CA-MRSA biofilm-forming in children with LRTI. **(A)** The higher ratio of patients with a duration of cough over two weeks in strong biofilm-forming group ( $P=0.037$ ). **(B)** The line graph indicates that CA-MRSA biofilm formation increased the duration of cough in pediatric patients with LRTI ( $P=0.0496$ ). **(C)** Higher proportion of patients needing to adjust antibiotics in the strong biofilm-forming group.

colonization or current infection,<sup>31,32</sup> as well as vertical transmission,<sup>33</sup> breast milk,<sup>34</sup> and household contacts.<sup>30,35</sup> Environmental cleaning, hand hygiene, contact precautions, and active screening for colonization are fundamental for the prevention of CA-MRSA infection.<sup>26,36</sup>

ST59 was the most predominant clone among the CA-MRSA isolates in our study as well as in China.<sup>37,38</sup> Owing to the clonal diversity of CA-MRSA strains and varied antimicrobial resistance patterns, understanding the antibiotic resistance trends of epidemic clones in different regions could be helpful for clinical treatment. In the current study, 88.7% (47/53) of the isolates were resistant to four or more antibiotics, mainly  $\beta$ -lactam antibiotics, lincosamides, and macrolides, similar to the antimicrobial profiles of a nationwide study conducted in China.<sup>37</sup> Clindamycin was once effective for treating CA-MRSA infections in the USA and Europe, where the most prevalent clones are USA300 and ST80; however, drug resistance has been increasing, not only to clindamycin, but also to other non- $\beta$ -lactams, such as levofloxacin and mupirocin, because of the carriage of the *mupA* gene and widespread antibiotics use.<sup>39–42</sup> Linezolid, vancomycin, teicoplanin, and daptomycin remain effective against MRSA infections. Unfortunately, some strains with reduced susceptibility to glycopeptides have been identified in a few cases.<sup>43,44</sup>

Biofilm formation has been implicated in recurrent pediatric upper respiratory tract infections, such as tonsillitis, adenoiditis, and chronic rhinosinusitis (CRS).<sup>45</sup> Since biofilm formation in the lower respiratory tract is difficult to observe directly and there are few sources of bioptic samples, biofilm studies in LRTI has not been fully explored. To our knowledge, this study firstly explored the correlation between the biofilm-forming ability of CA-MRSA strains isolated from the respiratory tract and the clinical characteristics of respiratory diseases in children. The results showed that all the isolated CA-MRSA strains could form biofilms, and patients with strong biofilm-forming isolates tended to have a higher incidence of LRTI, more severe chest imaging, higher antibiotic change rate, and prolonged cough duration. Previous studies have shown that biofilms not only serve as barriers for bacteria but also exhibit high virulence potential. Biofilm proteome studies have revealed that the extracellular proteins primarily consist of functional virulence factors, including hemolysins, leukotoxins, and lipases.<sup>46,47</sup> In addition, excreted intracellular proteins, such as FbaA, can promote binding to cell surfaces and exhibit high cytotoxicity toward host cells.<sup>48</sup> Polysaccharides and eDNA enhance biofilm formation and mediate numerous virulence traits.<sup>49,50</sup> Furthermore, biofilm-related intracellular inflammation has an indispensable role in infections. Tan et al observed intracellular *S. aureus* infection in CRS

patients and identified its association with surface biofilm.<sup>51</sup> Jardeleza et al analyzed the biofilm features of sinonasal tissue samples from CRS patients and pointed out that an increasing intracellular inflammasome response with the upregulation of *AIM2* was involved in the formation of nasal polyps and *S. aureus* biofilms.<sup>52</sup>

Several studies have proposed promising strategies for inhibiting or eliminating the formation of biofilms,<sup>53–55</sup> such as antibiofilm coatings for implants, ultrasonic eradication, and functional compounds blocking biofilm-forming signalling pathways. However, these methods are more applicable to device-related biofilms, but not to infection tissues, for which antibiotics remain one of the few choices to combat biofilm-related infections. Nevertheless, previous studies have revealed that exposure to sub-inhibitory concentrations of antibiotics can enhance biofilm formation and promote the transmission of antibiotic resistance genes, thereby increasing bacterial tolerance to antibiotics.<sup>56,57</sup> Similarly, we found that the CA-MRSA strains in the strong biofilm-forming group had a much higher percentage of macrolides medication history. Early improper use of antibiotics may be one factor contributing to the development of biofilms, although further research with a larger sample size is needed to verify this assumption.

## Conclusion

ST59-t437 is the most prevalent clone of CA-MRSA respiratory isolates among children in Children's Hospital at Zhejiang University School of Medicine and also belongs to the predominant lineage in China. Most of these strains showed multi-drug resistance, and all were able to form biofilms. Their biofilm-forming ability correlated well with clinical characteristics. The stronger the biofilm-forming ability, the more serious and prolonged were the respiratory symptoms. More studies are needed to confirm this relationship and develop applicable biofilm control measures.

## Ethics Approval and Consent to Participate

This study complies with the Declaration of Helsinki and was approved by the ethics committee of the Children's Hospital, Zhejiang University School of Medicine, (EC) approval number: 2022-IRB-005. The informed consent was waived as the retrospective and anonymous nature of this study.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare no potential conflicts of interest in this work.

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