Fungaemia caused by *Candida glabrata* with reduced susceptibility to fluconazole due to altered gene expression: risk factors, antifungal treatment and outcome

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**Background:** The role of *Candida glabrata* in fungaemia is attributed in part to its reduced susceptibility to azoles, usually due to altered expression of genes encoding drug efflux pumps. The aims of this study were to identify risk factors for fungaemia due to *C. glabrata* isolates with decreased susceptibility to fluconazole and to analyse the response to antifungal treatment and the clinical outcome of *C. glabrata* infections in hospitalized patients.

**Methods:** A retrospective case–case–control study was conducted at a university hospital from 2000 to 2006. Three patient groups were studied: 14 patients infected by a fluconazole-less-susceptible isolate [susceptible-dose-dependent (SDD) or resistant]; 21 patients infected by a fluconazole-susceptible (FS) isolate; and 70 uninfected controls. We measured expression of the drug efflux pump-encoding *CgCDR1* and *CgCDR2* genes in isolates of the two infected groups using quantitative real-time PCR.

**Results:** Multivariable analysis found that patients with prior fluconazole use [odds ratio (OR) 12.24, 95% confidence intervals (CIs) 1.77–84.39, *P* = 0.01], diabetes (OR 10.47, 95% CI 1.96–55.96, *P* = 0.006) and a central venous catheter (CVC) (OR 8.48, 95% CI 1.82–39.36, *P* = 0.006) were more likely to develop fungaemia due to a less-susceptible isolate. Previous surgery (OR 7.73, 95% CI 2.18–27.41, *P* = 0.002) was an independent risk factor for fungaemia due to a susceptible isolate, in addition to the presence of a CVC (OR 5.48, 95% CI 1.69–17.72, *P* = 0.004). The crude 30 day mortality rate was high for both case groups. Seven patients received inadequate antifungal treatment, including five infected by a fluconazole-resistant isolate but empirically treated with fluconazole; six of these seven patients died. Expression of the *CgCDR* genes was up-regulated in all fluconazole-resistant and, to a lesser extent, SDD isolates, but not in the FS isolates.

**Conclusions:** Our data suggest that when candidaemia is suspected or detected, a more broad-spectrum antifungal drug (i.e. echinocandins or amphotericin B) should be considered as initial treatment for patients with prior azole exposure.

Keywords: candidaemia, antifungal resistance, mortality, inadequate therapy, drug efflux pumps

**Introduction**

*Candida glabrata* has emerged as a leading cause of fungal infection and is the second most common *Candida* organism responsible for fungaemia in the United States.¹ It is known to exhibit intrinsically low susceptibility to azoles and to develop resistance after exposure to these drugs,²⁻⁹ through the *CgPDR1*-dependent¹⁰ up-regulation of the genes encoding the ATP-binding cassette (ABC) transporters *CgCDR1* and *CgCDR2*,³⁻⁵,¹¹⁻¹⁶ and also *CgSNQ2*.¹⁷

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The aims of this study were to identify specific variables associated with fungaemia due to C. glabrata isolate with reduced susceptibility to fluconazole (RFS) and to assess how the effectiveness of antifungal therapy for C. glabrata fungaemias caused by less-susceptible isolates compares with that for susceptible isolates. Molecular studies were also performed to investigate the mechanisms underlying the azole less-susceptibility or resistance of the fungal isolates.

Patients and methods

Study site, patients and design

This retrospective study was performed at the Catholic University Hospital, a 1700 bed university hospital located in Rome, Italy. It offers a full range of clinical services and admits ~55 000 patients per year.

The microbiology laboratory historical database was used to identify patients, hospitalized from January 2000 through December 2006, who had at least one positive blood culture for C. glabrata. Patients could be entered into the study only once, at the time of the initial positive blood culture.

A case–case–control study design was used. This approach involves two parallel case–control studies with a common control group to give a more precise estimate of risk.18–20 Two case groups were studied: the first case group included patients with C. glabrata fungaemia due an isolate with RFS (MIC ≥16 mg/L) and the second case group consisted of patients with C. glabrata fungaemia due a fluconazole-susceptible (FS) isolate (MIC ≤8 mg/L). The RFS isolates included either susceptible-dose-dependent (SDD; MICs 16–32 mg/L) or resistant isolates (MICs ≥64 mg/L) (see below). A third group, the control group, consisted of randomly selected patients who had been hospitalized in our centre during the same periods of time and in the same wards as the case patients, but who did not have C. glabrata fungaemia. Patients were included as controls only if complete data series could be obtained from their medical records. The distribution of the control admissions throughout the study period was similar to that of the case admissions.29

Variables and definitions

Patient data were collected from computerized laboratory and medical records. The following variables were assessed as possible risk factors: age; sex; location of patient at the time of diagnosis in an intensive care unit (ICU) or a non-ICU; underlying co-morbidities; invasive procedures [including insertion of a central venous catheter (CVC) or nasogastric tube, endoscopy, bronchoscopy and urinary catheterization] and total parenteral nutrition (TPN) administration within 72 h prior to the onset of candidaemia; and prior use of immunosuppressive agents, surgery, bacteraemia or exposure to antibiotics or antifungal agents within 30 days of the onset of candidaemia (or, for controls, at any point during hospitalization).20 The Charlson score was used to provide a composite score of co-morbid conditions.21 Risk days were determined as the number of hospital days from admission to the date of the first positive blood culture for case patients or total days in the hospital for control patients.18 Antibiotic use was examined, both in terms of whether any antibiotics were used and by aggregate classes (glycopeptides, macrolides, fluoroquinolones, expanded-spectrum cephalosporins, aminoglycosides, carbapenems and β-lactam-β-lactamase inhibitors).18 The type, dose and duration of antifungal therapy, as well as the 30 day mortality, were also recorded.

If patients had positive blood cultures after 48 h of hospital admission, C. glabrata fungaemias were defined as hospital-acquired; also, C. glabrata fungaemias were defined as catheter-related if: (i) the semi-quantitative catheter tip culture yielded more than 15 cfu of the same Candida species; or (ii) simultaneous quantitative cultures of blood samples showed a ratio ≥5:1 in cfu of blood samples obtained through the catheter and a peripheral vein, as reported previously.22 Inadequate C. glabrata fungaemia therapy was defined as a lack of antifungal treatment; administration of antifungal treatment 48 h after drawing the first positive blood sample for culture, administration of antifungal treatment with isolation of an organism found in vitro to be resistant to the antifungal agent or antifungal administration for <5 days.22 The 30 day mortality was used as the outcome measure.

Microbiological studies

For each patient studied, blood samples were inoculated into aerobic media and processed using the BACTEC 9240 automated system (Becton–Dickinson Microbiology Systems, Sparks, MD, USA). The first isolate from each patient was studied. Identification was performed using micromorphology analysis23 and biochemical tests (Vitek 2 Yeast Identification, bioMérieux, Marcy l’Étoile, France). The in vitro antifungal activity of amphotericin B, fluconazole, itraconazole, voriconazole and posaconazole was determined, at least three times, using the broth microdilution procedure recommended by the CLSI (formerly the NCCLS).24 Testing for posaconazole susceptibility was performed using the Sensititre YeastOne system according to the manufacturer’s instructions (Trek Diagnostic Systems, Inc., Cleveland, OH, USA)6 on three separate occasions. To classify the isolates, available interpretive CLSI MIC breakpoints for fluconazole, voriconazole and itraconazole were used: isolates were defined as susceptible (≤8, ≤1 and ≤0.12 mg/L, respectively), SDD (16–32, 2 and 0.25–0.5 mg/L, respectively) or resistant (≥64, ≥4 and ≥1 mg/L, respectively).32 Interpretive criteria are not available for amphotericin B, caspofungin and posaconazole; however, in agreement with previous studies,25–27 isolates for which the amphotericin B, caspofungin or posaconazole MIC was ≤1 mg/L were defined as susceptible in this study. Isolates were maintained as frozen stocks and subcultured on Sabouraud dextrose agar plates at 37°C as required.

The clonality of the isolates was assessed using the repetitive probes CgB and Cg1229 and by multilocus sequence typing,29 as described elsewhere.15,30

The biofilm-forming capability of the isolates was determined in microtitre plates as described previously27 by measuring the amount of light blocked passing through the wells (%Tblo).31 and by using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyle)-5-[(phenylamino) carbonyl]-2H-tetrazodium hydroxide) reduction assay.32

Quantitative expression of the azole resistance-related genes CgCDR1, CgCDR2 and CgSNQ2 was performed by real-time RT–PCR, as described previously.15 Genes with a ΔCt value, calculated as Ct [test gene]–Ct [URA3 reference gene], that fell over the 3-SD range measured in the azole-susceptible isolates were considered overexpressed.33

Statistical analysis

Variables with normal or near normal distributions were described with means and SD, and compared using the Student t-test. Medians with interquartile ranges (IQRs) were used to describe non-normally distributed variables and compared using the Mann–Whitney U-test. Categorical variables were compared using the two-tailed χ² test or Fisher’s exact test, as appropriate; crude odds ratios (ORs)
and 95% confidence intervals (CIs) were calculated. Multivariate analysis was performed by means of multiple logistic regression, including all variables with a \( P < 0.2 \) in the univariate analysis. Two-tailed tests of significance \( (P < 0.05) \) were used to determine statistical significance. All statistical analyses were performed with Intercooled Stata software (version 8) for Windows (Stata Corp., College Station, TX, USA).

Results

Patient characteristics

Of 35 case patients studied, 14 (40%) were infected by an RFS isolate (i.e. fluconazole-SDD or -resistant) and the remaining 21 (60%) by an FS isolate (see below for isolate categorization). During the study period, the proportion of \( C. \) glabrata fungaemias due to RFS isolates increased from 25% in 2001 to 75% in 2006 (Figure 1). At the time of diagnosis, 17 patients (48.6%) were in a surgical ward, 14 patients (40%) in a medical ward and 4 patients (11.4%) in the ICU. Thirty-two patients (91.4%) had hospital-acquired fungaemia. While the primary source of infection was unknown for 22 patients (62.9%), it was identified as a CVC or surgical wound for 9 (25.7%) and 4 (11.4%) patients, respectively. The median length (IQR) of hospitalization in patients infected by an RFS isolate was 32.5 (25–55) days, and 32 (24–73) days in patients infected by an FS isolate. Compared with patients with fungaemia due to \( F. \) candida, patients in the RFS \( C. \) glabrata group were more likely to be male \( (P = 0.02) \), older \( (P = 0.06) \) and admitted to a medical ward \( (P = 0.001) \).

Risk factors for fungaemia due to RFS \( C. \) glabrata or FS \( C. \) glabrata

The results of univariate and multivariable analyses for RFS or FS \( C. \) glabrata fungaemia are shown in Tables 1 and 2.

Characterization of \( C. \) glabrata isolates

As noted previously, the eight fluconazole-SDD and six fluconazole-resistant isolates were arbitrarily grouped into a unique fluconazole-reduced susceptibility category and designated as RFS isolates. All of the six fluconazole-resistant isolates were also resistant to itraconazole and voriconazole. Finally, by using an MIC of \( \geq 1 \) mg/L to define resistance to amphotericin B, posaconazole and caspofungin (see the Patients and methods section), all 35 isolates were found to be susceptible to amphotericin B and caspofungin, although not to posaconazole. While 29 isolates had posaconazole MICs of \( < 1 \) mg/L, the 6 azole-resistant isolates exhibited posaconazole MICs ranging from 4 to \( > 8 \) mg/L and were thus considered resistant to this compound [Table S1, available as Supplementary data at \( JAC \) Online (http://jac.oxfordjournals.org/)].

All isolates were examined for expression of \( CgCDR1, CgCDR2 \) and \( CgSNQ2 \) relative to that of the \( C. \) glabrata control isolate DSY562 (fluconazole MIC, 8 mg/L).\(^\text{12} \) The six fluconazole-resistant isolates had up-regulated \( CgCDR1, CgCDR2 \) and/or \( CgSNQ2 \) expression. The eight fluconazole-SDD isolates also up-regulated \( CgCDR1, CgCDR2 \) or \( CgSNQ2 \), but the relative expression levels were much lower than those of the six fluconazole-resistant isolates. In contrast, no increase in the expression of \( CgCDR1, CgCDR2 \) and \( CgSNQ2 \) was observed for the 21 FS isolates studied (Table S1).

Nine of the 35 isolates (25.7%) were able to form biofilms, as assessed by the \( %T \) method (Table S1). For all the isolates tested, \( %T \) determinations correlated well with XTT absorbance measurements (data not shown). When biofilm production of the isolates was analysed according to fluconazole susceptibility, the percentages of isolates that were able to form biofilms among the RFS and FS isolates were 35.7% (5/14 patients) and 19% (4/21 patients), respectively; there was no statistically significant difference between the two groups \( (P = 0.26) \).

Finally, when the 35 \( C. \) glabrata isolates were genotyped, 28 sequence types were found, of which 10 had not been published previously.\(^\text{29} \) Isolates with identical sequence types were studied by the \( Cg6 \) and \( Cg12 \) hybridization method and yielded different patterns, indicating that these isolates are epidemiologically unrelated strains (data not shown). Except for 3 patients who were diagnosed with community-acquired fungaemia, we excluded any nosocomial transmission of \( C. \) glabrata isolate among the remaining 32 patients.

Antifungal treatment and outcome

Twenty-eight of the fungaemic patients (80%) received adequate systemic antifungal therapy for a median length (IQR) of 12 (6–20) days. Therapy consisted of fluconazole in 13 cases (46.4%), amphotericin B (conventional or lipidic formulations) in 8 cases (28.6%), caspofungin in 5 cases (17.9%) and voriconazole in 2 cases (7.1%). Therapy was considered inadequate for seven patients (20%). Of these seven patients, five who were treated with fluconazole were infected by a fluconazole-resistant isolate, one patient received antifungal treatment with caspofungin 4 days after a positive blood sample was drawn for culture and the remaining patient was never treated; except for two patients who were already dead at the time that the susceptibility testing results were known, for the remaining three of the five fluconazole-treated patients therapy was changed to amphotericin B (two patients) or caspofungin (one patient). Among the adequately treated patients, 8 patients who were infected by a fluconazole-SDD isolate received antifungal treatment with amphotericin B (3 patients) or caspofungin (5 patients), whereas 20 patients who were infected by a fluconazole-susceptible isolate were treated with fluconazole (13 patients), voriconazole (2 patients) or amphotericin B (5 patients). Among the last
patients, amphotericin B was switched to fluconazole in three patients, based on the susceptibility testing results.

Fifteen of the 35 fungaemic patients died (30 day crude mortality rate, 42.8%). The mortality rate was 38.1% for the 21 patients with fungaemia due to an FS isolate versus 50% among the 14 patients with fungaemia due to an RFS isolate \((P = 0.48)\). Among the seven patients who did not receive adequate therapy, the mortality rate was 85.7%. This rate differed significantly \((P = 0.01)\) from the rate (32.1%) among the 28 patients who received adequate therapy.

### Discussion

We identified two unique risk factors for fungaemia due to RFS *C. glabrata*: prior fluconazole exposure and diabetes mellitus. The selective pressure of fluconazole has often been cited as the major driver of low-susceptibility *Candida* species (*C. glabrata* and *C. krusei*),\(^34\) raising concerns about the use of azole-based drugs for prophylaxis, pre-emptive therapy and empirical therapy in high-risk hospital settings.\(^35\) In *vitro* work by Borst et al.\(^16\) demonstrated that acquisition of azole resistance in
C. glabrata can occur in the absence of prior exposure to these drugs and is associated with the increased expression of ABC transporter genes. However, another study of azole resistance mechanisms in C. glabrata isolates from transplant recipients receiving fluconazole prophylaxis showed that the MICs doubled, on average, every 31 days. Expression of CgCDR1, CgCDR2 and CgSNQ2 was up-regulated to different extents in the fluconazole-resistant (6 patients) or fluconazole-SDD (8 patients) isolates, but not in the FS isolates (21 patients). Our findings seem to be in discord with those of Lin et al., who reported the lack of an association between fluconazole use and the development of C. glabrata (or C. krusei) candidaemia. Nevertheless, in that study, C. glabrata isolates were assumed to be inherently more resistant than C. albicans to fluconazole, since no antifungal susceptibility testing was routinely performed for yeast isolates during the time period; this raises the possibility of a misclassification bias. For reasons we cannot explain, diabetes mellitus was an independent predictor of C. glabrata fungaemia due to RFS isolates but not of fungaemia due to FS isolates. Diabetes has previously been reported to be a risk factor for candiduria due to C. glabrata in renal transplant recipients, but association of hyperglycaemia with overall fungaemia has never been reported. In addition, people with diabetes are at increased risk for oesophageal candidiasis and vulvovaginal candidiasis.

Prior surgery was a risk factor for fungaemia due to FS C. glabrata but not for fungaemia due to RFS C. glabrata. Interventions that affect the intestine are highly likely to create a conduit for yeast to enter the bloodstream. The association between surgical procedures involving the bowel and fungaemia caused by FS strains can be explained either by greater fitness of the FS strains or by local factors in our hospital.

Treatment failure can be attributed to a combination of factors related to the host, the antifungal agent or the pathogen, indicating that in vitro susceptibility testing alone is not sufficient for predicting clinical success. On the other hand, fluconazole susceptibility testing shows promise: isolates in which MICs are ≥64 mg/L are significantly less likely to respond clinically to fluconazole therapy than isolates with low MICs (i.e. susceptible). Excluding the 15 case patients (42.9%) who died, 20 of 35 fungaemic patients had a favourable outcome, probably as a result of the receipt of appropriate antifungal therapy. In contrast, 6 of the 7 inadequately treated patients died (mortality rate of 85.7% versus 32.1% for the 28 adequately treated patients), including 5 who were empirically started with fluconazole before their isolates were known to have elevated MICs to fluconazole (and other azoles). The last finding supports the conclusion that reduced susceptibility of C. glabrata to fluconazole contributes to a poor outcome after invasive infection, especially in conjunction with inadequate empirical antifungal treatment. Although we cannot exclude the possibility that modifying treatment based on the susceptibility testing results contributes to improved clinical outcome, it should be noted that even a minimal delay in implementing the appropriate antifungal therapy in patients hospitalized with candidaemia has a significant impact on mortality. In this regard, new strategies such as early identification of resistance in clinical isolates (i.e. ‘real-time’ detection of CgPDR1 mutations) or identification of unique risk factors are needed to reduce infection-related deaths.

In conclusion, azole antifungal non-susceptibility in C. glabrata remains an especially acute problem in the management of diseases caused by this organism. Further studies evaluating the relationship between non-susceptibility and clinical outcome will be critical for determining the most effective treatment approach for patients with invasive candidiasis. For now, we advise caution when considering azole therapy for C. glabrata fungaemia in patients with prior azole drug exposure.

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Transparency declarations

No financial conflicts of interest to declare.

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Supplementary data

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


