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Evaluation of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Identification of Clinically Important Yeast Species[▽]

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We evaluated the use of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the rapid identification of yeast species. Using Bruker Daltonics MALDI BioTyper software, we created a spectral database library with *m/z* ratios of 2,000 to 20,000 Da for 109 type and reference strains of yeast (44 species in 8 genera). The database was tested for accuracy by use of 194 clinical isolates (23 species in 6 genera). A total of 192 (99.0%) of the clinical isolates were identified accurately by MALDI-TOF MS. The MALDI-TOF MS-based method was found to be reproducible and accurate, with low consumable costs and minimal preparation time.

Invasive fungal infections due to opportunistic pathogens are a significant cause of morbidity and mortality (2, 5, 8). The current rise in fungal infections correlates with the widespread use of broad-spectrum antibacterial agents, prolonged hospitalization of critically ill patients, and the increased number of immunocompromised patients. *Candida* species comprise the fourth most common cause of nosocomial bloodstream infections, and *Cryptococcus neoformans* is the most common cause of fungus-related mortality in HIV-infected patients (15, 19). While *Candida albicans* is still involved in more than half of all *Candida*-related bloodstream infections, an increase in recovery of non-*C. albicans Candida* spp., *Rhodotorula* spp., *Trichosporon* spp., and *Malassezia* spp. has occurred (2, 29). Treatment with amphotericin B may be useful for these organisms and inefficient for those belonging to other genera (5, 8). While many *Candida* species remain susceptible to fluconazole, it is important to differentiate the more resistant organisms, namely, *Candida glabrata*, *Candida krusei*, *Rhodotorula* spp., and some members of the genus *Trichosporon*. Additionally, *Rhodotorula* spp. have an innate resistance to voriconazole, and *Trichosporon*, *Cryptococcus*, and *Rhodotorula* are intrinsically resistant to the echinocandins (1, 15). These organisms present new challenges not only to treatment but also to standard identification methods used in the clinical laboratory (4, 8, 28).

Commercially available biochemical test systems identify most of the commonly isolated species of yeast accurately but may result in no identification or misidentification of more-unusual isolates (4, 21, 28). Additionally, samples for these tests must be incubated for 1 to 3 days before results are obtained. To overcome the inaccuracies of biochemical identification methods, nucleic acid-based tests have been developed. These tests amplify and then sequence a target gene, such as the rRNA genes or the internal transcribed spacer (ITS) region (9, 10, 14, 17). While these assays are highly accurate, they require considerable processing time and costly reagents.

As an alternative to biochemical and genome-based identification schemes, proteomic profiling by mass spectral analysis was recently evaluated for use in species differentiation of a variety of microorganisms. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) is emerging as a rapid and accurate tool for identifying pathogens, including Gram-positive and Gram-negative bacteria, mycobacteria, molds, and yeast species (3, 6, 11–13, 16, 18, 22, 23, 27). The technique can be performed rapidly, with minimal consumable expenses, and produces reproducible, species-specific spectral patterns that are not dependent upon the age of culture, growth conditions, or medium selection (7, 13, 20, 26).

In this work, we present the development of a yeast database library consisting of 109 type and reference strains (44 species in 8 genera), and we tested the robustness and accuracy of this library by using 194 well-characterized clinical isolates (23 species in 6 genera).

MATERIALS AND METHODS

Type and reference strains and clinical challenge isolates. In this study, type and reference strains from the American Type Culture Collection (ATCC), the University of Alberta Microfungus Collection and Herbarium (UAMH), and the National Institutes of Health (NIH) were used to construct the yeast database library. The accuracy of this test library for MALDI-TOF identification of clinical isolates of yeast was then evaluated with 194 isolates collected at the NIH Clinical Center (initially identified using the API 20C AUX yeast identification system [bioMerieux, Durham, NC]) or provided by M. Pfaller and D. Diekema (identified using Vitek 2 yeast cards [bioMerieux, Durham, NC]). For all organisms, isolates were cultured from frozen stock onto Sabouraud dextrose agar and incubated for 2 to 3 days at 30°C before being tested. Discrepancies in identification of reference strains and clinical isolates were resolved by Sanger sequencing of the ITS region (ITS1-5.8S-ITS2) or the secondary alcohol dehydrogenase gene (14, 24, 25).

Sample preparation. A single colony or multiple small colonies (enough to fill one-third of a 10-μl inoculating loop) were suspended in 1 ml of 70% ethanol (EtOH), briefly vortexed, and centrifuged in an Eppendorf 5415D centrifuge at 13,000 rpm for 2 min. The liquid was removed, and the pellet was quickly spun

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TABLE 1. Reference strains removed from the database library due to discordant identification results or unacceptable spectra

Reference organism	Identification[a]	
	Sequencing	MALDI-TOF
Candida periphelosum ATCC 20314	*Candida guilliermondii*	*Candida guilliermondii*
Candida periphelosum ATCC 20317	*Candida guilliermondii*	*Candida guilliermondii*
Candida rugosa ATCC 96275	*Candida pararugosa*	*Candida pararugosa*
Candida zeylanoides ATCC 15585	*Candida guilliermondii*	*Candida guilliermondii*
Candida zeylanoides ATCC 20393	*Candida lipolytica*	*Candida lipolytica*
Cryptococcus luteolus ATCC 44440	ND	No acceptable spectra
Cryptococcus luteolus ATCC 42279	*Cryptococcus podzolicus*	No acceptable spectra
Cryptococcus uniguttulatus ATCC 34143	*Candida glabrata*	*Candida glabrata*
Geotrichum candidum ATCC 10834	ND	No acceptable spectra
Geotrichum candidum ATCC 74169	ND	No acceptable spectra
Malassezia sympodialis ATCC 96803	ND	No acceptable spectra
Trichosporon asahii UMAH 10278	*Trichosporon asteroides*	*Trichosporon asteroides*
Trichosporon ovoides UMAH 563	*Trichosporon asahii*	*Trichosporon ovoides/asahii*
Trichosporon ovoides UMAH 672	*Trichosporon asahii*	*Trichosporon ovoides/asahii*
Trichosporon ovoides UMAH 635	*Trichosporon asahii/faecale*	*Trichosporon faecale*

[a] ND, not done.

again, followed by removal of residual EtOH. The pellet was then resuspended in 50 μl 70% formic acid, 50 μl of acetonitrile was added, the sample was vortexed briefly, and the mixture was centrifuged for 2 min at 3,000 rpm. The supernatant was either tested immediately or stored at −20°C until ready for MALDI-TOF analysis. A portion of each extracted sample was subcultured onto Sabouraud dextrose agar and incubated at 30°C to ensure that no viable organisms remained.

MALDI-TOF MS. The yeast sample to be analyzed was warmed to room temperature, 1 μl was spotted onto a steel target plate (Bruker Daltonics Inc., Billerica, MA) and allowed to evaporate to dryness on a 37°C plate warmer (model XH-2002 Premiere slide warmer; Daigger, Vernon Hills, IL), and then 2 μl matrix solution was applied to the spot and dried. The MALDI matrix, α-cyano-4-hydroxycinnamic acid, was prepared daily as a saturated solution in 50% acetonitrile and 2.5% trifluoroacetic acid. After complete drying, the mixture was exposed to laser pulses, resulting in energy transfer from the matrix to the nonvolatile analyte molecules, with desorption of analyte into the gas phase. The ionized molecules were accelerated by electrical potentials through a flight tube to the mass spectrometer, with separation of the biomarkers determined by their mass/charge ratio (m/z; z typically is 1). The profiles of biomarkers were then compared with profiles of a collection of well-characterized organisms. Each sample was assayed in quadruplicate.

Samples were evaluated on an UltraFlex I TOF-TOF mass spectrometer (Bruker Daltonics Inc.) in linear positive-ion mode across the m/z range of 2,000 to 20,000, with gating of ions below m/z 400 and a delayed extraction time of 450 ns. For these studies, each spot was measured using 1,000 laser shots at 25 Hz in groups of 50 shots per sampling area of the spot. The data sampling rate was 0.5 GHz. Each plate was calibrated before being run, using a mixture of protein standards I and peptide standards II from Bruker Daltonics. Total processing time and spectral analysis took approximately 15 min for a single isolate, and additional samples increased this time by 1 to 2 min per sample.

Spectral analysis by Bruker MALDI BioTyper. Acceptable spectra for reference strains were defined by Bruker Daltonics as having a minimum of 25 peaks with a resolution of >400, among which 20 peaks must have a resolution of >500, where resolution is defined as the peak m/z divided by the peak width at 50% of the peak maximum. Spectra of clinical challenge isolates were analyzed using the reference strain database and MALDI BioTyper software (BioTyper Library v 2.0.4; Bruker Daltonics), a proprietary algorithm for spectral pattern matching resulting in a logarithmic score of 0 to 3. As part of this study, we assessed the minimum spectral score that accurately predicted species identification of all yeast isolates.

RESULTS

A total of 124 type and reference strains were evaluated for construction of the yeast database library. Acceptable spectra were not obtained with 5 reference organisms, that is, insufficient numbers of quality peaks were observed for inclusion of the reference organisms in the database library. Additionally, identifications of 10 organisms by MALDI-TOF and gene sequencing were concordant but differed from the identifications assigned by ATCC or UAMH (Table 1). Therefore, these 15 organisms were eliminated, and the final yeast database library consisted of 109 strains representing 44 species in 8 genera (Table 2).

A set of 197 yeast clinical isolates, previously identified by biochemical analysis, was used to challenge the spectral library. A limited number of commonly isolated yeast and a broad spectrum of less common yeast were evaluated. Three organisms identified as *Candida famata* by biochemical tests were identified by gene sequencing as *Pichia caribbica* (2 isolates) or *Pichia mississippiensis* (1 isolate). These organisms are not in the database and gave no acceptable MALDI-TOF spectra, so the organisms were excluded from this analysis. Table 3 depicts the results of tests with the remaining 194 yeast isolates (23 species in 6 genera). Two organisms had MALDI-TOF spectral scores of <1.8 (for *Candida rugosa*, 1.5; and for *C. neoformans*, 1.3); all other organisms had scores of ≥1.8. Of the 192 organisms with scores of ≥1.8, the MALDI-TOF identification and initial identification were in agreement for 165 isolates, with 27 discordant identifications. Sequencing of the ITS region was used to resolve the discrepancies, with MALDI-TOF and sequencing results being in agreement for all 27 isolates. Thus, MALDI-TOF correctly identified 192 (99.0%) isolates, and all spectral scores of ≥1.8 accurately predicted the species identity.

During this study, the clinical isolates were assayed in quadruplicate, with each protein extract applied to 4 spots on the steel target plate. This allowed us to determine the number of assays per isolate that needed to be performed for acceptable identification. Of the 192 correctly identified species, the spectral score was ≥1.8 with the first spot for 172 (89.6%) isolates, with the first two spots for 187 (97.4%) isolates, and with the first three spots for 189 (98.4%) isolates. Low MALDI-TOF scores (i.e., <1.8) requiring repeat testing were most commonly observed with *Candida guilliermondii*, *C. krusei*, *Candida lusitaniae*, and *C. neoformans*.

TABLE 2. Reference strains and gene sequence-confirmed NIH clinical isolates evaluated for inclusion in the MALDI-TOF MS fungal database library

Reference isolate	Strain(s)
Candida albicans	ATCC 18804^T, NIH 513, NIH 528, NIH 540
Candida catenulata	ATCC 10565^T, ATCC 20117, ATCC 22872, ATCC 42214, ATCC 200552
Candida dubliniensis	ATCC MYA-646^T, ATCC MYA-583, ATCC MYA-579, ATCC MYA- 580, ATCC MYA-582
Candida glabrata	ATCC 2001^T, NIH 517, NIH 555, NIH 546, NIH 512
Candida guilliermondii	ATCC 6260^T
Candida haemulonii	ATCC 22991^T
Candida kefyr	ATCC 90902, ATCC 204093, ATCC 42265^T, ATCC 200553, ATCC 64885
Candida krusei	ATCC 6258^T
Candida lambica	ATCC 24750^T
Candida lipolytica	ATCC 18943^T, ATCC 18942, ATCC 20776, ATCC 20496, ATCC 201089
Candida lusitaniae	ATCC 34449^T, NIH 526
Candida metapsilosis	ATCC 96144^T
Candida orthopsilosis	ATCC 96139^T
Candida parapsilosis	ATCC 22019^T, NIH 521, NIH 534
Candida pararugosa	ATCC 38774^T
Candida periphelosum	ATCC 36239
Candida pintolopesii	ATCC 22987^T, ATCC 22998, ATCC 36231, ATCC 64177, ATCC 66058
Candida rugosa	ATCC 200555, ATCC 238772, ATCC 58964, ATCC 10571^T, NIH 574
Candida tropicalis	ATCC 750^T, NIH 515, NIH 536
Candida zeylanoides	ATCC 26899, ATCC 7351^T, ATCC 96278
Cryptococcus laurentii	ATCC 18803^T, ATCC 34142, ATCC 36833, ATCC 26024, ATCC 10668
Cryptococcus luteolus	ATCC 32044^T
Cryptococcus gattii	ATCC 32269^T
Cryptococcus neoformans	ATCC 32268, ATCC 14248, ATCC 32267, ATCC 56991, ATCC 32045^T, ATCC 208821
Cryptococcus podzolicus	ATCC 34208^T
Cryptococcus terreus	ATCC 11799^T, ATCC 32046, ATCC 32422, ATCC 34145
Cryptococcus uniguttulatus	ATCC 66033, ATCC 38298, ATCC 32047^T, ATCC 32048
Geotrichum candidum	ATCC 22601^T, ATCC 204307
Geotrichum capitatum	ATCC 10663^T, ATCC 62964
Kodamaea ohmeri	ATCC 26861^T
Malassezia furfur	ATCC 96809^T
Malassezia pachydermatis	ATCC 14522^T
Pichia anomala	ATCC 8168^T
Rhodotorula glutinis	ATCC 16726^T
Rhodotorula mucilaginosa	ATCC 11799^T
Trichosporon asahii	ATCC 90039^T, UAMH 7654, UAMH 7655
Trichosporon asteroides	ATCC 90043^T, UAMH 4261, UAMH 7656
Trichosporon coremiiforme	UAMH 7658, ATCC 90042^T
Trichosporon faecale	ATCC 90041^T
Trichosporon inkin	ATCC 18020^T, UAMH 4260, UAMH 7663, UAMH 7664
Trichosporon jirovecii	ATCC 34499^T
Trichosporon moniliiforme	ATCC 90045
Trichosporon mucoides	ATCC 90046, UAMH 7670
Trichosporon ovoides	ATCC 90040, UAMH 7671

DISCUSSION

In this work, we evaluated the use of MALDI-TOF analysis for the identification of clinically important yeast species. Traditional biochemical testing for yeast identification is inexpensive but time-consuming and sometimes inaccurate (4, 21, 28). Gene sequencing is highly accurate but is expensive, time-consuming, and technically demanding (17). The aim of this study was to determine if MALDI-TOF would be suitable for rapid yeast identification. Working with 109 reference and type strains representing 44 species in 8 genera, we created a MALDI-TOF spectral library and then challenged the library with 197 clinical isolates. Three clinical isolates gave no spectral score and could not be identified because they were not in the spectral library. Two organisms (*C. rugosa* and *C. neoformans*) gave consistently low spectral scores and could not be identified. Thus, of the 194 isolates (23 species in 6 genera) included in the spectral library, 192 (99.0%) were identified correctly at the species level by MALDI-TOF. These results compare favorably with the studies of Marklein et al. (12) and van Veen et al. (27). Marklein et al. (12) evaluated 18 reference strains representing 8 species of yeast and 267 clinical isolates (28 species in 7 genera). All reference organisms and 92.5% of their clinical isolates were identified correctly. The organisms that were not identified were not in the database library. When appropriate reference organisms were added to the database, all clinical isolates were identified correctly. van Veen et al. (27) evaluated the accuracy of MALDI-TOF for the identification of 80 yeast isolates (14 species in 7 genera). A total of 97.5% of isolates were identified correctly at the genus level, with 87.5% identified correctly at the species level, with most of the cases of unidentified organisms being due to insufficient entries in the database and to species-level identification being accepted only if the MALDI-TOF spectral score was >2.0. If we applied the same scoring criterion to the

TABLE 3. Identification of clinical isolates by MALDI-TOF MS

Organism (no. of isolates tested)	No. of isolates with spectral score of:			
	<1.8	1.8–<1.9	1.9–<2.0	>2.0
Candida albicans (20)				20
Candida catenulata (2)				2
Candida dubliniensis (12)				12
Candida glabrata (11)				11
Candida guilliermondii (15)		3	2	10
Candida haemulonii (2)				2
Candida kefyr (10)				10
Candida krusei (9)		1		8
Candida lipolytica (9)				9
Candida lusitaniae (10)		5	3	2
Candida metapsilosis (8)			1	7
Candida orthopsilosis (21)		2		19
Candida parapsilosis (17)				17
Candida pelliculosa (10)			1	9
Candida rugosa (7)	1			6
Candida tropicalis (8)				8
Cryptococcus neoformans (6)	1	1	1	3
Geotrichum candidum (2)				2
Malasezzia pachydermatis (2)				2
Rhodotorula mucilaginosa (5)				5
Trichosporon asahii (4)		1	1	2
Trichosporon coremiiforme (1)			1	
Trichosporon mucoides (3)				3
Total (194)	2	13	10	169

isolates tested in the current study, then the same proportion of clinical isolates (87.1%) would be identified correctly at the species level (Table 3); however, as stated above, spectral scores between 1.8 and 2.0 were consistently accurate for identification of yeast species in the present study. In the current study and earlier reports (12, 27), organisms not identified by MALDI-TOF resulted from (i) a low spectral score due to an insufficient number of peaks or (ii) the absence of the organism in the database. In contrast to the case for biochemical identification tests, no yeast isolate in any of these studies was misidentified as another species.

For studies validating a new identification system, it is important that the database be constructed with a comprehensive collection of accurately identified reference strains. Although we selected type and reference strains from the American Type Culture Collection and the University of Alberta Microfungus Collection, 11 ATCC reference organisms were identified incorrectly, as determined by gene sequencing (Table 1). Ten of these organisms were identified correctly by MALDI-TOF, with no acceptable spectra obtained for one isolate, *Cryptococcus luteolus* (*Cryptococcus podzolicus*) ATCC 42279. No acceptable MALDI-TOF spectra were obtained for four additional organisms (*C. luteolus* ATCC 42279, *Geotrichum candidum* ATCC 10834 and ATCC 74169, and *Malassezia sympodialis* ATCC 96803). This was most likely due to the rigorous standards used for inclusion of a reference strain in the MALDI-TOF database (refer to Materials and Methods), because both clinical isolates of *G. candidum* were identified correctly.

Spectra of clinical isolates were analyzed using the reference strain database and MALDI BioTyper software (BioTyper Library v 2.0.4; Bruker Daltonics), a proprietary algorithm for spectral pattern matching resulting in a logarithmic score of 0 to 3. The results reported in this study demonstrate that all organisms identified at the species level had a spectral score of 1.8 or greater. Five clinical isolates were not identified in this study: two organisms had unacceptably low spectral scores, 1.3 and 1.5, and three organisms had spectral scores of 0 because the species were not in the reference library database.

All isolates were tested in quadruplicate, that is, each sample preparation was spotted four times on the steel target plate. Because 89.6% and 97.4% of the isolates were identified with the first spot and the first two spots, respectively, we believe that testing should routinely be performed in duplicate, with retesting in duplicate if the spectral scores are <1.8. This is a practical approach to integrating this technology into the routine laboratory because there are no additional reagent costs for duplicate testing, the analysis of individual spots requires less than 5 min, and the steel plates are washed and reused.

In summary, use of MALDI-TOF mass spectrometry for the identification of clinically important yeast is rapid and accurate. Although the instrument cost is significant (a Bruker Daltonics Microflex LT mass spectrometer presently costs approximately $110,000), the reagent cost per test is pennies, and there are no consumable costs. Additionally, we have found that this technology can be used to identify a broad spectrum of organisms, including virtually all Gram-positive and Gram-negative bacteria, *Nocardia* and related *Actinomycetes* bacteria, mycobacteria, and fungi. Integration of this technology into the routine workflow of the laboratory allows us to identify most bacterial and fungal organisms within a few hours of initial detection and to eliminate the need for biochemical testing or to perform gene sequencing for the identification of most problem organisms.

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