Infection and Drug Resistance

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ORIGINAL RESEARCH

Comparison of in vitro Susceptibilities of *Talaromyces marneffei* in Mold and Yeast Forms in Malaysia

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Purpose: This study was aimed to determine minimum inhibitory concentration (MIC) differences between yeast and mold forms of *T. marneffei* in Malaysia.

Patients and Methods: Ninety-seven clinical strains of *T. marneffei* were received from various Malaysian hospitals from the year 2020 until 2022. Their identities were determined using microscopic, macroscopic and molecular methods. Next, the susceptibility of yeast and mold forms of each isolate against amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole, terbinafine, caspofungin and micafungin were tested according to the broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) M38 and M27 guidelines. The geometric means of minimal inhibitory concentration (GM MIC), MIC_{50}, and MIC_{90} were determined for each antifungal. Additionally, Wilcoxon signed-rank test was used to compare the significant difference of GM MICs for each antifungal, GM MIC, MIC_{50} and MIC_{90} for the combined nine antifungals against different growth forms of *T. marneffei*. The significance was set at $p<0.05$.

Results: Micafungin had the highest GM MIC, MIC_{50} and MIC_{90} for mold form of *T. marneffei*. For yeast form, amphotericin B achieved the highest GM MIC and MIC_{50} while micafungin achieved the highest MIC_{90}. However, the GM MIC, MIC_{50} and MIC_{90} of terbinafine and azole antifungals on *T. marneffei* were similar to each other, namely between 0.03 and 0.60µg/mL. The difference of GM MIC of all tested antifungals except caspofungin and micafungin was insignificant. Overall, GM MIC, MIC_{50} and MIC_{90} of the combined nine antifungals against two growth forms were insignificant.

Conclusion: The findings suggested either yeast or mold form can be used in the susceptibility testing of *T. marneffei* against amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole and terbinafine.

Keywords: *Talaromyces marneffei*, susceptibility, yeast, mold

Introduction

Talaromyces marneffei is a fungus that can cause a lethal fungal infection known as talaromycosis.[1] The common clinical presentations of talaromycosis are weight loss, fever, cough, skin lesions, lymphadenopathy, hepatomegaly and diarrhoea.[1] The disease is endemic in Southeast Asia in HIV-infected patients.[2] The annual incidence of talaromycosis in HIV-infected patients in Malaysia was estimated at 0.22–1.1 per 100,000 cases.[1,3] The mortality rate is 75% in those with delayed diagnosis and administration of antifungal therapy.[4,5]

Talaromyces marneffei is a dimorphic species. It grows as yeast at 37ºC but exists as a mold at 25ºC.[6] The inhalation of spores of the mold form was considered the route of transmission of *T. marneffei*.[7–9] On the other hand, the yeast form is pathogenic as it can produce proteins or toxins that can evade the immune defense of the host.[10] Furthermore, the yeast form is commonly isolated from the infected tissues and observed in the intracellular infection of the macrophages.[7,11–13]

Received: 23 November 2022
Accepted: 9 February 2023
Published: 22 March 2023

However, the protocol of susceptibility testing for *T. marneffei* has not been evaluated by any party. Moreover, the form to be used in the susceptibility test for *T. marneffei* remains a debate. As a result, this study was conducted to examine if a significant difference in minimum inhibitory concentrations (MIC) existed between mold and yeast forms of *T. marneffei*.

Materials and Methods

Isolate

The minimum number of samples calculated for this study was 97, following the calculation recommended by Ariffin.[14] In the year 2020 until 2022, 97 clinical isolates of *T. marneffei* which isolated on potato dextrose agar (PDA) were received from various hospitals in Malaysia. Their identities were confirmed by both macroscopic, microscopic and molecular methods. The internal transcribed spacer (ITS) region of the nuclear rDNA was amplified with PCR and detected with direct DNA sequencing to determine the species.[15]

Susceptibility Testing

Since no existing guidelines were available for susceptibility testing of *T. marneffei*, the minimum inhibitory concentration (MIC) was following the broth microdilution test mentioned in CLSI M38[16] and M27[17] for susceptibility testing of mold and yeast, respectively. The antifungals tested were amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole, terbinafine, caspofungin and micafungin. All antifungals were purchased from Sigma-Aldrich, Missouri, United States. Each microdilution well contained 100 µL of antifungal. The final concentrations of each antifungal ranged from 0.0313 to 16.0 µg/mL.

The mold and yeast inocula of *T. marneffei* were obtained as mentioned by Sar et al.[18] Briefly, 7-day-old slant cultures of *T. marneffei* mold were flooded with deionized distilled water. After the suspension had been ground, it was adjusted to 0.4×10^4 to 5×10^4 colony-forming unit (CFU)/mL. To obtain yeast inocula, the suspension was inoculated into brain heart infusion broth and incubated at 37ºC until yeast cells were grown. After broth cultures were centrifuged at 15,000 × g for 20 min, the sediment was washed three times successively with sterile deionized distilled water. Finally, the washed yeast cells were suspended in deionized distilled water and adjusted to 0.4×10^4 to 5×10^4 CFU/mL.

Following that, 100 µL of diluted inoculum suspension was added to the microdilution well. The mold mixture was then incubated at 25ºC for 96 h while the yeast mixture was incubated at 37ºC for 72 h.[6]

Quality Control

Each test included three reference strains; *Candida parapsilosis* ATCC 22019, *Aspergillus flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 to ensure that the MIC obtained was within the reference range.

Data Analysis

For each antifungal test, the GM MIC, MIC_{50} and MIC_{90} were calculated. MIC_{50} was defined as 50% of the isolates were inhibited, whereas MIC_{90} is the MIC at which 90% of the isolates were inhibited. Comparisons between the GM MIC of each antifungal, GM MIC, MIC_{50} and MIC_{90} of the combined nine antifungals, against the yeast and mold forms of *T. marneffei* were evaluated by the Wilcoxon test using SPSS 20.0 (IBM®, Armonk, New York). *P*-values less than 0.05 were considered statistically significant.

Results

T. marneffei was initially isolated from blood (n=89), pleural fluid (n=2), tracheal aspirate (n=3) and skin biopsy specimen (n=3). All isolates were obtained from HIV-infected patients. The age of patients ranged from 3 to 53 years where the male (87%) is more than the female (13%). The most common clinical manifestations were fever (n=65), cough (n=66) and diarrhea (n=47).

The GM MICs for amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole, terbinafine, caspofungin and micafungin against the mold form were 1.85 µg/mL, 0.07 µg/mL, 0.04 µg/mL, 0.05 µg/mL, 0.05

μg/mL, 0.04 μg/mL, 0.10 μg/mL, 2.23 μg/mL and 4.67 μg/mL, respectively; and against the yeast form were 0.37 μg/mL, 0.06 μg/mL, 0.05 μg/mL, 0.05 μg/mL, 0.05 μg/mL, 0.04 μg/mL, 0.07 μg/mL, 0.11 μg/mL and 0.20 μg/mL, respectively (Table 1). For mold form, the GM MIC of micafungin was the highest and followed by caspofungin and amphotericin B. For yeast form, the GM MIC of amphotericin B was the highest followed by micafungin and caspofungin. The GM MICs of other antifungals were similar to each other. Furthermore, the GM MIC of micafungin and caspofungin was shown significantly different, namely 3×10^{-4} and 2×10^{-4}, respectively. In general, the GM MIC results of the combined nine antifungals showed similar effectiveness against mold and yeast forms (p=0.058) (Table 2).

The MIC_{50} for amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole, terbinafine, caspofungin and micafungin against the mold form were 1 μg/mL, 0.03 μg/mL, 0.03 μg/mL, 0.03 μg/mL, 0.03 μg/mL, 0.03 μg/mL, 0.06 μg/mL, 4 μg/mL and 32 μg/mL, respectively; and against the yeast form were 0.5 μg/mL, 0.03 μg/mL, 0.03 μg/mL, 0.03 μg/mL, 0.03 μg/mL and 0.03 μg/mL, 0.03 μg/mL, 0.03 μg/mL and 0.03 μg/mL, respectively (Table 1). Similarly, the MIC_{50} of micafungin was the highest and followed by caspofungin and amphotericin B. For yeast form, the MIC_{50} of amphotericin B was the highest while readings of other antifungals were the same. There was no significant difference (p=0.068) in the MIC_{50} for all tested antifungals between the mold and yeast forms (Table 2).

The MIC_{90} for amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole, terbinafine, caspofungin and micafungin against the mold form was 8 μg/mL, 0.60 μg/mL, 0.03 μg/mL, 0.20 μg/mL, 0.13

Table 1 Minimum Inhibitory Concentrations (MIC) of 97 Clinical Isolates of *Talaromyces Marneffei* in Mold Form (MF) and Yeast Form (YF) to Antifungal Drugs

Antifungal	GM MIC (μg/mL)		MIC_{50} (μg/mL)		MIC_{90} (μg/mL)		Significant value (p)
	MF	YF	MF	YF	MF	YF	
Amphotericin B	1.85	0.37	1	0.5	8	8	0.082
Itraconazole	0.07	0.06	0.03	0.03	0.60	0.50	0.592
Voriconazole	0.04	0.05	0.03	0.03	0.03	0.05	0.546
Posaconazole	0.05	0.05	0.03	0.03	0.20	0.03	0.357
Ketoconazole	0.05	0.05	0.03	0.03	0.13	0.13	0.742
Isavuconazole	0.04	0.04	0.03	0.03	0.13	0.03	0.387
Terbinafine	0.10	0.07	0.06	0.03	0.50	0.50	0.061
Caspofungin	2.23	0.11	4	0.03	16	4	2×10^{-4}
Micafungin	4.67	0.20	32	0.03	32	32	3×10^{-4}

Abbreviations: GM, geometric mean; MF, mold form; MIC, minimum inhibitory concentration; μg/mL, micrograms per milliliter; MIC_{50}, the lowest concentration of the tested antifungal at which 50% of the isolates were inhibited; MIC_{90}, the lowest concentration of the antifungal at which 90% of the isolates were inhibited; YF, yeast form.

Table 2 Significant Difference of GM MIC, MIC_{50} and MIC_{90} of Combined Antifungals Between Mold Form (MF) and Yeast Form (YF)

Parameter	Significant value (p)
GM MIC	0.058
MIC_{50}	0.068
MIC_{90}	0.078

and 0.13 µg/mL, 0.50 µg/mL, 16 µg/mL and 32 µg/mL, respectively, and against the yeast form were 8 µg/mL, 0.50 µg/mL, 0.05 µg/mL, 0.03 µg/mL, 0.13 µg/mL, 0.03 µg/mL, 0.50 µg/mL, 4 µg/mL and 32 µg/mL, respectively (Table 1). For mold form, micafungin had the highest MIC_{90} and followed by caspofungin and amphotericin B. On the other hand, micafungin had the highest MIC_{90} and followed by amphotericin B and caspofungin in the yeast form. All tested antifungals showed insignificant difference (p=0.078) in efficiency against both mold and yeast forms of *T. marneffei*.

Discussion

The growth form of *T. marneffei* to be used in susceptibility testing remains a debate. This matter is more complicated when the protocol for the susceptibility testing of *T. marneffei* is currently unavailable. Therefore, this study applied the protocol from available standard guidelines namely CLSI M38 and M27 to determine the existence of significant differences in MIC which resulted from mold and yeast form of *T. marneffei*. This is due to CLSI M38 and M27 being the references for broth dilution antifungal susceptibility testing of mold and yeasts, respectively.[16,17] If the significant difference does not exist, the mold form could be used in susceptibility testing as it can grow easily in the laboratory compared to the yeast form. However, if a significant difference exists, further study has to be performed to determine which form is suitable and reflective of the treatment outcome.[19]

In this study, the overall GM MIC, MIC_{50} and MIC_{90} of mold form against the combined tested antifungals showed insignificant differences compared to the yeast form of *T. marneffei*. Specifically, the GM MIC, MIC_{50} and MIC_{90} of echinocandins and amphotericin B were the three highest records in both mold and yeast form. In comparison, the GM MIC, MIC_{50} and MIC_{90} of other antifungals were lower and their reading was almost similar.

The finding of amphotericin B in this study was similar to Sar et al from Cambodia.[18] This is due to the MIC of the mold form being found as high as the MIC of the yeast form. In contrast, Sekhon et al[20] reported higher MIC was observed in the yeast form of *T. marneffei* which was isolated from America and Europe. The MIC of >3µg/mL was found in 80% and 27% of yeast and mold forms, respectively. Furthermore, the MIC_{90} of amphotericin B against mold and yeast forms in this study recorded a high reading compared to other antifungals. This could be due to the production of melanin in *T. marneffei*.[21] Melanin is important for the virulence of *T. marneffei* by protecting it from solar, UV or gamma radiation.[22] In addition, it is also recognized as an antifungal resistance factor[23] and able to make *T. marneffei* resistant to antifungals including amphotericin B.[24]

On the other hand, the majority of the studies including Sar et al[18] and Sekhon et al[20] reported low GM MIC of itraconazole, ≤0.5 µg/mL, against yeast and mold forms which is similar to this study. The active activities of itraconazole against *T. marneffei* had been reported in previous reports.[6,25–27]

Similar to itraconazole, the low MIC of voriconazole against both forms was also reported by other researchers such as Liu et al,[9] Lau et al[6] and Singh and Devi.[25] Liu et al[28] reported the GM MIC of voriconazole against the yeast form of the isolate was ≤0.05 µg/mL, whereas Singh and Devi[25] reported the GM MIC of voriconazole against the mold form of the isolate was 0.125µg/mL.

Similar to the present study, the low MIC of posaconazole, <0.1 µg/mL, against both yeast and mold forms of *T. marneffei* were also found in Lau et al.[6] In addition, the MIC_{50} and MIC_{90} of the mold form were 0.016 µg/mL and 0.031 µg/mL, whereas the MIC_{50} and MIC_{90} of the yeast form were equal, namely 0.002µg/mL.

On the other hand, Supparatpinyo et al[26] reported a low GM MIC of ketoconazole, namely 0.027µg/mL against the yeast form of *T. marneffei*. This finding was parallel with the finding in this study.

Furthermore, terbinafine is a member of the allylamine class of antifungals.[29] It possesses fungicidal activity to yeast and filamentous fungi.[30] Unlike other classes of antifungals, it can block the fungal enzyme, namely squalene epoxidase, which is a component of the manufacturing route for fungus cell walls, hence preventing the formation of ergosterol.[31] The GM MIC of mold and yeast forms of *T. marneffei* in Liu et al[28] and Mcginnis et al[32] respectively was parallel with the finding of this study.

The overall finding of echinocandin in this study was comparatively high compared with other antifungals when tested against both forms of *T. marneffei*. This phenomenon is consistent with the finding of Fang et al[33] and Lei et al.[34]

The present findings supported the claim that echinocandins might have little to no effect on *T. marneffei* yeast as proposed by Fang et al.[33]

The variations in the pattern of susceptibility can be due to the unique mechanisms of action of each class of antifungal. For an instance, the polyene class of amphotericin B can disrupt fungal cell membranes via ergosterol binding, pore formation and leakage of cellular ions and eventually lead to fungal cell death.[35] On the other hand, azoles prevent the C14α demethylation of lanosterol in fungi, which in turn stops the formation of ergosterol in the fungal cell membrane.[36] Furthermore, echinocandins work by inhibiting the production of β-(1,3)-D-glucan, which is a component of the fungal cell wall.[37] In addition, many other factors including various affinities of different antifungals to their target can also affect the susceptibility of activity.[36]

The antifungals were selected according to the preferred therapy for penicilliosis in the Malaysian national antimicrobial guidelines and previous publications.[38] To our knowledge, this is the first study comparing the susceptibility pattern of clinical yeast and mold forms of *T. marneffei* in Malaysia. Apart from that, this study also presented the first insight into the susceptibility of isavuconazole against both growth forms of this pathogen. However, this study suffers a limitation where the results were unable to be interpreted as susceptible or resistant as there are no official breakpoints for *T. marneffei* according to the CLSI method.

Conclusion

In conclusion, the GM MIC of each antifungal except caspofungin and micafungin were found not significantly different between the two different growth forms. Therefore, we conclude that either yeast or mold form could be used in the susceptibility testing of *T. marneffei* against amphotericin B, itraconazole, voriconazole, posaconazole, ketoconazole, isavuconazole and terbinafine. However, further studies are necessary to evaluate these findings with the clinical outcomes.

Ethics Approval

An ethical review was conducted and approved by the Medical Research and Ethics Committee, Ministry of Health of Malaysia, Malaysia (NMRR-20-207-53067).

Acknowledgments

The authors would like to thank the Director-General of Health, Malaysia, for his permission to publish this article. The authors would also like to express their gratitude to the Director of the Institute for Medical Research for supporting this study.

Disclosure

The authors report no conflicts of interest in this work.

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