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Optimization of Routine Identification of Clinically Relevant Gram-Negative Bacteria by Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry and the Bruker Biotyper

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) might complement and one day replace phenotypic identification of bacteria in the clinical microbiology laboratory, but there is no consensus standard regarding the requirements for its validation prior to clinical use in the United States. The objective of this study was to assess the preanalytical variables influencing Gram-negative identification by use of the Bruker Biotyper MALDI-TOF MS system, including density of organism spotting on a stainless steel target plate and the direct overlay of organisms with formic acid. A heavy smear with formic acid overlay was either superior or equivalent to alternative smear conditions. Microbiological preanalytical variables were also assayed, such as culture medium, growth temperature, and use of serial subculture. Postanalytical analysis included the application of modified species-level identification acceptance criteria. Biotyper identifications were compared with those using traditional phenotypic methods, and discrepancies were resolved with 16S rRNA gene sequencing. Compared to the recommended score cutoffs of the manufacturer, the application of optimized Biotyper score cutoffs for species-level identification increased the rate of identification by 6.75% for the enteric Gram-negative bacteria and 4.25% for the nonfermenting Gram-negative bacteria. Various incubation temperatures, growth medium types, and repeat subcultures did not result in misidentification. We conclude that the Bruker MALDI Biotyper is a robust system for the identification of Gram-negative organisms in the clinical laboratory and that meaningful performance improvements can be made by implementing simple pre- and postanalytical techniques.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) employs soft ionization to detect individual intact biomolecules within complex solutions. Practical use of MALDI-TOF has been facilitated by the development of matrices, such as α-cyano-4-hydroxycinnamic acid (1). While the potential for the identification of bacteria by their individual mass spectrometric "fingerprints" has long been appreciated (2), the adoption of MALDI-TOF MS in clinical microbiology laboratories in the United States has been hindered until recently by a lack of available platforms with databases of bacterial whole-cell MALDI-TOF reference spectra.

Recent studies using the Bruker Biotyper MALDI-TOF MS platform have revealed that this system might correctly identify bacteria to the species level 95% of the time, with the remaining 5% comprising unidentified or erroneously identified isolates (3, 4). These studies invariably used Bruker's recommended scoring cutoffs (a Biotyper score of ≥2.0 for species-level identification and ≥1.7 for genus-level identification) to define the confidence with which a correct identification had been made. Alatoom and colleagues (5) noted that the preparatory extraction of the protein fraction of Gram-positive organisms was necessary to obtain the species-level identification score recommended by Bruker. This raised questions of how often extraction would be used in routine practice compared to spotting whole cells directly from culture medium onto MALDI-TOF target plates and whether the cutoffs supplied by the manufacturer are optimal for all classes of bacteria.

Subtleties of the MALDI-TOF analytical techniques have the potential to modulate performance. The objective of this study was to validate the Bruker Biotyper system for clinical use in identifying Gram-negative enteric and non-glucose-fermenting organisms, while also assessing the impact of variables routinely encountered in the clinical laboratory. We focused on variables that are encountered in routine clinical practice in order to derive a comprehensive protocol for how Gram-negative clinical isolates might be optimally identified by use of MALDI-TOF MS. An accompanying paper by McElvania TeKippe et al. (6) focuses on the optimization of the Bruker Biotyper system for identification of Gram-positive bacteria.

(This work was presented in part at the 22nd Annual European Congress of Clinical Microbiology and Infectious Diseases, London, England, April 2012.)

MATERIALS AND METHODS

Clinical isolates. The clinical isolates tested in this study were recovered in routine clinical workflow from specimens submitted to the St. Louis Children's Hospital Microbiology Laboratory from April 2011 to August 2011; unusual isolates from freezer stocks were also used (Tables 1 and 2). Cultures were processed per standard laboratory practices and, once pure culture was obtained, enteric Gram-negative bacteria (EGNB) and non-glucose-fermenting/fastidious Gram-negative bacteria (NFGNB) were

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TABLE 1 Identity and analysis of isolates for enteric Gram-negative bacteria

Genus	Species	No. (%) of isolates	Total no. in genus	Isolates requiring (*n*): 16S rRNA gene sequencing[a]	Isolates requiring (*n*): Repeat analysis
Enterobacter		1	41		
	E. cloacae	34		*	2
	E. aerogenes	1			
	E. gergoviae	4			
	E. amnigenus	1			1
Proteus		0	8		
	P. mirabilis	8			
Klebsiella		0	42		
	K. oxytoca	14			
	K. pneumoniae	28			
Citrobacter		0	21		
	C. freundii complex	20		*	
	C. braakii	1			
Aeromonas		0	4		
	A. caviae	4			
Shigella		0	7		
	S. sonnei	7			
Plesiomonas		0	4		
	P. shigelloides	4			1
Morganella		0	1		
	M. morganii	1			
Providencia		0	2		
	P. rettgeri	1			
	P. stuartii	1			
Rahnella	*R. aquatilis*	2	2		
Escherichia		0	39		
	E. vulneris	1			
	E. coli	36			
	E. hermannii	2		2	2
Salmonella		10	10		
Pantoea		9	12	8	9
	P. agglomerans	2			2
	P. ananatis	1			
Serratia		0	15		
	S. marcescens	15			
Total		208	208	10 (*)	17
Genera			14		
Species			22		
Extent of analysis performed					
No extraction		191 (91.8)			
Repeat analysis		17 (8.2)			
16S sequencing		12 (5.8)			

[a] Each asterisk indicates that one *E. kobei* and one *C. freundii* isolate (see Table 5) had correct MALDI-TOF identifications to the species level but were sequenced because of an inadequate phenotypic identification. These did not require repeat analysis; by definition, the rest of the isolates for which 16S rRNA gene sequencing was performed required repeat analysis. Other isolates (right column) were identified on repeat analysis, averting 16S rRNA gene sequencing.

TABLE 2 Identity and analysis of isolates, non-glucose-fermenting Gram-negative bacteria

Genus	Species	No. (%) of isolates	Total no. for the genus	Isolates requiring (*n*):	
				16S rRNA gene sequencing[a]	Repeat analysis
Achromobacter		5	17		4
	A. denitrificans	1			
	A. xylosoxidans	11			1
Acinetobacter		9	14	2	3
	A. radioresistens	1			
	A. ursingii	4			1
Alcaligenes		0	1		
	A. faecalis	1			
Bordetella		0	2		
	B. holmesii	2			2
Brevundimonas		1	1		
Burkholderia		0	23		
	B. cepacia	2			
	B. cenocepacia	1			
	B. gladioli	13			
	B. multivorans	6		*	
	B. pyrrocinia	1			
Campylobacter		0	2		
	C. jejuni	2			
Chryseobacterium		0	15		
	C. indologenes	15			7
Cupriavidus		1	1	*	
Delftia		0	3		
	D. acidovorans	1			
	D. tsuruhatensis	2		2	2
Eikenella		1	5		1
	E. corrodens	4			2
Gardnerella		0	5		
	G. vaginalis	5			1
Haemophilus		0	54		
	H. influenzae	25			1
	H. parahaemolyticus	1			
	H. parainfluenzae	27			2
	H. paraphrophilus	1			1
Herbaspirillum	*H. huttiense*	1	1	*	
Moraxella		1	7		1
	M. catarrhalis	6			
Neisseria		0	8		
	N. gonorrhoeae	3			
	N. lactamica	1			
	N. macacae	1			
	N. weaveri	3		*	
Ochrobactrum		0	5		
	O. anthropi	2		1	1
	O. intermedium	1			
	O. tritici	1		*	

(Continued on following page)

TABLE 2 (Continued)

Genus	Species	No. (%) of isolates	Total no. for the genus	Isolates requiring (*n*): 16S rRNA gene sequencing[a]	Isolates requiring (*n*): Repeat analysis
Oligella	*O. ureolytica*	1	1		1
Paracoccus	*P. yeei*	1	1		1
Pasteurella		0	2		
	P. canis	1			
	P. multocida	1			
Plesiomonas		0	2		
	P. shigelloides	2			
Pseudomonas		0	40		
	P. aeruginosa	31			3
	P. fluorescens	5		2	2
	P. monteilii (*putida group*)	2			
	P. oryzihabitans	2			2
Rhizobium		0	3		
	R. radiobacter	2			
	R. larrymoorei	1		1	1
Roseomonas		2	4	2	2
	R. mucosa	2		*	
Sphingomonas	*S. paucimobilis*	1	1	*	
Stenotrophomonas	*S. maltophilia*	35	35		2
Total		252	252	10 (*)	44
Genera			26		
Species			44		
Extent of analysis performed					
No extraction		207 (82.1)			
Repeat analysis		44 (17.5)			
16S sequencing		17 (6.8)			

[a] Each asterisk indicates that one isolate each of *C. pauculus*, *B. multivorans*, *N. weaveri*, *S. paucimobilis*, *R. mucosa*, *H. huttiense*, and *O. tritici* (see Table 5) had correct MALDI-TOF identifications to the species level but were sequenced because of an inadequate or incorrect phenotypic identification. These did not require repeat analysis; by definition, the rest of the isolates for which 16S rRNA gene sequencing was performed required repeat analysis. Other isolates (right column) were identified on repeat analysis, averting 16S rRNA gene sequencing.

identified according to the standard operating procedures (SOPs) of our laboratory. This included a variety of phenotypic, automated, and commercial methods, such as Vitek 2 (bioMérieux, St. Louis, MO), Phoenix (Becton-Dickson, Sparks, MD), API 20 NE (bioMérieux), and other manual identification methods. In parallel to routine processing, colonies were applied to a MALDI-TOF target as part of the normal workflow and were batch processed for MS analysis at the end of the workday. MALDI-TOF operators were blinded to the phenotypic identities of the organisms. The Biotyper scoring system involves a pattern-matching algorithm that queries a database of spectra to generate a score reflecting the probability that an identification is correct. Per the recommendations of the manufacturer, a score of ≥2.0 is considered an accurate species-level identification, a score from 1.7 to 1.99 is considered accurate to the genus level and a score of <1.7 is considered unreliable. For our study, positive identifications were defined as those with a Biotyper score of ≥2.0 when analyzed in automatic mode under any of four spotting conditions (described below); isolates with lower scores were reanalyzed in manual mode. If a score of ≥2.0 was still not achieved, the sample was subcultured for reprocessing the next day. Failure to obtain an identification after replating prompted chemical extraction of the isolate. Failure to identify the organism after extraction, or any MALDI-TOF/phenotypic discrepant identification, prompted 16S rRNA gene sequencing of the isolate. Isolate characteristics and the extent of analysis are summarized in Tables 1 and 2.

Individual MALDI-TOF analyses. Two hundred eight EGNB isolates (97.6% from the regular clinical workflow) and 252 NFGNB isolates (88.9% from the regular clinical workflow) were spotted using four methods for MALDI-TOF analysis as follows. For a "heavy smear," individual colonies were picked with the tip of a sterile wooden toothpick and spotted with a circular motion onto a 96-spot reusable Bruker stainless steel MALDI target plate (part no. 224990, Bruker Daltonics, Billerica, MA). Without picking up more material from the original colony, the isolate was spotted again to the adjacent spot on the MALDI target, for a "light smear." Heavy and light smears were repeated, allowed to dry, and overlaid with 1 μl 100% formic acid. Once dry, all four spots were overlaid with 1 μl of an α-cyano-4-hydroxycinnamic-acid matrix (part no. 255344, Bruker). Two microliters of bacterial test standard (BTS) (part no. 255343, Bruker), prepared according to the instructions of the manufacturer, were plated in duplicate for each run for calibration and quality control purposes. Identifications were assigned using the Bruker Biotyper software v3.0.

TABLE 3 Temperature studies of enteric[a] and non-glucose-fermenting[b] Gram-negative bacteria

Bacterial type and identification	% of isolates, by temp and spotting condition[c]					
	4°C		Room temp		35°C	
	DIR	FA	DIR	FA	DIR	FA
Enteric Gram-negative						
Misidentified	0	0	0	0	0	0
Unidentified	31.2	25.0	6.3	6.3	6.3	6.3
Correct, score > 2.0	68.8	75.0	93.8	93.8	93.8	93.8
Nonfermenting Gram-negative						
Misidentified	0.0	2.1	0.0	0.0	0.0	0.0
Unidentified	68.7	72.9	64.6	52.1	29.2	43.7
Correct, score > 2.0	31.3	25.0	35.4	47.9	70.8	56.3

[a] Twenty-four isolates consisting of the following (number of isolates in parentheses if more than one): *A. caviae*, *C. freundii* (2), *E. aerogenes*, *E. cloacae* (2), *E. gergoviae*, *E. coli* (5), *K. oxytoca* (2), *K. pneumoniae* (3), *M. morganii*, *P. mirabilis* (2), *Salmonella* spp. (2), and *S. marcescens* (2).

[b] Twenty-five isolates consisting of the following (number of isolates in parentheses if more than one): *A. xylosoxidans* (2), *B. cenocepacia*, *B. cepacia*, *B. gladioli* (2), *C. indologenes* (2), *H. influenzae* (2), *H. parainfluenzae*, *M. catarrhalis* (2), *P. ananatis*, *P. canis*, *P. shigelloides*, *P. aeruginosa* (5), and *S. maltophilia* (4).

[c] DIR, direct spotting without formic acid treatment; FA, formic acid treated. Data from heavy and light spotting conditions are pooled.

Medium, temperature, and subculture studies. A subset of 24 diverse isolates of EGNB (Table 3, footnote *a*) were cultured on sheep blood, MacConkey, chocolate, Hektoen enteric, blood with ampicillin, and MacConkey-sorbitol agars and were analyzed as described above after overnight incubation at 35°C (under 5% CO_2 atmosphere for sheep blood and chocolate agars and room air for all others). Twenty-five diverse isolates of NFGNB (Table 3, footnote *b*) were cultured on sheep blood, MacConkey, chocolate, oxidation-fermentation polymyxin-bacitracin-lactose (OFPBL), and *Haemophilus* isolation plate (HIP) agars and were analyzed as described above after overnight incubation at 35°C (under the atmosphere conditions described above).

For temperature studies, the isolates from the medium studies were subcultured to MacConkey agar, incubated at 35°C in room air for 24 h, and then incubated at 35°C, 4°C, and room temperature for 5 consecutive days and analyzed using the four direct smear techniques described above. For subculture studies, the isolates from the medium studies were subcultured to MacConkey agar once per day for 5 consecutive days and were analyzed four times as described above on days 3, 4, and 5. All media for the medium, temperature, and subculture studies were purchased from Remel (Lenexa, KS).

Organism extraction. For the extraction protocol (5, 7), a single large colony was transferred to a microcentrifuge tube containing 300 μl of molecular-grade water. To this, 900 μl of 100% ethanol was added, and the solution was vortexed and centrifuged at 13,780 × *g* for 2 min. The supernatant was decanted completely and the pellet was resuspended in 50 μl of 70% formic acid and vortexed, and then it was mixed with 50 μl of 100% acetonitrile and vortexed again. This mixture was centrifuged, and 1 μl of the supernatant was plated to a steel target, allowed to dry, overlaid with matrix, and analyzed as described above. All reagents were high-performance liquid chromatography (HPLC) grade or better.

16S rRNA gene sequencing and sequence analysis. Samples that were not assigned a Biotyper score of ≥2.0 using any of the four direct smear techniques, or those whose MALDI-TOF identification was discrepant with the identification determined by traditional phenotypic methods, were identified by use of standard 16S rRNA gene sequencing (8, 9). The resulting sequences were aligned with the GenBank nonredundant/nucleotide collection (nr/nt) (10), Ribosomal Database Project (RDP) (11), and GreenGenes databases (12). Interpretation of sequencing results was in accordance with the criteria of the CLSI MM18-A guideline (9).

Supplemental testing and taxonomic resolution. For EGNB, the isolates identified by MALDI-TOF as *Escherichia coli* were confirmed by an evaluation of lactose fermentation and the use of a spot indole test; if an isolate was lactose and indole negative, its identity was changed to *Shigella* sp. (based on our local epidemiology) (13). Any isolate identified as *Raoultella ornithinolytica* was tested for ornithine decarboxylase activity (14) and, if negative, its identity was changed to *Klebsiella oxytoca* (13, 15). *Citrobacter* and *Enterobacter* species identified by MALDI-TOF were grouped as "*Citrobacter freundii* complex" or "*Enterobacter cloacae* complex," respectively, if it was appropriate in accordance with how these organisms were routinely reported in the clinical laboratory.

For NFGNB, the genomovars of *Burkholderia cepacia* were grouped as "*B. cepacia* complex" for reporting and interpretation purposes, and subspecies within the *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas aeruginosa* groups were each reported to the group level, respectively.

Statistical analysis. Tests of statistical significance (specified where they are presented) were performed with Systat 12 software (Systat Software, Inc., Chicago, IL). A *P* value of ≤0.05 was considered significant.

RESULTS

MALDI-TOF performance using manufacturer's scoring cutoffs. For EGNB, Biotyper scores for both direct light (average score, 2.17) and heavy smear with formic acid overlay (average score, 2.19) were statistically superior to those for standard direct spotting with a heavy smear (average score, 2.07), according to a repeated-measures analysis of variance (ANOVA) comparing all possible combinations of groups ($P < 0.01$); the heavy smear with formic acid group demonstrated the highest rate of identification to the species level (Table 4). Notably, formic acid treatment reduced the number of unidentified isolates from 36 to 27. For NFGNB, a Biotyper score for a light formic acid-treated smear was statistically inferior ($P < 0.01$) to the heavy-smear formic acid-treated isolates, but otherwise, all other pairs of treatments were not statistically different by repeated-measures ANOVA comparing the scores for all possible combinations of groups. Superior scores for the heavy-smear formic acid-overlaid group were reflected by a higher rate of species-level identification using the cutoff of the manufacturer (70.6% versus 63.9%, for heavy- and light-smear formic acid-overlaid groups, respectively [Table 4]). This is in contrast to EGNB, where formic acid treatment did not result in a statistically different rate of identification for this organism group (Table 4). There was only one misidentification in this data set, which was in the formic acid-treated heavy-smear group of the NFGNB. An isolate of *Stenotrophomonas maltophilia* was falsely identified as *Cupriavidus pauculus*, with a Biotyper score of 2.08.

TABLE 4 Classification statistics using a cutoff of ≥2.0 for species identification and >1.7 to <2.0 for genus identification

Classification of identification	No. (%) of isolates, by method			
	Direct smear		Smear with formic acid overlay	
	Heavy	Light	Heavy	Light
Enteric Gram-negative bacteria				
To species level	169 (81.3)	172 (82.7)	181 (87.0)	175 (84.1)
Only genus-level resolution	3 (1.4)	0	0	0
Unidentified	36 (17.3)	36 (17.3)	27 (13.0)	33 (15.9)
Misidentified	0	0	0	0
Nonfermenting Gram-negative bacteria				
To species level	179 (71.0)	168 (66.7)	178 (70.6)	161 (63.9)
Only genus-level resolution	8 (3.2)	4 (1.6)	3 (1.2)	3 (1.2)
Unidentified	65 (25.8)	80 (31.7)	70 (27.8)	88 (34.9)
Misidentified	0	0	1 (0.4)	0

Improved MALDI-TOF performance with optimized scoring cutoffs. For EGNB, all isolates with a Bruker Biotyper score of ≥1.9 were correctly identified to the species level. When the score was <1.9, identifications were a mix of incorrect and correct, rendering the identification "to the genus level" irrelevant in this context. Therefore, we applied a single Bruker Biotyper cutoff for a correct identification to the species level of 1.9, which resulted in a rate of identification of 88.0% (a 1% improvement) for a formic acid-treated heavy smear, a total improvement of 6.75% over the use of Bruker's recommended species-level score cutoff with a non-formic-acid-treated smear. Nine of 16 additional isolates identified were members of the *E. cloacae* group, which is genetically homogeneous (16) and is a difficult group to identify by use of MALDI-TOF analysis (3).

For the NFGNB, formic acid treatment had no statistical effect on scores and no appreciable effect on the rates of identification when this group of organisms was analyzed together (Table 4). Therefore, we sought to improve the rate of identification by dividing the NFGNB into "fastidious" isolates (those that do not grow on MacConkey agar) and "nonfastidious" isolates (those that do grow on MacConkey agar) and then reanalyzing the data by constructing modified receiver operating curves for the heavily smeared spots to choose a score for clinical reporting that maximized the rate of correct identifications while minimizing misidentifications (data not shown). The highest yield of identifications to the species level without any misidentifications was achieved with a heavy smear without formic acid treatment and with Biotyper score cutoffs of ≥2.0 for fastidious NFGNB and ≥1.9 for nonfastidious NFGNB. This raised the rate of identification to 77% for the NFGNB as a group, an improvement of 4.25% over direct heavy spotting with a cutoff of ≥2.0.

Subanalysis of failed and discrepant identifications. Most unidentified isolates were random, i.e., one of four spots of a set failed to be identified. Seventeen EGNB samples required repeat analysis, resulting in 5 additional identifications. Twelve isolates were selected for 16S rRNA gene sequencing, as they were not identified by use of MALDI-TOF (i.e., none of the four replicates had a score of >2.0) and/or there was a discrepancy with the phenotypic method or the isolate was not identified by use of phenotypic means. Of these 12 samples, phenotypic identification was always correct, while MALDI-TOF agreed with the phenotypic identification twice but failed to identify 8 *Pantoea* spp. and 2 *Escherichia hermannii* isolates (Table 5). A single *C. freundii* isolate and a single *E. cloacae* isolate were identified correctly by MALDI-TOF, phenotypic methods, and 16S sequencing (Table 5). Of these isolates, all were present in the Biotyper 3.0 database except *E. hermannii*.

Repeat analysis of 45 NFGNB isolates (17.9%) resulted in 28 additional identifications. Seventeen isolates (6.8%) were then selected for 16S sequencing, and of these, MALDI-TOF was correct to the species level 7 times (*Cupriavidus pauculus*, *Burkholderia multivorans*, *Sphingomonas paucimobilis*, *Roseomonas mucosa*, *Ochrobactrum tritici*, *Herbaspirillum huttiense*, and *Neisseria weaveri*), while phenotypic identification was correct 5 times, but generally with less resolution when identified to the species level (*Cupriavidus* sp., two *Delftia* spp., *Sphingomonas* sp., and *Ochrobactrum anthropi*); both were correct 4 times, and neither was correct 5 times (Table 5). All organisms identified in this group were present in the Biotyper database.

Medium, temperature, and subculture studies. A subset of 24 EGNB and 25 NFGNB isolates was analyzed on a number of different medium types to assess the impact of selective medium on MALDI-TOF MS identification of Gram-negative bacteria. For EGNB, performance on most media, with or without formic acid treatment, was similar. Hektoen enteric medium resulted in a rate of successful identification that was approximately 20% less than rates of the other medium types, but there were no misidentifications on any medium type.

For NFGNB, organisms that did not grow on a particular medium were excluded from analysis. There was one misidentification from MacConkey agar (*Burkholderia gladioli* was identified as *Chryseobacterium indologenes* by MALDI-TOF MS, with a relatively high score of 2.33) with formic acid treatment and a heavy smear. Otherwise, the rates of identification ranged widely, from over 90% on sheep blood agar to less than 60% on OFPBL agar.

The isolates from the medium studies were subcultured to MacConkey agar at 35°C for 24 h and then analyzed once after 5 days of further incubation at 4°C, 25°C, and 35°C to assess the impact of growth temperature on the rate of MALDI-TOF MS identification. The rate of identification decreased with decreasing temperature. The rate of identification for EGNB was identical from 35°C to room temperature (93.8%) and dropped by 25% at 4°C. This effect was more pronounced for NFGNB; the rate of

TABLE 5 16S rRNA gene sequencing for enteric and nonfermenting Gram-negative bacteria

16S rRNA gene sequencing ID[a]	MALDI-TOF ID[a]	MALDI-TOF score	Organism in Biotyper database?	Phenotypic ID[a]
Enteric Gram-negative bacteria				
Escherichia hermannii	Not reliable	1.59	No	*E. hermannii*
Escherichia hermannii	*Salmonella* sp.	1.80	No	*E. hermannii*
Citrobacter/Enterobacter/Pantoea	*C. freundii*[b]	2.08	Yes	*Citrobacter* sp.
Pantoea spp.	*K. oxytoca*	1.78	Yes	*P. agglomerans*
Pantoea spp.	Not reliable	1.48	Yes	*P. agglomerans*
Pantoea spp.	*R. aquatilis*	1.73	Yes	*P. agglomerans*
Pantoea spp.	Not reliable	1.67	Yes	*P. agglomerans*
Pantoea spp.	Not reliable	1.29	Yes	*P. agglomerans*
Pantoea spp.	Not reliable	1.37	Yes	*P. agglomerans*
Pantoea spp.	Not reliable	1.34	Yes	*P. agglomerans*
Pantoea spp.	Not reliable	1.31	Yes	*P. agglomerans*
E. cloacae	*E. kobei*[c]	2.11	Yes	*E. cloacae* complex
Nonfermenting Gram-negative bacteria				
O. anthropi/lupini/tritici/cytisi	*O. tritici*	1.71	Yes	*O. anthropi*
Cupriavidus spp.	*C. pauculus*	2.29	Yes	*Cupriavidus* sp.
Acinetobacter spp.	Not reliable	1.53	Yes	Not reliable
Acinetobacter spp.	Not reliable	1.49	Yes	*Acinetobacter* sp.
B. cepacia complex	*B. multivorans*	2.26	Yes	*Acinetobacter* sp.
N. weaveri	*N. weaveri*	2.12	Yes	None
Roseomonas	Not reliable	1.59	Yes	None
Roseomonas	Not reliable	1.43	Yes	None
P. fluorescens group	Not reliable	1.56	Yes	*P. putida*
P. fluorescens group	Not reliable	1.59	Yes	*P. putida*
D. tsuruhatensis	*D. acidovorans*	1.91	Yes	*Delftia* sp.
Sphingomonas spp.	*S. paucimobilis*	2.47	Yes	*Sphingomonas* sp.
Roseomonas spp.	*R. mucosa*	2.11	Yes	None
Herbaspirillum spp.	*H. huttiense*	2.33	Yes	None
O. tritici	*O. tritici*	2.09	Yes	*O. anthropi*
R. larrymoorei	Not reliable	1.39	Yes	*R. radiobacter*
D. tsuruhatensis	*D. acidovorans*	1.76	Yes	*Delftia* sp.

[a] ID, identification.
[b] A second ID of *C. youngae* (score 2.02) was obtained, prompting rRNA gene sequencing.
[c] While *E. kobei* is in the *E. cloacae* complex, a second ID of *E. asburiae* (score of 2.07, also in the *E. cloacae* complex) was obtained, prompting rRNA gene sequencing that could not distinguish these two species.

identification at 35°C was cut in half at room temperature and declined an additional 4% to 31.3% at 4°C (Table 3). In spite of the low rate of identification, there was only one misidentification in the heavy smear formic acid-treated NFGNB group at 4°C, which again was a *B. gladioli* isolate that was called *C. indologenes* (Biotyper score, 2.08).

On serial subculture of 24 (EGNB) or 25 (NFGNB) isolates to sheep blood agar once per day for 5 days, the mean scores and rates of identification were essentially identical day-to-day (data not shown). We were therefore able to calculate day-to-day scoring coefficients of variation for the different spotting conditions, which ranged from 3.0 to 5.6% for the enterics and 11.0 to 28.9% for the nonfermenters.

DISCUSSION

Currently, phenotypic identification methods consist of a variety of rapid benchtop, tubed, manual parallel, and automated parallel biochemical tests that are used in concert with Gram stain to obtain species-level identification. Identification of nonfermenting Gram-negative bacteria is frequently protracted and expensive. This difficulty is usually attributed to the mucoid nature of these isolates and/or the fact that they may be biochemically inert (17–19). This situation is possibly improved by MALDI-TOF, but because no system is currently cleared by the FDA, laboratories are presented with only generic scoring cutoffs for species-level identification and a lack of a specific procedures or clear framework providing detailed information about related technical nuances.

In building this framework for Gram-negative organisms, we found that the rate of identification to the species level was 88% for EGNB (heavy smear and formic acid overlay) and 77% for NFGNB (heavy smear and no formic acid overlay) and our optimized Biotyper cutoffs. While these rates are consistent with published data for EGNB (4, 13, 20, 21) and NFGNB (22–24), this study is unique in its assessment of preanalytical (temperature, media type, and subculture), analytical (spotting density, formic acid overlay, and database completeness), and postanalytical (Biotyper scoring cutoffs, additional biochemical testing required, and sequencing analysis) variables and in its focus on the identification of organisms directly from the routine clinical workflow. For this reason, we believe the approach outlined here might serve as a template for laboratories that are considering validation and optimization of MALDI-TOF systems.

Using our optimized procedure, the Bruker MALDI Biotyper system made only two erroneous identifications across all studies,

which examined a total of 460 isolates four times each. Our rate of identification to the species level falls between that of 16S rRNA gene sequencing and that of standard phenotypic identification systems, such as Vitek 2 or API 20 NE (17, 25), with MALDI-TOF being markedly faster than either of these other methods. An exception where phenotypic methods were superior was in the identification of organisms that form durable, adherent, or mucoid colonies, such as *Pantoea agglomerans* and mucoid *Pseudomonas* spp.; this is consistent with previous findings that mucoid organisms can represent a challenge for MALDI-TOF analysis (7, 26, 27).

Many clinical specimen types, such as stool and respiratory specimens, are plated to a variety of selective and differential media to minimize the overgrowth of normal flora and facilitate the recovery of pathogens. The ability to analyze isolates directly on these medium types, rather than subculturing to enriched medium first, can improve the time it takes to identify these isolates. It is intuitively understood that incubation on different media may alter the MALDI-TOF spectral profile for a given isolate (26, 28, 29), and others have noted that Hektoen enteric medium results in poor performance or requires a full extraction (13, 30). However, we found that this medium type, as well as other selective media, such as MacConkey and OFPBL, reduced the rate of identification but induced no misidentifications, verifying that MALDI-TOF analysis can be performed from primary subcultures on selective agar.

A final practical consideration was the burden of additional testing required in order to obtain reliable identifications from the isolates recovered in routine workflow. Among Gram-negative organisms, *Shigella* spp. and *E. coli* are indistinguishable from each other using 16S rRNA gene sequencing or MALDI-TOF (20, 31, 32), which is attributed to the taxonomic proximity of these two organisms (33); individual laboratories will need procedures to reconcile this based on local epidemiology and the availability of additional biochemical testing methods. An unanticipated but not surprising (14) tendency to call *K. oxytoca* isolates *R. ornithinolytica* is easily remedied using ornithine decarboxylase.

In routine practice, the need to perform 16S rRNA sequencing on ~6% of isolates might be balanced by the reduced need for additional biochemical testing and the previously reported low cost of consumables for MALDI-TOF testing (4, 27). Others (34) have also found that a time savings of many days is possible with biochemically inert nonfermenting Gram-negative organisms.

Bruker's generic scoring cutoff of ≥2.0 for a species-level identification was overly conservative for EGNB and nonfastidious NFGNB. When the threshold for species-level identification was reduced to ≥1.9 for EGNB, this resulted in the identification of 17 additional isolates, 9 of which were *Enterobacter* spp. In clinical use, unless other tests have been performed to identify an isolate as Gram-negative enteric or nonfermenting Gram-negative, we suggest that a cutoff of ≥1.9 be used for any Gram-negative organism that grows on MacConkey agar and ≥2.0 be used for those that do not or that were not grown on MacConkey agar in primary culture. From the findings of our medium, temperature, and subculture studies, we recommend that if an initial identification is >0.2 Biotyper scoring units below the cutoff, the isolate should be subcultured to sheep blood agar, incubated at 35°C, and reanalyzed the next day.

In conclusion, we provide a comprehensive assessment of the major variables that influence MALDI-TOF MS identification of clinically relevant Gram-negative bacteria. The major finding of this study is that the use of a single spot overlaid with formic acid and the application of less-stringent scoring cutoffs for EGNB and nonfastidious NFGNB improved the rates of identification without inducing misidentifications. As data acquisition, spectral databases, and algorithms for spectral pattern matching evolve, performance is likely to improve and the specific failings of the present system might be remedied. With simple procedural optimizations, the rates of correct identification and misidentification (higher and lower, respectively) for even the most fastidious organisms are superior to those using biochemical methods (35), positioning MALDI-TOF to supplant them in the clinical microbiology laboratory.

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