Species distribution and antifungal susceptibility patterns of *Candida* species : is low susceptibility to itraconazole a trend in Malaysia ?

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SUMMARY

Resistance to antifungal agents has increased in *Candida* spp., especially in non-albicans species. Recent findings reported a strikingly low susceptibility in *Candida* spp. towards itraconazole in Malaysia. In this study, a colorimetric broth dilution method was utilized to determine the susceptibility of *Candida* spp. isolated in Kuala Lumpur Hospital within a six month period. A total of 82 isolates from blood, peritoneal and other fluids were tested against 8 antifungal agents using the Sensititre Yeast One method. These comprised of 32 (39%) *C. albicans*, 17 (20.7%) *C. glabrata*, 15 (18.3%) *C. tropicalis*, 13 (15.9%) *C. parapsilosis*, two (2.4%) *C. sake* and 1 (1.2%) each of *C. pelliculosa*, *C. rugosa* and *Pichia etchellsii/carsonii*. Overall, susceptibility of all isolates to caspofungin was 98.8%, amphotericin B, 97.6%; 5-flucytosine, 97.6%; voriconazole, 97.6%; posaconazole, 87.8%; fluconazole, 82.9%; ketoconazole, 79.3%; and itraconazole, 56.1%. A total of 18 *Candida* spp. isolates (22 %) were resistant to at least one antifungal agent tested, and half of these were resistant to three or more antifungal agents. *C. glabrata* was the most frequently identified resistant species (10 isolates), followed by *C. tropicalis* (4 isolates), *C. parapsilosis* (3 isolates) and *C. albicans* (1 isolate). Resistance was highest against ketoconazole (20.9%), followed by itraconazole (13.4%). However, 30.5% of isolates were susceptible-dose dependent towards itraconazole. Long-term usage of itraconazole in Malaysia and a predominance of non-*albicans* species may account for the results observed in this study. In conclusion, susceptibility to antifungal drugs is species-dependent among *Candida* spp.; reduced susceptibility to itraconazole is concomitant with the high number of non-*albicans Candida* species isolated in Malaysia.

KEY WORDS:
Candida, susceptibility, antifungal, Malaysia, itraconazole

INTRODUCTION

Candida spp. have been reported to be the fourth most commonly isolated organisms from nosocomial bloodstream infections, with the highest mortality rate, in United States hospitals[1]. Although *Candida albicans* is the predominant cause of mycoses in hospitalized patients, the rate of candidaemia caused by non-*albicans Candida* spp. is increasing[2,3]. Among these candidiases, *Candida glabrata* and *Candida krusei* infections are frequently reported (15-25% of bloodstream *Candida* isolates worldwide) and difficult to treat because of their low susceptibility to azole antifungal agents[4]. The emergence of antifungal resistance mechanisms and the different susceptibility to antifungal drugs among *Candida* spp. highlight the importance of accurate species identification and rapid antifungal MIC determination in the clinical setting. In addition, geographic variations are apparent in the distribution of *Candida* species, which emphasizes the importance of epidemiological data[5,6].

A recent report in Malaysia found a strikingly low susceptibility (40%) towards itraconazole among *Candida* spp. isolates[7]. This was the first such report in Malaysia which underlines the need for knowing susceptibility trends in local settings. Studies on antifungal susceptibility patterns in this region (South East Asia) are very limited, most likely due to the high cost involved.

The interpretation of antifungal susceptibility data was revised in 2011 by the Clinical Laboratory Standards Institute (CLSI) with the introduction of new breakpoints which are species dependent. These values indicate that the prevalence of antifungal resistance is higher than previously thought, with probable poorer clinical outcomes[8].

In Malaysia, antifungal susceptibility testing is carried out only in large, tertiary-care hospitals, and is restricted to fungal isolates from blood or other sterile sites. The turnaround time to obtain an antifungal MIC is at least 4 days, following specimen collection. As rapid initiation of antifungal therapy results in a better prognosis for the patient, empirical treatment needs to be prescribed to patients with high risk factors for serious infections. Therefore, this study aimed to provide susceptibility profiles for Malaysian *Candida* spp. isolates to both newer antifungal drugs and those which have long been used.

MATERIALS AND METHODS

Study site

This study was carried out at Hospital Kuala Lumpur, which is the largest public referral hospital in Malaysia, with 82 wards and 2502 beds.

Isolates

All *Candida* spp. isolates from blood and other body fluids

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obtained from January until July 2009 were evaluated in this study.

Identification of Yeast Isolates
Species determination was initially performed using the germ tube test to identify C. albicans isolates, following which, identity of all isolates was confirmed with ID 32C (bioMérieux, France).

Antifungal susceptibility testing
Susceptibility testing was carried out using the Sensititre Yeast One 08 broth microdilution method (TREK Diagnostic Systems, East Grinstead, West Sussex, England). The panels, containing dried antifungals (dilution range, µg ml^{-1}) posaconazole (0.008-8), amphotericin B (0.008-16), fluconazole (0.125-256), itraconazole (0.008-16), ketoconazole (0.008-16), 5-flucytosine (0.03-64), voriconazole (0.008-16), caspofungin (0.008-16) and alamar blue as an indicator dye were inoculated and read according to the manufacturer's instructions. In short, yeast isolates from a 24 hour culture on Sabouraud Dextrose Agar (SDA) were suspended in water to the equivalent of a 0.5 McFarland turbidity standard. After inoculation and incubation at 35°C for 24 hours, panels were read and colorimetric minimum inhibitory concentrations (MICs) were interpreted as the lowest concentration of antifungal agent preventing the development of a red color (the first blue well). Interpretation of the MIC was determined by the values given in the CLSI document M27-A3 (2008). Quality control was performed as stated by the manufacturer using *Candida parapsilosis* (ATCC 22019). For C. parapsilosis isolates, including the control strain, MIC values were obtained after 48 hours incubation, as per instructions provided by the manufacturer.

Interpretation of MIC values
Isolates were identified as susceptible, susceptible-dose dependent, intermediate (only for 5-flucytosine) and resistant, according to CLSI breakpoints except for amphotericin B, ketoconazole and posaconazole, for which breakpoints have not been indicated. However, based on experiments on animal models[10], isolates with MIC > 1.0 µg ml^{-1} for amphotericin B were categorized as resistant and based on previous studies[4,9], MIC > 0.125 µg ml^{-1} for ketoconazole and MIC > 1.0 µg ml^{-1} for posaconazole were considered resistant. As this study was carried out in 2009, interpretation of MIC values was carried out according to the CLSI published guidelines in 2008. However, to address the current situation, a comparison with the new breakpoints introduced in 2011 is discussed.

RESULTS

Specimen
A total of 82 *Candida* spp. isolates were obtained from 82 patients from January until July 2009. *Candida* spp. accounted for 2.8 % (82 out of 2908) of all positive blood and other body fluid culture specimens obtained during this period at Kuala Lumpur Hospital. The source of *Candida* spp. isolates was predominantly blood, yielding 56 (68.3%) isolates, while 17 (20.7%) isolates were from peritoneal fluid, 3 (3.7%) from bronchial washing, 2 (2.4%) from synovial fluid and one isolate (1.2%) each from CSF, pleural fluid, nephrotomy fluid and urine. The urine specimen was from a nine month old child admitted to the paediatric ICU ward with acute gastroenteritis and hypovolaemic shock.

Patients' data
Age range of patients who were positive for *Candida* spp. was from 5 days till 86 years. Mean age was 55. Twenty patients were being treated at ICU wards while 62 were from non-ICU wards.

Species distribution
Of a total of 82 *Candida* spp. isolates obtained throughout the study period, 32 (39%) isolates were identified as C. albicans, while 50 (61%) isolates were non-*albicans Candida* which comprised of 17 (20.7%) *C. glabrata*, 15 (18.3%) *Candida tropicalis*, 13 (15.9%) *C. parapsilosis*, 2 (2.4%) *Candida sake* and 1 (1.2%) each of *Candida pelliculosa*, *Candida rugosa* and *Pichia etchellsii/carsonii*. Of 20 isolates from patients in ICU wards, 10 isolates (50%) were *C. albicans*. Therefore, more non-*albicans* species were isolated from non-ICU wards (40 isolates; 64.5%). *C. albicans* was the predominant species causing candidaemia (33.9% of blood stream isolates), peritoneal (58.8%), bronchial (66.7%) and CSF (100%) infections, while *C. tropicalis* was the only species isolated from urine, pleural and nephrotomy fluids (one of each specimen) and *C. parapsilosis* was isolated from two synovial fluid specimens. *C. glabrata* accounted for 23.2% of blood stream isolates, followed by *C. tropicalis* (17.9%) and *C. parapsilosis* (16%).

Susceptibility profiles
The MIC values were higher for the non-*albicans Candida* compared to *C. albicans* isolates. All *C. albicans* isolates were susceptible to all antifungal agents except for two isolates. Overall, susceptibility of all isolates to caspofungin was 98.8%, amphotericin B, 97.6%; 5-flucytosine, 97.6%; voriconazole, 97.6%; posaconazole, 87.8%; fluconazole, 82.9%; ketoconazole, 79.3%; and itraconazole, 56.1% (Table I). MIC values were read after 24 hours incubation except for the two *C. parapsilosis* isolates where MIC was read after 48 hours as the growth control only showed a positive result after 48 hours. The MIC for the control strain *C. parapsilosis* ATCC 22019 was within the recommended CLSI range after 48 hours incubation.

Good susceptibility was noted towards caspofungin, amphotericin B, 5-flucytosine and voriconazole, among all *Candida* spp., only two isolates were resistant and these were multi-drug resistant (described below). Dose dependant susceptibility or resistance was noted for itraconazole, fluconazole, ketoconazole and posaconazole. Although resistance was highest against ketoconazole with 17% of isolates resistant, low susceptibility (dose dependant) or resistance against itraconazole was evident in 43.9 % of isolates. Overall, resistance against antifungals was mostly seen in *C. glabrata*, but all *C. glabrata* isolates were susceptible to caspofungin, amphotericin B, 5-flucytosine and voriconazole.

Table I: In vitro susceptibilities (MIC range and MIC90) of *Candida* spp. isolates towards eight antifungal agents as determined by Sensititre Yeast One method

Organisms (No. of isolates)	Antifungal agent	MIC range (µg ml-1)	MIC90 (µg ml-1)	No. of isolates S* (%)	No. of isolates R (%)	No. of isolates S-DD (%)
C. albicans (32)	posaconazole	<0.008- >8	0.06	31 (96.9)	1 (3.1)	-
	amphotericin B	0.25 - >16	1.0	31 (96.9)	1 (3.1)	-
	fluconazole	<0.12- >256	1.0	31 (96.9)	1 (3.1)	0 (0)
	itraconazole	0.008- >16	0.12	30 (93.8)	1 (3.1)	1 (3.1)
	ketoconazole	<0.008- >16	≤ 0.008	31 (96.9)	1 (3.1)	-
	5-flucytosine	<0.03-0.5	0.25	32 (100)	0 (0)	0 (0)
	voriconazole	<0.008- >16	0.015	31 (96.9)	1 (3.1)	0 (0)
	caspofungin	0.03- 16	0.12	31 (96.9)	1 (3.1)	-
C. glabrata (17)	posaconazole	0.008- >8	2.0	9 (52.9)	8 (47.1)	-
	amphotericin B	0.25 - 1	1.0	17 (100)	0 (0)	-
	fluconazole	0.25- 64	32	7 (41.2)	1 (5.9)	9 (52.9)
	itraconazole	0.03- >16	1.0	3 (17.6)	7 (41.2)	7 (41.2)
	ketoconazole	<0.008- 4	1.0	7 (41.2)	10 (58.8)	-
	5-flucytosine	<0.03-0.5	0.5	17 (100)	0 (0)	0 (0)
	voriconazole	<0.008- 1	0.5	17 (100)	0 (0)	0 (0)
	caspofungin	0.03- 0.12	0.12	17 (100)	0 (0)	-
C . tropicalis (15)	posaconazole	0.06 - >8	0.5	14 (93.3)	1 (6.7)	-
	amphotericin B	0.5 - 2	1.0	14 (93.3)	1 (6.7)	-
	fluconazole	0.5- >256	32	13 (86.6)	1 (6.7)	1 (6.7)
	itraconazole	0.12 - >16	1.0	1 (6.7)	2 (13.3)	12 (80.0)
	ketoconazole	<0.008- 8	1.0	11 (73.3)	4 (26.7)	-
	5-flucytosine	<0.03- >64	0.12	14 (93.3)	1 (6.7)	0 (0)
	voriconazole	<0.03- >16	0.5	14 (93.3)	1 (6.7)	0 (0)
	caspofungin	0.03- 0.25	0.12	15 (100)	0	-
C. parapsilosis (13)	posaconazole	0.015- 1	0.5	13 (100)	0 (0)	-
	amphotericin B	0.5 - 1	1.0	13 (100)	0 (0)	-
	fluconazole	0.5- 16	8.0	12 (92.3)	0 (0)	1 (7.7)
	itraconazole	0.06- 1	0.5	8 (61.5)	1 (7.7)	4 (30.8)
	ketoconazole	0.015- 0.5	0.25	11 (84.6)	2 (15.4)	-
	5-flucytosine	0.06- >64	0.25	12 (92.3)	1 (7.7)	0 (0)
	voriconazole	0.015- 0.25	0.25	13 (100)	0 (0)	0 (0)
	caspofungin	0.012- 1	1.0	13 (100)	0 (0)	-
Other species# (5)	posaconazole	<0.008- 0.5	0.5	5 (100)	0 (0)	-
	amphotericin B	0.25 - 1	1.0	5 (100)	0 (0)	-
	fluconazole	<0.12- 8	8.0	5 (100)	0 (0)	0 (0)
	itraconazole	0.015- 0.25	0.25	3 (60)	0 (0)	2 (40)
	ketoconazole	<0.008- 0.12	0.12	5 (100)	0 (0)	-
	5-flucytosine	<0.03-1	1.0	5 (100)	0 (0)	0 (0)
	voriconazole	<0.008- 0.5	0.5	5 (100)	0 (0)	0 (0)
	caspofungin	0.015- 2	2.0	5 (100)	0 (0)	-
All organisms (82)	posaconazole	<0.008- >8	2.0	72 (87.8)	10 (12.2)	-
	amphotericin B	0.25 - >16	1.0	80 (97.6)	2 (2.4)	-
	fluconazole	<0.12- >256	32	68 (82.9)	3 (3.6)	11 (13.4)
	itraconazole	0.008- >16	1.0	46 (56.1)	11 (13.4)	25 (30.5)
	ketoconazole	<0.008- >16	0.5	65 (79.3)	17 (20.7)	-
	5-flucytosine	<0.03- >64	0.25	80 (97.6)	2 (2.4)	0 (0)
	voriconazole	<0.008- >16	0.5	80 (97.6)	2 (2.4)	0 (0)
	caspofungin	0.015- 16	0.5	81 (98.8)	1 (1.2)	-

S, susceptible; R, resistant; S-DD, susceptible–dose dependent. S-DD categorization is not indicated for posaconazole, amphotericin B, ketoconazole and caspofungin.

* : Susceptibility breakpoints (µg ml-1): posaconazole > 1 (R); amphotericin B > 1 (R); fluconazole ≤ 8 (S), 16–32 (S-DD), ≥ 64 (R); itraconazole ≤ 0.125 (S), 0.25–0.5 (S-DD), ≥ 1 (R); ketoconazole > 0.125 (R); flucytosine ≤ 4 (S), 8–16 (S-DD), ≥ 32 (R); voriconazole ≤1 (S), 2 (S-DD), ≥4 (R); caspofungin >2 (Not Susceptible).

: C. sake (n=2), C. pelliculosa (n = 1), C. rugosa (n=1), Pichia etchellsii/carsonii (n=1). S-DD isolates were C. pelliculosa and Pichia etchellsii/carsonii.

Table II : In vitro susceptibilities (MIC) of *Candida* spp. isolates resistant towards antifungal agents listed according to increasing order of resistance

No.	Isolate	MIC (µg ml^{-1})							
		posaco-nazole	ampho-tericin B	fluconazole	itraconazole	ketoco-nazole	5-flucy-tosine	vorico-nazole	caspo-fungin
1	*C. tropicalis*	0.12 (S)	1 (S)	2 (S)	0.25 (SDD)	0.5 (R)	0.06 (S)	0.12 (S)	0.06 (S)
2	*C. parapsilosis*	0.12 (S)	1 (S)	4 (S) [SDD]	0.25 (SDD)	0.12 (S)	>64 (R)	0.12 (S)	0.5 (S)
3	*C. glabrata*	1 (S)	1 (S)	8 (S) [SDD]	0.5 (SDD)	0.5 (R)	0.06 (S)	0.25 (S)	0.1 (S)
4	*C. parapsilosis*	1 (S)	1 (S)	16 (SDD) [R]	0.5 (SDD)	0.5 (R)	0.12 (S)	0.25 (S) [I]	1 (S)
5	*C. glabrata*	1 (S)	1 (S)	32 (SDD)	0.5 (SDD)	0.5 (R)	<0.03 (S)	0.5 (S)	0.12 (S)
6	*C. tropicalis*	0.25 (S)	1 (S)	32 (SDD) [R]	0.5 (SDD)	0.5 (R)	>64 (R)	0.25 (S) [I]	0.25 (S)
7	*C. tropicalis*	0.5 (S)	0.5 (S)	2 (S)	1 (R)	1 (R)	0.06 (S)	0.5 (S) [I]	0.06 (S)
8	*C. parapsilosis*	0.5 (S)	1 (S)	8 (S) [R]	1 (R)	0.25 (R)	0.06 (S)	0.25 (S) [I]	0.5 (S)
9	*C. glabrata*	4 (R)	1 (S)	16 (SDD)	0.5 (S-DD)	0.5 (R)	0.06 (S)	0.5 (S)	0.06 (S)
10	*C. glabrata*	2 (R)	1 (S)	16 (SDD)	1 (R)	0.5 (R)	0.06 (S)	0.5 (S)	0.12 (S)
11	*C. glabrata*	4 (R)	1 (S)	16 (SDD)	1 (R)	0.5 (R)	0.06 (S)	0.5 (S)	0.12 (S)
12	*C. glabrata*	2 (R)	1 (S)	32 (SDD)	1 (R)	1 (R)	<0.03 (S)	0.5 (S)	0.06 (S)
13	*C. glabrata*	2 (R)	1 (S)	32 (SDD)	1 (R)	0.5 (R)	0.06 (S)	0.5 (S)	0.12 (S)
14	*C. glabrata*	2 (R)	1 (S)	32 (SDD)	1 (R)	1 (R)	0.03 (S)	1 (S)	0.12 (S)
15	*C. glabrata*	>8 (R)	0.5 (S)	32 (SDD)	>16 (R)	4 (R)	<0.03 (S)	1 (S)	0.06 (S)
16	*C. glabrata*	2 (R)	1 (S)	64 (R)	1 (R)	1 (R)	0.06 (S)	0.5 (S)	0.12 (S)
17	*C. tropicalis*	>8 (R)	2 (R)	>256 (R)	>16 (R)	8 (R)	0.06 (S)	>16 (R)	0.12 (S)
18	*C. albicans*	>8 (R)	>16 (R)	>256 (R)	>16 (R)	>16 (R)	0.06 (S)	>16 (R)	16 (R)

S, susceptible; R, resistant; SDD, susceptible–dose dependent.. Interpretation of MIC values using new breakpoints are indicated in square brackets (shown only for those that differ). I, intermediate. New breakpoints are not yet available for posaconazole and voriconazole (for C. glabrata).

A total of 18 isolates (22%) were resistant to at least one antifungal agent; of these, nine isolates were resistant to three or more antifungals (Table II). Multi-drug resistance was observed in two isolates. One isolate of *C. albicans* was resistant to all antifungal agents except 5-flucytosine and one isolate of *C. tropicalis* was only susceptible to 5-flucytosine and caspofungin. Repeated identification and susceptibility testing of these isolates revealed the same results. The *C. albicans* was a bloodstream isolate from a month-old baby, while the *C. tropicalis* was from blood of a 53 year old male patient. Both patients were admitted at non-ICU wards.

DISCUSSION

This study provides data on species distribution and susceptibility of *Candida* spp. isolated from patients at Hospital Kuala Lumpur within a six month period. In line with other published studies, *C. albicans* was the most common species isolated from blood and other sites[11-12] accounting for 39 % of isolates and *C. glabrata* was the second most commonly isolated species comprising 20.7% of isolates[13-14]. The predominance of non-albicans isolates such as *C. tropicalis*, *C. glabrata* and *C. parapsilosis* has been reported in previous studies conducted in Singapore[12] and Malaysia[7,15].

Most of the isolates were obtained from blood (68.3 %), followed by peritoneal fluid (20.7%) indicating a prevalence of candidaemia and peritoneal infection most likely due to dissemination of *Candida* from the gut. The non-albicans *Candida* spp. accounted for 66.1% of blood stream infections which emphasizes the importance of selecting appropriate antifungal drug for empirical or preemptive therapy.

Sensititre Yeast One method is a simple method for routine susceptibility testing. It is based on the CLSI reference method and has been shown to have high agreement with CLSI method[16,17]. Due to its ease of use this method was chosen for this study. However a previous study reported low agreement for susceptibility results with fluconazole for *C. glabrata* isolates where MIC values were higher with the Sensititre Yeast One test compared to the CLSI reference method[18]. As a comparative analysis of susceptibility testing methods was not carried out in the present study, this could not be determined.

In previous studies *C. glabrata* has been reported to show low susceptibility or resistance to azole drugs including triazole drugs such as fluconazole, itraconazole, voriconazole & posaconazole[6]. However, while many studies report concomitant resistance against fluconazole and itraconazole[4,6], other studies including the present study found greater resistance against itraconazole compared to fluconazole in *C. glabrata* isolates[18,19]. This may be due to azole specific resistance mechanisms in *C. glabrata* strains[20]. Increased incidence of infections caused by *C. glabrata* has been reported worldwide, which is to be expected as antifungal therapy over a long period of time would select for this resistant species.

In the present study *Candida* isolates were least susceptible to itraconazole; 56.1% of isolates were susceptible while the remaining were either susceptible dose-dependent (SDD) or resistant. Low susceptibility to itraconazole was noted not only in C. glabrata, but also in *C. tropicalis* and *C. parapsilosis* isolates (Table I). A similar finding was reported at the UKM Medical Centre in Kuala Lumpur for *Candida* spp. isolates obtained from 2005 to 2006 and tested using Sensititre Yeast One method[7]. A study in Singapore found susceptibility to itraconazole at 62.5% using the same method[12]. However, another recent study which used the Etest method to test *Candida* spp. isolates obtained in Hospital Kuala Lumpur from 2006 to 2008 reported only 7.2% of isolates either SDD or resistant to itraconazole[21]. This may be due to species distribution where a higher number of *C. glabrata* isolates were tested in the present study and susceptibility testing method variability. Although the earlier study also tested isolates from Hospital Kuala Lumpur, it was not a prospective study, unlike the present study which included all *Candida* spp. isolated from systemic sites within a six-month period. This accounts for the difference in *Candida* species distribution in the two studies.

Itraconazole had been administered in Hospital Kuala Lumpur as empirical therapy for suspected invasive fungal infections in high risk patients (such as bone marrow transplant recipients) for more than 10 years, at the time this study was conducted. Long-term exposure to itraconazole may explain the development of reduced susceptibility to the drug in clinical fungal isolates. Although no early data exists for susceptibility of *Candida* spp. towards itraconazole, in a study carried out between 1997 and 1999 in Malaysia, all 102 *Candida* spp. isolates (82% non-albicans species) tested were susceptible to ketoconazole and 97% were susceptible to fluconazole[15]. This indicates a marked change in the susceptibility pattern of *Candida* spp. isolates after 10 years of antifungal drug therapy, which is also due to an increased incidence of *C. glabrata* infections.

When analyzing the susceptibility data according to the new breakpoint values which are species dependent, higher rates of resistance or reduced susceptibility towards fluconazole and voriconazole were noted (Table II). These results underline the global trend of increasing resistance to azole drugs, in *Candida* species which is also associated with higher mortality rates in patients[22].

In conclusion, the present study found susceptibility to azole drugs is species dependent, with low susceptibility towards itraconazole noted in the non-*albicans Candida* species isolated.

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