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High-resolution subtyping of *Staphylococcus aureus* strains by means of Fourier-transform infrared spectroscopy

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ABSTRACT

Staphylococcus aureus causes a variety of serious illnesses in humans and animals. Subtyping of *S. aureus* isolates plays a crucial role in epidemiological investigations. Metabolic fingerprinting by Fourier-transform infrared (FTIR) spectroscopy is commonly used to identify microbes at species as well as subspecies level. In this study, we aimed to assess the suitability of FTIR spectroscopy as a tool for *S. aureus* subtyping. To this end, we compared the subtyping performance of FTIR spectroscopy to other subtyping methods such as pulsed field gel electrophoresis (PFGE) and *spa* typing in a blinded experimental setup and investigated the ability of FTIR spectroscopy for identifying *S. aureus* clonal complexes (CC). A total of 70 *S. aureus* strains from human, animal, and food sources were selected, for which clonal complexes and a unique virulence and resistance gene pattern had been determined by DNA microarray analysis. FTIR spectral analysis resulted in high discriminatory power similar as obtained by *spa* typing and PFGE. High directional concordance was found between FTIR spectroscopy based subtypes and capsular polysaccharide expression detected by FTIR spectroscopy and the *cap* specific locus, reflecting strain specific expression of capsular polysaccharides and/or other surface glycopolymers, such as wall teichoic acid, peptidoglycane, and lipoteichoic acid. Supervised chemometrics showed only limited possibilities for differentiation of *S. aureus* CC by FTIR spectroscopy with the exception of CC45 and CC705. In conclusion, FTIR spectroscopy represents a valuable tool for *S. aureus* subtyping, which complements current molecular and proteomic strain typing.

Introduction

Subtyping of *Staphylococcus aureus*, an organism causing a wide range of life-threatening infections and toxinoses, is crucial to epidemiological investigations and phylogenetic studies [5,23]. Common techniques used for subtyping of *S. aureus* are pulsed field gel electrophoresis (PFGE), *spa* typing, and multilocus sequence typing (MLST) [1,11,34]. However, PFGE exhibits grave performance discrepancies when used in inter-laboratory comparisons [44], *spa* typing may fail to recognize new lineages as a result of inherent homoplasy and differing evolutionary rates of *spa* alleles [33], and MLST has been shown to offer only moderate discriminatory power for epidemiological studies at comparatively high cost [15,16]. In addition, these techniques rely on molecular genotyping and do not allow insights into phenotypic features.

In contrast, Fourier-transform infrared spectroscopy (FTIR) represents a technique that enables not only bacterial identification, but also phenotypic characterization. FTIR spectroscopy measures the overall chemical composition of a sample, thus creating a spectrum that can be used as a fingerprint and that is analyzed by pattern recognition algorithms [41]. It is a promising tool used for microbial identification and was shown to yield resolution power sufficient for typing of bacteria and yeast below the species level [14,28,42]. For instance, FTIR was used for identification of methicillin-resistant *S. aureus* [2], capsular serotypes [18], the examination of small-colony variants [4], and for the rapid discrimination of strains involved in an outbreak investigation [23].

In the presented study, the *S. aureus* subtyping performance of FTIR spectroscopy, PFGE, and *spa* typing was compared using discriminatory power (Simpson's index of diversity) and directional

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congruence (adjusted Wallace coefficient) [7,28] as performance criteria.

Material and methods

Bacterial strains

The 70 *S. aureus* strains used in this study originate from a collection of well-characterized strains isolated from human, animal, and food sources by the Institute for Food Safety and Hygiene, University of Zurich. DNA microarray profiles, including assignment to clonal complexes (CC) and virulence and resistance gene patterns of all strains, were generated in previous studies [13,22,25,38]. All 70 strains showed unique DNA microarray hybridization patterns. The strain set comprises 10 strains each of CC5, CC8, CC30, CC45, CC398, and CC705 (former CC151), as well as two strains each of CC9, CC12, CC15, CC22, and CC97. Clonal complexes were selected to represent *S. aureus* commonly isolated from asymptomatic and infected humans (CC5, CC8, CC30, and CC45) and animals (CC705, CC398). Strains of CC705 cause severe losses to the dairy industry due to mastitis in cattle and strains assigned to CC398 have been linked to livestock associated MRSA infections in humans. To be able to test the subtyping performance of the different methods for a more diverse strain set, two strains each of five additional clonal complexes (CC9, CC12, CC15, CC22, CC97) were included. Strains belonging to CC45 and CC705 exhibited the highest degree of homogeneity regarding virulence and resistance gene profiles determined by DNA microarray. Other clonal complexes such as CC5, CC8, CC22, and CC398 exhibited a higher degree of variation with regard to the presence/absence of virulence and resistance genes. A comprehensive overview of strain sources, clonal complexes, as well as virulence and resistance gene profiles is provided as a supplemental file (Supplement 1). The diversity of the strain set is illustrated by a SplitsTree calculated as previously described [38] from DNA microarray hybridization profiles (Supplement 2). To assure unbiased processing, strains were assigned random numbers before being blinded analyzed by FTIR spectroscopy. *S. aureus* Reynolds prototype strain CP5 and its isogenic mutants Reynolds CP8 and Reynolds CP- (nonencapsulated) were used as controls for genotyping as well as for FTIR spectroscopic biotyping [39].

FTIR spectroscopic measurement and spectral preprocessing

FTIR spectroscopic measurements, spectral quality determination, spectral preprocessing, and chemometric analysis were performed as previously reported [18,24]. Three independent experiments were performed on different days. Isolates were grown as a bacterial lawn on tryptone soy agar plates (Oxoid) for 24 h at 30 °C and measured by FTIR spectroscopy. One loopful of bacterial cells was suspended in 100 μl sterile deionized water. An aliquot of 30 μl of the suspension was spotted on a zinc selenite (ZnSe) optical plate and dried at 40 °C for 40 min to yield transparent films, and subsequently submitted to FTIR spectroscopic measurement. FTIR spectra were recorded in transmission mode in the spectral range of 4000–500 cm^{-1} with an HTS-XT microplate adapter coupled to a Tensor 27 FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany). OPUS software (Version 6.5; Bruker Optics GmbH) was used for FTIR spectroscopic data analysis. Original spectra were preprocessed: (1) second derivatives were calculated over the whole spectral range using a second-order 9-point Savitzky–Golay algorithm, (2) spectra were vector normalized. In order to obtain the levels of reproducibility among the replicate samples, the averages ±2 standard deviations (SD) of the *D*-value, which is based on the Pearson's product moment correlation coefficient [8,21,30] were calculated in the spectral range used for typing (1200–800 cm^{-1}). Assuming that by strictly following the sample protocol a *D*-value below 0.50 is achievable, spectra with sufficient reproducibility can be obtained [40,42].

Unsupervised and supervised chemometrics

For hierarchical cluster analysis (HCA), the spectral region that offers the maximum information and discriminatory power (1200–800 cm^{-1}) was selected, which is dominated by C—O—C and C—O—P stretching vibrations of various oligosaccharides and polysaccharides and their specific types of glycosidic linkages. The dendrogram was generated using Ward's algorithm at repro-level 30. Strains were considered to be distinguishable, if one or more strain(s) formed a subcluster that comprised all three repetitive measurements and was clearly separated from subclusters of other strains [40]. Capsular serotypes (CP5, CP8, NT) were determined using artificial neuronal network (ANN) analysis [18]. The determination of capsule types included *S. aureus* strain Reynolds CP5 and its isogenic mutants Reynolds CP8 and Reynolds CP- as control strains for CP expression [39]. To investigate the ability of FTIR spectroscopy for identifying *S. aureus* clonal complexes, unsupervised principal component analysis (PCA), supervised principal component and linear discriminant analysis (PCA-LDA), and support vector–machine classification (SVMC) was performed using the software Unscrambler X (CAMO Software, Oslo, Norway) applying the same spectral windows as mentioned above. PCA computation was based on the NIPALS algorithm and three components were projected for PCA-LDA. SVMC used a radial basis function as Kernel type and the optimal *C*-value and Gamma was predetermined using the grid search function. For PCA-LDA and SVMC, 70% of randomly selected isolates of each CC were used to set up the model and 30% for external validation.

cap *gene determination*

The presence of the respective *cap* genes defining the CP5 and CP8 serotypes was determined by PCR amplification using primers and conditions described elsewhere [19,37].

spa *typing*

The polymorphic X region of *spa* was determined as previously described [38]. Briefly, *spa* was amplified using the following primers: spa-1113f (5′-TAA AGA CGA TCC TTC GGT GAG C-3′) and spa-1514r (5′-CAG CAG TAG TGC CGT TTG CTT-3′) [1]. The GoTaq PCR system (Promega AG, Dübendorf, Switzerland) was used at the following reaction conditions: (i) 5 min at 94 °C; (ii) 35 cycles of 45 s at 94 °C, 45 s at 60 °C and 90 s at 72 °C; and (iii) 10 min at 72 °C. Amplicons were purified (MinElute PCR Purification Kit, Qiagen, Hilden, Germany) and sequencing was outsourced (Microsynth, Balgach, Switzerland). The *spa*-server (http://spa.ridom.de/) was used to assign nucleotide sequences to *spa* types [20].

PFGE analysis

Chromosomal DNA was prepared and PFGE analysis of *Sma*I digested fragments was performed as described elsewhere [3]. Electrophoresis was carried out in a Bio-Rad CHEF-DR III electrophoresis cell and *Salmonella enterica* serovar Braenderup strain H9812 digested with 50 U *Xba*I for 12 h at 37 °C was used as a molecular size standard. Gels were analyzed with Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium) using the dice coefficient and were represented by unweighted pair grouping by mathematical averaging (UPGMA) with an optimization of 0.5%,