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ORIGINAL ARTICLE

Risk factors and molecular analysis of Panton-Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus* colonization and infection in children

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Panton-Valentine leukocidin (PVL)

Background and Purpose: Limited information is available regarding the role Panton-Valentine leukocidin (PVL) plays in methicillin-susceptible *Staphylococcus aureus* (MSSA). In this study, we compared the frequency of the *PVL* gene between MSSA strains isolated from patients with MSSA infections and MSSA strains isolated from patients with evidence of MSSA nasal colonization. We also explored the role that the PVL toxin plays in the ability of MSSA to cause disease as well as the phylogenetic relationship between these *S aureus* strains.
Methods: The presence of MSSA strains was screened among children aged <18 years during routine health maintenance visits and among children aged <7 years at daycare centers or kindergartens during the 2003 to 2008 period. At the same time, clinical MSSA isolates were recovered from patients with various types of bacterial infections. Polymerase chain reaction was applied to detect the presence of the *PVL* and *SEB* genes in these strains. The strains were also subjected to pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) studies. Clinical features were compared between patients with PVL-positive and those with PVL-negative isolates.
Results: A total of 495 colonizing MSSA and 71 clinical MSSA isolates were used. The prevalence of PVL-positive *S aureus* was significantly higher among clinical isolates than among colonizing isolates (14/71, 19.7%; 5/495, 1.0%; $p < 0.05$). In addition, we found that patients with PVL-positive MSSA infections had a significantly longer duration of fever and tended to have higher C-reactive protein levels than patients with PVL-negative MSSA infections. MLST typing of the 19 PVL-positive MSSA isolates revealed ST59, a strain that is similar to the MLST type of

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community-associated methicillin-resistant *Staphylococcus aureus* found in Taiwan. The PFGE typing of PVL-positive/ST59 MSSA isolates revealed multiple pulsotypes.
Conclusion: The prevalence of the *PVL* gene was significantly higher among clinical strains of MSSA (19.7%) than among colonizing strains (1.0%). In addition, patients infected with PVL-positive MSSA strains had fever for a significantly longer duration and tended to have higher C-reactive protein levels than patients with PVL-negative MSSA infections. Our findings imply that PVL may play an important role in the pathogenesis of *S aureus* infection.

Introduction

Staphylococcus aureus plays an important role in various infections including skin and soft tissue infections, pneumonia, bacteremia, meningitis and sepsis. Many community-associated methicillin-resistant *S aureus* (CA-MRSA) strains carry genes encoding Panton-Valentine leukocidin (PVL), a bicomponent pore-forming cytotoxin that has been shown to target polymorphonuclear cells, monocytes and macrophages in humans and rabbits.[1] Clinically, PVL-producing *S aureus* is primarily associated with skin and soft tissue infections, such as furunculosis and skin abscesses, as well as severe necrotizing community-acquired pneumonia.[2–6] Recent studies have shown that *PVL* genes are commonly detected among CA-MRSA strains that cause infection.[7] In fact, research has demonstrated that between 70% and 100% of CA-MRSA isolates that cause infection carry *PVL* genes.[8–11] The most common site of *S aureus* carriage is the nose.[12] The results of our previous study and those of other studies conducted in Taiwan have also found that almost all of the CA-MRSA strains that cause skin and soft tissue infections, necrotizing pneumonia, and bone and joint infections carry the *PVL* gene.[13–21]

The epidemiology and the pathogenic potential of PVL-positive strains of methicillin-susceptible *S aureus* (MSSA) are not well known in Taiwan. The proportion of MSSA strains carrying the *PVL* gene has been reported to range from 12% to 81%.[22,23] In this study, we compared the frequency of the *PVL* gene between MSSA strains isolated from patients with MSSA infections and from patients with evidence of MSSA nasal colonization. We also explored the role that the *PVL* gene plays in the ability of MSSA to cause disease and the phylogenetic relationship between these *S aureus* strains. In our previous studies, high Staphylococcal Enterotoxin B (*SEB*) gene carriage rate in *S aureus* was noted.[16,19] In this study, we wanted to find the definite carriage rate of community-associated (CA)-MSSA in Taiwan.

Methods

Study design and specimen collection

In this retrospective cohort study we evaluated all children aged <18 years who presented to the Tri-Service General Hospital (TSGH), Taipei, Taiwan during the period from January 2003 to December 2008, with culture-proven evidence of CA-MSSA infection. Non-duplicate CA-MSSA isolates were recovered from various clinical specimens that had been obtained from patients who had sought treatment in inpatient or outpatient wards. The definition states that to be CA-MSSA, the infection must be diagnosed in an outpatient or within 48 hours of hospitalization. Patient characteristics and clinical information including age, gender, underlying medical conditions, infection site and type, dates of admission, discharge, last visit, clinical course, duration of fever, white cell count, C-reactive protein (CRP) level, and appropriateness of antibiotic therapy were obtained from the patient medical records. At the same time, to ascertain the circulation of *S aureus* in nearby communities in Taipei, nasal swabs were collected from the nares of infants and children aged 1 month to 6 years during health maintenance visits or physical examinations at daycare centers or kindergartens with informed consent.

Laboratory methods

Preparation of chromosomal DNA

S aureus was cultured in Muller Hinton agar medium and stored in 20% glycerol containing trypticase soy broth at −70°C. The isolates were confirmed as being *S aureus* by colonial morphology, Gram staining, catalase activity and coagulation of citrated rabbit plasma, using the Gentra Puregene Bacteria Kit (Qiagen, Germany) for purification and extraction of DNA.

Polymerase chain reaction

The presence of the *lukS*-PV and *lukF*-PV genes encoding components of PVL was determined by a polymerase chain reaction (PCR)-based method with the primer pair and thermocycler conditions as previously described in Lina et al.[2] The expected size of the *PVL* gene product was 433 base pairs. Sequences specific for *SEB* were detected using methods described by Jarraud et al.[24] Positive and negative controls were included in each assay.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed using the CHEF Mapper XA system (Bio-Rad Laboratories, Hercules, CA, USA) according to a published protocol.[25] Findings were interpreted on the basis of standard criteria. The pulsotypes were designated in alphabetical order. PFGE patterns with fewer than four band differences from one existing pulsotype were defined as subtypes and labeled with Arabic numbered suffixes.

To identify PFGE polymorphisms, band patterns were analyzed using Molecular Analyst Fingerprinting, Fingerprinting Plus, and Fingerprinting DST software (Bio-Rad Laboratories, Richmond, CA, USA). The grouping method was performed to deduce a dendrogram from the matrix using the unweighted pair group method, with the arithmetic averages clustering technique after calculation of similarities using the Pearson correlation coefficient between each pair of organisms; the PFGE patterns were distinguished at the 70% similarity level.

Multilocus sequence typing

The PVL-positive MSSA strains were studied using multilocus sequence typing (MLST). MLST was performed by PCR amplification and sequencing of housekeeping genes using primers designed by Enright et al.[26] Each sequence was submitted to the MLST database website for assignment of the allelic profile and sequence type (ST).

Statistical analysis

Statistical analyses were performed using the SAS statistical package for Windows (Version 9.1, SAS Institute, NC, USA). All categorical variables were compared by two-sided chi-squared test Fisher's exact test. Continuous variables were compared by the Wilcoxon rank sum test. A p value of <0.05 was defined as statistically significant.

Results

Population characteristics

A total of 495 nasal colonizing MSSA isolates were recovered from 3305 infants and kindergarten children ranging in age from 1 month to 6 years. Overall, a total of 71 non-duplicate MSSA clinical isolates were cultured from samples including blood (n = 5, 7%), eye discharge (n = 4, 5.6%), ear discharge (n = 2, 2.8%), and purulent fluid obtained from wounds, carbuncles, furuncles and cellulitis wounds (n = 60, 84.5%). The gender distribution and median age were similar in both groups.

PVL and SEB toxins

The *PVL* gene was detected in five of the 495 colonizing *S aureus* isolates (1%) and in 14 of the 71 clinical *S aureus* isolates (19.7%). The prevalence of PVL-positive *S aureus* was significantly higher among clinical isolates than among colonizing isolates (5/495, 1.0% *versus* 14/71, 19.7%; $p < 0.05$). On the other hand, the *SEB* gene was detected in 56 of the 495 colonizing *S aureus* isolates (11.3%) and in 11 of the 71 clinical *S aureus* isolates (15.5%). There was no significant difference in prevalence of the *SEB* gene between the two groups of children. Only three out of five PVL-positive colonizing strains (60%) carried the *SEB* gene; whereas 10 of 14 PVL clinical strains (71.4%) harbored the *SEB* gene (Table 1).

Table 1 Annual distribution of PVL and SEB genes among 566 colonizing and clinical MSSA isolates

Year	MSSA in healthy carriers (N = 495)		MSSA in patients with infection (N = 71)	
	PVL(+)	SEB(+)	PVL(+)	SEB(+)
2003	3	19	7	7
2004	0	12	0	0
2005	0	1	1	1
2006	2	15	1	0
2007	0	5	1	0
2008	0	4	4	3
Total	5	56	14	11

MSSA = methicillin-sensitive *Staphylococcus aureus*; PVL = *Panton-Valentine leukocidin* gene; *SEB* = *Staphylococcus aureus enterotoxin B* gene.

The role of PVL toxin in clinical infection

The second part of the study compared the differences in clinical manifestations between patients with PVL-positive and patients with PVL-negative MSSA strains. We found that patients with PVL-positive MSSA infections had a fever for a significantly longer time and tended to have higher CRP levels than patients with PVL-negative MSSA infections. There were no significant differences in age, gender or duration of hospitalization between the two groups. In addition, there were no significant differences in major sites of infection between the two groups. Furthermore, there were no significant differences in white blood cell counts, treatment counts, treatment with incision and drainage, or duration of hospitalization between the two groups (Table 2).

MLST typing

MLST typing of the PVL-positive MSSA isolates revealed that all of the PVL-positive MSSA strains were ST59, which is similar to the major MLST type of CA-MRSA found in Taiwan.

PFGE typing

PFGE typing revealed a wide diversity of pulsotypes among the 19 MSSA strains (Fig. 1). Three clusters, which included 13 (68.4%) strains, were distinguished at the 70% similarity level. Pulsotype A (three subtypes) was the most common and accounted for two (40%) of the five colonizing strains and four (28.6%) of the 14 clinical strains. Pulsotype C (two subtypes) was detected in one clinical stain and one colonizing strain. It was indistinguishable from TSGH-17, which is a prototype of CA-MRSA; ST59/PFT USA1000/PVL (+)/SCCmecV_T.[27]

Discussion

The epidemiology of PVL-positive MSSA is not well known and the pathogenic potential is probably underestimated. The proportion of MSSA strains containing the *PVL* gene has been reported to range from 12% to as high as 81%.[22,23] Some animal studies have suggested that PVL is a major virulence determinant.[5,28] In addition, the *PVL* gene has been reported to be more common in MRSA than in MSSA isolates.[1,17–21]

Table 2 Demographic and clinical characteristics of 71 children with MSSA infection

Variable	Number of patients		*P* value
	PVL(-) patients (n = 57)	PVL(+) patients (n = 14)	
Demographic information			
Age, median months (range)	62 month (1d–17y)	80 month (5m–16y)	0.5531
Sex, M:F (ratio)	1.59 (35:22)	3.6 (11:3)	0.3507
OPD/admission	22/35	7/7	0.4367
Clinical diagnosis			
Bacteremia	5/57 (8.8%)	0/14 (0%)	0.2036
Conjunctivitis	4/57 (7%)	0/14 (0%)	
Otitis media with discharge	1//57 (1.8%)	1/14 (7.1%)	
Scarlet fever	6/57 (10.5%)	2/14 (14.3%)	
Skin and soft tissue infection (carbuncle, cellulites)	41/57 (71.9%)	11/14 (78.6%)	
Infection site			
Thigh	21/57 (36.8%)	7/14 (50%)	0.2028
Head and neck	6/57 (10.5%)	3/14 (21.5%)	
Buttock	3/57 (5.3%)	2/14 (14.3%)	
Trunk	18/57 (31.6%)	1/14 (7.1%)	
Others (blood, ear, eye discharge)	9/57 (15.8%)	1/14 (7.1%)	
Laboratory findings*			
WBC count, median cells _ $10^3/mm^3$ (range)	9950 (3900–20,930)	10,100 (5900–39,200)	0.6279
CRP level, median mg/dL (range)	0.64 (0.04–11.93)	2.64 (0.06–28.7)	0.0795
Treatment and outcome*			
Antibiotic treatment	31/35 (88.6%)	7/7 (100%)	0.9999
No active antibiotic	4/35 (11.4%)	0/7 (0%)	
Incision and drainage	10/35 (28.6%)	3//7 (42.9%)	0.6567
No incision and drainage	25/35 (71.4%)	4/7 (57.1%)	
Duration of fever after antibiotic, median days (range)	1.00 (0–19)	5.00 (1–26)	0.0083[a]
Total hospital stay, median days (range)	5.00 (1–28)	6.00 (3–29)	0.5617

NOTE. Data are number (%) of patients, unless otherwise indicated. CRP = C-reactive protein, MSSA = methicillin-sensitive *Staphylococcus aureus*, MRSA = methicillin-resistant *S. aureus*, OR = odds ratio, WBC = white blood cell. CRP levels were determined for patients.* 42 admission patients profiles.

[a] Statistically significant association after analysis ($p < 0.05$). All categorical variables were compared by two-sided chi-squared or Fisher's exact tests. Continuous variables were compared using the Wilcoxon rank sum test.

In the present study, nasal colonization of MSSA was found in 495 (15%) of the 3305 children tested; a finding that is consistent with data in our previous report conducted during 2004 to 2009, which showed that the prevalence of nasal colonization by MSSA was 14.2%.[19]

In our previous study[16] we found that 71.4% of clinical MSSA isolates and 60% of colonizing MSSA isolates harbored the *SEB* gene; whereas 100% of the CA-MRSA strains carried the gene. The findings in this study, which show that the *SEB* gene had high carriage rate by *S aureus* in Taiwan, support the findings reported in numerous studies.[13,16–21] Previous studies in Taiwan have found that ST59 MRSA isolates are the most common causes of community-associated infections and are the most frequently found isolates in nasal colonization.[13–21] In this study, molecular analysis indicated that all PVL-positive MSSA colonizing and clinical isolates had the same ST59 type. This means that there is a strong association between ST59 CA-MRSA clinical strains and community-associated ST59 MSSA strains.

The PVL toxin plays an important role in inflammatory response and is known to be involved in the development of some diseases, such as skin and soft tissue infection and necrotizing pneumonia.[2–4] Our study showed that only 19.7% of clinical MSSA isolates harbored the *PVL* gene, and only 1% of colonizing isolates were PVL-positive. This finding is similar to that in our previous study[16] which showed that almost all clinical CA-MRSA isolates were PVL-positive, whereas only 16.9% of colonizing strains contained the *PVL* gene. The prevalence of the *PVL* gene was higher in CA-MRSA isolates than in MSSA isolates and was higher in clinical isolates than in colonization isolates. The finding suggests that the *PVL* gene plays an important role in the pathogenesis of *S aureus* infections.

To the best of our knowledge, this is the first case series to compare the distribution of PVL-positive and -negative genotypes in children with MSSA infections in Taiwan. In our univariate analysis, we found that only longer duration of fever and higher CRP levels were associated with PVL-positive clinical MSSA isolates; whereas in our previous study of CA-MRSA strains that caused staphylococcal scarlet fever,[18] PVL-positive isolates were associated with significantly larger abscess sizes, higher white blood cell counts, higher CRP levels, longer duration of fever, generalized rash and longer hospital stay.

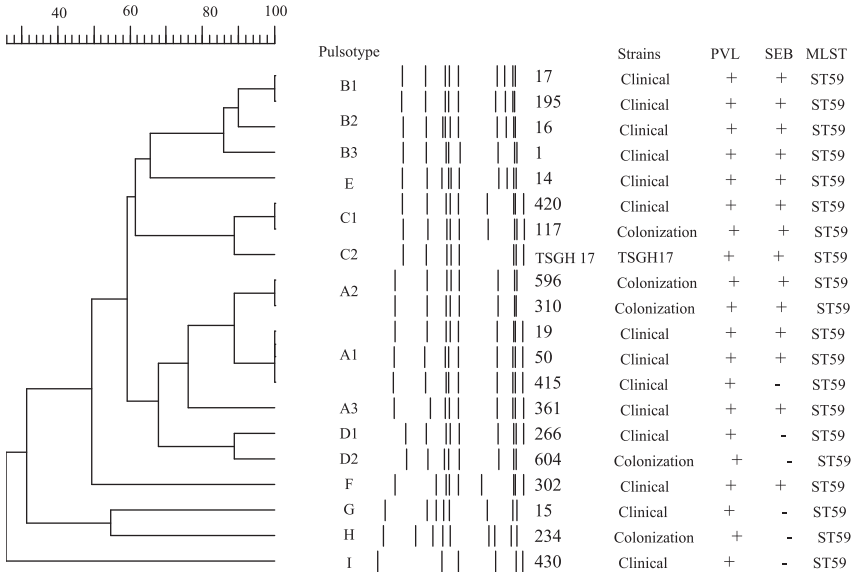

Figure 1. Characterization of 19 PVL-positive MSSA clinical or colonization strains, which were classified into nine pulsotypes. TSGH-17 was a prototype ST59/PFT USA1000 SCCmecV_T CA-MRSA strain.

PFGE revealed one clinical strain and one colonizing strain of MSSA that were indistinguishable from TSGH-17, which is a prototype of CA-MRSA (ST59/PFT USA1000/PVL (+)/SCCmecV_T) circulating in Taiwan. We hypothesize that the *PVL* gene might have been independently integrated into the genomes of many different ancestral MSSA clones, including ST59 MSSA. The ST59 MSSA strains might then have transformed into PVL-positive ST59 MSSA strains. After that, a few of the most evolutionarily successful PVL-positive ST59 MSSA strains might have received a small cassette conferring methicillin resistance (*mecA* gene) for a successful transition to an CA-MRSA genotype comprising the *Staphylococcal Cassette Chromosome mec* (*SCCmec*) and the *PVL* gene.

The prevalence of the *PVL* gene was significantly higher among clinical strains of MSSA (19.7%) than colonizing strains (1%). Patients infected with PVL-positive MSSA strains had significantly longer durations of fever and tended to have higher CRP levels than patients with PVL-negative MSSA infections. Our findings imply that PVL may play a role in the pathogenesis of *S aureus* infection.

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