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Identification of Clinical Isolates of α-Hemolytic Streptococci by 16S rRNA Gene Sequencing, Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Using MALDI Biotyper, and Conventional Phenotypic Methods: a Comparison

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Fifty-six α-hemolytic streptococcal isolates were identified using MALDI Biotyper MS (Bruker Daltonics), API 20 Strep (bioMérieux), and BD Phoenix (Becton, Dickinson). The gold standard for identification was 16S rRNA gene sequence analysis with 16S-23S rRNA intergenic spacer sequencing. The following percentages of isolates were correctly identified to the species level: MALDI Biotyper, 46%; BD Phoenix, 35%; and API 20 Strep, 26%.

Identification of α-hemolytic streptococci is clinically important (3, 15, 26, 27, 33, 34). The aim of this study was to evaluate and compare the reliability of API 20 Strep, the automated BD Phoenix system, and MALDI Biotyper MS for clinically identifying significant isolates of α-hemolytic streptococci to the species and group levels. MALDI Biotyper has been found to give high-confidence identifications for β-hemolytic streptococci (8). As a gold standard, 16S rRNA gene sequencing was used (4, 5, 7, 15, 22, 31), with a sequence homology of 99% or greater with published species sequences considered to denote the same species, in accordance with CLSI guidelines (10). For *Streptococcus mitis* group species, better discrimination was obtained by sequence analysis of the 16S-23S rRNA intergenic spacer (ITS) region (7). Taxonomy and nomenclature followed that outlined by Facklam at the CDC's Streptococcal Laboratory, Atlanta, GA (15), and subsequently cited and expanded upon by others (11, 24). Unlike previous studies of phenotypic methods (5, 6, 12, 16, 19, 20), this was a direct comparison of phenotypic methods versus the gold standard.

Bacterial isolates. Forty-nine clinically relevant isolates (40 blood culture, 2 cerebrospinal fluid (CSF), 3 urine, 1 each from ascitic fluid, joint fluid, and an infected wound, and 1 from an unspecified site but referred as clinically significant) of α-hemolytic streptococci (except *Streptococcus pneumoniae*) were collected prospectively from sterile sites cultured in three clinical laboratories. Additionally, a selection of fully characterized strains was used, including NCTC 11086 (*Streptococcus sanguinis*), NCTC 11427 (*Streptococcus parasanguinis*), NCTC 10904 (*S. sanguinis*), M99 and SK12 (both *Streptococcus gordonii*) (23), SK36 (*S. sanguinis*) (35), and UA159 (*Streptococcus mutans*) (1).

API 20 Strep and BD Phoenix automated identification. API 20 Strep (bioMérieux) and BD Phoenix (Becton, Dickinson) tests were carried out according to the manufacturers' instructions, with Apiweb 1.2.1 software used to identify API codes. BD Phoenix SMIC/ID streptococcal identification panels were used. Following the manufacturers' recommendations, identification with a score of <80% for API 20 Strep or <90% for BD Phoenix was considered unacceptable. If a similar result was obtained on repeat testing, the strain was assigned as unidentified by that method.

MALDI Biotyper identification. The MALDI Microflex (matrix-assisted laser desorption ionization–time of flight mass spectrometry [MALDI-TOF MS]; Bruker Daltonics GmbH, Bremen, Germany) with MALDI Biotyper software 2.0 was used. Identifications were performed according to the manufacturer's instructions, using the full extraction procedure as described previously (18) with isolates from chocolate blood agar (Oxoid, Basingstoke, United Kingdom). The extraction method is superior to direct colony testing for Gram-positive cocci (2). Samples on the target plate were left to air dry before adding the matrix solution (saturated solution of alpha-4-cyano-hydrocycinnamic acid in 50% acetonitrile and 2.5% trifluoracetic acid solution, all LC-MS quality; Sigma) and placing the plate in the MALDI Biotyper system. According to the manufacturer, a score of >2 indicates secure genus and probable species identification (18). These recommended evaluation criteria have been scrutinized in other studies which have found that acceptable identifications can be obtained by using scores ≥1.9 (25) or ≥1.7 (9, 13, 32) in most genera. Nonetheless, for isolates with a score of <2, the test was repeated. Where repeat testing still resulted in a score of <2 but ≥1.7, the result was recorded together with the score.

16S rRNA gene and 16S-23S ITS spacer region amplification and sequencing. The 16S rRNA gene was amplified using the bacterium-specific universal primers LPW57 (5′ AGTTTGATCCTG GCTCAG) and LPW58 (5′AGGCCCGGGAACGTATTCAC) to amplify an ~1.5-kb fragment (34). PCR amplification was performed on a Rotorgene 6000 real-time PCR machine (Qiagen,

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TABLE 1 Comparison of BD Phoenix, API 20 Strep, and MALDI Biotyper for identification of clinical ($n = 39$) and reference ($n = 7$) α-hemolytic streptococci at the group and species levels

Species by 16S rRNA sequencing	No. of isolates (%)	Agreement by BD Phoenix (%)	Agreement by API 20S (%)	Agreement using Biotyper >2 (%)	Agreement using Biotyper >1.7 (%)
Mitis group	28 (61)[a]	15 (54)[c]	21 (75)[c]	17 (61)[c]	18 (64)[c]
S. mitis	20 (71)[b]	3 (18)[d]	9 (45)[d]	0 (0)[d]	0 (0)[d]
S. oralis	7 (25)	2 (22)	0 (0)	4 (57)	4 (57)
S. infantis	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)
Sanguinis group	11 (24)[a]	8 (73)[c]	3 (27)[c]	8 (73)[c]	10 (91)[c]
S. sanguinis	4 (36.5)[b]	3 (75)[d]	1 (17)[d]	3 (75)[d]	4 (100)[d]
S. gordonii	5 (45.5)	2 (40)	0 (0)	5 (100)	5 (100)
S. parasanguinis	2 (18)	2 (100)	0 (0)	0 (0)	1 (50)
Bovis group	2 (4)[a]	0 (0)[c]	2 (100)[c]	2 (100)[c]	2 (100)[c]
Streptococcus lutetiensis	2 (100)[b]	0 (0)[d]	0 (0)[d]	2 (100)[d]	2 (100)[d]
Anginosus group	2 (4)[a]	2 (100)[c]	1 (50)[c]	2 (100)[c]	2 (100)[c]
Streptococcus anginosus	2 (100)[b]	2 (100)[d]	0 (0)[d]	2 (100)[d]	2 (100)[d]
Salivarius group	1 (2)[a]	1 (100)[c]	1 (100)[c]	0 (0)[c]	1 (100)[c]
Streptococcus salivarius	1 (100)[b]	1 (100)[d]	1 (100)[d]	0 (0)[d]	1 (100)[d]
Mutans group	1 (2)[a]	1 (100)[c]	1 (100)[c]	1 (100)[c]	1 (100)[c]
S. mutans	1 (100)[b]	1 (100)[d]	1 (100)[d]	1 (100)[d]	1 (100)[d]
Granulicatella adiacens[e]	1 (2)[a]	0 (0)	0 (0)	0 (0)	1 (100)[d]
Total	46				
Total agreement by group[e]		27 (59)	29 (63)	30 (65)	34 (74)
Total agreement by species		16 (35)	12 (26)	17 (37)	21 (46)

[a] Percentage of all isolates in each streptococcal group.
[b] Percentage of each species within each streptococcal group.
[c] Percentage of each group assigned to correct group by a test.
[d] Percentage of each species assigned to correct species by a test.
[e] *Granulicatella adiacens* isolate not included when considering results by group.

Crawley, United Kingdom) (21) under the following conditions: an initial incubation at 95°C for 4 min, 35 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 1 min, followed by a final incubation at 72°C for 10 min. Primers and excess nucleotides were removed using a PCR cleanup kit (Qiagen). DNA sequencing was performed by Source BioScience (Cambridge, United Kingdom) using the PCR primers. 16S rRNA gene sequences were subjected to BLAST analysis against the NCBI nucleotide database. When an isolate was from the *S. mitis* group and 16S rRNA BLAST analysis could not identify it to the species level, PCR amplification and sequencing of the shorter (<400 bp) ITS region of the 16S-23S rRNA genes were performed as described, using a second pair of bacterium-specific universal primers, 13BF (5′-GTGAATAC GTTCCCGGGCCT-3′) and 6R (5′-GGGTTTCCCCGTTCGGA AAT-3′) (7). Identification of other isolates was solely by analysis of the 16S rRNA gene sequence against the NCBI database, with verification against the curated Greengenes database (http://greengenes.lbl.gov) not revealing any differences in identification.

Results. Of the 49 clinical isolates, 39 gave 16S rRNA gene sequencing results with ≥99% similarity to published species (10). An additional 6 isolates had a similarity of >97% but <99%, reportable under CLSI guidelines such as "*Streptococcus* species most closely related to (likely species name)." These are not included in the table. The species distribution of these significant (mainly bloodstream) α-hemolytic streptococcal isolates is similar to that described previously by others (17). The species identifications of the 39 clinical isolates definitively identified by molecular methods and the reference isolates are shown in Table 1, along with the relative performance of each test method. Separate columns show the MALDI Biotyper results when using scores of 2 and 1.7 as a cutoff. A score of <2 but ≥1.7 was recorded for four clinical isolates and one reference isolate. None of the phenotypic methods used were reliable for identification to the species level: the MALDI Biotyper system performed best. Among the reference strains alone, the MALDI Biotyper again performed best, correctly identifying all seven of the strains when a cutoff score of >1.7 was applied or 6 (86%) with a cutoff score of >2, followed by BD Phoenix, which correctly identified 4/7 (57%), and API 20 Strep, which correctly identified 2/7 (29%). For the clinical isolates alone, all methods scored less well than for the reference isolates: when the seven reference isolates were excluded from the analysis, the MALDI Biotyper correctly identified 36% (cutoff score of >1.7) or 28% (cutoff score of >2), BD Phoenix correctly identified 31%,and API 20 Strep correctly identified 23%. The MALDI Biotyper would have performed better overall were it not for its particularly poor performance with *S. mitis* isolates, failing to correctly identify any of the 20 *S. mitis* isolates, naming 13 as *Streptococcus oralis* (all with scores of >2) and 7 as *Streptococcus pneumoniae* (all with

scores of >2). This led to much better success with identification at the group level than at the species level. The overidentification of *S. pneumoniae* by the MALDI Biotyper has been previously noted (30): the manufacturers give a "matching hint," advising further testing when an identification of *S. pneumoniae* is obtained. API 20 Strep was let down by a poor ability to identify *S. oralis*, all bar one of which it identified as *S. mitis*. For group level identification, there was greater similarity between methods than for exact species identification (Table 1).

Gene sequence analysis remains the most reliable method by far for the laboratory identification of α-hemolytic streptococci. Although MALDI Biotyper identification performed slightly better than the conventional identification methods tested, it does not yet display the generally excellent identification seen with other genera of microorganisms (9, 14, 28, 29, 32). Further development of the database may help to improve its performance; in particular, an improvement in its ability to identify *S. mitis* group streptococci would greatly enhance its reliability.

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