Rapid Identification of Molds by MALDI-TOF MS

Brittany O'Brien, Yanchun Zhu, Bryanna Freitas, Sudha Chaturvedi
Mycology Laboratory, Wadsworth Center, New York State Department of Health, Albany, NY

Abstract

Background: Invasive mold infections are rising among immunocompromised patients resulting in life-threatening illnesses. The species-level identification (ID) of molds is essential for rapid diagnosis and timely implementation of antifungal therapy. The gold-standard morphological identification methods of molds are slow, laborious, and rely on intense training and expertise. The molecular identification of molds by ribosomal genes (ITS & D1/D2) PCR and Sanger sequencing followed by BLAST search is an excellent technique, but they are both labor and time intensive. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has revolutionized the diagnostic mycology laboratory by successfully identifying yeasts but not molds to the same extent. The difficulty of mold ID by MALDI-TOF MS was attributed to variable growth, pigmentation, and agar penetration. In this investigation, we evaluated the use of dialysis membrane on agar surfaces for mold growth, sample incubation at -20°C overnight, and bead-beating techniques for library enrichment, followed by MALDI-TOF MS identification.

Methods: Mold isolates were grown on Sabauraud dextrose agar (SDA) slant implanted with SnakeSkin™ dialysis membrane at 30°C. Mold growth on the membrane was removed carefully with a needle, added to 75% ethanol, mixed thoroughly, and either processed immediately or stored at -20°C overnight. The mold-ethanol mixture was centrifuged at 12,000 rpm, ethanol was removed, and pellet was dried for 10 min. The pellet was mixed with a 1:1 ratio of 40 µL each of 70% formic acid (FA) and 100% acetonitrile (ACN) mixture, vortexed, and centrifuged at 12,000 rpm for 2 min. Additionally, a representative of mold pellets were mixed with 70% FA, bead-beating for 15 sec at 4,700 RPM followed by ACN addition and rest of the procedures as described above. One µL of supernatant was loaded onto the target plate, and after drying, 1 µL of HCCA matrix was added, followed by analysis by MALDI-TOF MS (models: Biotyper Microflex LT and Biotyper MBT Smart).

Results: Molds incubated at -20°C in 75% ethanol overnight yielded better spectra with a 75% success rate of mold ID compared to 59% without incubation overnight. Mold growth on the snakeskin improved mold ID to 93% with no interference from agar. Addition of bead-beating further improved mold spectra to 100%. In total, 317 mold strains have been added to Wadsworth Center Mycology library database including 62 genera and 120 species.

Conclusions: The growth over snakeskin in SDA slant, incubation overnight in 75% ethanol, bead-beating in 70% FA, and sufficient representation of each mold species in the database are all crucial for the successful identification of molds by MALDI-TOF MS. These results holds promise as a fast and accurate identification tool for molds, which can improve patient care.

Introduction

Emerging, drug-resistant and invasive mold infections are rising, and they are the leading cause of morbidity and mortality in immunocompromised patients. Currently, mold identification relies on the macroscopic and microscopic observation of colonies grown on mycological media. Adequate phenotypic identification of molds requires highly skilled mycologists, a specialty diminishing slowly. The PCR followed by sequencing of the ribosomal genes (ITS & D1/D2) are excellent technologies but are time consuming and require at least 5 to 7 days in the routine clinical laboratory setting. This delay negatively impacts the patients' prognosis. Finally, only some clinical laboratories routinely use a molecular approach for microorganism identification. Therefore, the identification of molds remains problematic, and misidentifications are likely to occur in routine settings. The MALDI-TOF MS has revolutionized microbial identification, including bacteria, yeasts, and molds. There are few reports of mold library database and its utility in rapid identification of molds by MALDI-TOF MS. Despite progress made, the mold ID by MALDI-TOF MS remains challenging. In the present investigation, we report a few advances made in the accurate identification of common, rare, and dimorphic molds.

References

1. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med **4**:165rv13. doi: 10.1126/scitranslmed.3004404.
2. Clark AE, Kaleta EJ, Arora A, Wolk DM. 2013. Matrix-assisted laser desorption ionization–time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev **26**:547–603. doi: 10.1128/CMR.00072-12.
3. Becker PT, de Bel A, Martiny D, et al. Identification of filamentous fungi isolates by MALDI-TOF mass spectrometry: clinical evaluation of an extended reference spectra library. *Med Mycol*. 2014;52(8):826-834. doi:10.1093/mmy/myu064
4. Choi Y, Kim D, Choe KW, et al. Performance Evaluation of Bruker Biotyper, ASTA MicroIDSys, and VITEK-MS and Three Extraction Methods for Filamentous Fungal Identification in Clinical Laboratories. *J Clin Microbiol*. 2022;60(11):e0081222. doi:10.1128/jcm.00812-22
5. Sanguinetti M, Posteraro B. Identification of Molds by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *J Clin Microbiol*. 2017;55(2):369-379. doi:10.1128/JCM.01640-16
6. Shao J, Wang Q, Wei L, Wan Z, Li R, Yu J. Limitations of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the identification of Aspergillus species. *Med Mycol*. 2022;60(2):myab084. doi:10.1093/mmy/myab084

Acknowledgments

This study was supported by the Clinical Laboratory Reference System, Wadsworth Center, NYSDOH, and Cooperative Agreement Grant (NU50CK000423), funded by the Centers for Disease Control and Prevention. We would also like to thank Wadsworth Center Applied Genomic Technologies and Media & Tissue Cultures Cores for DNA sequencing and culture media, respectively.

Methods

Figure 1. Mold Protein Extraction Workflow for Bruker MALDI-TOF MS

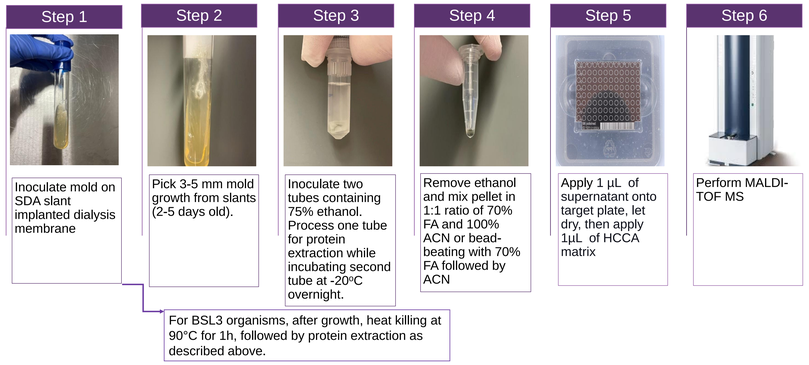
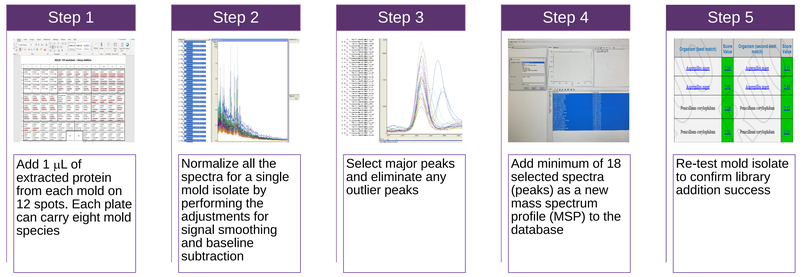


Figure 2. Library Creation for Molds using Bruker MALDI-TOF MS



Results

Figure 3. Stepwise Improvement of Mold Spectra following Incubation with 75% Ethanol at -20°C Overnight, Growth on Dialysis Membrane, and Bead-beating with a Representative Sample Set

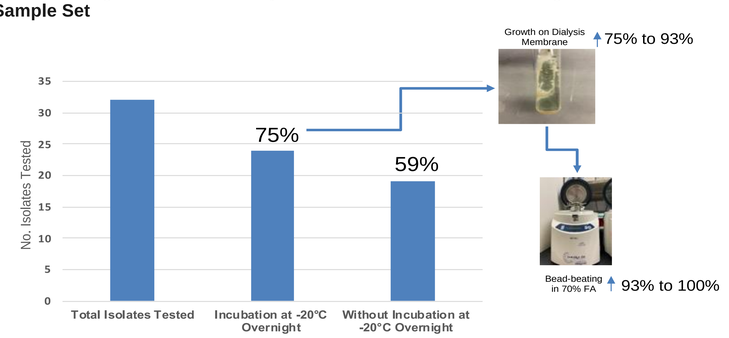


Table 1. Successful Identification of Molds to Species Level by Bruker MALDI-TOF MS

Category	Mold ID	# Samples tested	Agreement between ITS and MALDI-TOF MS	MALDI-TOF MS score range	Bruker library	Wadsworth library
Dematiaceous fungi	*Alternaria alternata*	3	3	1.94-2.21	5	-
	Alternaria chartarum	1	1	1.9	1	-
	Curvularia geniculata	2	2	1.87-2.09	-	4
	Epicoccum nigrum	2	2	1.98-2.34	7	1
	Exophiala dermatitidis	6	5	1.67-2.18	2	5
	Exophiala lecanii-corni	4	3	1.59-2.07	-	4
	Exophiala oligosperma	3	2	1.22-2.15	-	3
	Scedosporium apiospermum	2	2	1.84-1.95	5	4
Dermatophyte	*Microsporum audouinii*	1	1	2.04	4	1
	Microsporum canis	1	1	2.22	9	4
	Trichophyton rubrum	7*	5	1.59-2.23	8	6
Hyaline fungi	*Arthrinium arundinis*	1	1	2.02	4	-
	Aspergillus calidoustus	6	6	1.90-2.27	2	3
	Aspergillus flavus	3*	2	1.90-2.0	16	6
	Aspergillus fumigatus	55*	50	No Peak-2.30	12	7
	Aspergillus nidulans	4	4	1.83-2.30	9	3
	Aspergillus niger	8*	3	1.19-2.19	12	2
	Aspergillus sydowii	4	4	1.86-2.20	2	5
	Aspergillus terreus	4*	2	1.22-2.13	12	4
	Aspergillus unguis	5	5	1.74-2.29	2	4
	Aspergillus ustus	1	1	2.16	1	-
	Fusarium proliferatum	2	1	1.61-1.90	6	-
	Fusarium solani	2	2	1.70-2.35	11	3
	Magnusiomyces clavatus	1	1	2.87	3	-
	Microascus gracilis	1	1	1.95	1	-
	Paecilomyces variotii	1	1	2.07	-	5
	Penicillium citrinum	2	2	1.85-1.97	4	2
	Penicillium corylophilum	2	2	2.12-2.14	5	-
	Penicillium digitatum	1	1	2.4	10	-
	Penicillium glabrum	2	2	1.94-2.11	3	2
	Purpureocillium lilacinum	2	2	2.17-2.34	5	6
	Talaromyces diversus	1	1	2.31	2	1
	Trpex lacteus	5	5	1.92-2.25	-	5
	Peroneutypa scoparia	6	6	1.30-2.11	-	5
	Schizophyllum commune	1	1	2.05	4	2
	Thanatephorus cucumeris	1	1	2.24	2	1
	Trametes versicolor	1	1	2.34	-	4
Mucormycete	*Absidia corymbifera*	1	1	2.09	8	5
	Mucor circinelloides	8	8	1.29-2.45	8	11
	Rhizopus microsporus	3	3	1.90-2.09	9	1
	Rhizopus oryzae	1	1	2.23	6	7
Dimorphic Fungi (BSL3)	*Blastomyces dermatitidis*	5	5	2.0-2.78	-	5
	Coccidioides immitis	3	3	2.69-2.72	-	3
	Coccidioides posadasii	4	4	2.11-2.79	-	4
	Histoplasma capsulatum	4	4	2.13-2.77	-	4
	Penicillium marneffei	6	6	2.68-2.82	-	6

*isolates with no ID by MALDI-TOF MS; possible reasons could be different genotype or no peaks (spectra)

Figure 4. Bead-beating further Improves MALDI Spectra for Mold ID to Species Level

Mold ID	MALDI-TOF MS score	
	Before bead-beating	After bead-beating
Aspergillus calidoustus	no peak	2.27
Aspergillus fumigatus	no peak	2.21
Aspergillus nidulans	no peak	2.02
Exophiala lecanii-cornii	no peak	2.19
Kneiffiella palmae	no peak	2.21
Microascus gracilis	no peak	1.95

Conclusions

- We report successful identification of molds to species level by Bruker MALDI-TOF MS.
- Culturing molds on dialysis membrane, incubation at -20°C overnight in 75% ethanol, and bead-beating in 70% FA were some technical advances to get optimal spectra for mold ID.
- Of 344 mold isolates tested prospectively, 319 (93%) produced good spectra upon incubation at -20ºC in 75% ethanol overnight. Bead-beating in 70% FA further improved the spectra of the remaining 25 molds (7%).
- There was high agreement between MALDI-TOF MS ID and 18S ribosome gene sequencing.
- The library creation using molds grown on solid media in the present study was vital to include mold heterogeneity, which follows routine clinical mycology processes.

Challenges and Limitations

- Some closely related molds could not be identified to species level by MALDI-TOF MS. Further work is currently underway to assess these discrepancies.
- Fresh growth seems to be crucial for MALDI-TOF MS ID, which can hamper patient care, especially for slow growing molds