Original Article

In Vitro Antifungal Activities against Moulds Isolated from Dermatological Specimens

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Abstract

Background: **This study aimed to determine the minimum inhibitory concentrations (MICs) of various antifungal agents against moulds isolated from dermatological specimens.**

Methods: **We identified 29 moulds from dermatological specimens between October 2012 and March 2013 by conventional methods. We performed antifungal susceptibility testing on six antifungal agents, amphotericin B, clotrimazole, itraconazole, ketoconazole, miconazole and terbinafine, according to the Clinical and Laboratory Standards Institute guidelines contained in the M38-A2 document.**

Results: **Most antifungal agents were active against the dermatophytes, except for terbinafine against *Trichophyton rubrum* (geometric mean MIC, MIC_{GM} 3.17 µg/mL). The dematiaceous moulds were relatively susceptible to amphotericin B and azoles (MIC_{GM} 0.17-0.34 µg/mL), but not to terbinafine (MIC_{GM} 3.62 µg/mL). Septate hyaline moulds showed variable results between the relatively more susceptible *Aspergillus* spp. (MIC_{GM} 0.25-4 µg/mL) and the more resistant *Fusarium* spp. (MIC_{GM} 5.66-32 µg/mL). The zygomycetes were susceptible to amphotericin B (MIC_{GM} 0.5 µg/mL) and clotrimazole (MICGM 0.08 µg/mL), but not to other azoles (MIC_{GM} 2.52-4 µg/mL).**

Conclusion: **Amphotericin B and clotrimazole were the most effective antifungal agents against all moulds excepting *Fusarium* spp., while terbinafine was useful against dermatophytes (except *T. rubrum*) and *Aspergillus* spp. However, a larger study is required to draw more solid conclusions.**

Keywords: *antifungal, dermatology, mold, amphotericin B, azoles, terbinafine*

Introduction

Apart from yeasts, moulds form another group of fungi that can cause infections in humans, ranging from superficial to disseminated systemic infections. These pathogenic moulds can be divided into dermatophytes, dematiaceous moulds and hyaline moulds. Dermatophytes, such as *Trichophyton, Microsporum* and *Epidermophyton* spp., cause superficial infections of skin (tineas), nail and hair, and are collectively known as dermatophytoses (1). Dematiaceous fungi, such as *Curvularia, Exophiala* and *Madurella* spp., are darkly pigmented fungi that produce melanin in their cell walls, explaining the dark colour of their conidia and hyphae. These fungi have increasingly caused human diseases including phaeohyphomycosis, chromoblastomycosis and mycetoma (2). Non-dermatophyte hyaline moulds, which are characterised by colourless or hyaline hyphae, may be further subdivided into septate hyaline moulds such as *Aspergillus*, *Fusarium* and Paecilomyces, which cause hyalohyphomycoses; and sparsely septate hyaline moulds known as zygomycetes, such as *Basidiobolus*, *Rhizopus* and Syncephalestrum, which cause zygomycosis or mucormycosis (3).

At present, these fungal infections are treated empirically. Much work has been done on yeast susceptibility, especially for the *Candida* species, but susceptibility data on moulds is still limited,

especially for those that are dermatologically isolated. This could be due to a lack of established breakpoints for moulds, the cost of antifungal reagents and laborious laboratory procedures.

Therefore, this study aimed to determine the minimum inhibitory antifungal concentrations against dermatologically isolated moulds in an attempt to increase susceptibility data for moulds, which, in turn, may serve as a useful guide for clinicians in providing more effective treatment for patients.

Materials and Methods

We isolated moulds from dermatological specimens from patients attending Universiti Kebangsaan Malaysia Medical Centre (UKMMC), a tertiary-care teaching hospital in Kuala Lumpur, from October 2012 to March 2013. We identified the moulds by both macroscopic and microscopic characteristics using standard conventional methods, i.e. by inoculating them on Sabouraud dextrose agar and potato dextrose agar, incubating them in air at 30°C and conducting daily inspection for growth. Once a mature colony was present, we recorded its macroscopic features and performed a lactophenol cotton blue preparation using the scotch-tape technique. A mycology-trained laboratory technician identified the species by observing its microscopic and macroscopic features, and a mycologist verified the findings. We prepared the fungal inoculations and performed antifungal susceptibility tests according to the CLSI document M38-A2, with slight changes where necessary (4). We cultured the isolates on potato dextrose agar (PDA) at 30°C until good conidial growth was present. We then covered the colonies with about 1 mL of sterile 0.85% saline and probed gently with the tip of a transfer pipette to loosen the conidia or sporangiospores. We then transferred the conidia or sporangiospores suspension to a sterile tube and allowed it to settle for 5 to 10 minutes, before transferring the upper homogenous suspension to another sterile tube and vortexing for 15 seconds. We counted the conidia with a hemacytometer (for dermatophytes) or determined the inoculum density spectrophotometrically at 530 nm and adjusted to an optical density that ranged from 0.09 to 0.13 for *Aspergillus* sp. and *Exophiala dermatitidis*, 0.15 to 0.17 for *Aureobasidium* sp., *Hormonema dematioides*, *Fusarium* sp., *Madurella* sp. and the zygomycetes, and 0.25 to 0.3 for *Curvularia* sp. (4, 5). The final suspension concentrations after adjustments were $1 - 3 \times 10^3$ CFU/mL for dermatophytes and $0.4 - 5 \times 10^4$ CFU/mL for non-dermatophytes. Finally, we verified the inoculum sizes by quantitative colony counts, i.e. by plating 0.01 mL of a 1:10 dilution of the adjusted inoculation on Sabouraud dextrose agar and incubating it at 30°C.

We tested six antifungal agents: amphotericin B (Sigma, Israel), clotrimazole (Sigma, Italy), itraconazole (Pharmaniaga, Malaysia), ketoconazole (Pharmaniaga, Malaysia), miconazole (Sigma, Italy) and terbinafine (Novartis, Switzerland). The manufacturers provided the antifungal agents in the standard powder form. We prepared stock solutions at 1600 µg/mL with 100% dimethyl sulfoxide (DMSO, Fisher Scientific Company, USA), followed by further dilutions to make a concentration series of working antifungal solutions from 32 to 0.06 µg/mL. We then inoculated 100 µl of the working antifungal solutions into 96-well round-bottomed microtiter plates, followed by 100 µl of the inoculum suspension. When combined with the inoculum suspension, the final concentration series ranged from 16 to 0.03 µg/mL. We included growth and sterility controls for each isolate tested, and used *Candida parapsilosis* ATCC 22019 as a reference quality control strain in every batch. Finally, we incubated the microdilution plates at 35°C until there was sufficient growth present in the growth control well (drug-free medium) for minimum inhibitory concentration (MIC) determination.

Amphotericin B MICs corresponded to the lowest antifungal concentrations that resulted in a 100% growth inhibition as compared to the growth control well. Itraconazole MICs corresponded to the lowest antifungal concentrations that resulted in an 80% growth reduction for dermatophytes and 100% growth inhibition for non-dermatophytes. Ketoconazole, clotrimazole and miconazole MICs corresponded to the lowest drug concentrations that caused an 80% growth reduction for dermatophytes and 50% growth reduction for non-dermatophytes. Terbinafine MICs corresponded to the lowest drug concentrations that caused an 80% growth reduction for both dermatophytes and non-dermatophytes (4). We performed all antifungal susceptibility tests in triplicates for each isolate, and took the median MIC as the final MIC for the isolate. We also noted the antifungal MIC range and calculated the geometric mean MIC (MIC_{GM}) by calculating the *n*th root of the product of n numbers of MICs.

Results

We identified 58 moulds from dermatological specimens of 53 patients from UKMMC. Of these, 40 (69%) were non-dermatophyte-hyaline moulds, 11 were dermatophytes (19%) and 7 were dematiaceous fungi (12%). Due to limited resources, we selected only 29 out of the 58 isolates (all obtained from skin and nail specimens) for antifungal susceptibility testing; of these, 11 (37.9%) were dermatophytes, 11 (37.9%) were non-dermatophyte hyaline moulds and 7 (24.1%) were dematiaceous fungi.

Amphotericin B and all azoles were active against all dermatophytes with MICs that ranged between 0.03 and 0.5 µg/mL, whereas terbinafine showed reduced activity against *Trichophyton rubrum* with MICs that ranged between 2 and 4 µg/mL. The antifungal activities against non-dermatophyte septate hyaline moulds were more variable, due to the presence of relatively more susceptible *Aspergillus* spp. and more resistant *Fusarium* spp. All antifungal agents tested showed elevated MICs against *Fusarium* spp. that ranged between 2 and >16 µg/mL, with amphotericin B showing the lowest MIC at 2 µg/mL. Amphotericin B also showed a lower MIC against *A. niger* than A. *flavus* (0.5 µg/mL vs. 1–2 µg/mL). In contrast, azoles and terbinafine showed higher MICs against *A. niger* than *A. flavus*. Among the zygomycetes, in general, *Basidiobolus* sp. was more resistant to all antifungal agents than *Syncephalastrum* spp. Interestingly, we noted that terbinafine was active against *Syncephalastrum* spp. but not *Basidiobolus* sp., and that clotrimazole was the most active azole against the zygomycetes. As for the dematiaceous moulds, amphotericin B and all azoles showed relatively good activities, with MICs that ranged between 0.03 and 2 µg/mL, except for one strain of *Aureobasidium* sp. (ketoconazole MIC of 4 µg/mL). Terbinafine, meanwhile, showed relatively poor activities against all dematiaceous moulds, with MICs that ranged between 4 and 16 µg/mL, except for another isolate of *Aureobasidium* sp. (MIC of 0.25 µg/mL).

An overall antifungal ranking based on geometric mean MICs (MIC_{GM}) from lowest to highest (most effective to least effective against all moulds) would be as follows: amphotericin B, clotrimazole, ketoconazole, miconazole, itraconazole and terbinafine (Table 1).

Discussion

Antifungal susceptibility for moulds is not as well established as for yeasts. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) prescribe standardised and validated methods for antifungal susceptibility testing; however, except for *Aspergillus* spp., interpretive clinical breakpoints have not been validated for any mould-drug combination (4, 6). In spite of this, data collection on minimum inhibitory concentrations (MICs) of antifungal agents against moulds still provides a valuable tool to guide clinicians in prescribing the best antifungal treatment. Therefore, this study was initiated as a first effort to build a local antifungal susceptibility database, which will not only benefit our institution but may also contribute to national, regional and global antifungal databases.

Dermatophytes

In this study, *in vitro* susceptibility testing showed that amphotericin B and all azoles were active against dermatophytes, which supports the use of azoles as a topical treatment for dermatophyte infection (1). Of all azoles tested, we found that clotrimazole had the lowest MIC against dermatophytes. Patankar et al. (7) reported similar findings in India, which showed that clotrimazole had the lowest minimum fungicidal concentration (MFC) against clinical isolates of dermatophytes, as compared to ketoconazole, miconazole and terbinafine. We also found that miconazole had low MICs against dermatophytes, similarly to findings by other studies (8, 9), which makes it an alternative to clotrimazole. Unfortunately, both clotrimazole and miconazole are available as topical formulations only, which limits their use in extensive dermatophytosis, where an oral antifungal agent such as ketoconazole or itraconazole may be needed (1). However, ketoconazole has fallen out of favour due to its many adverse effects, and has been superseded by the more effective and tolerable itraconazole (10). Our study showed that itraconazole had low MICs against dermatophytes *in vitro*, which is reflected by its *in vivo* efficacy in other studies (11, 12). Other studies reported that terbinafine was the most effective drug against dermatophytes (8, 9); however, we found that this is only true for some dermatophyte species. While the terbinafine MICs were low against most dermatophytes, we found

Table 1: Antifungal minimum inhibitory concentrations (MICs) against dermatologically isolated moulds

Clinical isolates	Antifungal MIC range (Geometric mean, MIC_{GM}) [values in µg/mL]					
	AMB[a]	CLO[a]	ITR[a]	KET[a]	MIC[a]	TER[a]
Dermatophytes *(n = 11)*	0.06-0.25 (0.15)	0.06-0.25 (0.10)	0.06-0.5 (0.16)	0.03-0.5 (0.17)	0.25-0.5 (0.41)	0.06-4 (0.21)
Epidermophyton floccosum (n = 1)	0.25	0.25	0.12	0.12	0.5	0.06
Trichophyton rubrum (n = 3)	0.06-0.12 (0.08)	0.06-0.12 (0.08)	0.5 (0.5)	0.25-0.5 (0.31)	0.5 (0.5)	2-4 (3.17)
Trichophyton spp. *(n = 7)*	0.06-0.25 (0.19)	0.06-0.12 (0.09)	0.06-0.25 (0.10)	0.03-0.25 (0.14)	0.25-0.5 (0.37)	0.06-0.12 (0.08)
Dematiaceous moulds *(n = 7)*	0.03-2 (0.25)	0.12-2 (0.34)	0.03-0.5 (0.17)	0.03-4 (0.19)	0.06-2 (0.23)	0.25-16 (3.62)
Aureobasidium spp. *(n = 2)*	0.03-0.12 (0.06)	0.12 (0.12)	0.03-0.06 (0.04)	0.03-4 (0.35)	0.06-0.5 (0.18)	0.25-4 (1)
Curvularia spp. *(n = 2)*	0.12-0.25 (0.18)	0.12-0.5 (0.25)	0.25 (0.25)	0.06-0.5 (0.18)	0.12-2 (0.5)	4 (4)
Exophiala dermatitidis (n = 1)	2	0.25	0.25	0.06	0.5	8
Hormonema dematioides (n = 1)	1	2	0.5	0.5	0.12	16
Madurella sp. *(n = 1)*	0.25	1	0.25	0.06	0.06	4
Septate hyaline moulds[b] *(n = 8)*	0.5-16 (1.41)	0.25-16 (1.3)	0.25->16 (1.54)[c]	1-8 (2.83)	2-16 (3.36)	0.06->16 (1.3)[c]
Aspergillus flavus (n = 3)	1-2 (1.59)	0.25-0.5 (0.4)	0.25-0.5 (0.31)	1(1)	2-16 (3.36)	0.06-0.5 (0.25)
Aspergillus niger (n = 3)	0.5 (0.5)	1(1)	1(1)	4 (4)	2-4 (3.17)	0.5-1 (0.79)
Fusarium spp. *(n = 2)*	2-16 (5.66)	8-16 (11.31)	>16 (32)	8 (8)	4-16 (8)	>16 (32)
Zygomycetes *(n = 3)*	0.25-1 (0.5)	0.03-0.5 (0.08)	0.5->16 (4)[c]	0.5-8 (2.52)	1-8 (2.52)	0.06->16 (0.5)[c]
Basidiobolus sp. *(n = 1)*	0.5	0.5	4	8	8	>16
Syncephalastrum spp. *(n = 2)*	0.25-1 (0.5)	0.03 (0.03)	0.5->16 (4)[c]	0.5-4 (1.41)	1-2 (1.41)	0.06 (0.06)
All moulds *(n = 29)*	0.03-16 (0.36)	0.03-16 (0.26)	0.03->16 (0.42)[c]	0.03-8 (0.5)	0.06-16 (0.77)	0.06->16 (0.75)[c]

[a]AMB, amphotericin B; CLO, clotrimazole; ITR, itraconazole; KET, ketoconazole; MIC, miconazole; TER, terbinafine
[b]excluding dermatophytes
[c]for MIC values of >16 µg/mL, a presumptive MIC of 32 µg/mL was taken to calculate the geometric mean of MIC

that they were high against *Trichophyton rubrum* (MIC_{GM} 3.33 µg/mL). Terbinafine resistance is most likely to occur through the squalene epoxidase pathway (13).

Dematiaceous fungi

Dematiaceous fungi cause various clinical syndromes in both immunocompromised and immunocompetent individuals, such as chromoblastomycosis, mycetoma and phaeohyphomycosis. Chromoblastomycosis and mycetoma have characteristic histologic features, whereas phaeohyphomycosis is a term used to include all other infections caused by dematiaceous fungi, ranging from superficial mycoses to brain abscesses (2). In our study, dematiaceous fungi were mostly isolated from superficial sites such as skin and nail. Amphotericin B showed good activity against all dematiaceous fungi tested with MIC values ≤ 2 µg/mL. Azoles generally showed good activity against dematiaceous fungi, except one out of two isolates of *Aureobasidium* spp. (ketoconazole MIC of 4 µg/mL). Our two isolates of *Curvularia* spp. were susceptible to amphotericin B and itraconazole, in contrast to another study in India where their MIC_{GM} to four *Curvularia lunata* isolates were 16 µg/mL and 128 µg/mL, respectively (14). Our study also showed that terbinafine did poorly against dematiaceous fungi with MICs that ranged between 0.25 and 16 µg/mL (MIC_{GM} 5.75 µg/mL). This contrasts with other reports in which terbinafine showed promising activity against dematiaceous fungi, either on its own or when combined with other antifungal agents (15).

Non-dermatophyte, septate hyaline moulds

Aspergillus spp., the most common non-dermatophyte mould in this study, was mostly recovered from nails. This finding correlates to earlier local studies (16, 17) that identified *Aspergillus* spp. as the most common hyalohyphomycete causing onychomycosis. We noted that the antifungal MICs in this group were more diverse, due to the presence of more susceptible *Aspergillus* spp. and more resistant *Fusarium* spp. We found that amphotericin B MICs against *A. niger* were lower than *A. flavus*, which corroborates another study from 2011 that showed many *Aspergillus* spp., including *A. terreus*, *A. flavus* and *A. nidulans*, to have reduced susceptibility to amphotericin B. The same study also reported that *A. niger* was known to have variable susceptibility patterns, with reduced susceptibility to azoles (18). We noted that the MICs of azoles against *A. niger* were consistently higher than *A. flavus*. Although designed to target dermatophytes, we found that terbinafine also had low MIC profiles against *Aspergillus* spp., with MIC_{GM} of 0.35 µg/mL and 0.83 µg/mL for *A. flavus* and *A. niger*, respectively. Similarly, Moore et al. (19) reported that terbinafine has potential activity *in vitro* against *A. niger*, *A. flavus* and *A. terreus* at low concentrations, compared with amphotericin B and itraconazole. *Fusarium* spp. are well known for their multidrug resistance including to amphotericin B, azoles and terbinafine (20). However, there are studies that reported favourable *in vitro* activity against *Fusarium* spp., or successful outcome of fusariosis that was treated with amphotericin B (20) or terbinafine (21), while treatment success was less consistent with azoles (22). Case reports of combination antifungal therapy for disseminated fusariosis in immunocompromised patients showed that 70% of cases responded positively, particularly those involving amphotericin B combined with voriconazole or terbinafine (23).

Zygomycetes

The phylum Zygomycota is divided into two orders, Mucorales and Entomophthorales. Mucorales, which include *Rhizopus*, *Mucor* and *Syncephalstrum* spp., are causative agents of mucormycosis and systemic zygomycosis. Meanwhile, entomophthorales, which include *Basidiobolus* and *Conidiobolus* spp., are causative agents of entomophthoromycosis and subcutaneous zygomycosis. According to Otcenask and Buchta (24), most zygomycetes are susceptible to amphotericin B but resistant to many antifungal azoles. This is consistent with our study, in which amphotericin B, clotrimazole and terbinafine showed low MICs against *Syncephalastrum* spp., while miconazole, ketoconazole and itraconazole showed elevated MICs. A study on global mucorales susceptibility showed that *Syncephalastrum* spp. were susceptible to, in increasing MIC order, amphotericin B, terbinafine, posaconazole and

itraconazole (25). As regards *Basidiobolus* sp., our study showed that it was a fairly resistant isolate with only amphotericin B and clotrimazole showing activity against it (MICs of 0.5 µg/mL), while other azoles and terbinafine MICs were ≥ 4 µg/mL. This contrasts with another study on four isolates of *Basidiobolus* spp. that caused gastrointestinal basidiobolomycosis, which showed that all four were susceptible to itraconazole, two were susceptible to fluconazole, one was susceptible to miconazole, one was susceptible to ketoconazole, and all four were resistant to amphotericin B and flucytosine (26). Apart from surgery and the standard antifungal agents, the addition of potassium iodide has been shown to accelerate healing of lesions in subcutaneous zygomycosis (27).

Conclusion

There are several limitations to this study. First, the small sample size may cause bias, especially to the geometric means of the MICs and, consequently, the overall picture of susceptibility. To address this, we performed the antifungal susceptibility tests in triplicate. Second, we were unable to interpret the MICs as there are no official clinically correlated breakpoints for moulds. Accordingly, the words 'susceptible' and 'resistant' were used to refer to the values of MICs that would most likely represent susceptible or resistant isolates. Although this is a fairly small study, this data represents a valuable addition to the limited antifungal database in Malaysia.

In conclusion, amphotericin B and clotrimazole were the broadest spectrum of antifungal agents against dermatologically isolated moulds in this study, with the exception of *Fusarium* spp. Itraconazole showed a similar spectrum of activity, except for zygomycetes. In addition, miconazole and ketoconazole showed reduced activity against *A. niger*. Terbinafine, meanwhile, was only effective against dermatophytes, excepting *T. rubrum*.

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Conflict of Interest

None.

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Author's Contribution

Conception and design : TMN, JS
Analysis and interpretation of the data: TMN, RAAB, SMS, TVK, JS
Drafting of the article: TMN, RAAB, SMS, TVK
Critical revision of the article for important intellectual content: TMN, JS
Final approval of the article: TMN, RAAB, SMS, TVK, MM, HS, HY, JS
Provision of study materials or patients: MM, HS, HY, JS
Statistical expertise: TMN, JS
Obtaining of funding: TMN, JS
Administrative, technical, or logistic support: RAAB, SMS, TVK, MM, HS, HY, JS
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