Journal of Associated Medical Sciences 2022; 55 (3): 44-50

JAMS
Journal of Associated Medical Sciences

Thai-Journal Citation Index Centre (TCI) & ASEAN Citation Index (ACI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index



Species characterization and antifungal susceptibility profile of yeast isolates from blood cultures of fungemic patients in Thammasat University Hospital, Thailand

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ARTICLE INFO

Article history:
Received 20 April 2022
Accepted as revised 23 June 2022
Available online 18 July 2022

Keywords:
Yeast, MALDI-TOF MS, Sensititre YeastOne

ABSTRACT

Background: *Candida albicans* is the most isolated fungal agent in a worldwide health system. Species distribution of *Candida* infection is different according to geographical regions. However, a shift in favor of non-albicans *Candid*a species with antifungals resistance have increased as an important cause of candidemia.

Objectives: This study aimed to identify the species of yeasts isolated from the blood samples of patients at the university hospital and to determine in vitro susceptibilities of three most common isolates against nine antifungal agents.

Materials and methods:In total, 130 yeast isolates from 130 patients were defined the species using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Antifungal susceptibility testing was carried out using broth dilution Sensititre YeastOne panels included amphotericin B, 5-fucytosine, fluconazole, itraconazole, voriconazole, posaconazole, anidulafungin, micafungin, and caspofungin.

Results: The most common species in all age groups was *C. tropicalis* (n=48, 36.9%), followed by *C. albicans* (n=38, 29.2%). *C. glabrata* (n=23, 17.7%) was more common among elderly patients, while *C. parapsilosis* (n=9, 6.9%) was more frequently isolated from younger patients. Antifungal susceptibility testing in *Candida* species expressed MIC in the low level of almost antifungal drug except for reduced fluconazole susceptibility against *C. glabrata and C. tropicalis* isolates.

Conclusion: *C. tropicalis* is the most common infection in candidemic patients. Fluconazole resistance strains were found in *C. glabrata* and *C. tropicalis*, respectively. In addition, voriconazole resistance strains were found in *C. tropicalis*.

Introduction

Bloodstream yeast infection (BYI) especially the genus *Candida* has increased worldwide in healthcare system because of the rising number of immunosuppressed patients leading to high disencouragement and fatality in the range of 40% to 60%.[1] From the ARTEMIS DISK Surveillance Program between 1997 and 2007, *Candida albicans* is the major species reported just before *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* (*Pichia kudriavzevi*i), respectively.[2] Species spreading of Candida infection is different according to geographical regions. However, a change of species distribution to non-albicans Candida (NAC) may be increasingly resistant to common antimicrobial agents.[3] Recently, 52 candidemic patients in a University Hospital Thailand have been identified as NAC (64%) and *Candida albicans* (36%). From 35 % of *C. tropicalis* isolates, 68.7% were high-level fluconazole

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doi: 10.12982/JAMS.2022.023
E-ISSN: 2539-6056

resistance.[4] Therefore, rapid and curate species identification have become important, especially in cases involving invasive infection.

The situation of non-*albicans Candida* was increasingly resistant to commonly used antimicrobial agents especially in azole agents.[5-6] Recently, fluconazole resistance in *C. glabrata* strains has been reported in Asia-Pacific region including Thailand.[7-8] Increasing resistance rates of fluconazole, voriconazole, micafungin, caspofungin and anidulafungin in non-albicans candidemia isolates especially *C. tropicalis* and *C. glabrata* isolates were reported in 5 tertiary hospitals of Taiwan.[9] In Thailand, *C. glabrata* and *C. tropicalis* isolates were increasingly resistant to azole agents and *C. glabrata* isolates were decreasingly susceptible to echinocandins especially caspofungin.[8, 10]

These research objectives were to identify yeasts in the species level and investigate their antifungal susceptibility patterns. All strains were isolated from blood samples of suspected yeast septicemic patients in 3.25 years period at a Thai University Hospital. In vitro antifungal susceptibility testing of three most common yeasts isolated from blood was conducted using nine antifungal agents in the SYO panel included amphotericin B, 5-fucytosine, fluconazole, itraconazole, voriconazole, posaconazole, anidulafungin, micafungin, and caspofungin.

Materials and methods

Sample collection

A total of 130 yeast isolates obtained from blood culture from January 2013 to March 2016 (3.25 years) in Microbiology Laboratory, Thammasat University Hospital located at the central region of Thailand were tested for identification and antifungal susceptibility test. The isolates were collected in sequential order without selection criteria. Duplicate isolates from the same patient were excluded. Reference strains of *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control. The study was approved by the Ethics Sub-Committee Board for Human Research Involving, Thammasat University, No.3, Thailand, Protocol code 132/2562.

Yeast identification using Matrix-assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI-TOF MS)

Yeast isolates were subcultured on Sabouraud dextrose agar (SDA) at 37°C for 24 to 48 hours and fresh growth was extracted using formic acid extraction method described, briefly, fresh cells were suspended in a 1.5 mL sterile tube and mix thoroughly in 300 µL of sterile distilled water. Then, Yeast suspension was added with 900 µL of absolute ethanol and centrifuged at 13,000 rpm for 2 min and discarded the supernatant. The air-dried pellet at room temperature was resuspended in 50 µL of 70% formic acid and mix thoroughly before the addition of 50 µL of acetonitrile. After 2-min centrifugation at 13,000 rpm, 1 µL of the supernatant was placed onto a single spot of the polished steel target plate and air-dried prior to MALDI-TOF analysis.[10] Each spot was covered by 1 µL of α–cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 2.5% trifluoroacetric acid (TFA) (Sigma-Aldrich, France) matrix. Identification was performed on an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Instrument calibration using the Bruker Bacterial Standard Escherichia coli (#8255343) was performed with Flex control program. Ionized spectra for each spot were acquired on linear mode at a laser frequency of 20 Hz within a mass range from 2000 to 20000 Da. MBT Compass software was automatically smoothing, normalization, baseline subtraction and peak selection of different mass spectra. Mass spectra of each sample were selected to create sample profiles that were compared with those of MBT 8468 MSP Library using pattern-matching algorithms. Identification was provided with accompanying scores as per the manufacturer's schemes: a score <1.7 indicated no reliable identity; a score between 1.7 and 1.9 indicated identity at genus level; a score ≥2.0 indicated identity at species level.

Antifungal Susceptibility testing with Sensititer YeastOne

Susceptibility testing, reading, and interpretations of the results using Sensititer YeastOne (SYO) was followed according to the manufacturer's instructions (Thermo Fisher Scientific, Cleveland, OH, USA). All yeast isolates were subcultured on SDA at 35°C for 24 hr to 48 hr to confirm their purity and viability. After that, standard 0.5 McFarland yeast suspensions were adjusted in sterile distilled water with Sensititre nephelometer, and the reference method was performed simultaneously. The plates contained serial two fold dilutions of amphotericin B (0.12 to 8 mg/L), 5-flucytosine (0.06 to 64 mg/L), fluconazole (0.12 to 256 mg/L), itraconazole (0.015 to 16 mg/L), voriconazole (0.008 to 8 mg/L), posaconazole (0.008 to 8 mg/L), anidulafungin (0.015 to 8mg/L), micafungin (0.008 to 8mg/L), and caspofungin (0.008 to 8mg/L) and MICs endpoints were read after 24 hr of incubation (72 hr for *C. neoformans*) by the color changed from blue indicating no growth to magenta indicating growth.[8]

MIC results interpretation

Interpretation of susceptibility was performed by applying the clinical break points (CBPs) defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical & Laboratory Standards Institute (CLSI).[11-13] In the results that CBPs absence of EUCAST and CLSI , isolates were defined as having a wild-type or a non-wild-type drug susceptibility phenotype including amphotericin, 5-fucytosine, itraconazole, posaconazole and voriconazole (only *C. glabrata*) according to the epidemiological cutoff values (ECV) determined by SYO,[14-15] as shown in Table 3.

Statistical analysis

Analytical data between age group and yeast species were explained as a mean with standard deviation and a 95% confidence interval. The level of significance was set at 0.05. The data were analyzed using the Kruskal-Wallis test to avoid random significance when comparing several groups. The statistical tests were conducted with the aid of SPSS Statistical software version 27.

Results

Species identification

Overall, 130 isolates were collected from blood samples of 130 patients (75 males and 55 females) from January 2013 to March 2016 (3.25 years). The mean and median ages were 57 and 63 years, respectively, with an age range of 1-92 years.

The yeast species suspected fungemia was as follows. *C. tropicalis*, 48 (36.9%); *C. albicans*, 38 (29.2%); *C. glabrata*, 23 (17.7%); *C. parapsilosis*, 9 (6.9%); other NAC [*C. krusei* (*Pichia kudriavzevii*), *C. rugose* (*Diutina rugosa*), *C. intermedia*, *C. haemulonii* and *C. nivariensis*, 5 (3.8%); *Cryptococcus neoformans*, 5 (3.8%); and others (*Loderomyces elongisporus* and *Trichospron asahii*), 2 (1.5%) (Table 1).

Table 1 Yeast species classification among 130 patients with positive blood cultures.

Yeast species	Patients or Isolates N (%)	Gender		Age Mean±SD
		Males (N)	Female (N)	
Candida albicans	38 (29.2)	21	17	58±25
Non-albicans Candida (NAC)				
Candida tropicalis	48 (36.9)	27	21	58±25
Candida glabrata	23 (17.7)	16	7	64±23
Candida parapsilosis	9 (6.9)	4	5	42±32
Candida krusei (Pichia kudriavzevii)	1 (0.8)	1	0	73
Candida rugosa (Diutina rugosa)	1 (0.8)	0	1	49
Candida intermedia	1 (0.8)	1	0	70
Candida haemulonii	1 (0.8)	0	1	92
Candida nivaniensis	1 (0.8)	0	1	31
Other yeasts species				
Cryptococcus neoformans	5 (3.8)	3	2	41±22
Lodderomyces elongisporus	1 (0.8)	1	0	40
Trichosporon asahii	1 (0.8)	1	0	14
Total	**130 (100)**	**75**	**55**	**57±25**

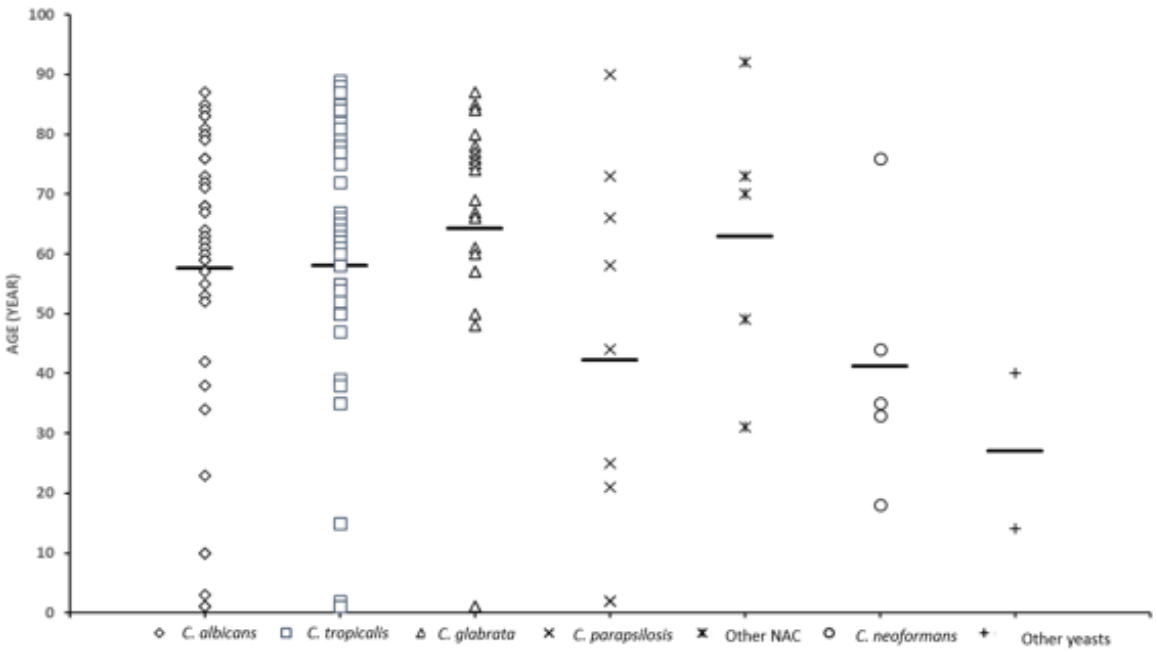


Figure 1. *Yeast species and an average patient's age (year) during the infection.*

From figure 1, *C. albicans*, *C. tropicalis*, *C. glabrata* and other NAC were more common among elderly patients (mean age >50 years), while *C. parapsilosis*, *C. neoformans* and other yeasts were more frequently isolated from younger patients (mean age<50 years). Notwithstanding, no statistically significant difference was found between yeast species and age groups.

The blood cultures were collected from patients who were admitted in different wards. Altogether, there were 43 patients (33.1%) at ICUs, 50 patients (38.5%) at medicine wards, 13 patients (10%) at surgical wards, 6 patients (4.6%) at pediatric wards, and 18 patients (13.8%) at other wards. Table 2 shows the yeast species distribution in relation to the hospitalized unit of the patient. Fungemia caused by yeasts were found in patients mostly admitted to medicine units (38.5%) and ICU (33.1%). However, yeast septicemia was uncommonly found in the pediatric units.

Table 2 Number of yeast species identified from patients in hospital wards.

Yeast species	Hospital Wards					
	ICU	Medicine	Surgery	Pediatric	Other	Total
C. albicans	*15*	*8*	7	1	6	38
C. tropicalis	*15*	*24*	1	1	7	48
C. glabrata	*8*	*8*	3	2	2	23
C. parapsilosis	*3*	*3*	1	1	1	9
Other NAC	*1*	*3*	1	0	0	5
C. neoformans	*0*	*2*	0	0	3	5
Other yeasts	*1*	*0*	0	1	0	2
Total N (%)	43 (33.1%)	50 (38.5%)	13 (10%)	6 (4.6%)	18 (13.8%)	130 (100%)

Antifungal susceptibility patterns

MICs were used to evaluate the efficacy of nine antifungal agents among *C. albicans, C. tropicalis and C. glabrata* from the blood samples summarized in Table 3. Following the EUCAST and CLSI clinical breakpoints (CBPs), we found that all *C. albicans* isolates were susceptible to fluconazole and voriconazole. Overall, 21(91.3%) of all *C. glabrata* isolates showed reduced susceptibility to fluconazole (MIC_{90}=32 µg/mL). Interestingly, 32 (66.7%) and 10 (20.8%) of *C. tropicalis* isolates showed susceptible and intermediate/SDD to fluconazole, whereas MIC_{90} indicated resistant level (MIC_{90}=16 µg/mL). The MIC values for posaconazole were overall low in *C. albicans*, but rather high in *C. tropicalis* when applying the EUCAST CBPs. The MIC values foranidulafungin, micafungin and caspofungin were overall low in all isolates when the CLSI CBPs were applied. However, 21 (55.3%) of *C. albicans* isolates were susceptible to andulafungin when applying EUCAST CBPs. Applying the epidemiological cutoff values (ECVs), all the isolates had wild type phenotype drug susceptibility to amphotericin, 5-flucytosine, micafungin and caspofungin. For triazole agents, all *C. albicans* isolates had wild type phenotype drug susceptibility to itraconazole and posaconazole, whereas 2 (4.2%) *C. tropicalis* isolates exhibited non-wild type phenotypes to itraconazole and only one isolate (4.4%) of *C. glabrata* exhibited non-wild type phenotypes to itraconazole, voriconazole and posaconazole.

Table 3 Antifungal susceptibility testing against Candida spp. isolated from blood samples.

					Susceptible isolates N (%)		ECV	Isolates N (%)	
		Range	MIC50	MIC90	EUCAST	CLSI	SYO	Wild type	Non-wild type
C. albicans (n=38)	Amphotericin B	0.12-0.5	0.5	0.5	38 (100)	ND	2	38 (100)	0 (0)
	5-Flucytosine	0.06-0.25	0.12	0.25	ND	ND	0.5	38 (100)	0 (0)
	Fluconazole	0.25-1	0.5	1	38 (100)	38 (100)			
	Itraconazole	0.015-0.12	0.12	0.12	16 (42.1)	ND	0.12	38 (100)	0 (0)
	Voriconazole	0.008-0.03	0.008	0.015	38 (100)	38 (100)			
	Posaconazole	0.008-0.06	0.03	0.06	38 (100	ND	0.06	38 (100)	0 (0)
	Anidulafungin	0.015-0.12	0.03	0.12	21 (55.3)	38 (100)			
	Micafungin	0.008-0.015	0.008	0.015	38 (100)	38 (100)			
	Caspofungin	0.015-0.25	0.03	0.06	ND	38 (100)	0.25	38 (100)	0 (0)

MIC50: minimal inhibitory concentration value at which ≥50% of the isolates in a test, MIC_{90}: maximum inhibitory concentration of an antibiotic, at which 90% of the isolates, ECV: epidemiological cutoff value, ND: not determined, S: susceptible, I: intermediate, SDD: susceptible dose-dependent.

Table 3 Antifungal susceptibility testing against Candida spp. isolated from blood samples. (continues)

					Susceptible isolates N (%)		ECV	Isolates N (%)	
		Range	MIC50	MIC90	EUCAST	CLSI	SYO	Wild type	Non-wild type
C. tropicalis (n=48)	Amphotericin B	0.12-1	0.5	1	48 (100)	ND	2	48 (100)	0 (0)
	5-Flucytosine	0.06-0.12	0.06	0.12	ND	ND	0.5	48 (100)	0 (0)
	Fluconazole	1-256	2	16	32 (66.7, S)	32 (66.7, S)			
					10 (20.8, I)	10 (20.8, SDD)			
	Itraconazole	0.12-1	0.25	0.5	7 (14.6)	ND	0.5	46 (95.8)	2 (4.2)
	Voriconazole	0.12-8	0.25	1	7 (14.6, S)	7 (14.6, S)			
					35 (72.9, I)	35 (72.9, I)			
	Posaconazole	0.12-1	0.25	0.5	0 (0)	ND	1	48 (100)	0 (0)
	Anidulafungin	0.015-0.25	0.12	0.25	18 (37.5)	48 (100)			
	Micafungin	0.015-0.06	0.03	0.03	ND	48 (100)	0.12	48 (100)	0 (0)
	Caspofungin	0.03-0.12	0.03	0.06	ND	48 (100)	0.25	48 (100)	0 (0)
C. glabrata (n=23)	Amphotericin B	0.25-1	0.5	1	23 (100)	ND	2	23 (100)	0 (0)
	5-Flucytosine	0.006-0.12	0.06	0.06	ND	ND	0.25	23 (100)	0 (0)
	Fluconazole	16-256	32	32	21 (91.3, I)	21 (91.3, SDD)			
	Itraconazole	0.12-16	1	2	ND	ND	2	22 (95.6)	1 (4.4)
	Voriconazole	0.06-8	1	2	ND	ND	2	22 (95.6)	1 (4.4)
	Posaconazole	0.12-8	2	2	ND	ND	4	22 (95.6)	1 (4.4)
	Anidulafungin	0.015-0.06	0.015	0.03	23 (100)	23 (100)			
	Micafungin	0.008-0.015	0.015	0.015	23 (100)	23 (100)			
	Caspofungin	0.03-0.12	0.06	0.12	ND	23 (100)	0.25	23 (100)	0 (0)

MIC50: minimal inhibitory concentration value at which ≥50% of the isolates in a test, MIC_{90}: maximum inhibitory concentration of an antibiotic, at which 90% of the isolates, ECV: epidemiological cutoff value, ND: not determined, S: susceptible, I: intermediate, SDD: susceptible dose-dependent.

Discussion

Hitherto, we reported that over 90%of yeast septicemic cases in the Thammasat University Hospital occurred from three *Candida* species including *C. tropicalis*, *C. albicans* and *C. glabrata*, respectively. This similar result from blood culture was conducted in the same geophysical area of Thailand during 2016 to 2017.[16] Likewise, the previous study in Pakistan and India found *C. tropicalis* was the most isolated candida in blood culture.[17] On the contrary, *C. albicans* isolates (46%) were reported as the most common cause of invasive fungal infection in a study in Srinagarind University Hospital, the Northeast Thailand between 2006 and 2011 and other countries in Asia-Pacific region including Australia, Japan, Korea, Hong Kong (China), Taiwan, Malaysia, Saudi Arabia and Singapore.[17-18] A predominance of *C. tropicalis* may cause from its pathogenic mechanism reported as a very strong biofilm producer,[19-20] the owner of various virulence factors such as hemolysin, proteinase activity and the ability to survive to high salt concentration.[19, 21] Besides, geographic variance especially in tropical countries including Thailand.[16-17, 22] Although no statistically significant difference between species of *Candida* and age group was seen, *C. glabrata* seem to be the most frequent detection among aged patients (mean age = 64±23 years) and *C. parapsilosis* seem to be more frequently isolated from younger patients (mean age = 42+32 years). Simultaneously, *C. glabrata* and *C. parapsilosis* were described in previous as common isolates in relation to different age groups studies.[23] The colonization of NAC including *C. glabrata* in the oral cavities of aged patients[24] lower urinary tract[23] or gastrointestinal tract[25] may support the opinion that NAC is more common midst aged patients. *C. tropicalis* and *C. albicans* were found in all age groups with the same mean age (58+25 years). Our study found that candidemia from *C. tropicalis* mostly occurred in medicine ward that was related to reported risk factors including the insertion of inherent urinary catheters, period of stay in hospitalized unit, diabetes mellitus, lengthened antibiotic therapy, immunosuppressive treatment, surgeries, and aging.[26]

In current work, we reported MIC values using the EUCAST and CLSI CBPs because no standard CBPs were determined by commercial antifungal susceptibility testing including the SYO method.[11] Reduced fluconazole susceptibility was perceived in *C. glabrata* and *C. tropicalis* isolates, 91.3% and 20.8%, respectively. This indicated that the use of fluconazole for treatment should be avoided in candidemic patients from non-albicans *Candida*. Our study found that *C. glabrata* is the second most common NAC in blood cultures. Fluconazole resistance in *C. glabrata* was reviewed in previous study.[27] Prolonged fluconazole exposure of this strain brought to develop its resistance[28] by upregulating *CgCDr1* and *CgCDR2* efflux pump expression significantly.[29] *ErG11* and *UPC2* overexpression level were related to the reduction of fluconazole susceptibility in *C. tropicalis* fungemia.[30] When applying the EUCAST CBPs, 21 (55.3%) of the *C. albicans* isolates and 18 (37.5%) of the *C. tropicalis* isolates were

categorized as intermediate anidulafungin susceptibility. However, according to the CLSI CBPs, these isolates were reported as anidulafungin susceptibility result. Anidulafungin intermediate susceptible result of *Candida* strains should be confirmed *FKS* mutation by *FKS* genes sequencing.[31] Resistance to all echinocandins including caspofungin anidulafungin or micafungin of *Candida* isolates possessed distinctive *FKS* hot spot mutations.[31]

Conclusion

In summary, *C. tropicalis* was the major species identified from the blood samples of patients with candidemia. Reduced susceptibility to antifungal drugs was not seen in *Candida albicans* isolated from blood, while triazole resistance was perceived in NAC including *C. glabrata and C. tropicalis.*

Conflicting interests

Authors declare that there are no commercial, personal, political any other potentially conflicting interests related to the submitted manuscript.

Funding Statement

We would like to thank National Research Council of Thailand (NRCT), 2019 and Thammasat University Hospital, 2018 for research fund.

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