

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Aspergillus* species

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Abstract

New *Aspergillus* species have recently been described with the use of multilocus sequencing in refractory cases of invasive aspergillosis. The classical phenotypic identification methods routinely used in clinical laboratories failed to identify them adequately. Some of these *Aspergillus* species have specific patterns of susceptibility to antifungal agents, and misidentification may lead to inappropriate therapy. We developed a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)-based strategy to adequately identify *Aspergillus* species to the species level. A database including the reference spectra of 28 clinically relevant species from seven *Aspergillus* sections (five common and 23 unusual species) was engineered. The profiles of young and mature colonies were analysed for each reference strain, and species-specific spectral fingerprints were identified. The performance of the database was then tested on 124 clinical and 16 environmental isolates previously characterized by partial sequencing of the β -tubulin and calmodulin genes. One hundred and thirty-eight isolates of 140 (98.6%) were correctly identified. Two atypical isolates could not be identified, but no isolate was misidentified (specificity: 100%). The database, including species-specific spectral fingerprints of young and mature colonies of the reference strains, allowed identification regardless of the maturity of the clinical isolate. These results indicate that MALDI-TOF MS is a powerful tool for rapid and accurate identification of both common and unusual species of *Aspergillus*. It can give better results than morphological identification in clinical laboratories.

Keywords: Aspergillosis, *Aspergillus*, fingerprint, identification, MALDI-TOF mass spectrometry, species complex, spectra

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Introduction

Invasive aspergillosis (IA) remains a severe complication of cytotoxic therapy. Despite the availability of new antifungal agents, overall mortality rates range from 29% to 42% [1]. Until recently, it was largely admitted that only a small number of species of *Aspergillus* were responsible for IA, i.e. *Aspergillus fumigatus*, the leading aetiological agent, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus nidulans* [2]. Several studies have reported cases of IA caused by

previously unrecognized *Aspergillus* species. These new, clinically relevant species, have been identified with methods based on multilocus sequencing (MLS) of genes coding for β -tubulin, calmodulin or actin [3]. Currently, the genus *Aspergillus* includes seven subgenera that are subdivided into 22 sections, each corresponding to a specific 'species complex', grouping related species [4]. For instance, the section *Fumigati* or '*A. fumigatus* complex' includes not only *A. fumigatus*, but also 34 other related species [5]. In clinical settings, identification of aspergilli is routinely based on determination of macroscopic and microscopic morphological characteristics, such as colour, shape, ornamentation, and recognition of asexual or sexual stages [6]. These conventional means of identification do not allow discrimination between morphologically close species of *Aspergillus* (i.e. species belonging to the same section), and many reports have stressed the occurrence of misidentification with

traditional phenotypic approaches [5,7–10]. Thus, the number of species responsible for IA has been underestimated.

Some of the newly described species responsible for IA have decreased susceptibilities to multiple antifungal drugs *in vitro* [8,10–13], and therapeutic failures have been reported in cases of infection with *Neosartorya fischeri* [14], *Neosartorya pseudofischeri* [15], *Neosartorya hiratsukae* [16], *Neosartorya udagawae* [17], *Aspergillus insuetus* [18], *Aspergillus calidoustus* [19] and *Aspergillus niveus* [20]. These species are difficult to separate from more susceptible *Aspergillus* species, sometimes from the same section, solely on the basis of morphological criteria.

The choice of appropriate antifungal therapy is strongly influenced by the inherent resistance phenotype of each *Aspergillus* species, and correct identification is therefore of paramount clinical importance [2,21]. Current recommendations include the use of molecular identification by MLS for identification of individual species within the various *Aspergillus* sections [3,22]. However, sequencing is time-consuming and expensive for the identification of all isolates recovered from clinical samples, stressing the need for rapid and accurate new strategies for identification of these recently described *Aspergillus* species.

Several studies have reported the efficiency of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) for fast and accurate identification of bacterial, yeast and mould species [23–27]. However, this method has not been assessed for most of the clinically relevant *Aspergillus* species. In our study, we designed and validated a rapid identification method, using MALDI-TOF-MS technology to discriminate clinically relevant intra-section species of *Aspergillus*. We demonstrate that MALDI-TOF MS technology is effective for accurate identification of clinical isolates of *Aspergillus* recovered from patients or from the environment.

Materials and Methods

Aspergillus strains

Twenty-eight reference strains, corresponding to 28 *Aspergillus* species from seven sections, were chosen to represent the vast majority of the species currently described as being responsible for infection in humans. They included both common and unusual *Aspergillus* species, and were used to engineer the MALDI-TOF MS database (Table 1). These strains were obtained from three different sources. Eight strains were purchased from the collections of BCCM-IHEM (Brussels, Belgium), 12 from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and eight from collections of clinical

TABLE 1. Reference strains used to establish the matrix-assisted laser desorption ionization time-of-flight mass spectrometry *Aspergillus* database

Section (n)	Species	Reference strain	
Fumigati (11)	<i>Aspergillus fumigatus</i>	IHEM 1246	
	<i>Aspergillus lentulus</i>	CBS 116879	
	<i>Neosartorya pseudofischeri</i>	CBS 208.92	
	<i>Neosartorya fischeri</i>	IHEM 660	
	<i>Aspergillus fumigatiaffinis</i>	CBS 117194	
	<i>Aspergillus fumisynnematus</i>	CNM-CM-4063	
	<i>Aspergillus viridinutans</i>	CBS 127.56	
	<i>Neosartorya udagawae</i>	CBS 114217	
	<i>Neosartorya hiratsukae</i>	CBS 109356	
	<i>Neosartorya spinosa</i>	CBS 483.65	
	<i>Neosartorya fennelliae</i>	CBS 598.74	
	Flavi (5)	<i>Aspergillus flavus</i>	IHEM 306
		<i>Aspergillus oryzae</i>	CBS 115.33
<i>Aspergillus tamarii</i>		FLA 17 ^a	
<i>Petromyces alliaceus</i>		CBS 536.65	
<i>Aspergillus parvisclerotigenus</i>		FLA 30 ^a	
Terrei (1)	<i>Aspergillus terreus</i>	IHEM 5857	
Nigri (3)	<i>Aspergillus niger</i>	IHEM 2864	
	<i>Aspergillus tubengensis</i>	NIG 11 ^a	
	<i>Aspergillus foetidus</i>	NIG 15 ^a	
Nidulantes (4)	<i>Emericella nidulans</i>	IHEM 3665	
	<i>Emericella quadrilineata</i>	CBS 426.77	
	<i>Aspergillus sydowii</i>	IHEM 566	
	<i>Aspergillus versicolor</i>	IHEM 2983	
Usti (3)	<i>Aspergillus calidoustus</i>	CBS 121601	
	<i>Aspergillus pseudodeflectus</i>	UST 26 ^a	
	<i>Aspergillus insuetus</i>	UST1 ^a	
Circumdati (1)	<i>Aspergillus ochraceus</i>	CIR 1 ^a	

CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CNM-CM, Centro Nacional de Microbiología, Servicio de Micología, Madrid, Spain; IHEM, BCCM-IHEM collection, Brussels, Belgium.
^aReference spectra established with clinical isolates identified by multilocus sequencing.

isolates, previously well characterized by MLS. The tested strains used to validate the databases were 124 clinical and 16 environmental fresh isolates of *Aspergillus* (see Supporting Information). The clinical isolates were mainly recovered from respiratory specimens (sputum and bronchoalveolar lavage) and from nail and skin biopsy specimens. The environmental isolates were from the air of our hospital. Clinical and environmental isolates were identified by morphological observation, and exact species were determined with a molecular method based on MLS, using β -tubulin and/or calmodulin genes as previously described [3] (see Supporting Information). All of the strains used in the study were stored at -80°C in trypticase soy broth supplemented with 15% glycerol.

MALDI-TOF MS

The strains were grown at 30°C on Sabouraud dextrose agar with chloramphenicol and gentamicin (BioRad, Marnes-la-Coquette, France) and checked daily for maturation (sufficient sporulation and mycelium development). Superficial material, a mixture of spores, conidiophores and mycelium, was collected gently at the surface of the colony, and mixed in 1 μL of pure water previously deposited on a target plate (Bruker Daltonics, Bremen, Germany). This mixture was

allowed to dry at room temperature. One microlitre of absolute ethanol was then added to each well, and the mixture was allowed to dry. One microlitre of matrix solution (2,5-dihydroxybenzoic acid, 80 mg/mL, 30% acetonitrile, 0.1% trifluoroacetic acid) was then added and allowed to co-crystallize with the sample. An internal control with *Pseudomonas aeruginosa* allowed us to validate the calibration for each experiment. Samples were processed in the MALDI-TOF MS spectrometer (Microflex; Bruker Daltonics) with the flex control software (Bruker Daltonics). Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. The analysis was performed with the flex analysis software. The presence and absence of peaks were considered to be fingerprints for a particular isolate. The profiles were analysed and compared by use of Andromas software (Andromas, Paris, France). Numerical data obtained from the spectrometer acquisition software (peak value and relative intensity for each peak) were sent to the Andromas software [24,28].

Results

Reference database construction

For each of the 28 reference strains, the MALDI-TOF MS profile obtained from ten different runs was analysed. As

some differences were observed between the spectra from young and mature colonies within a same species, two different reference spectra were created for each reference strain: one from young colonies (<2 days of sporulation) and the other from mature colonies (between 4 and 10 days of sporulation). Fig. 1 presents a selection of the specific spectral fingerprints of young and mature colonies for three species. The standard deviation for each conserved peak did not exceed 10 *m/z*. The spectral fingerprints were specific for each selected reference strain.

Validation of the database

We next aimed at determining whether the above database could be used for identification of *Aspergillus* species. The database was blindly tested with the set of 124 fresh clinical and 16 environmental *Aspergillus* isolates.

The Andromas software identified the number of common peaks between the spectra of the tested isolate and the species-specific fingerprints of the reference strains in the database (i.e. Aspergilli database). For each isolate, all peaks with an intensity >0.01 were retained and were compared with that of the species-specific fingerprints of each reference strain, as described above, taking into account possible variations of ± 10 *m/z*. Then, the percentage of common peaks was obtained ($100 \times$ number of common peaks between the tested isolate and the peaks of the

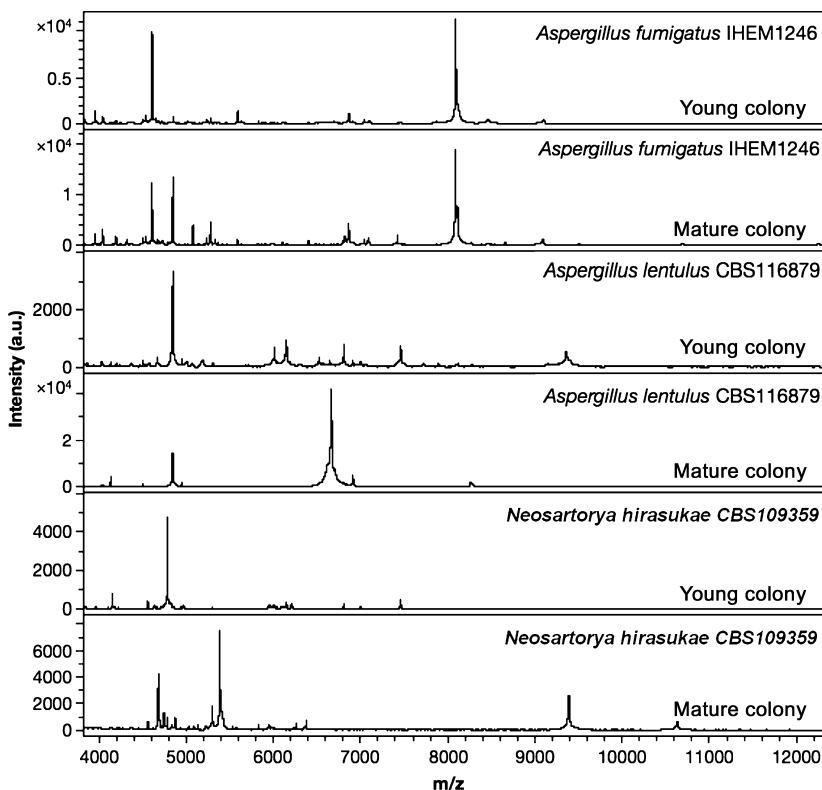


FIG. 1. Representation of the specific spectral fingerprints of young and mature colonies for three selected species.

species-specific spectral fingerprint/total number of peaks specific for the species-specific spectral fingerprint). Only the first and second best matches were retained. Acceptable identification of a tested strain corresponds to the species having $\geq 66\%$ of common peaks with the reference strains in the database. A difference of at least 10% between the first and the second match was required. When the identification was not acceptable (i.e. $<66\%$ of common peaks with reference strain or $<10\%$ of difference between first and second matches), a second run was performed and analysed in a similar manner.

With our database, 127 isolates were identified after a single run, 11 were identified after two runs, and two could not be identified. The results of MALDI-TOF MS identification are presented for each section in Table 2. Identification was correct for 138 of 140 (98.6%) isolates according to our identification strategy. Two isolates for which MALDI-TOF MS failed to give a spectrum were finally identified as *A. fumigatus* by MLS (GenBank accession number AY048754), whereas conventional morphological criteria did not allow accurate identification, owing to the absence of conidiogenesis for one and of atypical sporulation for the other. No isolate was misidentified, leading to 100% specificity.

TABLE 2. Identification results for the 124 clinical and 16 environmental isolates of *Aspergillus* obtained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Section	Species	No. of isolates tested	No. of isolates correctly identified ^a	% of isolates correctly identified ^b
Fumigati	<i>Aspergillus fumigatus</i>	50	48	96
	<i>Aspergillus lentulus</i>	7	7	100
	<i>Neosartorya pseudofischeri</i>	5	5	100
	<i>Neosartorya fischeri</i>	1	1	–
	<i>Aspergillus fumigatiifinis</i>	2	2	–
	<i>Aspergillus fumisynnematus</i>	1	1	–
	<i>Aspergillus viridinutans</i>	2	2	–
	<i>Neosartorya udagawae</i>	1	1	–
	<i>Neosartorya hiratsukae</i>	2	2	–
	Flavi	<i>Aspergillus flavus</i>	13	13
<i>Aspergillus tamarii</i>		2	2	–
<i>Aspergillus parvisclerotigenus</i>		1	1	–
Terrei	<i>Aspergillus terreus</i>	8	8	100
Nigri	<i>Aspergillus niger</i>	9	9	100
Nidulantes	<i>Aspergillus tubengensis</i>	1	1	–
	<i>Aspergillus foetidus</i>	2	2	–
	<i>Emericella nidulans</i>	4	4	–
	<i>Emericella quadrilineata</i>	1	1	–
Usti	<i>Aspergillus sydowii</i>	7	7	100
	<i>Aspergillus versicolor</i>	3	3	–
	<i>Aspergillus calidoustus</i>	14	14	100
Circumdati	<i>Aspergillus pseudodeflectus</i>	1	1	–
	<i>Aspergillus insuetus</i>	1	1	–
Circumdati	<i>Aspergillus ochraceus</i>	2	2	–

^aIn comparison with identification obtained by partial gene sequence-based method.

^bCalculated only when the number of isolates was at least five.

Discussion

We have developed and validated a MALDI-TOF database that allows precise identification of an extensive number of *Aspergillus* species currently isolated in clinical settings, including both common and recently described unusual species.

This identification procedure can be performed from single colonies on Sabouraud dextrose agar whenever the microbiologist suspects an *Aspergillus* mould from the presence of a characteristic conidial head by morphological observation. We have developed a very simple and fast experimental protocol that involves depositing the superficial material collected from the surface of the mould colonies directly onto the MALDI-TOF MS target plate without sub-culture or colony preparation. Indeed, this avoids many laborious sample preparation steps that are currently used to pre-extract proteins, such as washings before using both ethanol and acid solution, or multiple centrifugations.

Because growth of the various *Aspergillus* species is neither uniform nor equivalent in terms of sporulation, we have developed a database that includes the species-specific spectral fingerprints from young and mature colonies for all species. Consequently, identification can be performed regardless of the maturity of the tested isolate, thus making MALDI-TOF MS a robust method for *Aspergillus* species identification. Our procedure allowed identification in <10 min and at very low cost. MALDI-TOF MS appears to be the first automatic phenotypic method available for accurate identification of the most important *Aspergillus* species. For daily identification of *Aspergillus* species in clinical microbiology laboratories, MALDI-TOF MS could be used as a first-line approach to confirm morphological findings.

In our (Bougnoux M-E, Alanio A, Lacroix C, unpublished data) experience, some clinical respiratory samples, especially from cystic fibrosis patients, frequently yield colonies of *Aspergillus* species with variable morphological macroscopic aspects. This raises the question of the presence of a mixture of different *Aspergillus* species or of variation in growth. Because of the low costs and speed of MALDI-TOF MS, which is much faster and cheaper to perform than alternative techniques for *Aspergillus* identification, such as the microsphere-based Luminex assay [27,29], it might be an appropriate tool to screen multiple colonies from a single clinical sample in order to confirm or rule out the presence of a mixture of different *Aspergillus* species isolates.

As a large number of new species involved in IA have been recently reported, *Aspergillus* species identification has become a real challenge in clinical laboratories. Indeed, a major shift has been observed in the recent epidemiology of

the species responsible for IA. Two distinct phenomena have been observed. First, recent studies have documented cases of IA caused by new *Aspergillus* species, such as *N. fischeri* [14], *N. pseudofischeri* [15], *N. hiratsukae* [16], *A. calidoustus* [19] and *A. niveus* [20]. These species, usually found in the environment (i.e. soil, food and air), had not been identified previously as potential pathogens in susceptible hosts (i.e. immunocompromised patients in a haematological setting). Second, new clinical species, previously unrecognized by phenotypic means, have been identified when collections of *Aspergillus* strains isolated from IA cases have been with the MLS method. These include species encompassing from same section, such as *Aspergillus* species from sections *Usti*, *Fumigati* or *Nidulantes* [10,17,30]. Indeed, these species display very close morphological features. For instance, among 36 isolates previously identified as *A. fumigatus*, it was recently found that MLS clearly distinguished four isolates of *N. uadagawae* from *A. fumigatus* sensu stricto [17]. Moreover, some of these new species seem to be responsible for a form of aspergillosis distinct from typical IA, as the clinical picture displays either chronic or refractory aspects [17]. These studies have stressed the fact that IA caused by unusual *Aspergillus* species may account for a significant proportion of aspergillosis cases, and highlighted the limitations of phenotypic methods for species identification within the *Aspergillus* sections when different antifungal profiles have been reported. Thus, different species from the same section have been shown to display different antifungal susceptibility patterns *in vitro* [11,30], proving that *Aspergillus* identification at the species level might have clinical consequences in terms of primary antifungal therapy. Indeed, recent guidelines recommend *Aspergillus* identification at the species level, to enable adaptation of the antifungal treatment for the *Aspergillus* species involved [2,21].

Our database includes fingerprints of the more relevant species of *Aspergillus* involved in these cases of IA. The panel of reference strains included in our study was concordant with the distribution of *Aspergillus* species isolated from patients. In addition, we have also studied and included the recently described species, which cannot be identified with traditional phenotypic approaches (non-*A. fumigatus* species from the section *Fumigati*, *Emericella quadrilineata* from the section *Nidulantes*, and *A. insuetus* or *Aspergillus pseudodeflectus* from the section *Usti*) [8,10,30]. Our database cannot be exhaustive, because of the extremely large number of *Aspergillus* species (more than 250). However, the database will be rapidly updated when new species involved in human pathology are described. Also, our protocol does not yield identification for atypical isolates of *Aspergillus*, such as sterile isolates. Indeed, in such

instances, the MLS strategy will remain the method of choice for identification.

We believe that MALDI-TOF MS technology provides a real opportunity to improve our knowledge of the epidemiology of medically relevant *Aspergillus* species, by providing fast and accurate identification of *Aspergillus* species isolated in clinical practice. It should be useful in enabling the early adaptation of antifungal therapy, in order to avoid microbiological therapeutic failure when species with natural resistance to some antifungal drugs are identified.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of the 140 isolates of *Aspergillus* spp.

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