Title: Evaluation of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) for the Rapid Identification of Fungi

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Abstract
The Food and Drug Administration (FDA) needs to identify fungi from regulated products and controlled areas of manufacturing environments for numerous reasons, i.e. to assess safety of product and manufacturing environment; provide epidemiological data for investigations related to outbreaks; and for quality assurance of laboratory analyses. In addition, recent outbreaks pertaining to medical products caused by fungal contamination have made it imperative that the FDA be prepared to identify fungi in order to quickly recall contaminated products and thereby protect public health. Conventional phenotypic methods used to identify fungi are generally time-consuming and some identification problems have arisen due to closely-related species having identical morphologies. In 2015, the FDA adopted ribosomal DNA (rDNA) sequencing, now the gold standard, to identify fungal isolates. While rDNA sequencing is accurate, the method is laborious, requiring several hands-on manipulations and many reagents. Here, a Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) based protocol is evaluated for identifying fungi, that is quicker and less labor intensive than either sequencing or phenotypic-based identification methods. MALDI-TOF MS measures microbe specific protein profiles and compares these spectra to libraries for identification. Protein spectra are considered 'fingerprints' or unique patterns that can be used to identify microbes to the species level. A comparative study of 135 fungi, confirmed by rDNA sequencing, has been performed in an effort to evaluate the use of MALDI-TOF MS for fungal identification; 82.3% were identified (46.7% genus and species identified, 35.6% genus only identified), 16% were unidentified and 1.5% were misidentified. An additional 50 environmental fungal isolates were analyzed, 74% were identified (46% genus and species identified, 28% genus only) and 26% were unidentified. MALDI-TOF MS has the potential to be used for an accurate, and more rapid identification of fungi.

Introduction

In recent years, there has been a growing number of fungal infections and fungal related outbreaks in the United States. Infections can originate from many places including the health-care environment, or from contaminated drugs or medical devices [1]. In order to fulfill its mission of protecting the public health, it would be greatly beneficial for the FDA to employ the most accurate and rapid methods to identify fungal contaminants. Accurate fungal identification is essential due to strain specific susceptibility to antifungals and increasing cases of antifungal drug resistance. Rapid identification is essential during the response to a fungal outbreak to limit the spread of infection, inform the public and healthcare providers and treat patients. Fungi that commonly cause opportunistic infections include *Candida* species, *Cryptococcus neoformans* and *Aspergillus* species [2, 3]. *Candida albicans*, a yeast, is the fourth leading cause of bloodstream infections (9% of all bloodstream infections) [4] and leading cause of catheter associated urinary tract infections (20%) [5]. A significant number of infections are caused by other *Candida* species including the emerging pathogen *Candida auris* [6, 7]. *C. neoformans* is

spread through aerosolization of spores and typically causes cryptococcal meningitis, which has a high mortality rate if left untreated [8, 9]. *Aspergillus fumigatus* causes most cases of aspergillosis [10]. Typically, exposure to *Aspergillus* conidia in the air leads to allergic reactions, but a more invasive infections can occur, such as pulmonary and disseminating infections, especially in immune compromised individuals [11, 12]. Other molds that are linked to human infection include *Fusarium* species and Zygomycetes like *Rhizopus* species and *Mucor* species [13]. *Fusarium* species are typically plant pathogens but can also cause cutaneous infections [14]. *F. solani* is the leading cause of fungal keratitis and has been linked to contamination of contact lenses and contact lens solutions [15]. Zygomycetes are found in the environment and can be spread in a hospital setting through inhalation of spores, which can lead to respiratory tract infections [16]. Zygomycetes are an emerging pathogen for organ transplant recipients [17].

Currently, methods for identifying fungi are morphologic identification and molecular identification rDNA sequencing. Morphological identification is the traditional method that uses macroscopic characteristics such as colony size, color, and growth rate and microscopic characteristics such as conidia size, conidia shape and hyphae branching to obtain identification [18]. Morphologic identification requires extensive training and experience to properly identify fungi. The disadvantage of this technique is that fungi with similar phenotypes are often misidentified and rare fungi are unidentifiable. Also, this technique requires growing fungi on various media to visualize characteristics and consequently an identification can take several days. Sequencing is now the gold standard method for identifying fungi. For this method, DNA is isolated from fungi and a specific region of DNA is amplified by PCR. Typically, a variable region within the ribosome sequence such as the D1/D2 region of the 28S large ribosomal subunit or internal transcribed sequence (ITS) is chosen because there is enough variation to differentiate between species. The amplicon is purified and a second sequencing reaction is used to prepare the sample. The sequence is then aligned to a known sequence in a reference database to obtain an identification [19-21]. This method is very accurate but requires at least 1 - 2 days for identification. This demonstrates that sequencing involves many steps, several reagents, and molecular biology experience. The VITEK®2 System (bioMérieux, Marcy l'Etoile, Fr.) is another method available for identifying bacteria and yeast. It utilizes biochemical tests prepackaged in cards to identify microbes [22]. Though VITEK® is convenient and requires very little training, identification by VITEK® is limited due to the small number of references in the library and molds cannot be identified using VITEK® [23].

MALDI-TOF MS has been developed over the last decade as a quicker and less labor-intensive method to identify bacteria and fungi [24]. Compared to rDNA sequencing, MALDI-TOF MS has a lower cost for reagents, less hands-on sample manipulation and reduced time to identification. MALDI-TOF MS requires few hours for identification compared to the day or multiple days required for sequencing or morphology-based identification, respectively. Quicker identification has been shown to improve healthcare outcomes in clinics by shortening the time to treat patients with appropriate antimicrobials [25, 26]. The increased use of MALDI-TOF MS in clinical settings has been aided by the FDA's 510(k) approval of the Mini-Flex and Auto-Flex series (Bruker Daltonic GmbH, Bremen, Germany) and the VITEK® MS (bioMérieux) mass spectrometers for identifying Gram negative bacteria, yeast and mold. Fungal identification via MALDI-TOF MS would allow for higher sample throughput during routine sterility testing and in the event of an outbreak.

Studies using MALDI-TOF MS to identify bacteria often utilize a formic acid extraction followed by the addition of acetonitrile prior to spotting protein extracts on target plate for analysis. Fungi, however, have a resilient cell wall that requires extra manipulation to lyse the cells. The National Institute of Health (NIH)-developed an extraction method, which allows for the growth of fungi on solid agar and uses silica beads to help lyse cells. Other studies using this extraction method have had varying levels of success. Additionally, the NIH curated a supplemental fungal library containing 365 spectra due to the low success rate when using the Bruker reference library

alone. Bruker Daltonics recommends a liquid extraction method that does not require the use of silica beads for extraction. At the time this study was completed the library used, consisted of Bruker's reference library (5627 spectra, 649 fungi), NIH's library (365 spectra), and our in-house library (35 spectra), which contained a total of 6027 spectra (1049 fungi and 4978 bacteria).

A previous study from this lab, demonstrated that MALDI-TOF MS can reliably identify Gram negative and Gram positive bacterial pathogens that commonly contaminate food, drugs and medical devices [27]. In the current study, an assessment of MALDI-TOF MS for the identification of fungi was utilized using optimal growth conditions and protein extraction method. Reference spectra from 35 fungi were added to the combined NIH and Bruker reference library to improve identification (**Supplementary Table 1**). We analyzed 135 known fungal strains, purchased from trusted sources (**Supplementary Table 2**), and 50 unknown fungal isolates using MALDI-TOF MS. Identifications of all fungi were confirmed by rDNA sequencing.

Materials and Methods

Fungal Strains and Growth Conditions

Fungal strains were obtained from the FDA Southwest Lab, the United States Department of Agriculture (USDA) Agricultural Research Services (ARS), the Centers for Disease Control and Prevention (CDC) Antibiotics Resistance Collection, BEI Resources and the American Type Culture Collection (ATCC). Strains analyzed in this study are listed in **Supplementary Table 3**. A total of 50 unknown environmental fungi were isolated from various sources. For isolation of environmental strains, microbes were collected using a cotton swab that was resuspended in yeast peptone dextrose broth. All environmental isolates were plated on yeast peptone dextrose agar (YPDA) for single colony isolation prior to storing strains at $\geq$-80º C. Fungal isolates were grown on Sabouraud dextrose agar (SDA) for 5-7 days at 22º C prior to MALDI-TOF MS analysis or rDNA sequencing, as indicated by the results of a growth condition study below.

Solid Agar Formic Acid Protein Extraction

A total 135 fungi grown on solid agar were extracted using a method developed by the NIH [28]. Briefly, for fungi, 5 mm of mold was taken from the surface of an agar plate using a sterile scalpel and placed in a microcentrifuge tube containing 500 µL of 70% (v/v) ethanol (Sigma-Aldrich, St. Louis, MO) and 50 µL of 0.1 mm diameter silica beads (BioSpec Products, Bartlesville, OK). Samples were vigorously vortexed for two minutes at 3000 rpm using BeadBug™ Mini Homogenizer (Benchmark Scientific, Inc., Edison, NJ) then centrifuged for two minutes at 9400 rpm (8500 x g) (ThermoFisher Scientific, Inc., Legend Micro 21). For yeasts, a 1µL loop of yeast was removed from the surface of the agar plate and resuspended in a microcentrifuge tube containing 500 µL of 70% ethanol. Yeast isolates were briefly vortexed to disperse cells. Yeast samples were centrifuged for 2 minutes at 13,000 rpm (11600 x g). For both molds and yeasts, ethanol was removed, and samples were dried completely to remove any residual ethanol. Additionally, 50 µL of 70% (v/v) formic acid (Fisher Chemical, Fair Lawn, NJ) was added to dried samples and vortexed vigorously for two minutes. Samples were briefly centrifuged to collect material at the bottom of the tube. Acetonitrile (100%) (Sigma-Aldrich) was added and the samples were vortexed for five minutes. The samples were centrifuged for two minutes at 9400 rpm (8500 x g). Extracts were either spotted on an MSP Big Anchor Chip ground steel target plate (Bruker Daltonic) for analysis via MALDI-TOF MS or stored for up to one week at -20º C.

Liquid Media Formic Acid Protein Extraction

A total of 8 fungal strains grown in liquid media were extracted using a method developed by Bruker Daltonics [29] in order to compare to results from solid media. A sampling of molds and yeasts (yeast, non-septate (or Mucorales), hyaline, dermatophyte and dematiaceous fungi) were grown in 8 mL of Sabouraud dextrose broth (SDB) at room temperature for 5 days. Cultures were

left on the bench to settle for 10 minutes. Next, 1.5 mL of sedimented culture was transferred to microcentrifuge tubes and centrifuged for 2 minutes at 13,000 rpm (11600 x g). If the culture formed a pellet, the spent media was removed and the pellet was washed with molecular grade sterile water. If the culture did not form a pellet, as much media as possible was removed without disturbing the fungus and spent media was replaced with sterile water and centrifuged for an additional 2 minutes at 13,000 rpm. After washing with water, pellets were washed with 500 µL of 70% ethanol. Ethanol was discarded and pellets were allowed to air dry to remove any residual ethanol. Formic acid (70%) was added, up to 50 µL (enough to cover the pellet), and tubes were vortexed vigorously. An equal volume of 100% acetonitrile was added to the pellets and samples were vortexed again. Samples were either spotted immediately onto the target plate for analysis or stored for up to one week at -20º C and then analyzed.

MALDI-TOF MS

Using 1 µL fungal extract, the target plate was spotted in triplicate and allowed to air dry. Then, 2 µL of α-cyano-4-hydroxycinnnamic acid (HCCA) matrix (Bruker Daltonic) dissolved in Standard Solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) (Fluka) (Sigma-Aldrich) was overlaid on top of dried extracts. The matrix was air dried and formed crystals for MALDI-TOF MS analysis. With each run a bacterial test standard (BTS) (Bruker Daltonic) and *Aspergillus ustus* were spotted on the target plate as positive controls. Extracts were analyzed on an Auto-flex Series instrument (Bruker Daltonic) in positive linear mode with laser frequency set to 1000 Hz and mass/charge range from 2000 to 20,000 Da. Each spectrum was obtained using 200 laser shots. Spectra were analyzed using Realtime Biotyper v3.1 software, which assigned a log score between 0 and 3. Bruker defines classification scores of ≥ 2.00 as identification of both genus and species, between $\geq$1.99 and $\geq$1.70 as identification of genus, and ≤ 1.69 as no reliable identification. Reference libraries used for identifications include in-house generated (35 spectra), NIH (365 spectra) and Bruker Daltonics (5627 spectra, 649 fungal spectra). The average of the top two scores was used to calculate the final score for each sample.

ITS rDNA Sequencing

Samples for fungal DNA sequencing were prepared using an Office of Regulatory Affairs (ORA) protocol described below. Fungal cell walls are more resilient and difficult to lyse. Therefore, a modified lysis step was used for DNA extraction. Approximately 3.5mm diameter of a well grown fungal colony (remove as much agar as possible) was transferred to a microcentrifuge tube containing 360 µL of DNA lysis buffer (10 mM Tris-Cl, 1 mM EDTA, 100 mM NaCl and 50 mM β-mercaptoethanol), 40 µL of Proteinase K (Qiagen, Hilden, Germany) and 50 µL of silica beads. Samples were vortexed vigorously for 1 minute. DNA isolation was completed using the DNeasy Blood & Tissue Kit (Qiagen). Following DNA extraction, ITS region was amplified by PCR using molecular grade sterile water, HotStarTaq Master Mix (Qiagen), forward primer (5' GGAAGTAAAAGTCGTAACAAGG) and reverse primer (5' TCCTCCGCTTATTGATATGC). Amplicons were purified with ExoSAP-IT™ PCR Product Cleanup Reagent (Affymetrix Inc., Cleveland, OH). The DNA sequencing reaction was prepared using the purified amplicons, BigDye Terminator v3.1 cycle sequencing kit (Qiagen) and ITS forward and reverse primers. DNA sequencing amplicons were purified using Performa DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD). Purified sequencing product was combined with HiDi™ formamide in a 96 well-plate and sequenced via an ABI 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed using NCBI BLAST search to obtain identifications using the top match, consistent with ORA procedure. *Aspergillus brasiliensis* (ATCC 16404) was used as a positive control for all sequencing runs.

Results and Discussion

Protein extraction from solid agar improves Biotyper scores

There are two standard methods that are used to extract proteins from fungi. Bruker Daltonics recommends extracting proteins from fungi grown in liquid culture and the NIH-developed method utilizes a solid agar. To determine if protein extraction from liquid culture or solid agar would improve identification, a selection of yeast, non-septate (or Mucorales), hyaline, dermatophyte and dematiaceous fungi were grown either in SDB or SDA and analyzed via MALDI-TOF MS. The scores from SDA protein extracts were significantly higher compared to fungi grown in liquid media (**Figure 1**). More fungi were identified to the species level, scores ≥ 2.00, when proteins were extracted from fungi grown on solid media (**Table 1**). This result compliments the current sterility method. During sterility testing of medical products, samples that test positive for microbial growth are transferred from liquid media to solid agar to ensure there is a pure culture prior to identification. Future work is required to assess the viability of this workflow process.

Organism	SDA		SDB	YPDA		PDA		TSA	
	22°	30°	22°	22°	30°	22°	30°	22°	30°
Candida albicans	2.01	2.08	2.00	2.04	1.96	1.97	2.03	1.94	2.00
Cryptococcus neoformans	2.28	2.28	2.10	2.44	2.46	2.47	2.38	2.24	2.25
Aspergillus fumigatus	2.14	1.74	1.88	1.82	1.48	1.96	1.95	1.88	1.52
Penicillium oslonii	2.10	1.65	1.63	1.65	1.57	2.07	1.53	1.49	1.39
Curvularia lunata	1.80	1.89	1.56	1.80	1.61	1.95	1.43	2.17	1.68
Exophiala dermatitidis	2.03	2.07	1.94	2.12	2.11	1.80	2.30	2.28	2.07
Mucor circinelloides	1.89	1.79	1.70	1.91	1.47	1.75	1.64	1.92	1.60
Rhizomucor variabilis var regularior	2.33	2.03	1.61	2.10	1.89	2.10	2.33	2.22	2.01
Microsporum gypseum	1.86	1.90	1.55	1.53	1.86	2.00	2.07	1.62	1.74

Table 1. *Assessment of temperature, growth media and extraction method on the log score. Sabouraud dextrose agar (SDA); Sabouraud dextrose broth (SDB); yeast peptone dextrose agar (YPDA); potato dextrose agar (PDA); tryptic soy agar (TSA).*

Growth media and temperature can affect Biotyper scores

Molds and yeasts were grown on various solid media to determine if growth on a specific medium significantly improved Biotyper scores. Overall scores ranged from 1.39 to 2.47, with an average score of 2.00 (**Table 1**). Fungi grown on SDA (at 22°C) gave the highest scores with an average score of 2.05 and YPDA and Tryptic soy agar (TSA) gave the lowest average scores 1.94 and 1.98, respectively. Except for fungi grown on SDA compared to YPDA at 22°C, there was no significant difference in scores obtained from fungi grown on the different media. However, there was impaired growth of some fungi on TSA that were not detected when using the other media. For most individual fungal strains, there were not significant differences in scores. Several studies have also suggested that culture conditions do not significantly affect MALDI-TOF MS analysis; even with different growth media, there are still a core set of peaks which are used to identify microbes [28, 30, 31]. The exceptions were *Rhizomucor variabilis* grown on SDA was significantly better

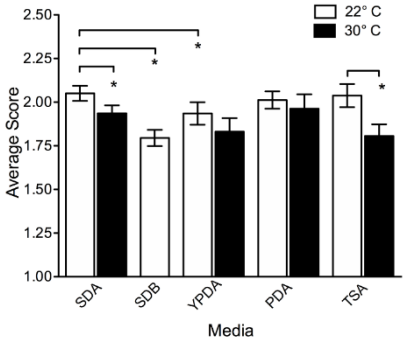

Figure 1*. Growth conditions affect analysis via MALDI-TOF MS. Error bars represent the standard error of the mean. Significance (*) was determined using paired two-sided t test with p value ≤ 0.05.*

than PDA and YPDA. *Curvularia lunata* grown on TSA gave a significantly higher score compared to SDA. Also, *Penicillium oslonii* grown on SDA and PDA gave higher scores compared to YPDA and TSA. During routine analysis, suspected fungal isolates are grown on both TSA and SDA; the data suggests that isolates should be taken from SDA for MALDI-TOF analysis.

In addition to growing fungi on various media, fungi were also grown at 30° C to determine if elevated temperature could significantly affect Biotyper scores. Overall scores decreased when fungi were grown at 30° C compared to 22° C (**Figure 1**). The average score for fungi grown at 30° C decreased from 2.00 to 1.88. There was a significant decrease in scores for fungi grown on SDA and TSA at 30° C compared to 22° C. Furthermore, the impaired growth on TSA that was detected at 22° C was more apparent at 30° C. *Candida albicans*, *Cryptococcus neoformans, Exophiala dermatitidis* and *Rhizomucor variabilis* were not affected by elevated temperature.

The most significant aspect to obtaining the best score for MALDI-TOF MS is to grow fungi under conditions where there is enough biomaterial for analysis. Based on our data, fungi were grown on SDA at 22° C for the remainder of the study. These growth conditions coincide with the ORA fungal sequencing method used by field labs to identify fungal contamination in regulated products. Utilizing the same growth conditions would streamline fungal identification for analysts.

Identifying fungi by MALDI-TOF MS

To evaluate MALDI-TOF MS for identifying fungi, 135 fungi that were previously identified via rDNA sequencing were analyzed. Of the 135 isolates analyzed, 64 (47.4%) were identified to the genus and species and an additional 48 (35.6%) were identified to the genus level. Additionally, 22 fungi (16.3%) were not reliably identified and 1 (0.7%) was misidentified (**Table 2**). Yeasts were identified at a higher rate compared to molds (**Figure 2**). Of the 58 yeasts, 38 identified to the species level (65.5%) and 18 (31.0%) identified to the genus level. Of the 77 molds, 26 (33.8%) were identified to the species level and 30 (39%) were identified to the genus. Only 2 yeast (3.4%), *Cryptococcus gatti and Candida catenulata*, were unidentified compared to 20 molds (26.0%). Also, no yeasts were misidentified compared to 1 mold (1.3%). This is probably due to better extraction of proteins from yeasts compared to molds. Protein extraction for yeasts is identical to most bacteria. Silica beads are not required to help lyse yeast and most bacteria cells.

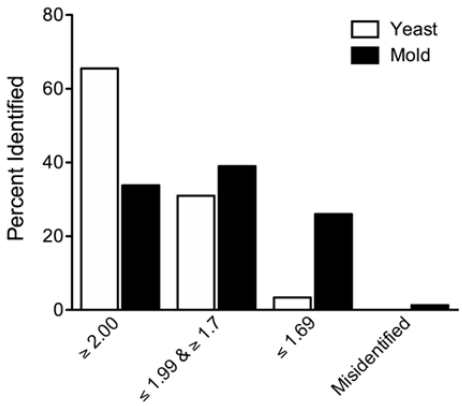

Figure 2. *Comparison of yeast and mold identification via MALDI-TOF MS. A total of 135 fungi (77 molds and 58 yeasts) were analyzed.*

Our results build on Biotyper studies performed by NIH. The initial NIH study identified 88.9% of molds to the species level, an additional 4.3% to the genus level and 7.8% were unidentifiable. This study compared the NIH curated library with the Bruker library. [28]. A subsequent NIH study identified 53.7% of molds to the species level, 24.5% to the genus level, and 21.7% were unidentifiable. That study focused on creating a more rapid extraction procedure. [32]. Our work fits within this structure by expanding upon current knowledge through combining the NIH and Bruker libraries with our own in-house library. By continuing to diversify the library, the success of future identifications may be improved.

Bruker Daltonics recommends threshold scores for identifying microbes using Realtime Biotyper. Currently, they advise any score ≥2.00 is a reliable identification of both genus and species. A score between 1.99 - 1.70 is likely identification of genus only and a score less than 1.69 is not reliably identified. However, in our study we have found that when the cut off was lowered to 1.70, 112 out of 135 (82.9%) isolates were identified to at least the genus level (105 of the 135 (77.7%) were identified to the species and 7 of the 135 (5.1%) were identified to the genus).This adjustment does not cause an increase false positives. Several studies have also demonstrated that reducing the cut off could improve identification of fungi [33-35]. Adjusting the cut off could improve the identification of genus and species for fungi for future analyses.

Scores	No. of fungi (%)
≥ 2.00	64 (47.4)
≤1.99 and ≥1.70	48 (35.6)
≤ 1.69	22 (16.3)
Misidentified	1 (0.7)

Table 2. *Evaluation of combined reference libraries with 135 fungi*

Organism (No. analyzed)	≥ 2.00 (%)	≤1.99 and ≥1.70 (%)	≤ 1.69 (%)
Acremonium zonatum (1)			NA
Alternaria sp. (1)		1 (100)	
Alternaria alternata (1)			1 (100)
Alternaria obovoidea (1)			NA
Aspergillus sp. (2)	2 (100)		
Aspergillus brasiliensis (1)		1 (100)	
Aspergillus fumigatus (1)	1 (100)		
Aspergillus brasiliensis (2)		2 (100)	
Aureobasidium pullulans (2)			2 (100)
Botrytis cinerea (4)			NA
Candida fermenticarens (1)			1 (100)
Candida galli (1)	1 (100)		
Cladosporium sp. (5)	3 (60)	1 (20)	1 (20)
Cladosporium cladosporoides (6)	1 (16.6)	5 (83.3)	
Cladosporium tenuissimum (1)			NA
Fusarium sp. (1)			1 (100)
Fusarium incarnatum-equiseti (1)		1 (100)	
Hypocreales sp. (1)			NA
Paradendryphiella salina (1)			NA
Penicillium sp. (6)	2 (20)		4 (80)
Penicillium biourgeianum (3)	1 (33.3)	2 (66.6)	
Penicillium camemberti (1)		1 (100)	
Penicillium commune (1)	1 (100)		
Penicillium crustosum (6)	6 (100)		
Penicillium expansum (2)			NA
Penicillium olsonii (2)	2 (100)		
Pestalotiopsis sp. (1)			NA
Ramularia sp. (2)			NA
Rhizopus stolonifer (2)	2 (100)		
Rhodotorula glutinis (1)			1 (100)
Rhodotorula mucilaginosa (1)	1 (100)		
Rhodotorula sphaerocarpa (1)			1 (100)
Saccharomycopsis fibuligera (1)			1 (100)

Table 3. *Biotyper log scores for environmental fungal isolates*

NA, reference spectra not present in combined reference libraries.

Assessing unknown fungal isolates using MALDI-TOF MS.

Environmental fungal isolates were collected from multiple sources and analyzed via MALDI-TOF MS. All isolate identifications were confirmed by rDNA sequencing using the top match. *Penicillium* and *Cladiosporium* species were the most commonly collected isolates (**Table 3**). Out of the 64 isolates analyzed 14 (21.9%) could not be identified because there was no reference spectrum present in the libraries. Evaluating the 50 remaining isolates where reference spectra were present, 46% could be identified to the species level, 28% could be identified to the genus and 26% of the environmental isolates could not be identified (**Table 4**). The most commonly unidentified fungi were *Penicillium* species and *Aureobasidium pullulans.* The lack of identification for some of the environmental isolates compared to known fungi is partially due to the configuration of the reference libraries which are composed mainly of spectra from clinically important fungi rather than environmental fungi. Therefore, many of the isolates did not have a reference spectrum in the library.

Scores	No. of fungi (%)
≥ 2.00	23 (46)
≤1.99 and ≥1.70	14 (28)
≤ 1.69	13 (26)

Table 4. *Summary of Biotyper log scores for environmental fungal isolates with reference spectra in library.*

Biotyper scores ≥ 2.00 signifies genus and species identification, scores ≤ 1.99 and ≥ 1.70 signifies genus identification and scores ≤ 1.69 signifies no identification.

Conclusions

Currently, the limitation of MALDI-TOF MS is due to the limited number of the reference spectra in the libraries used for identification, especially for fungi. In this study, we utilized the Bruker Biotyper Library containing 649 fungal spectra references, the NIH fungal reference library containing 365 fungal spectra and 35 spectra in WEAC's in house library. In total, we had 1049 spectra in the fungal library compared to the 4970 bacteria reference spectra in the Bruker library alone. Research groups continue to build specific reference spectra libraries to suit their lab's needs. Additionally, Bruker Daltonics continues to create more reference libraries for microbes. Bruker has a specific fungal reference library that currently contains 130 spectra, which focuses on medically important fungi. While the NIH fungal library did improve identifications, there are spectra in the library that are defined to the genus only. Ensuring reference spectra are defined for both genus and species is ideal for identification. Throughout this study, the MALDI-TOF results were compared to rDNA sequencing (**Supplementary Table 2**). MALDI-TOF is currently used in clinical settings however its accuracy is highly dependent on the library used for analysis which can vary. The MALDI-TOF library used for this study was limited and continues to grow. Considering the complex method for determining an identification following rDNA sequencing of fungal isolates, MALDI-TOF could be used as an orthogonal technique to increase the confidence of sequencing results. Additionally, because if its speed MALDI-TOF has the potential to be employed as a screening technique in times that rapid results are necessary. Currently, MALDI-TOF analysis does not outperform rDNA sequencing for fungal identification with WEAC's library. However, as libraries improve, becoming more extensive and accurate, the technology should be reevaluated.

Acknowledgements

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Supplementary Table1. List of strains in FDA fungal reference library.

Classification	Organism
Yeast (9)	*Candida auris* *Candida fermenticarens* *Candida galli* *Cryptococcus ater* *Rhodotorula glutinis* *Rhodotorula mucilaginosa* *Rhodotorula sphaerocarpa* *Saccharomycopsis fibuligera* *Saprochaete clavata*
Hyaline (14)	*Aspergillus candidus* *Bjerkandera adusta* *Chrysosporium tuberculatum* *Chrysosporum* sp. *Fusarium* sp. *Coniochaeta* sp. *Penicillium biourgeianum* *Penicillium brevicompactum* *Penicillium commune* *Penicillium crustosum* *Penicillium olsonii* *Phialemonium* sp. *Sarocladium (Acremonia) kiliense* *Sporotrichum pruinosum*
Dematiaceous (4)	*Bipolaris spicifera* *Cladosporum cladosporioides* (2) *Cladophialophora carrionii*
Non-septate or Mucorales (5)	*Cokeromyces* sp. *Lichtheimia* sp. *Rhizomucor variabilis var regularior* *Rhizopus arrhizus* *Rhizopus oryzae*
Dermatophyte (2)	*Microsporum audouinii* *Trichophyton terrestre*
Dimorphic (1)	*Sporothrix globrosa*

Supplementary Table 2. MALDI-TOF Results compared to Sequencing Results

Name	Score 1	Score 2	Average Score	MALDI Classification	Sequencing Top Match
Aspergillus candidus	1.386	1.376	1.38	not reliable identification	*Aspergillus candidus*
Aspergillus flavipes	1.683	1.546	1.61	not reliable identification	*Aspergillus flavipes*
Aspergillus flavipes	1.493	1.533	1.51	not reliable identification	*Aspergillus sp.*
Aspergillus flavus	1.85	1.943	1.90	*Aspergillus flavus*	*Aspergillus flavus*
Aspergillus flavus	2.32	2.229	2.27	*Aspergillus flavus*	*Aspergillus flavus*
Aspergillus fumigatus	2.301	2.278	2.29	*Aspergillus fumigatus*	*Aspergillus fumigatus*
Aspergillus fumigatus	2.214	2.207	2.21	*Aspergillus fumigatus*	*Aspergillus fumigatus*
Aspergillus fumigatus	2.087	2.053	2.07	*Aspergillus fumigatus*	*Aspergillus fumigatus*
Aspergillus nidulans	1.757	1.72	1.74	*Aspergillus nidulans*	*Aspergillus nidulans*
Aspergillus niger	1.909	1.873	1.89	*Aspergillus niger*	*Aspergillus niger*
Aspergillus niger	1.81	1.892	1.85	*Aspergillus niger*	*Aspergillus niger*
Aspergillus sydowii	2.017	2.019	2.02	*Aspergillus sydowii*	*Aspergillus sydowii*
Aspergillus terreus	1.875	1.849	1.86	*Aspergillus terreus*	*Aspergillus terreus*
Aspergillus terreus	1.568	1.598	1.58	not reliable identification	*Aspergillus terreus*
Aspergillus terreus	1.564	1.7331	1.65	*Aspergillus terreus*	*Aspergillus terreus*
Aspergillus ustus	2.115	2.138	2.13	*Aspergillus ustus*	*Aspergillus ustus strain*
Aspergillus versicolor	1.778	1.922	1.85	*Aspergillus versicolor*	*Aspergillus versicolor*
Aureobasidium pullulans	1.605	1.584	1.59	not reliable identification	*Aureobasidium pullulans*
Beauveria sp.	1.821	1.813	1.82	*Beauveria bassina*	*Beauveria sp.*
Bipolaris spicifera	1.745	1.812	1.78	*Bipolaris spicifera*	*Curvularia spicifera (syn. Bipolaris spicifera)*
Bjerkandera adusta	2.097	2.125	2.11	*Basidiomycete (Bjerkandera; Thanatephorus)*	*Bjerkandera adusta*
Candida albicans	2.268	2.227	2.25	*Candida albicans*	*Candida albicans*
Candida albicans	2.173	2.23	2.20	*Candida albicans*	*Candida albicans*
Candida auris	2.287	2.269	2.28	*Candida auris*	*Candida albicans*
Candida auris	2.339	2.395	2.37	*Candida auris*	*Candida albicans*
Candida auris	2.19	2.265	2.23	*Candida auris*	*Candida albicans*
Candida auris	2.406	2.521	2.46	*Candida auris*	*Candida albicans*
Candida auris	2.095	2.155	2.13	*Candida auris*	*Candida albicans*
Candida auris	1.882	1.933	1.91	*Candida auris*	*Candida albicans*
Candida auris	2.226	2.171	2.20	*Candida auris*	*Candida albicans*
Candida auris	2.137	2.028	2.08	*Candida auris*	*Candida albicans*
Candida auris	2.149	2.208	2.18	*Candida auris*	*Candida albicans*
Candida auris	2.129	1.981	2.06	*Candida auris*	*Candida albicans*
Candida boidinii	1.707	1.807	1.76	*Candida boidinii*	*Candida boidinii*
Candida catenulata	1.78	1.579	1.68	*Candida catenulata*	*Candida catenulata*

Name	Score 1	Score 2	Average Score	MALDI Classification	Sequencing Top Match
Candida glabrata	2.611	2.575	2.59	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.536	2.556	2.55	*Candida parapsilosis*	*Candida glabrata*
Candida glabrata	2.51	2.554	2.53	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.511	2.538	2.52	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.48	2.545	2.51	*Candida glabrata*	*[Candida] glabrata*
Candida glabrata	2.5	2.492	2.50	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.478	2.5	2.49	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.485	2.487	2.49	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.502	2.463	2.48	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.488	2.453	2.47	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.467	2.449	2.46	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.427	2.368	2.40	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.358	2.418	2.39	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.389	2.37	2.38	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.34	2.388	2.36	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.4	2.283	2.34	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.291	2.356	2.32	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.319	2.236	2.28	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.284	2.212	2.25	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.26	2.211	2.24	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.164	2.181	2.17	*Candida glabrata*	*Candida glabrata*
Candida glabrata	2.013	1.972	1.99	*Candida glabrata*	*Candida glabrata*
Candida intermedia	1.838	1.879	1.86	*Candida intermedia*	*Candida intermedia*
Candida krusei	2.402	2.374	2.39	*Candida krusei*	*Pichia kudriavzevii*
Candida metapsilosis	1.86	1.949	1.90	*Candida metapsilosis*	*Candida metapsilosis*
Candida orthopsilosis	1.922	1.841	1.88	*Candida orthopsilosis*	*Candida orthopsilosis*
Candida parapsilosis	2.323	2.329	2.33	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	2.152	2.174	2.16	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.929	1.92	1.92	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.979	1.834	1.91	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.826	1.899	1.86	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.811	1.908	1.86	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.832	1.78	1.81	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.798	1.81	1.80	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.773	1.824	1.80	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	1.753	1.749	1.75	*Candida parapsilosis*	*Candida parapsilosis*
Candida parapsilosis	2.061	2.045	2.05	*Candida parapsilosis*	*Candida parapsilosis*
Candida tropicalis	1.852	1.85	1.85	*Candida tropicalis*	*Candida tropicalis*
Candida tropicalis	1.893	1.758	1.83	*Candida tropicalis*	*Candida tropicalis*
Chaetomium sp.	1.528	1.67	1.60	not reliable identification	*Chaetomium sp.*

Name	Score 1	Score 2	Average Score	MALDI Classification	Sequencing Top Match
Chrysosporium sp. (Microsporum gypseum)	1.974	1.971	1.97	*Microsporum gypseum*	*Microsporum gypseum*
Chrysosporium tuberculatum	1.819	1.867	1.84	*Sarocladium (Acremonia)*	*Sarocladium kiliense*
Cladophialophora bantiana	1.473	1.576	1.52	not reliable identification	*Cladophialophora bantiana*
Cladophialophora bantiana	1.438	1.555	1.50	not reliable identification	*Cladophialophora bantiana*
Cladophialophora carrionii	1.722	1.692	1.71	*Cladophialophora carrionii*	*Cladophialophora carrionii*
Cladosporium herbarum	1.593	1.846	1.72	*Cladosporum cladosporioides*	*Cladosporium sp.*
Cladosporium sp.	1.807	1.792	1.80	*Cladosporium species*	*Cladosporium sp.*
Cladosporum cladosporioides	2.278	2.303	2.29	*Cladosporum cladosporioides*	*Cladosporum cladosporioides*
Cryptococcus gatti	1.555	1.524	1.54	not reliable identification	*Cryptococcus gattii*
Cryptococcus laurentii	1.716	1.715	1.72	*Cryptococcus laurentii*	*Cryptococcus laurentii*
Cryptococcus magnus	1.871	1.826	1.85	*Cryptococcus magnus*	*Filobasidium magnum*
Cryptococcus neoformans NIH306	2.053	2.113	2.08	*Cryptococcus neoformans*	*Cryptococcus neoformans*
Cryptococcus neoformans NIH398	2.349	2.432	2.39	*Cryptococcus neoformans*	*Cryptococcus neoformans*
Curvularia lunata	2.017	2.059	2.04	*Curvularia lunata*	*Curvularia lunata*
Curvularia sp.	2.004	2.048	2.03	*Curvularia species*	*Curvularia sp.*
Exophiala dermatitidis	2.066	2.075	2.07	*Exophiala dermatitidis*	*Exophiala dermatitidis*
Exophiala jeanselmei	1.407	1.55	1.48	not reliable identification	*Exophiala jeanselmei*
Exserohilum rostratum	1.976	1.938	1.96	*Exserohilum rostratum*	*Exserohilum rostratum*
Exserohilum sp.	2.114	2.216	2.17	*Exserohilum rostratum*	*Exserohilum sp.*
Fusarium incarnatum-equiseti	1.362	1.387	1.37	not reliable identification	*Fusarium equiseti*
Fusarium oxysporum	1.421	1.359	1.39	not reliable identification	*Fusarium oxysporum*
Fusarium solani	1.784	1.724	1.75	*Fusarium solani*	*Fusarium solani*
Lichtheimia corymbifera	2.183	2.205	2.19	*Lichtheimia species*	*Lichtheimia corymbifera*
Microsporum audouinii	1.831	1.94	1.89	*Microsporum audouinii*	*Microsporum audouinii*
Microsporum canis	2.086	2.063	2.07	*Microsporum canis*	*Microsporum canis*
Microsporum gypseum	2.633	2.654	2.64	*Microsporum_gypseum*	*Arthroderma gypseum*
Microsporum gypseum	1.82	1.736	1.78	*Microsporum_gypseum*	*Arthroderma gypseum*
Mucor (Rhizomucor) pusillus	1.852	1.841	1.85	*Rhizomucor sp.*	*Rhizomucor pusillus*
Mucor circinelloides	2.18	2.177	2.18	*Rhizomucor variabilis*	*Rhizomucor variabilis var. regularior*
Mucor circinelloides	1.939	1.809	1.87	*Mucor circinelloides*	*Mucor circinelloides*

Name	Score 1	Score 2	Average Score	MALDI Classification	Sequencing Top Match
Mucor sp.	1.949	1.97	1.96	*Mucor circinelloides*	*Mucor sp.*
Neosartorya fischeri	1.751	1.884	1.82	*Neosartorya fischeri*	*Neosartorya fischeri*
Ochroconis gallopava	2.108	2.165	2.14	*Ochroconis gallopava*	*Ochroconis gallopava*
Paecilomyces sp.	2.138	2.058	2.10	*Paecilomyces variotii*	*Paecilomyces sp.*
Penicillium biorgeianum	2.033	2.049	2.04	*Penicillium biorgeianum*	*Penicillium brevicompactum*
Penicillium brevicompactum	2.338	2.374	2.36	*Penicillium brevicompactum*	*Penicillium brevicompactum*
Penicillium camembertii	1.945	1.869	1.91	*Penicillium camemberti*	*Penicillium sp.*
Penicillium chrysogenum	2.037	2.092	2.06	*Penicillium sp.*	*Penicillium chrysogenum*
Penicillium crustosum	1.767	1.744	1.76	*Penicillium crustosum*	*Penicillium crustosum*
Penicillium olsonii	2.181	2.208	2.19	*Penicillium olsonii_USDA*	*Penicillium olsonii*
Phialemonium sp.	1.501	1.645	1.57	not reliable identification	*Uncultured Acremonium*
Phialophora verrucosa	1.938	1.879	1.91	*Phialophora species*	*Phialophora verrucosa*
Phoma sp.	1.402	1.556	1.48	not reliable identification	*Phoma sp.*
Pseudallescheria boydii	2.156	2.273	2.21	*Pseudallescheria boydii*	*Pseudallescheria boydii*
Purpereocillium lilacinum	1.884	1.753	1.82	*Purpureocillium lilacinum*	*Purpereocillium lilacinum*
Rhizopus arrhizus	1.529	1.492	1.51	*not reliable identification*	*Rhizopus delemar/Rhizopus oryzae*
Rhizopus delemar	1.533	1.527	1.53	*not reliable identification*	*Rhizopus oryzae*
Rhizopus sp.	1.412	1.523	1.47	*not reliable identification*	*Rhizopus sp.*
Saccharomyces cerevisiae	1.789	1.815	1.80	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*
Scedosporium prolificans	2.264	2.263	2.26	*Scedosporium prolificans*	*Scedosporium prolificans*
Scopulariopsis sp.	1.966	1.97	1.97	*Scopulariopsis brevicaulis*	*Scopulariopsis sp.*
Sporothrix schenckii	2.02	2.128	2.07	*Sporothrix schenkii*	*Sporothrix schenckii*
Sporotrichum pruinosum	1.918	1.731	1.82	*Sporotrichum pruinosum*	*Sporotrichum pruinosum (syn. Phanerochaete chrysosporium)*
Syncephalastrum sp.	1.744	1.773	1.76	*Syncephalastrum species*	*Syncephalastrum sp.*
Trichophyton mentagrophytes	1.563	1.748	1.66	*Trichophyton mentagrophytes*	*Trichophyton mentagrophytes*
Trichophyton mentagrophytes	1.534	1.415	1.47	*not reliable identification*	*Trichophyton quinckeanum*
Trichophyton rubrum	2.078	1.836	1.96	*Trichophyton rubrum*	*Trichophyton rubrum*
Trichophyton terrestre	1.958	1.874	1.92	*Trichophyton terrestre*	*Trichophyton terrestre*
Trichophyton tonsurans	2.135	2.094	2.11	*Trichophyton tonsurans*	*Trichophyton tonsurans*
Trichophyton verrucosum	1.611	1.737	1.67	*Trichophyton verrucosum*	*Trichophyton verrucosum*

Supplementary Table 3. List of fungi analyzed in this study.

Classification	Organism	No. of fungi
Yeast (58)	*Candida* spp.	53
	Candida albicans (2)	
	Candida auris (10)	
	Candida boidinii (1)	
	Candida catenulata (1)	
	Candida glabrata (22)	
	Candida intermedia (1)	
	Candida krusei (1)	
	Candida metapsilosis (1)	
	Candida orthopsilosis (1)	
	Candida parapsilosis (11)	
	Candida tropicalis (2)	
	Cryptococcus spp.	5
	Cryptococcus gatti (1)	
	Cryptococcus laurentii (1)	
	Cryptococcus magnus (1)	
	Cryptococcus neoformans (2)	
	Saccharomyces cerevisiae	1
Hyaline (38)	*Aspergillus* spp.	18
	Aspergillus candidus (1)	
	Aspergillus flavipes (2)	
	Aspergillus flavus (2)	
	Aspergillus fischeri (1)	
	Aspergillus fumigatus (3)	
	Aspergillus nidulans (1)	
	Aspergillus brasiliensis (2)	
	Aspergillus sydowii (1)	
	Aspergillus terreus (3)	
	Aspergillus ustus (1)	
	Aspergillus versicolor (1)	
	Beauveria sp.	1
	Bjerkandera adusta	1
	Chaetomium sp.	1
	Chrysosporium tuberculatum	1
	Fusarium spp.	3
	Fusarium incarnatum-equiseti (1)	
	Fusarium oxysporum (1)	
	Fusarium solani (1)	
	Paecilomyces sp.	1
	Penicillium spp.	6
	Penicillium biourgeianum (1)	
	Penicillium brevicompactum (1)	
	Penicillium camembertii (1)	
	Penicillium chrysogenum (1)	
	Penicillium crustosum (1)	
	Penicillium olsonii (1)	
	Phialemonium sp.	1
	Pseudallescheria boydii	1
	Purpereocillium lilacinum	1

	Scedosporium prolificans	1
	Scopulariopsis sp.	1
	Sporotrichum pruinosum	1
Dematiaceous (17)	*Aureobasidium pullulans*	1
	Bipolaris spicifera	1
	Cladophialophora bantiana	2
	Cladophialophora carrionii	1
	Cladosporium spp.	4
	Cladosporium cladosporioides (1)	
	Cladosporium herbarum (1)	
	Curvularia spp.	2
	Curvularia lunata (1)	
	Exophiala spp.	2
	Exophiala dermatitidis (1)	
	Exophiala jeanselmei (1)	
	Exserohilum spp.	1
	Exserohilum rostratum (1)	
	Ochroconis gallopava	1
	Phialophora verrucosa	1
	Phoma sp.	1
Non-septate or Mucorales (9)	*Lichtheimia corymbifera*	1
	Mucor spp.	3
	Mucor circincelliodes f. circinelloides (2)	
	Rhizomucor pusillus	1
	Rhizopus spp.	3
	Rhizopus arrhizus (1)	
	Rhizopus delemar (1)	
	Syncephalastrum sp.	1
Dermatophyte (11)	*Microsporum* spp.	5
	Microsporum audouinii (1)	
	Microsporum canis (1)	
	Microsporum gypseum (3)	
	Trichophyton spp.	6
	Trichophyton mentageophytes (2)	
	Trichophyton rubrum (1)	
	Trichophyton terrestre (1)	
	Trichophyton tonsurans (1)	
	Trichophyton verrucosum (1)	
Dimorphic (1)	*Sporothrix schenckii*	1