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Original Article

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Identification of filamentous fungi isolates by MALDI-TOF mass spectrometry: clinical evaluation of an extended reference spectra library

Pierre T. Becker[1,*], Annelies de Bel[2], Delphine Martiny[3,4], Stéphane Ranque[5,6], Renaud Piarroux[5,6], Carole Cassagne[5,6], Monique Detandt[1] and Marijke Hendrickx[1]

[1]BCCM/IHEM fungal collection, Mycology and Aerobiology Laboratory, Scientific Institute of Public Health, Brussels, Belgium, [2]Department of Microbiology and Infection Control, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel (VUB), Brussels, Belgium, [3]Department of Immunology and Microbiology, Université Libre de Bruxelles, Brussels, Belgium, [4]Department of Microbiology, Saint-Pierre University Hospital and Jules Bordet Institute, Brussels, Belgium, [5]APHM, CHU Timone-Adultes, Parasitology and Mycology, Marseille, France and [6]Aix Marseille Université, IP-TPT UMR MD3, Marseille, France

*To whom correspondence should be addressed. P. Becker, Scientific Institute of Public Health, Brussels, Belgium; Tel.: +3226425618; Fax: +3226425541; Email: pierre.becker@wiv-isp.be

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Abstract

The identification of filamentous fungi by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) relies mainly on a robust and extensive database of reference spectra. To this end, a large in-house library containing 760 strains and representing 472 species was built and evaluated on 390 clinical isolates by comparing MALDI-TOF MS with the classical identification method based on morphological observations. The use of MALDI-TOF MS resulted in the correct identification of 95.4% of the isolates at species level, without considering LogScore values. Taking into account the Brukers' cutoff value for reliability (LogScore >1.70), 85.6% of the isolates were correctly identified. For a number of isolates, microscopic identification was limited to the genus, resulting in only 61.5% of the isolates correctly identified at species level while the correctness reached 94.6% at genus level. Using this extended in-house database, MALDI-TOF MS thus appears superior to morphology in order to obtain a robust and accurate identification of filamentous fungi. A continuous extension of the library is however necessary to further improve its reliability.

Indeed, 15 isolates were still not represented while an additional three isolates were not recognized, probably because of a lack of intraspecific variability of the corresponding species in the database.

Key words: database, filamentous fungi, identification, MALDI-TOF MS.

Introduction

Infections caused by filamentous fungi are generally opportunistic and constitute a growing threat for the rising number of immunocompromised patients, especially those with neutropenia. The latter include people undergoing chemotherapy, corticosteroid treatment, or hematopoietic stem cells transplantation. Other at-risk patients are persons with diabetes, hematologic malignancies, iron overload, human immunodeficiency virus (HIV) infection, solid organ graft, or extensive burns [1,2]. A high mortality and morbidity is associated with fungal infections, and a fast and reliable identification of clinical isolates is therefore of great interest.

Currently, identifications performed in routine microbiological laboratories rely mainly on the morphological characters of the isolates and require specialized knowledge in medical mycology. Molecular assays based on DNA, such as polymerase chain reaction (PCR), real-time PCR, or hybridization techniques, have been developed, but they are unable to identify a large panel of species. This limitation does not apply to DNA sequencing, which is considered as the gold standard for fungal identification. However, this method is expensive, labor-intensive, and is subjected to a high risk of environmental contamination due to its multistep workflow.

During the last decade, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) arose as a powerful, reproducible, and accurate diagnosis tool to identify microbial agents, especially bacteria [3,4]. It analyzes the protein content of an unknown isolate under the form of a species-specific spectrum, typically between 2 and 20 kDa, which is then identified by comparison with the reference spectra of a database. This technology uses a simple, fast, and cost-effective protocol allowing the identification of any species, provided that it is properly represented in the library.

In recent years the use of MALDI-TOF MS has also emerged to identify isolates of fungi, particularly yeasts [5–10], but also a few genera of filamentous fungi, including *Aspergillus* [11–15], *Penicillium* [11,16,17], *Fusarium* [11,12,18], *Scedosporium* [11,19], *Trichoderma* [20], *Verticillium* [21], Mucorales [12], and dermatophytes [22–26], which are among the most frequently encountered fungal pathogens. The most recent libraries developed by Lau *et al.* [27] and Ranque *et al.* [28] contained, respectively, 152 and 63 species. Their clinical evaluation demonstrated correct identifications at species level reaching about 89%. However, several isolates were still mis- or not identified because of the absence of important reference spectra in the databases [27,28]. It also appeared that the identity of some reference strains was limited to the genus [27]. We hypothesized that a more extended and accurate library covering the vast majority of pathogenic filamentous fungi would therefore increase the robustness and reliability of the MALDI-TOF MS-based identification. In the present study, we constructed and evaluated a new reference database containing 760 strains, which would permit the identification of 472 species belonging to 147 genera. The clinical validation of the database, the largest reported to date, was performed to assess its added-value to traditional methods by comparing the MALDI-TOF MS results with the identifications obtained using the conventional procedure (i.e., based on morphology) commonly used by the clinical laboratories. Discrepancies between both approaches were resolved by DNA sequencing.

Materials and methods

Fungal strains and construction of the database

The in-house database of reference spectra, defined as MSP (Main SPectra) by Bruker, was built with strains of the BCCM/IHEM fungal collection. This library contains 760 strains representing 472 species and 147 genera (Table S1), all being independent from the clinical isolates used for the clinical validation. The identity of all reference strains was confirmed by DNA sequencing (multilocus if required), and the specificity of their MSP was controlled by challenging a subculture of each reference strain against the database. Dermatophyte species were excluded from the library as the sample preparation generally requires greater growth and thus not fit the standard protocol and should be considered in a separate survey with a relevant procedure.

For the clinical evaluation, isolates were collected between July 2012 and July 2013 from the routine activities of two university hospitals, namely, the CHU Saint-Pierre and the Universitair Ziekenhuis Brussel, both located in Brussels, Belgium. The morphological identifications were performed in the clinical laboratories of these hospitals by

skilled technicians, according to the macroscopic and microscopic features of the isolates, and following the keys of the Atlas of Clinical Fungi [29]. The MALDI-TOF MS analyses were conducted in the laboratory of the BCCM/IHEM collection, which is hosted by the Scientific Institute of Public Health.

MALDI-TOF MS analyses and interpretation

Reference spectra of the in-house database were obtained using the standardized methodology described previously that combined 4 biological and 10 technical replicates [30,31]. Spectra of the clinical isolates were performed in quadruplicates (technical repeats) using the same protocol.

The spectra were obtained after 240 shots in positive linear mode with a Microflex LT MALDI-TOF MS instrument (Bruker Daltonics), with an m/z range of 2–20 kDa and an accelerating voltage of 20 kV. A bacterial test standard (Bruker Daltonics) was used for calibration. The data were automatically acquired by the FLEXCONTROL version 2.4 software using the autoexecute default settings. In case of failure of the automatic analysis (53% of the cases), a manual analysis was performed, which slightly increased the analytical duration.

For the clinical validation, identification of the spectra, with related logscore values, was performed with the BIOTYPER version 3.0 software using either the in-house library or the commercially-available Bruker database dedicated to fungi. The following four criteria were applied to interpret the MALDI-TOF MS identification [30]:

1. If less than three out of the four spots tested from one isolate matched MSPs of the same species, the result of the MALDI-TOF MS identification was considered non-interpretable. In this case, the isolate was submitted to a new extraction and analysis. If this second identification was still not concordant, the isolate was considered unidentified by MALDI-TOF MS.
2. If at least three of the four spots tested matched the MSPs of the same species, the MALDI-TOF MS identification was considered interpretable. The highest logscore (of the 3 or 4 spots) was assigned to each interpretable identification.
3. If the MALDI-TOF MS identification gave the same result as the one obtained by conventional methods (i.e., morphology), the clinical isolate was considered correctly identified by MALDI-TOF MS, but in instances in which this criterion was not met, the isolate was further identified by DNA sequencing (see below).
4. If the genetic and MALDI-TOF MS analyses provided the same identification, the clinical isolate was considered correctly identified by MALDI-TOF MS; if not, it was considered misidentified by MALDI-TOF MS.

Logscore values were therefore not strictly used as interpretative criteria for the reliability of the identification but were provided to indicate that according to the manufacturer, scores <1.70 indicate unreliable identification, while scores of 1.70–1.99 and ≥ 2.0 indicate acceptable genus and species level identification, respectively.

DNA sequence analyses

Isolates with discrepant identification (MALDI-TOF vs. morphology) and those with identification limited to genus level using microscopy were subjected to sequencing. Genes were amplified by PCR using primer sets targeting the internal transcribed spacer, the beta-tubulin, the actin, the translation elongation factor 1 alpha, or the large subunit ribosomal DNA [32–35]. Sequences were analyzed with the DNASTAR LASERGENE 8 software, and their identification was performed by comparison with the MycoBank database (http://www.mycobank.org) and the GenBank database using the BLAST tool [36]. The best-matched organism and a minimal 99% sequence similarity were chosen as criterion for identification. Moreover, multilocus sequencing was performed if the discrimination was insufficient with a single gene.

Results

A total of 390 isolates of clinical filamentous fungi were collected in the two hospitals over the indicated one-year period. The majority of the samples were obtained from patients with cystic fibrosis (37%) or onychomycosis (23%); respiratory diseases including asthma, chronic obstructive pulmonary disease, pneumonia, and aspergillosis (13%); cancer (7%); otomycosis (4%); and cutaneous infections (3%). Nail and skin infections were caused by nondermatophyte species. Noteworthy, 14 isolates (3.6% of the total isolates) required more than the standard 72 h to reach an optimal growth, of which 6 belonged to the *Scedosporium* genus.

Results of the MALDI-TOF MS and morphological identifications are presented in Tables 1 and 2. The isolates belonged mainly to *Aspergillus* (66%), followed by *Penicillium* (9%), *Fusarium* (5%), *Scedosporium* (4%), and *Scopulariopsis* (3%). Noteworthy was the fact that isolates of *A. tubingensis* (n = 13) were identified as *A. niger* by microscopy, but the morphological identification was nevertheless considered as correct because the morphology of both species was similar. The designation of *Aspergillus* section *Nigri* was therefore used in order to avoid

Table 1. Identification by species of the 390 clinical isolates using either MALDI-TOF MS or microscopy.

	MALDI-TOF MS ID				ID based on morphology		
Species (number of isolates)	No ID or mis-ID at genus level	Correct at genus level	Correct at species level	Logscore range (median score)	No ID or mis-ID at genus level	Correct at genus level	Correct at species level
Acremonium strictum (6)	1 (ni[a])		5	1.37–2.04 (1.58)		6	
Alternaria alternata (3)			3	1.99–2.28 (2.20)		3	
Alternaria infectoria (3)	1 (ni)		2	1.72–1.74 (1.73)	1 (ni)	2	
Aspergillus calidoustus (2)			2	2.09–2.75 (2.42)		2	
Aspergillus caesiellus (1, NA[b])	1 (ni)					1	
Aspergillus flavus (16)			16	1.59–2.48 (2.28)	1 (ni)	2	13
Aspergillus fumigatus (177)			177	1.42–2.45 (2.03)		1	176
Aspergillus hollandicus (3)			3	1.83–2.17 (2.01)		3	
Aspergillus nidulans (14)			14	1.86–2.38 (2.11)		9	5
Aspergillus section *Nigri* (27)			27	1.58–2.34 (2.00)			27
Aspergillus sclerotiorum (1)			1	1.83		1	
Aspergillus terreus (4)			4	1.61–2.07 (1.97)		1	3
Aspergillus unguis (2)			2	1.93–2.37 (2.15)		2	
Aspergillus versicolor (9)			9	1.55–2.38 (1.83)	1	7	1
Cladosporium bruhnei (1, NA)		1		1.46		1	
Cladosporium cladosporioides (1, NA)		1		1.44		1	
Cladosporium halotolerans (2, NA)	1 (ni)	1		1.47		2	
Cladosporium pseudocladosporioides (3)			3	1.49–1.89 (1.81)		3	
Cladosporium ramotenellum (1)			1	1.80		1	
Cladosporium sphaerospermum (2)			2	1.62–1.79 (1.71)		2	
Colletotrichum lineola (1, NA)	1 (ni)				1		
Coniochaeta sp. (1, NA)	1 (ni)				1		
Eurotium repens (2)		1 (2.32)	1 (2.37)			2	
Exophiala dermatitidis (1)			1	1.43		1	
Fusarium dimerum (1)			1	1.89		1	
Fusarium oxysporum (13)			13	1.48–2.10 (1.79)		13	
Fusarium proliferatum (1)			1	1.82		1	
Fusarium solani (4)			4	1.42–1.96 (1.79)		4	
Fusarium verticilloides (1)			1	1.76		1	
Gallactomyces geotrichum (2)			2	1.72–2.00 (1.86)	1 (ni)	1	
Geosmithia pallida (1)			1	2.51	1		
Lecythophora sp. (2, NA)	2 (ni)				2		
Mucor circinelloides (3)			3	1.71–2.09 (1.83)	3		
Mucor hiemalis (1)	1 (ni)				1		
Oidiodendron sp. (1, NA)	1 (ni)				1		
Penicillium atramentosum (1)			1	1.95		1	
Penicillium brasilianum (1)			1	1.87		1	
Penicillium cecidicola (1, NA)	1 (ni)					1	
Penicillium chrysogenum (11)			11	1.67–2.34 (2.24)		11	
Penicillium citrinum (4)			4	1.85–2.21 (1.91)	1 (ni)	3	
Penicillium concentricum (1, NA)	1 (ni)					1	
Penicillium corylophilum (2)			2	2.07–2.28 (2.18)		2	
Penicillium crustosum (2)			2	2.13–2.22 (2.18)		2	
Penicillium diversum (1)			1	2.28		1	
Penicillium glabrum (2)			2	1.92–2.24 (2.08)		2	

Table 1. *Continued.*

Species (number of isolates)	MALDI-TOF MS ID				ID based on morphology		
	No ID or mis-ID at genus level	Correct at genus level	Correct at species level	Logscore range (median score)	No ID or mis-ID at genus level	Correct at genus level	Correct at species level
Penicillium oxalicum (1)			1	1.51		1	
Penicillium polonicum (6)			6	1.56–2.29 (1.98)		6	
Penicillium raistrickii (1)			1	1.40		1	
Penicillium rubrum (2)			2	1.53–2.07 (1.80)		2	
Penicillium rugulosum (1)			1	1.84		1	
Phaeosphaeria avenaria (1, NA)	1 (ni)				1 (ni)		
Pithomyces chartarum (3)			3	1.76–1.92 (1.81)	3		
Pleospora papaveracea (1, NA)	1			1.76	1		
Rhizopus microsporus (1)			1	2.56			1
Rhizopus oryzae (3)			3	1.58–1.77 (1.61)		3	
Scedosporium apiospermum complex (15)			15	1.22–2.31 (1.85)			15
Scedosporium prolificans (1)			1	2.17		1	
Scopulariopsis brevicaulis (12)			12	1.66–2.62 (2.29)		12	
Scopulariopsis candida (1)			1	1.80		1	
Trichoderma longibrachiatum (2)			2	2.03–2.24 (2.14)	1 (ni)	1	
TOTAL (390)	**14**	**4**	**372**		**21**	**129**	**240**

[a]ni: No interpretable identification (less than three of the four tested spots matched MSPs of a same species).
[b]NA: Not available in the MALDI-TOF MSP database.

misinterpretation. The same remark also applied to *S. aurantiacum* (n = 6) within the *S. apiospermum* complex.

Using the in-house database, we noted that 10 out of the 390 clinical isolates were not identified by MALDI-TOF MS as a corresponding MSP was not available in the database. Moreover, an *Acremonium strictum*, an *Alternaria infectoria,* and a *Mucor hiemalis* were not recognized although represented in the library. Of the remaining 377 interpretable identifications, 372 (95.4% of the total samples) were correctly identified at species level by MALDI-TOF MS with variable scores ranging from 1.22 to 2.62, of which 334 (85.6% of the total samples) had a score above the manufacturer cutoff for reliability (i.e., 1.70). Correct identification was limited to genus level for an additional four isolates including *Cladosporium bruhnei*, *C. cladosporioides,* and *C. halotolerans*, all absent from the database. They were considered as *C. pseudocladosporioides* or *C. macrocarpum* with a low score (<1.5). The fourth isolate was an *Eurotium repens* that, although represented in the library, was identified as an *E. amstelodami* with a score of 2.32. Finally, the last sample was a *Pleospora papaveracea*, absent from the database, misidentified as *A. chlamydospora* with a score of 1.76, both species being representatives of the family Pleosporaceae.

MALDI-TOF MS identification was also performed using the Bruker reference database but only within the in-house library. Therefore, a subset of 296 isolates belonging to 15 species were analyzed, of which 105 (35.5%) were not recognized. The remaining 191 isolates were correctly identified to the species level but with low scores. Indeed the latter were below 1.70 for 142 of them, while the 49 other isolates had scores ranging from 1.70 to 2.11. In comparison, correct identification was obtained for (100%) of these 296 isolates using the in-house database, with scores exceeding 1.70 for 273 (92.2%) of them.

Traditional identification was performed using microscopic features of which 247 of the 390 isolates were identified at species level and resulted in correct identification for 240 of them (61.5% of the total samples). However, of the remaining seven isolates, six were correctly identified at genus level, while a *Lecythophora* sp. was considered as a *Scedosporium apiospermum*. For an additional 135 isolates, the morphological identification was limited to the genus, of which 123 were correct. A total of 129 samples (33.1% of all clinical isolates) were thus correctly identified

Table 2. Details of the 22 isolates incorrectly identified by MALDI-TOF MS or by microscopy.

Correct identification (based on sequencing)	Identification provided by	
	MALDI-TOF MS (score[a])	Microscopy
Aspergillus nidulans		*A. fumigatus*
Aspergillus versicolor		*Penicillium* sp.
Cladosporium bruhnei (A[b])	*C. pseudocladosporioides* (1.46)	
Cladosporium cladosporioides (A)	*C. pseudocladosporioides* (1.44)	
Cladosporium halotolerans (A)	*C. macrocarpum* (1.47)	
Colletotrichum lineola		*Fusarium* sp.
Coniochaeta sp.		*Acremonium* sp.
Eurotium repens (P[c])	*E. amstelodami* (2.32)	*Aspergillus nidulans*
Fusarium verticilloides		*F. oxysporum*
Geosmithia pallida		*Acremonium* sp.
Lecythophora sp.		*Acremonium* sp.
Lecythophora sp.		*Scedosporium apiospermum*
Mucor circinelloides		*Rhizopus* sp.
Mucor circinelloides		*Rhizomucor* sp.
Oidiodendron sp.		*Aspergillus* sp.
Pithomyces chartarum (3 isolates)		*Alternaria* sp.
Pleospora papaveracea (A)	*Alternaria chlamydospora* (1.76)	*Alternaria* sp.
Rhizopus oryzae		*R. microsporus*
Scedosporium prolificans		*S. apiospermum*
Trichoderma longibrachiatum		*T. viride*

[a]Highest score out of the spots
[b]A: Absent from the MALDI-TOF MSP database
[c]P: Present in the MALDI-TOF MSP database

at the genus level. Moreover, no name could be given to six isolates, and a *Mucor circinelloides* and a *M. hiemalis* were noted as "Mucorales" without further identification.

Discussion

The MALDI-TOF MSP library developed in the present study offers the most extended, yet comprehensive, diversity to date for identification of moulds recovered from clinical samples. Its utilization for the identification of filamentous fungi was evaluated and compared to the classical method based on morphological observations. Correct identification at species level was obtained for 95.4% of the total samples using the in-house database, while only 61.5% of the isolates could be identified with the classical method, namely microscopy. This difference is mainly due to the fact that morphological identification was limited to genus level for more than one-third (34.6%) of the isolates. Correct genus-identification rate was indeed similar using microscopy or MALDI-TOF MS, reaching 94.6% and 96.4%, respectively. MALDI-TOF MS therefore provides reliable identifications but, most importantly, showed a higher accuracy than traditional approach. MALDI-TOF MSP libraries of filamentous fungi described by Lau *et al.* [27] or by Ranque *et al.* [28] included less species and provided lower performance with, respectively, 88.9% and 89% of the total isolates correctly identified at species level. Therefore, increasing the diversity of the database seems to improve the robustness and precision of the MALDI-TOF MS-based identification. However, this conclusion should be tempered by the fact that logscore cutoff values were not taken into account in the present study. An accurate identification to species level is not only important for treatment purpose (i.e., different antifungal susceptibilities among species belonging to the same genus) but also to answer epidemiological issues. The precision provided by MALDI-TOF MS was further illustrated by the possibility to identify isolates within certain species complexes provided an adequate representation of the species were in the reference database. *Aspergillus niger* and *A. tubingensis* in the *A. niger* section, together with *Scedosporium apiospermum* and *S. aurantiacum* in the *S. apiospermum* complex, displayed distinct spectra allowing their differentiation, which was not possible based on morphological features. Such a differentiation was important because *A. tubinge*nsis and *S. aurantiacum* are more resistant to various antifungal agents than other species within their own complexes [37–39].

The present database was built using a standardized methodology evaluated previously [30–31] and based on the use of solid media cultures. This protocol differs from the liquid culture procedure recommended by the manufacturer as the latter is generally considered cumbersome. Extraction from solid media makes MALDI-TOF MS identification more suitable for routine diagnostic laboratories but requires a library constructed using similar cultures as growth conditions greatly influence mass spectra [30,40,41]. Indeed, challenging spectra obtained from solid media culture against the Bruker database resulted in a correct species level identification rate of <65%, with much lower scores. In a previous work, Lau *et al.* made the same observation with <2% of their isolates correctly identified while using the liquid culture based library [27].

Using MALDI-TOF MS, 18 isolates were not or incorrectly identified, of which 4 were represented in the database. An *Eurotium repens* was first considered as an *E. Amstelodami*, but it should be noted that both species are members of the *Aspergillus* section *Aspergillus* [42], which probably explains why a misidentification with a high score was obtained. Additionally, no interpretable identification could be obtained for an *Acremonium strictum*, an *Alternaria infectoria* and a *Mucor hiemalis*, possibly due to the poor quality of the spectra, as revealed by criteria described by Stevenson *et al.* [10].

MALDI-TOF MS analyses provide identification scores that provided information on their reliability. According to the manufacturer, scores <1.70 indicate unreliable identification. In this work, 38 isolates (<10% of the total samples) were correctly identified at species level but with scores below 1.70. The confidence of these identifications was enhanced by the fact that each isolate was analyzed in quadruplicate. Using this procedure, it was possible to avoid most misidentifications. However, for diagnosis purpose, identifications with unreliable score should be completed through the use of other identification tools and by the clinical manifestations of the infections. It appeared that the quality of the spectra would be partially responsible for the scores observed with these 38 correctly identified isolates, as half of their spectra displayed a low quality [10]. In comparison, only 10.5% of the 38 isolates with the highest scores (≥2.27) showed poor grade spectra (Fig. S1). Pigmentation is a possible cause of inferior spectra, as it is known to decrease or suppress ion signal by inhibiting the desorption/ionization process during MALDI-TOF MS [43]). Several dematiaceous isolates analyzed in this study yielded scores lower than 1.70. Besides pigmentation, further interferences could result from scraps of agar, matrix clusters, unclean MALDI-TOF instrument generating contaminating cationic components, or quaternary ammonium compounds observed as strong background ions [44].

General performance of the spectral database developed in the present research suggests MALDI-TOF MS as a promising and powerful tool for rapid identification of filamentous fungi. Moreover, the library is continuously extended in order to include atypical strains but also species that are still not or insufficiently represented. This should further improve the reliability of the database. The latter being intended for end users such as clinicians, it will be publically available. For quality reasons (e.g., taxonomic changes, additional MSP), it is important that we keep the control of the library content. The database will thus not be delivered as such but available through on online system that is currently developed.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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