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Clonal Analysis of Methicillin-Resistant *Staphylococcus aureus* Strains in Korea

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In this study, the distribution of the *mec* regulator genes and the presence of the mutation in *mecI* gene and *mec* promoter region among 50 MRSA clinical isolates derived from a single university hospital in Korea were analyzed. Among 50 MRSA strains, 13 strains had a deletion of *mecI* gene, and 37 strains were found to have mutations in *mecI* gene or *mecA* promoter region corresponding to a presumptive operator of *mecA*, i.e., the binding site of the repressor protein. Furthermore, in order to track the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA) distributed in Korea, we determined the MRSA clonotype by combined use of genetic organization patterns of *mec* regulator genes, ribotype, and coagulase type. As the result, 48 of 50 MRSA strains could be classified into four distinct clones. Clonotype I is characterized by the coagulase type 3, deletion of *mecI* gene, and ribotype 1 shared by NCTC10442, the first reported MRSA isolate in England (9 strains). Clonotype II is characterized by the coagulase type 4, C to T substitution at position 202 of *mecI* gene, and ribotypes 2, 3 and 4 shared by 85/3619 strain isolated in Austria (10 strains). Clonotype III is characterized by the coagulase type 2, mutations of *mecA* promoter region and/or *mecI*, and ribotypes 4, 5, and 6 shared by N315 strain isolated in Japan (25 strains). Clonotype IV is characterized by the coagulase type 4, deletion of *mecI* gene, and ribotype 7 (4 strains). The clonality of two strains could not be determined due to their undefined ribotype.

Key Words: MRSA, Mutation in *mec* regulator gene, Coagulase type, Ribotype, Clonotype

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as a nosocomial pathogen in the early 1960s and has become a major nosocomial pathogen in community hospitals, long-term-care facilities, and tertiary care hospitals. In Korea, a rapid increase of MRSA was reported from the mid-1980s in large university hospitals and the prevalence was increased from 24.2% in 1988 to 74.2% in 1995 (3,5). In the early 1990s, the prevalence reached 50% in most hospitals. Nowadays, MRSA is one of the most commonly isolated endemic nosocomial pathogens in many hospitals in Korea.

More than 90% of the methicillin-resistant clinical staphylococcal strains share a common

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feature of carrying the *mecA* gene on their chromosome. The *mecA* gene encodes PBP2a, a penicillin-binding protein with low binding affinity to practically all β-lactam antibiotics clinically available today (5,21,27). The *mecA* gene is a component of a large DNA fragment designated *mec* DNA, which is located at the specific site of the *S. aureus* chromosome and has been suggested to be transmitted from other bacterial species (1,2,4,9,28).

Expression of PBP2a is controlled by two regulator genes on *mec* DNA, *mecI* and *mecR1*, located upstream of *mecA*, which encode *mecA* repressor protein and signal transducer protein, respectively (15,18,26). It has been accepted that intact *mec* regulator genes strongly repress the transcription of *mecA* gene, whereas the repression activity is lost by mutation or deletion which occurred in *mec* regulator genes, resulting in constitutive production of PBP2a (6,15, 22). Indeed, the deletion or point mutations in the *mecI* gene has been found in a number of clinical MRSA isolates (6,10,14,24). In some strains, point mutations were detected in the *mecA* promoter region corresponding to a presumptive operator of *mecA*, i.e., the binding site of the repressor protein (6,14). Therefore, an analysis of the genomic diversity found in *mec* regulator genes of staphylococci is important to understand the molecular basis for methicillin resistance.

On the other hand, the genetic alteration of *mec* regulator region has been associated with the clonal emergence of MRSA (2,6,9,28). Archer et al. (2) found that there were at least two classes of genetic organization of the *mec* gene that cannot be related easily to each other. The earlist MRSA isolate, COL, contained a deletion of *mecR1* and *mecI*, the regulatory genes commonly found in recently isolated MRSA strains. In addition, the early isolate contained new DNA added at the point of deletion that was not found in recent isolates. All of the isolates that examined from the 1960s contained the identical deletion junction; intact *mecR1* and *mecI* sequences did not appear in MRSA isolates until 1970s. Later isolates that had an intact *mecR1-mecI* region by DNA hybridization also expressed phenotypic methicillin-resistance, but in this case, the regulatory sequences may be dysfunctionl because of mutations. Recently, by combined use of *mec* DNA typing and ribotyping, Hiramatsu et al. (9) suggested that there are at least five distinct MRSA clones in the world.

In this study, the distribution of the *mec* regulator genes and the presence of the mutation in *mecI* gene and *mecA* promoter region among 50 MRSA clinical isolates derived from a single hospital were analyzed. Furthermore, the MRSA clonotype was inferred by combined use of genetic organization pattern of *mec* regulator genes, ribotype, and coagulase type.

MATERIALS AND METHODS

Bacterial isolates

A total of 50 strains of methicillin-resistant *Staphylococcus aureus* isolated from Kyungpook National University Hospital in Korea between 1994 and 1995 were studied. *S. aureus* ATCC 43300 (American Type Culture Collection, Rockville, MD, USA) was used as a positive control strain for detection of *mecA* gene.

Antimicrobial susceptibility test and coagulase typing

Minimal inhibitory concentrations (MICs) of the eight antimicrobial agents were determined by the agar dilution method according to the recommendation of NCCLS (19). Tested antimicrobial agents were chloramphenicol and tetracycline (Chong Kun Dang Co., Seoul, Korea), kanamycin (Dong-A Pharm. Co., Korea), gentamicin (Choong Wae Pharm. Co., Korea), amikacin (Young Il Pharm. Co., Korea), trimethoprim and rifampin (Sigma Chemical Co, USA), and ciprofloxacin (Bayer Korea Ltd. Seoul, Korea).

Table 1. Oligonucleotide primers used for PCR and sequencing

Amplified gene*	Oligonucleotide primer		Size of PCR product (bp)
	Pair	Sequences[a] (5' to 3')	
mecA	mecA1	AAAATGGATGGTAAAGGTTGGC	533
	mecA2	AGTTCTGCAGTACCGGATTTGC	
mecR1 (MS)	mecR1	GTCTCCACGTTAATTCCATT	310
	mecR2	GTCGTTCATTAAGATATGACG	
mecR1 (PB)	mecR3	CAGGGAATGAAAATTATTGGA	319
	mecR4	CGCTCAGAAATTTGTTGTGC	
mecI	mecI1	AATGGCGAAAAAGCACAACA	481
	mecI2	GACTTGATTGTTTCCTCTGTT	
mecA promoter	Pr-mecR	TTCTACACCTCCATATCACAA	277
	Pr-mecA	CTTCACCATTCTCGCTTTT	

*_mecR1_ (MS) and _mecR1_ (PB) represent half portion of the 5' and 3' end of _mecR1_ gene, respectively.

MICs for methicillin (Sigma Chemical Co., USA) were also determined by the agar dilution method using Mueller-Hinton agar plates supplemented with 2% NaCl with an inoculum size of 5×10^4 CFU of bacteria according to the recommendation of NCCLS (19). *S. aureus* ATCC25923 was used as a control strain. For coagulase typing, a kit purchased from Denka Seiken (Tokyo, Japan) was used. The results were read after incubation at 35℃ for 2, 4, 24 and 48 hours.

PCR and DNA sequencing

DNA samples for PCR were prepared with achromopeptidase by the method described previously (17). Five sets of primers indicated in Table 1 were used for PCR to detect *mecA*, *mec* regulator genes (*mecR1* and *mecI*), and presumptive *mecA* promoter region. Primer pairs mecR1 and mecR2, and mecR3 and mecR4 amplified the regions located on the half portion of 5' end (transmembrane domain; MS) and 3' end (penicillin-binding domain; PB) of *mecR1* gene, respectively. A primer set, Pr-mecA and Pr-mecR, was used to amplify the promoter region of *mecA* and *mecR1*. DNA amplification was performed on a thermal cycler (Gene Cycler™, Bio-Rad) in a 25 μl of reaction mixture containing 200 μM each of dATP, dCTP, dGTP, and dTTP; 20 pM each of a pair of primers; 1 U of Taq DNA polymerase (Promega); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; and 1.5 mM $MgCl_2$. DNA fragments were amplified for 30 cycles, with each cycle consisting of 1 min at 94℃ for denaturation, 1 min at 55℃ for annealing, and 1 min at 72℃ for primer extension. With the PCR-amplified DNA fragments as templates, nucleotide sequences of the *mecI* gene (481 bases) and the promoter region of *mecA* and *mecR1* (277 bases) were determined by the dideoxynucleotide chain termination method with the Sequenase kit, version 2.0 (United States Biochemical Corp., Cleveland, Ohio).

Ribotyping

Preparation of genomic DNA of *S. aureus* was performed using the guanidium extraction method (20). Extracted DNAs were digested with the *Hind* III restriction enzyme and *Hind* III-digested DNAs were electrophoresed in a 1% agarose gel. The gel was transferred to a nitrocellurose membrane and the blot was hy-

bridized using a 23S ribosomal DNA probe labeled with digoxigenin (Boerhinger Mannheim, Germany).

RESULTS AND DISCUSSION

Susceptibility of MRSA to antimicrobial agents

Multiresistance is a characteristic of MRSA. Forty-nine strains (98%) among fifty MRSA were resistant to chloramphenicol, tetracycline, kanamycin and gentamicin. Thirty-six strains (72%) and nine strains (18%) were resistant to ciprofloxacin and rifampin, respectively. All strains were susceptible to amikacin and trimethoprim, so these antibiotics may be useful for the treatment of MRSA infection. All strains were highly resistant to methicillin, with MICs ranging from 128 to $\geq$ 512 μg/ml.

Coagulase type

Coagulase is one of the virulent factors of *S. aureus*, and can be grouped into eight different types (12). In this study, three of eight coagulase types were detected among 50 MRSA strains: type-2 in 26 strains (52%), type-3 in 9 (18%), and type-4 in 15 (30%).

Coagulase types have been widely used for the epidemiologic study in Japan (11) and Tanaka et al. (25) suggested the transition of MRSA types connected with the change in the predominant coagulase type. The investigators found that type 4 coagulase producing strains declined drastically in the 1990s, instead type 2 coagulase producing strains increased and has become the most dominant MRSA strain which accounts for over 70% of all MRSA isolates in the 1990s throughout Japan (25). Similar transition was also reported in Korea (16). The incidence of coagulase type 2 isolates increased from 8.9% in the 1980s to 30% in 1994, while the proportion of coagulase type 3 strains decreased from 75.6% to 57.5% in the same period (16). This study also showed the prevalence of coagulase type 2 as detected in 26 of 50 MRSA strains. In Japan, the increased isolation of type 2 coagulase producing MRSA was coincident with an increase of highly methicillin-resistant isolates (18).

Distribution of *mec* regulator genes in MeSA clinical strains

Although *mecA* is present in all MRSA, there is a considerable variation in the presence of the other genes involved in the expression of methicillin resistance. Previous studies have detected *mecR1* and *mecI* in 60~95% of *mecA*-positive *S. aureus* (14,24).

In this study, a total of 50 MRSA strains were analyzed by PCR with specific primers to determine whether they carried *mecA* and *mec* regulator genes. The *mecA* gene and *mecR1* MS gene were detected in all isolates tested, but the *mecR1* PB gene and the *mecI* gene were detected in only 37 strains. Therefore, 13 of 50 strains had a deletion of *mecI* and 3'-end of *mecR1* gene, and these isolates were able to express methicillin-resistance in the absence of its repressor effect.

Mutations detected in *mecI* gene and *mecA* promoter region

Thiry-seven MRSA strains were found to have no deletions of *mec* regulator genes, and the possibility of mutation in the *mecI* and *mecA* promoter/operator region was suggested. So, we analyzed the genetic diversity of mutations in the *mecI* and *mecA* promoters and the results obtained were compared with the sequence obtained from pre-MRSA strain N315 (4), a prototype strain possessing intact *mec* regulator genes.

In all the MRSA strains, a point mutation was detected in one of the two gene sequences, except in three strains which had mutations in both sequences. As shown in Table 2, the mutations detected in MRSA strains were classified into six groups (M1 to M6).

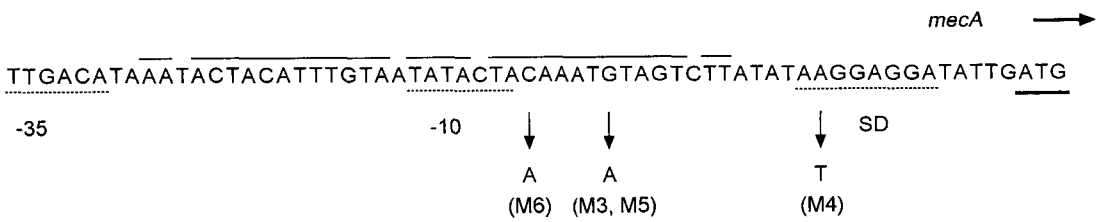

Figure 1. Nucleotide sequence of the *mecA* promoter region. Positions of base substitution are shown by arrows. Putative -35 and -10 promoter sequences and Shine-Dalgano (SD) sequences are shown by dotted lines, and a pair of palindrome sequences are indicated by solid lines above the sequence. The direction of *mecA* gene transcription is shown by a solid arrow, with the initiation codon indicated by solid underlining.

Table 2. Mutations detected in *mecI* gene and *mecA* promoter region of MRSA strains

Mutation group[a]	MRSA strain(s)	Mutation in *mecI* gene		Mutation in *mecA* promoter region[b]	Coagulase types
		Nucleotide position	Codon change change		
M1(3)	S4013, S4067, S4114	43 (G→T)	Val15→Phe	None	type 2
M2(11)	S4003, S4016, S4031, S4032, S4046, S4056, S4061, S4064, S4066, S4069, S4073	202 (C→T)	Gln68→stop codon	None	type 4
M3(2)	S4039, S5005	202 (C→T)	Gln68→stop codon	G→A	type 2
M4(1)	S4020	202 (C→T)	Gln68→stop codon	A→T	type 2
M5(19)	S4001, S4008, S4017, S4019, S4021, S4022, S4024, S4027, S4029, S4030, S4040, S4043, S4057, S4071, S5002, S5004, S5006, S5009, S5010	None		G→A	type 2
M6(1)	S4045	None		C→A	type 2

[a] Parentheses are the number of isolates included. [b] Positions of nucleotide substitutions are shown in Figure 1.

Among 37 strains, 17 strains showed point mutations in *mecI* gene: C to T at position 202 (M2-M4) in 14 strains and G to T substitution at position 43 (M1) in 3 strains. A nucleotide substitution at position 43 in *mecI* generated an amino acid change and this mutation has been reported in one strain by Kobayashi et al (14). A nucleotide substitution at position 202 in *mecI* generated an in-frame stop codon in the middle of the open reading frame, and this has been noted previously amongst British and European MRSA (6,14,24).

Twenty-three strains showed point mutation in *mecA* promoter region: G to A substitution (M3 and M5) in 21 strains, A to T (M4) in 1 strain, and C to A (M6) in 1 strain (Table 2 and Fig. 1). G to A and C to A substitutions are located downstream of the *mecA* promoter sequence (-10) on a palindrome structure corresponding to the presumptive operator of the *mecA* gene and has been known to cause a decrease in the stacking energy of the stem (6,14, 23). While, A to T substitution (M4), identified for the first time in the present study, is located on a Shine-Dalgano sequence. We did not study the role of this mutation on the transcription of *mecA* gene, but this strain has another mutation in *mecI* gene allowing full expression of methicillin resistance.

Table 3. Association between coagulase types, organization of the *mec* regulator genes, and degree of methicillin resistance in MRSA strains

Coagulase type (No. of isolates)	Mutation pattern of *mec* regulation genes	MIC_{90} of methicillin (μg/ml)
type-2 (26)	Intact *mecI* & mutation in *mecA* promoter region (20)	1024
	Mutation in *mecI* at position 43 (3)	512
	Mutation in both sequences (3)	512
type-3 (9)	Deletion of *mecI* and 3'-end of *mecR1* (9)	512
type-4 (15)	Mutation in *mecI* at position 202 (11)	1024
	Deletion of *mecI* and 3'-end of *mecR1* (4)	256

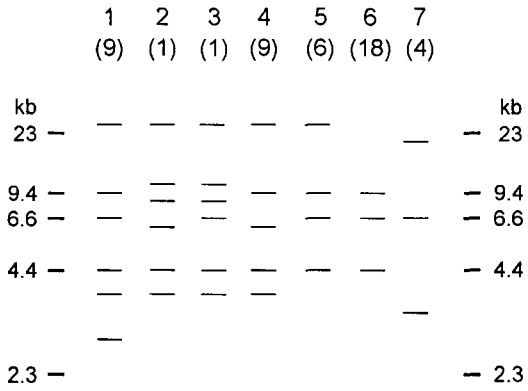

Figure 2. Ribotypes of 48 clinical strains of MRSA isolated from Korea. A total of 7 banding patterns were obtained by Southern blot hybridization of *Hind*III-digested genomic DNA with a 23S ribosomal DNA probe and *Hind*III-digested lambda DNA was used as a molecular weight marker. Parenthese are the number of isolates belonging to each ribotype.

Association between coagulase types, the genetic organization of *mec* regulator genes, and degree of methicillin resistance

As shown in Table 3, it was noted that all MRSA strains of coagulase type 3 were found to have a deletion of *mecI* and 3'-end of *mecR1* (*mecR1* PB), but all strains of coagulase type 2 had intact *mec* regulator genes. Most strains of coagulase type 4 (11 of 15) were found to have a mutation in *mecI*, but most strains of coagulase type 2 (20 of 26) had mutation in *mecA* promoter region not in *mecI*. Three strains of coagulase type 2 had mutations in both sequences.

Strains of coagulase type 2 revealed the high MIC_{90} of $\geq$ 512 μg/ml. Strains of coagulase type 4 revealed the MICs ranging from 128 to 1024 μg/ml, but the strains showing a deletion of 3'-end of *mecR1* revealed lower methicillin resistance than the strains carrying the gene. So, the important role of 3'-end of *mecR1* in determining resistance level was suggested.

Ribotyping of MRSA strains

As shown in Fig. 2, seven ribotype patterns were observed in 48 MRSA strains. (Ribotype of two strains could not be determined.) The most prevalent banding pattern was the type 6, which shared by 18 strains. Six strains had the type 5 banding pattern, in which the top band was absent in the type 6. Twenty-four of twenty-six coagulase type-2 strains were belonged to ribotype 5 or 6. All nine coagulase type-3 strains were corresponded to ribotype 1, and coagulase type-4 strains were distributed among ribotype 2, 3, 4 and 7.

We compared the above ribotypes with those of MRSA strains isolated in various countries. The ribotypes 1, 4 and 5 are the same to those of NCTC10442 strain, 85/3619 strain, and N 315 strain, respectively (6).

Inference of clonotype

As shown in Fig. 3, grouping based on the genotypes (mutation pattern of *mec* regulator

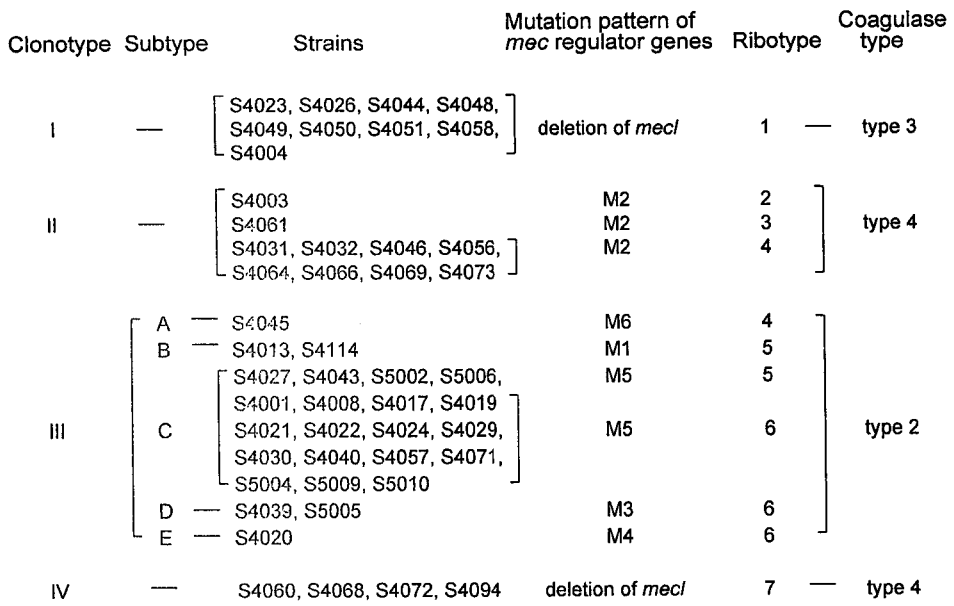

Figure 3. Inference of clonotype in clinical isolates of MRSA. Clonotype has been inferred with genetic organization of *mec* DNA, ribotypes and coagulase types. Subtyping of clonotype III is based on the mutation patterns of *mecI* gene and *mecA* promoter region. Mutation pattern and ribotype number refer to those of Table 2 and Figure 2, respectively.

genes and ribotypes) correlates well with coagulase type. Therefore, using the genetic organization of *mec* regulator genes, ribotypes and coagulase types, we could infer MRSA clonotypes for most of the strains listed in Fig. 3.

Forty-eight of fifty MRSA strains could be classified into 4 distinct clones. The clonality of two strains could not be determined due to their undefined ribotypes. Clonotype I is characterized by the coagulase type 3, deletion of *mecI* gene, and ribotype 1 shared by NCTC 10442 (6), the first reported MRSA isolate in England (9 strains). Clonotype II is characterized by the coagulase type 4, C to T substitution at position 202 of *mecI* gene, and ribotypes 2, 3 and 4 shared by 85/3619 strain (6) isolated in Austria (10 strains). Clonotype III is characterized by the coagulase type 2, mutations of *mecA* promoter region and/or *mecI*, and ribotypes 4, 5, and 6 shared by N315 strain (6) isolated in Japan (25 strains). This type was further divided into 5 subtypes based on the polymorphism of mutations in *mecI* and *mecA* promoter region. Clonotype IV is characterized by the coagulase type 4, deletion of *mecI* gene, and ribotype 7 similar to those of 93/H44 and 81/108 (6), prevalent strains in the 1980s in Japan (4 strains). Although clonotype IV strains were also distributed among coagulase type 4 producing strains with the clonotype II, their *mec* DNA and ribotype pattern were completely different from those of the clonotype II.

Recently, Hiramatsu et al. (6,9) described five distinct clones (I-A, II-A, II-B, II-C, and III-A) of MRSA differentiated by combining the types of *mec* DNA and the types of chromosome into which *mec* DNA is integrated (ribotype). Clonotype I-A is the oldest MRSA strains which prevailed in the 1960s in U.K. and clonotype III-A is disseminated in the countries having geographical or historical ties to U.K. (9). Clonotype II-A is the one prevailing in Japan as well as in USA (9). In comparison of these clones with our clones based on their genotype and coagulase type, our clonotype I, II, and III are well corresponded to clonotype I-A, III-A,

and II-A, respectively. Therefore, it is suspected that several MRSA clones, not the one prevailed, are distributed in Korea and the determination of the clonality of MRSA based on the genetic organization of *mec* regulator region, coagulase type and ribotype may be useful to track the evolution of MRSA.

Clonotype II-A is the most ubiquitous clone in Japan in 1990s and characterized by the production of coagulase type 2 and TSST-1 toxin (6,9). Interestingly, the first isolate (Mu50) of vancomycin-resistant *S. aureus* (VRSA) and the hetero-VRSA strains currently isolated in Japan have PFGE banding patterns similar to clonotype II-A (7). So, it was suggested that hetero-VRSA strains seem to have originated from this clonotype of MRSA and Japanese MRSA strains, represented by clonotype II-A, had the potential to generate VRSA more readily than MRSA strains from other parts of the world (7). Although this study was performed for the MRSA strains isolated from only one hospital in Korea, it showed the prevalence of the clonotype III, corresponding to the clonotype II-A. In addition, a VRSA strain (AMC11094) (MIC, 8 mg/L) was isolated from a surgical patient from 2,200-bed university hospital in Korea in 1999 (10). The emergence of the VRSA strain and the prevalence of clonotype II-A in Korea are warning to us that VRSA may become a global problem soon unless prudent use of antibiotics is practiced.

In conclusion, we now know that there are at least four kinds of clones of MRSA in Korea with a prevalence of the clonotype II-A (9): clonotype I considered to be the same to the clonotype I-A, the oldest MRSA strains prevailed in the 1960s in U.K., clonotype II considered to be the same to the clonotype III-A disseminated in the countries having close geographical or historical ties to U.K., clonotype III considered to be the same to the clonotype II-A, prevailing in Japan, and clonotype IV maybe evolved from the same one distributed mainly in Japan in 1980s.

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