JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Azole non-susceptible *C. tropicalis* and polyclonal spread of *C. albicans* in Central Vietnam hospitals

Thi Minh Chau Ngo[1], Antonella Santona[2], Maura Fiamma[2], Phuong Anh Ton Nu[1], Thi Bich Thao Do[1], Piero Cappuccinelli[2,3], Bianca Paglietti[2]

[1] Department of Parasitology, Hue University of Medicine and Pharmacy, Hue University, Hue, Vietnam
[2] Department of Biomedical Science, University of Sassari, Sassari, Italy
[3] Department of Microbiology and Carlo Urbani Center – Hue University of Medicine and Pharmacy, Hue University, Hue, Vietnam

Abstract

Introduction: *Candida* spp. are responsible for infections ranging from local to systemic, and resistance to antifungal first-line therapy is increasing in non-albicans *Candida* species. We aimed to determine the etiology of candidiasis and the antifungal resistance of *Candida* spp. isolated in Hue hospitals, Central-Vietnam.

Methods: Species identification was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry supported by fungal internal-transcribed-spacer amplification and sequencing. Antifungal susceptibility testing was performed by disk diffusion method and minimum inhibitory concentrations of azoles, caspofungin, and amphotericin B against *C. tropicalis* were determined by broth microdilution. Polymorphism of *erg11* gene associated with fluconazole resistance was carried out by polymerase chain reaction and sequencing. Multilocus sequence typing (MLST) was used for typing selected *C. albicans* isolates.

Results: Overall, 196 *Candida* isolates were detected, mostly *C. albicans* (48%), followed by *C. tropicalis* (16%), *C. parapsilosis* (11%), *C. glabrata* (9%), *C. orthopsilosis* (6%) and to a lesser extent another eight species. High rates of resistance to fluconazole and voriconazole (18.8%) were observed in *C. tropicalis* with five isolates co-resistant to both agents. Y132F and S154F missense mutations in the ERG11 protein were associated with fluconazole-resistance in *C. tropicalis* (67.7%). Resistance to caspofungin was found in one isolate of *C. albicans*. MLST identified a polyclonal population of *C. albicans* with multiple diploid sequence types, and with few lineages showing potential nosocomial spread.

Conclusions: Resistance to triazole agents should be considered in *C. tropicalis* infections in the studied hospitals, and surveillance measures taken to avoid *Candida* diffusion.

Key words: Candida, azole resistance, MLST, Central Vietnam.

J Infect Dev Ctries 2023; 17(4):550-558. doi:10.3855/jidc.17574

(Received 23 October 2022 – Accepted 02 March 2022)

Introduction

Candida species are human commensals, commonly found on the mucosal surfaces of gastrointestinal and genitourinary tracts, skin, under fingernails, lung, and gut mycobiota [1]. However, *Candida* spp can act as opportunistic invaders capable of causing different diseases, ranging from commonly encountered superficial infections to systemic diseases in humans [2]. The switch from commensalism to pathogenesis in *Candida* spp. is influenced by both fungal and host factors [3], including immune status, co-morbidities, and underlying conditions, as well as exposure to certain medications and medical devices [4].

C. albicans represents one of the most common cause of hospital-acquired bloodstream infections (BSI), which leads to increased morbidity and mortality, prolongation of hospital stays, and increased hospital costs [5,6]. However, an increasing trend of invasive infections caused by non-*albicans Candida* (NAC) group has been observed, including several *Candida* species, such as *C. tropicalis, C. parapsilosis, C. krusei, C. glabrata and C. stellatoidea* [4,7,8]. In Vietnam, fungal opportunistic diseases are growing due to the increased number of immunocompromised patients [9,10]. Although physicians have dealt with *Candida* infections more frequently, there are limited systematic epidemiological data on *Candida* in the country [11-13], even less from Central Vietnam.

In the last few years, the frequency of resistance to antifungal therapy continued to increase, and data reported globally indicated higher levels of resistance

from NAC species than *C. albicans* [14-17]. Among antifungal drugs, azoles are widely used to treat almost all superficial and deep mucosal and disseminated fungal infections caused by *Candida* spp. [18]. As a consequence, the extensive use of fluconazole led to the development of resistance, resulting in therapeutic failures [19], which makes the treatment of candidemia a huge challenge for physicians [20].

The most common mechanisms leading to *Candida* spp. resistant to fluconazole as well as other azole compounds include alteration of the target enzyme, the cytochrome P-450 lanosterol 14α-demethylase (ERG11), which is responsible for the synthesis of ergosterol. Mutations in the *erg11* gene can alter the enzyme's structure and reduce its binding affinity for the drugs, making the fungal cells less susceptible to their effects [18]. Thus, monitoring for the presence of *ERG11* mutations in *Candida* isolates can inform antifungal therapy decisions and guide the selection of alternative treatment options, such as echinocandins or polyenes, which are not affected by *ERG11*-mediated resistance. Understanding the molecular mechanisms of azole resistance can also aid in the development of new antifungal agents that target alternative pathways or proteins, thereby mitigating the impact of *ERG11* mutations on antifungal therapy efficacy. The development of resistance to fluconazole and voriconazole can also occur through overexpression of efflux pump genes such *mdr* or *cdr* by reducing the intracellular concentrations of fluconazole and voriconazole [21].

The main purpose of this study was to investigate the etiology of candidiasis and the antifungal susceptibility of *Candida* spp. isolated from patients of the Hue University of Medicine and Pharmacy Hospital (HUMPH), and Hue Central Hospital (HCH) in Central Vietnam during October 2012 and June 2016. Moreover, molecular methods were utilized to investigate *erg11* mutations in fluconazole-resistant isolates and to type selected *C. albicans* isolates.

Methodology

Study population

Sampling was conducted during October 2012 and June 2016 from 163 patients in 10 departments at HUMPH (Dermatology, Endoscopy, Intensive Care, Internal Medicine, Obstetrics, Oncology, Ophthalmology, Otorhinolaryngology, Pediatrics, and Surgery) and in two departments at HCH (Hematology and Pediatric). *Candida* isolates were classified into four groups depending on the patient's status, including *Candida* colonization, cutaneous candidiasis, mucosal candidiasis, or systemic candidiasis.

Candida colonization is typically asymptomatic and it was classified through the isolation of *Candida* from clinical specimens, such as urine, sputum, stool, gastric, external ear, bronchoalveolar from patients without clinical signs and symptoms of candidiasis that recovered without antifungal therapy. In contrast *Candida* infections were classified by the presence of clinical signs and symptoms, such as fever, pain, inflammation, and discharge, along with the detection of *Candida* in relevant clinical specimens, such as blood, endotracheal aspiration, urine or tissue samples, and the patients were cured with antifungal therapy. Mucosal candidiasis included oral, vaginal, esophageal and cornea candidiasis. Onychomycosis and skin candidiasis patients belonged to cutaneous candidiasis. Systemic candidiasis included candidemia, peritoneal candidiasis, and pulmonary candidiasis.

Samples were first cultured onto Sabouraud Dextrose Agar medium plates (Oxoid Ltd, Basingstoke UK), yeast colonies were then subcultured in Brilliance *Candida* agar plates (Oxoid Ltd, Basingstoke UK) to screen mixed isolates. Seventeen samples had mixed isolates, of which 15 samples were two mixed isolates, and two samples were three mixed isolates. Therefore, 196 *Candida* isolates were collected from 177 samples. All *Candida* isolates were stored at -80 °C and subcultured in Sabouraud media for antifungal susceptibility testing.

Identification of Candida species

Species identification was performed by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics Inc. - Billerica, USA), at San Francesco Hospital, Nuoro, Italy, and isolates with a score < 1.7 were identified by polymerase chain reaction (PCR) and sequencing using universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for the whole nuclear ribosomal internal transcribed spacer (ITS1-2) region [22,23]. Fungal DNA was extracted using the thermolysis method according to the protocol of Zhang *et al.* [22]. PCRs were performed in a 25 µL volume with 0.2 mmol/L of dNTPs, 1.5 mmol/L $MgCl_2$, 0.2 µmol/L of primers, and 1.0 U *Taq* polymerase (Invitrogen, Waltham, USA) in a thermal cycler (Hybaid, Altrincham, UK). PCR products were visualized in 1% agarose gel in TAE 1X buffer containing GelRed™ (Biotium, Fremont, USA) on a UV trans-illuminator. The amplicons were then purified

by the DNA Clean and ConcentratorTM-5 (Zymo Reaserch, Irvine, USA) columns, quantified by comparison of bands to low molecular mass ladder (Invitrogen, Waltham, USA), and sent for sequencing to the Sequencing Service LMU Munich, Germany (http://www.gi.bio.lmu.de/sequencing). Sequences were analyzed by Geneious 4.8.4 version and blasted on nucleotides Genebank (http://blast.ncbi.nlm.nih.gov/Blast.cg) for species identification.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed by disk diffusion method using Mueller-Hinton agar (Liofilchem Laboratories, Teramo, Italy) supplemented with 2% dextrose and 0.5 µg/mL methylene blue according to the Clinical and Laboratory Standards Institute document (CLSI) M44 A-Ed3 [24], and interpreted following CLSI M60 - Ed2 [25]. Fluconazole 25 µg/disk, caspofungin 5 µg/disk, and voriconazole 1 µg/disk (Liofilchem Laboratories, Teramo, Italy) were used. The *C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750 were used as standard reference strains. The zone of inhibition was recorded after 24 hours.

The minimal inhibitory concentration (MIC) values of fluconazole, itraconazole amphotericin B, caspofungin (Sigma-Aldrich, St. Louis, USA) and voriconazole (AK Scientific, Inc, Union City, USA) in *C. tropicalis* were determined by broth microdilution following CLSI M27-Ed4 [26]. The MICs were interpreted according to M60-Ed2 [25], while itraconazole and amphotericin B were categorized based on the epidemiological cut-off values (ECV) according to CLSI M59-Ed3 [27]. *C. krusei* ATCC 6258 was used as a standard reference strain.

Detection of erg11 gene mutations in C. tropicalis

PCR and sequencing were used to detect polymorphisms in the *erg11* (Lanosterol 14α-demethylase) gene in fluconazole-resistant *C. tropicalis* strains. In addition, one fluconazole susceptible strain was included as a control. We designed two pairs of primers, erg11a-F 5'-TCTTTTGTCAACACAGTAATGGC-3' and erg11a-R 5'- GGATCAATATCACCGCTTTCTC-3' and erg11b-F 5'-GCGGTGATATTGATCCAAAGAG- 3' and erg11b-R 5' - GGGATTTTTCTAGCTACTCCATGG- 3', on the *erg11* gene sequence of *C. tropicalis* reference strain IHEM 21234 (AY942645.1). Two separate PCRs were performed in a 25 µL volume with 12.5 µL of 2x PCR SuperMix LeGene (Twin Helix, Milano, IT) and 0.2 µmol/L of each primer.

Amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles, each consisting of 30 sec at 94 °C for denaturation, 40 sec at 50 °C for annealing, and 50 sec at 72 °C for elongation, and a final elongation step of 10 min at 72 °C. The erg11a and erg11b amplicons, of 834 bp and 816 bp respectively, were then quantified, purified, and sequenced as described above. The nucleotide sequences were translated into amino acid sequences, aligned, and compared with a *C. tropicalis ERG11* reference sequence (NCBI GenBank accession: AY942645) using Geneious 4.8.

C. albicans Multilocus Sequence Typing (MLST)

The internal regions of seven housekeeping genes (*aat1a, acc1, adp1, mpib, sya1, vps13,* and *zwf1b*) were amplified using the specific sets of primers and PCR cycle included in the *C. albicans* MLST scheme (http://pubmlst.org/calbicans). PCR was carried out in a 25 µL reaction volume (PCR LeGene PCR Mix 2X), and 0.4 µmol/L primer mix. Purified amplicons (DNA Clean and ConcentratorTM-5, Zymo Reaserch, Irvine, USA) were sent to LMU, Germany (http://www.gi.bio.lmu.de/sequencing) for sequencing. The nucleotide sequences were trimmed and analyzed using Geneious 4.8, diploid sequence types (DST)s were assigned according to the *C. albicans* MLST database (http://pubmlst.org/calbicans/), clades and singletons according to a previous publication [28].

Data analysis

The data were analyzed using Window SPSS 20.0. A *p* value less than 0.05 was considered to be statistically significant.

Ethical approval

This study was approved by the Ethics Committee of Hue University of Medicine and Pharmacy (code DHH2015-04-44).

Results

Species identification and distribution in samples source

Overall, 196 strains of *Candida* spp. were isolated from 15 clinical sample sources including oral mucosa (24.5%), sputum (14%), vaginal discharge (13%), urine (9%), gastric drainage fluid (8.7%), stool (7%), nail (7%), endotracheal aspiration fluid (6%), skin wound (4%), external ear (3%), gastric biopsy (1.5%), corneal

Table 1. Distribution of *C. albicans* and non-*albicans Candida* in *Candida* colonization and candidiasis.

Candida aspects (number of isolates)	*C. albicans*	Non- *albicans Candida*	*p* value
Candida colonization (90)	33 (35.1%)	57 (55.9%)	**0.0036**
Cutaneous candidiasis (20)	6 (6.4%)	14 (13.7%)	0.0924
Mucosal candidiasis (72)	46 (48.9%)	26 (25.5%)	**0.0007**
Systemic candidiasis (14)	9 (9.6%)	5 (4.9 %)	0.2033
Total (196)	**94 (48%)**	**102 (52%)**	

ulcer (1%), blood (0.5%), bronchoalveolar lavage (0.5%), and peritoneal fluid (0.5%).

Out of 196 *Candida* isolates, 186 were correctly identified by MALDI-MS with a score ≥ 1.7; the other 10 isolates were further identified by internal transcribed spacer sequencing as NAC spp., including *C. parapsilosis* (n = 2), *C. orthopsilosis* (n = 1), *C. digboiensis* (n = 3), *C. famata* (n = 2), *C. mesorugosa* (n = 1) and *C. blankii* (n = 1). In total, the identified species were *C. albicans* (48%), *C. tropicalis* (16%), *C. parapsilosis* (11%), *C. glabrata* (9%), *C. orthopsilosis* (6%), *C. krusei* (3%), *C. metapsilosis* (1.5%), *C. guilliermondii* (1,5%), *C. digboiensis* (1.5%), *C. famata* (1%), *C. mesorugosa* (0.5%), *C. novergensis* (0.5%), and *C. blanki* (0.5%).

The numbers of isolates from *Candida* colonization, mucosal candidiasis, cutaneous candidiasis, and systemic candidiasis were 90, 72, 20, and 14, respectively (Table 1). With regards to *C. albicans* species versus non-*albicans*, we observed a higher proportion of NAC spp. (55.9%) in *Candida* colonization compared to *C. albicans* (35.1%) (p = 0.0036).

In contrast, *C. albicans* (48.9%) was more represented in mucosal candidiasis compared to NAC spp. (25.5%) (p = 0.0007). No statistically significant differences between *C. albicans* and NAC spp. were found in cutaneous and invasive candidiasis (Table 1).

Among NAC colonization, 8 different species were detected as showed in Table 2, with *C. albicans* and *C. tropicalis* the most encountered species. Also, the distribution of *Candida* species by disease types is summarized in Table 2, with the dominance of *C. albicans* from mucosa diseases. On the contrary, NAC spp. showed a higher frequency of isolation from onychomycosis, and some species caused invasive candidiasis (Table 2). Predominant *Candida* species from intensive care unit (ICU) patients (n = 27) were *C. albicans* (n = 13), *C. tropicalis* (n = 13) and *C. parapsilosis* (n = 1).

Antifungal susceptibility of Candida spp. isolates

The antifungal susceptibility results by disk diffusion method are shown in Table 3. *C. albicans* and *C. parapsilosis* isolates were susceptible to fluconazole. Resistance to fluconazole was found in *C. tropicalis* (12.5%) and *C. glabrata* (11.7%). *C. tropicalis* isolates were also resistant to voriconazole (18.8%) compared to the other *Candida* spp. One isolate of *C. tropicalis* was identified from blood and found to be susceptible to all antifungal drugs tested, with MIC values of 0.25 µg/mL for amphotericin B, 2 µg/mL for fluconazole, 0.008 µg/mL for itraconazole, 0.06 µg/mL for voriconazole, and 0.125 µg/mL for caspofungin.

Table 2. Distribution of *Candida* species by type of diseases and colonization.

Type of diseases	Species (number of isolates)
Skin and nails	
Interdigital candidiasis (7)	*C. albicans* (3), *C. tropicalis* (1), *C. parapsilosis* (2), *C. glabrata* (1)
Onychomycosis (13)	*C. albicans* (3), *C. tropicalis* (1), *C. parapsilosis* (5), *C. orthopsilosis* (1), *C. digboiensis* (1), *C. krusei* (1), *C. metapsilosis* (1)
Mucosa	
Thrush (43)	*C. albicans* (30), *C. tropicalis* (5), *C. parapsilosis* (3), *C. orthopsilosis* (2), *C. norvegensis* (1), *C. digboiensis* (1), *C. mesorugosa* (1)
Vulvovaginal (26)	*C. albicans* (16), *C. parapsilosis* (1), *C. glabrata* (8), *C. metapsilosis* (1)
Esophageal candidiasis (2)	*C. albicans* (1), *C. orthopsilosis* (1)
Keratitis (2)	*C. parapsilosis* (2)
Invasive diseases	
Septicemia (1)	*C. tropicalis* (1)
Pneumonia (11)	*C. albicans* (7), *C. tropicalis* (2), *C. parapsilosis* (1), *C. orthopsislopsis* (1)
Peritonitis (1)	*C. albicans* (1)
Colonization (90 isolates)	*C.albicans* (33), *C. tropicalis* (22), *C. glabrata* (8), *C. parapsilosis* (7), *C. orthopsilosis* (6), *C. krusei* (6), *C. guilliermondii* (3), *C. famata* (2), *C. metapsilosis* (1), *C. digboiensis* (1), *C. blankii* (1)
Total	**196**

Table 3. Antifungal susceptibility results of *Candida* species by disk diffusion following CLSI M60-Ed2 interpretation.

Antifungal agent	Species (number of isolates)	Antifungal susceptibility testing			
		S	I	SDD	R
Fluconazole	*C. albicans* (94)	94 (100%)			
	C. tropicalis (32)	26 (81.3%)		2 (6.2%)	4 (12.5%)
	C. glabrata (17)			15 (88.3%)	2 (11.7%)
	C. parapsilosis (22)	22 (100%)			
Voriconazole	*C. albicans* (94)	94 (100%)			
	C. tropicalis (32)	26 (81.2%)			6 (18.8%)
	C. parapsilosis (22)	22 (100%)			
	C. krusei (7)	7 (100%)			
Caspofungin	*C. albicans* (94)	93 (98.9%)			1 (1.1%)
	C. tropicalis (32)	32 (100%)			
	C. parapsilosis (22)	22 (100%)			
	C. krusei (7)	7 (100%)			
	C. guilliermondii (3)	3 (100%)			

S: susceptible; I: intermediate; SDD: susceptible dose-dependent, R: resistant.

Table 4. Antifungal susceptibility of *C. tropicalis*.

Antifungal agents	MIC range	MIC50 - MIC 90	Geometric Mean	Interpretation of Antifungal susceptibility testing		
				S	I	R
Fluconazole[a]	0.125 - 128 (µg/mL)	2 -128 (µg/mL)	4.122 (µg/mL)	26 (81,2 %)		6 (18.8%)
Voriconazole[a]	0.03 - 8 (µg/mL)	0.125 -8 (µg/mL)	0.294 (µg/mL)	26 (81,2 %)		6 (18.8%)
Itraconazole[b]	0.008 - 0.25 (µg/mL)	0.008 - 0.015 (µg/mL)	0.009 (µg/mL)	30 (93.8%)		2 (6.2%)
Caspofungin[a]	0.06 - 0.25 (µg/mL)	0.125 - 0.125 (µg/mL)	0.125 (µg/mL)	32 (100%)		
Amphotericin B[b]	0.125 - 0.5 (µg/mL)	0.125 - 0.5 (µg/mL)	0.144 (µg/mL)	32 (100%)		

S: Susceptible; I: Intermediate; R: Resistance; MIC: minimum inhibitory concentrations; MIC values according to breakpoints interpretation; [a]: CLSI M60-Ed2; [b]: CLSI M59-Ed3.

Table 5. *C. albicans* isolation information and Multilocus Sequence Typing loci, Diploid Sequence Type (DST) Clonal Cluster and Clades assignments.

Strain code	Isolation date	Department	Source	AAT1a	ACC1	ADP1	MPIAb	SYA1	WPS13	ZWP1b	DST	Clade
19A	26.12.2013	Inter[1]	Sputum	4	17	21	19	27	83	22	299	12
91	08.04.2014	Inter[1]	Stool	4	17	21	19	27	13	22	459	12
34	29.10.2013	Inter[1]	Bronchoalveolar lavage	3	3	6	4	53	109	13	2726	14
176	19.05.2015	ICU[1]	Endotracheal aspiration fluid	3	7	6	1	34	76	15	3069**	15
177	19.05.2015	ICU[1]	Gastric drainage fluid	3	7	6	1	34	76	15	3069**	15
17	16.4.2014	Ped[2]	Stool	1	7	15	6	61	105	112	693	11
5	11.12.2012	Onc[1]	Oral	8	29	4	4	207*	279	266*	2936***	4
179	21.05.2015	Sur[1]	Gastric drainage fluid	8	29	4	4	207*	279	266*	2936***	4
49	23.11.2013	ICU[1]	Oral	5	4	6	3	93	189	22	2933**	1
75	20.02.2014	Hem[2]	Oral	5	32	21	34	7	55	5	732	18
69	28.02.2014	Hem[2]	Oral	5	5	5	27	2	6	146	2934**	1
32	25.10.2013	Der[1]	Skin	5	78	5	9	2	6	5	2935**	1
108	17.01.2014	Onc[1]	Oral	5	5	5	4	2	6	5	2445	1
60	18.02.2014	Ped[2]	Oral	59	5	21	21	80	108	15	2937**	17
89	28.02.2014	ICU[1]	Endotracheal aspiration fluid	59	5	21	21	80	108	15	2937**	17
26	25.10.2013	Obs[1]	Vaginal secretion	4	4	6	6	96	111	15	2932**	5
104	21.02.2014	Oto[1]	Sinus	4	4	6	6	96	111	15	2932**	5
33	18.10.2013	Sur[1]	Sputum	4	4	6	2	96	111	15	768	5
174	15.5.1015	ICU[1]	Endotracheal aspiration fluid	4	4	6	2	96	111	15	768	5
175	15.5.2015	ICU[1]	Gastric drainage fluid	4	4	6	2	96	111	15	768	5
120	21.10.2014	End[1]	Gastric biopsy	47	35	4	21	74	118	105	2477	S

Department: Oto: Otorhinolaryngology; Obs: Obstetric; ICU: Intensive Care Unit; Hem: Hematology; Onc: Oncology; Ped: Pediatric; Int med: Internal Medicine; Sur: Surgery; End: Endoscopy; Der: Dermatology. Department[1]: from Hue Medicine and Pharmacy University Hospital; Department[2]: from Hue Central Hospital. *: New allele; **: DST with new allele combinations; ***: DST with new alleles; S: Singletone.

Resistant isolates of *C. tropicalis* to azoles were detected in various samples, including nails, sputum, endotracheal aspirate, urine, gastric drainage, and oral mucosa. 15.63% of isolates were co-resistant to both fluconazole and voriconazole, (urine and gastric isolates), while 6.2% of isolates (nail and urine isolates), were resistant to all triazole agents (Table 4). Only one isolate of *C. albicans* showed resistance to caspofungin by both disk diffusion and broth microdilution (MIC = 2 µg/mL). The MICs results of fluconazole, voriconazole, itraconazole, caspofungin and amphotericin B in *C. tropicalis* isolates are summarized in Table 4.

Mutations in ERG11 protein associated with fluconazole resistance in C. tropicalis

The 67.7% of fluconazole-resistant isolates of *C. tropicalis* showed two mutations in the *erg11* gene (A395T and C461T), corresponding to Y132F and S154F amino acids substitution in the ERG11 protein.

C. albicans MLST

Twenty-one *C. albicans* isolates selected from different sources and departments were typed by MLST as shown in Table 5. MLST identified a total of 15 different diploid sequence types (DST) of which 8 DST lineages were already present in *C. albicans* MLST database: DST768 (n = 3), DST299, DST459, DST693, DST732, DST2445, DST2477, and DST2726. Seven were new DSTs, six of which, DST2932 (n = 2) Single locus Variant (SLV) of DST768, DST2933, DST2934, DST2935 Double Locus Variant (DLV) of DST2445, DST2937 (n = 2) and DST3069 (n = 2), had a new combination of alleles already present in the database. The new DST2936 (n = 2), showed two new SYA1 (277) and ZWP1b (266) alleles. Same DST lineages were detected in different wards of HUMPH, even after years (Table 5). Nine clades plus 1 singleton were identified, with clade 5 (n = 5) and clade 1 (n = 3) the most common (Table 5).

Discussion

During the course of this study, we determined the etiology and the antifungal resistance of *Candida* spp. that have been isolated from hospitalized patients in two hospitals in Hue, Central Vietnam between October 2012 and June 2016. Among the 196 isolates, a large variety of *Candida* species was detected, with *C. albicans*, being the most prevalent followed by *C. tropicalis, C. parapsilosis, C. glabrata, C. orthopsilosis,* and *C. krusei*, as also previously reported from other countries [7,17,29,30]. Moreover, *C. digboiensis, C. blankii*, and *C. norvegensis* species were detected in Vietnam for the first time; the last two rarely detected in other countries [31-33].

C. albicans remains the most common *Candida* species reported worldwide [6,7,16,17,30,34], while the distribution relative to other species varies in different geographical areas with *C. glabrata* the most frequent species in Northern Europe and the USA [6], *C. parapsilosis* in Italy, Spain and Brazil [6,29], and *C. tropicalis* in Asian countries [17,30,35], especially in tropical southeast Asian countries [13,36-38]. In this study, *C. albicans* was dominant in mucosal diseases and it was the most isolated species in invasive candidiasis, even if no a statistically significant difference was observed compared to NAC. The presence of NAC species (*C. tropicalis, C. parapsilosis* and *C. orthopsilosis*) was higher in *Candida* colonization.

C. albicans isolates were susceptible to all antimycotics tested; only one isolate was resistant to caspofungin. We found remarkable resistance of *C. tropicalis* to fluconazole and voriconazole (18.8%) and also to a lesser extent to itraconazole (6.2%). Our results are in line with studies conducted in other Asian countries such as China (2011-2021) [17], Asia-Pacific region (2016) [37], Australia (2017) [38], and Japan (2019) [16]. The isolates of *C. tropicalis* in our hospitals were less resistant than those in Ho Chi Minh and Ha Noi cities of Southern and Northern Vietnam, respectively [37]. *C. tropicalis* is reported as one of the four major *Candida* species responsible for candidemia worldwide [39]. It has been described as the first species in Vietnam [12]. It has increased dramatically in the last years due to the development of resistance to fluconazole [34,40], especially in Asia than in North America or Europe [35,41], resulting in higher mortality compared to *C. albicans* [40]. Nevertheless, some studies have indicated that fluconazole resistance has been increasing in *C. tropicalis* in Europe [42,43]. Fluconazole is usually used to treat systemic mycosis in Vietnam [44], particularly in ICU [11], where impaired immunity of patients and prolonged stay in hospitals facilitate the development of invasive mycoses. When comparing disk diffusion and broth microdilution methods in testing *C. tropicalis*, there was a good concordance for voriconazole. Conversely, compared to disk diffusion testing, a greater level of resistance to fluconazole was determined by broth microdilution in the case of *C. tropicalis* isolates that displayed susceptibility-dependent doses and were classified as resistant by broth microdilution. In nearly 70% of *C. tropicalis*, fluconazole-resistance was associated with

ERG11 protein missense mutations Y132F and S154F, in accordance with previous studies [45,46]. Regarding resistance to *C. glabrata*, our results were similar to reports from European countries [42,43] and Japan [16].

Although few numbers of isolates of *C. krusei* and *C. guilliermondii* were isolated in this study, we found that *C. krusei* was susceptible to voriconazole and caspofungin, while *C. guilliermondii* was susceptible to caspofungin.

In 2018, *C. tropicalis* isolated from ICUs of our hospitals showed a higher fluconazole resistance (data not shown). Thus, these hospitals should be aware of possible *Candida* treatment failures because of a noticeable resistance to azole observed in NAC isolates (*C. tropicalis, C. glabrata*). Moreover, MLST highlighted a polyclonal nature of selected *C. albicans* isolates, belonging to several DSTs and clades, with few detected in subsequent years within the departments, suggesting their possible nosocomial spread.

Some studies have indicated that clade 1 was the most common *C. albicans* clade in Asiatic countries [47-49], with other dominant clades varying by countries; clades 6 and 17 in China [49], clade 4, 12 and 18 in Korea [48], clades 3, and 17 in Taiwan and Thailand [50,51]. Thus, the current study described the circulation of "Asian clades" (clades 1, 3, 4, 5, 12 and 17 and 18) in Central Vietnam hospitals, including the presence of isolates from clade 5, DST 2933 and DST 768, previously detected in Japan [52]. A better understanding of the risk factors associated with nosocomial transmission of *Candida* infections by healthcare personnel will significantly contribute to limiting their spread.

Conclusions

Our results highlighted a variety of *Candida* species in Hue hospitals, with *Candida albicans* prevailing in the majority of cases. The study underscores that healthcare professionals in Hue hospitals should be vigilant in managing *Candida* colonization and infections, particularly in light of the emergence of fluconazole resistance in *C. tropicalis* and *C. glabrata*. Rational use of azole drugs and improved surveillance can help prevent *Candida* infections and reduce their spread in healthcare settings, ultimately improving patient outcomes.

Acknowledgements

We would like to thank our colleagues from Hue University of Medicine and Pharmacy and Carlo Urbani Center-Hue University of Medicine and Pharmacy, Vietnam; and the University of Sassari, Italy and the Microbiology lab of San Francesco Hospital, Nuoro, Italy.

The study was supported by Hue University (Grant code: DHH2015-04-44), and was partially supported by the Italian Agency for International Cooperation (AICS) AID 8487 and AID 9922. BP is funded by Bando Fondazione di Sardegna 2022-23, Progetti di ricerca di base dipartimentali.

Authors' contributions

Investigation: Thi Minh Chau Ngo, Antonella Santona. Maura Fiamma, Phuong Anh Ton Nu, Thi Bich Thao Do. Methodology: Thi Minh Chau Ngo, Phuong Anh Ton Nu, Antonella Santona. Writing the original draft: Thi Minh Chau Ngo, Antonella Santona, Phuong Anh Ton Nu. Review and editing: Antonella Santona, Thi Minh Chau Ngo, Bianca Paglietti, Piero Cappuccinelli. All authors have read and agreed to the published version of the manuscript.

References

1. Dignani MC, Solomkin JS, Anaissie EJ (2009) Chapter 8 - *Candida*. In Pfaller E, editor. Clinical Mycology (second edition). Edinburgh: Churchill Livingstone. 197-229.
2. Kabir MA, Ahmad Z (2013) *Candida* infections and their prevention. ISRN Preventive Medicine 2013: 763628.
3. Schulze J, Sonnenborn U (2009) Yeasts in the gut: from commensals to infectious agents. Deutsches Arzteblatt International 106: 837-842.
4. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K (2018) *Candida albicans* - biology, molecular characterization, pathogenicity, and advances in diagnosis and control - an update. Microb Pathog 117: 128-138.
5. Puig-Asensio M, Padilla B, Garnacho-Montero J, Zaragoza O, Aguado JM, Zaragoza R, Montejo M, Muñoz P, Ruiz-Camps I, Cuenca-Estrella M, Almirante B (2014) Epidemiology and predictive factors for early and late mortality in *Candida* bloodstream infections: a population-based surveillance in Spain. Clin Microbiol Infect 20: O245-254.
6. Guinea J (2014) Global trends in the distribution of *Candida* species causing candidemia. Clin Microbiol Infect 20 Suppl 6: 5-10.
7. Pfaller M, Diekema D, Gibbs D, Newell V, Ellis D, Tullio V, Rodloff A, Fu W, Ling T (2010) Results from the ARTEMIS DISK global antifungal surveillance study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J Clin Microbiol 48: 1366-1377.
8. Chang A, Neofytos D, Horn D (2008) Candidemia in the 21st century. Future Microbiol 3: 463-472.
9. Hoy D, Rao C, Nhung NT, Marks G, Hoa NP (2013) Risk factors for chronic disease in Viet Nam: a review of the literature. Prev Chronic Dis 10: 120067.
10. Mwangi J, Kulane A, Van Hoi L (2015) Chronic diseases among the elderly in a rural Vietnam: prevalence, associated socio-demographic factors and healthcare expenditures. Int J Equity Health 14: 134.

11. Beardsley J, Denning DW, Chau NV, Yen NTB, Crump JA, Day JN (2015) Estimating the burden of fungal disease in Vietnam. Mycoses 58 Suppl 5: 101-106.
12. Bac ND, Anh LT, Quang LB, Luc NK, Nga TTT, Nagi M, Yoshitsugu M, Ha HTT, Anh DD, Quyet D, Anh DN (2019) Prevalence of *Candida* bloodstream isolates from patients in two hospitals in Vietnam. Iran J Microbiol 11: 108-113.
13. Sinh CT, Loi CB, Minh NTN, Lam NN, Quang DX, Quyet D, Anh DN, Hien TTT, Su HX, Tran-Anh L (2021) Species distribution and antifungal susceptibility pattern of *Candida* recovered from intensive care unit patients, Vietnam National Hospital of Burn (2017-2019). Mycopathologia 186: 543-551.
14. Berkow EL, Lockhart SR, Ostrosky-Zeichner L (2020) Antifungal susceptibility testing: current approaches. Clin Microbiol Rev 33: e00069-19.
15. Bassetti M, Vena A, Bouza E, Peghin M, Muñoz P, Righi E, Pea F, Lackner M, Lass-Flörl C (2020) Antifungal susceptibility testing in *Candida*, *Aspergillus* and *Cryptococcus* infections: are the MICs useful for clinicians? Clin Microbiol Infect 26: 1024-1033.
16. Kajihara T, Yahara K, Nagi M, Kitamura N, Hirabayashi A, Hosaka Y, Abe M, Miyazaki Y, Sugai M (2022) Distribution, trends, and antifungal susceptibility of *Candida* species causing candidemia in Japan, 2010-2019: a retrospective observational study based on national surveillance data. Med Mycol 60: myac071.
17. Bilal H, Shafiq M, Hou B, Islam R, Khan MN, Khan RU, Zeng Y (2022) Distribution and antifungal susceptibility pattern of *Candida* species from mainland China: a systematic analysis. Virulence 13: 1573-1589.
18. Noël T (2012) The cellular and molecular defense mechanisms of the *Candida* yeasts against azole antifungal drugs. J Mycol Med 22: 173-178.
19. Canuto MM, Rodero FG (2002) Antifungal drug resistance to azoles and polyenes. Lancet Infect Dis 2: 550-563.
20. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD (2016) Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 62: e1-50.
21. Pfaller MA (2012) Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med 125 Suppl 1: S3-S13.
22. Zhang Y, Zhang S, Liu X, Wen H, Wang M (2010) A simple method of genomic DNA extraction suitable for analysis of bulk fungal strains. Lett Appl Microbiol 51: 114-118.
23. White T, Bruns T, Lee S, Taylor J, Innis M, Gelfand D, Sninsky J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis M, Gelfand D, Sninsky J, White T, editors. PCR protocols: a guide to methods and applications. Orlando, Fla: Academic Press. 315-322.
24. Clinical and Laboratory Standards Institute (CLSI) (2018) Method for antifungal disk diffusion susceptibility testing of yeasts, 3rd ed. CLIS guideline M44-Ed3 (ISBN 978-1-68440-030-0).
25. Clinical and Laboratory Standard Institute (CLSI) (2020) Performance standards for antifungal susceptibility testing of yeasts. CLSI supplement M60-ED2 (ISBN 978-1-68440-082-09).
26. Clinical and Laboratory Standards Institute (CLSI) (2017) Reference method for broth dilution antifungal susceptibility testing of yeasts, 4th ed. CLSI standard M27-Ed4 (ISBN 1-56238-826-6).
27. Clinical and Laboratory Standards Institute (CLSI) (2020) Epidemiological Cutoff Values for Antifungal Susceptibility Testing, 3rd ed. CLSI supplement M59-Ed3 (ISBN 1-56238-840-1).
28. Gong Y-B, Jin B, Qi H, Zhang R, Zhang X-Y, Yuan P, Zhao T-X, Geng X-H, Zhang M, Zheng J-L (2018) Multilocus sequence typing of *Candida albicans* isolates from the oral cavities of patients undergoing haemodialysis. Sci Rep 8: 16413.
29. Bassetti M, Merelli M, Righi E, Diaz-Martin A, Rosello EM, Luzzati R, Parra A, Trecarichi EM, Sanguinetti M, Posteraro B, Garnacho-Montero J, Sartor A, Rello J, Tumbarello M (2013) Epidemiology, species distribution, antifungal susceptibility, and outcome of candidemia across five sites in Italy and Spain. J Clin Microbiol 51: 4167-4172.
30. Verma R, Pradhan D, Hasan Z (2021) A systematic review on distribution and antifungal resistance pattern of *Candida* species in the Indian population. Med Mycol 59: 1145-1165.
31. Chowdhary A, Stielow JB, Upadhyaya G, Singh PK, Singh A, Meis JF (2020) *Candida blankii*: an emerging yeast in an outbreak of fungaemia in neonates in Delhi, India. Clin Microbiol Infect 26: 648.e5-.e8.
32. Kollu VS, Kalagara PK, Islam S, Gupte A (2021) A report of *Candida blankii* fungemia and possible endocarditis in an immunocompetent individual and the review of literature. Cureus 13: e14945.
33. Kumar S, Kumar A, Roudbary M, Mohammadi R, Černáková L, Rodrigues CF (2022) Overview on the infections related to rare *Candida* species. Pathogens 11: 963.
34. Brescini L, Mazzanti S, Orsetti E, Morroni G, Masucci A, Pocognoli A, Barchiesi F (2020) Species distribution and antifungal susceptibilities of bloodstream *Candida* isolates: a nine-years single center survey. J Chemother 32: 244-250.
35. Tan BH, Chakrabarti A, Li RY, Patel AK, Watcharananan SP, Liu Z, Chindamporn A, Tan AL, Sun PL, Wu UI, Chen YC (2015) Incidence and species distribution of candidaemia in Asia: a laboratory-based surveillance study. Clin Microbiol Infect 21: 946-953.
36. Boonyasiri A, Jearanaisilavong J, Assanasen S (2013) Candidemia in Siriraj hospital: epidemiology and factors associated with mortality. Journal of the Medical Association of Thailand 96 Suppl 2: S91-S97.
37. Tan TY, Hsu LY, Alejandria MM, Chaiwarith R, Chinniah T, Chayakulkeeree M, Choudhury S, Chen YH, Shin JH, Kiratisin P, Mendoza M, Prabhu K, Supparatpinyo K, Tan AL, Phan XT, Tran TT, Nguyen GB, Doan MP, Huynh VA, Nguyen SM, Tran TB, Van Pham H (2016) Antifungal susceptibility of invasive *Candida* bloodstream isolates from the Asia-Pacific region. Med Mycol 54: 471-477.
38. Chapman B, Slavin M, Marriott D, Halliday C, Kidd S, Arthur I, Bak N, Heath CH, Kennedy K, Morrissey CO, Sorrell TC, van Hal S, Keighley C, Goeman E, Underwood N, Hajkowicz K, Hofmeyr A, Leung M, Macesic N, Botes J, Blyth C, Cooley L, George CR, Kalukottege P, Kesson A, McMullan B, Baird R, Robson J, Korman TM, Pendle S, Weeks K, Liu E, Cheong E, Chen S (2017) Changing epidemiology of candidaemia in Australia. J Antimicrob Chemother 72: 1103-1108.
39. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ (2018) Invasive candidiasis. Nat Rev Dis Primers 4: 18026.

40. Pristov KE, Ghannoum MA (2019) Resistance of *Candida* to azoles and echinocandins worldwide. Clin Microbiol Infect 25: 792-798.
41. Pfaller MA, Messer SA, Jones RN, Castanheira M (2015) Antifungal susceptibilities of *Candida*, *Cryptococcus neoformans* and *Aspergillus fumigatus* from the Asia and Western Pacific region: data from the SENTRY antifungal surveillance program (2010-2012). J Antibiot 68: 556-561.
42. Adam KM, Osthoff M, Lamoth F, Conen A, Erard V, Boggian K, Schreiber PW, Zimmerli S, Bochud PY, Neofytos D, Fleury M, Fankhauser H, Goldenberger D, Mühlethaler K, Riat A, Zbinden R, Kronenberg A, Quiblier C, Marchetti O, Khanna N (2021) Trends of the epidemiology of Candidemia in Switzerland: a 15-year FUNGINOS survey. Open Forum Infect Dis 8: ofab471.
43. Trouvé C, Blot S, Hayette MP, Jonckheere S, Patteet S, Rodriguez-Villalobos H, Symoens F, Van Wijngaerden E, Lagrou K (2017) Epidemiology and reporting of candidaemia in Belgium: a multi-centre study. Eur J Clin Microbiol Infect Dis 36: 649-655.
44. Kneale M, Bartholomew JS, Davies E, Denning DW (2016) Global access to antifungal therapy and its variable cost. J Antimicrob Chemoth 71: 3599-3606.
45. Tan J, Zhang J, Chen W, Sun Y, Wan Z, Li R, Liu W (2015) The A395T mutation in *ERG11* gene confers fluconazole resistance in *Candida tropicalis* causing candidemia. Mycopathologia 179: 213-218.
46. Jiang C, Dong D, Yu B, Cai G, Wang X, Ji Y, Peng Y (2013) Mechanisms of azole resistance in 52 clinical isolates of *Candida tropicalis* in China. J Antimicrob Chemother 68: 778-785.
47. Odds FC (2010) Molecular phylogenetics and epidemiology of *Candida albicans*. Future Microbiol 5: 67-79.
48. Shin JH, Bougnoux M-E, d'Enfert C, Kim SH, Moon C-J, Joo MY, Lee K, Kim M-N, Lee HS, Shin MG (2011) Genetic diversity among Korean *Candida albicans* bloodstream isolates: assessment by multilocus sequence typing and restriction endonuclease analysis of genomic DNA by use of BssHII. J Clin Microbiol 49: 2572-2577.
49. Wu K, Luo T, Li L, Zhang Q, Zhu J, Gao Q, Chen M, Zhu M (2015) Multilocus sequence typing of pathogenic *Candida albicans* isolates collected from a teaching hospital in Shanghai, China: a molecular epidemiology study. PloS One 10: e0125245.
50. Wang SH, Shen M, Lin HC, Sun PL, Lo HJ, Lu JJ (2015) Molecular epidemiology of invasive *Candida albicans* at a tertiary hospital in northern Taiwan from 2003 to 2011. Med Mycol 53: 828-836.
51. Pham LTT, Pharkjaksu S, Chongtrakool P, Suwannakarn K, Ngamskulrungroj P (2019) A predominance of clade 17 *Candida albicans* isolated from hemocultures in a tertiary care hospital in Thailand. Front Microbiol 10: 1194.
52. Jolley KA, Bray JE, Maiden MCJ (2018) Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 3: 124. Available: https://pubmlst.org/organisms/candida-albicans. Accessed: 8 June 2016.

Corresponding author

Antonella Santona
Department of Biomedical Science, University of Sassari,
Viale S. Pietro 43/b, 07100 Sassari, Italy
Tel.: +39 3270109249
Fax +39 079212345
Email: asantona@uniss.it

Conflict of interests: No conflict of interests is declared.