Original Article

Characterization of *Staphylococcus aureus* Isolated from Clinical Specimens by Matrix Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

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Abstract

Objective To develop a matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) approach to identify *Staphylococcus aureus* (*S. aureus*) and differentiate methicillin-resistant *S. aureus* (MRSA) from methicillin-sensitive *S. aureus* (MSSA).

Methods A total of 100 *S. aureus* strains isolated from clinical specimens and farm workers were collected and analyzed by MALDI-TOF-MS. And data obtained were interpreted with biotyper software.

Results Ninety-two strains were identified by MALDI-TOF-MS as *S. aureus* at a level of secure genus and probable species, and 4 strains were identified at probable genus after their cultivation, spectral collection and data preprocessing. One strain was identified as *S. aureus* with lower score. It was revealed that identification of *S. aureus* by MALDI-TOF-MS was highly correlated with typing by biochemical and serological methods with an accuracy as high as 97%. The biotyper cluster analysis showed that 100 isolates were divided into 2 types at the distance level of 400. Higher peak intensity in the mass of both 3784 Da and 5700 Da was observed in MRSA, whereas that was absent from MSSA.

Conclusion MALDI-TOF-MS is considered as a simple, rapid and highly reproducible technique with high-throughput and accuracy for the identification of *S. aureus* and it can reliably differentiate MRSA from MSSA.

Key words: *Staphylococcus aureus*; Characterization; MRSA; MSSA; MALDI-TOF-MS

Biomed Environ Sci, 2013; 26(6):430-436 *doi: 10.3967/0895-3988.2013.06.003* *ISSN:0895-3988*

www.besjournal.com(full text) *CN: 11-2816/Q*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) causes serious infections in animals and human beings. This bacterium produces several types of heat-stable enterotoxins causing food poisoning. As reported by U.S. CDC, a total of 796 food poisoning outbreaks with 33 206 cases affected were caused by bacterial pathogens from 1988 to 1992[1]. And among them, 50 (6.28%) outbreaks and 1678 cases (5.05%) were due to the consumption of foods heavily contaminated with enterotoxins from *S. aureus*. An extensive outbreak of food poisoning caused by drinking low-fat milk and powdered skim milk contaminated with *Staphylococcal* enterotoxin A occurred in Kansai district, Japan in the spring of

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Received: September 14, 2012; Accepted: February 23, 2013

2000 and as many as 13 420 cases were affected[2]. Isolation and characterization of *S. aureus* from food samples is therefore crucial for epidemiological investigations and clinical treatment.

Methicillin-resistant *S. aureus* (MRSA) was first identified in the 1960s. It is usually considered a nosocomial pathogen and can also be acquired in the community[3]. The vast majority of the estimated 94 000 life-threatening annual MRSA infections were associated with medical care practice, and *S. aureus* isolates resistant to methicillin rose from 20% in 1992 to 75% in 1996[4]. Hospital-based epidemiological studies conducted in USA demonstrated that 28%-41% of adults with MRSA infection acquired the organism before admission[5]. Identification and characterization of MRSA is therefore a high priority for both public health and medical institutions.

Identification and classification of *S. aureus* are traditionally based on morphological and physiological properties of the bacteria. And the morphological identification of typical and atypical colonies on selective culture medium is usually followed by biochemical assays of virulence factors such as thermonuclease and coagulase. This approach is time-consuming and expensive, and it is not yet been routinely used in differentiating MRSA from methicillin-sensitive *S. aureus* (MSSA). Chemotaxonomic and molecular approaches including peptidoglycan and lipids analysis, biochemical analysis together with polymerase chain reaction (PCR) very much helped extend the range of phenotypic characters of this bacterium. A potential improvement is matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

MALDI-TOF-MS is a powerful tool that has attracted worldwide attention for its direct and rapid discrimination, identification and characterization of different microorganisms[6-10]. It is characterized by easy operation, large-scale simultaneous sample analysis, high-resolution, accuracy, sensitivity and reproducibility, and its detection limits is superior to traditional biochemical methods. It has been successfully applied in characterizing various microbial agents such as bacteria, viruses, protozoa, and fungi[11-12]. Our present study aims to develop a MALDI-TOF-MS method for the identification of *S. aureus* isolated from foods and clinical specimens and also a method to differentiate MRSA from MSSA in order to provide a new option for rapid and effective identification and control of *Staphylococcal* foodborne illness and clinical treatment of serious staphylococcal infections.

MATERIALS AND METHODS

Microorganisms

A total of 100 *S. aureus* strains isolated from clinical specimens and farm workers originally identified and characterized by conventional methods such as Gram staining, catalase test, ability to coagulate rabbit plasma as well as API STAPH (BioMerieux Inc., France) were collected and used for the study. Susceptibility to methicillin was tested using the disk diffusion method in accordance with recommendations by the Clinical and Laboratory Standards Institute (CLSI,U.S.). *S. aureus* ATCC 25923 was used as quality control in each set of tests. Forty-eight MRSA isolates and 52 methicillin-sensitive *S.aureus* (MSSA) isolates were identified based on the results of antimicrobial susceptibility test, latex agglutination test and the presence of the *mecA* gene was analyzed by PCR. All isolates were stored at -70 °C in glycerol brain heart infusion broth and recovered on cooked meat medium at 37 °C prior their use. Primary sub-cultures were used for MALDI-TOF-MS analysis.

Reagents

The matrix solution α-cyano-4-hydroxycinnamic acid as well as formic acid, trifluoroacetic acid and acetonitrile were purchased from Sigma-Aldrich (U.S.). Tap water was directly purified into Milli-Q quality water with a conductivity of greater than 18.2 MΩ.cm at 25 °C with a Millipore water purification system (Millipore, Bedford, MA, U.S.). Yeast extract, beef powder and peptone were from OXOID Company (U.K.) and glucose, NaOH, and NaH_2PO_4 from Chemical Regent Beijing Co., Ltd. All organic solvents used for sample extraction and preparation were of LC grade. *Escherichia coli* DH5a (*E.coli* DH5a) was selected as calibration strain and obtained from the Chinese Academy of Military Medical Sciences.

Cooked meat medium was prepared with 3 g peptone, 0.5 g beef powder, 0.5 g yeast extract, 0.3 g glucose and 0.5 g NaH_2PO_4 in 100 mL distilled water, adjusted pH to 7.6 with NaOH and autoclaved at 112 °C for 20 min.

Preparation of the Intact Bacterial Cell

All *S. aureus* isolates were sub-cultured in Mueller-Hinton medium at 37 °C for 24 h and were

transferred into cooked meat medium and incubated at 37 °C for another 24 h. 500 μL of the fresh bacterial suspension was loaded into an Eppendorf tube and centrifuged at 16 873 g for 2 min. The precipitate was washed twice with distilled water (300 μL each time) and centrifuged at 16 873 g for 2 min. The cells were suspended in 300 μL of distilled water and 900 μL of 100% ethanol was added (LC grade, J. T. Baker, U.S.). Then, the mixture was centrifuged at 16 873 g for another 2 min (centrifuged once again if necessary in order to remove the ethanol completely). The pellet was re-suspended in 20 μL of 70% formic acid and 20 μL of acetonitrile. An aliquot of 1 μL of the suspension was applied directly onto the MALDI sample target, spread evenly and allowed to air dry for 5 min before being overlaid with 2 mL matrix solution containing acetonitrile, trifluoroacetic acid and water (50:2.5:47.5, *v:v:v*) saturated with α-4-cyano-hydroxycinnamic acid (LC-MS quality, Sigma). The prepared target was air dried prior to MALDI-TOF analysis. Mass spectra were analyzed in triplicate batches from each strain. The calibration standard containing 11 standard peptides of *E.coli* DH5a ranging in molecular mass from 4364.33 to 10 299.09 Dalton was manually spotted to a separate anchor of the MALDI target plate.

MALDI-TOF-MS Analysis

The Bruker Daltonics MALDI-Microflex (MALDI-TOF-MS, Bruker Daltonics GmbH, Bremen, Germany) with the MALDI Biotyper software was employed for the analysis of the intact bacterial cell. All analyses were performed in linear-delayed extraction positive ion mode with a sampling rate of 0.5 ns, using an accelerating voltage of 20 kV and the ion accelerating voltage in MS/MS mode of 18.6 kV. Resolution for the peak with ion reflector was required to be higher than 300. Peptides were measured as $[M+H]^+$. For MS/MS, dominant parent peaks in the MS spectrum were selected for further fragmentation in MS/MS mode. The resulting spectra were analyzed in an m/z range between 2000 Dalton and 20 000 Dalton. The acceptance criteria were based on 50 laser shots per spot.

Data Acquisition

By comparing the unknown spectrum with a best-fitting spectrum from the library, a final log score value for the unknown spectrum was calculated in the range from 0 to 3. In order to determine the accuracy of the species identification, the manufacturer's recommendations were followed. Highly probable species identification was obtained with the log score range from 2.300 to 3.000, while secure genus identification probable species identification with 2.000-2.299, probable genus identification with 1.700-1.999. The log score range of 0.000-1.699 means that the identification is not reliable.

The dendrogram setting in the MALDI Biotyper 2.0 database, containing 132 main spectra of staphylococci encompassing 132 Staphylococcus species and sub-species, was used to group species, as the analysis was based on the pattern matching of spectra and log score values. A dendrogram was also generated from the new minimal spanning tree data set. The predicted drug resistance from MALDI-TOF-MS were compared with the antimicrobial susceptibility testing. And the latter was performed as described above.

RESULTS

Species Identification of Microorganisms by MALDI-TOF-MS

The test strains were analyzed by MALDI-TOF-MS and the resulting spectra of their ionisable cell surface components were compared to spectra in the release database provided with the MALDI Biotyper software. The Bruker Daltonics MALDI Biotyper database and software were 100% correct in assigning the correct species to the tested isolates. Evaluation criteria to be applied were suggested by the manufacturer in conjunction with the score values, and the resulting dendrogram differentiated the 100 analyzed *S. aureus* isolates accurately into the expected 3 species groups (Figure 1). A total of 92 isolates were correctly identified as *S. aureus* at a level of secure genus and probable species identification (score ≥2.0; Figure 1 and Table 1) and another 4 isolates were identified at probable genus identification. One strain was identified as *S. aureus* with the score under 1.700. One strain was identified as *Stenotrophomonas maltophilia* and two as *Pseudomonas geniculata*. It was demonstrated that MALDI-TOF-MS identification for *S. aureus* was highly correlated with biochemical and serological identification, with an accuracy of 97%. The MALDI-TOF analysis was completed within 30 min per isolate from plate to obtain final results and it took approximately 2 h to analyze a full 96-spot target plate. A total of 32 isolates could be identified

within 2 h if triplicate samples were analyzed per isolate. Therefore, MALDI-TOF-MS was considered a rapid, high-throughput and highly accurate tool for the identification of *S. aureus*.

In order to clarify if the MALDI Biotyper software could recognize clonally related strains at the species level, spectra from 100 well-characterized *S. aureus* strains were analyzed as described above. The spectra were then used to generate a new cross-wise minimal spanning tree (MSP) data set. A dendrogram was also generated from the new MSP data set (Figure 1). It was noted that the resulting dendrogram differentiated the 100 analyzed isolates accurately into the 3 species groups: one strain of *Stenotrophomonas maltophilia*, two strains of *Pseudomonas geniculata* and 97 strains of *S. aureus*.

Table 1. Identification of 100 Strains by MALDI-TOF-MS

Category	Log Score Range	No. of *S. aureus*	No. of Others
1	2.300-3.000	71	2
2	2.000-2.299	21	0
3	1.700-1.999	4	1
4	0.000-1.699	1	0

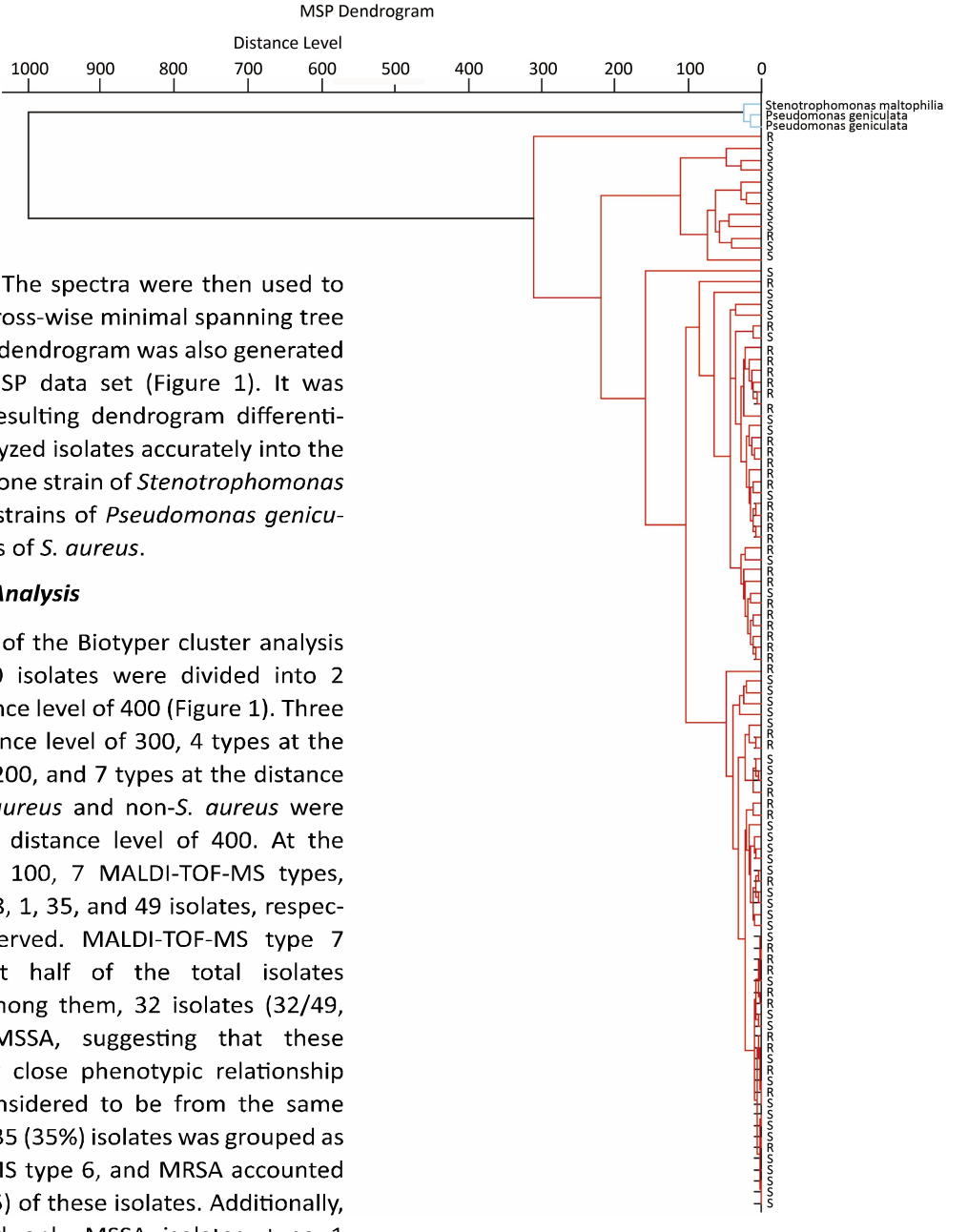

Figure 1. Dendrogram of the differentiation among species analyzed (S and R represent MSSA and MRSA, respectively).

Biotyper Cluster Analysis

The results of the Biotyper cluster analysis showed that 100 isolates were divided into 2 types at the distance level of 400 (Figure 1). Three types at the distance level of 300, 4 types at the distance level of 200, and 7 types at the distance level of 100. *S. aureus* and non-*S. aureus* were separated at the distance level of 400. At the distance level of 100, 7 MALDI-TOF-MS types, grouping 3, 1, 3, 8, 1, 35, and 49 isolates, respectively, were observed. MALDI-TOF-MS type 7 contained almost half of the total isolates analyzed. And among them, 32 isolates (32/49, 65.31%) were MSSA, suggesting that these isolates had very close phenotypic relationship and could be considered to be from the same matrix. A total of 35 (35%) isolates was grouped as the MALDI-TOF-MS type 6, and MRSA accounted for 74.29% (26/35) of these isolates. Additionally, type 3 contained only MSSA isolates, type 1 contained 3 strains other than *S. aureus,* type 2 contained 1 strain of MRSA, type 4 included 7

strains of MSSA and 1 strain of MRSA. One stain in type 5 was MSSA.

Results of ClinProTools Analysis

For the interpretation of the MALDI-TOF-MS spectra derived from 100 isolates of *S. aureus*, the ClinProTools bioinformatics package, that combined efficient visualization with automated data pretreatment as well as intuitive statistical analysis, was employed. A list of all peaks sorted according to the statistical separation strength was created as an output file. The classical statistics of peak signal intensities combined with the genetic algorithm was used to detect spectra pattern differences. The individual peak pattern included peak intensity distribution and peak frequency of the respective microorganism generated by extracting the typical peak information. Genetic algorithm was used in preprocessing one batch via the batch processing command. Peaks used in models generated by the genetic algorithm were highlighted with red (MRSA) or green (MSSA) lines in the viewer (Figure 2).

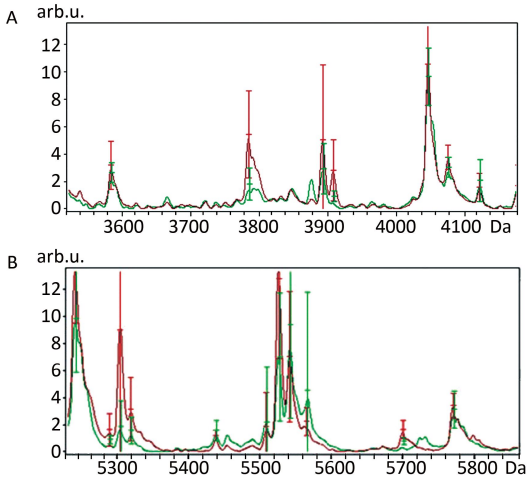

Figure 2. Chromatograms of 48 isolates of MRSA (red) and 52 isolates of MSSA (green) at two time intervals. A and B represent two different observation time point respectively.

ClinProTools provided a list of peaks sorted according to the statistical significance in order to differentiate MRSA from MSSA. The spectra of *S. aureus* at two time intervals indicated that key biomarkers within the spectrum remained unchanged, but minor changes in mass intensities were apparent. Several mass regions with differences between MRSA and MSSA were found upon visual inspection. It was worth pointing out that the obvious region of multiple signals, in the range from 3500 to 6000 Da, revealed different intensities between MRSA and MSSA. The probability of the observed intensity differences in the individual peaks was calculated using a Welch's *t*-test. Peaks with high separation power in the Welch's *t*-test might be used to generate a biomarker pattern model. Accordingly, the peaks labeled as significant to distinguish between MRSA and MSSA were directly controlled using the visualization features. The specialized views were zoomed into the region of approximately 3784 Da and 5700 Da, respectively. In both cases, the peaks of interest showed different signal intensities between MRSA and MSSA. Higher peak intensity in the mass of both 3784 Da and 5700 Da was observed in MRSA than in MSSA. It should be pointed out that there was no significant difference for the new mass ion in the range between 5700 Da and 5800 Da that was present in MSSA only. Therefore, both biomarkers allowed for an easy and efficient identification of MRSA from MSSA.

A virtual gel view overviewing representation of data derived from 100 *S. aureus* strains is shown in Figure 3. All individual spectra are shown on a density scale and Figure 3 shows the intensity distribution of the relevant signals in the different samples. It was noted that two potential biomarkers located at 3784 Da and 5700 Da with a difference in signal intensities could be especially well visualized between MRSA and MSSA. The bands generated by MRSA at the mass of 3784 Da and 5700 Da were obviously deeper in color than those generated by MSSA. This indicated that the distribution of both biomarkers on the cell surface of MRSA was more pronounced than on that of MSSA.

Two-dimensional clustering profiling of the charged cell surface-associated biomarkers at the masses of 3784 Da and 5700 Da from intact cells between MRSA and MSSA generated by the genetic algorithm is shown in Figure 4. The vertical and horizontal axes represent the abundant distribution of the biomarkers at the masses of 3784 Da and 5700 Da on the cell surface of each bacterial isolate, respectively. Each MSSA isolate expressed as green circle and MRSA is expressed as red crosses. It could be clearly noted that the majority of MSSA had lower abundance in the masses of 3784 Da and 5700 Da, and cluster separately from MRSA. Two large rings with red and green were labeled automatically based the genetic algorithm. There are statistical differences between the two rings with the protein abundance. This analysis also indicated that the biomarkers on the

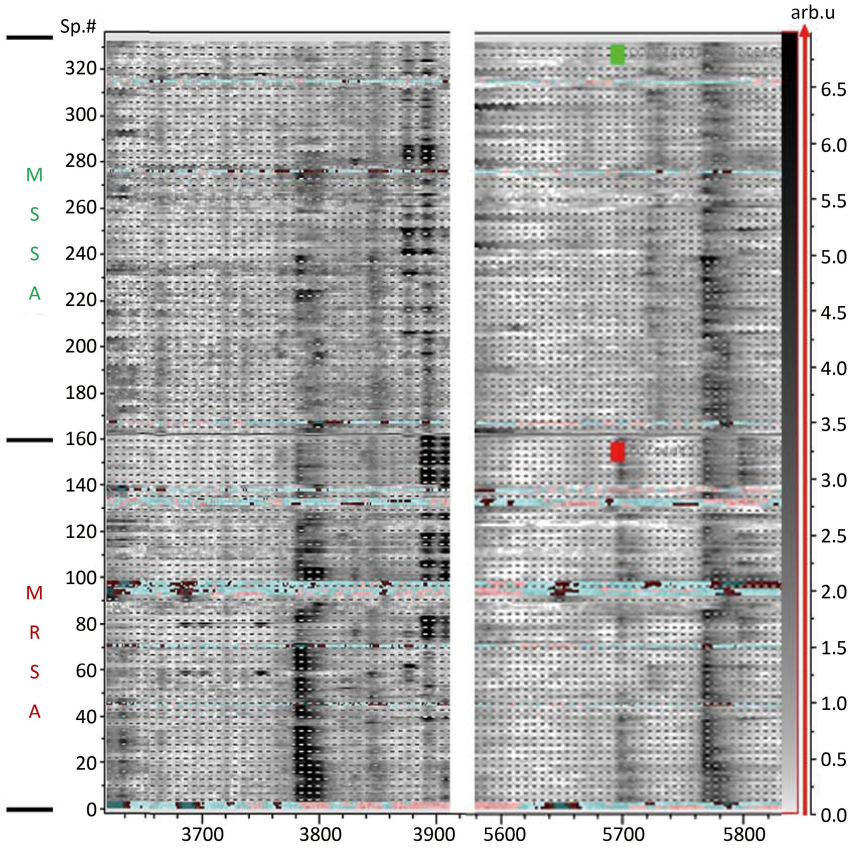

Figure 3. Gel view of biomarkers derived from 100 *S. aureus* isolates at two time intervals.The different color of spots were the gathering of proteins with different contents.

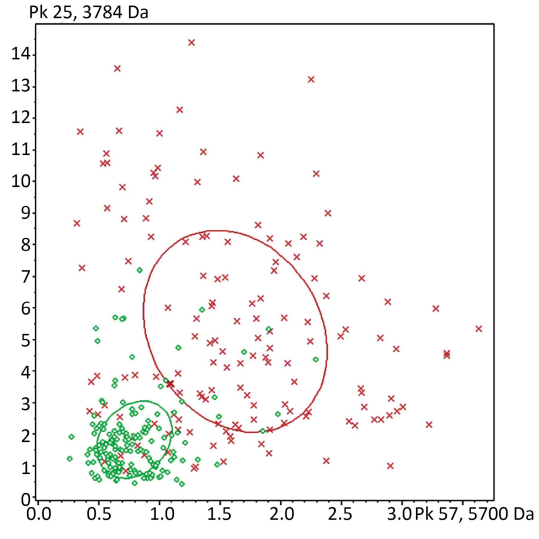

Figure 4. Two-dimensional profiling of MRSA and MSSA. MSSA isolate: green circle； MRSA isolate: red crosses. Two large rings with red and green indicated the tendency of gathering between MRSA and MSSA.

cell surface of MRSA were much more than on that of MSSA.

DISCUSSION

S. aureus has become a common pathogen of hospital-acquired infections, particularly of those being resistant to methicillin. Failing to correctly identify MRSA-infected patients in the early stages of infection may result in a more protracted and costly treatment and convalescence. In this context, accurate, fast and early identification of specimens from MRSA positive patients is extremely important. Conventional methods for identifying *S. aureus* and differentiating between MRSA and MSSA are slow, costly, and are barely reliable[5,11,13-14]. Therefore, an accurate, rapid and cost-effective tool is urgent needed. MALDI-TOF-MS plays an increasingly important role in infection control strategies and the clinical management of disease caused by *S. aureus*.

A MALDI-TOF-MS method for the identification of *S. aureus and* differentiation of MRSA from MSSA was developed in this study. Identification of 100 *S.*

aureus strains was performed and the rate of correct identification at the species level (relative sensitivity) was 97%. The lower initial scores for 3 isolates at the species level with the score between 1.700 and 2.000 might be related to sample preparation as the proportion of matrix to sample in the first measurement which was not optimal. Discrepant results regarding species identification were consistent in the triplicate samples. And this suggests that it is necessary to validate the conventional identification of these three strains with methods other than MALDI-TOF-MS.

The time to obtain results for a MALDI-TOF MS-based identification was clearly less than that for conventional and molecular methods. The approach was rapid and took approximately 30 min per isolate from plate to obtaining final results, and approximately 2 h to analyze a full 96-spot target plate. Reliable recognition of identical, closely related, possibly related, and unrelated isolates among epidemiologically related *S. aureus* within the time frame of the MALDI Biotyper analysis would be advantageous to any other typing methodology currently available. Compared to the fast agglutination tests, the use of the MALDI-TOF MS-based approach for a routine *S. aureus* identification is reliable which is characterized by easy operation, large-scale sampling, high-resolution and accuracy, more sensitivity and reproducibility.

Many researchers have investigated MALDI-TOF-MS typing profiles. Some studies have verified the relationship between MALDI-TOF-MS profiles generated by both MRSA and MSSA. Some scientists have made efforts to differentiate among isogenic teicoplanin-susceptible and teicoplanin-resistant strains of methicillin-resistant *S. aureus* by MALDI-TOF-MS[15]. And it has been found that MALDI-TOF-MS could successfully distinguish the drug resistant strains of *S. aureus* among the same genotypes. In the present study, the selected biomarkers at the masses of 3784 Da and 5700 Da provided direct evidence for the differentiation of MRSA and MSSA. We believe that there is a need to further improve the accuracy of the method developed in our present study as well as to explore different modeling algorithms in the follow-up studies.

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