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Detection of *MecA* Gene in Clinical Isolates of *Staphylococcus aureus* by Multiplex-PCR, and Antimicrobial Susceptibility of MRSA

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Abstract Multiplex-PCR protocols were designed in order to make a rapid identification of MRSA. *MecA*, *femB*, and 16S rRNA genes were amplified for making a detection of MRSA. The incidence of MRSA in the clinical isolates of *Staphylococcus aureus* was examined by using a multiplex-PCR assay. The *mecA* gene was detected in 266 strains out of 336 clinical isolates of *S. aureus*, thus the incidence of MRSA was approximately 76.5%. The MRSAs of 247 strains (96.1%) showed resistance to more than eight species of the antimicrobial agents tested. The isolates of MRSA showed 27 different antimicrobial-resistant patterns. The results indicate that many different MRSA strains having high multidrug resistance are actually prevalent in Korea. Also, VISA was screened from the MRSA. Two strains were grown on the BHI agar plate supplemented with 8 μg/ml of vancomycin at a frequency of $1/10^8$ colony forming units or higher.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), *mecA*, multiplex-PCR, vancomycin-intermediate *Staphylococcus aureus* (VISA)

Staphylococcus aureus is one of the major causes of both community and hospital-acquired infections [2, 27, 34]. It produces numerous toxins, including superantigens that cause unique disease entities such as toxic-shock syndrome and staphylococcal scarlet fever [5, 28]. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major clinical problem. MRSA strains, which are resistant to multiple antimicrobial agents, had spread worldwide during the late 1980s and the 1990s, which limited the therapeutic options in various infections caused by these strains [2, 4, 13, 18, 27]. Moreover, MRSA strains are isolated with high incidence from the clinical patients in Korea [3, 13, 20, 21]. The *S. aureus* genome is composed of a complex mixture of genes, many of which seem to have been acquired by a lateral gene transfer [2, 10, 20]. Most of the antimicrobial resistance genes are carried either by plasmids or by mobile genetic elements [2, 10, 20]. Methicillin resistance of staphylococci is due to the production of a novel penicillin-binding protein, PBP 2a, with decreased binding affinity for methicillin [8, 29]. This is coded by the chromosomal gene *mecA*, which is located in an externally acquired *mec* region. The *mec* region has insertional sites of some antimicrobial-resistant genes, thus many MRSA strains show multidrug resistance [2, 4, 10]. The sequence of *mecA* gene is conserved in all MRSA and methicillin-resistant coagulase-negative staphylococci (CNS) [2, 4, 10]. Due to the fact that intrinsic resistance of both *S. aureus* and CNS appeared with no exception due to PBP 2a production, PCR and probe techniques have been developed to identify the *mecA* genetic determinant coding this protein [1, 6, 7, 12, 14, 15, 25, 31, 32]. These techniques show a high degree of correlation with susceptibility tests which make it possible to accurately classify highly resistant strains as well as borderline-resistant strains. Therefore, multiplex-PCR seems to be a good method with a certain degree of accuracy and time efficiency to detect MRSA. Vancomycin is recognized as a reliable antimicrobial agent in the final treatment of MRSA infections. However, vancomycin therapy, which is the final option in the clinical setting, is challenged with the appearance of VISA. Ten cases of vancomycin-intermediate *S. aureus* (VISA) have been reported worldwide until 2001 [24, 26].

In this study, multiplex-PCR protocols were designed for rapid identification of MRSA. The incidence of MRSA in the clinical isolates of *Staphylococcus aureus* was examined by using the multiplex-PCR method. The antimicrobial susceptibilities of MRSA strains were examined to classify

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them into antimicrobial-resistant patterns. Also, VISA was screened from the tested MRSAs.

MATERIALS AND METHODS

Bacterial Isolates and Reagents

During the period between September and November 2001, 326 strains of *S. aureus* were collected from the isolates of the clinical specimens at the clinical laboratory, Wonju Christian Hospital, Korea. The isolates were identified as *S. aureus* by identification kits. The isolates of *S. aureus* were cultivated on Brain Heart Infusion agar plates (BHI).

Oxacillin, vancomycin, lysostaphin, proteinase K, and agarose type IV were purchased from Sigma chemical Co. (St. Louis, MO, U.S.A.). *Taq* polymerase and PCR kit were purchased from Bionics Co. (Seoul, Korea). BHI was purchased from Difco (Detroit, MI, U.S.A.). PCR primers were synthesized by Genotech (Daejon, Korea).

Preparation of DNA

S. aureus cells from a single colony of overnight growth were washed and suspended in 400 µl of lysis solution [50 mM of Tris, 50 mM EDTA (pH 8), 50 mM NaCl]. Lysostaphin was added to the final concentration of 20 mg/l. The suspension was incubated at 37°C with gentle shaking for 60 min. The mixture was further incubated for 2 h at 50°C after the addition of 80 µl of proteinase solution [50 mM of Tris, 0.4 M of EDTA (pH 8), 0.5% sodium dodecyl sulfate containing 0.5 mg proteinase K]. DNA was extracted with 200 µl each of phenol and chloroform, and the mixture was centrifuged at 13,000 ×g for 5 min. The top layer was set aside, and the bottom layer was then mixed with an equal volume of ice-cold ethanol, and centrifuged as before. The DNA pellet was resuspended in 25 µl TE (10 mM of Tris, 1 mM of EDTA, pH 8) buffer and stored at -20°C until needed.

Multiplex-PCR

The specific primers for *mecA*, *femB*, and 16S rRNA gene were used for the multiplex-PCR assay. Bacterial 16S rRNA universal sequence was used as an internal control to identify potential false-negative results. *FemB* gene unique sequence to *S. aureus* was used as a marker to identify *S. aureus*. The sequences of 16S rRNA-specific primers were 5'-GGA ATT CAA A(T/G)G AAT TGA CGG GGG C (X) and 5'-CGG GAT CCC AGG CCC GGG AAC GTA TTC AC (Y) [31]. The primers gave rise to a 479 bp product that indicated the presence of eubacteria. The sequences of *mecA*-specific primers were 5'-GTA GAA ATG ACT GAA CGT CCG ATA A (mecA1) and 5'-CCA ATT CCA CAT TGT TTC GGT CTA A (mecA2) [9]. The primers gave rise to a 310 bp *mecA*-specific product that indicated the presence of methicillin-resistant gene. The sequences of *femB*-specific primers were 5'-TTA CAG AGT TAA CTG TTA CC (FemB1) and 5'-ATA CAA ATC CAG CAC GCT CT (FemB2) [17]. The primers gave rise to a 651 bp *femB*-specific product that identified *S. aureus*. To amplify the three genes, DNA templates were diluted to 10-folds. A 20 µl reaction mixture contained 2 µl of DNA template, 200 µM of each dNTP, 2.5 pmol 16S rRNA primers, 20 pmol *mecA* primers, 30 pmol *femB* primers, 1 unit of *Taq* polymerase, and 1× *Taq* polymerase reaction buffer. The cycling profile was composed of an initial step of 94°C for 1 min, followed by 35 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 1 min, and then 72°C for 30 sec. PTC-100 programmable thermal controller (MJ Research Inc, U.S.A.) was used for amplification of the genes. Each PCR batch was controlled with a known positive, a known negative strain, and an organism-free sample. Ten microliters of PCR product was separated on a 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Antimicrobial Susceptibility Test

The antimicrobial susceptibilities of *S. aureus* with *mecA* gene were determined using the Vitek system, following National Committee for Clinical Laboratory Standards recommended procedures. Random screening of vancomycin resistance was performed with a two-fold agar dilution method. VISA, which grows greater than or equal to 8 µg/ml of vancomycin, was screened from the tested MRSAs. MRSA isolates were cultured on the BHI agar plate supplemented with 8 µg/ml of vancomycin at a frequency of $1/10^8$ colony forming units or higher. The culture was incubated at 35°C for 48 h, and the growth was then examined by the naked eye.

RESULTS AND DISCUSSION

Multiplex-PCR and Incidence of MRSA

Conventional microbiological culture and antimicrobial susceptibility test require several days to confirm the presence of MRSA. Several multiplex-PCR protocols have been proposed to rapidly identify MRSA [1, 6, 12, 14, 25, 30, 31]. DNA sequences unique to both the species and the methicillin resistance were amplified simultaneously. These methods detect the *mecA* gene as the resistance marker, but use different target sequences to identify the species, such as *nuc*, *coaA*, *femA*, or *femB* [1, 12, 14, 25].

In this study, bacterial 16S rRNA universal sequence was amplified as the false-negative control. To determine both the species and the methicillin resistance, *femB* and *mecA* genes were simultaneously amplified with specific primers by multiplex-PCR. *Fem* (factor essential for methicillin resistance) genes are not linked to the *mec* determinant, and are found in both resistant and susceptible *S. aureus*

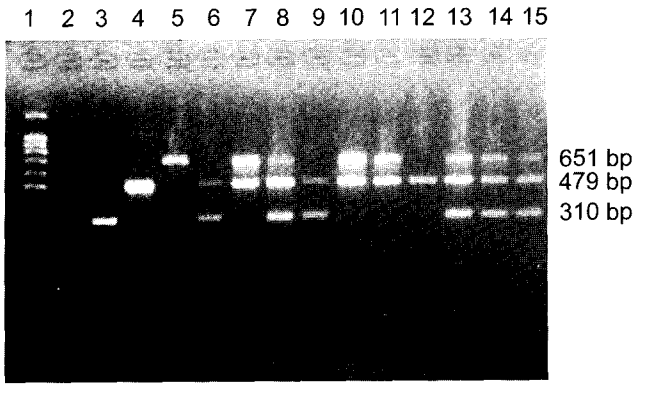

Fig. 1. The electrophoresis patterns of multiplex-PCR products on the agarose gel.
The DNA band of 310 bp, 479 bp, and 651 bp indicates *mecA*-specific product (lane 3), 16S rRNA-specific product (lane 4), and *femB*-specific product (lane 5), respectively. MRSA shows three DNA bands (lanes 8, 13, 14, 15), and MSSA shows 479 bp and 651 bp DNA bands (lane 7, 10, 11). Lanes 6 and 9 are *mec*-positive CNS. Lane 2 is internal negative control, and lanes 4 and 12 are internal positive controls.

strains. Enzymes coded for *femA* and *femB* are important in cross-linking peptidoglycan of staphylococci. *S. aureus*-specific primers of *femB* were designed to indicate *S. aureus* [9, 17]. The agarose gel electrophoresis patterns of the multiplex-PCR products are shown in Fig. 1, and 3 DNA bands are seen at 310 bp, 479 bp, and 651 bp in the *S. aureus* with *mecA* gene.

The *mecA* gene was analyzed to investigate the incidence of *mecA* in the 336 clinical isolates of *S. aureus* by the multiplex-PCR protocol. The *mecA* gene was detected in 257 out of 336 *S. aureus* strains (Fig. 2), indicating that the incidence of *mecA* gene was approximately 76.5%.

A rapid increase of MRSA has been reported since the mid-1980s in large university hospitals in Korea. The proportion increased from 24.5% in 1988 to 74.2% in 1995 [3, 30]. According to other reports, the proportion of MRSA already rose over 50% at most large hospitals in the early 1990s [13, 18, 20], and MRSA is one of the most commonly isolated endemic nosocomial pathogens in Korean hospitals today. In Germany, Austria, and Switzerland, 12.9% to 15.2% of the isolates of *S. aureus* were recognized as methicillin resistant in the mid-1990s [18], showing the highest rate of MRSA, as compared with earlier reports [3, 13, 18, 20, 30]. MRSA has become one of the most challenging nosocomial pathogens in the world [33], and is

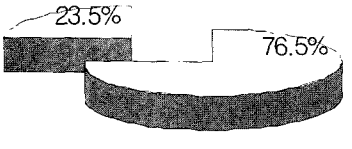

Fig. 2. The rate of MRSA in the clinical isolates of *S. aureus*.
The *mecA* was detected in 257 (76.5%) out of the 336 clinical isolates.

especially a serious problem in Korea due to its high incidence.

Antimicrobial Susceptibility

Antimicrobial susceptibility of MRSA isolates was investigated by Vitek system or disc diffusion method. The susceptibility of MRSAs was tested to 15 antimicrobial agents. The antimicrobial resistance was determined with standard minimum inhibitory concentration (MIC), which was greater than or equal to each of the following examples; 32 μg/ml teicoplanin, 32 μg/ml vancomycin, 16 μg/ml tetracycline, 80 μg/ml trimethoprim, 4 μg/ml rifampin, 16 μg/ml penicillin-G, 8 μg/ml oxacillin, 16 μg/ml gentamycin, 64 μg/ml nitrofurantoin, 8 μg/ml erythromycin, 4 μg/ml ciprofloxacin, 32 μg/ml cephalothin, 8 μg/ml clindamycin, 32 μg/ml ampicillin/sulbactam, and 16 μg/ml ampicillin. MRSAs from the result were classified into antimicrobial-resistant patterns as shown in Table 1.

Multifarious Antimicrobial-Resistant Patterns

The drug-resistant patterns of the clinical isolates seem to be extremely complex and diverse. The MRSAs of 254 strains (96.1%) have resistance to more than at least eight kinds of the antimicrobial agents tested (Fig. 3). The results indicate that most of the MRSA has a high multiple antimicrobial resistance. The isolates of MRSA showed 27 different antimicrobial-resistant patterns, and the results indicate the diversity of prevalent strains in Korea. The clinical control of MRSA has been challenged in many countries, therefore, the need for novel antimicrobial agents in the clinic has been increasing.

Numerous researches have been performed to identify a drug-target gene in MRSA [23]. Moreover, the whole genome sequence of pre-MRSA was reported in 2001 [19]. As a result, new drug-target genes have been suggested. However, only a few trials on the drug targets or the antimicrobial agents have been performed in Korea [9, 11, 22]. Greater effort is needed to develop novel antimicrobial agents against MRSA in order to overcome a crisis of antimicrobial therapy. Moreover, this research promises great potential for economic benefit.

The Rate of Resistance Against an Antimicrobial

The resistance rate to penicillin-G, oxacillin, gentamycin, erythromycin, ciprofloxacin, cephalothin, ampicillin/sulbactam, and ampicillin was higher than 92% (Fig. 4). The resistance rate to tetracycline and clindamycin was higher than 83%. Unfortunately, these antimicrobial agents cannot be expected to have therapeutic effect on most MRSAs in Korea. The strains tested were relatively susceptible to rifampin (86.4%) or trimethoprim (91.8%). All of the tested MRSA strains were found to be susceptible to nitrofurantoin. Two strains showed resistant to 8 μg/ml vancomycin and teicoplanin.

Table 1. Antimicrobial-resistant patterns of MRSA isolates.

Antimicrobial-resistant patterns	Resistant strains	
	Number	%
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Gen, Oxa, Pen, Rif, Tri, Tet	4	1.56
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Gen, Oxa, Pen, Rif, Tet	26	10.12
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Gen, Oxa, Pen, Tri, Tet	16	6.22
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Gen, Oxa, Pen. Tet	144	56.03
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Gen, Oxa, Pen. Rif	1	0.39
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Oxa, Pen, Rif, Tet	1	0.39
Amp, Amp/Sul, Cep, Cip, Ery, Gen, Oxa, Pen, Tet	1	0.39
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Gen, Oxa, Pen	6	2.33
Amp, Amp/Sul, Cep, Cip, Ery, Gen, Oxa, Pen, Tet	19	7.39
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Oxa, Pen, Tet	3	1.17
Amp, Amp/Sul, Cli, Cep, Cip, Gen, Oxa, Pen, Tet	4	1.56
Amp, Amp/Sul, Cep, Cip, Gen, Oxa, Pen, Rif, Tet	2	0.78
Amp, Amp/Sul, Cep, Cip, Gen, Oxa, Pen, Tri, Tet	2	0.78
Amp, Amp/Sul, Cli, Cep, Cip, Gen, Oxa, Pen. Rif	1	0.39
Amp, Amp/Sul, Cep, Cip, Gen, Oxa, Pen, Tet	7	3.39
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Oxa, Pen	2	0.78
Amp, Amp/Sul, Cli, Cep, Ery, Gen, Oxa, Pen	4	1.56
Amp, Amp/Sul, Cli, Cep, Cip, Ery, Oxa, Pen	2	0.78
Amp, Amp/Sul, Cep, Cip, Ery, Oxa, Pen, Tet	1	0.39
Amp, Amp/Sul, Cep, Ery, Gen, Oxa, Pen, Tet	1	0.39
Amp, Amp/Sul, Cli, Cep, Ery, Oxa, Pen	1	0.39
Amp, Amp/Sul, Cep, Ery, Oxa, Pen	2	0.78
Amp, Amp/Sul, Cep, Gen, Oxa, Pen	3	1.17
Amp, Amp/Sul, Cep, Oxa, Pen	2	0.78
Amp, Cip, Gen, Pen, Tet	1	0.39
Amp, Gen, Pen	1	0.39
Cli	1	0.39

The antimicrobials tested and abbreviations: Tie, teicoplanin; Van, vancomycin; Tet, tetracycline; Tri, trimethoprim; Rif, rifampin; Pen, penicillin-G; Oxa, oxacillin; Gen, gentamycin; Nit, nitrofurantoin; Ery, erythromycin; Cip, ciprofloxacin; Cep, cephalothin; Cli, clindamycin; A/S, ampicillin/sulbactam; Amp, ampicillir.

VISA Screening

VISA, which grows better in 8 μg/ml of vancomycin, was screened from the MRSAs tested. Two strains in the MRSA isolates were grown on the BHI agar plate supplemented with 8 μg/ml of vancomycin at a frequency of $1/10^5$ colony forming units or higher.

Ten cases of VISA were reported worldwide until 2001 and, in all cases, the resistant strains were isolated from the patients who repeatedly received vancomycin therapy [24, 26]. Only one case of VISA isolated in 1997 was reported in Korea [16]. Vancomycin is recognized as a reliable antimicrobial agent in the final treatment of MRSA

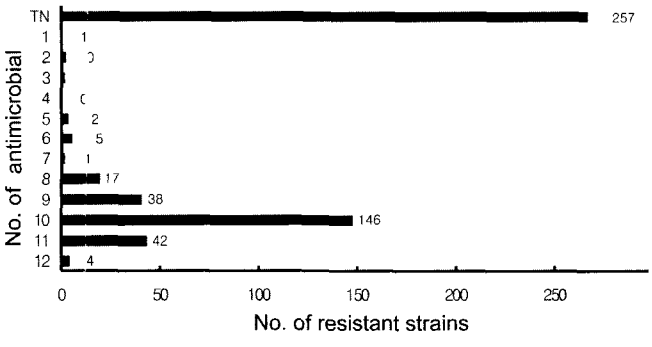

Fig. 3. The number of multifarious antimicrobial-resistant strains.
No. of antimicrobial means the number of resistant antimicrobial agent of a MRSA. and TN is the number of total MRSA strains.

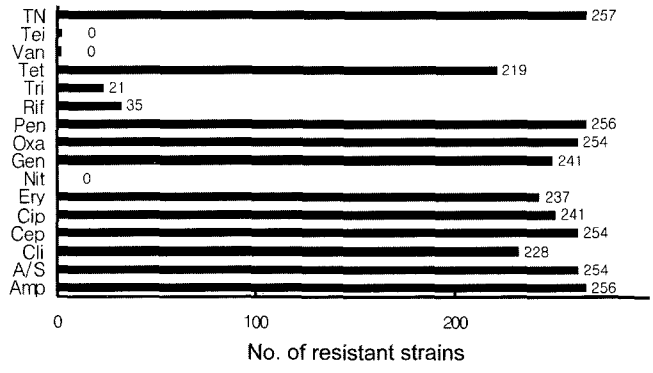

Fig. 4. The number of resistant strains against an antimicrobial agent.
TN, total number; Tie, teicoplanin; Van, vancomycin; Tet, tetracycline; Tri, trimethoprim; Rif, rifampin; Pen, penicillin-G; Oxa, oxacillin; Gen, gentamycin; Nit, nitrofurantoin; Ery, erythromycin; Cip, ciprofloxacin; Cep, cephalothin; Cli, clindamycin; A/S, ampicillin/sulbactam; Amp, ampicillin.

infections. However, vancomycin therapy, which is the final option in the clinic setting, is challenged with the appearance of VISA. We are now in the process of elucidating the physiological and genetic properties of VISA and rifampin-resistant strains.

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