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# Antifungal Resistance of *Candida glabrata* Vaginal Isolates and Development of a Quantitative Reverse Transcription-PCR-Based Azole Susceptibility Assay▽

Scott E. Gygax,[1]†* John-Paul Vermitsky,[1]† Sean G. Chadwick,[1] Matthew J. Self,[1] Jessica A. Zimmerman,[3] Eli Mordechai,[3] Martin E. Adelson,[3] and Jason P. Trama[2]

*Antimicrobial Resistance Division[1] and Molecular and Cellular Biology Division,[2] Medical Diagnostic Laboratories, L.L.C.,[3] 2439 Kuser Road, Hamilton, New Jersey 08690*

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**A multiplex quantitative reverse transcription-PCR assay was developed to detect azole resistance in *Candida glabrata*, an important opportunistic pathogen that develops resistance rapidly. Resistance was defined as a ≥3-fold increase in *CDR1* expression by this assay, which proved to be 100% sensitive and 95% specific in comparison to the gold standard broth microdilution assay.**

In the United States, *Candida* fungal infections have increased significantly over the past 3 decades, particularly those due to non-*albicans* species (2, 3, 6). The emergence of non-*albicans* species, especially *Candida glabrata*, is problematic for both immunocompetent and immunocompromised populations. *C. glabrata* is now recognized as the second most common cause of *Candida* infections (10 to 30%) and the primary species isolated from diabetic patients (61.3%) and the elderly (51.2%), with mortality rates up to 51% (1, 4, 5, 13, 21). This organism exhibits intrinsically low susceptibility to azole antifungals, as shown in this study and one by Richter et al. (15), in which a majority of isolates are susceptible-dose dependent (S-DD) or resistant (R). Additionally, this organism can rapidly develop resistance in both the clinical setting and within the laboratory (14, 19).

Identifying the species and susceptibility of an isolate involved in infection is imperative for determining the proper course of treatment. Surveillance programs performed over the past few decades have demonstrated that although azole resistance is rare in *Candida albicans* isolates (<1%), it is becoming very common among isolates of *C. glabrata* (up to 15%) and other non-*albicans* species (10, 15, 16). Current practices for determining the susceptibility of a particular isolate involve the microbroth dilution assay (MBD) outlined in the M27-2A document of the Clinical Laboratory Standards Institute (CLSI) (9). Although this assay is quite effective, results can take upwards of a week to obtain and the need to develop a more rapid, yet still reliable, method for determining the susceptibility of yeast isolates is warranted.

The purpose of this study was to examine the susceptibility of and establish azole resistance markers for *C. glabrata.* The predominant mechanism of azole resistance in *C. glabrata* is the increased expression of multidrug transporter genes *CDR1* and *PDH1* under the transcriptional regulation of *PDR1* (8, 14, 17, 18, 19, 20). In this study, all three genes were examined for upregulated expression by a quantitative reverse transcription (qRT)-PCR assay. Recent publications have established similar assays for *C. albicans* (7, 11) but not for *C. glabrata*, for which resistance is far more prevalent.

*C. glabrata* isolates were retrieved from cervicovaginal swabs that were tested using species-specific in-house real-time PCR assays and confirmed on BBL CHROMagar *Candida* plates. Azole susceptibility testing was performed on 175 clinical isolates by MBD for fluconazole, voriconazole, and miconazole according to the CLSI guidelines outlined in document M27-A2 (9). Our *C. glabrata* isolates demonstrated R and S-DD profiles (26.9% and 42.2%, respectively) (Table 1) similar to those of previously reported vulvovaginal candidiasis isolates (15.2% and 51.8%) (15).

A qRT-PCR assay was developed to detect *C. glabrata* azole resistance. RNA from cells grown in culture to mid-log phase was isolated using the Qiagen RNeasy kit and treated with RQ1 DNase (Promega). A total of 50 ng of RNA was used in the qRT-PCRs. The primers and probes used are listed in Table 2. The levels of mRNA of the multidrug resistance (MDR) genes (*CDR1*, *PDH1*, and *PDR1*) and the actin housekeeping gene (*ACT1*) were measured using a One-Step qRT-PCR on the Stratagene Mx3000P QPCR system (Stratagene). The expression levels of the *ACT1* gene were found to be consistent among the susceptible (S), S-DD, and R isolates. The qRT-PCR assay was performed in triplicate, with independent amplifications using the same RNA for the gene of interest (*CDR1*, *PDH1*, or *PDR1*) and the *ACT1* control in a duplex reaction. The expression levels of the MDR genes were found to be consistent among the S isolates (Fig. 1). Five S clinical isolates (MICs of 8, 8, 4, 4, and 2 μg/ml) were chosen to determine a consistent baseline expression profile for each assay. The gene expression level relative to this panel of S isolates was expressed as $2^{-\Delta\Delta CT}$. Furthermore, the qRT-PCR assay was specific for *C. glabrata* as it did not cross-react with a panel of 88 different fungal, bacterial, and viral pathogens, including other *Candida* species (data not shown).

* Corresponding author. Mailing address: Antimicrobial Resistance Division, Department of Research and Development, Medical Diagnostic Laboratories, L.L.C., 2439 Kuser Road, Hamilton, NJ 08690. Phone: (609) 570-1015. Fax: (609) 570-1030. E-mail: sgygax@mdlab.com.

† These authors have contributed equally to this work.

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TABLE 1. Susceptibility of 175 *C. glabrata* isolates

| Antibiotic | No. (%) of isolates[a] | | |
|---|---|---|---|
| | S | S-DD | R |
| Fluconazole | 54 (30.9) | 74 (42.2) | 47 (26.9) |
| Voriconazole[b] | 109 (62.3) | 24 (13.7) | 42 (24.0) |
| Miconazole[c] | 175 (100.0) | 0 (0.0) | 0 (0.0) |

[a] Total number of *C. glabrata* isolates tested, 175. Susceptibility values were as determined by the CLSI M27-A2 MBD method (9).
[b] Proposed breakpoints were used to determine interpretive categories for voriconazole (9, 12).
[c] Miconazole is active in vitro at relatively low concentratoins (<0.6 μg/ml) (15).

Ten swabs from each of the three susceptibility groups (S, S-DD, and R) previously tested by MBD (Table 1) were randomly chosen. Three isolates from each swab were used in the qRT-PCR azole susceptibility assay. Nonsusceptible isolates (S-DD and R) were defined by a ≥2-fold increase in any of the three MDR genes. Compared to MBD, the qRT-PCR assay detected nonsusceptible isolates with 80% sensitivity, 100% specificity, 100% positive predictive value, and 71% negative predictive value. For determining resistance, a more stringent cutoff of a ≥3-fold increase in expression of *CDR1* alone could be used as the predominant molecular marker with 100% sensitivity, 95% specificity, 91% positive predictive value, and 100% negative predictive value. All of the isolates tested from the 10 resistant swabs showed ≥3-fold-increased expression levels of *CDR1*. The single false positive was an S-DD swab with a MIC of 32 μg/ml. All 10 of the susceptible swabs tested had increases of less than twofold for all three molecular markers. The three isolates tested from each of 29 swabs demonstrated a consistent susceptibility phenotype, as characterized by both MBD and qRT-PCR assays. Interestingly, one swab sample contained two isolates that tested susceptible and one isolate that was highly resistant. Although a rare occurrence, this result supports the need for selecting multiple colonies per swab when the qRT-PCR assay is performed.

Apart from providing the susceptibility profile of *C. glabrata* cervicovaginal isolates, our study proposes the use of molecular markers to determine the susceptibility of a particular isolate to azole antifungals. qRT-PCR is now recognized as a reliable method for evaluating gene expression. In this study, we found that a ≥3-fold change in the expression of the *C. glabrata CDR1* gene based on a qRT-PCR assay determined azole resistance with 100% sensitivity and 95% specificity. Moreover, the qRT-PCR assay provides a simple, efficient, and cost-effective method for early detection, identification, and

TABLE 2. Primers and dually labeled probes developed in this study

| Primer/probe | Sequence (5′–3′)[a] |
|---|---|
| ACT1F | CGCTTTGGACTTCGAACAAGAA |
| ACT1R | GTTACCGATGGTGATGACTTGAC |
| ACT1 probe | Cy5-AACCGCTGCTCAATCTTCCTCCAT-BHQ-2 |
| PDH1F | CAGACCCGGTTCCGGTTGTA |
| PDH1R | CGGTAGTGCTTCTTGATCTCGTT |
| PDH1 probe | FAM-ACGCTGCTGAAGTCCATCTCCTCG-TAMSp |
| CDR1F | TTAAAAGTTCAAGCCAGTATTTCC |
| CDR1R | AAATTTGATAACCATCGTAAAGCA |
| CDR1 probe | FAM-ACGCTGCTGCTACTGTGGCTATCT-TAMSp |
| PDR1F | TACATGGAACATCTGTTGCTTCTT |
| PDR1R | CGACTCTTCATAGCCGACGT |
| PDR1 probe | FAM-TGAAGAACAGCTTGCTCTCGACGA-TAMSp |

[a] FAM, 6-carboxyfluorescein; BHQ-2, black hole quencher 2; TAMSp, 6-carboxytetramethylrhodamine.

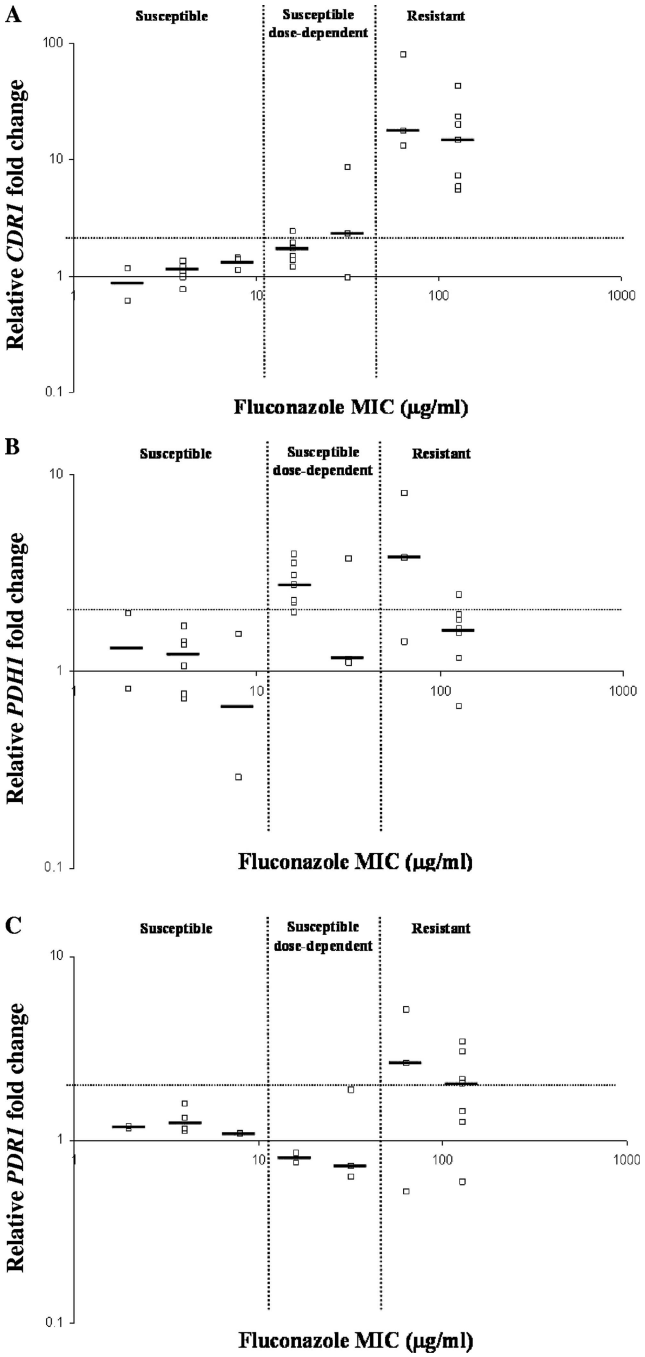


FIG. 1. Gene expression profiles within the susceptibility groups. Clinical isolates were tested for fluconazole susceptibility by MBD (9), and the results were compared to qRT-PCR expression levels of *CDR1* (A), *PDH1* (B), and *PDR1* (C). The heavy solid horizontal bars represent the median values. The horizontal dotted line represents a twofold increase in expression level above that for the susceptible panel. The vertical dotted lines distinguish the three susceptibility groups as determined by CLSI breakpoints for *Candida* species.

azole susceptibility testing in as little as 30 h for a particular *C. glabrata* isolate. Conversely, the conventional MBD (9) is labor-intensive, requiring colony isolation, identification to the species level, and culturing that can take upwards of 72 h in addition to the time of the assay itself. Therefore, the qRT-PCR assay would assist in expediting a proper course of treatment and would minimize the likelihood of selecting for resistant isolates through treatment with ineffective levels of azole antifungals.

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