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 echinocandins, 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.
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 \(\text{Zn}_2\text{Cys}_6\) transcription factor \(\text{PDR1}\), 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 \(\text{FKS1}\) and \(\text{FKS2}\) genes encoding glucan synthases. Regarding the clinical prognosis, some studies indicate that sequencing of HSs in \(\text{FKS1/2}\) more accurately predicts therapeutic failure (TF) of echinocandins than phenotypic antifungal susceptibility testing (AFST).\(^23,25\)

\(C\) 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.

\(C\) 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...
centres in Turkey including Ege (n = 54), Gulhane (n = 25), Dokuz Eylül (n = 18), Selcuk (n = 4) 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 Eylül 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

**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

| Amino acid change | Resistance % | Fluconazole minimum inhibitory concentration (µg/mL) |
|-------------------|--------------|--------------------------------------------------|
| Wild type         | 0            | 0.5 1 1 2 4 8 16 32 64 Total |
| S76P, V91I, L98S, T143P, 226-Ins_KLTQAVN-227<sup>a</sup> | 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 |
| S76P, V91I, L98S, T143P, M774I, V775L | 0            | 1 1 |
| V91I, T143P, E590D | 0            | 1 1 |
| T143P, E590V | 0            | 1 1 |
| T143P, D243N | 0            | 1 2 2 |
| T143P, E590D, R593P | 0            | 1 |
| S76P, V91I, L98S, T143P, E590V | 0            | 1 |
| Total | 4/107        | 1 22 30 47 1 2 4 107 |

<sup>a</sup>Seven amino acids were inserted between amino acids 226-227.
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 antibiotic 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.

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. 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. 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. 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. MCF- and AND-resistant isolates were defined at the MICs ≥ 0.25 µg/mL and ≥0.5 µg/mL, respectively. Resistance to VRZ, ITZ and AMB was reported based on epidemiological cut-off values, and MICs > 0.5 µg/mL and ≥2 µg/mL, respectively, were considered to indicate non-wild-type isolates.
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. Sequences were assembled and edited using SeqMan Pro software (DNASTAR) and aligned using MEGA 7.0. 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. Isolates that differed in a single locus among the six alleles tested were considered to belong to the same genotype. Biolnumerics 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 as $\alpha < 0.05$.

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, 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, USA, South Korea, India and Iran, 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. 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 and India, 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 but much higher than those in Iran and the USA. 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 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 $\geq 64 \mu$g/mL); furthermore, FLZ MIC = 32 $\mu$g/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 $\mu$g/mL and 0.25 $\mu$g/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 as well as reports from several other Asian, South American and European countries; however, it is in contrast with the data from USA, where echinocandin resistance in C. glabrata is a major public health problem.

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%, 34B/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, whereas mutations F439I and D554E 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, whereas mutations F439I and D554E occurred between the inhibitory and middle homology domains of Pdr1p (Figure 1). 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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 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; therefore, the combination of AFST and HS-FKS sequencing may more accurately predict echinocandin TF than either techniques alone. Consistent with previous studies, 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 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 2016–2019, 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,
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.

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