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Susceptibility of Methicillin-resistant *Staphylococcus aureus* Clinical Isolates to Various Antimicrobial Agents. II. Isolation of Arbekacin-resistant Strain.

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ABSTRACT

Resistance patterns against 24 antimicrobial agents were examined for 50 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated at Hiroshima University Hospital during the period October 1990 and July 1991. Overall resistance (the percentage of highly and moderately resistant strains) to the antimicrobial agents is summarized as follows: methicillin 100%, flomoxef 100% (β-lactams); kanamycin 94%, tobramycin 94%, amikacin 100%, isepamicin 94%, gentamicin 80%, dibekacin 94%, arbekacin 2% (basic oligosaccharide group/aminocyclitols); ofloxacin 96%, temafloxacin 96%, levofloxacin 96% (fluoroquinolones); erythromycin 98%, clarithromycin 98%, josamycin 30% (macrolides); vancomycin 0% (glycopeptide); tetracycline 94%, minocycline 94% (tetracyclines); fosfomycin 100%; mikamycin B 30%, nosiheptide 0% (peptide); rifampicin 2% (ansamycin); streptomycin 2% (basic oligosaccharide group); chloramphenicol 2%.

Arbekacin resistance was observed in one case: the cross resistance was complete among the aminocyclitol antibiotics tested in this study and streptomycin, probably due to the ribosomal alteration.

Key words: *Methicillin-resistant Staphylococcus aureus (MRSA), Antibiotic resistance, Vancomycin, Arbekacin, Toxic shock syndrome toxin I (TSST-1)*

Methicillin (DMPPC)-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961[5]. Since 1980, MRSA has caused increasing problems in hospitals worldwide[10,17,19].

The low-affinity penicillin binding protein (PBP), designated PBP 2'[21], PBP 2a[4] or MRSA PBP[15] encoded by DMPPC-resistance determinant *mec*A, a 2,130-bp segment of foreign DNA[1], is responsible for the intrinsic resistance to β-lactams.

Furthermore, many MRSA strains are resistant to a variety of antibiotics including kanamycin (KM), tobramycin (TOB), gentamicin (GM), erythromycin (EM), clindamycin and tetracycline (TC)[10].

This study aimed to examine the incidence of multi-drug resistance in MRSA isolated at Hiroshima University Hospital during the period October 1990 to July 1991. Although arbekacin (ABK) still remains to be one of the most effective antibiotics against the MRSA strains, a single strain isolated in April 1991 showed significant resistance to ABK. The circumstances surrounding the appearance of this strain will be discussed.

MATERIALS AND METHODS

Fifty MRSA strains were isolated at Hiroshima University Hospital between October 1990 and July 1991. DMPPC-susceptible *S. aureus* FDA 209P was used as a reference.

The antibiotics used and their manufacturers or distributors were as follows: DMPPC (SIGMA Chemical Co.); flomoxef (FMOX) and vancomycin (VCM) (Shionogi & Co., Ltd.); isepamicin (ISP) (Toyo Jozo Co., Ltd.); EM and clarithromycin (CAM) (Taisho Pharmaceutical Co., Ltd.); TC and minocycline (MINO) (Lederle Japan, Ltd.); fosfomycin (FOM) (Meiji Seika Kaisha, Ltd.); nosiheptide (NH) (Mitsubishi Kasei Corporation); josamycin (JM), ofloxacin (OFLX) and levofloxacin (LVFX) (Daiichi Pharmaceutical Co., Ltd.); temafloxacin (TMFX) (Tanabe Seiyaku Co., Ltd.); mikamycin B (MKM-B) (Kanegafuchi Chemical Ind. Co., Ltd.); KM, TOB, dibekacin (DKB), amikacin (AMK), GM and ABK (Inst. Microb. Chem.); chloramphenicol

Table 1. Incidence of antibiotic resistance in the MRSA strains isolated at Hiroshima University Hospital from October 1990 to July 1991 (50 strains)

Antimicrobial agent	Resistant strains		Moderately resistant strains		Susceptible strains		*S. aureus*
	No. of strains (MIC, μg/ml)	%	No. of strains (MIC, μg/ml)	%	No. of strains (MIC, μg/ml)	%	FDA 209P
Methicillin (DMPPC)	45 (≧100)	90	5 (12.5–25)	10	0	0	(0.10)
Flomoxef (FMOX)	44 (≧25)	88	6 (0.39–0.78)	12	0	0	(0.20)
Kanamycin (KM)	47 (≧50)	94	0	0	3 (0.78–3.13)	6	(0.78)
Tobramycin (TOB)	47 (≧25)	94	0	0	3 (0.20–0.78)	6	(0.10)
Dibekacin (DKB)	40 (≧25)	80	7 (3.13–6.25)	14	3 (0.20–0.78)	6	(0.20)
Gentamicin (GM)	40 (≧12.5)	80	0	0	10 (0.20–0.39)	20	(0.10)
Amikacin (AMK)	47 (≧6.25)	94	3 (1.56–3.13)	6	0	0	(0.39)
Isepamicin (ISP)	47 (≧3.13)	94	–	–	3 (0.78–1.56)	6	(1.56)
Arbekacin (ABK)	1 (>100)	2	0	0	49 (0.20–1.56)	98	(0.20)
Streptomycin (SM)	1 (>100)	2	0	0	49 (0.78–3.13)	98	(1.56)
Erythromycin (EM)	49 (≧12.5)	98	0	0	1 (0.39)	2	(0.20)
Clarithromycin (CAM)	42 (≧25)	84	7 (1.56–6.25)	14	1 (0.39)	2	(0.10)
Josamycin (JM)	13 (≧50)	26	2 (6.25–12.5)	4	35 (0.39–1.56)	70	(0.78)
Tetracycline (TC)	47 (50–100)	94	0	0	3 (0.10–0.39)	6	(0.10)
Minocycline (MlNO)	47 (6.25–25)	94	0	0	3 (0.05–0.10)	6	(0.05)
Fosfomycin (FOM)	48 (≧50)	96	2 (12.5–25)	4	0	0	(0.78)
Vancomycin (VCM)	0	0	0	0	50 (0.39–1.56)	100	(0.39)
Ofloxacin (OFLX)	46 (6.25–25)	92	2 (0.78–3.13)	4	2 (0.39)	4	(0.10)
Levofloxacin (LVFX)	46 (3.13–12.5)	92	2 (0.78)	4	2 (0.20–0.39)	4	(0.10)
Temafloxacin (TMFX)	46 (6.25–12.5)	92	2 (0.78)	4	2 (0.10–0.20)	4	(0.05)
Mikamycin B (MKM-B)	9 (≧100)	18	6 (12.5–50)	12	35 (3.13–6.25)	70	(3.13)
Nosiheptide (NH)	0	0	0	0	50 (0.003–0.012)	100	(0.003)
Rifampicin (RFP)	1 (1.56)	2	0	0	49 (0.024–0.20)	98	(0.10)
Chloramphenicol (CP)	1 (100)	2	0	0	49 (1.56–6.25)	98	(3.13)

(CP) (Wako Pure Chemical Ind., Ltd.); rifampicin (RFP) (Kanto Chemical Co., Inc.); streptomycin (SM) (Irvin Scientific).

The minimum inhibitory concentration (MIC) was measured by two-fold agar dilution method with Mueller-Hinton agar (DIFCO Laboratories). Test strains grown overnight at 37°C in 5 ml of Meuller-Hinton broth (MHB) (DIFCO Laboratories) were 10^2-fold diluted with fresh MHB, and about 5 x 10^3 CFU was applied with multipoint plating apparatus on the surface of agar plates. The plates were incubated at 37°C for 20 hr.

The production of β-lactamase by individual MRSA strains was monitored by using BBL cefinase (Becton Dickinson Microbiology Systems).

The production of toxic shock syndrome toxin I (TSST-1) was monitored by the polymerase chain reaction using synthetic primers. The details will be reported elsewhere.

RESULTS

Fifty MRSA strains were classified as: resistant, moderately resistant or susceptible to each antimicrobial agent depending on their MICs according to the definitions of Maple et al[10] and the British Society for Antimicrobial Chemotherapy[2]. Resistance patterns of all the MRSA strains against 24 antimicrobial agents as well as the MIC distribution of individual compounds are shown in Tables 1 and 2, respectively. Many of the MRSA strains showed resistance to more than 15 antibiotics. The antibiotics, to which more than 90% strains were resistant, included: DMPPC, FMOX, KM, TOB, DKB, AMK, ISP, EM, CAM, TC, MINO, FOM, OFLX, LVFX and TMFX. The resistance against GM was slight lower (80%) than these antibiotics and 30% strains were resistant to JM and MKM-B. Forty-nine of 50 strains were susceptible to the antimicrobial activity of ABK, RFP, CP or SM (resistance, 2%) and no resistance was observed to VCM and NH.

The resistance of MRSA to aminocyclitol antibiotics was determined by three inactivating enzymes: bifunctional 6'-acetyltransferase/2''-phosphotransferase AAC(6')/APH(2''), 4'-adenyltransferase AAD(4') and 3'-phosphotransferase APH(3')[20]. The

Table 2. MICs of 24 antibiotics against the MRSA strains isolated at Hiroshima University Hospital (50 strains)

Antimicrobial agent	MIC (μg/ml)			
	Range	50%	90%	FDA 209P
DMPPC	12.5 – >100	>100	>100	0.10
FMOX	0.39 – >100	50	100	0.10
KM	0.78 – >100	>100	>100	0.78
TOB	0.20 – >100	50	100	0.10
DKB	0.20 – >100	25	50	0.20
AMK	1.56 – >100	6.25	12.5	0.39
GM	0.20 – >100	50	50	0.10
ISP	0.78 – >100	12.5	25	0.39
ABK	0.20 – >100	0.78	0.78	0.20
SM	0.78 – >100	3.13	3.13	1.56
EM	0.39 – >100	>100	>100	0.20
CAM	0.39 – >100	100	>100	0.10
JM	0.39 – >100	1.56	>100	0.78
TC	0.10 – 100	100	100	0.10
MINO	0.05 – 25	12.5	12.5	0.05
FOM	12.5 – >100	>100	>100	0.78
VCM	0.39 – 1.56	0.78	1.56	0.39
OFLX	0.39 – 25	12.5	12.5	0.10
LVFX	0.20 – 12.5	6.25	6.25	0.10
TMFX	0.10 – 12.5	6.25	12.5	0.05
MKM-B	3.13 – >100	6.25	>100	3.13
NH	0.003 – 0.012	0.006	0.012	0.003
RFP	0.024 – 1.56	0.05	0.1	0.1
CP	1.56 – 100	3.13	6.25	3.13

Table 3. Distribution of aminocyclitol aminoglycoside inactivating enzymes in the MRSA strains

Phenotype	Aminocyclitol aminoglycoside-modifying enzyme	Number of strains
$KM^sTOB^sGM^sAMK^sABK^sSM^s$	–	3
$KM^rTOB^sGM^sAMK^sABK^sSM^s$	APH (3')	0
$KM^rTOB^rGM^sAMK^rABK^sSM^s$	AAD (4')	7
$KM^rTOB^rGM^rAMK^sABK^sSM^s$	AAC (6')/APH (2'') or AAC (6')/APH (2'')+APH (3')	1
$KM^rTOB^rGM^rAMK^rABK^sSM^s$	AAC (6')/APH (2'')+AAD (4') or AAC (6')/APH (2'')+AAD (4')+APH (3')	38
$KM^rTOB^rGM^rAMK^rABK^rSM^r$	not classified	1
	Total	50

Table 4. Type distribution of MLS-resistance in the MRSA strains

Phenotype	Type of resistance	Number of strains
$EM^sCAM^sJM^sMKM\text{-}B^s$	none	1
$EM^rCAM^rJM^sMKM\text{-}B^s$	MLS-resistance/inducible	34
$EM^rCAM^rJM^rMKM\text{-}B^r$	MLS-resistance/constitutive	15
	Total	50

When MLS-resistance is inducible, the strains are resistant to 14-membered macrolides (e.g., EM, CAM), but susceptible to 16-membered maclorides (e.g., JM), lincosamides and streptogramin B-type antibiotics (e.g., MKM-B).

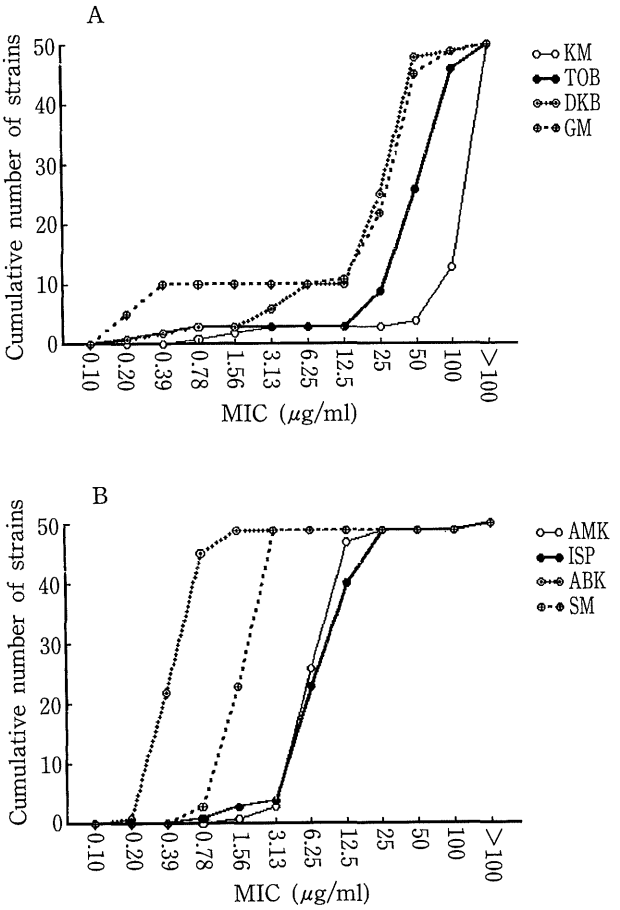

Fig. 1. Cumulative susceptibility of the MRSA strains to aminoglycoside-group antibiotics

The MICs of *S. aureus* FDA 209P were as follows (μg/ml): KM, 0.78; TOB, 0.10; DKB, 0.20; GM, 0.10; AMK, 0.39; ISP, 1.56; ABK, 0.78; SM, 1.56.

divergent phenotypes with respect to the resistance patterns to KM, TOB, GM and AMK were accounted for by the expression of these inactivating enzymes in individual MRSA strains: $KM^sTOB^sGM^sAMK^s$, no enzyme; $KM^rTOB^sGM^s AMK^s$, APH(3'); $KM^rTOB^rGM^sAMK^r$(so-called TOB^r type), AAD(4'); $KM^rTOB^rGM^rAMK^s$ (so-called GM^r type), AAC(6')/APH(2'') or AAC(6')/APH(2'') + APH(3'); $KM^rTOB^rGM^rAMK^r$ (so-called Mix^r type), AAC(6')/APH(2'') + AAD(4') or AAC(6')/APH(2'') + AAD(4') + APH(3'). As can be seen in Table 3, 45 strains expressed AAD(4') alone (7 strains) or in combination with AAC(6')/APH(2'') (38 strains), three strains were not protected by any inactivating enzymes, one strain expressed APH(3') and one strain was ABK resistant. The resistance pattern of the ABK^r strain could not be explained by these inactivating enzymes. The MIC distribution of each aminocyclitol antibiotic or SM is shown in Fig. 1.

The MLS resistance was inducible in 34 strains with a $EM^rCAM^rJM^sMKM\text{-}B^s$ drug-resistance phenotype and constitutive in 15 strains with a

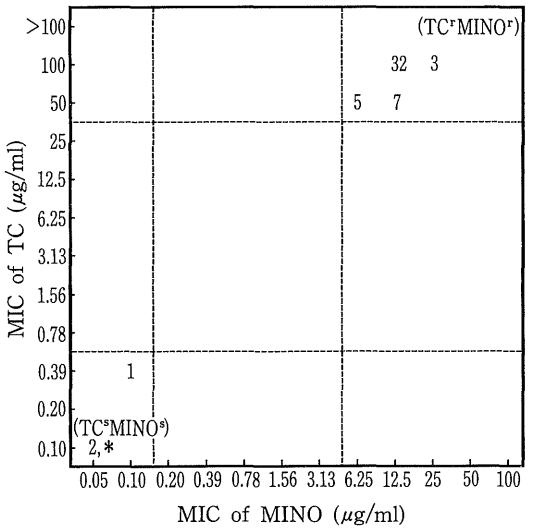

Fig. 2. Relationship between resistance to TC and MINO of the MRSA strains isolated at Hiroshima University Hospital

Figures represent the number of MRSA strains with the corresponding MICs. The MICs of TC and MINO for the ABK^r strain are 50 and 12.5 μg/ml, respectively, whereas those for the coagulase type IV strain are 0.1 and 0.05 μg/ml, respectively.
* represents the MICs of TC and MINO for *S. aureus* FDA 209P.

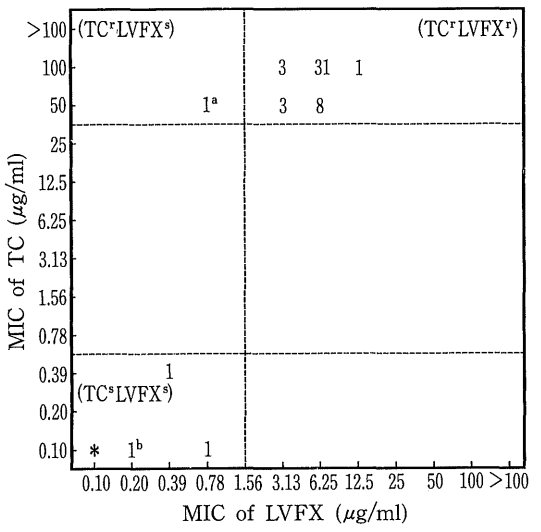

Fig. 3. Relationship between resistance to TC and LVFX of the MRSA strains isolated at Hiroshima University Hospital

Figures represent the number of MRSA strains with the corresponding MICs.
* represents the MICs of TC and LVFX for *S. aureus* FDA 209P. [a], ABK^r strain; [b], coagulase type IV strain.

$EM^rCAM^rJM^rMKM\text{-}B^r$ phenotype (Table 4). Only one strain was susceptible to any antibiotics in the MLS group.

As for tetracyclines and fluoroquinolones, the resistance was not expressed independently. The correlation between the resistances to TC and MINO and that between TC and LVFX is shown in Figs. 2 and 3, respectively.

DISCUSSION

In a previous study covering the period 1984 to September 1990[16], there was reported a vital turning point with respect to the drug-resistance of MRSA strains isolated at Hiroshima University Hospital. MRSA strains isolated before and after this turning point were tentatively designated groups A and B, respectively. MRSA strains isolated during the period covered in the present study, i.e., from October 1990 to July 1991, were, therefore, classified as group C. The number of strains were 22 (group A), 12 (group B) and 50 (group C).

MRSA strains harbor *mec*A gene coding for low-affinity PBP responsible for their intrinsic resistance to β-lactams, whereas the β-lactamase is thought to contribute to borderline resistance to β-lactams. The coagulase types and productivities of TSST-1 were characterized for 50 MRSA strains in group C. As for the coagulase type, type II strains amount to 49 with one type IV strain. TSST-1 was produced by all the type II strains but not by the type IV strain. Nakayama et al[12] reported an inverse correlation between the productivities of TSST-1 and β-lactamase, a fact confirmed in this study. The frequencies of β-lactamase-positive strains in groups A, B and C were 64% (14 of 22), 8% (1 of 12) and 2% (1 of 50), respectively. The aforementioned turning point defined by the drug resistance was also concerned with the production of β-lactamase or TSST-1.

The resistance pattern of MRSA strains to aminocyclitol antibiotics can be conveniently accounted for by the function of three different inactivating enzymes, AAD(4'), APH(3') and AAC(6')/APH(2'') (Table 3) encoded by *aad*D, *aph*A and *aac*A-*aph*D, respectively. Two types of MRSA strains were recognized by Ubukata et al differing in the length of HindIII fragments carrying *mec*A gene: 4.3- and 4.0-kb fragments[23]. The HindIII fragment of TOB-resistant (TOB^r) type MRSA strains containing both *mec*A and *aad*D genes was confined to the longer 4.3-kb fragment[23] and this type of MRSA had rapidly become dominant in Japan since its first report in 1983[7]. The linkage between *mec*A and *aad*D in the MRSA strains in group C was as high as 92% (45 of 49).

AAC(6')/APH(2'') is also frequently detected in MRSA strains either alone (GM-resistant type, GM^r type) or together with APH(3') and/or AAD(4'). The incidence of coexpression of AAC(6')/APH(2'') and AAD(4') (Mix-resistant type, Mix^r type) has been extremely high since April 1990[16]. In this study, the numbers of TOB^r-type MRSA and Mix^r-type MRSA strains were 7 and 38, respectively (45 altogether) with one GM^r-type strain, three inactivating enzyme-free strains including the coagulase type IV strain, and one ABK^r strain (Table 3). Most of them (45 of 50) were highly resistant to DMPPC (MICs, $\geqq 100$ μg/ml). The five strains consisting of the GM^r-type strain, three aminocyclitol-susceptible strains and one Mix^r-type strain showed low level resistance to DMPPC (MICs, 6.25 – 50 μg/ml).

An ABK^r strain was isolated, for the first time, with the MIC of ABK higher than 100 μg/ml; in contrast, the reported MICs of ABK for ABK^r strains ranged from 6.25 to 50 μg/ml[11]. Furthermore, the ABK^r strain was resistant to all the aminocyclitol antibiotics tested as well as SM (MIC^s, >100 μg/ml; Fig.1), implying that the resistance resulted from the ribosomal alteration. The drug-resistance properties of the ABK^r strain were not only unique among the MRSA strains but also far different from those of another MRSA strain isolated from the same patient. Properties of the ABK^r strain included the following: resistant to tetracyclines such as TC and MINO but rather susceptible to fluoroquinolones (Fig. 3), and exceptionally resistant to RFP. Taking these results into consideration, a small population of ABK^r strains might have existed before ABK was clinically used and the ABK^r strain was isolated by chance in the presence of ABK.

The coagulase type IV strain was susceptible to all the antibiotics tested except for DMPPC and FOM. On the other hand, no resistance was observed against VCM and NH. The efficacy of CP and RFP was satisfactory (resistance, 2%); RFP resistance was only encountered with the ABK^r strain.

There are two well-characterized mechanisms of MRSA resistance to typical tetracyclines such as TC and MINO, one of which involves the efflux of antibiotics from bacteria (e.g., plasmid-encoded Tet K determinant) and the other involving ribosomal protection (e.g., chromosomal Tet M determinant)[9,14]. The Tet K determinant mediated high-level resistance to TC but not to MINO. In contrast, the Tet M determinant expressed resistance to TC and MINO. In 47 of 50 MRSA strains, the resistance to tetracyclines seemed accounted for by the Tet M determinant, since they showed a TC^rMINO^r phenotype (Fig. 2). The remaining 3 strains were susceptible to tetracyclines with a TC^sMINO^s phenotype.

The incidence of tetracycline and fluoroquinolone resistance increased simultaneously since April 1990[16]. Moreover, these two types of drug resistance were coexpressed as seen in Fig. 3 (phenotype, TC^rLVFX^r). Only one exception to this general rule was the ABK^r strain (TC^rLVFX^s). The resistance to a number of structurally unrelated drugs was often conferred by efflux transport-

ers such as *Bacillus subtilis* Bmr[13], Tet K in *S. aureus*[14], Tet A, B and C in gram-negative bacteria[9] and NorA responsible for fluoroquinolone resistance in MRSA[6,22]. Based on these findings, the authors propose the existence of common efflux protein for TC, MINO and LVFX, which can be distinguished from Tet K and NorA.

Macrolide-lincosamide-streptogramin B (MLS) resistance was first described in *S. aureus*[3] and is now common in this and other species of staphylococci. MKM-B is structurally related to streptogramin B and is included in this group of antibiotics. The MLS resistance is conferred by the function of methylase which converts an adenosine residue of 23S ribosomal RNA to 6-*N*-dimethyladenosine, thereby reducing the affinity of the ribosome for all the MLS-group antibiotics[8]. The classical MLS resistance is inducible. When expression is inducible, MRSA is resistant to 14-membered and 15-membered macrolides only with a $EM^rCAM^rJM^sMKM\text{-}B^s$ phenotype. In contrast, constitutive MLS-resistance gives a $EM^rCAM^rJM^rMKM\text{-}B^r$ phenotype. Tillotson et al[18] suggested that the acquision of TC resistance resulted in a phenotypical change from constitutive MLS resistance to inducible one. In agreement with those findings, the TC resistant MRSA strains in groups A and B exhibited inducible MLS resistance and the marked phenotypical change in MLS resistance was observed between March and April 1990 from constitutive type to inducible one (in other words, from TC^s to TC^r). MRSA strains with a $TC^rEM^rCAM^rJM^sMKM\text{-}B^s$ phenotype were often isolated until March 1991. Since April 1991 constitutive MLS resistance has become dominant again without losing TC resistance (phenotype, $TC^rEM^rCAM^rJM^rMKM\text{-}B^r$).

Because of the isolation of ABK^r strain and a progressive increase in the number of antibiotics to which MRSA clinical isolates are resistant, the continued surveillance for the drug resistance seems important.

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