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Evaluation of conventional methods for species identification of *Staphylococcus aureus* using MALDI-TOF MS, protein identification and quantification

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ABSTRACT

Objectives: This study aimed to evaluate conventional methods for species identification of Staphylococcus aureus by MALDI-TOF MS. Additionally, the representatives of different species were used to analyze protein expression.

Materials and methods: A total of 185 non-duplicated clinical *S. aureus* identified using the conventional method (colony morphology, Gram's stain, slide coagulase test, tube coagulase test, catalase test, and mannitol fermentation) was confirmed the identification by MALDI-TOF MS and analyzed by Mass spectral profiling (MSP) and Principal component analysis (PCA). The representatives of different species reported by both methods were confirmed using *16SrRNA* sequence and analyzed proteins expression by timsTOF MS.

Results: All *S. aureus* suspected isolates could discriminate among species by MALDI-TOF MS including *S. aureus* (N=151, 81.6%), *S. argenteus* (N=32, 17.3%), *S. hominis* (N=1, 0.5%), and S. *haemolyticus* (N=1, 0.5%). Using *16S rRNA* gene-based analysis, *S. aureus* and *S. argenteus* could not differentiate from each other. Protein expression of *S. aureus* was similar to *S. argenteus.* These genes including *rpsT, Huti, pyrF, atpD.1, cpfC, SAUA300_0786, atl.1,* and *MW2416* showed higher expression in *S. aureus* (MS076) than *S. argenteus* (MS060), *S. haemolyticus* (MS095) and *S. hominis* (MS060).

Conclusion: MALDI-TOF MS provides an excellent tool for accurately species identification of staphylococci. *S. aureus* expressed protein analyzed higher than the other 3 species. The highest protein expression in *S. aureus* implies the most virulence of this strain.

Introduction

Staphylococci including *Staphylococcus aureus* constitute the microbiota of humans and animals but are the critical cause of hospital-acquired infection, particularly methicillin-resistant *S. aureus* (MRSA) in intensive care units.[1] The high pathogenicity is associated with multidrug resistance and their adaptation to a variety of environmental conditions.[2] *S. aureus*, one of the most important clinical pathogens, causes various diseases such as skin and soft tissue, bacteremia, pneumonia, endocarditis, or osteomyelitis which conducts an increasing number of morbidity and mortality in the world.[3] The conventional culture method had a limitation to differentiate some species of Staphylococci. For the species identification of staphylococci, slide coagulase,

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tube coagulase, and mannitol salt agar have been used for presumptive isolation among *S. aureus* and other staphylococci, however, these biochemical tests are not sufficient to distinguish between *S. aureus* and other staphylococci.[4]

Molecular analysis for the *Staphylococcus* identification into species level has been performed manually with electrophoresis, taking hours to obtain the result and resulting in a heavy workload for technicians. Several methods have been developed to be a rapid and effective tool to profile bacteria at the genus and species level with high reproducibility such as more recently the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).[5] MALDI-TOF MS with library-based and/or bioinformatics-enabled methods has been successfully applied in many areas. For example, in clinical microbiology, MALDI-TOF MS has been used to identify pathogens, which can be rapidly reported by comparing spectra to a database and this technique has been used to accurately identify various microorganisms including *S. aureus.*[6] Kim *et al.* identified 100% *of S. aureus* 89.5% of *Staphylococcus* spp. other than *S. aureus* isolated from food samples by MALDI-TOF MS. PCA and MSP-based dendrogram of each species of *Staphylococcus* isolates were clearly clustered.[7] This study aimed to evaluate conventional methods for species identification of *Staphylococcus aureus* by MALDI-TOF MS. Additionally, the representatives of different species were used to analyze protein expression.

Materials and methods

Study design, Sample Collection, and Ethical Approval

The study was designed as a cross-sectional study; 185 non-duplicated clinical samples *S. aureus* were collected from Prapokklao Hospital, a tertiary healthcare setting, during the period 2018-2020. The bacterial isolates were frozen at -80 °C until use. This study was approved by the Ethics Committee of Prapokklao Hospital, Chanthaburi (Approval Number: CTIREC 048).

Bacterial Isolation and Identification

A total of 185 non-duplicated clinical *S. aureus* suspected isolates were recovered and identified using conventional biochemical tests. The isolates underwent colony morphology, Gram staining, catalase test, coagulase test, and mannitol salt fermentation.

MALDI-TOF MS identification

The bacterial colony was extracted using the formic acid extraction method. Briefly, the bacterial isolates were grown on Mueller-Hinton agar (Becton, Dickinson and Company, USA) plates overnight at 35±2 °C. Fresh bacterial cells 4-5 colonies were transferred into 300 µL of deionized water, stir and mix with 900 µL of ethanol thoroughly until the material was completely in suspension. Sediment was obtained after centrifugation 13,000xg for 2 min and repeated this step until the supernatant was completely removed. Air-dry the pellet for at least 15 min at room temperature. Added 50 µL 70% aqueous formic acid and 50 µL 100% acetonitrile and mix with a vortex mixer for 1 min. Centrifuged the mixture 13,000xg for 2 min. One µL supernatant was directly spotted on a MALDI polish steel target plate (Bruker Daltonics). The preparation was overlaid with 1 µL of 4-hydroxy-α-cyanocinnamic acid (HCCA) in Standard solvent (2.5% trifluoroacetic acid/50% acetonitrile). The colony was then air-dried at room temperature to allow co-crystallization with the experimental sample. MALDI-TOF MS was performed on an autoflex maX™ TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with smartbeam-II laser with FlexControl™ software 3.4 (Bruker Daltonics) for automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kD. Each spectrum was acquired with 2,000 laser impulses at a frequency of 200 Hz. The sample was triplicate collecting the spectra. The mass spectrometer was periodically calibrated using a Bacterial Test Standard (BTS) (*Escherichia coli* ATCC 25922). All isolates were confirmed species by comparing to the MBT compass explorer, library version 4.1 (9,999 entries; Bruker Daltonics) at the species level with a score value of >2.0. For this library version could identify 45 species of staphylococci *eg*. *S. aureus, S. argenteus, S. schweitzeri, S. hominis, S. haemolyticus*, *etc*.

Analysis of mass spectrometry data

BioTyper required summarization of mass spectra of biological and/or technical replicates of bacteria. The resulting composite mass spectra are called main spectral profiles (MSPs) Dendrogram. With regard to the strain-level characterization, BioTyper has been suggested to be useful in characterizing bacteria at the strain level, but typically with the assistance of additional software, such as ClinProTools™.[5] Analysis of the entire spectrum with rigorous analytical tools is often required to obtain reliable strain identification. The ClinProTools™ provides a Principal component analysis (PCA) that also can differentiate between closely related samples, recalibrate to generate the necessary alignment, normalize each peak, and then can contribute equally to model generation.[8] PCA is a widely used mathematical technique designed to extract, display, and rank the variance within a data set.[9]

16S rRNA gene sequence

Representative different species were investigated using *16SrRNA* gene sequence. Briefly, the bacterial isolates were grown on Mueller-Hinton agar (Becton, Dickinson and Company, USA) plates overnight at 35±2 °C. The single bacterial 3-5 colonies were re-suspended in 1x TAE buffer (sterile)200 µL and heated in the chamber at 95 °C for 15 min. After centrifugation at 12,000xg for 5 min, the supernatant was used as a DNA template for PCR. The total PCR reaction volume was 20 µL containing 10x PCR buffer minus $MgCl_2$, 2 µL of DNA template, 0.2 U of Taq DNA polymerase, 0.05 µM of each primer (forward primer: 5'AGAGTTTGATCCTGGCTCAG 3' and reverse primer: 5'GCGTGGACTACCAGGATATC 3'), 200 µM of each dNTP per and 2 mM of $MgCl_2$.[10] Afterwards, amplification was performed in a PCR Thermo Cycler (BioRad) under the following

conditions: 94 °C for 5 min, followed by 30-cycles using parameters: denaturing at 94 °C for 30 sec, annealing at 53 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 5 min. The PCR products were 802 bp analyzed using 1% (w/v) agarose gel stained with loading dye. The PCR product was purified using QIAquick gel extraction kit (Qiagen, Germany). Fifteen µL of purified product was sent to U2Bio company, Thailand for sequencing. Thailand for sequencing. The obtained sequences were analyzed using nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and aligned to the reference sequences deposited in the GenBank (v. 253.0 ; Jan 12, 2023)

Protein identification and quantification

Representative different species were identified proteins by timsTOF MS. Bacterial pellets were first resuspended in a lysis buffer that contained 2 M thiourea, 7M urea, 4% CHAPS, and 1% protease inhibitors cocktail. The bacterial cells were disrupted using a sonicator with an amplitude of 60 and 0.5 cycles, while keeping the samples on ice. The resulting lysate was then centrifuged at 14,000 rpm for 30 minutes at 4 °C. This centrifugation step would have caused the lysate to separate into different components based on their density. The protein concentration of the resulting pellet was measured using Bradford's method. The protein samples were then collected and stored at -80 °C for later use in downstream applications.[11]

The first step involved conducting acetone precipitation to transition from the lysis buffer to 8 M urea. To prepare each sample, 100 µg of protein was reduced with 100 mM dithiothreitol in 100 mM TEAB at room temperature for 30 min. Next, an alkylating buffer containing 100 mM iodoacetamide in 100 mM TEAB was added, and the sample was incubated in the dark at room temperature for 30 min. After quenching with reduction buffer for 15 min, the sample was treated with ice-cold acetone and incubated overnight at -20 °C. The pellet was collected by centrifugation, resuspended in 8 M urea in 100 mM TEAB, and digested using Trypsin, Gold (mass spectrometry grade; Promega, USA) for 16 hrs at 37 °C. The resulting sample was dried using a CentriVap DNA Concentrator (Labconco Co., Kansas City, Missouri, USA), cleaned up with a C18 Zip tip, and then dried again in the CentriVap before storage at -80 °C until further processing. Finally, the peptide concentration was measured using NanoDrop 1000 (Thermo Fisher Scientific, Bremen, Germany) after resuspending the sample in 0.1% formic acid.[12]

A nanoElute nano-flow chromatography system was coupled online to a hybrid trapped ion mobility spectrometry - quadrupole time of flight mass spectrometer (timsTOF Pro flex, Bruker Daltonics, Bremen, Germany) with a modified nano-electrospray ion source (CaptiveSpray, Bruker Daltonics). Liquid chromatography was performed at 50 °C and with a constant flow of 400 nL/min on a reversed-phase column (15 cm × 75 µm i.d.) packed with 1.9 µm C18-coated porous silica beads. Mobile phases A and B were 98/2/0.1 water/ACN/formic acid (v/v/v) and 99.9/0.1% ACN/formic acid (v/v/v), respectively. In 42-min experiments, peptides were separated with a linear gradient from 2 to 32% B within 30 min followed by a washing step at 95% B for 12 min, and re-equilibration.

In the timsTOF Pro Flex mass spectrometer ions are generated in a captive spray source and 20 µm tapered emitter, transferred into the vacuum system through a glass capillary, and then deflected by 90° into the TIMS device where ions are accumulated and released from the device based on their size-to-charge ratio. The quadrupole switches mass position extremely quickly in sync with the elution time of the precursor ion packages from the TIMS device, isolating the precursors for subsequent fragmentation in the collision cell. This PASEF method significantly increases the sequencing speed up to >120 Hz and therefore is ideally suited to short gradient measurements.[13,14] Data were collected over an m/z range of 100 to 1,700 for MS and MS/MS on the timsTOF Pro instrument using an accumulation time and ramp time of 100 msec. The data acquisition cycle times of 1.1 s were used.

Data processing and analysis of Protein identification and quantification

The protein sequence database was the combination of unreviewed *Staphylococcus* genus proteins (accessed 01/31/2023 from UniProt; 13,591 entries), with decoy sequences and contaminants added. IonQuant (version 1.8.10) was used to perform quantitative analysis. MSFragger (version 3.7) coupled with FragPipe (version 19.1) and Philosopher (4.6.0) was used to perform a closed search.[14,15] FragPipe-Analyst (http://fragpipe-analyst.nesvilab.org) was employed for data visualization.

Results

The Identification of S. aureus suspected isolates

Using MALDI-TOF MS, 185 isolates were correctly identified as *S. aureus* (N=151, 81.6%), *S. argenteus* (N=32, 17.3%), *S. hominis* (N=1, 0.5%), and S. *haemolyticus* (N=1, 0.5%) at the species level with a score>2.0. With ≥99% average nucleotide identity, *16S rRNA* gene sequence could classify *S. haemolyticus and S. hominis from S. aureus* whereas *S. argenteus* isolates were misidentified as *S. aureus* (data not shown).

PCA and MSPs Dendrogram analysis

The Biotyper program (version 4.1) was used to create the dendrogram based on the main spectrum profiles (MSPs). The dendrogram of 185 *Staphylococcal* isolates showed that *S. aureus, S. argenteus, S. haemolyticus,* and *S. hominis* were completely separated from each other (Figure 1). Using ClinPro Tool program, the distribution of the isolates was plotted according to the peak intensity of m/z 6905 (x-axis) and *m/z* 6889 (y- axis) (Figure 2). The peaks at m/z 6905 and m/z 6889 were the top two characteristic peaks among staphylococcal isolates. The peak at m/z 6905 was found in *S. argenteus* S060 whereas *m/z* 6889-6895 was *S. aureus* S076 and *S. haemolyticus* S096. Both of these peaks were not found in *S. hominis* S080. In addition, *m/z* 2305 in *S. aureus* and *S. argenteus* could be used to separate from *S. haemolyticus* and *S. hominis* (Figure 3).

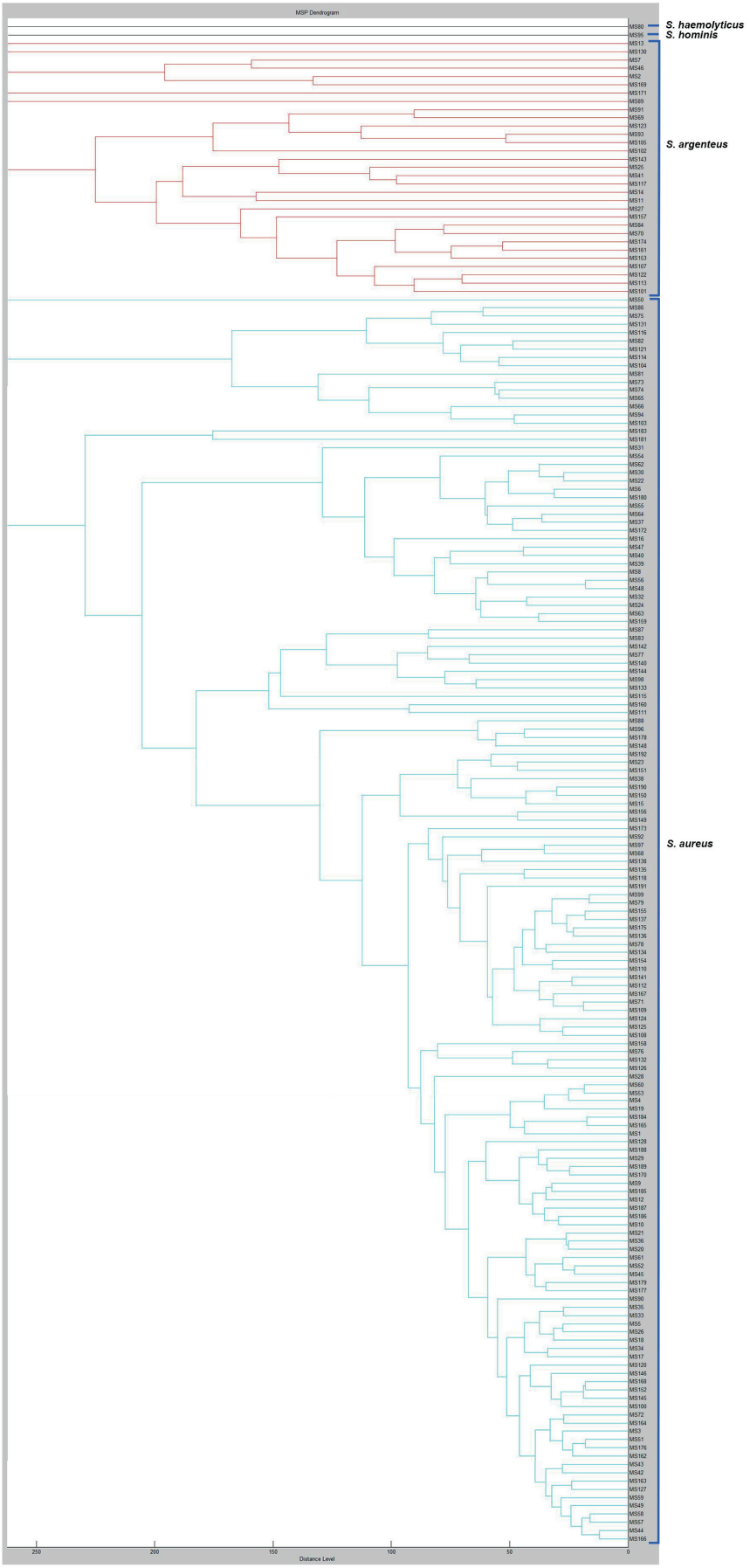

Figure 1 MALDI-TOF MS-based main spectrum profile (MSP) dendrogram showing the relatedness of 185 staphylococcal isolates. Top: *S. haemolyticus*) followed by *S. hominis*, Red lines: *S. argenteus isolates*, Blue lines: *S. aureus isolates*.

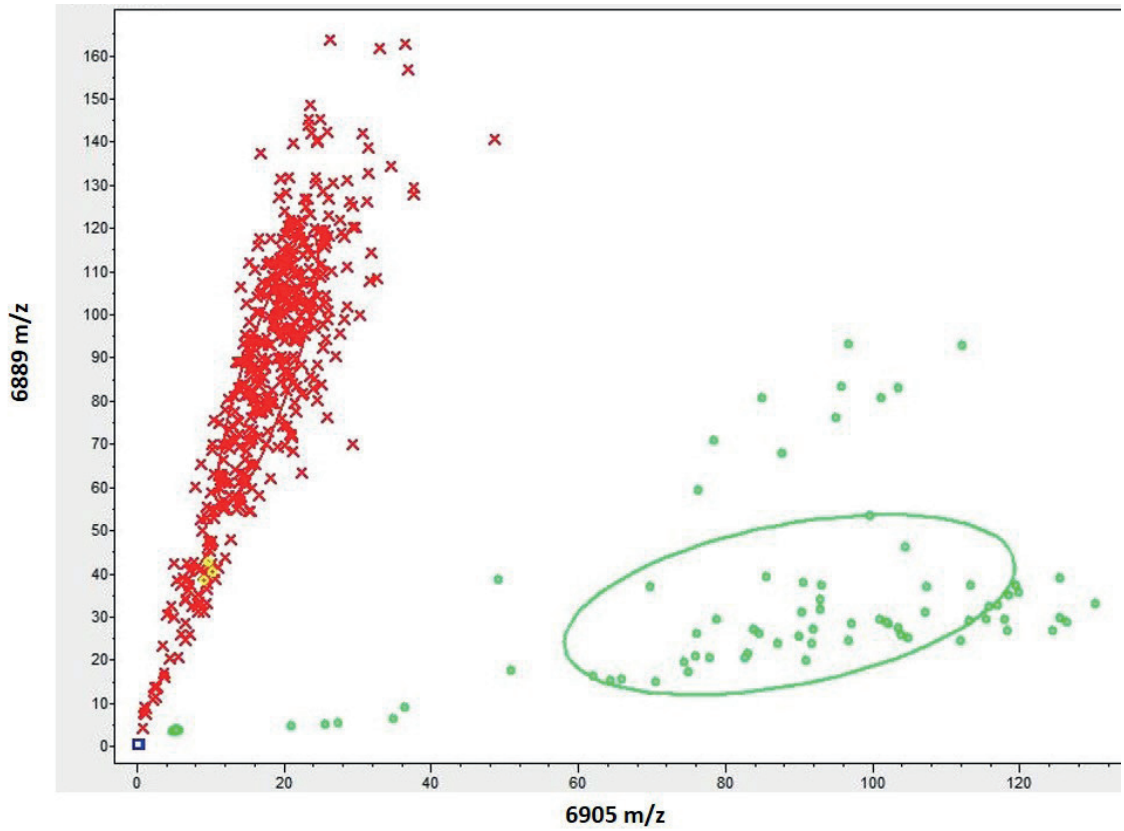

Figure 2 Scatter plot of various Staphylococcal isolates. The peaks at m/z 6905 and m/z 6889 served as the X- and Y- axis, respectively. The intensities of the characteristic peaks were expressed in arbitrary intensity units. The ellipses represent the 95% confidence intervals of peak intensities for each isolate. In the preliminary analysis, *S. hominis* (blue squares) could not be separated based only on these two characteristic peaks. The peak at or near *m/z* 6889 was expressed in *S. aureus* (red crosses) and *S. haemolyticus* (yellow diamonds) whereas the peak at or near *m/z* 6905 were expressed in *S. argenteus* (green diamond).

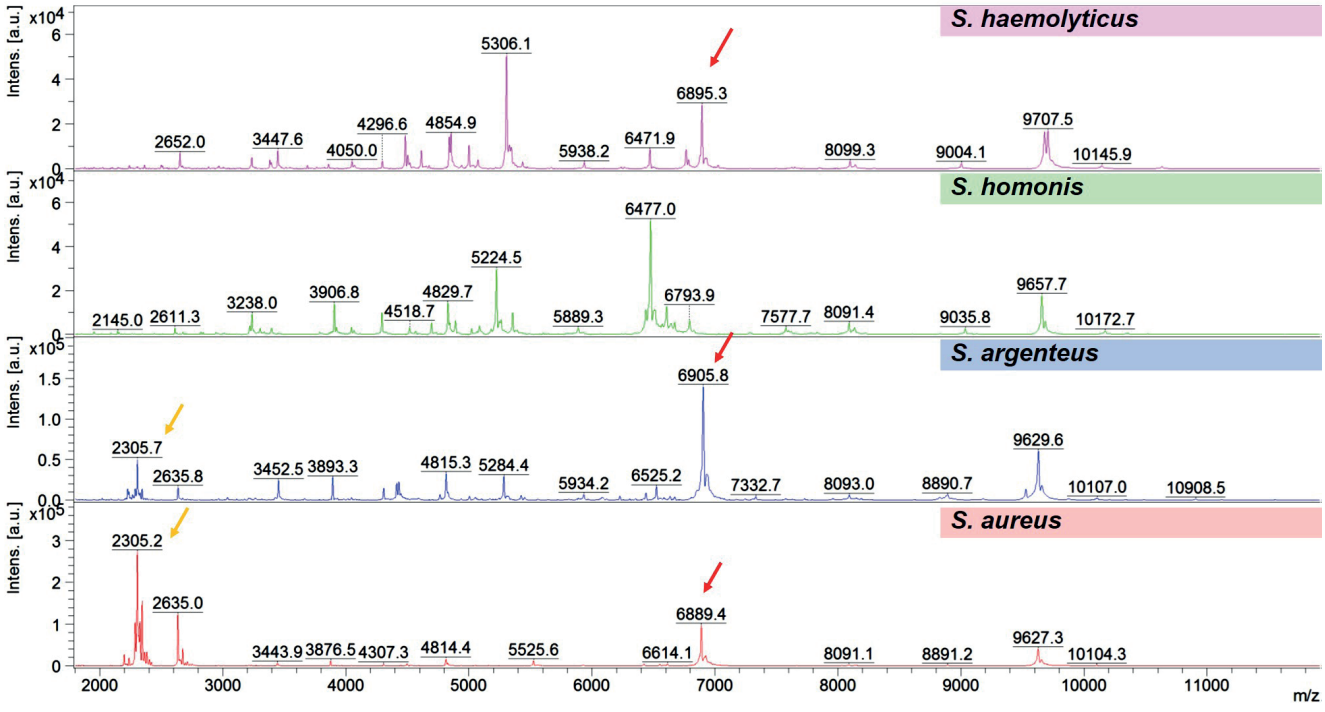

Figure 3 Comparison of MALDI-TOF MS representative spectra of 4 species of staphylococci. *m/z* 6889 -6895 (red arrow) were presented in *S. aureus* and *S. haemolyticus*. m/z 6905 were presented in *S. argenteus.* *m/z* 2305 (yellow arrow) was presented *S. aureus* and *S. argenteus*.

Principal component analysis (PCA) was conducted on the selected features to illustrate the distribution of various staphylococcal isolates. Each spot referred to a protein spectrum and the different colors illustrate the considered cluster involvement in which each spot is considered as one measured protein spectrum profile. The results of the PCA cluster views of the staphylococcal protein mass spectrum peaks showed that most of the peaks completely separated the species of staphylococci from each other (Figure 4). In the 3D scatter profile, each species group was located to each other in the same species (Figure 4).

***The comparison of protein expression among Staphylococcus argenteus* MS06·, *Staphylococcus aureus* MS076, *Staphylococcus haemolyticus* MS09ㄱ *and Staphylococcus hominis* MS080**

Staphylococcus aureus MS076 has fewer proteins than the other 3 species (Figure 5). In the univariate analysis (fold-change >2, p<0.01), the volcano plot identified eight genes including *rpsT, Huti, pyrF, atpD.1, cpfC, SAUA300_0786, atl.1,* MW2416 to be of higher expression in *S.aureus* MS076 than *S. argenteus* MS060, *S. haemolyticus* MS096, and *S. hominis* MS080 (Figure 6). The corresponding proteins of *rpsT, Huti, pyrF, atpD.1, cpfC, SAUA300_0786, atl.1, MW2416* genes were 30S ribosomal protein S20, imidazolonepropionase, orotidine 5'-phosphate decarboxylase, ATP synthase subunit beta, coproporphyrin iii ferrochelatase, organic hydroperoxide resistance protein-like, Bifunctional autolysin, and putative surface protein MW2416, respectively.

Discussion

S. aureus is a major cause of hospital and community-acquired infection that can result in worse clinical outcomes, although it is possible to increase morbidity and mortality due to MRSA infections. In this investigation, the clinical specimens were collected and processed based on the standard microbiological procedure. It takes a long time consuming, labored intensity, and defective reproduction to identify *S. aureus* from a cultured colony using conventional and PCR methods, whereas MALDI-TOF MS enables a few minutes of identification from a colony.

For the conventional method, the isolates underwent colony formation and its morphological analysis, gram staining, catalase test, coagulase tube test, mannitol salt fermentation. The PCR assay was performed using a single set of primers for the amplification of *16S rRNA* gene. When comparing the conventional method with MALDI-TOF MS (species level identification) and PCR for *16S rRNA* gene, the results showed 34 of 185 strains (18.4%) were mistakenly identified as *S. haemolyticus, S. hominis* and *S. argenteus*. Previous studies report misidentified *S. haemolyticus* and *S. hominis* as *S. aureus* resulted from an intermediate result in the test for the utilization of D-mannose [16]. Being closely related species, *S. aureus* and *S. argenteus* resembled each other as they were reported previously in northeast Thailand between 2010 and 2013, 19% were infected with *S. argenteus* and 81% with *S. aureus* [17] because *S. argenteus* shares a pathogenicity island, bacteriophages, virulence genes, and resistance genes with *S. aureus* [18]. The nearly

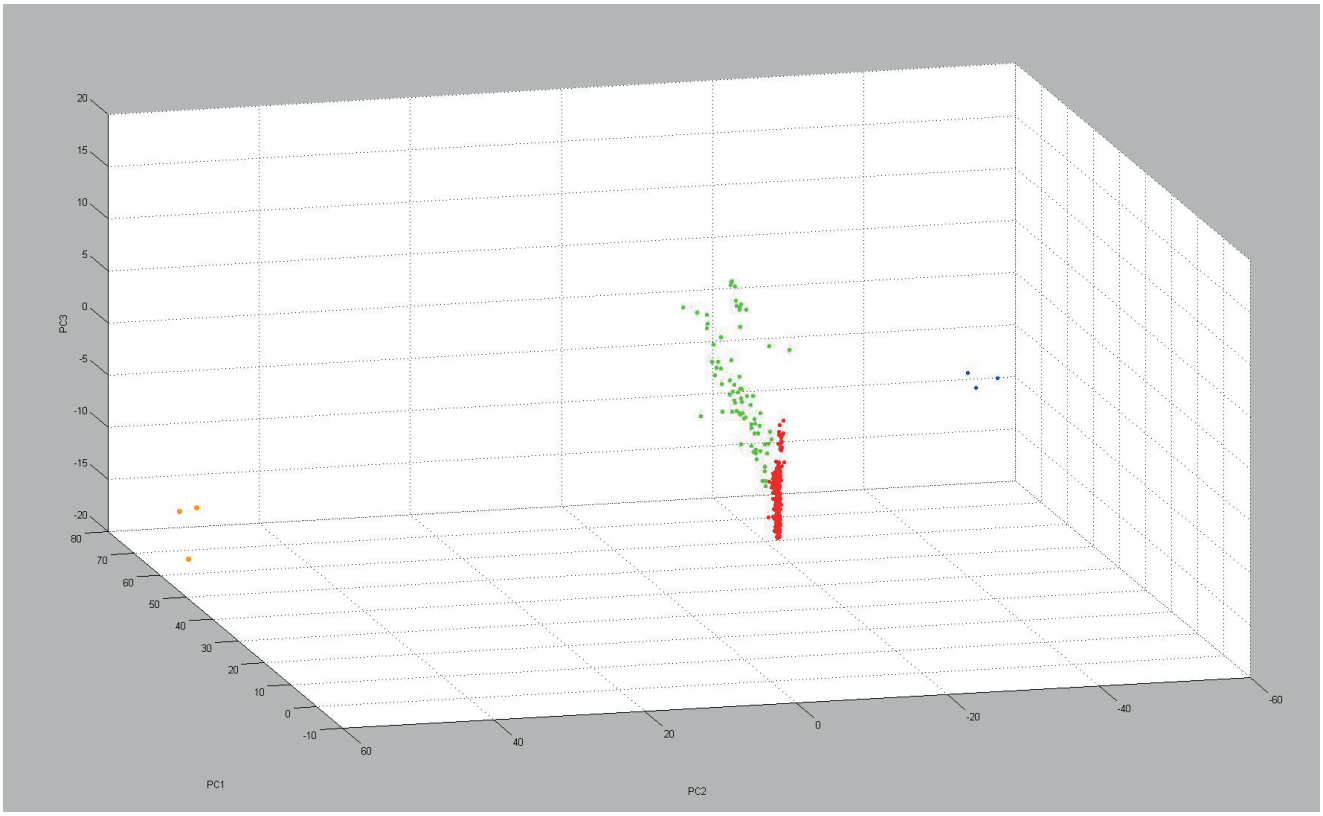

Figure 4 3D scatter profile of 185 isolates of Staphylococci. The spot represents one spectrum, and the plots were generated by PCA. *S. aureus*: red spots, *S. argenteus*: green spots, *S. haemolyticus*: blue spots, and *S. hominis*: yellow spots.

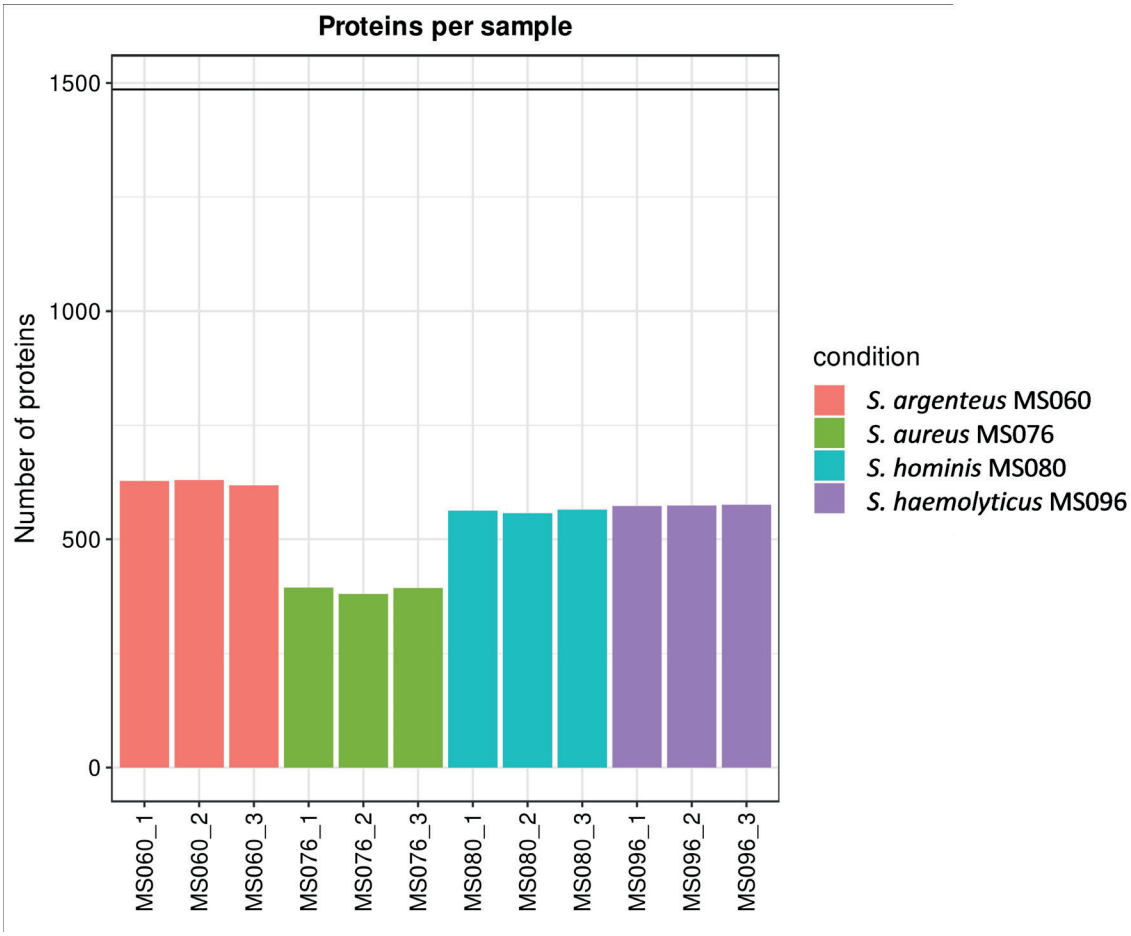

Figure 5 Protein Groups for each group after pre-processing.

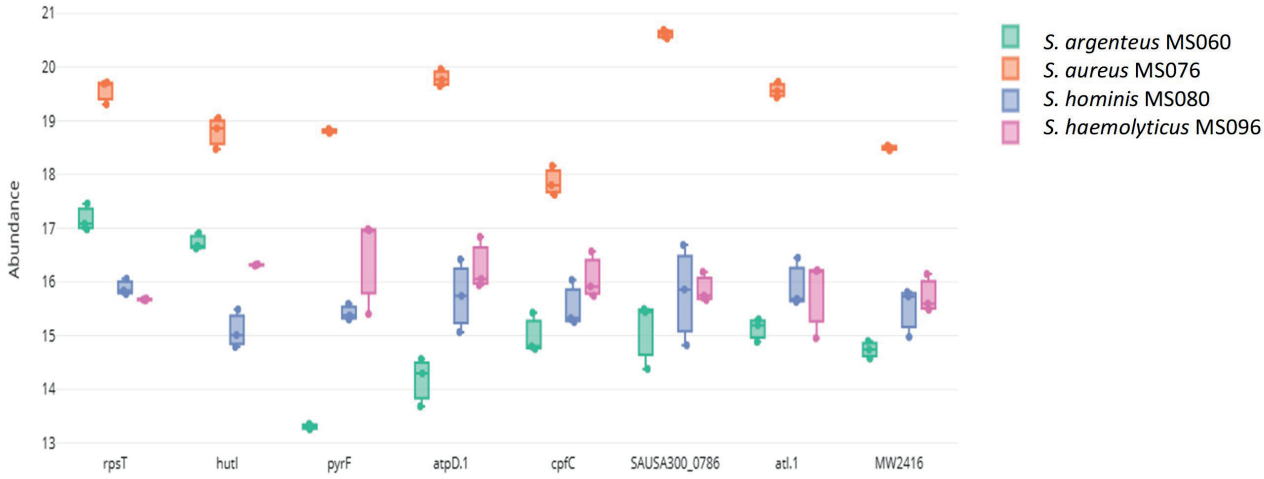

Figure 6 **Box plot for protein expression**. The vertical axis indicates abundance on the $\log_2$ scale. The horizontal axis indicates protein expressed group. Numbers in the plot indicate the MS experiment in which the protein was detected. Orange box: *S. aureus* MS076, Green box: *S. argenteus* MS060, Pink box: *S. haemolyticus* MS096, Blue box: *S. hominis* MS080, *rpsT*: 30S ribosomal protein S20, *hutI*: imidazolonepropionase, *pyrF*: orotidine 5'-phosphate decarboxylase, *atpD.1*: ATP synthase subunit beta, *cpfC*: coproporphyrin iii ferrochelatase, *SAUSA300_0786*: organic hydroperoxide resistance protein-like, *atl.1*: Bifunctional autolysin, *MW2416*: putative surface protein MW2416.

identical *16S rRNA* gene sequences could not classify *S. argenteus* from *S. aureus* whereas the protein mass spectral profile on a database of MALDI-TOF MS could successfully differentiate *S. argenteus* from *S. aureus*. Kosecka-Strojek reported that sanger sequencing of the *16S rRNA* gene could identify 27 (54%) species level of Staphylococci.[19] The strains of *S. aureus* and *S. argenteus* indistinguishable by sanger sequencing of *16S rRNA* gene could be identified with sanger sequencing of *rpoB*, *sodA*, or *tuf* genes and NGS of the *16S-23S rRNA* gene region.[19] S. *argenteus* formerly reported as *S. aureus* clonal complex 75 could differentiate from S. aureus using MALDI-TOF MS.[20] In addition, MALDI-TOF MS technique was used to identify *Staphylococcus* species isolated from food samples at the highest performance by formic acid extraction method (83.3-89.5% accuracy) which *S. aureus, S. haemolyticus, S. hominis* were 100% accurate identification at the species level.[7] Our study observed that using MALDI-TOF MS, mass spectral peak m/z 6905 was used to differentiate *S. argenteus* from *S. aureus*, *S. haemolyticus* and *S. hominis*. When the proteins expression among *S. aureus, S. argenteus, S. haemolyticus* and *S. hominis* was analyzed, *S. aureus* was similar to *S. argenteus* containing fewer proteins group than S*. argenteus, S. haemolyticus* and *S. hominis*. PCA analysis can clearly separate each group from each other (Figure 4). *S. aureus* expressed protein analyzed higher than the other 3 species. The 5 types of protein expressed (Figure 6) including organic hydroperoxide resistance protein-like (SAUSA300_0786), beta subunit of ATP synthase (*atpD.1*), bifunctional autolysin (*atl.1*), putative surface protein MW2416 (MW2416) and imidazolonepropionase (*huti*) are virulence factors of staphylococci which are the highest expression in *S. aureus*. In the reports of the organic hydroperoxide resistance (*ohr*) gene, it might be associated with multiple mechanisms such as the expression control of virulence genes in *Vibrio cholerae* and *Burkholderia thailandensis*, the regulation of bactericides production in *Streptomyces avermitilis.*[21-23] The *atpD* gene encodes beta subunit of the ATP synthase catalytic core which *atpA* is upstream of *atpD* and *atpG*. The *atpA* mutant (Δ*atpA*) biofilm extracts analysis decreased levels of ATP synthase subunit alpha (*atpA*), beta (*atpD*), gamma (*atpG*) and Δ*atpA* biofilm supernatant analysis reduced many virulence factors and toxin including serine protease, alpha-hemolysin and leucocidin-like proteins and biofilm growth.[24] Moreover, the major autolysin (encoded by *atl* gene) is a cell surface-associated peptidoglycan hydrolase with amidase and glucosamininidase domains involved in *Staphylococcus* pathogenesis during colonization and infection such as adherence with multiple host cellular components (fibronectin, gelatin, and heparin), biofilm formation and bacterial cell wall degradation and cell separation during cell division.[25] The putative surface protein or cell wall surface anchor family protein MW 2416 encoded with *sasG* gene was correlated with biofilm formation.[26,27] The *huti* (encoding imidazolonepropionase) and *hutU* (encoding urocanate hydralase) involved in the synthetic pathway of L-glutamate from L-histidine were found upregulate of *gltS* gene which played an important role in biofilm formation in MRSA.[28] Two types of protein expression including coproporphyrin iii ferrochelatase (encoded by *cpfC*) and orotidine 5'-phosphate decarboxylase (encoded by *pyrF*) played important role in the survival of *S. aureus* during infection.[29,30] In addition, Ribosomal protein S20 (encoded by *rpsT* gene) regulates the translation initiation of ribosomal protein synthesis. Mutations of *rpsT* were defective in many steps of translation initiation and ribosomal assembly.[31] In this study, the infections from *S. aureus*. imply more virulence than those from *S. argenteus, S. hominis* and *S. haemolyticus.*

Based on the Eshaghi study, the recommendation for a clinical report of *S. argenteus* accurately using MALDI-TOF MS because of its potential severity of the infection and incorrect species reports resulting in incorrect breakpoint for cefoxitin susceptibility results.[32,33] MALDI-TOF MS provides an excellent tool for the accurate and rapid identification of staphylococci and can potentially supplant labor-intensive and high-complexity molecular testing, thus increasing in-house capabilities and allowing information to be provided to physicians in a timely manner.[34]

Conclusions

MALDI-TOF MS provides an excellent tool for the accurate and rapid identification of staphylococci. *S. aureus* expressed protein analyzed higher than the other 3 species. The highest protein expression in *S. aureus* implies the most virulence of this strain.

Conflicts of Interest

All of the authors declare that there are no commercial, personal, political any other potentially conflicting interests related to the submitted manuscript.

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