

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for rapid identification of fungal rhinosinusitis pathogens

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Abstract

Purpose. Filamentous fungi are among the most important pathogens, causing fungal rhinosinusitis (FRS). Current laboratory diagnosis of FRS pathogens mainly relies on phenotypic identification by culture and microscopic examination, which is time consuming and expertise dependent. Although matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has been employed to identify various fungi, its efficacy in the identification of FRS fungi is less clear.

Methodology. A total of 153 FRS isolates obtained from patients were analysed at the Clinical Laboratory at the Beijing Tongren Hospital affiliated to the Capital Medical University, between January 2014 and December 2015. They were identified by traditional phenotypic methods and Bruker MALDI-TOF MS (Bruker, Biotyper version 3.1), respectively. Discrepancies between the two methods were further validated by sequencing.

Results. Among the 153 isolates, 151 had correct species identification using MALDI-TOF MS (Bruker, Biot 3.1, score ≥ 2.0 or 2.3). MALDI-TOF MS enabled identification of some very closely related species that were indistinguishable by conventional phenotypic methods, including 1/10 *Aspergillus versicolor*, 3/20 *Aspergillus flavus*, 2/30 *Aspergillus fumigatus* and 1/20 *Aspergillus terreus*, which were misidentified by conventional phenotypic methods as *Aspergillus nidulans*, *Aspergillus oryzae*, *Aspergillus japonicus* and *Aspergillus nidulans*, respectively. In addition, 2/2 *Rhizopus oryzae* and 1/1 *Rhizopus stolonifer* that were identified only to the genus level by the phenotypic method were correctly identified by MALDI-TOF MS.

Conclusion. MALDI-TOF MS is a rapid and accurate technique, and could replace the conventional phenotypic method for routine identification of FRS fungi in clinical microbiology laboratories.

INTRODUCTION

Fungal rhinosinusitis (FRS) is characterized by inflammation of the nasal and paranasal sinus mucosae. With increase in the usage of antibiotics, immunoagents, chemotherapy and radiotherapy, FRS has been reported with increasing frequency over recent decades worldwide [1]. The disease occurs in two distinct forms: noninvasive and invasive FRS. Acute invasive FRS, one subgroup of the latter form, predominantly occurs in immunocompromised patients and is associated with a mortality rate of above 50 % [2–4]. Misdiagnosis of FRS may lead to treatment failure or even death. *Aspergillus fumigatus* accounts for up to 95 % of FRS cases. Other pathogens responsible are primarily filamentous fungi, including *Aspergillus flavus*, *Aspergillus niger*,

dematiaceous hyphomycetes, *Pseudallescheria boydii*, *Fusarium* spp. and *Mucorales* [2, 3]. Currently, FRS diagnosis is primarily based on clinical manifestations, endoscopy, imaging, pathological examination and morphological mould identification. Nevertheless, identification of the specific FRS fungus involved is often challenging in clinical laboratories with these conventional examinations, since the wide variety of fungal pathogens is often phenotypically similar despite their genetically and/or pathogenically different from their counterparts [5–7]. On the other hand, although nucleic acid-based methods such as PCR and sequencing of conserved genes of fungal species can provide definitive identification of FRS pathogens, these are expensive and require special expertise.

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Keywords: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; fungal rhinosinusitis; filamentous fungi; rapid identification.

Abbreviations: FRS, fungal rhinosinusitis; ITS, internal transcribed spacer; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight. Seven supplementary figures are available with the online Supplementary Material.

In recent years, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has emerged as a powerful tool for rapid and accurate identification of microbes. The method is based on identification of the protein composition of the microbial cell, and is robust, inexpensive and rapid [8, 9]. Although MALDI-TOF MS has been proven extremely useful for the identification of a wide range of bacteria and yeasts [10–16], very few recent studies have focused on its clinical application in the identification of FRS pathogens. Correct species-level identification of FRS fungi, however, is urgently needed for understanding the clinical significance of these isolates and effective treatment of the disease.

As one of the top departments of otolaryngology in China, our hospital treats approximately 400 cases of FRS annually. According to our statistics covering the past 10 years, the majority of FRS pathogens detected in our patients are *Aspergillus* spp., followed by *Alternaria alternata*, *Scedosporium apiospermum*, *Schizophyllum* spp. and *Rhizopus* spp. (data not shown). In this study, we evaluated the performance of MALDI-TOF MS in identifying 153 FRS fungal isolates.

METHODS

Clinical isolates

All fungal strains were prospectively isolated from positive cultures obtained from sinus specimens of FRS patients that were analysed at the Clinical Laboratory at Beijing Tongren Hospital affiliated to the Capital Medical University, between January 2014 and December 2015. Fungal ball FRS and allergic FRS were initially diagnosed by clinical symptoms, nasal endoscopy (Olympus) and sinus CT scan (GE), and confirmed by post operative maxillary sinus puncture and positive fungal culture. Invasive FRS was diagnosed based on clinical symptoms, sinus CT scan and positive fungal culture, and confirmed by frozen section biopsy of the suspected tissues. These isolates were stored at -70°C for subsequent concurrent identification. This study was approved by the Research Ethics Committee at Beijing Tongren Hospital affiliated to the Capital Medical University.

Phenotypic identification

All isolates were prospectively identified using phenotypic methods following the routine diagnostic flow of the laboratory. Briefly, isolates were recovered on Sabouraud dextrose agar plates at 28°C for 3–10 days. The size, colour, shape, texture and growth rate of each isolate were recorded. Following spore germination, fungal smears were subjected to lactic acid phenol Medan and 10% KOH staining, and observed under an Olympus IX53 inverted microscope (Olympus). The phenotypic features of each fungal isolate were recorded, including the shape and colour of hyphae and vesicles, spore arrangement, the range of vesicles covered by spores as well as the colour and size of meristem spores. The fungi were identified according to criteria described previously [17, 18]. Phenotypic identification was performed

independently by two experienced pathologists in the pathology lab, and by a third pathologist in case of discrepancy.

MALDI-TOF MS

The fungal isolates were cultured and prepared for MALDI-TOF MS according to the manufacturer's recommendations, with modifications. Briefly, the fungal isolates were incubated in liquid Sabouraud medium on a shaker at 28°C for 24–48 h until abundant mycelia were observed. The same fungal isolates were also inoculated onto Sabouraud agar plate and incubated at 28°C in order to determine which cultivation method yielded better MALDI-TOF MS results. The fungi were collected by centrifugation at $16\,438\text{g}$ for 2 min. The pellet was washed three times with 1 ml of 75% ethanol, and centrifuged at $16\,438\text{g}$ for 2 min. The pellet was completely air-dried and mixed with an equal volume of 0.1 mm beads. The pellet was resuspended in 50 μl of 70% formic acid (v/v) and 50 μl of acetonitrile, vortexed until completely dissolved and centrifuged at $16\,438\text{g}$ for 2 min. The supernatant (1 μl) was applied in duplicate onto the steel target plate, and allowed to air-dry. Bruker Matrix HCCA (α -Cyano-4-hydroxycinnamic acid) solution (1 μl) was applied to the spot and allowed to dry at room temperature. Measurements were performed with a MALDI-TOF Microflex LT system (Bruker Daltonics). Protein mass fingerprints were recorded in linear positive mode at a laser frequency of 60 Hz within a mass range of 2 to 20 kDa. Each spectrum was obtained from 300 shots in 100-shot steps from different positions of the sample. The device was calibrated by standard substance (Bruker Daltonics). Quality assurance was done before each analysis using an *Aspergillus flavus* strain (CGMCC3.1398) and a *Aspergillus nidulans* strain (CGMCC3.1322) obtained from the Chinese General Microbiological Culture Collection Center (www.cgmcc.net/english/). Each spectrum was analysed using the Biotyper version 3.1 and Bruker filamentous fungi library V1.0. We used a lower threshold of 1.7 for genus identification and 2.0 for species identification. The identification was considered definitive when no discrepancy between two replicates was detected.

Sequencing

The isolates with identification discrepancy between phenotypic and MALDI-TOF MS were further confirmed by sequencing as described previously [19]. The internal transcribed spacer (ITS) region and β -tubulin gene were amplified by PCR (TaKaRa PCR Thermal Cycler Dice) using the following primers: ITS1, 5'-TCCGTAGGTGAACCTGCGG-3', ITS4, 5'-TCCTCCGCTTATTGATATG-3'; and β -tubulin forward βtub1 , 5'-AATTGGTGCCGCTTCTGG-3', reverse βtub2 , 5'-AGTTGTCGGGACGGAATAG-3'. The PCR system (30 μl) was prepared: 15 μl 2 $\times$ easyTaq Super-Mix (Takara), 1 μl forward primer, 1 μl reverse primer, 3 μl extracted fungal genome (100–120 ng μl^{-1}) and 10 μl H_2O . The reaction conditions were as follows: denaturation at 94°C for 5 min, followed by 35 cycles of 94°C 30 s, 55°C 45 s and 72°C 1 min, and extension at 72°C for 10 min. The PCR fragments were sequenced using a GenomeLab TM GeXP

Genetic Analysis System (Beckman Coulter). Sequences were analysed with BLAST software against GenBank, the European Molecular Biology Laboratory nucleotide sequence database (EMBL) and DNA Data Bank of Japan (DDBJ). Strains were identified based on a 99 % similarity or higher.

RESULTS

FRS patients and fungal isolates

Among the 153 patients, 110 were fungal ball FRS, 40 were allergic FRS and 3 were invasive FRS with bone destruction and invasion as suggested by both CT scan and histopathological exam. All three invasive FRS patients had concurrent disease (two had diabetes and one had Wegener's granulomatosis). Each patient had infection by only one fungal species, and no patient had two or more species. The number of the fungal species isolated from different FRS forms is summarized in Table 1. Altogether, this study enrolled a total of 153 isolates including 110 (20 *Aspergillus fumigatus*, 10 *Aspergillus flavus*, 15 *Aspergillus terreus*, 11 *Aspergillus nidulans*, 6 *Aspergillus versicolor*, 10 *Aspergillus niger*, 9 *Alternaria alternata*, 10 *Scedosporium apiospermum*, 9 *Schizophyllum commune*, 5 *Fusarium moniliforme* and 5 *Fusarium solani*) from fungal ball FRS, 40 (10 *Aspergillus fumigatus*, 10 *Aspergillus flavus*, 5 *Aspergillus terreus*, 4 *Aspergillus nidulans*, 4 *Aspergillus versicolor*, 5 *Aspergillus niger*, 1 *Alternaria alternata* and 1 *Schizophyllum commune*) from allergic FRS and 3 (2 *Rhizopus oryzae* and 1 *Rhizopus stolonifer*) from invasive FRS patients.

Identification by phenotypic methods

Conventional phenotypic methods gave a correct species identification for 143 of the 153 FRS isolates (93.5 %). As shown in Table 1, seven isolates (4.6 %) including 2/30 *Aspergillus fumigatus*, 3/20 *Aspergillus flavus*, 1/20 *Aspergillus terreus* and 1/10 *Aspergillus versicolor*, were misidentified

as *Aspergillus japonicus*, *Aspergillus oryzae*, *Aspergillus nidulans* and *Aspergillus nidulans*, respectively, due to close phenotypic characteristics. Three isolates (2.0 %) were identified to the genus level only, including 2/2 *Rhizopus oryzae* and 1/1 *R. stolonifer*. These 10 isolates were then re-identified by sequencing ITS.

Identification by MALDI-TOF MS

In this study, we compared the MALDI-TOF MS results of fungal isolates cultivated in solid and liquid Sabouraud media. As shown in Table 2, the liquid Sabouraud method had correct species identification for 30 *Aspergillus fumigatus*, 18 *Aspergillus flavus*, 20 *Aspergillus terreus*, 15 *Aspergillus nidulans*, 10 *Aspergillus versicolor*, 15 *Aspergillus niger*, 10 *Alternaria alternata*, 10 *Scedosporium apiospermum*, 10 *Schizophyllum commune*, 2 *Rhizopus oryzae*, 1 *R. stolonifer*, 5 *F. moniliforme* and 5 *Fusarium solani*. In contrast, MALDI-TOF MS using samples grown on Sabouraud agar plate yielded a much lower rate of correct species identification (49 %), and 76 isolates (49.7 %) were identified only to the genus level, suggesting that rotation cultivation in liquid Sabouraud medium is a more effective culture method for the MALDI-TOF MS test compared with the conventional static cultivation on solid medium.

The performance of MALDI-TOF MS (using samples in liquid Sabouraud medium) and conventional phenotypic method was also compared. As shown in Table 3, 151 out of the 153 FRS isolates had correct species identification using MALDI-TOF MS. Among these, *Aspergillus fumigatus* ($n=30$), *Aspergillus flavus* ($n=20$), *Aspergillus terreus* ($n=20$) and *Aspergillus niger* ($n=15$) had a score above 2.3 while the others had a score above 2.0. MALDI-TOF MS enabled identification of some very closely related species that were indistinguishable with conventional phenotypic methods, including 1/10 *Aspergillus versicolor*, 3/20 *Aspergillus flavus*, 2/30 *Aspergillus fumigatus*, 1/20 *Aspergillus terreus*, 2/2 *Rhizopus oryzae* and 1/1 *Rhizopus stolonifer*. Two out of the 20 *Aspergillus flavus* were misidentified as *Aspergillus oryzae* due to very close MALDI-TOF mass spectra.

Representative phenotypic images and MALDI-TOF mass spectra of FRS isolates

Representative phenotypic images and MALDI-TOF mass spectra of the major FRS isolates are available in Figs S1–S7 (available in the online Supplementary Material). These spectra exhibited significant differences among the eight different fungal species, indicating that MALDI-TOF MS is accurate in differentiating these FRS fungal species.

DISCUSSION

Currently, phenotypic identification such as culture is the most commonly used diagnostic method for FRS fungi. Nevertheless, the results are often affected by culture methods and conditions, and sometimes even the patients' medication. Accurate identification of FRS fungi has become the major concern in the diagnosis and treatment of the disease. In our preliminary study, we have developed a

Table 1. Numbers of fungal species collected from different FRS types

Fungal species	Fungal ball FRS (n)	Allergic FRS (n)	Invasive FRS (n)
<i>Aspergillus fumigatus</i>	20	10	0
<i>Aspergillus flavus</i>	10	10	0
<i>Aspergillus terreus</i>	15	5	0
<i>Aspergillus nidulans</i>	11	4	0
<i>Aspergillus versicolor</i>	6	4	0
<i>Aspergillus niger</i>	10	5	0
<i>Alternaria alternata</i>	9	1	0
<i>Scedosporium apiospermum</i>	10	0	0
<i>Schizophyllum commune</i>	9	1	0
<i>Rhizopus oryzae</i>	0	0	2
<i>Rhizopus stolonifer</i>	0	0	1
<i>Fusarium moniliforme</i>	5	0	0
<i>Fusarium solani</i>	5	0	0
Total	110	40	3

Table 2. Comparison of identification by MALDI-TOF Microflex LT system using samples cultured in liquid and solid Sabouraud media

Definitive identification	No. of total isolates	Sabouraud medium			
		Liquid		Solid	
		Species	Genus	Species	Genus
<i>Aspergillus fumigatus</i>	30	30	0	20	10
<i>Aspergillus flavus</i>	20*	18	0	12	6
<i>Aspergillus terreus</i>	20	20	0	10	10
<i>Aspergillus nidulans</i>	15	15	0	7	8
<i>Aspergillus versicolor</i>	10	10	0	3	7
<i>Aspergillus niger</i>	15	15	0	5	10
<i>Alternaria alternata</i>	10	10	0	3	7
<i>Scedosporium apiospermum</i>	10	10	0	5	5
<i>Schizophyllum commune</i>	10	10	0	5	5
<i>Rhizopus oryzae</i>	2	2	0	1	1
<i>Rhizopus stolonifer</i>	1	1	0	0	1
<i>Fusarium moniliforme</i>	5	5	0	2	3
<i>Fusarium solani</i>	5	5	0	2	3
Total	153	151 (98.7 %)	0	75 (49 %)	76 (49.7 %)

*Two *Aspergillus flavus* isolates were misidentified as *Aspergillus oryzae*.

culture procedure during which the homogenized specimen was incubated on Sabouraud dextrose agar, potato dextrose agar, Czapek agar plates or semi-solid Sabouraud medium at 28 or 37 °C until the formation of mature colonies. The sample was subjected to lactic acid phenol Medan and 10 % KOH staining for microscopic examination. Although the developed culture method was generally effective, it often

has poor performance in cases of subspecies, new species or isolates with close morphology such as *Aspergillus fumigatus* vs *Aspergillus japonicus*, *Aspergillus flavus* vs *Aspergillus oryzae* and *Aspergillus nidulans* vs *Aspergillus terreus*. In this study, seven isolates (4.4 %) were misidentified including 1/10 *Aspergillus versicolor*, 3/20 *Aspergillus flavus*, 2/30 *Aspergillus fumigatus* and 1/20 *Aspergillus terreus*.

Table 3. Comparison of the identification of the 153 FRS isolates obtained by conventional phenotyping and the MALDI-TOF Microflex LT system

Definitive identification	No. of total isolates	Conventional phenotypic method			Bruker biotyper		
		Correct identification to the level of		Mis ID	Correct identification to the level of		Mis ID
		Species	Genus		Species (score)	Genus	
<i>Aspergillus fumigatus</i>	30	28	0	2	30 (2.300–2.600)	0	0
<i>Aspergillus flavus</i>	20	17	0	3	18 (2.300–2.500)	0	2*
<i>Aspergillus terreus</i>	20	19	0	1	20 (2.300–2.400)	0	0
<i>Aspergillus nidulans</i>	15	15	0		15 (2.000–2.370)	0	0
<i>Aspergillus versicolor</i>	10	9	0	1	10 (2.000–2.356)	0	0
<i>Aspergillus niger</i>	15	15	0	0	15 (2.300–2.473)	0	0
<i>Alternaria alternata</i>	10	10	0	0	10 (2.200–2.401)	0	0
<i>Scedosporium apiospermum</i>	10	10	0	0	10 (2.200–2.389)	0	0
<i>Schizophyllum commune</i>	10	10	0	0	10 (2.200–2.375)	0	0
<i>Rhizopus oryzae</i>	2	0	2	0	2 (2.200–2.400)	0	0
<i>Rhizopus stolonifer</i>	1	0	1	0	1 (2.317)	0	0
<i>Fusarium moniliforme</i>	5	5	0	0	5 (2.200–2.400)	0	0
<i>Fusarium solani</i>	5	5	0	0	5 (2.200–2.401)	0	0
Total	153	143 (93.5 %)	3 (1.9 %)	7 (4.6 %)	151 (98.7 %)	0	2 (1.3 %)

*Two *Aspergillus flavus* were misidentified as *Aspergillus oryzae*.

MALDI-TOF MS is a newly developed diagnostic tool that is increasingly being used for rapid and accurate identification of microbes, including various clinically relevant pathogens [20–24]. MALDI-TOF MS has also been employed to identify a wide range of fungi [25–28], including filamentous fungi [29] whereas its performance regarding fungal pathogens associated with FRS is less well understood, largely due to the lack of a FRS fungal library in most hospitals. Moreover, since the structures of these fungi are much more complex than bacteria, the cultivation of fungi and preparation of samples markedly affects MALDI-TOF MS results. In this study, we found that rotation cultivation in liquid Sabouraud medium yielded a much higher correct species identification rate for MALDI-TOF MS (98.7 %) when compared with conventional static cultivation on solid medium (49 %). More importantly, it is highly recommended that fungal mycelia are completely dissolved to achieve optimal MALDI-TOF MS results. Beads may be used to improve the quality of protein extraction. In this study, fungi were cultured in liquid Sabouraud medium on a shaker at 28 °C for 24–48 h, and proteins were extracted using beads using the formic acid and acetonitrile method. As a result, 151 out of the 153 FRS isolates had correct species identification (≥ 2.0 or 2.3). The majority of the FRS isolates were *Aspergillus* spp. (110/153, 71.9 %), most of which were *Aspergillus fumigatus* (30/110, 27.2 %) and *Aspergillus flavus* (20/110, 18.2 %). The results were consistent with a previous study in that *Aspergillus* was the main genus of FRS fungi, among which *Aspergillus fumigatus* and *Aspergillus flavus* were the most common species [30]. Moreover, MALDI-TOF MS identified some very closely related species that were misidentified by phenotypic methods, including one *Aspergillus versicolor*, three *Aspergillus flavus*, two *Aspergillus fumigatus* and one *Aspergillus terreus*. Nevertheless, detection of certain species within a close spectrum is problematic with MALDI-TOF MS. For instance, 2/2 *Aspergillus flavus* were misidentified as *Aspergillus oryzae*. Future improvements are needed to increase the identification accuracy of MALDI-TOF MS.

In summary, the performance of MALDI-TOF MS in the identification of FRS fungi was excellent when using samples cultivated with rotation in liquid Sabouraud medium. The method is more accurate, allowing identification of closely related species that are indistinguishable with conventional phenotypic methods. MALDI-TOF MS is accurate, easy, rapid and cheap, and is thus highly promising as a routine identification method for filamentous FRS fungi in a clinical microbiology laboratory.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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