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Analysis of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* by a molecular typing method based on coagulase gene polymorphisms

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SUMMARY

A molecular typing method for *Staphylococcus aureus* based on coagulase gene polymorphisms (coagulase gene typing) was evaluated by examining a total of 240 isolates which comprised 210 methicillin-resistant *S. aureus* (MRSA) and 30 methicillin-susceptible *S. aureus* (MSSA) collected from a single hospital. By *Alu*I restriction enzyme digestion of the PCR-amplified 3′-end region of the coagulase gene including 81-bp repeated units, the MRSA and MSSA isolates examined were divided into 6 and 12 restriction fragment length polymorphism (RFLP) patterns, respectively, whereas five patterns were commonly detected in MRSA and MSSA. MRSA isolates that showed a particular RFLP pattern were considered to be predominant in the hospital. Coagulase typing with type-specific antisera was also performed for all *S. aureus* isolates for comparison. Coagulase types II and VII were most frequently detected and included isolates with four and five different *Alu*I RFLP patterns, respectively, whereas each of the other coagulase types corresponded to a single RFLP pattern. These results indicated that RFLP typing was more discriminatory than serological typing, for typing *S. aureus* and demonstrated its utility in epidemiologic investigation of *S. aureus* infection in hospitals.

INTRODUCTION

S. aureus is an important human pathogen in both nosocomial and community-acquired infections. Methicillin-resistant *S. aureus* (MRSA) has caused serious problems in many countries on account of its high degree of antimicrobial resistance [1]. A number of methods for typing *S. aureus* have been elaborated to date, although they do not necessarily exhibit consistent results [2–4]. It has recently been recognized that molecular typing has an advantage over conventional typing based on phenotypic markers, such as biotypes or antimicrobial susceptibility [5–8], in terms of both its reproducibility and discriminatory power.

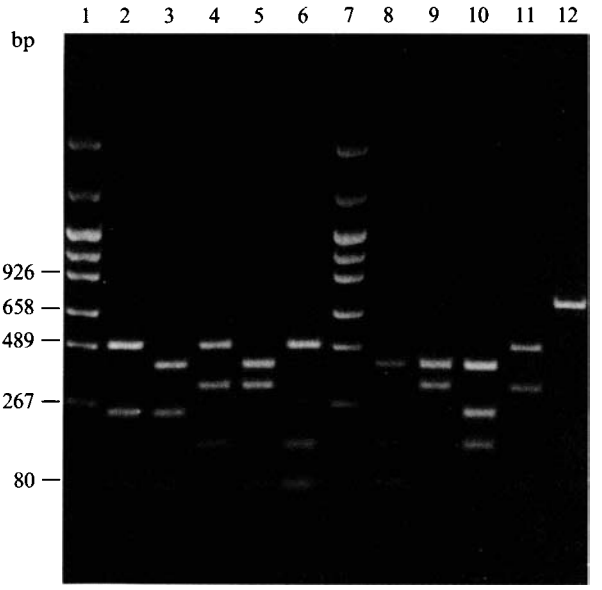

Fig. 1. RFLP patterns of *Alu*I restriction enzyme digestion of the PCR-amplified coagulase gene product. Lanes 1, 7, molecular weight marker. RFLP patterns correspond to those described in Table 1; lane 2, pattern A; lane 3, pattern B; lane 4, pattern D; lane 5, pattern C; lane 6, pattern E; lane 8, pattern F; lane 9, pattern J; lane 10, pattern H; lane 11, pattern G; and lane 12, pattern I.

Coagulase gene typing was designed as one of the molecular methods readily applicable to clinical laboratories [6]. This method utilizes the heterogeneity of the region containing 81-bp tandem repeats at the 3′-end of the coagulase gene [9–11]. In this method, DNA fragments corresponding to the variable region of the coagulase gene were amplified by PCR followed by *Alu*I restriction enzyme digestion and analysis of the restriction fragmentation (RFLP) pattern. The potential usefulness of this typing method has been indicated by several studies [4, 6, 12, 13]. However, efficacy of this method in epidemiologic studies of MRSA infection has not been well investigated, because in these previous reports, methicillin-susceptibility of *S. aureus* was not thoroughly examined and, even when examined, MRSA isolates could only be divided into a few types. Furthermore, most previous studies employed *S. aureus* isolates obtained from different hospitals or outbreaks, and usefulness of the coagulase RFLP typing for the analysis of *S. aureus* infections in a single hospital has not yet been reported.

On the other hand, a serological coagulase typing based on the antigenicity of coagulase has been employed as one of the markers in epidemiologic study, and eight (I–VIII) distinct types have been identified to date [14, 15]. Although extensive data on coagulase typing have been accumulated for years [16–18], correlation between serological and genetic typing is not well established.

In the present study, *S. aureus* strains isolated from a single university hospital were examined in order to assess the efficacy of the coagulase gene typing method for analysis of MRSA and MSSA circulating in the hospital environment, and to clarify the relationship between serological and genetic typing.

Table 1. *Molecular typing of* S. aureus *isolates based on coagulase gene DNA fragment polymorphism*

*Alu*I restriction pattern	PCR product (approx. bp)	Size of *Alu*I restriction digest fragments (bp)*	No. of isolates†		
			Total	*mecA*-positive	*mecA*-negative
A	820	81, 243, 486	185	182 (53)	3
B	740	81, 243, 405	20	17 (3)	3
C	820	81, 324, 405	7	6 (1)	1
D	980	162, 324, 486	3	0	3
E	820	81, 162, 486	2	2	0
F	740	81, 162, 405	2	0	2
G	900	81, 324, 486	1	0	1
H	820	162, 243, 405	3	1	2
I	820	81, 729	1	0	1
J	740	324, 405	3	0	3
K	820	162, 648	2	0	2
L	740	243, 486	1	0	1
M	820	None	9	1	8

* Results of only 81 bp-fragment or its multiples are shown.

† Numbers of colonizing MRSA strains isolated from environment or medical staff are shown in parentheses.

MATERIALS AND METHODS

Bacterial strains

A total of 240 *S. aureus* strains obtained from clinical specimens, hospital environments and medical staff were analysed. One hundred and eighty-three strains were isolated from 156 patients admitted to a university hospital in Sapporo, Japan, between January and June in both 1993 and 1994. In 27 patients, two strains each of *S. aureus* were isolated from different specimens. In the 1994 study, the presence of MRSA in hospital environment (floors and sinks of wards in five clinical departments) and medical staff (hand and nasal cavity) was investigated. Samples collected using sterile swabs were subjected to bacterial culture on mannitol salt agar plates, and species of the bacteria were identified. A total of 51 (detection rate; 46·9%) and 6 (detection rate; 17·6%) strains of MRSA isolated from the environmental sources and medical staff, respectively, were also analysed in this study.

Identification of *S. aureus* and antimicrobial susceptibility test were performed by the use of MicroScan WalkAway 96 (Baxter Diagnostics Inc., West Sacramento, USA). Essentially MRSA was defined by the production of penicillin-binding protein(PBP)-2a encoded by *mecA* gene [19, 20]. In addition to susceptibility test to oxacillin, the presence of *mecA* gene in the *S. aureus* strains was examined by PCR as described previously [21]. In this study, *S. aureus* possessing *mecA* gene was designated MRSA.

Coagulase gene typing

Bacterial DNA was extracted using achromopeptidase as described previously [21]. The 3′-end region of coagulase gene containing the 81-bp tandem repeats was

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Table 2. *Coagulase types and* Alu*I restriction patterns based on coagulase genes*

Coagulase type*	No. of isolates†	*mecA* gene		*Alu*I restriction patterns												
		Positive	Negative	A	B	C	D	E	F	G	H	I	J	K	L	M
I	2	0	2											2		
II	190 (53)	184	6	185				2				1	2			
III	2	0	2						2							
IV	7 (1)	6	1			7										
V	3	0	3				3									
VII	34 (3)	19	15		20					1	3		1			9
VIII	1	0	1												1	
Clinical isolates																
1993 (Jan.–June)	96	74	22	58	12	5	1	1	2	1	2	1	1	2	1	8
				[55]‡	[11]	[5]		[1]			[1]					[1]
1994 (Jan.–June)	87	78	9	74	5	1	2	1	0	0	1	0	2	0	0	1
				[74]	[3]			[1]								

† Numbers of colonizing MRSA strains are shown in parentheses.
* Coagulase type VI was not detected in this study.
† Numbers of colonizing MRSA strains are shown in parentheses.
‡ Numbers of MRSA are shown in square brackets.

amplified by PCR using primers C2 and C3 as described by Goh and colleagues [6] with minor modifications. Briefly, reaction mixture (100 µl) containing 10 µl of template DNA, 1·0 U of Replitherm™ Thermostable DNA polymerase (Bokusui Brown Inc.), 10 mM-Tris-HCl (pH 8·3), 50 mM-KCl and 1·5 mM-$MgCl_2$, was subjected to 25 PCR cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and primer extension (72 °C, 2 min). The PCR products were analysed on 1% agarose gels stained with ethidium bromide.

Restriction analysis of PCR product was performed with *Alu*I (Takara Biomedicals) according to manufacturer's instructions. The *Alu*I digests were electrophoresed on 1·5% NuSieve 3:1 agarose gels (FMC BioProduct) and visualized by staining with ethidium bromide.

Coagulase test and coagulase typing

Production of free coagulase was examined by Staphyslide test (bioMérieux Inc., France) and tube testing method using rabbit plasma. Coagulase type was determined by neutralization test using antisera against each of different coagulase types (I–VIII) (Denka Seiken Inc., Japan) as described previously [14, 15].

RESULTS

A single fragment between 740 and 980 bp was obtained by amplification with PCR from coagulase gene of 239 *S. aureus* strains. However, no PCR product was obtained from one MSSA strain (SH-41) derived from a clinical specimen (pharyngeal swab), even when nested PCR was performed using primers C1 and C4 which covered the region amplified with primers C2 and C3 as described previously [6].

After the restriction analysis of PCR product using *Alu*I, 13 different restriction fragment length polymorphysm (RFLP) patterns designated A–M were distinguished among the 239 *S. aureus* strains. Figure 1 shows representative RFLP

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patterns, and Table 1 summarizes the sizes of *Alu*I restriction digest fragments in each pattern. PCR product representing pattern M was not digested by *Alu*I.

In total, pattern A was most frequent (185/240; 77·1%), followed by patterns B (20/240; 8·3%), M (9/240; 3·8%), and C (7/240; 2·9%). *MecA* gene was detected by PCR in 152 strains from clinical specimens and 57 isolates derived from environmental sources and medical staff. These MRSA strains were classified into six RFLP patterns (A, B, C, E, H, M), and most strains (87·1%) were grouped into pattern A. In contrast, 30 MSSA strains were typed into 12 patterns which included 5 patterns (A, B, C, H, M) observed among MRSA (Table 1).

Coagulase type was determined for all *S. aureus* strains except for one MSSA strain (SH-41). The coagulase produced by strain SH-41 was not neutralized by any of the antisera to coagulase types I–VIII. As shown in Table 2, coagulase type II showed the highest frequency (79·2%), and 96·8% of strains that belonged to this coagulase type were MRSA. In contrast, the second frequent was coagulase type VII (14·2%) which contained MRSA at a lower rate (55·9%). When coagulase types were compared with *Alu*I restriction patterns, coagulase types II- and VII-strains were subdivided into 4 (A, E, I, J) and 5 (B, G, H, J, M) RFLP patterns, respectively. However, each of the other coagulase types contained a single RFLP pattern.

Most MRSA from clinical specimens (129/152; 84·9%) belonged to coagulase type II-RFLP pattern A. Similarly, this type of MRSA was detected at the highest rate in isolates from the hospital environment (48/51; 94·1%) and medical staff (5/6; 83·3%). Other colonizing strains belonged to coagulase type VII-RFLP pattern B (3 strains from the environment) and coagulase type IV-RFLP pattern C (1 strain from a medical staff).

When RFLP patterns of clinical isolates of MRSA were compared between the two study periods, a smaller number of RFLP patterns was detected in 1994 (3 types) than in 1993 (6 types) (Table 2). Moreover, detection rate of RFLP pattern A was higher in 1994 (74/78, 94·9%) than in 1993 (55/74, 74·3%).

DISCUSSION

A variety of systems, such as antimicrobial susceptibility patterns (antibiogram) [2, 4], phage typing [22, 23], capsular polysaccharide serotyping [24], zymotyping [25], protein analysis [26] and plasmid typing [5] have been used to type *S. aureus*. Antibiogram and phage typing are commonly used in epidemiological study, however, the former is often inadequate to differentiate strains, and the latter is limited by reproducibility and the frequent occurrence of untypable strains [3, 4]. Molecular typing procedures were developed to overcome the disadvantages of these conventional methods, and include chromosomal DNA analysis by pulsed-field gel electrophoresis (PFGE) [3, 27], ribotyping [28, 29], and RFLP analyses of staphylococcal genes amplified by PCR [6, 8]. Among these, RFLP analysis after PCR amplification may have an advantage in its ease for practice and interpretation of results, and therefore may be suited to routine examination in a hospital.

Results of coagulase gene typing demonstrated that the gene sequences were stable after subcultivation and *Alu*I digestion yielded the most diverse RFLP

patterns [13]. In the first attempt of coagulase gene typing of *S. aureus* strains isolated from a local hospital, MRSA was classified into only two RFLP patterns, while MSSA was divided into eight patterns [6]. In the present study, in contrast, higher diversity in RFLP patterns was observed; MRSA and MSSA were classified into 6 and 12 RFLP patterns, respectively. Furthermore, compared with serological typing, RFLP analysis of the coagulase gene provided greater discrimination among strains. However, since our present study indicated that MRSA and MSSA cannot be precisely differentiated by coagulase gene typing only, simultaneous examination for methicillin resistance or the presence of *mecA* gene may be necessary for detailed characterization of *S. aureus*.

It was of note that 84·9% of clinical MRSA strains and most MRSA strains derived from the hospital environment and medical staff were classified into the RFLP pattern A. This result suggested that MRSA with this pattern was predominant in the hospital during the study period. In contrast, clinical isolates of MSSA were typed into 12 different patterns and none of them was considered a predominant type. This finding was similar to that in coagulase gene typing described by Goh and colleagues [6]. Alternatively, analyses of MRSA and MSSA isolated from a single hospital based on the genetic diversity of plasmids [30] or chromosomal DNA [7] revealed that genomic variation was lower in MRSA than in MSSA. Similar observations were described in analysis of MRSA in a Spanish hospital [31]. Together with these results, our present analysis also showed that the genomes of MRSA from a single hospital are highly conserved compared with MSSA. These findings are consistent with the view that MRSA strains arose from a single or limited number of clones [7, 32].

In our present study, comparison of RFLP patterns obtained from the two study periods indicated that genetic variation among MRSA strains in 1994 was lower than in 1993 and almost all (95%) of MRSA strains in 1994 were of pattern A.

It has been reported that whereas MRSA with coagulase type IV was predominant in Japan before the middle of 1980s, the prevalence of MRSA with coagulase type II increased thereafter [16–18] as was observed in this study. Although the reason for a sharp increase of coagulase type II-MRSA in Japan is still not clear, it was shown that the coagulase type II-MRSA comprised of at least three subpopulations on the basis of production patterns of enterotoxins (A–E) and toxic shock syndrome toxin-1 [18]. Similarly, genetic methods including coagulase gene typing will provide further characterization of those coagulase type II-MRSA, and may help to clarify dissemination of coagulase type II strains.

Amino acid sequence analysis of coagulase genes revealed that the coagulase molecule consists of three distinct regions, i.e. a divergent N-terminus, a highly conserved central region, and a C-terminal region including repeated units of 27 amino acids [11]. The N-terminal region contains the prothrombin-binding site and was suggested to be responsible for antigenic difference among strains, while the function of C-terminal region with repeated units and the reason for divergence of this region are unknown. In our present study, strains with coagulase types II and VII were divided into 4 and 5 distinct RFLP patterns, respectively (Table 2). This finding may suggest that the divergence in C-terminal region hardly affect the antigenicity of coagulase.

Although several strains were untypable by coagulase gene typing in previous reports [4, 6], there was only one MSSA isolate in this study, from which no coagulase gene-derived PCR product was obtained. Since the serological coagulase type of this strain could not be determined, it seems likely that coagulase gene of this strain has deviated considerably from those of common *S. aureus* strains. Further sequencing analysis may reveal the genomic diversity of the coagulase gene and present useful information for improvement of coagulase gene typing.

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