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Optimization of the score cutoff value for routine identification of *Staphylococcus* species by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry☆,☆☆

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ABSTRACT

Staphylococcus species are important pathogens. We evaluated 2 score cutoffs (2.0 and 1.7) and the replicate number (a single or a duplicate test) on the identification of staphylococci using the Bruker matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS). A collection of 440 clinical isolates (11 species) and 144 reference strains (36 species) was evaluated. For clinical isolates using a cutoff of 2.0 and duplicate tests, the rates of species, genus, and unreliable identifications were 93.4%, 5.7%, and 0.9% respectively, while the respective values were 99.3%, 0.2%, and 0.5% when the cutoff was 1.7. The species identification rates were significantly higher ($P < 0.01$) when a cutoff of 1.7 or a duplicate test was used. Similar results were obtained for reference strains. In conclusion, a cutoff of 1.7 and duplicate tests are recommended for identification of staphylococci using MALDI-TOF MS.

1. Introduction

Currently, there are 49 species and 26 subspecies in the genus *Staphylococcus* on the online encyclopedia of Bergey's Manual of Systematics of Archaea and Bacteria (http://www.bacterio.net/index.html). Although *Staphylococcus aureus* is clinically most relevant, coagulase-negative staphylococci (CoNS) are increasingly recognized as pathogens causing hospital-acquired infections and intravascular or prosthetic device–related infections (Szabados et al., 2012; von Eiff et al., 2002). Treatment of infections caused by CoNS can be challenging as many species in this group carry genes for multiple antibiotic resistances. Methicillin resistance was found in approximately 55–75% of nosocomial isolates of CoNS, and glycopeptide resistance was reported in strains of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (Biavasco et al., 2000; Piette and Verschraegen, 2009). This emphasizes the need of an accurate and simple identification method of staphylococci to the species level in clinical microbiology laboratory.

Molecular methods, especially DNA sequencing techniques, are more accurate than phenotypic tests for identification of *Staphylococcus* spp. (Bergeron et al., 2011; Heikens et al., 2005; Layer et al., 2006). The targets used for species identification include genes of *tuf* (Bergeron et al., 2011; Carpaij et al., 2011; Ghebremedhin et al., 2008; Heikens et al., 2005; Martineau et al., 2001), *gap* (Bergeron et al., 2011; Ghebremedhin et al., 2008), *hsp60* (Ghebremedhin et al., 2008; Goh et al., 1996), *sodA* (Ghebremedhin et al., 2008; Poyart et al., 2001), *rpoB* (Drancourt and Raoult, 2002; Ghebremedhin et al., 2008, Mellmann et al., 2006; Spanu et al., 2011), and 16S rRNA (Becker et al., 2004; Ghebremedhin et al., 2008). The *gap* and *tuf* genes are most discriminative for differentiating closely related species of *Staphylococcus* (Bergeron et al., 2011; Ghebremedhin et al., 2008).

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has been used for years; it serves as an accurate, rapid, paradigm-shifting, and robust method for identification of clinical microorganisms (Clark et al., 2013). Species-level identification rates of *Staphylococcus* using MALDI-TOF MS ranged from 60% to ≥99% (Bergeron et al., 2011; Clerc et al., 2014; Dupont et al., 2010; Matsuda et al., 2012; Prod'hom et al., 2010; Richter et al., 2012; Spanu et al., 2011; Tan et al., 2012). The large variation in identification rate was caused by different protein extraction methods, sample types (pure isolate or positive blood culture), replicate number for each isolate, the database used, species tested (rare or common species), and particularly the log score thresholds when using the Bruker Microflex instrument (Bruker Daltonik, Bremen, Germany) (Bergeron et al., 2011; Clerc et al., 2014; Dupont et al., 2010; Matsuda et al., 2012; Richter et al., 2012; Spanu et al., 2011). The manufacturer of Bruker MALDI-TOF MS recommends score values of ≥2.0, <2.0 but ≥1.7, and <1.7, respectively, for species, genus, and unreliable identifications using the

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Biotyper database (Bruker Daltonik). These thresholds (cutoffs) are generally adopted in clinical practice (Elamin et al., 2015; Matsuda et al., 2012; Schulthess et al., 2013; Spanu et al., 2011; Tan et al., 2012). However, a score cutoff of 2.0 for species identification was considered too stringent by some authors (Neville et al., 2011; Richter et al., 2012; TeKippe et al., 2013). Several studies found an adjustment of the score cutoff from 2.0 to 1.7 (Clerc et al., 2014; Prod'hom et al., 2010; TeKippe et al., 2013) or even 1.5 (Saffert et al., 2012) can improve bacterial identification rate. Richter et al. (2012) found that 13.7% of staphylococcal isolates failed to reach a score of 2.0, even a standard protein preparation protocol (ethanol/formic acid extraction method) was used. Besides, the replicate number (a single or duplicate test) for each isolate can impact the identification rate (Dupont et al., 2010; Matsuda et al., 2012; Tan et al., 2012). A single test for each isolate is preferred in routine clinical laboratory, as this can reduce the analysis time and cost.

The database and the underlying algorithm used for microorganism identification are also important. The current Bruker Biotyper database (version 4.0.0.1, 2013; Bruker Daltonik, Taipei, Taiwan) includes 201 reference spectra for 39 species of *Staphylococcus*. However, only about 10 staphylococcal species are common clinical isolates (>1% of all staphylococci) (Dupont et al., 2010; Richter et al., 2012). Other non-clinical species are present in various environments, used in the food industry as starter cultures, animal pathogens (Tomazi et al., 2014), or colonizers of the skin and mucous membranes of animals (Bergeron et al., 2011; Corbiere Morot-Bizot et al., 2007). The objectives of this study were to optimize the score cutoff and to evaluate the replicate number (a single or a duplicate test) on the identification of staphylococci.

2. Materials and methods

2.1. Bacterial strains

A collection of 147 staphylococcal reference strains (37 species) were evaluated, with the type strain of each species being included (Supplemental Table S1). Reference strains were obtained from the Bioresources Collection and Research Center (BCRC, Hsinchu, Taiwan), the Culture Collection of the University of Göteborg (CCUG, Göteborg, Sweden), and the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). Of the 37 species of reference strains, only *Staphylococcus gallinarum* was not included in the Bruker Biotyper database (version 4.0.0.1). The species designations of all reference strains were reconfirmed by sequence analysis of the near complete 16S rRNA gene (Relman, 1993) and the partial *gap* gene (931 bp) (Bergeron et al., 2011) when an unambiguous identification was not obtained by 16S rRNA gene sequencing. In addition, 440 clinical isolates of *S. aureus* and CoNS identified by biochemical tests or the VITEK 2 GP identification card (VITEK bioMérieux, Taipei, Taiwan) were analyzed. Clinical isolates were obtained from the Division of Microbiology, Department of Pathology, National Cheng Kung University Hospital (Tainan, Taiwan). All clinical isolates were further identified to species level by sequencing of the partial *gap* gene (Yugueros et al., 2001) and the *tuf* gene (660 bp) when a reliable species name was not obtained by *gap* sequencing or the *gap* gene amplification failed (Bergeron et al., 2011; Ghebremedhin et al., 2008). Species names determined by gene sequencing were considered the gold standard. Subspecies-level identification was not considered since gene sequencing is unable to identify staphylococci to the subspecies level (Ghebremedhin et al., 2008; Poyart et al., 2001) in many instances. All strains were subcultured on blood agar plates and incubated at 35 °C for 18–24 h before MALDI-TOF MS analysis.

2.2. MALDI-TOF MS analysis

Bacterial proteins were extracted on-plate by the direct transfer–formic acid method (Schulthess et al., 2013). A single colony was smeared as a thin film on a 96-spot, polished, stainless steel target plate (Bruker Daltonik, Taipei, Taiwan) using a toothpick. The spot was overlaid with 1 μL of 70% formic acid, air dried, overlaid with 1 μL of a saturated α-cyano-4-hydroxycinnamic acid matrix solution in 50% acetonitrile–2.5% trifluoroacetic acid (Bruker Daltonik), and air dried at room temperature. A duplicate test (2 spots on the target plate) was conducted for each isolate, and the score value of the first spot was considered the result of "a single test". MALDI-TOF MS measurement was performed with the MicroFlex LT mass spectrometer (Bruker Daltonik) using Biotyper database (version 4.0.0.1) and standard pattern matching algorithm (default settings) against the spectra in the database. Mass spectra were daily calibrated using the test standard of *Escherichia coli* DH5α supplemented with myoglobin and RNase A and were acquired in a linear positive ion mode at a laser frequency of 60 Hz across a mass/charge ratio (m/z) of 2000 to 20,000. The pattern-matching results were expressed as log (score) values.

2.3. Data analysis

The highest score value of a match against the Biotyper database was applied for identification. Identification rates were calculated either at a score cutoff of 2.0 or 1.7. When using a cutoff of 2.0, scores of ≥2.0 indicated species-level identification, scores of <2.0 and ≥1.7 indicated genus-level identification, and scores of <1.7 indicated unreliable identification. When using a cutoff of 1.7, scores ≥1.7 indicated species-level identification, scores of <1.7 and ≥1.5 indicated genus-level identification, and scores <1.5 indicated unreliable identification. If a duplicate test resulted in different results, such as a species-level identification and a genus-level identification, the species result was used. A misidentification was defined as a species determined by MALDI-TOF MS being different from that obtained by DNA sequencing. An identification to the genus level was not considered as a misidentification.

2.4. Statistical analysis

Statistical calculations were done using 2-sided χ^2 test. Comparison of identification rates at each level (species, genus, unreliable, and incorrect identifications) were made between the 2 cutoffs (score 2.0 versus score 1.7) and between the replicate number (a single test versus a duplicate test) at the 2 cutoffs. Comparison of identification rate was not made between reference strains and clinical isolates. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Identification of clinical isolates

A total of 440 clinical isolates of *Staphylococcus* was analyzed by MALDI-TOF MS. Using a score cutoff of 2.0 and duplicate tests, the numbers of isolates with species, genus, unreliable, and incorrect identifications (misidentification) were 411 (93.4%), 25 (5.7%), 4 (0.9%), and 0 (0%), respectively (Table 1). If using a cutoff of 1.7 and duplicate tests, the numbers of isolates with species, genus, unreliable, and incorrect identifications were 437 (99.3%), 1 (0.2%), 2 (0.5%), and 0 (0%) respectively (Table 1). No misidentification was found at both cutoffs. Three of the 8 isolates of *Staphylococcus cohnii* were not identified to species level even when a score threshold of 1.7 was used (Table 1). Of the 440 clinical isolates, 4 had score values of ≤1.7; they were *S. cohnii* 5256N (score 1.567), *S. cohnii* 6598N (score 1.389), *S. cohnii* 8665A (score 1.462), and *S. epidermidis* 3069 (no peaks found). Comparing the 2 cutoffs (1.7 versus 2.0), the species identification rate was significantly higher (99.3% versus 93.4%, $P < 0.01$), and the genus identification rate was significantly lower (0.2% versus 5.7%, $P < 0.01$) at the lower cutoff (Table 2). No significant differences in the rates of unreliable (0.5% versus 0.9%, $P = 0.413$) and incorrect identifications (0% versus 0%)