Nationwide epidemiological study revealed the dissemination of meticillin-resistant *Staphylococcus aureus* carrying a specific set of virulence-associated genes in Japanese hospitals

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To study comprehensive toxin profiles and the chromosomal diversity of current Japanese hospital-associated meticillin-resistant *Staphylococcus aureus* (HA-MRSA) strains, we conducted PCR-based identification of 28 toxin genes, and staphylococcal cassette chromosome *mec* (SCC*mec*) typing and PFGE analysis of 208 MRSA strains isolated from 100 hospitals throughout Japan. Of the tested HA-MRSA strains, 80.3 % were *tst*-positive. The most frequent toxin gene profile was characterized by the carriage of 13 genes, *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hly*, *hld* and *hlg-2*. Ninety of the 208 strains had this profile, which was named pattern A. Among the 118 non-pattern A strains, 100 had similar toxin gene profiles, the concordance rates to pattern A of which were more than 80 %. Consequently, 91.3 % of the examined HA-MRSA strains carried similar toxin profiles, although PFGE patterns showed a wide variation. These strains belonged to SCC*mec* type II, *agr* II and coagulase type II. We concluded that, unlike MRSA from many other countries, most of the Japanese HA-MRSA strains belonged to, or were related to, a specific group carrying the set of 13 toxin genes, irrespective of chromosomal diversity. In addition, among the 13 toxin genes, the coexistence rates of *tst*, *sec* and *sel*, and those of *seg*, *sei*, *sem*, *sen* and *seo*, were higher than for the other toxin genes. High coexistence rates of *tst*, *sec* and *sel* genes suggested the presence of the pathogenicity island SaPI_{n1} in these strains.

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INTRODUCTION

Meticillin-resistant *Staphylococcus aureus* (MRSA) still remains the most important hospital-associated pathogen in many countries. The rate of MRSA incidence among *S. aureus* isolates has been approximately 70 % in most Japanese hospitals over the past 20 years (Kimura *et al.*, 1992; Diekema *et al.*, 2001). The exotoxin gene profiles of MRSA have been used as molecular epidemiological markers. Moreover, the profile of exotoxin genes in a strain can be a potential factor of the virulence aspect of the strain.

Abbreviations: HA-MRSA, hospital-associated MRSA; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*.

Molecular epidemiological studies of MRSA have also been conducted by using methods such as PFGE, multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. By using PFGE, Oliveira *et al.* (2002) identified five major MRSA clones. Among these clones, USA100 (New York/Japan) was characterized as a clonal complex 5:ST5-MRSA-II by MLST and SCC*mec* typing (Enright *et al.*, 2002; McDougal *et al.*, 2003). The USA100 clone includes the largest group of isolates from throughout the United States (McDougal *et al.*, 2003). These were characterized as enterotoxin gene cluster (*egc*) locus-positive and *tst*-negative (Diep *et al.*, 2006), whereas previous studies suggested that Japanese MRSA isolates which were supposed to belong to the

USA100 clone (New York/Japan clone) were *tst*-positive and *egc* locus-positive (Piao *et al.*, 2005; Zaraket *et al.*, 2007; Nishi *et al.*, 2002; Hu *et al.*, 2008), although strains from only a limited area were analysed in these studies. MLST- and PFGE-based epidemiological classification therefore is likely insufficient for the characterization of isolates regarding their virulence aspects. An investigation of toxin profiles would provide fundamental information about the possible virulence of MRSA strains. The toxin gene profiles of Japanese MRSA isolates determined in the previous studies were based on local, not nationwide, surveillance. Therefore, the results of these studies did not necessarily represent the overall toxin characteristics of Japanese MRSA isolates.

In this study, we investigated the genotypic characteristics using a PCR method with reference to 28 virulence-associated exotoxin genes and carried out chromosomal PFGE analysis of MRSA strains which were uniformly collected from hospitals located throughout Japan.

METHODS

Bacterial isolates. Two hundred and eight Japanese MRSA strains were analysed in this study. These strains were randomly collected from 100 different hospitals located throughout Japan during 2002–2004 by a nationwide surveillance study conducted by the National Institute of Infectious Diseases, Japan. In order to investigate the general features of the toxin gene carriage of Japanese MRSA strains, the 100 hospitals were selected to cover as much as possible of the area of Japan (Fig. 1). In addition, only one to four strains were collected from each hospital. Further, these strains were isolated from various clinical specimens from epidemiologically unrelated patients, which included carriers and infected patients. The specimens included blood ($n=34$), intracatheter ($n=2$), sputum ($n=78$), tracheal tube ($n=1$), pharyngeal mucosa ($n=16$), nasal mucosa ($n=13$), faeces ($n=10$), urine ($n=4$), otorrhoea ($n=10$), skin ($n=3$), pus ($n=28$), soft tissue ($n=1$), cerebrospinal fluid ($n=1$), pleural effusion ($n=1$), ascites ($n=1$), vaginal discharge ($n=1$) and others ($n=4$).

The strains were identified as MRSA in each hospital. In our laboratory, all collected strains were reconfirmed as MRSA by conventional biological tests and by the presence of the *mecA* gene using PCR with specific primers (Bignardi *et al.*, 1996).

Template preparation and PCR procedure. Template DNA of *S. aureus* was prepared as described previously (Louie *et al.*, 2000). Primers that were previously described for the *tst*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sem*, *sen*, *seo*, *edinA*, *lukPV*, *lukED*, *lukM*, *hla*, *hlb*, *hld*, *hlg* and *hlg-2* genes were used (Jarraud *et al.*, 2002). Primers for other genes were designed in this study from the sequences deposited in GenBank (NCBI) as shown in Table 1. Strains used for positive controls are listed in Table 2. The presence of the toxin genes in these positive controls of clinical origin was confirmed by PCR followed by DNA sequencing, and, when necessary, gene products were identified by proteomic analysis (Nakano *et al.*, 2002a).

PCR was performed in a 10 µl reaction mixture containing 0.4 µl template DNA, 1 µl 10 × *Taq* buffer, 0.8 µl dNTP mixture, 0.5 µM of each primer and 0.25 U *Taq* polymerase (Takara Bio). The following amplification cycles were repeated 30 times: denaturation for 30 s at 94 °C, annealing of primers for 30 s at suitable temperature for each amplification, and extension for 60 s at 72 °C. Amplified products were analysed by 1.4% agarose gel electrophoresis in 1 × TAE buffer at 135 V for 20 min. The gel was stained with ethidium bromide and

Fig. 1. Distribution map of 100 Japanese hospitals. Filled circles represent the locations of hospitals where the 208 MRSA strains were isolated.

then exposed to UV light to visualize the amplified products. When the bands were not clear, we repeated the experiment a few times to confirm the reproducibility.

Typing of *SCCmec*, *agr* and coagulase. *SCCmec* typing was performed by PCR using previously published primers that identified the class of *mec* gene complex and the type of cassette chromosome recombinase (*ccr*) gene (Ito *et al.*, 2001). The *agr* class was determined by PCR using specific primers as previously described (Gilot *et al.*, 2002). Coagulase typing was performed with a staphylococcal coagulase typing antiserum kit (Denka Seiken).

Chromosomal DNA fingerprinting by PFGE. *Sma*I macrorestriction patterns of chromosomal DNA were obtained by use of the CHEF-DR III System (Bio-Rad) with the following settings: voltage, 6 V cm⁻¹; temperature, 14 °C; initial time, 1 s; final time, 40 s; and duration, 20 h. Chromosomal patterns were examined visually, and digitally analysed with Finger Print II (Bio-Rad) to generate a dendrogram based on Dice coefficients. PFGE patterns were grouped according to the criterion of Tenover *et al.* (1995) and using a dendrogram similarity of more than 80% to assign strain relatedness (McDougal *et al.*, 2003).

RESULTS AND DISCUSSION

Carriage profile of superantigenic toxin genes in Japanese hospital-associated MRSA (HA-MRSA)

The toxin genes were detected with the aforementioned published primers and the primers that we newly designed

Table 1. Primers newly designed in this study

Gene	Primer	Sequence (5'–3')	Annealing temperature (°C)	Product length (bp)
<i>sek</i>	<i>sek</i> -f	AACTTGATGTTTTGGTATT	56.0	237
	<i>sek</i> -r	AAATGTTGTATTCTTCTTGA		
<i>sel</i>	<i>sel</i> -f	TGATACATTGTATGCTGAAT	56.0	427
	<i>sel</i> -r	GTAAAATAAATCATACGTGT		
<i>eta</i>	<i>eta</i> -f	CTATTTACTGTAGGAGCTAG	47.5	741
	<i>eta</i> -r	ATTTATTTGATGCTCTCTAT		
<i>etb</i>	<i>etb</i> -f	ATACACACATTACGGATAAT	50.5	629
	<i>etb</i> -r	CAAAGTGTCTCCAAAAGTAT		
<i>edinB</i>	<i>edinB</i> -f	CATAAAATACTCCTCTAAG	54.0	444
	<i>edinB</i> -r	GCATATTCTGTCCCTCTA		
<i>edinC</i>	<i>edinC</i> -f	TATTAAGCATTCAATCAA	44.0	626
	<i>edinC</i> -r	GTAGTCTGTTCTCTA		

in this study based on the DNA sequence databases. As shown in Table 3, the positive carriage rates of *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen* and *seo* were 76.4–94.2 %. The carriage rates of these eight genes were much higher than those of *sea* (9.6 %) and *seb* (21.2 %). The other superantigenic toxin genes, *sed*, *see*, *seh*, *sej* and *sek*, showed low carriage rates of less than 2.4 %. Two hundred and four out of 208 strains carried at least one of the 15 superantigenic toxin genes tested in this study, which corresponds to a carriage rate of 98.1 %.

Carriage profile of other toxin genes

The exfoliative toxin genes *eta* and *etb* and the epidermal cell differentiation inhibitor genes *edinA*, *edinB* and *edinC* were rarely detected. Only two strains were *eta*-positive. These two strains carried none of the superantigenic toxin genes. Pantone–Valentine leukocidin component genes *lukPV* were not detected in any of the strains. The *lukED* genes showed a high carriage rate of 96.6 %. Haemolysin genes *hla*, *hly*, *hld* and *hlg-2* variant were also frequently

detected. The γ -haemolysin gene variant *hlg-2* was dominant in Japanese MRSA strains.

Toxin patterns of HA-MRSA

Fifty-eight different toxin patterns were identified in the 208 HA-MRSA strains. The major patterns are shown in Table 4. The most frequent pattern, pattern A, was characterized by the carriage of the following 13 genes: *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hly*, *hld* and *hlg-2*. Ninety strains showed this pattern, which corresponds to 43.3 % of all tested strains. The next most frequent major patterns were patterns B, C, and D. Only one out of 28 genes was different in these three patterns compared to pattern A. Therefore, these patterns showed high concordance rates of 96.4 % to pattern A. The minor toxin patterns also showed high concordance rates. One hundred strains had concordance rates of more than 82.1 % to pattern A, which corresponds to 84.7 % of the MRSA strains other than of pattern A; i.e. 190 strains (91.3 %) carried quite similar toxin profiles.

Table 2. Strains used for positive controls

Strain	Gene	Source
MRSA N315	<i>tst</i> , <i>sec</i> , <i>seg</i> , <i>sei</i> , <i>sel</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>lukED</i> , <i>hla</i> , <i>hly</i> , <i>hld</i> , <i>hlg-2</i>	Kuroda <i>et al.</i> (2001)
<i>S. aureus</i> ATCC 27664	<i>see</i>	Sergeev <i>et al.</i> (2004)
<i>S. aureus</i> ATCC 49775	<i>lukPV</i> , <i>hlg</i>	Lina <i>et al.</i> (1999)
<i>S. aureus</i> ATCC 31890	<i>lukM</i>	Kaneko <i>et al.</i> (1997)
MRSA MF330	<i>sea</i> , <i>seb</i> , <i>seh</i> , <i>sek</i>	Clinical isolate*
MRSA GG28	<i>sed</i> , <i>sej</i>	Clinical isolate*
MRSA MH	<i>eta</i>	Clinical isolate†
MRSA NA1	<i>etb</i> , <i>edinC</i>	Clinical isolate†
MRSA C9	<i>edinA</i>	Clinical isolate‡
MRSA B11	<i>edinB</i>	Clinical isolate‡

*Food poisoning.

†Staphylococcal scalded skin syndrome.

‡Skin region of atopic dermatitis.

Fig. 2. Dendrogram of PFGE results based on the Dice coefficients and schematic representation of the *SmaI* restriction pulsotype of Japanese MRSA strains. Arrows represent strains belonging to the most frequent toxin gene pattern A group. Strain names are available from the corresponding author.

Table 3. Identification of toxin genes in Japanese MRSA strains

Pathogenic factor	Gene	MRSA (<i>n</i> =208)	
		<i>n</i>	%
Toxic shock syndrome toxin 1	<i>tst</i>	167	80.3
Enterotoxins	<i>sea</i>	20	9.6
	<i>seb</i>	44	21.2
	<i>sec</i>	168	80.8
	<i>sed</i>	2	1.0
	<i>see</i>	0	0.0
	<i>seg</i>	186	89.4
	<i>seh</i>	3	1.4
	<i>sei</i>	189	90.9
	<i>sej</i>	3	1.4
	<i>sek</i>	5	2.4
	<i>sel</i>	159	76.4
	<i>sem</i>	196	94.2
	<i>sen</i>	184	88.5
	<i>seo</i>	195	93.8
More than one of the superantigens		204	98.1
Exfoliative toxins	<i>eta</i>	2	1.0
	<i>etb</i>	0	0.0
Epidermal cell differentiation inhibitors	<i>edinA</i>	0	0.0
	<i>edinB</i>	0	0.0
	<i>edinC</i>	0	0.0
Leukocidins	<i>lukPV</i>	0	0.0
	<i>lukED</i>	201	96.6
	<i>lukM</i>	0	0.0
Haemolysins	<i>hla</i>	202	97.1
	<i>hlb</i>	176	84.6
	<i>hld</i>	203	97.6
	<i>hlg</i>	4	1.9
	<i>hlg-2</i>	202	97.1

SCCmec type, *agr* class and coagulase type of strains with major toxin patterns

The types of SCCmec, *agr* and coagulase were determined for ten strains with major toxin patterns which were selected randomly. Of ten strains, three were from toxin pattern A, two from pattern B, two from pattern C, one from pattern D, one from pattern E, and one from pattern F. We found that all strains were SCCmec type II, *agr* class II and coagulase type II. Irrespective of differences in toxin pattern, these strains belonged to a single group of SCCmec, *agr* and coagulase types. The SCCmec region and most of the toxin genes analysed in this study were supposed to be extrinsic in origin (Kuroda *et al.*, 2001; Novick, 2003). It is therefore likely that the acquisition of the SCCmec region and the toxin genes were genetically independent events.

PFGE profiles

PFGE typing was performed for all of the 208 HA-MRSA strains. Of these, 202 were successfully analysed (Fig. 2).

Table 4. Major patterns among the 58 toxin patterns in Japanese MRSA strains

+, Gene is present; blank cells, gene is absent.

Gene	Pattern [no. (%) of strains]					
	A [90 (43.3)]	B [21 (10.1)]	C [12 (5.8)]	D [7 (3.4)]	E [7 (3.4)]	F [6 (2.9)]
<i>tst</i>	+	+	+	+		+
<i>sea</i>				+		+
<i>seb</i>		+			+	
<i>sec</i>	+	+	+	+		+
<i>sed</i>						
<i>see</i>						
<i>seg</i>	+	+	+	+	+	+
<i>seh</i>						
<i>sei</i>	+	+	+	+	+	+
<i>sej</i>						
<i>sek</i>						
<i>sel</i>	+	+	+	+		+
<i>sem</i>	+	+	+	+	+	+
<i>sen</i>	+	+	+	+	+	+
<i>seo</i>	+	+	+	+	+	+
<i>eta</i>						
<i>etb</i>						
<i>edinA</i>						
<i>edinB</i>						
<i>edinC</i>						
<i>lukPV</i>						
<i>lukED</i>	+	+	+	+	+	+
<i>lukM</i>						
<i>hla</i>	+	+	+	+	+	+
<i>hlb</i>	+	+		+	+	
<i>hld</i>	+	+	+	+	+	+
<i>hlg</i>						
<i>hlg-2</i>	+	+	+	+	+	+
Concordance rate with pattern A (%)						
	—	96.4	96.4	96.4	85.7	92.9

The PFGE profiles of the 202 strains were classified into 74 PFGE types using a cut-off of 80 % similarity (McDougal *et al.*, 2003) as well as the criterion of Tenover *et al.* (1995). Among the 202 strains, 89 shown with arrows in Fig. 2 belonged to the toxin gene pattern A group and these strains were classified into 39 PFGE types, corresponding to 52.7 % of all the identified PFGE types. This indicated that the strains carrying the set of pattern A toxin genes differentiated without changing stable carriage of these toxin genes or rather that extrinsic genetic elements such as phages and plasmids which carried the toxin genes were disseminated by horizontal transmission among various HA-MRSA strains in Japan.

In a previous study, Piao *et al.* (2005) determined toxin patterns of Japanese MRSA isolated from a hospital in Tokyo. Similar studies were also conducted in other hospitals (Zaraket *et al.*, 2007; Nishi *et al.*, 2002; Hu *et al.*, 2008). These four studies commonly reported the high carriage rate of *tst*, *sec* and the *egc* locus (*seg*, *sei*, *sem*, *sen* and *seo*), although MRSA strains for the analysis in each

study were collected from hospitals within a narrow geographical area. Our study also presented similar but more conclusive results for strains collected throughout Japan. The toxin patterns that we have determined therefore could represent those of MRSA isolates from throughout Japan having various genetic backgrounds as determined by PFGE.

Coexistence of toxin genes

As shown in Table 4, while most of the major patterns had high concordance rates with pattern A, pattern E showed somewhat lower concordance. This was due to a lack of *tst*, *sec* and *sel* genes. We also observed the same for the minor toxin patterns, which are not shown in Table 4. Regardless of whether they were major or minor, the toxin patterns showing relatively lower concordance rates often lacked *tst*, *sec* and *sel* genes at the same time. Similarly, there were some strains which lacked a set of *seg*, *sei*, *sem*, *sen* and *seo* genes. In order to evaluate the coexistence of toxin genes

Table 5. Rates of coexistence among the 13 genes

Cells marked with dark-grey shading contain genes relating to SaPin1. Cells marked with light-grey shading contain genes relating to SaPin3. Unmarked cells contain genes that do not relate to these pathogenicity islands.

<i>tst</i>		<i>sec</i>		<i>sel</i>		<i>seg</i>		<i>sei</i>		<i>sem</i>		<i>sen</i>		<i>seo</i>		<i>lukED</i>		<i>hla</i>		<i>hlb</i>		<i>hld</i>		<i>hlg-2</i>	
Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%	Gene	%
<i>sel</i>	92.3	<i>sec</i>	92.8	<i>sel</i>	92.8	<i>seg</i>	96.6	<i>sei</i>	96.6	<i>sem</i>	97.6	<i>sen</i>	93.8	<i>seo</i>	97.6	<i>lukED</i>	96.6	<i>hla</i>	95.7	<i>hlb</i>	83.2	<i>hld</i>	95.7	<i>hlg-2</i>	96.6
<i>sec</i>	90.9	<i>tst</i>	90.9	<i>tst</i>	92.3	<i>sem</i>	94.2	<i>sem</i>	96.6	<i>sei</i>	96.6	<i>sem</i>	93.3	<i>sei</i>	95.2	<i>hld</i>	95.2	<i>lukED</i>	94.7	<i>sem</i>	82.7	<i>lukED</i>	95.2	<i>hld</i>	94.7
<i>sei</i>	84.6	<i>sei</i>	88.9	<i>seg</i>	84.1	<i>seo</i>	93.8	<i>seo</i>	95.2	<i>seg</i>	94.2	<i>sei</i>	92.8	<i>seg</i>	93.8	<i>hla</i>	94.7	<i>hlg-2</i>	94.2	<i>hla</i>	82.7	<i>hlg-2</i>	94.7	<i>hla</i>	94.2
<i>seg</i>	83.2	<i>seg</i>	88.5	<i>sei</i>	83.7	<i>lukED</i>	92.8	<i>sen</i>	92.8	<i>sen</i>	93.3	<i>seg</i>	92.3	<i>sen</i>	93.8	<i>seg</i>	92.8	<i>seo</i>	91.8	<i>hlg-2</i>	81.7	<i>seo</i>	92.3	<i>sem</i>	91.3
<i>seo</i>	82.7	<i>seo</i>	86.1	<i>seo</i>	81.7	<i>sen</i>	92.3	<i>lukED</i>	92.3	<i>hld</i>	91.8	<i>lukED</i>	88.9	<i>hld</i>	92.3	<i>sei</i>	92.3	<i>sem</i>	91.3	<i>seo</i>	81.3	<i>sem</i>	91.8	<i>sei</i>	90.9
<i>sem</i>	82.2	<i>sem</i>	85.6	<i>sem</i>	80.3	<i>hlg-2</i>	90.4	<i>hlg-2</i>	90.9	<i>hla</i>	91.3	<i>hlg-2</i>	87.5	<i>hla</i>	91.3	<i>seo</i>	91.3	<i>sei</i>	89.9	<i>lukED</i>	81.3	<i>sei</i>	89.4	<i>seo</i>	90.9
<i>lukED</i>	81.7	<i>sen</i>	85.6	<i>sen</i>	80.3	<i>hld</i>	88.9	<i>hla</i>	89.9	<i>hlg-2</i>	91.3	<i>hld</i>	87.0	<i>lukED</i>	91.3	<i>sem</i>	90.9	<i>seg</i>	88.5	<i>seg</i>	79.8	<i>seg</i>	88.9	<i>seg</i>	90.4
<i>sen</i>	81.3	<i>lukED</i>	82.2	<i>lukED</i>	78.8	<i>sec</i>	88.5	<i>hld</i>	89.4	<i>lukED</i>	90.9	<i>hla</i>	86.5	<i>hlg-2</i>	90.9	<i>sen</i>	88.9	<i>sen</i>	86.5	<i>sei</i>	79.3	<i>sen</i>	87.0	<i>sen</i>	87.5
<i>hlg-2</i>	81.3	<i>hld</i>	81.3	<i>hlg-2</i>	77.4	<i>hla</i>	88.5	<i>sec</i>	88.9	<i>sec</i>	85.6	<i>sec</i>	85.6	<i>sec</i>	86.1	<i>sec</i>	82.2	<i>hlb</i>	82.7	<i>sen</i>	78.8	<i>hlb</i>	83.2	<i>hlb</i>	81.7
<i>hla</i>	80.3	<i>hla</i>	80.8	<i>hld</i>	76.9	<i>sel</i>	84.1	<i>tst</i>	84.6	<i>hlb</i>	82.7	<i>tst</i>	81.3	<i>tst</i>	82.7	<i>tst</i>	81.7	<i>sec</i>	80.8	<i>sec</i>	73.1	<i>sec</i>	81.3	<i>tst</i>	81.3
<i>hld</i>	78.8	<i>hlg-2</i>	80.8	<i>hla</i>	76.4	<i>tst</i>	83.2	<i>sel</i>	83.7	<i>tst</i>	82.2	<i>sel</i>	80.3	<i>sel</i>	81.7	<i>hlb</i>	81.3	<i>tst</i>	80.3	<i>tst</i>	71.6	<i>tst</i>	78.8	<i>sec</i>	80.8
<i>hlb</i>	71.6	<i>hlb</i>	73.1	<i>hlb</i>	69.7	<i>hlb</i>	79.8	<i>hlb</i>	79.3	<i>sel</i>	80.3	<i>hlb</i>	78.8	<i>hlb</i>	81.3	<i>sel</i>	78.8	<i>sel</i>	76.4	<i>sel</i>	69.7	<i>sel</i>	76.9	<i>sel</i>	77.4

quantitatively, we calculated the coexistence rates between 13 genes in each strain (Table 5). The 13 genes were *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hlb*, *hld* and *hlg-2*, which belonged to pattern A. As already mentioned above, these 13 genes showed high carriage rates among the examined strains, resulting in high coexistence rates with each other. However, as shown in Table 5, if we arrange them in order of the coexistence rate, *tst*, *sec*, *sel* genes showed the highest coexistence rates between each other (marked with dark grey). Likewise, *seg*, *sei*, *sem*, *sen* and *seo* genes showed higher coexistence rates (marked with light grey). Consequently, our results indicate that the set of three genes *tst*, *sec* and *sel*, and the set of five genes *seg*, *sei*, *sem*, *sen* and *seo*, highly coexist in Japanese HA-MRSA. According to the genome analysis of an early Japanese hospital isolate, MRSA N315, two major pathogenicity islands, SaPin1 and SaPin3, were identified on the chromosome (Kuroda *et al.*, 2001). SaPin1 was characterized as the carrier of *tst*, *sec* and *sel* genes and SaPin3 was the carrier of the *egc* locus and *lukED* genes. In the *egc* locus of N315, *seg*, *sei*, *sem*, *sen* and *seo* genes were identified. In our study, we confirmed that the toxin pattern of MRSA N315 belongs to the most frequent pattern A. In addition, our results showed that the correlation of existence between *tst*, *sec* and *sel*, and also between *seg*, *sei*, *sem*, *sen* and *seo*, was higher than for the other toxin genes, which suggested that a majority of Japanese HA-MRSA strains probably carried pathogenicity islands SaPin1 and SaPin3.

Characterization of Japanese MRSA isolates regarding their virulence aspects

The pathogenicity of MRSA depends, at least partially, on the toxin repertoire of each MRSA strain. Therefore, the manifestations of MRSA infection may vary in different countries. The most remarkable difference between the toxin patterns of Japanese strains and those of MRSA strains isolated in European countries and North America was high carriage and high coexistence rates of *tst*, *sec* and *sel* genes in the Japanese strains. These three genes have been rarely observed in European and North American MRSA strains. We reported in a previous study that MRSA strains isolated from patients with neonatal toxic shock syndrome-like exanthematous disease (NTED) produced a large amount of TSST-1 and SEC (Nakano *et al.*, 2002b). Moreover, MRSA strains associated with enterocolitis, which was sometimes accompanied by toxic shock syndrome and multiorgan failure, were also TSST-1- and SEC-positive (Watanabe *et al.*, 2001). The patients with NTED have been reported only in Japan (Takahashi *et al.*, 1998; Takahashi, 2003). MRSA-associated enterocolitis has been occasionally observed in Japanese patients receiving surgical operations, while it has rarely been reported in other countries. It is therefore likely that Japanese MRSA isolates that were *tst*- and *sec*-positive were involved in the aetiology of these infections. As for the Japanese HA-MRSA strains, most of the *S. aureus* isolates from

European countries also carried an identical *egc* locus (Jarraud *et al.*, 2001). The typical *egc* locus encodes five intact enterotoxins, for which superantigenic activities have been proven (Jarraud *et al.*, 2001). These enterotoxins, however, are unlikely to be involved in the pathogenesis of severe infections, since their expression level is very low (Omoe *et al.*, 2002) and, moreover, the prevalence of the *egc* locus in *S. aureus* decreases significantly with severity of infection (Ferry *et al.*, 2005).

The conclusive result in our study was that the most of the Japanese HA-MRSA isolates had specific toxin patterns which were characterized by a high carriage rate of *tst*, *sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *lukED*, *hla*, *hlb*, *hld* and *hlg-2* genes, although their PFGE patterns showed a wide variation. Compared with strains isolated in European countries and North America, the most remarkable difference was that Japanese MRSA isolates carried *tst*, *sec* and *sel* genes at high frequencies.

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