Identification of dermatophytes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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In this study we evaluated the suitability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of dermatophytes in diagnostic laboratories. First, a spectral database was built with 108 reference strains belonging to 18 species of the anamorphic genera Epidermophyton, Microsporum and Trichophyton. All strains were well characterized by morphological criteria and ITS sequencing (gold standard). The dendrogram resulting from MALDI-TOF mass spectra was almost identical with the phylogenetic tree based on ITS sequencing. Subsequently, MALDI-TOF MS SuperSpectra were created for the identification of Epidermophyton floccosum, Microsporum audouinii, M. canis, M. gypseum (teleomorph: Arthroderma gypseum), M. gypseum (teleomorph: A. incurvatum), M. persicolor, A. benhamiae (Tax. Entity 3 and Am-Eur. race), T. erinacei, T. interdigitale (anthropophilic and zoophilic populations), T. rubrum/T. violaceum, T. tonsurans and T. terrestr. Because T. rubrum and T. violaceum did not present enough mismatches, a SuperSpectrum covering both species was created, and differentiation between them was done by comparison of eight specific peptide masses. In the second part of this study, MALDI-TOF MS with the newly created SuperSpectra was tested using 141 clinical isolates representing nine species. Analyses were done with 3-day-old cultures. Results were compared to morphological identification and ITS sequencing; 135/141 (95.8%) strains were correctly identified by MALDI-TOF MS compared to 128/141 (90.8%) by morphology. Therefore, MALDI-TOF MS has proven to be a useful and rapid identification method for dermatophytes.

Keywords dermatophytes, morphology, phylogeny, taxonomy, ITS sequencing, MALDI-TOF MS

Introduction

Classical identification of dermatophytes relies on culture characteristics, microscopic morphology, physiological tests and clinical data [1]. Their overlapping phenotypic characteristics, however, may be confusing [2] and the identification requires growth of the organisms in culture for at least one week, which delays the diagnosis.

Molecular techniques allow a fast and reliable identification of dermatophytes [3–5]. ITS sequencing is presently considered the gold standard for molecular identification and phylogenetic analyses of dermatophytes. However, DNA sequencing is expensive and at least 2–3 days are required before a sequence is obtained from a culture.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is now used routinely in clinical diagnostic laboratories as it is faster than PCR and requires little sample handling. It is a cost-effective and reliable technique for the identification and typing of microbial pathogens including bacteria [6–11], yeasts [12–14], and filamentous fungi [11,15–18]. MALDI-TOF
MS has already been used for the identification of dermatophytes, either after their isolation and cultivation [19–21] or directly from clinical material [22]. However, the number of dermatophyte species analyzed was limited in two studies [19,20], and in one of them [20] only 59.6% of 171 strains were identified at the species level, which is insufficient for the routine application. A recent study [21] analyzed 12 dermatophyte species, but some important ones (Microsporum audouinii, M. gypseum, Arthroderma benhamiae, Trichophyton verrucosum) were not included. Moreover, the time of incubation was 3 weeks, making the MALDI-TOF MS system not attractive for diagnostics.

Our study aimed to test MALDI-TOF MS with most of the important dermatophyte species, analyzing them after only 3 days of incubation. First, a database was built with 108 reference strains and SuperSpectra were created. In a second step, 141 clinical isolates were tested and the results compared with those obtained from morphological and ITS sequencing studies.

**Materials and methods**

**Fungal strains**

A total of 108 reference strains belonging to 18 species of *Arthroderma*, *Epidermophyton*, *Microsporum* and *Trichophyton* were included to construct the reference database (Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). They were obtained from culture collections (CBS, Utrecht, The Netherlands; Micoteca da Universidade do Minho (MUM), Braga, Portugal), external quality control programs or laboratories own collections (Z. Zürich; bM, Bellinzona). For the MALDI-TOF MS identification study, 141 clinical isolates belonging to nine species were used (Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476), all of them originated from Zurich (*z*) and Bellinzona (*M*).

**Morphological identifications**

All 249 isolates were grown on potato dextrose agar (Becton Dickinson, France) plates at 25–30°C in the dark until sporulation. Physiological tests (Christensen’s urea agar, BCP milk glucose agar, *in vitro* hair perforation test, and temperature enhancement analysis) were conducted when morphology alone did not allow reliable identification. Identifications were made using standard keys in the literature [23–25], those isolates that did not demonstrate all typical features were reported as species-like (e.g., *T. interdigitale*-like).

**ITS sequencing**

All 249 isolates were inoculated on Sabouraud gentamycin chloramphenicol 2 agar plates (bioMérieux, France) and incubated at 25–30°C for 3–7 days. DNA was extracted by phenol-chloroform or using the Nexttec Genomic DNA Kit (Nexttec, Germany). The ITS region was amplified using primers ITS1 and ITS4 [26]. Sequences were generated with the ITS4 primer and compared to the publicly available database (http://www.cbs.knaw.nl/dermatophytes/BioIoMICSID.aspx) using the pairwise sequence alignment tool (Supplementary Tables 1 and 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). A phylogenetic tree was constructed using Mega 4.0 (Neighbor-Joining method, Kimura two-parameter distances, bootstrap with 1,000 replicates).

**MALDI-TOF MS**

All strains were grown on Sabouraud gentamycin chloramphenicol 2 agar plates (bioMérieux SA, Marcy l’Etoile, France) and incubated at 30°C for 3 days. Young, growing mycelium was extracted using 25% formic acid, followed by a step of lipid elimination with methanol and chloroform to avoid any competition between proteins and lipids during the phase of protonization (Guido Vogel, Mabritec AG, personal communication). The supernatant obtained after centrifugation was mixed with a matrix containing 30–40 mg of α-cyano-4-hydroxy-cinnamic acid (CHCA) in acetonitrile/ethanol/water (1:1:1) supplemented with 3% trifluoroacetic acid (Sigma-Aldrich, Switzerland). For each isolate, 1 μl was spotted in quadruplicate onto wells of a 48-position stainless steel FLEXImass target plate (Shimadzu Biotech, Kyoto, Japan). MS analyses were performed in positive linear mode in the range of 2,000–20,000 mass-to-charge ratio (*m/z*) with delayed, positive ion extraction (delay time: 104 ns with a scale factor of 800) and an acceleration voltage of 20 kV (pulse width: 3 nS). Averaged profile spectra fulfilling the quality criteria were collected from 5 laser shot cycles. For every sample, 50 averaged profile spectra were used for analysis. All spectra were processed using the MALDI-TOF MS Launchpad 2.8 software (Shimadzu Biotech, Kyoto, Japan) with baseline correction, peak filtering and smoothing.

The resulting peak lists were imported into the BioNumerics software package (Version 6.0, Applied Maths NV, Sint-Martens-Latem, Belgium). Distances were computed using the Pearson’s correlation (0.5% tolerance) and the UPGMA agglomeration algorithm to produce a dendrogram (one mass spectrum per isolate, mass range from 3,000–20,000 m/z).

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**MALDI-TOF MS identification strategy**

Reference spectra were imported into the SARAMIS software package (bioMérieux, La Balme, France). SuperSpectra™ containing the most representative peptide masses were created for the taxonomic clusters and integrated in the SARAMIS database for future rapid identification of clinical samples.

All 141 clinical isolates were submitted to identification by SuperSpectra but if identification with SuperSpectra was not possible (<70%), spectra were compared directly with the reference spectra of the database (COMPARE function of the SARAMIS software). Identifications through the COMPARE function were considered valid when the similarity was ≥40%. As *T. rubrum* and *T. violaceum* presented too few mismatches to be distinguishable, a strategy of comparison of specific masses was created to discriminate them.

**Results**

**Reference strains**

The morphological identification of 91/108 (84.3%) of the isolates was in accord with ITS sequencing, in 4/108 (3.7%) identification was not possible due to the lack of spore formation in culture, and in 13/108 (12.0%) morphological results were discrepant with ITS sequencing (Table 1, Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). The ITS sequences of the 108 reference strains were 535–686 nucleotides long. The best and second best data (2012.746476).

The ITS sequences of the 108 reference strains were 535–686 nucleotides long. The best and second best database matches are presented in Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). The ITS sequences of the 108 reference strains were 535–686 nucleotides long. The best and second best database matches are presented in Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). More than 94% of the strains were identified by MALDI-TOF MS, morphology and ITS sequencing (Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). Identifications through the COMPARE function were considered valid when the similarity was ≥40%. As *T. rubrum* and *T. violaceum* presented too few mismatches to be distinguishable, a strategy of comparison of specific masses was created to discriminate them.

**Creation of MALDI-TOF MS SuperSpectra**

Species-specific biomarkers (SuperSpectra) identifying *E. floccosum*, *M. audouinii*, *M. canis*, *M. gypseum* (*A. gypseum*), *M. gypseum* (*A. incurvatum*), *M. persicolor*, *A. benhamiae* (covering Tax. Entity 3 and Am-Eur. race), *T. erinacei*, *T. interdigitale* (covering both anthropophilic and zoophilic populations), *T. tonsurans* and *T. terrestrae* were created.

No species-specific SuperSpectra could be created for *T. rubrum* and *T. violaceum* as a result, a combined SuperSpectrum for both species was generated. For further discrimination, the reference spectra of *T. rubrum* and *T. violaceum* were compared mass by mass and eight specific peptides (m/z 5,052, 5,436, 5,575, 5,706, 6,647, 7,550, 8,607, 11,409) were selected. m/z 7,550 was prevalent in *T. violaceum* (96.8%) and the others were prevalent in 68.4–95.7% of all *T. rubrum* reference spectra. Subsequently, when a clinical strain was identified as *T. rubrum*/ *T. violaceum* the presence/absence of the eight masses described above was checked, giving to each other a value proportional to the frequencies found in the reference spectra. After multiplying all the values, the frequencies were normalized by dividing each by their sum and multiplying by 100 to give the *T. rubrum* and *T. violaceum* identification percentages as previously described [28,29].

**Validation of the MALDI-TOF MS identification method on clinical strains**

A total of 141 clinical isolates belonging to nine species were identified by MALDI-TOF MS, morphology and ITS sequencing (Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). Identification by MALDI-TOF MS allowed an overall correct identification of 95.8% of all clinical isolates (Table 2) which included the correct identification of all strains belonging to *M. audouinii* (five), *M. canis* (16), *M. gypseum* (*A. gypseum*) (two), *T. erinacei* (one), and *T. tonsurans* (four) (Table 2, Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). Four *T. interdigitale* and two *T. rubrum* isolates required the use of the COMPARE function. In two of 12 *A. benhamiae* three of

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Genus, species</th>
<th>% ID</th>
<th>Mismatches</th>
<th>MALDI-TOF MS cluster</th>
<th>Morphological identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS 100.64</td>
<td><em>Microsporum gypseum</em></td>
<td>100</td>
<td>0/585</td>
<td><em>Microsporum gypseum</em> complex</td>
<td><em>Trichophyton rubrum</em> (african pop.): sterile, colony orange-yellow, corresponding to former <em>Trichophyton soudanense</em></td>
</tr>
<tr>
<td>bM 134</td>
<td><em>Microsporum canis</em></td>
<td>100</td>
<td>0/656</td>
<td><em>Arthroderma otae</em> complex</td>
<td><em>Chrysosporium inops</em>: thick walled aleurospores present, no macroconidia.</td>
</tr>
<tr>
<td>CBS 318.56</td>
<td><em>Trichophyton mentagrophytes</em></td>
<td>100</td>
<td>0/604</td>
<td><em>Arthroderma vanbreuseghemii</em> complex</td>
<td><em>Trichophyton erinacei</em>: no spiral hyphae seen</td>
</tr>
<tr>
<td>CBS 101546</td>
<td><em>Trichophyton mentagrophytes</em></td>
<td>100</td>
<td>0/604</td>
<td><em>Arthroderma vanbreuseghemii</em> complex</td>
<td>Atypical <em>Trichophyton tonsurans</em>: microconidia variable, tear-drop-shaped to clavate, colony flat, white with some brown colour, hair perforation test negative.</td>
</tr>
<tr>
<td>bM 126</td>
<td><em>Trichophyton erinacei</em></td>
<td>100</td>
<td>0/598</td>
<td><em>Arthroderma benhamiae</em> complex</td>
<td>No identification: many diverse microconidia, colony white, cottony</td>
</tr>
<tr>
<td>CBS 623.66</td>
<td><em>Arthroderma benhamiae</em> (Am-Eur race)</td>
<td>99.8</td>
<td>1/598</td>
<td><em>Arthroderma benhamiae</em> complex</td>
<td>Atypical <em>Trichophyton erinacei</em>: microconidia slender, clavate, colony flat, cottony, reverse yellow.</td>
</tr>
<tr>
<td>bM 123</td>
<td><em>Arthroderma benhamiae</em> (tax. entity 3)</td>
<td>100</td>
<td>0/599</td>
<td><em>Arthroderma benhamiae</em> complex</td>
<td>Atypical <em>Trichophyton tonsurans</em>: microconidia abundant and of variable size, cylindrical to clavate or balloon-shaped. Colony white, flat.</td>
</tr>
<tr>
<td>bM 132</td>
<td><em>Trichophyton verrucosum</em></td>
<td>99.8</td>
<td>1/597</td>
<td><em>Arthroderma benhamiae</em> complex</td>
<td><em>Trichophyton concentricum</em>: no chlamydospores as illustrated for <em>Trichophyton verrucosum</em>. Seems to have better growth than typical <em>Trichophyton verrucosum</em>.</td>
</tr>
<tr>
<td>MUM 09.15</td>
<td><em>Trichophyton rubrum</em> (African pop.)</td>
<td>100</td>
<td>0/589</td>
<td><em>Trichophyton rubrum</em> complex</td>
<td>No identification: microconidia variable, slender to short-clavate, macroconidia cylindrical, 2–7-celled, colony slow-growing, reverse yellow.</td>
</tr>
<tr>
<td>CBS 518.63</td>
<td><em>Trichophyton rubrum</em> (african pop.)</td>
<td>99.5</td>
<td>3/609</td>
<td><em>Trichophyton rubrum</em> complex</td>
<td>No identification: sterile, colony white, fluffy</td>
</tr>
<tr>
<td>Neqas 9649</td>
<td><em>Trichophyton rubrum</em> (african pop.)</td>
<td>100</td>
<td>0/541</td>
<td><em>Trichophyton rubrum</em> complex</td>
<td><em>Trichophyton tonsurans</em>: microconidia abundant and of variable size, cylindrical to clavate or balloon-shaped. Colony yellowish with red-brown reverse.</td>
</tr>
</tbody>
</table>
Fig. 1  (A) Phylogenetic tree based on ITS sequencing. Neighbor-Joining method, Kimura 2-parameter, bootstrap from 1,000 replicates. (B) Dendrogram based on MALDI-TOF mass spectra. Mass range (m/z): 3,000–20,000, Pearson’s correlation, UPGMA. Indications of complex memberships according to [2].
Identifi cation of dermatophytes by MALDI-TOF MS was not possible. The other 47 T. rubrum and all seven T. violaceum isolates were submitted to the T. rubrum versus T. violaceum differentiation system using the eight discriminating peptide masses. All were correctly assigned to their respective species, with percentages of reliability between 95.6 and 99.9% for T. violaceum (Table 2).

Some 128 of the 141 (90.8%) isolates were correctly identifi ed by morphology at the species level, whereas nine (6.4%) could not be identifi ed due primarily to the lack of conidial formation and four (2.8%) were discrepant compared to sequencing and mass spectrometry (Supplementary Table 2).

Specificity, sensitivity, positive predictive values and negative predictive values were computed for the outcomes of identifi cations by morphology and MALDI-TOF MS for each of the nine species, using ITS sequencing as the gold standard. MALDI-TOF MS was always comparable to morphology in terms of the parameters computed.

### Discussion

This study has shown that MALDI-TOF MS is a reliable, rapid and effective method for the routine identifi cation of dermatophytes. Overall, 95.8% of the clinical isolates were correctly identifi ed by MALDI-TOF MS. In addition, it was very powerful in discriminating closely related and morphologically indistinguishable species, as it allowed for the differentiation of A. gypseum and A. incurvatum, T. interdigitale and T. mentagrophytes and the two A. benhamiae groups.

Morphological identifi cation is straightforward for those isolates forming typical macro- and/or microconidia or other characteristic features. However, it relies on the experience of the staff and in daily practice the mycology laboratory is often confronted with strains that do not produce identifiable diagnostic morphologies. ITS sequencing is currently the gold standard for the identifi cation of dermatophytes, and MALDI-TOF MS is not directly comparable to sequencing and mass spectrometry. Some 128 of the 141 (90.8%) isolates were correctly identifi ed by morphology at the species level, whereas nine (6.4%) could not be identifi ed due primarily to the lack of conidial formation and four (2.8%) were discrepant compared to sequencing and mass spectrometry (Supplementary Table 2).

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Table 2. Comparison of identifi cations between ITS sequencing, morphology and MALDI-TOF MS for the 141 clinical strains.

<table>
<thead>
<tr>
<th>Genus, species</th>
<th>No. of isolates investigated</th>
<th>Morphology (no.)</th>
<th>MALDI-TOF MS (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correct identification</td>
<td>No identification</td>
<td>Misidentifi cation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SuperSpectra TM (% ID)</td>
<td>COMP</td>
</tr>
<tr>
<td>A. gypseum</td>
<td>2</td>
<td>10 (75.0 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>M. audouinii</td>
<td>5</td>
<td>4 (78.0 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>M. canis</td>
<td>16</td>
<td>12 (87.0 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>M. gypseum</td>
<td>2</td>
<td>1 (99.9)</td>
<td>0</td>
</tr>
<tr>
<td>M. audouinii</td>
<td>12</td>
<td>10 (75.0 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>A. benhamiae</td>
<td>46</td>
<td>42 (78.2 – 99.9)</td>
<td>4</td>
</tr>
<tr>
<td>T. interdigitale</td>
<td>48</td>
<td>47 (82.0 – 99.9)</td>
<td>2</td>
</tr>
<tr>
<td>T. mentagrophytes</td>
<td>7</td>
<td>4 (95.5 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>T. violaceum</td>
<td>7</td>
<td>6 (93.0 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>T. rubrum</td>
<td>48</td>
<td>47 (91.6%)</td>
<td>6 (4.2%)</td>
</tr>
<tr>
<td>T. tonsurans</td>
<td>4</td>
<td>4 (91.6%)</td>
<td>6 (4.2%)</td>
</tr>
<tr>
<td>T. erinacei</td>
<td>1</td>
<td>1 (97.0)</td>
<td>0</td>
</tr>
<tr>
<td>T. interdigitale</td>
<td>46</td>
<td>42 (78.2 – 99.9)</td>
<td>4</td>
</tr>
<tr>
<td>T. rubrum</td>
<td>48</td>
<td>47 (82.0 – 99.9)</td>
<td>2</td>
</tr>
<tr>
<td>T. tonsurans</td>
<td>4</td>
<td>4 (95.5 – 99.9)</td>
<td>0</td>
</tr>
<tr>
<td>T. erinacei</td>
<td>1</td>
<td>1 (97.0)</td>
<td>0</td>
</tr>
</tbody>
</table>

a As identifi ed by ITS sequencing; b % ID, percentage given by identifi cation with the SuperSpectra TM; c COMP, COMPARE function from the SARAMIS TM system.

discussion

46 T. interdigitale and one of 48 T. rubrum isolates identifi cation by MALDI-TOF MS was not possible. The other
which allows the identification at two levels, i.e., (i) directly reference spectra and SuperSpectra can be easily added, and (ii) with the less rapid (yet still quite quick and robust) COMPARE function that allows comparison of an unknown spectrum with reference spectra in the database. In difficult cases, the use of both steps allowed an identification of 95.8%, which is higher compared to a one step identification system [20].

In this study, an open database was used, to which new reference spectra and SuperSpectra can be easily added, which allows the identification at two levels, i.e., (i) directly and rapidly with SuperSpectra or (ii) with the less rapid (yet still quite quick and robust) COMPARE function that allows comparison of an unknown spectrum with reference spectra in the database. In difficult cases, the use of both steps allowed an identification of 95.8%, which is higher compared to a one step identification system [20]. The use of an open database has the additional advantage of customisation by each laboratory [10,17].

Our study has some limitations. The 141 clinical dermatophytes only included nine species and were dominated by T. rubrum, T. interdigitale, M. canis and by the American-European strains of A. benhamiae. M. gypseum (A. gypseum), T. erinacei and T. tonsurans were under-represented with only 2, 1 and 4 isolates, respectively. SuperSpectra™ were created for E. floccosum, M. gypseum (A. incurvatum), M. persicolor, and T. terrestré but could not be tested, because no clinical strains were obtained during the study period.

Conclusions

This study has shown that MALDI-TOF MS is a fast, robust and reliable method for the identification of the most frequent dermatophyte species isolated in the clinic. Under-represented and uncommon taxa, however, must be included in further studies to confirm the present results and to cover the whole dermatophyte species spectrum.

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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.

References


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Supplementary material available online

Supplementary Tables 1–3.

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