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ORIGINAL ARTICLE

Antifungal susceptibility profile and biofilm-producing capability of *Candida tropicalis* isolates in a tertiary medical centre

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Abstract

Background: Candida tropicalis is a globally distributed yeast that has been popping up in the medical literature lately, albeit for unenviable reasons. *C. tropicalis* is associated with substantial morbidity, mortality as well as drug resistance. The aims of this study were to ascertain the antifungal susceptibility profile and the biofilm-producing capability of this notorious yeast in our centre. *Methods: C. tropicalis* isolates from sterile specimens were collected over a 12-month period. Conclusive identification was achieved biochemically with the ID 32 C kit. Susceptibility to nine antifungal agents was carried out using the colourimetric broth microdilution kit Sensititre YeastOne YO10. Biofilm-producing capability was evaluated by quantifying biomass formation spectrophotometrically following staining with crystal violet. *Results:* Twenty-four non-repetitive isolates of *C. tropicalis* were collected. The resistance rates to the triazole agents were 29.2% for fluconazole, 16.7% for itraconazole, 20.8% for voriconazole and 8.3% for posaconazole—the pan-azole resistance rate was identical to that of posaconazole. No resistance was recorded for amphotericin B, flucysosine or any of the echinocandins tested. A total of 16/24 (66.7%) isolates were categorized as high biomass producers and 8/24 (33.3%) were moderate biomass producers. None of our isolates were low biomass producers. *Conclusion:* The *C. tropicalis* isolates from our centre were resistant only to triazole agents, with the highest resistance rate being recorded for fluconazole and the lowest for posaconazole. While this is not by itself alarming, the fact that our isolates were prolific biofilm producers means that even azole-susceptible isolates can be paradoxically refractory to antifungal therapy.

Keywords: antifungal, biofilm, biomass, *Candida tropicalis*, fluconazole, triazole

INTRODUCTION

Since the dawn of the new millennium, *Candida* species have rapidly surfaced as a foremost cause of both healthcare-associated and opportunistic infections, as a direct consequence of the ever-rampant use of intravenous catheters, total parenteral nutrition, cytotoxic chemotherapeutic agents, and last but not least, broad-spectrum antibiotics.[1] The upsurge in the human population of individuals with compromised immune systems have further contributed to the increase in these infections. Although in excess of 200 species of *Candida* have been described hitherto, 90% of invasive human fungal infections are attributable to only five species: *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* and *Pichia kudriavzevii* (formerly known as *Candida krusei*).[2] The prevalence of non-albicans *Candida* (NAC) appears to be rising in certain geographical areas, with *C. tropicalis* taking the pole position from *C. albicans* in tropical countries, where it can account for up to 66% of candidaemia cases.[3]

The designation of a yeast as a NAC is not done merely for academic purposes but also because of the stigma of antifungal (namely fluconazole) resistance attached to it.[4] Therefore, it is conceivable that *C. tropicalis* is also notorious for its diminished susceptibility to

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fluconazole.[1] While antifungal susceptibility is most conveniently inferred from reading drug minimal inhibitory concentration (MIC) values, this practice may underestimate drug resistance *in-vivo*. The presence of biofilms has been known to bestow upon organisms the extraordinary ability to withstand the effects of antifungals. Thus, coupled with high antifungal MICs, biofilms help to orchestrate the 'perfect storm' for treatment failure. Additionally, biofilms formed on indwelling medical devices can be released periodically into the bloodstream to facilitate the establishment of disseminated candidiasis that can further thwart antifungal treatment efforts.[5] *C. tropicalis* is putatively a prolific biofilm producer, outdoing *C. albicans* in some studies.[6] Therefore, the objectives of this study were to determine the antifungal susceptibility profile and the biofilm-producing capability of the *C. tropicalis* isolates in our local setting.

MATERIALS AND METHODS

Study design

This cross-sectional study over a period of 12 months was conducted from November 2020 until November 2021. Non-duplicate *C. tropicalis* isolates were collected from sterile specimens (e.g., blood, joint aspirate, bile and pleural fluid) from patients who presented to Hospital Canselor Tuanku Muhriz (HCTM) for various medical or surgical conditions. HCTM is a 1000-bedded tertiary medical centre with diverse specialities and subspecialities (such as intensive care, infectious diseases and oncology) in the capital city of Malaysia.

Isolate identification

During routine culture of sterile clinical specimens, any yeast isolate found to produce branched pseudohyphae with numerous ovoid blastoconidia on cornmeal agar were morphologically identified as *C. tropicalis* and set aside for further identification. Definitive identification was achieved biochemically using ID 32 C (bioMérieux SA, France), a commercially available yeast identification kit with 32 miniaturised carbohydrate assimilation tests. The identification was carried out in accordance with the manufacturer's instructions. To identify each *C. tropicalis* isolate, 1-3 identical colonies that had been growing on artificial media for between 24-48 hours were removed to prepare a suspension with a turbidity equivalent to 2 McFarland in an ampule containing 2 mL of API Suspension Medium. Following this, 250 µL of the suspension was transferred into an ampule containing API C Medium. The inoculated API C Medium was then homogenized and 135 µL of the suspension was transferred into each cupule of the ID 32 C strip. Finally, a transparent lid was placed on the strip before incubating it at 30°C for 24-48 hours. As a form of purity assurance, the remaining inoculated API C Medium was also used to inoculate a CHROMagar plate and a Sabouraud dextrose agar (SDA) plate. If the CHROMagar culture yielded colonies other than blue or the SDA culture produced colonies with obviously different morphologies, the sample was deemed impure/contaminated and the corresponding ID 32 C strip was discarded. Each ID 32 C strip with acceptable purity plates was inspected visually after 24 hours of incubation, and cupules more turbid than the control were recorded as positive. The cupule reactions obtained were coded into a 10-digit numerical profile and interpreted using the api*web* identification software. Identifications reported by the software as 'very good' or 'good' were taken as correct. A second reading at 48 hours was performed only for strips with 'low discrimination', 'unacceptable or doubtful profile' or 'identification not valid before 48 hours of incubation'.

Antifungal susceptibility testing (AFST)

Our *C. tropicalis* isolates were tested against a total of nine antifungal agents (amphotericin B, anidulafungin, caspofungin, micafungin, flucytosine, itraconazole, voriconazole, posaconazole and fluconazole). Susceptibility testing was carried out using the commercially available colorimetric broth microdilution kit Sensititre YeastOne YO10 (Trek Diagnostic Systems, UK), as per the manufacturer's instructions. The kit contains desiccated antifungal agents and a colorimetric indicator in a 96-well microplate format. To perform the test for each isolate, yeast colonies from a 24-hour-old culture were emulsified in sterile saline and vortexed for 15 seconds to get a homogenised suspension. The turbidity of the suspension was adjusted to 0.5 McFarland and 20 µL of this suspension was transferred into 11 mL of YeastOne broth. The inoculated broth was then poured into a sterile seed trough. Using a pipette, 100 µL of the inoculated broth was then transferred into each well of the Sensititre plate. An adhesive seal was used to cover all the inoculated wells, with attention to avoid creases. The inoculated plate was then incubated

at 35°C for 24-25 hours. The following day, if the growth well was red, the MIC for each antifungal was read manually as the lowest antifungal concentration that inhibited fungal growth (i.e. the first blue well). However, if the growth well was still blue (or faintly purple), the plate was re-incubated for an additional 24 hours before being re-examined. The relevant Clinical & Laboratory Standards Institute (CLSI) documents were used to interpret the MIC results. Although we tested each isolate against nine antifungal agents, the CLSI only provided breakpoints for five antifungals (i.e., anidulafungin, caspofungin, micafungin, voriconazole and fluconazole) in its most recently published guideline.[7] Thus, for the remaining four antifungal agents, we referred to a set of older CLSI documents.[8-9]

Quantification of biofilm-producing capability

The biofilm-producing capability of our *C. tropicalis* isolates was evaluated through the quantification of biomass formation using crystal violet stain as described by Marcos-Zambrano *et al*.[6] For each isolate, a loopful of yeast colonies from a 24-hour-old culture was inoculated in 20 mL of yeast peptone dextrose broth, which was then incubated with shaking at 160 rpm at 35°C overnight. Following centrifugation, the cells were re-suspended in 20 mL of phosphate buffered saline and centrifuged at 3000 × g for five minutes for washing – this step was executed twice, and the washed cells were re-suspended in 10 mL of RPMI 1640 broth medium; the suspension's turbidity was adjusted to 0.35 McFarland. A total of 100 µL of the suspension was inoculated in a 96-well microtiter plate and incubated at 37°C for 24 hours. Planktonic cells were then discarded after three washes with 100 µL of phosphate buffered saline solution. The microtiter plate with the preformed biofilm (containing sessile cells) was then turned upside down until it was completely dry. Each *C. tropicalis* isolate was tested in triplicate. Next, 125µL of 0.1% crystal violet (CV) solution was added to each well of the microtiter plate that contained the dry preformed biofilms and the plate was incubated at room temperature for 15 minutes. The solution was then removed by submerging the plate in a container with distilled water – this step was replicated until all the unbound CV was removed. The plate was then overturned again until it was completely dry, and 125 µL of a 30% acetic acid solution was added to each well to solubilise the biofilm-bound CV following incubation at room temperature for 15 minutes. At the end of the incubation, 100 µL of the solution was transferred onto a new 96-well microtiter plate that was spectrophotometrically read at a wavelength of 540 nm. *C. albicans* ATCC 14053, *C. parapsilosis* ATCC 22019 and 30% acetic acid solution served as our high biomass, low biomass and negative controls, respectively. The biomass formation was classified as 'low' if the optical density reading at 540 nm (OD_{540}) was < 0.44, 'moderate' if between 0.44–1.17, and 'high' if > 1.17. Figure 1 depicts the appearance of 'low' and 'high' biomasses under an inverted light microscope.

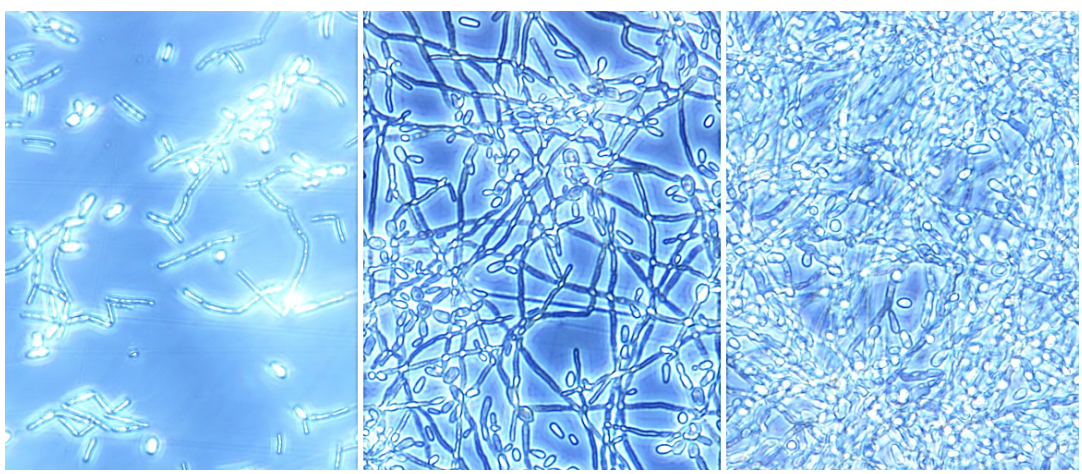

FIG. 1. The microscopic image on the left is that of our *C. parapsilosis* control strain with low biomass formation (because none of our clinical *C. tropicalis* isolates had low biomass). The images in the middle (isolate no. 16 in Table 1) and on the right (isolate no. 20 in Table 1) represent clinical *C. tropicalis* isolates with moderate and high biomass formation, respectively. From left to right, the images show progressively higher biomass formation, as evidenced by increasingly thicker and denser hyphal structures. All images are shown at 400x magnification.

RESULTS

A total of 24 non-duplicate biochemically confirmed *C. tropicalis* isolates were cultured from sterile clinical specimens during the study period. Twenty (83.3%) of our specimens were blood and the remaining four (16.7%) were one specimen each of bile, pleural fluid, joint aspirate and bronchoalveolar lavage.

The antifungal MIC distributions for individual isolates are displayed in Table 1. Where amphotericin B and all three echinocandins are concerned, our resistance rate was 0% to each drug (with resistance to any one agent being defined as an MIC reading of > 1.0 μg/mL). Likewise, our isolates' resistance rate to flucytosine was 0% (with resistance being defined as an MIC reading of ≥ 32.0 μg/mL). A total of 7/24 isolates were classified as being fluconazole-resistant (defined as an MIC reading of ≥ 8.0 μg/mL), resulting in a fluconazole

Table 1. Antifungal MICs and biomass quantification

Isolate code	Antifungal MICs (μg/mL)									Biomass (OD_{540})*
	AmB	Ani	Mic	Cas	5-F	Pos	Vor	Itr	Flu	
1	1.0	0.12	0.030	0.030	0.06	8.00	8.00	16.00	128.0	1.417
2	0.5	0.12	0.030	0.120	0.06	0.25	0.25	0.25	2.0	2.678
3	0.5	0.06	0.030	0.015	0.06	0.12	0.12	0.12	1.0	0.596
4	1.0	0.12	0.060	0.060	0.25	0.12	0.12	0.50	2.0	1.216
5	1.0	0.12	0.030	0.060	0.12	8.00	8.00	16.00	128.0	2.035
6	1.0	0.12	0.030	0.015	0.06	0.50	0.50	0.50	4.0	0.539
7	0.5	0.06	0.015	0.030	0.06	0.06	0.06	0.12	1.0	1.890
8	1.0	0.06	0.030	0.030	0.06	0.25	0.12	0.25	2.0	0.890
9	1.0	0.06	0.030	0.030	0.06	0.12	0.06	0.25	1.0	1.835
10	1.0	0.06	0.015	0.030	0.06	0.25	0.25	0.25	4.0	1.274
11	1.0	0.12	0.030	0.500	0.12	0.50	0.50	0.50	8.0	1.522
12	1.0	0.06	0.015	0.060	0.06	1.00	0.25	0.50	2.0	1.336
13	1.0	0.12	0.030	0.060	0.12	0.50	0.50	1.00	4.0	1.036
14	1.0	0.12	0.015	0.120	0.06	0.25	0.12	0.25	2.0	1.487
15	1.0	0.06	0.015	0.030	0.06	0.12	0.25	0.25	2.0	1.102
16	1.0	0.12	0.030	0.250	0.12	0.50	4.00	0.50	128.0	0.785
17	1.0	0.12	0.015	0.250	0.25	0.50	0.25	0.50	2.0	1.539
18	0.5	0.12	0.030	0.030	0.06	0.25	0.25	0.25	16.0	1.251
19	1.0	0.12	0.030	0.250	0.25	0.50	1.00	0.50	8.0	1.095
20	1.0	0.12	0.030	0.030	0.06	0.12	0.12	0.25	1.0	2.579
21	1.0	0.12	0.015	0.060	0.06	0.25	0.25	0.25	2.0	1.253
22	0.5	0.12	0.015	0.120	0.12	0.03	0.03	0.06	0.5	1.455
23	1.0	0.12	0.015	0.030	0.06	1.00	8.00	1.00	128.0	1.296
24	0.5	0.12	0.030	0.060	0.06	1.00	0.25	0.50	2.0	1.112
CAl	-	-	-	-	-	-	-	-	-	1.678
CPa	-	-	-	-	-	-	-	-	-	0.267

MICs, minimal inhibitory concentrations; AmB, amphotericin B; Ani, anidulafungin; Mic, micafungin; Cas, caspofungin; 5-F, flucytosine; Pos, posaconazole; Vor, voriconazole; Itr, itraconazole; Flu, fluconazole; OD_{540}, optical density at 540 nm; CAl, *C. albicans* ATCC 14053; CPa, *C. parapsilosis* ATCC 22019

* mean of three OD_{540} readings

resistance rate of 29.2%. Voriconazole resistance (defined as an MIC reading of ≥ 1.0 µg/mL) was detected in 5/24 isolates (20.8%). Voriconazole resistance seems to be a suitable surrogate marker for fluconazole resistance, as all five of our voriconazole-resistant isolates were also fluconazole-resistant. Only 2/24 isolates (8.3%) were resistant to posaconazole (defined as an MIC reading of > 1.0 µg/mL). Posaconazole resistance appears to serve as an appropriate surrogate marker for fluconazole resistance as well, as both of our posaconazole-resistant isolates were also fluconazole-resistant. Lastly, where itraconazole is concerned, 4/24 of our isolates (16.7%) tested resistant (defined as an MIC reading of ≥ 1.0 µg/mL). Its utility as a surrogate marker for resistance to other azoles (particularly to fluconazole) is impeded by the occurrence of itraconazole monoresistance (isolate no. 13). Resistance to all four azoles tested was observed in just two isolates (8.3%). Coincidentally, these two isolates (no. 1 and no. 5) were also posaconazole-resistant, indicating the applicability of posaconazole resistance as a surrogate marker for pan-azole resistance.

Where biofilm production is concerned, all our *C. tropicalis* isolates were either moderate or high biomass producers, with the lowest mean OD_{540} reading being 0.539 (isolate no. 6) and the highest 2.678 (isolate no. 2). A total of 16/24 isolates (66.7%) were high biomass producers while the remaining 8/24 isolates (33.3%) were moderate biomass producers. The 16 isolates categorised as high biomass producers were cultured from patients with fungaemia (13), pneumonia (1), cholangitis (1) and pleural effusion (1). However, since the total number of blood specimens with *C. tropicalis* was 20, only 65% of patients with *C. tropicalis* fungaemia had high biomass-producing strains. Conversely, 100% of patients with pneumonia, cholangitis or pleural effusion associated with *C. tropicalis* had high biomass-producing strains but we only had one specimen each of bronchoalveolar lavage, bile fluid and pleural fluid. There was no statistically significant association between the amount of biomass produced and susceptibility to fluconazole, which is the agent with the highest resistance rate (Table 2). Taking isolates no. 2 and no. 6 as examples again, it can be appreciated that despite the higher biomass formation (by a factor of five) by the former, its azole MICs were in fact lower across the board than those of the latter.

DISCUSSION

Fluconazole is a first-generation triazole antimycotic agent which has been available for use since the 1990s.[10] It is still favoured today because of its low cost (partly because of easy access to generic brands), excellent bioavailability and good side-effect profile. Fluconazole is 'strongly recommended' by the Infectious Diseases Society of America (IDSA) as an alternative therapy for candidaemia in non-neutropenic patients, provided that a fluconazole-resistant *Candida* sp. is unlikely.[11] Unfortunately, with our *C. tropicalis* isolates, we have grim news to report, as 29.2% of our isolates tested as fluconazole-resistant. Although our rate is not the lowest in the Asian region, it is equivalent (if not lower) compared to those recently reported by other investigators from key Asian nations. For instance, investigators from China and Nepal reported markedly higher fluconazole resistance rates of 39.5% and 54.6% among their *C. tropicalis* isolates, respectively.[12,13] Our resistance rate is comparable to those reported by investigators from Thailand (i.e., 28.6%) and Japan (i.e., 30.2%).[14,15] Researchers from Singapore, another immediate neighbour of Malaysia, reported a lower fluconazole non-susceptibility rate of 22% for their *C. tropicalis* isolates.[16] The true fluconazole resistance rate in the Singaporean study is possibly lower, as the authors also included isolates which tested as 'susceptible-dose dependent' to fluconazole in their analysis.

Although itraconazole is also a first-generation triazole, it is not explicitly recommended for treating invasive candidiasis—it is

TABLE 2: Association between fluconazole susceptibility and biomass formation

Fluconazole susceptibility	Biomass formation		*p*-value*
	Moderate	High	
Susceptible	6	11	1.000
Resistant	2	5	

* two-tailed *p*-value derived from Fisher's exact test

largely reserved for the treatment of mucosal candidiasis.[11] Hence, although the itraconazole resistance rate of our *C. tropicalis* isolates is noticeably lower compared to that of fluconazole (16.7% vs. 29.2%), it is of limited clinical impact. Moreover, the CLSI did not update the breakpoints for itraconazole when it issued a revised antifungal susceptibility testing guideline for yeasts in 2020, raising the prospect that we could have overcalled susceptibility (or underreported resistance) to itraconazole. Where the second-generation triazoles are concerned, the resistance rates of our *C. tropicalis* isolates are generally low (i.e., 20.8% for voriconazole and 8.3% for posaconazole). Our finding of a reasonably lower voriconazole resistance rate (compared to fluconazole) contrasts with those of other investigators, who have reported that these two triazoles share near parallel resistance rates.[14,15] Thus, the 'second-generation' label accorded to voriconazole does not necessarily reflect its enhanced activity against yeasts (since voriconazole is still fungistatic rather than fungicidal for yeasts), but more its broader antifungal spectrum which encompasses moulds.[17] Posaconazole, on the other hand, potentially inhibits the 14α-demethylase enzyme responsible for ergosterol synthesis in yeast cell membranes to a greater extent than fluconazole and is even fungicidal for *Candida* spp.[18] Therefore, it is not surprising that posaconazole was the triazole with the lowest resistance rate for our cohort of *C. tropicalis* isolates and owing to this, it is also an apt marker for pan-azole resistance.

For AmB (the only polyene antifungal agent tested), we opted for an MIC breakpoint of > 1.0 μg/mL to denote AmB resistance because this was once proposed in a CLSI document.[8] However, like itraconazole, CLSI did not update the AmB breakpoints in its revised antifungal susceptibility testing guideline. Owing to this, some investigators employ the epidemiological cut-off value (ECV) instead to determine if an isolate is resistant to AmB. This is essentially > 2.0 μg/mL for AmB (i.e. one dilution higher compared to our breakpoint) and any isolate with an AmB MIC above this is considered 'non-wild type' and thus AmB-resistant.[19] Nonetheless, irrespective of the criteria used, all our *C. tropicalis* isolates were susceptible to AmB. Similarly, all our *C. tropicalis* isolates were susceptible to all three echinocandin agents tested (i.e. micafungin, anidulafungin and caspofungin). This finding of ours lends support to the IDSA guideline on candidiasis which 'strongly recommends' an echinocandin for the treatment of candidaemia in both neutropenic and non-neutropenic patients.[11] Alas, due to cost issues (generic echinocandin brands are yet to be available in our hospital's formulary at the time of writing and a vial of an echinocandin can easily cost more than 50 times that of fluconazole), it is doubtful that the IDSA recommendation on echinocandins can be adopted in its entirety by hospitals with limited resources. In addition, echinocandins are also the first-line therapy for specific NAC species (e.g. *Candida auris*)—this would further diminish its already limited availability to treat infections caused by *C. tropicalis*.[20]

To further complicate matters, it is an 'open secret' that a susceptible *in-vitro* AFST result does not guarantee *in-vivo* treatment success with the antifungal agent concerned—this is despite the daily task of performing AFST by many diagnostic mycology laboratories throughout the world. This can be elucidated (at least in part) by the inherent capability of *Candida* spp. to produce biofilms. A yeast biofilm is fundamentally composed of cell communities attached to either a biotic (living) or an abiotic (non-living) surface and embedded within an exopolymeric matrix fabricated by these same cells.[5,21] Besides facilitating adherence of cells onto surfaces, the extracellular polymeric matrix also shields the cells from environmental insults and is believed to assist in the retention of nutrients. The matrix can also sequester antifungal drugs, effectively thwarting them from reaching their intended intracellular targets.[22] It is therefore of no coincidence that *C. tropicalis* has been implicated in device-related infections (which can be particularly recalcitrant) since this yeast can form extensive biofilms on the surface of polyvinyl chloride and polystyrene materials.[21] Hence, unless eradicated, the cells in the biofilm will continue to serve as a source of reinfections. Unfortunately, antifungal therapy by itself cannot eradicate biofilms, mandating the ultimate removal of an infected medical device (which is frequently critical for patient care) to achieve fungal clearance.[22]

Sessile yeast cells within the biofilm have also been shown to possess a modified phenotype related to growth and gene transcription rates compared to that of their planktonic counterparts.[23] Although this altered phenotype contributes to drug resistance, this resistance does not entail the procurement of genetic mutations,

because sessile biofilm cells resuspended or recultured in planktonic conditions will once again be susceptible to antifungal agents.[22] Thus, it is imperative to realize that diagnostic mycology laboratories that perform *in-vitro* AFST only report antifungal MICs valid for planktonic cells (which have yet to form biofilms). Investigators have reported that sessile cells are more resistant to antifungals compared to their planktonic counterparts by a factor ranging from two (for echinocandins) to a thousand (for triazoles).[22] Echinocandins appear to be comparatively more effective against sessile cells because they inhibit a synthase enzyme tasked with the production of β-1,3 glucan, a key matrix carbohydrate.[24] Our study has unequivocally confirmed that *C. tropicalis* is indeed a prolific biofilm producer, with high biomass readings being recorded in two-thirds of our isolates. While it is not impossible to determine antifungal MICs for sessile cells, this is largely impractical for diagnostic laboratories as it is more costly, laborious and time-consuming. We have also shown in Table 2 that attempting to predict high biomass production by considering the planktonic cell drug susceptibility to fluconazole (i.e., the drug with the highest calculated resistance rate) is futile—a fully susceptible *C. tropicalis* isolate is just as likely to be a high biomass producer as a drug-resistant one.

The main limitation of this study was the relatively small number of *C. tropicalis* isolates. This single-centre study only included yeast isolates cultured from sterile clinical specimens and was conducted over a brief period of 12 months.

In conclusion, the *C. tropicalis* isolates from our centre have documented *in-vitro* resistance only to triazole antifungal agents, with the highest resistance rate being recorded for fluconazole and the lowest for posaconazole. However, AFST reports for *C. tropicalis* should not be taken at face value, particularly if the isolates are susceptible to azoles. The very fact that *C. tropicalis* is a prolific biofilm producer implies that the peril of treatment failure with azoles is real, especially if indwelling medical devices are also present. Thus, in centres that favour an azole as first-line therapy for infections associated with *C. tropicalis*, the threshold to switch to an echinocandin in the event of treatment failure should be low. Endeavours to develop novel drugs that can hamper yeast biofilm synthesis should also be urgently undertaken.

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Author contributions: Ding CH, Neoh HM and Tan TL conceptualised and supervised the study, and participated in manuscript writing. Navarathinam SD performed literature review, data collection, data analysis and manuscript writing. Wahab AA and Tzar MN reviewed and revised the manuscript.

Conflict of interest: All authors have no conflicts of interest to declare.

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