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Increased species-assignment of filamentous fungi using MALDI-TOF MS coupled with a simplified sample processing and an in-house library

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Abstract

In this study we evaluated the capacity of MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) to identify clinical mould isolates. We focused on two aspects of MALDI-TOF MS identification: the sample processing and the database. Direct smearing of the sample was compared with a simplified processing consisting of mechanical lysis of the moulds followed by a protein extraction step. Both methods were applied to all isolates and the Filamentous Fungi Library 1.0 (Bruker Daltonics) was used for their identification. This approach allowed the correct species-level identification of 25/34 *Fusarium* spp. and 10/10 *Mucor circinelloides* isolates using the simplified sample processing. In addition, 7/34 *Fusarium* spp. and 1/21 *Pseudallescheria/Scedosporium* spp. isolates were correctly identified at the genus level. The remaining isolates–60–could not be identified using the commercial database, mainly because of the low number of references for some species and the absence of others. Thus, an in-house library was built with 63 local isolates previously characterized using DNA sequence analysis. Its implementation allowed the accurate identification at the species level of 94 isolates (91.3%) and the remaining nine isolates (8.7%) were correctly identified at the genus level. No misidentifications at genus level were detected. In conclusion, with improvements of both the sample preparation and the feeding of the database, MALDI-TOF MS is a reliable, ready to use method to identify moulds of clinical origin in an accurate, rapid, and cost-effective manner.

Key words: filamentous Fungi, routine identification, MALDI-TOF MS, gene sequencing.

Introduction

Rapid and reliable identification of moulds to the species level together with an early onset of effective antifungal therapy have demonstrated to be key factors for a better outcome of patients with mould infections.[1] Recent taxonomical changes showed that phenotypic identification of moulds is cumbersome, challenging, and imprecise as emerging cryptic species causing disease in humans are commonly misidentified.[2–4] Thus, alternative reliable and rapid methods for mould identification are needed.[5]

Identification of moulds based on DNA sequence analysis is nowadays considered the reference method.[6] The internal transcribed spacer (ITS) region has been shown useful to achieve accurate identification of the most clinically relevant moulds at complex-level.[7] Additional sequencing of other targets—the beta-tubulin gen for the genus *Scedosporium* and *Aspergillus*[8] or the elongation factor 1 alpha (EF-1alpha) in the case of the *Fusarium* genus[9]—is mandatory for species level identification. Unfortunately, DNA sequencing analysis is time-consuming, requires highly experienced staff, and specific equipment that may not be available in many clinical microbiology laboratories.

In recent years, MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) has emerged as a very useful methodology to obtain rapid identification of bacteria, mycobacteria and yeasts.[10–12] Its implementation for the identification of filamentous fungi has been hampered by the availability of very few extensive databases where the most common moulds species causing human infections are well represented.[13] As a consequence, some laboratories have implemented their own in-house databases containing local moulds isolates.[14–17] This approach although cumbersome, since it requires accurate identification of moulds at species levels by molecular procedures, seems to be the best way so far to overcome the shortfall of ad-hoc commercial databases.

Studies reporting the use of different sample processing methods and the benefits of in-house libraries with MALDI-TOF are still scarce even for groups of moulds commonly isolated in clinical practice. In this study we applied MALDI-TOF MS technology for the identification of a collection of well characterized, *Fusarium* spp., *Scedosporium/Pseudallescheria* spp., and Mucorales clinical isolates using two different sample preparation methods and also comparing the commercial Filamentous Fungi Library 1.0 alone or in combination with an in-house database built with local isolates.

Methods

Isolates and species identification

A total of 166 stored mould isolates (one per patient) morphologically identified from patients admitted to Hospital Gregorio Marañón, Madrid (Spain) between 1980 and 2016 were retrieved from the hospital collection (70°C) and cultured on Potato Dextrose Agar (PDA) or Sabouraud Dextrose Agar (SDA) and incubated at 35°C for up to 6 days.[5] Isolates were identified by molecular identification after amplification and sequencing of the ITS region (Mucorales)[18], *β-tubulin* gene (*Scedosporium/Pseudallescheria* spp.),[19] and α-elongation factor (*Fusarium* spp.).[20] A BLAST search of all the sequences was performed to identify the isolates; reference sequences were included to construct a phylogenetic tree using MEGA 4 software to confirm molecular identification[21]. A total of eight genera and 20 species were found (Table 1). Sixty-three isolates belonging to 15 mould species and eight genera, previously characterized by DNA sequence analysis, were included in the HGM in-house library (Table 2). The remaining 103 isolates were analysed by MALDI-TOF MS using the commercial database alone and in combination with the in-house library.

MALDI-TOF MS analysis

MALDI-TOF MS identification was performed using a Microflex LT bench-top mass spectrometer (Bruker Daltonics, Bremen, Germany). FlexControl 3.3 and MALDI Biotyper 3.1 (Bruker Daltonics, Germany) were applied for spectra acquisition and comparison with reference spectra from the database, respectively. The commercial BDAL database—updated with 6903 Main Spectra (MSP) entries—plus the Filamentous Fungi Library 1.0 (FFL1.0 Bruker Daltonics) was used. In addition, an in-house database was created and evaluated in parallel with the commercial database (Table 2).

Default settings (acquisition of mass spectra in the linear positive mode within the 2–20 kDa range, ion source 1 (IS1) 20 kV, IS2 18.05 kV, lens 6.0 kV, linear detector 2,560 V) were applied.

Two methods were applied for sample preparation: (i) a direct protocol that consisted of mixing a small portion of the colony surface, scraped off with a metallic, sterile loop with 1.5 μl of 70% formic acid as described by Sitterlé et al.[22] The spots were overlaid with 1 μl of matrix (α-cyano-4-hydroxy-cinnamic acid [HCCA] solution in 50% acetonitrile and 2.5% trifluoroacetic acid), prepared

Table 1. List of isolates included in the study, their identification provided by MALDI-TOF MS using the commercial database and the Filamentous Fungi Library 1.0 and with the in-house HGM database. The score values are also stated.

		Identification by MALDI-TOF MS with BDAL+ FFL1.0							Identification by MALDI-TOF MS with BDAL+ HGM in-house library				
Identification by DNA sequencing analysis	Number of isolates	Species Level	Genus Level	Not Reliable ID	Score ≥1.8	Score 1.79-1.6	Score <1.6	Discordant or no ID	Species Level	Genus Level	Score ≥1.8	Score 1.79-1.6	Score <1.6
Fusarium													
Fusarium dimerum	1	–	–	1	–	–	–	1	–	1	1	–	–
Fusarium falciforme	1	–	1	–	–	1	–	–	–	1	1	–	–
Fusarium fujikuroi	1	–	1	–	1	–	–	–	–	1	1	–	–
Fusarium oxysporum	8	6	1	1	3	4	1	–	8	–	8	–	–
Fusarium petroliphilum	2	–	2	–	2	–	–	–	2	–	2	–	–
Fusarium proliferatum	19	18	1	–	17	2	–	–	19	–	19	–	–
Fusarium solani	1	1	–	–	–	1	–	–	1	–	1	–	–
Fusarium verticillioides	1	–	1	–	–	–	1	–	1	–	–	1	–
Total	**34**	**25**	**7**	**2**	**23**	**8**	**2**	**1**	**31**	**3**	**33**	**1**	**0**
Scedosporium/Pseudallescheria													
Pseudallescheria boydii	7	–	–	7	–	–	–	7	7	–	6	1	–
Pseudallescheria minutispora	1	–	–	1	–	–	–	1	–	1	–	–	1
Scedosporium apiospermum	9	–	1	8	–	–	1	8	6	3	6	3	–
Scedosporium aurantiacum	1	–	–	1	–	–	–	1	–	1	–	1	–
Scedosporium prolificans	3	–	–	3	–	–	–	3	3	–	3	–	–
Total	**21**	**0**	**1**	**20**	**0**	**0**	**1**	**20**	**16**	**5**	**15**	**5**	**1**
Mucorales													
Cunninghamella bertholletiae	4	–	–	4	–	–	–	4	4	–	3	1	–
Lichtheimia corymbifera	5	–	–	5	–	–	–	5	5	–	4	1	–
Lichtheimia ramosa	12	–	–	12	–	–	–	12	11	1	12	–	–
Mucor circinelloides	10	10	–	–	–	7	3	–	10	–	7	3	–
Rhizomucor pusillus	5	–	–	5	–	–	–	7	5	–	5	–	–
Rhizopus arrhizus	9	–	–	9	–	–	–	13	9	–	9	–	–
Rhizopus microsporus	3	–	–	3	–	–	–	5	3	–	3	–	–
Total	**48**	**10**	**0**	**38**	**0**	**7**	**3**	**46**	**47**	**1**	**43**	**5**	**0**

BDAL, Bruker Daltonics Database; FFL1.0, Filamentous Fungi Library 1.0; HGM, Hospital Gregorio Marañón.

according to the manufacturer's instructions. When the spots were dried, spectra were acquired using default settings and compared with the database; (ii) a protocol that included a mechanical lysis step in order to break the fungal cell wall and make the ribosomal proteins easier to detect by MALDI-TOF MS, followed by a standard protein extraction procedure. In this case we applied the protocol developed by Lau et al.[23] Briefly, a 5-mm diameter piece of mould was mixed with 250 μl of 100% ethanol and 0.5 mm diameter zirconia-silica beads and vortexed for 15 min. Then, the tubes were centrifuged, and the pellets were subjected to a standard protein extraction protocol using 50 μl of 70% formic acid and acetonitrile. For both methods, isolates were handled in a biosafety cabinet until the matrix was dry. Samples were analysed in duplicates, and the higher score was recorded and a bacterial test standard (BTS) provided by the manufacturer was included in every run for calibration purposes.

In this study, identifications obtained with MALDI-TOF MS were compared at the species and genus level with those provided by DNA sequencing analysis regardless of their score value (Table 1). In addition, score values ≥1.8 were considered sufficient for species level and ≥1.6 for genus level identification.[24] MALDI-TOF MS identifications with score values below 1.6 were only considered when the four top identifications were identical, otherwise they were considered as "not reliable."

Database construction

Sixty-three isolates from 15 species belonging to the genera *Fusarium*, *Scedosporium/Pseudallescheria* and the order Mucorales were processed according to the manufacturer's instructions and included to the in-house database (HGM library) as individual MSP (Table 2). The isolates were grown on SDA plates for 1–2 days in the case of *Scedosporium/Pseudallescheria* spp. and Mucorales isolates

Table 2. List of isolates included in the HGM in-house database.

Identification by DNA sequencing analysis	Number of isolates
Fusarium	
Fusarium oxysporum	6
Fusarium petroliphilum	4
Fusarium proliferatum	5
Fusarium solani	2
Fusarium verticillioides	2
Scedosporium/Pseudallescheria	
Pseudallescheria boydii	6
Scedosporium apiospermum	9
Scedosporium prolificans	9
Mucorales	
Cunninghamella bertholletiae	2
Lichtheimia corymbifera	2
Lichtheimia ramosa	6
Mucor circinelloides	2
Rhizomucor pusillus	2
Rhizopus arrhizus	4
Rhizopus microsporus	2
Total	63

and 3–4 days for *Fusarium* spp. All isolates included in the HGM library had been previously identified by DNA sequence analysis. Freshly prepared BTS was spotted onto one position on the target plate in order to calibrate the instrument before spectra acquisition. Isolates were submitted to mechanical lysis plus a protein extraction step and subsequently spotted onto eight positions in the target plate. Each position was read three times. At least 20 identical spectra from each isolate were required by the software for a MSP to be added to the in-house library. Due to this requirement, none of the isolates could be included in the library when spotted directly.

The number of isolates added depended on their availability in the stored isolates collection. To corroborate the accuracy of the MSPs added, the library was challenged with the remaining 103 isolates.

Statistical analysis

Comparison for both databases was performed using the McNemar test for paired samples, with two tails. Data were analyzed using SPSS software package 18.0 (IBM, Chicago, IL, USA).

Ethics statement

The hospital Ethics Committee approved this study and gave consent for its performance (Code: MICRO.HGUGM.2017-003). Since only microbiological samples were analyzed, not human products, all the conditions to waive the informed consent have been met.

Results

Identification based on the classical simple processing

Direct application of the hyphae on the MALDI target plate allowed the identification of only 2/34 *Fusarium* spp. isolates and 3/48 *Mucorales* spp. isolates at the genus level. Besides, the quality of the protein profiles obtained with this method was so poor that they were not accepted by the software to be used as reference spectra. Thus, the protocol consisting of a mechanical lysis step followed by a standard protein extraction procedure[23] was applied throughout this study.

Identification of *Fusarium* spp. isolates

Thirty-four isolates belonging to eight *Fusarium* species were analyzed. The implementation of the protocol developed by Lau et al.[23] allowed the correct identification of 25 isolates (73.5%) by MALDI-TOF MS at the species level using the Filamentous Fungi Library 1.0. Seven isolates (20.6%) were identified by MALDI-TOF at the genus level (Table 1). *F. falciforme* ($n = 1$) and *F. fujikuroi* ($n = 1$), both absent in the library and misidentified as *F. solani* (log score 1.65) and *F. proliferatum* (log score 1.86), respectively; *F. oxysporum* ($n = 1$) identified as *F. proliferatum* (log score 1.89); all *F. petroliphilum isolates* ($n = 2$) identified as *F. solani; F. proliferatum* ($n = 1$) identified as *F. oxysporum* (log scores 2.05) and *F. verticillioides* ($n = 1$) identified as *F. proliferatum* (log score 1.50). In addition, one *F. dimerum* isolate and one *F. oxysporum* isolates could not be reliably identified by MALDI-TOF MS. The use of our in-house database containing 19 isolates from five *Fusarium spp*. species allowed the species level identification of 31/34 isolates (91.2%); the three remaining isolates, two of them—*F. falciforme* and *F. fujikuroi*—not represented in any database, were identified at the genus level (Table 1). All isolates were identified, at least as *Fusarium* spp. and the score values for all but one isolate was above 1.8 using this improved database.

Identification of *Scedosporium/Pseudallescheria* spp. isolates

The commercial databases (BDAL+FFL1.0), which contain one *S. aurantiacum*, two *S. prolificans* and four *S. apiospermum* MSPs, identified *S. apiospermum* ($n = 1$) as *S. aurantiacum* (score value 1.22). The remaining 20/21 isolates were unreliably identified with score values between

1.2 and 1.3. However, when 24 well-characterized isolates from three species (*P. boydii, S. apiospermum,* and *S. prolificans*) were added to the in-house database, 16 out of 21 isolates (76.2%) were accurately identified at species level with scores values ≥1.8 (Table 1). Three *S. apiospermum* isolates were wrongly identified as *P. boydii* (n = 1) and *S. prolificans* (n = 2). Moreover, *P. minutispora* and *S. aurantiacum*—species not included in the in-house database—were misidentified as *S. prolificans* and *P. boydii*, respectively.

Identification of *Mucorales* isolates

The use of the commercial libraries allowed the correct species assignment of the 10 isolates of *Mucor circinelloides* with score values ranging between 1.2 and 1.7. The addition of 20 isolates from seven species to the in-house database increased the correct species level identifications to 47/48 isolates (97.9%). Only one *Lichteimia ramosa* isolate was misidentified as *L. corymbifera* (score value of 1.92) by MALDI-TOF MS with the improved database.

Overall, the commercial databases allowed the correct species assignment of 25/34 *Fusarium* spp. isolates (73.5%), 0/21 *Scedosporium/Pseudallescheria* spp. isolates (0%), and 10/48 Mucorales isolates (20.8%) versus 31/34 (91.2%) *Fusarium* spp., 16/21 *Scedosporium/Pseudallescheria* spp. (76.2%), and 47/48 Mucorales (97.9%) when the in-house database was implemented. These differences were statistically significant ($p < 0.001$). The sample preparation method consisting of mechanical lysis of the isolates plus a subsequent protein extraction step yielded high-quality protein spectra from moulds grown on PDA and SDA (data not shown) and was the method of choice in this study over the direct smearing method. Its turnaround time is 30 minutes.

The reliability of MALDI-TOF MS identification at the species level is shown in the protein dendrogram (Fig. 1) where different isolates from a single mould species cluster together, clearly separated from other close-related species. Only the two *Fusarium verticillioides* reference spectra clustered with *F. proliferatum* spectra due to their close-relatedness.

Discussion

Filamentous fungi belonging to the genus *Fusarium* and *Scedosporium/Pseudallescheria* and to the order *Mucorales* are considered as clinically significant emerging pathogens.[5,25] Their correct and rapid identification to the species level is highly important due to their frequent lack of susceptibility to the most commonly used antifungal drugs.

In our study, the comparison of the direct smearing of the sample vs the implantation of a mechanical lysis step followed by protein extraction[23] demonstrated that the latter method yielded higher quality profiles with an average of 80 peak (data not shown). Thus, this was the method of choice, and it was applied to all isolates throughout the study.

Previous studies have evaluated the capacity of MALDI-TOF MS for the identification of *Fusarium* species using in-house databases, reporting 80–100% successful identification.[15,26–28] MALDI-TOF MS only failed to obtain correct species level identification for isolates belonging to species absent from the databases. In this study, using solely the BDAL database plus the Filamentous Fungi Library 1.0, which contains 36 MSPs from different *Fusarium* species, 25 (73.5%) correct species-level identification was obtained vs. 31 (91.2%) with the in-house database. The three isolates identified at the genus level with the in-house library belonged to two species not represented in any of the databases evaluated—*F. falciforme* and *F. fujikuroi*—and to a species, *F. dimerum,* represented in the commercial database by three MSPs. In addition, no isolate was unreliably identified or remained unidentified using the in-house database, in contrast with the Filamentous Fungi Library 1.0 and it allowed the increase of the score values with values above 1.8 in all but one case.

The identification of the *Scedosporium/Pseudallescheria* genus using MALDI-TOF MS and an in-house database has shown to range between 94.0 and 100%.[17,22,28–30] In this study, the inclusion of 24 references from three species to our in-house database allowed the identification of 16 isolates at the species level (76.2%) (Table 1), with score values ranging between 1.65 and 1.9. However, five isolates remained identified only at genus level: two isolates from two different species not represented in the in-house database (*P. minutispora* and *S. aurantiacum*) and three *S. apiospermum* isolates misidentified by MALDI-TOF MS as *P. boydii* and *S. prolificans*. This fact underscores the need to increment the number of reference strains in the database in order to provide a good resolution among closely related species.[23]

Regarding the *Mucorales* isolates analyzed, their identification by MALDI-TOF MS using our in-house library provided 97.9% species-level identification while the remaining 2.1% of the isolates was reliable identified at the genus level. The addition to the in-house library of more *Lichteimia corymbifera* isolates would prevent misidentifications of this species as *L. ramosa* in the future. The score values obtained were above 1.8 in all cases, except for five isolates identified with score values over 1.7. Similar results were obtained by other authors,[15,31–32] pointing to the accuracy of MALDI-TOF MS for correct species assignment of different species within this order. This has proved to be a key factor for rapid onset of directed therapy for

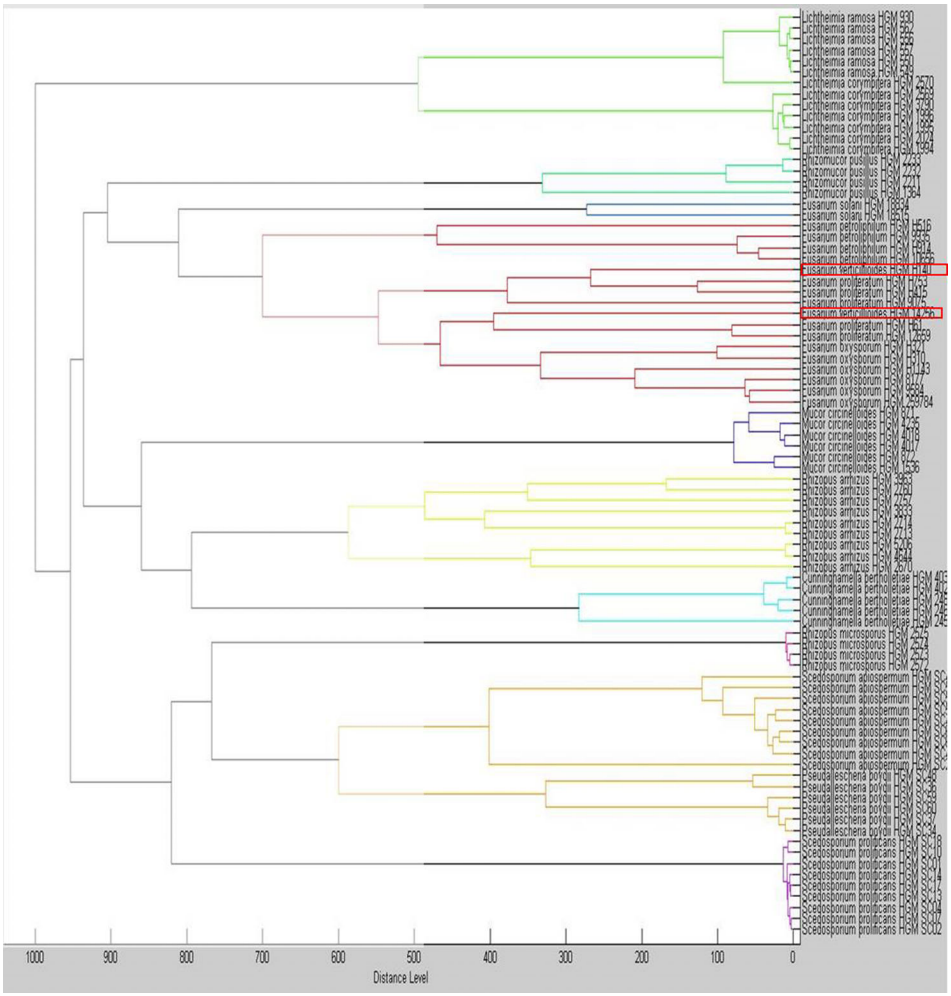

Figure 1. Protein dendrogram of the isolates included in the study. Every single species groups in different clusters, distinctively separated from each other, except for the *F. verticilloides* isolates that lie within the *F. proliferatum* cluster due to their close-relatedness. This Figure is reproduced in color in the online version of *Medical Mycology*.

mucormycosis.[31,33] In the case of *Fusarium* and *Scedosporium*, as there is no difference in terms of antifungal susceptibility among the species misidentified, the identification reached by MALDI-TOF was suitable for the clinical microbiology laboratory. However, further addition of well-characterized strains belonging to these genera, especially from those still lacking or underrepresented, will allow a higher rate of correct species assignment in the future.

A high correlation between the identification provided by DNA sequencing analysis and MALDI-TOF MS has been shown for all species of moulds analyzed regardless the score values. However, when the cut-off for reliable species identification was established as ≥1.8 in our study, the identifications provided by MALDI-TOF MS was identical to those obtained by DNA sequencing analysis in 96.7% of the cases, showing a high robustness. Besides, correlation at the species-level has also been found with score values below 1.6 as shown by other authors.[24]

This study highlights, in the first place, the importance of implementing an efficient sample processing to improve the capacity of MALDI-TOF MS to detect proteins from moulds and provide high-quality protein spectra. Only spectra with a good number of peaks will allow a reliable identification of moulds. Second, the achievement of high-quality protein profiles is not enough if the available databases do not contain those profiles and therefore are not able to provide identification for them. There is an urgent need of more complete databases containing the most important moulds of clinical importance. As shown in this study, the commercial database only provided species level identification for 35/103 isolates (34.0%), which correlated with the species well represented in the Filamentous Fungi 1.0 library—*Fusarium* spp and *Mucor circinelloides*—and with the fact that the commercial database was built with mould isolates grown on liquid medium.

In our opinion, the implementation of an effective and standardized methodology for sample preparation will

allow the exchange of both protein profiles from unknown moulds and databases between research centers. This is a practical approach to overcome the low number of available commercial databases and implement MALDI-TOF MS as a rapid and accurate tool for the identification of moulds in routine laboratory practice.

Our recommendation for routine identification of moulds using MALDI-TOF MS is to optimize the growth conditions (culture in PDA or SDA for 2–5 days depending on the mould species) and sample preparation procedure for MALDI-TOF MS identification. Score values ≥1.8 and ≥1.6 could be reliably used for species- and genus-level identification, respectively. If the profile obtained is a high-quality one with over 50–60 peaks and no identifications are obtained by MALDI-TOF MS, most probably it belongs to a mould species not included in the commercial database. Those isolates are suitable to be identified by molecular methods and subsequently added to the database. Since building an in-house library is a cumbersome process, our database will be soon available for validation in other laboratories.

In conclusion, MALDI-TOF MS has proved to be a rapid and reliable technology for mould identification provided that an efficient sample preparation method is implemented and an improved library containing clinically important mould species is available. These two factors have shown paramount importance for a rapid and reliable identification of these microorganisms. When both of them are applied, the identification of an unknown mold isolate can be performed in approximately 30 minutes and, according to the results from this study, it may provide correct species assignment in 91.3%, avoiding further delays and extra costs derived from confirmatory tests.

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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.

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