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High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories[▽]

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is suitable for high-throughput and rapid diagnostics at low costs and can be considered an alternative for conventional biochemical and molecular identification systems in a conventional microbiological laboratory. First, we evaluated MALDI-TOF MS using 327 clinical isolates previously cultured from patient materials and identified by conventional techniques (Vitek-II, API, and biochemical tests). Discrepancies were analyzed by molecular analysis of the 16S genes. Of 327 isolates, 95.1% were identified correctly to genus level, and 85.6% were identified to species level by MALDI-TOF MS. Second, we performed a prospective validation study, including 980 clinical isolates of bacteria and yeasts. Overall performance of MALDI-TOF MS was significantly better than conventional biochemical systems for correct species identification (92.2% and 83.1%, respectively) and produced fewer incorrect genus identifications (0.1% and 1.6%, respectively). Correct species identification by MALDI-TOF MS was observed in 97.7% of *Enterobacteriaceae*, 92% of nonfermentative Gram-negative bacteria, 94.3% of staphylococci, 84.8% of streptococci, 84% of a miscellaneous group (mainly *Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Eikenella*, and *Kingella* [HACEK]), and 85.2% of yeasts. MALDI-TOF MS had significantly better performance than conventional methods for species identification of staphylococci and genus identification of bacteria belonging to HACEK group. Misidentifications by MALDI-TOF MS were clearly associated with an absence of sufficient spectra from suitable reference strains in the MALDI-TOF MS database. We conclude that MALDI-TOF MS can be implemented easily for routine identification of bacteria (except for pneumococci and viridans streptococci) and yeasts in a medical microbiological laboratory.

Identification of bacteria and yeasts is generally based on conventional phenotypic methods, encompassing culture and growth patterns on specific media, Gram staining, and morphological and biochemical characteristics. Although results of Gram staining can be achieved within minutes, complete identification usually takes 1 or more days. In addition, tests may be difficult to interpret or inconclusive and require specialized staff. Recent molecular methods for microbial identification, such as real-time PCR, sequence analysis, or microarray analysis, have found some application in bacteriology. However, these methods do not provide the complete solution in routine bacterial identifications. To optimize care of patients with infectious diseases, there still is an urgent need for rapid and simple techniques for microbial identification.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been used to analyze many different biological molecules. The application of microbial identification based on species-specific spectra of peptides and protein masses by mass spectrometry was first reported about 30 years ago (1). By further improvement of the technique, a rapid, accurate, easy-to-use, and inexpensive method has become available for identification of microorganisms (4, 14, 27). MALDI-TOF MS can be used for accurate and rapid identification of various microorganisms, such as Gram-positive bacteria (2, 3, 9, 10, 22, 26), *Enterobacteriaceae* (5), nonfermenting bacteria (6, 19–21), mycobacteria (12, 16, 24), anaerobes (10, 23), and yeasts (18, 25). Most studies have reported on MALDI-TOF MS identification of a single strain or family of microorganisms in a research setting. Only one study applied MALDI-TOF MS for identification of bacteria—but not yeasts—in conventional microbiology settings but did not evaluate the results for individual bacteria at the species level (27). In the present study, identification of bacteria by MALDI-TOF MS was extensively evaluated for both bacterial and yeast species identification in an academic medical microbiologic laboratory.

MATERIALS AND METHODS

Bacterial and yeast isolates. All isolates were recovered from routine examination of clinical specimens submitted to the microbiological laboratory, such as blood, urine, pus, biopsy, swab from any site of the body, cerebrospinal fluid, respiratory tract, and wound specimens. For the retrospective study, 327 isolates were selected in such a manner that a wide range of bacteria and yeasts, including strains highly resistant to antibiotics, was obtained with representative numbers of isolates per strain (Table 1). All isolates were recovered from glycerin-broth and cultured on 5% sheep blood agar or chocolate agar (*Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Eikenella*, and *Kingella* [HACEK] bacteria). For the prospective study, all isolates recovered from clinical specimens were prospectively included over a 5-week period (Table 1).

Culture conditions and sampling. The isolates were recovered after aerobic, microaerophilic, or anaerobic incubation at 35°C on 5% sheep blood agar, chocolate agar, cysteine-lactose electrolyte-deficient (CLED) agar, and Sab-

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TABLE 1. List of bacterial and yeast strains evaluated for identification by MALDI-TOF MS

Group	Retrospective study strains	Prospective study strains
Enterobacteriaceae	*Citrobacter freundii*, *Citrobacter koseri*, *Citrobacter murliniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella ornithinolytica Klebsiella oxytoca*, *Klebsiella/Raoultella planticola*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella* group *B/C/D*, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Typhimurium, *Serratia marcescens*, *Yersinia enterica*, *Yersinia rohdei*	*Citrobacter amalonaticus*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter aerogenes*, *Enterobacter asburiae*, *Enterobacter cloacae* (complex), *Escherichia coli*, *Hafnia alveii*, *Klebsiella ornithinolytica*, *Klebsiella oxytoca*, *Klebsiella/Raoultella planticola*, *Klebsiella pneumoniae*, *Morganella morganii*, *Pantoea agglomerans*, *Proteus mirabilis*, *Proteus vulgaris*, *Providentia rettgeri*, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhi, *Serratia marcescens*, *Yersinia enterica*
Nonfermentative Gram-negative rods	*Achromobacter xylosoxidans*, *Acinetobacter* species, *Chryseobacterium indologenes*, *Leclercia adecarboxylata*, *Pseudomonas aeruginosa*, *Pseudomonas oryzihabitans*, *Pseudomonas putida*, *Sphingomonas paucimobilis*, *Stenotrophomonas maltophilia*	*Acinetobacter baumannii*, *Acinetobacter johnsonii*, *Acinetobacter junii*, *Acinetobacter ursingii*, *Alcaligenes faecalis*, *Bordetella bronchiseptica*, *Brevundimonas diminuta*, *Brevundimonas vesicularis*, *Burkholderia cepacia*, *Comamonas acidovorans*, *Moraxella lacunata*, *Moraxella osloensis*, *Ochrobactrum antropi*, *Pseudomonas aeruginosa*, *Pseudomonas montelli/Pseudomonas putida*, *Pseudomonas stuzeri*, *Stenotrophomonas maltophilia*
Gram-positive cocci	*Aerococcus urinae*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Leuconostoc* sp., *Micrococcus* sp., *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, *Staphylococcus schleiferi*, *Staphylococcus warneri*, *Streptococcus agalactiae* (group B), *Streptococcus anginosus*, *Streptococcus dysgalactiae*, *Streptococcus gallolyticus*, *Streptococcus pyogenes* (group A), *Streptococcus milleri*, *Streptococcus mitis*, *Streptococcus. oralis*, *Streptococcus pneumoniae*, *Streptococcus sanguis/sanguinis*	In groups: *Rothia mucilaginosus*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus pasteuri*, *Staphylococcus saprophyticus*; in chains: *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus agalactiae* (group B), *Streptococcus dysgalactiae*, *Streptococcus gallolyticus*, *Streptococcus massiliensis*, *Streptococcus milleri* group (*Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus intermedius*), *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* (group A), *Streptococcus sanguinis group*, *Streptococcus viridans* group
Miscellaneous bacteria	*Aeromonas* sp., *Aeromonas caviae*, *Bacillus cereus/Bacillus thuringiensis*, *Campylobacter coli*, *Campylobacter jejuni*, *Haemophilus aphrophilus* (*Aggregatibacter aphrophilus*), *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Listeria monocytogenes*, *Moraxella catarrhalis*, *Neisseria* sp., *Neisseria gonorrhoeae*, *Neisseria meningitidis*	*Aggregatibacter aphrophilus*, *Arthrobacter albus*, *Bacillus cereus*, *Bacillus silvestris*, *Clostridium perfringens*, *Corynebacterium pseudodiphtheriticum*, *Corynebacterium amycolatum*, *corrodens*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Lactococcus species*, *Listeria monocytogenes*, *Moraxella catarrhalis*, *Neisseria flavescens*, *Neisseria meningitides*, *Neisseria mucosa*, *Propionibacterium acnes*
Yeast	*Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida kefyr*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Saccharomyces cerevisiae*	*Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida lusitaniae*, *Candida parapsilosis*, *Candida tropicalis*, *Galactomyces geotrichum*, *Geotrichum* species, *Magnusiomyces capitatus*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, *Trichosporon mucoides*

ouraud's agar (bioMérieux, France) media. A single colony of a (sub)culture was directly deposited in duplicate on a MALDI-TOF plate (Bruker Daltonik GmbH, Germany). Simultaneously, biochemical identification was performed. Technicians performing one method of identification were unaware of the results obtained from the other method.

Biochemical identification. After Gram staining and determination of catalase and oxidase activities, isolates were identified by appropriate Vitek identification card using a Vitek-2 apparatus (bioMérieux) or an appropriate API identification strip (bioMérieux), both according to the manufacturer's instructions. *Staphylococcus aureus* was also identified by Slidex Staph Plus (bioMerieux) according to manufacturer's instructions. Hemolytic streptococci were identified based on the combination of colony morphology, Gram staining, and rapid latex agglutination test (Streptex; Remel, Lenexa, KS). Murex (Remel) was used for species identification of *Candida albicans*; all other yeast were identified by API.

Sequence data. Isolates that yielded discrepant results between routine phenotypical and MALDI-TOF MS identifications were subjected to partial 16S rRNA gene sequencing. A species was attributed when the highest sequence homology (>99%) was obtained with a single species sequence in GenBank (15).

Mass spectrometry. A thin smear of bacteria or yeast was deposited on a MALDI plate. Microorganisms that could not be identified directly by MALDI-TOF MS underwent pretreatment and were retested. For pretreatment, one colony was suspended in 300 μl of distilled RNase-free water. Ethanol absolute (900 μl) was added and mixed carefully, and then the sample was centrifuged (14,000 × *g* for 2 min). Supernatant was discarded, and the pellet was air dried. Formic acid (70%; 25 μl) and acetonitrile (25 μl) were added to the pellet, mixed thoroughly, and then the sample was centrifuged again (14,000 ×*g* for 2 min). The supernatant (1 μl) was deposited on the MALDI plate. Pretreated and untreated samples were overlaid with 1 μl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). The matrix sample was cocrystallized by air drying at room temperature. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) using FlexControl software (version 3.0). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV; mass range, 2,000 to 20,000 Da). For each spectrum 240 shots in 40-shot steps from different positions of the target spot (automatic mode) were collected and analyzed.

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TABLE 2. Retrospective analysis of identification of bacteria and yeasts by MALDI-TOF MS

Organisms	Sample data (no.)			MALDI-TOF MS analysis (no. [%])[a]					
	Isolates	Genera	Species	Genus correct	Species correct	Major error	Minor error	No ID	No uniform ID
Enterobacteriaceae	89	9	23	89 (100)	86 (96.6)	0 (0)	3 (3.4)	0 (0)	0 (0)
Nonfermentatitve Gram-negative bacteria	55	7	9	45 (81.8)	41 (74.5)	2 (3.6)	1 (1.8)	2 (3.6)	6 (10.9)
Gram-positive cocci	87	6	26	85 (97.7)	70 (80.5)	0 (0)	1 (1.1)	2 (2.3)	0 (0)
Miscellaneous bacteria	77	7	13	73 (94.8)	65 (84.4)	1 (1.3)	2 (2.6)	0 (0)	1 (1.3)
Yeasts	19	2	8	19 (100)	18 (94.7)	0 (0)	0 (0)	0 (0)	0 (0)
Total	327	29	80	311 (95.1)	280 (85.6)	3 (0.9%)	7 (2.1)	6 (1.8)	7 (2.1)

[a] MALDI-TOF MS identifications were compared to final identification (ID) based on biochemical phenotypic methods and/or 16S sequencing. Isolates were tested in duplicate for MALDI-TOF MS identification. A major error occurred when the genus was incorrect; a minor error occurred when the genus was correct but the species was incorrect. No uniform results between duplicate results were scored.

Spectra were internally calibrated by using *Escherichia coli* ribosomal proteins every day. The spectra were imported into the BioTyper software (version 2.0; Bruker, Germany) and were analyzed by standard pattern matching with default settings. The software (including the database) and the MALDI-TOF MS apparatus are an integrated system. The BioTyper database contains spectra of approximately 2,881 species and is regularly updated by the Bruker Company. Results of the pattern-matching process were expressed as proposed by the manufacturer with scores ranging from 0 to 3. For each isolate, the highest score of a match against a spectrum in the database was used for identification. Scores below 1.7 were considered not to have generated a reliable identification; a score of $\geq$1.7 was considered identification to genus, and a score of $\geq$2.0 was used for species identification. When MALDI-TOF MS identifications between duplicate tests matched exactly (identical genus and/or species), MALDI-TOF MS identification was considered final. Discrepant results were regarded as follows: identifications with scores of $\geq$2.0 overruled duplicate identifications with scores of $<$2.0 (e.g., a *Klebsiella* species score of 1.9 was overruled by a *Klebsiella pneumoniae* score of 2.3 on a duplicate test). When scores of discrepant duplicate tests were within the same range (e.g., *K. pneumoniae* score of 2.2 and *Klebsiella oxytoca* score of 2.2), the isolate was retested. If the discrepancy persisted, the identification was considered not uniform even when genus identifications were identical with discrepant species identifications (e.g., *Klebsiella ornithinolytica* versus *K. oxytoca*).

Criteria for identification and discrepant analysis. When routine biochemical phenotype and MALDI-TOF MS had exactly the same identification to the species level, the identification was considered final. In the case of discrepant results or no identification with one or both methods, the result of sequencing of the 16S rRNA indicated the final identification. Incorrect genus identification was considered a major error. Correct genus identification with incorrect species identification was considered a minor error. For comparisons between conventional and MALDI-TOF MS identification methods, a chi-square test was used. For correlations, a Spearman test was used.

RESULTS

Initial experiments revealed that neither culture medium (blood, chocolate, CLED, Sabouraud's agar medium, and colistin-neomycin medium), incubation temperature (4, 30, or 35°C), incubation conditions (O_2 or 5% CO_2), nor length of incubation (up to 72 h) affected the accuracy of MALDI-TOF MS identifications (data not shown). Pretreatment of yeasts was essential for correct identification and was therefore always applied. When nonfermentative Gram-negative rods had a low score, with only genus identification ($<$2.0) or no identification ($<$1.7), pretreatment increased the level of identifications in 37% of the 19 isolates tested. In general, pretreatment was not necessary for Gram-positive bacteria and *Enterobacteriaceae*.

Retrospective study (i) MALDI-TOF MS identifications. Of 327 isolates encompassing 29 species and 80 genera, 311 isolates (95.1%) were correctly identified by MALDI-TOF MS. Of these isolates, 280 (85.6%) had correct species identification (Table 2). Results of MALDI-TOF MS identifications for *Enterobacteriaceae*, nonfermentative Gram-negative rods, Gram-positive cocci, a group of miscellaneous bacteria, and yeasts are depicted separately in Table 2. *Enterobacteriaceae* and yeasts yielded high percentages of correct species identifications (96.6% and 94.7%, respectively), whereas for nonfermentative Gram-negative bacteria there were fewer correct species identifications (74.5%) and higher numbers of isolates with incorrect (5.4%) or no (3.6%) identification.

(ii) MALDI-TOF MS versus conventional identification. MALDI-TOF MS and conventional identification were concordant in 82.3% (Spearman's rho, -0.114; $P = 0.039$) (Table 3) of isolates. Twenty-four isolates (7.3%) yielded MALDI-TOF MS identifications to the genus level only (score of $<$2.0), with identifications consisting of 1 of 4 isolates of *Aeromonas* species, 1 of 3 *Bacillus cereus*/*Bacillus thuringiensis*, 3 of 10 *Campylobacter coli*, 1 of 2 *Chryseobacterium* species, 1 of 3 *Haemophilus parainfluenzae*, 1 of 2 *Sphingomonas paucimobilis*, 1 of 36 (2.8%) *Staphylococcus* species (*Staphylococcus warneri*), 1 of 34 (2.9%) *Stenotrophomonas* species (*Stenotrophomonas maltophilia*), 1 of 1 *Streptococcus dysgalactiae*, 5 of 10 *Streptococcus milleri* group, 3 of 3 *Streptococcus mitis*, 3 of 4 *Streptococcus oralis*, 1 of 5 *Streptococcus sanguinis* group, and 1 of 1 *Candida dubliniensis*.

(iii) Incorrect identifications. Ten isolates were misidentified by MALDI-TOF MS: three isolates (0.9%) had the wrong genus identification (major error), and seven (2.1%) had the correct genus with incorrect species identification (minor error). Major errors were one identification as *Acinetobacter* species instead of *Campylobacter jejuni*, and two *Sphingomonas* spp. instead of *Paracoccus yeei*. The latter species was not present in the BioTyper database. Both isolates were erroneously identified as *Sphingomonas* species (genus identification only) and *Sphingomonas aerolata*. The seven minor errors encompassed *Achromobacter ruhlandii* instead of *Achromobacter xylosoxidans*, *C. jejuni* instead of *C. coli*, *Citrobacter koseri* and *Citrobacter freundii* instead of *Citrobacter murliniae*, *Enterococ-*

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TABLE 3. Concordance between conventional phenotypic biochemical identification and MALDI-TOF MS identification for the retrospective analysis and prospective analysis[a]

Analysis type and phenotypic biochemical identification parameter	Retrospective MALDI-TOF analysis (no. of isolates)					Prospective MALDI-TOF analysis (no. of isolates)						
	Species ID	Genus ID	No ID	Misidentification	Total	Species ID	Genus ID	Major error	Minor error	No ID	No uniform ID	Total
Retrospective												
Species identification	263	20	10	5	298							
Genus identification	7	3	3	0	13							
No ID	1	0	0	2	3							
Misidentification	9	1	0	3	13							
Total for group	280	24	13	10	327							
Prospective												
Species ID						783	26	1	2	0	2	814
Genus ID						99	17	0	12	3	1	132
Major error						7	5	0	0	3	0	15
Minor error						11	1	0	2	0	0	14
No ID						2	1	0	0	2	0	5
Total for group						902	50	1	16	8	3	980

[a] Isolates were tested in duplicate by MALDI-TOF MS and by conventional methods according to manufacturers descriptions (Vitek, API, and bioMerieux) both retrospectively and prospectively. For MALDI-TOF analysis, species identification (score of >2.0), genus identification (score of <2.0 but >1.7), major error (incorrect genus), minor error (correct genus with incorrect species), no identification (score of <1.7) and nonuniform identifications between duplicates were scored. Misidentification applies to either the genus or species level. ID, identification.

cus phoeniculicola for *Enterococcus casseliflavus*, *Klebsiella varicola* for *K. pneumoniae*, and *Listeria innocua* for *Listeria monocytogenes*.

(iv) No identification. Of 13 isolates that, despite pretreatment, could not be identified by MALDI-TOF MS, 6 isolates (1.8% of all isolates) had no identification at all (score of <1.7), and 7 isolates (2.1%) had no uniform identification by MALDI-TOF MS between duplicates: 1 of 5 (20%) *A. xylosoxidans* (*A. xylosoxidans* and *A. ruhlandii*), 1 of 4 (25%) *Acinetobacter* species (*Achromobacter* species and *Acinetobacter* species), 1 of 10 (10%) *C. coli* (*C. coli* and *C. jejuni*), and 4 of 34 (11.8%) *S. maltophilia* (*S. maltophilia* and *Pseudomonas hibiscicola* or *Pseudomonas geniculata* or *Pseudomonas beteli*).

Prospective study. (i) MALDI-TOF MS versus conventional identification. During a 5-week period, 980 isolates—of which 919 were bacteria and 61 were yeasts—were identified by both conventional phenotypic biochemical methods and MALDI-TOF MS. Of these 980 isolates (encompassing 46 genera and 88 species), 953 (97.2%) had identical genus identifications by MALDI-TOF MS and conventional methods, and of these 783 (79.9%) yielded exactly the same species identification (Spearman's rho, 0.283; $P < 0.01$) (Table 3). Conventional methods resulted in fewer species identifications (83.1% versus 92%; $P < 0.01$), more identifications to genus level only (13.5% versus 5.1%; $P < 0.01$), and more major errors (1.5% versus 0.1%; $P < 0.01$) than MALDI-TOF MS. On the other hand, slightly fewer minor errors (1.4% versus 1.6%; P = not significant [NS]) were obtained by conventional techniques than by MALDI-TOF MS, and no identifications were made (0.5% versus 0.8%; P = NS) (Table 3). Two isolates could not be identified by MALDI-TOF MS or by classical biochemical methods: one isolate was identified as *Moraxella osloensis* by 16S sequencing, and one isolate could not be identified by 16S sequencing either.

(ii) Analysis for different groups of bacteria and yeasts. Performance of MALDI-TOF MS was further analyzed for different groups of microorganisms (Table 4). For *Enterobacteriaceae*, MALDI-TOF MS had correct species identification (97.7%), whereas nonfermentative Gram-negative rods yielded correct identifications to species and genus level in 92.0% and 94.3%, respectively. One major error was made by MALDI-TOF MS: *S. maltophilia* was erroneously identified as *P. beteli* in two of six *S. maltophilia* isolates. The low percentage of species identifications (63.2%) among Gram-positive cocci in clusters by conventional identification is due to the fact that routine species identification of coagulase-negative staphylococci (CoNS) is performed only in specific situations. Gram-positive cocci in chains are discussed in detail below. The group of miscellaneous bacteria included 51 isolates belonging to the group of HACEK bacteria (three genera and four species). Fifty isolates (98%) were correctly identified to species level by MALDI-TOF MS; one isolate (*Haemophilus parainfluenzae*) could be correctly identified only to the genus level (score of 1.76). Conventional methods also resulted in correct identification in 50 of 51 isolates, whereas one major error occurred by mistaking *Eikenella corrodens* for a nonfermentative Gram-negative rod. The performance of yeast identification by MALDI-TOF MS did not differ from the conventional method (API).

(iii) Gram-positive cocci in chains. This group included 64 enterococci (36 *Enterococcus faecalis* and 28 *Enterococcus faecium*), 49 hemolytic streptococci (24 S*treptococcus agalactiae*, 15 *S. dysgalactiae*, and 10 S*treptococcus pyogenes*), 9 *Streptococcus milleri* group, 22 pneumococci, and 21 viridans streptococci (*S. mitis* group and *S. sanguinis* group). Enterococci and hemolytic streptococci were correctly identified by MALDI-TOF MS to species level in 98.4% and 100% of instances, respectively, and by conventional identification in 95.3% and 100% of instances, respectively (Table 5). One *E. faecalis* was identified

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TABLE 4. MALDI-TOF MS identifications and routine biochemical phenotypic identifications compared to final identifications[a]

Organism group and identification parameter (isolate data)	MALDI-TOF MS identification (no. of isolates [%])	Routine biochemical phenotypic identification (no. of isolates [%])[d]	*P* value[e]
All isolates (n = 980; 42 genera, 92 species)			
Genus correct	968 (98.8)	960 (98.0)	NS
Species correct	902 (92.0)	814 (83.1)	<0.01
Major error	1 (0.1)	16 (1.6)	<0.01
Minor error	16 (1.6)	14 (1.4)	NS
No identification	8 (0.8)	5 (0.5)	NS
No uniform result	3 (0.3)	NA	
Enterobacteriaceae (n = 311; 14 genera, 21 species)			
Genus correct	311 (100)	311 (100)	NS
Species correct	304 (97.7)	304 (97.7)	NS
Major error	(0)	(0)	
Minor error	1 (0.3)	7 (2.3)	0.05
No identification	(0)	(0)	
No uniform result	(0)	NA	
Nonfermentative Gram-negative rods (n = 88; 10 genera, 17 species)			
Genus correct	83 (94.3)	82 (93.2)	NS
Species correct	81 (92.0)	77 (87.5)	NS
Major error	1 (1.1)	2 (2.3)	NS
Minor error	(0)	1 (1.1)	NS
No identification	2 (2.3)	4 (4.5)	NS
No uniform result	2 (2.3)	NA	
Gram-positive cocci in cluster (n = 261; 2 genera, 9 species)[b]			
Genus correct	261 (100)	259 (99.2)	NS
Species correct	246 (94.3)	165 (63.2)	<0.01
Major error	(0)	2 (0.8)	NS
Minor error	1 (0.4)	(0)	NS
No identification	(0)	(0)	
No uniform result	(0)	NA	
Gram-positive cocci in chains (n = 165; 2 genera 16 species)[c]			
Genus correct	163 (98.8)	165 (100)	NS
Species correct	140 (84.8)	145 (87.9)	NS
Major error	(0)	(0)	
Minor error	12 (7.3)	3 (1.8)	0.03
No identification	2 (1.2)	(0)	NS
No uniform result	(0)	NA	
Miscellaneous bacteria (n = 94; 12 genera, 17 species)			
Genus correct	91 (96.8)	83 (88.3)	0.03
Species correct	79 (84.0)	76 (80.9)	NS
Major error	(0)	11 (11.7)	<0.01
Minor error	(0)	1 (1.1)	NS
No identification	3 (3.2)	1 (1.1)	NS
No uniform result	(0)	NA	
Yeasts (n = 61; 7 genera, 12 species)			
Genus correct	59 (96.7)	60 (98.4)	NS
Species correct	52 (85.2)	47 (77.0)	NS
Major error	(0)	1 (1.6)	NS
Minor error	2 (3.3)	2 (3.3)	NS
No identification	1 (1.6)	(0)	NS
No uniform result	1 (1.6)	NA	

[a] Isolates were tested by MALDI-TOF MS (duplicate) and conventional biochemical phenotypic methods. Definite identification was set when phenotypic biochemical methods exactly matched MALDI-TOF MS identification or by 16S sequencing. See Table 3 footnote and the text for an explanation of MALDI-TOF MS scoring.

[b] Genera are *Rothia* and *Staphylococcus*.

[c] Genera are *Enterococcus* and *Streptococcus*.

[d] NA, not applicable.

[e] *P* values are calculated by comparison of MALDI-TOF MS identification with conventional identification (chi-square test). NS, not statistically significant.

to genus level only (score 1.999) by MALDI-TOF MS. Routine biochemical methods misidentified 3 (5%) out of 64 isolates of *E. faecium* as *E. faecalis* (two times) and as *Enterococcus gallinarum*. Seven of nine isolates belonging to the *S. milleri* group (eight *S. anginosus* and one *S. constellatus*) were correctly identified to species level, whereas conventional biochemical methods identified all isolates as belonging to the *S. milleri* group without further specification. Two isolates of the *S. milleri*

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TABLE 5. Identification of Gram-positive cocci in chains by MALDI-TOF MS and routine biochemical phenotypic methods[a]

Organism group and identification parameter (no. of isolates)	MALDI-TOF MS identification (no. of isolates [%])	Routine biochemical phenotypic identification (no. of isolates [%])[b]	*P* value[c]
Enterococci (64)			
Genus correct	64 (100)	64 (100)	NS
Species correct	63 (98.4)	61 (95.3)	NS
Major error	(0)	(0)	
Minor error	(0)	3 (4.7)	NS
No identification	(0)	(0)	
No uniform result	(0)	NA	
Hemolytic streptococci (49)			
Genus correct	49 (100)	49 (100)	NS
Species correct	49 (100)	49 (100)	NS
Major error	(0)	(0)	
Minor error	(0)	(0)	
No identification	(0)	(0)	
No uniform result	(0)	NA	
Streptococcus milleri group (9)			
Genus correct	9 (100)	9 (100)	NS
Species correct	7 (77.8)	9 (100)	NS
Major error	(0)	(0)	
Minor error	(0)	(0)	
No identification	(0)	(0)	
No uniform result	(0)	NA	
Pneumococci (22)			
Genus correct	22 (100)	22 (100)	NS
Species correct	19 (86.4)	22 (100)	NS
Major error	(0)	(0)	
Minor error	(0)	(0)	
No identification	(0)	(0)	
No uniform result	(0)	NA	
Viridans streptococci (21)			
Genus correct	19 (90.5)	21 (100)	NS
Species correct	2 (9.5)	4 (19.0)	NS
Major error	(0)	(0)	
Minor error	12 (57.1)	(0)	<0.01
No identification	2 (9.5)	(0)	
No uniform result	(0)	NA	

[a] Isolates were tested by MALDI-TOF MS (duplicate) and conventional biochemical methods. Definite identification was set when phenotypic biochemical methods exactly matched MALDI-TOF MS identification or by 16S sequencing. See Table 3 footnote and the text for an explanation of MALDI-TOF MS scoring.
[b] NA, not applicable.
[c] *P* values were calculated by comparison of MALDI-TOF MS identification with conventional identification (chi-square test). NS, not significant.

group had scores of 1.86 and 1.83, respectively, and thus were identified only to the genus level. Most pneumococci—19 out of 22 (86.4%) isolates—had correct species identification; 3 isolates (13.6%) had scores of 1.937, 1.827, and 1.769 and could be identified only to the genus level. MALDI-TOF MS erroneously identified 12 out of 21 (57.1%) viridans streptococci as *S. pneumoniae*. One isolate was correctly identified as *Streptococcus massiliensis*, and one was identified as *Streptococcus mutans*. Five isolates (23.8%) had only genus-level identification: two isolates were identified as *Streptococcus* species (scores of 2.042 and 2.078) without further genus specification; three isolates had a score of <2.0 (1.831, 1.88, and 1.859, respectively) and therefore were identified to genus only. By conventional biochemical methods 4 isolates (19.0%) could be identified as *S. mitis* (1 isolate), *S. mutans* (1 isolate), and *S. oralis* (1 isolates); the remaining 17 isolates (81.0%) were all correctly identified as viridans streptococci without precise species identification.

DISCUSSION

MALDI-TOF MS has now been implemented in our laboratory for efficient, cost-effective, rapid, and routine identification of bacterial and yeast isolates. Application of MALDI-TOF MS is dependent on the reference strains included in the database. When a given strain is tested, the species of the reference strain with the closest match is retained for identification of the tested strain. This approach eliminates strong influences of culture medium and culture conditions, as shown in the first experiments. The results of the retrospective analysis, encompassing 327 clinical isolates, revealed an excellent performance of MALDI-TOF MS in comparison with conventional identification techniques and stimulated us to perform a prospective validation study. In total, 980 clinical isolates of bacteria and yeasts were tested by conventional identification systems and MALDI-TOF MS. Significantly more bacterial isolates could be identified to the species level by MALDI-

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TOF MS, with better performance of MALDI-TOF MS especially for staphylococci and bacteria from the HACEK group.

Until now, most studies reported on the proof-of-concept of MALDI-TOF MS for specific microorganisms (2, 7–11, 17, 21–23, 26, 31). The majority of these studies included strains from reference and culture collections. Recently, a study by Seng et al. included clinical isolates and concluded that MALDI-TOF MS can replace conventional systems for identification of bacteria in a conventional laboratory (27). In our study, the performance of MALDI-TOF MS was specified in detail for different groups of microorganisms and also included yeasts. In accordance with the results of Seng et al., >95% of all clinical isolates could be identified to genus and species levels by MALDI-TOF MS. *Enterobacteriaceae* and HACEK bacteria were accurately identified to species level (>98%) even for very closely related species (5, 11). *Enterobacteriaceae* and HACEK bacteria together account for the large majority of aerobic Gram-negative rods in a conventional medical microbial laboratory.

In the prospective study, MALDI-TOF MS performed equally as well as conventional systems for genus and species identification of nonfermentative Gram-negative bacteria. In the retrospective analysis about three-quarters of all isolates had correct species identifications while in the prospective study >90% were correctly identified to species level. This difference can be explained by the observation that in the latter study, the majority (61 of 95 isolates) of isolates were *Pseudomonas aeruginosa*, which was easily identified as the species by MALDI-TOF MS. Additionally, eight of nine *Acinetobacter* isolates belonging to five different species were correctly identified. Five of 41 isolates of *S. maltophilia* were identified erroneously as *P. geniculata*, *P. beteli*, or *P. hibiscicola* by MALDI-TOF MS, and this was considered a major error. We hypothesized that this discordance resulted from a mislabeling of bacterial species in the BioTyper database. Indeed, *P. beteli* and *P. hibiscicola* are invalid names for *S. maltophilia* in the database (13, 29). These results clearly demonstrate that an up-to-date database is essential for bacterial identification and that the current database needs improvement, with the addition of more spectra from correctly identified nonfermentative rods.

An important advantage of MALDI-TOF MS is rapid identification of *S. aureus* and of separate species belonging to CoNS. Most commercial identification systems allow a rapid identification of only *S. aureus*, but MALDI-TOF MS recognizes correctly spectra of various species of CoNS (3, 7, 28). A recently performed comparative study between MALDI-TOF MS and two rapid-identification automated systems (BD Phoenix from BD Diagnostic Systems, France, and Vitek-2 from bioMérieux, France) for identification of 234 CoNS representing 20 different species revealed significantly better performance of MALDI-TOF MS (93.2%) than Phoenix (75.6%) and Vitek-2 (75.2%), with fewer misidentifications (7). Previously, in our laboratory not all CoNS were routinely identified to the species level, but introduction of MALDI-TOF MS simplified CoNS identifications considerably. It has now been implemented for all CoNS in our daily routine. Identification to the exact species level may be very useful as some CoNS can cause serious infections, and it may help to differentiate contaminated cultures from true infections by staphylococcus species (30).

Although enterococci and hemolytic streptococci were correctly identified by MALDI-TOF MS, viridans streptococci and pneumococci revealed too many misidentifications. Seng et al. found that nearly 50% of *S. pneumoniae* isolates were misidentified as *Streptococcus parasanguinis* because the database included only three *S. pneumoniae* and two *S. parasanguinis* reference spectra (27). Therefore, the database also needs improvement, with more spectra of well-identified streptococcal species. Until then, MALDI-TOF MS is inappropriate for identification of pneumococci, especially since erroneous identification has important clinical consequences.

MALDI-TOF MS has performed well for identification of yeasts in our prospective study, with correct identification of 85% of 61 isolates encompassing 12 different species without major errors occurring. An extraction method prior to analysis in the mass spectrometer was shown to be mandatory to obtain appropriate spectra. During the past decade, the clinical impact of severe infections with yeasts and yeast-like fungi has increased, especially in immunocompromised hosts. Rapid and reliable species identification is essential for antifungal treatment, for which conventional biochemical methods are too time-consuming. Additionally, high-resolution DNA-based molecular techniques, such as 16S or 18S rRNA or internal transcribed spacer (ITS) DNA sequencing and real-time PCR assays, are expensive and time-consuming. A recent German study of 267 clinical isolates and 18 collection strains also concluded that MALDI-TOF MS is a rapid and reliable tool for the identification of yeasts and yeast-like fungi, with low expenditure of consumables, easy interpretation of results, and a fast turnaround time (18). Misidentifications in our study and the German study were associated with the use of an incomplete database, such as for *C. dubliniensis*.

The strengths of our study are the implementation of MALDI-TOF MS in a routine setting, the comparison of MALDI-TOF MS with conventional identification systems on clinical isolates, the use of 16S DNA and ITS sequencing for analysis of discrepancies, and the inclusion of yeasts. A limitation of our study is the lack of inclusion of sufficient anaerobic bacteria and Gram-positive aerobic rods. During the study period, anaerobic bacterial species and aerobic Gram-positive rods were isolated sporadically ($n = 4$ and $n = 13$ isolates, respectively) and were mostly considered part of a mixed flora and therefore not identified further. Conventional identification methods for anaerobic bacteria are cumbersome and time-consuming and require a specific location (e.g., anaerobic chamber) at the laboratory. MALDI-TOF MS for anaerobic identification would certainly increase the number of species identifications since it can be applied directly from bacterial colonies on the primary culture plates. So far, one study reported on MALDI-TOF for routine identification of anaerobic bacteria (27). Despite the above-mentioned advantages, the investigators concluded that a more accurate database is required since *Fusobacterium nucleatum* was not present and more than 50% of 46 isolates with no identification by MALDI-TOF MS consisted of anaerobic bacteria. For those anaerobic species (e.g., *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides uniformis*) of which sufficient

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spectra are present in the database, MALDI-TOF MS performs better than conventional biochemical methods (23).

To summarize, MALDI-TOF MS is a rapid, simple, inexpensive, and high-throughput proteomic technique for identification of both bacteria (except for identification of viridans streptococci and pneumococci or anaerobic bacteria) and yeasts and can be implemented in a conventional laboratory setting in its current application. Although the technique has a high accuracy for microbial identification in general and performs equally as well as or better than conventional techniques, the performance can be significantly improved when more spectra of appropriate reference strains are added to the database.

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