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Epidemiological Studies on the Methicillin Resistant *Staphylococcus aureus* Isolated from Clinical Samples

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Abstract: A total of 45 *Staphylococcus aureus* strains from clinical samples were tested for the biochemical test and antibiotic susceptibility test. Forty-five *S. aureus* strains were subjected to the molecular epidemiological study by susceptiblity test, antibiogram, bacteriophage typing, polymerase chain reaction and *mec*-associated hypervariable region gene in order to detect of *mec*A gene which was one of the structural gene related to antibiotic resistant expression factors. Three of 15 *mec*A-negative *S. aureus* isolates were classified as oxacillin resistant despite borderline minimal inhibitory concentration values. Methicillin susceptiblities were completely consistent with PCR results for these strains. On the other hand, 4 of 30 *mec*A-positive isolates yielded results in the oxacillin and methicillin susceptibility tests which were discrepant from those of PCR analysis. Except for SA6, the methicillin resistant *S. aureus* strains tested were highly resistant to penicillin, oxacillin, gentamicin, and chloramphenicol. In the phage typing, 27 strains were typable. The lytic group III was as many as 12 strains, and 7 of 12 were 75/83A/84 type. In the PCR of specific *mec*A gene probe with chromosomal DNA of 30 methicillin resistant *S. aureus*, the amplified DNA band of 533 bp was confirmed in 30 strains and not in methicillin sensitive *S. aureus*. The single amplified band of hypervariable region related to *mec* was investigated in all of 30 methicillin resistant *S. aureus*, but in methicillin sensitive *S. aureus* it was amplified. The size of PCR products was between 200 bp and 600 bp. Four units was directly repeated.

Key Words: *mec*A, Hypervariable region polymerase chain reaction

INTRODUCTION

There has been a steady increase in the incidence of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in recent years[1]. These infections complicate the treatment of patients, prolong hospitalization, increase the cost of medical care, and are sometimes life threatening[16]. Although MRSA strains have not been shown to be more virulent than other strains, the multiple antimicrobial resistance of these isolates makes them responsible for high mortality rates in compromised patients. In hospital units, these strains are spread by patients, staffs, and environmental factors. Strains need to be distinguished from each other for epidemiological purposes. Reducing the number of MRSA infections by detec-

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ting and eradicating the sources of the organisms or by interrupting their path to the patient are important goals[24]. Since MRSA often colonize hospital personnels, attaining these goals depends, in turn, upon techniques for characterizing epidemic MRSA and for distinguishing them from resident strains. Antibiograms and phage typing are commonly used to characterize MRSA in hospital epidemiologic studies.

The mechanism of methicillin resistance (Mt^r) in *S. aureus* has been considered to be the production of a low-affinity penicillin-binding protein (PBP) variously termed PBP2'[8]. PBP 2a, or MRSA PBP cloned from the chromosomal DNA of MRSA in *E. coli* and sequenced[13]. Recently, this study recloned the *mec*A gene to methicillin senstive *S. aureus* (MSSA) and confirmed that expression of PBP 2' encoded by the gene results in Mt^r. Moreover, it has been demonstrated that a similar PBP was present in Mt^r *S. epidermidis* and was present in Mt^r *S. epidermidis* and was encoded by a gene identical to that from MRSA[17,19]. In Mt^r *S. haemolyticus*, DNA hybridizing with a probe prepared from MRSA has also been reported[20]. These findings suggest that *mec*A genes are found widely in different species of staphylococci. The *mec* DNA is over 30 kb long and integrates into a specific site of the *Sma*I G fragment of the *S. aureus* NCTC 8325 chromosome. MSSA lacks an allelic site for *mec*[22]. The *mec* determinant carries the *mec*A gene, which codes for the low-affinity penicillin binding protein PBP2', the component required for expression of methicillin resistance.

The *mec*A gene has been cloned and sequenced by different researchers[5]. Transformation of Mt staphylococci with a plasmid bearing *mec*A renders the recipient strain resistant to methicillin. On the *mec* determinant, Barberis-Maino *et al.*[2] have identified an insertion sequence-like element, termed IS*431*, which is associated with *mec*A. The intervening DNA sequencing between IS*431 mec* and *mec*A of various *mec* determinants has been claimed to be hypervariable, because the IS*431 mec-mec*A region shows DNA restriction length polymorphism[18]. Virtually no other sequences on *mec*, except *mec*A and IS*431 mec*, have been determined[21].

In staphylococci, many resistance determinants, located either on plasmids or on the chromosome, are flanked by single or multiple copies of insertion sequence-like elements. Insertion sequence or transposon attachment sites on the chromosome may function as target sites for recombination of different genetic elements such as, e.g., resistance genes, plasmids, bacteriophages, and transposons[2]. Therefore, the possible involvement of IS*431 mec* in gene transfer is a tempting hypothesis. The region comprising *mec*A and IS*431 mec* seems indeed to be active in promoting rearrangements of *mec*-associated DNA segments[23]. The amplified DNA region involved in these events comprised IS*431*. The site of excision was often located within or near IS*431*. All of these phenomena account for the instability of *mec*-associated sequences and appear to share a common site, namely, the DNA comprising IS*431*[9]. The aim of this work was to investigate the nature of this interesting DNA region and to an epidemiological study of nosocomial MRSA infections.

MATERIALS AND METHODS

Bacterial strains, Identification and Culture conditions

S. aureus organisms were identified as gram-positive cocci that were facultative anaerobes producing free coagulase tested by the tube coagulase test with reconstituted citrate rabbit plasma (Bio Merieux, Marcy l'Etoile, France), and aceton and that were deficient for β-galactosidase production[7]. Testing for methicillin resistance was performed by inoculating Mueller Hinton Broth (Difco. Lab., Detroit, Mich., USA)-5% NaCl agar plates according to the recommendations of Barry and Thornnsberry[3]. Resistance to methicillin was recorded when

the diameter of the zone of inhibition was less than 20 mm for 5 μg oxacillin disks (BBL. Becton Dickinson Microbiology Systems, Cockeysville, USA), as defined by the National Committee for Clinical Laboratory Standards (NCCLS)[15]. MRSA isolates were kept frozen in brain heart infusion medium containing 10% horse serum and 10% glycerol. Present study collected 30 MRSA isolates, 15 MSSA isolates, 5 Mt^r and 2 Mt^s *S. epidermidis* isolates and 5 Mt^r and 3 Mt^s *S. haemolyticus* isolates. The identification of all isolates used in the study was confirmed by using gram-positive identification card VITEK GPI (bioMerieux Vitek, Inc., St. Louis, USA). All bacterial strains were grown in LB broth at 37℃. Methicillin was added where needed[15].

Susceptibility Test

Minimal inhibitory concentrations (MICs) of methicillin against these strains were determined by the agar plate dilution method using Mueller-Hinton agar plates supplemented with 4% NaCl with an inoculum size of 1×10^4 to 5×10^4 CFU of bacteria according to the recommendation of NCCLS[15]. Bacterial growth was evaluated after incubation for 24 hours at 32℃. The antibiotics used in this study were tested for chloramphenicol, erythromycin, vancomycin, penicillin, rifampin, tetracycline, cephalothin (Sigma Co, St., Louis, USA).

Antibiogram

Antibiograms were determined by disk diffusion[4] by using the following antimicrobial agent-containing disks: chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), oxacillin (1 μg), penicillin (10 μg), rifampin (5 μg) and tetracycline (30 μg). Plates were inoculated and zone sizes were interpreted as described by the NCCLS[15]. The mean zone diameter was determined for each set of 20 organisms, and all zone diameters within ±2 mm of the mean were arbitrarily considered identical. Zone sizes of >2 mm from the mean were considered indicative of a different strain. Strains were given different letter designations if two or more of the antimicrobial agents tested had zone diameters of >2 mm from the mean for that drug. Strains that differed by a single antimicrobial agent were numbered as subtypes. Oxacillin MICs were determined by the broth microdilution method with Mueller-Hinton agar as described previously. The susceptibilities to antimicrobial agents were determined by the standard disk method[11].

Bacteriophage typing

Bacteriophage typing[6] was performed as described previously by using the international bacteriophage typing set at the routine test dilution and ×100 the routine test dilution. A plus sign indicates the presence of additional strong phage reactions. Phage types that differed by the presence or absence of one phage were considered to be related. Differences by the presence or absence of two or more phages were considered to be unrelated strains.

Primers for polymerase chain reaction

The primers used for the *mec*A gene amplification were (5'-AAAATCGATGGTAAAGGTTGGC) corresponded to nucleotides 1282 to 1303, and the outer (5'-AGTTCTGCAGTACCGGATTTGC) was complementary to nucleotides 1793 to 1814. The primers used for amplification of the *mec*-associated HVR were 5'-ACTATTCCCTCAGGCGTCC-3' (HVR1, location 338-356) and 5'-GGAGTAATCTACGTCTCATC-3' (HVR2, location 892-912). The sequence numbers indicate the position of the primers according to GeneBank Nucleotide Sequence Data Library (accession numbers X52593 and X52594, respectively)[14].

Template DNA preparation for PCR

Each isolate was subcultured overnight in 1 ml of trypticase soy broth and washed by centrifugation (1,000 x g) in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM disodium

EDTA. The cell pellet was resuspended in 500 μl of TE containing 15 U of lysostaphin per ml and was incubated at 37℃ for 1 hour. To this was added 1 ml of lysing buffer (composed of 0.45% Nondiet P-40, 0.45% Tween 20, and 0.6 μg of proteinase K per ml in PCR buffer [50 mM KCl-1.5 mM $MgCl_2$-1 mg of gelatin per ml - 10 mM Tris-HCl, pH 8.3]). The sample was further incubated for 1 hour at 56℃. Proteinase K was inactivated by being heated at 95℃ for 10 min. DNA purification was performed by phenol-chloroform extraction and ethanol precipitation. Extracted DNA was suspended in TE buffer.

PCR amplification

Extracted DNA (0.1 to 1.0 μg) was added to a PCR mixture containing 1 μM/L of each primer, 250 μM/L of each deoxynucleotide triphosphate, 2 U of *Taq* DNA polymerase, 50 mM/L KCl, 10 mM/L Tris-HCl (pH 9.0 at 25℃), 1.5 mM/L $MgCl_2$, and 0.1% Triton X-100 in a final volume of 50 μl of deionized water. Each sample was subjected to 30 cycles-consisting of 1 minute at 94℃, 1 minute at 55℃, and 2 minutes at 72℃-in a thermocycler (Ericomp, Inc., Conerstone Court West, San Diego, CA, USA). The reaction mixture (5 μl) was loaded onto 2% agarose gel with ethidium bromide. The band of amplified DNA was visualized under ultraviolet light.

Table 1. Correlation of *mec*A gene PCR results and methicillin resistance of *S. aureus*

Characteristic	No. of strains[a]			
	Oxacillin		Methicillin	
	S	R	S	R
*mec*A negative	12	3	10	5
*mec*A positive	4	26	4	26

[a] S: susceptible, R: resistant, MICs were determined by broth microdilution assay. The breakpoints for susceptibility and resistance were ≤2 and ≥4 μg/ml for oxacillin and ≤8 and ≥16 μg/ml for methicillin, respectively

Sequencing of DNA frgments

The PCR-amplified *mec*-associated HVR fragments were isolated and subcloned into the pGEM-T vector (Promega Corp. MI, USA). The nucleotide sequences of the dideoxy-nucleotide method with the Uniplex DNA Sequence Kit (Millipore Co., Bedford, USA).

RESULTS

Susceptibility Test

Determination of MICs of oxacillin and methicillin were performed for 45 clinical isolates of *S. aureus*. Table 1 summarizes the results. Three of 15 *mec*A-negative *S. aureus* isolates were classified as oxacillin resistant

Table 2. Susceptibility of *S. aureus* to 7 antibiotics

Antibiotics	MIC ranges (μg/ml)[a]	MIC_{50}[b]	MIC_{90}[b]
Chloramphenicol	8-128	8	128
Erythromycin	8-256	128	256
Oxacillin	4-512	4	256
Cephalothin	0.25-128	32	64
Penicillin	16-512	256	256
Rifampin	4-512	256	512
Tetracycline	8-64	32	32

a: Criteria of resistance was determined as described in NCCLS.
b: MIC_{50} and MIC_{90} are MICs required to inhibit 50% and 90% of the strains (μg/ml), respectively

Table 3. Classification of methicillin resistant *S. aureus* strains on the basis of antibiotics

Strain No.	Antibiotics*							Antibiotic pattern
	Pc	Ox	Ch	Er	Gm	Rf	Tt	
SA1	R	R	R	R	R	S	S	A-1
SA3	R	R	R	R	R	I	S	A-2
SA4	R	R	R	R	R	I	I	A-3
SA6	R	R	I	I	R	I	S	A-4
SA12	R	R	R	I	R	I	S	A-5
SA13	R	R	R	R	R	R	R	A-6
SA14	R	R	I	R	R	R	R	A-7
SA22	R	R	I	R	R	S	S	A-8
SA27	R	R	R	R	R	R	S	A-9

*Pc: penicillin, Ox: oxacillin, Ch: chloramphenicol, Er: erythromycin, Gm: gentamicin, Rf: rifampin, Tt: tetracycline, R: resistant, S: susceptible, I: intermediate

Table 4. Phage types of methicillin resistant *S. aureus*

Lytic group	Phage type	No. of strain	Subtotal
I	52	2	5
	29/80	1	
	79/80	2	
II	3A	1	4
	3A/55	2	
	3A/71	1	
III	42E	1	12
	83A	1	
	42E/53	2	
	83A/84	1	
	75/83A/84	7	
Miscellaneous	94/96	4	4
Mixed	80/81	1	2
	3C/81	1	
Nontypable		3	3
Total	30	30	

despite borderline MIC values (oxacillin MIC, 4 μg/ml). Methicillin susceptiblities were completely consistent with PCR results for these strains. On the other hand, 4 of 30 *mec*A-positive isolates yielded results in the oxacillin and methicillin susceptibility tests which were discrepant from those of PCR analysis. The susceptibilities of SA2, SA5, SA9, and SA11 to β-lactams were similar to those of *mec*A-negative strains (the MIC of oxacillin is shown in Table 2).

Antibiogram

The antibiograms are shown in Table 3. Except for SA6, the MRSA strains tested were highly resistance to penicillin, oxacillin, gentamicin, and chloramphenicol. The susceptibilities to the other antibiotics varied among strains. The resistance to rifampin was characteristic in strains SA13, SA14 and SA27. Accordingly, 9 types of antibiotic patterns were exhibited.

Bacteriophage typing

Since phage typing has been the standard epidemiologic tool for *S. aureus* for many years, present study was animal to test the ability of the various techniques to differentiate strains with a common phage type that were known to be epidemiologically unrelated. In the phage typing, 27 strains were typable. The lytic group III was as many as 12 strains, and 7 of 12 were 75/83A/84 type (Table 4).

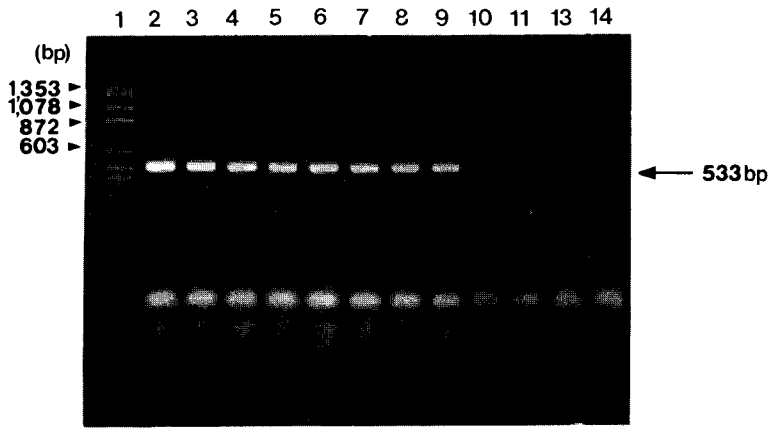

Fig. 1. Electrophoretic analysis of PCR-amplification products from *S. aureus*. PCR was performed with a lysate of MRSA of MSSA as a templates. Lane 1: *Hae*III digest of φX174 DNA, lane 2 to 9: MRSA, lane 10 to 12: MSSA, lane 13: negative control a *mec*A-negative strains. The size of DNA fragment in the bands are indicated in bp.

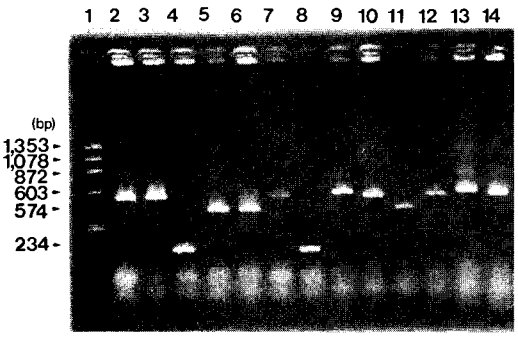

Fig. 2. Agarose gel electrophoresis of HVR-PCR products amplified from clinical isolates. Lane 1: size marker (*Hae*III digest of φX174 DNA), lane 2 to 7: MRSA isolates, lane 8 to 9: Mt^r *S. hamolyticus* isolates, lane 10 to 11: Mt^r *S. epidermidis* isolates, lane 12 and 13: Mt^s *S. epidermidis* isolates, lane 14: Mt^s *S. hamolyticus*.

Table 5. Molecular typing of MRSA and Mt^r *Staphylococci* isolates acording to the size of the HVR-PCR products

Strain	Type	PCR product	No. of isolates
MRSA	A	590	5
	B	510	9
	C	410	10
	D	194	6
Mt^r *S. epidermidis*	E[1)]	410	4
	F	310	1
Mt^r *S. hamolyticus*	G	310	3
	H[2)]	194	2

1) same as MRSA type C.
2) same as MRSA type D.

Detection of the *mec*A gene and the *mec*-associated HVR by PCR amplification

The PCR technique was applied to 8 strains of MRSA and 3 strains of MSSA. The DNA fragment of 533 bp was amplified from DNA of all MRSA and was absent from the susceptible strains. No background DNA bands were observed on the gel. The amplified DNA fragment of the *mec*A gene with a predicted size of 533 bp was detected in the 30 MRSA isolates, 5 Mt^r *S. epidermidis* isolates, and 5 Mt^r *S. haemolyticus* isolates. No DNA amplification was observed in methicillin sensitive strains (Fig. 1).

And a single amplified fragment of the *mec*-associated HVR was also detected in all MRSA, Mt^r *S. epidermidis*, and Mt^r *S. haemolyticus* isolates but not in methicillin-sensitive strains. Although the predicted size of the PCR product of the *mec*-associated HVR was 574 bp, products ranging from 200 to 600 bp were observed (Fig. 2). The 30 MRSA isolates were

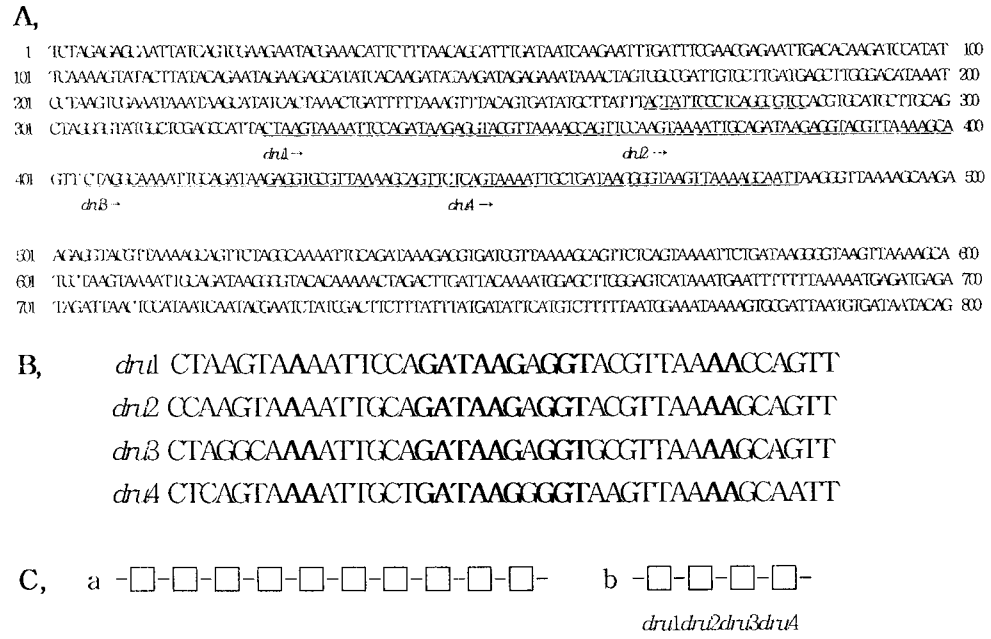

Fig. 3. A: Nucleotide sequence of the HVR-PCR product of MRSA isolats (HVR genotype B). Primers are boxed. The *dru*'s are indicated by arrow and are numbered in sequence. **B:** Alignment of 6 *dru* sequences. The underlined sequences the *dru* consensus sequence described by Ryffel *et al.* **C:** Schematic comparison of the *dru* element in the sequence described by Ryffel *et al.* (a) and *dru* element in present HVR-PCR products (b). Solid boxes indicate the *dru* sequences.

grouped into four HVR genotypes on the basis of the size of the PCR product (Table 5). This study observed two HVR genotypes in Mt^r *S. epidermidis* isolates and Mt^r *S. hamolyticus* isolates respectively.

Sequencing of the amplified HVR

The nucleotide sequence of the HVR genotype C of MRSA isolates had a 410 bp size (Fig. 3A). The DNA fragment contained 40 bp repeated consensus sequences (*dru*) in the HVR. The *dru* sequence was directly repeated four times in our DNA fragment (Fig. 3B) while Ryffel *et al*[18] detected 10 direct repeats.

DISCUSSION

The PCR technique described here required a bacterial suspension of more than 4×10^5 CFU/ml for the preparation of template DNA for PCR to detect the *mec*A gene, which was equivalent to 2×10^3 bacterial cells per PCR tube. Because of the relatively high limit for *mec*A detection, it was unlikely that contamination of a small number of the resistant bacterial cells led to the false-positive results. This detection limit seemed to be due to the low yield of template DNA prepared from methicillin-resistant *S. aureus*, since 10 PFU of λ phage per reaction tube yielded positive results for phage sequence detection by PCR performed under conditions similar to those for *mec*A detection. In testing for the *mec*A gene. This study used a bacterial suspension of 3×10^8 CFU/ml to prepare bacterial lysate, which was sufficient to obtain very clear results, as shown in Fig. 1. Moreover, one colony 1 mm in diameter was enough to prepare a bacterial suspension of this density, though there was a possibility that a single colony from a clinical specimen yield fasle negative results in clinical laboratories. Furthermore, definite results could

be obtained within several hours after isolation of the colony to be tested. Another potential advantage of this method is that agarose gel electrophoresis for identification of amplified DNA fragments enables detection of several genetic markers in the same experiment by using appropriate primers which amplify different sizes of DNA regions. The cryptically methicillin-resistant strains were most probably derived from typically resistant strains. They were first selected as methicillin-resistant strains by β-lactam antibiotics but later stopped the production of PBP2' with loss of their resistance. The unstable nature of methicillin resistance has been reported previously[10], and a recent report described *mec*A-positive but pheontypically susceptible subclones, as well as *mec*A-positive but phenotypically susceptible subclones as well as *mec*A-negative ones, that arose from a methicillin-resistant *S. aureus* susceptible to β-lactams. These findings support the derivation of cryptically methicillin-resistant strains from typically resistant ones. Another possible explanation is that the cryptic strains continued to be methicillin susceptible even after acquisition of the *mec*A gene but were later selected by resistance to drugs other than β-lactams.

Ryffel *et al*[18] characterized the DNA sequence intervening between *mec*A and IS*431 mec*, which they labeled the "hypervariable region". Investigators have suggested that IS*431 mec* is involved in gene transfer and that the *mec* determinant is acquired onto the chromosomal gene. This study amplified the *dru* element in the HVR of the *mec* deteminant of Mt^r staphylococcal strains by using PCR. The present study detected both the *mec*A gene and the HVR of *mec* determinant only in Mt^r strains, suggesting that the HVR was also specific for Mt^r Staphylococcus. In the present study, MRSA isolates were classified into four HVR genotypes according to the size of HVR-PCR products. These typing method would be easier and more rapid to perform than recently developed molecular typing methods, such as genomic DNA fingerprinting by pulsed-field gel electrophoresis[10], because present method dose not require the specialized electrophoretic equipment and restriction enzyme digestion. The present study takes about 8 hours to perform this method, whereas it takes at least 2 or 3 days to perform genomic DNA fingerprinting by pulsed-field gel electrophoresis. The distribution of HVR genotypes of MRSA isolates and the pattern of antibiograms differed among hospitals, suggesting that this molecular typing method may be useful for epidemiologic investigation of nosocomial infections caused by MRSA.

The nucleotide sequence of the HVR-PCR product of MRSA isolates in the present study was similar to that of the *mec*-associated HVR described by Ryffel *et al.*[18], although the number of *dru* sequences differed. The similarity between the two sequences confirmed that the HVR-PCR products represented the amplified *dru* element of the *mec*-associated HVR and that the length polymorphism of this region was due to an unusual cluster of *dru* sequences. The *mec*A gene was present in different species of staphylococci. However, it remains to be determined how staphylococci acquire the *mec*A gene on the chromosomal gene and how the *mec*A gene was transferred among different species of staphylococci. Most of the HVR-PCR products in *S. epidermidis* isolates, but not in *S. haemolyticus* isolates, were the same type as those in MRSA isolates (HVR genotype C, D).

In conclusion, methicillin-resistant staphylococci could be successfully detected by the HVR-PCR technique employed here. This method could also be used to detect cryptically methicillin-resistant strains which yielded a typically methicillin resistant subpopulation. From the viewpoint of clinical practice, these cryptic strains should not be classified as methicillin susceptible in spite of their susceptiblilty to β-lactam antibiotics, because of the possibility that typically methicillin-resistant variants appeared

during chemotherapy with β-lactam antibiotics.

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=국문초록=

임상가검물에서 분리한 Methicillin내성 *Staphylococcus aureus*의 분자역학적 연구

부산대학교 대학원 의학과

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임상가검물에서 생화학 검사와 항생제 감수성 검사를 통하여 45주의 *Staphylococcu aureus*를 분리하여 역학적 연구를 위한 형별 분류로써 항생제 감수성 검사, bacteriophage typing, 효소중합 연쇄반응 등을 실시하였으며, methicillin 내성과 관련이 있는 *mec*A 유전자를 검출 및 역학적 변별력이 있는 가변지역의 중합효소증폭반응을 실시하여, 약제 내성과 관련된 구조 유전자의 발현 양상을 비교하여 특정 유전자 배열의 상동성을 밝히고자 하였다. 45주의 *Staphylococcus aureus*중에서 *mec*A 유전자 양성주는 30주였으며, 그 중에서 26주가 methicillin에 내성을 보였다. 약제 내성양상에 따라 9개의 antibiogram으로 분류되었으며, SA6을 제외한 균주에서 penicillin, oxacillin, gentamicin 및 chloramphenicol에서는 높은 내성을 보였으며, SA13, SA14 및 SA27에서는 rifampin에 내성을 보였다. 27주에서 bacteriophage 형별 분류가 가능하였으며, lytic group III가 12주로 가장 많았다. *mec*A 유전자와 *mec*관련 가변부위의 polymerase chain reaction을 실시한 결과 모든 methicillin resistant *Staphylococcus aureus*에서는 533 bp의 증폭 band가 관찰되었으나, methicillin 감수성 균주에서는 증폭된 band가 관찰되지 않았다. *mec*관련 가변부위의 polymerase chain reaction에서는 200 bp에서 600 bp사이에 분포하여 4개의 유형으로 분류되었으며, 410 bp인 C형이 10균주로 가장 많았다. C형 가변부의의 DNA sequence에서 40 bp가 반복되는 *dru* sequence를 관찰할 수 있었으며, 이러한 *dru* sequence는 4 unit가 직접적으로 중복됨을 알 수 있었다.

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