MALDI-TOF MS for the rapid identification and drug susceptibility testing of filamentous fungi

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Abstract. The present study aimed to evaluate the applicability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for identifying filamentous fungi and assessing the *in vitro* activities of common antifungal drugs against different kinds of filamentous fungi that are commonly encountered in a clinical setting. A total of 123 strains of filamentous fungi (24 species) were submitted for identification by MALDI-TOF MS, and the findings were compared with those obtained by conventional methods. The discrepancies were further investigated by internal transcribed spacer (ITS) sequence analysis. Then, 79 strains were randomly selected for further testing by the minimum inhibitory concentration Etest method. MALDI-TOF MS correctly identified 114 (92.70%) of the 123 filamentous fungi and failed to identify six isolates (4.9%). By contrast, the conventional identification methods made 113 (91.9%) correct identifications. In addition, 15 isolates of filamentous fungi were further identified by ribosomal DNA-ITS sequencing. In the *in vitro* antifungal susceptibility test, voriconazole showed the strongest antifungal activity among the tested drugs against a broad range of filamentous fungi. Caspofungin showed a better *in vitro* antifungal activity than fluconazole, itraconazole, and amphotericin B. MALDI-TOF MS offers a cost/time-saving, high-throughput and accurate working protocol for identifying filamentous fungi. Voriconazole could still serve as the first-line drug for treating serious infections caused by filamentous fungi, while caspofungin may be another treatment option for fungal infections.

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Introduction

The increase in antimicrobial abuse, bone marrow and organ transplantation, invasive treatments, and incidences of HIV infections and cancer in the past decades has resulted in an increase in the incidence of mycoses due to emerging filamentous fungi (1). Most filamentous fungi grow slowly, therefore it often takes several days for the laboratory diagnosis of a fungal infection (2-4). Furthermore, some filamentous fungi, such as Sporothrix schenckii, *Sporothrix globosa*, *Trichophyton interdigitale*, *Trichophyton mentagrophytes*, *Arthroderma benhamiae* and *Trichophyton rubrum* cannot be clearly differentiated by microscopic examination after traditional culturing and staining. Their overlapping phenotypic characteristics can be confusing, and identification requires growth of the organisms in culture for at least one week, thus delaying the diagnosis (5-9).

It is crucial to develop rapid identification methods and effective treatments for infections by filamentous fungi (10). Molecular techniques such as PCR, ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence analysis, or 18S ribosomal RNA (rRNA) gene sequencing can accurately identify many filamentous fungi at the species level (11). However, these methods are not only time-consuming and expensive but also require expertise (12,13). Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (MALDI-TOF MS) offers a time-saving and highly accurate means for rapid identification of microbial agents, especially bacteria (14-17). In recent years, the application of MALDI-TOF MS has also emerged as a means to identify isolates from fungi, and it is a faster and more robust diagnostic technique compared with the other established protocols for the identification of yeasts isolated from carious clinical specimens (18,19). After the successful MALDI-TOF MS identification of bacteria and yeasts in clinical laboratories, increased interest has emerged in applying this method to filamentous fungi (20,21). There are however some disadvantages to this method. MALDI-TOF identification has been hindered by the absence of filamentous fungi from in-house MALDI-TOF MS libraries. Consequently, several isolates have been mis-identified or unidentified because of the absence of the important reference spectra in the databases (22,23). Additionally, the high cost of equipment prevents this technique from being used in developing

countries (24). In light of this, this present study attempted to identify 123 strains of filamentous fungi by using conventional methods and MALDI-TOF MS. When these methods gave discordant results, the discrepancies were further confirmed by ITS sequence analysis.

The Clinical and Laboratory Standards Institute has developed a reference broth (M38A2) microdilution (BMD) approach for testing the antifungal susceptibility of various filamentous fungi (25). Unfortunately, this method is cumbersome. By contrast, commercially available Etest strips containing defined concentration gradients of drugs provide a simple and easy approach for testing the susceptibility of filamentous fungi. In the present study, the Etest method was employed to investigate the antifungal activities of five antifungal agents (fluconazole, amphotericin B, voriconazole, itraconazole and caspofungin) against 79 strains of filamentous fungi *in vitro*.

Materials and methods

Fungal isolates. A total of 123 filamentous fungi were collected in this study. Of these 123 fungi, 19 standard strains were purchased from The China Medical Culture Collection Center (Table I). The remaining 104 isolates were from clinical specimens: 21 isolates were obtained from The Clinical Laboratory of the Third Affiliated Hospital of Soochow University, and the remaining 83 strains were kindly provided by the Clinical Laboratory of The Jiangsu Province Hospital and Nanjing General Hospital. These clinical isolates consisted of sputum, urine, bronchial washing fluid and skin.

Phenotypic identification. All standard strains and clinical isolates were subcultured on Sabouraud dextrose agar (SDA) at 28˚C for 2-6 days. The fungi were first identified by skilled mycologists on the basis of macroscopic and light microscopic features using conventional methods, including lactophenol cotton blue and KOH staining. Briefly, the size, shape, pigment, texture and growth conditions of each isolate were recorded. Subsequently, the spores and hyphae were smeared on glass slides, stained with lactophenol cotton blue (Baso Diagnostic, Inc.) or 10% KOH, and observed under an Olympus IX53 microscope at a final magnification of x1,000.

Identification by MALDI-TOF MS. Protein analysis was performed using a MALDITOF Microflex LT mass spectrometer with a N2 laser set at 337 nm. The spectra were recorded in positive ion mode at a frequency of 60 Hz laser within a m/z range of 2,000 to 20,000. For each spectrum, a total of 240 shots from different positions of the metal plate were collected. The acceleration voltage, extraction voltage, lens voltage and delayed extraction time were set as 20 kV, 18.00 kV, 6 kV and 150 ns, respectively. In addition, the flow rate was maintained at 70 l/min. The fungal isolates were cultured and prepared for MALDI-TOF MS (Bruker Corporation) in accordance with the manufacturer's instructions. Briefly, the fungal isolates were incubated in liquid Sabouraud medium (Hangzhou Binhe Microbial Reagent Co., Ltd.) on a shaker at 28˚C for 24-48 h until abundant mycelia were observed. Subsequently, the mixture was transferred into a microcentrifuge tube and centrifuged at 25˚C for 2 min at

15,493 x g to remove the supernatant. Next, the pellet was vortexed first with 300 µl of water and then with 900 µl of absolute ethanol (Sigma-Aldrich; Merck KGaA). The suspension was then centrifuged at 25˚C for 2 min at 15,493 x g to remove the supernatant. The pellet was air dried for 5 min and then vortexed first with ~10-30 µl of 70% formic acid (Sigma-Aldrich; Merck KGaA) and then with the same amount of acetonitrile. The suspension was then centrifuged for at 25˚C 2 min at 15,493 x g. A total of 1.0 µl of the supernatant was added to a 96-spot polished steel target plate and allowed to dry. A saturated solution of 1.0 µl of MALDI-TOF MS matrix (a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied to each sample, and the mixture was allowed to dry at room temperature (26-28). Mass spectra were acquired by MALDI-TOF MS and analysed using FlexControl 3.1 and the Bruker filamentous fungi library 1.0 (Bruker Daltonics). The spectrometer was calibrated using the bacterial test standard (BTS; Bruker Daltonics). Each sample was analysed in triplicate. Before each analysis, quality assurance was performed by using *Aspergillus fumigatus* (ATCC® 3626™; American Type Culture Collection) and *Aspergillus niger* (CMCC (F)A3; National Center for Medical Culture Collections) reference strains. Identification scores ≥2.000 indicated species-level identification, scores of 1.700-1.999 indicated genus-level identification, while scores <1.700 indicated no identification (29-31). Results showing 'no peaks' required reanalysis.

ITS sequence analysis. Isolates with identification discrepancy between phenotyping and MALDI-TOF MS findings were further analysed by ITS sequencing. A total of 15 filamentous fungi were identified by rDNA-ITS sequence analysis. Fungal genomic DNA was extracted from mycelium with a HiPure Fungal DNA kit (Magen) according to the manufacturer's instructions. The total DNA was amplified by PCR using forward ITS1, 5'-TCCGTAGGTGAACCTGCGG-3' and reverse ITS4, 5'-TCCTCCGCTTATTGATATGC-3' universal primers (32) synthesized by Genewiz, Inc. PCR was performed with the following conditions: An initial denaturation at 95˚C for 5 min followed by 35 cycles of 30 sec at 94˚C, 30 sec at 52˚C and 30 sec at 72˚C, with a final extension step at 72˚C for 5 min. All reactions were performed in 2 µl volumes composed of 10X reaction buffer, 1 µl of template, 0.5 µl of each dNTP, 0.5 µl of each primer, 0.2 µl of TransStart Tag DNA Polymerase (Beijing Transgen Biotech Co., Ltd.) and water added to a total volume of 20 µl. The products were electrophoresed in 1% agarose gel containing 0.01% Genecolour™ nucleic acid stain (Gene Bio Tech Co., Ltd.). All PCR-amplified products were sequenced with an ABI 3730 Sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The ITS sequences of the isolates were compared with reference sequences from GenBank by using the associated BLAST online tool (National Center for Biotechnology Information). Strains were identified on the basis of 99% similarity or higher.

Antifungal susceptibility testing. Etest gradient strips with fluconazole (range, 0.016-256 µg/ml), amphotericin B, voriconazole, itraconazole and caspofungin (range, 0.002-32 µg/ml) were purchased from Autobio Diagnostics Co., Ltd. The Etest assay was performed in accordance with the manufacturer's

Table I. A total of 19 standard strains obtained from China Medical Culture Collection Center.

Standard strain	Preservation number
Aspergillus clavatus	CMCC(F)A4a
Aspergillus chevalieri	CMCC(F)A28
Aspergillus flavus	CBS13161
Aspergillus fumigatus	ATCC 3626
Aspergillus nidulans	CMCC(F)A7a
Aspergillus niger	CMCC(F)A3
Aspergillus tamarii	CMCC(F)A13
Aspergillus terreus	ATCC 3633
Aspergillus ustus	CMCC(F)A15a
Aspergullus versicolor	CBS 245.65
Beauveria bassiana	CMCC(F)B13b
Microsporum gypseum	CBS 118893
Paecilomyces lilacinus	CMCC 35539
Penicillium chrysogenum	CMCC(F)B31
Rhizopus microsporus	CMCC(F)B46
Sporothrix schenckii	ATCC 49.12
Talaromyces marneffei	CMCC(F)B33r
Trichophyton rubrum	ATCC 4438
Trichophyton tonsurans	CBS 171.65

instructions. Fungi were grown on SDA at 35°C for 3-7 day(s) to ensure adequate sporulation from mature fungi. Conidial suspensions were created from harvested cultures, and the turbidity was adjusted to 0.5 McF. These suspensions were directly inoculated onto the surface of the antimicrobial test fungal medium (BIO-KONT Co., Ltd.) using swabs, and the plates were incubated at 35°C until confluent lawns of filamentous fungi were formed. Subsequently, the surface of each of the plates was dried, and the antifungal agent strips were placed on the plates. The Etest minimum inhibitory concentration (MIC) was recorded as the lowest concentration of an antifungal drug for which the elliptical zone of growth inhibition intersected the Etest strip. Microcolonies within the ellipse were ignored. The Etest MIC was recorded after incubation for 24 or 48 h at 35°C. For most fungal species, the final Etest MIC readings were recorded at 24 h for echinocandins (caspofungin) and at 48 h for other drugs. Seventy-nine strains were randomly selected and further tested using the Etest MIC method, and quality control for this assay was performed using *Candida parapsilosis* (ATCC® 22019™; American Type Culture Collection).

Statistical analysis. Distinct sequences were annotated by Blast search against the NCBI non-redundant database with a cut-off E-value of 10^{-5}. A low E-value represents high confidence in the annotation.

Results

Identification of filamentous fungi by phenotypic methods. For most of the evaluated filamentous fungi, the conventional methods take an average of 4 days and cost $2 each for completing the identification process. After 3-7 days of incubation, all isolates showed different sizes, shapes, pigments and textures on SDA plates. The phenotypic characteristics of filamentous fungi varied among each other. The *Aspergillus* species (spp.) grew rapidly and formed loose colonies on SDA plates, with most species exhibiting characteristic colours and shapes. For example, *Aspergillus flavus* formed fluffy greyish-green colonies consisting predominantly of vegetative hyphae, while *A. niger* usually formed characteristic black colonies on SDA plates. The *Penicillium* spp. grew more slowly than the *Aspergillus* spp. On SDA, most *Penicillium* spp. produced radiated sulcata with a velvety colony surface, and some displayed coloured exudates. For example, *Talaromyces marneffei* produced a soluble red pigment, which diffused into the agar, lending a red or pink appearance to the reverse side of the colony. Dermatophytes showed radial growth, and many typical isolates of common dermatophytes were identified directly on the SDA plates. *Microsporum canis* secreted a yellow pigment, and *T. rubrum* also usually secreted a yellow pigment. Fig. 1 shows 12 typical colony morphologies of filamentous fungi. Of the 123 filamentous fungi analysed in this study, 113 (91.9%) were correctly identified by phenotypic methods, while 5 *T. interdigitale*, 3 *S. globosa* and 2 *Trichophyton tonsurans* were morphologically confused with *T. mentagrophytes*, *S. schenckii* and *T. rubrum*, respectively.

Identification by MALDI-TOF MS. The MALDI-TOF MS method took an average of 15 min and US$0.3 for identifying filamentous fungi (starting from the time the culture became available for testing). Of the 123 filamentous fungi analysed by MALDI-TOF MS, 114 (92.70%) isolates could be identified at the genus level (identification scores, 1.700-1.999), and 80 (65.0%) were correctly identified at the species level (identification scores ≥2.000). However, this method failed to identify six strains (4.9%; scores <1.700). *Penicillium chrysogenum* and *T. mentagrophytes* were misidentified as *Penicillium camemberti* and *Trichophytum equinum*, respectively (Table II). Fig. 2 shows characteristic MALDI Biotyper spectra of five representative filamentous fungi. These results showed that MALDI-TOF MS rapidly and accurately identified most filamentous fungi and is, therefore, a reliable tool for the diagnosis and treatment of fungal infections.

ITS sequence analysis. Fifteen isolates of filamentous fungi were further identified by rDNA-ITS sequencing analysis. The fungal rDNA ITS were amplified by PCR and then subjected to agarose electrophoresis (Fig. 3). Of the 15 isolates, five were confirmed as *T. interdigitale*, three as *S. globosa*, two as *T. mentagrophytes*, two as *T. tonsurans*, and the remaining as *T. marneffei*, *Aspergillus chevalieri* and *Beauveria bassiana* (Table III). These results indicated that rare or ambiguous fungi were accurately identified by rDNA-ITS sequencing.

Antifungal susceptibility analysis. The results of the quality control tests were within the control limits for the five antifungal drugs. Fluconazole showed the highest MIC that caused 90% inhibition (MIC_{90}) values against the 79 evaluated filamentous fungi: >256 µg/ml against *A. fumigatus*, *A. flavus*, *A. niger*, *T. rubrum*, *T. tonsurans*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus tamarii*, *Aspergillus clavatus*, *Aspergillus ustus*, *Aspergillus versicolor* and *Microsporum*

Table II. Results of identification of filamentous fungi by MALDI-TOF MS (n=123).

| | | MALDI-TOF MS results, no. (%) | | | |
| | | Unreliable ID | | | |
Strain	No. of isolates	No ID generated	Incorrect ID (ID generated)	Genus level ID only	Species level ID
Aspergillus fumigates	31	0	0	0	31 (100)
Aspergillus flavus	16	0	0	0	16 (100)
Aspergillus niger	7	0	0	0	7 (100)
Aspergillus nidulans	1	0	0	0	1 (100)
Aspergillus terreus	1	0	0	0	1 (100)
Aspergillus tamarii	1	0	0	1 (100)	0
Aspergillus clavatus	1	0	0	0	1 (100)
Aspergillus ustus	1	0	0	0	1 (100)
Aspergillus versicolor	2	0	0	0	2 (100)
Trichophyton rubrum	23	0	0	16 (69.6)	7 (30.4)
Epidermophyton floccosum	5	0	0	5 (100)	0
Microsporum canis	5	0	0	4 (80)	1 (20)
Trichophyton tonsurans	6	0	0	3 (50)	3 (50)
Microsporum gypseum	6	0	0	3 (50)	3 (50)
Sporothrix schenckii	1	0	0	1 (100)	0
Sporothrix globosa	3	3	0	0	0
Rhizopus microspores	1	0	0	0	1 (100)
Penicillium chrysogenum	1	0	1 (*Penicillium camemberti*)	0	0
Purpureocillium lilacinus	1	0	0	1 (100)	0
Beauveria bassiana	1	1	0	0	0
Aspergillus chevalieri[a]	1	1	0	0	0
Talaromyces marneffei[a]	1	1	0	0	0
Trichophyton interdigitale	5	0	0	0	5 (100)
Trichophyton entagrophytes	2	0	2 (*Trichophytum equinum*)	0	0
Total	123	6 (4.9)	3 (2.4)	34 (27.6)	80 (65.0)

[a]Species not included in the database. ID, identification; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

gypseum; 24 µg/ml against *M. canis*; and 12 µg/ml against *Epidermophyton floccosum*. By contrast, voriconazole showed the lowest MIC_{90} values against the 79 filamentous fungi: ≤0.38 µg/ml against *A. fumigatus*, *A. flavus*, *A. niger*, *T. rubrum*, *T. tonsurans*, *M. canis*, *E. floccosum*, *A. nidulans*, *A. terreus*, *A. tamarii*, *A. versicolor* and *M. gypseum*; 1.5 µg/ml against *A. clavatus*; and 2 µg/ml against *A. ustus*. The MIC_{90} values of caspofungin against the 79 filamentous fungi were the second lowest, next to those of voriconazole: ≤1 µg/ml against *A. fumigatus*, *A. flavus*, *A. niger*, *T. rubrum*, *T. tonsurans*, *M. canis*, *E. floccosum*, *A. nidulans*, *A. terreus*, and *A. tamarii*; 6 µg/ml against *M. gypseum*; 8 µg/ml against *A. ustus* and *A. versicolor*; and 12 µg/ml against *A. clavatus*. For *T. tonsurans*, the MIC_{90} values of itraconazole corresponded with those of caspofungin. Against *E. floccosum*, the MIC_{90} and MIC that caused 50% inhibition (MIC_{50}) of amphotericin B both corresponded with those of caspofungin. Against *A. fumigatus* and *T. rubrum*, the MIC_{90} values of amphotericin B corresponded with those of itraconazole. Additionally, the MIC of amphotericin B against

A. terreus and that of itraconazole against both *A. niger* and *A. clavatus* were >32 µg/ml. The *in vitro* susceptibilities of the 79 filamentous fungi are summarized in Table IV.

Discussion

Because of immune deficiency, some individuals, such as those with malignant tumours, HIV infection/acquired immune deficiency syndrome or infectious diseases treated with extended-spectrum antimicrobials as well as transplant recipients, are susceptible to fungal infections (33,34). These infections can be classified as exogenous and endogenous types on the basis of the pathogen source. *Candida* spp. are still the main pathogens in endogenous opportunistic fungal infections, whereas *Aspergillus* spp., *Cryptococcus* spp., dermatophytes, zygomycetes and dimorphic fungi are the predominant sources of exogenous fungal infections (35,36).

At present, identification of fungi, especially filamentous fungi, is very challenging. Conventional methods such as
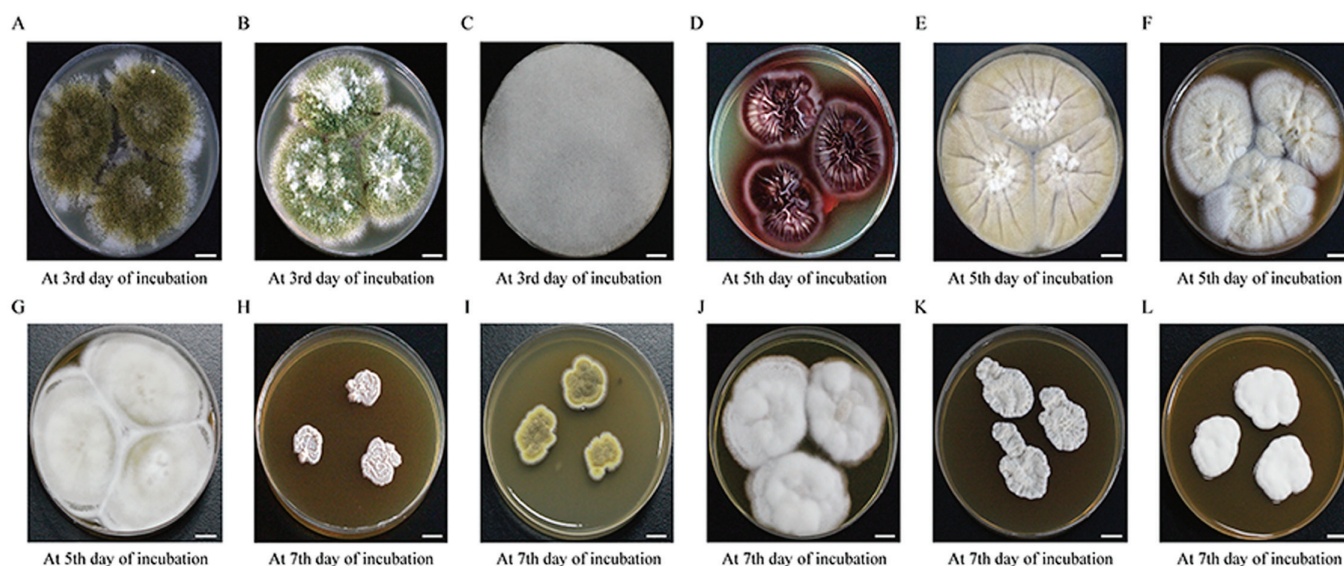
Figure 1. Twelve types of colony morphology of filamentous fungi. (A) *Aspergillus tamarii* (B) *Aspergillus flavus*, (C) *Rhizopus microsporus*, (D) *Talaromyces marneffei*, (E) *Aspergillus nidulans*, (F) *Aspergillus terreus*, (G) *Aspergillus ustus*, (H) *Sporothrix schenckii*, (I) *Aspergillus chevalieri*, (J) *Trichophyton rubrum*, (K) *Purpureocillium lilacinus*, (L) *Beauveria bassiana*. Scale bar, 1 cm.
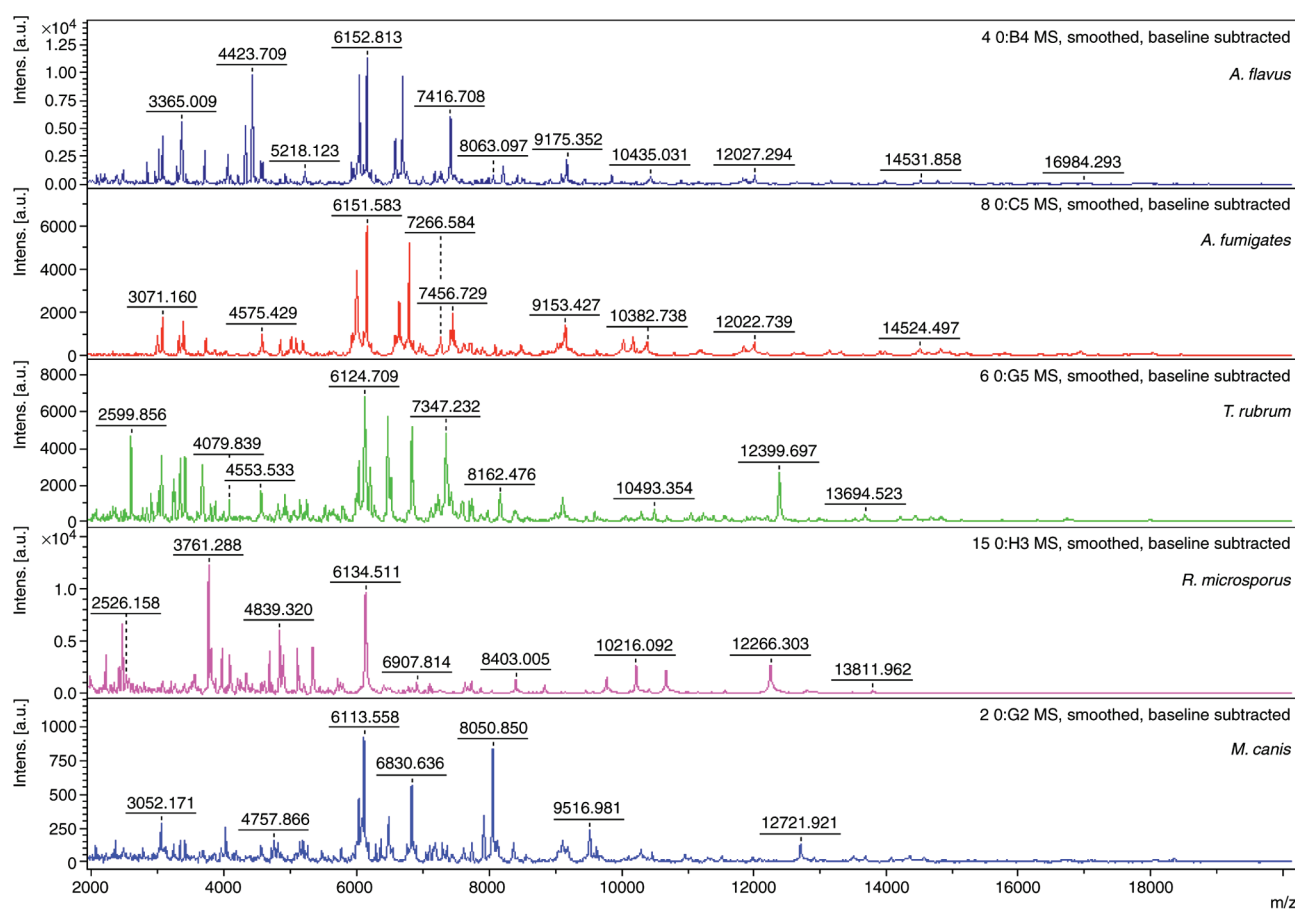

Figure 2. Spectra of *Aspergillus flavus*, *Aspergillus fumigatus*, *Trichophyton rubrum*, *Rhizopus microsporus* and *Microsporum canis* generated by the matrix-assisted laser desorption/ionization time-of-flight Bruker Biotyper. The absolute intensities and masses of the ions are shown on the Y and X-axes, respectively. The m/z values represent the mass-to-charge ratio. MS, mass spectometry.

macroscopic observation (phenotyping on the basis of growing conditions, colony shape, pigments and texture) and direct or post-staining microscopic examination, are not only time consuming but can also easily misidentify the fungi. Although β-1,3-D glucan and galactomannan tests can rapidly provide evidence of fungal infection, the specificities of these tests

Table III. Filamentous fungi identified by ITS sequencing analysis (n=15).

Strain	Result	Max score	Query cover	E-value	Identity	Accession
134-194V$_o$	*Trichophyton interdigitale*	1262	100%	0.0	99%	KP308373.1
20157639	*T. interdigitale*	1260	100%	0.0	99%	KP308373.1
23-20101126	*Sporothrix globosa*	835	99%	0.0	100%	JX997737.1
24-20101149	*S. globosa*	837	99%	0.0	100%	JX997737.1
25-20134489	*S. globosa*	835	99%	0.0	99%	LC317795.1
10-2157180	*Trichophyton tonsurans*	1249	99%	0.0	99%	AB220045.1
130-048 V$_o$	*T. interdigitale*	1254	100%	0.0	99%	LC317434.1
131-104 V$_o$	*T. interdigitale*	1157	100%	0.0	100%	JX122224.1
2157649	*Trichophyton mentagrophytes*	1253	99%	0.0	99%	KM355551.1
133-053 V$_o$	*T. interdigitale*	1266	99%	0.0	100%	KP308373.1
2157536	*T. mentagrophytes*	1256	99%	0.0	99%	AB566303.1
13-2157226	*T. tonsurans*	1188	99%	0.0	99%	AB220045.1
CMCC (f)B13b	*Beauveria bassiana*	1042	99%	0.0	99%	JF429894.1
CMCC (f)B33r	*Talaromyces marneffei*	1046	100%	0.0	100%	KY115196.1
CMCC (f)A28	*Aspergillus chevalieri*	1022	100%	0.0	99%	KX463363.1

ITS, internal transcribed spacer.

are limited because of false negative or false positive results. MALDI-TOF MS has been proven to be a remarkably flexible means for rapid identification and classification of bacteria and yeasts in clinical microbiology (37), and therefore presents a strong challenger to established microscopic and molecular biology methods. However, it has not been widely applied for identifying filamentous fungi because of the complex phylogenetic relationships between species and even more complicated morphology, which can make it difficult to extract fungal proteins.

To the best of the authors' knowledge, there is no standardised procedure for routine identification of filamentous fungi in the clinical laboratory. The limited number of existing studies in this regard have reported varying extraction procedures for fungi (26). Current extraction procedures include bead beating, ultrasonication, and chemical lysis. Here, a simple, economic, and efficient method for extracting filamentous fungi in a clinical laboratory setting has been reported in order to achieve mass spectra of good quality. In the present study, fungi were propagated in liquid cultures and extraction was performed using the ethanol-formic acid method.

In the present study, MALDI-TOF MS achieved 65.0% (80/123) identification of filamentous fungi at the species level, with a particular high identification rate for *Aspergillus* spp. (96.8%; 60/62), which is in agreement with the findings of previous studies (17,38). However, the identification rate of non-*Aspergillus* spp. was lower; of the 61 non-*Aspergillus* isolates, only 20 (32.8%) were correctly identified at the species level. The low performance of MALDI-TOF MS in identifying non-*Aspergillus* spp. may be because of the difficult steps involved in protein extraction. Therefore, it is necessary to identify novel techniques for protein extraction from non-*Aspergillus* spp. MALDI-TOF MS could not identify some filamentous fungi such as *S. globosa* and *T. marneffei*, which is likely to be due to their limited reference spectra in

the Bruker spectrum database or the highly conserved nature of ribosomal proteins among these species. MALDI-TOF MS also failed to identify other species such as *B. bassiana* and *A. chevalieri* because they do not exist in the Bruker spectrum database. Furthermore, the MALDI-TOF Bruker Biotyper generated wrong identifications for *P. chrysogenum* and *T. mentagrophytes*. Therefore, it is vital to update the database in order to extend the identification to all fungal species.

The Etest method overcomes the drawback of the BMD method in that the BMD method can be a tedious operation. It also offers other advantages, including direct and easy operation and that it is likely to be available at clinical laboratories that test relatively few filamentous fungi. Consequently, it would be beneficial to validate the Etest method for antifungal susceptibility testing of filamentous fungi. In testing the susceptibility of filamentous fungi against amphotericin B, triazoles and echinocandins, Etest findings show a strong correlation with BMD results (39). In the present study, it was found that incubating the filamentous fungi in RPMI basal medium for 24 or 48 h was sufficient for visual interpretation of the MIC without cumbersome microscopy.

In the present study, voriconazole (a triazole) showed the most efficient *in vitro* activity against *A. ustus*, with a MIC of 2 µg/ml. However, Lamoth and Alexander (39) have previously reported a voriconazole MIC of >16 µg/ml against *A. ustus*. In the present study, the antifungal activity of caspofungin was inferior to that of voriconazole, which contradicts the results reported by Shi *et al* (40). Itraconazole and amphotericin B exhibited slightly higher MIC values against most filamentous fungi except *A. terreus*, *A. niger* and *A. clavatus*. The MIC value of amphotericin B against *A. terreus* and those of itraconazole against *A. niger* and *A. clavatus* were >32 µg/ml, which suggested that the Etest method effectively detected drug-resistant isolates. The MIC of amphotericin B against *A. terreus* in this study (>32 µg/ml) was similar to

Table IV. Results of the Etest *in vitro* susceptibility assay for filamentous fungi.

Strain	MIC$_{50}$/MIC$_{90}$, μg/ml (range)				
	Amphotericin B	Itraconazole	Voriconazole	Fluconazole	Caspofungin
Aspergillus fumigatus (n=19)	2/4 (1-6)	3/4 (1.5-6)	0.094/0.125 (0.064-0.125)	>256/>256 (>256)	0.5/1 (0.25-2)
Aspergillus flavus (n=14)	3/>32 (1->32)	1.5/3 (0.5-3)	0.125/0.19 (0.048-0.25)	>256/>256 (>256)	0.38/0.75 (0.38-1)
Aspergillus niger (n=5)	4/4 (2-4)	16/>32 (16->32)	0.25/0.25 (0.25-1)	>256/>256 (>256)	0.25/0.75 (0.19-0.75)
Trichophyton rubrum (n=18)	1.5/2 (1-4)	0.75/2 (0.25-2)	0.048/0.38 (0.004-0.64)	16/>256 (3->256)	1/1 (0.25-2.00)
Trichophyton tonsurans (n=6)	3/3 (2-3)	0.38/0.38 (0.25-0.38)	0.024/0.024 (0.016-0.024)	16/>256 (16->256)	0.25/0.38 (0.25-0.38)
Microsporum canis (n=5)	1/2 (1-2)	0.38/0.38 (0.19-0.38)	0.094/0.094 (0.064-0.094)	24/24 (12-24)	0.5/0.5 (0.048-0.5)
Epidermophyton floccosum (n=5)	0.125/0.19 (0.094-0.19)	0.19/0.25 (0.125-0.25)	0.004/0.006 (0.002-0.006)	8/12 (6-12)	0.125/0.19 (0.002-0.19)
Aspergillus nidulans (n=1)	3	0.75	0.048	>256	1
Aspergillus terreus (n=1)	>32	1	0.25	>256	0.25
Aspergillus tamarii (n=1)	2	0.5	0.25	>256	1
Aspergillus clavatus (n=1)	6	>32	1.5	>256	12
Aspergillus ustus (n=1)	8	12	2	>256	8
Aspergillus versicolor (n=1)	8	1.5	0.125	>256	8
Microsporum gypseum (n=1)	3	2	0.006	>256	6

MIC$_{50}$, MIC causing inhibition of 50% of the strains tested; MIC$_{90}$, MIC causing inhibition of 90% of the strains tested. MIC, minimum inhibitory concentration.
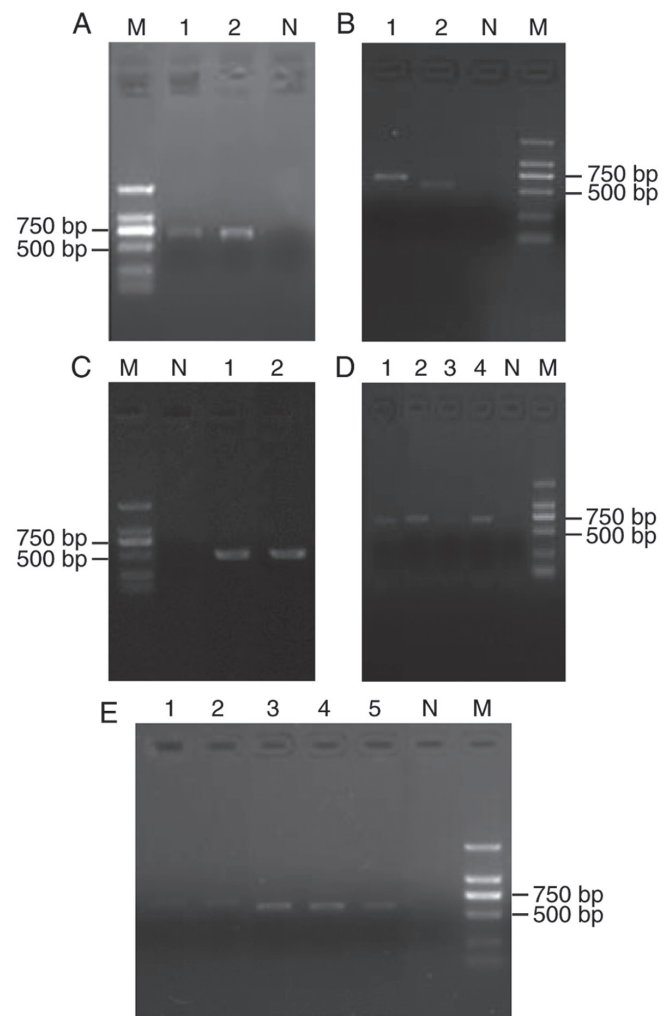

Figure 3. PCR products of the fungal ribosomal DNA internal transcribed spacer sequence. Lane M, Marker. (A) Lane 1, *Trichophyton interdigitale*; lane 2, *Trichophyton mentagrophytes*; lane N, negative control. (B) Lane 1, *Trichophyton tonsurans*; lane 2, *Beauveria bassiana*; lane N, negative control. (C) Lane 1, *Aspergillus chevalieri*; lane 2, *Talaromyces marneffei*; lane N, negative control. (D) Lane 1, *T. tonsurans*; lane 2, *T. interdigitale*, lane 3, *T. interdigitale*; lane 4, *T. mentagrophytes*; lane N, negative control. (E) Lane 1, *T. interdigitale*; lane 2, *T. interdigitale*; lane 3, *Sporothrix globosa*; lane 4, *S. globosa*; lane 5, *S. globosa*; lane N, negative control. bp, base pairs.

that reported in a previous study (41). Furthermore, the data from the present study were similar to the BMD results for the testing of *A. terreus* against amphotericin B that were found in a study by Vaezi *et al* (42). A large number of tests have shown that *A. terreus* is a poor target for amphotericin B and can, therefore, be reported as being amphotericin-B-resistant without further antifungal testing (43).

Fluconazole is by no means the most commonly used antifungal drug for treating filamentous fungi. Although clinical isolates of most filamentous fungi show a broad *in vitro* resistance to fluconazole, fluconazole is still second-line drugs of choice for prevention and treatment of certain mycoses, including sporotrichosis (9,44,45). Fluconazole showed the highest MIC$_{50}$ and MIC$_{90}$ values against all filamentous fungi tested in the present study, which might indicate that the majority of commonly used drugs offer greater resistance than fluconazole. It is interesting to note the activity of azoles against filamentous fungi is variable. Although fluconazole shows

weak activity against most filamentous fungi, new-generation azoles such as voriconazole might still exhibit favourable activity, highlighting the need for guidance in drug selection. In general, the *in vitro* activities of voriconazole and caspofungin in the present study were more effective than those of fluconazole, itraconazole and amphotericin B, consistent with previous findings (39,46). On the basis of these observations, the Etest method appears to be suitable for testing the *in vitro* activity of voriconazole, caspofungin, amphotericin B and itraconazole against filamentous fungi.

In conclusion, it was found that MALDI-TOF MS-based identification of filamentous fungi is less expensive and easier than phenotypic identification and ITS sequence analysis, and its fast turnaround time allows the analysis to be performed more rapidly. As indicated above, it is suitable as a first-line test for identifying filamentous fungi in routine clinical laboratories. However, conventional identification cannot be abandoned and will continue to be an alternative when MALDI-TOF MS fails to provide a definitive result. With the increasing use of MALDI-TOF MS in diagnostic laboratories and the further expansion of online databases of filamentous fungi libraries, a transition to more accurate and rapid identification of filamentous fungi is likely to occur. Etests for direct susceptibility testing for common filamentous fungi has been reported as a rapid antifungal susceptibility testing tool that can provide results in 24-48 h (47). The present *in vitro* antifungal susceptibility results showed that voriconazole possessed the strongest antifungal activity among the tested drugs and can be used against a broad range of filamentous fungi, while caspofungin possessed better *in vitro* activity than fluconazole, itraconazole and amphotericin B. Therefore, voriconazole can still be used as the first-line drug for treating serious infections caused by filamentous fungi, while caspofungin could serve as a treatment option for fungal infections. The Etest method is considered an appropriate alternative to guide directed antifungal therapy for routine clinical laboratories. It is important to assess the MIC values of more drugs by comparing clinical outcomes and perfecting interpretive clinical breakpoints, which can help determine whether the use of an antifungal drug is appropriate.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YP performed the experiments and prepared the manuscript. QZ and CX analyzed the data. WS was responsible for study conception and design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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