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ORIGINAL ARTICLE

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Evaluation of three MALDI-TOF mass spectrometry libraries for the identification of filamentous fungi in three clinical microbiology laboratories in Manitoba, Canada

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Summary

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is commonly used by clinical microbiology laboratories to identify bacterial pathogens and yeasts, but not for the identification of moulds. Recent progress in extraction protocols and the composition of comparative libraries support potential application of MALDI-TOF MS for mould identification in clinical microbiology laboratories. We evaluated the performance of the Bruker Microflex™ MALDI-TOF MS instrument (Billerica, MA, USA) to identify clinical isolates and reference strains of moulds using 3 libraries, the Bruker mould library, the National Institutes of Health (NIH) library and the Mass Spectrometry Identification (MSI) online library, and compared those results to conventional (morphological) and molecular (18S/ITS; gold standard) identification methods. All 3 libraries demonstrated greater accuracy in genus identification (≥94.9%) than conventional methods (86.4%). MALDI-TOF MS identified 73.3% of isolates to species level compared to only 31.7% by conventional methods. The MSI library demonstrated the highest rate of species-level identification (72.0%) compared to NIH (19.5%) and Bruker (13.6%) libraries. Greater than 20% of moulds remained unidentified to species level by all 3 MALDI-TOF MS libraries primarily because of library limitations or imperfect spectra. The overall identification rate of each MALDI-TOF MS library depended on the number of species and the number of spectra representing each species in the library.

KEYWORDS

filamentous fungi, identification, mass spectrometry identification online library, matrix-assisted laser desorption ionisation-time of flight mass spectrometry, moulds

1 | INTRODUCTION

Over the past decade the identification of bacteria by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) has increasingly replaced traditional identification methods that were most frequently based on biochemical reactions.[1-3] However, comprehensive MALDI-TOF MS databases for filamentous fungi have been established only recently, and apart from commercial libraries, were not widely available. Thus, clinical microbiology laboratories that implemented MALDI-TOF MS were

required to establish local in-house databases by a process that is both time consuming and labour intensive.[4-7] Fortunately, efforts to make large databases available for public access are in progress and will pave the way for clinical microbiology laboratories to more broadly implement MALDI-TOF MS for mould identification.[4,8]

The first comprehensive mould library for use with the Bruker Biotyper in North America was published in 2013 by investigators at the National Institutes of Health (NIH, Bethesda, MD, USA), and contained 294 strains from 152 species and 76 genera.[4] The NIH library not only contained twice as many strains as the concurrently available Bruker mould library, but also simplified the sample preparation method. The NIH library was created by extracting the moulds directly from agar medium rather than using broth culture, the method used to generate the Bruker mould library. In 2014, 2 more comprehensive mould libraries containing 374 and 472 fungal species, respectively, were published by European investigators.[6,9] Building on the strain collection used to generate the library described by Gautier et al,[6] a new library, the Mass Spectrometry Identification (MSI) library, has recently been published by Normand et al,[8] and currently contains 938 species belonging to 246 genera (11 815 spectra for 1913 strains).[8] This library has been established as an online identification system and has recently been made accessible to the medical mycology community free of charge.[8] In addition, many comprehensive fungal in-house libraries have been described for certain genera or groups of moulds including dermatophytes, *Fusarium* and *Scedosporium*.[5,10,11] Unfortunately, libraries for the identification of bacteria and fungi by MALDI-TOF MS have been created and validated for specific instruments like the Bruker Microflex or the bioMérieux VITEK® MS only and cannot be used across platforms. Proprietary issues and differences in the algorithms applied to match peaks for identification purposes have hampered the generation of libraries that are compatible with different mass analysers.

Mould identification rates vary depending on the library used and the variety of isolates tested. In the study by Lau et al,[4] the NIH mould database yielded a species-level identification rate of 88.9% (370/421) for clinical isolates compared with the Bruker mould library that identified only 3 of the 421 isolates. Using their respective in-house libraries, Gautier et al,[6] described a species-level identification rate for clinical isolates of 98.8% (1094/1107), whereas Becker et al,[9] reported an identification rate of 95.4% (372/390) but an identification rate of only 65% using the Bruker library.

High accuracy in the identification of moulds using MALDI-TOF MS has also been reported for another MALDI-TOF MS platform, the VITEK® MS instrument. Using the VITEK® MS v3.0 system, Rychert et al,[12] identified 92% (1387/1519) of isolates that were represented in the library with an accuracy of 98%. The accuracy was highest for dimorphic fungi (100%) and lowest for dermatophytes (85%), reflecting MALDI-TOF MS identification problems with this organism group across various libraries and platforms. Species-level identification is notoriously difficult for dermatophytes, even with molecular methods. As such, taxonomy remains more controversial than for other fungal groups, with more frequent reclassifications.[13] Despite this, MALDI-TOF MS is emerging as a highly accurate and useful tool for the identification of clinically relevant moulds.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates tested

Three hospital clinical microbiology laboratories in Manitoba, Canada (2 laboratories in Winnipeg and one laboratory in Brandon) prospectively characterised at least 50 local clinical isolates each as well as 15 to 30 reference/characterised fungal strains each. In total, 221 mould isolates were tested. These isolates were comprised of 158 clinical isolates and 63 reference/characterised strains (15 American Type Culture Collection [ATCC] strains, 25 Centraalbureau voor Schimmelcultures [CBS, Westerdijk Fungal Biodiversity Institute] strains and 23 external proficiency testing strains from the College of American Pathologists [CAP]). The clinical isolates and reference/characterised strains tested in this study were members of 5 major groups of fungi: dermatophytes, hyaline moulds, dematiaceous moulds, *Mucorales* and a dimorphic mould (*Blastomyces dermatitidis*). Clinical isolates were collected and analysed at each hospital laboratory in the chronological order they had been received. All specimen types that are currently accepted by our laboratory for culture of filamentous fungi were analysed, including sterile sites, respiratory sites, blood, skin, nails, etc.). Therefore, the investigated spectrum of fungi was representative for a typical clinical microbiology laboratory. Repeat isolates obtained from the same patient were excluded.

2.2 | Fungal cultures

Clinical isolates and reference/characterised strains were grown on Potato Flake Agar, Mycosel, Inhibitory Mould Agar or Sabouraud's Agar incubated at 30°C for 1-7 days.

2.3 | DNA sequencing

All clinical isolates were identified by Sanger DNA sequencing. DNA sequencing was performed at McGill University and the Genome Quebec Innovation Center (Montréal, Canada). DNA sequencing targeted the rRNA ITS2 region (primer sequences: ITS3 – GCA TCG ATG AAG AAC GCA GC and ITS4c – TCC TCC GCT TAT TGA TAT GC). For certain taxa, additional genes were sequenced including the partial β-tubulin gene (primer sequences: Bt2A – GGT AAC CAA ATC GGT GCT GCT TTC and Bt2B – AAC CTC AGT GTA GTG ACC CTT GGC) or translation elongation factor 1-α (primer sequences: EF1 – ATG GGT AAG GAR GAC AAG AC and EF2 – GGA RGT ACC AGT SAT CAT GTT). Validation of the species identity required sequencing reads to be longer than 300 bp, with at least 99% homology using the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the CBS nucleotide database (http://www.westerdijkinstitute.nl/Collections/BioloMICSSequences.aspx). If 2 or more species fulfilled these criteria, the mould was reported to the species complex level or as a species combination.

2.4 | Conventional identification of filamentous fungi

All clinical isolates were identified using conventional methods, including evaluation of colony morphology, microscopy of lactophenol cotton blue stained scotch-tape preparations and slide cultures and biochemical testing (urease, and Trichophyton Agar 1-4 (BD Difco). Morphological identification was performed before identifications resulting from DNA sequencing or MALDI-TOF analysis were known.

2.5 | MALDI-TOF MS sample preparation

All moulds were extracted as described previously.[5,7] In short, following incubation on solid agar medium, pieces of mould containing conidia and hyphae were transferred to a microcentrifuge tube, washed in 100% ethanol and extracted with 70% formic acid and an equal volume of acetonitrile. One microlitre of extract supernatant was spotted on the MALDI-TOF MS target plate in triplicate, air dried and overlaid with 1 μL of HCCA (α-cyano-4-hydroxycinnamic acid) matrix. MALDI-TOF MS spectra were generated at each of the 3 hospital clinical microbiology laboratory testing sites using the Bruker Microflex™ system (Billerica, MA, USA). If an acceptable score was not obtained with any of the 3 libraries, the mould was re-extracted after 24-48 hours of additional growth and re-analysed by MALDI-TOF MS.

2.6 | Mass spectrometry software and reference databases

Two software applications were used for identification of the mass spectra, the MALDI Biotyper version 3.1 software (Bruker) with a genus threshold of 1.7 and a species threshold of 2.0, and the MSI online platform using a species threshold of 17 (no genus threshold).[8] Three mould libraries were used: the Bruker Filamentous Fungi Library 1.0 (containing 127 species), the NIH library database (containing 294 strains belonging to 152 species and 76 genera),[4] and the MSI database (2016 version) (containing 2,013 strains belonging to 909 species and 246 genera).[8]

3 | RESULTS

3.1 | Comparison of library performance

While all 3 libraries had limitations, the MSI online library provided the best identification rate (Table 1 and Table 2). Overall, 72% (159/221) of isolates were correctly identified to the species level by the MSI library (MSI score ≥17), compared to 13.6% (30/221) by the Bruker library (Bruker score ≥2) and 19.5% (43/221) by the NIH library (Bruker score ≥2). When only genus-level identification was considered (a Bruker or NIH score of ≥1.7) the overall identification rate for the Bruker and NIH libraries increased to 33.5% (74/221) and 50.2% (111/221) respectively. Since the Bruker and NIH libraries can be locally installed and accessed simultaneously by the Bruker software for peak analysis, we also evaluated the identification rates for both libraries combined (Table 2). This improved the species identification rate to 29% (64/221) and the genus identification rate to 59.7% (132/221). Twelve isolates that could not be identified using the MSI online library were identified using the combined Bruker and NIH libraries (3 species and 9 genus identifications), improving the overall identification rate to the genus or species level from 72% for the MSI library alone to 77.4% for all 3 libraries combined. Thus, 22.6% of moulds were not identified by MALDI-TOF MS using any library. Reasons for this relatively high number of unidentified moulds were either the lack of reference spectra in the libraries or, in 12 cases, a suboptimal extraction procedure resulting in the absence of spectral peaks. No significant trend for specific genera that may more likely be associated with suboptimal extraction was identified. The overall identification rate for conventional identification was 31.7% (70/221) to species level and 86.4% (191/221) to genus level (Table 2).

The overall agreement (accuracy) between the gold standard using molecular gene sequencing of the ITS region and, where indicated, sequencing of fragments of the β-tubulin gene or elongation factor gene, and MALDI-TOF MS was 94.9% (4 errors in 78 identifications) for the Bruker library, 98.2% (2 errors in 113 identifications) for the NIH library, 96.4% (5 errors in 137 identifications) for the Bruker and NIH libraries combined, and 97% (5 errors in 164 identifications) for the MSI library (Table 2). Most of these discrepancies were related to nomenclature changes, the inability to discriminate between genetically very closely related species, or were associated with scores near the cut-off value (Table 3). The most concerning misidentification was a *Fusarium oxysporum* isolate that was identified by the MSI library as *Fusarium petroliphilum*. The latter belongs to the *Fusarium solani* species complex indicating possible problems to correctly identify *Fusarium* species to the correct species complex. All MALDI-TOF MS misidentifications detected in this study, except one, could have been avoided by further investigating results that were discrepant between the different libraries. The agreement between conventional identification methods (morphology, microscopy, biochemical tests) and molecular identification on the genus level was 86.4% (30 errors in 221 identifications).

3.2 | Inter-laboratory variability

While performance trends for each library were similar in all 3 clinical microbiology laboratories, significant variation in identification rates were observed ranging from 65%-84% for the MSI library, 40%-59% for the NIH library and 20%-45% for the Bruker library (data not shown). The highest and lowest values for each library were consistently obtained by the same laboratory.

3.3 | Identification scores

While the MSI library does not provide a cut-off value for genus versus species level identification, the Bruker and NIH database

use values from 1.7 to <2 for genus only identification and ≥2-3 for species level identification. As mentioned above, applying the species threshold, we obtained identification rates of 13.6% (30/221) and 19.5% (43/221) for the Bruker and NIH libraries respectively (Table 2). However, our results indicated that most genus-level identifications (scores 1.7 to <2) were also correct to the species level, suggesting that a score of ≥ 1.7 could be acceptable for species identification. By applying the lower 1.7 cut-off score for

TABLE 1 Species identification rates for 221 mould isolates tested using 3 MALDI-TOF MS libraries[a]

				MALDI-TOF MS Library				
				Percent of isolates correctly identified to species and genus level				
				Bruker		NIH		MSI
Genus	Number of isolates per genus (percent of all isolates)	Species	Number of isolates per species (percent of all isolates)	Species level	Genus level	Species level	Genus level	Species level
Absidia	4 (1.81)	*Absidia (Lichtheimia) corymbifera*	4 (1.81)	0	0	0	0	100
Alternaria	7 (3.17)	*Alternaria alternata*	6 (2.71)	33.3	66.7	0	100	100
		Alternaria chlamydospora	1 (0.45)	0	0	0	0	0
Aspergillus	50 (22.62)	*Aspergillus basiliensis*	2 (0.90)	0	100	50	0	100
		Aspergillus chevalieri clad.	2 (0.90)	0	0	0	0	100
		Aspergillus clavatus	2 (0.90)	0	0	0	0	100
		Aspergillus flavus/oryzae	3 (1.36)	33.3	0	33.3	0	100
		Aspergillus fumigatus	14 (6.33)	28.6	35.7	64.3	21.4	100
		Aspergillus glaucus	1 (0.45)	0	0	0	0	0
		Aspergillus hollandicus - Eurotium amstelodami	2 (0.90)	0	0	50	50	100
		Aspergillus nidulans	5 (2.26)	60	40	40	40	100
		Aspergillus niger	3 (1.36)	66.7	0	33.3	0	100
		Aspergillus sydowii	3 (1.36)	0	0	0	66.7	100
		Aspergillus terreus	5 (2.26)	20	0	0	40	80
		Aspergillus ustus	1 (0.45)	0	0	0	100	0
		Aspergillus versicolor complex	7 (3.17)	0	42.9	42.9	42.9	42.9
Beauveria	3 (1.36)	*Beauveria bassiana*	3 (1.36)	0	0	0	0	33.3
Blastomyces	1 (0.45)	*Blastomyces dermatitidis*	1 (0.45)	0	0	0	100	0
Cadophora	2 (0.90)	*Cadophora luteo-olivacea*	1 (0.45)	0	0	0	0	0
		Cadophora malorum	1 (0.45)	0	0	0	0	0
Cephalotrichum	2 (0.90)	*Cephalotrichum/ Doratomyces/Trichurus*	2 (0.90)	0	0	0	0	0
Chaetomium	4 (1.81)	*Chaetomium globosum*	4 (1.81)	0	33.3	0	0	33.3
Cladosporium	1 (0.45)	*Cladosporium cladosporioides*	1 (0.45)	0	0	0	0	100
Cryptendoxyla	1 (0.45)	*Cryptendoxyla hypophloia*	1 (0.45)	0	0	0	0	0
Cunninghamella	1 (0.45)	*Cunninghamella bertholletiae*	1 (0.45)	0	0	0	100	100
Curvularia	2 (0.90)	*Curvularia lunata*	2 (0.90)	0	50	50	0	100
Epicoccum	3 (1.36)	*Epicoccum nigrum*	3 (1.36)	0	0	0	0	100
Exophiala	7 (3.17)	*Exophiala cancerae/salmonis*	1 (0.45)	0	0	0	0	0
		Exophiala dermatitidis	2 (0.90)	0	50	0	50	100
		Exophiala phaeomuriforms	1 (0.45)	0	0	0	100	0
		Exophiala pisciphilia/ lecanii-corni	3 (1.36)	0	0	0	33.3	0

(*Continues*)

TABLE 1 (Continued)

Genus	Number of isolates per genus (percent of all isolates)	Species	Number of isolates per species (percent of all isolates)	MALDI-TOF MS Library				
				Percent of isolates correctly identified to species and genus level				
				Bruker		NIH		MSI
				Species level	Genus level	Species level	Genus level	Species level
Exserohilum	1 (0.45)	*Exserohilum rostratum*	1 (0.45)	0	0	0	0	100
Fusarium	14 (6.33)	*Fusarium oxysporum*	5 (2.26)	0	60	20	40	80
		Fusarium proliferatum	1 (0.45)	0	0	0	0	0
		Fusarium sambucinum	1 (0.45)	0	0	0	0	0
		Fusarium solani	4 (1.81)	0	33.3	0	33.3	100
		Fusarium solani complex	2 (0.90)	33.3	0	0	20	66.6
		Fusarium verticilloides	1 (0.45)	0	0	0	0	0
Lecanicillium	1 (0.45)	*Lecanicillium aphanocladii*	1 (0.45)	0	0	0	0	0
Microascus	1 (0.45)	*Microascus cirrosus*	1 (0.45)	0	0	0	0	0
Microsporum	9 (4.07)	*Microsporum canis* (=*Nannizia otae*)	6 (2.71)	16.7	50	33.3	33.3	66.6
		Microsporum gypseum	2 (0.90)	0	50	0	0	50
		Microsporum persicolor	1 (0.45)	0	0	0	0	0
Mucor	2 (0.90)	*Mucor circillenoides*	2 (0.90)	0	0	0	50	100
Neoscytalidium	1 (0.45)	*Neoscytalidium dimidiatum*	1 (0.45)	0	0	0	0	100
Paecilomyces	5 (2.26)	*Paecilomyces variotii*	4 (1.81)	75	0	25	50	100
		Paecilomyces variotii or *formosus*	1 (0.45)	0	0	0	0	100
Penicillium	9 (4.07)	*Penicillium camemberti* or *oxalicum*	2 (0.90)	0	0	0	0	0
		Penicillium citrinum	2 (0.90)	0	50	0	100	100
		Penicillium digitatum	1 (0.45)	100	0	0	0	100
		Penicillium glabrum	2 (0.90)	0	0	0	0	100
		Penicillium janthinellum	1 (0.45)	0	0	0	0	100
		Penicillium rubens or *Penicillum chrysogenum*	1 (0.45)	0	100	0	100	100
Phoma	2 (0.90)	*Phoma herbarorum*	2 (0.90)	0	0	0	0	0
Pseudallescheria	1 (0.45)	*Pseudallescheria boydii*	1 (0.45)	0	0	0	0	100
Purpureacillium	2 (0.90)	*Purpureacillium lilacinum*	2 (0.90)	0	50	0	100	100
Ramularia sp. or *Glomerella sp.*	1 (0.45)	*Ramularia sp.* or *Glomerella sp.*	1 (0.45)	0	0	0	0	0
Rhizomucor	2 (0.90)	*Rhizomucor pusillus*	2 (0.90)	0	100	0	100	100
Rhizopus	5 (2.26)	*Rhizopus oryzae*	2 (0.90)	50	0	0	50	100
		Rhizopus stolonifera	3 (1.36)	0	100	0	50	100
Sarocladium	1 (0.45)	*Sarocladium bacillisporum* or *Sarocladium implicatum*	1 (0.45)	0	0	0	0	0
Scediosporum	9 (4.07)	*Scediosporum apiospermum*	6 (2.71)	33.3	33.3	0	33.3	100
		Scedosporium ellipsoidea	1 (0.45)	0	0	0	100	100
		Scedosporium prolificans	2 (0.90)	100	0	100	0	100
Scopulariopsis	5 (2.26)	*Scopulariopsis brevicaulis*	5 (2.26)	60	20	60	20	80
Sporothrix	2 (0.90)	*Sporothrix schenckii*	2 (0.90)	0	0	50	50	100

(*Continues*)

TABLE 1 (Continued)

Genus	Number of isolates per genus (percent of all isolates)	Species	Number of isolates per species (percent of all isolates)	MALDI-TOF MS Library				
				Percent of isolates correctly identified to species and genus level				
				Bruker		NIH		MSI
				Species level	Genus level	Species level	Genus level	Species level
Stachybotrys	1 (0.45)	*Stachybotrys echinata*	1 (0.45)	0	0	0	0	0
Syncephalastrum	2 (0.90)	*Syncephalastrum racemosum*	2 (0.90)	0	0	0	100	0
Talaromyces	2 (0.90)	*Talaromyces amsteltolkiae*	1 (0.45)	0	0	0	0	0
		Talaromyces pinophilus	1 (0.45)	0	0	0	0	0
Thanatophorus or *Bjerkandera*	3 (1.36)	*Thanatophorus cucumeris* or *Bjerkandera adjusta*	3 (1.36)	0	0	0	66.7	66.7
Trametes	1 (0.45)	*Trametes versicolor*	1 (0.45)	0	0	0	0	0
Trichoderma	1 (0.45)	*Trichoderma harzianum - Hypocrea lixii*	1 (0.45)	0	0	0	100	100
Trichophyton	50 (22.62)	*Trichophyton interdigitale* or *Trichophyton mentagrophytes*	12 (5.43)	8.3	16.7	0	33.3	83.3
		Trichophyton rubrum	26 (11.76)	0	19.23	38.5	34.6	92.3
		Trichophyton rubrum or *violaceum*	8 (3.62)	12.5	12.5	25	25	87.5
		Trichophyton verrucosum/ Arthroderma benhamiae	4 (1.81)	0	0	0	0	0
Total number of isolates	221		221					

[a]Species identification referred to a MALDI-TOF MS score of ≥2.0 (Bruker Filamentous Fungi Library 1.0 or NIH library database) or ≥17 (MSI database) and genus identification referred to a MALDI-TOF MS score of ≥1.7-<2 (Bruker Filamentous Fungi Library 1.0 or NIH library database).

species identification, we increased the identification rate to 30.3% (67/221) and 48.9% (108/221) while reaching an accuracy of 85.9% and 95.6% for the Bruker and NIH libraries respectively (Table 4). Although the accuracy for the Bruker library using this score was reduced from 95.9% to 85.9% and for the NIH library from 98.2% to 95.6%, using both libraries combined increased the accuracy for the ≥1.7 species cut-off value to 96.7% (4 errors in 122 identifications) and the species identification rate to 53.4% (118/221) (Table 3).

4 | DISCUSSION

The implementation of MALDI-TOF MS for the identification of bacteria has revolutionised clinical microbiology. As it has been adopted over the past decade, it has improved laboratory workflow, reduced time to identification, allowed more reliable identification of rare or previously difficult to identify organisms, and reduced overall cost for laboratories. By comparison, reliable identification of moulds using MALDI-TOF MS has become a reality in few clinical laboratories only recently, once more comprehensive mould libraries and less complex extraction protocols became available.

This study evaluated the performance of 3 mould libraries that have been described for the identification of moulds using the Bruker Biotyper system. As expected, a major determining factor in the successful identification of moulds was the comprehensiveness of the libraries. Intra-species morphological variability and molecular diversity is higher for fungi compared to bacteria.[14] Therefore, the inclusion of more strains representing a single species and different spectra of the same strain using different growth conditions is more important for fungi than for bacteria when creating a library. This requirement likely accounts for the poor performance of the Bruker library compared to the NIH and the MSI libraries. At the time of testing, the Bruker Filamentous Fungi Library 1.0 (2012) contained 127 strains (127 species belonging to 43 genera), the NIH library (2013) contained 294 strains (152 species belonging to 76 genera)[4] and the MSI online library contained 2013 strains belonging to 909 species and 246 genera.[8] It is important to note that the MSI library version we used for this study was a 2016 version that contained less spectra than the current version.[8] Thus, we can assume that the newest version of the MSI library would increase the identification rate above the 72% obtained in this study.

Considering there are 196 511 fungal taxa with over 350 000 species described (listed in Mycobank as of October 26, 2017), it is

TABLE 2 MALDI-TOF MS library mould species and genus identification rates and accuracy[a]

Cumulative data from all three testing laboratories	MALDI-TOF MS Library					
	Bruker	NIH	Bruker +NIH	MSI	Bruker +NIH+MSI	Conventional identification
Number of isolates with no identification	143	108	84	57	45	0
Number of isolates with species-level identification	30	43	64	159	162	70
Number of isolates with genus-level identification	44	68	68	N/A[b]	9	121
Number of isolates with species- or genus-level identification	74	111	132	159	171	191
Number of isolates that were misidentified	4	2	5	5	4	30
Total number of identifications	78	113	137	164	175	221
Total number of isolates tested	221	221	221	221	221	221
Percentage of total number of identifications that were misidentifications	5.1%	1.8%	3.7%	3.1%	2.3%	13.6%
Species identification rate for the total number of isolates tested	13.6%	19.5%	29%	72%	73.3%	31.7%
Genus identification rate for the total number of isolates tested	33.5%	50.2%	59.7%	72%	77.4%	86.4%
Percentage of the total number of identifications made that agreed with the DNA sequencing result	94.9%	98.2%	96.4%	97%	97.7%	86.4%

[a]Species identification referred to a MALDI-TOF MS score of ≥2.0 (Bruker Filamentous Fungi Library 1.0 or NIH library database) or ≥17 (MSI database) and genus identification referred to a MALDI-TOF MS score of ≥1.7-<2 (Bruker Filamentous Fungi Library 1.0 or NIH library database).
[b]N/A, not applicable.

not surprising that even the most comprehensive library tested was still unable to identify approximately 30% of the isolates. Since there are relatively few clinically relevant moulds (approximately 600 species listed in the 4th version of the Atlas of Clinical Fungi),[15,16] which are well represented in the NIH and MSI libraries, it is likely that some of the non-identifiable strains represent environmental contaminants that are less abundant in the these libraries, and are mostly absent from the Bruker library. This was supported by identifying the moulds using DNA sequencing as the gold standard in our study. Environmental isolates that remained unidentified by all libraries included *Stachybotrys echinata*, *Lecanicillium aphanocladii*, *Cryptendoxyla hypophloia* and *Microascus cirrosus* (Table 3). Therefore, moulds remaining unidentified after analysis through these libraries are less likely to be clinically relevant. Other isolates were represented in the libraries but would repeatedly generate insufficient spectra, suggesting a possibility of suboptimal extraction. A third group of unidentified isolates had unacceptable low scores close to the cut-off but were actually identified correctly.

When comparing the performance of the 3 libraries, it is important to note that the growth condition and extraction method used in our study was adopted from Cassagne et al[7] and was the method used to create the MSI online database. The NIH protocol also used a similar extraction method from growth on agar plates but included the use of beads in the mechanical lysis of the moulds. This step was deemed unnecessary by Cassagne et al[7] and was shown to not significantly improve mould identification rates in their studies. The Bruker library was created using growth of moulds in liquid medium, which, in addition to the limited number of spectra in the library, could explain why it had the lowest identification rate (33.5%) in our study. Consistently, 2 previous studies using the Bruker mould library with agar-grown isolates showed identification rates of less than 65% and less than 2%.[4,9]

One problem we encountered during our study, which extended to all 3 libraries, was the difficulty to keep up-to-date with the current nomenclature of moulds. Some of the type reference strains or clinically relevant species that have been included in the libraries have since been reclassified. In our study, this resulted in misidentification of *Aspergillus brasiliensis* (ATCC 16404) as *Aspergillus niger* (Bruker library) and *Neoscytalidium dimidiatum* as *Scytalidium sp.* (Bruker and NIH library). Molecular methods are driving the identification of countless new cryptic species that could not be easily differentiated, if at all, from their closest relatives by conventional methods. Tracking these changes and modifying the libraries to reflect them is a daunting task and will undoubtedly lag behind, especially since synonyms for many moulds remain in use and nomenclature is rapidly undergoing revision. For example depending on the library, the same isolate was either reported as *Aspergillus hollandicus* (anamorph) or *Eurotium amstelodami* (teleomorph), or *as Paecilomyces lilacinus* (former name) or *Purpureocillium lilacinum* (new name). *Lichtheimia corymbifera* was reported by the MSI online

TABLE 3 Misidentifications by conventional identification methods and individual MALDI-TOF MS libraries

Identification Method	Isolate identification	MALDI-TOF MS Score	Molecular Identification (Gold Standard)
Conventional identification	*Mucor species*	N/A[a]	*Absidia (Lichtheimia) corymbifera*
	Aspergillus niger	N/A	*Aspergillus brasiliensis*
	Aspergillus niger	N/A	*Aspergillus brasiliensis*
	Gliocladium species	N/A	*Aspergillus chevalieri clade*
	Aspergillus sydowii	N/A	*Aspergillus fumigatus*
	Aspergillus glaucus	N/A	*Aspergillus hollandicus - Eurotium amstelodami*
	Trichophyton mentagrophytes	N/A	*Beauveria bassiana*
	Scedosporium species	N/A	*Blastomyces dermatitidis*
	Phaeoacremonium parasiticum	N/A	*Cadophora luteo-olivacea*
	Acremonium sp.	N/A	*Cadophora malorum*
	Scopulariopsis species	N/A	*Chaetomium globosum*
	Hormographiella sp.	N/A	*Cryptendoxyla hypophloia*
	Graphium sp.	N/A	*Exophiala cancerae/salmonis*
	Beauveria sp.	N/A	*Lecanicillium aphanocladii*
	Chaetomium species	N/A	*Microascus cirrosus*
	Trichophyton tonsurans	N/A	*Microsporum persicolor*
	Scydalidium sp.	N/A	*Neoscytalidium dimidiatum*
	Pseudallescheria boydii	N/A	*Scedosporium ellipsoidea*
	Scopulariopsis brumptii	N/A	*Stachybotrys echinata*
	Penicillium sp.	N/A	*Talaromyces amsteltolkiae*
	Penicillium species	N/A	*Talaromyces pinophilus*
	Geotrichum sp.	N/A	*Thanatephorus cucumeris*
	Geotrichum species	N/A	*Thanatophorus cucumeris/Bjerkandera adjusta*
	Geotrichum species	N/A	*Thanatophorus cucumeris/Bjerkandera adjusta*
	Malbranchea species	N/A	*Trametes versicolor*
	Trichophyton violaceum	N/A	*Trichophyton rubrum*
	Trichophyton mentagrophytes	N/A	*Trichophyton rubrum*
	Trichophyton tonsurans	N/A	*Trichophyton rubrum*
	Trichophyton mentagrophytes	N/A	*Trichophyton verrucosum/Arthroderma benhamiae*
	Trichophyton tonsurans	N/A	*Trichophyton verrucosum/Arthroderma benhamiae*
Bruker MALDI-TOF MS library	*Curvularia pallescens*	2.046	*Curvularia lunata*
	Mucor ramosissimus	2.13	*Mucor circinelloides*
	Scydalidium sp.	1.799	*Neoscytalidium dimidiatum*
	Trichophyton tonsurans	2.101	*Trichophyton interdigitale/Trichophyton mentagrophytes*
NIH MALDI-TOF MS library	*Scydalidium sp.*	1.791	*Neoscytalidium dimidiatum*
	Emericella quadrilineata	2.074	*Aspergillus nidulans*
MSI MALDI-TOF library	*Chalastospora gossypii*	18.615	*Alternaria chlamydospora*
	Eurotium minus	18.66	*Aspergillus glaucus*
	Fusarium petroliphilum	28.463	*Fusarium oxysporum*
	Fusarium-Acremonium falciforme	25.192	*Fusarium solani*
	Aphanocladium album	31.021	*Lecanicillium aphanocladii*

[a]N/A, not applicable.

TABLE 4 Library species identification rate and accuracy (score ≥1.7)

Cumulative data from all three testing laboratories	MALDI-TOF MS Library		
	Bruker	NIH	NIH + Bruker
Number of species not identified	143	108	99
Number of species correctly identified	67	108	118
Number of species misidentified	11	5	4
Total number of identifications	78	113	122
Percentage of total number of identifications that were misidentifications	14.1%	4.4%	2.9%
Total number of isolates tested	221	221	221
Species identification rate for the total number of isolates tested	30.3%	48.9%	53.4%
Species identification accuracy for the total number of isolates tested	85.9%	95.6%	96.7%

library as *Absidia corymbifera*. The definition of a species in mycology is less well defined than in bacteriology, and this often leads to a lack of consensus concerning the proper species name. Because of these issues, based on the library used, the same isolate may be identified with different names complicating reporting rules for clinical laboratories.

Even after combining all 3 fungal databases, we were still unable to identify more than 20% of isolates This number is higher compared to recent literature describing identification rates up to 98.8%.[17,18] One reason for this discrepancy is that we may have analysed more environmental isolates (contaminants) than other studies and that these local contaminants are less well represented in the libraries. As well, our study contained a comparably very high number of dermatophyte cultures (26.7%) which form a complex group of moulds that is historically difficult to speciate. It is also possible that the local isolates from our Canadian province (Manitoba) match the spectra of the libraries less perfectly. This geographical limitation can be overcome by increasing the spectral diversity in the libraries, as illustrated by the better performance of the MSI library, which contains significantly more isolates compared to the NIH library. Finally, our lower identification rates could partially reflect the fact that we included results with no peaks (12/221), whereas other studies either obtained peaks for all isolates or used only moulds that were known or shown to generate peaks. Excluding no peak results would have increased the species identification rates for the MSI library from 71.95% to 76.08% (159/209) and for all 3 libraries combined from 73.8% to 78% (163/209). The overall identification rate for all 3 libraries on the genus level would have increased from 77.8% to 82.3% (172/209).

Another factor that increased the identification rate for the Bruker and NIH libraries was the cut-off chosen for species identification. While Lau et al follow Bruker recommendations and suggest ≥2.0 as a cut-off, our study suggested that using ≥1.7 as a cut-off increases the identification rate for both libraries combined from 29% to 53.4% while not significantly increasing the number of misidentification. Indeed, recent publications also suggest this score for species level identification using the Bruker Biotyper software in conjunction with the Bruker mould library.[19,20]

We also observed that one of our clinical microbiology laboratories had higher identification rates than the other 2 suggesting that slight variations between mass spectrometers and/or experience of laboratory staff may influence the quality of spectra obtained and therefore the identification rate. Interestingly, when the same target plate with spotted mould extracts was analysed by 2 different laboratories there was significant variability in the spectra obtained, the quality of spectra, and thus the ability to identify the mould. This finding points to intra-instrument variability or inherent reproducibility problems when collecting spectra. Significant variability has previously been described for replicates of the same extract spotted on the same target plate, which led Gautier et al, to suggest spotting extracts in quadruplicate.[6]

Another consideration that needs to be addressed by each laboratory is how MALDI-TOF MS is most efficiently used in the workup of specimens. The required extraction protocol for moulds can be time-consuming and it may therefore not be practical to use MALDI-TOF for all specimen types and every isolate. Indeed, the most frequently encountered and rapidly sporulating moulds may be more quickly (not earlier) identified by experienced technologists, albeit with a higher overall error rate, based on growth and microscopy. This holds true for at least genus-level identification, which, depending on the specimen type and the clinical context, may be sufficient for many isolates for appropriate clinical management. The clinical relevance of species identification for most fungal genera is still unclear. However, emerging data highlight the importance of speciation for some clinically important moulds such as *Aspergillus*, *Fusarium*, *Scedosporium* and *Sporothrix*, because different susceptibility profiles have been observed at the species level that would merit different treatment choices. For example *Aspergillus lentulus* (section *Fumigati*, ie *Aspergillus fumigatus* complex), although infrequently isolated from patients, is more frequently resistant to commonly used antifungal agents than *A. fumigatus*. Finally, although MALDI-TOF MS can be used before sporulation is observed and therefore

may lead to earlier identification compared to conventional methods, it may not be as clinically valuable for slowly progressing fungal infections (eg dermatophytes) as it is for fungi isolated from sterile sties or for acute bacterial infections.

Another concern from a clinical laboratory perspective is the safety for laboratory staff during the workup of moulds. Clinical specimens may contain risk group 3 pathogens like *Cladophialophora bantiana* or the dimorphic fungus *Coccidiodes immitis*, and studies to examine complete inactivation of moulds after the extraction process are sparse. The few studies that have been published on this subject show complete inactivation of moulds but use less pathogenic moulds as representatives, rather than risk group 3 organisms[4] (see Poster Hall 2014 Bruker; Validation of the MALDI Biotyper for identification of filamentous fungi, available at www.bruker.com). Whether these inactivation data can be extrapolated to dimorphic fungi, remains a concern for laboratories since incomplete inactivation may unnecessarily expose laboratory staff. Furthermore, the few inactivation data that have been published have used different extraction procedures, making extrapolation of safety even more difficult.[4] Due to the paucity of comprehensive safety studies, each laboratory may be advised as has been suggested previously to carry out a careful risk assessment and testing of an inactivation extraction method, including risk group 3 moulds, before implementing MALDI-TOF MS identification.[21]

In conclusion, the overall identification rate of moulds in this study was 77.4%. We considered this rate of identification to be very promising and triggered our 3 clinical microbiology laboratories to implement MALDI-TOF MS for mould identification in the clinical workup of patient specimens. However, for the time being, conventional identification cannot be abandoned and will continue to be an alternative for those instances when MALDI-TOF MS fails to provide a definitive result. Furthermore, to minimise the chance of reporting errors MALDI-ToF identification results should always be evaluated critically and compared to growth characteristics and microscopy. As fungal libraries continue to expand their databases and as these resources are more widely accessed by diagnostic laboratories worldwide, we will see a transition to more rapid and cost-effective identification of moulds. As with bacterial identification, MALDI-TOF MS will undoubtedly also revolutionise the field of clinical mycology.

CONFLICTS OF INTEREST

The authors do not have any conflicts of interest to declare.

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