Application of a MALDI-TOF analysis platform (ClinProTools) for rapid and preliminary report of MRSA sequence types in Taiwan

Hsin-Yao Wang 1,2, Tsui-Ping Liu 1, Frank Lien 1, Chun-Hsien Chen 3, Chao-Jung Chen Corresp. 4,5, Jang-Jih Lu Corresp. 1,6,7

1 Department of Laboratory Medicine, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan
2 Ph.D. Program in Biomedical Engineering, Chang Gung University, Taoyuan, Taiwan
3 Department of Information Management, Chang Gung University, Taoyuan, Taiwan
4 Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan
5 Proteomics Core Laboratory, China Medical University Hospital, Taichung, Taiwan
6 Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Taoyuan, Taiwan
7 School of Medicine, Chang Gung University, Taoyuan, Taiwan

Corresponding Authors: Chao-Jung Chen, Jang-Jih Lu
Email address: cjchen@mail.cmu.edu.tw, jjlpcp@cmgh.org.tw

Background

The accurate and rapid preliminarily identification of the types of methicillin-resistant Staphylococcus aureus (MRSA) is crucial for infection control. Currently, however, expensive, time-consuming, and labor-intensive methods are used for MRSA typing. By contrast, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a potential tool for preliminary lineage typing. The approach has not been standardized, and its performance has not been analyzed in some regions with geographic barriers (e.g., Taiwan island).

Methods

The mass spectra of 306 MRSA isolates were obtained from multiple reference hospitals in Taiwan. The multilocus sequence typing (MLST) types of the isolates were determined. The spectra were analyzed for the selection of characteristic peaks by using the ClinProTools software. Furthermore, various machine learning (ML) algorithms were used to generate binary and multiclass models for classifying the major MLST types (ST5, ST59, and ST239) of MRSA.

Results

Ten peaks with the highest discriminatory power (m/z range: 2082–6594) were identified and evaluated. All the single peaks revealed significant discriminatory power during MLST typing. Moreover, the binary and multiclass ML models achieved sufficient accuracy (82.80%–94.40% for binary models and >81.00% for multiclass models) in classifying the major MLST types.

Conclusions

A combination of MALDI-TOF MS analysis and ML models is a potentially accurate, objective, and efficient tool for infection control and outbreak investigation.
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Hsin-Yao Wang†1,2, Tsui-Ping Liu†1, Frank Lien†1, Chun-Hsien Chen3, Chao-Jung Chen*4,5, Jang-Jih Lu*1,6,7

1 Department of Laboratory Medicine, Chang Gung Memorial Hospital at Linkou, Taoyuan City, Taiwan
2 Ph.D. Program in Biomedical Engineering, Chang Gung University, Taoyuan City, Taiwan
3 Department of Information Management, Chang Gung University, Taoyuan City, Taiwan
4 Graduate Institute of Integrated Medicine, China Medical University, Taichung City, Taiwan
5 Proteomics Core Laboratory, China Medical University Hospital, Taichung City, Taiwan
6 Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Taoyuan City, Taiwan
7 School of Medicine, Chang Gung University, Taoyuan City, Taiwan

† Contributed equally to this work

* Corresponding Author:

1. Jang-Jih Lu
   No. 5, Fuxing Street, Guishan District, Taoyuan City, Taiwan
   Email address: jjlpcp@cgmh.org.tw

2. Chao-Jung Chen
   No. 2, Yude Road, North District, Taichung City, Taiwan
   Email address: cjchen@mail.cmu.edu.tw
Abstract

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Introduction

Since their emergence in the 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been a major health care concern worldwide (Chen & Huang 2014; Walter et al. 2015; Wang et al. 2010). Many epidemiological studies have revealed that different multilocus sequence types (MLST) present specific characteristics such as virulence gene profiles (Recker et al. 2017; Schuenck et al. 2012; Wang et al. 2010; Wang et al. 2012; Wang et al. 2009). Understanding the evolution of MRSA lineages and the origin of infection is crucial in outbreak investigation. Molecular typing methods, such as pulsed-field gel electrophoresis and MLST, are highly expensive and labor intensive for epidemiological studies. Hence, the application of these methods in clinical practice is limited (Struelens et al. 2009). Sequence based typing methods have been widely used since the past decade, and it provides adequately high resolution for confirming transmission. However, in regions with few medical resources and financial constraints, it remains relatively impracticable. (Harris et al. 2013; Koser et al. 2012; Schwarze et al. 2018)

Recently, matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) has been used in many clinical microbiology laboratories. This method can be used to identify bacterial species effectively and rapidly (Ge et al. 2016). The peptide or protein MS fingerprint of each bacterium can be generated and stored as a in a bacterial library for species identification. In addition, MALDI-TOF MS also provides an alternative solution for molecular typing methods (e.g., MLST) (Lartigue 2013; Lu et al. 2012) and has the potential to offer lineage typing up to the subspecies level.

Studies that have adopted the MALDI-TOF MS approach (Lasch et al. 2014; Sauget et al. 2017; Ueda et al. 2015) have reported varying results; it may be due to several reasons. First, the
predominant MRSA lineages in different areas are different and the discriminatory power of MALDI-TOF might therefore differ when compared between different MRSA lineages. Second, the bacterial MS fingerprints of isolates from one area may not match those of isolates from other areas. Third, in many of the published works, the MALDI-TOF mass spectra have been assessed manually. An objective, standardized, and automated protocol has not been widely applied thus far (Camoez et al. 2016). Finally, the data obtained from MALDI-TOF mass spectra are relatively complicated and may be analyzed by a wide variety of bioinformatics tools. A manual approach cannot ensure a consistently high-quality output, because of the potential for large interindividual or intraindividual variation in the interpretation of the data. Therefore, a reliable analysis platform is necessary to ensure comparability between reports in clinical practice. Although Staphylococcus protein A (spa) typing provides comparable performance as MLST typing with less cost and time, our study has adopted MLST typing because we aim to demonstrate the possibility to implement our method beyond S. aureus. (Crisostomo et al. 2001; O'Hara et al. 2016) To validate the use of MALDI-TOF mass spectra in classifying MLST types of MRSA in Taiwan, we used the ClinProTools software for analyzing MALDI-TOF mass spectra to generate classification models of MRSA lineages. Accordingly, clinical microbiology laboratories may rapidly provide preliminary typing reports of MRSA, which may be further confirmed using a sequence-based method, thus enabling clinical practitioners to exclude an outbreak or transmission in time.

Materials & Methods

Bacterial lineages

This study included 306 convenience non-duplicate MRSA lineages isolated from multiple
reference hospitals in Taiwan, mainly through the Surveillance of Multicenter Antimicrobial
Resistance in Taiwan (SMART) program (Wang et al. 2012). The SMART program
consecutively collected MRSA isolates from ten medical centers throughout Taiwan from March
to August 2003. (Ho et al. 2010) All the lineages in this study were convenience samples which
had been recovered from blood cultures. All duplicate isolates were removed from the study.
After procuring the cultures from bacterial banks, tests were performed again to confirm the
characteristics of each lineage. The identification of S. aureus was based on colony morphology,
microscopic examination, a coagulase test, a catalase test, and MALDI-TOF mass spectra.

Bacterial identification through MALDI-TOF MS

The fresh bacterial colonies that were grown on blood agar plates for 24 hours were picked
up and smeared onto a MALDI steel target plate, forming a thin film of colonies. Next, 1 µL of
70% formic acid was introduced on the film and dried at room temperature. Subsequently, 1 µL
of the matrix solution (i.e., 50% acetonitrile containing 1% α-cyano-4-hydroxycinnamic acid and
2.5% trifluoroacetic acid) was introduced on the film again. The sample-matrix was dried at
room temperature before analyzing it through MS for data acquisition. Mass spectrum analysis
was performed using a MicroFlex LT mass spectrometer (Bruker Daltonik GmbH, Bremen,
Germany) with linear positive model, and the analytic region was 2000–20000 Da. For each
sample, 240 laser shots (at frequency of 20 Hz) were collected, and a Bruker Daltonics Bacterial
test standard (Bruker Daltonik GmbH) was used for calibration and as the control with the linear
positive model. The procedures were conducted according to the manufacturer’s instructions,
which have been detailed in previous studies (Ge et al. 2016; Lu et al. 2012). The results of mass
spectrum from the MALDI Biotyper 3.1 software (Bruker Daltonik GmbH) were compared with
those in the database and assigned scores. Peaks with scores >2 were further selected for peak
signal analysis. The lineages were randomly divided into batches, and the analyses were
carried out on different days to avoid a possible batch effect.

**MLST**

We sequenced the lineages for seven housekeeping genes, namely carbamate kinase (*arcC*),
shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate
acetyltransferase (*pta*), triosephosphateisomerase (*tpi*), and acetyl coenzyme A acetyltransferase
(*yqiL*). The sequencing results of these genes were compared with those in the *S. aureus* MLST
database ([http://saureus.mlst.net/](http://saureus.mlst.net/)) to acquire an allelic number and a sequence type (Enright et al. 2000).

**MALDI-TOF MS Spectra Analysis**

MALDI-TOF mass spectra of the MRSA lineages were fed into the ClinProTools™
software (version 3.0, Bruker Daltonik GmbH) in batches. The data preprocessing steps,
including baseline subtraction, smoothing, and recalibration, were set as default for all analyses
(Bruker Daltonik GmbH 2011; Camoez et al. 2016; Zhang et al. 2015). ClinProTools is a widely
used software developed by Bruker (Bruker Daltonik GmbH). It has been used in MALDI-TOF
data analysis for MRSA lineages typing (Camoez et al. 2016; Zhang et al. 2015). Characteristic
peaks among various MLST types were selected and sorted through several statistical tests,
including the t-test, analysis of variance (ANOVA), the Wilcoxon or Kruskal–Wallis (W/KW)
test, and the Anderson–Darling (AD) test. A *P* value of 0.05 was set as the cutoff. If *P* was <0.05
in the AD test, a characteristic peak was selected if the corresponding value of *P* in the W/KW
test was also <0.05. When $P$ was $\geq 0.05$ in the AD test, then a characteristic peak was selected if the corresponding value of $P$ in ANOVA was also <0.05 (Stephens 1974).

**Generation and validation of classification models**

Classification models of the major MLST types (ST5, ST59, and ST239) were generated using the ML algorithms in ClinProTools, namely QuickClassifier (QC), Supervised Neural Network (SNN), and Genetic Algorithm-K Nearest Neighbor (GA-KNN). The description and setting of the ML models are detailed in the ClinProTools user manual (Bruker Daltonik GmbH 2011). All the peaks in the spectra were used in model generation. The W/KW test was used to sort peaks during selection. For GA-KNN, GA was used as the method for feature selection, where the maximum number of best peaks was set as 30, and the maximum number of generations was set as 50. The numbers of nearest neighbors evaluated in the GA-KNN algorithm were 1, 3, and 5 to 7 for each binary classification. To avoid overfitting, we used 5-fold cross validation to obtain an unbiased statistical measurement of performance. Accordingly, the data were split into five subsets in a randomized manner. Each subset would serve as the validation set for the model trained by the remaining four subsets iteratively. Classification accuracy was obtained from the average of the five evaluations. Consequently, the bias of overfitting could be avoided using 5-fold cross validation.

**Statistical analysis**

The AD test is used to test for a normal distribution of peak intensity in ClinProTools. In the AD test when $P \leq 0.05$ (i.e., the data distribution did not follow normal distribution), the W/KW test was used as the statistical method to select discriminative peaks. In the W/KW
test, if \( P \leq 0.05 \), a rank-based multiple test procedure was used for post-hoc analysis to conduct paired comparisons in the nonnormal distributed data and calculate the simultaneous confidence intervals with Tukey-type contrasts (Konietschke et al. 2012). For evaluating the performance of various ML models, accuracy, sensitivity, and specificity were used as the metrics.

\[
\text{Accuracy} = \frac{TP + TN}{TP + FP + TN + FN}
\]

\[
\text{Sensitivity} = \frac{TP}{TP + FN}
\]

\[
\text{Specificity} = \frac{TN}{TN + FP}
\]

where TP, TN, FP, and FN represent the number of true positives, true negatives, false positives, and false negatives, respectively.

**Results**

Six MLST types of MRSA were identified in the isolates, namely ST5 (\( n = 40 \)), ST45 (\( n = 8 \)), ST59 (\( n = 62 \)), ST239 (\( n = 179 \)), ST241 (\( n = 12 \)), and ST573 (\( n = 5 \)). The isolates ST5, ST59, and ST239 were considered the major MLST types of MRSA because they exhibited numerous lineages. The isolates ST45, ST241, and ST573 were categorized together as other ST types.

**Characteristic peaks for discrimination among various MLST types**

The characteristic peaks were sorted using the corresponding \( P \) values obtained in the (W/KW) test because the AD test revealed \( P < 0.05 \) (Table 1). The top 10 characteristic peaks, sorted using the W/KW test results, were selected for further statistical evaluation. The 10 peaks ranged from \( m/z \) 2082–6594 (Table 1). All the 10 selected peaks revealed \( P < 0.000001 \) in the W/KW test, thus indicating that they were informative and discriminative peaks in the MLST type classification.

In Table 1, the distribution of these peaks over various MLST types was further evaluated.
High expression levels of \(m/z\) 2430 and 3893 and a low expression level of \(m/z\) 3877 indicated the fingerprint of ST5; low expression levels of \(m/z\) 2416, 2980, and 3893 indicated the fingerprint of ST59; high expression levels of \(m/z\) 2416 and 2880 and low expression levels of \(m/z\) 3277 and 3893 indicated the fingerprint of ST239. Based on 10 informative peaks, the odds ratio (OR) of different ST-pairs was calculated to determine the association between the peaks and respective ST-pairs (Supplemental Table S1). Furthermore, the distribution of the isolates was plotted according to peak intensity of \(m/z\) 3277 (x axis) and \(m/z\) 6594 (y axis) (Fig. 1). The peaks at \(m/z\) 3277 and 6594 were the top two characteristic peaks among the ST types (Fig. 1). The scatter plot figures may be seen as a projection of a high-dimensional scatter space with various dimensions of the \(m/z\) peaks.

The average intensities of the 10 peaks of these lineages are further illustrated in Fig. 2. These results demonstrate the specific characteristics and patterns of peaks in the different MLST types. Specifically, the distribution of the ST59 lineages and ST239 lineages showed satisfactory separation (Fig. 1); ST5 lineages could also be distinguished from ST239 lineages based on distribution (Fig. 1). By contrast, ST5 lineages and ST59 lineages could not be satisfactorily discriminated using information from the peaks \(m/z\) 3277 and 6594 only (Fig. 1). Similarly, the lineages of other minor ST types mixed with other major ST types (i.e., ST5, ST59, and ST239) on the scatter plot (Fig. 1).

**ML models for classification of various MLST types of MRSA**

Various MLST types showed specific patterns of peaks expression, as presented in Table 1 and Fig. 3-5. To generate a comprehensive and objective classification, ML algorithms were used. In the ST5 binary classification models, the peaks at \(m/z\) 3877 and \(m/z\) 3893 showed
satisfactory discriminative power (Fig. 3). The QC algorithm attained the highest cross-
validation values (94.40%, Table 2(a)).

In ST59 binary classification models, peaks at $m/z$ 2980 and $m/z$ 2416 also showed
satisfactory discriminative power (Fig. 4). The GA-KNN algorithm attained the highest cross-
validation values among all the algorithms. The GA-KNN algorithm showed highest
performance when the number of nearest neighbor was set as 5 (85.00%, Table 2(b)).

For the binary classification of ST239 versus non-ST239, the peaks at $m/z$ 3277 and $m/z$
6553 showed moderate discriminative power (Fig. 5). The GA-KNN algorithm showed higher
performance over other algorithms when the number of nearest neighbor was set as 7 (82.80%,
Table 2(c)).

In this study, multiclass models for classifying an isolate into one of four lineages were
designed. Generally, the GA-KNN algorithm outperformed the other algorithms; more
specifically, this algorithm was suitable for detecting ST239 (Table 3). The GA-KNN algorithm
could successfully detect the ST5, ST59, and ST239 lineages with an accuracy of >81.00%.
However, none of the multiclass models showed reliable accuracy in detecting isolates of the
other ST lineages (i.e., ST45, ST241, and ST573).

Discussion

In this study, the major ST types (i.e., ST5, ST59, and ST239) of blood stream MRSA in
Taiwan could be classified through ML-based MALDI-TOF mass spectra analysis with high
accuracy. The analysis was conducted using a standardized system (i.e., ClinProTools) to obtain
objective and consistent results. Moreover, the ST types of MRSA could be predicted accurately
through MALDI-TOF mass spectra analysis before additional molecular typing methods (e.g.,
MLST). Biomarker peaks of various MRSA ST types were discovered through MALDI-TOF
mass spectra analysis by using the ClinProTools software. Some of the biomarker peaks have been previously reported (Camoez et al. 2016; Josten et al. 2013; Sauget et al. 2017; Zhang et al. 2015) and validated in the study (namely m/z 3277, 3877, 3893, 6553, and 6594), whereas some of them have not yet been widely validated (namely m/z 2082, 2416, 2430, 2880, and 2980).

Nevertheless, reports on the MS characteristics of MRSA are inconsistent (Lasch et al. 2014; Sauget et al. 2017; Ueda et al. 2015). Although the accuracy of typing is sufficient in individual studies, the general patterns of specific lineages are not available (Sauget et al. 2017). Currently, bacterial lineages obtained from geographically diverse areas cannot be clearly discriminated using MALDI-TOF MS. However, more robust characteristic patterns of various types may be available in regions with geographic barriers (e.g., Taiwan island) than in regions without these barriers. The characteristic patterns of specific lineages may be the result of the disseminated lineages in local areas. By contrast, establishing a localized solution by using an appropriate method of interpreting mass spectra may be more practical and crucial than establishing a generalized pattern. The isolates in this study were obtained from multiple reference hospitals in Taiwan. Consequently, the isolates used in the study can represent the molecular characteristics of MRSA in the local region. The localized molecular characteristics of MRSA may be sufficiently useful for clinical practice in regions with geographic barriers (e.g., Taiwan island).

Currently, clinical microbiology laboratories generally use MALDI-TOF MS for bacterial identification because of its advantages in accuracy and effectiveness over traditional biochemical methods (Lartigue 2013). Before analytical measurement by using MALDI-TOF MS, a protein extraction process is necessary. In-tube extraction or the direct deposit method are two common extraction methods used in clinical microbiology laboratories. In-tube extraction provides more purified intracellular components than the direct deposit method does, which results in high quality MALDI-TOF mass spectra and low noise. By contrast, the direct
deposition of bacteria onto a steel plate is considered a less labor-intensive and more rapid preanalytical process than in-tube extraction. The time for the entire process and the turnaround time of MALDI-TOF MS can be considerably reduced by using the direct deposit method. Consequently, considering the relevance in routine practice, the direct deposit method was evaluated in this study.

Knowledge of the bacterial molecular type of MRSA is crucial while performing epidemiological studies on bacterial outbreak. In this study, specific peaks were identified for major clonal lineages of MRSA. The peaks $m/z$ near 3277, 3877, 3893, 6553, and 6594 identified in this study have been reported in previous studies as characteristic peaks in discriminating MRSA clonal complexes (CCs). (Camoez et al. 2016; Josten et al. 2013; Wolters et al. 2011). Zhang et al. reported the highest expression of the peaks at $m/z$ 3277 and 6554 in ST59 (Zhang et al. 2015). The peak at $m/z$ 6594 was recognized as the SA1452 protein and as a biomarker of the clonal complex 8 (CC8) and USA-300 lineages; both these MRSA lineages are prevalent in communities and hospitals (Boggs et al. 2012; Josten et al. 2013; Wolters et al. 2011). In this study, the peak at $m/z$ 6594 was characteristic for ST239, which were the most prominent ST type of bloodstream MRSA and prominent HA MRSA (Table 1). By contrast, some of the characteristic peaks have not previously been reported and validated as discriminative peaks (i.e., peaks at $m/z$ 2082, 2416, 2430, 2880, and 2980) (Sauget et al. 2017); however, they play a crucial role in MLST type classification. For example, the peak at $m/z$ 2980 was a distinguishing peak for the ST59 MRSA lineages (Fig. 4), which is one of the characteristic ST types of the CA MRSA in Taiwan (Huang & Chen 2011). Moreover, the peaks at $m/z$ 2416 and 2430 were noted as characteristic peaks in the ST5 and ST239. In the QC models, the peak at $m/z$ 2416 was proven to represent $psm\text{-}mec$, which is strongly associated with SCC$mec$ III and VIII (Queck et al. 2009). Briefly, although the peak at $m/z$ 2416 is commonly expressed peak in MRSA, the
expression level, in addition to its presence, may serve as an informative feature in classification of MRSA lineages.

Furthermore, for identifying subtle differences in the MALDI-TOF mass spectra for preliminary reporting of AST or subspecies results, not only single characteristic peaks but also specific combinations of characteristic peaks may be beneficial. Single MS peaks have provided some characteristics of each major ST type (Table 1, Fig. 1 to Fig. 5). Additional integration of these characteristic peaks by using ML models may generate a more comprehensive and robust result for ST typing of MRSA (Table 2) than that generated using single peaks. Consequently, the accuracy of the subspecies classification by using a combination of peaks may be higher and more resistant to variations in analysis than that using a single peak because of comprehensive interpretation. Zhang et al. reported the successful use of ML models (by ClinProTools) in analyzing MALDI-TOF mass spectra to classify various MLST types of MRSA (Zhang et al. 2015). High performance of the binary models (including ST5, ST45, ST59, and ST239 binary classification models) were described. However, the high performance might have resulted from overfitting because only one specific combination of training sets and validation sets was used for performance evaluation.

This study had several limitations. Firstly, our study did not adopt nucA PCR or other sequencing methods to exclude S. argenteus, which were previously identified as S. aureus using molecular typing (Thaipadungpanit et al. 2015; Tong et al. 2015). The MRSA isolates were collected from an island with geographic barriers, thus resulting in a relative simple ST distribution. Possibly, our method might be unsuitable in other country with wide variety of bacterial lineage. We thus recommend researchers who intend to adopt our approach to train and validate their own model by using regional MRSA lineages. Another limitation of this study is that the MRSA isolates in this study are all from previously collected samples under the SMART
program. This indicates the model may not exhibit sufficient accuracy in performance on new MRSA isolated in the future. Moreover, only six MLST types (namely ST5, ST45, ST59, ST239, ST241 and ST573) of MRSA were included for models training and validation. Based on the study design, ML learning models can be used for detecting the major lineages of MRSA in Taiwan only, but they cannot be used for detecting new clones, which were not included in model training. Consequently, the models are designed to be used for reporting preliminary lineage information in outbreak investigation. When a new clone emerges in the future, the ML models could be tuned and updated using the newly collected datasets. Our study has demonstrated that in regions with limited medical resources, an MALDI-TOF-based MLST typing model may serve as a valuable method for timely intervention in the transmission or outbreak of MRSA.

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References

Boggs SR, Cazares LH, and Drake R. 2012. Characterization of a Staphylococcus aureus USA300 protein signature using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Med Microbiol* 61:640-644. 10.1099/jmm.0.037978-0

Bruker Daltonik GmbH. 2011. ClinProTools 3.0: User Manual. Bremen, Germany: Bruker Daltonik GmbH.

Camoez M, Sierra JM, Dominguez MA, Ferrer-Navarro M, Vila J, and Roca I. 2016. Automated categorization of methicillin-resistant Staphylococcus aureus clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry. *Clin Microbiol Infect* 22:161 e161-161 e167. 10.1016/j.cmi.2015.10.009

Chen CJ, and Huang YC. 2014. New epidemiology of Staphylococcus aureus infection in Asia. *Clin Microbiol Infect* 20:605-623. 10.1111/1469-0691.12705
Crisostomo MI, Westh H, Tomasz A, Chung M, Oliveira DC, and de Lencastre H. 2001. The evolution of methicillin resistance in Staphylococcus aureus: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. Proc Natl Acad Sci U S A 98:9865-9870. 10.1073/pnas.161272898

Enright MC, Day NP, Davies CE, Peacock SJ, and Spratt BG. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. J Clin Microbiol 38:1008-1015.

Ge MC, Kuo AJ, Liu KL, Wen YH, Chia JH, Chang PY, Lee MH, Wu TL, Chang SC, and Lu JJ. 2016. Routine identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Success rate, economic analysis, and clinical outcome. J Microbiol Immunol Infect. 10.1016/j.jmii.2016.06.002

Harris SR, Cartwright EJP, Török ME, Holden MTG, Brown NM, Ogilvy-Stuart AL, Ellington MJ, Quail MA, Bentley SD, Parkhill J, and Peacock SJ. 2013. Whole-genome sequencing for analysis of an outbreak of meticillin-resistant Staphylococcus aureus: a descriptive study. The Lancet Infectious Diseases 13:130-136. 10.1016/s1473-3099(12)70268-2

Ho CM, Hsueh PR, Liu CY, Lee SY, Chiueh TS, Shyr JM, Tsao SM, Chuang YC, Yan JJ, Wang LS, Wang JH, Ho MW, Tien N, and Lu JJ. 2010. Prevalence and accessory gene regulator (agr) analysis of vancomycin-intermediate Staphylococcus aureus among meticillin-resistant isolates in Taiwan--SMART program, 2003. Eur J Clin Microbiol Infect Dis 29:383-389. 10.1007/s10096-009-0868-4

Huang YC, and Chen CJ. 2011. Community-associated meticillin-resistant Staphylococcus aureus in children in Taiwan, 2000s. Int J Antimicrob Agents 38:2-8. 10.1016/j.ijantimicag.2011.01.011

Josten M, Reif M, Szekat C, Al-Sabti N, Roemer T, Sparbier K, Kostrzewa M, Rohde H, Sahl HG, and Bierbaum G. 2013. Analysis of the Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrum of Staphylococcus aureus Identifies Mutations That Allow Differentiation of the Main Clonal Lineages. J Clin Microbiol 51:1809-1817. 10.1128/jcm.00518-13

Konietschke F, Hothorn LA, and Brunner E. 2012. Rank-based multiple test procedures and simultaneous confidence intervals. Electronic Journal of Statistics 6:738-759. 10.1214/12-EJS691

Koser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, Hsu LY, Chewapreecha C, Croucher NJ, Harris SR, Sanders M, Enright MC, Dougan G, Bentley SD, Parkhill J, Fraser LJ, Betley JR, Schulz-Trieglaff OB, Smith GP, and Peacock SJ. 2012. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N Engl J Med
Lartigue MF. 2013. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for bacterial strain characterization. Infect Genet Evol 13:230-235. 10.1016/j.meegid.2012.10.012

Lasch P, Fleige C, Stämmler M, Layer F, Nübel U, Witte W, and Werner G. 2014. Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of Enterococcus faecium and Staphylococcus aureus isolates. Journal of Microbiological Methods 100:58-69. 10.1016/j.mimet.2014.02.015

Lu J-J, Tsai F-J, Ho C-M, Liu Y-C, and Chen C-J. 2012. Peptide biomarker discovery for identification of methicillin-resistant and vancomycin-intermediate Staphylococcus aureus strains by MALDI-TOF. Analytical chemistry 84:5685-5692.

O'Hara FP, Suaya JA, Ray GT, Baxter R, Brown ML, Mera RM, Close NM, Thomas E, and Amrine-Madsen H. 2016. spa Typing and Multilocus Sequence Typing Show Comparable Performance in a Macropadiologic Study of Staphylococcus aureus in the United States. Microb Drug Resist 22:88-96. 10.1089/mdr.2014.0238

Queck SY, Khan BA, Wang R, Bach T-HL, Kretschmer D, Chen L, Kreiswirth BN, Peschel A, DeLeo FR, and Otto M. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS Pathog 5:e1000533.

Recker M, Laabei M, Toleman MS, Reuter S, Saudersson RB, Blane B, Torok ME, Ouadi K, Stevens E, Yokoyama M, Steventon J, Thompson L, Milne G, Bayliss S, Bacon L, Peacock SJ, and Massey RC. 2017. Clonal differences in Staphylococcus aureus bacteraemia-associated mortality. Nat Microbiol 2:1381-1388. 10.1038/s41564-017-0001-x

Sauget M, Valot B, Bertrand X, and Hocquet D. 2017. Can MALDI-TOF Mass Spectrometry Reasonably Type Bacteria? Trends Microbiol. 10.1016/j.tim.2016.12.006

Schuenck RP, Cavalcante FS, Emery E, Giambiagi-de Marval M, and dos Santos KR. 2012. Staphylococcus aureus isolates belonging to different multilocus sequence types present specific virulence gene profiles. FEMS Immunol Med Microbiol 65:501-504. 10.1111/j.1574-695X.2012.00958.x

Schwarze K, Buchanan J, Taylor JC, and Wordsworth S. 2018. Are whole-exome and whole-genome sequencing approaches cost-effective? A systematic review of the literature. Genet Med. 10.1038/gim.2017.247

Stephens MA. 1974. EDF Statistics for Goodness of Fit and Some Comparisons. Journal of the American Statistical Association 69:730-737. 10.1080/01621459.1974.10480196

Struelens MJ, Hawkey PM, French GL, Witte W, and Tacconelli E. 2009. Laboratory tools and strategies for methicillin-resistant Staphylococcus aureus screening, surveillance and typing: state of the art and unmet needs. Clin Microbiol Infect 15:112-119.
Thaipadungpanit J, Amornchai P, Nickerson EK, Wongsuvan G, Wuthiekanun V, Limmathurotsakul D, and Peacock SJ. 2015. Clinical and molecular epidemiology of Staphylococcus argenteus infections in Thailand. *J Clin Microbiol* 53:1005-1008.

Tong SY, Schaumburg F, Ellington MJ, Corander J, Pichon B, Leendertz F, Bentley SD, Parkhill J, Holt DC, Peters G, and Giffard PM. 2015. Novel staphylococcal species that form part of a Staphylococcus aureus-related complex: the non-pigmented Staphylococcus argenteus sp. nov. and the non-human primate-associated Staphylococcus schweitzeri sp. nov. *Int J Syst Evol Microbiol* 65:15-22.

Ueda O, Tanaka S, Nagasawa Z, Hanaki H, Shobuike T, and Miyamoto H. 2015. Development of a novel matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF-MS)-based typing method to identify meticillin-resistant Staphylococcus aureus clones. *J Hosp Infect* 90:147-155.

Walter J, Haller S, Blank HP, Eckmanns T, Abu Sin M, and Hermes J. 2015. Incidence of invasive meticillin-resistant Staphylococcus aureus infections in Germany, 2010 to 2014. *Euro Surveill* 20. 10.2807/1560-7917.ES.2015.20.46.30067

Wang JL, Wang JT, Sheng WH, Chen YC, and Chang SC. 2010. Nosocomial meticillin-resistant Staphylococcus aureus (MRSA) bacteremia in Taiwan: mortality analyses and the impact of vancomycin, MIC = 2 mg/L, by the broth microdilution method. *BMC Infect Dis* 10:159.

Wang WY, Chiueh TS, Sun JR, Tsao SM, and Lu JJ. 2012. Molecular typing and phenotype characterization of meticillin-resistant Staphylococcus aureus isolates from blood in Taiwan. *PLoS One* 7:e30394. 10.1371/journal.pone.0030394

Wang WY, Lee SY, Chiueh TS, and Lu JJ. 2009. Molecular and phenotypic characteristics of meticillin-resistant and vancomycin-intermediate staphylococcus aureus isolates from patients with septic arthritis. *J Clin Microbiol* 47:3617-3623. 10.1128/jcm.00539-09

Wolters M, Rohde H, Maier T, Belmar-Campos C, Franke G, Scherpe S, Aepfelbacher M, and Christner M. 2011. MALDI-TOF MS fingerprinting allows for discrimination of major meticillin-resistant Staphylococcus aureus lineages. *Int J Med Microbiol* 301:64-68.

Zhang T, Ding J, Rao X, Yu J, Chu M, Ren W, Wang L, and Xue W. 2015. Analysis of meticillin-resistant Staphylococcus aureus major clonal lineages by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). *J Microbiol Methods* 117:122-127.
Figure 1

Scatter plot of various MLST types isolates

The peaks at $m/z$ 3277 and $m/z$ 6594 served as the x and y axes, respectively. The intensities of the characteristic peaks were expressed in arbitrary intensity units. The ellipses represent the 95% confidence intervals of peak intensities for each MLST type. In the preliminary analysis, the various MLST types could not be satisfactorily separated based only on two characteristic peaks. ST5: red crosses, ST59: green circles, ST239: blue squares, and other ST types: yellow diamonds.
Figure 2

Average spectra of characteristic peaks among various MLST types

Intensities of characteristic peaks ($m/z$ 2082, 2416, 2430, 2880, 2980, 3277, 3877, 3893, 6553, and 6594, from A to J, respectively) in ST5 (red), ST59 (green), ST239 (blue), and other ST types (yellow) expressed in arbitrary intensity units.
Figure 3

Scatter plot of the ST5 and non-ST5 isolates

The peaks at m/z 3893 and m/z 3877 served as the x and y axes, respectively. Intensities of the characteristic peaks were expressed in arbitrary intensity units. The ellipses represent the 95% confidence intervals of peak intensities for ST5 (red ellipse) or non-ST5 (green ellipse). ST5: red crosses, non-ST5: green circles.
Figure 4

Scatter plot of the ST59 and non-ST59 isolates

The peaks at $m/z$ 2416 and $m/z$ 2980 served as the $x$ and $y$ axes, respectively. The intensities of the characteristic peaks were expressed in arbitrary intensity units. The ellipses represent 95% confidence intervals of the peak intensities for ST59 (red ellipse) or non-ST59 (green ellipse). ST59: red crosses, non-ST59: green circles.
**Figure 5**

Scatter plot of the ST239 and non-ST239 isolates

The peaks at \( m/z \) 3277 and \( m/z \) 6553 served as the x and y axes, respectively. The intensities of the characteristic peaks were expressed in arbitrary intensity units. The ellipses represent 95% confidence intervals of the peak intensities for ST239 (red ellipse) or non-ST239 (green ellipse). ST239: red crosses, non-ST239: green circles.
Table 1 (on next page)

Characteristic MALDI-TOF MS peaks in different MLST types of MRSA

Abbreviations: DAve: difference between the maximal and the minimal average peak intensity of all classes; PW/KW: P value obtained through Wilcoxon/Kruskal-Wallis test; PAD: P value obtained through Anderson–Darling test, MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry
Table 1:

Characteristic MALDI-TOF MS peaks in different MLST types of MRSA

| Mass   | DAve | PW/KW   | PAD     | Average of peak Intensity |
|--------|------|---------|---------|---------------------------|
|        |      |         |         | ST5 | ST59 | ST239 | Other ST |
| 2082.13| 4.28 | < 0.000001| < 0.000001| 6.13 | 3.84 | 1.85 | 3.24 |
| 2415.79| 38.19| < 0.000001| < 0.000001| 24.57 | 6.13 | 44.32 | 19.72 |
| 2430.49| 18.13| < 0.000001| < 0.000001| 20.12 | 3.86 | 21.99 | 6.23 |
| 2880.03| 11.2 | < 0.000001| < 0.000001| 1.15 | 1.88 | 12.35 | 5.38 |
| 2980.45| 6.85 | < 0.000001| < 0.000001| 7.4 | 2.47 | 9.32 | 7.88 |
| 3276.8 | 2.94 | < 0.000001| < 0.000001| 2.61 | 3.73 | 0.79 | 2.08 |
| 3876.96| 6.24 | < 0.000001| < 0.000001| 0.75 | 5.5 | 6.33 | 7 |
| 3892.91| 4.91 | < 0.000001| < 0.000001| 5.61 | 0.69 | 0.87 | 2.77 |
| 6553.03| 6.23 | < 0.000001| < 0.000001| 6.28 | 9.32 | 3.1 | 6.15 |
| 6593.54| 4.33 | < 0.000001| < 0.000001| 1.98 | 3 | 6.3 | 5.48 |

Abbreviations: DAve: difference between the maximal and the minimal average peak intensity of all classes; PW/KW: P value obtained through Wilcoxon/Kruskal-Wallis test; PAD: P value obtained through Anderson–Darling test, MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
Table 2 (on next page)

Test performance of various lineage typing ML models

QC, QuickClassifier; SNN, supervised neural network; GA, genetic algorithm; KNN, K-Nearest Neighbor. The number following “GA-KNN” indicates the number of nearest neighbors used in models.

Selected peaks: number of peaks selected by the models; Acc, accuracy; Sen, sensitivity; Spe, specificity. Performance metrics are expressed as mean ± standard error.
| ST  | Model    | Selected peaks | Acc       | Sen       | Spe       |
|-----|----------|----------------|-----------|-----------|-----------|
|     | QC       | 8              | 0.94±0.04 | 0.94±0.05 | 0.95±0.05 |
|     | SNN      | 24             | 0.63±0.05 | 0.91±0.05 | 0.34±0.04 |
| ST5 | GA_KNN1  | 30             | 0.83±0.03 | 0.68±0.03 | 0.98±0.05 |
|     | GA_KNN3  | 30             | 0.84±0.03 | 0.71±0.03 | 0.97±0.02 |
|     | GA_KNN5  | 19             | 0.83±0.03 | 0.68±0.03 | 0.98±0.02 |
|     | GA_KNN7  | 14             | 0.88±0.03 | 0.79±0.03 | 0.97±0.02 |
|     | QC       | 22             | 0.76±0.03 | 0.85±0.04 | 0.67±0.04 |
|     | SNN      | 5              | 0.68±0.04 | 0.57±0.05 | 0.79±0.04 |
| ST59| GA_KNN1  | 30             | 0.78±0.03 | 0.62±0.04 | 0.94±0.03 |
|     | GA_KNN3  | 29             | 0.82±0.02 | 0.70±0.02 | 0.94±0.03 |
|     | GA_KNN5  | 29             | 0.85±0.03 | 0.74±0.03 | 0.96±0.02 |
|     | GA_KNN7  | 22             | 0.82±0.02 | 0.67±0.02 | 0.97±0.02 |
|     | QC       | 6              | 0.81±0.02 | 0.91±0.01 | 0.72±0.02 |
|     | SNN      | 1              | 0.58±0.03 | 0.72±0.03 | 0.44±0.04 |
| ST239| GA_KNN1 | 30             | 0.80±0.02 | 0.88±0.03 | 0.72±0.02 |
|     | GA_KNN3  | 30             | 0.80±0.02 | 0.85±0.02 | 0.74±0.02 |
|     | GA_KNN5  | 29             | 0.81±0.02 | 0.89±0.01 | 0.72±0.02 |
|     | GA_KNN7  | 28             | 0.83±0.02 | 0.90±0.02 | 0.76±0.02 |

QC, QuickClassifier; SNN, supervised neural network; GA, genetic algorithm; KNN, K-Nearest Neighbor. The number following “GA-KNN” indicates the number of nearest neighbors used in models.

Selected peaks: number of peaks selected by the models; Acc, accuracy; Sen, sensitivity; Spe, specificity. Performance metrics are expressed as mean ± standard error.
Table 3 (on next page)

Accuracy of various multiclass models in classifying different ST lineages

QC, QuickClassifier; SNN, supervised neural network; GA, genetic algorithm; KNN, K-Nearest Neighbor, the number following “GA-KNN” indicated the number of nearest neighbor used in models; Selected peaks: number of peaks selected by the models. Accuracies were expressed as mean ± standard error.
Table 3. Accuracy of various multiclass models in classifying different ST lineages.

| Model   | Selected peaks | ST5    | ST59   | ST239  | Others  |
|---------|----------------|--------|--------|--------|---------|
| QC      | 24             | 0.65±0.05 | 0.85±0.03 | 0.79±0.03 | 0.09±0.07 |
| SNN     | 25             | 0.65±0.05 | 0.34±0.05 | 0.25±0.06 | 0.13±0.09 |
| GA_KNN1 | 30             | 0.58±0.05 | 0.63±0.04 | 0.84±0.02 | 0.09±0.07 |
| GA_KNN3 | 26             | 0.73±0.03 | 0.78±0.03 | 0.90±0.02 | 0.13±0.08 |
| GA_KNN5 | 27             | 0.73±0.03 | 0.78±0.03 | 0.94±0.02 | 0.04±0.07 |
| GA_KNN7 | 22             | 0.92±0.03 | 0.81±0.03 | 0.94±0.02 | 0.04±0.07 |

QC, QuickClassifier; SNN, supervised neural network; GA, genetic algorithm; KNN, K-Nearest Neighbor, the number following “GA-KNN” indicated the number of nearest neighbor used in models; Selected peaks: number of peaks selected by the models. Accuracies were expressed as mean ± standard error.