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ORIGINAL ARTICLE

Concomitant genotyping revealed diverse spreading between methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *Staphylococcus aureus* in central Taiwan

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multilocus sequence

Background: *Staphylococcus aureus* is a versatile bacterium, which can lead to various infectious diseases. Various molecular typing methods are applied to the evolution and epidemiology surveys of *S. aureus*, mostly for methicillin-resistant *S. aureus* (MRSA). However, methicillin-susceptible *S. aureus* (MSSA) is still an important pathogen, but their molecular typing is evaluated infrequently.
Methods: Pulsed-field gel electrophoresis (PFGE), *spa* typing, and detection of five virulent genes for 95 MRSA and 56 MSSA isolates (July—December 2008 and July 2008—December 2009, respectively) during an overlapping period were performed.
Results: More diversity was found in MSSA isolates (23 pulsotypes and 25 *spa* types, excluding 4 new-type and 1 nontypable isolates for *spa* typing) than in MRSA isolates (19 pulsotypes and 16

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typing;
pulsed-field gel electrophoresis;
spa;
typing

spa types, excluding 1 new-type and 1 nontypable isolates for *spa* typing). By *spa* typing, t002 ($n = 30$), t037 ($n = 23$), t437 ($n = 21$), t234 ($n = 3$), t1081 ($n = 3$), and t1094 ($n = 3$) were the six major MRSA clones. For MSSA isolates, t189 ($n = 13$), t437 ($n = 4$), t084 ($n = 3$), t213 ($n = 3$), t701 ($n = 3$), and t7200 ($n = 3$) were the six major types. Combining PFGE and spa typing, there were five combinations (pulsotype + spa type) that contained both MRSA and MSSA isolates (pulsotype 9-t437, pulsotype 15-t037, pulsotype 19-t002, pulsotype 21-t002, and pulsotype 28-t1081). For all 151 *S. aureus* or 95 MRSA isolates, the PFGE typing had more discrimination power, but *spa* typing had larger discrimination index for 56 MSSA isolates.

Conclusion: In conclusion, there were different predominant MRSA and MSSA clones clinically. Continuing longitudinal tracking of molecular typing is necessary for elucidating the evolution of this important clinical pathogen.

Introduction

Staphylococcus aureus is a versatile human pathogen, which can cause numerous infectious diseases, ranging from skin, soft tissue, joint and bone infections to food poisoning and pneumonia, even endocarditis, septicemia, and toxic shock syndromes.[1] Besides its high virulence, *S. aureus* is also notorious for its ability to develop resistances to various antibiotics rapidly, including penicillin, methicillin, and even vancomycin.[2] Various molecular typings, including pulsed-field gel electrophoresis (PFGE), *spa* sequencing typing, multilocus sequence typing (MLST), and so on, are applied to the evolution and epidemiology surveys of *S. aureus*, mostly for methicillin-resistant *S. aureus* (MRSA).[3–6] Only a few studies put emphasis on methicillin-susceptible *S. aureus* (MSSA).[7] However, MSSA is still an important pathogen for community or health-care-associated and invasive infections.[8–10] In Taiwan, MRSA-related infections were always a rampant problems, and clonal spreading of specific MRSA strains had been demonstrated.[11,12] However, the molecular epidemiology of MSSA in Taiwan is still limited.[13,14] In this study, we want to elucidate the relationship between clinical MRSA and MSSA isolates from an overlapping period.

Materials and methods

Clinical MRSA and MSSA isolates

As reported in previous studies, 95 MRSA and 56 MSSA isolates were collected from blood culture of different patients.[12,15] The collection periods for MSSA and MRSA were July 2008—December 2009 and July—December 2008, respectively. Identification of clinical isolates was processed initially with a Bactec 9000 system (Becton Dickinson, Sparks, MD, USA). The positive samples were streaked across Trypticase soybean agar with 5% sheep blood (TSA II)/Levine EMB agar (Becton Dickinson) and incubated at 37°C for appropriate periods. Bacterial isolates were identified as *S. aureus*, and the susceptibility to oxacillin was determined using a BD Phoenix automated microbiology system (Becton Dickinson). The minimal inhibitory concentration (MIC) interpretive standards for oxacillin susceptibility were those recommended by the Clinical Laboratory Standards Institute.[16]

DNA extraction

Briefly, isolates were grown on BAP agar plate (BBL Microbiology Systems, Becton Dickinson). Three to five bacterial colonies were suspended in 600 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and centrifuged briefly. The Genomic DNA Mini Kit (Geneaid, New Taipei City, Taiwan) was used to extract DNA from pelleted cells.

spa typing

The X region of the *spa* gene contains a variable number of repeats of 21—27 bp.[17] The size of the most common repeat is 24 bp. The X region of each MRSA isolate was amplified by polymerase chain reaction (PCR) with primers 1095F: 5′-AGACGATCCTTCGGTGAGC-3′ and 1517R: 5′-GCTTTTGCAATGTCATTTACTG-3′, as described previously.[18] The amplified products were sequenced, and the sequences obtained were analyzed using Ridom Staph Type software [version 1.4; Ridom GmbH, Wurzburg, Germany (http://spa.ridom.de/index.shtml)] to determine the repeat profile and *spa* type of each isolate.[18]

PFGE typing

All bacterial isolates were genotyped using PFGE according to the manual protocol using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA). PFGE analysis was carried out as described previously.[19] The bacterial genomic DNA was prepared and digested with *SmaI* (New England Bio Labs, Beverly, MA, USA). The digested DNA fragments were subjected to PFGE, which was conducted at a voltage of 6.0 V/cm for 21 hours at switch times ramped from 5 seconds to 40 seconds. The gel was stained and analyzed using BioNumerics software (Applied Maths, Kortrijik, Belgium).

Pulsotypes were assigned to same clusters with >80% similarity from the dendrograms.

SCC*mec* typing

Detection and identification of SCC*mec* were performed by multiplex PCR using the genomic DNA from each MRSA isolate as the template, as described previously.[20] The amplified products were analyzed by agarose gel electrophoresis (100 V, 30 minutes) and stained with ethidium bromide for photography. Types V and V_T SCC*mec* were distinguished using the following primers[21]: F: 5′-GAA CATTGTTACTTAAATGAGCG-3′ and R: 5′-TGAAAGTTG TACCCTTGACACC-3′. The amplification was carried out with a 1-minute denaturation step at 94°C, followed by 30 cycles of 30 seconds at 94°C for denaturation, 60 seconds at 55° for annealing, and 60 seconds at 72°C for extension and then 5 minutes at 72°C for the final extension. The size of the PCR products of SCC*mec* type V was 325 bp and that of SCC*mec* V_T was 1600 bp.

MLST typing

Seven housekeeping genes (*arc*, *aroE*, *glp*, *gmk*, *pta*, *tpi*, and *yqiL*) of *S. aureus* were used for the MLST typing. Amplification of a portion of each gene was performed as described previously.[22] The amplified products were sequenced, and the sequences thus obtained were analyzed using the software available at http://saureus.mlst.net/sql/multiplelocus.asp.

Detection of virulent genes

Detection of virulent genes, including enterotoxin A (*sea*), enterotoxin B (*seb*), enterotoxin C (*sec*), toxic shock toxin-1 (*tst*), and Panton—Valentine leukocidin (PVL) (*lukS/F*), was performed by PCR with the primers and conditions as previously reported.[23]

Discriminatory power

Discriminatory powers of *spa* typing and PFGE were calculated using the Hunter—Gaston discriminatory index (HGDI):[24]

$$\mathrm{HGDI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

where N is the total number of isolates examined, s the total number of types identified, and nj the total number of isolates belonging to the jth type.

Results

The results of PFGE typing, *spa* typing, SCC*mec* typing (MRSA only), and MLST typing (36 strains only), and virulent genes for 95 MRSA and 56 MSSA isolates are shown in Fig. 1. Table 1 shows the six most common *spa* types of MRSA and MSSA isolates along with their corresponding pulsotypes, SCC*mec* types, MLST types (36 strain only), virulent genes, and percentage. The results of *spa* typing, SCC*mec* typing, MLST typing, and virulent genes for MRSA isolates have been described in our previous report.[12] By PFGE typing, there were 35 pulsotypes for all 151 *S. aureus* isolates, including 19 pulsotypes for 95 MRSA and 23 pulsotypes for 56 MSSA isolates. The most frequent pulsotype of MRSA was type 15 ($n = 22$), followed by type 19 ($n = 17$); type 18 ($n = 16$); type 9 ($n = 7$); type 7 ($n = 6$); type 3 ($n = 4$); types 5, 16, and 28 ($n = 3$); types 6, 10, 11, and 21 ($n = 2$); and types 12, 14, 23, 29, 33, and 35 ($n = 1$). For MSSA isolates, the most abundant one was type 20 ($n = 11$), followed by type 25 ($n = 10$); type 21 ($n = 7$); type 23 ($n = 4$); type 24 ($n = 3$); types 9, 26, and 27 ($n = 2$); and types 1, 2, 4, 8, 13, 15, 17, 19, 22, 28, 29, 30, 31, 32, and 34 ($n = 1$). Pulsotypes containing both MRSA and MSSA isolates were types 9 ($n = 7$ and $n = 2$, respectively), 15 ($n = 22$ and $n = 1$, respectively), 19 ($n = 17$ and $n = 1$, respectively), 21 ($n = 2$ and $n = 7$, respectively), 23 ($n = 1$ and $n = 4$, respectively), 28 ($n = 3$ and $n = 1$, respectively), and 29 ($n = 1$ and $n = 1$, respectively).

As identified by *spa* typing, there were 37 *spa* types for all 144 *S. aureus* isolates (excluding 1 nontypable MRSA, 1 nontypable MSSA, 1 new-type MRSA, and 4 new-type MSSA isolates), including 16 and 25 different types for 93 MRSA and 51 MSSA isolates, respectively. The most common *spa* type of MRSA was t002 ($n = 30$), followed by t037 ($n = 23$); t437 ($n = 21$); t234, t1081, and t1094 ($n = 3$); and t138, t186, t214, t441, t824, t932, t1212, t1751, t3527, and t3528 ($n = 1$). All t002 strains were SCC*mec* type II, and those undergoing MLST typing analysis ($n = 10$) were all ST5. More than half of these t002 strains also harbored *sec* ($n = 20$, 66.7%) and *tst* ($n = 24$, 80%) virulent genes. All t037 strains were SCC*mec* type III, except the MRSA4103 strain that was SCC*mec* type II, and those underwent MLST typing analysis ($n = 7$) were all ST239. Most of these t037 strains also carried *sea* ($n = 20$, 87%) virulent gene. All t437 strains were either SCC*mec* type IV ($n = 14$) or type V_T ($n = 7$), and those undergoing MLST typing analysis ($n = 5$) were all ST59. More than half of these t437 strains also harbored *seb* ($n = 16$, 76.2%), and most ($n = 8$, 80%) MRSA strains carried PVL toxic gene belonged to t437. Among MSSA isolates, the most common was t189 ($n = 13$), followed by t437 ($n = 4$); t084, t213, t701, and t7200 ($n = 3$); t002, t267, and t593 ($n = 2$); and t037, t073, t091, t094, t127, t160, t164, t269, t286, t338, t359, t796, t1081, t2883, t2949, and t5078 ($n = 1$). Three isolates of each t189 and t437 strains and two of each t084, t213, t701, and 7200 strains underwent MLST analysis. The MLST types of each *spa* type analyzed isolates were ST188, ST59, ST15, ST12, ST6, and ST15 respectively. All ($n = 4$) t437 MSSA isolates carried virulent gene *seb* and 75% ($n = 3$) had PVL toxic gene; *spa* types containing both MRSA and MSSA isolates were t002 ($n = 30$ and $n = 2$, respectively), t037 ($n = 23$ and $n = 1$, respectively), t437 ($n = 21$ and $n = 4$, respectively), and t1081 ($n = 3$ and $n = 1$, respectively).

Combining pulsotype and *spa* type, those with both same molecular typings and containing both MRSA and MSSA isolates were pulsotype 9-t437 (MRSA4047, MRSA4084, MRSA4116, MSSA13, and MSSA53), pulsotype 15-t037 (17 MRSA isolates and 1 MSSA isolate, MSSA38), pulsotype 19-t002 (12 MRSA isolates and 1 MSSA isolate, MSSA34), pulsotype 21-t002 (MRSA4161 and MSSA10), and pulsotype 28-t1081 (MRSA4141, MRSA4046, and MSSA02). The

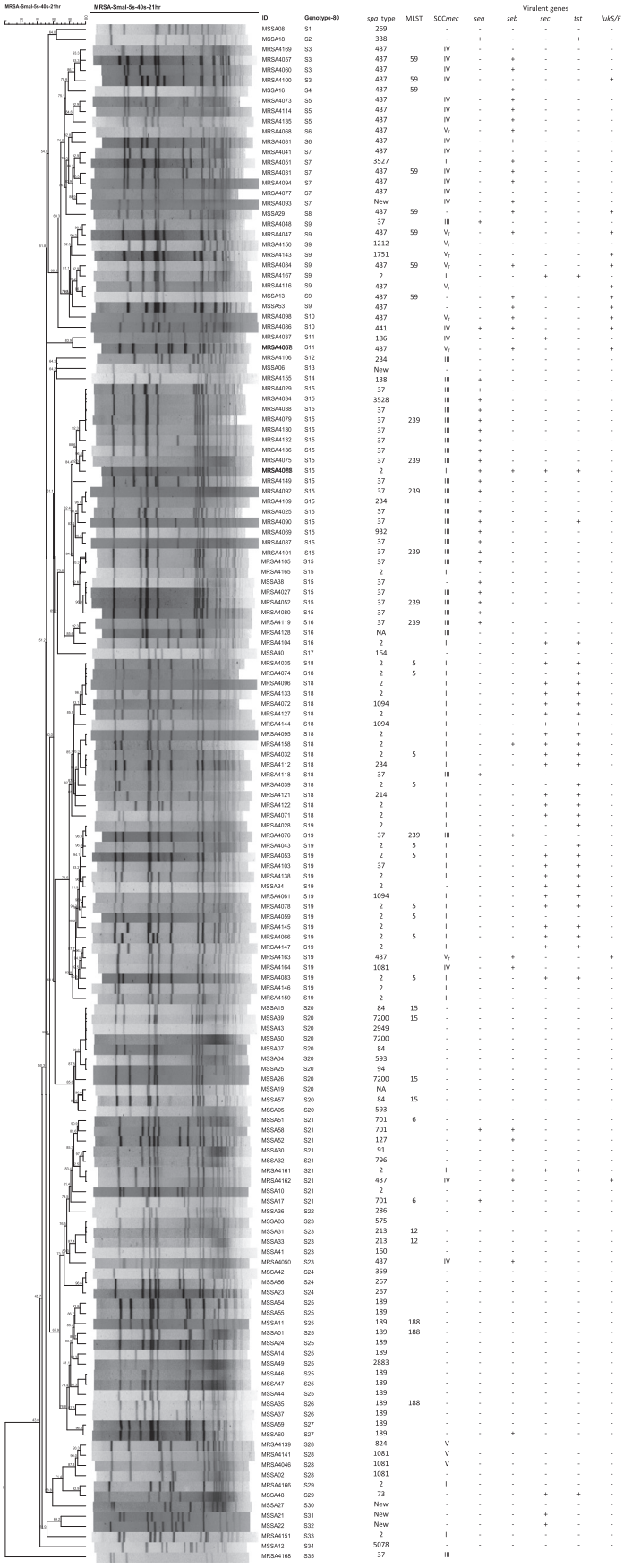

Figure 1. PFGE typing, *spa* typing, SCC*mec* typing, MLST typing (36 strains only) and virulent genes of 95 MRSA and 56 MSSA isolates. MRSA = methicillin-resistant *Staphylococcus aureus*; MSSA = methicillin-susceptible *S. aureus*.

Table 1 Six most frequent *spa* types and their pulsotypes, SCC*mec* types, MLST types, and virulent genes among MRSA and MSSA isolates

Rank	*spa* type (no.)	Pulsotype (no.)	SCC*mec* type (no.)	MLST type[a]	Virulent genes, *n* (%)					%	Cumulative %
					sea	*seb*	*sec*	*tst*	*lukS/F*		
MRSA (n = 95)											
1	t002 (30)	19 (12), 18 (11), 15 (2), 9 (1), 16 (1), 21 (1), 29 (1), 33 (1)	II (30)	5 (10/10)	1 (3.3)	3 (10)	20 (66.7)	24 (80)	0	32.3	32.3
2	t037 (23)	15 (17), 19 (2), 9 (1), 16 (1), 18 (1), 35 (1)	III (22), II (1)	239 (7/7)	20 (87)	1 (4.3)	1 (4.3)	2 (8.7)	0	24.7	57.0
3	t437 (21)	3 (4), 7 (4), 5 (3), 9 (3), 6 (2), 10 (1), 11 (1), 19 (1), 21 (1), 23 (1)	IV (14), V_T (7)	59 (5/5)	0	16 (76.2)	0	0	8 (38.1)	22.6	79.6
4	t234 (3)	12 (1), 15 (1), 18 (1)	III (2), II (1)	—	0	0	1 (33.3)	1 (33.3)	0	3.2	82.8
4	t1081 (3)	28 (2), 19 (1)	V (2), IV (1)	—	0	1 (33.3)	0	0	0	3.2	86.0
4	t1094 (3)	18 (2), 19 (1)	II (3)	—	0	0	3 (100)	3 (100)	0	3.2	89.2
—	Others[b] (12)	7 (2), 9 (2), 15 (2), 10 (1), 11 (1), 14 (1), 16 (1), 18 (1), 28 (1)	II (2), III (4), IV (3), V (1), V_T (2)	—	4 (33.3)	3 (25)	2 (16.7)	1 (8.3)	2 (16.7)	10.8	100
MSSA (n = 56)											
1	t189 (13)	25 (9), 26 (2), 27 (2)	—	188 (3/3)	0	1 (7.7)	0	0	0	23.2	23.2
2	t437 (4)	9 (2), 4 (1), 8 (1)	—	59 (3/3)	0	4 (100)	0	0	3 (75)	7.1	30.3
3	t084 (3)	20 (3)	—	15 (2/2)	0	0	0	0	0	5.4	35.7
3	t213 (3)	23 (3)	—	12 (2/2)	0	0	0	0	0	5.4	41.1
3	t701 (3)	21 (3)	—	6 (2/2)	2 (66.7)	1 (33.3)	0	0	0	5.4	46.5
3	t7200 (3)	20 (3)	—	15 (2/2)	0	0	0	0	0	5.4	51.9
—	Others[c] (27)	—	—		2 (7.4)	1 (3.7)	4 (14.8)	3 (11.1)	0	48.2	100

[a] Numbers in parentheses represent the number of isolates that underwent MLST analysis.
[b] Including one new-type strain and one nontypable strain.
[c] Including four new-type strains and one nontypable strain.
MLST = multilocus sequence typing; MRSA = methicillin-resistant *Staphylococcus aureus*; MSSA = methicillin-susceptible *S. aureus*.

discrimination power of PFGE typing and *spa* typing is shown in Table 2. For all 151 *S. aureus* isolates or 95 MRSA isolates, the discrimination index of PFGE was better than that of *spa* typing. However, for MSSA isolates, the discrimination index of *spa* typing was somewhat more than that of PFGE typing.

Discussion

The features of different molecular typing methods were applicable to various conditions. For microvariation and short-period intervals, such as single-unit outbreak, PFGE was suitable due to its high discrimination power. For macrovariation, and long-term and large-scale evolution, MLST was good due to easy interlaboratory comparability, standardized nomenclature, and slow variation accumulation.[2,6] Based on the sequencing of a hypervariable repeat region within protein A (*spa*), the *spa* typing was always considered as a middle ground between PFGE and MLST, with the ability to detect both genetic micro- and macrovariation.[25,26] In spite of the fact that not all isolates underwent MLST analysis, the MLST type could be deduced from the *spa* type because isolates with the same *spa* type always shared the same MLST type but not vice versa.[26] This phenomenon was also observed in random selection of MRSA or MSSA isolates that underwent MLST analysis. By PFGE and *spa* typings, more diversity was observed in MSSA isolates (23 pulsotypes and 25 spa types, excluding 4 new-type and 1 nontypable isolates for spa typing) than in MRSA isolates (19 pulsotypes and 16 spa types, excluding 1 new-type and 1 nontypable isolates for spa typing), similar to the findings of a previous study conducted in Europe based on *spa* typing.[10] Lesser evolution time and higher antibiotic selection pressure of MRSA isolates than those of MSSA isolates were suspected to attribute to this diversity difference.[10] Major clones of MRSA (t002 with *sec* and *tst*, t037 with *sea*, and t437 with *seb*) were spreading in Taiwan.[12,27,28] There were also major spreading of MSSA clones, although MSSA is more divergent than MRSA. Excluding one nontypable and four new *spa* types, six *spa* types (t189, t437, t084, t213, t701, and t7200) occupied more than a half (29/51 = 56.9%) MSSA isolates, especially t189 isolates (13/51 = 25.5%). Except t084 isolates, these most common six *spa* types among MSSA isolates were different from those found in the United States and Europe, as reported in previous studies, which showed that the most prevalent one is t002, followed by t008, t012, t084, t216, t021, t084, t051, t091, t012, and t127.[7,10] The t189 isolates, which were the most common in our study, and t701 isolates ranked seventh and 30th, respectively, in the United States, but both were not among the most common 20 *spa* types found in Europe.[7,10]

Table 2 Hunter–Gaston discriminatory index of *spa* typing and PFGE of 151 *Staphylococcus aureus* isolates

	PFGE typing	*spa* typing
MRSA (n = 95)	0.8786	0.7880[a]
MSSA (n = 56)	0.9136	0.9224[b]
Total (n = 151)	0.9338	0.8858[a,b]

[a] Excluding one nontypable strain (MRSA4128) and one new-type strain (MRSA4093).
[b] Excluding one nontypable strain (MSSA19) and four new-type strains (MSSA6, MSSA21, MSSA26, and MSSA27).
MRSA = methicillin-resistant *S. aureus*; MSSA = methicillin-susceptible *S. aureus*; PFGE = pulsed-field gel electrophoresis.

The methicillin resistance of MRSA came from the horizontal transfer of a mobile element, SCC*mec*, among staphylococcal species.[3,4] The predominant clones of MRSA in Taiwan were New York/Japan strain (USA100, ST5-t002 with type II SCC*mec*), Brazilian/Hungarian strain (ST239-t037 with type III SCC*mec*), and ST59-t437 strains (with type IV or V SCC*mec*).[12,27] The New York/Japan strains were derived by the acquisition of type II SCC*mec* in ST5 MSSA.[3,4] The Brazilian/Hungarian strains was suspected to originate from the acquisition of type III SCC*mec* in ST8 MSSA, and then recombined with ST30 isolate to become ST 239 MRSA.[3,29] The source of community-acquired MRSA isolates was debatable; acquisition of type IV or V SCC*mec* by PVL-positive MSSA strain of animal origin has been mentioned.[30,31] Combining pulsotype and *spa* type, five combinations (pulsotypes 19-t002, 21-t002, 15-t037, 9-t437, and 28-t1081) containing both MRSA and MSSA isolates were detected. As for pulsotype 19-t002 isolates (n = 13), all were MRSA with type II SCC*mec* and suspected to be New York/Japan strains, except for one MSSA isolate (MSSA34). Both virulent genes *sec* and *tst* were found in the MSSA34 strain and most of the others in pulsotype 19-t002 MRSA isolates (n = 7, 58.3%). According to the evolution model and the same PFGE typing result, these 12 MRSA isolates and MSSA34 might have originated from the same ancestor and most of them picked up type II SCC*mec* thereafter, except MSSA34. A similar scenario was also suspected between MRSA (MRSA4047, MRSA4084, and MRSA4116) and MSSA (MSSA13 and MSSA53) isolates of pulsotype 9-t437. Of interest, all these five isolates carried PVL toxic gene, and four isolates (except MRSA4116) also harbored the virulent gene *seb*. As to pulsotype 15-t037 isolates (n = 18), all isolates carried *sea* virulent gene and the only one MSSA isolate (MSSA38) was suspected to come from MRSA strain after the loss of type III SCC*mec*. Acquisition of type III SCC*mec* of ST8 MSSA was assumed to occur earlier than the transfer a 557-kb chromosome fragment from ST 30 MSSA into the ST8 background in Brazilian/Hungarian strains.[3,29] However, another possible evolution model could not be excluded. Relationships between MSSA and MRSA isolates in each same pulsotype-*sap* type should be elucidated based on more evidence of longitudinal and horizontal molecular epidemiology of *S. aureus*.

Different molecular typings between MRSA and MSSA isolates were also found in a previous study. In the previous study, three dominant MRSA clones in Portuguese people were different from MSSA isolates and it was suspected that they came from abroad and not from the pediatric MRSA clone, which was suspected to originate from a local MSSA clone acquiring type IV SCC*mec* thereafter.[32] This observation was consistent with our findings that the only same molecular type in the most common six *spa* typings between MSSA and MRSA isolates was t437, which were always belonging to "molecular" community-acquired MRSA (CA-MRSA), SCC*mec* type IV or V, in our previous study.[12] The same MLST (ST59), but different *spa* type (t437) from the USA 1000 clone (t216), and similar number of MRSA (n = 3) and MSSA (n = 2) isolates

of pulsotype 9-t437 also implied local origination of these molecular CA-MRSA isolates. This speculation was supported partly by the finding that all three MRSA (MRSA4047, MRSA4084, and MRSA4116) of pulsotype 9-t437 were SCC*mec* V_T, which is specific for CA-MRSA isolates in Taiwan.[33]

One MRSA isolate and one MSSA isolate could not be defined by *spa* typing, and mutations at *spa* primer binding sites in these nontypable strains had been proved.[34] As PFGE was always considered as a high discriminative typing method,[6] discrimination indexes higher than those of *spa* typing were expected for all *S. aureus* and MRSA isolates. However, the Hunter—Gaston discriminatory indexes of *spa* typing for MSSA isolates were somewhat more than those of PFGE typing, and this interesting result was rarely described by previous studies. Because clonal spreading was found in these MRSA isolates,[12] molecular typing methods with more discrimination power (like PFGE) would have shown higher discrimination indexes. For MSSA isolates, higher diversity by itself would reduce the differences in discrimination indexes between two molecular typing methods with dissimilar discrimination powers.

In conclusion, concomitant genotyping by PFGE and *spa* typing revealed that clonal spreading was different between MRSA and MSSA isolates. More diversity was found for MSSA isolates. Some MRSA and MSSA isolates shared the same pulsotypes (>80% similarity in the dendrograms) and *spa* types. A long-term longitudinal epidemiological survey was necessary for elucidating the evolutional correlation between these isolates.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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