

# A 25-year trace of methicillin-resistant *Staphylococcus aureus* dissemination in a geriatric hospital in Japan

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**Abstract:** We analyzed 218 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from the septicemia patients in a geriatric hospital for 25 years. These strains were classified into 11 major DNA types, A through K, and 27 minor types. The strains belonging to group A and B isolated before 1990 were susceptible to imipenem (IPM), fluoroquinolone, and most other antibiotics tested, except that they were markedly resistant to gentamicin. Strains mostly isolated in 1985 and thereafter were classified into group C through K, and they were mainly resistant to IPM, fluoroquinolones, and clindamycin. Analysis of the MRSA marker gene, staphylococcal cassette chromosome *mec* (SCC*mec*), of these strains revealed that the strains in groups A and B had mainly type IV and type I, respectively, and that strains in groups C through J had mainly type II. These results suggested that the strains holding type II SCC*mec* were resistant to IPM, fluoroquinolone, and clindamycin and they were dominant-resistant type after late 1980s. The antibiotic resistance profiles of MRSA dramatically changed during late 1980s, and these were correlated with the SCC*mec* types. The lesson from this study would be that consistent execution of surveillance study is needed to update the resistant profiles.

**Keywords:** methicillin-resistant *Staphylococcus aureus*, DNA typing, antibiotic resistance, survey, geriatric hospital

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major causative agent of nosocomial and community-acquired infections, which bring about variety of clinical symptoms, including endocarditis, osteomyelitis, pneumonia, toxic shock syndrome, food poisoning, carbuncles, and boils.<sup>1</sup> The nosocomial outbreak of MRSA was first reported in England in 1961 and that had been reported all over the world soon after.<sup>2-4</sup> The isolation frequency of MRSA strains dramatically increased in ~1960s thereafter.

Hospitalization of geriatric patients in general hospital due to bloodstream infection (BSI) has been increasing from 1980s through 2000s in the United States.<sup>5-7</sup> The major pathogenic organisms causing septicemia are *Escherichia coli*, *Staphylococci*, *Streptococci*, and *Pneumococci*.<sup>6</sup> Overall BSI-associated mortality has been reported to be 30%–60% in elderly patients, and the highest mortality rate is associated with *S. aureus* infection.<sup>7-9</sup> Associated risk factors causing BSI are high incidence of comorbid conditions, such as chronic heart or lung disease, hypertension, and diabetes mellitus.<sup>6,7</sup> It is increasingly reported in the health care-associated acquisition of MRSA in elderly patients.<sup>8</sup>

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For the monitoring of antibiotic susceptibilities, it is helpful to determine the minimum growth inhibitory concentration (MIC) of antibiotics. More recently, the gene(s) responsible for the resistance, such as the staphylococcal cassette chromosome *mec* (SCC*mec*) element, have been analyzed.<sup>10</sup> It is also powerful to use the molecular grouping technique to classify MRSA into subgroup by the pulsed-field gel electrophoresis (PFGE) of the chromosomal DNA; such study revealed the presence of highly virulent MRSA, such as USA300. The studies contribute both to the control of nosocomial infection and to a better understanding of the evolutionary relationships among MRSA.<sup>11–13</sup>

The data lacking is a long-term trace of antibiotic-resistant MRSA in a geriatric hospital. To analyze the fate of antibiotic-resistant MRSA, we collected MRSA from the patients in a geriatric hospital through two decades. And they were subjected to the SCC*mec* typing, the antibiotic susceptibility test, and the DNA typing. The results revealed that MRSA with unique DNA type were carried into the hospital probably with incoming patients and causing the nosocomial dissemination and forming the resistant clones.

## Material and methods

### Bacterial strains used

A total of 218 strains of MRSA were collected from the patients in a single geriatric hospital in Tokyo from 1978 through 2002. Information on patient's background and clinical symptoms could not be collected due to the law on the protection of privacy regarding the processing of personal data. All the *S. aureus* strains were isolated from the blood specimens on the mannitol–NaCl agar plate, and the colonies grown on the medium were subjected to the methicillin and oxacillin susceptibility test, the *mecA* gene amplification by Tsuchizaki et al method.<sup>14</sup> The *mecA*-positive strains with MIC of methicillin  $\geq 16$  mg/L or that of oxacillin  $\geq 4$  mg/L were classified as MRSA. These strains were kept frozen at  $-80^{\circ}\text{C}$  in the brain heart infusion broth (Nippon Becton Dickinson, Tokyo, Japan) supplemented with 40% glycerol.

### Antibiotics

Imipenem (IPM) was a gift from Banyu Pharmaceutical Co (Tokyo, Japan). Arbekacin (ABK) was purchased from Meiji Seika Kaisha (Tokyo, Japan). Gentamicin (GM), minocycline (MINO), rifampicin (RFP), and sulfamethoxazole were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ciprofloxacin (CPFX), clindamycin (CLDM),

trimethoprim, teicoplanin (TEIC), and linezolid (LZD) were a gift from Bayer Yakuhin (Osaka, Japan), Dainippon Sumitomo Pharma Co (Osaka, Japan), Shionogi and Co (Osaka, Japan), Astellas Pharmaceutical Inc (Tokyo, Japan), and Pfizer Japan Inc (Tokyo, Japan), respectively. Vancomycin (VCM) was purchased from Sigma-Aldrich (Saint Louis, MO).

### Determination of the MIC of antibiotic

The MIC of the antibiotics was determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline as described previously.<sup>15</sup>

### Typing of the SCC*mec* gene

Multiplex polymerase chain reaction (PCR) was carried out using a pair of primers according to the method of Oliveira and de Lencastre.<sup>16</sup> The DNA polymerase used was Phusion High-Fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland). The reaction mixture contained the formula recommended for the DNA polymerase. The thermal cycler was set to  $95^{\circ}\text{C}$  for 30 sec, a total 30 cycles of  $98^{\circ}\text{C}$  for 10 sec,  $57^{\circ}\text{C}$  for 10 sec, and  $72^{\circ}\text{C}$  for 15 sec, and  $72^{\circ}\text{C}$  for 5 min at the end using a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems Japan, Tokyo, Japan). The products were subjected to electrophoresis in 4% agarose gel in EDTA buffer at 100 V for 80 min. A 100-bp DNA ladder (New England Biolabs, Hitchin, UK) was used as a size maker. Since on time PCR over 25 years is not practical, all the samples were kept frozen at  $-80^{\circ}\text{C}$  and subjected to PCR within about half a year in 2007.

### PFGE

Chromosomal DNA was extracted from the MRSA cells and was digested with *Sma*I according to the method described by Bannerman et al.<sup>17</sup> The DNA plugs sliced at a thickness of 1–4 mm were placed in 170  $\mu\text{L}$  of a solution containing 10 units of *Sma*I, 20  $\mu\text{L}$  of T-buffer (0.1 M tris-HCl, pH 8.0, 700 mM  $\text{MgCl}_2$ , 0.2 M KCl, 700 mM 2-mercaptoethanol), and 20  $\mu\text{L}$  of 0.1% bovine serum albumin. The mixture was incubated at  $25^{\circ}\text{C}$  for 4 h. Samples were loaded on 1% agarose gel prepared in  $0.5 \times \text{TBE}$  buffer containing 44.5 mM tris, 44.5 mM boric acid, and 1 mM EDTA pH 8.0. The wells were sealed with 1% agarose in the same buffer. PFGE was carried out with a CHER-DRIII electrophoresis cell (Bio-Rad, Hercules, CA) at 6 V/cm for 20 h at  $14^{\circ}\text{C}$  with initial and final pulses conducted for 5.3 and 34.9 sec, respectively. The gel was stained with GelRed (Biotium,

Hayward, CA) according to the manufacturer's manual and visualized under a 254-nm UV light.

The digital images were analyzed with PDQuest™ software (version 4.5; Bio-Rad), according to the unweighted pair-group matching analysis clustering algorithm. The strains with over ~90% identity were classified into the same group. Those groups that consisted of less than four isolates were excluded from further study. Similar to PCR, PFGE of all strains was carried out in a few months.

## Results

### Grouping of MRSAs

Because all the MRSA strains subjected to this study were collected through two decades, 1978–2002, they might be suitable for the model study of chronological changes of MRSA with regard to antibiotic susceptibility, DNA type, and the MRSA marker gene. All 218 strains were subjected to PFGE fingerprinting, and 11 major groups (more than five strains per group) and 27 minor groups (less than four strains per group) were found (Figure 1). Group A through K was aligned along chronologic order of sampling (Figure 2). Strains isolated from 1978 through 1989 were classified into group A and B. Strains isolated ~1985 through 2002 were classified into the group C through K. Remaining 47 strains in 27 minor groups were not studied any further.

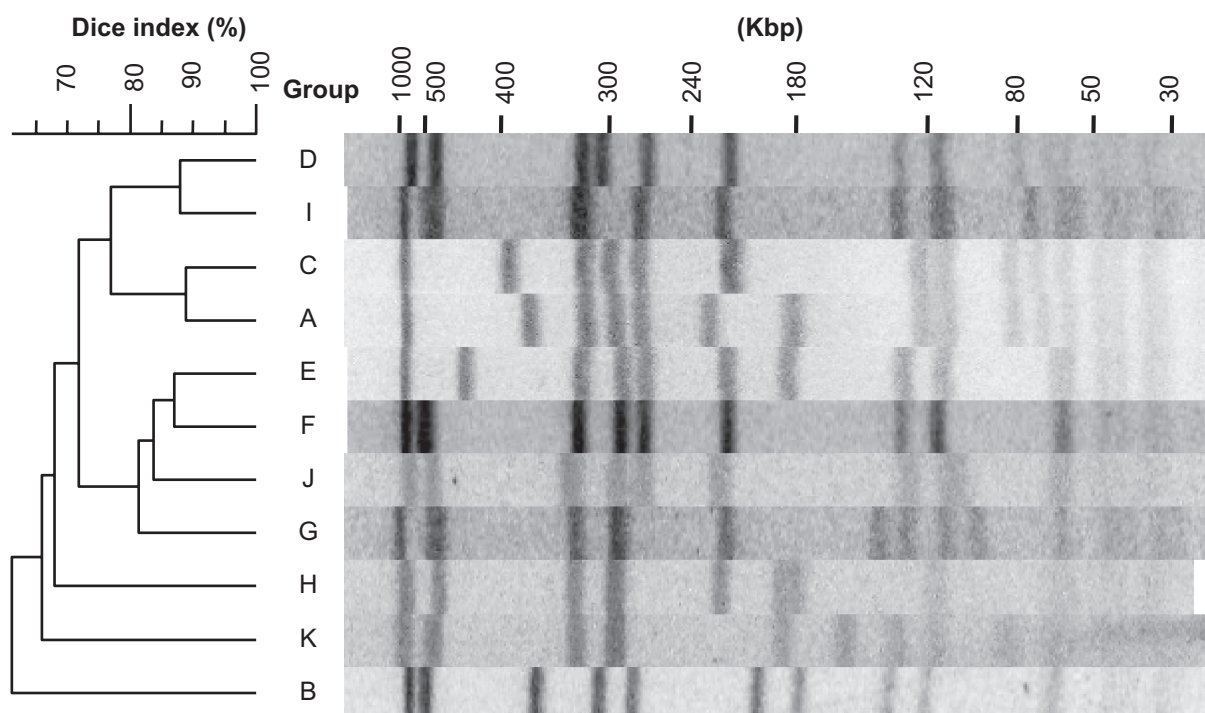
### Strains isolated from 1978 through 1989, the groups A and B

As seen in Figure 2, six strains in the group A, which was isolated from 1978 through 1989 had the type IV *SCCmec* without exception. Strains in the group B, consisting of 38 strains, isolated in 1980–1989, showed predominantly the type I *SCCmec* with only exception of two strains.

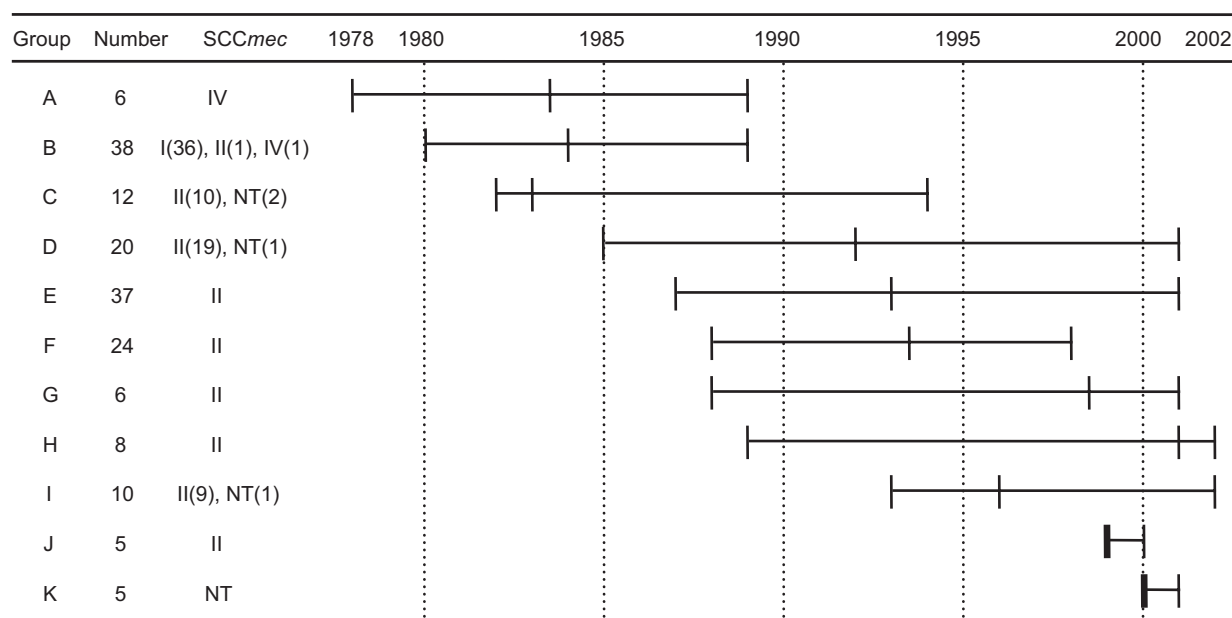
MIC<sub>50</sub> values were the antibiotic concentration that inhibits the growth of 50% of strains and the ranges were shown in Table 1. The MIC<sub>50</sub> of antibiotics in the respective groups was determined and the results were shown in Table 2. The strains in the groups A and B showed susceptibility to all the antibiotics tested with an exception against GM. These strains were collected during the period of 1980 through 1989 and were having the type IV or type I *SCCmec* predominantly. The strains in the group A and B were resistant to GM and that turned to be susceptible in the strain in the groups C, E, F, G, and K. The strains in the groups A and B were susceptible to MINO.

### Strains isolated ~1985 through 2002, the groups C through K

All the strains in the groups C through K isolated during the period of ~1985–2002 showed the type II *SCCmec* excepting



**Figure 1** PFGE profiles and a phylogeny of MRSAs grouped into 11 unique DNA types. The PFGE profiles were digitized, and the degree of homology was calculated by the unweighted pair-group method. The DNA profiles that showed over ~90% identity were classified into the same group. The groups of MRSA that showed unique DNA profiles but consisted of less than four strains were excluded from this figure. A dendrogram was derived from PFGE analysis of *Sma*I-digested chromosomal DNA.



**Figure 2** Length and the chronology of the MRSA isolation and their SCCmec type. Length and the chronology of the MRSA isolation and their SCCmec type A through K represents the groups of DNA type and number represents numbers of strain belonging to each type of DNA group. The SCCmec type was given. A horizontal bar shows duration, in which the same types of MRSA were isolated. A vertical cross bar within the horizontal bar is a median value.

nine nontypeable strains. Strains in group K, isolated in 2000–2001, had all nontypeable SCCmec by the present method. This seems to be understandable because the MRSA in this group were isolated in the latest period during this study. Therefore, the strains in the group K likely had the new type of SCCmec. The MINO-susceptible strains were found in the groups C, D, F, and I and the strains in the rest of groups were MINO resistant.

The strains classified into the group C, which was isolated during the period of 1982 through 1994, suddenly became resistant to the  $\beta$ -lactam antibiotics (IPM) and this trend has been kept for the strains in the groups D through K with only an exception that strains in the group G showed IPM susceptibility. Strains in the groups C through K, which were isolated during the period of 1985 through 2002, showed marked resistance to CPFX, though the strains in the group C isolated in 1982–1994 were intermediately resistant. These results suggested that strong  $\beta$ -lactam antibiotics and fluoroquinolone antibiotics might be introduced for clinical use in this period.

The GM-resistant strains in group A and B turned out to be more susceptible than those strains in group C, E, F, and G. However, reemergence of the GM-resistant MRSA in the groups C, H, I, and J suggested that GM-resistant clones were selected and spread within the hospital. The strains in the group E, G, H, J, and K were resistant to MINO suggesting that tetracycline derivatives might be used during the period for the groups G through J. The

strains in the group C through K except the group I were CLDM susceptible.

## Antibiotics effective against MRSA

All the strains tested showed the susceptible range of MIC<sub>50</sub> of ABK, RFP, ST, TEIC, VCM, and LZD, whereas they were resistant to IPM, GM, and CLDM according to the CLSI criteria. Thus, the analysis clearly revealed that the susceptibility to these drugs was unrelated with the PFGE fingerprinting and the SCCmec types.

## Discussion

PFGE is a useful method for genomic fingerprinting of microorganisms, and the data could be used for epidemiological study of virulent organisms. SCCmec typing is also used for the epidemiological study of MRSA.<sup>18–21</sup> However, it might be difficult to use the SCCmec typing for the clonal identification of MRSA because the SCCmec typing only analyzes a small fraction of the chromosome. Our investigation was conducted to analyze clonal identity of 218 blood-isolated MRSA from 1978 through 2002, and the results were used for the interpretation of the origins of the strain, possible dissemination, and persistency in the hospital. Attempts have been made to link the MIC of antibiotics with the DNA types of the strain and SCCmec type.

The DNA typing of the strain revealed four important facts: 1) Three quarters of the isolates, which belonged to only 11 DNA types among 39, were inhabited and spread

Table 1 MIC<sub>50</sub> of antibiotics in MRSA grouped to II DNA types by pulsed-field gel electrophoresis

| Group | IPM                        | CPFX              | GM                | ABK            | MINO               | RFP                | CLDM               | ST           | TEIC           | VCM         | LZD        |
|-------|----------------------------|-------------------|-------------------|----------------|--------------------|--------------------|--------------------|--------------|----------------|-------------|------------|
| A     | MIC <sub>50</sub><br>Range | ≤0.25<br>≤0.25–32 | 16<br>≤0.25–128   | ≤0.5<br>≤0.5–1 | ≤0.25<br>≤0.25     | ≤0.25<br>≤0.25     | ≤0.25<br>≤0.25–128 | 1<br>1       | ≤0.5<br>≤0.5–1 | 1<br>1–2    | 2<br>2–4   |
| B     | MIC <sub>50</sub><br>Range | 0.5<br>≤0.25–64   | >128<br>≤0.25–128 | 8<br>≤0.5–8    | ≤0.25<br>≤0.25     | ≤0.25<br>≤0.25     | ≤0.25<br>≤0.25–128 | 2<br>1–4     | 1<br>≤0.5–2    | 1<br>≤0.5–2 | 2<br>1–4   |
| C     | MIC <sub>50</sub><br>Range | 64<br>0.5–128     | 0.5<br>≤0.25–64   | ≤0.5<br>≤0.5–1 | ≤0.25<br>≤0.25–0.5 | ≤0.25<br>≤0.25–4   | >128<br>≤0.25–128  | 1<br>1       | 1<br>1–8       | 2<br>≤0.5–2 | 2<br>2–4   |
| D     | MIC <sub>50</sub><br>Range | 32<br>≤0.25–128   | 32<br>≤0.25–128   | ≤0.5<br>≤0.5–2 | 2<br>≤0.25–16      | ≤0.25<br>≤0.25–128 | >128<br>≤0.25–128  | 1<br>0.5–2   | ≤0.5<br>≤0.5–2 | 1<br>≤0.5–2 | 2<br>1–4   |
| E     | MIC <sub>50</sub><br>Range | 16<br>1–64        | 0.5<br>≤0.25–128  | ≤0.5<br>≤0.5–2 | 16<br>≤0.25–16     | ≤0.25<br>≤0.25–128 | >128<br>≤0.25–128  | 0.5<br>0.5–8 | 1<br>≤0.5–2    | 1<br>≤0.5–2 | 1<br>0.5–2 |
| F     | MIC <sub>50</sub><br>Range | 16<br>≤0.25–128   | 0.5<br>≤0.25–128  | ≤0.5<br>≤0.5–1 | ≤0.25<br>≤0.25–16  | ≤0.25<br>≤0.25     | >128<br>≤0.25–128  | 1<br>≤0.25–2 | 1<br>≤0.5–8    | 1<br>≤0.5–2 | 1<br>1–4   |
| G     | MIC <sub>50</sub><br>Range | 4<br>0.5–32       | 0.5<br>≤0.25–64   | ≤0.5<br>≤0.5   | 16<br>≤0.25–16     | ≤0.25<br>≤0.25     | >128<br>≤0.25–128  | 1<br>0.5–8   | 1<br>≤0.5–1    | 1<br>≤0.5–2 | 1<br>1–2   |
| H     | MIC <sub>50</sub><br>Range | 16<br>4–128       | 32<br>0.5–128     | ≤0.5<br>≤0.5–2 | 16<br>8–16         | ≤0.25<br>≤0.25     | >128<br>≤0.25–128  | 0.5<br>0.5–1 | 1<br>≤0.5–1    | 1<br>≤0.5–1 | 1<br>1–4   |
| I     | MIC <sub>50</sub><br>Range | 32<br>≤0.25–64    | 128<br>≤0.25–128  | ≤0.5<br>≤0.5–1 | 4<br>≤0.25–16      | ≤0.25<br>≤0.25     | ≤0.25<br>≤0.25–128 | 1<br>0.5–8   | 1<br>≤0.5–4    | 1<br>≤0.5–1 | 2<br>1–4   |
| J     | MIC <sub>50</sub><br>Range | 64<br>16–128      | 64<br>≤0.25–128   | ≤0.5<br>≤0.5–2 | 16<br>≤0.25–16     | ≤0.25<br>≤0.25–128 | >128<br>≤0.25–128  | 0.5<br>0.5   | 2<br>1–8       | 1<br>≤0.5–1 | 1<br>1–2   |
| K     | MIC <sub>50</sub><br>Range | 16<br>16–64       | ≤0.25<br>≤0.25    | ≤0.5<br>≤0.5   | 16<br>8–16         | ≤0.25<br>≤0.25     | >128<br>≤0.25–128  | 0.5<br>0.5–1 | 1<br>≤0.5–1    | 1<br>≤0.5–1 | 1<br>1     |

**Abbreviations:** IPM, imipenem; CPFX, ciprofloxacin; RFP, rifampicin; ST, sulfamethoxazole-trimethoprim; GM, gentamicin; ABK, arbekacin; MINO, minocycline; CLDM, clindamycin; TEIC, teicoplanin; VCM, vancomycin; LZD, linezolid.



**Table 2** The antibiotic susceptibility and SCCmec type of the strains grouped by the DNA type

| Group | Major type of SCCmec | IPM | CPFX | GM | ABK | MINO | CLDM | RFP | ST | TEIC | VCM | LZD |
|-------|----------------------|-----|------|----|-----|------|------|-----|----|------|-----|-----|
| A     | IV                   | S   | S    | R  | S   | S    | S    | S   | S  | S    | S   | S   |
| B     | I                    | S   | S    | R  | S   | S    | S    | S   | S  | S    | S   | S   |
| C     | II                   | R   | I    | S  | S   | S    | R    | S   | S  | S    | S   | S   |
| D     | II                   | R   | R    | R  | S   | S    | R    | S   | S  | S    | S   | S   |
| E     | II                   | R   | R    | S  | S   | R    | R    | S   | S  | S    | S   | S   |
| F     | II                   | R   | R    | S  | S   | S    | R    | S   | S  | S    | S   | S   |
| G     | II                   | S   | R    | S  | S   | R    | R    | S   | S  | S    | S   | S   |
| H     | II                   | R   | R    | R  | S   | R    | R    | S   | S  | S    | S   | S   |
| I     | II                   | R   | R    | R  | S   | S    | S    | S   | S  | S    | S   | S   |
| J     | II                   | R   | R    | R  | S   | R    | R    | S   | S  | S    | S   | S   |
| K     | NT                   | R   | R    | S  | S   | R    | R    | S   | S  | S    | S   | S   |

**Notes:** S, I, and R represent susceptible, intermediate, and resistant judged by the CLSI MIC<sub>50</sub> criteria except ABK, of which susceptibility was followed to that of AMK, AMK ≤ 16 mg/L. The criteria of susceptibility/resistance were as following: IPM ≤ 4 mg/L, CPFX ≤ 1 mg/L, GM ≤ 4 mg/L, MINO ≤ 4 mg/L, CLDM ≤ 0.5 mg/L, RFP ≤ 1 mg/L, ST ≤ 38/2 mg/L, TEIC ≤ 8 mg/L, VCM ≤ 2 mg/L, and LZD ≤ 4 mg/L.

within the hospital. 2) The strains inhabited in the hospital persisted for a long period of time causing the presence of multiple types of MRSA clones of different genotype in the same time. 3) Roughly, one quarter of strains were either successfully treated or phased out. 4) Group A (SCCmec type I) and B (type IV) were highly susceptible to both carbapenem and fluoroquinolone than group C–J (type II) and group K (nontype). In addition, it was generally said in Japan that the strains resistant to less numbers of antibiotics tend to be SCCmec type I and IV.

It was well recognized that the recently isolated MRSA from septicemia were found to have SCCmec type II.<sup>22</sup> However, the result shown in Figure 2 suggested that septicemia in early 1980s was caused by the strain having SCCmec type I (group B) and IV (group A). The strains having SCCmec type I and IV seemed to disappear from hospital probably because they were highly susceptible to multiple antibiotics except against GM. Because the SCCmec typing provides information mainly on β-lactam antibiotic susceptibility, drastic change of the SCCmec types might be related to the use of β-lactam. In fact, the SCCmec type of strains in the group A and B has been changed largely from type IV and I to type II in the group C through J that is consistent with the approval of carbapenem. Close examination of the dendrogram also showed drastic change of the DNA types in groups A, B, and C through K. Moreover, the CPFX susceptibility profile has been markedly changed between the strains in the group A (SCCmec type IV), B (SCCmec type I), and C through K (SCCmec type II and nontype) that is consistent with the approval of the fluoroquinolone antibiotics. One other thing notable in this study would be the GM susceptibility. The groups A and B, those isolated in the early phase of this study, were GM-resistant and the groups C, E,

F, G and K turned out to be GM-susceptible. This is probably due to the fact that the isolation frequency of the strain producing aminoglycoside-modifying enzyme decreased markedly.<sup>23</sup> The GM-resistant clones reemerged again later, which could be interpreted in two ways: 1) the susceptible strain gained GM resistance, or 2) new GM-resistant clone(s) were carried in from outside. Eventually, the strains resistant to GM, IPM, and CPFX, the multiantibiotic-resistant MRSA survived in the hospital.

## Conclusions

The conclusions of this study are as follows: 1) SCCmec type had been changed from type I and IV to type II in the late 1980s. 2) The strains having SCCmec type I or IV were more susceptible to antibiotics than type II. 3) The strains having SCCmec type II were highly antibiotic resistant and therefore they could be persisted in the hospital. To eradicate MRSA from hospitals, a long-term usage of antimicrobial agents must be designed cautiously. Therefore, preventing the dissemination of MRSA in the hospital environments may be the first priority among the infection-control programs. Active surveillance of MRSA, proper use of antibiotics, and hygienic hand-washing are recommended for prevention of nosocomial spread of MRSA.

## Disclosure

The authors report no conflicts of interest in this work.

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