Molecular Typing of Methicillin-Resistant *Staphylococcus aureus* by Polymerase Chain Reaction: Distribution of the Typed Strains in Hospitals

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Three methods for molecular typing of methicillin-resistant *Staphylococcus aureus* using polymerase chain reaction (PCR) were compared. The method which amplified the variable number of tandem repeat of *dru* sequences grouped the isolates into six types. Whereas, the method examining restriction fragment length polymorphism of coagulase gene and the method using arbitrarily primed-PCR showed poor diversity in typing. We investigated the distribution of *dru* types in two hospitals. Obvious concentration of a type in one ward was not recognized in our hospital. In the other hospital, a rare type was detected from the inpatients in the pediatrics ward. It suggested that the infection was an epidemic. We also found that some patients were infected with more than two strains. Even if two isolates show the same type, it does not necessarily mean that they originated from one clone. However, this method brings meaningful information on nosocomial infection, more easily than other genotyping. (Internal Medicine 36: 694–699, 1997)

Key words: *dru* sequence, nosocomial infection, coagulase gene

Introduction

Nosocomial infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has been a serious problem in hospitals (1). To prevent infection, it is imperative to determine whether the infection is epidemic, sporadic, or endogenous; if it is epidemic, it is helpful to know how the organisms spread in the hospital. To investigate this, it is necessary to differentiate the strains isolated from patients. Molecular typing of the strains is useful for this purpose.

For genotyping of MRSA, several methods have been reported. Plasmid analysis was reported by McGowan et al (2). Although this method has strong discriminatory power, about 10% of strains can not be analyzed as they have no plasmid (3). Ribotyping (4) is also useful to discriminate isolates. However, this method takes a few days to perform. DNA fingerprinting by pulsed-field gel electrophoresis (PFGE) (5) has been also reported to have strong discriminatory power. Ichiyama et al (5) reported identification of 31 different patterns by PFGE. However, this method requires special equipment and technique and takes several days.

Recently, some types of DNA polymorphism by means of polymerase chain reaction (PCR) have been reported. This

method is easier and takes much less time than other methods described above. In the present study, we performed the genotyping of a large number of MRSA strains isolated from patients using three kinds of PCR-based methods; variable number of tandem repeat (VNTR) of *dru* sequences (6, 7), restriction fragment length polymorphism (RFLP) of coagulase gene (8, 9), and arbitrarily primed (AP) – PCR method reported by van Belkum et al (10). We examined the usefulness of the three PCR-base genotyping methods with clinical samples. Furthermore, we investigated the distribution of the typed strains among the wards in hospitals.

Materials and Methods

MRSA strains and DNA preparation

All the isolates of MRSA were collected from clinical samples at the clinical microbiology laboratory in our hospital in Tokyo from March to May in 1996. The sources of the samples included sputum, throat swabs, blood, stool, urine, pus, ascites, and catheters. A total of 120 isolates were collected from 75 patients in our hospital. For reference, 39 isolates were also collected from 24 patients in Toride Kyodo Hospital (T hosp.), a local general hospital located 50 kilometers north of

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Tokyo. For DNA preparation, a colony on MRSA screen agar plate containing 6 µg/ml oxacillin and 4% NaCl (Becton Dickinson, Lincoln Park, NJ) was picked up, put into 500 µl of water in microtube and boiled for 15 minutes. After centrifugation, the supernatant was used as a DNA sample for PCR.

PCR-VNTR of *dru* element

The *dru* element consists of a repeated sequence of 40 base pairs (bp). This element is located near the *mecA* gene (6). The *dru* element was amplified by PCR according to the method reported by Nishi et al (7). Briefly, cell lysate (5 µl) was added to a PCR mixture containing 1 µmol of each primer, 200 µmol deoxynucleotide triphosphate, and 0.5 U of *Taq* polymerase (Takara Shuzo, Kyoto) in a final volume of 100 µl. The primers were sense 5'ACTATTCCTCAGGCGTCC3' and antisense 5'GGAGTTAATCTACGTCTCATC3'. After denaturation at 94°C for 3 minutes, reactions were performed for 30 cycles consisting of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C, followed by final elongation at 72°C for 7 minutes in a GeneAmp PCR system 2,400 thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR products were electrophoresed through an agarose gel and stained with ethidium bromide. Band sizes were determined by comparison with 100 bp DNA ladder standards (BRL Life Technology, Bethesda, MD) run concurrently. Each examination was performed in duplicate to ascertain the reproducibility.

PCR-RFLP of coagulase gene

The 3' end region of the coagulase gene was amplified by nested PCR according to the method reported by Goh et al (8) and Yamashita et al (9) with modification. The amplified region contains 81 bp tandem repeat sequences. Cell lysate was added to a PCR mixture as described above, and subjected to 30 PCR cycles, each consisting of 1 minute at 94°C, 2 minutes at 59°C, and 3 minutes at 72°C. For nesting, 2 µl of the first PCR product was added to a fresh PCR mixture and subjected to 30 PCR cycles, each consisting of 1 minute at 94°C, 2 minutes at 56°C, and 3 minutes at 72°C. The outer primers were 5'ATACTC-AACCGACGACACCG3' and 5'GATTTTGGATGAAGC-GGATT3'. The inner primers were 5'GCAAGACCAAGATTCAACAA3' and 5'AAAGAAAACCACTCACATCA3'. The nested PCR product was digested with the restriction endonuclease *Alu*I (Takara). Both the nested PCR products and the restriction digest fragments were electrophoresed through an agarose gel and stained with ethidium bromide.

AP-PCR

Cell lysate was added to the PCR mixture described above, and subjected to 40 PCR cycles using both the arbitrary primer and the primer which was originally designed for ERIC2 region, according to the method reported by van Belkum et al (10). The sequences of the primers were 5'TACATTCTGA-GGACCCCTAAGTG3' and 5'AAGTAAGTGACTGGGG-TGAGCG3', respectively. The cycles consisted of 1 minute at 94°C, 1 minute at 25°C, and 2 minutes at 74°C. The PCR products were electrophoresed through an agarose gel and

stained with ethidium bromide.

Results

Preliminary experiments

First, we examined whether the *dru* type of the colonies on a plate is generally uniform or diverse. If it is diverse, it is necessary to pick up many colonies from one plate to investigate. We selected five plates randomly and eight colonies were picked up from each plate. Then, the *dru* type of each colony was examined. As a result, eight colonies from each plate showed the identical *dru* type (data not shown).

Next, we examined the stability of the *dru* element. Because, if the *dru* type of a strain changes quickly within a short period, the genotyping is meaningless. Briefly, a colony was picked up and cultured in 5 ml of liquid medium. Then, 50 µl of the suspension was transferred into 5 ml of fresh medium. This procedure was repeated five times, which meant 10¹⁰ fold amplification, followed by plating on agar. Six colonies from the plate and the original colony were analyzed. The seven samples showed the same genotype (data not shown). This result denied the changeability of the *dru* element within a short period.

Typing of VNTR of *dru* element

In the 120 MRSA isolates from our hospital, we observed 5 different sizes of PCR products for *dru* element: approximately 410, 450, 490, 530, and 570 bp (Fig. 1). In addition, a PCR product of 210 bp was observed from the isolates in T hospital. In this paper, we assigned the strain from which PCR product was 410, 450, 490, 530, 570, and 210 bp, types A, B, C, D, E,

Figure 1. PCR-VNTR of *dru* element. PCR products from representative MRSA isolates were electrophoresed. Each type is assigned a letter from A to F. Molecular size markers are indicated on both sides.

and F, respectively.

The distribution of *dru* types among wards in our hospitals is shown in Table 1. If an identical type was detected repeatedly from multiple samples from one patient, these were counted as one strain in this table. Accordingly, the total number of strains totaled 90. For reference, the distribution of the types in T hospital and Kagoshima University hospital (K hosp.) located at the south end of Japan is also shown. The data in the latter hospital is cited from the paper reported by Nishi et al (7). As the sizes of the PCR products they reported were slightly different from ours, we adapted our standard to their data in this table.

From twenty-two patients in our hospital, plural samples were sent to the laboratory during this period. From ten patients out of twenty-two, two types of MRSA were detected. In seven patients out of these ten, two types of MRSA were detected from the same site of isolation. Moreover, three patients had three types of MRSA detected. The details are shown in Table 2.

Table 3 shows the relationship between the antibiogram and *dru* typing. For types B, E, and F, the strains in each type showed the identical antibiogram. The strains in the other types showed diverse antibiogram.

RFLP of coagulase gene

All the isolates in our hospital showed 810 bp of nested PCR product for coagulase gene (Fig. 2; right side). For all the strains except one, the *Alu* I restriction fragment length pattern consisted of 81, 243, and 486 bp. Several faint bands seen in the lane seemed to be the fragments due to partial digestion. One strain, which was categorized in *dru* type E, showed the fragments of 162, 243, 405 bp (Fig. 2; left).

All the isolates in T hospital also showed 810 bp of PCR products. One strain, which was categorized in *dru* type E, showed the fragments of 162 and 648 bp. All the rest showed the fragments of 81, 243, and 486 bp (data not shown).

AP-PCR

Five different banding patterns of PCR fragments, assigned types I to V, were obtained by AP-PCR (Fig. 3). All the types had the bands of approximately 600, 410, 360, 160, 120 bp. In addition to these five bands, type II had the bands of 180, 100, and 80 bp and type III had the bands of 490, 300, 250, and 220 bp. In type IV, the band of 490 bp was visible and the band of 410 bp was faint compared with other bands in the lane. In type V, the bands of 600 and 160 bp were faint or invisible. However, as we considered that types IV and V were essentially identical

Table 1. Distribution of MRSA Types among Wards

Bldg.	Floor	Department	No. of isolates of type					
			A	B	C	D	E	F
A	6	ICU	0	0	0	1	0	0
A	8	Pediatrics	2	0	0	1	0	0
A	9	Intern. Surg.	4	2	1	5	0	0
A	10	Neurosurgery	3	0	0	2	0	0
A	11	Orthopedics	0	0	0	1	0	0
A	12	Surgery	2	0	1	5	0	0
A	13	Radiology	2	0	0	1	0	0
A	15	Internal	3	1	0	6	0	0
A		subtotal	16	3	2	22	0	0
B	2	Surgery	0	0	0	1	0	0
B	5	Gynecology	2	0	0	1	0	0
B	6	Urology	3	0	1	1	0	0
B	7	Dermatol. ENT.	9	1	0	6	1	0
B	8	Respiratory	3	2	0	1	0	0
B		subtotal	17	3	1	10	1	0
Outpatient clinic			4	0	1	10	0	0
Total			37	6	4	42	1	0
T hosp.			0	0	0	21	1	3
*K hosp.			56	1	26	0	22	0**

The types were determined by PCR-VNTR of *dru* element. *The data in K hosp. are cited from the paper reported by Nishi et al (7) with modification for reference. **Another type showing 250 bp of PCR product was reported in their paper.

Genotyping of MRSA by PCR

to type I, we grouped the strains into three types. Consequently, 84, 2, and 2 isolates were grouped into types I, II and III, respectively. As the remaining 32 isolates showed an obscure banding pattern, typing was impossible. No clear relationship was recognized between typing of A to E and typing of I, II and III.

For the isolates in T hospital, 31 isolates showed type I pattern. The remaining eight isolates could not be grouped as their banding patterns were obscure.

Discussion

Here, we examined the usefulness of the three PCR-based

Table 2. List of the Patients Infected with Three Types of MRSA

Patient	Ward	Date	Source	Type
N.M.	A 9	Mar. 26	sputum	D
		Apr. 1	ascites	D
		Apr. 1	stool	D
		Apr. 8	pus	A
		Apr. 9	stool	C
		May 8	stool	D
		May 9	pus	D
		May 10	throat	D
M.N.	B 8	Mar. 18	sputum	A
		Apr. 10	sputum	B
		Apr. 15	decubitus	D
		May 9	decubitus	A
M.S.	A 15	Apr. 11	throat	A
		Apr. 15	stool	D
		Apr. 22	throat	B

The types were determined by PCR-VNTR of *dru* element.

genotyping methods with a large number of clinical materials. As shown above, the method of VNTR of *dru* element grouped the MRSA isolates into six different types. This method is easy to perform, takes only a few hours to complete and is clearly interpretable as it gives only one band. Moreover, as the target region is linked to *mec A* gene (6), the detection of this PCR product can verify that this isolate is methicillin-resistant. In

Figure 2. RFLP of coagulase gene. The nested PCR products for coagulase gene from representative MRSA isolates are shown in the right half. *Alu I* restriction patterns of the nested PCR products are shown in the left half. Molecular size markers are indicated on both sides.

Table 3. Relationship between Antibigram and *dru* Typing

Antibiogram			No. of isolates of type							
IPM	MINO	OFLX	Our Hospital					T Hospital		
			A	B	C	D	E	D	E	F
R	S	R	17	0	2	19	0	2	0	0
R	I	R	17	6	1	23	0	0	0	3
R	R	R	0	0	0	0	0	19	0	0
R	I	I	2	0	1	0	0	0	0	0
I	S	R	1	0	0	0	0	0	0	0
S	S	S	0	0	0	0	1	0	1	0

IPM: imipenem, MINO: minocycline, OFLX: ofloxacin, R: resistant, I: intermediate, S: susceptible.

Figure 3. AP-PCR products from representative MRSA isolates are shown. Each banding pattern is assigned a number from I to V. Molecular size markers are indicated on both sides.

this study, the diversity of *dru* types was slight. However, the strain showing 250 bp of PCR product was reported by Nishi et al (7) and the strains showing 331 bp and 611 bp of PCR products were reported by Kagawa et al (11). Accordingly, a total of 9 different types have been observed until now. Based on the nucleotide sequence of this region (6, 7), the size of the PCR product is expected to be $40 \times N + 171$ bp. N indicates the number of repeats. Therefore, some more types which show a different size of the PCR product should exist, and thus, the discriminatory power of this method is not so poor.

Regarding RFLP of coagulase gene, Goh et al (8) reported observation of 10 RFLP patterns. However, almost all the strains in our hospital and T hospital showed the same pattern. Goh et al (8) examined both MRSA and methicillin-sensitive SA and the strains were collected from many hospitals. This may be the reason why their investigation showed such diversity. At any rate, our results show that even if many MRSA isolates show the same RFLP pattern of coagulase gene, it does not mean they originate from a single clone.

As for AP-PCR method, although we observed five banding patterns, most of the isolates showed the type I pattern. For several isolates, some of the bands were so faint that it was impossible to decide the type, as was often the case with the AP-PCR method. Accordingly, this method was not useful for us. van Belkum et al (12) undertook a multicenter evaluation of AP-PCR for genotyping of MRSA. They reported that their method could discriminate 16 to 30 different types. However, they also mentioned that this method had problems in interlaboratory reproducibility and interpretation of DNA fingerprints.

In the present study, we also investigated the distribution of the *dru* types among the wards in our hospital. As shown in Table 1, type A and type D strains were predominant in our hospital. Type A strains were predominant in building A and type D strains were predominated in building B, although these were not significant differences. The obvious concentration of a single type in one ward was not recognized in our hospital.

In T hospital, MRSA was detected from three inpatients who had become feverish successively in the pediatrics ward. We found that these patients had type F MRSA. This type was not detected in the other wards and it was a rare type according to our investigation. These findings supported the supposition that the episode was not sporadic or endogenous, but epidemic in the ward.

Regarding the cause of the predominance of type A and type D strains in our hospital, two hypotheses are considered. The first is that type A and type D strains have gradually prevailed by nosocomial infection in our hospital. The second is that type A and type D strains are universally predominant. To examine the second hypothesis, we compared the distribution in our hospital and in T hospital and K hospital. We found that the predominant types were different among the three. Accordingly, the second hypothesis seems less possible. To solve this problem, continuous investigation at numerous hospitals is needed.

In this study, we found that some patients were infected with more than two kinds of strains. As for the cause, the following interpretations are considered: 1) A patient may have various MRSA at one focus. 2) Different kinds of MRSA may exist at different foci or organs. 3) Some strains may be replacing sequentially. 4) The length of the *dru* element itself may change although an identical strain stays on. The first interpretation seems less possible because more than two types of strains were not detected from one agar plate according to the sampling examination (data not shown). We denied the fourth hypothesis in the section of Preliminary experiments in Results. To conclude these four interpretations, much further investigation is required.

The analysis of the relationship between antibiogram and genotyping was difficult because the diversity of antibiogram for MRSA was poor. Clear correspondence between the two was not recognized. Interestingly, type E strains isolated in our hospital and in T hospital showed identical and distinctive antibiograms. The fact that the isolates in type A showed four different antibiograms may suggest that the isolates in type A consisted of at least four clones. By combining the genotyping and antibiogram, we may be able to make the discriminatory power stronger.

We conclude that PCR-VNTR of *dru* element is the best discriminatory method of the three methods examined. It should be performed as the first step in the hospitals in which other molecular typing such as PFGE can not be performed. Even if two isolates show the same *dru* type, it does not necessarily mean that they originated from one clone. However, this method brings meaningful information for nosocomial infection, more easily than other genotyping.

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