Medical Mycology 2013, **Early Online:** 1–8

informa
healthcare

A MALDI-TOF MS procedure for clinical dermatophyte species identification in the routine laboratory

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The conventional identification of dermatophytes requires a long turnaround time and highly skilled mycologists. We have recently developed a standardized matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) assay to routinely identify molds of potential clinical significance. This study objective was to determine if this same assay could also be employed to identify clinical dermatophytes in the routine laboratory setting. The effects of the inclusion of cycloheximide in the culture medium and incubation time were tested after building a reference spectra library that included 48 well-characterized isolates of 17 dermatophyte species. Then these same isolates were prospectively identified using this library. MALDI-TOF MS-based identification was effective regardless of the presence of cycloheximide or incubation time as 130/133 (97.8%) of the clinical isolates were appropriately identified. Two *Microsporum canis* isolates yielded uninformative spectra and one *M. audouinii* isolate was misidentified. Since one only requires a small colony for MALDI-TOF MS analysis, accurate identifications were obtained in 3–6 days and, specifically, before the appearance of their characteristic morphological features. Consequently, identification turnaround time was dramatically reduced as compared to that needed for conventional morphological identification. In conclusion, this standardized MALDI-TOF MS-based identification procedure for filamentous fungi effectively identifies clinical dermatophyte isolates and drastically reduces the response times in the routine clinical laboratory.

Keywords clinical dermatophytes, MALDI-TOF mass spectrometry, standardized procedure, routine, identification

Introduction

Dermatophytoses in humans are chiefly superficial fungal infections restricted to skin, nails and hair. Dermatophytes are distributed worldwide and species vary according to geographic area [1]. According to Emmons [2] and despite recent significant changes in the taxonomy, around 30 clinically important dermatophyte species can be grouped into three anamorphic genera, i.e., *Trichophyton*, *Microsporum* and *Epidermophyton* [3]. However, the

Received 17 July 2012; Received in final form 18 January 2013; Accepted 26 February 2013

This study was presented in part at the 5th Trends in Medical Mycology (TIMM), 2–5 October 2011 in Valencia, Spain, and at the 18th Congress of the International Society for Human and Animal Mycology (ISHAM), 11–15 June 2012 in Berlin, Germany.

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DOI: 10.3109/13693786.2013.781691

taxonomy, identification and nomenclature of the pathogenic species have been amended by the recent phylogenetic species concept, based on DNA sequence analysis [4].

Classically, the major dermatophytes are identified by skilled mycologists on the basis of macroscopic and microscopic of colonies growing in culture. Sometimes, complementary identification techniques, e.g., subculturing on specific culture media (lactrimel, potato-dextrose, malt extract, etc.) or the ability to degrade keratin, are needed to successfully identify the isolate. Since dermatophytes grow slowly, reliable identification may require several weeks. Moreover, conventional identification techniques cannot be employed with some isolates which may not produce characteristic morphological features in culture. Most of the clinical dermatophytes can otherwise be identified to the species level by using either DNA sequencing [5,6] or specific PCR assays [5,7]. However, DNA sequence-based identification, the recommended gold standard, is relatively costly and time-consuming in the routine clinical laboratory setting. Thus matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for rapid, simple, reproducible and reliable identifications has raised considerable interest in the clinical microbiology community. This assay generates spectra, comparable to protein fingerprint signatures of microorganisms which can then be identified within minutes by comparing their spectra with those in a reference spectra database. For the past few years, MALDI-TOF MS has enhanced routine bacterial identification in the clinical microbiology laboratory [8–14]. More recently, MALDI-TOF MS has been applied to the routine identification of yeasts [15] and molds [1,10]. We have recently developed a standardized MS-based assay suited to identifying a comprehensive array of molds species in the routine clinical laboratory setting [16] but without addressing dermatophyte identification. The present study thus aimed to evaluate this standardized MALDI-TOF MS-based identification assay for the identification of dermatophytes in the routine clinical laboratory setting.

Materials and methods

Dermatophytes reference spectra library

A reference spectra (MSP) library was built that included 48 isolates of 17 dermatophyte species, as detailed in Table 1. All were identified using both conventional and molecular procedures, as described below. This library included *Trichophyton mentagrophytes* complex members with *T. mentagrophytes*, *T. interdigitale*, *T. erinacei* and *T. simii* and *Arthroderma otae*-related species including *Microsporum canis*, *M. audouinii* and *M. langeronii*. Two non-pathogenic dermatophytes, *T. terrestre* and *T. ajelloi*, were also included in the investigation.

Table 1 Characteristics of the dermatophyte strains included in the reference spectra library.

Species	*N*	Strain no.	GenBank ref. with ≥99% identity
Epidermophyton flocossum	2	50600264	
		59700858	
Microsporum audouinii	8	xTA	AJ252334.1
		1043189	AJ252334.1
		013M	AJ252334.1
		001M	AJ252334.1
		027M	FJ479802.1
		171M	AJ252334.1
		50500597	
		50600031	
M. audouinii var. *langeronii*	2	50002007	
		59700523	
Microsporum canis	3	1100085	
		50500921	
		249D	AY213709.1
Microsporum cookei	2	IHEM 14180	
		94.548	
Microsporum gypseum	4	250D	EU151494.1
		50500961	
		93.629	
		94.446	
Microsporum nanum	1	IHEM 21111	
Microsporum persicolor	2	92.136	
		59900598	
Onychocola canadensis	1	161D	AY123784.2
Trichophyton mentagrophytes	5	1036900	AB566295.1
		1039316	HQ014710.1
		1036279	AB566295.1
		1042599	AB566299.1
		IHEM 4270	
T. mentagrophytes var. *ajelloi*	1	IHEM 17776	
T. mentagrophytes var. *erinacei*	2	IHEM 15931	
		IHEM 20118	
T. mentagrophytes var. *interdigitale*	1	IHEM 620	
Trichophyton rubrum	3	1043024	FM178326.1
		235D	AJ270806.1
		1041710	FM178326.1
Trichophyton schoenleinii	1	1100084	
Trichophyton simii	1	IHEM 15735	
Trichophyton soudanense	5	243D	FJ409221.1
		1100082	
		110 31 66 05	
		110 32 11 05	
		115 33 73 57	
Trichophyton terrestre	1	LMA 92 347	
Trichophyton tonsurans	2	90D	AB220045.1
		1100081	
Trichophyton verrucosum	1	IHEM 5480	
Trichophyton violaceum	1	IHEM 13459	

Clinical dermatophyte isolates

From November 2009 to December 2011, a total of 133 isolates were recovered from nail, hair or skin samples of individual patients in the medical mycology laboratories of university hospitals in three French cities (Angers, Marseille and Nancy; Table 2). They were identified to the

Table 2 Identification of 134 clinical dermatophyte isolates. (NB. *Onychocola canadensis* is a non-dermatophyte fungus implicated in nail infections).

Species	Number of clinical isolates tested	Number of strains in the library	Number of corrrect MALDI-TOF MS identifications
Epidermophyton flocossum	1	2	1
Microsporum audouinii	32	8	31 (97%)
M. audouinii var. *langeronii*	2	2	2
Microsporum canis	6	3	4 (67%)
Microsporum cookei	0	2	-
Microsporum gypseum	3	4	3
Microsporum nanum	0	1	-
Microsporum persicolor	3	2	3
Onychocola canadensis	1	1	1
Trichophyton mentagrophytes	21	4	21
T. mentagrophytes var. *erinacei*	0	2	-
T. mentagrophytes var. *porcellae*	0	1	-
Trichophyton rubrum	52	3	52
Trichophyton schoenlenii	0	1	
Trichophyton soudanense	12	5	12
Trichophyton terrestre	0	1	-
Trichophyton tonsurans	2	2	2
Trichophyton verrucosum	0	1	-
Total	134	45	129

species level using both conventional methods and MALDI-TOF MS.

Growth conditions

The fungi were cultivated at 30°C for three or 15 days on both Sabouraud gentamicin-chloramphenicol (SGC; Bio-Rad, France) and Sabouraud gentamicin-chloramphenicol with cycloheximide (SGCc; Bio-Rad) agar plates. This allowed testing the effects of culture conditions, e.g., culture medium, incubation time on MALDI-TOF identification results.

Conventional morphological identification

All fungi were evaluated both macroscopically, in terms of colony growth and pigment production and microscopically to detect the presence and morphology of macro- and/or microconidia and any other key characteristics following the keys of the *Atlas of Clinical Fungi* [17].

DNA sequence-based identification

Fungal DNA was isolated with NucliSENS EasyMAG (bioMérieux, France) according to the manufacturer's recommendations which yielded approximately 1 µg of high molecular weight DNA. Internal transcribed spacer (ITS) 2 region of the rDNA were amplified using ITS3 (gCATC gATgAAgAACgCAgC) and ITS4 (TCCTCCgCTTATT gATATgCTTAAgT) primers [18–20] in PCR reactions of 50 µl containing 10 ng template DNA, 20.0 pmol of each primer, 0.2 mM dNTPs, 2.5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1 U Taq polymerase (Applied Biosystems, France). Fragments were amplified in a T1 thermocycler (Biometra, Göttingen, Germany) with the following reaction profile: 10 min 94°C, then 40 cycles of: 30 s, 94°C; 30 s, 55°C; and 60 s, 72°C. The resulting amplicons were purified with UltraClean GelSpin DNA purification kit® (MO BIO laboratories, Carlsbad, CA, USA) and sequenced with Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, France) in a 3130 Genetic analyser (Applied Biosystems, France). Homology searching with the ITS region sequences was performed against sequences registered in GenBank/NCBI using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Protein extraction and target plate preparation

Dermatophyte proteins were extracted using the procedure previously described for molds [16]. Briefly, a piece of mycelium was gently scraped from the culture plate with a scalpel and suspended in 900 µl absolute ethanol (ethyl alcohol anhydrous, Carlo Erba SDS, Val de Reuil, France) and 300 µl HPLC water (Water HPLC, Prolabo BDH, Fontenay-sous-Bois, France). The sample was centrifuged at 13,000 *g* for 10 min, with the resulting pellet resuspended in 12.5 µl of 70% formic acid (Sigma-Aldrich, Lyon, France) and the solution then incubated for 5 min at room temperature. Subsequently, 12.5 µl of 100% acetonitrile (Prolabo BDH, Fontenay-sous-Bois, France) was added during 10 min at room temperature and the sample then centrifuged at 13,000 *g* for 10 min. One µl of supernatant

was spotted onto a MTP 384 target plate polished steel TF (Bruker Daltonics GmbH, Bremen, Germany) and allowed to air dry. Then, the spot was covered with 1 µl matrix solution [alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Lyon, France) saturated in 50 acetonitrile: 25 HPLC water: 25 10% TFA and was allowed to air dry. A bacterial test standard (Bruker Daltonics) was used for instrument calibration.

Mass spectra data acquisition

The MALDI-TOF MS assays were performed on an UltraFlex (Bruker Daltonics) mass spectrometer, according to the manufacturer's instructions, and analyzed using the MaldiBioTyper v2.1 software (Bruker Daltonics). The spectra were acquired after 650 shots in linear mode in the ion-positive mode with a 337 nm nitrogen laser, using the following adjustments: delay time: 170 ns; acceleration voltage: 20 kV; ion source 2 voltage: 18.5 kV; mass spectral range: 2–20 kDa, acquisition time: 30–60 s per laser pulse. The TOF measurements were converted to m/z values and all raw spectra were automatically processed by the Flexcontrol v2.4 (Bruker Daltonics) AutoXecute software. The resulting peak lists were exported to the MaldiBioTyper v2.1 software (Bruker Daltonics) but only peaks with a signal/noise ratio ≥ 10 were considered.

Reference spectra (MSP) library creation

Reference spectra were created from fungal isolate cultured at 30°C for three days using the procedure described above. The library included four MSPs (each of them derived from 10 raw spectra of four culture replicate using the 'MSP creation' function of the MaldiBiotyper software) of each of 48 strains (Table 1) grown under two conditions, i.e., SGC/3-days and SGCc/3-days. To assess the validity of this library, the 48 test isolates were sub-cultured again, with four spots prepared for each isolate and submitted for MS identification using this 48 reference spectra library. Results of the matching process were expressed as LogScore (LS) values, computed by the MaldiBiotyper software, ranging from 0 (no spectrum match) to 3.0 (perfect match). Two LS values, indicative of identification strength, were recorded for each spot which included the best-match LS value for the 1st identified species and the best-match LS value for the 2nd identified species. A cut-off value for reliable species identification was defined taking into account both LS values.

MALDI-TOF MS-based identification of clinical isolates

In parallel to the conventional identification, clinical isolates grown on SGC or SGCc agar were analyzed in quadruplicate (four spots each) by MS. An LS value was obtained by comparing the unknown fungus spectra with the reference spectra in the MS library using the 'start identification' function of the MaldiBiotyper software. MS identification was considered concordant (or informative) when at least three out of the four spots matched with the reference spectra of the same species and at least one of these spots yielded a best-matched LS value above the defined cut-off. MALDI-TOF MS-based identification results were considered adequate when they agreed with those of morphological identification. When the initial LS value was below the cut-off, the isolate was re-extracted 2–4 days later and reanalyzed using an identical procedure.

Statistical analysis

Data were expressed as mean ($\pm$ SD) LS; or proportion of correct identifications. Effect of the culture medium was analyzed by comparing the LS values obtained from dermatophytes grown on both SGC and SGCc culture media to a library of reference spectra obtained on SGC medium. Incubation time effect was analyzed by comparing the LS values obtained for 3–6 or 15-day-old cultures to a library of reference spectra obtained from 3-day-old cultures. Generalized estimating equations were used to account for the non-independence of spectra from the same isolate. All statistical analyses were performed with the SAS 9.2 (Cary, NC, USA) statistical software. All statistical tests were two-sided and $P < 0.05$ was considered significant.

Results

Reference spectra library validation

Spectra of the 48 strains (Table 1) grown on both SGC and SGCc agar plates for three days were included in the library resulting in 96 reference spectra. The validity of this library was controlled using the raw MALDI-TOF spectrum of a subculture of each of these 48 strains on both culture media. The use of 380/384 spots tested (eight spots per strain; four spots per each of the two media) led to exact identification at the species level with high best-match LS (mean LS = 2.33 ± 0.32 and 2.15 ± 0.34 from SGC and SGCc plates, respectively). In contrast, the second best-match LS score corresponding to a misidentification (i.e., the best-match LS score with a reference spectrum from a distinct species) was much lower (mean LS = 1.37 ± 0.26 or 1.40 ± 0.22 from SGC or SGCc plates, respectively). Four of the eight spots for *T. violaceum* IHEM 13459 strain were misidentified with a high best-match LS (2.27 and 2.23) with the *T. violaceum* reference strain being found to be *T. soudanense*. Therefore, as

plotted in Figure 1, the distributions of exact and false best-match LS values were distinct and allowed us to choose a LS cut-off ≥ 1.9 for species identification in subsequent experiments. It is noteworthy to indicate that the LS scores obtained by comparing the spectra of all the *T. mentagrophytes* complex's strains were very similar.

Culture medium effect

When using a reference library of spectra obtained from isolates grown only on SCG culture it should be noted that the identification of the isolates grown on SGCc was significantly better ($P = 0.0036$). However, the magnitude of this discrepancy was of minimal biological significance as the mean LS were 2.25 (± 0.32) and 2.32 (± 0.33) for SGC and SGCc, respectively. As a LogScore (LS) > 2 indicates a very good match between the analyzed spectrum and the reference spectrum in the library, these findings indicate that the presence of cycloheximide in the culture medium only marginally altered MS-based identification results for the evaluated species. Noticeably, we found that the spectra of fungi grown with or without cycloheximide were visually similar.

Incubation-time effect

The effect of incubation-time on MS-based identification results was tested on 15 isolates of seven species: *M. audouinii* (n = 3); *M. canis* (n = 1); *T. mentagrophytes* (n = 2); *T. rubrum* (n = 2); *T. soudanense* (n = 5); *M. gypseum* (n = 1) and *T. tonsurans* (n = 1). The mass spectra obtained from three- or 15-day-old cultures on SGC medium were visually very similar. When using a reference library of spectra obtained after three days of incubation, identification of isolates was significantly better ($P = 0.0119$) than those obtained for 15-day-old cultures. However, the magnitude of this discrepancy was of minimal biological significance as the mean ($\pm$ SD) LS were 2.30 (± 0.32) and 2.09 (± 0.27) for three- or 15-day-old cultures, respectively, which suggests that MS-based identification was effective, irrespective of incubation time. More importantly, because a small colony was enough for MALDI-TOF MS analysis, dermatophytes isolates could be identified after only three days of culture and, specifically, before the appearance of their characteristic morphological features.

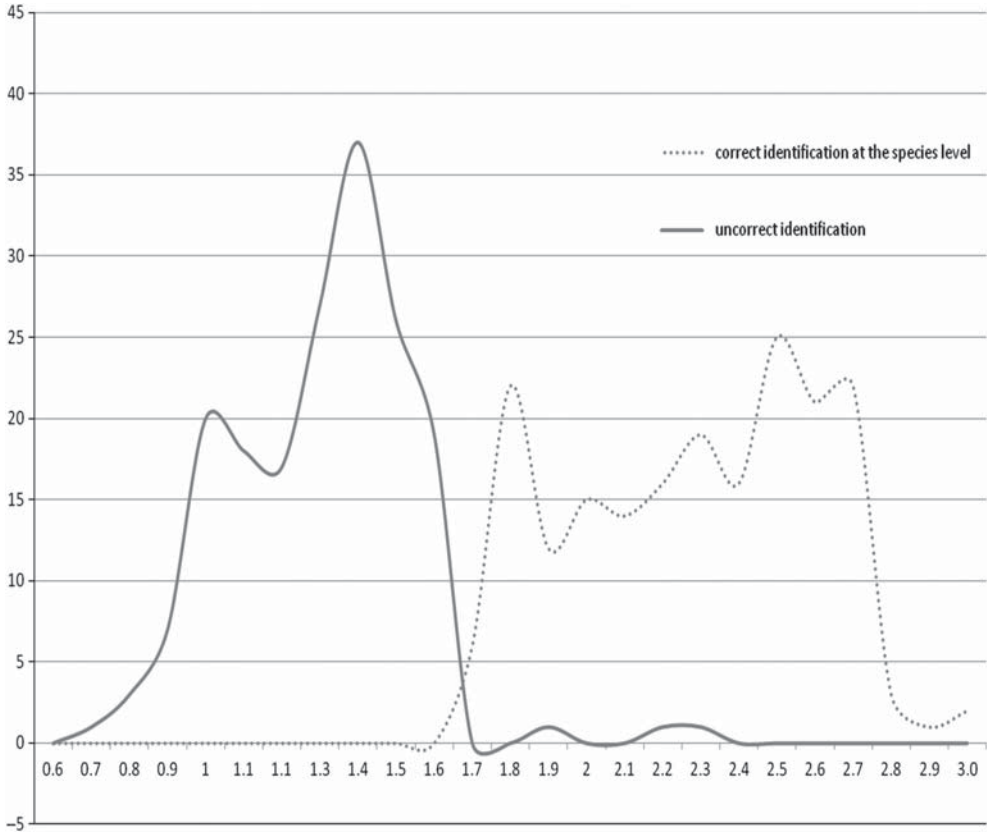

Fig. 1 Distribution of the best-match LogScore (LS) values issued from the MALDI-TOF MS-based identification of the 48 reference library strains. The dotted line plots the best-match LS values of concordant spots whereas the solid gray line plots those of the discordant spots. Concordant and discordant spots best-match LS value distributions were almost distinct and allowed us to choose an LS cut-off ≥ 1.7 for species identification.

MALDI-TOF MS identification of sequential clinical isolates

From the 133 clinical isolates collected, the spectra generated for 130 (97.76%) allowed for their identification at the species level with a sufficient LS score (Table 2). The failures of MALDI-TOF MS identification corresponding to three isolates are detailed in Table 3. The LS-score was below the 1.7 threshold in two that were identified, by both conventional and DNA sequence-based identifications, as *Microsporum audouinii*. The last isolate was falsely identified as *Microsporum audouinii* (with a mean best-match LS of four concordant spots = 1.757 ± 0.08) whereas phenotypic and DNA sequence-based identification yielded *Microsporum canis*. Although MS-based species identification was accurate in 130 isolates, it could not discriminate varieties among species. Indeed, *Trichophyton mentagrophytes* complex members also matched together especially *T. mentagrophytes* with *T. interdigitale* and *T. simii*.

Discussion

The present study demonstrates that the same standardized MALDI-TOF procedure that has been used for mold identification in the routine clinical laboratory setting [16], is also effective in the identification of clinical dermatophyte isolates. Thus, the two reference libraries validated in Marseille's University Hospital mycology laboratory can be combined into a unique comprehensive library suitable to identify clinical filamentous fungi, including dermatophytes. The major benefits of the use of MS-based identification in the routine clinical laboratory are that it allows an accurate recognition to the species level, without the need of a skilled mycologist and with a dramatic reduction of turnaround times. MALDI-TOF is available in a growing number of microbiology laboratories, and starting from pure cultures, MS-based identification procedure is straightforward, faster and less expensive than DNA-sequencing [8,15].

The first investigations of the identification of clinical dermatophytes using MALDI-TOF MS demonstrated that *T. rubrum*, *T. interdigitale*, *T. tonsurans* and *Arthroderma benhamiae* could be appropriately recognized after one month of culture [21]. Then, Theel *et al*. [22] prospectively identified clinical dermatophyte isolates to the species level with a sensitivity of 59.6% using the commercial standard Bruker library (MBL), supplemented with additional dermatophyte spectra obtained from clinical isolates and 20.5% using the MBL alone. The comprehensive reference spectra library used in the present study included 384 spectra of 48 strains of 17 dermatophytes species. It allowed identifying 97.8% of studied clinical isolates belonging to nine distinct dermatophyte species. Species identification by MALDI-TOF MS is largely limited by the number of spectra in the reference library. In the present library, dermatophytes species commonly isolated in the clinical laboratory, e.g., *T. rubrum*, *M. audouinii* and *T. mentagrophytes* have more reference spectra than those isolates infrequently seen in the clinical setting. Theoretically, a higher number of spectra entries per species would encompass more intraspecies diversity occurring due to variable protein expression, growth conditions, or age of culture [8]. In this way, we chose to build the reference spectra library by including multiple references derived from four subculture replicate under two culture conditions. In the study of Theel *et al.*, the reference supplemented library was limited in replicate number of spectra per strains and per species, and some important factor, as the age of the culture used by the manufacturer to build the original MBL are not mentioned. The better percentage identification elicited with our study is likely due to a number of factors. As mentioned above, the added dermatophyte spectra per strains likely enhanced the species-specific diversity covered by the library. Surprisingly, neither culture medium nor incubation time, both factors that are known to alter fungal protein profiles, significantly impacted the dermatophyte MALDI-TOF MS identification.

Table 3 Details of the best-match and second-match identifications and LogScore (LS) values (one for each of the four spot tested) of the three MALDI-TOF MS-based identification failures.

Isolate	Best-match LS	Species	Second match LS	Species
Microsporum audouinii	1.45	*Microsporum canis*	0.976	*Trichophyton rubrum*
	1.07	*Microsporum canis*	1.048	*Trichophyton rubrum*
	1.37	*Microsporum canis*	0.871	*Trichophyton rubrum*
	0.94	*Microsporum audouinii*	0.915	*Trichophyton rubrum*
Microsporum canis	1.727	*Microsporum audouinii*	1.609	*Microsporum canis*
	1.817	*Microsporum audouinii*	1.444	*Microsporum canis*
	1.662	*Microsporum audouinii*	1.607	*Microsporum canis*
	1.823	*Microsporum audouinii*	1.533	*Microsporum canis*
Microsporum canis	1.590	*Microsporum nanum*	1.13	*Microsporum audouinii*
	1.599	*Microsporum audouinii*	1.416	*Trichophyton mentagrophytes*
	1.017	*Microsporum audouinii*	0.905	*Trichophyton mentagrophytes*
	1.343	*Trichophyton mentagrophytes*	1.293	*Microsporum canis*

Reference spectra included in our library were usually processed after three days of culture and for some strains after three and 15 days. In our study, clinical strains were processed when an adequate colony size, corresponding to a young culture, was present to sample for MALDI-TOF. This may explain the high level of identification obtained with our library as the protein spectra of clinical samples may be significantly identical to those found in the library.

To note that among the clinical isolates, 21 were not correctly identified by a first MS-based identification assay, but only after re-extraction and reprocessing the MS-based identification assay. This limitation has previously been described [22] by Nenoff *et al*. [23] who constructed a mass spectra database with 285 isolates of which 164 of the species identification were confirmed by sequence analysis. The study showed that conventional identification and DNA-based identification matched the results of MALDI-TOF MS for 78.2% and 99.3%, respectively, of the 285 isolates tested. *T. violaceum* could not be identified by the MALDI-TOF MS technique as during the validation step of our reference database library, this dermatophyte was misidentified as *T. soudanense* in about half (4/8) of the tests. Yet, *T. violaceum* and *T. soudanense* are very close phylogentically and whether they are distinct species is disputed [4]. The findings from two recent studies, using various MALDI-TOF MS systems, indicated that the procedure is a fast and very specific method for species differentiation of dermatophytes grown in culture [23,24].

In this study, we could not differentiate species within the *T. mentagrophytes* complex. Since the complex includes both anthrophilic and zoophilic specie, it is important to have a reliable method of identifying the human-pathogenic species. The taxonomy of this complex was largely debated [27–29] because the species members were phylogenetically closely related together. The taxonomy was based among other factors on the dichotomy between zoophilic and anthropophilic species indicating a preference for a particular host. However, this feature is not convincing as it does not provide for sufficient differentiation as does habitats. It is known that *T. simii* was closely related to *T. mentagrophytes* [4] and MALDI TOF results yielded similar LS values for both species.

Preliminary data indicate that this limitation of MALDI-TOF-based dermatophyte identification can be addressed by increasing the number of reference strains of these species in the library. It is now well known that the mass spectra are influenced by culture conditions, protein extractions procedures, the type of the matrix and the spectrometer used [25,26]. It was thus very encouraging that MALDI-TOF MS-based dermatophyte identification performed well with colonies grown on both SGC and SGCc agar as they are the most widely employed for recovery of dermatophytes in the clinical setting. Interestingly, dermatophyte identification from selective SGCc agar plates was efficient when molds were concomitantly present in the clinical sample. More importantly, dermatophyte species identification was obtained within 3–6 days turnaround time with MALDI-TOF MS, which was considerably faster than the conventional morphological identification that may take up to two or three weeks, in particular when complementary techniques requiring a subculture are used. The MALDI-TOF MS identification method was easily applicable to a routine laboratory because regents' costs and hands-on time are considerably lower than with the conventional method. At this time, the major limitation of this method was the architecture of the references spectra database. Identification results are improved when the spectra included in the reference database and those obtained from the sample to be identified are processed with the same culture and extraction protocol.

In conclusion, MALDI-TOF MS-based identification proved efficient and dramatically reduced response time for identification of clinical dermatophytes. This standardized procedure is suited to the first-line identification of filamentous fungi, including dermatophytes. Ongoing work aims to enhance MALDI-TOF MS-based identification of filamentous fungi by increasing the number of quality-controlled reference spectra in the library.

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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This paper was first published online on Early Online on xx xx xxxx.