AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

MINIREVIEW

Update on Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Identification of Filamentous Fungi

Laura S. Wilkendorf,[a] Edmée Bowles,[a] Jochem B. Buil,[a,b] Henrich A. L. van der Lee,[a,b] Brunella Posteraro,[c,d] Maurizio Sanguinetti,[c,e] Paul E. Verweij[a,b]

[a]Radboud University Medical Center, Department of Medical Microbiology, Nijmegen, the Netherlands
[b]Center of Expertise in Mycology Radboudumc/CWZ, Nijmegen, the Netherlands
[c]Dipartimento di Scienze Biotecnologiche di Base, Cliniche Intensivologiche e Perioperatorie, Università Cattolica del Sacro Cuore, Rome, Italy
[d]Dipartimento di Scienze Gastroenterologiche, Endocrino-Metaboliche e Nefro-Urologiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy
[e]Dipartimento di Scienze di Laboratorio e Infettivologiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy

ABSTRACT Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS)-based species identification has found its place in many clinical routine diagnostic laboratories over the past years, allowing significantly reduced turnaround times and high-precision results. With regard to MALDI-TOF MS for filamentous fungi, here, we discuss different approaches for sample processing and growth conditions before analysis. In particular, we review the performances of different commercially available databases as well as the potential of complementary (self-constructed) in-house databases.

KEYWORDS Biotyper, Bruker, filamentous fungi, MALDI-TOF MS, identification, in-house database, Vitek

Fast and reliable identification of fungal pathogens is crucial for the initiation of appropriate antifungal treatment of patients with fungal diseases. Commonly, mold identification is based on micro- and macroscopic characteristics of cultured colonies in clinical microbiology laboratories. This requires time to obtain mature growth and technicians who are highly skilled in mycology. Furthermore, identification to the species *sensu stricto* level cannot be obtained using phenotypic methods alone and requires DNA sequencing (1). Overall, phenotypic and molecular methods for the identification of molds are time-consuming and not widely available.

In the last few years, proteomics has emerged as a potent method for the identification of microorganisms, which is based on MALDI-TOF MS analysis. The acronym MALDI-TOF MS stands for matrix-assisted laser desorption ionization–time of flight mass spectrometry. Microorganisms of interest are transferred to a target plate, and an organic matrix is placed on top. They are then ionized with a nitrogen laser. To separate the ionized molecules, they are accelerated through a magnetic field and migrate with a velocity according to their mass-to-charge ratio (*m/z*). At the end of the vacuum tube, a detector measures their time of flight and abundance over time. With these data, a raw spectrum is created and compared with a database of reference spectra. The identification is then based on the similarity of the sample spectrum to the reference spectrum (2). MALDI-TOF MS is currently replacing traditional microbiological identification methods, especially in the field of bacteriology, and the technique is highly reliable, fast, and easy to perform.

Although the use of MALDI-TOF MS is highly accepted for identifying yeasts, there are still some problems when it comes to filamentous fungi. First, identification may be

Citation Wilkendorf LS, Bowles E, Buil JB, van der Lee HAL, Posteraro B, Sanguinetti M, Verweij PE. 2020. Update on matrix-assisted laser desorption ionization–time of flight mass spectrometry identification of filamentous fungi. J Clin Microbiol 58:e01263-20. https://doi.org/10.1128/JCM.01263-20.

Editor Colleen Suzanne Kraft, Emory University

Address correspondence to Jochem B. Buil, jochem.buil@radboudumc.nl.

Accepted manuscript posted online 16 September 2020
Published 18 November 2020

hampered by the presence of a more robust cell wall (mostly being composed of glucans and chitin) than that of bacteria (3). Second, fungi have a fast-changing morphology (mycelium/conidia), which also results in different spectra (4). Third, commercially available fungal reference libraries are currently not as comprehensive as the bacterial ones. In this review, we provide an overview of the MALDI-TOF MS system, sample preparation, databases, cutoff levels, and the potential use of MALDI-TOF MS for antifungal resistance testing.

Four different MALDI-TOF MS benchtop platforms are currently approved and commercialized in Europe for the routine identification of fungi in clinical microbiology laboratories: the Bruker Biotyper (Bruker Daltonics, Bremen, Germany), Vitek MS (bioMérieux, Marcy l'Etoile, France), Axima@Saramis (Shimadzu/AnagnosTec, Duisburg, Germany), and the Andromas system (Andromas SAS, Paris, France) (5). The first two systems are also approved in the United States, but the Bruker system is limited to the clinical identification of bacteria and yeasts (3), while the Vitek MS system is also approved for the identification of fungi (6). Each manufacturer has developed a peak-matching algorithm to compare an unknown spectrum to its database (2).

MALDI-TOF MS-BASED IDENTIFICATION OF FILAMENTOUS FUNGI

Sample preparation. There are two main methods to obtain a spectrum by MALDI-TOF MS. The basic method is the direct deposition of cells onto a target plate, fixated with a suitable matrix. This method is called the intact-cell (IC) method because the cells remain intact (although mold cells need to be inactivated with ethanol first) (5). To enhance the spectrum quality, a short target extraction step may be beneficial (3). However, the IC method may be hampered by the presence of a more robust cell wall in fungi (3). The second method, which counteracts this, is a complete extraction (complete lysis [CL]) method. Here, the cells are lysed using an ethanol-formic acid (FA) procedure to allow complete protein extraction. Direct colony deposition is faster than the CL method, but it has a lower discriminatory power because the spectra are more influenced by the culture media, and identification problems can occur with melanized fungi (7).

Growth conditions and extraction methods. Different growth conditions as well as extraction methods are used to extract all fungal proteins. This is mainly because the MALDI-TOF MS platform providers recommend different growth methods (Sabouraud broth for Bruker and solid plates for Vitek), while growth on (selective) Sabouraud agar, oatmeal agar, or other agars is generally preferred by clinical laboratories. This preference is because growth on agar allows the evaluation of the morphological characteristics of the isolates. To optimize existing protocols, different growth conditions and extraction methods were tested on different molds by Cassagne et al. (1). They compared spectra after a standard FA extraction step, a centrifugation step followed by FA extraction, or a lysing step with microbeads followed by FA extraction. These three different procedures gave no significant difference in spectra, which led to the recommendation to use the simplest extraction methods to save time and resources. In their favored method, the fungi of interest were grown for 72 h on Sabouraud gentamicin-chloramphenicol agar plates followed by formic acid-acetonitrile extraction (1). However, Coulibaly et al. studied the *Pseudallescheria-Scedosporium* species complex by extracting mycelia grown on malt agar with trifluoracetic acid (TFA) and on Sabouraud agar with FA extraction. Both methods gave no significant difference in correct identifications, but this study revealed that a longer incubation probably leads to better identification results (8). For *Fusarium* species, a 72-h incubation at 27°C on malt agar gave more distinguishable peaks than Sabouraud gentamicin-chloramphenicol agar or potato dextrose agar, but these small differences did not prevent correct identifications on the three different agar plates (9). An overview of the sample preparation methods resulting in the most distinguishable spectra for different fungi is provided in Table 1. Nevertheless, if the extraction protocol differs from the one used to build the reference library, different spectra might be obtained, and the fungi might not be correctly identified.

TABLE 1 Growth conditions for filamentous fungi generating the most distinguishable peaks[a]

Growth conditions	Method for harvest of material	Extraction step(s)	Species (no. of isolates)	Reference
72 h at 27°C on Sabouraud gentamicin-chloramphenicol agar plates	Harvested by scraping with a sterile plastic device	FA extraction	*Aspergillus* spp. (43), *Penicillium* spp. (16), *Scedosporium* spp. (18), *Fusarium* spp. (11), other (68)	1
24 h at 27°C in Sabouraud broth	10-min centrifugation at 13,000 × *g*	Pellet washed 3 times with 1 ml of sterile water and suspended with 300 μl of HPLC sterile water and 900 μl of anhydrous ethyl alcohol; FA extraction	*Aspergillus* spp. (43), *Penicillium* spp. (16), *Scedosporium* spp. (18), *Fusarium* spp. (11), other (68)	1
72 h at 27°C on Sabouraud gentamicin-chloramphenicol agar plates	Harvested by scraping with a sterile plastic device	Hydroalcoholic suspension of fungal material lysed by 3 cycles of microbeads with a FastPrep-24 instrument; FA extraction	*Aspergillus* spp. (43), *Penicillium* spp. (16), *Scedosporium* spp. (18), *Fusarium* spp. (11), other (68)	1
72 h at 27°C on Sabouraud dextrose agar with antibiotics		FA extraction	*Pseudallescheria-Scedosporium* species complex	8
72 h at 27°C on malt agar		TFA extraction	*Pseudallescheria-Scedosporium* species complex	8
72 h at 27°C on malt agar	Surfaces scraped using a sterile scalpel	TFA extraction	Clinical *Fusarium* isolates	9

[a]Abbreviations: FA, formic acid; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

Although the use of mycelia is recommended for the Bruker Biotyper system, studies are focusing on the identification of fungi just by using spores. Welham et al. (10) demonstrated that relatively simple spectra can be obtained from spores of *Penicillium* spp. These spectra could then be distinguished from different *Aspergillus* species spores. Discrimination between aflatoxic and nonaflatoxic strains was also possible (11).

Melanized fungi are a group of fungi that are difficult to discriminate morphologically as well as by MALDI-TOF MS analysis. This is mostly because the dark fungal pigments of melanized fungi may inhibit the formation of distinguishable spectra (12). This can be overcome by growth in liquid media that suppress pigment formation (12) or by preanalytical washing steps (13). Furthermore, they are underrepresented in commercial databases; thus, better database representation will likely improve the percentage of correct identifications of melanized fungi (14, 15).

Databases. The species coverage in the database might be the greatest drawback of each available MALDI-TOF MS system. The Bruker Filamentous Fungi Library contains just 127 species (V1) or 152 species (V2) (Bruker Daltonics, Bremen, Germany), while Vitek MS Knowledge Base version 3.0 contains only 79 clinically relevant mold strains (16). This results in low identification rates when only the manufacturer's database is used. When the Bruker database (version 3.3.1.0) was used, Lau et al. (14) identified only 1.9% of fungal isolates correctly to the species level, while Stein et al. (17) identified 13.6%, Normand et al. (18) identified 23.9%, Schulthess et al. (19) identified 54.2%, and Sleiman et al. (20) identified 63% of fungal isolates correctly to the species level with Bruker Filamentous Fungi Library V. 1.0. When Vitek MS Knowledge Base version 3.0 was used, Rychert et al. (16) identified 91%, McMullen et al. (21) identified 68.8%, and Pinheiro et al. (22) identified 52% of fungal isolates correctly to the species level. However, the identification rate is highly influenced by the fungal isolates used in the study, and no direct comparison was performed.

One way to avoid the limitations of the commercial databases from the manufacturers is to build an in-house reference database with well-characterized clinical isolates. Therefore, several spectra must be combined with a reference metaspectrum (MSP) using the software provided with the system. In most cases, spectra from 4 biological and 10 technical replicates are used (1, 23, 24). Additionally, the quality of

each spectrum should be assessed beforehand. It should contain at least 25 peaks with a resolution of >400 and 2 peaks with a resolution of >500 (14).

Many laboratories have established their in-house databases to complement the manufacturers' ones. The most extensive, publicly available databases established by Lau et al. (also known as the NIH mold database), Gautier et al., and Becker et al. contain 152, 347, and 472 fungal species, respectively (14, 23, 24). In tests with clinical isolates, these databases yielded 87.9%, 98.8%, and 95.5% correct identifications. The in-house database of the BCCM/IHEM culture collection and the Mycology Laboratory of Marseille Hospital comprises 1,913 strains, representing 938 fungal species and 246 fungal genera, and is linked to a Web application to compare it to the obtained spectra. This new algorithm (in combination with the database) outcompeted the Bruker system in both identification time and accuracy. The only misidentifications concerned closely related species or *Basidiomycetes*, which were not present in the reference database (18). The BCCM/IHEM reference database and MSI software are publicly available at https://biological-mass-spectrometry-identification.com/msi/ and are updated constantly. Smaller in-house reference databases often focus on increasing the reference spectra of different species within a few fungal genera (Table 2). Nevertheless, they achieved identification rates ranging from 82.1% (25) to 100% (26–29) for the studied species. This shows that a small-scale expansion of manufacturers' databases can significantly increase the number of correct identifications, especially when a clinical laboratory frequently encounters samples of the same species. The accuracy of the identification of the isolates included in the in-house database is of utmost importance, as false identifications of these isolates may automatically result in false identifications of clinical isolates that are compared to the database. The isolates included in the NIH mold database were identified using sequencing of the internal transcribed spacer (ITS) with additional loci (D1/D2 domains of the 23S ribosomal DNA [rDNA] complex, β-tubulin, and elongation factor Iα) if needed (14). The isolates included in the database by Gautier et al. were identified morphologically in combination with multilocus sequencing of both the D1-D2 variable region of the 28S gene and the ITS2 region (24). For the construction of the BCCM/IHEM reference database, sequencing was also used to confirm the identifications of the isolates, and the internal transcribed spacer, beta-tubulin, actin, translation elongation factor 1 alpha, or large-subunit ribosomal DNA was used when required (23). As fungal taxonomy rapidly changes, in-house databases should be reviewed regularly and should be updated if needed to ensure correct identification based on recent literature.

In the clinical setting, it is challenging, but crucial, to identify cryptic fungal species for the correct treatment. Cryptic *Aspergillus* species of the same section are indistinguishable by morphological characteristics. The in-house reference database of Vidal-Acuna et al. (30) includes 19 cryptic *Aspergillus* species, resulting in a rate of correct identifications to the species level of 70.7%. The MSI online platform was also evaluated for the identification of cryptic *Aspergillus* species in a multicentric study. In this study, 5,108 isolates were identified as *Aspergillus* species, of which 1,477 (28.9%) belonged to cryptic *Aspergillus* species. Of the 1,477 cryptic *Aspergillus* isolates, 245 were additionally identified by DNA sequencing. The agreement between sequencing and MSI-based identification was 99.6% (244/245 species) (31). This indicates that the correct distinction of cryptic *Aspergillus* isolates at the sectional level is possible with MALDI-TOF MS.

Furthermore, melanized fungi are currently still underrepresented in commercially available databases (15), but recent studies have shown that the addition of melanized fungi to an in-house database increased the number of correct identifications (15, 32).

Cutoff levels. The manufacturer-recommended cutoff levels, in terms of the Bruker Biotyper identification score, are ≥2.0 for a correct species-level and ≥1.7 for a correct genus-level identification. Several studies analyzed the impact of lowering the cutoff level on the number of correct identifications (19, 30, 33–36). Lowering the cutoff levels to ≥1.7 for species-level and ≥1.5 for genus-level identifications resulted in an increase in accurate species-level identifications of 9.55% on average (range, 4% to 16.9%;

TABLE 2 Studies evaluating the performance of in-house MALDI-TOF MS databases for identification of filamentous fungi[a]

MALDI system(s)	Order(s) and/or group(s)	Organism(s) studied (no. of species)	No. of species (no. of strains) in DB	Acceptance criterion for ID (Bruker Biotyper ID score)	No. of correctly identified isolates while challenging DB/total no. of isolates while challenging DB	Accuracy (%)	Comparative method(s)	Sequence(s) used for ID	Reference
Bruker Daltonics	*Eurotiales*, *Mucorales*, *Hypocreales*	*Aspergillus* (sections *Fumigati*, *Flavi*, *Terrei*, *Nigri*, *Nidulantes*, *Usti*, *Circumdati*, *Aspergillus*, *Candidi*) (33), *Mucor* spp. (5), *Lichtheimia* spp. (2), *Rhizopus* spp. (2), *Rhizomucor* spp. (2), *Fusarium* spp. (12)	55	≥2.0; 1.7–2.0	91/94; 3/94	96.8; 3.2	MB	ITS1-5.8S-ITS2, β-tubulin, calmodulin, elongation factor Iα	42
Bruker Daltonics	*Eurotiales*, *Hypocreales*, *Mucorales*, *Microascales*, dermatophytes, dimorphic, other	*Aspergillus* spp. (63), *Penicillium* spp. (10), *Paecilomyces* spp. (15), *Fusarium* spp. (15), *Pseudallescheria* spp. (11), *Scedosporium* spp. (11), *Mucor* spp. (15), other (154)	152 (294)	≥2.0; 1.7–2.0	370/421; 18/421	87.9; 4.3	MB, MO	ITS, D1/D2 domains of the 23S rDNA complex, β-tubulin, elongation factor Iα	14
Bruker Daltonics	*Eurotiales*, *Hypocreales*, other	*Aspergillus* spp. (77), *Penicillium* spp. (45), *Paecilomyces* spp. (7), *Fusarium* spp. (14), dematiaceous species (11), dermatophytes (46), other (130)	347 (708)	NR	257/262	98.1	MB, MO	D1-D2 variable region of the 28S gene; ITS2	24
Bruker Daltonics	*Eurotiales*, *Hypocreales*, *Mucorales*, *Microascales*, other	*Aspergillus* spp. (256), *Penicillium* spp. (37), *Fusarium* spp. (20), *Scedosporium* spp. (16), *Cladosporium* spp. (10), other (39)	472 (760)	≥2.0; 1.7–2.0	372/390	95.4	MO, MB	ITS, β-tubulin, elongation factor Iα, LSU rDNA	23
Bruker Daltonics	*Eurotiales*, *Hypocreales*, *Microascales*, other	*Aspergillus* spp. (43), *Penicillium* spp. (16), *Scedosporium* spp. (18), *Fusarium* spp. (11), other (68)	63 (146)	≥2.0; 1.7–2.0	150/156	96.15	MO, MB	ITS1-5.8S-ITS2, D1-D2 domains of the 28S rDNA complex	1
Bruker Daltonics	*Eurotiales*	*Aspergillus* spp. (23)	14	≥2.0	24/24	100	MB, MO	Calmodulin, β-tubulin	29
Bruker Daltonics	*Hypocreales*	*Fusarium* spp. (19)	40 (289)	≥2.0	222/268	82.8	MB, MO	ITS, partial ribosomal LSU, β-tubulin, elongation factor Iα	36
Bruker Daltonics	*Mucorales*	*Rhizopus arrhizus* *Rhizopus microsporus*	2 (38)	NR NR	25/25 13/13	100 100	MB MB	ITS, actin, elongation factor Iα	27
Bruker Daltonics	*Eurotiales*	*Talaromyces marneffei*	1 (21)	≥2.0	39/39	100	MB	MPI, PM-ATPase, PK	28
Bruker Daltonics	*Eurotiales*	*T. marneffei*	1 (4)	≥2.0	23/28	82.1	MB, MO	ITS1, ITS4	25
Bruker Daltonics	*Eurotiales*	*Paecilomyces* spp. (4)	8 (8)	≥2.0; ≥1.8	67/71	94.30	MB, MO	ITS1-5.8S-ITS2, D1/D2 region, β-tubulin	33
Bruker Daltonics	Dermatophytes	*Trichophyton* spp. (6), *Microsporum* spp. (4), *Epidermophyton floccosum*	13 (24)	>2.0; 1.7–2.0	64/64	100	MB, MO	5.8S-ITS2 rDNA	26
Bruker Daltonics	*Eurotiales*, *Hypocreales*, *Mucorales*, *Microascales*, *Onygenales*, other	*Aspergillus* spp. (115), *Fusarium* spp. (33), *Penicillium* spp. (60, *Paecilomyces* spp. (5), *Scedosporium* spp. (20), *Trichophyton* spp. (183), other (85)	938	≥20	435/501; 26/501	87.35; 5.22	MB	ITS, β-tubulin, elongation factor	18[b]
Bruker Daltonics	*Pleosporales*, *Chaetothyriales*, *Capnodiales*, other	*Alternaria* spp. (9), *Cladophialophora* spp. (10), *Cladosporium* spp. (8), *Fonsecaea pedrosoi* (10), *Bipolaris* spp. (10), other (70)	29 (59)	≥2.0; 1.75–1.99	75/117; 42/117	64.1; 35.9	MB	ITS, 28S rDNA	15

(Continued on next page)

TABLE 2 (Continued)

MALDI system(s)	Order(s) and/or group(s)	Organism(s) studied (no. of species)	No. of species (no. of strains) in DB	Acceptance criterion for ID (Bruker Biotyper ID score)	No. of correctly identified isolates while challenging DB/total no. of isolates while challenging DB	Accuracy (%)	Comparative method(s)	Sequence(s) used for ID	Reference
Bruker Daltonics	*Eurotiales*, *Hypocreales*, *Microascales*	*Aspergillus* spp. (55), *Fusarium* spp. (45), *Scedosporium* spp. (17)	28 (117)	≥2.0; 1.7–1.99	105/117; 8/117	90; 6.8	MB, MO	ITS, β-tubulin, elongation factor Iα	20
Bruker Daltonics	*Eurotiales*	*Aspergillus* (sections *Fumigati*, *Flavi*, *Terrei*, *Nigri*, *Nidulantes*, *Usti*, *Circumdati*, *Versicolores*)	53 (23)	≥2.0; 1.7–2.0	165/190; 25/190	86.8; 13.2	MB, MO	β-Tubulin, calmodulin	30
Bruker Daltonics	*Eurotiales*, *Hypocreales*, *Microascales*	*Aspergillus* spp. (55), *Fusarium* spp. (45), *Scedosporium* spp. (17)	6 (13)	≥2.0; 1.7–2.0	90/111; 14/111	81.8; 12.6	MB	ITS1, ITS4	43
Bruker Daltonics	*Hypocreales*, *Microascales*, *Mucorales*	*Fusarium* spp. (19), *Scedosporium* spp. (24), *Lichtheimia* (8), other (12)	15 (63)	≥1.8; ≥1.6	94/101; 7/101	91.3; 8.7	MB	ITS, β-tubulin, elongation factor Iα	44
Bruker Daltonics, Andromas	*Eurotiales*	*Aspergillus* (sections *Fumigati*, *Flavi*, *Terrei*, *Nigri*, *Nidulantes*, *Usti*, *Circumdati*)	28	≥66% of common peaks of the reference strains	138/140	98.6	MB	β-Tubulin, calmodulin	45

[a]Within a row where there is a pair of entries separated by semicolons in the "Acceptance criterion for ID" column, there is a one-to-one correspondence between that pair, the pair in the "No. of correctly identified isolates while challenging DB/total no. of isolates while challenging DB" column, and the pair in the "Accuracy" column. Abbreviations: DB, database; ID, identification; MB, molecular biology; MO, morphology; NR, not reported; LSU, large subunit; MPI, mannose phosphate isomerase; PM-ATPase, plasma membrane H^+ ATPase; PK, pyruvate kinase.

[b]A combination of an in-house database and online software (MSI) was used.

median, 7.2%). Surprisingly, no increase in the number of misidentifications was reported even when cryptic *Aspergillus* species were studied (30). These results show that reliable species identification using MALDI-TOF MS can also be achieved by using a log score of 1.7.

OUTLOOK

MALDI-TOF MS has already changed the routine of fungal identification in many clinical laboratories due to its fast identification process. However, the species coverage of the reference databases is still the most significant drawback for the identification of fungi using MALDI-TOF MS systems. Manufacturers and researchers should continue to expand and update the various available reference databases. When different species within a section have distinguishable susceptibility patterns (e.g., *Aspergillus* species) or when it is important to differentiate between pathogenic and nonpathogenic species (e.g., *Talaromyces* species), the method should be able to identify the isolates to the species level using the reference database. Additionally, the databases should keep track of the fast-changing fungal nomenclature.

There may be new MALDI-TOF systems available in Europe soon. In China, the Xiamen MALDI-TOF MS system is already available. In a comparative study, Xiamen MALDI-TOF MS showed a performance nearly identical to that of the Bruker Daltonics system (37).

The MALDI-TOF MS system may also be able to differentiate azole-resistant *Aspergillus fumigatus* isolates. A proof-of-concept proteomic study of azole-susceptible and -resistant *A. fumigatus* strains revealed proteomic differences in proteins associated with resistance, virulence, and the host response. However, only previously identified strains were used in this study, and the isolates were not exposed to antifungal drugs (38). Another method that was used to detect caspofungin resistance in *Aspergillus* species relies on the assumption that the protein composition of a microorganism will vary at different drug concentrations. Therefore, the minimal profile change concentration (MPPC) was defined as the value where the lowest drug concentration resulting in a profile change can be detected. To determine the MPCC, MALDI-TOF MS spectra of isolates were collected after exposure to high, intermediate, or null drug concentrations used to generate a composite correlation index (CCI) (39, 40).

In a recent publication, Lau et al. (46) demonstrated that the optimization of acquisition parameters (e.g., peak selection, minimum intensity threshold, and sum of acquired shots) significantly decreased intercenter variability in their study. This implies that the acquisition parameters as well as the state and maintenance of the MALDI-TOF MS instrument play an important part in the identification of fungi using MALDI-TOF MS. However, there is currently no calibration standard for molds that could be tested during maintenance to ensure reproducibility.

Furthermore, there may be a simpler sample processing option. The French company Conidia (Quincieux, France) recently developed new agar plates, called ID Fungi plates, which are designed specifically for the identification of fungi by MALDI-TOF MS (http://conidia.fr/en/id-fungi-plates/). With these plates, enough mycelia for MALDI-TOF MS should be grown within 24 h, and the direct transfer of fungal material, followed by TFA extraction on the target, should be sufficient to obtain high-quality spectra. One study showed that the identification score values were higher after fungal culture on ID Fungi plates than after culture on Sabouraud dextrose agar (41).

REFERENCES

1. Cassagne C, Ranque S, Normand AC, Fourquet P, Thiebault S, Planard C, Hendrickx M, Piarroux R. 2011. Mould routine identification in the clinical laboratory by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. PLoS One 6:e28425. https://doi.org/10.1371/journal.pone.0028425.
2. Cassagne C, Normand AC, L'Ollivier C, Ranque S, Piarroux R. 2016. Performance of MALDI-TOF MS platforms for fungal identification. Mycoses 59:678–690. https://doi.org/10.1111/myc.12506.
3. Sanguinetti M, Posteraro B. 2017. Identification of molds by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 55:369–379. https://doi.org/10.1128/JCM.01640-16.
4. Bizzini A, Greub G. 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin Microbiol Infect 16:1614–1619. https://doi.org/10.1111/j.1469-0691.2010.03311.x.
5. Bader O. 2013. MALDI-TOF-MS-based species identification and typing

approaches in medical mycology. Proteomics 13:788–799. https://doi.org/10.1002/pmic.201200468.
6. US Food and Drug Administration. 2017. 510(k) document K162950: substantial equivalence determination decision summary, 2017. US Food and Drug Administration, Silver Spring, MD.
7. Vermeulen E, Verhaegen J, Indevuyst C, Lagrou K. 2012. Update on the evolving role of MALDI-TOF MS for laboratory diagnosis of fungal infections. Curr Fungal Infect Rep 6:206–214. https://doi.org/10.1007/s12281-012-0093-y.
8. Coulibaly O, Marinach-Patrice C, Cassagne C, Piarroux R, Mazier D, Ranque S. 2011. *Pseudallescheria/Scedosporium* complex species identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Med Mycol 49:621–626. https://doi.org/10.3109/13693786.2011.555424.
9. Marinach-Patrice C, Lethuillier A, Marly A, Brossas JY, Gene J, Symoens F, Datry A, Guarro J, Mazier D, Hennequin C. 2009. Use of mass spectrometry to identify clinical *Fusarium* isolates. Clin Microbiol Infect 15:634–642. https://doi.org/10.1111/j.1469-0691.2009.02758.x.
10. Welham KJ, Domin MA, Johnson K, Jones L, Ashton DS. 2000. Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 14:307–310. https://doi.org/10.1002/(SICI)1097-0231(20000315)14:5<307::AID-RCM823>3.0.CO;2-3.
11. Li TY, Liu BH, Chen YC. 2000. Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 14:2393–2400. https://doi.org/10.1002/1097-0231(20001230)14:24<2393::AID-RCM178>3.0.CO;2-9.
12. Buskirk AD, Hettick JM, Chipinda I, Law BF, Siegel PD, Slaven JE, Green BJ, Beezhold DH. 2011. Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi. Anal Biochem 411:122–128. https://doi.org/10.1016/j.ab.2010.11.025.
13. Dong H, Kemptner J, Marchetti-Deschmann M, Kubicek CP, Allmaier G. 2009. Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry. Anal Bioanal Chem 395:1373–1383. https://doi.org/10.1007/s00216-009-3067-3.
14. Lau AF, Drake SK, Calhoun LB, Henderson CM, Zelazny AM. 2013. 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. J Clin Microbiol 51:828–834. https://doi.org/10.1128/JCM.02852-12.
15. Paul S, Singh P, Sharma S, Prasad GS, Rudramurthy SM, Chakrabarti A, Ghosh AK. 2019. MALDI-TOF MS-based identification of melanized fungi is faster and reliable after the expansion of in-house database. Proteomics Clin Appl 13:e1800070. https://doi.org/10.1002/prca.201800070.
16. Rychert J, Slechta ES, Barker AP, Miranda E, Babady NE, Tang YW, Gibas C, Wiederhold N, Sutton D, Hanson KE. 2018. Multicenter evaluation of the Vitek MS v3.0 system for the identification of filamentous fungi. J Clin Microbiol 56:e01353-17. https://doi.org/10.1128/JCM.01353-17.
17. Stein M, Tran V, Nichol KA, Lagace-Wiens P, Pieroni P, Adam HJ, Turenne C, Walkty AJ, Normand AC, Hendrickx M, Piarroux R, Karlowsky JA. 2018. Evaluation of three MALDI-TOF mass spectrometry libraries for the identification of filamentous fungi in three clinical microbiology laboratories in Manitoba, Canada. Mycoses 61:743–753. https://doi.org/10.1111/myc.12800.
18. Normand AC, Becker P, Gabriel F, Cassagne C, Accoceberry I, Gari-Toussaint M, Hasseine L, De Geyter D, Pierard D, Surmont I, Djenad F, Donnadieu JL, Piarroux M, Ranque S, Hendrickx M, Piarroux R. 2017. Validation of a new Web application for identification of fungi by use of matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 55:2661–2670. https://doi.org/10.1128/JCM.00263-17.
19. Schulthess B, Ledermann R, Mouttet F, Zbinden A, Bloemberg GV, Bottger EC, Hombach M. 2014. Use of the Bruker MALDI Biotyper for identification of molds in the clinical mycology laboratory. J Clin Microbiol 52:2797–2803. https://doi.org/10.1128/JCM.00049-14.
20. Sleiman S, Halliday CL, Chapman B, Brown M, Nitschke J, Lau AF, Chen SC. 2016. Performance of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of *Aspergillus*, *Scedosporium*, and *Fusarium* spp. in the Australian clinical setting. J Clin Microbiol 54:2182–2186. https://doi.org/10.1128/JCM.00906-16.
21. McMullen AR, Wallace MA, Pincus DH, Wilkey K, Burnham CA. 2016. Evaluation of the Vitek MS matrix-assisted laser desorption ionization–time of flight mass spectrometry system for identification of clinically relevant filamentous fungi. J Clin Microbiol 54:2068–2073. https://doi.org/10.1128/JCM.00825-16.
22. Pinheiro D, Monteiro C, Faria MA, Pinto E. 2019. Vitek MS v3.0 system in the identification of filamentous fungi. Mycopathologia 184:645–651. https://doi.org/10.1007/s11046-019-00377-0.
23. Becker PT, de Bel A, Martiny D, Ranque S, Piarroux R, Cassagne C, Detandt M, Hendrickx M. 2014. Identification of filamentous fungi isolates by MALDI-TOF mass spectrometry: clinical evaluation of an extended reference spectra library. Med Mycol 52:826–834. https://doi.org/10.1093/mmy/myu064.
24. Gautier M, Ranque S, Normand AC, Becker P, Packeu A, Cassagne C, L'Ollivier C, Hendrickx M, Piarroux R. 2014. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: revolutionizing clinical laboratory diagnosis of mould infections. Clin Microbiol Infect 20:1366–1371. https://doi.org/10.1111/1469-0691.12750.
25. Chen Y-S, Liu Y-H, Teng S-H, Liao C-H, Hung C-C, Sheng W-H, Teng L-J, Hsueh P-R. 2015. Evaluation of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry Bruker Biotyper for identification of *Penicillium marneffei*, *Paecilomyces* species, *Fusarium solani*, *Rhizopus* species, and *Pseudallescheria boydii*. Front Microbiol 6:679. https://doi.org/10.3389/fmicb.2015.00679.
26. Calderaro A, Motta F, Montecchini S, Gorrini C, Piccolo G, Piergianni M, Buttrini M, Medici MC, Arcangeletti MC, Chezzi C, De Conto F. 2014. Identification of dermatophyte species after implementation of the in-house MALDI-TOF MS database. Int J Mol Sci 15:16012–16024. https://doi.org/10.3390/ijms150916012.
27. Dolatabadi S, Kolecka A, Versteeg M, de Hoog SG, Boekhout T. 2015. Differentiation of clinically relevant *Mucorales Rhizopus microsporus* and *R. arrhizus* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). J Med Microbiol 64:694–701. https://doi.org/10.1099/jmm.0.000091.
28. Lau SK, Lam CS, Ngan AH, Chow WN, Wu AK, Tsang DN, Tse CW, Que TL, Tang BS, Woo PC. 2016. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for rapid identification of mold and yeast cultures of *Penicillium marneffei*. BMC Microbiol 16:36. https://doi.org/10.1186/s12866-016-0656-0.
29. Masih A, Singh PK, Kathuria S, Agarwal K, Meis JF, Chowdhary A. 2016. Identification by molecular methods and matrix-assisted laser desorption ionization–time of flight mass spectrometry and antifungal susceptibility profiles of clinically significant rare *Aspergillus* species in a referral chest hospital in Delhi, India. J Clin Microbiol 54:2354–2364. https://doi.org/10.1128/JCM.00962-16.
30. Vidal-Acuna MR, Ruiz-Perez de Pipaon M, Torres-Sanchez MJ, Aznar J. 2018. Identification of clinical isolates of *Aspergillus*, including cryptic species, by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Med Mycol 56:838–846. https://doi.org/10.1093/mmy/myx115.
31. Imbert S, Normand AC, Gabriel F, Cassaing S, Bonnal C, Costa D, Lachaud L, Hasseine L, Kristensen L, Schuttler C, Raberin H, Brun S, Hendrickx M, Stubbe D, Piarroux R, Fekkar A. 2019. Multi-centric evaluation of the online MSI platform for the identification of cryptic and rare species of *Aspergillus* by MALDI-TOF. Med Mycol 57:962–968. https://doi.org/10.1093/mmy/myz004.
32. Singh A, Singh PK, Kumar A, Chander J, Khanna G, Roy P, Meis JF, Chowdhary A. 2017. Molecular and matrix-assisted laser desorption ionization–time of flight mass spectrometry-based characterization of clinically significant melanized fungi in India. J Clin Microbiol 55:1090–1103. https://doi.org/10.1128/JCM.02413-16.
33. Barker AP, Horan JL, Slechta ES, Alexander BD, Hanson KE. 2014. Complexities associated with the molecular and proteomic identification of *Paecilomyces* species in the clinical mycology laboratory. Med Mycol 52:537–545. https://doi.org/10.1093/mmy/myu001.
34. Park JH, Shin JH, Choi MJ, Choi JU, Park YJ, Jang SJ, Won EJ, Kim SH, Kee SJ, Shin MG, Suh SP. 2017. Evaluation of matrix-assisted laser desorption/ionization time-of-fight mass spectrometry for identification of 345 clinical isolates of *Aspergillus* species from 11 Korean hospitals: comparison with molecular identification. Diagn Microbiol Infect Dis 87:28–31. https://doi.org/10.1016/j.diagmicrobio.2016.10.012.
35. Theel ES, Hall L, Mandrekar J, Wengenack NL. 2011. Dermatophyte identification using matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 49:4067–4071. https://doi.org/10.1128/JCM.01280-11.
36. Triest D, Stubbe D, De Cremer K, Pierard D, Normand AC, Piarroux R,

Detandt M, Hendrickx M. 2015. Use of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of molds of the *Fusarium* genus. J Clin Microbiol 53:465–476. https://doi.org/10.1128/JCM.02213-14.
37. Huang Y, Zhang M, Zhu M, Wang M, Sun Y, Gu H, Cao J, Li X, Zhang S, Wang J, Lu X. 2017. Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for the identification of clinical filamentous fungi. World J Microbiol Biotechnol 33:142. https://doi.org/10.1007/s11274-017-2297-3.
38. Vermeulen E, Carpentier S, Kniemeyer O, Sillen M, Maertens J, Lagrou K. 2018. Proteomic differences between azole-susceptible and -resistant *Aspergillus fumigatus* strains. Adv Microbiol 8:77–99. https://doi.org/10.4236/aim.2018.81007.
39. De Carolis E, Vella A, Florio AR, Posteraro P, Perlin DS, Sanguinetti M, Posteraro B. 2012. Use of matrix-assisted laser desorption ionization–time of flight mass spectrometry for caspofungin susceptibility testing of *Candida* and *Aspergillus* species. J Clin Microbiol 50:2479–2483. https://doi.org/10.1128/JCM.00224-12.
40. Marinach C, Alanio A, Palous M, Kwasek S, Fekkar A, Brossas JY, Brun S, Snounou G, Hennequin C, Sanglard D, Datry A, Golmard JL, Mazier D. 2009. MALDI-TOF MS-based drug susceptibility testing of pathogens: the example of *Candida albicans* and fluconazole. Proteomics 9:4627–4631. https://doi.org/10.1002/pmic.200900152.
41. Robert MG, Romero C, Dard C, Garnaud C, Cognet O, Girard T, Rasamoelina T, Cornet M, Maubon D. 2020. Evaluation of ID Fungi plates medium for identification of molds by MALDI Biotyper. J Clin Microbiol 58:e01687-19. https://doi.org/10.1128/JCM.01687-19.
42. De Carolis E, Posteraro B, Lass-Florl C, Vella A, Florio AR, Torelli R, Girmenia C, Colozza C, Tortorano AM, Sanguinetti M, Fadda G. 2012. Species identification of *Aspergillus*, *Fusarium* and *Mucorales* with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 18:475–484. https://doi.org/10.1111/j.1469-0691.2011.03599.x.
43. Shao J, Wan Z, Li R, Yu J. 2018. Species identification and delineation of pathogenic *Mucorales* by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 56:e01886-17. https://doi.org/10.1128/JCM.01886-17.
44. Zvezdanova ME, Escribano P, Ruiz A, Martinez-Jimenez MC, Pelaez T, Collazos A, Guinea J, Bouza E, Rodriguez-Sanchez B. 2019. Increased species-assignment of filamentous fungi using MALDI-TOF MS coupled with a simplified sample processing and an in-house library. Med Mycol 57:63–70. https://doi.org/10.1093/mmy/myx154.
45. Alanio A, Beretti JL, Dauphin B, Mellado E, Quesne G, Lacroix C, Amara A, Berche P, Nassif X, Bougnoux ME. 2011. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Aspergillus* species. Clin Microbiol Infect 17:750–755. https://doi.org/10.1111/j.1469-0691.2010.03323.x.
46. Lau AF, Walchak RC, Miller HB, Slechta ES, Kamboj K, Riebe K, Robertson AE, Gilbreath JJ, Mitchell KF, Wallace MA, Bryson AL, Balada-Llasat J-M, Bulman A, Buchan BW, Burnham C-AD, Butler-Wu S, Desai U, Doern CD, Hanson KE, Henderson CM, Kostrewza M, Ledeboer NA, Maier T, Pancholi P, Schuetz AN, Shi G, Wengenack NL, Zhang SX, Zelazny AM, Frank KM. 2019. Multicenter study demonstrates standardization requirements for mold identification by MALDI-TOF MS. Front Microbiol 10:2098. https://doi.org/10.3389/fmicb.2019.02098.