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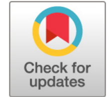

Improved Differentiation of *Streptococcus pneumoniae* and Other *S. mitis* Group Streptococci by MALDI Biotyper Using an Improved MALDI Biotyper Database Content and a Novel Result Interpretation Algorithm

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ABSTRACT Reliable distinction of *Streptococcus pneumoniae* and viridans group streptococci is important because of the different pathogenic properties of these organisms. Differentiation between *S. pneumoniae* and closely related *Sreptococcus mitis* species group streptococci has always been challenging, even when using such modern methods as 16S rRNA gene sequencing or matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. In this study, a novel algorithm combined with an enhanced database was evaluated for differentiation between *S. pneumoniae* and *S. mitis* species group streptococci. One hundred one clinical *S. mitis* species group streptococcal strains and 188 clinical *S. pneumoniae* strains were identified by both the standard MALDI Biotyper database alone and that combined with a novel algorithm. The database update from 4,613 strains to 5,627 strains drastically improved the differentiation of *S. pneumoniae* and *S. mitis* species group streptococci: when the new database version containing 5,627 strains was used, only one of the 101 *S. mitis* species group isolates was misidentified as *S. pneumoniae*, whereas 66 of them were misidentified as *S. pneumoniae* when the earlier 4,613-strain MALDI Biotyper database version was used. The updated MALDI Biotyper database combined with the novel algorithm showed even better performance, producing no misidentifications of the *S. mitis* species group strains as *S. pneumoniae*. All *S. pneumoniae* strains were correctly identified as *S. pneumoniae* with both the standard MALDI Biotyper database and the standard MALDI Biotyper database combined with the novel algorithm. This new algorithm thus enables reliable differentiation between pneumococci and other *S. mitis* species group streptococci with the MALDI Biotyper.

KEYWORDS MALDI-TOF, *Streptococcus pneumoniae*, algorithm, *mitis* group streptococci, phenotypic identification, pneumococcus

Received 29 September 2016 **Returned for modification** 23 October 2016 **Accepted** 27 December 2016

Accepted manuscript posted online 4 January 2017

Citation Harju I, Lange C, Kostrzewa M, Maier T, Rantakokko-Jalava K, Haanperä M. 2017. Improved differentiation of *Streptococcus pneumoniae* and other *S. mitis* group streptococci by MALDI Biotyper using an improved MALDI Biotyper database content and a novel result interpretation algorithm. J Clin Microbiol 55:914–922. https://doi.org/10.1128/JCM.01990-16.

Editor Sandra S. Richter, Cleveland Clinic

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Traditional classification of streptococci was based on the potential to cause hemolysis on sheep blood agar. The term "viridans" is used to refer to the greenish coloring (alpha-hemolysis) of the medium around the colonies due to partial lysis of red blood cells. *Streptococcus pneumoniae*, one of the most common causative agents of bacterial meningitis, community-acquired pneumonia, and otitis media, is clinically the most important alpha-hemolytic streptococcus. Because of the different pathogenic potentials of *S. pneumoniae* and other alpha-hemolytic streptococci, the practical dichotomy in clinical laboratories has been between *S. pneumoniae* and viridans group

streptococci (VGS), the latter referring to nonpneumococcal alpha-hemolytic streptococci as a group. VGS form an important part of the normal flora of the human respiratory, gastrointestinal, and urogenital tracts. Although mostly commensal, VGS have also been associated with various infections, especially endocarditis in patients with predisposing factors (1–4).

The classification of streptococci has changed significantly in the last few decades, and hemolysis is no longer a valid basis for streptococcal taxonomy. Accordingly, many "VGS species" also form nonhemolytic or beta-hemolytic colonies (1, 2). The taxonomy within VGS has also seen several upheavals since the 1990s, due mainly to the advent of modern molecular typing methods (1, 2, 5, 6). Current classification divides VGS into five major groups, i.e., the *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus mitis*, *Streptococcus mutans*, and *Streptococcus salivarius* groups (1, 7). Of these groups, the *S. mitis* species group containing closely related species of different pathogenic potentials has posed great challenges for reliable species level identification. In addition to commensal species, the major pathogen *S. pneumoniae* taxonomically belongs to this group. Besides *S. pneumoniae*, the *S. mitis* species group currently includes the following species: *S. australis*, *S. cristatus* (formerly *S. crista*), *S. gordonii*, *S. infantis*, *S. massilienis*, *S. mitis*, *S. oligofermentans*, *S. oralis*, *S. orisratti*, *S. parasanguinis* (formerly *S. parasanguis*), *S. peroris*, *S. pseudopneumoniae*, *S. sanguinis* (formerly *S. sanguis*), and *S. sinensis* (1). Furthermore, two recently described species, *S. dentisani* (8) and *S. tigurinus* (9), seem to belong to the *S. mitis* species group as well.

In clinical microbiology laboratories, VGS have traditionally been differentiated from pneumococci on the basis of their lack of bile solubility. Optochin resistance testing is also often used for the preliminary identification of pneumococci, despite its limitations. Wessels and coworkers (10) reported that optochin testing in a CO_2 atmosphere and tube bile solubility testing give consistent results for *S. pneumoniae*. However, in another study (11), 21 optochin-susceptible isolates were identified as *S. mitis* by multilocus sequence analysis (MLSA). Also, commercial biochemical test panels have limitations in identifying streptococci to the species level (12–16).

In the last few decades, molecular methods have enabled more accurate genotypic identification of bacteria. The close relationship between *S. pneumoniae* and other *S. mitis* species group streptococci renders analysis of 16S rRNA gene sequences incapable of definitive species identification within the *S. mitis* species group (13). Therefore, several other streptococcal genes have been targeted in identification and phylogenetic studies, sometimes using an MLSA approach (17–22). Some of the most commonly used targets have been housekeeping genes *ddl* (21, 23), *groESL* (24), *rnpB* (25–27), *rpoB* (28), *sodA* (29, 30), *tuf* (31), and *recA* (32). However, currently, MLSA is too time-consuming and expensive to be used routinely in a clinical microbiology laboratory but is a useful tool for taxonomic studies. In recent years, the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) technique has emerged as an alternative for clinical microbiology laboratories for the identification of a wide range of bacteria and fungi (33–35). Identification of *S. pneumoniae* and VGS by MALDI-TOF MS has been widely studied previously (27, 35–49). The close relationship between *S. pneumoniae* and other *S. mitis* species group streptococci, especially *S. mitis* and *S. oralis*, poses considerable challenges for the reliable distinction between species of these groups by this technique as well.

In recent comparisons of two commercially available MALDI-TOF instruments, use of the MALDI Biotyper (Bruker Daltonics) system resulted in misidentifications of nonpneumococcal *S. mitis* species group strains as *S. pneumoniae*, while use of the Vitek MS (bioMérieux) system often resulted in the identification of these strains as *S. oralis*/*S. mitis* (48, 49). Expansion of the databases of these MALDI-TOF systems to include recently described species of the *S. mitis* species group such as *S. tigurinus* (28) and improvement of the algorithms used to calculate closest matches (48, 50) have both been suggested as means of obtaining a more reliable distinction between *S. pneumoniae* and different species of the *S. mitis* species group. In this work, we present an improved distinction between *S. pneumoniae* and other *S. mitis* species group strains by

TABLE 1 Differences in the database contents and in identification results between different MALDI Biotyper database versions tested in this study

MALDI Biotyper database version	No. of microbial strains in database				*S. pneumoniae* strains correctly identified to the species level		Nonpneumococcal strains correctly identified to the group level[a]	
	Total	*S. pneumoniae*	*S. mitis*	*S. oralis*	n^b	%	*n*	%
3.3.1 (DB_4613) with log(score) algorithm	4,613	8	1	4	188/188	100	35/101[c]	35
4.0.0.1 (DB_5627) with log(score) algorithm	5,627	30	39	38	188/188	100	100/101[c]	99
4.0.0.1 (DB_5627) with list(score) algorithm	5,627	30	39	38	188/188	100	101/101	100

[a]As nonpneumococcal species in the *S. mitis* species group.
[b]*n*, number of strains.
[c]All misidentified strains were misidentified as *Streptococcus pneumoniae*.

using a novel algorithm utilizing list(scores) combined with the expanded MALDI Biotyper database. This would allow MALDI-TOF mass spectrometry to be utilized for the identification of both pneumococci and *S. mitis* species group streptococci, resulting in more rapid identification than that of traditional biochemical methods.

RESULTS

MALDI-TOF analysis of all strains yielded adequate spectra, and they could all be used for species identification. The identification results obtained by use of MALDI Biotyper database versions DB_4613 and DB_5627 with a classical log(score) algorithm, as well as those obtained using version DB_5627 with the list(score) algorithm, are summarized in Table 1. All the identification results obtained by MALDI Biotyper are shown in Table S1 in the supplemental material for pneumococcal strains, including the log(score) ranking lists and the list(scores). When database versions DB_4613 and DB_5627 and the classical log(score) algorithm were used, none of the 437 measured *S. pneumoniae* spectra were identified as species of the *S. mitis* species group other than *S. pneumoniae* (Table 1). All the pneumococcal strains were also clearly identified as *S. pneumoniae* when the list(score) algorithm was used in connection with database version DB_5627 (Table 1).

Examples of the identification results for nonpneumococcal *S. mitis* species group strains are shown in Table 2. All the identification results obtained by MALDI Biotyper for nonpneumococcal *S. mitis* species group strains are shown in Table S2, including the log(score) ranking lists and the list(scores). When the earlier database version DB_4613 and classical log(score) algorithm were used, 66 of 101 nonpneumococcal *S. mitis* species group strains were misidentified as *S. pneumoniae* (Table 1). When database version DB_5627 and the classical log(score) calculation were used, only one isolate (62680), putatively identified as *S. infantis* by pyrosequencing, was incorrectly identified as *S. pneumoniae*, with a log(score) of 2.03 (Table S2). When the new algorithm based on weighted list(scores) was employed in addition to database version DB_5627, none of the nonpneumococcal *S. mitis* species group strains tested were misidentified as *S. pneumoniae* (Table 1).

Interestingly, even when the earlier database DB_4613 with the classical log(score) algorithm was used, only one of the strains putatively identified as members of the *S. sanguinis* species group (*S. gordonii*, *S. parasanguinis*, or *S. sanguinis*) by pyrosequencing was misidentified as *S. pneumoniae*. With the updated version DB_5627 and the classical log(score) algorithm, none of these strains were misidentified as *S. pneumoniae* and 19 of the 22 strains could be identified as members of this species group using the classical log(score) algorithm (Table S2). All of the remaining three strains putatively identified by pyrosequencing as belonging to the *S. sanguinis* group were identified by MALDI-TOF as *S. oralis*. Therefore, a separate list(score) calculation for these three species provided no further benefit.

The species identification of *S. pneumoniae* and *S. oralis* appeared very clear when using the list(scores). All 10 positions in the ranking list were almost always occupied

TABLE 2 Examples of identification of nonpneumococcal *S. mitis* group isolates

		recA sequencing			4613[a]		5627[a]		list(score)[b]				
									Sum of weighted log(scores) for species sorted[e]				
Strain	Pyrosequencing results	%	NCBI[c]	BIBI[d]	Best match	log(score)	Best match	log(score)	p	m	o	sp.	Best match
93	*S. mitis/oralis/pneumoniae/ pseudopneumoniae*	99.8	*S. mitis*	*S. mitis*	*S. pneumoniae*	2.08	*S. mitis*	2.45	9	85	34		*S. mitis*
2790	*S. mitis/oralis/pneumoniae/ pseudopneumoniae*	99.8	*S. oralis*	*S. oralis*	*S. oralis*	1.74	*S. oralis*	2.24			119		*S. oralis*
3002	*S. mitis*				*S. pneumoniae*	2.06	*S. mitis*	2.30		90	33		*S. mitis*
20542	*S. mitis/oralis*				*S. pneumoniae*	2.00	*S. oralis*	2.39			125		*S. oralis*
21429	*S. mitis/infantis*				*S. pneumoniae*	1.94	*S. oralis*	2.04	12	32	65		*S. oralis*
24173	*S. mitis/tigurinus*				*S. gordonii*	2.27	*S. gordonii*	2.27		2	67	43	*S. oralis*
593	*S. oralis*				*S. pneumoniae*	1.89	*S. oralis*	2.33		13	112		*S. oralis*
2805	*S. oralis/gallinaceus*				*S. parasanguinis*	2.08	*S. parasanguinis*	2.08				37	sp.
3981	*S. dentisani*				*S. pneumoniae*	1.95	*S. mitis*	2.55		124	9		*S. mitis*
333	*S. infantis*				*S. pneumoniae*	2.00	*S. mitis*	2.35		95	25		*S. mitis*
20837	*S. infantis/cristatus*				*S. pneumoniae*	2.24	*S. mitis*	2.45		83	47		*S. mitis*
23958	*S. infantis/dentisani*				*S. pneumoniae*	1.97	*S. mitis*	2.37	6	46	69		*S. oralis*
2017	*S. tigurinus*				*S. pneumoniae*	1.98	*S. oralis*	2.33			125		*S. oralis*
195	*S. sanguinis*				*S. sanguinis*	2.24	*S. sanguinis*	2.24				107	sp.
62013	*S. parasanguinis*				*S. parasanguinis*	2.35	*S. parasanguinis*	2.35				57	sp.
3446	*S. gordonii*				*S. gordonii*	2.25	*S. gordonii*	2.25				55	sp.

[a]4613, MALDI Biotyper database version 4613; 5627, MALDI Biotyper database version 5627.
[b]The list(score) is defined as the sum of weighted log(scores) for each single species (Fig. 2).
[c]NCBI, species identification using the *recA* gene and the NCBI database.
[d]BIBI, species identification using the *recA* gene and the BIBI database.
[e]p, *S. pneumoniae*; m, *S. mitis*; o, *S. oralis*; sp., any other species in the Biotyper database.

by the respective species only. Identification of the isolates putatively identified as *S. mitis* by pyrosequencing delivered in most cases a much more diverse mixture of all three species (*S. mitis*, *S. oralis*, *S. pneumoniae*). Nevertheless, the list(score) showed the highest value for *S. mitis* in all cases (Table S2). For all isolates identified as *S. tigurinus* by pyrosequencing, the list(score) showed the highest value for *S. oralis*, as shown in Table S2. For other nonpneumococcal *S. mitis* species group isolates, the highest valued match given by the list(score) algorithm was either *S. mitis*, *S. oralis*, or other *S. mitis* species group strains but never *S. pneumoniae* (Table S2).

None of the 24 strains identified only ambiguously by pyrosequencing as *S. pneumoniae/S. pseudopneumoniae/S. mitis/S. oralis* were most closely matched to *S. pneumoniae* or *S. pseudopneumoniae* by *recA* sequencing, whereas all were most closely matched to either *S. mitis* or *S. oralis* (Table S2). Seven of these strains were unambiguously identified as *S. oralis* and 10 as *S. mitis* both by *recA* sequencing and by using MALDI Biotyper. For two strains, the closest *recA* sequence match was to *S. mitis*, but MALDI Biotyper identified them as *S. oralis*. For four strains, the closest *recA* sequence match was to *S. mitis*, but MALDI Biotyper identifications were ambiguous (*S. mitis/S. oralis*). One strain was identified as *S. mitis* both by *recA* sequencing and by the first two matches using the standard MALDI Biotyper database but gave ambiguous identification results (*S. mitis/S. oralis*) when the list(score) algorithm was used.

DISCUSSION

Using the classical interpretation rules of the MALDI Biotyper, the database update from version DB_4613 to DB_5627 was already a considerable improvement in *S. mitis* species group identification. The addition of more *S. pneumoniae*, *S. mitis*, and *S. oralis* strains in the database led to much better "classical" identification rates using log(scores) and ranking lists. The rate at which nonpneumococcal *S. mitis* species group streptococci were misidentified as pneumococci was reduced from 66 of 101 to 1 of 101 within this group in this study. The only misidentification as *S. pneumoniae* when the DB_5627 version of MALDI Biotyper was used with the classical log(score) algorithm was one *S. infantis* isolate that was misidentified as *S. pneumoniae*. The new list(score) algorithm together with MALDI Biotyper version

DB_5627 resulted in an even better capacity to distinguish between pneumococci and other *S. mitis* species group strains, with no false identifications of *S. pneumoniae* for any of the nonpneumococcal isolates. The paucity of misidentifications of *S. gordonii*, *S. parasanguinis*, and *S. sanguinis* as *S. pneumoniae* compared to other nonpneumococcal *S. mitis* species group strains seems to be in line with the views of those taxonomists who have separated *S. gordonii*, *S. parasanguinis*, and *S. sanguinis* from *S. mitis* species group streptococci and grouped them in the *Streptococcus sanguinis* group (2).

The other nonpneumococcal isolates in the *S. mitis* species group could generally not be reliably identified to the species level even when MALDI Biotyper version DB_5627 was used. This is not surprising, considering that many of the nonpneumococcal isolates could not be identified to the species level even by pyrosequencing, and even the clearer species identifications provided by pyrosequencing should be considered only putative identifications. Nevertheless, for clinical laboratories, the exact identification to the species level among nonpneumococcal *S. mitis* species group strains is by far less important than their discrimination from *S. pneumoniae*. However, reliable identification of other viridans group streptococci to the species level would enable researchers to gain new insights into their pathogenic properties and antimicrobial resistance patterns.

As a final conclusion, it can be stated that adding further *S. mitis* species group isolates (*S. mitis*, *S. oralis*, and *S. pneumoniae*) to the MALDI Biotyper database and using the new list(score) algorithm to interpret MALDI Biotyper results led to significantly improved differentiation of pneumococci from other species of the *S. mitis* species group. MALDI Biotyper version DB_5627 together with the new algorithm might thus provide routine clinical microbiological laboratories with a rapid and affordable method for accurately distinguishing between these bacteria. Bruker Daltonics is currently planning to introduce the list(score) result interpretation for users of the Research Use Only (RUO) version of MALDI Biotyper software but not the In Vitro Diagnostics (IVD) version.

Reliable identification to the species level within nonpneumococcal *S. mitis* species group streptococci remains a challenge, as demonstrated recently for *S. pseudopneumoniae* (51). This is perhaps not surprising, given that exact species lineation within this group is elusive even with the most sophisticated molecular techniques. For the newly described species *S. tigurinus* and *S. dentisani*, the addition of spectra from reference strains of these species to the MALDI Biotyper database might enable more accurate species level identifications.

MATERIALS AND METHODS

Bacterial strains. In this study, 188 *S. pneumoniae* strains and 101 strains belonging to other species of the *S. mitis* species group were analyzed. All the strains had been isolated from human clinical specimens at the Clinical Microbiology Laboratory of Turku University Hospital, Turku, Finland. The identification process for the strains is outlined in Fig. 1.

All *S. pneumoniae* strains were identified as pneumococci by optochin sensitivity testing using optochin discs (optochin, 10 μg, Diatabs; ROSCO Diagnostica A/S, Taastrup, Denmark). All *S. pneumoniae* strains were subsequently serotyped and confirmed as *S. pneumoniae* by optochin sensitivity and bile solubility testing at the National Institute for Health and Welfare (Helsinki, Finland). Also, a multiplex PCR with primers specific for *S. pneumoniae* was performed at the National Institute for Health and Welfare for all the *S. pneumoniae* strains as described by Siira et al. (52). The serotyping results for the *S. pneumoniae* strains are shown in Table S2.

Fifty of the *S. mitis* species group strains other than *S. pneumoniae* belonged to the collection used in references 13 and 48. The remaining 51 *S. mitis* species group strains were new isolates. The isolates had been presumptively identified as members of the *S. mitis* species group by the Vitek 2 instrument (bioMérieux, Grenoble, France) (13). An optochin resistance test (optochin, 10 μg, Diatabs; ROSCO Diagnostica A/S, Taastrup, Denmark) was also performed. The strains were then identified by pyrosequencing of the 16S rRNA gene at the Finnish National Institute for Health and Welfare as described by Haanperä et al. (13). Of the *S. mitis* species group strains tested, there were 24 strains for which the pyrosequencing results of the 16S rRNA differed by the same number of nucleotides (1 to 3 nucleotides) from the 16S rRNA sequences of the type strains of *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis*, and *S. oralis*. In order to confirm whether these isolates belonged to the species *S. pseudopneumoniae*, *recA* sequencing according to reference 32 was performed.

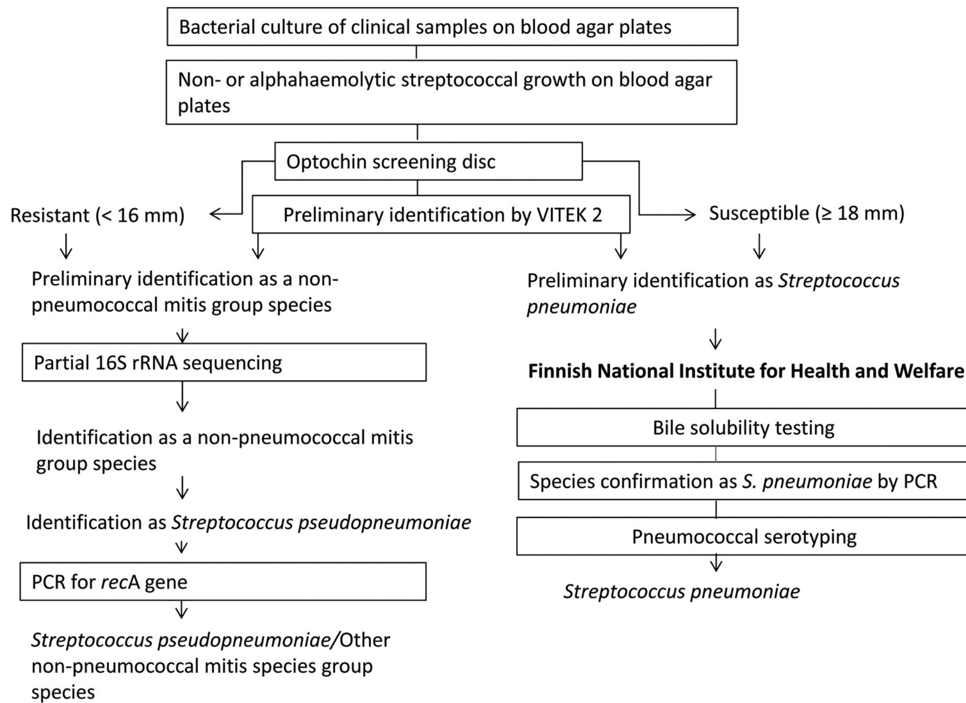
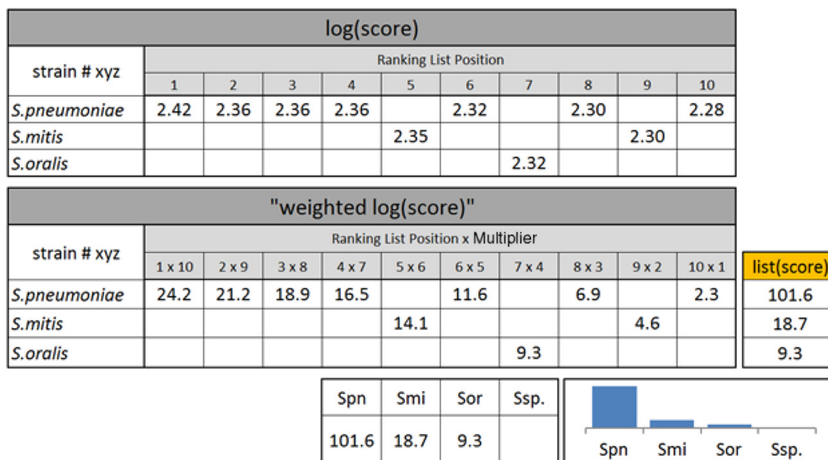


FIG 1 Identification process for *Streptococcus pneumoniae* strains and the *S. mitis* species group strains used in this study.

Cultivation and storage. All isolates were stored in a mixture of skim milk and glycerol at −70°C before they were initially cultured on BD BBL Trypticase soy agar with 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ, USA). Pure cultures were inoculated on the same plates from these initial culture plates. The resulting pure cultures were incubated at 35°C with 5% CO_2 for 17 to 24 h before analysis by mass spectrometry.

Sample preparation. MALDI-TOF sample preparation of all strains was carried out as described in reference 47. Direct application was first carried out to generate MALDI-TOF MS spectra, and if it did not result in a score value of ≥2.0 (designated good identification to the species level by MALDI Biotyper software version 3.0 [RUO]), ethanol/formic acid extraction was performed. All strains were



log(score)

strain # xyz	Ranking List Position									
	1	2	3	4	5	6	7	8	9	10
S.pneumoniae	2.42	2.36	2.36	2.36		2.32		2.30		2.28
S.mitis					2.35				2.30	
S.oralis							2.32			

"weighted log(score)"

strain # xyz	Ranking List Position x Multiplier										
	1 x 10	2 x 9	3 x 8	4 x 7	5 x 6	6 x 5	7 x 4	8 x 3	9 x 2	10 x 1	list(score)
S.pneumoniae	24.2	21.2	18.9	16.5		11.6		6.9		2.3	101.6
S.mitis					14.1				4.6		18.7
S.oralis							9.3				9.3

Spn	Smi	Sor	Ssp.
101.6	18.7	9.3	

FIG 2 List(score) calculation. "Strain # xyz" represents the spectrum of an unknown isolate; Spn, *S. pneumoniae*; Smi, *S. mitis*; Sor, *S. oralis*; Ssp, *Streptococcus* spp. In the first step, each log(score) of the first 10 positions of a ranking list is multiplied by a factor of 10 (1st position), a factor of 9 (2nd position), . . ., a factor of 2 (9th position), and a factor of 1 for the 10th position to calculate weighted log(scores). In the second step, the list(scores) are calculated by totaling weighted log(scores) for each single species appearing in the ranking list. List(scores) can be compared between all species appearing in the ranking list. In the example above, the highest list(score), 101.6, was observed for *S. pneumoniae*.

measured on a Microflex LT instrument (Bruker Daltonics, Bremen, Germany) using Flex Control 3.0 software.

Data analysis. Spectra were analyzed using MALDI Biotyper software version 3.0 (RUO) for both database versions 3.3.1 (4,613 reference entries) and 4.0.0.1 (5,627 reference entries). The differences in these databases are summarized in Table 1. For result interpretation, the standard data interpretation rules [using log(scores) for the best and second best matches of the ranking list] were applied to both database versions.

MALDI Biotyper database version 3.3.1 (4,613 entries; RUO) contained 1, 4, and 8 *S. mitis*, *S. oralis*, and *S. pneumoniae* strains, respectively. For the updated database version 4.0.0.1 (5,627 entries; RUO), the spectra of several hundred well characterized strains of *S. mitis*, *S. oralis*, and *S. pneumoniae* from reference centers and routine laboratories covering strains from a broad variety of geographical and other origins were analyzed, and several strains of each of these species were added in order to improve or optimize the species resolution between these three species. In DB_5627 as well as in the newer MALDI Biotyper database version DB_5989, the three species were represented by 39, 38, and 30 isolates, respectively (Table 1). When version DB_5627 was used, the first 10 positions of the ranking lists were also used to calculate a new supplementary list(score). The process of calculating the list(scores) is outlined in Fig. 2. At the first step, each log(score) of the first 10 positions of a ranking list is multiplied by a factor of 10 (1st position), a factor of 9 (2nd position), . . ., a factor of 2 (9th position), and a factor of 1 for the 10th position to calculate weighted log(scores). In the second step, each weighted log(score) is summarized for each single species appearing in the ranking list. List(scores) were compared between all species appearing in the ranking list. For example, if only *S. pneumoniae* appears in the ranking list, the final list(score) is a sum of all 10 weighted log(scores). If only the first position of a ranking list reports *S. pneumoniae* and the remaining positions report only *S. oralis*, the influence of the first position is much lower in the overall summary. The use of such list(scores) as an additional tool to differentiate closely related microorganisms requires a database content preferably of more than 10 strains per species. The most abundant species in the ranking list is finally emphasized.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01990-16.

TEXT S1, PDF file, 0.9 MB.

ACKNOWLEDGMENTS

This work was supported by the Research Fund of Hospital District of Southwest Finland. Inka Harju has also received travel grants from the American Society for Microbiology and from the University of Helsinki in order to present preliminary results of this work as a poster at the General Meeting of American Society for Microbiology.

Inka Harju, Kaisu Rantakokko-Jalava, and Marjo Haanperä declare they have no conflicts of interest. Cristoph Lange, Markus Kostrzewa, and Thomas Maier are employed by Bruker Daltonik GmbH.

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