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ORIGINAL RESEARCH

MALDI-TOF MS-Based Clustering and Antifungal Susceptibility Tests of *Talaromyces marneffei* Isolates from Fujian and Guangxi (China)

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Introduction: *Talaromyces marneffei* is a life-threatening pathogen that causes systemic talaromycosis in immunocompromised and acquired immunodeficiency syndrome (AIDS) patients. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a tool to cluster *T. marneffei* isolates is rarely reported and the data on antifungal susceptibility of *T. marneffei* isolated in the southern region of China, especially in Fujian, is hardly found.

Methods: MALDI-TOF MS was used to cluster 135 *T. marneffei* isolates, and the minimum inhibitory concentration (MIC) values of amphotericin B, itraconazole, posaconazole, voriconazole, fluconazole, anidulafungin, micafungin, caspofungin and 5-fluorocytosine with Sensititre YeastOne™ YO10 assay were measured during January 2017 to October 2020 in Fujian and Guangxi.

Results: MALDI-TOF MS correctly identified 100% of the *T. marneffei* isolates. Hierarchical clustering of MALDI-TOF peak profiles identified four different clusters. MICs for itraconazole, posaconazole, voriconazole and amphotericin B were as follows: ≤0.015–0.03 μg/mL, ≤0.008–0.03 μg/mL, ≤0.008–0.06 μg/mL, ≤0.12–1 μg/mL, respectively. MICs for echinocandins and fluconazole were comparatively high.

Conclusion: Since only simple sample preparation is required and since results are available in a short period of time, MALDI-TOF MS can be considered as a method for identification and clustering of *T. marneffei*. Itraconazole, posaconazole, voriconazole and amphotericin B can be used to treat *T. marneffei* infected patients due to the low MICs.

Keywords: *Talaromyces marneffei*, MALDI-TOF, antifungal susceptibility, HIV-infected, non-HIV-infected

Introduction

Talaromyces marneffei, a temperature dimorphic fungus, belongs to division Ascomycota, class Eurotiomycetes, order Eurotiales, family Trichocomaceae, genus *Talaromyces*. *T. marneffei* is considered an emerging life-threatening pathogenic fungus that causes systemic talaromycosis in Southeast Asia and the southern region of China.[1] *T. marneffei* infection is not only the third most common secondary disease of AIDS but also an emerging opportunistic mycosis in non-HIV-infected and immunocompromised patients.[2,3] *T. marneffei* infections can cause different clinical symptoms in HIV-infected and non-HIV-infected patients,[4] requiring distinct treatments.[5,6] Since the disease has a high mortality rate and is associated with relapse, early identification of the fungus and quick diagnosis of disease are essential. Further, improvements in treatment options are especially important for non-HIV-infected patients.[7]

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be used to successfully identify the most clinically relevant fungi[8,9] and is an effective method by which to cluster microorganism.[10–14]

T. marneffei strains may develop resistance during prolonged therapy with the antifungal drugs, as a result of relapse and/or treatment failure. However, as an important guideline for the clinical antifungal drugs use, antifungal susceptibility results of *T. marneffei* are rarely reported in the southern regions of China as far as we have searched. Such results would be very useful for clinical antifungal treatment of patients. The Sensititre YeastOne™ YO10 assay has been shown to be suitable for antifungal drug susceptibility testing of the yeast phase of *T. marneffei* by Lei et al.[15]

Herein, we report results of a *T. marneffei* study in the southern regions of China with the following objectives: (1) evaluate the application performance of MALDI-TOF MS for identification of *T. marneffei* isolates and (2) determine *T. marneffei* minimal inhibitory concentrations (MICs) for amphotericin B, itraconazole, posaconazole, voriconazole, fluconazole, anidulafungin, micafungin, caspofungin, and 5-fluorocytosine.

Materials and Methods

Ethics Statement

This study was approved by the local Ethics Committee of The First Affiliated Hospital of Xiamen University and complied with the Declaration of Helsinki (2008). Written and informed consent was obtained from all participants.

Isolation of *T. marneffei* Strains

A total of 135 *T. marneffei* isolates were collected from the infected patients between January 2017 and October 2021 in Fujian and Guangxi, China. Among them, 78 strains were from Guangxi, and 57 strains were from Fujian. All the 135 *T. marneffe* strains were stored at −80°C, thawed for 24 hours at 20°C, transferred to Sabouraud dextrose agar (SDA) medium, and incubated for 96 hours at 25°C. Multiple colonies were transferred to potato dextrose agar(PDA) medium for cultivation at 35°C and enrichment for later use. All *T. marneffei* strains were identified by the following criteria: (1) demonstration of thermal dimorphism by showing a conversion from the yeast form at 37°C to the mold form at 25°C, (2) production of a diffusible red pigment from the mold form when cultured at 25°C on SDA, (3) microscopic morphology of mycelia, including the presence of conidiophore-bearing biverticillate penicillin and each penicillus being composed of four or five metulae with smooth walled conidia,[16] as well as (4) sequencing of the internal transcribed spacer (ITS).[33]

DNA Extraction, PCR Amplification and Sequencing

DNA extraction was from 1- to 2-week-old colonies grown on malt extract agar using an Ultraclean™ Microbial DNA isolation Kit (MoBio, Solana Beach, USA) with extracts stored at −20°C. For PCR, a standard thermal cycle was used, which ran 35 cycles and had a 55°C annealing temperature. The ITS region was amplified using the primers pair V9G and LS266. For the DNA-dependent RNA polymerase II, the second largest subunit (RPB2), the primer-pair *RPB2*-5F and *RPB2*-7Cr, was used with a step-up PCR that started with 5 cycles and an annealing temperature of 48°C, followed by 5 cycles at 50°C, and 25 cycles at 52°C. β-tubulin (BenA) was amplified with primer pair Bt2a and Bt2b. Primer pair, T10 and Bt2b, was used with a 52°C annealing temperature for Islandici species. Sequencing reactions used the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, CA) with the same primer sets used for PCR amplification. Sequences were determined with an ABI PRISM 3730xl genetic analyser (Applied Biosystems, California, USA). Sequence contigs were assembled using Seqman Pro v. 9.0.4 (DNAstar Inc.) and newly generated sequences deposited into GenBank. Accession numbers are included in the phylogenetic trees, with accession numbers of ex-type sequences provided in the accepted species list.

Sample Preparation for Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS

Autof (Autof KIT): One or two isolated colonies were picked with a moistened swab and the fungal material placed in a 1.5 mL micro-centrifuge tube containing 300 µL deionized water, and thoroughly mixed. Next, 900 µL of anhydrous ethanol was added and mixed. The supernatant was completely removed after centrifugation (12,000 rpm for 2 min),

tubes were dried 2–5 min until there was no residual ethanol, 10 μL of 70% formic acid was added, and mixed. Next, 10 μL acetonitrile was added, mixed, and the supernatant containing extracted protein was obtained after centrifugation (12,000 rpm for 2 min). One microliter of the protein extract was placed onto a plate, dried at room temperature, and 1 μL α-cyano-4-hydroxycinnamic acid (CHCA; Zybio Inc., Chongqing, China) matrix solution was added. The plate was dried at room temperature and then placed into the instrument for analysis.

MALDI-TOF MS

Mass spectrometry analysis was performed using MALDI-TOF MS EXS3000 (Zybio Inc., Chongqing, China). MS spectra were obtained in linear mode within a range of 2000–20,000 Da. *E. coli* ATCC 25922 was used for mass calibration and instrument parameter optimization, with an average deviation of molecular weight less than 300 ppm after correction. MS data were analyzed using MDT Master (version 1.1). As specified by the manufacturer's instructions, log scores ≥2.0 were accepted for the identification at the species level, and log scores <2.0 and ≥1.7 were used for identification at the genus level or the presumptive species level. Log scores below 1.7 were considered unreliable.

Each sample was coated with two targets. High-quality spectra with stable baseline, abundant protein peaks, and even distribution were selected. The iDBac (version 1.1.10) software was used to create the dendrogram based on the main spectrum projection (MSP), using the algorithm of the unweighted pair-group method with arithmetic means (UPGMA).

Cluster analysis was performed by spectra comparison within the database according to the manufacturer's instructions (Zybio Inc., Chongqing, China). Mass spectrometry analysis was performed with MDT Master to calculate the height and area of spectrum peaks. Welch's *t* test was used to determine peaks with statistical differences. Finally, an output file was generated. The spectra of peaks from different types of strains were collected together. Hierarchical clustering of the spectra was performed by applying the ward's method and cosine distance between all pairs of peak profiles.

Antifungal Susceptibility Testing

The Sensititre YeastOne™ YO10 assay has been shown to be suitable for antifungal testing of the yeast phase of *T. marneffei* by Lei et al.[15] In order to investigate the antifungal susceptibility of *T. marneffei* strains, the Sensititre YeastOne™ YO10 assay (ThermoFisher Scientific, Cleveland, OH, USA) was used according to manufacturer's instructions. The Disposable Sensititre panels, which are 96-well plates, contain serial twofold dilutions of dried antifungal agents including amphotericin B, itraconazole, posaconazole, voriconazole, fluconazole, anidulafungin, micafungin, caspofungin and 5-fluorocytosine. MICs were in the range of 0.12–8 μg/mL, 0.015–16 μg/mL, 0.008–8 μg/mL, 0.008–8 μg/mL, 0.12–256 μg/mL, 0.015–8 μg/mL, 0.008–8 μg/mL, 0.008–8 μg/mL, 0.06–64 μg/mL, respectively.

A 0.5 McFarland sterile saline suspension of the sporangiospores was prepared using the spectrophotometric method. Then, 20 μL of the suspension was added into 11 mL YeastOne micro-inoculum broth, with a final concentration of 1.5–8 × 10^3 CFU/mL. 100 μL of the micro-inoculum broth was dispensed into each well of the dried panels for rehydration. Finally, the panels were enclosed with adhesive seals and incubated for 72 hours at 37°C in a non-CO_2 atmosphere (the yeast form of *T. marneffei* grows better at 37°C than at 35°C). Quality control strains of *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included with each assessment.

MICs for amphotericin B were read as having the lowest drug concentration with no visible growth. But for the other antifungal drugs, the MICs were defined as the lowest concentrations necessary to inhibit 80% of growth compared to the positive control. Antifungal susceptibility testing was performed on three different days, with MIC values reported as the median of the results for the 3 days.

Results

Clinical Data and Specimen Distribution

Of the 135 *T. marneffei* isolates, 82.22% (111/135) of the strains were isolated from HIV-infected patients and 17.78% (24/135) were from non-HIV-infected patients. Of the 24 *T. marneffei* isolates from HIV negative patients, 54.2% (13/24) were from Fujian, and the other 45.8% (11/24) were from Guangxi.

Most *T. marneffei* strains were isolated from blood, accounting for 67.41% (91/135). Respiratory specimens were the second most prevalent, accounting for 21.48% (29/135), followed by pus, which accounted for 5.93% (8/135) (Figure 1).

MALDI-TOF MS

MALDI-TOF MS correctly identified 100% of the *T. marneffei* isolates. Hierarchical clustering of MALDI-TOF peak profiles identified four different clusters. Interestingly, all *T. marneffei* strains, except for strain 5, from HIV-negative patients belonged to cluster II in Fujian, which was different in Guangxi. The strains of non-HIV-infected patients belonged to cluster III and IV from Guangxi (Figure 2).

Antifungal Susceptibility Results

Results for the in vitro antifungal susceptibility of the 135 *T. marneffei* isolates are listed in Table 1. The MIC range for amphotericin B, itraconazole, posaconazole, voriconazole, fluconazole, anidulafungin, micafungin, caspofungin and 5-fluorocytosine were ≤0.12–1 μg/mL, ≤0.015–0.03 μg/mL, ≤0.008–0.03μg/mL, ≤0.008–0.06 μg/mL, 0.25–16 μg/mL, ≤0.015- ≥8 μg/mL, ≤0.008- ≥8 μg/mL, ≤0.008- ≥8 μg/mL, ≤0.06–4 μg/mL, respectively. No difference in antifungal susceptibility between Fujian and Guangxi was found. There was no difference between the HIV group and non-HIV group (Supplementary Tables 1 and 2).

Amphotericin B was found to have low MIC for all of the isolates with a MIC ≤ 1 μg/mL. MIC_{50} and MIC_{90} were 0.25 μg/mL and 0.5 μg/mL, respectively. MICs for triazoles, except fluconazole, were generally in congruence and low. More than 90% of the *T. marneffei* isolates had MICs ≤ 0.015 μg/mL for itraconazole, posaconazole, and voriconazole. MIC_{50} and MIC_{90} for itraconazole were the lowest of the three evaluated azoles (≤0.015 μg/mL). Fluconazole had a reduced antifungal effect for the *T. marneffei* yeast form (MIC range, 0.25–≥16 μg/mL), with MIC_{50} and MIC_{90} of 2 μg/mL and 4 μg/mL, respectively. The susceptibilities results indicated that echinocandins might have lower activity against *T. marneffei* yeasts, 68.89% (93/135) isolates against anidulafungin with MIC ≥ 4 μg/mL and 70.37% (95/135) strains to micafungin with MIC ≥8 μg/mL. The activity of caspofungin against *T. marneffei* yeasts was comparatively effective with an MIC_{50} of 2 μg/mL. 5-fluorocytosine may be active against some *T. marneffei* yeasts with an MIC_{50} of 0.25 μg/mL and an MIC_{90} of 0.5 μg/mL.

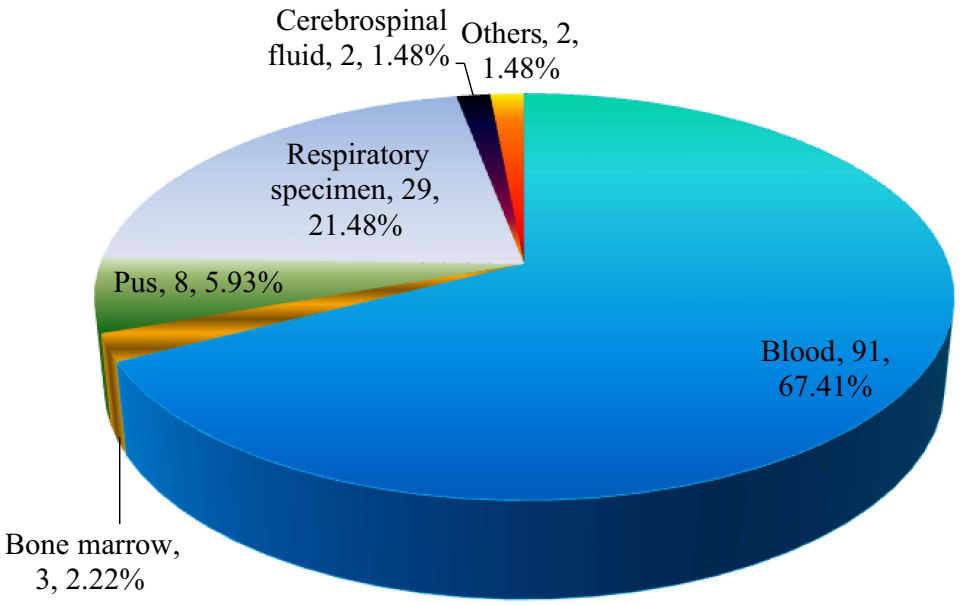

Figure 1 The specimen distribution of 135 *T. marneffei* strains. Most *T. marneffei* strains were isolated from blood. Respiratory specimen was in the second place, followed by pus.
Notes: blood; bone marrow; pus; Respiratory specimen; cerebrospinal fluid; others.

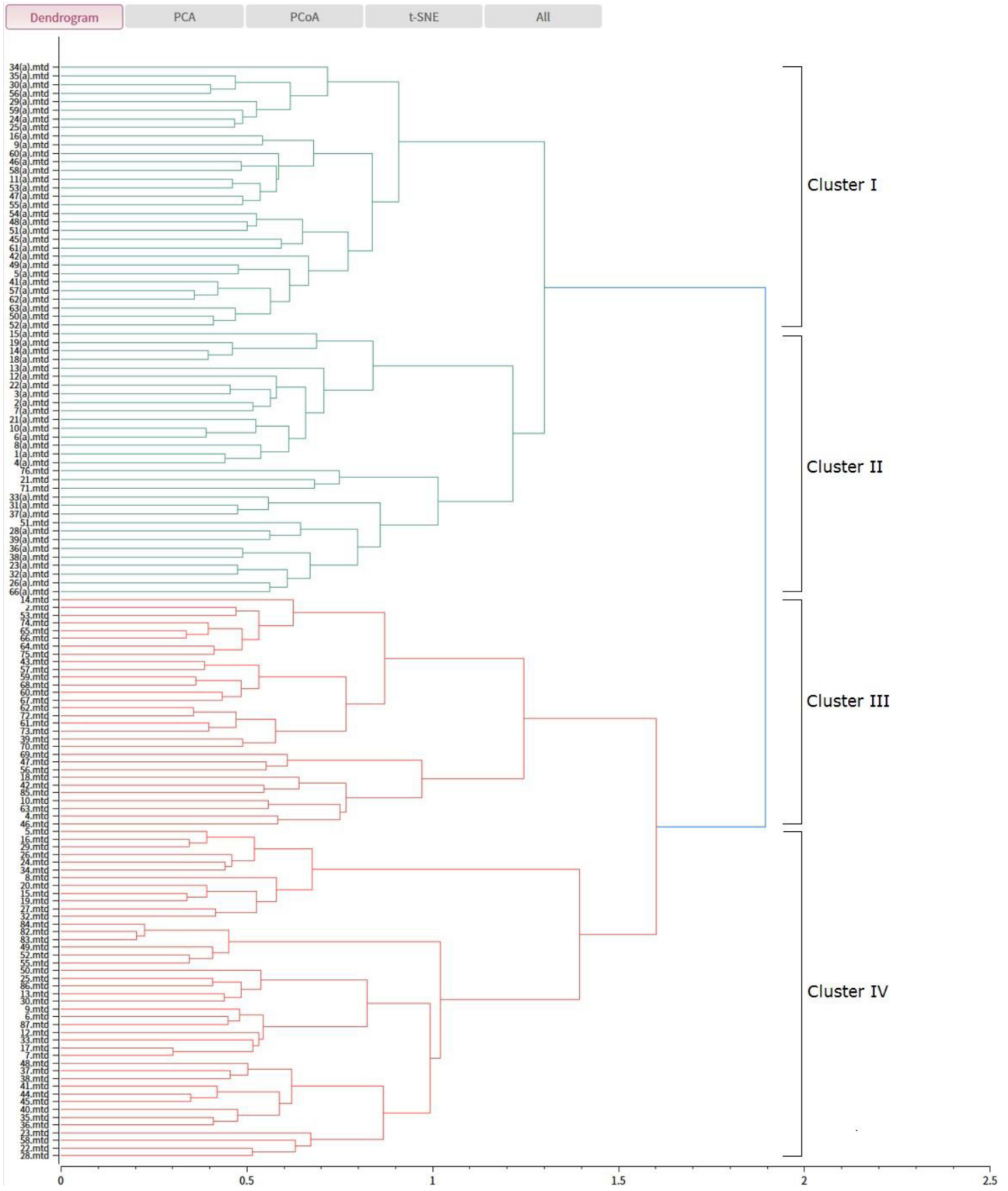

Figure 2 Hierarchical clustering of *Talaromyces marneffei* isolates by MALDI-TOF MS. The hierarchical clustering of MALDI-TOF peak profiles identified four different clusters. All *T. marneffei* strains, except for strain 5, from HIV-negative patients belonged to cluster II in Fujian, which were different in Guangxi. The strains of non-HIV-infected patients were in cluster III and IV in Guangxi.

Table 1 In vitro MIC (μg/ml) of the 135 *T. marneffei* Strains Against 9 Antifungals as Determined by YeastOne Method

Antifungal Agent	No. of Isolates with MIC (μg/mL)												MIC_{50}	MIC_{90}
	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16		
Amphotericin B					52	56	24	3					0.25	0.5
Itraconazole		131	4										≤0.015	≤0.015
Posaconazole	114	19	2										≤0.008	≤0.008
Voriconazole	94	36	4	1									≤0.008	0.015
Fluconazole						5	16	32	53	20	8	1	2	4
Anidulafungin		4	2	1			3	6	26	65	28		4	≥8
Micafungin	5	11	3	5	3		2	7	1	3	95		≥8	≥8
Caspofungin	3	2	4	6	10	15	3	17	24	44	7		2	4
5-fluorocytosine				15	37	41	31	10		1			0.25	0.5

Abbreviations: MIC, minimum inhibitory concentration; MIC_{50}, 50% minimum inhibitory concentration; MIC_{90}, 90% minimum inhibitory concentration.

Discussion

Bacterial typing is an important method to identify the route of pathogen transmission. For fungal identification, MALDI-TOF MS provides an incomparable advantage.[8] Dhieb et al[11] reported that MALDI-TOF geographical clustering of *C. glabrata* was congruent with microsatellite length polymorphism (MLP) genotyping. Usbeck et al[12] reported that mass fingerprints of 33 *Saccharomyces* strains, which are commonly used in wine fermentations, were generated by MALDI-TOF MS once sample preparation and instrument settings were optimized. As a reference method, delta-PCR was chosen to study the genetic diversity of the employed strains. Finally, MALDI-TOF MS, acting at the level of the proteome, provides valuable information about the relationship between yeast strain and biological applications. Theresa Bartosch et al obtained distance dendrograms for MALDI-TOF MS analysis of *T.verrucosum* and other keratinophilic fungi strains. Results demonstrated the dermatophyte, T. *benhamiae*, to have the highest similarity to T. *verrucosum*. Two clusters of T. *verrucosum* isolates were identified.[13]

In this study, MALDI-TOF MS correctly identified 100% of the *T. marneffei* isolates. Hierarchical clustering of MALDI-TOF peak profiles identified four distinct clusters. However, due to small sample size, further studies are needed to confirm this observation.

T. marneffei, was originally identified as *Penicillium marneffei*. It was renamed as *Talaromyces marneffei* by R.A. Samson based on gene sequencing analysis in 2011.[17] Today, it is an important thermal dimorphic fungus causing systemic mycosis in south-east Asia.[1,18,19] Furthermore, *T. marneffei* infections often have a high mortality rate and are associated with relapse, even though antifungal susceptibility results for *T. marneffei* are rarely reported.

The Sensititre YeastOne method is usually used to study the antifungal AST in *Candida* species and has been used for the analysis of other fungi. Hsuan-Chen Wang[20,21] reported that the YeastOne method could be used as an alternative for azole susceptibility testing of *Aspergillus* species and for detection of *A. fumigatus* TR34/L98H isolates. Jesu's Guinea[22] reported that Sensititre YeastOne was suitable for the analysis of A. *fumigatus* itraconazole and voriconazole MICs when results were assessed at 48 h of incubation. Fatima Zohra Delma[23] reported essential agreement between Sensititre YeastOne and EUCAST/CLSI for amphotericin B, 5-flucytosine, fluconazole, and voriconazole with >89%/>93% for MTS and for EUCAST/CLSI 57%/>75%. Major error rates are low for amphotericin B and fluconazole (<3%) and a bit higher for the other drugs (<8%). The YeastOne™ YO10 assay was shown to be suitable for susceptibility testing of the yeast phase of *T. marneffei* by Lei et al.[15] For dimorphic fungi, antifungal drug susceptibility can differ between mycelial and yeast forms.[24,25] The activity of antifungal drugs against the parasitic form of this dimorphic fungus (yeast) was evaluated in this study.[26,27] There is no standard method by which to evaluate dimorphic fungi. The Sensititre method performed in the study was modified from M27-A guidelines approved by the National Committee for Clinical Laboratory Standards (NCCLS).[28]

The data in Table 1 show MIC values without susceptibility categories, whether intermediate or resistant, in that there are no established *T. marneffei* breakpoints for antifungal agents. Herein, outcomes for antifungal drug sensitivity are in

accordance with previous reports.[15,29] Among the tested azoles, posaconazole had the lowest MIC_{50} and MIC_{90} for *T. marneffei* yeasts. Results are consistent with previous reports demonstrating the activity of posaconazole against the yeast phase of *T. marneffei*.[30,31] The yeast forms of the *T. marneffei* isolates were also inhibited by itraconazole and voriconazole with MIC_{50} and $MIC_{90} \leq 0.015$ μg/mL. These results suggest that amphotericin B had good antifungal activity against the *T. marneffei* isolates, which is consistent with the report of Liu et al.[32] However, toxicity should be considered when using amphotericin B for clinical management of talaromycosis. MICs for fluconazole, 5-fluorocytosine, echinocandins, anidulafungin, micafungin, and caspofungin were high, which suggests that these antifungal agents have little or no activity against the *T. marneffei* at yeast phase. Overall, an in vitro analysis of antifungal susceptibilities found the antifungal activity of triazoles to be superior to echinocandins and 5-fluorocytosine for the yeast phase of *T. marneffei*.

There are limitations to this study. First, this is a study with a relatively small study population. Second, a broader range of clinical isolates from multiple settings would be useful. Third, clinical information should be included in future studies. Fourth, there is no standard cluster method by which to compare the results derived herein by MALDI-TOF MS. Each of these limitations should be addressed by future investigations.

Conclusions

Since only simple sample preparation is required and since results are available in a short period of time, MALDI-TOF MS can be considered as a method for identification and clustering of *T. marneffei*. Itraconazole, posaconazole, voriconazole, and amphotericin B can be used to treat *T. marneffei* infected patients based on their relatively low MICs in vitro.

Abbreviations

AIDs, acquired immune deficiency syndrome; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *T. marneffei, Talaromyces marneffei*; MIC, the minimum inhibitory concentration; HIV, human immunodeficiency virus; SDA, Sabouraud dextrose agar; PDA, potato dextrose agar; ITS, the internal transcribed spacer; CHCA, α-cyano-4-hydroxycinnamic acid; MLP, microsatellite length polymorphism; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute; NCCLS, the National Committee for Clinical Laboratory Standards.

Ethics Statement

This study was approved by the local Ethics Committee of The First Affiliated Hospital of Xiamen University and complied with the Declaration of Helsinki (2008). Written and informed consent was obtained from all participants.

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Disclosure

The authors declare that they have no conflicts of interest.

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