Original

Molecular characterization of *Staphylococcus epidermidis* bloodstream isolates from two hospitals in Tokyo

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ABSTRACT

Dissemination of multidrug-resistant *Staphylococcus epidermidis*, especially methicillin-resistant *S. epidermidis* (MRSE), with enhanced pathogenicity is a serious global public health concern. We characterized the antimicrobial/biocide susceptibility and virulence of 105 *S. epidermidis* bloodstream isolates from two hospitals in Tokyo to expand on the limited information available in Japan.

The phenotypic and genetic features of antimicrobial resistance, biocide tolerance, SCC*mec* type, and biofilm development or adhesion were analyzed.

In total, 76 (72.4%) isolates were identified as MRSE, which showed higher resistance rates to most antimicrobial classes, except for vancomycin, than methicillin-susceptible *S. epidermidis* (MSSE). MRSE was classified into 16 sequence types (STs), including the most prevalent ST2, which is a global high-risk *S. epidermidis* clone. In addition, our MRSE isolates possessed higher rates of *qacA/B* than the MSSE isolates, resulting in a higher tolerance to the three low-level antiseptics compared to MSSE isolates. Among these, ST6 and ST2 isolates showed higher *qacA/B* positivity rates. Furthermore, SCC*mec* type IV was predominant in the MRSE isolates. MRSE isolates possessed *aap* and IS*256* more frequently than MSSE isolates. Moreover, *sesI* and *icaA* were found in the ST2 isolates. The proportion of biofilm producers in MRSE tended to be higher than that in MSSE, and strong biofilm producers were concentrated in ST2 isolates among the four predominant STs.

Collectively, our findings provide the first evidence that highly virulent, multidrug-resistant *S. epidermidis* isolates, including the global ST2 MRSE lineage, may have already spread and persisted for a long time in healthcare facilities in Japan.

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Key Words
Staphylococcus epidermidis, methicillin resistance, *mecA*, biocide tolerance, biofilm, sequence type

I. Introduction

Staphylococcus epidermidis is the most common species of coagulase-negative staphylococci (CoNS) and a normal human commensal bacterium in the skin and mucous membranes. Although it is an important opportunistic pathogen, it is also a leading cause of healthcare-associated infections (HAIs) involving medical devices such as catheters and prosthetic joints[1)2)]. In Japan, *S. epidermidis* is the most frequently identified pathogen associated

with catheter-associated bloodstream infections[3].

S. epidermidis can acquire determinants conferring antimicrobial resistance and biocide tolerance, which can be horizontally transferred to other *Staphylococcus* species[1)4)]. The recent emergence and dissemination of multidrug-resistant *S. epidermidis*, especially methicillin-resistant *S. epidermidis* (MRSE) with staphylococcal cassette chromosome *mec* (SCC*mec*), has become a global concern because of the difficulty in treating infections, prolonged hospitalization, and increased healthcare costs[5)]. In addition, *S. epidermidis* has enhanced pathogenicity due to acquired virulence factors, such as biofilm development and adherence, and has been isolated globally[2)6)]. Furthermore, among several global hospital-adapted HAI-associated *S. epidermidis* lineages, sequence type (ST) 2 of clonal complex 2 (CC2) has been one of the most prevalent MRSEs in recent decades [2)5)7)].

Although these factors may have contributed to the MRSE lineage becoming a globally successful clone in healthcare facilities, this phenomenon is not yet fully understood. Despite the frequent isolation of HAIs globally, knowledge regarding the phenotypic and genomic characteristics, such as antimicrobial resistance or virulence, of *S. epidermidis* isolates from Japan is still limited. Therefore, in this study, we aimed to clarify the profiles of antimicrobial and biocidal susceptibilities, biofilm formation, and genetic features, including the SCC*mec* type, of *S. epidermidis* bloodstream isolates from Japan.

II. Methods

A. Bacterial samples and growth conditions

A total of 105 non-duplicate *S. epidermidis* isolates recovered from at least two sets of positive blood cultures per patient, collected between 2007 and 2015 (n = 43) at the Tokyo Metropolitan Toshima Hospital and between 2014 and 2015 (n = 62) at the Tokyo Medical and Dental University Hospital, were used in this study. Microbial identification was confirmed using a MALDI Biotyper (Bruker Daltonics, Karlsruhe, Germany). The *S. epidermidis* strains were grown at 37℃ under aerobic conditions on tryptone soya agar plates and tryptone soya broth (Oxoid, Hampshire, United Kingdom).

B. Antimicrobial susceptibility testing and minimal inhibitory concentrations of biocides

Minimal inhibitory concentrations (MICs) of 12 antimicrobials (penicillin, oxacillin, ampicillin, cefazolin, imipenem, gentamicin, erythromycin, clindamycin, minocycline, levofloxacin, vancomycin, and sulfamethoxazole/trimethoprim), were determined by microdilution using commercial plates (Eiken Chemical, Tokyo, Japan). The detailed MIC of oxacillin in all ST2 isolates was also measured using Etest (bioMérieux Marcy-l'Étoile, France). All the results were interpreted according to the CLSI M100- ED30 guidelines.

Similarly, the MICs of chlorhexidine, benzalkonium chloride, and olanexidine gluconate were determined using the broth doubling microdilution method according to the CLSI M100-ED30 guidelines[8)].

C. Antimicrobial resistance, biocide tolerance, and biofilm development or adhesion genes screening

DNA was extracted using the Cica Geneus™ DNA Extraction Reagent (Kanto Chemical, Tokyo, Japan). *mecA* and seven biofilm development- or adhesion-associated genes (*sesI*, *icaA*, *bhp*, *aap*, *atlE*, *arcA*, and IS*256*) identified in *Staphylococcus* species were screened using PCR as previously described[9)-14)]. PCR screening was also conducted for two major multidrug efflux pump-encoding genes for biocide tolerance: *qacA/B* and *smr*[15)].

D. Multi-locus sequence typing

Multilocus sequence typing (MLST) was performed using seven loci (*arcC*, *aroE*, *gtr*, *mutS*, *pyrR*, *tpi*, and *yqiL*), as previously described[16)], with some modifications. Briefly, *aroE* was amplified with the primers aroE-F2 (5'-TCAGCACCTTGATGAACGAA-3) and aroE-R2 (5'-GAACGTATTATTCCGTACCTAGATG-3). PCR was performed using the 2 × EmeraldAmp MAX PCR Master Mix (Takara Bio, Shiga, Japan), and the products were purified and sequenced. STs were defined using the PubMLST *S. epidermidis* genome database (https://pubmlst.org/organisms/staphylococcus-epidermidis). The minimum spanning tree for genetic relatedness of the MLST data was visualized with goeBURST Full MST using PHYLOVIZ 2.0[17)].

E. PCR-based SCC*mec* typing

PCR-based SCC*mec* typing of 76 MRSE strains was performed according to previously described methods [18)19)], and the SCC*mec* type was interpreted based on the guidelines of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements[20)]. When the combined results for *mec* class and *ccr* type were not reported before, or when no *mec* or *ccr* was detected, they were classified as non-typeable.

F. Biofilm formation quantification

Biofilm formation assays were conducted in 96-well polystyrene plates (AS ONE Corporation, Osaka, Japan), as previously described[21)22)], with some modifications. Briefly, 3-h pre-cultured *S. epidermidis* isolates were inoculated into 2 ml fresh 1% glucose containing TSB (OD550 = 0.01–0.04), and then 200 μL of these cultures were transferred into each well, followed by overnight

incubation at 37℃. Following incubation, all liquids were removed and the wells were washed three times with distilled water. The plates were stained with 1% (w/v) crystal violet solution, washed three times, and dried for 10–15 minutes. Finally, 200 μL 99.5% ethanol was added to each well, and the stained biofilms were measured at 570 nm using a MULTISKAN FC (Thermo Fisher Scientific, Waltham, MA, USA). Each isolate was tested at least six times; the mean results are presented.

Biofilm formation was assessed as described previously[23]. Briefly, the isolates were categorized as strong, moderate, weak, or non-biofilm producers according to the following equations:

OD < ODC (average OD of negative control) = non-biofilm producer

ODC < OD ≤ (2 × ODC) = weak biofilm producer

(2ODC) < OD ≤ (4 × ODC) = moderate biofilm producer

(4 × ODC) < OD = strong biofilm producer.

S. epidermidis ATCC 12228, which lacks biofilm formation, was used as a negative control.

G. Statistical analysis

Categorical variables were evaluated using the chi-square test. Statistical differences were determined using the Mann-Whitney U test. $p < 0.05$ was set as the threshold of significance.

III. Results

A. Antimicrobial susceptibility profile and its relationship with *mecA* prevalence

Among 105 *S. epidermidis* isolates, 76 (72.4%) were identified as MRSE, and 29 (27.6%) were identified as methicillin-susceptible *S. epidermidis* (MSSE) (**Table 1**). All MRSE isolates harbored *mecA*, whereas the MSSE isolates did not. The resistance rates to most other antimicrobial classes were significantly higher in MRSE than

Table 1 Antibiotic susceptibility profiles of 105 S. *epidermidis* isolates

Antibiotic	MSSE (n=29)				MRSE (n=76)				p-value
	MIC (μg/mL)			%R	MIC (μg/mL)			%R	
	Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}		
Penicillin	≤ 0.03->8	2	>8	55	2->8	>8	>8	100	<0.001
Oxacillin	≤ 0.25	≤ 0.25	≤ 0.25	0	2->2	>2	>2	100	<0.001
Ampicillin	≤ 0.12->8	0.25	2	0	2->8	>8	>8	100	<0.001
Cefazolin	≤ 8	≤ 8	≤ 8	0	<4->16	≤ 8	>16	100	<0.001
Imipenem	≤ 1	≤ 1	≤ 1	0	≤ 1->8	8	>8	100	<0.001
Gentamicin	≤ 1->8	≤ 1	>8	31	≤ 1->8	>8	>8	66	<0.01
Erythromycin	≤ 0.25->4	≤ 0.25	>4	21	≤ 0.25->4	>4	>4	62	<0.001
Clindamycin	≤ 0.5-2	≤ 0.5	≤ 0.5	0	≤ 0.25->2	≤ 0.5	>2	36	<0.001
Minocycline	≤ 2	≤ 2	≤ 2	0	<1->8	≤ 2	≤ 2	4	0.278
Levofloxacin	≤ 0.5->4	≤ 0.5	4	24	≤ 0.5->4	4	>4	79	<0.001
Vancomycin	≤ 0.5-2	1	2	0	1-2	2	2	0	ND
ST	≤ 1->2	≤ 1	2	7	≤ 1->2	≤ 1	>2	26	<0.05

ST, sulfamethoxazole-trimethoprim. R, resistant. ND, not determined.

%R categorical variables were evaluated by the Chi-square test.

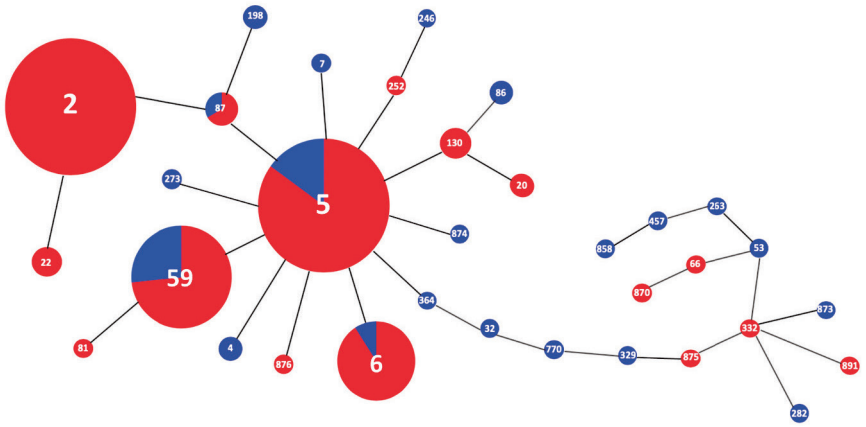

Figure 1 Minimum spanning tree based on the allelic profiles of 33 STs identified in 105 *S. epidermidis* isolates. Node sizes are proportional to the number of isolates for each ST and the numbers on connecting lines indicate the number of locus variants determined by pair-wise comparison. Red and blue areas represent MRSE and MSSE isolates, respectively.

in MSSE. No vancomycin resistance was detected in any isolate.

The 105 isolates were classified into 33 STs, including six newly assigned STs (ST870, ST873, ST874, ST875, ST876, and ST891) (**Figure 1**). Twenty-nine MSSE isolates were more diverse and were classified into 21 STs while the 76 MRSE isolates were classified into 16 STs. ST5 (n = 21, 20%) was the most prevalent of 33 STs, followed by ST2 (n = 19, 18%), ST59 (n = 15, 14%) and ST6 (n = 11, 10%). Among these four STs, MRSE isolates accounted for the largest proportion (100% for ST2, 86% for ST5, 91% for ST59, and 73% for ST6).

Table 2 Biocide susceptibility profiles of 105 *S. epidermidis* isolates

Biocides	MIC (μg/mL)				p-value
	Range	MIC_{50}	MIC_{90}	GM	
chlorhexidine					
MSSE (n=29)	≤ 0.5-2	1	2	1.05	0.001
MRSE (n=76)	≤ 0.25-4	1	2	1.44	
benzalkonium chloride					
MSSE (n=29)	≤ 0.5-8	2	8	2.48	0.004
MRSE (n=76)	≤ 1-8	4	8	4.30	
olanexidine gluconate					
MSSE (n=29)	≤ 0.5-2	2	2	1.40	<0.001
MRSE (n=76)	≤ 1-4	2	4	2.11	

GM, geometric mean of MICs. GMs were compared using the Mann-Whitney U-test.

Table 3 Prevalence of biocide resistance and biofilm development/adhesion-associated genes of 105 *S. epidermidis* isolates

	No. (%) of isolates		p-value
	MSSE	MRSE	
Biocide resistance genes			
qacA/B	3 (10.3)	39 (51.3)	<0.01
smr	12 (41.4)	33 (43.4)	0.85
Biofilm development/adhesion-associated genes			
sesI	1 (3.4)	12 (15.8)	0.086
icaA	7 (24.1)	25 (32.9)	0.38
bhp	10 (34.5)	24 (31.6)	0.78
aap	10 (34.5)	48 (63.2)	<0.01
atlE	29 (100)	76 (100)	ND
arcA	13 (44.8)	25 (32.9)	0.26
IS*256*	7 (24.1)	42 (55.3)	<0.01

ND, not determined. Categorical variables were evaluated by the Chi-square test.

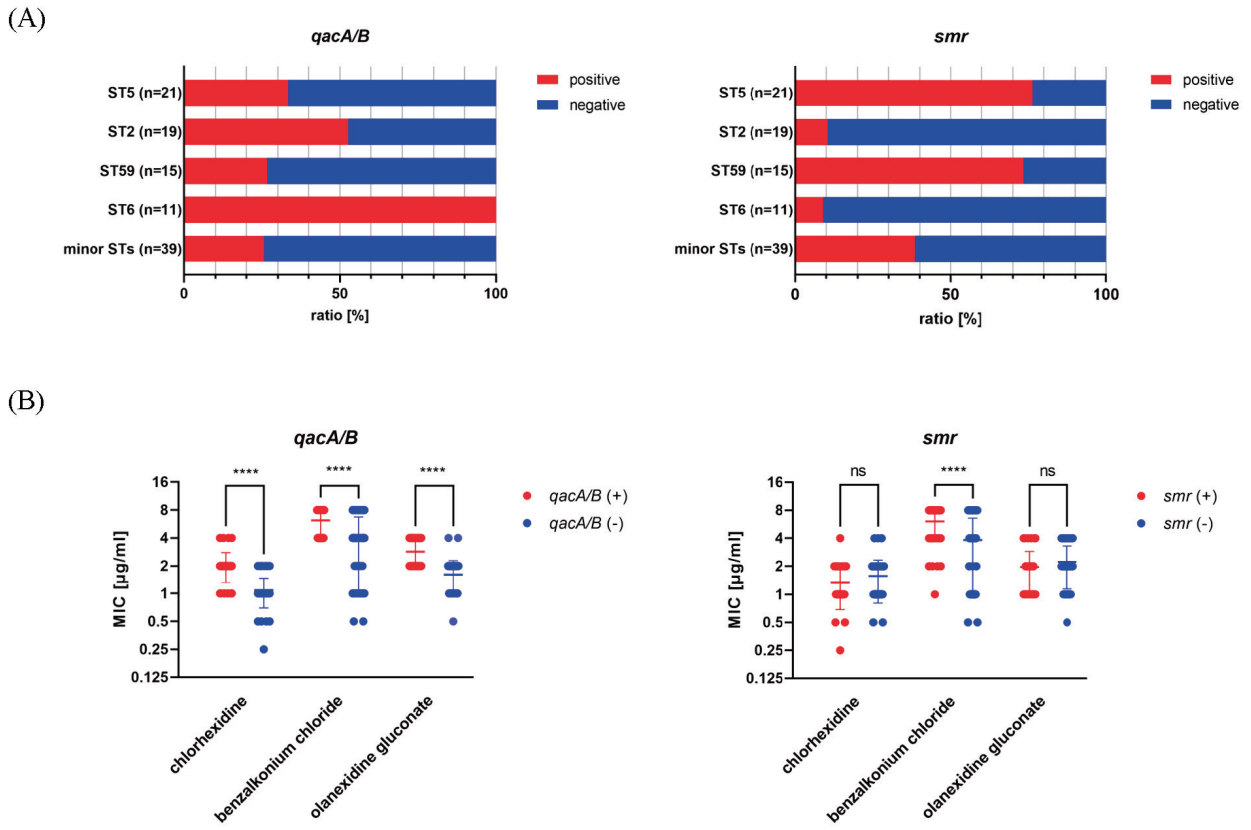

Figure 2 Prevalence of multidrug efflux pump-encoding genes and their association with biocide tolerance in 105 *S. epidermidis* isolates. (**A**) Prevalence of *qacA/B* and *smr* among each ST, and (**B**) the relationship between the presence of *qacA/B* and *smr* and three biocide MICs. Geometric means of biocide MICs were compared using the Mann-Whitney U-test. ****$p < 0.0001$.

B. Biocide MIC profiles and their association with STs and biocide tolerance genes

We investigated the relationship between the biocide MICs and STs. The geometric mean MICs of chlorhexidine, benzalkonium chloride, and olanexidine gluconate in the MRSE isolates were significantly higher than those in the MSSE isolates (**Table 2**). The prevalence of *qacA/B* was higher among the MRSE isolates (n = 39, 51%) than among the MSSE isolates (n = 3, 10%), whereas the prevalence of *smr* was similar between the MRSE and MSSE isolates (**Table 3**). ST6 and ST2 showed higher *qacA/B* positive rates of 100% and 53%, respectively, whereas the lowest frequencies of *smr* were observed in these two STs (9% and 11%, respectively) (**Figure 2A**).

The correlation between biocidal MICs and tolerance genes revealed that *qacA/B*-positive isolates showed significantly higher MICs for the three biocides than *qacA/B*-negative isolates (**Figure 2B**). However, *smr*-positive isolates exhibited significantly higher MIC for benzalkonium chloride.

C. SCC*mec* analysis and its association with STs

Our 76 MRSE isolates were classified into 15 different types of SCC*mec* including non-typeable types. Among the SCC*mec* elements detected and classified, SCC*mec* Type Ⅳ (2B) (n = 29, 38%) was predominant, followed by Type Ⅳ (2B & 5) (n = 11, 14%) (**Table 4**). However, 10 out of 15 different SCC*mec* types were classified as non-typeable SCC*mec* due to an unclassified combination of *mec* class and *ccr* type, and comprised 31 MRSE isolates (41%). Moreover, in terms of SCC*mec* types among the four major STs, the SCC*mec* types of ST6 isolates tended to be less diverse than those of the ST2, ST5, and ST59 isolates (**Table 4**).

D. Prevalence of biofilm development or adherence-associated genes

Next, we evaluated the frequency of virulence-associated genes, as *S. epidermidis* possesses several factors that are responsible for adhesion to host cells and biofilm formation. Among the MRSE strains, the frequency of *aap*, which is related to biofilm development through accumulation on polymer surfaces, and IS*256*, which is involved in biofilm production through the inactivation of *ica* operon-mediated phase variation by its insertion into *S. epidermidis*, was significantly higher than that of MSSE (**Table 3**). However, there was no significant difference in the prevalence of the other five genes between MRSE and MSSE.

sesI, which functions in cell-to-cell adhesion through the synthesis of a polysaccharide intercellular adhesin, and *icaA*, which is composed of the *ica* operon, were found in ST2 isolates (58% and 100%, respectively) (**Figure 3**). All ST2 strains were negative for *bhp*, a cell-wall anchored protein, whereas higher frequencies were found in ST5 and ST59 (52% and 67%, respectively). There were higher frequencies of *aap* among the three major strains, ST5, ST2, and ST59, whereas the prevalence of *arcA*, which encodes an arginine catabolic mobile element responsible for facilitating staphylococcal colonization of

Table 4 SCC*mec* profiles and its association with STs

SCC*mec*	*ccr*	*mec*	total MRSE (n=76)	Sequence type				
				ST2 (n=19)	ST5 (n=18)	ST59 (n=11)	ST6 (n=10)	other STs (n=18)
Type II	2	A	2	0	1	0	0	1
Type III	3	A	3	3	0	0	0	0
Type Ⅳ (2B)	2	B	29	1	4	8	9	7
Type Ⅳ (2B&5)	2, 5	B	11	4	3	1	0	3
Type XIV (5A)	5	A	1	1	0	0	0	0
Non-typeable 1	5	B	1	1	0	0	0	0
Non-typeable 2	2, 4	B	1	0	0	1	0	0
Non-typeable 3	2, 5	C	4	0	0	0	0	4
Non-typeable 4	3, 5	A	8	7	1	0	0	0
Non-typeable 5	4, 5	C	2	0	1	0	0	1
Non-typeable 6	2, 3, 5	A	1	1	0	0	0	0
Non-typeable 7	2	ND	7	0	7	0	0	0
Non-typeable 8	2, 5	ND	1	0	1	0	0	0
Non-typeable 9	ND	A	3	1	0	0	0	2
Non-typeable 10	ND	B	2	0	0	1	1	0

ND, not detected.

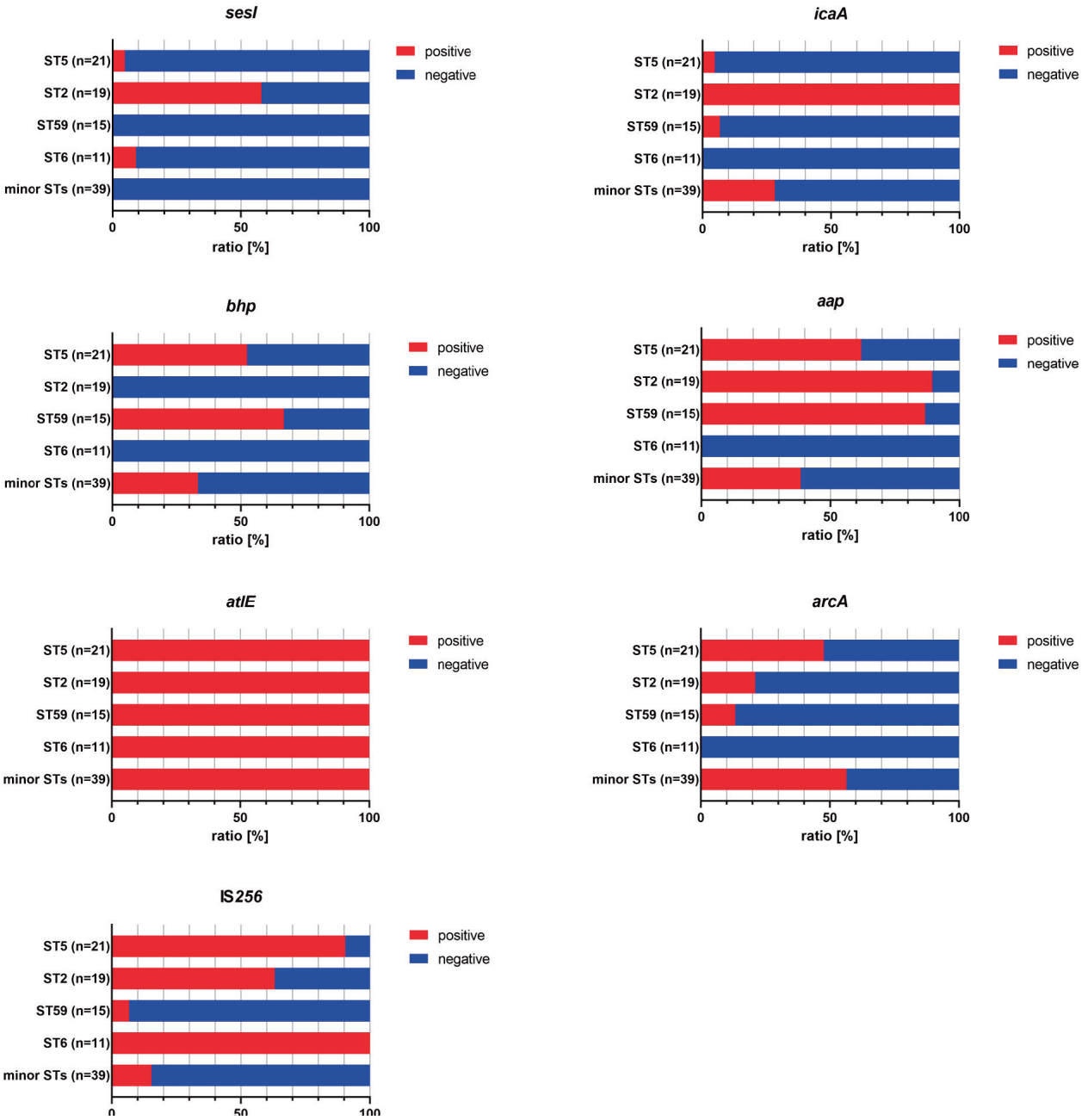

Figure 3 Prevalence of major biofilm development- or adhesion-associated genes among each ST. *sesI*, *icaA, bhp*, *aap, atlE*, *arcA*, and IS*256* identified in *Staphylococcu*s species were screened in 105 *S. epidermidis* isolates, as described in section C of Methods.

the skin and mucous membranes, was higher in the minor STs than in the three major STs. *atlE*, which mediates attachment to the polystyrene surface, was present in all isolates. Interestingly, ST6 was 100% positivity for IS*256* and 100% negativity for *icaA*, *aap*, *bhp*, and *arcA*.

E. Biofilm development ability

We further characterized the biofilm phenotypes of the 105 *S. epidermidis* isolates. Although the prevalence of strong producers in MRSE strains tended to be higher than that in MSSE, the total ratio of isolates with weak to strong biofilm formation was not significantly different between MRSE and MSSE ($p > 0.05$, **Figure 4A**).

Strong producers were concentrated in ST2 isolates among the four predominant ST groups but were also found in other STs (**Figure 4B**). Interestingly, all ST6 and many ST59 isolates were categorized as non-biofilm producers, indicating that there were different distributions of biofilm phenotypes among each ST.

IV. Discussion

S. epidermidis clinical isolates tend to be multidrug-resistant, with oxacillin resistant-MRSE ranging from

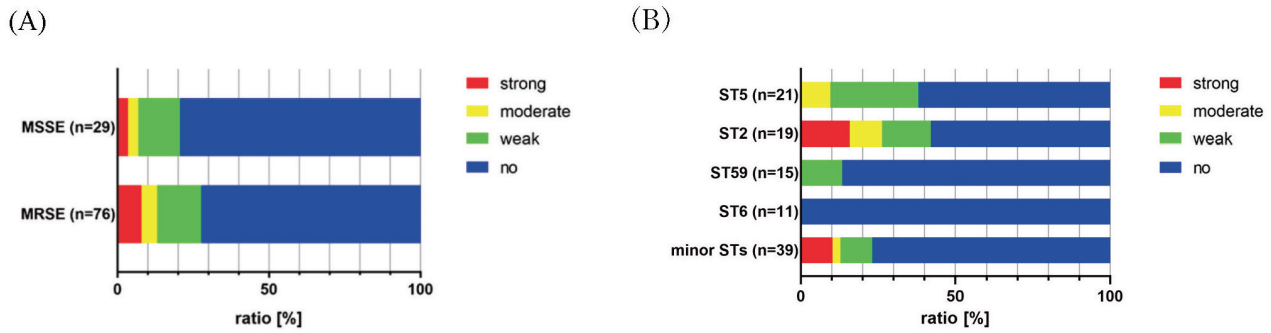

Figure 4 Biofilm development ability of 105 *S. epidermidis* isolates. (**A**) Prevalence of each biofilm phenotype in MSSE and MRSE isolates, and (**B**) relationship between the biofilm phenotype and STs. The phenotype was categorized, as described in section D of Methods.

70–90% prevalence, including data from nationwide surveillance in Japan[1)3)24)]. Our results agree with this, highlighting that MSSE remains highly susceptible to several antimicrobials and that vancomycin is still recommended for treating MRSE infections. Our findings support the need to monitor antimicrobial resistance to effectively treat *S. epidermidis* infections.

S. epidermidis, especially MRSE, is a major reservoir of antimicrobial and biocidal resistance determinants[4)]. In this study, MRSE isolates possessed higher rates of *qacA/B* than MSSE isolates and were associated with a higher tolerance to three low-level antiseptics. These results, coupled with those of previous studies on *Staphylococcus* species[2)25)], demonstrate that antimicrobial and antiseptic co-resistant/tolerant isolates may be spreading in Japanese hospitals. However, as QacA/B and Smr cannot functionally exhaust many intermediate-level antiseptics/disinfectants that are often used in healthcare facilities, such as ethanol[26)], from inside bacterial cells, appropriate antiseptics/disinfectants may be efficient in eliminating antiseptic/disinfectant-tolerant isolates.

Methicillin-resistant CoNS, including *S. epidermidis*, possesses multiple SCC*mec* elements, resulting in diverse structural variants[19)27)]. Consistent with previous reports[19)27)], our results demonstrated that diverse SCC*mec* elements, including SCC*mec* type IV, were predominant, suggesting that CoNS in Japan may constitute a reservoir of SCC*mec* with methicillin resistance. Moreover, a previous study reported that SCC*mec* islands could evolve through site-specific recombination at this locus during adaptation to the human host[28)]. Therefore, we may need to characterize SCC*mec* islands at the whole-genome level to clarify the global diversification of *S. epidermidis* used in this study.

We demonstrated that MRSE isolates frequently possessed the biofilm-forming or adherence-associated genes *aap* and IS*256*; these higher rates did not result in significant differences in phenotypic biofilm formation between MRSE and MSSE. This may be because major biofilm development- or adhesion-associated genes were not fully upregulated in the planktonic cells used in this study or because other determinants regulating the biofilm production process were present in our isolates. However, coupled with the results of antimicrobial/antiseptic co-resistance/tolerance, our findings suggest that highly pathogenic multidrug-resistant *S. epidermidis* isolates have already been disseminated in Japan. In addition, a previous study from China demonstrated that ST2 isolates were exclusively *ica*- and IS*256*-positive and biofilm-forming[6)]. Our results showed a similar tendency, indicating for the first time that ST2 MRSE isolates might have higher pathogenicity among the four major STs in our isolates. Extracellular biofilms inhibit the action of most antimicrobials, thereby impairing the treatment of *Staphylococcus* medical-device infections. This indicates that the consistent application of infection prevention measures at an appropriate point, such as the periprocedural period, is crucial, in concordance with previous studies[2)13)]. Moreover, previous studies have demonstrated that exopolysaccharides, such as polysaccharide intercellular adhesins, protect *S. epidermidis* from host defense mechanisms such as neutrophil killing[1)29)]. Therefore, further investigation is needed to elucidate the relationship between STs, prevalence of virulence factors, and pathogenicity.

Among global high-risk *S. epidermidis* clones, ST2 belongs to the CC2 strain, which is the most widespread healthcare-associated strain[2)5)6)]. However, there are limited reports focusing on the genetic features of ST2 MRSE, none of which are from Japan. The results of our MLST analysis provide novel insights that multidrug-resistant *S. epidermidis* CC2 isolates, including ST2 and ST5, may already be predominant and have adapted to healthcare facility environments in Japan. Further de-

tailed genetic analysis using whole-genome sequencing may contribute to the understanding of genetic diversity and features that remain unidentified in antimicrobial resistance, virulence, and pathogenicity in CC2 MRSE healthcare-associated strains in Japan.

The current study had some limitations. For example, the number of clinical isolates collected from the two hospitals was small, and they were not compared with the isolates from healthy participants, and detailed information such as the isolated numbers and wards of this organism involved in horizontal transmission in each hospital was not investigated.

V. Conclusion

Highly virulent, multidrug-resistant *S. epidermidis* isolates have emerged in Japan, complicating infection prevention and treatment. Our findings provide novel insights and reveal that the global ST2 MRSE clone and its close genetic lineages may have already been disseminated and have persisted for a long time in Japanese healthcare facilities. Therefore, robust surveillance systems and infection control and prevention measures based on continuous monitoring of their lineages are crucial for their elimination.

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Disclosures

We declare that we have no conflicts of interest. All authors read and approved the final manuscript.

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