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Original Article

High hemolytic activity of the *Staphylococcus aureus spa* t1081 among clonal complex 45 in Taiwan

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Abstract *Background:* Methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type (ST) 45 was first reported in Taiwan in 2006. Since then, the prevalence of ST45 MRSA in clinical isolates has increased. This study was carried out to understand the changes in the proportions, evolutionary relationships, and infection advantages of ST45 and its related clones.
Materials and methods: *S. aureus* including MRSA and MSSA (methicillin-sensitive *S. aureus*), and clonal complex (CC) 45 blood isolates were collected in 2000, 2005, and from January 2010 to August 2014. Molecular typing, multiple-locus variable-number tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP)-based phylogenetic analysis were performed. Fitness and virulence analyses were used to understand the infection advantages of the isolates.
Results: Among the 67 CC45 isolates, only MSSA ST508 isolates were found in 2000 and 2005. Since 2010, the prevalence of MRSA has increased, t1081/ST45 has become dominant, and MRSA ST508 has been found. Phylogenetic analysis indicated that most of the ST45 isolates were located in a cluster distinct from those of ST508 and ST929. However, the t026 isolates clustered with the ST508 isolates rather than with the other ST45 isolates. Moreover, fitness and virulence analyses revealed that the t1081 isolates had higher hemolytic activity than the t026 and ST508 isolates did.

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Conclusion: Our findings indicated that the increased prevalence of ST45 MRSA isolates from blood cultures in Taiwan was due to the t1081 isolates, and their high hemolytic activity may provide an infection advantage.

Introduction

Staphylococcus aureus is a major pathogen known for causing a broad spectrum of infections, such as skin and soft tissue infections (SSTIs), bacteremia, pneumonia, endocarditis, and osteomyelitis.[1] The emergence of methicillin-resistant *S. aureus* (MRSA), a significant drug-resistant pathogen, has contributed to a considerable global disease burden.[2]

MRSA is traditionally classified into community-associated MRSA (CA-MRSA) and healthcare-associated MRSA (HA-MRSA), which differ in their epidemiology, microbiological characteristics, and populations they affect.[3,4] In most reports, HA-MRSA and CA-MRSA strains usually carry different SCC*mec* types. HA-MRSA, which is the main cause of opportunistic infections, is often associated with multidrug resistance and most commonly carries SCC*mec* I, II, or III.[5,6] In contrast, CA-MRSA strains usually carry stronger virulence factors such as Panton-Valentine leucocidin (PVL), with SCC*mec* types IV and V being the most common.[7,8] Over the past two decades, three major MRSA clones have been prevalent in Taiwan, sequence type (ST) 239, ST5, and ST59.[9] While ST239 and ST5 are predominantly linked to healthcare settings, ST59, which was initially a community-associated MRSA clone, has increasingly infiltrated healthcare environments.[10] However, in recent years, the prevalence of ST8 and ST45 among MRSA colonization and infection isolates has increased.[11,12] In Taiwan, ST239 primarily carries SCC*mec* III, ST5 carries SCC*mec* II, and ST8 carries SCC*mec* IV.[12,13] In contrast, ST59 and ST45 have more diverse SCC*mec* types, including SCC*mec* IV and V_T [SCC*mec* V (5C2&5)].[13]

ST45, also known as the 'Berlin clone,' was first observed in Berlin in 1993 and has since spread globally.[14,15] In Taiwan, ST45 was first identified in 2006.[16] Since then, ST45 has been reported in epidemiologic investigations of nasal carriage, infections, and environmental sources. ST45 was the most common nasal MRSA carriage clone in the 2012 survey of nursing home residents and staff.[17] It has since remained the predominant nasal MRSA clone.[18—20] In 2015, a nasal MRSA carriage survey of emergency department patients and healthcare workers in Taiwan revealed that ST45 exceeded 30% for the first time,[18] and in 2016, a similar survey in nursing homes and long-term care facilities suggested that ST45 accounted for nearly 50% of the total.[19] ST45 has also been found in invasive and superficial infections, including cellulitis, osteomyelitis, and bacteremia.[9,11,12,21—24] Among these reports, ST508, which belongs to the same clonal complex (CC) as ST45, was found and was sometimes classified in the same or a closely related pulsotype as ST45.[20,21]

ST45 is a highly diverse lineage with characteristics that vary across different global regions and clinical performance.[15] ST45 isolates carrying different SCC*mec* types (including SCC*mec* II, IV, or V) have been reported, and many MSSA (methicillin-sensitive *S. aureus*) ST45 isolates have also been identified.[15] It is traditionally recognized that there are significant differences in the prevalent lineages of MSSA and MRSA. A previous study indicated that ST59 was the only common ST for MSSA and MRSA in Taiwan.[25] However, some studies have suggested that specific MSSA lineages may be the ancestors of contemporary MSSA and MRSA.[26] A global evolutionary analysis revealed that ST45 originated in Europe and diverged into different branches by acquiring different types of SCC*mec*.[15] Previous genomic analyses revealed that ST45 in Taiwan can be divided into two distinct clones, carrying SCC*mec* IV and SCC*mec* V, each originating from different regions and associated with different diseases.[24,27]

Owing to the high diversity of ST45 and its similarity to ST508, we focused on CC45 isolates in this study. We retrospectively analyzed the lineage changes in CC45, including shifts in the percentages of MSSA and MRSA, before and after 2006, when ST45 was first reported.

Materials and methods

Bacterial isolates

A total of 67 *S. aureus* CC45 isolates recovered from blood cultures were collected from the Bacteriology Laboratory at National Taiwan University Hospital (NTUH). Among these isolates, 56 were collected between January 2010 and August 2014, 4 were collected in 2005, and 7 isolates were collected in 2000. All the isolates were identified via a VITEK 2 automated system (bioMérieux, La Balme les Grottes, France).

Detection of methicillin resistance

All the *S. aureus* isolates were tested first for antibiotic susceptibility to cefoxitin via the VITEK 2 automated system and then confirmed via the disc diffusion method. The MIC values for oxacillin were determined via the standard agar dilution method. Both the disc diffusion method and standard agar dilution method were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines,[28] and *S. aureus* ATCC 25923 and 29213 were used as the reference strains. The *mecA* gene was detected via PCR as previously described.[29] In accordance with the CLSI guidelines, isolates with a cefoxitin inhibition zone $\leq$21 mm, an oxacillin MIC $\geq$4 μg/mL or *mecA* gene positivity were defined as MRSA.

SCC*mec* typing, *agr* typing, *spa* typing and multilocus sequence typing

The SCC*mec* types were determined via multiplex PCR as described previously.[30] The SCC*mec* V_T was additionally detected with the primer pair ccrC2-F2 and ccrC2-R2.[31] The determination of the four *agr* types was performed via the multiplex PCR method previously described with the forward primer pan-agr and the four reverse primers agr1, agr2, agr3 and agr4.[32] The *spa* type was determined via PCR and sequencing as previously described.[33] Multilocus sequence typing (MLST) was conducted as previously described.[34]

Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA)

MLVA-10_{Orsay} was applied to the typing of all the isolates with the PCR primer set described previously.[35] The MLVA codes are provided in the order Sa0122, Sa0266, Sa0311, Sa0704, Sa1132, Sa1194, Sa1291, Sa1729, Sa1866, and Sa2039 corresponding to the genome position in the reference strain Mu50 (accession number NC_002758). The MLVA repeat numbers for the 10 loci were analyzed with BioNumerics v.7.6 (Applied Maths, Sint-Martens-Latem, Belgium) following the manufacturer's instructions to create a UPGMA (unweighted pair−group method with arithmetic mean) dendrogram or a minimum spanning tree.

Whole genome sequencing (WGS) and phylogenetic analysis

The WGS of selected isolates was performed via the PacBio Sequel II platform (Pacific Biosciences, Menlo Park, CA, USA). PacBio HiFi reads were *de novo* assembled and polished via hifiasm v0.15.3 and GCpp v2.0.2. The assembled genome quality was evaluated via QUAST v4.6.3. The WGS sequences were then analyzed via CSI Phylogeny 1.4 (https://cge.food.dtu.dk/services/CSIPhylogeny/)[36] and the phylogenetic tree was visualized via iTOL (https://itol.embl.de/). All sequencing data of selected CC45 isolates have been deposited in GenBank under accession number PRJNA1141196.

Virulence gene detection

The detection of virulence genes including *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sem*, *sen*, *seo*, *pvl*, *lukDE* and *lukM* was performed via PCR.[37]

Growth curve

The growth curves were generated under different conditions, including 42 °C, pH 3.5, or with the addition of 10% human serum. Isolates incubated at 37 °C in tryptone soy broth (TSB) at pH 7.4 served as the control. The overnight cultures were diluted with TSB resulting in an OD600 of 0.05, and then incubated at 180 rpm. The OD 600 was measured after 2, 4, 6, 8, and 24 h.

Biofilm formation

Biofilm formation was analyzed via a microtiter plate assay.[38] The isolates were cultured overnight at 37 °C in TSB, washed three times with phosphate-buffered saline (PBS) and adjusted to McFarland 1.0 with TSB, TSB with 1% glucose, or TSB with 4% plasma. After 1:50 dilution, 100 μl of bacteria were placed in 96-well flat-bottom microtiter plates and then incubated at 37 °C for 24 h. The culture medium was gently removed, and the wells were washed with PBS and then air dried overnight. The biofilms were stained with 0.6% crystal violet for 1 min. To remove excess dye, the wells were washed with water twice and dried. The stained biofilms were resuspended in 100 μl of 25% acetone, and the OD595 was measured.

H_2O_2 killing assay and human whole blood killing assay

The overnight cultures were washed with PBS and adjusted to an OD600 of 1.5 (the approximate cell density was 10^9 cells/ml). The cells were incubated with 0.75% H_2O_2 at 37 °C for 1 h for the H_2O_2 killing assay. For the whole blood killing assay, the cells were incubated with 30% serum at 37 °C for 30 min. A total of 10^4 CFU in 100 μl were mixed with 300 μl of human blood treated with heparin as an anticoagulant and then incubated at 37 °C for 2.5 h. Before plating, 300 μl of 1% Triton X-100 was added. After treatment and incubation, serially diluted cells were plated on TSB agar media, and the plates were incubated overnight at 37 °C for both the H_2O_2 killing assay and the human whole blood killing assay. Both assays were evaluated on the basis of colony-forming ability of the cells.

Hemolytic activity

The hemolytic activity assay was performed as previously described.[38] The supernatant of 500 μl of overnight cultures was collected, and an equal volume of 2% rabbit RBCs (rRBCs) in PBS was added to 96-well V-bottom plates. After 15 min of incubation at 37 °C, the rRBCs were pelleted at 1000 rpm for 5 min and the supernatant fluid was measured according to the OD410. Triton X-100 (1%) was used as a positive control, while PBS with 2% bovine serum albumin served as a negative control. A hemolytic unit (HU) was defined as the reciprocal of the highest dilution that exhibited complete hemolysis.

Statistical analysis

The statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software Inc.; San Diego, CA, USA). A Mann-Whitney *U* test was used to compare the differences in fitness and virulence between the t1081 isolates and non-t1081 isolates. A probability of $p < 0.05$ was considered statistically significant.

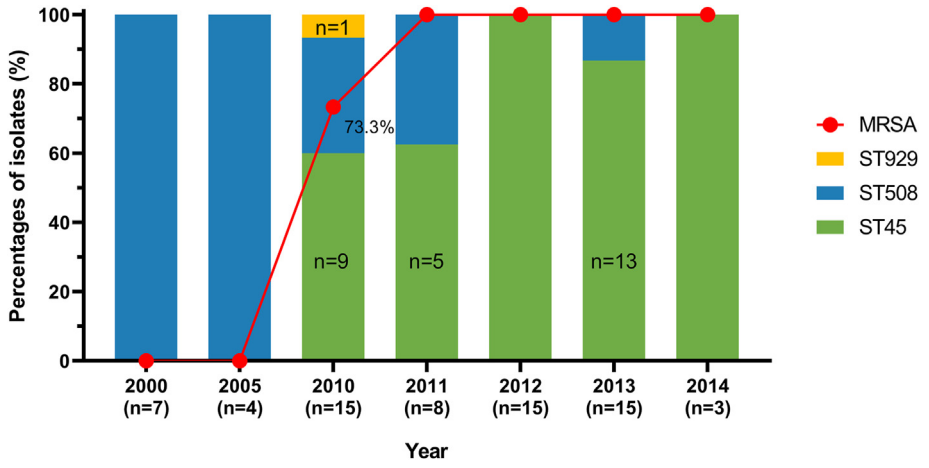

Figure 1. Changes in the proportions of STs and MRSA isolates among CC45 blood isolates over time. The proportions of sequence types are displayed in a 100% stacked bar chart. The line chart shows the percentage of MRSA isolates.

Results

Lineages of CC45

Among the 67 CC45 isolates, three STs, namely, ST45 (n = 44), ST 508 (n = 21) and ST929 (n = 1), were found. The ST45 isolate has become predominant since 2010 and was not detected in 2000 or 2005, when only the ST508 isolate was found. The ST508 isolate was last detected in 2013 (Fig. 1 and Table S1). There was only one ST929 isolate found in 2010. Three *spa* types were found in the ST45 isolates, most of which belonged to t1081, except for one t026 isolate and one isolate of unknown type (UT2, which is one nucleotide different from t1081). The *spa* types found in ST508 were diverse, with a total of 8 different types, including an unknown type, UT1. The predominant *spa* types in ST508 were t015 (38.1%, 8/21) and t073 (23.8%, 5/21). Only one *spa* type, t589, was found in ST929. As a result, among *S. aureus* isolates from blood cultures in Taiwan, the proportion and number of ST45 isolates increased, whereas those of the ST508 isolates decreased significantly after 2010. This phenomenon was mainly due to a significant increase in the number of t1081 isolates.

SCC*mec* and *agr* types

According to the results of the antibiotic susceptibility tests and detection of the *mecA* gene, 77.6% (52/67) of the isolates were MRSA (Table 1). Of these, 84.6% (44/52) were ST45, and 15.4 % (8/52) were ST508 (Table 1). The only ST929 isolate was MSSA. Moreover, all but one of the ST45 isolates were MRSA. The *spa* types of the MRSA ST45 isolates were t1081, t026, and UT2, whereas the *spa* types of the MRSA ST508 isolates were t015, t073, t550, t772, and t2171 (Fig. S1). As the percentage of ST45 increased, the percentage of MRSA increased from 0% in 2000 and 2005 to 73.3% (11/15) in 2010 and reached 100% in 2011–2014 (Fig. 1). Additionally, the composition of the ST508 isolates shifted from MSSA to MRSA. Two SCC*mec* types were found among these 53 MRSA isolates. Most of the MRSA ST45 isolates carried SCC*mec* V_T (93.2%, 41/44), whereas the rest carried SCC*mec* IV (6.8%, 3/44). All the MRSA ST508 isolates carried SCC*mec* IV. Moreover, the results of *agr* typing revealed that all the ST508 and ST929 isolates belonged to *agr*-I. However, two types, *agr*-I and *agr*-IV, were found among the ST45 isolates. The only *agr*-I-ST45 isolate was t026, and this isolate contained SCC*mec* IV. Combining the results, four types of isolates were found among the MRSA ST45 isolates: t1081/SCC*mec* V_T/*agr*-IV (n = 39), t1081/SCC*mec* IV/*agr*-IV (n = 3) UT2/SCC*mec* V_T/*agr*-IV (n = 1) and t026/SCC*mec* IV/*agr*-I (n = 1). Among the MRSA ST45 isolates, the combination of SCC*mec* and *agr* types in t026 (SCC*mec* IV/*agr*-I) was distinct from that in t1081 (SCC*mec* V_T/*agr*-IV was the majority) but similar to that in ST508 (SCC*mec* IV/*agr*-I).

Phylogenetic analysis

MLVA was performed on all 67 isolates to further understand the phylogenetic relationships among the different *spa* types of the CC45 isolates. The MLVA results can be divided into two major clusters: Cluster 2, consisting of t1081 and

Table 1 Prevalence of MSRA, *agr* types and SCC*mec* types among CC45 isolates.

ST (No.)	No. of MRSA	*agr* type (No.)	SCC*mec* type (No.)
ST45 (45)	44 (97.8%)	*agr*-I (1), *agr*-IV (44)	SCC*mec* IV (4), SCC*mec* V_T (40)
ST508 (21)	8 (38.1%)	*agr*-I (21)	SCC*mec* IV (8)
ST929 (1)	0 (0%)	*agr*-I (1)	–
Total (67)	52 (77.6%)	*agr*-I (23), *agr*-IV (44)	SCC*mec* IV (11), SCC*mec* V_T (41)

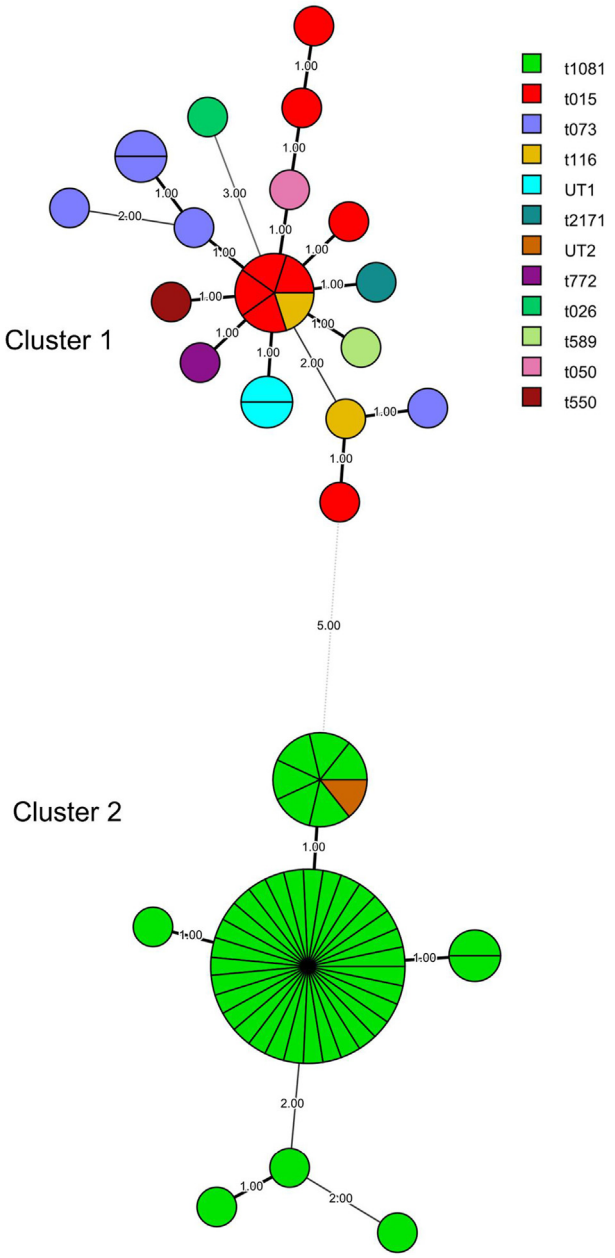

Figure 2. Minimum spanning tree of 67 CC45 isolates based on 10 VNTR loci. Each circle represents an MLVA type, with the size corresponding to the number of isolates sharing that identical MLVA type. The numbers on the lines connecting different MLVA types represent the number of VNTRs that differ between them. Each color represents a different *spa* type. UT, unknown type.

UT2; and Cluster 1, containing the remaining 10 *spa* types (Fig. 2 and Fig. S1). In Cluster 2, 31 t1081 isolates, including the MSSA and MRSA isolates, were located in the core. UT2 was located in a subcluster with six t1081 isolates, and its MLVA code differed from that of the isolates located in the core of Cluster 2 by only one repeat. Three t1081 isolates carrying SCC*mec* IV were clustered together, distinct from the core of Cluster 2 (carrying SCC*mec* V_T in the case of MRSA), with a difference of two to four loci. The isolates in Cluster 1 were more diverse, with the core consisting of four t015 isolates and one t116 isolate. Isolates of the same *spa* type, such as t015 and t073, may be distributed across different subclusters. Moreover, although classified as ST45, similar to the t1081 isolates, the t026 isolate clustered with non-ST45 isolates in Cluster 1, confirming the dissimilarity between t026 and t1081.

To understand the genomic evolutionary relationships of t1081 and t026, as well as non-ST45 isolates, 14 isolates from different STs were randomly selected for WGS. These included seven t1081 isolates, three t015 isolates, two t073 isolates, one t026 isolate, and one t589 isolate. The single nucleotide polymorphism (SNP)-based phylogenetic trees were similar to those of MLVA, with the isolates divided into two major branches (Fig. 3). All the t1081 isolates were in close branches, whereas the t026 isolates of ST45, t073 and t015 isolates of ST508, and t589 isolates of ST929 isolates were located in nearby branches. These results showed that t026 was closer to ST508 and ST929 than to t1081.

Detection of virulence factors

Given the elevated prevalence of the t1081 lineage, detection of common virulence factors that may contribute to pathogenesis was performed. The results are shown in Table 2 and Fig. S1. The *sei*, *sem* and *seo* genes were present in all 67 isolates, whereas none of the isolates contained the *sea*, *seb*, *sed*, *see*, *sek*, *lukM* or *pvl* genes. Nearly all the isolates carried the *seg* gene, except for seven that did not. The *seh* gene was detected in most of the isolates, except for one ST45 isolate and five ST508 isolates. In contrast, the *lukDE* gene was found in only a small number (n = 6) of the t1081 isolates. The *sen* gene was found in all the ST508 and ST929 isolates, but some t1081 isolates either did not carry it (n = 7) or had it truncated by IS elements (n = 10). Notably, only the t1081 and UT2 isolates contained the *sej* gene, whereas only the t026, ST508 and ST929 isolates carried the *sec* gene. We found once again that t026 was relatively similar to ST508 and ST929. As a result, the isolates were separated into two groups, t1081 and non-t1081, for the following experiments.

Fitness and virulence analysis

To further understand whether t1081 has a clinical advantage, we selected some isolates for fitness and virulence analysis. The results of the growth curve under different conditions are displayed in Figs. 4 and S2. There was no significant difference in the growth of the t1081 and non-t1081 isolates under normal culture conditions (37 °C, pH 7.4), high temperatures (42 °C, pH 7.4), acidic environments (37 °C, pH 3.5), or in the serum. For biofilm formation, whether induced with 1% glucose or 4% plasma, there was no significant difference between the two groups (Fig. 5A and B). The results of the H_2O_2 killing assay indicated that the resistance of the isolates to oxidative stress was similar (Fig. 5C). Although the survival rate of the non-t1081 isolates was greater than that of the t1081 isolates in the human whole blood killing assay, there was no statistically significant difference between the two groups (Fig. 5D). However, the hemolytic activities of the t1081

Figure 3. Phylogenetic tree of 14 isolates based on SNPs in genomes. The light and dark green background colors represent the ST45 isolates, the light and dark blue background colors represent the ST508 isolates, and the yellow background color represents the ST929 isolates. Four ST45 isolates from other countries, CA-347 (t004, CP006044) from the USA, 198 (t015, CP077925) from Germany, C331 (t026, CP127657) from Australia, and SA0907 (t1081, CP121204) from China, were included for comparison.

isolates were significantly greater than those of the non-t1081 isolates, including the t026 isolates (Fig. 5E). The higher hemolytic activities may give the t1081 isolates an advantage in causing infections.

Discussion

In this study, early CC45 isolates from blood cultures in Taiwan were primarily ST508 and were MSSA. Since 2010, ST45 isolates have become predominant, and the proportion of MRSA has significantly increased. Among the ST45 isolates, the t026 isolates presented greater similarities with the ST508 and ST929 isolates in terms of molecular typing, genomic analysis, and virulence factor genes than did the t1081 isolates. Moreover, t1081 isolates exhibited stronger hemolytic activity than did t026 isolates, as well as the ST508 and ST929 isolates.

Effelsberg et al. collected ST45 strains from various regions worldwide for evolutionary analysis.[15] They concluded that different branches of ST45 (*agr*-I and *agr*-IV) emerged, each acquiring distinct SCC*mec* fragments over time, leading to regional differences. Their study found that ST45 originated in Europe and subsequently branched into different lineages. One branch acquired SCC*mec* II when it spread to the USA, forming USA600, whereas another branch in Australia acquired SCC*mec* V, and SCC*mec* IV entered the ST45 genome multiple times. However, the number of Asian strains in this study was insufficient to clarify the situation in Asia. A phylogenetic analysis performed by Huang et al., in 2021 categorized the MRSA ST45 isolates in Taiwan into two clades: clade I and clade II.[27] They concluded that clade I, which carried mainly SCC*mec* IV and was more similar to isolates from the USA, was predominantly found in immigrant populations. Clade II, which carried mainly SCC*mec* V and was more similar to isolates from Singapore, was found primarily in patients and healthcare workers. We analyzed the *spa* types of the isolates in the study by Huang et al. and found that all the isolates belonging to clade I were t026 (data not shown). Moreover, the *sec* gene was found only in all clade I isolates, and the *sej* gene was found only in all clade II isolates. In this study, all the t1081 and UT2 isolates carried the *sec* gene, whereas the t026, ST508, and ST929 isolates did not; the opposite was true for the *sej* gene (Table 2). The situation is similar in both cases. Another phenotypic analysis of MRSA ST45 isolates from cellulitis (CL-MRSA) and osteomyelitis (OM-MRSA), conducted by Peng et al., in 2024, also divided those isolates into two major groups, I and II.[24] The isolates in Group I were closely related to those from Singapore and Australia, whereas the isolates in group II were closely related to those from the USA and Europe. All the OM-MRSA isolates were t1081/SCC*mec* V/Group I. There were two clones of CL-MRSA isolates: t026/SCC*mec* IV/Group II and t1081/SCC*mec* IV/Group I. Our clustering results were similar to those of the above two studies. We also showed that t026 was significantly different from t1081 in terms of the genome. Additionally, we further confirmed the similarity of t026 with ST508 and ST929.

ST508 is a single variant of ST45 and is one of the main nasal carriage MSSA clones in Taiwan.[25] It was also reported as an MRSA clone, even in nasal carriage or infection.[12,18,21–23] A phylogenetic analysis based on the repeats of *spa* types indicated that the t015/ST508 isolates were distinct from the t1081/ST45 and t026/ST45 isolates.[18] However, in pulsed-field gel electrophoresis (PFGE), the t015/ST508, t550/ST508, and t026/ST45 isolates were grouped into the same pulsotype, AK, whereas the t1081/ST45 isolates were located in another pulsotype, BM.[21] In our study, phylogenetic analysis based on both MLVA and SNPs revealed that the t026 isolate was in the same cluster as ST508 and ST929 (Figs. 2 and 3). We hypothesize that for CC45, a highly divergent clone, *spa* typing and MLST may not accurately reflect its evolutionary relationships. According to the findings of Effelsberg et al., ST45 isolates with different *agr* types might have appeared around 1917 and 1945.[15] The t026 and ST508 isolates in the present study were *agr*-I, whereas the t1081 isolates were *agr*-IV. Considering the similarity between ST45 and ST508 in MLST, our study suggested that ST508 may have evolved

Table 2 Distribution of virulence factors genes of different lineages among CC45 isolates.

ST/*spa* type	No. of isolates carrying different virulence genes (%)														
	sea	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*sej*	*sek*	*sem*	*sen*	*seo*	*lukDE*	*lukM*	*pvl*
ST45 (45)	0 (0)	0 (0)	1 (2.2)	0 (0)	0 (0)	38 (84.4)	44 (97.8)	45 (100)	44 (97.8)	0 (0)	45 (100)	28 (62.2)	45 (100)	6 (13.3)	0 (0)	0 (0)
t1081 (43)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	36 (83.7)	42 (97.7)	43 (100)	43 (100)	0 (0)	43 (100)	26[a] (60.5)	43 (100)	6 (14.0)	0 (0)	0 (0)
t026 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
UT2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
ST508 (21)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	21 (100)	16 (76.2)	21 (100)	0 (0)	0 (0)	21 (100)	21 (100)	21 (100)	0 (0)	0 (0)	0 (0)
t015 (8)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	8 (100)	7 (87.5)	8 (100)	0 (0)	0 (0)	8 (100)	8 (100)	8 (100)	0 (0)	0 (0)	0 (0)
t073 (5)	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	5 (100)	3 (60)	5 (100)	0 (0)	0 (0)	5 (100)	5 (100)	5 (100)	0 (0)	0 (0)	0 (0)
t116 (2)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	2 (100)	2 (100)	0 (0)	0 (0)	2 (100)	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)
UT1 (2)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	2 (100)	2 (100)	0 (0)	0 (0)	2 (100)	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)
t050 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
t550 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
t772 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
t2171 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
ST929 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
t589 (1)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
Total (67)	0 (0)	0 (0)	23 (34.3)	0 (0)	0 (0)	60 (89.6)	61 (91.0)	67 (100)	44 (65.7)	0 (0)	67 (100)	50 (74.6)	67 (100)	6 (9.0)	0 (0)	0 (0)

[a] An additional 10 isolates had the *sen* gene truncated by insertion sequences (IS).

from the ST45/*agr*-I clone. However, this hypothesis needs to be confirmed by further studies.

There was a t1081 MSSA isolate (CL16) in this study (Table 1 and Fig. S1), which has been rarely reported previously. The results of phylogenetic analysis indicated that this isolate was closely related to other t1081 isolates. The difference between CL16 and the closest isolate in PFGE was two bands, with the *orfX* gene located within these bands, as confirmed by Southern blotting (data not shown). This suggests that CL16 may have originated from an MRSA isolate that lost the SCC*mec* fragment and became MSSA. A similar situation has been reported in other studies, where t1081 MSSA isolates and MRSA isolates have similar PFGE patterns.[39] T026 MSSA was reported in a study on nasal carriage and community-onset infection MSSA, and its PFGE pattern was related to that of ST508.[25] In our study, both t026 and ST508 carried SCC*mec* IV. According to the conclusion of Effelsberg et al. that the ST45 population acquired SCC*mec* IV multiple times,[15] it is possible that ST508 evolved from a t026-related clone, with both clones subsequently acquiring SCC*mec* IV fragments separately.

In addition to differences in methicillin resistance, ST45 isolates exhibit a higher rate of ciprofloxacin resistance (41/45, 91.1%), whereas all ST508 and ST929 isolates are ciprofloxacin-susceptible (data not shown). This finding is consistent with previous literature reporting high rates of ciprofloxacin resistance among ST45 isolates.[40] Notably, the four ciprofloxacin-susceptible ST45 isolates include one t026 isolate and three t1081 isolates carrying SCC*mec* IV.

Peng et al. established that OM-MRSA ST45 isolates (with nine t1081 isolates) had greater biofilm formation ability and higher cellular infectivity and caused more severe diseases in mice than CL-MRSA ST45 isolates (with four t026 and two t1081 isolates) did.[24] In our study, there was no significant difference in biofilm formation ability and between the t1081 and non-t1081 (t026, ST508, and ST929) isolates.

The t1081 isolates in our study displayed higher hemolysis activity than the other isolates did. The hemolysis activity of *S. aureus* is caused by the production of several exotoxins, such as α-hemolysin (Hla), β-hemolysin (Hlb), γ-hemolysin (Hlg), δ-hemolysin (Hld), phenol-soluble modulins (PSMs) and some leukocidins.[41] These exotoxins are associated with the pathogenicity of *S. aureus*.[42] The *hla*, *hlg*, *hld* and *psms* genes can encode pore-forming toxins and the expression of these genes is regulated by the *agr* quorum-sensing system.[43] The *hlb* gene is usually truncated by the ϕSa3int family prophage, which can encode several types of immune escape cluster (IEC) genes. The expression of the *hlb* gene is regulated by phage regulatory switches.[44] The mechanisms underlying the high hemolytic activity among the t1081 isolates require further investigation.

There were several limitations in our study. First, because the stock of isolates did not include isolates from 2001 to 2004 and 2006—2009, we were unable to observe continuous changes over time. Second, there was only one t026 isolate in our collection, so the characteristics it exhibited may not represent the overall profile of the t026 isolates. Third, we selected only a subset of isolates for fitness and virulence analysis, and did not include all the isolates or *spa* types. Finally, without reviewing patients' medical histories or using animal models, we cannot

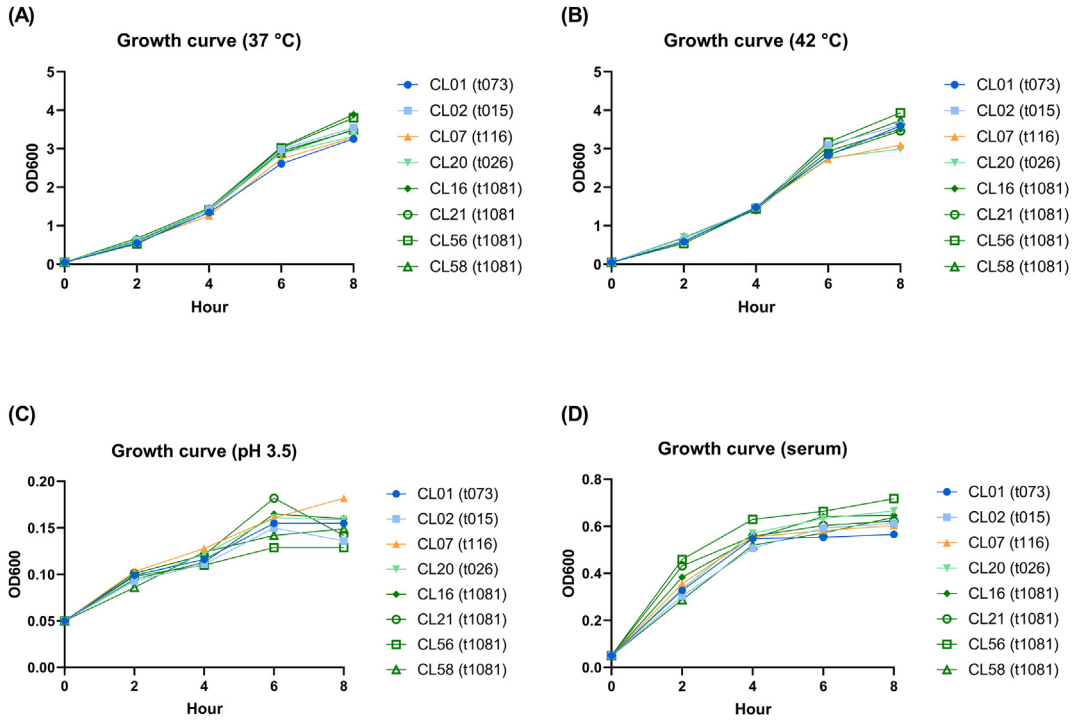

Figure 4. Growth curves of selected isolates under different conditions. Isolates were incubated: (A) in tryptone soy broth (TSB) at 37 °C and pH 7.4 (B) in TSB at 42 °C and pH 7.4 (C) in TSB at 37 °C and pH 3.5 (D) in serum at 37 °C. The light and dark green symbols represent the ST45 isolates, the light and dark blue symbols represent the ST508 isolates, and the yellow symbols represent the ST929 isolates.

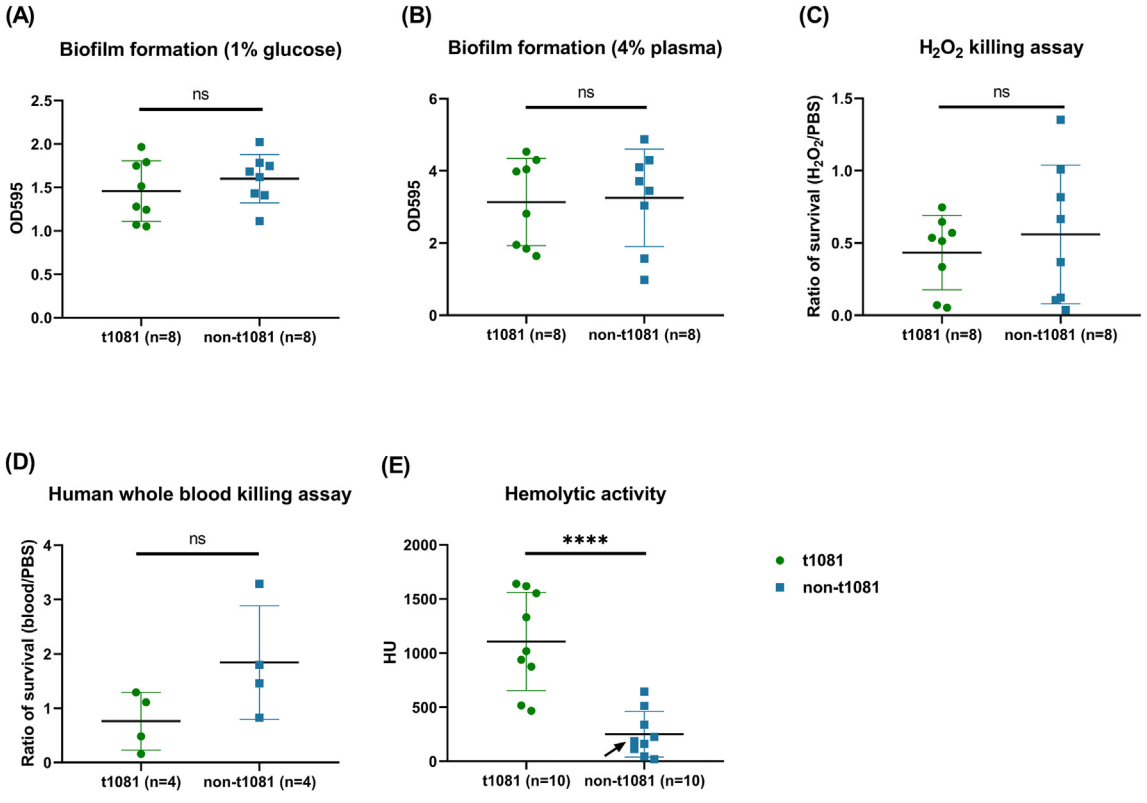

Figure 5. Fitness and virulence analysis of selected isolates. (A) Biofilm formation induced by 1% glucose. (B) Biofilm formation induced by 4% plasma. (C) H_2O_2 killing assay. (D) Human whole blood killing assay. (E) Hemolytic activity. The green symbols represent t1081 isolates, and the blue symbols represent nont-1081 isolates. The arrow indicates the hemolytic activity of the t026 isolate. ns, not significant. ****, $p < 0.0001$.

determine whether the specific characteristics of t1081 isolates in terms of virulence factors and hemolytic activity are reflected in their clinical manifestations.

Conclusions

We found that the increase in the number of MRSA CC45 isolates causing bacteremia was due primarily to the increasing proportion of t1081/ST45 isolates since 2010 and the transformation of ST508 from MSSA to MRSA. Additionally, the evolutionary and virulence characteristics of the t026/ST45 isolates were more similar to those of the ST508 isolates. Furthermore, we observed that the t1081 isolates presented stronger hemolytic activity than the other CC45 isolates did, which may explain why the t1081 isolates presented a greater infection advantage.

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CRediT authorship contribution statement

Yu-Tzu Lin: Writing — review & editing, Writing — original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chun-Li Lee:** Writing — original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Chin-Yun Lin:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Tai-Fen Lee:** Resources, Methodology, Investigation. **Po-Ren Hsueh:** Writing — review & editing, Supervision, Resources.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.08.012.