Direct Identification of Aerobic Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Is Accurate and Robust

Javier Mestas,¹ Teeophany Quias,¹ and Jennifer Dien Bard^{1,2*}

¹Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California

²Keck School of Medicine at the University of Southern California, Los Angeles, California

Background: Bacterial identification in the clinical laboratory can be laborious and expensive. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid and cost-effective diagnostic tool for the identification of organisms routinely found in the microbiology laboratory. The objective of this study was to demonstrate that identification of aerobic Gram-positive and Gram-negative organisms could be performed accurately and efficiently by MALDI-TOF MS and the Bruker Biotyper system without the use of time-consuming extraction methodologies. **Methods:** Isolates previously recovered by routine culture and workup from clinical specimens were cultured to appropriate media, identified directly by MALDI-TOF MS, and compared to results from

various biochemical identification methods. **Results:** Using the direct-smear method, 99.5% and 98.0% of aerobic Gram-negative and Gram-positive bacteria, respectively, were identified to the genus level. At a score of ≥ 1.9 , 97.6% Gram-negative organisms and 94.6% Gram-positive organisms were correctly identified to the species level by direct-smear method. Only 1.1% of isolates required further reflex to direct-plate extraction. The direct-smear method proved to be robust, as various growth temperatures, media, culture age, and different operators had no notable impact on the bacterial identification rate. **Conclusion:** The direct-smear method is an accurate and time-saving method for routine species-level bacterial identification. *J. Clin. Lab. Anal.* 30: 543–551, 2016. © 2015 Wiley Periodicals, Inc.

Key words: MALDI-TOF MS; bacteria identification; direct smear; extraction; Gram-positive; Gram-negative

INTRODUCTION

The application of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the characterization of bacteria and yeast by unique protein profiles was first introduced a few years ago (1,2). Since then, MALDI-TOF MS has been proven to be a rapid and cost-effective diagnostic tool for routine use in the clinical microbiology laboratory (3–8). MALDI-TOF MS technology uses ionizing laser for structural elements of the isolate to generate isolate-derived spectra, which is then compared to a reference database. Numerous studies have extensively evaluated the performance of Bruker MALDI-TOF MS Biotyper and confirm that this system can reliably identify 95% of all isolates cultured on media in the clinical laboratory using Bruker's recommended

confidence scoring of ≥ 2.0 for species-level and 1.7–1.99 for genus-level identification (7,9–11).

The majority of unreliable spectra scores can be attributed to limited species coverage in the existing software or variability in sample preparation of the isolate (12–14). There are typically two methods by which a sample may be prepared for MALDI-TOF MS—direct

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*Correspondence to: Jennifer Dien Bard, PhD, D(ABMM), Department of Pathology and Laboratory Medicine, Keck School of Medicine, University of Southern California, 4650 Sunset Blvd, Mailstop #32, Los Angeles, CA 90027. E-mail: jdienbard@chla.usc.edu

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smearing of intact cells onto the target plate or protein extraction of isolate prior to smearing onto target plate. Although extraction of the protein fraction of isolates increases the robustness and yield of species-level identification, particularly in Gram-positive organisms (8, 12, 15, 16), the extraction method itself is cumbersome and contradictory to the revolutionary concept of rapid identification by MALDI-TOF MS. Recent studies on Gram-positive organisms have demonstrated that an alternate extraction method, the direct-plate extraction or formic acid overlay method, can yield comparable species-level identification to the traditional extraction method (17, 18). In contrast, direct smearing of Gram-negative organisms does not yield significantly different identification rates over the extraction method (19), prompting the need for two different sample preparation methods on a routine basis. Therefore, we sought to determine whether the direct-smear method can produce accurate and robust results for both Gram-negative and Gram-positive organisms in our institution, allowing for the optimal efficiency and improved workflow.

In this study, we aim to evaluate the ability of MALDI-TOF MS and Bruker Biotyper system to accurately identify aerobic Gram-positive and Gram-negative organisms routinely identified in the clinical laboratory using the direct-smear method. Variables commonly encountered in the laboratory, including temperatures and media, will also be assessed.

MATERIALS AND METHODS

Clinical Samples

Clinical isolates previously recovered by routine culture and workup from specimens submitted to the Clinical Microbiology Laboratory at Children's Hospital Los Angeles (CHLA) between September 2012 and June 2013 were included in the study. Clinical specimens were processed according to standard laboratory practices and isolated colonies were tested by routine methodologies and MALDI-TOF MS. A total of 996 aerobic Gram-positive and Gram-negative organism isolates were included in the study. The study was approved by the Institutional Review Board at CHLA.

Bacterial Identification

Clinical specimens were cultured to appropriate media, including but not limited to blood agar, MacConkey, and chocolate agar and incubated for 18–24 h at 37°C with or without 5% CO₂. Isolated colonies of bacteria growing on media were identified using a variety of phenotypic, biochemical, and commercial identification systems, including Vitek II (bioMérieux, Durham, NC), APIs

(bioMérieux), and numerous other manual identification methods. The isolated colonies were concurrently spotted on the MALDI-TOF MS 96-well target plate using sterile swab. Direct identification method was performed on all bacterial isolates: bacterial colonies were spotted onto the target plate, allowed to air dry, followed by the addition of 1 µl of α -cyano-4-hydroxycinnamic-acid matrix to each spot. Direct-plate extraction with formic acid was performed on any isolate that yielded a score <1.7 using the direct-smear method. Colonies were spotted onto the target plate, allowed to air dry, followed by extraction with 1 µl of 70% formic acid, prior to the addition of 1 µl of α -cyano-4-hydroxycinnamic-acid matrix. All MALDI-TOF MS testing was performed in duplicate with the same swab spotted onto two spots on the target plate, one spot representing a "heavy" inoculum and the second spot representing a "light" inoculum. Acquisition and analysis of mass spectra was performed using the Microflex LT mass spectrometer (Bruker Daltonics, Fremont, CA, USA.).

Variability Studies

Clinical bacteria isolates were subjected to different conditions throughout the course of the study. For media studies, 84 Gram-negative bacteria were cultured on blood agar, chocolate agar, and MacConkey agar; 74 Gram-positive bacteria were cultured on blood agar, chocolate agar, and colistin nalidixic acid (CNA) agar. For temperature and stability studies, four Gram-negative bacteria and four Gram-positive bacteria were cultured onto blood agar and incubated at 37°C under 5% CO₂ for 24 h, followed by subsequent incubation at 37°C, 4°C, and room temperature for up to 7 days. For interoperator studies, 69 Gram-negative bacteria and 48 Gram-positive bacteria were cultured onto blood agar and spotted in duplicate on target plates by two Clinical Laboratory Scientist (CLS).

MALDI-TOF MS Data Interpretation

Following the recommendations of the manufacturer, a score of ≥ 2.0 is equivocal to a reliable species-level identification, a score of 1.7–1.99 is considered reliable to the genus level and a score of <1.7 is considered unreliable for bacteria identification. Bacterial isolates with scores of <1.7 were repeated by direct method followed by direct-plate extraction method and tube extraction method, if required. Results of MALDI-TOF MS were compared to routine methods performed in the microbiology laboratory. All discordant identifications were confirmed by additional biochemical or 16S rRNA sequencing.

Data Analysis

Chi-square and Fisher's exact tests were used for calculating any statistical significant difference demonstrated in the variability studies. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

Optimization of Scoring Cutoffs

A total of 996 bacterial isolates, 597 Gram-negative organisms and 399 Gram-positive organisms, were tested in this study and performance of direct-smear method for genus- and species-level identification was evaluated at cutoff scores between ≥ 1.7 and ≥ 2.0 to determine an optimal score that will allow the highest identification success rate without compromising accuracy (Table 1). All isolates that resulted in a score < 1.7 were repeated using the direct-plate extraction method. Overall, using the manufacturer's recommended cutoff of ≥ 1.7 for genus-level identification, we correctly identified 994 of the 996 isolates (99.8%) to the genus-level and the only isolates not identified were two Gram-positive organisms. Changing the cutoff score to ≥ 1.8 , ≥ 1.9 , or ≥ 2.0 decreased genus-level identification by 1.2% ($P = 0.006$), 3.3% ($P = 0.0001$), and 10.2% ($P = 0.0001$), respectively. A cutoff of ≥ 1.7 was more beneficial for the Gram-positive organisms than Gram-negative organisms, particularly the *Streptococcus* group that demonstrated a 20.6% increase in genus-level identification ($P = 0.0001$) compared to the ≥ 2.0 cutoff.

Further species-level identification was required on 921 of the 994 isolates (92.7%), 552 Gram-negative organisms and 369 Gram-positive organisms, and using the manufacturer's recommended cutoff of ≥ 2.0 for species-level identification, 818 isolates (88.8%) were correctly identified. A higher number of Gram-negative bacteria (94.6%) were correctly identified compared to Gram-positive bacteria (80.2%). Similarly to genus-level identification, reducing the cutoff score to ≥ 1.9 , ≥ 1.8 , and ≥ 1.7 resulted in an increase in species-level identification rate by 6.3% ($P = 0.0001$), 7.6% ($P = 0.0001$), and 8.4% ($P = 0.0001$), respectively. Reduction of cutoff scores was particularly beneficial for the Gram-positive organisms, with a 10.3% increase noted at ≥ 1.9 cutoff compared to 3.6% for the Gram-negative organisms ($P = 0.0001$). Of the discrepant results, 20 viridans group streptococci were misidentified as *Streptococcus pneumoniae*, which is not surprising as similar findings have been previously reported in the literature (5, 6, 17, 20, 21). Thus, omission of these 20 isolates dramatically increased the species-level identification rate of Gram-positive organisms from 84.8% to 95.7% at a ≥ 1.9 cutoff.

Direct Identification of Gram-Negative and Gram-Positive Bacteria by MALDI-TOF MS

A cutoff scoring of ≥ 1.9 was implemented in our laboratory since 97.8% (876/896) of all isolates were correctly identified to the species-level at this score, and the correct identification rate increased to 99.7% when the discrepant *S. viridans* group isolates were omitted. A total of 597 aerobic Gram-negative organisms were included in the study, of which 42 different genus and 57 different species were tested (Table 2). Using the direct-smear method, 594 of 597 (99.5%) aerobic Gram-negative bacteria and 391 of 399 (98.0%) Gram-positive organisms were identified at spectra score of ≥ 1.7 for genus-level identification and 100% of the isolates were correctly identified. At a score of ≥ 1.9 , 97.6% (539/552) Gram-negative organisms, and 94.6% (330/349) Gram-positive organisms were correctly identified to species-level by direct-smear method. Due to the known identification issues for *S. pneumoniae*, all *S. viridans* group isolates were eliminated from the species-level analysis in this study.

The direct-plate extraction was utilized on isolates with a score of < 1.7 by direct-smear method. A total of 11 of 996 (1.1%) isolates were subjected to direct-plate extraction; three Gram-negative organisms and eight Gram-positive organisms (Table 2). Extraction allowed for further identification of eight isolates to the species-level, three Gram-negative and five Gram-positive bacteria, and an additional Gram-positive organism to genus-level. Of the remaining two isolates, one was incorrectly identified by MALDI-TOF MS and the final isolate was an *Aerococcus viridans* that had no reliable spectral score by MALDI-TOF MS. Thus, after direct-plate extraction was performed, the species-level identification rate at a score of ≥ 1.9 increased to 98.2% for Gram-negative organisms and 95.7% for Gram-positive organisms (Table 1).

Analysis of Discrepant Results Between MALDI-TOF MS and Conventional Methods

Discrepant results were reported on 8 (1.3%) Gram-negative organisms and 27 (6.8%) Gram-positive organisms, when MALDI-TOF MS was compared to conventional methods. 16S rRNA sequencing was performed on all eight Gram-negative isolates and identification of five isolates concurred with MALDI-TOF MS. Scores of ≥ 1.9 were obtained for seven of the eight Gram-negative isolates, including the five isolates that were correctly identified. The other two isolates were incorrectly identified to the species-level by MALDI-TOF MS as *Achromobacter denitrificans* and *Elizabethkingia miricola*. The final isolate had a score of 1.868 and was misidentified as *Enterobacter cloacae* by MALDI-TOF MS. However, using our set cutoff score of ≥ 1.9 for

TABLE 1. Performance Characteristics Using Alternative Score Cutoffs

Organism (no.)	Cutoff score	No. (%) of isolates correctly identified to		No. of isolates incorrectly identified
		Genus	Species ^a	
Gram-negative (597)	2.0	562 (94.1)	522 (94.6)	1
	1.9	583 (97.7)	542 (98.2)	1
	1.8	589 (98.7)	545 (98.7)	1
	1.7	597 (100)	549 (99.5)	0
	≤ 1.7	n/a	n/a	0
Gram-positive (399)	2.0	330 (82.7)	296 (80.2)	13
	1.9	378 (94.7)	334 (90.5)	5
	1.8	393 (98.5)	343 (93.0)	3
	1.7	397 (99.5)	346 (93.8)	0
	≤ 1.7	n/a	n/a	1

n/a, not applicable.

^a 552 Gram-negative and 369 Gram-positive organisms were identified to the species level by both MALDI-TOF MS and conventional methods.

species-level identification, this isolate would have only been reported out as *Enterobacter* spp.

Of the 27 Gram-positive organisms with discrepant results, 19 isolates were identified as *S. pneumoniae* by MALDI-TOF MS with scores ≥ 1.85 , and were confirmed to be *S. viridans* group by negative bile solubility and resistant optochin results. 16S rRNA gene sequencing was performed on all of the remaining eight isolates and all the results were concordant with MALDI-TOF MS, with the exception of one *S. mitis* isolate that was also identified as *S. pneumoniae* by MALDI-TOF MS, and one isolate that was identified as *Leuconostoc mesenteroides* by MALDI-TOF MS but confirmed as *Weissella* spp. by sequencing.

Analysis of Media, Temperature, and Interoperator Variability on MALDI-TOF MS Bacterial Identification

To determine the influence of different media on direct-smear bacterial identification by MALDI-TOF MS, 84 Gram-negative and 73 Gram-positive organisms were cultured on a variety of media. In addition to performing direct smear from blood agar and chocolate agar, Gram-negative organisms were also identified from MacConkey media and Gram-positive organisms from CNA agar after 18- to 24-h incubation at 37°C and analyzed by the direct-smear method and MALDI-TOF MS. Using the cutoff scoring of ≥ 1.9 for species-level identification, Gram-negative organisms from blood and chocolate agar yielded the highest identification rate (100%), followed by MacConkey media (97.6%). Similarly, the highest identification rate for Gram-positive organisms was on CNA (100%) and the lowest identification rate was seen when isolates were grown on chocolate agar (88.9%).

Several commonly isolated Gram-negative and Gram-positive organisms were chosen to determine the stability of isolates post 24-h culture at 37°C and the effects of different growth temperatures on MALDI-TOF MS identification. The organisms were cultured on blood agar and incubated at 4°C, 22°C, and 35°C for 1, 2, 4, 5, and 7 days, and subsequently analyzed by MALDI-TOF MS using direct-smear method. The majority of isolates gave a spectra score of ≥ 2.0 , regardless of the incubation temperature and age of the isolates. Two isolates failed to produce a spectra score at day 7 of incubation, *Enterococcus faecalis* at 22°C and *Streptococcus agalactiae* at 4°C.

Analysis of interoperator variability in obtaining correct bacterial identification by MALDI-TOF MS was also performed using 69 Gram-negative and 48 Gram-positive organisms. Two individual CLSs carried out the analysis on the same isolates and on the same day. Using the same cutoff scoring as above, both operators obtained species-level identification rates of 93.2%. The differences in identification rate between the two operators were 1.5% and 2.1% for the Gram-negative and Gram-positive organisms, respectively.

DISCUSSION

Traditional methods of bacterial identification in the clinical laboratory can be laborious and expensive, particularly when multiple methods are required for definitive identification. MALDI-TOF MS offers a rapid, accurate, and cost-effective way of identifying bacteria through the generation of unique spectral data, derived from ionized protein elements and compared to a reference database (2, 22–25). While extraction of cellular content prior to analysis by MALDI-TOF MS increases

TABLE 2. Distribution of Gram-Negative and Gram-Positive Microorganisms Identified by MALDI-TOF MS With a Score ≥ 1.9

Gram-negative organism (no.)	No. (%) correctly identified				No. (%) correctly identified					
	Genus	Species	No. (%) incorrectly identified	No. (%) requiring extraction	No. (%) requiring sequencing	Genus	Species	No. (%) incorrectly identified	No. (%) requiring extraction	No. (%) requiring sequencing
<i>Achromobacter xylosoxidans</i> (35)	3 (100)	34 (97.1)	1 (2.9)		1 (2.9)		<i>Abiotrophia</i> spp. (1)	1 (100)		
<i>Acinetobacter</i> spp. (3)		17 (100)			1 (5.9)		<i>Actinomyces</i> spp. (3)	3 (100)		2 (50)
<i>Acinetobacter baumannii</i> complex (17)		1 (100)					<i>Actinomyces odontolyticus</i> (2)			
<i>Acinetobacter iwoffi</i> (1)		3 (100)					<i>Aerococcus urinae</i> (1)		1 (100)	
<i>Acinetobacter junii</i> (3)		1 (100)					<i>Aerococcus viridans</i> (1)			
<i>Acinetobacter radioresistens</i> (1)		5 (100)					<i>Bacillus</i> spp. (3)	3 (100)	1 (100)	
<i>Acinetobacter ursingii</i> (5)		1 (100)					<i>Brevibacterium casei</i> (2)		2 (100)	
<i>Aeromonas hydrophila</i> (1)		3 (100)			1 (33.3)		<i>Corynebacterium</i> spp. (10)	10 (100)		
<i>Aeromonas veronii</i> (3)	1 (100)						<i>Corynebacterium amycolatum</i> (1)			2 (100)
<i>Aggregatibacter</i> spp. (1)		2 (100)					<i>Corynebacterium jeikeium</i> (2)		2 (100)	
<i>Aggregatibacter aphrophilus</i> (2)		1 (100)					<i>Corynebacterium striatum</i> (1)		1 (100)	
<i>Alcaligenes faecalis</i> (1)		1 (100)					<i>Dermabacter hominis</i> (1)		1 (100)	
<i>Atopobium parvulum</i> (1)		11 (100)					<i>Enterococcus</i> spp. (2)	2 (100)		
<i>Burkholderia cepacia</i> complex (11)		8 (100)					<i>Enterococcus avium</i> (2)		2 (3)	
<i>Campylobacter</i> spp. (1)	1 (100)						<i>Enterococcus faecalis</i> (44)	44 (100)		
<i>Campylobacter jejuni</i> (8)		1 (100)					<i>Enterococcus faecium</i> (26)	26 (100)		1 (3.8)
<i>Capnocytophaga</i> spp. (1)		1 (100)					<i>Enterococcus gallinarum</i> (4)	4 (100)		
<i>Capnocytophaga sputigena</i> (1)		1 (100)					<i>Exiguobacterium</i> spp. (1)	1 (100)		1 (100)
<i>Chryseobacterium gleum</i> (2)		4 (100)					<i>Gardnerella</i> spp. (4)	4 (100)		
<i>Chryseobacterium indologenes</i> (4)		4 (100)					<i>Gardnerella vaginalis</i> (1)		1 (100)	
<i>Citrobacter amalonaticus</i> (4)		6 (100)					<i>Gemella</i> spp. (1)	1 (100)		
<i>Citrobacter braakii</i> (6)		11 (100)			1 (9.1)		<i>Granulicatella</i> spp. (1)	1 (100)		
<i>Citrobacter freundii</i> (11)		4 (100)					<i>Lactobacillus</i> spp. (5)	5 (100)		
<i>Citrobacter koseri</i> (4)		3 (75)	1 (25)				<i>Lactobacillus paracasei</i> (1)		1 (100)	
<i>Cupriavidus gilardii</i> (1)		9 (90)	1 (10)				<i>Lactobacillus reuteri</i> (1)		1 (100)	
<i>Delftia acidovorans</i> (2)		37 (100)					<i>Lactobacillus rhamnosus</i> (2)		2 (100)	
<i>Eikenella corrodens</i> (2)		65 (100)					<i>Micrococcus luteus</i> (4)		4 (100)	
<i>Elizabethkingia meningoseptica</i> (4)							<i>Rothia mucilaginosa</i> (2)		2 (100)	
<i>Enterobacter aerogenes</i> (10)							<i>Staphylococcus</i> spp. (3)	3 (100)		
<i>Enterobacter cloacae</i> complex (37)							<i>Staphylococcus aureus</i> (65)		65 (100)	
<i>Escherichia coli</i> (65)							<i>Staphylococcus caprae</i> (1)		1 (100)	
<i>Haemophilus</i> spp. (3)	3 (100)						<i>Staphylococcus epidermidis</i> (27)		27 (100)	
<i>Haemophilus influenzae</i> (32)		8 (100)					<i>Staphylococcus haemolyticus</i> (2)		2 (100)	
<i>Haemophilus parahaemolyticus</i> (8)							<i>Staphylococcus hominis</i> (8)		8 (100)	
<i>Haemophilus parainfluenzae</i> (8)							<i>Staphylococcus lugdunensis</i> (3)		3 (100)	

(Continued)

TABLE 2. Continued

Gram-negative organism (no.)	No. (%) correctly identified			No. (%) correctly identified		
	Genus	Species	No. (%) incorrectly identified	No. (%) requiring extraction	No. (%) requiring sequencing	Gram-positive organism (no.)
<i>Hafnia alvei</i> (1)		1 (100)				<i>Staphylococcus simulans</i> (2)
<i>Klebsiella oxytoca</i> (16)		16 (100)				<i>Staphylococcus warneri</i> (2)
<i>Klebsiella pneumoniae</i> (41)		41 (100)				6 (100)
<i>Kluyvera ascorbata</i> (1)		1 (100)				<i>Streptococcus agalactiae</i> (22)
<i>Kocuria</i> spp. (1)	1 (100)					<i>Streptococcus anginosus</i> (18)
<i>Ledercia</i> spp. (1)	1 (100)					<i>Streptococcus constellatus</i> (17)
<i>Moraxella</i> spp. (2)	2 (100)					<i>Streptococcus gallolyticus</i> (1)
<i>Moraxella catarrhalis</i> (19)		19 (100)				<i>Streptococcus intermedius</i> (2)
<i>Morganella morganii</i> (16)		16 (100)				<i>Streptococcus mitis</i> (1)
<i>Neisseria</i> spp. (3)	3 (100)					<i>Streptococcus pneumoniae</i> (17)
<i>Neisseria flavescens</i> (5)		5 (100)				<i>Streptococcus pyogenes</i> (35)
<i>Neisseria macacae</i> (1)		1 (100)			1 (100)	<i>Streptococcus saprophyticus</i> (3)
<i>Neisseria mucosa</i> (1)		1 (100)				<i>Streptococcus viridans</i> group (32)
<i>Neisseria sicca</i> (1)		1 (100)				<i>Trueperella bernardiae</i> (1)
<i>Ochrobactrum</i> spp. (2)	2 (100)					<i>Weissella</i> spp. (1)
<i>Oligella urethralis</i> (1)		1 (100)				
<i>Pantoea</i> spp. (2)	2 (100)					
<i>Pasteurella canis</i> (1)		1 (100)				
<i>Pasteurella multocida</i> (1)		1 (100)				
<i>Pasteurella</i> spp. (1)	1 (100)					
<i>Plesiomonas shigelloides</i> (2)		2 (100)				
<i>Proteus mirabilis</i> (22)		22 (100)				
<i>Providencia alcalifaciens</i> (4)		4 (100)				
<i>Providencia rettgeri</i> (1)		1 (100)				
<i>Providencia stuartii</i> (1)		1 (100)				
<i>Pseudomonas aeruginosa</i> (53)		53 (100)				
<i>Pseudomonas alcaligenes</i> (1)		1 (100)				
<i>Ralstonia pickettii</i> (1)		1 (100)				
<i>Salmonella</i> spp. (25)	25 (100)					
<i>Serratia liquefaciens</i> (1)		1 (100)				
<i>Serratia marcescens</i> (29)		29 (100)		1 (3.4)		
<i>Shewanella putrefaciens</i> (1)		1 (100)				
<i>Shigella</i> spp. (5)	5 (100)					
<i>Sphingobacterium multivorum</i> (1)		1 (100)				
<i>Stenotrophomonas maltophilia</i> (32)		32 (100)				
<i>Yersinia enterocolitica</i> (2)		2 (100)				
Total (597)	51	543	3			
					1 (50)	
						Total (399)
						40
						337
						22

spectral scores and the rate of species-level identification (12, 15), it involves additional hands-on steps, which can become laborious for the CLS performing batch setup of multiple isolates (15), increasing the preparation and identification time by up to fivefold per specimen (15, 17). Previous studies have demonstrated that although the direct-smear method is sufficient for Gram-negative bacteria identification to the species level, the yield for species-level identification of Gram-positive is low and typically requires an extraction step (12, 19, 21). In this study, we successfully demonstrated that identification of aerobic Gram-positive and Gram-negative organisms could be performed accurately and efficiently by MALDI-TOF MS and Bruker Biotyper system using the direct-smear method alone and a decreased score of ≥ 1.9 for species-level identification of both Gram-negative and Gram-positive organisms.

The cutoff score recommended by Bruker for genus-level and species-level identification are ≥ 1.7 and ≥ 2.0 , respectively. Using these parameters, studies have shown that approximately 95% of bacterial isolates in the clinical laboratory can be reliably identified to the species level (6, 7). However, these cutoff scores may be considered conservative and the rate of bacterial isolates correctly identified to the species level can be increased by lowering the scoring threshold (19, 21, 26–28). Ford and Burnham (19) found that all Gram-negative isolates with a spectra score of ≥ 1.9 were correctly identified to the species level with an improvement of 6.75% in identification rate compared to the manufacturer's cutoff of ≥ 2.0 . Another recent study reported a 21.6% increase in species-level identification of Gram-positive organisms using the direct-smear method and scores of ≥ 1.7 as compared to using a ≥ 2.0 cutoff score (18). Similarly, in our laboratory, a reduction in cutoff scores to ≥ 1.9 yielded a species-level identification rate of 96.4% as compared to 90.1% at a cutoff of 2.0, for both Gram-negative and Gram-positive organisms using the direct-smear method. The reduction in score threshold resulted in the correct identification of 57 additional isolates and one misidentified isolate. Lowering the cutoff was most beneficial in identifying Gram-positive organisms, as 37 additional isolates were identified compared to 20 Gram-negative isolates. *Streptococcus* spp. made up 19 of 37 isolates with scores between 1.9 and 1.99, whereas 14 of 37 isolates were *Staphylococcus* spp.

Multiple preparatory techniques exist for the generation of MALDI-TOF MS data from bacterial isolates, most commonly, the direct-smear, direct-plate extraction, and tube extraction methods. Previous studies have evaluated the use of direct-plate extraction for bacterial and yeast identification (18, 19, 29, 30). For the Gram-negative organisms, direct-plate extraction increased the identification rate by approximately 0.7% over the direct-smear method alone (19), whereas the results were more

striking for Gram-positive bacteria, with an increase of 14.3–17.2% in identification rates following direct-plate extraction (17, 18). In contrast, we determined that identification of Gram-negative and Gram-positive bacteria using direct-smear method alone is sufficient for routine testing in the clinical laboratory, as reflex to direct-plate extraction was required in only 1.1% (11/966) of isolates. Of the isolates that failed to produce spectral score using the direct-smear method, seven were Gram-negative and Gram-positive bacteria routinely isolated in the clinical laboratory, and most likely failed due to insufficient sample on the target plate, given that the same organisms were successfully identified by direct smear multiple times throughout the study. Direct-plate extraction further identified 8 of 11 isolates (three Gram-negative and five Gram-positive organisms) at a score of ≥ 1.9 . Overall, using a score cutoff of 1.9, the direct-smear method had a minimal effect on bacterial identification rates, with a 0.6% decrease for both Gram-negative (98.2–97.6%) and Gram-positive (95.7–95.1%) organisms, but allowed for an efficient streamlined workflow for routine testing.

Although bacterial identification by MALDI-TOF MS has proven to be very accurate, one limitation is the misidentification of viridans group streptococci, namely *S. mitis/oralis* (5, 6, 17, 18, 20). MALDI-TOF MS analysis of *S. mitis/oralis*, and other closely related members of the viridans group streptococci have invariably resulted in the misidentification of these isolates as *S. pneumoniae* with high spectral scores. Stevenson et al. reported misidentification of all *S. mitis* isolates analyzed as *S. pneumoniae* with scores > 1.9 (6). In our study, 20 viridans group streptococci isolates were misidentified as *S. pneumoniae*, 16 of which had spectral scores ≥ 1.9 , and 3 with scores between 1.8 and 1.89. The final isolate was identified as *S. mitis* by 16S rRNA gene sequencing. Members of the *S. viridians* group have been shown to have very high ($> 99\%$) sequence homology at the 16S rRNA locus (31), making it difficult to differentiate between the species (32). Other nucleic acid based methods have also exhibited low specificity in identifying members of the viridans group streptococci (33, 34). However, studies have demonstrated that discrimination between *S. pneumoniae* and other members of the *S. mitis* group is possible by analyzing characteristic MALDI-TOF MS peak profiles within the different species (35). In addition, analysis of spectral data using the Vitek MS database (bioMérieux) has been shown to be more specific for the identification of viridans group streptococci, compared to Bruker Biotyper system (36, 37). Bile solubility and/or optochin susceptibility testing is typically performed to discriminate between these organisms in the clinical laboratory (15).

Testing several variables in the preanalytical process, namely bacterial growth media, growth temperature, age of isolates, and interoperator variability confirmed the

robustness of Bruker Biotyper using direct-plate method. At a cutoff score of ≥ 1.9 , there were negligible effects on bacterial identification to the species level when these variables were introduced. Most notably, Gram-positive organisms grown on chocolate agar were found to have the lowest identification rate, albeit only 6.2% lower than Gram-positive organisms grown on blood agar throughout the study. Growth temperature and age of the isolates and different operators had no notable impact on identification rate, with the majority of isolates having spectral scores of ≥ 2.0 . In addition, there was no significant difference between light and heavy spotting on the target plate in our laboratory, which differs from a recent study that demonstrated statistically higher spectral scores for direct spotting with a light smear compared to heavy smear (19).

In conclusion, the direct-smear method was accurate and effective in streamlining the workflow of Gram-positive and Gram-negative bacterial identification in our clinical laboratory. For simplicity, we recommend using the direct-smear method for the majority of specimens commonly found in the clinical laboratory and an optimized spectral score cutoff of ≥ 1.9 for enhanced species-level identification rates. In the infrequent cases in which direct smear does not result in spectral data with high scoring, the direct-plate extraction method may be utilized with minimal effort to obtain improved scores.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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