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Identification of *Staphylococcus aureus* and Determination of Its Methicillin Resistance by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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To evaluate the performance of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in identifying *Staphylococcus aureus* and in determining its methicillin resistance, we analyzed 76 *S. aureus* clinical isolates using a linear MALDI-TOF MS. Spectral profile data obtained were compared with the database provided with the instrument, and 74% of the isolates were identified as *S. aureus*, as confirmed by a *nuc*-based PCR test. The determination of the methicillin resistance in *S. aureus* is based on the fact that the spectral profiles of methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) differ greatly from each other. Replicate spectral profiles obtained from each isolate were combined to be a representative spectrum of it, and representative spectral profiles from all the isolates constitute a user's self-established database. All the spectral profiles in the database were classified into two groups based on clustering analysis, and one is for MSSA and another MRSA. There was a little discrepancy between the results from MALDI-TOF MS and from PCR. Seven isolates that are negative for the *mecA* gene by PCR were identified as MRSA by MALDI-TOF MS. The discrepancy may be partially explained by the heterogeneous nature of methicillin resistance in *S. aureus*. Our results suggested that comparison of MALDI-TOF MS spectral profiles of microorganism could serve as a simple and rapid method for bacterial identification and antibiotic susceptibility analysis.

The rapid and accurate identification of microorganisms in environmental surveillance, food production, and pathogenic bacteria detection is vital for prevention of health hazards and human morbidity. The goal to find a rapid and specific method for microorganism identification has long been pursued. Most commonly used methods that rely on assessment of various bacterial metabolic activities are usually complicated and time-consuming. Recent progress in mass spectrometry makes it possible to analysis complex mixtures of microorganisms, which interests scientists in the field of microorganism identification. In a series of pioneering studies, a variety of mass spectrometric techniques coupled with fast atom bombardment, pyrolysis, laser desorption, or plasma desorption have been investigated for bacterial chemotaxonomy and microorganism identification.[1−5] These approaches primarily detect small-molecule biomarkers, such as phospholipid, instead of macromolecular biomarkers because the latter are not volatile or stable enough to survive the ionization process. Soft ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionzation (MALDI), however, circumvent this problem. For example, MALDI-TOF MS has been used to detect macromolecules in complex mixtures without any purification of the specimens. While some have used MALDI-TOF MS in conjunction with other separation techniques such as HPLC[6] or with digestion or previous extractions,[7,8] whole-cell analysis of bacteria offers a simple and straightforward protocol. Holland and co-workers demonstrated that whole-cell detection of microorganisms with MALDI-TOF MS provided distinctive and reproducible spectral profiles, which can be compared with standards to identify unknown samples.[9] Reports from other groups have also indicated that whole-cell analysis of bacteria with MALDI-TOF MS is feasible.[10,11] Most of studies typically analyzed laboratory isolates and focused on technique. However, the clinically isolated bacteria had not been analyzed to evaluate MALDI-TOF MS as a tool to identify unknown bacteria.

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Staphylococcus aureus is a common pathogen that causes a wide variety of hospital-acquired infections, ranging from abscesses to bacteremia. Therefore, rapid and specific determination of *S. aureus* and its antimicrobial susceptibility is essential for disease diagnosis and timely treatment. Edwards-Jones et al. reported that spectra obtained in intact cell mass spectrometry (ICMS) of methicillin-susceptible *S aureus* (MSSA) and methicillin-resistant *S aureus* (MRSA) were readily discriminated, suggesting that MALDI-TOF MS has the potential for MRSA identification and subtyping.[12] In one of their follow-up studies, the intra-and interlaboratory reproducibility of ICMS and the effect of culture media on the spectral profiles were evaluated.[13] In this study, we evaluated the performance of MALDI-TOF MS in detecting clinical isolates of *S. aureus* and its methicillin susceptibility based on searching the database and software analysis.

EXPERIMENTAL SECTION

Materials. The MALDI matrixes used were 5-chloro-2-mercaptobenzothiazole (CMBT) and α-cyano-4-hydrocinnamic acid (α-cyano), purchased from Aldrich Chemie GmbH (Steinheim, Germany). Trifluoroacetic acid (TFA) and 18-crown-6 ether were purchased from Acros Organics. Columbia agar (CBA) was purchased from Difco Laboratory (Detroit, MI). Seven peptides and proteins for instrument calibration, bradykinin (1060.2 u), angiotensin I (1296.5 u), Glu-fibrino peptide B (1570.6 u), renin sustrate tetradecapeptide (1759.6 u), ACTH (18−39) (2465.7 u), insulin (bovine) (5733.5 u), ubiquitin (8564.9 u), were purchased from Sigma Chemical Co. (St. Louis, MO).

The matrix solutions were saturated solutions of CMBT or α-cyano dissolved in acetonitrile/methanol/water (1:1:1, v/v/v), containing 0.1% formic acid and 0.01 M 18-crown-6 ether, and were freshly made before use. The CBA blood agar plate was made by adding 5% defibrinated sheep blood to CBA at 45 °C and stored at 4 °C until use. Adequate amounts of MALDI-TOF MS calibrants were dissolved in 0.1% TFA/water solutions, and each mixture was used for instrumental calibration after being mixed with an equal volume of α-cyano matrix solution.

All the bacterial isolates came from specimens of clinical patients and were purified by three continuous subcultures of a single colony. All the isolates were previously characterized as Gram-positive, catalase-positive, coagulase-positive cocci and were identified as *S. aureus* by the Vitek System (BioMérieux Vitek Inc., Hazelwood, MO).

Sample Preparation. The bacteria were incubated aerobically on a 5% CBA plate at 37 °C for 24 h. Single colonies on the plates were transferred to wells on a 96-well stainless target plate with a sterilized toothpick. Each isolate was inoculated to 12 individual wells. After 15 min, an aliquot of 1 μL of CMBT matrix was added to each well and allowed to dry at ambient temperature.

Instrument. The analysis was performed on a linear MALDI-TOF MS (Micromass UK Ltd., Manchester U.K.) equipped with a nitrogen laser light (wavelength 337 nm).The instrument was supported by a Microbelynx software system, which provides a database containing spectral profiles of NCTC-type strains and allowed users to establish the database by themselves. All spectral profiles were acquired at an accelerating voltage of 15 kV. The mass range for data acquisition is from m/z 500 to 10 000 Da. The instrument was externally calibrated using a mixture of seven peptides and proteins described in Materials.

PCR Amplification. PCR primers specific for *S. aureus nuc* gene (accession number V01281) and *mecA* gene (accession number E09771) were designed according to the sequences published in DDBJ (http://www.ddbj.nig.ac.jp). The primer sequences were as follows: *nuc* forward primer, nuc1, 5′ AATTAATGTACAAAGGTCAAC 3′ and reverse primer, nuc2, 5′ TGATAAATATGGACGTGGCT 3′; *mecA* forward primer, mecA1, 5′ TGGCTATCGTGTCACAATCG 3′ and reverse primer, mecA2, 5′ CTGGAACTTGTTGAGCAGAG 3′; the two pairs of primers amplify 195-bp *mecA* gene fragment and 310-bp *nuc* gene fragments, respectively.

Colonies of bacteria were suspended in 100 μL of distilled water. After boiling and centrifugation, the supernatant was subjected to PCR amplification. PCR was performed on a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Norwalk, CT). The reaction mixture with a volume of 30 μL contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% BSA, 0.1 μM of each primer, 0.08 mM dNTPs, 1 unit of Taq DNA polymerase, and 2 μL of template. The reaction profile is as follows: 3-min predenatureation at 95 °C, 35 cycles of PCR (denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s), and 5-min extension at 72 °C. An aliquot of 10 μL of the product was subjected to agarose gel electrophoresis in 0.5× TBE (0.045 mM Tris-boracic acid, 1 mM EDTA, pH 8.0). The gels were stained with ethidium bromide to be visualized under UV light.

RESULTS AND DISCUSSION

Under the current experimental conditions, most spectral peaks observed were in the mass range of m/z 800−3500 Da, which is similar to what was reported by Edwards-Jones et al.[12,13] This range is relatively narrow compared with the results from other studies of microorganism identification using MALDI-TOF MS. For example, previous MALDI-TOF MS studies had reported the analysis of protein samples from solvent suspension or whole-cell extraction yielding a number of peaks in the 3−18-kDa mass range. The difference in mass range of spectra peaks might be due to the difference in sample preparation methods. In our study, a method similar to what Edwards-Jones et al. used[12,13] was employed. Briefly, the colonies of bacteria were inoculated directly on the target plate and appropriate matrix solution was dropped on the bacterial spots and dried. The whole process of sample preparation was simple and time-saving, taking only ∼20 min for each isolate. The sample preparation methods used in others studies were more complicated, having involved procedures such as bacteria collection, extraction with solvents, and so on.

The presence of mass spectra peaks indicates some of the substances on the cell surfaces of the bacteria being tested. These substances can be cocrystallized with the matrix after falling off the surface and detected by MALDI-TOF MS.[14] It was reported that the 0.5−3-kDa mass range was indicative of the presence of lipids and glycolipids on the bacterial cell surfaces.[10]

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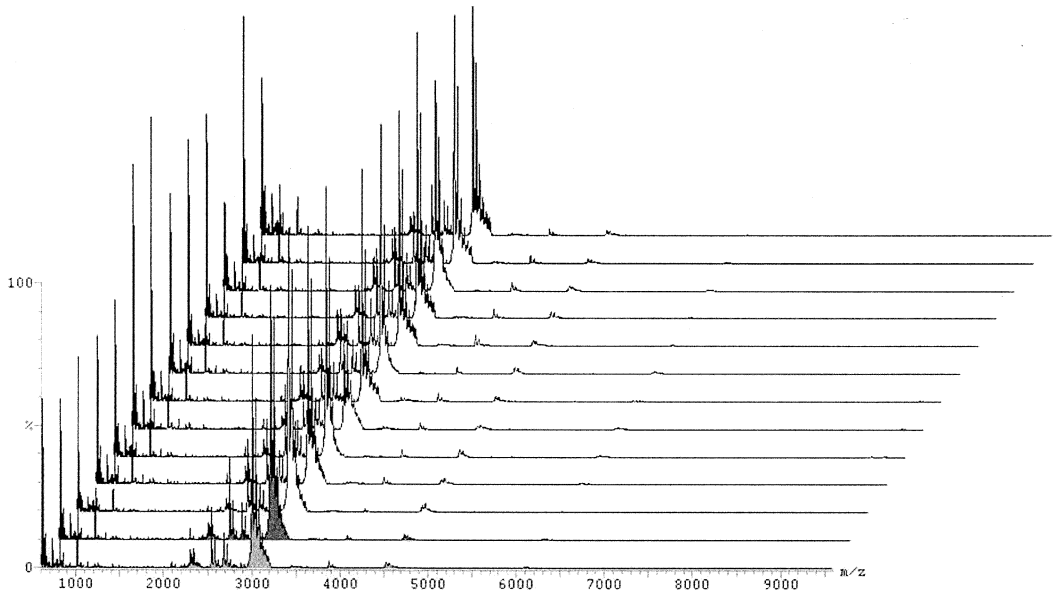

Figure 1. Spectra of 12 replicates of *S. aureus* isolate no. 43.

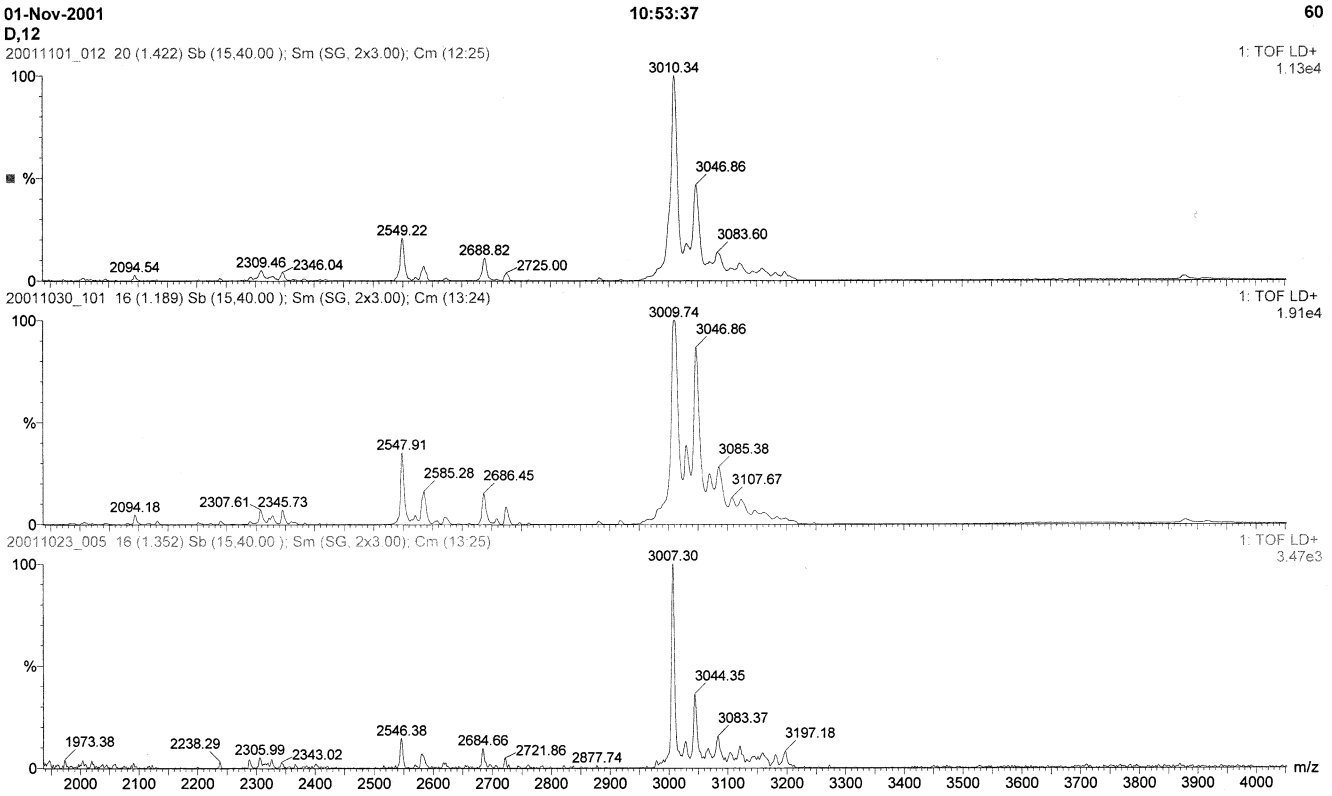

Figure 2. Representative spectra for three batches of isolate no. 43 cultured at 10-day intervals.

Reproducibility. Reproducibility is of great importance for reliable bacterial identification using MALDI-TOF MS. To evaluate the reproducibility of MALDI-TOF MS, analyses of 12 replicates of each isolate and repetitious analysis of 3 isolates at 10-day intervals were performed. The mean root square (rms) value of the spectra for each of the 12 replicates was obtained by comparing the spectra of each replicate in turn with the average of the other 11 replicates. An rms rejection value of 3 is used to identify the outliers at the 0.1% significance level. Replicates with rms value below 3 were accepted, and their spectra will be combined to a representative spectrum for the isolate. The combined spectral data constituted the user's self-established database, which can be used to classify unknown sample based on clustering analysis.[15] The result of this study showed that reproducibility of MALDI-TOF MS was acceptable (Figure 1). For most of the isolates, spectra of 11 or 12 replicates were usable for database construction.

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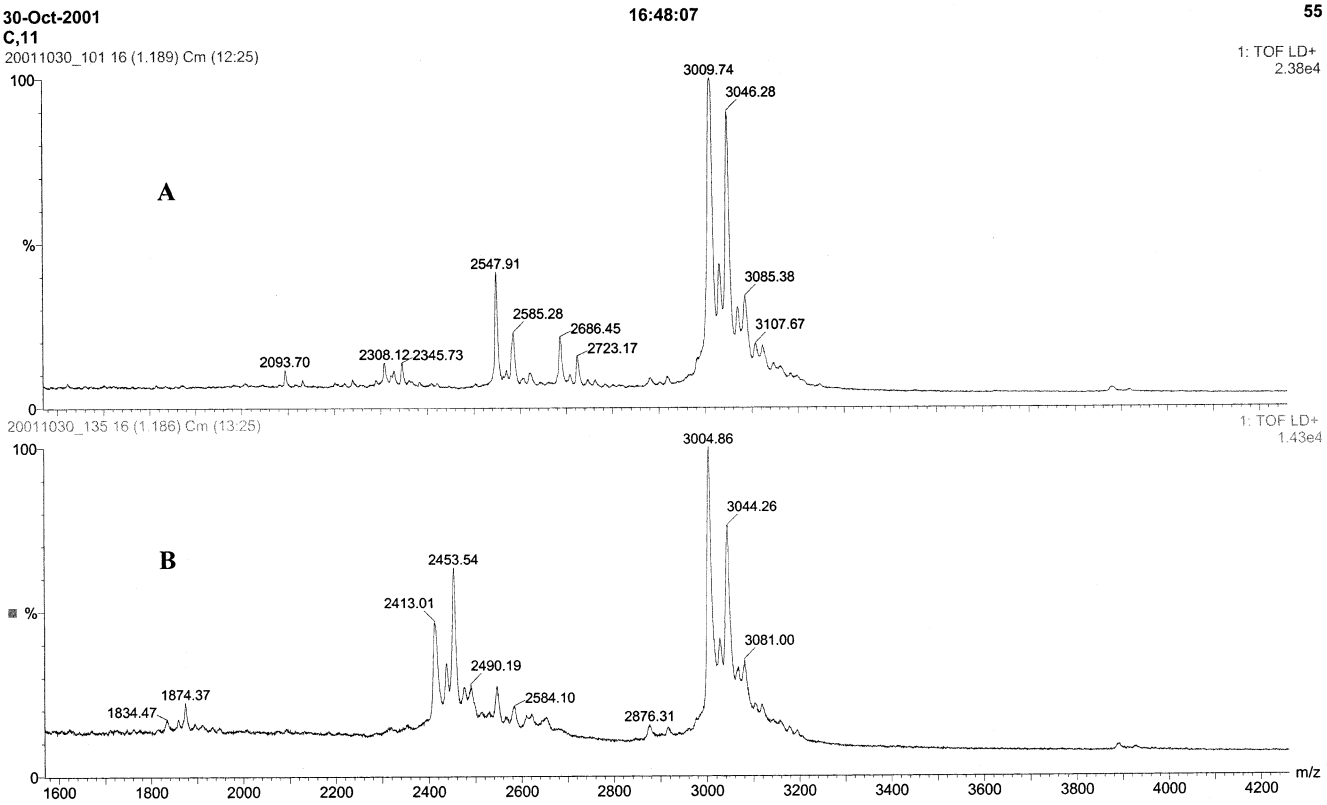

Figure 3. Typical spectra of *S. aureus* methicillin-susceptible strain (A) and methicillin-resistant strain (B).

Isolate 43, 44, and 65 were analyzed three times at 10-day intervals under controlled experimental conditions. Batch rms values were obtained by comparison of the spectra from each experiment, and they were small enough to be accepted (rms < 3). No significant differences in spectral profile between three batches were observed, and overall spectral profiles obtained were steady and reproducible (Figure 2).

It has been demonstrated that experimental conditions, such as incubation time and types of culture media, influence the bacterial mass spectral profiles greatly.[13,16] As the techniques were standardized, reproducible results were achievable. To achieve satisfactory reliability, the protocol used in the establishment of the database (established by Micromass UK. Ltd.) was carefully followed in this study.

Identification of *S. aureus*. *Nuc* gene exists only in *S. aureus* and is a common target gene for specific detection of *S. aureus*;[17] therefore, *nuc*-based PCR detection was used as a reference method for MALDI-TOF MS analysis in this study. The results of *nuc*-based PCR detection demonstrated that all the 76 isolates in this study were *S. aureus*.

The mass spectral data were analyzed with software provided with the instrument. The isolates were identified by comparing their spectral profiles with the database, which is composed of more than 300 strains of ~60 genera from NCTC, including 18 species of genus *Staphylococcus*. The pattern recognition algorithm uses all the mass intensity data in the mass spectrum to give the best database match.[15]

However, only 74% of these isolates were identified as *S. aureus* by MALDI-TOF MS. Some of the isolates yielded very weak signals in the mass spectrum, which led to failure of identification. Repeated analysis of those isolates demonstrated similar results. This indicated that a much different spectrum could be obtained from different strains of *S. aureus* using the current method. It might be caused by some substances, such as metabolites, existing on the surface of cell wall of some isolates that can interfere with the cocrystallization process during sample preparation.[6] The nature of this interference needs further investigation. A special sample preparation procedure is needed for strains that yielded weak spectral profiles, and we will go on with the investigation to gain the insight into this intractable problem.

The relatively low accuracy of MALDI-TOF might be due to the following reasons. First, the database used in this study is not complete; therefore, *S. aureus* strains whose data are not in this database will not be identified. Sample preparation procedures and instrument parameters used in the database protocol provided with the instrument were followed in our study except that the horse blood in CBA media was replaced by sheep blood, and CBA was purchased from a different manufacturer. This alteration would produce some influence on the mass spectral patterns. It was demonstrated that the same culture media from different manufacturers or different batches of culture media from the same manufacturer will alter the spectral patterns of the same isolate.[13]

Methicillin-Resistance Determination. *MecA* gene is the genetic determinant of methicillin resistance in *Staphylococci*, and the presence of *MecA* indicates the existence of methicillin resistance. Therefore, a *mecA*-based PCR assay was used in this

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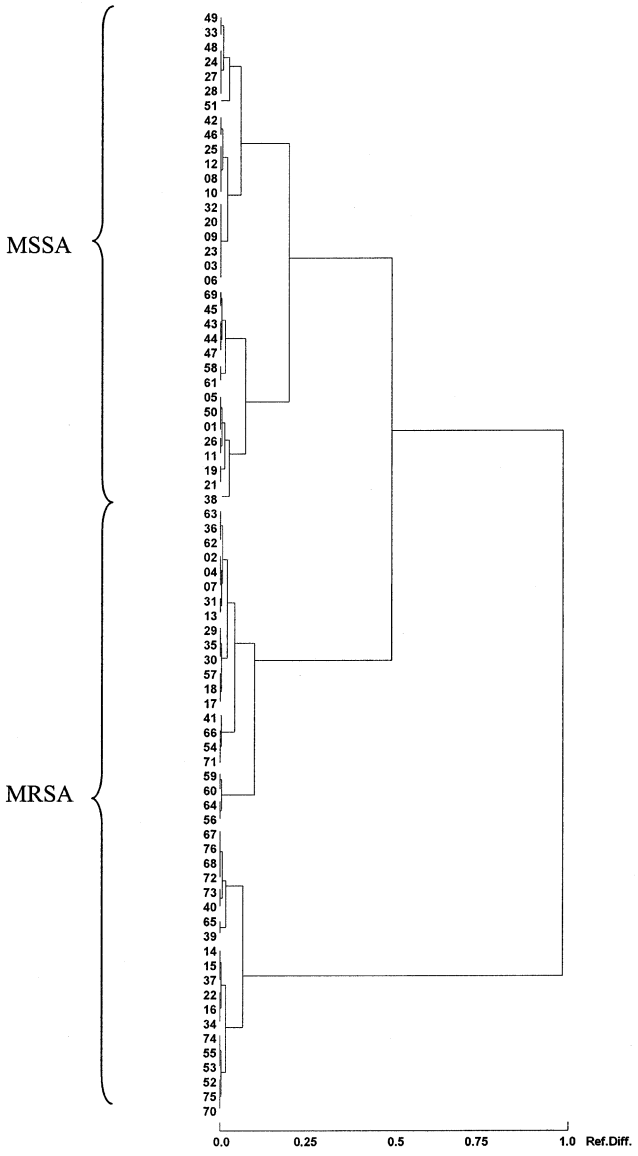

Figure 4. Cluster analysis of mass spectra of 76 *S. aureus* isolates. Numbers stand for the strain number of the isolates, Rel.Diff represents relative difference, a relative scale normalized between 0 and 1. A difference of 0 indicates the clusters are exactly the same. A difference of 1 indicates that the clusters are the least similar clusters in the current data set.

study to confirm the identification of the methicillin-resistant *S. aureus* isolates at the gene level.[18]

This study demonstrated that MSSA and MRSA differed in the spectral profiles in the mass range of m/z 500−3500 Da under the experimental conditions, as illustrated in Figure 3. All spectral profiles in the database can be clustered into a dendrogram. After the user's self-established database was generated as described in reproducibility evaluation, cluster analysis could be performed with the aid of the software. All the entries in this database were clustered into two separate groups that were composed of MRSA and MSSA strains (Figure 4).

Thirty-three out of 76 strains were positive in PCR amplification of *mecA* gene, and all of them were classified into MRSA groups by MALDI-TOF MS analysis. Seven strains negative for *mecA* gene

Table 1. Comparison of PCR, Conventional Susceptibility Testing, and MALDI-TOF Analysis Results of Seven *S. aureus* Strains

strain no.	Nuc PCR[a]	Mec PCR[b]	conventional susceptibility assay	MALDI-TOF analysis
02	+	−	R[c]	R
30	+	−	S[d]	R
41	+	−	S	R
54	+	−	S	R
57	+	−	R	R
59	+	−	S	R
60	+	−	S	R

[a] PCR detection based on *nuc* gene. [b] PCR detection based on *mecA* gene. [c] MRSA. [d] MSSA.

PCR amplification were classified into the MRSA group by MALDI-TOF MS analysis, but the phenotype susceptibility test reveals that two of the seven isolates (nos. 2 and 57) were MRSA and the other five strains were MSSA (Table 1). There are some discrepancies among results from PCR amplification, MALDI-TOF analysis, and the conventional phenotype susceptibility test. Although *mecA*-based PCR detection or DNA hybridization is considered as the "gold standard" for methicillin resistance determination, *S. aureus* strains negative for the *mecA* gene may have a methicillin-resistant phenotype due to mutation of PBPs or overproduction of β-lactam. Whether the strains no. 2 and 57 belong to this group needs further study.

A distinctive feature of methicillin resistance is the heterogeneity in the resistance level, which vary depending on the culture conditions, including the concentration of β-lactam antibiotic in the culture.[18] Differences in culture conditions used in MALDI-TOF MS analysis and in the conventional susceptibility assay may cause the different phenotypes of the same *S. aureus* isolates observed in this study.

A method to identify bacteria using a spectral profile from MALDI-TOF MS is reported here. The method is rapid, simple, and specific and could be used to identify clinical isolates of *S. aureus*. Nevertheless, further improvement of the method is needed. Since an ideal methodology should allow the identification of unknown bacteria (not blind numbered) by its spectral profiles, regardless of the culture conditions. Some papers had reported the use of an algorithm method in identifying unknown microorganisms. Since this method exploits the abundant genomic information of an increasing number of bacteria, it is a promising strategy to combine with MALDI-TOF MS in bacterial identification.[19−21]

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