Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry

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Abstract: Identification of dermatophytes is currently performed based on morphological criteria and is increasingly supported by genomic sequence comparison. The present study evaluates an alternative based on the analysis of clinical fungal isolates by mass spectrometry. Samples originating from skin and nail were characterized morphologically and by sequencing the internal transcribed spacer 1 (ITS1), ITS2 and the 5.8S rDNA regions of the rDNA clusters. In a blind comparative study, samples were analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF MS). The mass spectra were compared to a database comprising of the spectral data of reference strains by applying the sᴀʀᴀᴍɪs software package. All fungal isolates belonging to the taxa *Trichophyton rubrum*, *T. interdigitale*, *T. tonsurans*, *Arthroderma benhamiae* and *Microsporum canis* were correctly identified, irrespective of host origin and pathology.

To test the robustness of the approach, four isolates were grown on five different media and analyzed. Although the resulting mass spectra varied in detail, a sufficient number of signals were conserved resulting in data sets exploitable for unequivocal species identification. Taken together, the usually widespread dermatophytes can be identified rapidly and reliably by mass spectrometry. Starting from pure cultures, MALDI-TOF MS analysis uses very simple sample preparation procedures, and a single analysis is performed within minutes. Costs for consumables as well as preparation time are considerably lower than for PCR analysis.

Key words: *Arthroderma* – dermatophyte – identification – MALDI-TOF mass spectrometry – *Trichophyton*

Introduction

Infections caused by dermatophytes belong to the most common endemic infectious diseases (1–4); in some geographical areas more than 30% of the population are affected (5,6). In most cases, dermatophytoses in humans remain superficial infections restricted to skin, nails and hair (7). Under appropriate conditions, deeper subcutaneous soft tissue infections may occur (8). The pathogen/host interaction depends largely on the fungal species; on immunocompetence and general health of the human host. Pathogen species determination can be performed classically by morphological analysis of the fungus in combination with fungal growth parameters on selective media. Also the capability to degrade keratin is helpful for determination of some dermatophytic fungi (9). Novel methods for species determination use isolated fungal DNA for fingerprinting or PCR analysis based on differential sequence elements (10–12).

As an alternative to DNA-dependent methods, mass spectral analysis and identification of micro-organisms has become increasingly recognized (13). In a number of studies, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has been applied for the rapid classification and identification of micro-organisms (14–16). This approach detects highly abundant proteins in a mass range between 2 and 20 kDa, serving as taxon-specific biomarkers. The striking advantage of mass spectral approaches over genetical or morphological procedures is the very simple and straightforward sample preparation procedure and the short time required for analysis. The complete analysis including

sample preparation and data evaluation is completed within minutes.

The main prerequisite for successful MALDI-TOF MS identification of micro-organisms is a database archiving the reference spectra of the relevant, taxonomically validated strains. At AnagnosTec (Potsdam, Germany) mass spectral data of 1500 microbial species have been collected. Dermatophytic fungi are presently being included into this database on a broad scale. The data were archived and can be searched by the microbial identification system software package SARAMIS.

Fast and reliable identification of bacteria and fungi is one of the prerequisites for adequate therapy. Efficient methods for microbial detection are characterized by robustness, simple handling, low cycle costs, high speed and high-throughput capability. In all these respects, mass spectrometry offers significant advantages over classical technologies for determining moulds and yeasts from various sources with minimal sample preparation.

In this study, the applicability of MALDI-TOF MS for identifying clinical dermatophytes was tested. This approach was validated by conventional morphological identification supported by sequencing the internal transcribed spacer (ITS) regions of the ribosomal DNA and the information for 5.8S RNA.

Materials and methods

Strains and growth conditions

All strains were clinical isolates obtained by cultivation of skin or nail specimens from different patients (Table 1). The samples were inoculated in two different culture media: in duplicate, on Sabouraud's 4% (w/v) dextrose agar (Oxoid GmbH, Wesel, Germany) to grow dermatophytes, yeasts and other fungi in duplicate on Dermasel selective agar plates (Oxoid GmbH). These media contain cycloheximide for selective growth of dermatophytes and certain other fungi. Bacterial growth was inhibited by 50 μg/ml chloramphenicol. Plates were incubated for 4 weeks at 28°C. The cultures were inspected for fungal growth several times a week. All fungi were evaluated both macroscopically in terms of growth characteristics and pigment production and microscopically to detect the formation of macro- and microconidia or other typical differentiation forms.

Around 2804 Strains A–D (Table 1) were used in comparative inter-laboratory tests (Ringversuch 491, 2006, INSTAND e.V. – Institut für Standardisierung und Dokumentation in medizinischen Laboratorien e. V), and species determinations were also validated by other laboratories. Additional substrates were used for testing the influence of growth media on mass spectral patterns: potato dextrose agar (Heipha GmbH, Eppelheim, Germany), malt extract agar (Oxoid GmbH) and Kimmig agar (Oxoid GmbH).

Table 1. Species identification of clinical dermatophyte isolates used in this study. Morphological identification, ITS1, ITS2 and 5.8S rDNA sequencing and MALDI-TOF analysis yield identical results. A–D are reference strains from a comparative inter-laboratory test

Strain	Host	Clinical diagnosis	Acc. no of database references with sequence 100% identity	MALDI-TOF/ SARAMIS
186	m	Tinea pedis	AF170472	Trichophyton rubrum[1]
276	m	Onychomycosis	AF168124	T. interdigitale[1]
389	f	Onychomycosis	AF170472	T. rubrum[1]
411	m	Inguinal	AF170472	T. rubrum[1]
483	m	Onychomycosis	AF170472	T. rubrum[1]
494	m	Onychomycosis	AF168124	T. interdigitale[1]
505	m	Onychomycosis	AF170472	T. rubrum[1]
539	m	Onychomycosis	AF170472	T. rubrum[1]
564	m	Onychomycosis	AF170472	T. rubrum[2]
582	m	Tinea pedis	AF170472	T. rubrum[1]
597	f	Tinea corporis	AF170472	T. rubrum[1]
620	m	Tinea corporis	AF170472	T. rubrum[1]
646	m	Tinea pedis	AF168124	T. interdigitale[1]
694	m	Onychomycosis	AF168124	T. interdigitale[1]
883	m	Tinea pedis	AF170472	T. rubrum[1]
899	m	Tinea corporis	AF170472	T. rubrum[1]
A	m	Onychomycosis	AF170472	T. rubrum[1]
B	f	Tinea corporis	AY213690	T. tonsurans[1]
C	m	Tinea capitis	Z98016	Arthroderma benhamiae[1]
D	cat	Mycosis	AB193649	Microsporum canis[1]

ITS, internal transcribed spacer; rDNA, recombinant deoxyribonucleic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
[1]99.9% confidence level.
[2]80% confidence level.

DNA preparation, PCR conditions and DNA sequencing

Fungal DNA was isolated from mycelium grown on Sabouraud's glucose plates according to a published method (17) with the following modifications: a piece of mycelium was broken down in 0.4 ml lysis buffer using a sterile needle and incubated for 15 min at 70°C. Lysis buffer contains 200 mM Tris–HCl pH 8.0, 50 mM EDTA, 150 mM NaCl, 1% (w/v) SDS. After lysis 0.4 ml 3 M sodium acetate, pH 4.8 was added and after gentle mixing, the tubes were cooled on ice for 20 min. The extract was centrifuged for 5 min at 13 krpm at room temperature. About 0.5 ml of the supernatant was mixed with 0.4 ml trichloromethane

and centrifuged for 1 min at 13 krpm. The supernatant was transferred to a new tube and mixed with 0.4 ml 100% (v/v) propanol-2. After incubation at room temperature for 20 min, the DNA was centrifuged for 5 min at 13 krpm, the pellet was washed once with 0.5 ml of ice-cold 70% (v/v) ethanol, dried *in vacuo* and resolved in 30 μl sterile, deionised water. The total amount in these preparations varied between 0.5 and 1 μg of high molecular weight DNA.

Internal transcribed spacer 1 and 2 regions and the 5.8S rDNA were amplified using the primer pair LR1 (5′GGTTGGTTTCTTTTCCT3′) and SR6R (5′AAG-TAAAAGTCGTAACAAGG3′). PCR reactions of 25 μl 10 ng genomic template DNA, 10 pmol of each primer, 0.2 mm dNTPs, 3 mm MgCl$_2$, 10 mm Tris–HCl, pH 8.3, 50 mm KCl, 0.01% (w/v) gelatine and 1 U Taq polymerase (Fermentas, St. Leon-Rot, Germany). Fragments were amplified in a thermocycler (Biometra, Göttingen, Germany) with the following reaction profile: 5 min, 94°C; 30 cycles of 45 s, 94°C; 45 s, 55°C; 80 s, 72°C. The resulting fragments were purified with glass milk from a 0.8% agarose gel according to the method described by Vogelstein and Gillespie (18) and sequenced directly (JenaGen GmbH, Jena, Germany; SEQLAB, Göttingen, Germany).

MALDI-TOF MS

A mass spectral database for dermatophytes was built up by analyzing dermatophytes that had been identified by morphological and genetic criteria. These samples were provided by P. Nenoff and Y. Gräser and included isolates of the following taxa: *Arthroderma benhamiae*, *A. persicolor*, *Microsporum audouinii*, *M. canis*, *M. cookei*, *M. fulvum*, *M. gypseum*, *M. racemosum*, *M. vanbreuseghemii*, *Trichophyton eboreum*, *T. equinum*, *T. interdigitale*, *A. benhamiae*, *T. rubrum*, *T. schoenleinii*, *T. tonsurans*, *T. verrucosum*, and *T. violaceum*. The fungi were analyzed after cultivation on different media and growth times. Data from individual species were subsequently summarized in consensus spectra or superspectra, containing only those mass signals that are present in at least 80% of the individual mass spectra.

Sample preparation

Approximately 50 μg fresh cells were transferred from plates on stainless steel templates, immediately extracted with 0.3 ml matrix solution [10 mg 2,5-dihydroxy benzoic acid in 1 ml water:acetonitrile (1:1), acidified with 1% trifluoroacetic acid] and air dried.

Analyses were performed in linear mode with delayed, positive ion extraction (delay time: 950 ns; acceleration voltage: 20 kV) on an Applied Biosystems, Darmstadt, Germany, Voyager DE Pro mass spectrometer equipped with a nitrogen laser ($\lambda = 337$ nm). Spectra were accumulated from automatically acquired 200 laser pulse cycles. All spectra were processed by the Data Explorer software (Applied Biosystems, Darmstadt, Germany) with baseline correction, filtering and smoothing. The resulting peak lists were exported to the saramis software package (AnagnosTec).

Peak lists of individual samples were compared with the superspectra database generating a ranked list of matching spectra. saramis uses a point system based on peak list with mass signals weighed according to their specificity. The weighting is based on empirical data from multiple samples of the reference strains.

Results

Morphological and genetic identification

In the first step, pure cultures were classified at the morphological level (Table 1). All strains morphologically classified as *T. rubrum* were confirmed by sequencing ITS1, ITS2 and the 5.8S rDNA and by comparing the results with the National center for biotechnology information (NCBI) database. Table 1 shows the accession numbers of reference sequences with 100 % identity at the nucleotide level. All *T. rubrum* ITS1 and 2 are completely identical with those of the reference strain *T. rubrum* ATCC 28188 (Acc. no. AF170472; 12).

Interestingly, mycelium of isolate 620 showed an unusual yellow pigmentation (data not shown), although DNA sequence data as well as MALDI-TOF MS analysis clearly identify this strain as *T. rubrum*.

Several morphological differences are found between *T. interdigitale* and *A. benhamiae*. According to the new species concept, *T. mentagrophytes* strain C should now be addressed as *A. benhamiae*. In addition, we have observed the formation of gymnothecia in this isolate (data not shown). This isolate is the cause of a tinea capitis (Kerion celsi), typical for keratinophilic fungi. All characterized *T. interdigitale* strains were found to lead to onychomycosis or tinea pedis (Table 1). DNA sequence analysis shows the significant deviation of the *T. interdigitale* ITS regions compared with the ITS sequences of *A. benhamiae*.

ITS sequences of strains identified as *T. interdigitale* were identical to the corresponding *T. interdigitale* DNA sequences of the strain in the database (Acc. no. AF168124) and isolate 200257 provided by Y. Gräser. As expected, the ITS1 and 2 of *A. benhamiae* (*T. mentagrophytes* strain C) were identical to the corresponding sequences of *A. benhamiae* strain 2354. Strain CBS 280.83 (Acc. no. Z98016, 11; *T. mentagrophytes*) served as reference sequence. Accordingly, the *A. benhamiae* strain 2354 (19) and a zoophilic isolate (provided by K. Büsing, University of Leipzig, Leipzig, Germany) showed the same sequences. The taxonomy of the *T. mentagrophytes/A. benhamiae* complex has recently been debated based on molecular data; the results from this study support a recent taxonomic system based on the origin of isolates, being either zoo- or anthropophilic (20,21).

The ITS regions of *T. tonsurans* (strain B) are identical with ITS regions of *T. tonsurans* strain ATCC 65186 (Acc. no. AY213690, 10).

M. canis (strain D) ITS regions are identical with those of *M. canis* strain IFM 46803 (Acc. no. AB193649).

Influence of growth conditions on mass spectra

Reliable MALDI-TOF MS species determination should ideally be independent of growth conditions of the fungi. Differential expression patterns on several media could change the observed mass spectra. In order to check the robustness of the method, *T. mentagrophytes*, *T. rubrum*, and *M. canis* were cultivated on different media and MALDI-TOF mass spectra were determined. Figure 1 shows mass spectra of an *A. benhamiae* sample (strain C) grown on five different media. The mass spectra vary with respect to individual peaks and relative intensity of peaks present in all spectra. This is not unexpected and has two major reasons: (i) Protein expression patterns depend on growth conditions, especially the type of substrate and the temperature, and (ii) the peptides contained in the media themselves may contribute to the spectra. In Fig. 1, no correction has been made for mass signals originating from the media. Nevertheless, a sufficient number of diagnostic mass signals were observed in all mass spectra, and an unequivocal superspectrum could be calculated by the SARAMIS software (Fig. 1, lower panel). The superspectrum contains 17 mass signals, a number being generally sufficient to resolve species. In the routine of clinical diagnosis, samples are usually cultivated on a single or very few standard media. Growth
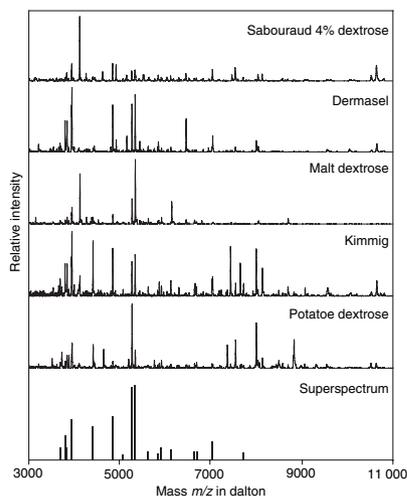
on five completely different media spans a wide range of culture conditions.

The intensity of individual mass signals varies among individual mass spectra. SARAMIS, however, converts peak lists to a binary data matrix not considering signal intensities. This technique has been approved for the identification of bacterial samples as it significantly reduces computing time and adds to the necessary robustness of the method.
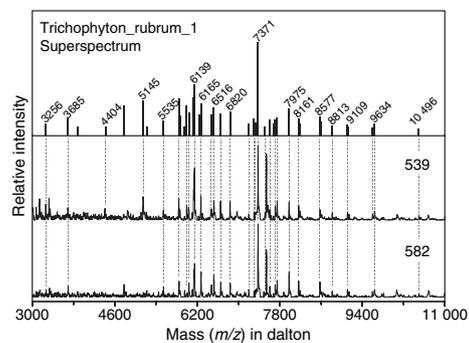
Comparable results were obtained with *T. rubrum*, *T. tonsurans* and *M. canis* strains A, B, and D, respectively, cultivated on several media (data not shown). In conclusion, dermatophyte species can be determined independently of cultivation conditions by comparing mass spectra from novel isolates with the SARAMIS superspectra.

## Identification of clinical isolates by mass spectrometry

All isolates produce mass spectra with 60 to 120 signals in a mass range between 2000 and 20 000 Da. The extraction and analysis procedures that were previously established for bacteria worked similarly and are reproducible for dermatophytes.

Figures 2 and 3 present examples of mass spectra from clinical isolates together with the corresponding identifying superspectra. Mass spectra of the two *T. rubrum* strains are essentially identical. The spectra show 30 (strain 539) and 25 (strain 582) mass signals matching the superspectrum of *T. rubrum*. This identifies both samples as *T. rubrum* with a level of confidence of 99.9%. The same level of confidence is reached for the other *T. rubrum* samples except for strain 564, for which mass spectral analysis results in the identification as *T. rubrum* with only 80% confidence.

For the two *T. interdigitale* isolates (Fig. 3), the number of matching masses was 17 (strain 646) and 15 (strain 694), respectively. This is sufficient to identify both isolates.

Figure 1. Mass spectra (size range: 3000–11 000 Da) of *Arthroderma benhamiae* strain C grown on different media. The lower panel presents the superspectrum containing only those mass signals common to all five spectra with averaged intensities. The superspectrum is sufficient to identify *A. benhamiae* with 99.9% confidence level.

Figure 2. Superspectrum of *Trichophyton rubrum* and mass spectra of two independent *T. rubrum* isolates (size range: 3000–11 000 Da). Numbers above the spectra describe strain numbers. Dotted lines indicate matching mass signals.
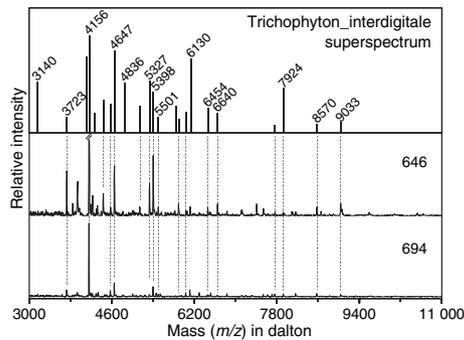
Figure 3. Superspectrum of *Trichophyton interdigitale* and mass spectra of two independent *T. interdigitale* isolates (3000–11 000 Da). Numbers above the spectra describe strain numbers. Dotted lines indicate matching mass signals.

In both mass spectra, a single prominent peak at 4 156 Da dominates; the peaks with the second and third height had relative intensities of approximately 30% or 20%, respectively. The same basic pattern was also found for two additional *T. interdigitale* isolates (strains 276 and 494) and thus seems to be characteristic for this species.

Strain C has been identified as *A. benhamiae* by morphological, genetic and mass spectral approaches. *Trichophyton interdigitale* and *A. benhamiae* can be differentiated unequivocally at the level of DNA sequences and by MALDI-TOF spectroscopy. Both approaches reveal high similarity, or identity in the case of ITS sequences between all isolates of *A. benhamiae*. *Trichophyton tonsurans* and *M. canis* (reference strains B and D) were identified with a statistical reliability of 99.9% by mass spectral analysis. For all species tested, MALDI-TOF analysis of peptides proved to be reliable and with respect to sample preparation and speed, it is the method of choice than amplifying the ITS region by PCR followed by DNA sequencing.

Discussion

The clinically most important dermatophyte species *T. rubrum*, *T. interdigitale*, *T. tonsurans* and *A. benhamiae* were successfully identified by MALDI-TOF MS analysis aided by the SARAMIS database and software package. These species comprise the most frequently encountered dermatophyte species from clinical samples. For all identifications by mass spectrometry, a high level of confidence of 99.9% was obtained except for one strain of *T. rubrum* that also showed several mass signals being typical for *T. violaceum*. This strain (564) was nevertheless identified correctly with a confidence level of 80%. *Trichophyton rubrum* and *T. violaceum* are closely related species and form the only group of dermatophytic fungi for which no teleomorph is known (20). The *T. rubrum* complex originally comprised 19 species and subspecies with strictly asexual reproduction that

can be divided into two clades based on molecular data (22).

Another example reflecting the current difficulties in species determination of dermatophytic fungi is an isolate that was initially identified as *T. mentagrophytes* based on morphological characters. Mass spectral as well as sequence analysis revealed the identity of this strain to *A. benhamiae*. According to a recent publication by Nenoff et al. (21), *A. benhamiae* is closely related to *T. erinacei* (formerly: *T. mentagrophytes* var. *erinacei*), a zoophilic variety or subspecies.

Taken together, species identification by MALDI-TOF MS and SARAMIS analysis was proven to be consistent with ITS and 5.8S rDNA sequence analysis; the technique has a resolving power comparatively as high as ITS sequence analysis. The analysis is performed rapidly within minutes by simple routine protocols without laborious sample preparation procedures, thus allowing convenient high throughput analysis of clinical samples.

Acknowledgements

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