Antifungal susceptibility, genotyping, resistance mechanism, and clinical profile of Candida tropicalis blood isolates

Arastehfar, A.; Daneshnia, F.; Hafez, A.; Khodavaisy, S.; Najafzadeh, M.-J.; Charsizadeh, A.; Zarrinfar, H.; Salehi, M.; Zare Shahrabadi, Z.; Sasani, E.; Zomorodian, K.; Pan, W.; Hagen, F.; Ilkit, M.; Kostrzewa, M.; Boekhout, T.

DOI
10.1093/mmy/myz124

Publication date
2020

Document Version
Final published version

Published in
Medical Mycology

ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2020, 58, 766–773
doi:10.1093/mmy/myz124
Advance Access Publication Date: 12 December 2019
Original Article

Original Article

Antifungal susceptibility, genotyping, resistance mechanism, and clinical profile of *Candida tropicalis* blood isolates

Amir Arastehfar[1,†], Farnaz Daneshnia[1,†], Ahmed Hafez[2], Sadegh Khodavaisy[3], Mohammad-Javad Najafzadeh[4], Arezoo Charsizadeh[5], Hossein Zarrinfar[6], Mohammadreza Salehi[7], Zahra Zare Shahrabadi[8], Elahe Sasani[9], Kamiar Zomorodian[10,*], Weihua Pan[11,*], Ferry Hagen[1,12,13], Macit Ilkit[14], Markus Kostrzewa[15] and Teun Boekhout[1,11,16]

[1]Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, [2]Biotechvana, 46980 Paterna, Valencia, Spain, [3]Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, [4]Department of Parasitology and Mycology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran, [5]Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran, [6]Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran, [7]Department of infectious diseases and Tropical Medicine, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [8]Department of Medical Mycology and Parasitology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, [9]Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, [10]Basic Sciences in Infectious Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, [11]Medical Mycology, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai 200003, China, [12]Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands, [13]Laboratory of Medical Mycology, Jining No. 1 People's Hospital, Jining, Shandong, People's Republic of China, [14]Division of Mycology, Department of Microbiology, Faculty of Medicine, University of Çukurova, Adana, Turkey, [15]Bruker Daltonik GmbH, Bremen, Germany and [16]Institute of Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam1012 WX, The Netherlands

*To whom correspondence should be addressed. Kamiar Zomorodian, PhD, Basic Sciences in Infectious Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. Tel: +989177144094; Fax: +987132349411; E-mail: zomorodian@sums.ac.ir, Weihua Pan, MD, PhD, Shanghai Key Laboratory of Molecular Mycology, Shanghai Chang zheng Hospital, Second Military Medical University, Shanghai 200003, People's Republic of China. Tel: +8602181885494; Fax: +8602181885493; E-mail: panweihua@smmu.edu.cn

†A.A. and F.D. contributed equally to this work.

Received 30 September 2019; Revised 14 November 2019; Accepted 6 December 2019; Editorial Decision 17 November 2019

Abstract

Candida tropicalis is one of the major candidaemia agents, associated with the highest mortality rates among *Candida* species, and developing resistance to azoles. Little is known about the molecular mechanisms of azole resistance, genotypic diversity, and the clinical background of *C. tropicalis* infections. Consequently, this study was designed to address those questions. Sixty-four *C. tropicalis* bloodstream isolates from 62 patients from three cities in Iran (2014–2019) were analyzed. Strain identification, antifungal susceptibility testing, and genotypic diversity analysis were performed by MALDI-TOF MS, CLSI-M27 A3/S4 protocol, and amplified fragment length polymorphism (AFLP) fingerprinting, respectively. Genes related to drug resistance (*ERG11*, *MRR1*, *TAC1*, *UPC2*, and *FKS1* hotspot9s) were sequenced. The overall mortality rate was 59.6% (37/62). Strains were resistant to micafungin [minimum inhibitory concentration (MIC) $\geq$1 μg/ml, 2/64], itraconazole (MIC $>$ 0.5 μg/ml, 2/64), fluconazole (FLZ; MIC $\geq$ 8 μg/ml, 4/64), and voriconazole (MIC $\geq$ 1 μg/ml, 7/64). Pan-azole and FLZ + VRZ resistance were observed in one and two isolates, respectively, while none of the patients were exposed to azoles. *MRR1* (T255P, 647S), *TAC1* (N164I, R47Q), and *UPC2* (T241A, Q340H,

T381S) mutations were exclusively identified in FLZ-resistant isolates. AFLP fingerprinting revealed five major and seven minor genotypes; genotype G4 was predominant in all centers. The increasing number of FLZ-R *C. tropicalis* blood isolates and acquiring FLZ-R in FLZ-naive patients limit the efficiency of FLZ, especially in developing countries. The high mortality rate warrants reaching a consensus regarding the nosocomial mode of *C. tropicalis* transmission.

Key words: *Candida tropicalis*, candidaemia, azole resistance, *ERG11*; *MRR1*; *TAC1*; *UPC2*, *FKS1*, genotyping.

Introduction

Candida tropicalis is the first or second common cause of candidaemia in developing countries such as India[1] and Brazil,[2] where the vast majority of cases are treated with fluconazole (FLZ) because of the high cost of echinocandins.[1,3] However, an increasing number of candidaemia studies have shown a significant increase in azole resistant *C. tropicalis* blood isolates[4–6] and some reported pan-azole[7,8] and pan-azole and amphotericin B (AMB) resistant isolates.[9] A comprehensive candidaemia study conducted in India revealed that the multidrug resistance (MDR) trait was equally seen for *C. tropicalis* and *Candida auris* isolates.[1] The isolation of azole-resistant *C. tropicalis* in azole-naive patients[4,8] will further limit the available treatment options and jeopardize the lives of patients, especially in developing countries. Furthermore, patients infected with *C. tropicalis* experience longer hospitalization and higher mortality compared to those infected with *Candida albicans*.[10] Surprisingly, over the course of 7 years surveillance of a *C. tropicalis* candidaemia study conducted in Taiwan, the authors noticed replacement of fluconazole susceptible dose-dependent isolates by those that are resistant to all azole drugs tested, including FLZ, voriconazole (VRZ), itraconazole (ITZ), and posaconazole (PSZ).[8] Collectively, these evidences show that *C. tropicalis* is not an innocuous azole-susceptible species and should be targeted by surveillance studies.

The major azole-resistant determinants in *C. tropicalis* are genes encoding for lanosterol 14-α-demethylase (*ERG11*), efflux pumps (*CDR1* and *MDR1*),[11,12] and the *ERG11* expression regulator (*UPC2*).[13] In *C. albicans*, specific gain-of-function mutations in *MRR1* and *TAC1*, that is, transcription regulators of *MDR1* and *CDR1*, are linked to the overexpression of the corresponding efflux pump genes and, therefore, azole-resistance.[14] However, no data on the occurrence of mutations in *MRR1* and *TAC1* in *C. tropicalis* azole-susceptible, azole-susceptible dose-dependent, and azole-resistant strains are available. In terms of echinocandin resistance, specific mutations at hotspots (HS) HS1 and HS2 of the *FKS1* gene encoding a 1,3-β-glucan synthase component are directly linked to the resistance in *C. tropicalis*.[15]

Although outbreaks[16,17] and clonal expansion of *C. tropicalis* in some clinical settings have been documented[18] and this species was found as a gut commensal in 46% of healthy individuals studied,[19] the other biological niches of the species yet remain to be discovered. Typing techniques permit identification of the source of infection, which may be followed by implementing appropriate preventive strategies, for example, initiation of antifungal prophylaxis or infection control, and may also facilitate the identification of genotypes that are associated with high mortality[3] and virulence.[20] While the typing resolution of multi-locus sequence typing is almost the same as that of microsatellite typing of six loci of *C. tropicalis* isolates,[21] the resolution of amplified fragment length polymorphisms (AFLP) genotyping is even better than the MLST when applied on clinical *C. albicans* isolates.[22] Moreover, despite the universality of this technique that obviates the need for previous knowledge about the genome of a target species,[23] AFLP has never been used for typing of *C. tropicalis* isolates.

Here we undertook a systematic multicenter study and retrospectively analyzed 64 *C. tropicalis* blood isolates recovered from candidaemia patients in Iran during 2014–2019. The isolates were characterized by MALDI-TOF MS, antifungal susceptibility testing (AFST), and sequencing of drug-resistance genes. AFLP analysis was used to assess their genotypic diversity. Since neutropenic patients and those suffering from leukemia have a high propensity for developing *C. tropicalis* candidaemia,[10] we also systematically analyzed the clinical data of patients included in the study.

Methods

Study design, isolates, and growth conditions

Sixty-four *C. tropicalis* blood isolates recovered from September 2014 to February 2019 from candidaemia patients admitted to 10 hospitals in three major cities of Iran (Mashhad, Shiraz, and Tehran) were included in the study. There was no restriction of age, sex, underlying conditions, and ward. The blood bottles were incubated in Bactec devices (Becton Dickinson, Franklin Lake, NJ, USA); 100 μl of positive blood cultures were inoculated onto Sabouraud dextrose agar and chromogenic media (Candiselect, Bio-Rad, Hercules, CA, USA) to ascertain the homogeneity of species involved, and incubated at 37°C for 24–48 hours. The candidaemia studies undertaken at each center had been approved by the ethical committee of the affiliated university, with the appropriate ethical approvals granted (approval numbers IR.SUMS.REC.1397.365, IR.MUMS.REC.1397.268,

and IR.TUMS.SPH.REC.1396.4195). Written consent was obtained from patients, and patient identity was blinded to the personnel performing data analysis. Antifungal naive patients were noted if a given patient did not receive any systemic antifungal 90 days prior to manifestation of candidaemia.

Isolate identification, DNA extraction, PCR, and sequencing

Strain identification was confirmed by MALDI-TOF MS (MALDI Biotyper; Bruker Daltonik, Bremen, Germany) using the full extraction method.[24] DNA was extracted using a CTAB-based extraction method.[25] Primers to amplify the full open reading frame of *MRR1*, *TAC1*, *UPC2*, and *ERG11*, and HS1 and HS2 of *FKS1* were designed (Table S1) using the genome of *C. tropicalis* MYA-3404 (AAFN00000000.2)[26] as a reference (wild-type sequences are listed at the end of Supplementary files). Amplification of each gene was performed using the program and conditions specified in Table S2. Amplicons were subjected to Sanger sequencing and the obtained sequences were analyzed by SeqMan Pro (DNASTAR, Madison, WI, USA). The analyzed sequences were aligned using MEGA v7.0,[27] the mutations were mapped to reference genes, and the corresponding mutations peaks were rechecked by using SeqMan Pro to assure the accuracy. Heterozygosity is defined when a double, clean, and decent peak representing two different nucleotides was observed at the same position.

Antifungal susceptibility testing (AFST)

AFST followed the CLSI M27-A3/S4 protocol.[28,29] The six antifungal agents tested were fluconazole (FLZ), voriconazole (VRZ), itraconazole (ITZ), and amphotericin B (AMB) (all from Sigma-Aldrich, St. Louis, MO, USA); micafungin (MFG; Astellas, Munich, Germany); and anidulafungin (AFG; Pfizer, NY, USA). Caspofungin was not tested because of the reported inter-laboratory variation.[30] Plates were incubated at 37°C for 24 hours and visually assessed. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included as quality controls. Minimum inhibitory concentration (MIC) values of FLZ, VRZ, AFG, and MFG were interpreted based on the species-specific clinical break points, with MIC $\geq$ 8 μg/ml denoting FLZ-resistance (R); and MIC $\geq$ 1 μg/ml denoting VRZ-R, AFG-R, and MFG-R;[31] MIC = 4 μg/ml and 0.25 $\leq$ MIC $\geq$ 0.5 μg/ml to indicate FLZ-susceptible dose-dependent (FLZ-SDD) and VRZ-intermediate phenotypes (VRZ-I), respectively. Because of the lack of clinical breakpoints, epidemiological cut-off values (ECV) were used for AMB and ITZ, with MIC values >2 μg/ml and >0.5 μg/ml considered non-wild type (NWT) for AMB and ITZ, respectively.[31]

AFLP genotyping

DNA samples were analyzed by using a previously described AFLP protocol.[3] Fluorescently labeled amplicons were resolved by capillary electrophoresis (ABI 3730xL Genetic Analyzer, Applied Biosystems, Palo Alto, CA, USA), and the data were analyzed using Bionumerics v7.6 (Applied Math, Sint-Martens-Latem, Belgium). The following reference and type strains were included in the AFLP analysis for comparative purposes: *C. tropicalis* CBS 433, CBS 643, CBS 2313, CBS 6862; *C. albicans* CBS 2704 and CBS 2705; and *Candida dubliniensis* CBS 7988.

Data availability

All sequences generated in the current study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers MK906127–MK906190 (*ERG11*), MK906052–MK906076 (*MRR1*), MK906077–MK906101 (*TAC1*), MK906102–MK906126 (*UPC2*), MK906191–MK906254 (HS1 of *FKS1*), and MK906255–MK906318 (HS2 of *FKS1*).

Statistical analysis

All statistical analyses were performed using SPSS v24 (SPSS Inc., Chicago, IL, USA) (Supplementary Files, statistical analysis section). The associations between genotypes, and FLZ and VRZ resistance were evaluated using two-tailed χ^2 test. Since the hospitalization duration data were not normally distributed, the association between genotypes and duration of hospitalization was evaluated using the Kruskal-Wallis test. To assess the direct and indirect influence of genotypes on mortality, the logistic multivariate regression and path analysis was used. P values < .05 were considered statistically significant.

Results

Clinical characteristics

Sixty-four *C. tropicalis* isolates were recovered from 62 patients, 42% (n = 26) of whom were male and 58% (n = 36) female, with a median age of 37 years (2 months to 90 year-old) (Table S3). Most isolates were obtained at Mashhad (n = 31, 48.4%), followed by Tehran (n = 28, 43.7%), and Shiraz (n = 5, 7.8%). Sepsis was observed in 31 patients (50%) when candidaemia was manifested. Pre-exposure to antibiotics (n = 64, 100%), central venous catheter insertion (n = 53, 84.6%), mechanical ventilation (n = 37, 59.7%), surgery (abdominal [n = 17, 27.1%] and non-abdominal [n = 8, 12.9%]), parenteral nutrition (n = 20, 32.2%), administration of immunosuppressive drugs (n = 14, 22.6%), and neutropenia (n = 12, 19.4%) were the major risk factors for the development of candidaemia (Table S3). AMB was the most widely used antifungal (n = 28, 45.2%), followed by FLZ (n = 16, 25.8%), CSP

Table 1. Antifungal susceptibility data for *Candida tropicalis* isolates obtained in the current study.

	Antifungal drugs					
Susceptibility data	FLZ	VRZ	ITZ	MCF	ANF	AMB
MIC values (μg/ml)						
≤0.016		5		**18**	**23**	
0.03		7		11	9	
0.06		**16**	2	17	17	
0.125	2	9	23	10	8	1
0.25	12	9	23	3	3	5
0.5	**18**	11	**14**	3	3	**29**
1	14	6	1	2		28
2	7					1
4	7	1				
8	3					
16			1			
32						
≥64	1					
Range	0.125–64	0.016–4	0.06–16	0.008–1	0.008–0.5	0.125–2
GM	0.878126	0.142408	0.2634	0.050506	0.038356	0.641435
MIC 50	0.5	0.125	0.25	0.062	0.025	0.5
MIC 90	4	1	1	0.25	0.125	1

MIC values denoted in boldface are modal values.
AMB, amphotericin B; ANF, anidulafungin; FLZ, fluconazole; GM, geometric mean value; ITZ, itraconazole; MCF, micafungin; MIC, minimum inhibitory concentration VRZ, voriconazole.

($n = 11$, 17.7%), and nystatin ($n = 4$, 6.4%), while nearly a quarter of patients ($n = 15$) did not receive any antifungals (Table S3). The overall mortality rate was nearly 60% ($n = 37$). The highest mortality rates were reported for Mashhad ($n = 21$, 67.7%) followed by Shiraz ($n = 3$, 60%) and Tehran 57.6% ($n = 15$) (Tables S3a and S3b).

AFST

Resistance to VRZ (MIC ≥ 1 μg/ml), FLZ (MIC ≥ 8 μg/ml), and MFG (MIC ≥ 1 μg/ml) was noted in seven (10.93%, 7/64), four (6.25%, 4/64), and two (3.12%, 2/64) isolates, respectively. Moreover, some isolates denoted VRZ-I (0.25 ≤ MIC ≥ 0.5 μg/ml, $n = 18$; 18/64) and FLZ-SDD (MIC = 4 μg/ml, $n = 7$; 7/64) (Table 1 and Table S4). All isolates were susceptible to AFG and AMB, while two were NWT for ITZ (MIC > 0.5 μg/ml, $n = 2$; 2/64). Three isolates were resistant to ≥2 azole drugs (4.7%); one showed pan-azole resistance to all azole drugs tested (1.6%); and two were cross-resistance to FLZ and VRZ (3.2%) (Tables 1 and 2, and Table S4). Except for two isolates (262E and N186), no multi-azole resistant isolates (to two or three azoles tested) represented a single genotype.

Mutation analysis of the isolates

We did not find previously known mutations in *ERG11* directly causing fluconazole-resistance in our fluconazole-resistant isolates.[11,12] Since FLZ MIC values depend on the heterozygosity and homozygosity status of the *MRR1*, *TAC1*, and *UPC2* genes[32] and in order to identify specific mutations for each MIC category, 26 isolates were categorised as control (C, MIC < 2 μg/ml) ($n = 12$), S (MIC = μg/ml) ($n = 3$), SDD (MIC = 4 μg/ml) ($n = 7$), and FLZ-R (MIC ≥ 8 μg/ml) ($n = 4$). Subsequently, target genes of those 26 isolates were sequenced (Table 2 and Table S4). Of those, T255P and A647S in *MRR1*, R47Q and N164I in *TAC1*, and T241A, Q340H, and T381S in *UPC2* were exclusively identified in FLZ-R isolates, while F571Y in *UPC2* and L430* (stop codon) in *TAC1* were only identified in an FLZ-SDD isolate (Table 2). The only pan-azole resistant isolate simultaneously carried FLZ-R specific mutations in both *UPC2* (Q340H and T381S) and *TAC1* (R47Q and N164I) genes. Although those ITZ-R isolates did not harbor any specific mutations, one of the VRZ-R isolates showed a unique mutation (A263T) in *UPC2*. No association between FLZ exposure and FLZ resistance was observed, as patients carrying FLZ-R strains had never been administered FLZ (Table S4).

AFLP genotyping of the isolates

AFLP analysis revealed five main genotypes (G2–G6) accounting for 89% of the isolates ($n = 57$) and seven minor genotypes, each represented by a single isolate (Fig. 1). Considering the major genotypes, G4 was the most prevalent ($n = 25$, 38.4%), followed by G6 ($n = 11$, 17.1%), G2 and G5 ($n = 9$ each, 14%), and G3 ($n = 3$, 4.6%) (Fig. 1). The isolates from Shiraz and a hospital from Tehran did not exhibit conspicuous accumulation of any specific genotype. However, 58% ($n = 18$) of Mashhad isolates represented G4, and 61% ($n = 11$) of those

Table 2. Sequences of the target genes in all FLZ-R ($n = 4$) and FLZ-SDD isolates ($n = 7$), and randomly selected FLZ-S isolates ($n = 15$).

Strain no.	FLZ (μg/ml)	VRZ (μg/ml)	ITZ (μg/ml)	*MRR1*	*TAC1*	*UPC2*	Genotype
Fluconazole-control isolates ($n = 12$)							
N8	0.5	0.125	0.5	A87T, V133A, M1022I, T1042N, T1044N, I1130M	WT	**A251T, Q289L, G392E**	G5
N15	0.5	0.125	0125	WT	L278S	N98S, L158V	G2
N71	0.125	0.016	0.125	S523F, K757E	L278S, D350N, F470C, D790N	**G392E**	MG
N104	1	0.25	0.125	M1022I, T1042N, T1044N, D1092E, I1130M	L278S	WT	G4
N147	0.5	0.031	0.125	M1022I, T1042N, T1044N, D1092E, I1130M	L278S	WT	G4
N195	0.25	0.031	0.125	S523F, K757E	L278S, D350N, F470C, D790N	**A297S***, **G392E**	G4
N210	0.125	0.062	0.125	WT	L278S	N98S, L158V, A251T	G2
SU-221	0.25	0.015	0.06	A87T), V133A, M1022I, T1042N, T1044N, D1092E, I1130M	L278S, F470C	WT	G5
SU-267	0.25	0.125	0.125	WT	L278S, F470C, S884G	N98S, L119F, A147T, L158V	MG
10BC	1	0.5	0.5	I408T, M1022I, T1042N, T1044N, D1092E, I1130M	L278S, F470C, D790N, D790N	A251T, Q289L, **A297S***	G6
24BC	0.5	0.5	0.125	S523F, K757E	L278S, F470C, D790N	**G392E**, T560N	G6
115-1BC	0.5	0.125	0.125	A87T, V133A, M1022I, T1042N, T1044N, I1130M	WT	A251T, Q289L	G5
Fluconazole-susceptible isolates ($n = 3$)							
N26	2	0.5	0.125	S523F, K757E	L278S, D350N, F470C, D790N	**A297S***, ***G392E***	G6
SU-235	2	0.125	0.125	WT	L278S	N98S, L158V, *A251T*	G2
85BC	2	0.25	0.125	WT	L278S	N98S, L158V	G2
Fluconazole-susceptible dose-dependent isolates ($n = 7$)							
SU-239	4	0.06	0.125	WT	L278S	L158V, N98S, L158V, **F571Y**	G2
N17	4	0.5	1	S523F, K757E, I1130M	L278S, **L340***, D350N, F470C, D790N	NA	
75BC	4	0.125	0.25	S523F, K757E, I1130M	L278S	WT	G4
82BC	4	1	0.25	I1130M	WT	A263T	G4
107BC	4	0.062	0.125	S523F, K757E	L278S, F470C, D790N	WT	G3
113-1BC	4	1	0.125	I1130M	L278S, F470C	A251T, Q289L	G4
115-2	4	0.5	0.06	A87T, V133A, M1022I, T1042N, T1044N, I1130M	WT	A251T, Q289L, **G392E**	G5
Fluconazole-resistant isolates ($n = 4$)							
99BC	8	1	0.5	V133A, **A647S**, M1022I, T1044N, D1092E, I1130M	L278S, F470C	WT	G5
113-2BC	8	0.062	0.25	WT	L278S	N98S, L158V, N230S, **T241A**	G2
262E	64	4	16	M1022I, T1042N, T1044N, D1092E, I1130M	**R47Q**, **N164I**, L278S	**Q340H**, **T381S**	G4
527E	8	1	0.5	**T255P**	**R47Q**, **N164I**, L278S, F470C, D790N	A147T, A251T, Q289L	MG

Underlined boldface amino acid substitutions were only identified in FLZ-R or FLZ-SDD isolates; asterisk-denoted boldface amino acids were exclusively identified in resistant isolates in previous studies; boldface italicized amino acids were exclusively found in susceptible isolates in the current and previous studies. All strains carried the *ERG11* WT sequence, except for SU-239 (K90I) and SU-267 (I25A). FLZ, fluconazole; ITZ, itraconazole; VRZ, voriconazole.

*Stop codon; NA, not amplified.

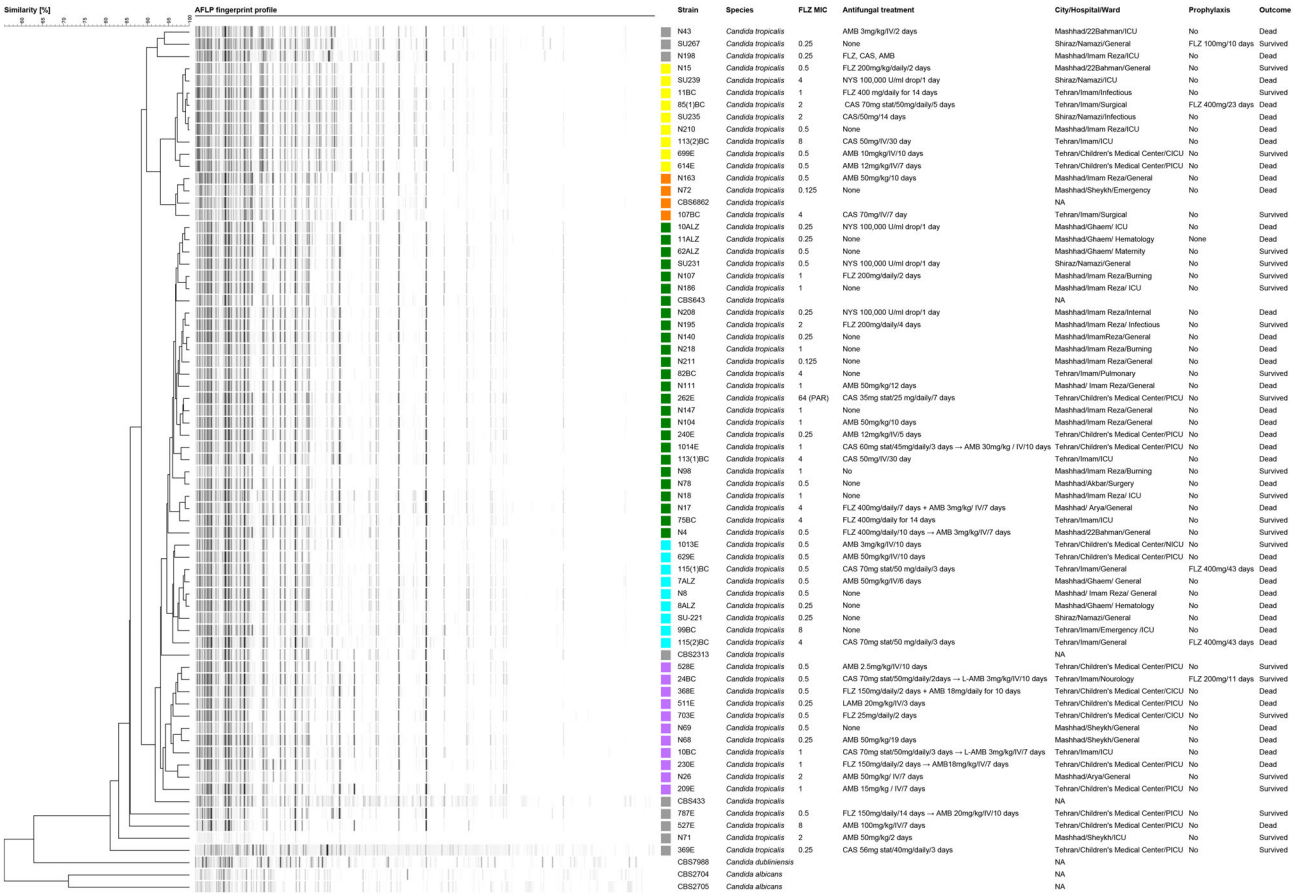

Figure 1. AFLP fingerprinting revealed five major and seven minor genotypes (shown with gray color). Each genotype (G) was defined by distinct color as follows: G2 = yellow, G3 = Orange, G4 = Green, G5 = Light blue, and G6 = Ultraviolet. G4 was the most predominant genotype found in all centers involved.

isolates were from different wards of a single hospital (Imam Reza, years 2015–2019). Furthermore, 37.5% ($n = 6$) of isolates from the Children's Medical Centre in Tehran represented G6 and all originated from the intensive unit wards (years 2015–2016). In case of three patients with duplicate isolates, except for 115-1 and 115-2BC that clustered in the same genotype, the isolates represented different genotypes (368 and 369E, and 113-1 and 113-2BC). Multivariate logistic regression, path analysis, and Kruskal-Wallis test did not indicate any association between the genotypes and patient mortality ($P = .47$), or genotypes and duration of hospitalization ($P = .6$) (Supplementary Files, Statistical analysis section). Further, as determined by using the two-tailed χ^2 test, the genotypes (G2–G6) and azole resistance were not significantly associated (Supplementary Files, Statistical analysis section).

Discussion

The patients included in the current study had common risk factors for the development of candidaemia, such as central venous catheter insertion, pre-exposure to antibiotics, mechanical ventilation, and abdominal surgery.[1] Even though leukemic patients show a high propensity for developing *C. tropicalis* candidaemia,[10] we found that, similar to a study from Italy,[18] other complications were the most prevalent underlying condition. This discrepancy could be explained by differences in the target populations examined. The mortality reported in the current study was even higher than that reported for *C. glabrata* (60% vs. 37.5%),[3] which is consistent with studies from Italy[33] and the United States,[34] and corroborates the highly virulent nature of *C. tropicalis* and its poor prognosis when compared to the other non-*albicans Candida* (NAC) species.[35,36]

Among the azoles tested in this study, we found the highest level of resistance to VRZ ($n = 7$, 7/64), followed by FLZ ($n = 4$, 4/64) and ITZ ($n = 2$, 2/64). In the current study, the observed low level of resistance to major antifungal drugs (except for ITZ) was comparable with that reported for Asian[1] and Middle Eastern countries,[37] and Italy and Spain,[38] and contrasted with the high reported resistance rates to FLZ and VRZ in China[5] and Taiwan.[8] Although previous and prolonged exposure is the main driving factor for emerging antifungal resistant isolates,[39,40] surprisingly, we did not find any association between FLZ-R and previous exposure with FLZ, as patients infected with FLZ-R isolates did not receive FLZ 90 days prior to candidaemia manifestation. This is in agreement with a previous study conducted in Japan[4] and Taiwan[8] where almost 50% of patients infected with fluconazole-resistant strains were azole-naive. We speculate that either host conditions triggered alternative

pathways leading to resistance[41] or the azole-resistant strains were acquired from the hands of healthcare workers (HCWs),[42] in addition to the possible link between antibiotic prophylaxis and FLZ-R.[43] Alternatively, a study in Taiwan noticed that a fruit-related azole resistant *C. tropicalis* isolate clustered with the fluconazole non-susceptible (FNS) blood isolates and this coincided with a fourfold increase in use of fungicides in agricultural applications in this country.[8] Therefore, the authors assumed that azole-naive patients might have acquired these FNS isolates from the environment,[8] the same as what was observed for *Aspergillus fumigatus*.[44]

Mechanistically, we did not identify any accountable mutations in the *ERG11* gene, but several suggestive mutations in *MRR1* (T255P, 647S), *TAC1* (N164I, R47Q), and *UPC2* (Q340H, T381S) were exclusively identified in FLZ-resistant isolates. Furthermore, unlike a previous report of A297S amino acid substitution found only in FLZ-R isolates,[13] we here identified this mutation exclusively in FLZ-S isolates. Although susceptible isolates were included in that study, the authors did not explore the occurrence of mutations in *MRR1* and *TAC1*; therefore, they might have been biased and other accountable mutations in those genes might have been overlooked. In our study, one VRZ-R isolate carried a unique mutation in *UPC2* (A263T); while this mutation was previously found in VRZ-S isolates,[13] hence it may not drive resistance to VRZ.

AFLP revealed that isolates from all the analyzed centers represented the predominant genotype G4, which might be an indication for intra-hospital and/or clonal transmission of *C. tropicalis*. Considering that 80% of yeasts isolated from the hands of HCWs are *C. tropicalis*,[42] a specific genotype was found to be enriched in Taiwan[17] and Italy,[18] and the same clone of *C. tropicalis* blood isolates was identified in a unit environment and on hands of HCWs,[18] thus likely suggesting indeed transmission may have occurred via the hands of HCWs. Interestingly, implementation of routine infection control strategies led to termination of an ongoing *C. tropicalis* outbreak,[18] which in view of the high mortality rate posed by this species further highlights the importance of application of typing techniques to assess the genotypic diversity of *C. tropicalis* in healthcare settings. The notable difference in typing protocols, study design, and patient size and isolates numbers hinder drawing a clear conclusion regarding the mode of transmission of *C. tropicalis* in the hospital settings and the current knowledge in this regard remained speculative. Therefore, application of standardized and resolutive typing techniques, such as whole genome sequencing, might address this question.

Although other studies reported a link between genotype and mortality,[3] we did not find such a link in the current study. Similarly, we did not find links between the genotype and duration of hospitalization, and genotype and azole susceptibility. Interestingly, two duplicate isolates from two patient belonged to different genotypes than the original isolate, which could be explained by either host and/or antifungal-triggered stress followed by minimal to gross chromosomal changes[45] or introduction of a new isolate into the bloodstream.

The current study has some limitations. For example, we did not analyze the expression of efflux pump genes, such as *CDR1* and *MDR1*, as an alternative azole resistance mechanism. Further, mutations identified in FLZ-R isolates are purely suggestive and heterologous expression in a susceptible *C. tropicalis* isolate is required to confirm involvement in FLZ-resistance.

The high mortality rate noted in the current study might be alleviated if resolutive typing techniques become part of a routine clinical procedure, considering the speculation that this species might be horizontally transferred. Furthermore, the presented data suggested that a full picture should be considered (*MRR1*, *TAC1*, and *UPC2* sequencing) to understand the underlying molecular azole-resistance mechanisms. Finally, the increasing risk of non-azole resistant *C. tropicalis* from blood isolates and FLZ-R isolates without previous exposure to this drug highlight the importance of species-specific candidaemia studies to extensively explore and highlight the clinical and microbiological differences between various *Candida* species, leading to better patient management strategies.

Supplementary material

Supplementary data are available at *MMYCOL* online.

Declaration of interest

M.K. is an employee of Bruker Daltonik GmbH, Bremen, Germany, the manufacturer of the MALDI-TOF MS system used for *Candida* identification in the current study. There are no other conflicts of interest to declare. The authors alone are responsible for the content and the writing of this paper.

Funding

This work was supported by the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie call [grant no. 642095]; National Health Department of China [grant no. 2018ZX10101003]; National Natural Science Foundation of China [grant no. 31770161]; Second Military Medical University [grant no. 2017JZ47]; Shanghai Science and Technology Committee [grants no. 14DZ2272900, 14495800500]; and Shiraz University of Medical Sciences [grant no. 98-01-43-21203].

References

1. Chakrabarti A, Sood P, Rudramurthy SM et al. Incidence, characteristics and outcome of ICU-acquired candidemia in India. *Intensive Care Med*. 2015; 41: 285–295.
2. Wille MP, Guimaraes T, Furtado GHC, Colombo AL. Historical trends in the epidemiology of candidaemia: analysis of an 11-year period in a tertiary care hospital in Brazil. *Mem Inst Oswaldo Cruz*. 2013; 108: pii: S0074-02762013000300288.
3. Arastehfar A, Daneshnia F, Zomorodian K et al. Low level of antifungal resistance in Iranian isolates of *Candida glabrata* recovered from blood samples in a multicenter study from 2015 to 2018 and potential prognostic values of genotyping and sequencing of *PDR1*. *Antimicrob Agents Chemother*. 2019; 63: pii: e02503-18.

4. Chong Y, Shimoda S, Yakushiji H et al. Fatal candidemia caused by azole-resistant *Candida tropicalis* in patients with hematological malignancies. *J Infect Chemother*. 2012; 18: 741–746.
5. Fan X, Xiao M, Liao K et al. Notable increasing trend in azole non-susceptible *Candida tropicalis* causing invasive candidiasis in China (August 2009 to July 2014): molecular epidemiology and clinical azole consumption. *Front Microbiol*. 2017; 8: 464.
6. Arendrup MC, Bruun B, Christensen JJ et al. National surveillance of fungemia in Denmark (2004 to 2009). *J Clin Microbiol*. 2011; 49: 325–334.
7. Xisto MIDS, Caramalho RDF, Rocha DAS et al. Pan-azole-resistant *Candida tropicalis* carrying homozygous *erg11* mutations at position K143R: a new emerging superbug? *J Antimicrob Chemother*. 2017; 72: 988–992.
8. Chen PY, Chuang YC, Wu UI et al. Clonality of fluconazole-nonsusceptible *Candida tropicalis* in bloodstream infections, Taiwan, 2011–2017. *Emerg Infect Dis*. 2019; 25: 1660–1667.
9. Eddouzi J, Parker JE, Vale-Silva LA et al. Molecular mechanisms of drug resistance in clinical *Candida* species isolated from Tunisian hospitals. *Antimicrob Agents Chemother*. 2013; 57: 3182–3193.
10. Kontoyiannis DP, Vaziri I, Hanna HA et al. Risk factors for *Candida tropicalis* fungemia in patients with cancer. *Clin Infect Dis*. 2001; 33: 1676–1681.
11. Fan X, Xiao M, Zhang D et al. Molecular mechanisms of azole resistance in *Candida tropicalis* isolates causing invasive candidiasis in China. *Clin Microbiol Infect*. 2019; 25: 885–891.
12. Jin L, Cao Z, Wang Q et al. MDR1 overexpression combined with *ERG11* mutations induce high-level fluconazole resistance in *Candida tropicalis* clinical isolates. *BMC Infect Dis*. 2018; 18: 162.
13. Choi MJ, Won EJ, Shin JH et al. Resistance mechanisms and clinical features of fluconazole-nonsusceptible *Candida tropicalis* isolates compared with fluconazole-less-susceptible isolates. *Antimicrob Agents Chemother*. 2016; 60: 3653–3661.
14. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. Azole antifungal resistance in *Candida albicans* and emerging non-*albicans Candida* species. *Front Microbiol*. 2016; 7: 2173.
15. Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E. Mutations in the *fks1* gene in *Candida albicans*, *C. tropicalis*, and *C. krusei* correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. *Antimicrob Agents Chemother*. 2008; 52: 3092–3098.
16. Li SY, Yang YL, Lin YH et al. Two closely related fluconazole-resistant *Candida tropicalis* clones circulating in Taiwan from 1999 to 2006. *Microb Drug Resist*. 2009; 15: 205–210.
17. Chou HH, Lo HJ, Chen KW, Liao MH, Li SY. Multilocus sequence typing of *Candida tropicalis* shows clonal cluster enriched in isolates with resistance or trailing growth of fluconazole. *Diagn Microbiol Infect Dis*. 2007; 58: 427–433.
18. Scordino F, Giuffre L, Barberi G et al. Multilocus sequence typing reveals a new cluster of closely related *Candida tropicalis* genotypes in Italian patients with neurological disorders. *Front Microbiol*. 2018; 9: 679.
19. Hallen-Adams HE, Suhr MJ. Fungi in the healthy human gastrointestinal tract. *Virulence*. 2017; 8: 352–358.
20. Ngamskulrungroj P, Serena C, Gilgado F, Malik R, Meyer W. Global VGIIa isolates are of comparable virulence to the major fatal *Cryptococcus gattii* Vancouver Island outbreak genotype. *Clin Microbiol Infect*. 2011; 17: 251–258.
21. Wu Y, Zhou H, Che J et al. Multilocus microsatellite markers for molecular typing of *Candida tropicalis* isolates. *BMC Microbiol*. 2014; 14: 245.
22. Asadzadeh M, Ahmad S, Al-Sweih N, Khan Z. Molecular fingerprinting studies do not support intrahospital transmission of *Candida albicans* among candidemia patients in Kuwait. *Front Microbiol*. 2017; 8: 247.
23. Restrepo CM, Llanes A, Lleonart R. Use of AFLP for the study of eukaryotic pathogens affecting humans. *Infect Genet Evol*. 2018; 63: 360–369.
24. Cassagne C, Cella AL, Suchon P, Normand AC, Ranque S, Piarroux R. Evaluation of four pretreatment procedures for MALDI-TOF MS yeast identification in the routine clinical laboratory. *Med Mycol*. 2013; 51: 371–377.
25. Theelen B, Silvestri M, Gueho E, van Belkum A, Boekhout T. Identification and typing of *Malassezia* yeasts using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE). *FEMS Yeast Res*. 2001; 1: 79–86.
26. Butler G, Rasmussen MD, Lin MF et al. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature*. 2009; 459: 657–662.
27. Kumar S, Stecher G, Tamura K. Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016; 33: 1870–1874.
28. Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard–Third Edition: M27-A3*. CLSI, Wayne, PA, USA, 2008.
29. Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Fourth Informational Supplement M27-S4*. CLSI, Wayne, PA, USA, 2012.
30. Espinel-Ingroff A, Arendrup MC, Pfaller MA et al. Interlaboratory variability of caspofungin MICs for *Candida* spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? *Antimicrob Agents Chemother* 2013; 57: 5836–5842.
31. Pfaller MA, Diekema DJ. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. *J Clin Microbiol*. 2012; 50: 2846–2856.
32. Morschhäuser J, Barker KS, Liu TT, BlaB-Warmuth J, Homayouni R, Rogers PD. The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in *Candida albicans*. *PLoS Pathog*. 2007; 3: e164.
33. Montagna MT, Caggiano G, Lovero G et al. Epidemiology of invasive fungal infections in the intensive care unit: results of a multicenter Italian survey (AURORA Project). *Infection*. 2013; 41: 645–653.
34. Andes DR, Safdar N, Baddley JW et al. The epidemiology and outcomes of invasive *Candida* infections among organ transplant recipients in the United States: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Transpl Infect Dis*. 2016; 18: 921–931.
35. Zuza-Alves DL, Silva-Rocha WP, Chaves GM. An update on *Candida tropicalis* based on basic and clinical approaches. *Front Microbiol*. 2017; 8: 1927.
36. Ko JH, Jung DS, Lee JY et al. Poor prognosis of *Candida tropicalis* among non-*albicans* candidemia: a retrospective multicenter cohort study, Korea. *Diagn Microbiol Infect Dis*. 2019: 95: 195–200.
37. Khan Z, Ahmad S, Al-Sweih N et al. Changing trends in epidemiology and antifungal susceptibility patterns of six bloodstream *Candida* species isolates over a 12-year period in Kuwait. *PLoS One*. 2019; 14: e0216250.
38. Bassetti M, Merelli M, Righi E et al. Epidemiology, species distribution, antifungal susceptibility, and outcome of candidemia across five sites in Italy and Spain. *J Clin Microbiol*. 2013; 51: 4167–4172.
39. Lewis JS, 2nd, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. *Antimicrob Agents Chemother*. 2013; 57: 4559–4561.
40. Perlin DS. Echinocandin resistance in *Candida*. *Clin Infect Dis* 2015; 61: 612–617.
41. Demers EG, Biermann AR, Masonjones S et al. Evolution of drug resistance in an antifungal-naïve chronic *Candida lusitaniae* infection. *Proc Natl Acad Sci U S A*. 2018; 115: 12040–12045.
42. Chakrabarti A, Chatterjee SS, Rao KLN et al. Recent experience with fungaemia: change in species distribution and azole resistance. *Scand J Infect Dis*. 2009; 41: 275–284.
43. Ben-Ami R, Olshtain-Pops K, Krieger M et al. Antibiotic exposure as a risk factor for fluconazole-resistant *Candida* bloodstream infection. *Antimicrob Agents Chemother*. 2012; 56: 2518–2523.
44. Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect Dis*. 2009; 9: 789–795.
45. Healey KR, Jimenez Ortigosa C, Shor E, Perlin DS. Genetic drivers of multidrug resistance in *Candida glabrata*. *Front Microbiol*. 2016; 7: 1995.