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Development of a Clinically Comprehensive Database and a Simple Procedure for Identification of Molds from Solid Media by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a powerful tool for the rapid and highly accurate identification of clinical pathogens but has not been utilized extensively in clinical mycology due to challenges in developing an effective protein extraction method and the limited databases available. Here, we developed an alternate extraction procedure and constructed a highly stringent database comprising 294 individual isolates representing 76 genera and 152 species. To our knowledge, this is the most comprehensive clinically relevant mold database developed to date. When challenged with 421 blinded clinical isolates from our institution, by use of the BioTyper software, accurate species-level (score of ≥2.0) and genus-level (score of ≥1.7) identifications were obtained for 370 (88.9%) and 18 (4.3%) isolates, respectively. No isolates were misidentified. Of the 33 isolates (7.8%) for which there was no identification (score of <1.7), 25 were basidiomycetes not associated with clinical disease and 8 were *Penicillium* species that were not represented in the database. Our library clearly outperformed the manufacturer's database that was obtained with the instrument, which identified only 3 (0.7%) and 26 (6.2%) isolates at species and genus levels, respectively. Identification was not affected by different culture conditions. Implementation into our routine workflow has revolutionized our mycology laboratory efficiency, with improved accuracy and decreased time for mold identification, eliminating reliance on traditional phenotypic features.

Gold-standard morphological mold identification in the clinical mycology laboratory is slow and laborious, relying on intense training and extensive experience for accurate identification of an increasingly widening spectrum of fungal pathogens. This decade alone has seen the emergence of numerous new species and species complexes, many of which (i) are resistant to antifungals or display atypical susceptibility profiles or (ii) are phenotypically similar but genetically and possibly pathogenically different from their counterparts (1–6). Such factors have complicated the accuracy of traditional phenotypic mold identification, compelling laboratories, where possible, to adopt methods such as DNA sequencing or matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) to enhance discriminatory power.

MALDI-TOF MS has become a powerful tool in the clinical microbiology setting and has revolutionized workflow in our laboratory, enabling rapid identification of bacteria (7, 8), yeasts (9), rapidly growing mycobacteria (10), and *Nocardia* (11). The accuracy of this technique compares favorably with that of genomic sequencing and is obtained at a significantly lower cost (12). However, its clinical application for the identification of filamentous fungi has lagged due to challenges in developing an efficient protein extraction method and the limited databases available. Consequently, many groups have developed in-house supplementary databases that target only select pathogens that are most prominent in their patient population (13–17).

We describe here the development and clinical evaluation of a comprehensive database for the identification of molds grown on solid media by MALDI-TOF MS. Our study highlights the many benefits of MALDI-TOF MS for rapid and unambiguous mold identification when an adequate database is available.

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MATERIALS AND METHODS

Reference strains and clinical isolates. A total of 294 fungal isolates comprising 58 reference strains obtained from the Centraalbureau voor Schimmelcultures (CBS), American Type Culture Collection (ATCC), and the National Institutes of Health (NIH) and 236 clinical strains obtained from NIH patients was used to construct the NIH mold database (Table 1; see also Table S1 in the supplemental material). The library included 76 genera and a total of 152 species comprised of 180 hyaline, 70 dematiaceous, 27 nonseptate, 7 dermatophyte, and 10 dimorphic mold isolates (Table 1). Organisms included in the NIH mold database were grown on Sabouraud dextrose agar at 28°C for 5 days prior to protein extraction. Five days was chosen as the optimal time point for growth, as

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TABLE 1 Isolates included in the NIH mold database

Group	Organism(s)[a]	Total no. of isolates represented (no. of reference strains)[b]
Hyaline ($n = 180$)	*Acremonium* sp.	4
	Acrodontium virellum	1 (1)
	Arthrinium sp.	1
	Arthrographis kalrae	1
	Ascomycetes (*Mycoshaerellaceae*, *Pestalotiopsis* sp.)	2
	Aspergillus spp. (*aculeatus*, *brevipes*, *chevalieri*, *duricaulis*, *flavipes*, *flavus*, *fumigatiaffinis*, *fumigatus*, *glaucus* group, *lentulus*, *nidulans*, *niger*, *novofumigatus*, *ochraceus*, *oryzae* var. *oryzae*, *sclerotiorum*, *sojae*, species, *sydowii*, *terreus*, *tubingensis*, *unguis*, *unilateralis*, *ustus*, *uvarum*, *versicolor*, *viridinutans*, *westerdijkiae*)	63 (14)
	Basidiobolus ranarum	1
	Basidiomycetes (*Agaricomycete*, mcr *Bjerkandera/Thanatephorus*, mcr *Hyphoderma* sp., mycorrhizal *Epipogium*, *Oxyporus* sp., *Peziza* sp., mcr *Polyporales/Oudemansiella*, *Trametes* sp.)	11
	Emericella spp. (*nidulans* var. *nidulans*, *quadrilineata*)	2 (2)
	Engyodontium sp.	1
	Eurotium amstelodami	1
	Eutypella spp. (*scoparia*, *vitis*)	4
	Fusarium spp. (*dimerum; incarnatum-equiseti* species complex; *oxysporum*, *proliferatum*, *solani*, *sporotrichioides*)	15 (1)
	Geosmithia argillacea	4 (1)
	Hamigera sp.	2 (1)
	Hyaline septate mold (mcr *Penicillium* sp., mcr *Pleosporales*)	3
	Hypocrea sp.	3
	Irpex sp.	1
	Leptosphaeria sp.	2
	Myrmecridium schulzeri	2
	Myrothecium	1
	Neosartorya spp. (*aureola*, *fischeri* var. *fischeri*, *hiratsukae*, *pseudofischeri*, *spinosa*, *udagawae*)	9 (7)
	Paecilomyces spp. (*formosus*, *lilacinus*, *saturatus*, species, *spectabilis*, *variotii*)	15 (3)
	Penicillium spp. (*decumbens*, species)	10
	Phanerochaete sordida	1
	Phellinus gilvus	1
	Phoma sp.	2
	Pithomyces sp.	2
	Pyrenochaeta romeroi	1
	Scopulariopsis brevicaulis	4
	Scytalidium sp.	1
	Sordariomycetes sp.	1
	Talaromyces spp. (*eburneus*, *helicus*)	3 (1)
	Theilavia terrestris	2
	Trichoderma viride	1
	Trichurus sp.	1
	Verticillium sp.	1
Dematiaceous ($n = 70$)	*Alternaria* sp.	2
	Bipolaris spp. (*hawaiiensis*, species)	3 (1)
	Chaetomium spp. (*globosum*, *nigricolor*)	2
	Cladophialophora spp. (*bantiana*, species)	2
	Cladosporium sp.	8
	Curvularia spp. (*geniculata*, *luntata*, species, *trifoli*)	6
	Dematiaceous *Scopulariopsis*	1
	Epicoccum sp.	2
	Exophiala spp. (*dermatitidis*, *phaeomuriformis*, *pisciphila*, species)	7
	Graphium penicillioides	1 (1)
	Ochroconis spp. (*gallopava*, *humicola*)	3
	Parascedosporium tectonae	1 (1)
	Periconia sp.	1
	Petriella spp. (*musispora*, *setifera*)	2 (2)
	Phaeoacremonium spp. (*aleophilum*, *parasiticum*)	3
	Phialophora spp. (species, *verrucoa*)	2
	Pseudallescheria spp. (*africana*, *angusta*, *boydii*, *desertorum*, *ellipsoidea*, *fusoidea*)	11 (11)
	Scedosporium spp. (*apiospermum*, *aurantiacum*, *prolificans*, species)	11 (3)
	Scopulariopsis chartarum	1
	Ulocladium sp.	1

(Continued on following page)

TABLE 1 (Continued)

Group	Organism(s)[a]	Total no. of isolates represented (no. of reference strains)[b]
Mucorales ($n = 27$)	*Cunninghamella* spp. (*bertholletiae*, species)	4 (1)
	Lichtheimia corymbifera	1
	Mucor spp. (*circinelloides*, *velutinosus*)	15 (1)
	Rhizomucor pusillus	1
	Rhizopus spp. (*oryzae*, species)	4
	Syncephalastrum sp.	1
	Zygomycete spp. (mcr *Syncephalastrum*)	1
Dermatophytes ($n = 7$)	*Microsporum* spp. (*canis*, *gypseum*)	3
	Trichophyton spp. (*mentagrophytes*, *rubrum*)	4 (1)
Dimorphic ($n = 10$)	*Blastomyces dermatitidis*	1 (1)
	Coccidioides immitis/posadasii	2
	Histoplasma capsulatum	4 (1)
	Paracoccidioides brasiliensis	1 (1)
	Penicillium marneffei	1 (1)
	Sporothrix schenkii	1

[a] mcr, most closely related.
[b] Total number of isolates, 294. Reference strains are listed in Table S1 in the supplemental material.

previously reported (16). A total of 421 clinically blinded isolates that were identified in our mycology laboratory by morphology alone were sent concurrently for MALDI-TOF identification. These molds were obtained from various media (Sabouraud dextrose, *Candida* chromogenic agar, buffered charcoal yeast extract, and brain heart infusion with blood, chloramphenicol, and gentamicin) prior to extraction, had been incubated at various temperatures (28 to 42°C), and were of various colony ages (2 to 7 days) depending on how soon mold was available for protein extraction (diameter of ~5 mm). Targeted Sanger sequencing was used to resolve discrepancies between MALDI-TOF results and morphological identification for four challenge isolates.

Protein extraction protocol. To extract proteins for MALDI-TOF MS, a small piece of mold (~5 mm in diameter) was excised from the culture plate inside a class II biosafety cabinet and placed into a 1.5-ml screw-cap Eppendorf tube containing 250 μl of 100% ethanol and 50 μl of 0.1-mm-diameter zirconia-silica beads (Cole-Palmer Instrument Co., Vernon Hills, IL). The mold was emulsified into a suspension using a sterile wooden stick and then vortexed for 15 min at top speed. Each tube was then centrifuged in an Eppendorf 5415D centrifuge at 9,447 × *g* for 2 min, and the ethanol was removed using a sterile fine-tip transfer pipette (Samco Scientific, San Fernando, CA). The pellet was resuspended in 50 μl of 70% formic acid and vortexed for 5 min. Following a brief 10-s centrifugation to remove any residual contents from the tube lid, 50 μl of acetonitrile was added, and the tube was vortexed again for 5 min and centrifuged at 9,447 × *g* for 2 min. The supernatant was either evaluated immediately by MALDI-TOF MS or was stored at −20°C until tested (within 1 week of being extracted). Subculturing the supernatant of several isolates onto Sabouraud dextrose agar did not reveal any viable organisms following extraction (data not shown). *Aspergillus ustus* (CBS 261.67T) was used as a positive quality control organism for each run. A log score of ≥2.0 for the control constituted an acceptable run (see "Spectral analysis" below).

MALDI-TOF MS. One microliter of supernatant from each isolate was spotted, in duplicate, onto a clean MALDI-TOF BigAnchorChip target plate on a 45°C slide warmer (Premiere slide warmer XH-2002; Daigger, Vernon Hills, IL), and 1 μl of calibration standard (protein calibration standard I and peptide calibration standard II; Bruker Daltonics, Inc., Billerica, MA) was pipetted onto a separate spot. After the samples had dried, 2 μl of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid prepared daily) was pipetted onto each of the samples and the calibration standard. Once dried, the plate was inserted into the MALDI-TOF MicroFlex LT mass spectrometer (Bruker Daltonics, Inc.), and spectra were acquired over a mass/charge (*m/z*) ratio ranging from 2,000 to 20,000. Each spot was measured using 1,000 laser shots at 60 Hz in groups of 50 shots per sampling area.

Spectral analysis. The NIH mold database was constructed using previously established criteria (9), in which each spectrum must contain at least 25 peaks with a resolution greater than 400 and 20 peaks with a resolution greater than 500 (FlexAnalysis software, version 3.0; Bruker Daltonics). For selected isolates in which criteria were not met due to an absence of protein peaks, the criteria were relaxed to 20 and 15 peaks, respectively. A minimum of 10 quality spectra per isolate was required for entry of that mold strain into the database and for creation of a mass spectral profile (MSP). The database was subsequently challenged against 421 clinical isolates that were run on duplicate spots. Spectra were analyzed against the Bruker database alone (Biotyper database V3.3.1.0_4110-4613; released 13 August 2012), the NIH database alone, and then both the NIH and Bruker databases combined using Biotyper software (version 3.0; Bruker Daltonics, Inc.) that assigned a logarithmic score ranging from 0 to 3. Manufacturer-recommended cutoff scores of ≥2.0 for species-level identification and ≥1.7 for genus-level identification were applied.

DNA extraction and sequencing. All 294 isolates included in the database were characterized morphologically with identification confirmed by targeted gene sequencing. Fungal DNA was extracted using an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. PCR was performed using Illustra PuReTaq Ready-to-Go PCR beads (GE Healthcare Life Sciences, Piscataway, NJ) in a 25-μl volume consisting of 22 μl of master mix and 3 μl of extracted DNA. Primers and PCRs for internal transcribed spacer (ITS), D1/D2 domains of the 23S ribosomal DNA (rDNA) complex, β-tubulin, and elongation factor-1α (EF-1α) amplification were performed in accordance with published protocols (2, 18–20). The entire ITS region was sequenced for all 294 molds built into the library, with additional genes sequenced for further taxonomic discrimination as needed. PCR products were visualized by 1% agarose gel electrophoresis and purified for sequencing using Amicon Ultra 0.5-ml 100K centrifugal filters (Millipore, Ltd., Ireland). Sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Data were assembled and edited using Lasergene SeqMan Pro technology (DNASTAR, Inc., Madison, WI). Sequences were run against the GenBank database using

TABLE 2 Overall performance of the NIH mold database and the Bruker library when challenged against 421 clinical isolates

Library(ies)	No. of isolates (%) with the indicated score[a]		
	≥2.0	≤1.99 and ≥1.7	<1.7
Combined NIH and Bruker	370 (88.9)	18 (4.3)	33 (7.8)
NIH alone	370 (88.9)	18 (4.3)	33 (7.8)
Bruker alone	3 (0.7)	26 (6.2)	392 (93.1)

[a] Scores represent the following: ≥2.0, species specific identification; ≤1.99 and ≥1.7, genus-specific identification; <1.7, no accurate identification.

NCBI BLASTn (http://blast.ncbi.nlm.nih.gov/) with an acceptable cutoff score of ≥99% similarity against reference strains, published sequences, or sequences deposited into GenBank by fungal reference centers.

RESULTS

Construction of the NIH mold database. A total of 294 fungal isolates was used to create the NIH mold database. The desired number of acceptable spectra was obtained for all but 11 of the 294 isolates (*Aspergillus sclerotiorum*, *Chaetomium nigricolor*, *Cladophialophora* sp., *Cladosporium* sp., *Exophiala dermatitidis*, *Exophiala pisciphila*, hyaline septate mold [no further differentiation], *Microsporum canis*, *Penicillium marneffei*, *Phialophora verrucosa*, and *Pithomyces* sp.) for which the number of quality peaks was lowered to a 20:15 ratio (see Materials and Methods) because of the limited number of peaks available for detection. A minimum of 10 quality spectra was obtained for all isolates before entry into the database, except for *Pyrenochaeta romeroi*, *Curvularia lunata*, *Histoplasma capsulatum*, *Penicillium marneffei*, and *Phialophora verrucosa*, for which only seven to nine spectra of sufficient quality were obtained.

To confirm specificity, all spectra included in the NIH mold database were analyzed against the NIH database plus the Bruker library (n = 5,118 MSPs). While all spectra matched to an exceptionally high degree with their own corresponding MSPs, cross-identifications at a score of ≥2.0 were noted for some isolates. These included bidirectional cross-identifications between the following: (i) members of the *Aspergillus* section Flavi (*Aspergillus flavus*, *Aspergillus oryzae*, and *Aspergillus sojae*), (ii) *Aspergillus nidulans* and *Emericella quadrilineata*, (iii) *Fusarium oxysporum* and *Fusarium proliferatum*, and (iv) *Paecilomyces spectabilis* and *Paecilomyces variotii*. *Aspergillus fumigatiaffinis*, *Aspergillus ochraceus*, and *Ulocladium* cross-identified with *Aspergillus viridinutans*, *Aspergillus westerdijkiae*, and *Alternaria*, respectively; however, no cross-identifications were observed in reverse. As expected for teleomorphs and anamorphs of the same mold, cross-matching patterns were observed between *Geosmithia argillacea*/*Talaromyces eburneus* and *Pseudallescheria boydii* complex/*Scedosporium apiospermum* (recently renamed *Pseudallescheria apiosperma* [21, 22]).

Clinical performance and validation. When blindly challenged against 421 clinical isolates, the NIH mold database provided species-level (score of ≥2.0) identification for 370 isolates (88.9%) while the most updated Bruker library alone (August 2012) identified only 3 isolates (0.7%) (Tables 2 and 3). Using the NIH mold database, an additional 18 isolates (4.3%) were identified to the genus level (score between 1.7 and 1.99) (Tables 2 and 3). No isolates were misidentified by MALDI-TOF MS. A total of 392 isolates (93.1%) failed to provide accurate identification (score of <1.7) when spectra were analyzed against the Bruker database alone (Tables 2 and 3).

Of the 236 clinical isolates included in the NIH mold database, 109 strains were subsequently added to the database after having been initially tested during the blinded clinical validation. To account for this possible bias, these strains were removed, and analysis was adjusted so that a total of 312 blinded clinical isolates were evaluated. Of these, 262 isolates (84%) were identified to the species level when analyzed against the NIH mold database alone, compared with the Bruker library that identified only 3 isolates (1%).

Four cases of morphological misidentification were detected. These included three *Aspergillus niger* and one *Aspergillus versicolor* isolates that were correctly reidentified by MALDI-TOF MS and confirmed by genomic sequencing as *Aspergillus aculeatus*, *Aspergillus sclerotiorum*, *Aspergillus tubingensis*, and *Aspergillus sydowii*, respectively. Of the 33 isolates (7.8%) for which there was no identification by MALDI-TOF MS, 25 were basidiomycetes not associated with clinical disease, and the remaining 8 were *Penicillium* species not represented in the database.

Because the NIH mold database was more diverse and contained more than twice the number of MSPs than the Bruker eukaryotic library (n = 133), numbers from the blinded challenge were adjusted to incorporate only those isolates for which there were representative spectra in the manufacturer's library (Table 4). Of the 156 isolates included, the NIH mold database provided species- and genus-level identifications for 144 (92.3%) and 4 (2.6%) isolates, respectively. The remaining eight strains were *Penicillium* sp. that failed to meet criteria for identification (score of <1.7). When the same 156 spectra were analyzed against the Bruker library alone, only 3 isolates (1.9%) were identified to the species level, and another 26 isolates (16.7%) were identified to the genus level. The remaining 127 (81.4%) were not identified despite having representative spectra in the Bruker library.

The new Bruker fungal library released in July 2012 was not available during clinical validation but was obtained post-manuscript submission. Retrospective analysis against this expanded library did show improved sensitivity as 68 (16.2%) and 80 (19%) isolates were identified to the species and genus levels, respectively. For the 287 isolates that had representative spectra in the new fungal library, 23.7% and 27.9% were identified to the species and genus levels, respectively. However, 48.4% of isolates failed to be identified despite having representative spectra in the new Bruker library.

Different culture conditions, including various media (Sabouraud dextrose, *Candida* chromogenic agar, buffered charcoal yeast extract, and brain heart infusion with blood, chloramphenicol, and gentamicin) that were incubated at various temperatures (27 to 42°C) and were of various colony ages (2 to 7 days), did not appear to affect the ability to obtain good identification results for the blinded isolates tested. Using the NIH mold database, the manufacturer's original cutoff scores of ≥2.0 for species and ≥1.7 for genus-level identifications were maintained so that specificity was not compromised to improve sensitivity. Matching threshold results between duplicate spots were achieved for 406 (96.5%) samples.

DISCUSSION

To our knowledge, we have developed the most comprehensive mold database to date to supplement the Bruker Biotyper library for the identification of filamentous fungi grown on solid media using MALDI-TOF MS. When challenged, the NIH mold data-

TABLE 3 Performance, by species, of the NIH mold database and the Bruker library when challenged against 421 clinical isolates

Organism (no. of isolates tested)	No. of isolates (%) identified by the indicated database and score:[a]			
	NIH mold database score of:		Bruker database score of:	
	≥2.0	≤1.99 and ≥1.7	≥2.0	≤1.99 and ≥1.7
Acremonium sp. (3)	3 (100)		NA	
Alternaria sp. (12)	11 (91.7)	1 (8.3)	0 (0)	1 (8.3)
Arthrographis kalrae (2)	2 (100)		0 (0)	2 (100)
Ascomycete	1 (100)		NA	
Aspergillus aculeatus (4)	4 (100)		NA	
Aspergillus flavipes (1)	1 (100)		NA	
Aspergillus flavus (10)	10 (100)		0 (0)	0 (0)
Aspergillus fumigatus (50)	50 (100)		3 (6)	16 (323)
Aspergillus nidulans (5)	5 (100)		NA	
Aspergillus niger (6)	6 (100)		0 (0)	1 (16.7)
Aspergillus ochraceus (1)	1 (100)		NA	
Aspergillus oryzae (2)	2 (100)		0 (0)	0 (0)
Aspergillus sclerotiorum (2)	2 (100)		NA	
Aspergillus sp. (1)	1 (100)		NA	
Aspergillus sydowii (8)	8 (100)		NA	
Aspergillus terreus (9)	9 (100)		0 (0)	0 (0)
Aspergillus tubingensis (2)	2 (100)		NA	
Aspergillus ustus (11)	11 (100)		NA	
Aspergillus uvarum (1)	1 (100)		NA	
Aspergillus versicolor (11)	9 (81.8)	2 (18.2)	0 (0)	1 (9)
Aspergillus viridinutans (2)	2 (100)		NA	
Aspergillus westerdijkiae (3)	3 (100)		NA	
Basidiomycete (37)	11 (29.7)	1 (2.7)	NA	
Chaetomium globosum (1)	1 (100)		0 (0)	0 (0)
Cladophialophora sp. (1)	1 (100)		NA	
Cladosporium sp. (14)	12 (85.7)	2 (14.3)	NA	
Curvularia geniculata (3)	3 (100)		NA	
Curvularia lunata (6)	5 (83.3)	1 (16.7)	NA	
Curvularia trifoli (1)	1 (100)		NA	
Dematiaceous *Scopulariopsis* (1)	1 (100)		NA	
Emericella quadrilineata (1)	1 (100)		NA	
Engydontium sp. (1)	1 (100)		NA	
Epicoccum sp. (5)	5 (100)		NA	
Eurotium amstelodami (2)	2 (100)		NA	
Eutypella scoparia (5)	5 (100)		NA	
Eutypella vitis (1)	1 (100)		NA	
Exherohilum rostratum (1)	NA		NA	
Exophiala dermatitidis (5)	5 (100)		0 (0)	0 (0)
Exophiala phaeomuriformis (1)	1 (100)		NA	
Exophiala pisciphila (1)	1 (100)		NA	
Exophiala sp. (1)	1 (100)		NA	
Fusarium incarnatum-equiseti species complex (5)	3 (60)	2 (40)	NA	
Fusarium oxysporum (3)	2 (77)	1 (33)	NA	
Fusarium proliferatum (3)	3 (100)		0 (0)	0 (0)
Fusarium solani (16)	16 (100)		NA	
Geosmithia argillacea (10)	9 (90)	1 (10)	NA	
Hamigera sp. (1)	1 (100)		NA	
Histoplasma capsulatum (5)	5 (100)		NA	
Hyaline septate mold, no further identification	2 (100)		NA	
Hypocrea sp. (4)	4 (100)		NA	
Irpex sp. (7)	6 (85.7)	1 (14.3)	NA	
Leptosphaeria sp. (2)	2 (100)		NA	
Microsporum canis (2)	2 (100)		0 (0)	0 (0)
Mucor circinelloides (1)	1 (100)		0 (0)	1 (100)
Mucor velutinosus (5)	5 (100)		NA	
Myrmecridium schulzeri (4)	4 (100)		NA	
Myrothecium sp. (1)	1 (100)		NA	
Neosartorya hiratsukae (1)	1 (100)		NA	
Ochroconis gallopava (4)	4 (100)		NA	
Ochroconis humicola (1)	1 (100)		NA	
Paecilomyces formosus (3)	3 (100)		NA	
Paecilomyces lilacinus (9)	9 (100)		0 (0)	0 (0)
Paecilomyces saturatus (2)	2 (100)		NA	
Paecilomyces sp. (2)	2 (100)		NA	
Paecilomyces variotii (8)	8 (100)		0 (0)	2 (25)
Penicillium sp. (15)	6 (40)	1 (6.7)	0 (0)	0 (0)
Periconia sp. (1)	1 (100)		NA	
Peziza sp. (1)	1 (100)		NA	
Phaeoacremonium aleophilum (1)	1 (100)		NA	
Phaeoacremonium parasiticum (4)	4 (100)		NA	
Phanerochaete sordida (1)	1 (100)		NA	
Phellinus gilvus (1)	1 (100)		NA	
Phialophora sp. (1)	1 (100)		NA	
Phialophora verrucosa (3)	1 (33)	2 (67)	NA	
Phoma sp. (2)	2 (100)		NA	
Pithomyces sp. (2)	2 (100)		NA	
Pseudallescheria boydii (9)	9 (100)		NA	
Rhizomucor sp. (1)	1 (100)		NA	
Rhizopus oryzae (3)	3 (100)		0 (0)	0 (0)
Rhizopus sp. (1)	1 (100)		0 (0)	0 (0)
Scedosporium apiospermum (11)	11 (100)		NA	
Scedosporium prolificans (4)	4 (100)		NA	
Scedosporium sp. (3)	3 (100)		NA	
Scopulariopsis brevicaulis (3)	3 (100)		NA	
Sordariomycetes sp. (1)	1 (100)		NA	
Syncephalastrum sp. (1)	1 (100)		NA	
Talaromyces helicus (1)	1 (100)		NA	
Thielavia terrestris (3)	3 (100)		NA	
Trametes sp. (3)	3 (100)		NA	
Trichoderma viridae (1)	1 (100)		NA	
Trichophyton rubrum (5)	5 (100)		0 (0)	2 (40)
Total	370 (88.9)	18 (4.3)	3 (0.7)	26 (6.2)

[a] NA, representative spectra not available in the Bruker database.

base provided accurate species-level identification for 370 isolates (88.9%), clearly outperforming the Bruker library, which identified only 3 isolates (0.7%) (Tables 2 and 3). This was partly due to the wider diversity of molds included in the NIH database (294 profiles) than in the Bruker Biotyper library (113 profiles). However, when 156 samples represented in the manufacturer's database were tested, strong performance was maintained by the NIH mold database (92.3% species-level identification), while the Bruker library continued to produce inadequate results (1.9%) (Table 4). Other investigators have also observed this phenomenon (13, 15, 17). This discrepancy may because Bruker utilized liquid mold cultures during database construction in an effort to minimize the effect of culture conditions and to aid in the production of uniform mycelium (23). Liquid mold cultures, however, are rarely employed in clinical mycology laboratories due to the increased risk of aerosolized spore contamination and the inability to visualize phenotypic macro- and microscopic characteristics. The discrepancy between methods used for clinical testing and database construction may explain why the Bruker database failed to identify 127 isolates (81.4%) for which there were representative data (Table 4). In July 2012, Bruker launched a separate library for the identification of molds grown in liquid media (23) that must be purchased separately from the primary library. Retrospective analysis against this expanded library (obtained post-

TABLE 4 Overall performance of the NIH mold database and the Bruker library when adjusted to evaluate only isolates ($n = 156$) for which there were representative spectra in the manufacturer's library

Library(ies)	No. of isolates (%) with the indicated score[a]		
	≥2.0	≤1.99 and ≥1.7	<1.7
Combined NIH and Bruker	144 (92.3)	4 (2.6)	8 (5.1)
NIH alone	144 (92.3)	4 (2.6)	8 (5.1)
Bruker alone	3 (1.9)	26 (16.7)	127 (81.4)

[a] Scores represent the following: ≥2.0, species specific identification; ≤1.99 and ≥1.7, genus-specific identification; <1.7, no accurate identification.

manuscript submission) did show improved sensitivity of the Biotyper due to its wider representation of fungal species; however, 48.4% of isolates were not identified despite having representative spectra in the new Bruker library. This, again, may be due to the library's reliance on liquid cultures.

Traditional phenotypic mold identification is laborious and requires considerable training and expertise. Identification is further complicated by sterile molds (24) and organisms that are genetically distinct from morphologically similar species. Several reports of mistaken identities have drawn attention (1–3, 25) because these masquerading molds are often refractory to antifungal agents (6, 24, 25). Correct identification is therefore imperative for appropriate disease management. In our study, *Aspergillus aculeatus*, *Aspergillus sclerotiorum*, and *Aspergillus tubingensis* were morphologically mistaken for *Aspergillus niger*, and *Aspergillus sydowii* was morphologically mistaken for *A. versicolor*. In most clinical laboratories, black aspergilli are generally reported as *Aspergillus niger* without further differentiation into *Aspergillus* section Nigri. Analysis of more *A. niger* isolates in our archive will likely reveal similar cases of mistaken identities and raise questions as to their clinical relevance. The phenotypic misidentification of *A. sydowii* for *A. versicolor* likely arose from the failure to wait for color formation of the colony, which typically requires an extended incubation for an additional 3 to 5 days. Our study showed that correct identification using the NIH mold database was obtainable from colonies as young as 2 days old on solid media, avoiding the time required for color production, sporulation, and/or temperature studies. This proved particularly useful for rapidly identifying *Fusarium solani* complex, *Histoplasma capsulatum*, *Coccidioides immitis/posadasii*, and members of the *Mucorales* during our blind validation. In addition, the number of isolates requiring DNA sequencing and molecular probes has decreased since MALDI-TOF MS has been incorporated into routine workflow.

Several cross-identifications were observed when all spectra included in the NIH mold database were analyzed against the entire NIH database plus the Bruker library (n = 5,118 MSPs). Although some bidirectional and unidirectional cross-identifications may be a cause for concern, accurate identification was always achieved at a higher score (at least 10%). We do not know whether other groups have observed a similar cross-identification problem as their databases included only some of the species listed above, and/or intralibrary specificity was not tested (13, 15, 16). However, neither of the aforementioned studies nor our study has documented any instances of mold misidentification by MALDI-TOF MS. In addition, unlike other investigators who lowered the score criteria to improve sensitivity (16, 17), we were able to maintain the manufacturer's original cutoff scores for species and genus identification without compromising sensitivity. Given time and the continual expansion of the database with more representative isolates, we predict that cross-identifications such as these will diminish.

While we strived to encompass both common and unusual isolates in our library, we recognize that the NIH mold database is not exhaustive and that some organisms (e.g., dermatophytes) were not widely represented due to their rarity at the NIH. We believe, however, that our database has the capacity to identify at least 90% of filamentous fungi isolated in most clinical mycology laboratories. The inherent expandability of the Biotyper software will also allow for inclusion of new species and complexes into our existing database. It is therefore not surprising that 25 nonpathogenic basidiomycetes and eight *Penicillium* species were not identified during clinical validation as many species in these groups were not represented in our database at all.

The use of extracted protein suspensions versus direct colony deposition ("toothpick method") for MALDI-TOF MS has remained controversial for many years. Comparative studies on bacteria have shown better identification scores from extracted protein suspensions (26, 27), presumably because cleaner spectra are produced without interference from salts, lipids, and other cell constituents. No comparative studies have been performed on molds thus far; however, MALDI-TOF MS from water suspensions of mycelia and/or conidia have shown promise (13, 15). Nonetheless, the risk of spore aerosolization and potential laboratory contamination will likely result in the continued use of protein extraction for filamentous fungi, with the added advantage of better quality spectra and enhanced sensitivity and specificity. While many different extraction procedures have been developed (14, 17, 28), our alternate extraction procedure is not restrictive to specific portions of mycelia or growth in liquid media. Future comparative versatility studies are needed to help standardize the application of MALDI-TOF MS for mold identification in a clinical setting. We also proved that duplicate spotting of protein extracts is not required since matching threshold results were achieved 96.5% of the time. Duplicate spotting, however, may be useful for assessing method accuracy and reproducibility during the clinical validation phase.

In summary, the NIH mold database is the most comprehensive library developed to date for the identification of molds from solid media by MALDI-TOF MS. Since implementation, laboratory efficiency has improved, with decreased turnaround time for identification and precision equivalent to genomic sequencing. Our protocol is easily adaptable, and the database can be made available to any clinical laboratory for future multicenter studies. We are optimistic that our procedure will be significantly beneficial, especially in laboratories with limited mycological expertise.

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