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Optimizing Identification of Clinically Relevant Gram-Positive Organisms by Use of the Bruker Biotyper Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry System

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) can be used as a method for the rapid identification of microorganisms. This study evaluated the Bruker Biotyper (MALDI-TOF MS) system for the identification of clinically relevant Gram-positive organisms. We tested 239 aerobic Gram-positive organisms isolated from clinical specimens. We evaluated 4 direct-smear methods, including "heavy" (H) and "light" (L) smears, with and without a 1-μl direct formic acid (FA) overlay. The quality measure assigned to a MALDI-TOF MS identification is a numerical value or "score." We found that a heavy smear with a formic acid overlay (H+FA) produced optimal MALDI-TOF MS identification scores and the highest percentage of correctly identified organisms. Using a score of ≥2.0, we identified 183 of the 239 isolates (76.6%) to the genus level, and of the 181 isolates resolved to the species level, 141 isolates (77.9%) were correctly identified. To maximize the number of correct identifications while minimizing misidentifications, the data were analyzed using a score of ≥1.7 for genus- and species-level identification. Using this score, 220 of the 239 isolates (92.1%) were identified to the genus level, and of the 181 isolates resolved to the species level, 167 isolates (92.2%) could be assigned an accurate species identification. We also evaluated a subset of isolates for preanalytic factors that might influence MALDI-TOF MS identification. Frequent subcultures increased the number of unidentified isolates. Incubation temperatures and subcultures of the media did not alter the rate of identification. These data define the ideal bacterial preparation, identification score, and medium conditions for optimal identification of Gram-positive bacteria by use of MALDI-TOF MS.

Phenotypic methods for the identification of bacteria in the clinical laboratory vary by laboratory and often include subjective test interpretation. An alternative method for bacterial identification that is emerging in clinical microbiology is matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS has been described as a rapid, cost-effective, and reliable method for the identification of bacteria in the clinical laboratory (1, 2, 3, 4, 5, 6, 7, 8, 9). During MALDI-TOF MS identification, a spectral profile representing a "fingerprint" of bacterial proteins is generated. The spectrum generated from a particular isolate can be compared to that of a reference database for organism identification. Ribosomal proteins are primarily used for identification due to their relative abundance in the bacterial cell (10).

Many studies have been published that demonstrate the utility of MALDI-TOF MS for the identification of specific groups of Gram-positive bacteria, including *Staphylococcus aureus*, coagulase-negative staphylococci, *Streptococcus agalactiae*, viridans group streptococci, atypical catalase-negative Gram-positive cocci, *Listeria* spp., and *Corynebacterium* spp. (11, 12, 13, 14, 15, 16, 17, 18). These publications have focused on two methods by which bacterial isolates can be prepared for MALDI-TOF MS analysis. The first is the direct application of a thin film or smear of bacteria onto the stainless steel target from an isolated colony, which is a rapid-identification method. Due to the thick peptidoglycan cell walls of Gram-positive bacteria, this method of preparation can sometimes result in poor MALDI-TOF MS spectra (6). To achieve better spectra, a protein extraction may be performed (1, 6). This process can be relatively time- and labor-intensive and can be disruptive to the workflow of the clinical laboratory. We sought to optimize the sample preparation process, from both accuracy and workflow standpoints. We evaluated a method of bacterial preparation that is an intermediate between these two well-described methods, a 1-μl formic acid overlay applied directly over the dried organism on the stainless steel target (19).

The objective of this study was to evaluate the ability of MALDI-TOF MS to accurately identify clinically relevant aerobic Gram-positive bacteria using a formic acid overlay method, and we focused on the optimization of methods to achieve the highest rate of identification without introducing misidentifications. In addition, this study evaluates the impact of different incubation temperatures, media types, and subculture frequency to determine if these conditions impact MALDI-TOF MS identification. An accompanying paper by Ford and Burnham (20) aims to validate the Bruker Biotyper system for clinical use for identification of Gram-negative bacteria.

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MATERIALS AND METHODS

Bacterial isolates. Two hundred thirty-nine aerobic Gram-positive organisms isolated from clinical specimens were included in this study. This

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TABLE 1 Organisms tested in this study

Organism	No. of isolates
Abiotrophia defectiva	1
Actinomyces spp.	2
Aerococcus urinae	1
Aerococcus viridans	1
Arthrobacter spp.	2
Bacillus cereus/thuringiensis	2
Bacillus megaterium	1
Bacillus spp.	9
Cellulosimicrobium spp.	3
Coagulase-negative *Staphylococcus* spp.	18
Corynebacterium pseudodiphtheriticum	1
Corynebacterium spp.	13
Corynebacterium striatum	5
Corynebacterium urealyticum	1
Dolosigranulum pigrum	1
Enterococcus avium	1
Enterococcus casseliflavus	1
Enterococcus faecalis	2
Enterococcus faecium	8
Enterococcus spp.	12
Granulicatella spp.	1
Group A alpha-hemolytic *Streptococcus* spp.	30
Group B alpha-hemolytic *Streptococcus* spp.	20
Group C alpha-hemolytic *Streptococcus* spp.	1
Group G alpha-hemolytic *Streptococcus* spp.	4
Kocuria spp.	1
Lactobacillus spp.	3
Lactococcus lactis	1
Listeria monocytogenes	1
Microbacterium spp.	1
Micrococcus spp.	3
Paenibacillus spp.	1
Rhodococcus equi	1
Rothia spp.	4
Saccharothrix spp.	1
Staphylococcus aureus	34
Staphylococcus epidermidis	11
Staphylococcus hominis	2
Staphylococcus lugdunensis	2
Staphylococcus saprophyticus	2
Streptococcus bovis group	2
Streptococcus mitis group	2
Streptococcus pneumoniae	9
Streptomyces spp.	2
Turicella otitidis	2
Viridans group *Streptococcus* spp.	13

TABLE 2 Summary of isolates, extent of analysis, and primary media from which isolates were tested

Category	No. of isolates (of a total of 239) in category	% of isolates in category
Source of isolates		
Fresh	222	92.9
Frozen	17	7.1
Extent of analysis		
Direct smear	219	91.6
Repeat direct smear	20	8.4
Full extraction	7	2.9
16S rRNA gene sequencing	17	7.1
Medium for isolate testing		
BAP	229	95.8
Chocolate	5	2.1
CNA	5	2.1

collection was comprised of *Streptococcus* spp. ($n = 81$), *Staphylococcus* spp. ($n = 69$), *Enterococcus* spp. ($n = 24$), *Bacillus* spp. ($n = 12$), *Corynebacterium* spp. ($n = 20$), miscellaneous Gram-positive rods ($n = 23$), and miscellaneous Gram-positive cocci ($n = 10$) (Table 1). Of these isolates, 222 (93%) were fresh isolates recovered from clinical specimens at St. Louis Children's Hospital between June and November 2011, and 17 were from frozen stocks (Table 2). Isolates were tested for growth on blood ($n = 229$), chocolate ($n = 5$), and colistin and nalidixic acid (CNA) agars ($n = 5$). Phenotypic identification of isolates was performed using conventional biochemical testing per the standard operating procedures for that organism type in the laboratory. 16S rRNA gene sequencing and additional biochemical testing were performed as required for discrepant resolution (21, 22, 23, 24). 16S rRNA gene sequencing was performed in our laboratory as described previously (23). All analyses in this study were performed to the same level of resolution used for the clinical reporting of these isolates.

Validation study design. To validate MALDI-TOF MS for use with Gram-positive bacteria in our laboratory, we planned to test at least 200 isolates, representing a minimum of 15 species, but using no more than 35 isolates of a single species. The isolates were to represent the regular flow of organisms in the clinical lab, with the supplementation of organisms rarely seen in the laboratory. All isolates were tested in quadruplicate as detailed below. Isolates that did not obtain an acceptable score (≥2.0) were reanalyzed, and if an acceptable score was not obtained a second time, the isolate was analyzed by MALDI-TOF MS following formic acid extraction.

Preparation of bacteria for MALDI-TOF MS analysis. To evaluate sample preparations, 4 direct-smear (DS) methods were performed. Using a toothpick, a very thin layer of bacteria from a single colony was applied to the stainless steel target ("heavy" smear [H]). Then, using the same toothpick, bacteria were applied to an adjacent spot on the target without returning to the plate for additional bacteria ("light" smear [L]). Heavy and light direct smears were repeated with the addition of a formic acid overlay (H+FA and L+FA, respectively), consisting of 1 μl of 100% formic acid placed directly on top of the dried organism on the stainless steel target. The formic acid overlay was left to air dry and, subsequently, 1 μl of matrix (α-cyano-4-hydroxycinnamic acid) (part no. 255344, Bruker Daltonics, Inc., Billerica, MA) was added to each spot on the target and left to dry at room temperature (RT).

MALDI-TOF mass spectrometry. Spectra were generated and analyzed using the MALDI Biotyper flexControl operating system and the Bruker Biotyper 3.0 system software and taxonomy library. MALDI-TOF MS analysis was performed in automatic mode, and a minimum of 240 laser shots were collected for each isolate. Bacterial test standard (BTS) (part no. 255343, Bruker Daltonics, Inc.) was used on each run as a calibrator and for quality control.

Formic acid extraction. A single large colony or 2 to 3 small colonies of bacteria underwent formic acid extraction as described previously (1). One microliter of the extract was spotted onto the MALDI-TOF MS stainless steel target and left to air dry, after which 1 μl of matrix was added to each direct bacterial smear and left to air dry before commencing MALDI-TOF MS analysis in automatic mode.

Media studies. A subset of 28 common isolates from the study was analyzed under different growth conditions to determine if preanalytic factors impact MALDI-TOF MS identification. The 28 isolates included *Bacillus* spp. ($n = 2$), coagulase-negative *Staphylococcus* spp. ($n = 1$), *Corynebacterium* spp. ($n = 2$), *Enterococcus faecalis* ($n = 1$), *Enterococcus faecium* ($n = 2$), *Enterococcus* spp. ($n = 1$), group A beta-hemolytic *Streptococcus* spp. ($n = 3$), group B beta-hemolytic *Streptococcus* spp. ($n = 3$), group G beta-hemolytic

TABLE 3 Mean score and standard deviation of MALDI-TOF MS analysis of Gram-positive isolates using four direct-smear methods

Organism	No. of isolates	Direct-smear type[a]			
		Heavy	Light	H+FA	L+FA
Overall	239	2.096 (0.188)	2.124 (0.186)	2.204 (0.193)[b]	2.110 (0.269)
Streptococcus spp.	81	2.100 (0.200)	2.150 (0.164)	2.234 (0.180)	2.100 (0.194)
Staphylococcus spp.	69	2.104 (0.138)	2.111 (0.142)	2.195 (0.173)	2.107 (0.187)
Enterococcus spp.	24	2.131 (0.272)	2.177 (0.313)	2.303 (0.228)	2.254 (0.263)
Bacillus spp.	12	2.015 (0.080)	1.990 (0.159)	2.056 (0.204)	2.019 (0.320)
Corynebacterium spp.	20	2.137 (0.212)	2.167 (0.191)	2.217 (0.200)	2.202 (0.213)
Miscellaneous Gram-positive rods	23	1.980 (0.164)	2.020 (0.166)	2.084 (0.169)	1.953 (0.216)
Miscellaneous Gram-positive cocci	10	2.037 (0.166)	2.117 (0.134)	2.135 (0.205)	2.091 (0.217)

[a] Data are presented as the means ± SD.
[b] H+FA achieved the highest overall score, which was statistically higher than all other direct-smear methods ($P < 0.0001$).

Streptococcus spp. ($n = 1$), *S. aureus* ($n = 3$), *Staphylococcus epidermidis* ($n = 2$), *Streptococcus mitis* group ($n = 2$), *Streptococcus pneumoniae* ($n = 3$), and viridans group *Streptococcus* spp. ($n = 2$). Isolates were analyzed following incubation for 5 days at various temperatures (35°C, 4°C, and RT), growth on various media (blood agar plate [BAP], chocolate agar, and CNA agar), and following daily subculture (1, 3, 4, and 5 days) prior to MALDI-TOF MS identification.

Statistical analysis. GraphPad Prism 5 software was used for statistical analysis. Analysis of variance (ANOVA), chi-square, and Fisher's exact tests were used to determine significance. A *P* value of 0.05 was considered statistically significant.

RESULTS

Modified formic acid overlay enhances MALDI-TOF identification. We performed four spotting techniques to determine which would provide the highest overall MALDI-TOF score for identification of the most clinical isolates. After analyzing all four direct-smear methods, we found that H+FA achieved the highest overall mean MALDI-TOF MS score (Table 3), which was statistically significant compared to all other spotting methods ($P < 0.0001$). H+FA also resulted in the highest number of correctly identified organisms (Table 4). Using the H+FA method and a cutoff of ≥2.0 for genus- and species-level identification, we identified 183 of the 239 isolates (76.6%) to the genus level, and of those phenotypically identified to the species level, we correctly identified 141 of the 181 isolates (77.9%). This was a 20% enhancement in identification to the genus level ($P < 0.0001$) and a 17% increase in species-level identification compared to heavy inoculation without the formic acid overlay ($P < 0.0006$). The use of formic acid was especially beneficial for the identification of *Streptococcus* spp. ($P = 0.0002$ for genus-level and $P = 0.0004$ for species-level identification) but did not significantly increase the number of *Staphylococcus* spp. identified. Other organism groups were not individually analyzed due to the low number of isolates in each group. Using a score of ≥2.0, all isolates were correctly identified to the genus level, and one isolate, *S. mitis* (1.3%), was misidentified by MALDI-TOF at the species level. These data show that the addition of a formic acid overlay enhanced Gram-positive bacterial identification by MALDI-TOF MS by improving the MS output score and resulted in identifying more isolates.

MALDI-TOF identification scores. The Bruker Biotyper expresses the identification of an organism as a "score" based on pattern matching. Rather than relying on the scoring scheme recommended by the manufacturer, we evaluated cutoffs between ≥2.0 and ≥1.7 for genus- and species-level identification. We optimized the reporting of Gram-positive bacteria based on this score such that we maximized the rate of correct identifications and minimized the number of incorrect identifications. In our data set, all isolates but one with a score of ≥2.0 were correctly identified by MALDI-TOF MS (*S. mitis* group). We analyzed our data by reducing the score for acceptable genus- and species-level identification by increments of 0.1, using scores ranging from ≥2.0 to ≥1.7 to determine how many additional isolates would be correctly identified and misidentified at each score. Using a score of ≥1.7, we identified 220 of the 239 isolates (92.1%) to the genus level, and of the 181 isolates requiring species-level identification per standard operating procedures, 167 isolates (92.2%) were correctly identified (Table 4). This is a 15.5% increase in genus-level identification ($P < 0.0001$) and a 14.3% increase in species-level identification compared to using a ≥2.0 cutoff score ($P < 0.0001$). Reducing the score from ≥2.0 to ≥1.7 was especially helpful in the identification of *Staphylococcus* spp. ($P = 0.0047$ for genus-level and $P = 0.0080$ for species-level identification) but less so for *Streptococcus* spp., where only a modest increase in identification was achieved. Other organism groups were not analyzed due to the low numbers of isolates in each group. Reducing the acceptable score to ≥1.7 resulted in only one additional misidentified isolate, a second isolate of the *S. mitis* group. These data show the importance of optimizing the acceptable MALDI-TOF MS confidence score for each organism group.

Unidentified and discrepant organisms. Six isolates were not identified by MALDI-TOF MS: *Dolosigranulum pigrum*, *Saccharothrix* sp., *Corynebacterium* sp., *Granulicatella* sp., and two *Streptomyces* spp. isolates. All genera except *Dolosigranulum* are present in the Bruker Biotyper MS 3.0 system database. It has been demonstrated previously that modified extraction techniques are needed for optimal MALDI-TOF MS identifications for aerobic actinomycetes; these techniques were not performed in this study and might explain why our *Streptomyces* isolates were not identified (25).

Of the 239 organisms tested, 19 isolates had discrepant results between the phenotypic and MALDI-TOF MS organism identifications (Table 5). 16S rRNA gene sequencing and additional biochemical testing showed that MALDI-TOF MS correctly identified 17 of the 19 discrepant isolates. Two isolates were phenotypically identified as *S. mitis* group by Gram stain, colony morphology, bile solubility testing, optochin susceptibility testing, and Vitek 2 automated identification system but were identified as *S. pneumoniae* by MALDI-TOF MS with high confidence scores (1.915 and 2.001). Problems with discriminating be-

TABLE 4 Postanalytical evaluation of MALDI-TOF scores for Gram-positive identification[a]

Organism type	Genus/species (*n*)	Identified to genus level			Identified to species level			No. (%) of misidentified isolates (DS/DS+FA) with a score of:	
		No. (%) of isolates (DS/DS+FA) with a score of:			No. (%) of isolates (DS/DS+FA) with a score of:				
		≥2.0	≥1.7	*P* value[c]	≥2.0	≥1.7	*P* value[c]	≥2.0	≥1.7
Overall	239/181	135/183* (56.4/76.6)	189/220* (79.0/92.1)	<0.0001	110/141* (60.7/77.9)	149/167* (82.3/92.2)	<0.0001	1/1 (0.4/0.4)	2/2[b] (0.8/0.8)
Streptococcus spp.	81/81	44/67* (54.3/82.7)	66/76 (81.5/93.8)	0.0485	43/65* (53.1/80.2)	64/73 (79.0/90.1)	NS[d]	1/1 (1.3/1.3)	2/2 (2.5/2.5)
Staphylococcus spp.	69/69	51/56 (73.9/81.2)	64/67 (92.8/97.1)	0.0047	51/55 (73.9/79.7)	63/66 (91.3/95.7)	0.0080	0/0 (0/0)	0/0 (0/0)
Enterococcus spp.	24/12	16/19 (66.7/79.2)	18/22 (75.0/91.7)		7/7 (58.3/58.3)	9/10 (75.0/83.3)		0/0 (0/0)	0/0 (0/0)
Bacillus spp.	12/3	4/7 (30.8/58.3)	8/11 (61.5/91.7)		1/2 (33.3/66.7)	2/3 (66.7/100)		0/0 (0/0)	0/0 (0/0)
Corynebacterium spp.	20/7	9/14 (45.0/70.0)	12/16 (60.0/80.0)		4/6 (57.1/85.7)	6/7 (85.7/100)		0/0 (0/0)	0/0 (0/0)
Miscellaneous Gram-positive rods	23/4	6/14 (26.1/60.9)	14/19 (60.9/82.6)		2/3 (50.0/75.0)	2/4 (50.0/100)		0/0 (0/0)	0/0 (0/0)
Miscellaneous Gram-positive cocci	10/5	5/6 (50.0/60.0)	7/9 (70.0/90.0)		2/3 (40.0/60.0)	3/4 (60.0/80.0)		0/0 (0/0)	0/0 (0/0)

[a] Each asterisk indicates that results of a comparison of direct smear (DS) versus DS+FA were statistically significant ($P < 0.05$).
[b] Two *S. mitis* group organisms were misidentified as *S. pneumoniae* by MALDI-TOF MS using both DS and DS+FA.
[c] *P* value column denotes statistical significance between an acceptable genus- and species-level cutoff value of ≥1.7 compared to ≥2.0.
[d] NS, nonsignificant.

tween *S. pneumoniae* and *S. mitis* group organisms by MALDI-TOF MS have been reported previously (5, 6, 7, 9, 16).

Based on reporting in the clinical laboratory, our study included nine isolates phenotypically identified as *S. pneumoniae*, two as *S. mitis* group, and 13 as viridans group *Streptococcus* (VGS). All nine isolates phenotypically identified as *S. pneumoniae* were also identified as *S. pneumoniae* by MALDI-TOF MS. Of the two isolates identified as *S. mitis* by phenotypic methods and as *S. pneumoniae* by MALDI-TOF MS, repeat biochemical testing showed these two isolates to be optochin resistant and bile solubility test negative, which is consistent with *S. mitis* group. Thirteen isolates in our study were identified and reported as VGS in our clinical laboratory. All of these isolates were optochin resistant and were not bile soluble, and five of the 13 isolates were incorrectly identified as *S. pneumoniae* by MALDI-TOF MS. These data highlight the necessity of confirming all isolates that were identified as *S. pneumoniae* by MALDI-TOF MS with additional methods, such as an optochin disk and/or a bile solubility test.

Full extraction for identification of Gram-positive isolates. Of the 239 isolates tested in this study, 20 isolates did not initially generate a score of ≥1.7 using any DS method (Table 2). Repeat MALDI-TOF MS testing was performed on 17 of the isolates (3 isolates could not be recovered for repeat analysis). Ten of the 17 isolates (58.8%) were successfully identified upon repeat MALDI-TOF MS using a score of ≥1.7. A full formic acid extraction was performed on 7 of the isolates with a score of <1.7 on repeat analysis. Only one of the seven isolates was identified by MALDI-TOF MS following full extraction (14.3%; 0.4% of all isolates in the study). These data suggest that full extraction is unlikely to be useful for Gram-positive organisms that are not identified using the 1-μl formic acid overlay method. If an organism is unidentified after repeat MALDI-TOF MS testing, phenotypic testing or sequence-based identification should be considered.

Media studies. A subset of 28 common isolates was analyzed after 5 days of incubation at 4°C, room temperature (RT), and 35°C (Fig. 1A). The number of correctly identified isolates was calculated using decreasing cutoff vales of ≥2.0, ≥1.9, ≥1.8, and ≥1.7 for acceptable genus- and species-level identification. The average MALDI-TOF scores for isolates identified at 35°C, RT, and 4°C were not significantly different from one another at 2.206, 2.254, and 2.231, respectively ($P = 0.7505$). Of interest, 4°C incubation gave the best results, correctly identifying 92.9% of isolates to the genus and species level using a cutoff score of ≥1.7. Incubation of organisms at RT and 35°C also produced good identification results; a score of ≥1.7 resulted in 82.1% and 85.7% of isolates being identified, respectively. No isolates were misidentified at any incubation temperature.

The same subset of 28 isolates was analyzed after growth on BAP, chocolate, and CNA agar plates (Fig. 1B). The average MALDI-TOF scores for isolates identified from BAP, chocolate, and CNA agars were not significantly different from one another at 2.282, 2.234, and 2.232, respectively ($P = 0.5224$). A score of ≥1.7 resulted in 100% of isolates being identified from CNA, 92.9% from BAP, and 89.3% identified from chocolate agar. No isolates were misidentified from any medium type.

These 28 isolates were then analyzed following daily subculture for 1, 3, 4, and 5 days to assay the impact of subculture frequency on MALDI-TOF MS identification (Fig. 1C). The average MALDI-TOF scores for the correct identification of isolates subcultured for 1, 3, 4, and 5 days were very similar to one another at

TABLE 5 Discrepant results with MALDI-TOF MS compared to phenotypic identification[a]

Phenotypic ID[b]	MALDI-TOF ID	MALDI-TOF score[c]	Methods for resolution/final ID[d]
Streptococcus mitis group	*Streptococcus pneumoniae*	1.915	Phenotypic: BS, P disk, Vitek
Streptococcus mitis group	*Streptococcus pneumoniae*	2.001	Phenotypic: BS, P disk, Vitek
Staphylococcus aureus	***Staphylococcus intermedius***	2.2	Phenotypic: PYR
Micrococcus sp. (Vitek)	***Rhodococcus equi***	2.269	16S rRNA gene sequencing: *Rhodococcus equi*
Sphingomonas paucimobilis (GS, Vitek)	***Cellulosimicrobium cellulans***	2.221	16S rRNA gene sequencing: *Cellulosimicrobium cellulans/funkei*
No ID (GS, motility, catalase, and colony morphology)	***Paenibacillus amylolyticus***	1.754	16S rRNA gene sequencing: *Paenibacillus* sp.
No ID (GS, catalase, and colony morphology)	***Rothia aeria***	2.063	16S rRNA gene sequencing: *Rothia* sp.
Sphingomonas paucimobilis (GS, Vitek)	***Arthrobacter oxydans***	2.16	16S rRNA gene sequencing: *Arthrobacter* sp.
No ID (Vitek, Phoenix NaCl, ESC, VP)	***Streptococcus equinus***	2.095	16S rRNA gene sequencing: *Streptococcus bovis* group
Streptococcus bovis group (Vitek, Phoenix, growth in 6.5% sodium chloride broth, ESC, VP)	***Streptococcus equinus***	2.056	16S rRNA gene sequencing: *Streptococcus bovis* group
Corynebacterium sp. (GS, catalase)	***Actinomyces neuii***	2.06	16S rRNA gene sequencing: *Actinomyces* sp.
VGS (Vitek, Phoenix)	***Streptococcus parasanguinis***	2.263	16S rRNA gene sequencing: *Streptococcus parasanguinis/mitis/oralis*
VGS (Phoenix)	***Streptococcus parasanguinis***	1.841	16S rRNA gene sequencing: *Streptococcus parasanguinis/mitis/oralis*
Coryneform bacteria (GS)	***Cellulosimicrobium cellulans***	2.042	16S rRNA gene sequencing: *Cellulosimicrobium cellulans*
Sphingomonas paucimobilis (GS, Vitek)	***Cellulosimicrobium cellulans***	2.164	16S rRNA gene sequencing: *Cellulosimicrobium cellulurum/funkei*
Group B beta-hemolytic *Streptococcus* spp. (GS, catalase, colony morphology, latex)	***Streptococcus dysgalactiae***	2.02	Repeat latex agglutination was positive for group G
Corynebacterium spp. (GS, CAT, Vitek, ESC, urea)	***Rothia dentocariosa***	2.242	16S rRNA gene sequencing: *Rothia dentocariosa*
Leuconostoc sp./*Aerococcus viridans* (Vitek/Phoenix)	***Leuconostoc lactis***	1.899	16S rRNA gene sequencing: *Leuconostoc lactis*
Coryneform bacteria (GS, catalase)	***Actinomyces radingae***	1.976	16S rRNA gene sequencing: *Actinomyces* sp.

[a] The correct identifications are in bold type.
[b] ID, identification.
[c] All isolates were identified by MALDI-TOF MS using the DS+FA method of bacterial preparation.
[d] BS, bile solubility; P disk, optochin disk; GS, Gram stain; ESC, esculin; PYR, pyrolidonyl arylamidase.

2.226, 2.229, 2.227, and 2.177, respectively ($P = 0.9930$). Although the scores of correctly identified isolates were nearly identical, the identification of bacterial isolates by MALDI-TOF MS decreased as subculture frequency increased. A score of ≥1.7 resulted in 100% of isolates being identified on day one, which fell to 78.6% after 5 days of subculture, a 21.4% decrease. This result is reflected in the coefficient of variation (CV), which rose from 9.1% on day one (scores ranging from 1.737 to 2.552) to 53.7% by day five of subculture (scores ranging from 0 to 2.480). Despite fewer isolates being identified following repeated subculture, there were no misidentifications when scores of ≥1.7 were obtained.

DISCUSSION

MALDI-TOF MS is a rapid and cost-effective way to identify bacteria in the clinical laboratory (2, 5, 8, 9, 26). This study evaluated a number of preanalytic and postanalytic factors that affect MALDI-TOF MS identification of Gram-positive bacteria to validate and optimize this method for use in the clinical laboratory. In an attempt to enrich the quality of spectra produced by MALDI-TOF MS, we used a 1-μl formic acid overlay. This improved genus- and species-level identification of isolates by 20% ($P < 0.0001$ and $P < 0.0006$, respectively). Our data allowed us to reduce the score for acceptable identification to genus and species level from ≥2.0 to ≥1.7 for this group of organisms, resulting in an additional 15.5% of isolates identified to the genus level and 14.3% of isolates identified to the species level. These data indicate that MALDI-TOF MS is a very effective way to identify Gram-positive bacteria in the clinical laboratory.

In this study, we evaluated the protocol modification of a direct 1-μl formic acid overlay on MALDI-TOF MS identification. Similar methods of rapid protein extraction have been reported in studies evaluating Gram-positive and Gram-negative organisms, as well as yeast (19, 27, 28). In all instances, the direct application of formic acid led to an overall increase in bacterial identification compared to direct spotting of bacteria without a formic acid overlay. In the past, formic acid extraction of bacterial isolates was used to improve MALDI-TOF MS spectra. This method is relatively labor-intensive and does not fit well into the workflow of the clinical laboratory. In contrast, a 1-μl formic acid overlay is easily adaptable to the clinical workflow. Bacteria are spotted on the stainless steel target as technologists read their cultures in real time. At set points throughout the day or when a target is full, the microbiology technologists add a 1-μl formic acid overlay to each spot of dried organism on the target. After a short drying period, matrix is added to each spot. In our hands, the addition of a 1-μl formic acid overlay adds only 5 to 10 min to the MALDI-TOF procedure, minimally impacts workflow, and improves the identification of Gram-positive bacteria by approximately 20%. With regard to the increased diagnostic yield of performing a full formic acid extraction, studies have shown mixed results (19, 27, 28). In our hands, full formic acid extraction resulted in an identification in only 1 of the 7 isolates not identified by MALDI-TOF MS using our DS methods. Due to the minimal diagnostic gains, we do not routinely perform formic acid extraction in our laboratory.

Reducing the acceptable score for genus- and species-level

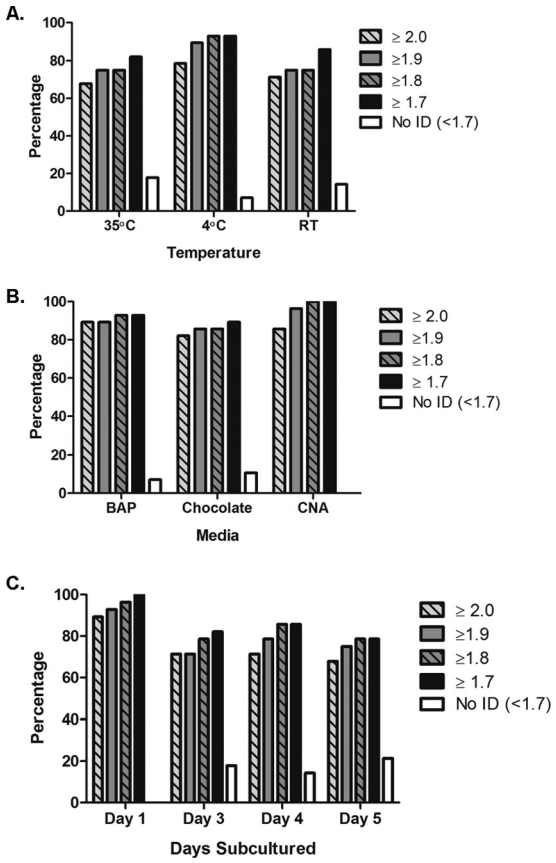

FIG 1 Impact of preanalytical factors on MALDI-TOF MS identification. Shown are the results of MALDI-TOF MS analysis of 28 common isolates after incubation at different temperatures (A), growth on different types of media (B), and daily subculture for up to 5 days (C). The number of correctly identified isolates was calculated using decreasing cutoff values between ≥2.0 and ≥1.7 for acceptable genus- and species-level identification. There were no statistical differences between isolates identified at different temperatures or off different media.

identification from ≥2.0 to ≥1.7 resulted in a 15.5% and 14.3% increase in genus- and species-level identification, respectively, while introducing only one misidentified isolate. This is similar to other published reports in which reducing the acceptable score for genus- and species-level identification for Gram-positive and Gram-negative bacteria and yeast resulted in increased identification of organisms while introducing very few misidentifications (27, 28, 29).

Our study was designed to reflect both the common and infrequent isolates seen in the clinical laboratory. Unusual isolates are more likely to be misidentified or unidentified using automated systems (13, 30, 31). MALDI-TOF MS has the potential to drastically streamline the identification of these organisms and is limited only by the completeness of the database which is used for identification. In this study, 82.6% of miscellaneous Gram-positive rods and 90% of miscellaneous Gram-positive cocci were identified (using a score of ≥1.7) with no misidentified isolates. These data indicate that MALDI-TOF MS identification is useful for the identification of infrequently encountered Gram-positive organisms.

Historically, Gram-positive bacteria have not been identified to the species level as frequently as Gram-negative organisms recovered from clinical specimens. Use of MALDI-TOF MS in the clinical laboratory will provide species-level identification in many situations where genus-level identification would have been reported in the past, such as for isolates of *Bacillus* and *Corynebacterium*. Additionally, organisms, such as the *Cellulosimicrobium cellulans* bacteria identified during this validation study, might be identified to the genus and species level while in the past they would have been identified only as *Corynebacterium* spp. or "coryneform bacteria." This increased species-level resolution might allow us to learn more about the clinical significance of specific taxa within these genera and might inform the pathogenic potentials of certain taxa that were previously unappreciated.

One of the benefits of MALDI-TOF MS analysis is that it does not rely on any upstream information for bacterial identification, and missteps occurring early on in the identification process do not impede accurate identification. This is different from automated identification systems, such as Vitek 2, which rely on the correct interpretation of a Gram stain to determine which identification method or panel should be used for organism identification. In our study, an isolate of *Cellulosimicrobium cellulans* was incorrectly interpreted as Gram-negative bacilli on Gram stain. This led to the use of a Vitek 2 Gram-negative card and resulted in a misidentification of *Sphingomonas* (Table 5). MALDI-TOF MS identification does not rely on interpretation of the Gram stain, which eliminates this type of error.

A known limitation of MALDI-TOF MS identification is its ability to accurately identify S. *pneumoniae* from *S. mitis* group organisms (5, 6, 7, 9, 16). Although they are genetically very similar, accurate identification of these isolates to the species level is clinically important. In keeping with previous studies, two of our isolates were identified phenotypically as non-*S. pneumoniae* members of the *S. mitis* group but were incorrectly identified as *S. pneumoniae* by MALDI-TOF MS. In our study, no *S. pneumoniae* isolates were assigned to a less-pathogenic species, but 5 of the 13 VGS in our study were incorrectly identified as *S. pneumoniae* using MALDI-TOF. Therefore, supplemental methods, such as bile solubility and/or optochin disk susceptibility, should be considered prior to reporting an isolate as *S. pneumoniae*. MALDI-TOF MS analysis cannot be used as a sole means of identification for these organisms.

We investigated several commonly encountered preanalytical factors and found that incubation at 35°C, RT, or 4°C or growing isolates on blood, chocolate, or CNA agars did not have a negative impact on MALDI-TOF MS identification. In a previous study, Anderson et al. (32) found that MALDI-TOF MS identification was slightly inferior if organisms were grown on CNA agar compared to blood agar. That study tested a collection of 20 staphylococci, while our study assayed a more diverse group of 28 Gram-positive organisms, which might account for the differences observed. Interestingly, we found that increasing subculture frequency had an inverse relationship with the likelihood of identification by MALDI-TOF MS. Therefore, it is suggested that Gram-positive bacterial isolates be tested following minimal subculture whenever possible.

In conclusion, MALDI-TOF MS was very effective in identifying Gram-positive bacteria found in the clinical laboratory, including infrequently isolated organisms. To correctly identify the highest number of Gram-positive organisms, we recommend using a 1-μl formic acid overlay, reducing the cutoff score for identification from ≥2.0 to ≥1.7, and using fresh isolates for MALDI-TOF MS whenever possible.

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