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Low level of antifungal resistance of *Candida glabrata* blood isolates in Turkey: Fluconazole minimum inhibitory concentration and *FKS* mutations can predict therapeutic failure

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Abstract

Background: *Candida glabrata* is the third leading cause of candidaemia in Turkey; however, the data regarding antifungal resistance mechanisms and genotypic diversity in association with their clinical implication are limited.

Objectives: To assess genotypic diversity, antifungal susceptibility and mechanisms of drug resistance of *C glabrata* blood isolates and their association with patients' outcome in a retrospective multicentre study.

Patients/Methods: Isolates from 107 patients were identified by ITS sequencing and analysed by multilocus microsatellite typing, antifungal susceptibility testing, and sequencing of *PDR1* and *FKS1/2* hotspots (HSs).

Results: *Candida glabrata* prevalence in Ege University Hospital was twofold higher in 2014-2019 than in 2005-2014. Six of the analysed isolates had fluconazole MICs ≥ 32 μg/mL; of them, five harboured unique *PDR1* mutations. Although echinocandin resistance was not detected, three isolates had mutations in HS1-Fks1 (S629T, n = 1) and HS1-Fks2 (S663P, n = 2); one of the latter was also fluconazole-resistant. All patients infected with isolates carrying HS-*FKS* mutations and/or demonstrating fluconazole MIC ≥ 32 μg/mL (except one without clinical data) showed therapeutic failure (TF) with echinocandin and fluconazole; seven such isolates were collected in Ege (n = 4) and Gulhane (n = 3) hospitals and six detected recently. Among 34 identified genotypes, none were associated with mortality or enriched for fluconazole-resistant isolates.

Conclusion: Antifungal susceptibility testing should be supplemented with HS-*FKS* sequencing to predict TF for echxinocandins, whereas fluconazole MIC ≥ 32 μg/mL may predict TF. Recent emergence of *C glabrata* isolates associated with antifungal TF warrants future comprehensive prospective studies in Turkey.

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KEYWORDS
antifungal agents, *Candida glabrata*, candidaemia, drug resistance, genotype, molecular typing

1 | INTRODUCTION

Candida glabrata is the second leading cause of candidaemia in USA,[1] Canada,[2] Australia[3] and some Scandinavian countries[4-9] and the first cause of candidaemia in intensive care units and patients with haematological malignancies and solid tumours.[10,11] Compared to the other *Candida* species, *C glabrata* is much more tolerant to antifungals,[12] which allows it to rapidly develop antifungal resistance during the course of antifungal therapy.[13-22] Indeed, *C glabrata* isolates resistant to azoles or echinocandins and even those demonstrating multidrug resistance are increasingly being identified in clinical settings.[6,8,23] It was shown that resistance to azoles and increase of virulence in *C glabrata* is mostly caused by gain-of-function mutations in the yeast Zn_2Cys_6 transcription factor *PDR1*, which lead to the overexpression of efflux pumps,[24] whereas resistance to echinocandins is associated with non-synonymous mutations in hotspots (HSs) 1 and 2 of the *FKS1* and *FKS2* genes encoding glucan synthases. Regarding the clinical prognosis, some studies indicate that sequencing of HSs in *FKS1/2* more accurately predicts therapeutic failure (TF) of echinocandins than phenotypic antifungal susceptibility testing (AFST).[23,25]

Candida glabrata is an endogenous opportunistic pathogen normally residing in the human gastrointestinal tract and causing bloodstream infections in immunocompromised hosts.[26] However, some molecular typing studies indicate that a possibility of horizontal transfer, suggesting that clonal enrichment of fluconazole-resistant *C glabrata* isolates cannot be excluded.[27,28] Furthermore, it has been shown that some *C glabrata* genotypes are associated with a higher mortality rate,[29,30] reinforcing the importance of strain profiling using genotyping techniques in clinical practice.

Candida glabrata is the third leading cause of candidaemia in Turkey, where it shows a low level of antifungal resistance as evidenced by a recent multicentre candidaemia study (1997-2017).[31] However, the correlation of important clinical parameters and microbiological properties such as genotypic diversity and molecular mechanisms underlying azole and echinocandin resistance has not been investigated. The current retrospective multicentre study was conducted to address these gaps in the knowledge regarding *C glabrata* blood isolates in Turkey.

2 | MATERIALS AND METHODS

2.1 | Isolates, growth conditions, and identification

Non-duplicate *C glabrata* blood isolates (n = 107) recovered from patients with candidaemia (n = 107) were collected in five clinical

TABLE 1 MICs of antifungal agents used against 107 *Candida glabrata* isolates

	Minimum inhibitory concentration (μg/ml)															
Antifungal agent	**0.016**	**0.032**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**MIC50**	**MIC90**	**GM**
Fluconazole						1	23	29	47	1		2	4	8	64	2.86
Voriconazole		106			1									0.032	0.125	0.032
Itraconazole		52	39	11	5									0.125	0.25	0.125
Micafungin	103	2		2										0.016	0.016	0.063
Anidulafungin	1	73	29	2	2									0.03	0.06	0.063
Amphotericin B				2	8	91	5	1						0.5	1	1

Note: Two isolates showing intermediate phenotype for micafungin (0.125 μg/mL) and anidulafungin (0.25 μg/mL) became resistant against both antifungals after 48 incubation (G53; 0.5 μg/mL and G103; 2 μg/mL).

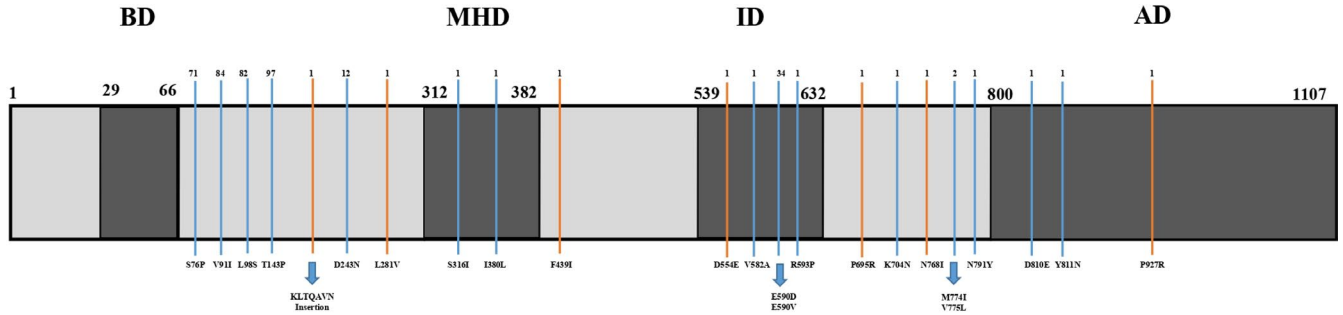

FIGURE 1 Mutations in the Pdr1p protein of *Candida glabrata* isolates. Amino acid changes observed in the isolates with high FLZ MIC values (≥32 μg/mL) are highlighted in orange colour. BD, DNA-binding domain; MHD, middle homology domain; ID, inhibitory domain; and AD, activator domain

TABLE 2 FLZ MICs for isolates carrying Pdr1p mutations, MICs ≥ 64 μg/mL was considered to indicate FLZ resistance

		Fluconazole minimum inhibitory concentration (μg/mL)								
Amino acid change	Resistance %	0.5	1	2	4	8	16	32	64	Total
Wild type	0			1	1					2
S76P, V91I, L98S, T143P, 226-Ins_KLTQAVN-227[a]	100								1	1
S76P, V91I, L98S, T143P, P695R	100								1	1
S76P, V91I, L98S, T143P, N768I	100								1	1
S76P, V91I, L98S, T143P, F439I, D554E, E590D, P927R	100								1	1
L98S, V91I, D243N, L281V, E590D	0							1		1
S76P, V91I, L98S, T143P	0		11	9	25			1		46
S76P, V91I, L98S, T143P, V582A, E590D	0					1				1
S76P, V91I, L98S, T143P, E590D	0		5	4	4					13
V91I, L98S, T143P, E590D	0			1	2					3
T143P, E590D	0		3	4	2					9
T143P	0	1		5	2					8
V91I, L98S, D243N	0		1	1	6					8
T143P, D243N, E590D	0				1					1
S76P, V91I, L98S, T143P, I380L, K704N	0				1					1
S76P, V91I, L98S, T143P, E590D, N791Y	0				1					1
S76P, V91I, L98S, T143P, D810E, Y811N	0				1					1
S76P, V91I, T143P	0				1					1
S76P, V91I, L98S, T143P, S316I	0			1						1
S76P, V91I, L98S, T143P, M774I, V775L	0			1						1
V91I, T143P, E590D	0			1						1
T143P, E590V	0			1						1
T143P, D243N	0			2						2
T143P, E590D, R593P	0		1							1
S76P, V91I, L98S, T143P, E590V	0		1							1
Total	4/107	1	22	30	47	1		2	4	107

[a]Seven amino acids were inserted between amino acids 226-227.

centres in Turkey including Ege (n = 54), Gulhane (n = 25), Dokuz Eylul (n = 18), Selcuk (n = 6) and Istanbul (n = 4) University Hospitals. Ege University Hospital with 1,800 beds was the largest institution, followed by Istanbul University Hospital, Gulhane Training and Research Hospital and Dokuz Eylul University Hospital (1,100 beds each), and Selcuk University Hospital (900 beds). Hospitals included in the current study typically use echinocandins as the first-line therapy for treatment of candidaemia due to *C glabrata*. Isolates from Ege

TABLE 3 Clinical characteristics of patients infected with *Candida glabrata* isolates showing FLZ MIC values ≥ 32 μg/mL and/or harbouring HS1-Fks1/2 mutations

Patient #	Age/sex (y)	Underlying diseases	Risk factors	Prophylaxis/Empiric	MAF
G6	NA (2006)	NA	NA	NA	NA
G29	56/M (2017)	Chronic viral hepatitis B, diabetes mellitus, atrophic left kidney	Abdominal and liver abscesses, CVC, BSAT	FLZ (first dosage 800 mg/d followed by 400 mg/d for 32 d), AND (unknown dosage for 7 d) → TF and PF	Ambisome (3 mg/kg for 20 d)
G51	60/M (2019)	Pancreatic cancer and chronic gastritis	CVC, BSAT	FLZ (first dosage 800 mg/d followed by 400 mg/d for 13 d) → TF and PF	AND (200 mg/d for 4 d)
G53	63/M (2019)	Diabetes mellitus, chronic obstructive pulmonary disease, hypertension, acute atrial fibrillation	Pleural puncture, PICVC, SVC, BSAT	MCF (100 mg/d for 3 d) → PF and TF but MCF was not changed	MCF (100 mg/d for 82 d)
G55	76/M (2016)	Ovarian cancer, cardiac problems, hypertension, chronic renal failure and bacterial infection	Cholecystectomy, CVC, BSAT	FLZ (200 mg/d for 31 d) → TF and PF	Ambisome (3 mg/kg) + AND (100 mg/d) for 13 d
G98	13/F (2017)	Acute myeloid leukaemia	Bone marrow transplantation, CVC, BSAT	Posaconazole (200 mg/d, for 3 d)	Caspofungin (50 mg/d for 40 d)
G103	22/F (2018)	Acute myeloid leukaemia	JVC, BSAT	Caspofungin (70 mg/d for 6 d) → TF and PF	FLZ (200 mg d for 4 d) → TF and PF
G107	78/M (2019)	Acute renal failure, upper gastrointestinal bleeding, and pneumonia	FC, UC, PICVC, BSAT	NO	FLZ (400 mg d for 3 d) → TF and PF

Note: Main treatment was defined as the first-choice antifungal therapy followed by blood culture; persistent fever was defined as TF despite antifungal therapy (prophylactic or main). Alternative antifungal treatments was provided in case of TF of the main treatment. Pdr1p mutations included only those occurring in the isolates with FLZ MIC ≥ 32 μg/mL. Risk factors do not include previous exposure to antifungals, which is mentioned separately.

Abbreviations: AA, Amino acid change; AAF, Alternative antifungal used due to therapeutic failure; AMB, Amphotericin B; AND, Anidulafungin; BSAT, Broad-spectrum antiobiotic therapy; C/G, Cluster/Genotype; CVC, Central venous catheter; FC, Femoral catheter; FLZ, Fluconazole; JVC, Jugular venous catheter; MAF, Main antifungal; MCF, Micafungin; MIC, Minimum inhibitory concentration; NA, Not available; NSAAC, No specific amino acid change; PF, Persistent fever; PICVC, Peripherally inserted central venous catheter; SVC, Subclavian venous catheter; TF, Therapeutic failure; UC, Urine catheter; WT, Wild type.

and Gulhane hospitals were collected from 2005– to 2019, whereas those from the other centres were collected from 2015– to 2019.

Isolates were cultured on Sabouraud dextrose agar (Merck, Darmstadt, Germany) plates for 48 h at 35°C and further verified by growth in CHROMAgar Candida medium (CAC, Becton Dickinson) to ensure their purity. Identification was performed by internal transcribed spacer (ITS) rDNA sequencing using ITS1 and ITS4 primers.[32] Persistence of fever and isolation of *C glabrata* from blood cultures despite antifungal treatment were considered as TF.

2.2 | AFST

All isolates (n = 107) were tested for drug sensitivity using the broth microdilution protocol suggested by CLSI M27-A3/S4.[33,34] The following drugs were used: fluconazole (FLZ), voriconazole (VRZ), itraconazole (ITZ), amphotericin B (AMB) (all from Sigma-Aldrich), micafungin (MCF; Astellas, Munich, Germany) and anidulafungin (AND; Pfizer); caspofungin (CSP) was not included in AFST experiments because of interlaboratory variations.[35] Plates were incubated at 35°C for 24 h, and drug minimum inhibitory concentrations (MICs) were determined by visual examination, and *Candida parapsilosis* (ATCC 22 019) and *Candida krusei* (ATCC 6258) were used for quality control purposes. FLZ resistance was scored at the MIC ≥ 64 μg/mL, and lower MIC values were considered to define susceptible-dose dependent isolates.[36] MCF- and AND-resistant isolates were defined at the MICs ≥ 0.25 μg/mL and ≥0.5 μg/mL, respectively.[36] Resistance to VRZ, ITZ and AMB was reported based on epidemiological cut-off values, and MICs > 0.5 μg/mL, 2 μg/mL and 2 μg/mL, respectively, were considered to indicate non-wild-type isolates.[36]

AAF	Outcome	C/G	Pdr1p AAC	Fksp AAC	MIC (μg/mL)			
					FLZ	MCF	AND	AMB
NA	Died	C5/G23	P695R	WT	64	0.016	0.032	0.5
NO	Died	C2/G11	NSAAC	S629T (HS1-Fks1)	1	0.016	0.064	0.5
NO	Died	C7/G28	NSAAC	WT	32	0.016	0.064	0.5
NO	Died	C8/G30	NSAAC	S663P (HS1-Fks2)	4	0.125	0.25	0.5
NO	Survived	C8/G32	N768I	WT	64	0.016	0.064	0.5
NO	Died	C8/G30	L281V	WT	32	0.016	0.032	0.5
AMB (200 mg/d first dosage and continued with 3 mg/kg for 7 d) → TF and PF → AMB (3 mg/kg) + voriconazole (dosage unknown) for 7 d	Died	C1/G1	226-Ins_ KLTQAVN-227	S663P (HS1-Fks2)	64	0.125	0.25	0.5
AMB (200 mg/d for 2 d)	Died	C3/G15	F439I, D554E, P927R	WT	64	0.016	0.064	0.5

2.3 | Sequencing of *PDR1* and HS1/HS2 of FKS1 and FKS2

PCR amplification and sequencing of the *PDR1* gene and HS1/2 regions of the *FKS1/2* genes were performed as previously described.[29,37] Sequences were assembled and edited using SeqMan Pro software (DNASTAR) and aligned using MEGA 7.0.[38] The genome of *C glabrata* CBS 138 (http://www.candidagenome.org) was used as a wild-type reference.

2.4 | Multilocus microsatellite genotyping (MMT)

The genotypic diversity of *C glabrata* isolates was evaluated by MMT based on three markers: MT1, RPM2 and ERG3.[39] Isolates that differed in a single locus among the six alleles tested were considered to belong to the same genotype. Bionumerics software v7.6 (Applied Math, Sint-Martens-Latem, Belgium) was used for data analysis and dendrogram construction by the unweighted-pair group method using average linkages.

2.5 | Statistical analysis

The data were statistically evaluated using SPSS v.24 (SPSS Inc). The two-tailed chi-square test and logistic regression were used to analyse the association between patient's outcome (death or survival) and clusters obtained by MMT. As each of the numerous identified genotypes was identified for only few isolates, clusters comprising similar genotypes were used to increase statistical power. To assess

the association between clusters and FLZ MIC values, which were not normally distributed, a non-parametric Kruskal-Wallis test was used. Statistical significance was defined at $\alpha < 0.05$.

2.6 | Availability of sequencing data

All sequences generated for *PDR1* and HSs of *FKS1* and *FKS2* were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: MN985836-MN985942 (*PDR1*), MN985943-MN986049 (HS1-*FKS1*), MN986050-MN986156 (HS2-*FKS1*), MN986157-MN986262 (HS1-*FKS2*), and MN986263-MN986369 (HS2-*FKS2*).

2.7 | Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval (number 20-2T/30) has been received.

3 | RESULTS AND DISCUSSION

In this study, we performed comprehensive evaluation of *C glabrata* blood isolates collected in five centres in Turkey; analysis included genotypic diversity, antifungal susceptibility and molecular features defining resistance to azoles and echinocandins. We found a low level of antifungal resistance among isolates, which is consistent with a previous study performed in Turkey,[31] and showed that sequencing together with AFST could provide more reliable data to guide clinicians in their choice of treatment for patients with *C glabrata* candidaemia. Together with similar studies conducted in China,[40,41] USA,[23] South Korea,[30] India[42] and Iran,[29] our study should contribute to better understanding of clinical and microbiological profiles of *C glabrata* bloodstream isolates.

Candidaemia was equally prevalent in men (n = 53) and women (n = 53) (data missing for one patient). The median age of the patients was 60 years (range, 0-87 years) (data missing for 12 patients), which is consistent with the fact that elderly patients are more prone to developing *C glabrata* candidaemia.[43,44] The vast majority of the patients were hospitalised in intensive care units (n = 28; 26.2%) and surgical wards (n = 25; 23.4%) followed by other wards (n = 54; 50.4%). The antifungal treatment data were unavailable for 46 patients; based on the available data, echinocandins were used in 39.2% of cases (n = 42; for 11 patients in combination with other antifungals), AMB in 14% (n = 15; for eight patients in combination) and azoles in 8.4% (n = 9; for three patients in combination). This statistics is in contrast with that in Iran[29] and India,[42] where the majority of patients with candidaemia are treated with azoles. The clinical outcome data were unavailable for 11 patients; based on the available data, the mortality rate was calculated as 55.1% (59/107), which is close to those observed in Austria and Germany[45] but much higher than those in Iran[29] and the USA.[44] Interestingly, the number of *C glabrata* isolates in Ege University Hospital almost doubled during 2015-2019 (n = 35) compared to 2005-2014 (n = 19). A similar increasing trend was reported in other studies[6,8] and was linked to the disproportionate use of antifungals, which, however, we could not prove because of the scarcity of antifungal treatment data for the 2005-2014 period.

Antifungal resistance was observed only for FLZ (n = 4, MIC ≥ 64 μg/mL); furthermore, FLZ MIC = 32 μg/mL was observed for two isolates (Table 1 and Table S1). All 107 isolates exhibited the susceptibility of the wild type for VRZ, ITZ and AMB and two isolates (G53 and G103) showed intermediate susceptibility to MCF and AND (0.125 μg/mL and 0.25 μg/mL, respectively). The low rate or apparent absence of antifungal resistance observed in our study is consistent with a previous multicentre candidaemia study conducted in Turkey[31] as well as reports from several other Asian,[29,30,41,42,46] South American[47] and European[45,48-50] countries; however, it is in contrast with the data from USA, where echinocandin resistance in *C glabrata* is a major public health problem.[12]

PDR1 sequencing showed that only two isolates (1.9%) were wild type; the rest harboured mutations leading to changes in the protein sequence (Figure 1, Table 2, and Table S1), most of which (87.6%, 348/397) occurred between the inhibitory and middle homology domains of Pdr1p (Figure 1). Among the changes exclusively found in FLZ-resistant isolates, the KLTQAVN insertion between residues 226 and 227 has been previously reported,[41] whereas mutations F439I + D554E + P927R, P695R and N768I were detected for the first time (Figure 1, Tables 2 and 3, and Table S1). One of the two isolates with the FLZ MIC = 32 μg/mL harboured a unique novel mutation (L281V) (Figure 1, Tables 2 and 3, and Table S1). Recent studies indicate that UPC2, a zinc cluster transcription factor, may contribute to FLZ resistance in *C glabrata*,[51,52] which may explain the high FLZ MIC for isolate G51 (32 μg/mL) without any exclusive amino acid changes in Pdr1p expected for this phenotype (Tables 2 and 3, and Table S1). Although AFST did not reveal echinocandin resistance in vitro, previously reported mutations, including S629T[53] (in isolate G29 susceptible to both MCF and AND) and S663P[54] (in isolates G53 and G103 intermediate for susceptibility to MCF and AND), were detected in HS1-Fks1 and HS1-Fks2, respectively (Table 3). As S633P has been associated with high MIC values for echinocandins,[54] we repeated the AFST of MCF and AND for the three isolates harbouring *FKS1* and *FKS2* mutations and obtained consistent results, suggesting that the S663P substitution could be present in isolates not resistant to echinocandins. Interestingly, one FLZ-resistant isolate (G103) simultaneously harboured the KLTQAVN insertion in Pdr1p and the S663P mutation in HS1-Fks2p (Tables 2 and 3, and Table S1). None of the isolates carried mutations in HS2 regions of *FKS1/2*.

We next evaluated factors potentially associated with TF in patients infected with isolates harbouring mutation in HS1 of *FKS1/FKS2* and those with FLZ MIC ≥ 32 μg/mL. Our data indicate that TF occurred in patients with isolates carrying HS1-*FKS*

mutations and those with FLZ MIC ≥ 32 μg/mL (n = 8; Table 3). Although S663P and S629T were not associated with echinocandin resistance in vitro, both of them corresponded to TF of MCF, AND and CSP (Table 3). This finding confirmed the notion that *FKS* sequencing is a more reliable approach to predict treatment outcome than phenotypic assays[25] and that AFST alone may be misleading in the selection of appropriate antifungal therapy. However, some echinocandin-resistant *C glabrata* isolates harbour mutations outside of the HS regions[55]; therefore, the combination of AFST and HS-*FKS* sequencing may more accurately predict echinocandin TF than either techniques alone. Consistent with previous studies,[56,57] our results indicated that development of abscesses and empiric/prophylactic treatment with echinocandins were associated with mutations in HS regions and echinocandin TF. Furthermore, we found that the isolates with FLZ MIC ≥ 32 μg/mL, which is below the clinical breakpoint of 64 μg/mL recommended by CLSI[36] and their respective mutations, could be associated with FLZ TF (Table 3). Considering that diverse mutations were detected throughout the entire Pdr1p sequence (Figure 1) and that one of the isolates with FLZ MIC = 32 μg/mL did not harbour any mutations in Pdr1p, AFST was more efficient in predicting FLZ TF compared to *PDR1* sequencing. Isolate #G103, which simultaneously harboured mutations in *PDR1* and *FKS2*, was responsible for TF with all azoles and echinocandins used. Among the eight isolates associated with TF, seven were detected in Ege (n = 4) and Gulhane (n = 3) hospitals; among these isolates, six were recovered between 2016– and 2019, including three recovered in 2019. Out of the eight patients with TF who were infected with isolates showing FLZ MIC ≥ 32 μg/mL and/or carrying *FKS* mutations, seven (87.5%) died (Table 3 and Appendix S1). Collectively,

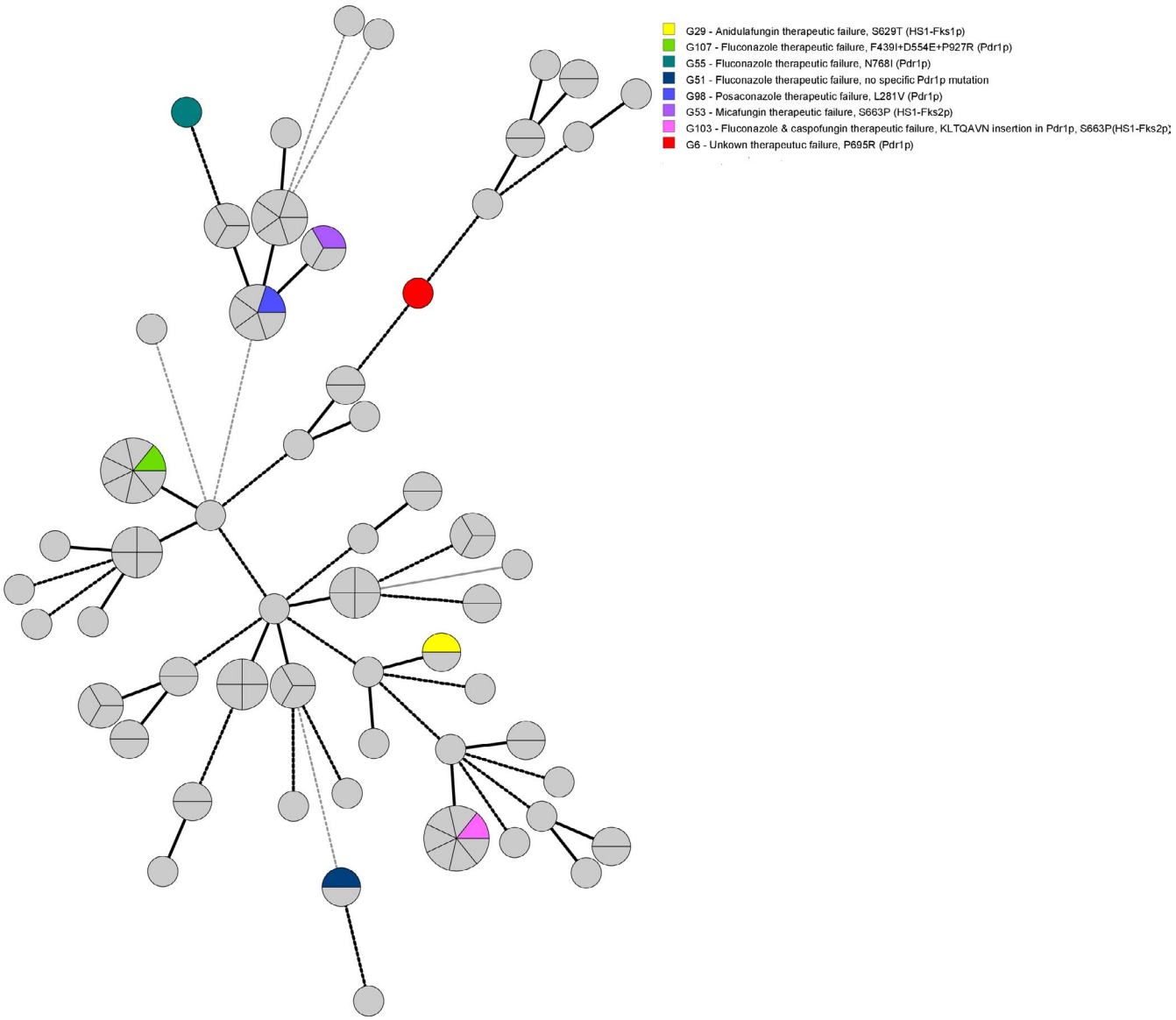

FIGURE 2 Minimum spanning tree illustrating the lack of clonal enrichment for isolates with FLZ MIC ≥ 32 μg/mL and/or mutations in HS1 of *FKS1/2*. All eight isolates, except for one without clinical data (marked red), showed azole and/or echinocandin TF

these data indicate the predictive potential of FLZ MIC ≥ 32 μg/mL for FLZ TF and of *FKS* sequencing combined with AFST data for echinocandin TF. However, it should be noted that TF cannot be exclusively attributed to microbiological characteristics of the isolates; other factors may be involved, including serum concentration of the antifungal which shows patient-to-patient variations, highlighting the importance of therapeutic drug monitoring to attain a favourable clinical outcome.[58] Moreover, considering that all patients with azole/echinocandin-resistant *C glabrata* isolates had a catheter inserted (except one patient infected with isolate #G6 without clinical data), it is plausible that catheter removal may have implications on clinical outcome.

MMT analysis revealed 34 genotypes and 10 clusters (Figure 2, Figure S1, and Table S1). As some isolates were clonal and/or belonged to the same genotype, horizontal transfer could be suggested; however, this hypothesis requires experimental confirmation by performing whole genome sequencing and environmental screening, which are beyond the scope of our study. Nowadays, various next-generation sequencing platforms have been increasingly employed to assess genotypic diversity as well as to identify mutations responsible for antifungal resistance, which may not be used for a particular gene but rather for numerous genes scattered throughout the genome.[59-61] In contrast to a previous study,[28] in our study we did not observe the phenomenon of clonal enrichment for FLZ-resistant *C glabrata* isolates as evidenced by the lack of statistical association between FLZ MIC values and cluster and MMT patterns (Figure 2). Moreover, statistical analysis did not reveal any link between genotype/cluster and mortality (Appendix S1, statistical analysis section), which, however, was detected in previous studies.[29,42]

In conclusion, we performed the first analysis of clinical and microbiological characteristics of *C glabrata* isolates from Turkish patients with candidaemia and updated the AFST data on a multicentre scale. Although the rate of antifungal resistance in vitro was low, TF was common and mostly observed in recent years. Fks mutations and FLZ MIC ≥ 32 μg/mL were predictive of echinocandin and FLZ TF, respectively.

This study was limited by its retrospective nature, which accounted for incomplete clinical data. Moreover, although it was a multicentre study, almost 50% of the isolates were from one institution (Ege University Hospital). Therefore, prospective comprehensive multicentre studies should be conducted in the future to more accurately determine the burden of antifungal resistance and its association with the clinical profile of *C glabrata*-infected patients in Turkey. It should also be noted that there were no repetitive isolates, which may ultimately result in underestimation of antifungal resistance. The same is true for mutations found in *PDR1*, which warrants future studies that should examine the expression of efflux pumps and determine if the identified mutations directly confer azole resistance. Other potentially relevant genes such as *MSH2* could be sequenced in azole/echinocandin-resistant and susceptible isolates to clarify their role in the sensitivity of *C glabrata* to antifungal drugs.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing.

AUTHOR CONTRIBUTIONS

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REFERENCES

1. Lockhart SR, Iqbal N, Cleveland AA, et al. Species identification and antifungal susceptibility testing of *Candida* bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. *J Clin Microbiol*. 2008;2012(50):3435-3442.
2. Remington TL, Isaac A, Vickers DM, Fuller J, Wrenn-Smith S. Epidemiology of candidemia at a tertiary Canadian hospital, 2004–2013. *Can J Infect Dis Med Microbiol*. 2018;3:14-23.
3. Chapman B, Slavin M, Marriott D, et al. Changing epidemiology of candidaemia in Australia. *J Antimicrob Chemother*. 2017;72:1103-1108.
4. Mari AH, Valkonen M, Kolho E, Friberg N, Anttila VJ. Clinical and microbiological factors associated with mortality in candidemia in adult patients 2007–2016. *Infect Dis (Lond)*. 2019;51:824-830.
5. Poikonen E, Lyytikäinen O, Anttila V-J, et al. Secular trend in candidemia and the use of fluconazole in Finland, 2004–2007. *BMC Infect Dis*. 2010;10:312.
6. Astvad KMT, Johansen HK, Røder BL, et al. Update from a 12-year nationwide fungemia surveillance: Increasing intrinsic and acquired resistance causes concern. *J Clin Microbiol*. 2018;56:e01564-e1617.
7. Lindberg E, Hammarström H, Ataollahy N, Kondori N. Species distribution and antifungal drug susceptibilities of yeasts isolated from the blood samples of patients with candidemia. *Sci Rep*. 2019;9:3838.
8. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. Twenty years of the SENTRY antifungal surveillance program: results for *Candida* species from 1997–2016. *Open Forum Infect Dis*. 2019;6:S79-S94.
9. Hesstvedt L, Gaustad P, Andersen CT, et al. Twenty-two years of candidaemia surveillance: Results from a Norwegian national study. *Clin Microbiol Infect*. 2015;21:938-945.
10. Farmakiotis D, Kontoyiannis DP. Epidemiology of antifungal resistance in human pathogenic yeasts: current viewpoint and practical recommendations for management. *Int J Antimicrob Agents*. 2017;50:318-324.
11. Aldardeer NF, Albar H, Al-Attas M, et al. Antifungal resistance in patients with candidaemia: a retrospective cohort study. *BMC Infect Dis*. 2020;20:55.
12. Healey KR, Perlin DS. Fungal resistance to echinocandins and the MDR phenomenon in *Candida glabrata*. *J Fungi*. 2018;4:105.
13. Lewis JS, 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.
14. Cleary JD, Garcia-Effron G, Chapman SW, Perlin DS. Reduced *Candida glabrata* susceptibility secondary to an FKS1 mutation developed during candidemia treatment. *Antimicrob Agents Chemother*. 2008;52:2263-2265.
15. Wright WF, Bejou N, Shields RK, Marr K, McCarty TP, Pappas PG. Amphotericin B induction with voriconazole consolidation as salvage therapy for FKS-associated echinocandin resistance in *Candida glabrata* septic arthritis and osteomyelitis. *Antimicrob Agents Chemother*. 2019;63:e00512–191-6.
16. Thompson GR, Wiederhold NP, Vallor AC, Villareal NC, Lewis JS, Patterson TF. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. *Antimicrob Agents Chemother*. 2008;52:3783-3785.
17. Grosset M, Desnos-Ollivier M, Godet C, Kauffmann-Lacroix C, Cazenave-Roblot F. Recurrent episodes of candidemia due to *Candida glabrata*, *Candida tropicalis* and *Candida albicans* with acquired echinocandin resistance. *Med Mycol Case Rep*. 2016;14:20-23.
18. Xiao M, Fan X, Hou X, et al. Clinical characteristics of the first cases of invasive candidiasis in China due to pan-echinocandin-resistant *Candida tropicalis* and *Candida glabrata* isolates with delineation of their resistance mechanisms. *Infect Drug Resist*. 2018;11:155-161.
19. Garcia-Effron G, Chua DJ, Tomada JR, et al. Novel FKS mutations associated with echinocandin resistance in *Candida* species. *Antimicrob Agents Chemother*. 2010;54:2225-2227.
20. Pfeiffer CD, Garcia-Effron G, Zaas AK, Perfect JR, Perlin DS, Alexander BD. Breakthrough invasive candidiasis in patients on micafungin. *J Clin Microbiol*. 2010;48:2373-2380.
21. Chapeland-Leclerc F, Hennequin C, Papon N, et al. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. *Antimicrob Agents Chemother*. 2010;54:1360-1362.
22. Costa-de-Oliveira S, Miranda IM, Silva RM, et al. FKS2 mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy. *Antimicrob Agents Chemother*. 2011;55:1312-1314.
23. Alexander BD, Johnson MD, Pfeiffer CD, et al. Increasing echinocandin resistance in *Candida glabrata*: Clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clin Infect Dis*. 2014;56:1724-1732.
24. Ferrari S, Ischer F, Calabrese D, et al. Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog*. 2009;5:e1000268.
25. Shields RK, Nguyen MH, Press EG, et al. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to *Candida glabrata*. *Antimicrob Agents Chemother*. 2012;56:4862-4869.
26. Healey KR, Nagasaki Y, Zimmerman M, et al. The gastrointestinal tract is a major source of echinocandin drug resistance in a murine model of *Candida glabrata* colonization and systemic dissemination. *Antimicrob Agents Chemother*. 2017;61:e01412-e1417.
27. Abbes S, Sellami H, Sellami A, et al. Microsatellite analysis and susceptibility to FCZ of *Candida glabrata* invasive isolates in Sfax Hospital, Tunisia. *Med Mycol*. 2010;49:10-15.
28. Goemaere B, Lagrou K, Spriet I, Hendrickx M, Becker P. Clonal spread of *Candida glabrata* bloodstream isolates and fluconazole resistance affected by prolonged exposure: a 12-year single-center study in Belgium. *Antimicrob Agents Chemother*. 2018;62:e00591-e618.
29. 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:e02503-e2518.
30. Byun SA, Won EJ, Kim M-N, et al. Multilocus Sequence Typing (MLST) genotypes of *Candida glabrata* bloodstream isolates in Korea: association with antifungal resistance, mutations in mismatch repair gene (Msh2), and clinical outcomes. *Front Microbiol*. 2018;9:1523.
31. Arikan-Akdagli S, Gülmez D, Doğan Ö, et al. First multicentre report of *in vitro* resistance rates in candidaemia isolates in Turkey. *J Glob Antimicrob Resist*. 2019;18:230-234.
32. Stielow JB, Lévesque CA, Seifert KA, et al. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia*. 2015;35:242-263.
33. Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Third Edition: M27–A3*. Wayne, PA: CLSI; 2008.
34. Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; fourth informational supplement. CLSI document M27–S4*. Wayne, PA: CLSI; 2012.
35. 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.

36. 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*. 2010;2012(50):2846-2856.
37. Vatanshenassan M, Arastehfar A, Boekhout T, et al. Anidulafungin susceptibility testing of *Candida glabrata* isolates from blood cultures by the MALDI Biotyper antibiotic (antifungal) susceptibility test rapid assay. *Antimicrob Agents Chemother*. 2019;63:e00554-e619.
38. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016;2016(33):1870-1874.
39. Foulet F, Nicolas N, Eloy O, et al. Microsatellite marker analysis as a typing system for *Candida glabrata*. *J Clin Microbiol*. 2005;43:4574-4579.
40. Hou X, Xiao M, Chen S-A, et al. Molecular epidemiology and antifungal susceptibility of *Candida glabrata* in China (August 2009 to July 2014): a multi-center study. *Front Microbiol*. 2017;8:880.
41. Hou X, Xiao M, Wang H, et al. Profiling of PDR1 and MSH2 in *Candida glabrata* bloodstream isolates from a multicenter study in China. *Antimicrob Agents Chemother*. 2018;62:e00153-e218.
42. Singh A, Healey KR, Yadav P, et al. Absence of azole or echinocandin resistance in *Candida glabrata* isolates in India despite background prevalence of strains with defects in the DNA mismatch repair pathway. *Antimicrob Agents Chemother*. 2018;62:e00195-e218.
43. McCarty TP, Pappas PG. Invasive candidiasis. *Infect Dis Clin North Am*. 2016;30:103-124.
44. Moran C, Grussemeyer CA, Spalding JR, Benjamin DKJ, Reed SD. Comparison of costs, length of stay, and mortality associated with *Candida glabrata* and *Candida albicans* bloodstream infections. *Am J Infect Control*. 2010;38:78-80.
45. Klotz U, Schmidt D, Willinger B, et al. Echinocandin resistance and population structure of invasive *Candida glabrata* isolates from two university hospitals in Germany and Austria. *Mycoses*. 2016;59:312-318.
46. Kiraz N, Dag I, Oz Y, Yamac M, Kiremitci A, Kasifoglu N. Correlation between broth microdilution and disk diffusion methods for antifungal susceptibility testing of caspofungin, voriconazole, amphotericin B, itraconazole and fluconazole against *Candida glabrata*. *J Microbiol Methods*. 2010;82:136-140.
47. Rodriguez L, Bustamante B, Huaroto L, et al. A multi-centric study of *Candida* bloodstream infection in Lima-Callao, Peru: species distribution, antifungal resistance and clinical outcomes. *PLoS One*. 2017;12:e0175172.
48. Bourgeois N, Laurens C, Bertout S, et al. Assessment of caspofungin susceptibility of *Candida glabrata* by the Etest®, CLSI, and EUCAST methods, and detection of *FKS1* and *FKS2* mutations. *Eur J Clin Microbiol Infect Dis*. 2014;33:1247-1252.
49. Tortorano AM, Prigitano A, Lazzarini C, et al. A 1-year prospective survey of candidemia in Italy and changing epidemiology over one decade. *Infection*. 2013;41:655-662.
50. 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.
51. Vu BG, Thomas GH, Moye-Rowley WS. Evidence that ergosterol biosynthesis modulates activity of the Pdr1 transcription factor in *Candida glabrata*. *mBio*. 2019;10:e00934-e1019.
52. Whaley SG, Caudle KE, Vermitsky JP, et al. UPC2A is required for high-level azole antifungal resistance in *Candida glabrata*. *Antimicrob Agents Chemother*. 2014;58:4543-4554.
53. Nunnally NS, Etienne KA, Angulo D, Lockhart SR, Berkow EL *In vitro* Activity of ibrexafungerp, a novel glucan synthase inhibitor against *Candida glabrata* isolates with FKS mutations. *Antimicrob Agents Chemother*. 2019;63: pii:e01692-19.
54. Pham CD, Iqbal N, Bolden CB, et al. Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob Agents Chemother*. 2014;58:4690-4696.
55. Hou X, Healey KR, Shor E, et al. Novel FKS1 and FKS2 modifications in a high-level echinocandin resistant clinical isolate of *Candida glabrata*. *Emerg Microb Infect*. 2019;8:1619-1625.
56. Cheng S, Clancy CJ, Hartman DJ, Hao B, Nguyen MH *Candida glabrata* intra-abdominal candidiasis is characterized by persistence within the peritoneal cavity and abscesses. *Infect Immun*. 2014;82:3015-3022.
57. Zhao Y, Prideaux B, Nagasaki Y, et al. Unraveling drug penetration of echinocandin antifungals at the site of infection in an intra-abdominal abscess model. *Antimicrob Agents Chemother*. 2017;61:e01009-e1017.
58. Vena A, Muñoz P, Mateos M, et al. Therapeutic drug monitoring of antifungal drugs: another tool to improve patient outcome? *Infect Dis Ther*. 2020;9:137-149.
59. Spettel K, Barousch W, Makristathis A, et al. Analysis of antifungal resistance genes in *Candida albicans* and *Candida glabrata* using next generation sequencing. *PLoS One*. 2019;14:e0210397.
60. Arastehfar A, Wickes BL, Ilkit M, et al. Identification of mycoses in developing countries. *J Fungi (Basel)*. 2019;5:pii:E90.
61. Consortium OPATHY, Gabaldón T. Recent trends in molecular diagnostics of yeast infections: from PCR to NGS. *FEMS Microbiol Rev*. 2019;43:517-547.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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