

Comparative molecular analysis of meticillin-resistant *Staphylococcus aureus* isolates from children with atopic dermatitis and healthy subjects in Taiwan

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Summary

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Conflicts of interest

None declared.

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Background Children with atopic dermatitis (AD) are more frequently colonized by *Staphylococcus aureus* than healthy children.

Objectives To assess whether any relationship exists between nasal meticillin-resistant *S. aureus* (MRSA) colonization and subsequent skin and soft-tissue infections (SSTI).

Patients and methods From 2005 through 2006, comparative molecular analyses of 23 MRSA-colonizing isolates from 133 children with AD, 44 MRSA-colonizing isolates from 490 healthy controls, and 12 MRSA-infecting isolates from 20 children with AD and concurrent SSTI were conducted.

Results Nasal MRSA colonization in children with AD was significantly higher compared with normal individuals (17.3% vs. 9.0%; $P = 0.01$). The molecular characteristics differed significantly between the MRSA isolates from children with AD and the MRSA-colonizing isolates from healthy controls. The clone characterized as sequence type (ST)59 (338)/pulsotype B/staphylococcal cassette chromosome *mec* (SCC*mec*) V_T/Panton–Valentine leucocidin (PVL)-positive/staphylococcal enterotoxin B (SEB)-positive accounted for half of the MRSA isolates from children with AD, and another clone, characterized as ST59/pulsotype A/SCC*mec* IV/PVL-negative/SEB-positive accounted for 61% of the MRSA-colonizing isolates from healthy controls.

Conclusions We found MRSA colonizing the anterior nares of a large number of Taiwanese children, especially among those with AD. Analysis of our data provides evidence that links MRSA-colonizing isolates to MRSA-infecting isolates from concurrent SSTI in children with AD.

Atopic dermatitis (AD) is the commonest chronic inflammatory dermatosis, with a reported global prevalence ranging from 1% to 20%, which is highest in northern Europe and becoming a major health burden.¹ It is defined by childhood onset, pruritis, chronicity, relapse and remissions, the presence of other atopic disorders, a family history of atopy and a characteristic pattern of skin involvement.² The exact pathogenesis for the progression of AD in children remains to be explained; nonetheless, it appears to be a complex interplay of genetics, environmental and immunological factors together with the competency of the skin as a barrier.^{3,4} Contact with allergens of various origins, such as food, aeroallergens and micro-organisms, leads to an allergic response with expansion of T-helper 2 (Th2) clones and subsequent production of IgE by specific B lymphocytes.⁵

Children with AD are more frequently colonized by *Staphylococcus aureus* in both AD lesions and normal-appearing skin than healthy children; the presence and quantity of *S. aureus* colonization increases with eczema severity.^{6,7} *Staphylococcus aureus* produces a multitude of factors with potential skin inflammatory properties including adhesins, cytotoxins, superantigens, capsule, epidermolytic toxins and a number of extracellular enzymes.⁸ Several previous studies suggest that a high proportion of *S. aureus* isolates from subjects with AD produce toxicogenic superantigens,^{9,10} but this finding is not universal.¹¹ Nonetheless, *S. aureus*-derived superantigens are important stimuli for T cells that have been recruited into AD skin.^{12,13}

Gilani et al.¹⁴ reported that three extracutaneous mechanisms by which *S. aureus* can re-colonize the skin in AD are antibiotic resistance, nasal carriage and treatment contamination.

Furthermore, nasal carriage of *S. aureus* plays a key role in the epidemiology and pathogenesis of disease.^{15–17} However, there has been no prospective study that has systematically collected and molecularly characterized the methicillin-resistant *S. aureus* (MRSA)-colonizing isolates in children with AD. Accordingly, the objective of this prospective study was to investigate the prevalence of MRSA nasal colonization among children with AD, to screen for a number of putative virulence factors, and to compare the molecular characteristics of MRSA isolates recovered from children with AD and healthy subjects in the community.

Patients and methods

Study design and location

The study proposal was reviewed and approved by the National Defense Medical Center Institutional Review Board. Two collections of *S. aureus* isolates were used. All children with AD ≤ 14 years of age with concurrent skin and soft-tissue infections (SSTI) in AD skin lesions who visited the Pediatric Department at Tri-Service General Hospital (TSGH) in Taipei as outpatients or hospital inpatients from 2005 through 2006 were recruited for the study. The diagnosis of SSTI was made via the definition described by Stevens *et al.*¹⁸ Affected children who were hospitalized for more than 48 h, who were discharged from any hospital within the 48 h before receiving the infection diagnosis or who had a prior history of MRSA infection were excluded from this study. AD was diagnosed following the criteria of Hanifin and Rajka,² which include pruritus, typical morphology and distribution of eczematous lesions, chronicity of disease, and personal or family history of atopy. Exclusion criteria included severe fungal infection or other skin diseases that might obscure the diagnosis; treatment with systemic corticosteroids or immunosuppressive drugs in the past 4 weeks; and treatment with topical antibiotics in the past 2 weeks or with systemic antibiotics in the past 4 weeks. There was no selection of children by sex or by location and severity of lesions. The first collection of 20 *S. aureus*-infecting isolates obtained from 20 children with AD with concurrent SSTI of AD skin lesions and isolated in the TSGH clinical microbiology laboratory were collected as part of this prospective surveillance programme.

A second collection of 237 *S. aureus*-colonizing isolates was obtained during the same time period by culturing samples from the anterior nares of 133 children with AD and 490 healthy children in the community. Eligible healthy subjects from the community were ≤ 14 years of age without personal or family history of AD, or other skin or allergic diseases who either visited the Pediatric Department at our hospital for a well-child check-up or attended one of two kindergartens in Taipei. We included healthy children who had not resided in a long-term care facility, had not been hospitalized in an acute care facility, had not been treated with central intravenous catheters or long-term venous-access devices, had not used urinary catheters, had not used other long-term percutaneous

devices, had not undergone prior surgical procedures, and were not receiving dialysis within 1 year before nasal sampling.¹⁹

Microbiological studies

After written informed consent was obtained, nasal samples were taken with a sterile cotton swab, placed in the transport medium (Venturi Transystem; Copan Diagnostics, Corona, CA, U.S.A.), and then transported to, and processed in, our microbiology laboratory within 4 h. Cotton swabs were plated on mannitol salt agar (MSA; BBL Microbiology Systems, Becton Dickinson, Company, Sparks, MD, U.S.A.). Each distinctive morphotype of mannitol-fermenting colony was selected from an MSA plate, subcultured to a trypticase soy agar plus 5% sheep blood agar plate (BAP; BBL Microbiology Systems, Becton Dickinson), and incubated at 37 °C in a humidified incubator, with 5% CO₂. Cultures on BAPs were screened using Slidex Staph Plus (bioMérieux, Marcy l'Etoile, France). MRSA identification and antimicrobial susceptibility testing were performed according to the Clinical Laboratory Standards Institute (CLSI; formerly known as the NCCLS) guidelines.^{20,21} All MRSA isolates were frozen at –70 °C for additional testing of organism characteristics.

Chromosomal DNA from three to five isolated colonies was prepared using the Puregene DNA purification kit (Gentra, Minneapolis, MN, U.S.A.) as recommended by the manufacturer, with lysostaphin at 2 mg mL^{–1} and RNase at 4 mg mL^{–1} for the lysis step. The presence of the *lukS*-PV and *lukF*-PV genes encoding Panton–Valentine leucocidin (PVL) components was determined by the polymerase chain reaction (PCR)-based method with the primer pair and thermocycler conditions as reported by Lina *et al.*²² Sequences specific for *sea* to *see*, *seg* to *sei*, *sek* and *seq*, *eta* and *etb*, and *tst*, encoding staphylococcal enterotoxins (SEA to SEE, SEG to SEI, SEK and SEQ), exfoliative toxins (ETA and ETB), and toxic shock syndrome toxin-1 (TSST-1), respectively, were detected using methods described by Jarraud *et al.* and Diep *et al.*^{23,24} Staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed using a multiplex PCR strategy with sets of region-specific primers as previously described.²⁵ Screening for SCC*mec* V_T was performed with the primer and thermocycler conditions reported by Boyle-Vavra *et al.*²⁶ Pulsed-field gel electrophoresis (PFGE) was performed using a CHEF Mapper XA system (BioRad Laboratories, Hercules, CA, U.S.A.) according to a published protocol.²⁷ Some isolates of representative PFGE patterns were subjected to multilocus sequence typing (MLST). MLST was performed by PCR amplification and sequencing of seven housekeeping genes using primers designed by Enright *et al.*²⁸ Each sequence was submitted to the MLST database website for assignment of the allelic profile and sequence type (ST).

Statistical analysis

To gain insight into the association between nasal MRSA-colonizing isolates and MRSA-infecting isolates from concurrent

SSTI in children with AD, we designed a comparative study in which the colonization and clinical samples were collected during the same period. Microbiological characteristics between three age- and sex-matched groups – children with AD, children with AD and concurrent SSTI, and healthy controls – were compared. Data were entered into Microsoft Access XP software and exported into SPSS statistical software, version 10.0 (SPSS Inc., Chicago, IL, U.S.A.), which was used for data analyses. The χ^2 test and Fisher's exact test were used for statistical analyses. All analyses were two-tailed, and a P-value of < 0.05 was defined as statistically significant.

Results

Distribution of *Staphylococcus aureus* isolates and prevalence of nasal methicillin-resistant *S. aureus* colonization

During the study period, 257 *S. aureus* isolates were identified from 643 children, and 79 of the isolates were methicillin-resistant (Table 1). Among them, 12 (60%) *S. aureus* isolates from 20 children with AD and SSTI were classified as MRSA. Of the 623 nares specimens, 67 (10.7%) were positive for MRSA, 170 (27.3%) were positive for methicillin-susceptible *S. aureus*, and 386 (62.0%) were culture negative. The percentage of MRSA isolates among all *S. aureus*-colonizing isolates was 28.3%. Additionally, the *S. aureus* colonization rate was significantly higher among children with AD than that of the healthy controls [67 of 133 (50.4%) vs. 170 of 490 (34.7%), $P = 0.002$]. Nasal cultures positive for MRSA occurred with greater frequency among children with AD than among the healthy controls [23 of 133 (17.3%) vs. 44 of 490 (9.0%), $P = 0.01$].

Table 1 Source of *Staphylococcus aureus* isolates in 643 children as related to methicillin resistance [values given as n (%)]

Source ^a	Meticillin-resistant	Meticillin-sensitive	Total
Children with AD	23 (34) ^b	44 (66)	67
Children with AD and SSTI	12 (60) ^c	8 (40)	20
Healthy controls	44 (26)	126 (74)	170

AD, atopic dermatitis; SSTI, skin and soft-tissue infections. ^aThe 67 and 170 nasal *S. aureus*-colonizing isolates were obtained from the anterior nares of 133 children with AD and 490 healthy controls, respectively. The 20 lesional *S. aureus*-infecting isolates were obtained from the AD skin lesions of 20 children with AD and concurrent SSTI. ^bThe rate of methicillin resistance among isolates from children with AD was significantly higher than among those from healthy controls ($P < 0.01$). ^cThe rate of methicillin resistance among isolates from children with AD and SSTI was significantly higher than among those from healthy controls ($P < 0.001$).

Microbiological characteristics of methicillin-resistant *Staphylococcus aureus* isolates

Distribution of Pantón–Valentine leucocidin genes

Table 2 illustrates the detailed distribution of SCCmec types, exotoxin profiles, PFGE patterns, sequence types and the presence of PVL genes among the MRSA isolates. Of the 79 MRSA isolates examined, PVL-producing isolates constituted 42% of all isolates analysed and 29% of isolates associated with nasal colonization. Among the 35 MRSA isolates from children with AD, 25 (71%) were PVL producers and were more likely to be found in children with AD and SSTI. In contrast, only eight (18%) of the 44 MRSA-colonizing isolates from healthy controls harboured the PVL locus ($P < 0.0001$).

SCCmec typing

Four SCCmec types were identified among the isolates. SCCmec V_T (20 of 35, 57%) was the most common SCCmec type among the MRSA isolates from children with AD; the remaining 15 (43%) isolates carried SCCmec IV. Conversely, MRSA-colonizing isolates from healthy controls were more likely to have SCCmec IV (80%) ($P = 0.0025$). No statistically significant differences were found between the three groups with respect to SCCmec II or SCCmec III type.

Possession of other toxins

Analysing the possession of genes that coded for other exotoxins, 60 of 67 (90%) MRSA-colonizing isolates were positive for at least one of the toxin genes tested, compared with 100% of the MRSA-infecting isolates from children with AD and SSTI. The most frequently encoded toxin gene among the MRSA-infecting isolates was *seb* (100%). While *seb* was encoded less frequently, *eta*, *etb*, *tst*, *sec*, *sed*, *see*, *seg/sei*, *sek* and *seq* were not present in any of the isolates.

Regarding single genes, MRSA-colonizing and -infecting isolates from children with AD were significantly more associated with the possession of genes that encoded SEB than were MRSA-colonizing isolates from healthy controls ($P = 0.0112$). In contrast, a *seg/sei*-positive PCR result was more likely to be present in MRSA-colonizing isolates from healthy controls ($P = 0.0231$).

Genotyping

A wide diversity of pulsotypes was found among the 79 MRSA isolates subjected to PFGE typing. In total, 13 PFGE patterns were identified; eight patterns were found among the 35 MRSA isolates from children with AD, while nine patterns were found among the 44 MRSA-colonizing isolates from healthy controls. PFGE pattern B, the most common pattern among the MRSA isolates from children with AD, accounted for 51% of the isolates. In contrast, pattern B accounted for only 11% of the MRSA-colonizing isolates from healthy

Table 2 Comparison of molecular characteristics of 79 MRSA isolates from 643 children

Characteristics	MRSA-colonizing isolates from children with AD (n = 23)	MRSA-infecting isolates from children with AD and SSTI (n = 12)	MRSA-colonizing isolates from healthy controls (n = 44)	P-value ^a
Presence of PVL genes, n (%)	15 (65)	10 (83)	8 (18)	< 0.0001
SCCmec type, n (%)				
II	0	0	1 (2)	0.6684
III	0	0	1 (2)	0.6684
IV	11 (48)	4 (33)	35 (80)	0.0025
V _T ^b	12 (52)	8 (67)	7 (16)	0.0004
Production of other toxins, n (%)				
TSST-1	2 (9)	0	7 (16)	0.2728
SEA	1 (4)	0	3 (7)	0.6230
SEB	20 (87)	12 (100)	28 (64)	0.0112
SEC	2 (9)	0	11 (25)	0.0576
SED	0	0	1 (2)	0.6684
SEG/SEI	1 (4)	0	11 (25)	0.0231
SEH	1 (4)	1 (8)	0	0.2136
PFGE pattern, n (%)				
A	5 (22)	2 (17)	28 (64)	0.0005
B	11 (48)	7 (58)	5 (11)	0.0004
C	2 (9)	1 (8)	2 (5)	0.7654
Other	5 (22)	2 (17)	9 (20)	0.9379
Sequence type	59, 338	59	59, 508, 89	

AD, atopic dermatitis; MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; PVL, Pantone–Valentine leucocidin; SCCmec, staphylococcal cassette chromosome mec; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C; SED, staphylococcal enterotoxin D; SEG, staphylococcal enterotoxin G; SEH, staphylococcal enterotoxin H; SEI, staphylococcal enterotoxin I; SSTI, skin and soft-tissue infections; TSST-1, toxic shock syndrome toxin-1. ^aP-value by χ^2 test (or Fisher's exact test) for the significance by comparison of children with AD, children with AD and SSTI, and healthy controls. ^bV_T refers to the SCCmec V_T element in strain TSGH 17 from Taiwan.²⁶

controls ($P = 0.0004$), while pattern A, the most common pattern among the MRSA-colonizing isolates from healthy controls, accounted for 64% of the isolates, but 20% of the MRSA isolates from children with AD ($P = 0.0005$).

MLST was performed on 26 isolates, and four STs were identified. ST59 was found in 22 (96%) of the 23 MRSA-colonizing isolates from children with AD, 12 (100%) of the 12 MRSA-infecting isolates from children with AD and SSTI, and 38 (86%) of the 44 MRSA-colonizing isolates from healthy controls. One MRSA-colonizing isolate from a child with AD was ST338, a single-locus variant of ST59 (one nucleotide difference in the *gmk* locus). ST89 and ST508, respectively, were found in four (9%) and two (5%) of the 44 MRSA-colonizing isolates from healthy controls. Altogether, two dominant clones were identified among these MRSA isolates; they were characterized as ST59 (338)/PFGE type B/SCCmec V_T/PVL(+)/SEB(+) and ST59/PFGE type A/SCCmec IV/PVL(-)/SEB(+) (Table 3). The ST59 (338)/PFGE type B/SCCmec V_T/PVL(+)/SEB(+) clone was significantly more frequently found among the MRSA isolates from children with AD than from healthy

controls ($P < 0.001$). In contrast, the ST59/PFGE type A/SCCmec IV/PVL(-)/SEB(+) clone was significantly more frequently found among the MRSA-colonizing isolates from healthy controls than from children with AD ($P < 0.0001$).

Antimicrobial susceptibility profiles

Antibiograms of 23 MRSA-colonizing isolates from children with AD did not differ significantly from those of 12 MRSA-infecting isolates from children with AD and SSTI with respect to clindamycin, erythromycin, gentamicin and chloramphenicol. All 35 MRSA isolates from children with AD were resistant to penicillin G, and none had reduced susceptibility to vancomycin, teicoplanin, trimethoprim-sulfamethoxazole, fusidic acid or ciprofloxacin.

Discussion

Nasal *S. aureus* colonization in patients with AD is a risk factor for more invasive infections, including bacteraemia, infective

Table 3 Distribution of two major clones of 79 MRSA isolates from 643 children [values are given as n (%)]

Origin	PFGE B/SCCmec V _T /PVL(+)/SEB(+) ^a	PFGE A/SCCmec IV/PVL(-)/SEB(+) ^a
Sequence type	59 (338) ^b	59
AD children (n = 23)	11 (48)	4 (17)
AD children with SSTI (n = 12)	7 (58)	1 (8)
Healthy controls (n = 44)	4 (9)	27 (61)

AD, atopic dermatitis; MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; PVL, Pantón–Valentine leucocidin; SCCmec, staphylococcal cassette chromosome mec; SEB, staphylococcal enterotoxin B; SSTI, skin and soft-tissue infections. ^aA significant difference was found between MRSA-colonizing, -infecting strains from children with AD and MRSA-colonizing strains from healthy controls in terms of the first and second clones ($P < 0.001$ and $P < 0.0001$, respectively). ^bST338 is a single-locus variant of ST59 and accounted for one colonizing strain of PFGE type B from children with AD.

endocarditis, osteomyelitis and septic arthritis.^{29,30} Recent trends indicate an increasing incidence of community-associated MRSA (CA-MRSA) in the general population, which is especially worrisome for clinicians caring for patients with AD.³¹ A previous study of nasal PVL-positive MRSA colonization supported a relationship between nasal strains of PVL-positive MRSA and CA disease.¹⁷ More recently, on the basis of univariate analysis, children with AD were at significant risk for PVL-positive MRSA colonization.³² These observations prompted us to assess the extent and molecular characteristics of MRSA colonization among children with AD during a period in which CA-MRSA infection was increasing. Nasal carriage of *S. aureus* in the general population is approximately 10–45% and is higher among patients with AD at 39–100%.^{15,33–35} In our study, the overall *S. aureus* and MRSA nasal colonization rates of children with AD were markedly higher than those of the controls (50.4% vs. 34.7% and 17.3% vs. 9.0%, respectively). Our findings are in agreement with previous reports that *S. aureus* colonization is increased in the anterior nares of patients with AD compared with normal individuals.^{34,35}

With regard to bacteriological studies, both nasal MRSA-colonizing isolates from children with AD and MRSA-infecting isolates from children with AD and SSTI shared a common pulsotype (type B). The type B pulsotype belonged to ST59 or its variant ST338, carried a newly identified subtype of SCCmec V_T (recently tentatively designated as type VII by Takano *et al.*³⁶), and had PVL genes, which were similar to CA isolates previously reported in Taiwan.^{17,26,37,38} This unique characteristic of the predominant CA-MRSA strain in Taiwan is totally different from those of isolates identified in the U.S.A. Furthermore, a clone characterized as ST59/PFGE type

A/SCCmec IV/PVL(-)/SEB(+), although accounting for fewer than 20% of the MRSA strains from children with AD in the current study, was the predominant clone among the colonizing isolates from healthy children without risk factors in Taiwan.³⁹ These results suggest that MRSA isolates from children with AD in Taiwan had not spread from the healthcare facility but originated *de novo* in the community.

The skin of patients with AD exhibits striking susceptibility to colonization and infection with *S. aureus*, which can secrete superantigens including SEA, SEB and SEC and TSST-1.⁴⁰ Of these, SEB is suggested to be the mediator inducing the AD lesion.³⁹ SEB stimulation results in sequential T-cell activation, proliferation, Fas expression and Th2 cytokine secretion, which is subsequently followed by apoptosis in a dose- and time-dependent manner.^{4,40,41} In our population samples, SEA, SEC and TSST-1 seem to be very scarcely produced among MRSA isolates from children with AD. Comparing children with AD with healthy subjects, MRSA isolates producing SEB were isolated significantly more frequently among children with AD, especially among the infecting isolates associated with SSTI. This supports evidence that SEB plays an important role in the mechanism of AD.

We identified five factors that support the relationship between nasal MRSA-colonizing isolates and MRSA-infecting isolates from concurrent SSTI in children with AD. First, most MRSA isolates from children with AD were PVL-positive, whereas fewer MRSA-colonizing isolates from healthy controls contained the PVL locus. Second, most of the MRSA isolates from children with AD had SCCmec V_T, which was different from those of the MRSA-colonizing isolates among healthy controls, whose SCCmec type was mainly type IV. Third, nasal MRSA-colonizing isolates from children with AD and MRSA-infecting isolates from children with AD and SSTI had consistent exotoxin profiles, which differed from those of MRSA-colonizing isolates among healthy controls. Fourth, genotype findings indicated that most MRSA isolates that colonized children with AD were clonally related to clinically isolated, MRSA-infecting isolates from children with AD and SSTI, especially those with SCCmec V_T, compared with SCCmec IV. Finally, an analysis of antibiotic sensitivity testing showed no significant differences between the MRSA isolated from children with AD compared with those from children with AD and SSTI.

Our data are subject to certain limitations. First, we were unable to demonstrate that greater nasal MRSA colonization among children with AD was associated with more severe AD, although nasal carriage and secondary infection of the affected skin are strongly associated. Second, some microbiological results are geographically distinct and may not be generalized to the global population. Third, we were unable to determine risk for SSTI because children with AD colonized with MRSA were not monitored in a longitudinal manner. Finally, our study is a snapshot in time because the molecular epidemiology of MRSA is constantly changing.

In conclusion, we demonstrated that *S. aureus* and MRSA colonize the anterior nares of a large number of children

with AD compared with healthy controls. Using the distribution of PVL genes, SCCmec typing, exotoxin profiling, genotyping and antimicrobial drug susceptibility testing, we provide evidence that supports the relationship between nasal MRSA-colonizing isolates and MRSA-infecting isolates from concurrent SSTI in children with AD. Further studies are needed to determine the therapeutic potential of eradicating MRSA from this secondary source and its effect on AD severity.

What's already known about this topic?

- Children with atopic dermatitis (AD) are more frequently colonized by *Staphylococcus aureus* than healthy children.

What does this study add?

- We found methicillin-resistant *S. aureus* (MRSA) colonize the anterior nares of a large number of Taiwanese children, especially those with AD. Data analysis provides evidence that links MRSA-colonizing isolates to MRSA-infecting isolates from concurrent skin and soft-tissue infections in children with AD.

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