

## Clinical Significance of Azole Antifungal Drug Cross-Resistance in *Candida glabrata*

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***Candida glabrata*, which can become resistant to fluconazole, is a common cause of bloodstream infection. This study was performed to determine the significance of cross-resistance to new azole drugs among *C. glabrata* isolates recovered as a cause of infection in azole-treated hematopoietic stem cell transplant (HSCT) recipients. Seven cases of invasive candidiasis caused by *C. glabrata* occurred in HSCT recipients who were receiving azole therapy between January 2000 and December 2004 in our institution. Case characteristics were ascertained. Sequential colonizing and invasive isolates were examined to determine susceptibilities to fluconazole, itraconazole, and voriconazole, and molecular relatedness by restriction fragment length polymorphism (RFLP) analysis. Twenty-three *C. glabrata* isolates were recovered from 4 patients who developed candidemia while receiving fluconazole and three patients who developed candidemia while receiving voriconazole. The mode MICs of fluconazole, itraconazole, and voriconazole for these isolates were  $\geq 64$   $\mu\text{g/ml}$  (range, 4 to  $\geq 64$   $\mu\text{g/ml}$ ), 2  $\mu\text{g/ml}$  (range, 0.25 to  $\geq 16$   $\mu\text{g/ml}$ ), and 1  $\mu\text{g/ml}$  (range, 0.03 to  $\geq 16$   $\mu\text{g/ml}$ ), respectively. Kendall  $\tau$  b correlation coefficients demonstrated significant associations between the MICs of voriconazole with fluconazole ( $P = 0.005$ ) and itraconazole ( $P = 0.008$ ). Colonizing and invasive isolates exhibiting variable susceptibilities had similar RFLP patterns. These observations suggest that *C. glabrata* exhibits considerable clinically significant cross-resistance between older azole drugs (fluconazole and itraconazole) and voriconazole. Caution is advised when considering voriconazole therapy for *C. glabrata* candidemia that occurs in patients with extensive prior azole drug exposure.**

*Candida glabrata* is currently the second most common cause of candidemia in the United States, and infection is associated with considerable mortality (6, 12). Microbial resistance to fluconazole readily develops; according to the 1993 to 2002 data from the ARTEMIS Global Antifungal Surveillance Program, 8.2% of *C. glabrata* isolates ( $n = 949$ ) had a fluconazole MIC of  $\geq 64$   $\mu\text{g/ml}$  and only 60% were truly susceptible by criteria outlined by previously published interpretive breakpoints (18, 19). Fluconazole use is thought to be one factor influencing the incidence of *C. glabrata* infections in certain geographic locations (16).

Voriconazole is an expanded-spectrum triazole derivative of fluconazole. The drug structurally resembles fluconazole except for the replacement of one of the triazole rings with a fluorinated pyrimidine and an additional  $\alpha$ -methyl group. It is approved for the primary treatment of acute invasive aspergillosis, salvage therapy for rare but serious fungal infections such as those caused by *Scedosporium apiospermum* and *Fusarium* spp., esophageal candidiasis, and invasive candidiasis (8). Evaluation of the susceptibilities of a large number of *C. glabrata* isolates suggests that voriconazole activity may be better than that of fluconazole (17, 18); however, the potential of clinically significant resistance has become evident with recent reports noting the development of *C. glabrata* candidemia in patients

receiving fluconazole (11) and voriconazole (7) therapy and the observation of cross-resistance developing in isolates recovered from the oral cavity and bloodstream (5). We performed this study to evaluate the clinical significance of azole cross-resistance in *C. glabrata* isolates recovered from colonizing and invasive sites of patients receiving azole therapy after hematopoietic stem cell transplantation (HSCT).

### MATERIALS AND METHODS

**Cases and definitions.** This study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board. To identify cases of fungal infections, HSCT recipients were monitored prospectively. A subset of patients had routine weekly screening for *Candida* isolates in mouthwash and stool samples. In addition, microbiology records were examined retrospectively for cases of bloodstream *Candida glabrata* infection in HSCT recipients between 1 January 2000 through 31 December 2004. During this period of time, patients received fluconazole (400 mg once daily) or itraconazole (2.5 mg/kg of body weight three times daily) as prophylaxis after HSCT (13) and voriconazole (3 to 4 mg/kg intravenously or 200 mg orally twice daily) for primary therapy of invasive aspergillosis. Voriconazole was also administered for prophylaxis in selected patients considered to have particularly high risks for invasive mold infection.

Candidemia was defined according to MSG/EORTC criteria (2). A *C. glabrata* invasive infection was considered to be an "azole breakthrough infection" if it developed after  $\geq 7$  days of azole therapy. Pharmacy records were reviewed for the use of antifungals prior to or during candidemia. Charts were reviewed for patient demographic information, underlying disease, HSCT type, comorbidity, immunosuppressive therapies, and outcome.

**Microbial testing: susceptibilities and typing.** *Candida glabrata* isolates were stored frozen at  $-70^\circ\text{C}$  until recovery by subculture. Fluconazole, itraconazole, and voriconazole susceptibilities were determined by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) M27-A microbroth dilution assay for yeast (14).

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TABLE 1. Cases of *C. glabrata* candidemia<sup>a</sup>

Case no.	Age (yr)	Underlying disease	Donor type	Day of candidemia <sup>b</sup>	Antifungal drug <sup>c</sup>
1	54	CML	MRD	41	Itraconazole (P)
2	12	MDS	MUD	82	Voriconazole (P)
3	21	AML	MUD	92	Voriconazole (P)
4	41	MM	MUD	64	Voriconazole (T)
5	44	HL	Autologous	13	Fluconazole (P)
6	31	MDS	MUD	16	Fluconazole (P)
7	53	NHL	MM-UD	173	Fluconazole (T)

<sup>a</sup> CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; MM, multiple myeloma; HL, Hodgkin's lymphoma; NHL, non-Hodgkin's lymphoma; MRD, human leukocyte antigen (HLA)-matched related donor; MUD, HLA-matched unrelated donor; MM-UD, HLA-mismatched unrelated donor.

<sup>b</sup> Relative to day of receipt of stem cells (day 0).

<sup>c</sup> Antifungal drugs received prior to or during candidemia. P, administered as part of a preventative algorithm; T, administered for treatment of a prior infection.

Molecular typing of sequential *C. glabrata* isolates obtained from patients was performed using a previously described restriction fragment length polymorphism method (10). Briefly, *C. glabrata* genomic DNA was isolated using the MasterPure yeast DNA purification kit (Epicenter, Madison, WI) according to the manufacturer's instructions. DNA was digested to completion with EcoRI, and Southern blots were hybridized with <sup>32</sup>P-labeled Cg12, a moderately repetitive DNA probe (provided by D. Soll, University of Iowa) using standard methods (21).

**Statistical analysis.** The strength of association between the MICs of fluconazole with itraconazole and voriconazole was assessed using the Kendall tau b rank correlation coefficient ( $\tau$ ). Bootstrapping and permutation methodologies were implemented to calculate 95% confidence intervals and *P* values, respectively, while appropriately accounting for correlated observations of isolates obtained within individuals.

RESULTS

During a 4-year period, 7 patients receiving azole therapy developed invasive *C. glabrata* infections. Case characteristics are listed in Table 1. The median time between the start of azole therapy and diagnosis of candidemia was 36 days (range, 13 to 173 days).

Colonizing and invasive isolates obtained from patients and corresponding susceptibilities are listed in Table 2. Three patients (cases 1 to 3) who had colonizing isolates available for analysis had developed colonization with isolates that had high MICs of multiple azoles prior to development of bloodstream infection. Colonizing isolates were not available for analysis from patients 4 to 7.

Isolates that had high fluconazole MICs ( $\geq 64$   $\mu\text{g/ml}$ ) appeared to also have relatively high MICs of itraconazole ( $\geq 2$   $\mu\text{g/ml}$ ) and voriconazole (1 to 2  $\mu\text{g/ml}$ ). Correlation coefficients suggest significant associations between the MICs of voriconazole and fluconazole ( $\tau$ , 0.58; 95% confidence interval, 0.43 to 0.85; *P*, 0.005) and itraconazole ( $\tau$ , 0.74; 95% confidence interval, 0.43 to 0.83; *P*, 0.008).

Molecular typing by restriction fragment length polymorphism demonstrated similar banding patterns among isolates recovered from the same patients but different patterns of isolates between patients (Fig. 1). Isolates recovered from case 1 were similar, except for colonizing isolate 11, which appeared to have a genotype similar to that of the isolate recovered from case 4. All other isolates were genetically dissimilar.

TABLE 2. Antifungal susceptibility of *C. glabrata* isolates

Case no.	Date collected (day/mo/yr) <sup>a</sup>	MIC ( $\mu\text{g/ml}$ ) of:		
		Fluconazole	Itraconazole	Voriconazole
1	1/11/2000	>64	0.25	<0.03
	1/25/2000	4	2	0.06
	1/30/2000	4	1	0.5
	2/8/2000	4	0.5	0.06
	<b>2/14/2000</b>	>64	2	1
	3/14/2000	>64	2	1
	3/21/2000	>64	2	1
	4/7/2000	>64	2	1
	4/11/2000	>64	2	2
	4/25/2000	>64	2	2
2	5/2/2000	>64	0.5	1
	5/9/2000	>64	2	1
	<b>8/5/2003</b>	>64	2	1
	<b>9/11/2004</b>	>64	2	1
	10/1/2004	>64	4	>16
	<b>12/2/2004</b>	>64	2	2
	3/22/2004	8	1	0.25
3	<b>7/10/2004</b>	>64	>16	2
	7/17/2004	>64	>16	2
	7/19/2000	>64	>16	4
	5	<b>11/28/2002</b>	8	1
6	<b>7/27/2003</b>	>64	>16	>16
	<b>1/29/2003</b>	>64	>16	4

<sup>a</sup> Bloodstream isolates are shown in bold.

DISCUSSION

We report a series of 7 cases of invasive *Candida glabrata* infections that developed during azole therapy, with several isolates exhibiting relatively high MICs of both the "old" (fluconazole) and "new" (voriconazole) drugs approved for treatment of candidemia. This observation has important therapeutic implications, as it suggests that *Candida glabrata* can exhibit clinically meaningful resistance across different azole drugs.

The spectrum of activity of voriconazole is increased compared to fluconazole, with several studies reporting that both *Candida albicans* and non-*C. albicans* *Candida* species exhibit low MICs of voriconazole (17, 18). The finding that MIC<sub>50</sub>s and MIC<sub>90</sub>s of voriconazole for *C. glabrata* are low, approximately 0.25 and 1  $\mu\text{g/ml}$  in large surveys of nonselected isolates (19), suggests that this drug retains good clinical activity. It is with this justification that investigators of a recent randomized trial evaluating voriconazole therapy for candidemia suggest that voriconazole (and caspofungin) should be considered better alternatives than fluconazole for primary therapy of candidemia (9). However, results of this and other recent case series (1, 11) suggest that these organisms may develop clinically meaningful cross-resistance among azole drugs, raising questions regarding the utility of voriconazole in individuals heavily pretreated with azole drugs.

*Candida glabrata* isolates for which MICs of both fluconazole and voriconazole are high have been observed. For instance, among the 46 *C. glabrata* isolates in the ARTEMIS study that were resistant to fluconazole (MIC,  $\geq 64$   $\mu\text{g/ml}$ ) and itraconazole (MIC,  $\geq 1$   $\mu\text{g/ml}$ ), 40 (87%) had a voriconazole MIC of  $\geq 2$   $\mu\text{g/ml}$  (18, 19). In a study evaluating voriconazole administered for "salvage" therapy, 90 *C. glabrata* isolates that exhibited high fluconazole MICs (mean, 128  $\mu\text{g/ml}$ ) also exhibited relatively high mean voriconazole MICs (4  $\mu\text{g/ml}$ ) (15).

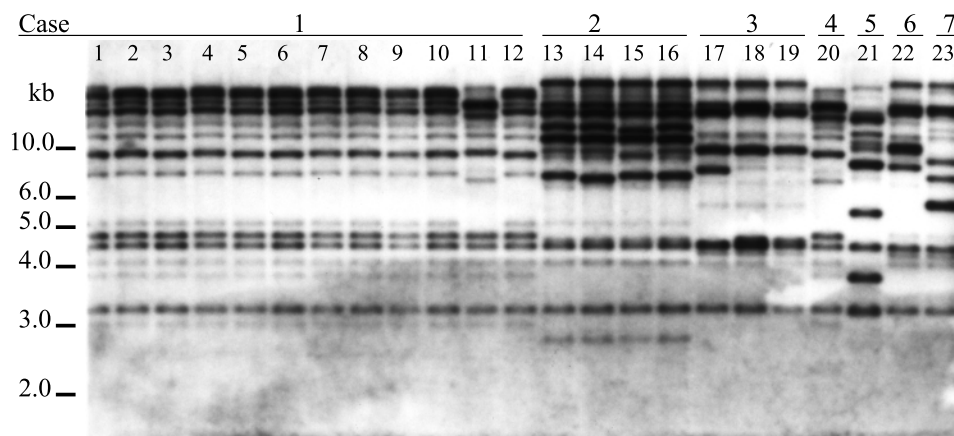


FIG. 1. Restriction fragment length polymorphism analysis of *Candida glabrata* isolates included in this study. Cases from which isolates were obtained are indicated.

In that study, the proportion of patients with *C. glabrata* infection who were treated successfully was approximately 25%; as clinical response is also dictated by host factors, success of “salvage” therapy may not be an optimal measure of the clinical impact of microbial resistance. Case series of what appear to be clinically significant microbial resistance are being reported (1, 11). Importantly, the characteristics and outcomes in these patients appear similar, as most of the patients have had a high amount of azole drug exposure in the setting of immunosuppressive conditions, such as after hematopoietic stem cell transplantation (1, 11).

Two recent studies have investigated the in vitro development of azole cross-resistance in detail. In one study, *C. glabrata* isolates recovered from clinical specimens during a 3-year hospital survey in Italy were evaluated (22). In this study, which included isolates that demonstrated both susceptible dose-dependent and -resistant fluconazole phenotypes, cross-resistance to voriconazole was noted. In another study, investigators observed rapid and stable acquisition of azole cross-resistance in *C. glabrata* isolates that were exposed to fluconazole upon serial culture in the laboratory (4). Our case series adds to our understanding of this phenomenon by demonstrating that colonizing *C. glabrata* isolates can develop increasingly high MICs to all three azoles during patient exposure to fluconazole, itraconazole, or voriconazole; these cases suggest clinical significance of cross-resistance in a heavily azole-pretreated population.

The mechanisms of resistance of *C. glabrata* to azoles are being elucidated. We have previously demonstrated that resistance to fluconazole in *C. glabrata* is associated with increased relative mRNA levels for ATP binding cassette transporters, *CgCDR1*, *CgCDR2*, and *PDH1*, which appeared to occur during patient exposure to fluconazole (3). More recently, evaluation of molecular mechanisms in matched and unmatched isolates recovered from hospitals in Italy provided evidence that increased mRNA of genes encoding *CgCDR1*, *CgCDR2*, and *CgSNQ2* was associated with high MICs of both fluconazole and voriconazole (22).

Statistical analysis demonstrated a significant association between the MIC of voriconazole and those of fluconazole and itraconazole, respectively. However, there were isolates in this

series that demonstrated variable MICs of the azole drugs; for instance, the first isolate from case 1 had a very high fluconazole MIC with low MICs of both itraconazole and voriconazole. Subsequent isolates demonstrated progressively increased MICs of both itraconazole and voriconazole. Evaluating the mechanism of azole resistance in each of these isolates will be of interest to determine whether efflux pumps, alterations in the azole target, or novel mechanism(s) are involved in sequential acquisition of increasing resistance in the series.

Results of molecular typing suggest that patients became infected with their colonizing strain, as was previously reported (20). Isolates infecting different people appeared to be different strains, having dissimilar patterns, with the exception of one isolate recovered from patients 1 and 4. Although both of those cases occurred in the year 2000, there was no epidemiological link noted, with consideration of overlapping space and time (data not shown). Also, the isolates recovered exhibited different susceptibility profiles. We cannot definitively state whether these isolates represent the same or similar strains.

Results of this study may have important implications when considering the definition of voriconazole “resistance.” Interpretive MIC breakpoints recently approved by CLSI to define voriconazole resistance are as follows: MIC of  $\leq 1$   $\mu\text{g/ml}$ , susceptible; MIC of 2  $\mu\text{g/ml}$ , susceptible dose dependent; MIC of  $\geq 4$   $\mu\text{g/ml}$ , resistant. In the three patients who developed *C. glabrata* candidemia during receipt of voriconazole, the isolate MICs were 1  $\mu\text{g/ml}$  (case 2), 2  $\mu\text{g/ml}$  (case 3), and 4  $\mu\text{g/ml}$  (case 4). Other reports noted *C. glabrata* isolates with MICs of  $> 2$   $\mu\text{g/ml}$  “breaking through” voriconazole therapy (1, 7).

This study demonstrates that colonizing *C. glabrata* isolates can acquire decreased susceptibility to multiple azole drugs, including voriconazole, during exposure to both voriconazole and “older” compounds, fluconazole and itraconazole. Future research should be performed to determine the molecular mechanisms by which isolates become sequentially resistant to numerous azoles. Caution is advised when considering voriconazole therapy for *C. glabrata* candidemia in settings that predict fluconazole resistance.

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## REFERENCES

- Alexander, B. D., W. A. Schell, J. L. Miller, G. D. Long, and J. R. Perfect. 2005. *Candida glabrata* fungemia in transplant patients receiving voriconazole after fluconazole. *Transplantation* **80**:868–871.
- Ascioglu, A., J. Rex, B. DePauw, J. Bennett, J. Bille, F. Crokaert, D. Denning, J. Donnelly, J. Edwards, Z. Erjavec, D. Fiere, O. Lortholary, J. Maertens, J. Meis, T. Patterson, J. Ritter, D. Selleslag, P. Shah, D. Stevens, T. Walsh, and J. Ritter. 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin. Infect. Dis.* **34**:7–14.
- Bennett, J. E., K. Izumikawa, and K. A. Marr. 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrob. Agents Chemother.* **48**:1773–1777.
- Borst, A., M. T. Raimer, D. W. Warnock, C. J. Morrison, and B. A. Arthington-Skaggs. 2005. Rapid acquisition of stable azole resistance by *Candida glabrata* isolates obtained before the clinical introduction of fluconazole. *Antimicrob. Agents Chemother.* **49**:783–787.
- Burn, A. K., A. W. Fothergill, W. R. Kirkpatrick, B. J. Coco, T. F. Patterson, D. I. McCarthy, M. G. Rinaldi, and S. W. Redding. 2004. Comparison of antifungal susceptibilities to fluconazole and voriconazole of oral *Candida glabrata* isolates from head and neck radiation patients. *J. Clin. Microbiol.* **42**:5846–5848.
- Fidel, P. L., Jr., J. A. Vazquez, and J. D. Sobel. 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin. Microbiol. Rev.* **12**:80–96.
- Imhof, A., S. A. Balajee, D. N. Fredricks, J. A. Englund, and K. A. Marr. 2004. Breakthrough fungal infections in stem cell transplant recipients receiving voriconazole. *Clin. Infect. Dis.* **39**:743–746.
- Johnson, L. B., and C. A. Kauffman. 2003. Voriconazole: a new triazole antifungal agent. *Clin. Infect. Dis.* **36**:630–637.
- Kullberg, B. J., J. D. Sobel, M. Ruhnke, P. G. Pappas, C. Viscoli, J. H. Rex, J. D. Cleary, E. Rubinstein, L. W. Church, J. M. Brown, H. T. Schlamm, I. T. Oborska, F. Hilton, and M. R. Hodges. 2005. Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. *Lancet* **366**:1435–1442.
- Lockhart, S. R., S. Joly, C. Pujol, J. D. Sobel, M. A. Pfaller, and D. R. Soll. 1997. Development and verification of fingerprinting probes for *Candida glabrata*. *Microbiology* **143**(Pt 12):3733–3746.
- Magill, S., C. Shields, C. Sears, M. Choti, and W. Merz. 2006. Triazole cross-resistance among *Candida* spp.: case report, occurrence among bloodstream isolates, and implications for antifungal therapy. *J. Clin. Microbiol.* **44**:529–535.
- Malani, A., J. Hmoud, L. Chiu, P. L. Carver, A. Bielaczyc, and C. A. Kauffman. 2005. *Candida glabrata* fungemia: experience in a tertiary care center. *Clin. Infect. Dis.* **41**:975–981.
- Marr, K. A., F. Crippa, W. Leisenring, M. Hoyle, M. Boeckh, S. A. Balajee, W. G. Nichols, B. Musher, and L. Corey. 2004. Itraconazole versus fluconazole for prevention of fungal infections in patients receiving allogeneic stem cell transplants. *Blood* **103**:1527–1533.
- National Committee for Clinical Laboratory Standards. 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard. NCCLS document M27-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
- Perfect, J. R., K. A. Marr, T. J. Walsh, R. N. Greenberg, B. DuPont, J. de la Torre-Cisneros, G. Just-Nubling, H. T. Schlamm, I. Lutsar, A. Espinel-Ingroff, and E. Johnson. 2003. Voriconazole treatment for less-common, emerging, or refractory fungal infections. *Clin. Infect. Dis.* **36**:1122–1131.
- Pfaller, M. A., and D. J. Diekema. 2004. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. *Clin. Microbiol. Infect.* **10**(Suppl. 1):11–23.
- Pfaller, M. A., D. J. Diekema, S. A. Messer, L. Boyken, and R. J. Hollis. 2003. Activities of fluconazole and voriconazole against 1,586 recent clinical isolates of *Candida* species determined by broth microdilution, disk diffusion, and Etest methods: report from the ARTEMIS Global Antifungal Susceptibility Program, 2001. *J. Clin. Microbiol.* **41**:1440–1446.
- Pfaller, M. A., S. A. Messer, L. Boyken, R. J. Hollis, C. Rice, S. Tendolkar, and D. J. Diekema. 2004. In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. *Diagn. Microbiol. Infect. Dis.* **48**:201–205.
- Pfaller, M. A., S. A. Messer, L. Boyken, S. Tendolkar, R. J. Hollis, and D. J. Diekema. 2004. Geographic variation in the susceptibilities of invasive isolates of *Candida glabrata* to seven systemically active antifungal agents: a global assessment from the ARTEMIS Antifungal Surveillance Program conducted in 2001 and 2002. *J. Clin. Microbiol.* **42**:3142–3146.
- Redding, S. W., K. A. Marr, W. R. Kirkpatrick, B. J. Coco, and T. F. Patterson. 2004. *Candida glabrata* sepsis secondary to oral colonization in bone marrow transplantation. *Med. Mycol.* **42**:479–481.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sanguinetti, M., B. Posteraro, B. Fiori, S. Ranno, R. Torelli, and G. Fadda. 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob. Agents Chemother.* **49**:668–679.