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☐ Review Article ☐

Acquisition of Methicillin Resistance and Progression of Multiantibiotic Resistance in Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant Staphylococcus aureus *(MRSA) produces specific penicillin-binding protein, PBP2′, which shows remarkably low affinities to most β-lactam antibiotics except those such as penicillin G and ampicillin. The region surrounding* mecA *has been called additional DNA or* mec *and is thought to be of extraspecies origin. From the study of* mec, *we found that* mec *is a novel mobile genetic element and designated as staphylococcal cassette chromosome* mec *(SCC*mec*). There are three types of SCC*mec. *In the past decades, MRSA has become resistant to many antibiotics, such as carbapenems, new quinolones, and minocycline etc. It seems to be a characteristic of MRSA to acquire multi-resistance by accumulating multiple resistance genes around the* mecA *gene inside SCC*mec.

Key Words: MRSA, SCC*mec*, *mecA*, *mecI*, *mecR1*, PBP2′, *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in England in 1961 shortly after the development of methicillin, the first penicillinase-stable semisynthetic penicillin (Jevons, 1961). MRSA produces a novel cell-wall synthesis molecule, penicillin-binding protein 2′ (PBP2′), that is not produced by methicillin-susceptible *S. aureus* (MSSA). MRSA is resistant to practically all β-lactam antibiotics, a class represented by penicillins and cephalosporins.

In Japan, hospital infection caused by MRSA became a problem in the 1980s when the use of third-generation cephalosporin became widespread. Since then, the properties of MRSA have continued to change, becoming highly resistant to β-lactam antibiotics, as well as multiantibiotic resistant.

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Acquisition of methicillin-resistance gene

S. aureus produces four PBPs involved in cell wall synthesis. In addition to these PBPs, a novel PBP was discovered in MRSA, which was termed PBP2′ (or PBP2a) because it was located between PBP1 and PBP2 when the membrane proteins bound with ^{14}[C]-penicillin G were separated by electrophoresis (Hartman and Tomasz, 1984; Reynolds and Brown, 1985; Utsui and Yokota, 1985).

The binding affinity of PBP2′ to β-lactam antibiotics except ampicillin and penicillin G is markedly lower than those of other PBPs. Therefore, MRSA can produce the cell wall using this exogenous cell wall-synthesizing enzyme, PBP2′, and it is capable of growing in the presence of β-lactam antibiotics. The structural gene for PBP2′, *mecA*, was first cloned from MRSA TK784 using the adjacent tobramycin-resistance gene as a marker (Matsuhashi *et al.* 1986). As a result of nucleotide sequencing, it was estimated that *mecA* is 2010 bp in size and that PBP2′ consists of 670 amino acids (Song *et al.* 1987). PBP2′ of other strains, N315 and

BB270, was slightly different in that the proteins consist of 668 amino acids, and they are 96.4% and 99.4% homologous to that of TK784, respectively. In addition to *S. aureus*, *mecA* is also present in a wide range of methicillin-resistant coagulase-negative staphylococci (MRC-NS) (Suzuki *et al.* 1992; Kawano *et al.* 1996). The amino acid sequence of MecA in a methicillin-resistant *S. epidermidis* strain reported by Tesch *et al.* was identical to that of N315 (Tesch *et al.* 1990). Wu *et al.* identified a PBP gene encoding a protein with 88% homology to MecA of MRSA on the chromosome of *S. sciuri*, a member of coagulase-negative staphylococci (Wu *et al.* 1996). From this finding, *mecA* is considered to have been derived from an as yet unknown species of staphylococci closely related to *S. sciuri.*

It has been known that the methicillin-resistance gene is located on the *S. aureus* chromosome (Kuhl *et al.* 1978). Then, it was shown that the region surrounding *mecA* on the chromosome is absent in MSSA (Beck *et al.* 1986). Furthermore, it was reported that other drug-resistance genes are also present around the *mecA* gene (Matthews *et al.* 1987). This MRSA-specific region, absent in MSSA, has been called additional DNA or *mec*, but its exact definition and properties have not been clarified. As well, the relationship between this region and the transmission of methicillin-resistance among staphylococcal species remains unclear. Thus, we cloned and determined the nucleotide sequence of the entire *mec* in the *S. aureus* strain N315, which was isolated in Japan in 1982.

By comparing the nucleotide sequence in N315 to that of MSSA type strain NCTC 8325, it was shown that *mec* in N315 is an approximately 52 kb long with 27 bp inverted repeats at both ends and 15 bp direct repeats, one of which was situated in the right extremity of *mec* and the other which was situated outside *mec* abutting its left boundary. There were clusters of antibiotic-resistance genes inside *mec* which were carried by transposon Tn*554* and an integrated copy of plasmid pUB110. Many of the open reading frames (*orfs*), apart from those encoding antibiotic resistance, were considered nonfunctional because of the acquired mutations or partial deletions found in the *orfs*.

Two *orfs* potentially encoding novel site-specific recombinases were found in *mec*. These two *orfs* were subcloned and their function was investigated. When a plasmid vector carrying the two *orfs* was introduced into a N315 cell, the *mec* was precisely excised out of the chromosome. The excision was accompanied by the appearance of an extrachromosomal DNA carrying a novel attachment sequence (*att*SCC) generated by a head-to-tail ligation of the excised *mec*. The experimental plasmid carrying the cloned *att*SCC, as well as the two *orfs* encoding presumptive recombinases, was found to integrate itself into a *S. aureus* chromosome, both site and orientation specific. From these results, it was considered that this *mec* was a novel mobile genetic element which had not been previously reported (manuscript submitted for publication). We designated this *mec* as staphylococcal cassette chromosome *mec* (SCC*mec*), and the two *orfs* involved in the movements of SCC*mec* were named as cassette chromosome recombinase A (*ccrA*) and B (*ccrB*).

As shown in Fig. 1, there are three types of SCC*mec*. The size of SCC*mec* is not uniform, ranging from approximately 30 kb to 60 kb or greater (Hiramatsu *et al.* 1996). This is extremely large and comprises 1% to 2% of the entire chromosome given that the size of the *S. aureus* chromosome is approximately 3,000 kb. In addition, all three types of SCC*mec* are integrated at the same site on the chromosome and the integration site is located in an open reading frame designated *orfX*. These findings strongly suggest that MRSA was generated in the past by the integration of SCC*mec* into MSSA chromosome.

Accumulation of drug-resistance genes in SCC*mec*

S. aureus resistant to multiple antibiotics, such as penicillin, tetracycline and aminoglycosides, was a problematic pathogen of hospital infection in the 1950s in Japan (Mitsuhashi *et al.* 1973). Penicillin is inactivated by a penicillin hydrolyzing-enzyme, penicillinase. Aminoglycosides are inactivated by aminoglycoside-modifying enzymes that acetylate, phosphorylate or adenylate them. Chloramphenicol is inactivated by acetyltransferase, and erythromycin is modified and inactivated by an enzyme that methylates adenine of 23S rRNA (Lyon and Skurray, 1987).

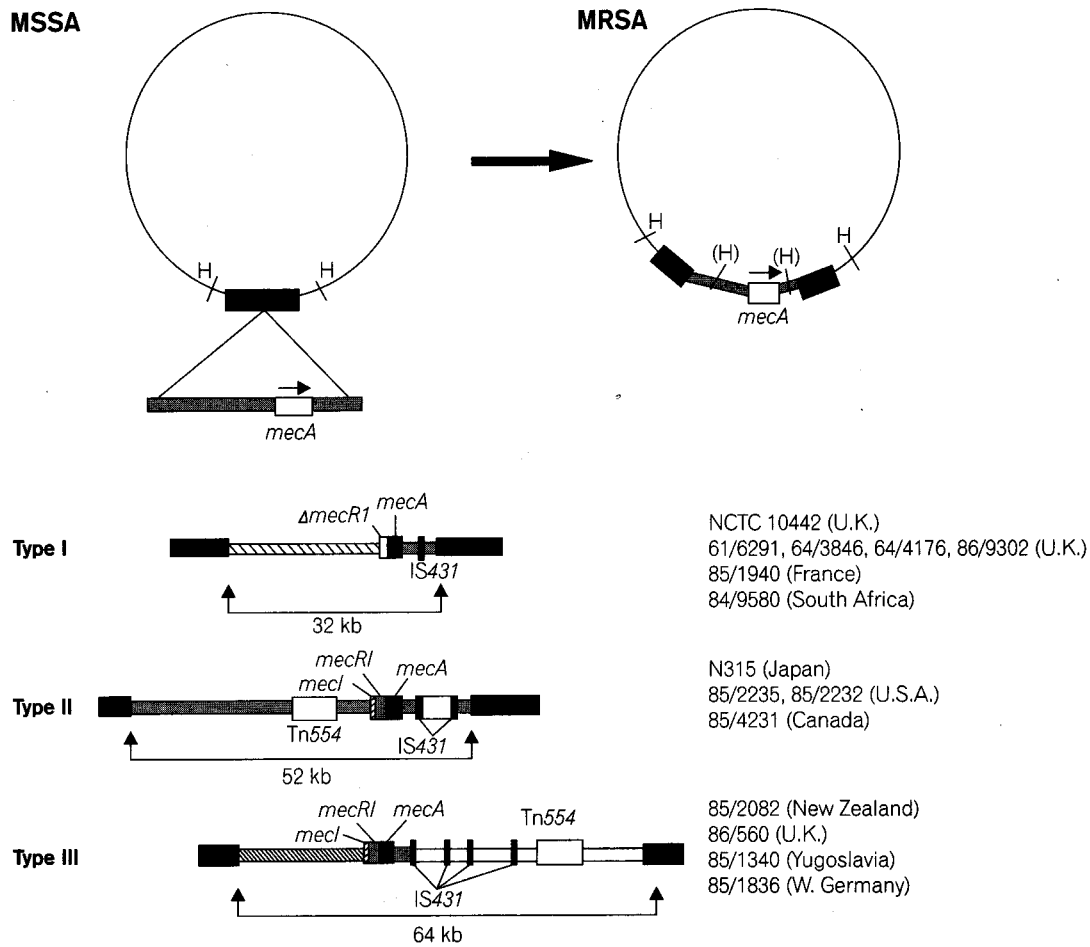

Fig. 1. *Three types of* SCCmec. ***MRSA*** *is generated by insertion of an exogenous mobile DNA,* SCCmec *into the chromosome of methicillin-susceptible* S. aureus. *There are at least three types of* SCCmec. *Type I* SCCmec *is found in MRSA isolated in England in the 1960s, represented by strain NCTC 10442. Type II* SCCmec *is found in N315. Type III* SCCmec *is present in MRSA isolated in the 1980s in Europe and countries having a close relation to United Kingdom such as New Zealand (strain 85/2082).*

Many of these resistance genes are located on transposons. They are present on either bacterial chromosomes or plasmids. These transposons include Tn*552* containing penicillinase gene, *bla* (Rowland and Dyke, 1989), Tn*554* containing *ermA* which encodes for erythromycin-resistance and *spc* which encodes for spectinomycin-resistance (Murphy *et al.* 1985), and Tn*4001* containing *aacA-aphD* which encodes for resistance to kanamycin, tobramycin, and gentamicin (Byme *et al.* 1990). IS*431*, an insertion sequence present in SCC*mec* of MRSA, is considered to be involved in the translocation of resistance genes, although by itself does not encode antibiotic resistance (Barberis-Maino *et al.* 1987). Due to the actions of these transposons and IS, the resistance genes located on small plasmids in the 1950s were by the 1980s frequently observed to form a cluster on a large molecular weight conjugative plasmid or on the chromosomal DNA (Byme *et al.* 1990). Many resistance genes are inserted and accumulated in SCC*mec* as well. In strain N315, a transposon Tn*554* encoding for re-

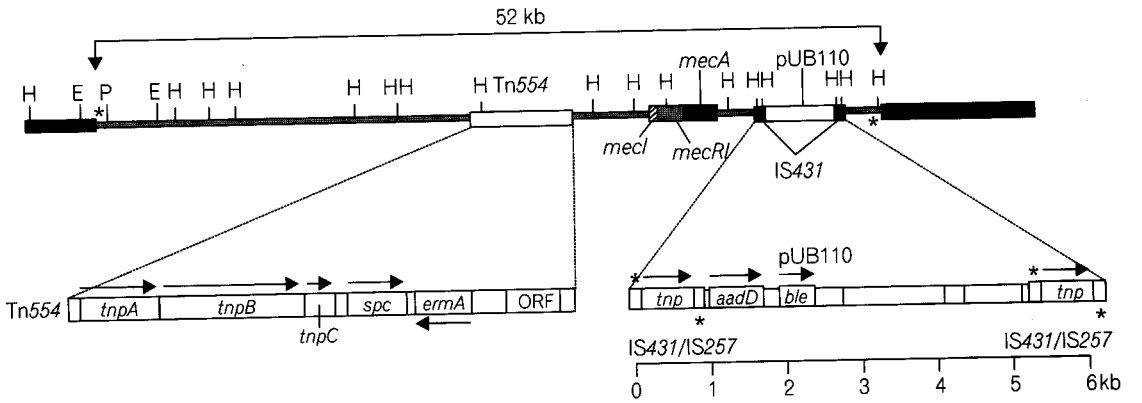

*Fig. 2. Drug-resistance genes present in N315-SCC*mec. *We constructed a chromosomal DNA library of strain N315. Starting with the* mecA *gene as a probe, SCC*mec *was cloned by successive chromosome walking. The sequences of both ends of SCC*mec *were determined by comparison with the corresponding nucleotide sequence of MSSA. The entire length of SCC*mec *in strain N315 is approximately 52 kb, and incomplete inverted repeats of 27 bp are present at both ends. A transposon, Tn*554 *is located upstream in the transcriptional direction of the* mecA *gene, which is not restricted to* S. aureus *and is widely found in genus* Staphylococcus. *Tn*554 *contains an erythromycin-resistance gene* (ermA) *and a spectinomycin-resistance gene* (spc) *in addition to three transposase genes,* tnpA, B, *and* C. *Two copies of IS*431 *are located downstream in the transcriptional direction of* mecA. *IS*431 *is an insertion sequence containing inverted repeats of 16 bp at both ends and 675 bp transposase* orf. B. subtilis*-derived plasmid, pUB110, is integrated between the two copies of IS*431 *on SCC*mec. *Resistance genes present in pUB110 are:* aadD, *kanamycin nucleotidyltransferase gene, and* ble, *a bleomycin-resistance protein gene.*

sistance to erythromycin and spectinomycin is located upstream in the transcriptional direction of *mecA*. Downstream, in the transcriptional direction of *mecA*, plasmid pUB110 is present on which *aadD* encoding for resistance to kanamycin and tobramycin and *ble* encoding for bleomycin-resistance are carried, flanked by the insertion sequence, IS*431* (McKenzie *et al.* 1986) (Fig. 2).

On investigating other MRSA strains in addition to N315, IS*431* was always found approximately 2 kb downstream of *mecA*. The number of IS*431* located on SCC*mec* is not always the same. One copy of IS*431* is located downstream in most MRSA strains containing type I SCC*mec* isolated in the 1960s, while in the majority of MRSA strains containing type II or type III SCC*mec* isolated in the 1980s, two or more copies of IS*431* are present. Dubin *et al.* reported that plasmid pT181 carrying *tet*, which encodes for resistance to tetracycline, and mercury-resistance operon are flanked by four copies of IS*431* in the SCC*mec* of strain ANS46 isolated in Australia (Dubin *et al.* 1992). Many strains containing type III SCC*mec*, including a New Zealand strain 85/2082, possess resistance genes similar to those of ANS46. Thus the IS*431*-mediated accumulation of resistance genes downstream of *mecA* have made the structure of SCC*mec* more polymorphic and complex.

Progression to high resistance and multiantibiotic resistance

PreMRSA strain N315 possesses *mecA* regulator genes, *mecI* and *mecRI* in their complete forms. It is called preMRSA because of its susceptibility to methicillin with a MIC of 1.56 μg/mL, which is due to a strong inhibition of *mecA* gene transcription (Hiramatsu *et al.* 1992; Kuwahara-Arai *et al.* 1996). The strain was subjected to selection in the presence of a low concentration of methicillin (3.13 μg/mL), and a resistant strain of N315LR3 was obtained. A colony of this strain was picked from the plate and cultured. Then the culture was inoculated on plates containing various concentrations of methicillin. As a result, although most of the cell population was inhibited by a relatively low concentration of methicillin, approximately 100 of 10^7 bacterial cells grew even in the presence of extremely high

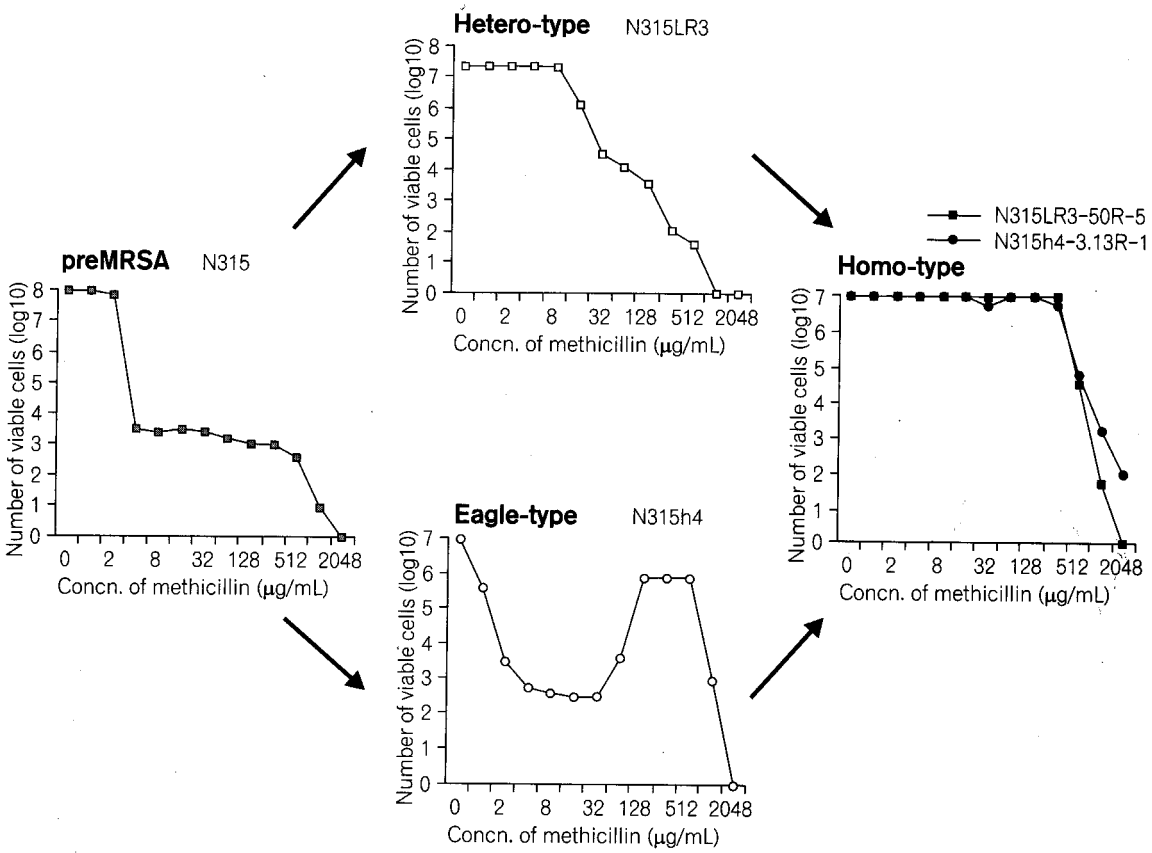

Fig. 3. Population analysis of resistant strains selected from N315 (Kuwahara-Arai et al. *1996). N315LR3 is a strain selected from N315 in the presence of 3.13 μg/mL of methicillin. N315LR3-50R is a strain obtained by re-selection in the presence of 50 μg/mL of methicillin from N315LR3. N315h4 is an Eagle-type resistance strain selected from N315 in the presence of 50 μg/mL of methicillin. N315h4-3.13R-1 is a homo-resistant strain selected from N315h4 in the presence of 3.13 μg/mL of methicillin.*

concentrations of methicillin (Fig. 3). Such a strain consisting of cell subpopulations with varying degrees of resistance is called hetero-type or heterogeneous resistance strain.

There were many hetero-type strains among the MRSAs isolated in the 1980s. However, when β-lactam antibiotics such as imipenem, which had a relatively greater antimicrobial activity against MRSA, became available in 1987, MRSA with higher level of methicillin resistance appeared. Such high-resistance strains consist of relatively homogeneous subpopulations with high methicillin resistance and are called homo-type or homogeneous resistance strains. When the N315LR3 described above was subjected to selection in vitro in the presence of higher concentrations of methicillin, homo-type strains such as N315LR3-50R were obtained.

In contrast, when N315 was cultured in the presence of high methicillin concentrations, 'Eagle-type' resistant strain was isolated, which was susceptible to methicillin at lower concentrations but resistant at high concentrations, as shown in Fig. 3. When the Eagle-type resistant strain, N315h4, was subsequently subjected to selection in the presence of low concentrations of methicillin, a homo-type resistance strain N315h4-3.13R-1 was obtained. In the experiments, acquisition of exogenous genes cannot be considered, indicating that the increase in resistance of MRSA is achieved by mutation of

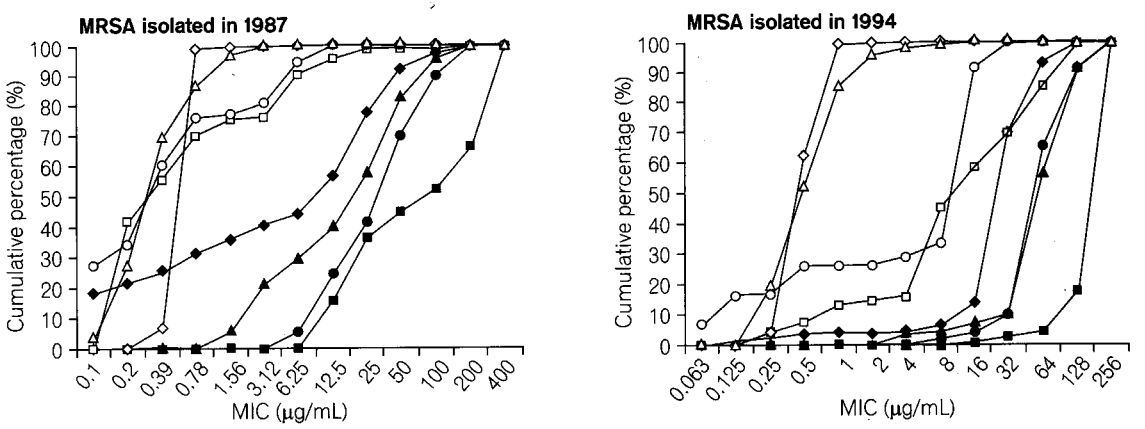

Fig. 4. *Drug-susceptibilities of 178 and 141 strains of MRSA isolated in 1987 and 1994, respectively. The cumulative percentage of the strains inhibited by various concentrations of drugs are shown (The data were provided by Ms. Toyoko Oguri, Clinical Laboratory, Juntendo University).* —■— : methicllin (1987), oxacillin (1994); —●— : cefmetazole; —▲— : flomoxef; —◆— : imipenem; —□— : ofloxacin; —○— : minocycline; —△— : arbekacin; —◇— : vancomycin.

chromosomal high methicillin resistance (*hmr*) gene (Berger-Bächi, 1994; Hiramatsu, 1995). This progression of the level of methicillin resistance seems to have occurred in clinical settings as shown in Fig. 3.

Fig. 4 presents the antibiotic susceptibilities of clinical MRSA strains isolated in Juntendo Hospital. The drug resistance of MRSA has greatly changed during 1987 to 1994 and progressed to higher and multiantibiotic resistance. While nearly half of the MRSA strains isolated in 1987 showed intermediate (or hetero) resistance to methicillin (oxacillin was used for the study in 1994), cefmetazole and flomoxef, most of these strains isolated in 1994 showed high resistance. Imipenem, which was active against MRSA in 1987, was hardly effective in 1994. The mechanism of progression to higher resistance to β-lactam antibiotics is not yet completely understood. However, it is thought that it is not due to the acquisition of exogenous resistance genes, but is due instead to mutation in staphylococcal genes. This hypothesis is based on the experimental in vitro generation of highly resistant strain from intermediate (or hetero) resistant MRSA (Kuwahara-Arai *et al.* 1996).

Fig. 4 also shows that multiantibiotic resistance has progressed in parallel. In 1987, there were only a few MRSA strains which were resistant to ofloxacin and minocycline. Vancomycin and arbekacin exerted good antimicrobial activity against these strains. However, in 1994, MIC of ofloxacin was increased to 8 μg/mL or greater and MIC of minocycline was increased to 16 μg/mL or greater in more than half of the MRSA strains, indicating that the bacteria acquired resistance to these drugs. The mechanism of acquiring resistance to these drugs has been considerably clarified.

New quinolones were successively developed in the 1980s beginning with norfloxacin in 1978. In new quinolone-resistant bacteria, mutation has been found on the DNA gyrase gene or topoisomerase gene or both on the chromosome, which are the target of these quinolones (Wiedemann and Heisig, 1994). Furthermore, a mechanism mediating the resistance by promoting quinolone efflux has been reported (Yoshida *et al.* 1990). Minocycline was developed as a drug active against tetracycline-resistant bacteria. The mechanism of minocycline resistance differ from the previously reported mechanism of tetracycline-resistance caused by *tetK* and other genes (promotion of tetracycline efflux). In minocycline resistance, a protein TetM, which is very similar to peptide elongation factor is synthesized and the protein synthesis system changes conferring resistance to tetracycline (Burdett, 1991). Arbekacin-resistant strains (MIC, equal to or greater than 12.5 μg/mL) were found in 2% to 8% of clinical isolates from 1990 to 1996 (Deguchi *et al.* 1997). Consequently, only vancomycin remained as a drug

which can be used to treat infections due to multiantibiotic, and highly resistant MRSA.

In Japan, vancomycin was approved for intravenous injection in 1991. However, vancomycin was not an exception for *S. aureus* to finally acquire resistance to it. Vancomycin-resistant MRSA (VRSA) showing the resistance level of MIC of 8 μg/mL was isolated in 1996 in Japan (Hiramatsu *et al.* 1997). It is increasingly important and necessary to use antibiotics prudently in the clinical settings, paying greater attention to the up-to-date trends of resistance in MRSA.

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