Original Article

In vitro Activities of Antifungal Drugs Against Yeasts Isolated from Blood Cultures and Moulds Isolated from Various Clinically Significant Sites in Singapore

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Abstract

Introduction: Fungaemia carries with it high mortality rates and appropriate as well as timely antifungal therapy has been shown to be life saving. Materials and Methods: We studied the in-vitro activities of antifungal agents using the Etest method, against 100 *Candida* isolates from blood cultures, 10 *Cryptococcus* isolates from blood or cerebrospinal fluid and 50 mould isolates from various clinically significant sites of patients in Singapore General Hospital, from June 2004 to December 2006. Results: Overall, the yeasts appeared to have low minimum inhibitory concentrations (MICs) for all the 5 antifungal drugs tested except for fluconazole. The overall high MIC_{90} values of the moulds against the azoles were largely attributed to the non-*Aspergillus* moulds. Posaconazole, itraconazole, voriconazole and caspofungin appear effective against local strains of *Aspergillus* species, although there are no interpretive breakpoints. Conclusions: The results show that the local fungal strains studied appear to be susceptible to the usual antifungal drugs recommended in the literature.

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Key words: Antifungal susceptibility, Aspergillus, Candida, Etest, Singapore

Introduction

Fungaemia carries with it high mortality rates and appropriate as well as timely antifungal therapy has been shown to be life saving.[1] Amphotericin B has the broadest coverage amongst the antifungal drugs against fungal infection, and was regarded the gold standard treatment for severe fungal infection. However, because of toxicity of amphotericin, and with the introduction of less toxic antifungal drugs, the azoles and echinocandins (e.g. caspofungin), the latter are now alternative treatment options. *Candida* remains the most common systemic fungal infection worldwide, followed by *Aspergillus*. High-risk patients for invasive fungal infections include solid-organ and haematopoietic stem cell transplantation, cancer, receipt of immunosuppressive therapy, AIDS, premature birth, advanced age and major surgery.[2] Unlike bacterial infections, antifungal susceptibility testing is not routinely done, because they are more costly, requires better standardisation, and often lack interpretative criteria.[3,4] Treatment has therefore largely been guided by results of published literature. There are limited antifungal susceptibility studies done in Singapore.[5,6] To the best of our knowledge, no studies have been carried out in Singapore, on the newer antifungal drugs such as posaconazole and caspofungin, which may used in certain difficult to manage cases. In addition, no local antifungal susceptibility studies have been done on moulds. Information on local strains will help clinicians in management of cases in Singapore.

The objective of this study was to look at the in vitro activities of antifungal drugs against selected yeast and mould strains isolated from blood cultures and other clinically significant sites of patients in the Singapore General Hospital from June 2004 to December 2006.

Materials and Methods

Archived yeast and moulds from patients in Singapore General Hospital were used in this study. One hundred *Candida* isolates from blood were selected, with roughly equal numbers from each of the 3 years. There were 24 *C. albicans*, 28 *C. tropicalis*, 27 *C. glabrata*, 12 *C. parapsilosis*, 7 *C. dubliniensis*, 1 *C. krusei* and 1 *C. famata* isolates. The isolates were identified using germ tube test, yeast morphology on Tween agar, and API 20 C AUX. In addition, 10 isolates of *Cryptococcus* species from blood or cerebrospinal fluid (CSF) were studied. Fifty mould isolates were studied, and were isolated from various sites and specimens. These were 34 *Aspergillus* species (12 *A. fumigatus*, 12 *A. niger*, 7 *A. flavus*, 3 *A. clavatus/A. nidulans*), 10 *Fusarium solani*, 2 *Syncephalastrum* species, 1 each of

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Table 1. Mould Isolates and Their Respective Sites of Isolation

Mould	No. of isolates	Source
Aspergillus sp.	34	BAL (26), pleural fluid (1), nail (1), pus (1), sputum (1), tissue (4)
A. fumigatus	12	BAL (9), breast tissue (1), pleural fluid (1), sputum (1), unknown (1)
A. niger	12	BAL (9), lung tissue (1), nasal tissue (1), pus (1) unknown (1)
A. flavus	7	BAL (5), Lung tissue (1), nail (1)
A. clavatus/A.nidulans	3	BAL (3)
Fusarium solani	10	Blood (1), cornea (7), leg wound (1), nail (1)
Syncephalastrum sp.	2	Nail (2)
Rhizopus sp.	1	Ethmoid sinus tissue (1)
Absidia sp.	1	Maxillary sinus tissue (1)
Acremonium sp.	1	Vitreous (1)
Paecilomyces sp.	1	Cornea (1)

BAL: bronchoalveolar lavage

Table 2. Comparative In vitro Activities (MIC in µg/mL) of Various Antifungal Drugs against *Candida* and *Cryptococcus*

Fungal isolates	No.	POS		FLU		VOR		AMB		CAS	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
All *Candida* sp.	100	0.064	1.5	0.38	16	0.032	0.38	0.38	0.75	0.094	0.25
C. albicans	24	0.032	0.064	0.125	0.19	0.006	0.008	0.094	0.125	0.032	0.125
C. tropicalis	28	0.094	0.125	0.38	0.75	0.047	0.094	0.5	1	0.094	0.19
C. glabrata	27	1	2	16	48	0.25	0.5	0.5	0.75	0.094	0.19
C. parapsilosis	12	0.023	0.047	0.38	0.75	0.012	0.023	0.125	0.5	0.25	1
C. dubliniensis	7	0.012	0.023	0.094	0.25	0.004	0.006	0.012	0.032	0.125	0.125
C. krusei	1	0.25	0.25	24	24	0.19	0.19	0.5	0.5	0.25	0.25
C. famata	1	0.006	0.006	0.094	0.094	0.003	0.003	0.016	0.016	0.19	0.19
Cryptococcus sp.	10	0.125	0.38	8	32	0.023	0.094	0.25	0.25	>32	>32
C. neoformans	8	0.125	0.38	4	32	0.016	0.094	0.25	0.38	>32	>32
C. gattii	2	0.19	0.5	8	32	0.064	0.125	0.25	0.25	>32	>32

Rhizopus, *Absidia*, *Acremonium* and *Paecilomyces* species. The source or site of isolation of the moulds is summarised in Table 1.

The minimum inhibitory concentrations (MICs) were performed and determined using Etest on RPMI agar (with 2% glucose and MOPS) in accordance to the instructions from the Etest manufacturer.[7] For the yeasts, the drugs tested were posaconazole, fluconazole, voriconazole, amphotericin B and caspofungin, and the plates were incubated at 35^{0}C in a moist incubator until good growth was observed. For most *Candida* species, the readings were taken at 24 hours of incubation. For *Candida tropicalis* and *Candida glabrata*, however, the readings were taken at 48 hours, as instructed in the Etest manufacturer's instructions.[8] For any of the *Candida* species not showing sufficient growth at 24 hours, notably *Candida parapsilosis*, the readings were taken at 48 hours of incubation. *Cryptococcus neoformans* Etests were read at 48 hours of incubation. For moulds, the drugs tested were posaconazole, itraconazole, voriconazole, amphotericin B and caspofungin. The inoculum was standardised using spectrophotometer at 530 nm wavelength to obtain counts of approximately 10^6 cfu/mL.[9,10] The RMPI plates (with 2% glucose and MOPS) were incubated at 35^{0}C for 1 to 3 days. Most moulds showed good growth and were read at 24 hours, while *Fusarium* species were read at 48 hours, and *Acremonium* and *Paecilomyces* at 72 hours.

The MIC readings were taken at the point where the ellipse of growth intersected the scale on the strip. For amphotericin B, the MIC was read at the point of complete (100%) inhibition. For the azoles and caspofungin, the MIC was read at the first point of significant inhibition/

Table 3. In vitro Activities of Antifungals Against 100 Strains of *Candida* sp. with Interpretive Breakpoints

Antifungal drugs	Breakpoints	No.	R	I	S	MIC_{50}	MIC_{90}	MIC mean	MIC range
Posaconazole	Unknown	100	NA	NA	NA	0.064	1.5	0.098	0.002-64
Fluconazole	S<=8 R>=64	100	3	19	78	0.38	16	0.794	0.032-512
Voriconazole	S<=1 R>=4	100	1	0	99	0.032	0.38	0.031	0.002-6
Amphotericin B	S<=1	100	0	0	100	0.38	0.75	0.197	0.004-1
Caspofungin	Unknown	100	NA	NA	NA	0.094	0.25	0.085	0.002-1

NA: not applicable, as no breakpoints are available.

Table 4. Comparative In vitro Activities (MICs in µg/mL) of Various Antifungal Drugs against Moulds

Fungal isolates	No.	POS		ITR		VOR		AMB		CAS	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
All moulds	50	0.047	>32	0.19	>32	0.094	4	0.25	3	0.016	>32
Aspergillus sp.	34	0.023	0.125	0.094	0.25	0.064	0.19	0.125	2	0.008	0.047
A. fumigatus	12	0.032	0.047	0.094	0.19	0.094	0.125	0.19	0.25	0.003	0.047
A. niger	12	0.012	0.032	0.064	0.19	0.032	0.064	0.125	0.19	0.012	0.064
A. flavus	7	0.094	0.19	0.19	0.25	0.125	0.19	2	4	0.008	0.023
A. clavatus/A. nidulans	3	0.016	1	0.25	0.38	0.094	0.25	0.032	0.38	0.016	0.032
Fusarium solani	10	>32	>32	>32	>32	2	4	3	4	>32	>32
Others	6	0.38	>32	1	>32	6	64	0.25	>32	>32	>32

marked decrease in growth intensity, using the principle of 80% inhibition to visually select the end point. For each isolate, the MIC readings were taken by at least 2 trained individuals where final consensus readings were obtained.

The results were tabulated and analysed with the WHONET software, which is available from http://www.who.int/drugresistance/whonetsoftware/en. Interpretative breakpoints are available only for fluconazole, voriconazole and amphotericin B against *Candida* species.

Quality Control

Quality control strains were used during each run. For runs involving yeasts, 2 control strains, *Candida parapsilosis* ATCC 22019 and *Candida albicans* ATCC 90028 were used, and their MICs were all within the range for amphotericin B, fluconazole, voriconazole, posaconazole and caspofungin. For runs involving moulds, the control strains used were *Aspergillus fumigatus* ATCC 204305 and *Candida parapsilosis* ATCC 22019, and MICs were within range.

Results

The MIC_{50} and MIC_{90} for the various antifungal agents are shown in Tables 2 to 4. The MIC distributions for the various fungi are presented in Figures 1 to 4.

Discussion

Interpretive breakpoints are available only for amphotericin B, fluconazole and voriconazole against *Candida* species, but not for posaconazole and caspofungin.[11] There are no known breakpoints for antifungal drugs against moulds. Where there are no breakpoints available, only MIC_{50} and MIC_{90} data are presented.

The yeasts appear in general to have low MICs for the 5 antifungal drugs tested, except for fluconazole. For antifungals with available breakpoints, the MIC_{90} values of voriconazole and amphotericin B against *Candida* species are within susceptible values. The value for fluconazole, however, is 16 µg/mL just beyond the susceptible value of 8 and this is contributed by *Candida glabrata* (MIC_{90} of 48 µg/mL) and *Candida krusei* which is innately resistant. The proportion of *Candida* species selected for this study may not be representative of the distribution in the clinical setting, hence the data in Table 3 should be used with this limitation in mind. Although there are no breakpoints for *Cryptococcus* species, amongst the azoles, posaconazole and voriconazole have low MICs, but fluconazole has the highest MIC values. The MIC results seem to indicate that *Cryptococcus* species are susceptible to amphotericin B and uniformly resistant to caspofungin, which is not unexpected as caspofungin is not active against *Cryptococcus* species. This is because caspofungin inhibits fungal cell wall synthesis of β-1-glucan, but the cell wall of *Cryptococcus* is β-4-glucan.

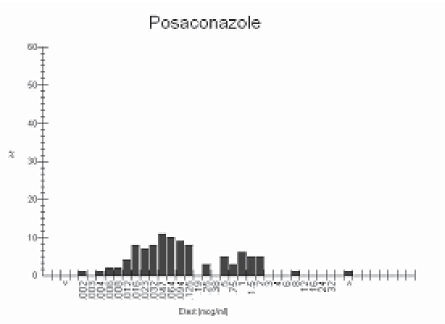

Fig. 1a.

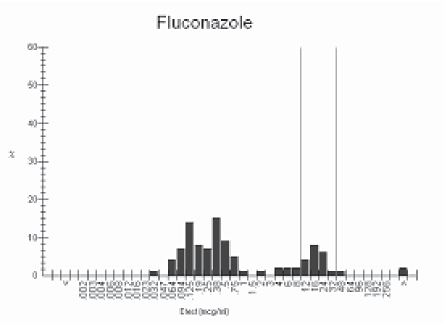

Fig. 1b.

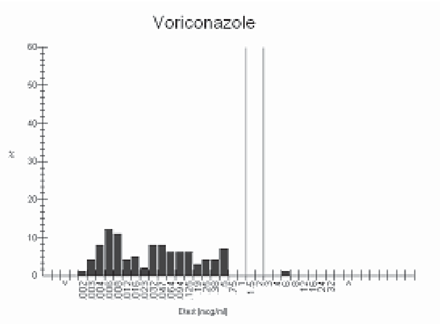

Fig. 1c.

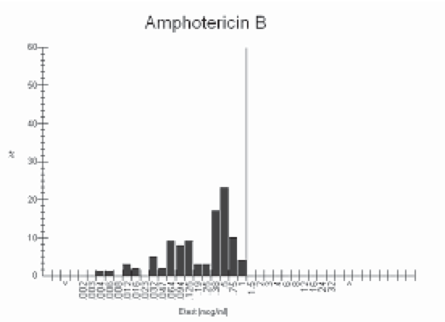

Fig. 1d.

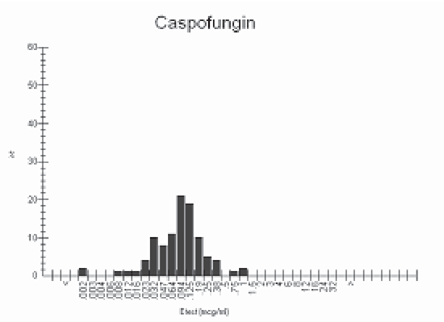

Fig. 1e.

Figs. 1a-1e. Antifungal MIC distribution for *Candida* sp. (n = 100).

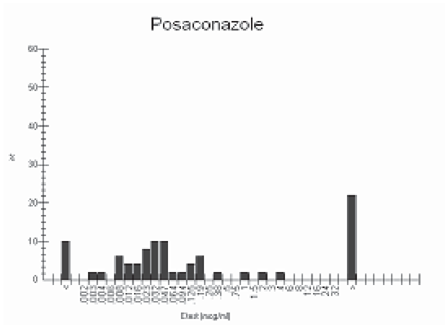

Fig. 2a.

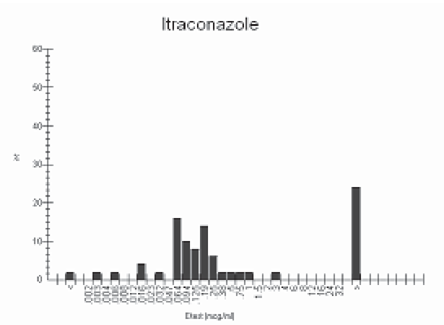

Fig. 2b.

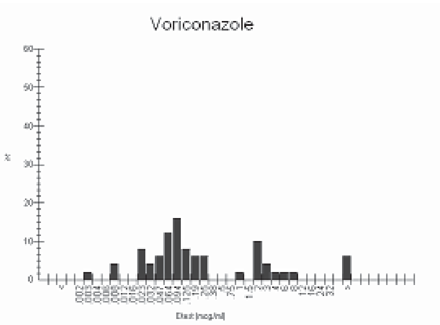

Fig. 2c.

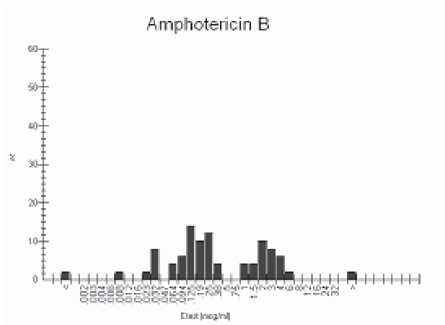

Fig. 2d.

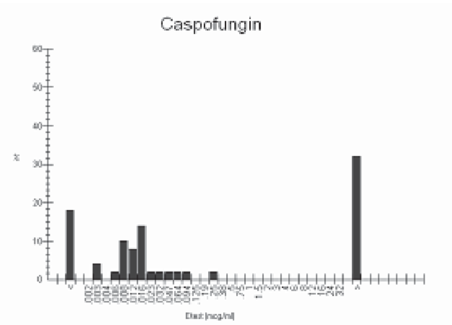

Fig. 2e.

Figs. 2a-2e. Antifungal MIC distribution for all moulds (n = 50).

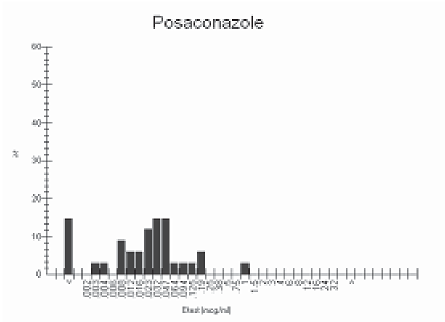

Fig. 3a.

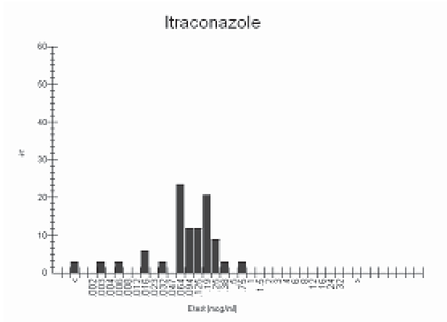

Fig. 3b.

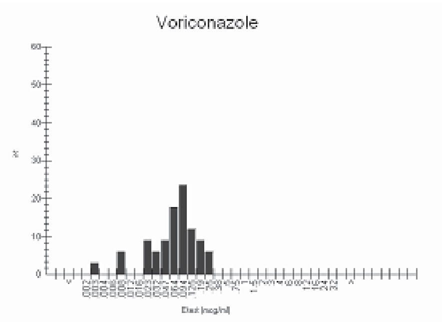

Fig. 3c.

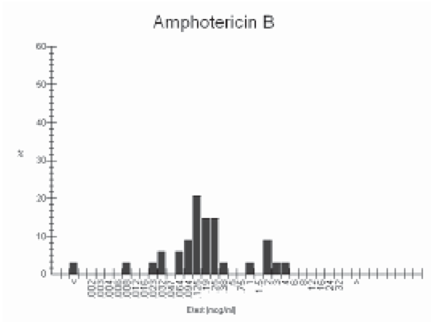

Fig. 3d.

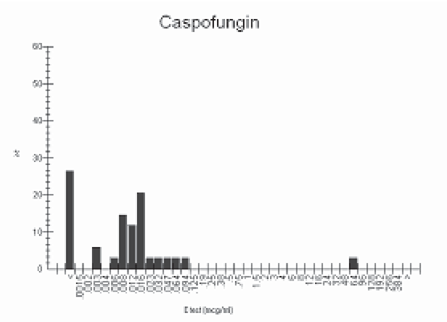

Fig. 3e.

Figs. 3a-3e. Antifungal MIC distribution for *Aspergillus* sp. (n = 34).

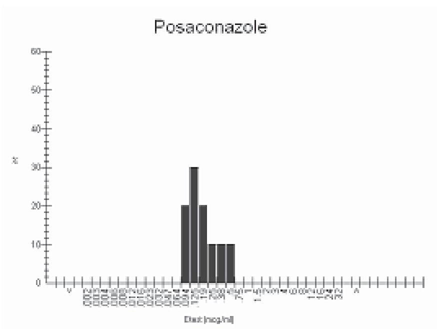

Fig. 4a.

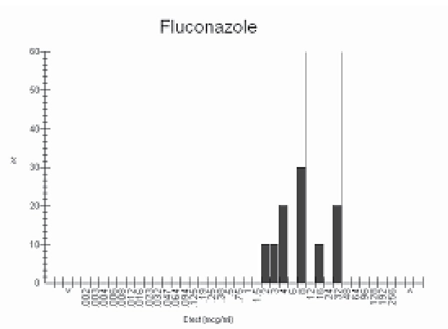

Fig. 4b.

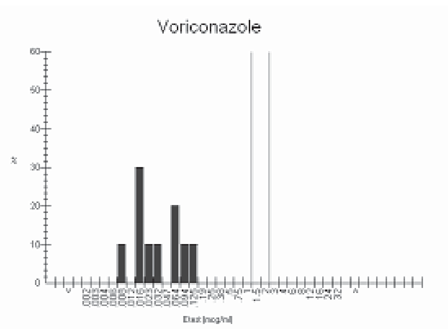

Fig. 4c.

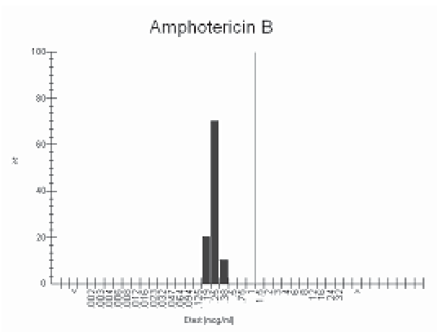

Fig. 4d.

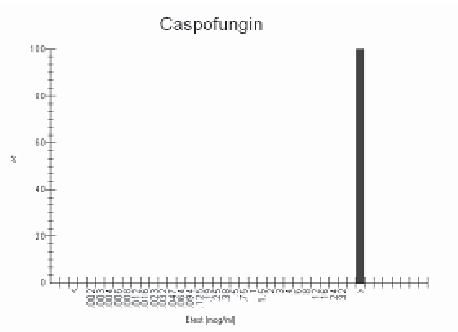

Fig. 4e.

Figs. 4a-4e. Antifungal MIC distribution for *Cryptococcus* sp. (n = 10).

The moulds in general have high MIC_{90} values, although their MIC_{50} appear low. The MIC_{90} value for amphotericin B for all moulds is 3 µg/mL and is close to the susceptible breakpoint, which is generally taken to be 1 µg/mL.[7] However for *Aspergillus*, the most common and significant mould isolate, the MICs are low for all the 5 antifungal drugs tested, which indicates that besides amphotericin B, posaconazole, itraconazole, voriconazole and caspofungin may be useful in treating *Aspergillus*. Note that *Aspergillus flavus* has high MIC value for amphotericin B. The overall high MIC_{90} values for moulds are therefore due to non-*Aspergillus* moulds consisting mainly of *Fusarium* species in this series.

Although there are no known breakpoints for posaconazole, the low MIC results suggest that posaconazole could be effective against *Candida*, *Cryptococcus* and *Aspergillus* species. Voriconazole, in our study, also appeared to have low MIC_{90} values against the same fungi, with an added advantage of relatively lower MIC_{90} values compared to posaconazole when tested against *Fusarium solani*. Caspofungin in this study appears to be effective against *Candida* species (except for *Candida parapsilosis* which corresponds to expected result), and the *Aspergillus* species. Nonetheless, the efficacy of these drugs would have to take into account their achievable serum levels (peak and sustained), other pharmacokinetic parameters, and would ultimately be determined by accumulation of clinical data.

One limitation of this study was the limited number of moulds available for testing. Although posaconazole has been demonstrated in vitro by Pfaller et al[12] to be more active against some of the zygomycetes compared to voriconazole, we were not able to validate this finding due to the limited number of zygomycetes in our study, as these are uncommonly isolated from our clinical specimens.

MIC testing *per se* is an in vitro test, while the MIC breakpoints for interpretative criteria have to take into account the pharmacokinetic and pharmacodynamic studies and clinical data. MICs may be used as a guide to treatment. Nonetheless, the efficacy of these drugs depends also on their achievable levels (peak and sustained) in serum or at site of infection, and other pharmacokinetic parameters.[13] More clinical data are needed to correlate MIC results with clinical outcome studies, in order to determine usefulness of the antifungal drug against specific fungi. Hence, interpretive criteria are lacking in many moulds and for the newer agents against *Candida* species.

Conclusion

Candida species in Singapore appear susceptible to the usual antifungal drugs recommended in the literature, that is amphotericin B, fluconazole (except for *Candida glabrata* and *Candida krusei*) and voriconazole. The other newer drugs, posaconazole and caspofungin (except for *Candida parapsilosis*), also appear to be suitable although there are no interpretive breakpoints.

Amphotericin B appears suitable against *Aspergillus* species, with perhaps the exception of *Aspergillus flavus*, which has a higher MIC_{90} value. Posaconazole, itraconazole, voriconazole and caspofungin also appear effective against local strains of *Aspergillus* species, although there are no interpretive breakpoints.

There are too few strains studied for the other fungal species, hence, no conclusion could be made.

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