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Original Article

Antimicrobial Resistance and Molecular Characteristics of Methicillin-Resistant *Staphylococcus aureus* Isolates from Child Patients of High-Risk Wards in Shenzhen, China

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SUMMARY: Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are responsible for high rates of mortality and thus pose a substantial burden to public health worldwide. Here, we investigated the antimicrobial susceptibility and molecular characteristics of MRSA isolated from child patients at Shenzhen Children's Hospital. We characterized 140 MRSA strains through antimicrobial susceptibility testing. We further performed *spa* typing, multilocus sequence typing (MLST), staphylococcal cassette chromosome *mec* (SCC*mec*) analysis, *pvl* gene analysis, and pulsed-field gel electrophoresis (PFGE). The analyzed MRSA strains were found to be sensitive to most non-β-lactam antimicrobial agents. Sequence type (ST) 59 was found to be the most common MLST lineage (54.3%). Most MRSA isolates belonged to the SCC*mec* IV (64.3%) and V (22.8%) types. The MRSA-ST59-SCC*mec* IV-t437 clone was the most predominant strain that infected 28.6% of all patients studied. Moreover, 50.7% of MRSA isolates were found to be *pvl*-positive. We report preliminary data on the prevalence and distribution of MRSA genotypes in Shenzhen Children's Hospital. We characterized MRSA colonization dynamics in child patients in China, and our findings can serve as the basis for the development of strategies to prevent MRSA infection and transmission.

INTRODUCTION

Staphylococcus aureus is a common pathogen that causes various forms of infectious diseases in humans (1). Methicillin-resistant *S. aureus* (MRSA) has been reported to cause skin and soft tissue infections, pneumonia, foreign-body infections, endocarditis, septic arthritis, blood-stream infections, sepsis, and osteomyelitis in both hospital and community settings (2). The health burden caused by high MRSA infection rate has contributed to increase globally (3).

Transmission of MRSA infection has been associated with healthcare facilities, especially large tertiary-care facilities (4). Children infected with MRSA act as potential reservoirs for the subsequent spread of MRSA in the community (5). Furthermore, immunologically immature infants and newborns, especially those born prematurely or who require specialized care, are the most susceptible to MRSA infection (6).

The latent shift of MRSA clones at both the local and global levels is of great interest, primarily because a detailed understanding of MRSA epidemiology will be necessary to establish of public health interventions to control the spread of MRSA. Molecular typing techniques including spa typing, multilocus sequence typing (MLST), staphylococcal cassette chromosome *mec* (SCC*mec*) analysis, and pulsed-field gel electrophoresis (PFGE), have been used to characterize the epidemiology and differentiation of MRSA isolates worldwide (7). Previous studies using these techniques demonstrated that the most prevalent MRSA clones have unique geographical distributions. For example, MRSA clones USA300, sequence type 80 (ST80), ST59, and ST30, known to cause community-acquired infections (CA-infections), were reported in the USA (8), Europe, the Asia-Pacific region, and worldwide, respectively (9). Currently, the most widespread MRSA clone responsible for skin and soft tissue infections (SSTIs) in mainland China is ST59-SCC*mec* IV (10). However, the epidemiology of MRSA in Chinese children has not been well studied (11,12). In this study, we isolated and characterized 140 clinical MRSA strains isolated from child patients in Shenzhen Children's Hospital, China. We aimed to identify the phenotypic and genotypic markers for these isolates, including antimicrobial resistance, spa type, SCC*mec* type, PFGE pattern, and sequence types of 7 unlinked housekeeping genes (*arcC*, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, and *yqil*). Finally, we also aimed to determine the association of these MRSA isolates with the *pvl* gene, which encodes the cytotoxin Panton-Valentine leukocidin (PVL). PVL is known to causes leukocyte destruction and is associated with increased of *S. aureus* virulence (13).

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MATERIALS AND METHODS

Strain collection, MRSA confirmation, and clinical information: A total of 140 non-duplicate MRSA isolates were collected from Shenzhen Children's Hospital neonatal department, pediatric intensive care

unit, and orthopedics department between January 2014 and October 2015. Samples were obtained from 58 female and 82 male patients (one isolate per patient). At the same time, 10 MRSA isolates were collected from paramedics working in these departments. Clinical data, including general demographic information, potential risk factors, laboratory results, clinical features, and treatments received were collected from medical records. Isolates were identified as *S. aureus* via Gram staining, coagulase test, positive catalase test, and Vitek microbiology analysis (BioMérieux, Durham, NC, USA). Methicillin resistance was screened using a cefoxitin disk (30 μg; Oxoid, Hampshire, UK). Species identification of *S. aureus* and determination of methicillin resistance were performed via detection of *mecA* and *nuc* genes using multiplex PCR (14). All patients were under 12 years of age. Ninety-two patients (65.7%) were < 1 year old; 26 patients (18.6%) aged 1~3 years; 14 patients (10.0%) aged 3~6 years, and 8 patients (5.7%) aged 6~12 years. The majority of patients were diagnosed with abscesses (91 patients, 65.0%). Fourteen patients (10.1%), 13 patients (9.3%), 10 patients (7.1%), 9 patients (6.4%), and 3 patients (2.1%) suffered from omphalitis of newborn, cellulitis, superficial skin infection, furuncle, or knee-joint effusion, respectively.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing of MRSA isolates was performed by agar dilution in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (15). A panel of antimicrobial agents (BioFosun; Shanghai Fosun, Shanghai, China), including oxacillin, nitrofurantoin, sulfamethoxazole/trimethoprim, ciprofloxacin, clindamycin, erythromycin, gentamicin, rifampin, tetracycline, penicillin, vancomycin, levofloxacin, moxifloxacin, and quinupristin, was used to determine the antimicrobial susceptibility profiles of the MRSA isolates. An MRSA isolate was considered to be multidrug-resistant (MDR) if it displayed resistance to ≥ 3 antimicrobial classes, according to the definition by the Chinese Center for Disease Control and Prevention.

***spa* typing:** The polymorphic region X of the *spa* gene was amplified from each isolate using previously designed primers (16). Amplified products were sent to Shanghai Invitrogen (Shanghai, China) for sequencing. The *spa* type was determined according to the number and arrangement mode of tandem repetitive sequences and confirmed using the *spa* server database <http://www.ridom.de/spaserver>.

MLST: MLST analysis of the MRSA isolates was performed according to previously reported procedures (17). The obtained sequence were compared against the sequence of 7 housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqil*) from the MLST database <http://saureus.mlst.net> through assignment of allelic numbers. MRSA STs were assigned according to the allelic profiles.

SCC*mec* typing: A battery of multiplex PCRs was performed as previously described (18). MRSA isolates that lacked fragments or harbor unanticipated fragments were considered to be non-typeable (NT). Control strains for SCC*mec* typing were kindly provided by Prof. Teruyo Ito from Juntendo University, Japan.

Detection of the *pvl* gene: The *pvl* gene was detected in all isolates using the forward and reverse primers, 5'-ACACACTATGCAATAGTTATTT-3' and 5'-AAAGCAATGCAATTGATGTA-3' (19). PCR amplification was performed according to the following profile: 1 cycle of 5 min at 80°C, 35 cycles of 45 s at 94°C, 45 s at 60°C, 90 s at 72°C, and a final extension step of 10 min at 72°C using Ex Taq (Takara Bio, Shiga, Japan). Amplified fragments were sequenced and compared to the EF571841.1 sequence.

PFGE typing: PFGE has been considered the gold standard technique used in molecular epidemiology studies (20). For PFGE, chromosomal DNA was digested with the *Sma*I restriction enzyme at 14°C as previously described and separated with a voltage of 6 volts/cm and an angle of 120º. Pulse increase from 5 s to 40 s was set as the switch time. A molecular weight standard was used for each gel to curate and normalize the DNA fragments. Optimization of 0.5% and band tolerance of 1.5% were selected during comparison of DNA profiles. Isolates were considered to be genetically related if the dice correlation coefficient was > 80%. Cluster analysis was performed using the unweighted pair group method using arithmetic mean (UPGMA). PFGE patterns were analyzed using Bionumerics version 6.6 (Applied-Maths, Sint-Martens-Latem, Belgium).

Statistical analysis: Statistical analyses were performed using SPSS (ver. 16; Chicago, IL, USA). Student's *t*-test was used for normally distributed variables, while and the χ^2 test was used for detecting the homogeneity of proportions for categorical data. All hypotheses were two-tailed. Results were considered statistically significant at *P*-value (*P*) < 0.05.

RESULTS

MRSA confirmation of isolates: *S. aureus* identification was performed using a combination of phenotypic tests, including microscopic examination, measurement of coagulase production, catalase activity, and Vitek microbiology analysis. MRSA isolates were screened using cefoxitin discs. All isolates tested positive for both the *mecA* and *nuc* genes. A total of 140 non-duplicate MRSA isolates from 140 patients were selected for further antimicrobial susceptibility testing and molecular typing.

Antimicrobial susceptibility profiles: Results of antimicrobial susceptibility testing indicated that all 140 MRSA isolates were resistant to oxacillin and penicillin (Table 1) but susceptible to nitrofurantoin, quinupristin, vancomycin, levofloxacin, and moxifloxacin. The majority of MRSA isolates were also resistant to erythromycin (77.8%), clindamycin (75.0%), and tetracycline (55.6%), while 7.4% and 3.7% were resistant to sulfamethoxazole/trimethoprim and gentamicin, respectively. Only 3.7% and 7.4% of isolates exhibited intermediate susceptibility to ciprofloxacin and rifampin, respectively. Susceptibilities to other antimicrobials are presented in Table 1. We further selected 43 MRSA isolates, including 29 CA-MRSA strains and 14 healthcare-associated MRSA (HA-MRSA) strains, for further antibiotic susceptibility analysis (sulfamethoxazole/trimethoprim, erythromycin, ciprofloxacin, clindamycin,

gentamicin, tetracycline, vancomycin, and linezolid). Results showed no significant differences in different isolate types among different antimicrobials (Table 2).

Molecular characteristics of MRSA isolates: *spa* typing identified 24 known *spa* types, out of which the most frequently detected were t437, t114, t5132, t324, t664, t116, t309, t441 and t034, which together account for 82.9% (116/140) of all *spa*-typed isolates. A total of 76 isolates (54.3%) were typed as t437; 11 isolates (7.9%) were typed as t114; and 6 isolates were typed as t324. t437 was the most frequently detected *spa* type in this cohort.

MLST test identified 23 distinct ST types. The most predominant ST type was ST59, which accounted for 54.3% (76/140) of all isolates, followed by ST1 (12.1%, 17/140). Eight isolates were ST45, 8 were ST338, 5 were ST398, and 5 were ST72. Three isolates were typed as ST88; only 2 were ST22, and 2 were ST25. The remaining isolates were identified as ST1, ST9, ST15, ST19, ST30, ST488, ST537, ST1507, ST2808, and ST3068, which corresponded to 1 isolate each. Notably, 4 STs that were characterized as ST2962, ST3185, ST3187, and ST3188, were observed in China for the first time. Thus, ST59 was the most frequently detected MLST type in this cohort.

Using the multiplex SCC*mec* typing method, 18 out of the 140 MRSA isolates were classified as non-typeable (NT). These isolates were distributed among t437 (6 isolates), t309 (4 isolates), and other *spa* types (8 isolates), including ST59 (6 isolates), ST1 (3 isolates), ST45 (1 isolate), and other of MLST types (8 isolates). Only SCC*mec* IV and SCC*mec* V clones were identified in the samples analyzed. A total of 90 isolates (64.3%) were identified as SCC*mec* IV, while 32 (22.9%) were SCC*mec* V.

The *pvl* gene was detected in 71 isolates (50.7%) that were SCC*mec* IV, SCC*mec* V, and non-typeable. A total of 35.6% (32/90) of SCC*mec* IV and 81.3% (26/32) of SCC*mec* V isolates were *pvl*-positive, 47.4% (36/76) of ST59 isolates carried the *pvl* gene. As shown in Table

Table 1. Antimicrobial resistance profiles of MRSA isolates from the child patients (n = 140)

Antimicrobial agent	R, %	I, %	S, %	MIC_{50}	MIC_{90}	MIC range
oxacillin	100	0	0	4	4	4–4
nitrofurantoin	0	0	100	16	16	16–32
sulfamethoxazole/trimethoprim	7.4	0	92.6	12	12	10–320
erythromycin	77.8	0	22.2	8	8	0.25–8
ciprofloxacin	0	3.7	96.3	0.5	1	0.5–2
clindamycin	75.0	0	25.0	8	8	0.25–8
quinupristin	0	0	100	0.25	0.25	0.25–0.5
rifampin	0	7.4	92.6	0.5	0.5	0.5–2
penicillin	100	0	0	0.5	0.5	0.25–0.5
gentamicin	3.7	0	96.3	0.5	0.5	0.5–16
tetracycline	55.6	0	44.4	16	16	1–16
vancomycin	0	0	100	0.5	1	0.5–1
levofloxacin	0	0	100	0.125	0.5	0.12–0.5
moxifloxacin	0	0	100	0.25	0.25	0.25–0.25

R, resistant; I, intermediate; S, susceptible; MIC, minimum inhibitory concentration (μg/ml); MIC_{50}, the minimal concentration of an antimicrobial agent necessary to inhibit the growth of 50% MRSA (μg/ml); MIC_{90}, the minimal concentration of an antimicrobial agent necessary to inhibit the growth of 90% MRSA (μg/ml).

Table 2. Antimicrobial susceptibility of partial methicillin-resistant *S. aureus* isolates

Antimicrobial agent	Resistant (%), n = 43	CA (%), n = 29	HA (%), n = 14
sulfamethoxazole/trimethoprim	2 (4.7)	2 (6.9)	0
erythromycin	36 (83.7)	24 (82.8)	12 (85.7)
ciprofloxacin	0	0	0
clindamycin	33 (76.7)	22 (75.9)	11 (78.6)
gentamicin	0	0	0
tetracycline	29 (67.4)	21 (72.4)	8 (57.1)
vancomycin	0	0	0
linezolid	0	0	0

CA, community-acquired; HA, healthcare-associated.

Table 3. Characteristics of methicillin-resistant *S. aureus* isolates

Origin	SCC*mec* type		
	IV (n = 90)	V (n = 32)	NT (n = 18)
CA-MRSA	63	25	15
HA-MRSA	27	7	3
spa	t437 (48), t114 (11), t324 (6), t5132 (2), t116 (4), t441 (2), other (17)	t437 (22), t5132 (2), t441 (2), t034 (4), other (2)	t437 (6), t309 (4), other (8)
ST	ST59 (52), ST1 (14), ST45 (7), ST398 (1), ST72 (5), other (11)	ST59 (18), ST338 (8), ST398 (4), other (2)	ST59 (6), ST1 (3), ST45 (1), other (8)
PVL-positive (CA + HA)[1)]	32 (21 + 11)	26 (20 + 6)	13 (10 + 3)

[1)]: No. of CA-MRSA with PVL-positive and HA-MRSA with PVL-positive.

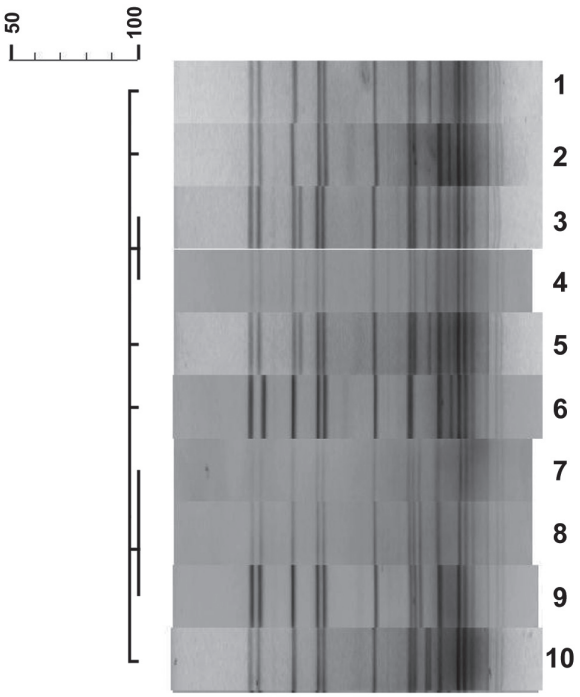

Fig. 1. PFGE patterns of methicillin-resistant *S. aureus* isolates identified as the same type. Lane 1 is the PFGE pattern of MRSA isolate (not included in the 140 MRSA isolates) from a paramedic in the neonatal department. The other 9 lanes are the PFGE patterns of MRSA isolates from neonatal patients in the neonatal department.

3, the *pvl* gene was detected in a significantly higher numbers of SCC*mec* IV ($\chi^2 = 10.23$, $P < 0.01$) and SCC*mec* V isolates ($\chi^2 = 12.7$, $P < 0.01$) than in other SCC*mec*-typed isolates. The number of *pvl*-positive SCC*mec* IV isolates was higher than that of SCC*mec* V isolates; however, the proportion of *pvl*-positive of SCC*mec* IV isolates (32/90, 35.6%) is significantly lower than that of SCC*mec* V isolates (26/32, 81.3%).

After PFGE, each isolate produced 16–20 electrophoresis bands. Ten isolates shared > 80% similarity and were considered to have the same PFGE type (Fig 1). Seven isolates showed 100% similarity to the PFGE type of an isolate collected from a paramedic working in the neonatal department (lane 1 in Fig 1).

DISCUSSION

Our analysis of MRSA isolates from child patients provided initial data on the prevalence and distribution of MRSA genotypes in Shenzhen Children's Hospital, China. First, results demonstrated that the high prevalence of MRSA among child patients < 1 year old. Second, abscess was the most frequent disease resulting from MRSA infection. Third, MRSA strains from child patients in Shenzhen were found to be most sensitive to non-β-lactam antimicrobial agents. Fourth, the MRSA-ST59-SCC*mec* IV-t437 clone was determined to be the most predominant strain among all MRSA isolates collected in this study

Previous molecular epidemiological studies have shown that MRSA strains spread via clonal transmission among different hospitals, cities, and countries (21). Characterizing epidemic MRSA clones can facilitate the development of effective strategies to control the spread of MRSA, optimize treatment, and identify the modes of pathogenicity (22). Modern genotyping techniques, such as sequencing of protein A (*spa* type), MLST, SCC*mec*, and PFGE typing, allow the evolution of prevalent MRSA clones to be monitored (21,23).

Shenzhen Children's Hospital is the only pediatric hospital in Shenzhen, China that treats more than 6,000 outpatients daily on average. Therefore, detailed understanding of the antimicrobial susceptibility and molecular characteristics of MRSA isolates in this hospital can help to devise control measures against MRSA infection, allow the investigation of suspected MRSA outbreaks, and prevent MRSA nosocomial transmission in Shenzhen.

Results from the present study showed that children younger than 1 year old comprised the highest number of child patients with MRSA visited the Shenzhen Children's Hospital. Immunologically immature infants and newborns, particularly those born prematurely or requiring specialized care, are especially susceptible to MRSA infections. Therefore, the high prevalence of MRSA infections detected in children under 1 year old in Shenzhen should be noted.

Antimicrobial susceptibility testing of the MRSA isolates in child patients at Shenzhen Children's Hospital revealed high rates of β-lactam resistance but greater sensitivity to most non-β-lactam antimicrobials, such as nitrofurantoin, quinupristin, vancomycin, levofloxacin, and moxifloxacin. A few isolates were found to be sensitive to erythromycin, clindamycin, or tetracycline. This situation could have been caused by the widespread use of antibiotics (particularly β-lactam antibiotics) in China (24) These results suggest that antimicrobial use exerted a strong selective pressure that facilitated the horizontal transfer of antibiotics-resistant genes within the hospital. Compared with the results of a previous study (25), our findings showed lower levels of susceptibility to erythromycin, clindamycin, penicillin, and tetracycline and revealed no significant difference in antimicrobial susceptibility among genetically distinct MRSA isolates (Table 2). Although sulfamethoxazole/trimethoprim and clindamycin are the appropriate first-line therapeutic options for the treatment of skin and soft-tissue infections in Europe, wherein CA-MRSA are widespread (26), our results suggest that these antibiotics are not likely to be successful for effective treatment of MRSA infections in Shenzhen, in China.

As previously reported, the *spa* types of MRSA isolates vary geographically. *spa* type t041 is a common strain type found in southern Germany (27). *spa* type t002 is also common in Israel (28), while *spa* type t044 is widespread in European countries (29). Our results indicate that t437 was the predominant *spa* type among MRSA isolates from Shenzhen, south China, and accounts for 57.1% of all MRSA isolates, consistent with a previous study of isolates collected in Beijing, northern China (30).

The MLST types of MRSA isolates have also been reported to vary geographically. For example, ST59 is mostly found in the Asia-Pacific region, and ST30 was reported worldwide, including in the USA, western Pacific region, Europe, Hong Kong, and Japan. In mainland China, ST59-MRSA was the predominant MRSA isolate detected in children (11,12). In our study,

the predominant MLST types were determined to be ST59 and ST338, both of which belong to CC59, which has previously been reported in Hungary, Denmark, England, Germany, USA, and the Asia-Pacific region (31).

The molecular characteristics of CA-MRSA isolates and clinical features of CA-MRSA infection differed from those of HA-MRSA. A mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*), plays an essential role in mediating the antibiotic susceptibility of *S. aureus* and is a major molecular hallmark for MRSA classification. MRSA strains with type I to III SCC*mec* elements, which are responsible for resistance to numerous classes of antibiotics, were associated with HA-MRSA, whereas type IV and V SCC*mec* elements were commonly identified in CA-MRSA strains (32). In fact, the distinction between CA-MRSA and HA-MRSA is not well defined, and CA-MRSA strains are also known to be transmitted in hospital settings. SCC*mec* IV and SCC*mec* V MRSA were reported in HA-MRSA (33), consistent with our current results, which identified only SCC*mec* IV and SCC*mec* V clones in MRSA isolates from child patients. These findings may be attributed to the fact that previous studies on CA- and HA-MRSA infections were based on clinical definitions and did not conduct molecular characterization.

The gene encoding PVL (*pvl* gene) is associated with higher *S. aureus* virulence (13). *S. aureus* strains harboring the *pvl* gene are frequently associated with skin and soft tissue infections, especially cellulitis, abscesses, and boils, and can even cause necrotizing pneumonia (34). So far, 5 major *pvl*-positive MRSA MLST types have been reported to have spread worldwide, namely, ST8, ST1, ST30, ST59, and ST80 (35). Our results showed that MLST type ST59 was the predominant strain among *pvl*-positive MRSA clones in Shenzhen, China.

PVL is highly associated with the presence of SCC*mec* IV and sporadically with SCC*mec* VI or V, but not with SCC*mec* types I, II, or III (34). Most MRSA isolates in the current study belong to SCC*mec* IV and V types. However, 35.6% of SCC*mec* IV and 81.3% of SCC*mec* V isolates were *pvl*-positive. By contract, 90.0% of SCC*mec* IV and 54.6% of SCC*mec* V isolates were *pvl*-positive in CA-MARS isolates collected from Wuhan, China (36). These findings suggest that PVL and SCC*mec* IV may confer a selective advantage for CA-MRSA pathogens (37).

Chambers (2005) reported that PVL is the primary virulence factor responsible for the epidemic caused by CA-MRSA strains (38). However, more than 80% of CA-MRSA ST59 clones detected in Taiwan were SCC*mec* VT-*pvl*-positive and SCC*mec* IV-*pvl*-negative (39), and most ST59 clones in Hong Kong were SCC*mec* IV-*pvl*-positive (9). The majority of ST59 clones isolated from Wuhan were SCC*mec* IV-*pvl*-positive, while the remaining isolates were SCC*mec* V-*pvl*-positive (36). Most MRSA isolates in the present study were SCC*mec* IV-*pvl*-positive ST59 isolates. Thus, the MRSA ST59 clones identified in Shenzhen potentially different from the isolates identified in Taiwan and are likely to be similar to those isolated from Hong Kong and Wuhan.

Repeated exposure to *S. aureus* in healthcare environments can result in frequent colonization of healthcare workers. In addition, *S. aureus* strains isolated from medical staff were reported to be closely related to those isolated from inpatients (40). Results of our study showed that 10 MRSA isolates from the neonatal department shared similar PFGE types, out of which 1 was isolated from a paramedic and the other 9 were isolated from neonatal patients. Epidemiological tracking revealed that the paramedic had direct contact with 1 neonatal patient but not with the other patients. We hypothesized that other patients may have been infected from a medical instrument that came into contact with the MRSA-positive paramedic. These findings suggest that healthcare workers may contribute to MRSA transmission among members of the community and in hospitals.

In summary, we validated the presence of 140 MRSA strains from 140 child patients treated in Shenzhen, China and investigated their antimicrobial susceptibilities and molecular characteristics. Our results provide baseline information on antimicrobial resistance and the molecular characteristics of MRSA strains infecting child patients in Shenzhen, China. These MRSA isolates were found to be sensitive to most non-β-lactam antimicrobial agents. The MRSA-ST59-SCC*mec* IV-t437 clone was the most predominant strain detected among all samples. Our findings verified the MRSA colonization dynamics in children and can contribute to the design of strategies and prophylactic measures that aim to prevent the spread of MRSA.

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Conflict of interest None to declare.

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