ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.02758.x

Use of mass spectrometry to identify clinical *Fusarium* isolates

C. Marinach-Patrice[1,2], A. Lethuillier[3], A. Marly[2], J.-Y. Brossas[4], J. Gené[5], F. Symoens[6], A. Datry[7], J. Guarro[5], D. Mazier[1,2,7] and C. Hennequin[1–3]

1) *Université Pierre et Marie Curie,* 2) *INSERM, UMR S 945,* 3) *APHP, Hôpital St Antoine, Service Parasitologie-Mycologie,* 4) *Centre d'Investigations Biomedicales, Groupe hospitalier Pitié-Salpétrière, Service Parasitologie-Mycologie, Paris, France,* 5) *Unitat de Microbiologia, Facultat de Medicina i Ciencies de la Salut, Universitat Rovira i Virgili, Reus, Spain,* 6) *Mycology Section, Scientific Institute of Public Health, Brussels, Belgium and* 7) *APHP, Groupe hospitalier Pitié-Salpêtrière, Service Parasitologie-Mycologie, Paris, France*

Abstract

Fusarium spp. have recently emerged as significant human pathogens. Identification of these species is important, both for epidemiological purposes and for patient management, but conventional identification based on morphological traits is hindered by major phenotypic polymorphism. In this study, 62 strains, or isolates, belonging to nine *Fusarium* species were subjected to both molecular identification *TEF1* gene sequencing and matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) analysis. Following stringent standardization, the proteomic-based method appeared to be both reproducible and robust. Mass spectral analysis by comparison with a database, built in this study, of the most frequently isolated species, including *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticilloides*, *Fusarium proliferatum* and *Fusarium dimerum*, correctly identified 57 strains. As expected, the four species (i.e. *Fusarium chlamydosporum*, *Fusarium equiseti*, *Fusarium polyphialidicum*, *Fusarium sacchari*) not represented in the database were not identified. Results from mass spectrometry and molecular identification agreed in five of the six cases in which results from morphological and molecular identification were not in agreement. MALDI–TOF yielded results within 1 h, making it a valuable tool for identifying clinical *Fusarium* isolates at the species level. Uncommon species must now be added to the database. MALDI–TOF may also prove useful for identifying other clinically important moulds.

Keywords: Diagnosis, fungal pathogens, *Fusarium*, MALDI–TOF, mass spectrometry
Original Submission: 16 May 2008; **Revised Submission:** 18 July 2008; **Accepted:** 21 August 2008
Editor: M. Drancourt
Article published online: 18 May 2009
Clin Microbiol Infect 2009; **15:** 634–642

Corresponding author and reprint requests: C. Hennequin, Service de Parasitologie-Mycologie, Hôpital St Antoine, 184 rue du Faubourg St Antoine, 75012 Paris, France
E-mail: christophe.hennequin@laposte.net

Introduction

The fungal kingdom is estimated to comprise some 1.5 million species, of which less than one-third have so far been identified. Since the 1980s, the incidence of fungal infections in humans has increased [1,2], as has the diversity of human fungal pathogens [3,4]. Among the most significant emerging pathogens are yeasts such as *Candida glabrata* and filamentous fungi (e.g. *Scedosporium* spp., *Fusarium* spp. and Mucorales). Approximately 500 different fungal species have now been implicated in at least one case of human infection [5]. These emerging fungal pathogens are difficult to identify, owing to a lack of specific tools, and tend to be less susceptible to available antifungal drugs. These two factors may contribute to high case fatality rates.

Identification of fungal pathogens is hindered by several factors. In particular, their taxonomy is sometimes complex and irrational. Indeed, the concept of biological species does not apply to many fungal pathogens (e.g. *Candida albicans*, *C. glabrata* and *Aspergillus fumigatus*) as no sexual stage has yet been described [5]. Approximately 85–95% of yeast isolates can be identified on the basis of biochemical and/or morphological traits. By contrast, the macroscopic and microscopic aspects of filamentous fungi are highly subjective, often leading to inconclusive or erroneous identification. These difficulties have led to the development of molecular approaches to fungal identification. Indeed, the phylogenetic species concept is probably the most reliable, but it requires in-depth analysis before it can be applied to a given group of organisms [6].

The genus *Fusarium* provides an excellent illustration of these difficulties. These filamentous fungi are widespread [7], notably causing cereal diseases with important economic repercussions. *Fusarium solani*, *Fusarium verticillioides* (syn. *Fusarium moniliforme*) and *Fusarium oxysporum* are the most frequently isolated species in the clinical setting, but at least 13 other species have been implicated in human infections. Some *Fusarium* species can cause both superficial mycoses (e.g. onychomycosis and keratitis) and life-threatening invasive infections, mainly in immunocompromised patients [8–10]. The taxonomy of the genus *Fusarium* is one of the most complex, notably because various classifications are used, including subgenus and subspecies categories such as sections and *formae speciales*, and because of the duality between teleomorph (sexual stage) and anamorph (asexual stage) taxonomies. Additionally, phenotypic variability in this genus is well known [11], placing *Fusarium* spp. among the species most difficult to identify by conventional methods (macroscopic and microscopic examination). Molecular approaches have made the situation even more complex as morphological species such as *F. solani* have been demonstrated to encompass not less than 45 distinct phylogenetic species, leading to the denomination of the *F. solani* species complex [12]. Direct sequencing methods targeting different DNA fragments have thus been developed for *Fusarium* species identification [13–16]. Among these targets, the translation elongation factor 1-α (TEF1) gene seems to be the most valuable, but has not been widely used to identify clinical isolates [15,17]. However, correct species identification is important, not only for epidemiological purposes, but also for therapeutic management because, for example, isolates of the *F. solani* species complex are significantly less susceptible than those of other species to new azole derivatives such as voriconazole and posaconazole [18,19].

Matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) analysis is a rapid and sensitive technique that has recently gained popularity as a tool for characterizing microorganisms [20]. Several studies have applied this approach to fungi [21–24], but no standardized protocol and no validated database have been proposed.

Here, we report the use of mass spectrometry to identify clinical *Fusarium* isolates at the species level. The objectives were: (i) to evaluate the reliability of the method compared with molecular identification; (ii) to determine the influence of factors such as growth temperature and culture medium on the mass spectrometry profile, and (iii) to validate a database for routine identification of clinical *Fusarium* isolates.

Materials and Methods

Strains

A set of 62 strains, or isolates, was available for testing (Table 1). Most strains were collected from superficial (skin, ungueal scrapings, cornea) or deep (blood, bronchoalveolar lavage fluid) sites of infection. Two environmental isolates collected within the Hôpital St Antoine, Paris were also included. Twenty-six strains were deposited in the Institute of Hygiene and Epidemiology (IHEM) collection in Brussels (http://bccm.belspo.be/). They were identified according to their macroscopic and microscopic characteristics (conidiogenesis, aspect of conidia), using a reference guide [25]. They belonged to eight different species, namely, *F. oxysporum*, *F. solani*, *F. verticillioides*, *Fusarium proliferatum*, *Fusarium dimerum*, *Fusarium equiseti*, *Fusarium chlamydosporum* and *Fusarium polyphialidicum*, corresponding to those predominantly involved in human infections. All strains were stored at − 80 °C in 10% glycerol until use.

Molecular identification

Molecular identification was based on direct sequencing of a fragment of the *TEF1* gene, as described by Geiser *et al.* [15]. Briefly, strains were sub-cultured on Sabouraud dextrose agar plates for 2 days at 27 °C. DNA was extracted by thermal lysis, followed by chelation of non-DNA cell components, which were discarded by centrifugation [13]. The PCR mix consisted of a 50-μL final volume containing 0.25 mM deoxynucleotide triphosphate, 0.25 μM each primer EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′), 1.5 units of Taq polymerase (New England Biolabs, Inc., Ipswich, MA, USA) and 10 μL DNA. Cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, and a final extension step at 72 °C for 10 min. Sequences were determined on both strands using the Big Dye Terminator protocol (Applied Biosystems, Inc., Courtaboeuf, France) with the primers used for amplification. Following manual correction, sequences were compared using blast software against the *Fusarium-Seq* Version 1.0 database (http://*Fusarium*.cbio.psu.edu) and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) limited to the genus *Fusarium*.

Mass spectrometry

Protein extraction and mass spectrum acquisition. Culture of malt agar plates (AES Laboratoire Groupe, Combourg, France) for 72 h at 27 °C was designated as the reference condition. The surfaces of the colonies were scraped using a sterile scalpel and suspended in 50 μL 80% trifluoroacetic acid (TFA) for

TABLE 1. ***Fusarium*** **strains used in this work with their respective identification using different typing methods**

			Molecular identification[a]		Mass spectrometry identification[b]	
Strain	**Site of isolation**	**Morphological identification**	**blast n first hit**	**% identity**	**Most frequent Biotyper first hit**	**% of Biotyper first hit**
IHEM10150	Reed stem (*Arundo donax*)	*F. chlamydosporum*	–	–	*F. solani*	50
26	Hospital environment	*F. dimerum*	*F. dimerum*	99	*F. dimerum*	75
IHEM10066[c]	Aortic valve	*F. dimerum*	*F. dimerum*	100	*F. dimerum*	100
IHEM19306	Soil	*F. equiseti*	*F. equiseti*	96	*F. solani*	50
4	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
10	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	75
11	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
14	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
20	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
21	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
30	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
32[c]	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
38	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
44[c]	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
52	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	75
53[c]	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
102	Nail	*F. oxysporum*	*F. oxysporum*	100	*F. oxysporum*	100
IHEM19748	Soil	*F. polyphialidicum*	–	–	*F. solani*	50
8[c]	Nail	*F. proliferatum*	*F. proliferatum*	99	*F. proliferatum*	100
47[c]	Cutaneous biopsy	*F. proliferatum*	*F. proliferatum*	99	*F. proliferatum*	100
49	Bronchial aspiration	*F. proliferatum*	*F. proliferatum*	99	*F. proliferatum*	100
101	Nail	*F. proliferatum*	*F. proliferatum*	100	*F. proliferatum*	100
IHEM3824	Not specified	*F. verticillioides*	*F. proliferatum*	99	*F. proliferatum*	100
IHEM5689	Cutaneous biopsy	*F. verticillioides*	*F. proliferatum*	99	*F. proliferatum*	100
IHEM6824	Carpet dust	*F. proliferatum*	*F. proliferatum*	100	*F. proliferatum*	100
IHEM7412	Cornea	*F. verticillioides*	*F. pseudonygamai*	99	*F. proliferatum*	100
IHEM10152[c]	Banana bud rot (*Musa* sp.)	*F. proliferatum*	*F. proliferatum*	100	*F. proliferatum*	100
IHEM20165	Cutaneous biopsy	*F. proliferatum*	–	–	*F. proliferatum*	100
IHEM20272	Sinus	*F. proliferatum*	*F. proliferatum*	100	*F. proliferatum*	100
2	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
3	Nail	*F. solani*	*F. solani*	100	*F. solani*	75
6	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
7	Nail	*F. solani*	*F. solani*	100	*F. solani*	75
16	Toeweb	*F. solani*	*F. solani*	100	*F. solani*	100
17	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
22	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
23[c]	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
24	Nail	*F. solani*	*F. solani*	100	*F. solani*	75
31	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
33	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
35	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
36	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
43	Nail	*F. solani*	*F. solani*	100	*F. solani*	100
46	Cutaneous biopsy	*F. solani*	*F. solani*	100	*F. solani*	100
50[c]	Toe web	*F. solani*	*F. solani*	100	*F. solani*	100
54	Nail	*F. solani*	*F. solani*	99	*F. solani*	100
IHEM15469[c]	Blood	*F. solani*	*F. solani*	99	*F. solani*	100
IHEM4194	Blood	*F. proliferatum*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM4195	Blood	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM4197[c]	Peritoneal fluid	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9526	Aortic valve	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9529	Drain	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9533	Pericardial fluid	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9576	Vitreous humour	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9577	Eye	*F. proliferatum*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9578	Hospital environment	*F. proliferatum*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM9835	Oral secretion	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM10153[c]	Maize stem (*Zea mays*)	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM18495[c]	Hospital environment (sink)	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
IHEM19667	Blood	*F. verticillioides*	*F. verticillioides*	99	*F. verticillioides*	100
IHEM20180	Sinus biopsy	*F. verticillioides*	*F. verticillioides*	100	*F. verticillioides*	100
103	Blood	*Fusarium* sp.	*F. sacchari*	99	*F. proliferatum*	50

All clinical strains were isolated from human; strains with identification discrepancies are underlined.
[a]Molecular identification based on *TEF1* gene sequence comparison.
[b]Mass spectrometry identification based on an algorithm (see Fig. 1).
[c]Selected strains used to construct the database.

30 min. Then 150 μL of distilled water was added, followed by 200 μL of acetonitrile. The mixture was centrifuged at 12 000 **g** for 2 min, and the supernatant was either vacuum-dried and stored at − 80 °C, or directly spotted (0.5 μL droplet) in duplicate onto a MALDI AnchorChip sample slide (Bruker-Daltonik GmbH, Bremen, Germany) and then air-dried. The α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix (Bruker-Daltonik GmbH), prepared at a concentration of

50 mg/mL in 50% acetonitrile, 50% water with 0.1% TFA, was sonicated for 5 min before being spotted (0.5 μL) over the dried sample. A DH5 alpha *Escherichia coli* protein extract (Bruker-Daltonik GmbH) was deposited on the calibration spot of the AnchorChip for external calibration.

MALDI analysis was performed on a Bruker Autoflex I MALDI–TOF mass spectrometer with a nitrogen laser (337 nm) operating in linear mode with delayed extraction (260 ns) at 20 kV accelerating voltage. Each spectrum was automatically collected in the positive ion mode as an average of 500 laser shots (50 laser shots at 10 different spot positions). Laser energy was set just above the threshold for ion production. Based on previous work [23], a mass range of 3000–20 000 m/z (ratio mass/charge) was selected to collect the signals with the AutoXecute tool of Flexcontrol acquisition software (Version 2.4; Bruker-Daltonik GmbH). Only peaks with a signal/noise ratio > 3 were considered. Spectra were eligible for further analysis when the peaks had a resolution better than 600.

Species identification process. Data were processed with Biotyper 1.0 software (Bruker-Daltonik GmbH) in order to obtain a normalized spectrum with respect to the highest peak. All peaks in the m/z mass range 3000–20 000, with a threshold of 0.01 (minimum 1% of the highest peak intensity), were considered. The spectrum profiles obtained were then transformed into tables, including mass peaks and their respective intensities, which were used for further comparison. Based on preliminary results, the number of relevant peaks in mass spectra was limited to 50 (data not shown). A database was built using main spectrum profiles (MSPs) of three strains of *F. oxysporum*, *F. solani*, *F. verticillioides* and *F. proliferatum* and one strain of *F. dimerum* (Table 1). The MSP consists of a general profile created from the peaks retrieved from at least 50% of 10 spectra collected under the same conditions (five extracts collected under the same conditions tested in duplicate). The comparison of new spectra against the database releases a first hit associated with two scores: real score (RS) and real p-number (RPN). Real score is defined as the number of peaks of the unknown species that can be attributed to an MSP, and RPN refers to the proportion of non-attributed peaks of the test strain in relation to the proportion of attributed peaks of the associated MSP. The closer the values of these scores are to 1, the more significant is the hit. Significant thresholds recommended by the manufacturer are 0.3 and 0.2, respectively.

Evaluation of the method. The robustness of the method to identify species was evaluated by testing a number of possible influencing factors. This was performed in five strains representative of each of the species most frequently isolated from human infections, namely, *F. oxysporum* (no. 32), *F. proliferatum* (no. 8), *F. verticillioides* (IHEM4197), *F. dimerum* (IHEM10066) and *F. solani* (IHEM15469). Each generated spectrum was compared with the MSP database, and the Biotyper first hit, RS and RPN were recorded. Repeatability was evaluated by testing colonies sampled from three different areas of the same culture plate. Results of duplicate assays of each extract were also analysed. Reproducibility was evaluated by comparing spectra obtained from two independent experiments using our reference protocol (malt agar, incubation at 27 °C for 72 h). To determine the effects of the culture medium, strains were plated on our reference medium of malt agar and two other media, Sabouraud chloramphenicol (200 mg/L) gentamicin (40 mg/L) agar (SCG) (Bio-Rad, Marnes la Coquette, France), and potato dextrose agar (PDA) (Difco Laboratories, Paris, France), with incubation at 27 °C for 72 h. The effect of temperature was evaluated by comparing spectra obtained from colonies cultured on malt agar for 72 h at 27 °C and 37 °C with the MSPs of the database. Three colonies per plate were tested in duplicate, thus providing six comparison scores which were used to establish a mean. Finally, the stability of the profiles over generations was tested using a series of extracts obtained from subcultures performed every 72 h over 6 weeks (11–13 extracts). For these experiments, spectra were generated from three colonies sampled from each plate (six spectra per strain).

To validate our database as a tool for routine species identification, a panel of 49 morphologically and molecularly identified strains was blindly tested using the MALDI–TOF procedure. For each strain, four spectra, obtained from two colonies tested in duplicate, were collected using our protocol, and compared with the MSPs of the database using Biotyper software. The species first hit, RS and RPN were collected for each spectrum. However, because the spectra obtained with *F. solani* strains showed a very limited number of peaks, we reduced the RPN score threshold for validated identification to 0.1. An algorithm for *Fusarium* species identification was thus developed, taking into account the results obtained for the four profiles associated with each strain and their respective RS and RPN scores (Fig. 1).

Results

A panel of 62 *Fusarium* strains was tentatively identified at the species level with both DNA sequencing and mass spectrometry.

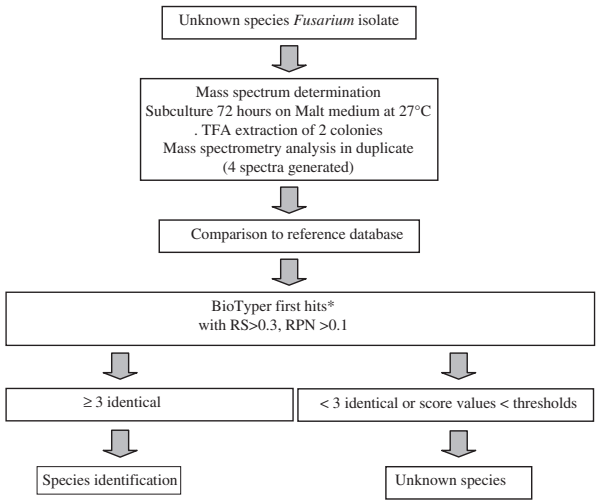

FIG. 1. Algorithm for identification of *Fusarium* species using a standardized mass spectrometry profiling protocol. *at the species level. RS, real score; RPN, real p-number (see text for explanation).

Molecular identification

A partial sequence of the *TEF1* gene was obtained for 59 strains and compared with the GenBank and *Fusarium*-Seq databases. Of these strains, 47 had 100% identity with at least one sequence in the databases. Eleven strains, of which six had the morphological aspect of *F. proliferatum*, exhibited 99% sequence identity with strains of this species and one *F. equiseti* strain (IHEM19306) achieved sequence identity of only 96%. Discrepancies between morphological and molecular identification were found for six strains; three of these with the morphological aspect of *F. proliferatum* were identified as *F. verticillioides* using sequencing, and three strains with the morphological aspect of *F. verticillioides* were identified as *F. proliferatum* (two strains) and *Fusarium pseudonygamai* (one strain) using sequencing (Table 1). One strain that could not be identified at species level by morphological methods was identified as *Fusarium sacchari* based on the nucleotide sequence of the *TEF1* gene.

Mass spectrometry identification

The majority of ions detected were in the range of 3000–10 000 Da. Figure 2 shows examples of spectra collected before the pre-processing step. For further comparisons, a database composed of MSPs from strains representative of the five species most frequently isolated in human infections (*F. solani*, *F. verticillioides*, *F. oxysporum*, *F. proliferatum* and *F. dimerum*) was constructed. No single peak could be identified as a marker of a given species, and no single peak was common to all the species tested.

The robustness of the MALDI–TOF method was assessed with five representative strains of the main species. Repeatability was evaluated at 100%, as testing colonies from three areas of the same plate and duplicate tests unambiguously identified the species, with RS and RPN values of 0.63–0.98 and 0.39–0.91, respectively. Interestingly, some profiles appeared to be strain-specific as the first match corresponded to the particular strain tested. Similar to repeatability, reproducibility reached 100%, with a high conservation of spectrum characteristics between extracts prepared from two different subcultures, and RS and RPN scores of 0.62–0.90 and 0.58–0.90, respectively. The stability of the spectra was tested on serial subcultures for five species (Table 2). Overall, the spectra exhibited minimal changes upon visual observation. Whatever the species, the six spectra obtained at each time-point were correctly identified up to subculture 9, with scores of 0.43–0.72 and 0.45–0.80 for RS and RPN, respectively. This remained true for subculture 11 of *F. oxysporum* and *F. solani* and for subculture 13 of *F. verticillioides* and *F. dimerum*, but was not confirmed for subculture 11 of *F. proliferatum*, for which only two-thirds of the spectra were correctly identified. In addition, the BIOTYPER first hit provided accurate species identification. Results obtained under different growth conditions are summarized in Table 3. Compared with malt agar at 27 °C, growth on PDA medium gave 100% correct species identification, with highly similar profiles (RS 0.66–0.88, RNP 0.38–0.64), independent of species. Similar results were obtained with SCG medium (RS 0.58–0.83, RNP 0.37–0.62), except for *F. verticillioides*. This latter species could not be identified when grown at either 37 °C on malt medium or 27 °C on SCG medium. Other thermotolerant species, such as *F. dimerum* and *F. proliferatum*, were correctly identified, but scores were generally lower.

Mass spectrometry-based identification relied on an algorithm developed for routine species identification (Fig. 1). Forty-five of the 49 strains were correctly identified as belonging to one of the five species represented in our database (Table 1). Among the 45 strains correctly identified, four spectra tested identical to one of the MSPs of the database in 39 strains, and three out of four spectra tested identical to one MSP in six strains.

The four strains with ambiguous results belonged to species not included in our database (*F. polyphialidicum*, *F. equiseti*, *F. chlamydosporum* and *F. sacchari*). For five of six strains showing discrepancies between morphological and molecular identification, mass spectrometry gave the same result as DNA sequencing. Finally, strain IHEM7412, identified morphologically as *F. verticillioides* and molecularly as *F. pseudonygamai* (99% sequence identity), was identified as *F. proliferatum* by mass spectrometry.

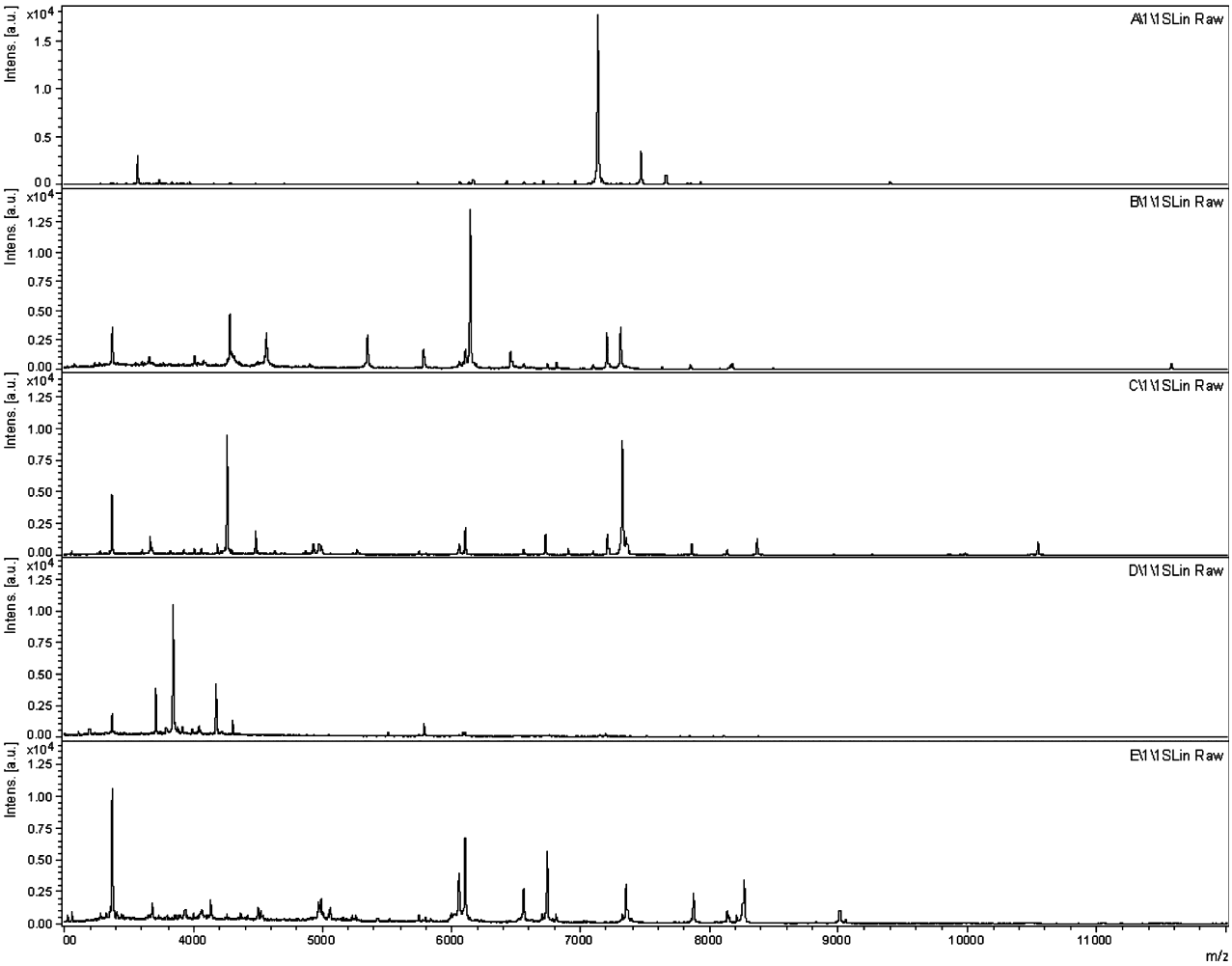

FIG. 2. Matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectra (raw data with no signal treatment) of strains (see Table 1) used for robustness experiments: (a) *Fusarium dimerum* IHEM10066; (b) *Fusarium oxysporum* no. 32; (c) *Fusarium proliferatum* no. 8; (d) *Fusarium solani* IHEM15469, and (e) *Fusarium verticillioides* IHEM4197.

TABLE 2. Stability testing comparing spectra collected from serial subcultures with the database

Fusarium strain	Subculture no.	BIOTYPER first hit	Mean RS ± SD[a]	Mean RPN ± SD[a]
F. dimerum IHEM10066	5	*F. dimerum* IHEM10066	0.91 ± 0.11	0.53 ± 0.06
	7	*F. dimerum* IHEM10066	0.72 ± 0.09	0.80 ± 0.11
	13	*F. dimerum* IHEM10066	0.59 ± 0.11	0.87 ± 0.07
F. oxysporum no. 32	3	*F. oxysporum* no. 32	0.75 ± 0.03	0.54 ± 0.03
	7	*F. oxysporum* no. 32	0.43 ± 0.06	0.63 ± 0.13
	11	*F. oxysporum* no. 32	0.35 ± 0.08	0.40 ± 0.04
F. proliferatum no. 8	3	*F. proliferatum* no. 8	0.58 ± 0.02	0.68 ± 0.09
	9	*F. proliferatum* no. 8	0.46 ± 0.11	0.50 ± 0.15
	11	Discrepancies[b]	NA	NA
F. verticillioides IHEM4197	5	*F. verticillioides* IHEM4197	0.46 ± 0.08	0.30 ± 0.08
	7	*F. verticillioides* IHEM4197	0.65 ± 0.05	0.45 ± 0.04
	13	*F. verticillioides* IHEM10153	0.61 ± 0.04	0.41 ± 0.01
F. solani IHEM15469	3	*F. solani* IHEM15469	0.47 ± 0.07	0.49 ± 0.05
	7	*F. solani* IHEM15469	0.58 ± 0.11	0.48 ± 0.08
	11	*F. solani* IHEM15469	0.74 ± 0.07	0.54 ± 0.06

RS, real score; RPN, real p-number; SD, standard deviation; NA, not applicable.
[a]Mean of scores obtained from BIOTYPER software, calculated for three colonies tested in duplicate.
[b]Two spectra were identified as *Fusarium proliferatum* no. 8 and the remaining four corresponded to 'unknown species'.

TABLE 3. Influence of culture conditions (medium and temperature) on mass spectrometry identification

Fusarium strain	Culture conditions: temperature, medium	Biotyper first hit	Mean RS ± SD[a]	Mean RPN ± SD[a]
F. dimerum IHEM10066	27 °C, PDA	*F. dimerum* IHEM10066	0.69 ± 0.10	0.38 ± 0.13
	27 °C, SCG	*F. dimerum* IHEM10066	0.83 ± 0.01	0.37 ± 0.04
	37 °C, Malt	*F. dimerum* IHEM10066	0.67 ± 0.09	0.39 ± 0.13
F. oxysporum no. 32	27 °C, PDA	*F. oxysporum* no. 32	0.80 ± 0.04	0.58 ± 0.04
	27 °C, SCG	*F. oxysporum* no. 32	0.74 ± 0.04	0.54 ± 0.02
	37 °C, Malt	Negative culture	–	–
F. proliferatum no. 8	27 °C, PDA	*F. proliferatum* no. 8	0.66 ± 0.04	0.43 ± 0.03
	27 °C, SCG	*F. proliferatum* no. 8	0.82 ± 0.04	0.62 ± 0.05
	37 °C, Malt	*F. proliferatum* no. 8	0.60 ± 0.06	0.36 ± 0.04
F. verticillioides IHEM4197	27 °C, PDA	*F. verticillioides* IHEM4197	0.66 ± 0.11	0.45 ± 0.08
	27 °C, SCG	Unknown species	–	–
	37 °C, Malt	Unknown species	–	–
F. solani IHEM15469	27 °C, PDA	*F. solani* IHEM15469	0.88 ± 0.01	0.64 ± 0.01
	27 °C, SCG	*F. solani* IHEM15469	0.58 ± 0.06	0.42 ± 0.06
	37 °C, Malt	Negative culture	–	–

RS, real score; RPN, real p-number; SD, standard deviation; PDA, potato dextrose agar; SCG, Sabouraud chloramphenicol gentamicin; Malt, malt agar.
[a]Mean of scores obtained from BIOTYPER software, calculated for three colonies tested in duplicate.

Discussion

In recent years, mass spectrometry has emerged as a powerful tool for the identification of bacteria [26–28]. Studies concerning fungal identification using this technology have involved a limited number of strains belonging to different species of a given genus [22–24], or have focused on non-human pathogens [21]. No validated databases have been developed for routine fungal identification.

Here, we investigated the value of MALDI–TOF for the identification of *Fusarium* spp. [3,29]. Molecular approaches are still time-consuming and targeting the *TEF1* gene remains uncertain for some *Fusarium* species. Indeed, using two different DNA databases, no perfect sequence matches were found for 12 (19%) of the 62 strains tested in the present study, although a 100% identity is recommended by the curators of the *Fusarium* database for species identification [15]. Thus, although our experience shows that 99% sequence identity fully agrees with morphological identification, other diagnostic tools are needed to facilitate the identification of *Fusarium* species.

Our undertaking was driven by the goal of providing a validated protocol and a database that can be widely used and shared. Our database is made up of 13 MSPs from representatives of the main species involved in human infections. The database can be integrated into the Biotyper software and can be easily implemented on other sites.

We first standardized pre-analytical steps such as the culture conditions and protein extraction. We selected a 72-h incubation period, corresponding to the stationary phase of growth, thus limiting the variability of the protein content. Malt medium was chosen as the reference medium because it promotes fructification, which forms the basis for morphological identification. Finally, the temperature was set at 27 °C, as growth may be inconsistent at 37 °C, notably for *F. oxysporum* [30]. As for bacteria [21,23], the choice of culture medium can affect the mass spectrometry profiles of *Fusarium*. Indeed, MALDI–TOF analysis produced more distinguishable peaks on malt agar and PDA media than on SCG medium. However, these subtle differences did not hamper identification, with the exception of *F. verticillioides*. The spectrum of this latter species was also strongly influenced by the incubation temperature, whereas other species were not significantly affected. Thus, the best growth conditions for MALDI–TOF analysis appear to be malt agar or PDA at 27 °C for 72 h. Finally, the spectra were significantly affected only after 11 subcultures; thus, subculturing should not hamper the identification of isolates using MALDI–TOF profiling.

Identification of bacteria using mass spectrometry is generally performed by comparison with a fingerprint database collection [26,28,31,32]. Such a strategy enables microbiologists to obtain correct identification rates of 75–100%. However, it has been pointed out that species-specific markers are difficult to demonstrate in spectra because overlapping signal ions increase along with the number of strains registered in the database. Identification using a protein sequence database has also been proposed, but this elegant method needs a complete annotated genome sequence and a high-resolution mass spectrometer to be effectively performed [33]. In our study, 44 of 48 strains were accurately identified in a first analysis, carried out blindly, and only one case of discrepancy was finally recorded. This shows that by selecting a set of representative strains and retaining the most conserved peaks for each MSP, a database can be reliably used for *Fusarium* species identification.

Indeed, mass spectrometry and molecular identification provide results that are consistent, whereas morphological identification appeared to misidentify at least five strains. This is not surprising because previous work on *Fusarium* [15,34] and other filamentous fungi [35] has shown similar discrepancies. Interestingly, all four strains belonging to species not included in our database, but identified from their nucleotide sequences, did not meet the conditions for correct identification (fewer than three spectra with the same results) and were thus considered as unidentified species. Reference strains belonging to these species should be tested to be included in our database. The only discrepancies between the molecular and MALDI–TOF identification involved the species *F. verticillioides*, *F. proliferatum* or *F. pseudonygamai*, three species known to be closely related.

One major advantage of MALDI–TOF analysis is that identification can be achieved within 1 h, including the extraction step. Indeed, MALDI–TOF analysis can include dozens of strains within the same run. By contrast, 1-week-old subculture on PDA medium is often needed to obtain discriminatory morphological characteristics, and molecular identification takes a minimum of 2 days.

As these data suggest that, in some instances, not only species, but also strains can be recognized, the capacity of MALDI–TOF analysis for infra-species discrimination should now be investigated. The use of MALDI–TOF analysis to identify more common fungal pathogens, notably *Aspergillus*, would also be of great interest. For example, it has recently been shown that *A. fumigatus*, the species most commonly involved in human aspergillosis, forms a complex of different species with distinct *in vitro* susceptibilities to antifungal drugs [36]. MALDI–TOF has the potential to become an essential tool for the identification of pathogenic moulds.

Acknowledgments

We thank J. L. Poirot, G. Buot and J. Dunand for sending us clinical isolates. We are indebted to our laboratory technicians, S. Vellaissamy and S. Thorez, for their excellent work.

Transparency Declaration

This investigation received financial support from Pierre et Marie Curie University, Paris (BQR 2006). The authors have no relationships (commercial or otherwise) that might constitute dual or conflicting interests.

References

1. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003; 348: 1546–1554.
2. Beck-Sague C, Jarvis WR. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. *J Infect Dis* 1993; 167: 1247–1251.
3. Marr KA, Carter RA, Crippa F, Wald A, Corey L. Epidemiology and outcome of mould infections in haematopoietic stem cell transplant recipients. *Clin Infect Dis* 2002; 34: 909–917.
4. Trick WE, Fridkin SK, Edwards JR, Hajjeh RA, Gaynes RP. Secular trend of hospital-acquired candidaemia among intensive care unit patients in the United States during 1989–1999. *Clin Infect Dis* 2002; 35: 627–630.
5. Guarro J, Gené J, Stchigel AM. Developments in fungal taxonomy. *Clin Microbiol Rev* 1999; 12: 454–500.
6. Taylor JW, Jacobson DJ, Kroken S *et al.* Phylogenetic species recognition and species concepts in fungi. *Fungal Genet Biol* 2000; 31: 21–32.
7. Nelson PE, Dignani MC, Anaissie EJ. Taxonomy, biology, and clinical aspects of *Fusarium* species. *Clin Microbiol Rev* 1994; 7: 479–504.
8. Hennequin C, Lavarde V, Poirot JL *et al.* Invasive *Fusarium* infections: a retrospective survey of 31 cases. The French 'Groupe d'Etudes des Mycoses Opportunistes' GEMO. *J Med Vet Mycol* 1997; 35: 107–114.
9. Dignani MC, Anaissie E. Human fusariosis. *Clin Microbiol Infect* 2004; 10 (Suppl 1): 67–75.
10. Nucci M, Anaissie E. *Fusarium* infections in immunocompromised patients. *Clin Microbiol Rev* 2007; 20: 695–704.
11. Guarro J, Gené J. *Fusarium* infections. Criteria for the identification of the responsible species. *Mycoses* 1992; 35: 109–114.
12. Zhang N, O'Donnell K, Sutton DA *et al.* Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. *J Clin Microbiol* 2006; 44: 2186–2190.
13. Hennequin C, Abachin E, Symoens F *et al.* Identification of *Fusarium* species involved in human infections by 28S rRNA gene sequencing. *J Clin Microbiol* 1999; 37: 3586–3589.
14. Summerbell RC, Schoers HJ. Analysis of phylogenetic relationship of *Cylindrocarpon lichenicola* and *Acremonium falciforme* to the *Fusarium solani* species complex and a review of similarities in the spectrum of opportunistic infections caused by these fungi. *J Clin Microbiol* 2002; 40: 2866–2875.
15. Geiser DM, del Mar Jimenez-Gasco M, Kang S *et al.* Fusarium-ID v1.0: a DNA sequence database for identifying Fusarium. *Eur J Plant Pathol* 2004; 110: 473–479.
16. Ninet B, Jan I, Bontems O *et al.* Molecular identification of *Fusarium* species in onychomycoses. *Dermatology* 2005; 210: 21–25.
17. Dornbusch HJ, Buzina W, Summerbell RC *et al. Fusarium verticillioides* abscess of the nasal septum in an immunosuppressed child: case report and identification of the morphologically atypical fungal strain. *J Clin Microbiol* 2005; 43: 1998–2001.
18. Diekema DJ, Messer SA, Hollis RJ, Jones RN, Pfaller MA. Activities of caspofungin, itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B against 448 recent clinical isolates of filamentous fungi. *J Clin Microbiol* 2003; 41: 3623–3626.
19. Azor M, Gené J, Cano J, Guarro J. Universal *in vitro* antifungal resistance of genetic clades of the *Fusarium solani* species complex. *Antimicrob Agents Chemother* 2007; 51: 1500–1503.
20. Lay JO Jr. MALDI-TOF mass spectrometry of bacteria. *Mass Spectrom Rev* 2001; 20: 172–194.

21. Chen HY, Chen YC. Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* 2005; 19: 3564–3568.
22. Li TY, Liu BH, Chen YC. Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2000; 14: 2393–2400.
23. Valentine NB, Wahl JH, Kingsley MT, Wahl KL. Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* 2002; 16: 1352–1357.
24. Welham KJ, Domin MA, Johnson K, Jones L, Ashton DS. Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2000; 14: 307–310.
25. de Hoog GS, Guarro J, Gené J, Figueras MJ. *Atlas of Clinical Fungi*, 2nd edn. Washington, DC: ASM Press, 2001.
26. Carbonnelle E, Beretti JL, Cottyn S *et al.* Rapid identification of *Staphylococci* isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 2007; 45: 2156–2161.
27. Hettick JM, Kashon ML, Slaven JE *et al.* Discrimination of intact mycobacteria at the strain level: a combined MALDI-TOF MS and biostatistical analysis. *Proteomics* 2006; 6: 6416–6425.
28. Keys CJ, Dare DJ, Sutton H *et al.* Compilation of a MALDI-TOF mass spectral database for the rapid screening and characterization of bacteria implicated in human infectious diseases. *Infect Genet Evol* 2004; 4: 221–242.
29. Sugar J. The contact lens/fusarium 'outbreak'. *Am J Ophthalmol* 2006; 142: 146.
30. Hennequin C, Ranaivoarimalala C, Chouaki T *et al.* Comparison of aerobic standard medium with specific fungal medium for detecting *Fusarium* spp. in blood cultures. *Eur J Clin Microbiol Infect Dis* 2002; 21: 748–750.
31. Jarman KH, Cebula ST, Saenz AJ *et al.* An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. *Anal Chem* 2000; 72: 1217–1223.
32. Mellmann A, Cloud J, Maier T *et al.* Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of non-fermenting bacteria. *J Clin Microbiol* 2008; 46: 1946–1954.
33. Amiri-Eliasi B, Fenselau C. Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. *Anal Chem* 2001; 73: 5228–5231.
34. Klaasen JA, Nelson PE. Identity of *Fusarium nygamai* isolates with long and short microconidial chains from millet, sorghum and soil in Africa. *Mycopathologia* 1997; 140: 171–176.
35. Kuhls K, Lieckfeldt E, Borner T, Gueho E. Molecular reidentification of human pathogenic *Trichoderma* isolates as *Trichoderma longibrachiatum* and *Trichoderma citrinoviride*. *Med Mycol* 1999; 37: 25–33.
36. Balajee SA, Nickle D, Varga J, Marr KA. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryot Cell* 2006; 5: 1705–1712.