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Genetic Diagnosis of Community-Acquired MRSA: A Multiplex Real-Time PCR Method for Staphylococcal Cassette Chromosome mec Typing and Detecting Toxin Genes

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Methicillin-resistant *Staphylococcus aureus* (MRSA) causes a wide range of infections in health care settings and community environments. In particular, community-acquired MRSA (CA-MRSA) is important for clinicians because many fatal cases in healthy populations have been reported. Staphylococcal cassette chromosome mec (SCCmec) is a mobile genetic element and carries the central determinant for broad-spectrum beta-lactam resistance encoded by the *mecA* gene. The emergence of MRSA is due to the acquisition and insertion of the SCC*mec* element into the chromosome. CA-MRSA is characterized as SCCmec type IV. Thus, we aimed to establish a novel multiplex real-time PCR method to distinguish SCCmec type, which enables us to evaluate the pathogenicity of MRSA. A total of 778 MRSA were isolated at Nagasaki University Hospital from 2000 to 2007. All isolates were subjected to minimal inhibitory concentration testing and PCR for SCCmec typing and detecting genes of toxins: *tst* (toxic shock syndrome toxin 1), *sec* (encoded enterotoxin type c), *etb* (exfoliative toxin type b), and *lukS/F-PV* (Panton-Valentine leukocidin). PCR was performed to amplify a total of 10 genes in the same run. The 667 MRSA clones detected from pus in 778 clones were classified as SCCmec type II (77.7%), type IV (19.2%), and type I (3.0%). 87.5% of SCCmec type II clone had *tst* and *sec* genes. No isolate was *lukS/F-PV* positive. The present study indicates the high rate of *lukS/F-PV*-negative SCCmec type IV in Nagasaki. Our PCR method is convenient for typing MRSA and detecting toxins in Japan.

Keywords: CA-MRSA/SCCmec type/pathogenicity/real-time PCR/rapid typing
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The widespread emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), particularly in nosocomial infections, is a serious clinical problem worldwide. MRSA causes various infections, for example, skin and soft tissue infections, blood-borne infections, and pneumonia (Gemmell et al. 2006). It is difficult to cure these infections because MRSA is multiple-drug resistant. MRSA strains emerge as the significant clinical problem around the world, and studies are moved ahead with the epidemiological analysis of typing of MRSA clones to mark a trend (Francois et al. 2004).

Staphylococcal cassette chromosome mec (SCCmec) typing analyzes a mobile genetic element, SCCmec, which contains the *mecA* gene encoding methicillin resistance. Until now, SCCmec can be classified into 8 types according to size and composition. MRSA is characterized as SCCmec type IV, which has recently emerged within the community setting of some countries. Numerous community-associated (CA)-MRSA infections have been seen in young, healthy populations without predisposing risk factors (Bonnstetter et al. 2007). As represented by the USA300 clone, CA-MRSA strains typically carry the Panton-Valentine leukocidin (*lukS/F-PV*) genes, which produce cytotoxins that cause leukocyte destruction and tissue necrosis (Genestier et al. 2005; Bonnstetter et al. 2007). These *lukS/F-PV* genes are also known to be involved in severe diseases, such as skin abscess formation, furunculosis and necrotizing pneumonia (Lina et al. 1999; Bonnstetter et al. 2007). In fact, there has been a particular increase in the number of reported *lukS/F-PV*-positive strains associated with severe necrotizing community-acquired pneumonia worldwide.

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In contrast, the NY/Japan clone is characterized as SCCmec type II and carries enterotoxin C (*sec*) and toxic shock syndrome toxin 1 (*tst*) genes that have been circulating among hospitals in Japan. Moreover, two *lukS/F-PV*-positive MRSA strains were isolated in 2003 and 2004 from Japanese hospitals (Takizawa et al. 2005), emphasizing the necessity to monitor *lukS/F-PV*-positive MRSA clones for preventing the spread of infection. SCCmec typing is useful for characterizing CA-MRSA clones. However, as previous methods are complicated, clinicians need a more rapid and simple detection system for CA-MRSA.

In this study, we therefore investigated genotypic characteristics of MRSA strains isolated in our hospital using SCCmec typing into four major types and analyzed various toxins based on antimicrobial susceptibility patterns to establish a convenient system for clinical application.

Materials and Methods

Clinical isolates

A total of 778 MRSA strains from our stock libraries isolated between January 2000 and December 2007 at the clinical microbiology laboratory of Nagasaki University Hospital were analyzed. Of these MRSA strains, 667 were isolated from pus (2000-2007), 93 from sputum (2006) and 18 from blood (2006). All strains were identified according to standard biochemical identification methods using standard culture media. Strains with an oxacillin MIC of ≥ 4 μg/ml were considered to be MRSA strains. Of the strains collected each year, those isolates from the same patient were excluded. As negative controls for the *mecA* and nuclease (*nuc*) genes (for identification of *S. aureus*), 10 methicillin-sensitive *S. aureus* (MSSA) strains and 10 coagulase-negative staphylococci (CNS) strains were used. As the *lukS/F-PV* and *vanA* genes and the SCCmec type III elements are rare in Japan, the following strains were used as positive controls in this study: NIpvl (*lukS/F-PV*), BM4147 (*vanA*) and 85/2028 (SCCmec type III element). NIpv was kindly provided by Prof. Yamamoto (Niigata Univ.), BM4147 (vancomycin-resistant-enterococci) was kindly provided by Reiko Kariyama (Okayama Univ.), and 85/2028 was kindly provided by Prof. Hiramatsu (Juntendo Univ.).

After strains were cultured overnight on blood-agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), bacterial DNA was extracted using Chelex (Bio-Rad Laboratories, Hercules, CA, USA), methanol, and boiling methods. Briefly, 200 μl of 10% Chelex solution and 100 μl of 250 U/ml achromopeptidase solution were placed in a 1.5-ml microcentrifuge tube and then mixed well with a colony of bacteria before incubation at 37-56°C for at least 15 min. The mixture was vortexed and incubated at 99°C for an additional 5 min and then chilled on ice for at least 1 min. After centrifugation at 12,000 rpm for 1 min, the supernatant was transferred to another 1.5-ml microcentrifuge tube and used for PCR amplification.

Susceptibility tests

MICs were determined by a broth microdilution assay according to Clinical and Laboratory Standard Institute reference methods. For susceptibility tests, we used custom-made frozen plates manufactured by Eiken Chemical Co. (Tokyo, Japan). Plates included nine antimicrobial agents: oxacillin (MPIPC; Sigma-Aldrich Japan Co., Tokyo, Japan), cefazolin (CEZ; Nichi-iko Pharmaceutical Co., Ltd., Toyama, Japan), cefmetazole (CMZ; Daiichi Sankyo Co., Ltd., Tokyo, Japan), cefpirome (CPR; Astellas Pharma Inc., Tokyo, Japan), imipenem (IPM; Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), gentamicin (GM; Schering-Plough K.K., Osaka, Japan), minocyclin (MINO; Takeda Pharmaceutical Co. Ltd., Osaka, Japan), levofloxacin (LVFX; Daiichi Sankyo Co., Ltd., Tokyo, Japan), and vancomycin (VCM; Shionogi & Co., Ltd., Osaka, Japan). Inoculum was adjusted to yield a cell density of 5×10^5 CFU/ml. Plates were incubated for about 24 h at 36°C and examined by visual observation. MIC was defined as the lowest concentration of antibiotic at which visible growth of the organism was completely inhibited.

Real-time PCR assay

PCR was performed using a LightCycler 480 (Roche Applied Science, Mannheim, Germany) to amplify a total of 10 genes in the same run. The PCR assay targeted SCCmec types I-IV, *nuc*, *mecA*, *vanA*, *tst*, *sec*, exfoliative toxin type b (*etb*) and *lukS/F-PV* (Table 1). For SCCmec typing, three gene elements were used, *SCCmec I*, *SCCmec II-III* and *SCCmec I-II-IV*. *SCCmec I* was specific for SCCmec type I, *SCCmec II-III* for SCCmec types II and III, and *SCCmec I-II-IV* for SCCmec types I, II and IV. The four major SCCmec types were easily distinguished (Table 2): SCCmec type I isolates were positive for *SCCmec I* and *SCCmec I-II-IV*; SCCmec type II isolates were positive for *SCCmec II-III* and *SCCmec I-II-IV*; SCCmec type III isolates were positive for *SCCmec II-III*; and SCCmec type IV isolates were positive for *SCCmec I-II-IV*.

The original PCR primers and probes were designed based on published primers(Johnson et al. 1991: Zhang et al. 2004; McClure et al. 2006), and sequence specificity was confirmed against all available data for genes in the GenBank database. All primers and probes were purchased from Nihon Gene Research Laboratories Inc. (Miyagi, Japan). The target probes for *SCCmec I-II-IV*, *mecA*, *vanA*, *tst* and *lukS/F-PV* were labeled at the 5´-end with FAM, and target probes for *SCCmec I*, *SCCmec II-III*, *nuc*, *etb* and *sec* were labeled at the 5´-end with LCRED610. Both sets of probes were labeled at the 3´-end with Black Hole Quencher (BHQ). The only four types of the eight known SCCmec types were detected in this study because SCCmec type V-VIII are not spread in Japan.

Reactions were performed in a 20-μl mixture of 0.5 μM primers, 0.2 μM TaqMan probes, 5 μl DNA template and 2x LightCycler 480 Probes Master (Roche Applied Science, Mannheim, Germany). Two target genes were detected in each well using the multicolor detection system. Gene pairs are shown in Table 1. Reaction conditions were 95°C for 10 min for activation of Taq polymerase, followed by 35 cycles of 10 s at 96°C (denaturation) and 50 s at 60°C (annealing and extension). Continuous fluorescence was monitored at 483-533 nm (FAM) and 550-610 nm (LCRED610). Fluorescence data were analyzed using LightCycler480R Software version 1.5.

Comparison of results with published PCR data

The multiplex PCR system for SCCmec typing established (Oliveira and de Lencastre 2002) was used for comparison of detection results. After isolates were characterized in our SCCmec typing system, 20 isolates of SCCmec type I, 30 isolates of SCCmec type II and 30 isolates of SCCmec type IV (total: 80 isolates) were chosen at random from the 776 MRSA isolates and used for comparison with published PCR data. Similarly, *mecA*, *tst*, *sec* and *etb* detection results were also compared with previous PCR data.

Table 1. List and characteristics of oligonucleotides used in the PCR assays.

primer and probe	sequence(5´-3´)	location	amplified product size (bp)	5´ dye	GeneBank accesion no.	PCR reaction mixture no.
mecA-S	atg tgg aag tta gat tgg ga	454 - 473				
mecA-AS	ttg gaa cga tgc cta tct c	598 - 616	163	FAM	AB221121	a
mecA-Taq	cca gga atg cag aaa gac caa agc	490 - 423				
SCC I-S	gag ttg ctg atg aag aag g	18401 - 18419				
SCC I-AS	ttt acc aca agg act acc ag	18872 - 18891	491	LC610	AB033763	a
SCC I-Taq	acc att act cat gca gct gaa aca cat	18424 - 18450				
SCC II III-S *	atc aag act tgc att cag gc	42428 - 42447				
SCC II III-AS *	gcg gtt tca att cac ttg tc	42617 - 42636	209	LC610	D86934	b
SCC II III-Taq	caa cga ctt gat tgt ttc ctc tgt ttt c	42488 - 42515				
SCC I II IV-S	tgc ttc cac ttc ctt gag	37905 - 37922				
SCC I II IV -AS *	cat cct atg ata gct tgg tc	37992 - 38011	107	FAM	B033763	b
SCC I II IV -Taq	ttc gct gtc ttc ctg tat ttc gtc tat gct	37959 - 37988				
nuc-S	tta cat aaa gaa cct gcg ac	225 - 244				
nuc-AS	gat gct ttg ttt cag gtg	328 - 345	121	LC610	EF529596	c
nuc-Taq	caa agg tca acc aat gac att cag act	287 - 313				
vanA-S	agg tct gtt tga att gtc cg	268 - 287				
vanA-AS	gca acg atg tat gtc aac g	342 - 360	93	FAM	EF206285	c
vanA-Taq	atc cct ttt gta ggc tgc gat att ca	290 - 315				
etb-S	acg gct ata tac att caa ttc	51 - 71				
etb-AS	ttg gat tat tag ggt att agg t	194 - 215	165	LC610	M17348	d
etb-Taq	tgg ata ttg tag aat gtg tca tgg tta tta cc	90 - 121				
tst-S	act ggt ata gta gtg ggt ctg a	152 - 173				
tst-AS	ggg cta taa taa gga ctc gg	262 - 281	130	FAM	AY074881	d
tst-Taq	agg ctg atg ctg cca tct gtg ttt	228 - 251				
sec-S	agg cga taa gtt tga cca at	700 - 719				
sec-AS	cat tct ttg ttg taa ggt gg	784 - 803	104	LC610	AB084256	e
sec-Taq	acg gtt gat tct aaa agt gtg aag ata gaa g	752 - 782				
lukS/F-PV-S	aaa tgt ctg gac atg atc c	590 - 608				
lukS/F-PV-AS	ctg tat tgg ata gca aaa gc	985 - 1004	415	FAM	AB245454	e
lukS/F-PV-Taq	aat ccg aga gac tat ttt gtg tca gac	643 - 669				

*These primers were not original. **All of probes were labeled at the 3´-end with BHQ (Black Hole Quencher).

Table 2. The four major SCCmec types were readily distinguishable.

Type of SCCmec	Detection of gene		
	SCCmec I	*SCCmec II III*	*SCCmec I II IV*
SCCmec typeI	+	–	–
SCCmec typeII	–	+	+
SCCmec type III	–	+	–
SCCmec typeIV	–	–	+

Results

Comparison of results with published PCR data

To evaluate our PCR system, we compared the detection results obtained in this study with those obtained from a previously published PCR method for SCCmec typing. These results, as well as those for detection of *nuc*, *mecA*, *tst*, *sec* and *etb*, were consistent with published data.

We used 10 MSSA and 10 CNS isolates as negative controls for *mecA* and *nuc*. The 10 MSSA isolates were negative for *mecA*, and the 10 CNS isolates were negative for *nuc*. Positive controls for *lukS/F-PV*, *vanA* and SCCmec type III element were positive using our PCR system.

Analysis of 778 MRSA isolates

The 667 MRSA isolated from pus were classified into

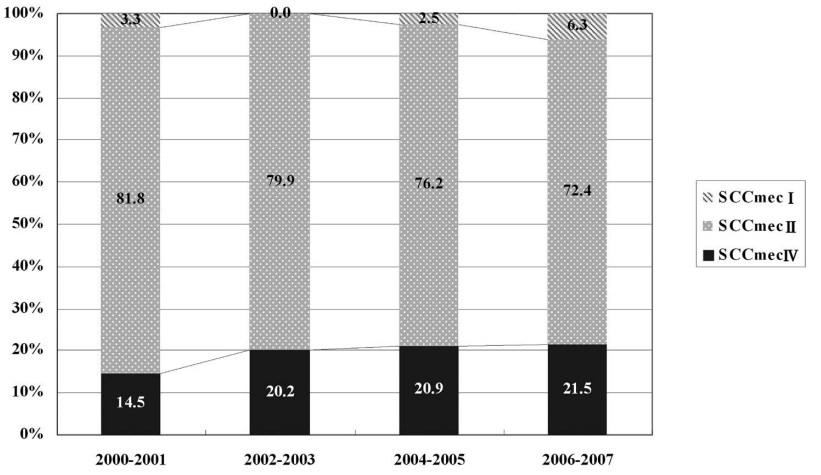

Fig. 1. Transition of SCCmec types of MRSA isolated from pus.
The transition of SCCmec types among MRSA isolates from pus over the 8-year period (2000 to 2007). Type II isolates decreased from 81.8% to 72.4%, while type IV isolates increased from 14.5% to 21.5%.

Table 3. Virulence genes in MRSA isolated from pus.

	etb	*lukS/F-PV*	*tst*	*sec*	/Total
2000-2001	3	0	148	145	/186
2002-2003	6	0	129	124	/155
2004-2005	4	0	134	134	/164
2006-2007	3	0	120	116	/162
Total	16 (2.2%)	0 (0%)	531 (79.6%)	519(77.8%)	/667

Table 4. MRSA isolates from pus having *sec* and *tst* between 2000 and 2007.

Year	Isolates of MRSA having *sec* + and *tst*+ /*total* of MRSA in each type		Total
	SCCmec II	SCCmec IV	
2000-2001	132/152	12/27	144/179
2002-2003	106/124	18/31	124/155
2004-2005	114/125	19/35	133/160
2006-2007	101/117	13/35	114/152
Total (%)	453/518 (87.5)	62/128 (48.4)	515/646 (79.7)

SCCmec type II (77.7%), type IV (19.2%) and type I (3.0%). Among MRSA isolates from 2006, those from pus comprised 3.5% SCCmec type I, 72.1% type II and 24.4% type IV; those from sputum comprised 4.3% type I, 80.6% type II and 15.1% type IV; and those from blood comprised no type I (0%), 83.3% type II and 16.7% type IV.

With regard to the transition of SCCmec types among MRSA isolates from pus over the 8-year period (2000 to 2007), type II isolates decreased from 81.8% to 72.4%, while type IV isolates increased from 14.5% to 21.5% (Fig. 1).

Detection of virulence genes is shown in Table 3. Sixteen isolates, all from pus, were *etb* positive. The *etb* gene was detected in 18.7% of type II and 81.3% of type IV isolates. No isolates were *lukS/F-PV* positive in this study. A total of 87.5% of type II and 48.4% of type IV isolates had the *tst* and *sec* genes (Table 4). Isolates from sputum (2006) possessing the *sec* and *tst* genes comprised 97.3% type II and 7.1% type IV, while those from pus comprised 88.5% type IV and 33.3% type II. Taken together, SCCmec type II isolates possessing *sec* and *tst* accounted for the majority of MRSA isolates from sputum, while MRSA isolates from pus had a wider variety of characteristics.

Susceptibility markers (MIC50 and MIC90) evaluated for each agent are shown in Table 5. Overall, the MICs for cephalosporin, IPM and LVFX among type IV isolates were lower than those for type II. In particular, isolates showing an MIC of $< 2\ \mu$g/ml for IPM comprised 10.4% type II and 85.9% type IV.

Table 5. MICs for SCCmec types II and IV.

	SCCmec II			SCCmec IV		
	Range	90%	50%	Range	90%	50%
MPIPC	8 - ≥ 32	≥ 32	≥ 32	4 - ≥ 32	≥ 32	≥ 32
CEZ	1 - ≥ 32	≥ 32	≥ 32	1 - ≥ 32	≥ 32	8
CMZ	8 - ≥ 32	≥ 32	≥ 32	2 - ≥ 32	16	8
CPR	≤ 0.5 - ≥ 32	≥ 32	≥ 32	1 - ≥ 32	≥ 32	2
GM	≤ 0.5 - ≥ 32	≥ 32	≤ 0.5	≤ 0.5 - ≥ 32	≥ 32	≥ 32
MINO	≤ 0.5 - 16	16	≤ 0.5	≤ 0.5 - ≥ 32	≤ 0.5	≤ 0.5
LVFX	≤ 0.5 - ≥ 32	≥ 32	≥ 32	≤ 0.5 - ≥ 32	8	≤ 0.5
VCM	≤ 0.5 - 4	2	1	≤ 0.5 - 2	1	1
IPM	≤ 0.5 - ≥ 32	≥ 32	≥ 32	≤ 0.5 - ≥ 32	2	≤ 0.5

MPIPC, oxacillin; CEZ, cefazolin; CMZ, cefmetazole; CPR, cefpirome; GM, gentamicin; MINO, minocyclin; LVFX, levofloxacin; VCM, vancomycin; IPM, imipenem.

Discussion

We established a convenient system for SCCmec typing that is also capable of detecting pathogenic genes. We selected four pathogenic genes for this study. The first of these was *etb*, as it encodes exfoliative toxins (ETs), which are known to cause skin and soft tissue infections such as bullous impetigo, abscesses, furunculosis and staphylococcal scalded skin syndrome (SSSS). Much attention is now focused on *etb* because it is widespread in CA-MRSA in Japan (Noguchi et al. 2006).

The second gene investigated in this study was *tst*, as it is present in *S. aureus* isolates, causing toxic shock syndrome in humans and animals. In Japan, *tst* is a commonly detected gene found in the NY/Japan clone, emphasizing the importance of identifying MRSA isolates with *tst* through Japanese epidemiologic studies.

The third gene investigated was *sec*. In addition to *tst*, *sec* is also present in the NY/Japan clone. Thus, to determine the type of clone, we must test for *sec*. Enterotoxins belong to a family of superantigens (Mehrotra et al. 2000) and are expressed by *S. aureus*, making it an important food-borne pathogen.

The final target gene was *lukS/F-PV*, as it encodes Panton-Valentine leukocidin, which has emerged in CA-MRSA strains. This exotoxin is involved in recurrent skin and soft tissue infections and lethal necrotizing pneumonia (Gillet et al. 2002). Detection of *lukS/F-PV* is essential in epidemiologic studies of MRSA outbreaks worldwide.

Vancomycin-resistant *S. aureus* (VRSA) has also been reported in various parts of the world, and such reports are increasing in frequency (Tiwari and Sen 2006). Detection of VRSA is important because infection by VRSA is difficult to treat. We therefore tested for the *vanA* gene using our PCR system.

The appearance of endemic CA-MRSA has recently been reported (Francois et al. 2004). In contrast to hospital-associated (HA)-MRSA, CA-MRSA is frequently isolated from healthy people. In addition, CA-MRSA tends to lead to serious and fatal outcomes. CA-MRSA isolates generally carry the SCCmec type IV element, which is much smaller than the SCCmec type I, II and III elements (Francois et al. 2004). It is thought that the SCCmec type IV element is prone to insertion into the MSSA in the community setting.

In Japan, about 4% of MRSA clones are classified as SCCmec type IV (Chongtrakool et al. 2006; Zaraket et al. 2007). However, in our study, we detected 19.2% to be SCCmec type IV, mostly isolated from hospitalized patients. We also observed numerous cases of nosocomial infection. As it has been reported that CA-MRSA was brought into hospitals from the community (Zaraket et al. 2007), we believe that SCCmec type IV was also brought into and spread throughout our hospital. The transition of SCCmec types of MRSA from pus between 2000 and 2007 indicates that SCCmec type IV has become more prevalent over the past 8 years in Nagasaki, and it is likely to continue increasing.

Under the selective pressure caused by extensive antibiotic use, MRSA strains predominant in the early 1980s appear to have been replaced by those carrying the SCCmec type II element, which is associated with several resistance genes, in response to environmental changes (Ma et al. 2006). On the other hand, SCCmec type IV clones are more sensitive than SCCmec type II clones, as CA-MRSA has a lower chance of exposure to antibiotics. In fact, our study showed differences in MIC50 and MIC90 with several agents, including cephem antibiotic, LVFX, and especially IPM. As MRSA is sensitive to IPM, it is more likely to be SCCmec type IV, indicating that IPM MIC is useful for SCCmec typing. In contrast, none of the MRSA strains detected from 2000 to 2007 possessed *vanA* or showed a VCM MIC of $> 8\ \mu$g/ml.

In Japan, no VRSA has been reported. Recently detected HA-MRSA clones in Japan are considered to be the NY/Japan clone, which is one of the major MRSA clones worldwide. The NY/Japan clone is characterized by a sequence type 5, SCCmec type II element, and possessing the *tst* and *sec* genes (Zaraket et al. 2007). In the present

study, we frequently detected MRSA clones both characterized as SCCmec type II and positive for *tst* and *sec* (70.1%). We assumed that these clones were the NY/Japan clones. However, 48.4% of SCCmec type IV MRSA isolates were also positive for *tst* and *sec*; of these, 33.3% were from pus in 2006 and 7.1% were from sputum (Table 5). In contrast, SCCmec type II isolates considered to be NY/Japan clones were detected more frequently in sputum than in pus.

A recent study reported that almost all *etb*-positive CA-MRSA strains isolated from outpatients with impetigo and SSSS carried SCCmec type IV elements (Noguchi et al. 2006). In this study, 81.2% of *etb*-positive strains were SCCmec type II, indicating that, currently, HA-MRSA clones frequently carry *etb*.

Although *lukS/F-PV* was not detected in this study, *lukS/F-PV*-positive MRSA strains are known as high-pathogenic CA-MRSA. Few strains have been detected in Japan; however, it is feared that high-pathogenic CA-MRSA may take hold throughout the world.

Our results indicate changing trends in the common forms of MRSA (CA-MRSA and HA-MRSA). Clear differences were seen between SCCmec type and retention of toxins among the isolates over the 8-year period; however, these differences are becoming less obvious due to shortcomings in epidemiologic analysis approaches in Japan. Therefore, improving epidemiologic analysis is necessary not only in Japan but also worldwide. Identifying trends among MRSA strains may be one approach, as this will prevent the establishment of high-pathogenic CA-MRSA.

Our rapid and simple PCR system is useful for determining epidemiologic information and it can be applied to screening of unusual MRSA strains for the prevention of outbreaks of new MRSA clones.

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