

Establishing Surrogate Markers for Fluconazole Resistance in *Candida albicans*

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ABSTRACT

Azole-resistant *Candida* can be a confounding factor for clinical management of opportunistic infections in immunocompromised patients, but rapid identification of such resistant organisms can improve patient outcome. New target-based molecular diagnostic strategies have the potential to identify resistant organisms faster than current culture-based assays. It was the objective of this study to determine whether target site mutations and/or drug pump over-expression are suitable surrogate markers of drug resistance that could aid new molecular-based diagnostic assays. A collection of 59 clinical isolates displaying a range of azole susceptibilities were assayed for mutations within the target gene *Erg11* and for over-expression of drug-efflux pumps *Cdr1*, *Cdr2*, *Flu1*, and *Mdr1*, as well as drug target gene *Erg11* by quantitative real-time PCR with molecular beacons. A fluconazole-resistant (MIC ≥ 64 $\mu\text{g/ml}$) phenotype was closely associated with over-expression of *Cdr1* ($p = 0.005$), *Cdr2* ($p = 0.01$), and *Mdr1* ($p = 0.03$) along with four mutations in *Erg11* (T229A, Y132F, S405F, G464S). Changes in expression levels for *Erg11* and *Flu1* were not statistically correlated with resistance ($p = 0.27$ and $p = 0.86$, respectively). Overall, these findings provide a statistical basis to establish *Erg11* mutations and drug pump over-expression as surrogate markers for phenotypic fluconazole resistance.

INTRODUCTION

TRIAZOLE ANTIFUNGAL AGENTS (e.g., fluconazole, itraconazole, etc.) are commonly used to treat *Candida* infections because of their high therapeutic index. Resistance to these agents, which were introduced in the early 1990s, is most notable among immunosuppressed patients.^{5,13} The molecular mechanisms of triazole resistance in *C. albicans* have been well characterized in recent years and include alteration of the triazole drug target enzyme, lanosterol 14 α -demethylase encoded by *Erg11*, and reduction in cellular drug accumulation.^{5,6,17,21,22} Alterations in Erg11p include mutations that result in reduced affinity to triazole antifungal drugs, as well as those that lead to over-expression of *Erg11*, which diminishes drug efficacy via target titration.^{4-6,8,15,16,22} A reduction of cellular drug accumulation has also been linked to over-expression of multidrug resistance (MDR) efflux transporter genes of the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) classes.^{4-6,11,14,16-18,20-22} ABC transporters require energy in the form of ATP and can expel a multitude of substrates in contrast to MFS transporters that use electrochemical proton gra-

dients as driving forces and have more limited substrate specificity. In *C. albicans*, the ABC transporter genes *Cdr1* and *Cdr2* and MFS gene *Mdr1* have been linked to clinical drug resistance.^{4-6,11,16-18,20-22} A novel MFS transporter gene *Flu1* has been identified in *C. albicans*, but its contribution to clinical drug resistance remains unclear.²

In an effort to identify potential surrogate markers for clinical drug resistance, the genes responsible for diverse resistance mechanisms were evaluated in a collection of clinical isolates displaying fluconazole susceptible (S), susceptible dose-dependent (SDD), and resistant (R) *in vitro* susceptibility phenotypes by DNA sequencing and molecular beacon-based transcriptional profiling.

MATERIALS AND METHODS

Strains and susceptibility testing

Candida albicans strains used in this study include a collection of 59 fluconazole-susceptible (MIC ≤ 8.0 $\mu\text{g/ml}$) ($n =$

14), susceptible dose-dependent (MIC 16–32 µg/ml) (*n* = 13), and resistant clinical isolates (MIC ≥ 64 µg/ml) (*n* = 32) as determined by the microdilution antifungal susceptibility testing method outlined by the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A.⁹ These strains were obtained from separate patients from diverse geographic regions and were not epidemiologically linked. Fluconazole was obtained from Pfizer (New York).

Isolation of RNA and DNA

Candida albicans strains were grown in YPD medium (Difco, Franklin Lakes, NJ) at 30°C until mid-log phase growth. Total DNA-free RNA was isolated using the RNeasy Minikit (Qiagen, Valencia, Calif.). For DNA extraction, an overnight culture grown at 30°C was used and total genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, Wis.).

PCR and DNA sequencing of Erg11

PCR and sequencing primers were designed to the coding region of Erg11 (GenBank accession number X13296) using Oligo 4.04 primer design software (Molecular Biology Insights, Inc., Cascade, CO). Primers used for PCR (5′–3′) were F52 (GACAAAGAAAGGGAATTCAATCG) and R1763 (CACTGAATCGAAAGAAAGTTGCCG). PCR amplification was carried out at 94°C for 1 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min and a final extension step of 72°C for 5 min. Primers used for DNA sequencing included F478 (GATGTTTCTGCTGAAGATGC), F947 (GTGGTGATATTGATCCAAATCG), F1363 (CCAGGTTATGCTCATACTAG), R498 (GCATCTTCAGCAGAAACATC), R968 (GATTTGGATCAATATCACCCAC), R1382 (CTAGTATGAGCATAACCTGG), and the PCR primers as described above. Automated DNA sequencing of the *Erg11*

coding region was performed using the DTCS Quick Start kit (Beckman Coulter, Fullerton, CA) and a CEQ 2000 XL capillary electrophoresis DNA sequencer (Beckman Coulter).

Primer and molecular beacon design for quantitative real-time PCR

PCR primers and molecular beacon probes were designed to amplify and identify *C. albicans* genes *Cdr1*, *Cdr2*, *Erg11*, *Flu1*, *Mdr1*, and *Pma1* as described by the molecular beacons synthesis protocol (<http://www.molecular-beacons.org>). Each molecular beacon was labeled with tetrachloro-6-carboxylfluorescein (TET) (BioSearch Technologies, Inc., Novato, CA). Primer and molecular beacon probe sequences are listed in Table 1. For each probe, a linear relationship was observed between the cycle threshold (Ct) value and the logarithm of known amount of initial template molecules (not shown). Furthermore, each molecular beacon was shown to be specific to its target and did not show any cross-interaction or nonspecific behavior.

RT assay followed by PCR

First-strand cDNA was synthesized using Omniscript RT PCR Kit (Qiagen) and 1 µg of total RNA. Samples incubated in the absence of reverse transcriptase were used as controls. Quantitative real-time PCR mixtures contained 0.2 µM of each molecular beacon, 10 pmol of each primer, 2 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.25 mM dNTPs, 4 mM MgCl₂, 1× PCR buffer, and 250 ng of cDNA. The thermal cycling program consisted of 10 min on a spectrofluorometric thermal cycler (Applied Biosystems 7700 Prism) at 95°C followed by 40 cycles of 20 sec at 95°C, 30 sec at 50°C, and 30 sec at 72°C, and real-time fluorescence was monitored during the reaction and plotted against the number of thermal cycles. All quantitative real-time PCR assays were

TABLE 1. PRIMERS AND MOLECULAR BEACON DESIGN FOR QUANTITATIVE REAL-TIME PCR

Primer	Sequence (5′–3′)	Amplicon size (bp)	MB Sequence (5′–3′)	GenBank accession number
CDR1F CDR1R	CTTAGTCAAACCACTGGATCG CCAAAAGTGATGAAAAGGC	85	cgaccGAGGTGCTG CCATGTTCTTTGgg gtcg	X77589
CDR2F CDR2R	CACGTCTTTGTGCGAACAGC ATGTTGTGACTTGCAGTAGC	105	ccgtggTGGGTGGAT GCACTGGACAATT ccacgg	U63812
ERG11F ERG11R	ACTAGATGGGATACTGCTGC CATCTATGTCTACCACCACC	137	ccgtggTGGGAAAGT TTCTAAAGGGGTT ccacgg	X13296
FLU1F FLU1R	TGTTGCCTTTGATGGTCCCG ACCGATAAGGCAGCAAGACC	102	ccgctgAAAATTTTA TATTGTGCATCTG cagcgg	AF188621
MDR1F MDR1R	TTCTTGGGTGGATTCTTCGC GCACCTAAACTCCAAGCGGC	113	ccgtggAGTCCTTGT TTGGCCACTGGTG ccacgg	X53823
PMA1F ^a PMA1R	GGCCAAGAAACAAGCTATTGT CGGAACACAAGATTTCAACAC	74	cgaccGTCTGCCAT TGAATCTTTGGggg tcg	M74075

^aConstitutively expressed internal control gene.

TABLE 2. SUMMARY OF *CANDIDA albicans* STRAINS USED IN THIS STUDY

Isolate number	Collection site ^a	FLU susceptibility ^b	FLU resistance gene over-expression ^c					ERG11p mutation(s) ^d
			CDR1	CDR2	ERG11	FLU1	MDR1	
1	OP	S						K128T
2	OP	S						D116E, G450E
3	OP	S						
4	OP	S						
5	OP	S						
6	OP	S						K128T
7	OP	S						
8	OP	S						F449S
9	OP	S						V437I, D446N
10	OP	S						V437I
11	Abscess	S						
12	Bronchial	S						
13	Unknown	S						
14	Unknown	S						
15	OP	SDD					+	
16	Vagina	SDD						
17	OP	SDD						S405F
18	OP	SDD						
19	OP	SDD						
20	OP	SDD						
21	OP	SDD						
22	Skin	SDD					+	
23	OP	SDD						
24	OP	SDD						
25	Vagina	SDD					+	
26	OP	SDD					+	
27	OP	SDD					+	R467K
28	OP	R					+	
29	OP	R					+	E266D, G464S, V488I
30	OP	R		+				
31	Unknown	R						
32	Vagina	R						
33	OP	R	+	+				D116E, G450E
34	OP	R						S405F
35	OP	R	+					K128T
36	OP	R						S405F
37	OP	R		+				V437I
38	OP	R					+	V437I
39	OP	R					+	
40	OP	R	+	+			+	
41	OP	R					+	Y132F
42	OP	R					+	Y132F
43	OP	R	+					Y132F
44	OP	R	+	+				Y132F
45	OP	R			+		+	F126L, K143R
46	OP	R			+		+	F126L, K143R
47	OP	R		+				F126L, K143R
48	OP	R						T229A
49	OP	R						T229A
50	OP	R	+	+				V437I, D446N
51	OP	R	+	+				G464S
52	Blood	R	+	+	+		+	
53	OP	R	+	+			+	
54	Eye	R	+		+			
55	OP	R						D116E, E266D
56	OP	R	+	+				
57	OP	R	+					D116E
58	OP	R		+				
59	OP	R	+					

^aOP, Oro-pharynx.^bS, Susceptible (MIC ≤ 8.0 μg/ml); SDD, susceptible dose dependent (MIC 16–32 μg/ml); R, resistant (MIC ≥ 64 μg/ml).^cOver-expression is defined as > 3 times the average FLU-susceptible strains values.^dRelative to GenBank Accession number X13296.

performed in triplicate and the essential gene *Pma1* was used as a highly-expressed internal normalization marker.

Statistical analysis

Association of individual *mdr* gene expression levels for *Cdr1*, *Cdr2*, *Mdr1*, *Erg11*, and *Flu1* relative to azole-susceptible, susceptible dose-dependent and resistant phenotypes was determined by one factor analysis of variance (ANOVA). *p* values less than 0.05 were considered significant.

RESULTS

Erg11 mutations

An epidemiologically diverse collection of 59 clinical isolates of *C. albicans* encompassing fluconazole susceptible, dose-dependent, and resistant phenotypes were evaluated by DNA sequence analysis of *Erg11*. Numerous mutations relative to the published *Erg11* sequence (GenBank accession number X13296) were identified resulting in 15 different amino acid substitutions, as follows: D116E, F126L, K128T, Y132F, K143R, T229A, E266D, S405F, V437I, D446N, F449S, G450E, G464S, R467K, and V488I (Table 2). The mutations ranged in frequency from 2.2 to 8.8% in the 45 R or SDD strains (Table 3) and clustered within four mutational “hot-spot” regions previously identified, which were located between amino acid residues 72 to 467.^{8,12} However, six amino acid mutations, D116E, K128T, G450E, V437I, D446N, and F449S, were not linked to triazole resistance as they were also found to be pres-

ent in fluconazole-susceptible strains (Table 2). The mutation S405F was only found in SDD and resistant strains, while other mutations also occurred in strains with SDD phenotypes (Table 2). Nine isolates had multiple amino acid mutations in *Erg11*.

Quantitative over-expression of *Erg11* and drug efflux genes

Molecular beacon probes were used to quantify the level of expression of azole target gene *Erg11*, and *mdr* pumps *Cdr1*, *Cdr2*, *Flu1*, and *Mdr1*. The 14 azole-susceptible strains were used to establish baseline values of transcripts for expression of the known resistance genes *Cdr1*, *Cdr2*, *Erg11*, *Flu1*, and *Mdr1*. As expected, a majority of the susceptible strains displayed low levels of expressed *mdr* genes, which remained closely grouped as shown in the scatter plot diagram (Fig. 1). The susceptible dose-dependent isolates similarly displayed low-level expression of *mdr* genes, with the exception of *Mdr1*, which revealed moderate to high increases (2- to 10-fold) of transcript. The azole-resistant isolates showed a 2- to 45-fold increase in *Mdr1* transcript levels when compared to the susceptible and susceptible dose-dependent isolates. For *Cdr1*, all of the susceptible dose-dependent isolates had similar expression profiles as the susceptible. However, *Cdr1* over-expression was found in ~34% (11/32) of the total R isolates tested with 2- to 15-fold increases in transcript levels. A majority of the isolates also co-expressed *Cdr2* (*n* = 11), supporting the previous notion that *Cdr1* and *Cdr2* are co-regulated.²¹ Overall, the frequency of gene over-expression for *Cdr1*, *Cdr2*, and *Mdr1* was 24.4%, 24.4%, and 33.3%, respectively, for all R and SDD isolates (*n* = 45) examined (Table 3). Gene over-expres-

TABLE 3. PREVALENCE AND ASSOCIATION OF SPECIFIC GENETIC MECHANISMS WITH RESISTANCE

<i>MDR</i> genotype	Isolates displaying phenotype			Frequency of SDD and R phenotype (%)	ANOVA <i>p</i> value ^a
	S (n = 14)	SDD (n = 13)	R (n = 32)		
<i>Cdr1</i> over-expression ^b	0	0	11	24.4	0.005
<i>Cdr2</i> over-expression	0	0	11	24.4	0.01
<i>Erg11</i> over-expression	0	0	4	8.8	0.27
<i>Flu1</i> over-expression	0	0	1	2.2	0.86
<i>Mdr1</i> over-expression	0	5	10	33.3	0.03
<i>Erg11</i> -D116E	1	0	3	6.6	ND ^c
<i>Erg11</i> -F126L	0	0	3	6.6	ND
<i>Erg11</i> -K128T	2	0	1	2.2	ND
<i>Erg11</i> -Y132F	0	0	4	8.8	ND
<i>Erg11</i> -T229A	0	0	2	4.4	ND
<i>Erg11</i> -E266D	0	0	2	4.4	ND
<i>Erg11</i> -S405F	0	1	2	6.6	ND
<i>Erg11</i> -V437I	2	0	3	6.6	ND
<i>Erg11</i> -D446N	1	0	1	2.2	ND
<i>Erg11</i> -F449S	1	0	0	0	ND
<i>Erg11</i> -G450E	1	0	1	2.2	ND
<i>Erg11</i> -G464S	0	0	2	4.4	ND
<i>Erg11</i> -R467K	0	1	0	2.2	ND
<i>Erg11</i> -V488I	0	0	1	2.2	ND

^aANOVA analyses were performed on the absolute expression levels values of the *mdr* genes.

^bOver-expression is defined as >3 times the average FLU susceptible strains values.

^cND, Not determined because population size was too limited for statistical significance.

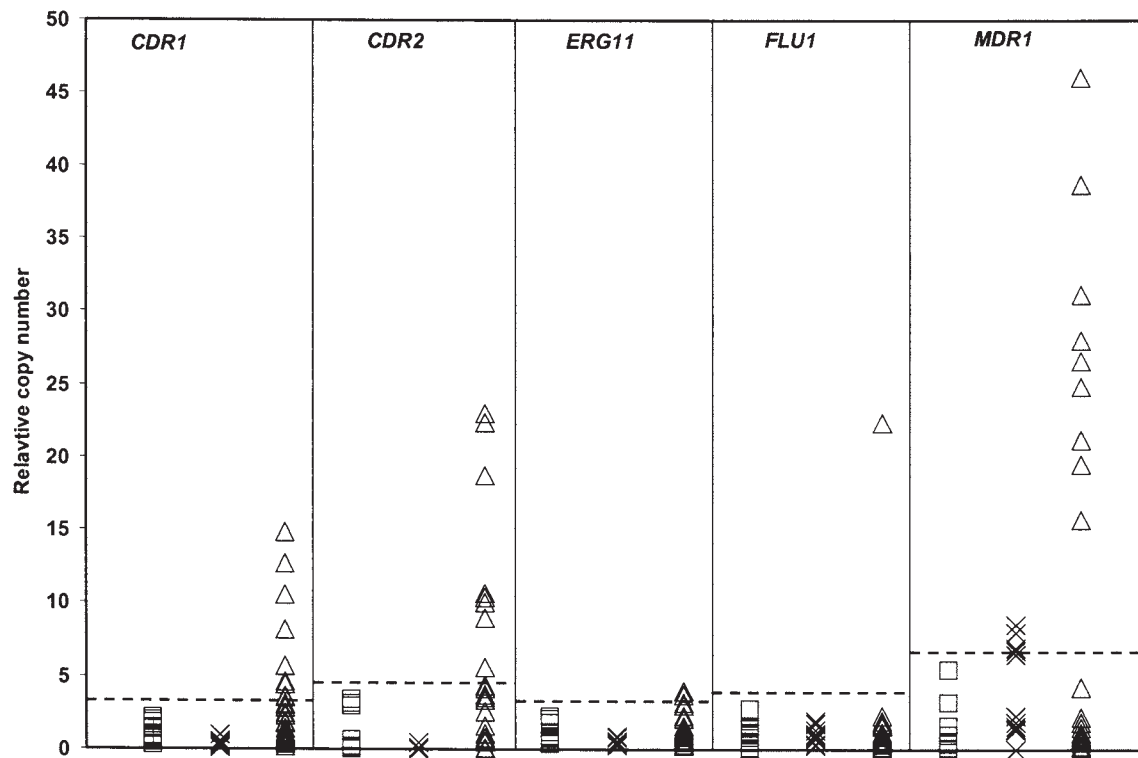


FIG. 1. Relative expression levels of *mdr* genes. Quantitative real-time PCR with molecular beacons of known *mdr* genes was performed on cDNA transcripts of a panel of *C. albicans* isolates ($n = 59$). The collection included 14 azole-susceptible (\square), 13 azole-susceptible dose-dependent (\times), and 32 resistant (\triangle) isolates. Variations in the initial amount of mRNA among the different samples were normalized to the level of *Pma1* mRNA, yielding a ratio between the specific *mdr* gene cDNA copy number and *Pma1* cDNA copy number.

sion was constitutively expressed in the strains, because there was no evidence for significant changes in expression due to drug exposure. *Erg11* over-expression was found in $\sim 12\%$ of the resistant isolates tested (4/32) whereas *Flu1* over-expression was found in one isolate, but neither *Erg11* nor *Flu1* over-expression were statistically linked to clinical drug resistance ($p = 0.27$ and $p = 0.87$, for *Erg11* and *Flu1*, respectively) (Table 3).

Five azole-susceptible dose-dependent strains had single mechanisms of resistance in contrast to 20 triazole-resistant isolates that displayed multiple mechanisms of resistance ($\sim 62\%$). A majority of strains showed multiple types (target site or drug efflux pump) of resistant mechanisms (Table 2). Of 10 isolates with a single resistance mechanism, five had target-based mutations and five showed efflux pump over-expression. Of the strains with target site mutations linked to resistance, a majority also showed over-expression of *mdr* pumps.

DISCUSSION

It is now well established that triazole resistance in *C. albicans* is multifactorial involving several different resistance mechanisms operating individually or collectively in a given strain.^{4,7,11,16,21,22} Such complexity implies that no one resistance mechanism plays a dominant role. Furthermore, high-

level resistance largely results from incremental susceptibility shifts mediated by resistance mechanisms induced in response to short- or long-term drug exposure.^{4,7,21} Nevertheless, there are subsets of the various resistance mechanisms that are more frequently associated with *in vitro* resistance and therefore have the potential to serve as diagnostic surrogates. Advances in molecular diagnostic approaches have made it possible to rapidly evaluate genetic changes (allele discrimination and gene over-expression) in an organism that result in a prominent phenotype, such as drug resistance.^{1,3,10} Most recently, it was shown that gene over-expression of multiple resistance targets could be profiled in a simple real-time assay,³ and at least seven different alleles associated with itraconazole resistance in *Aspergillus fumigatus* could be readily detected in a single multiplexed assay.¹

In this report, the most commonly associated resistance mechanisms include *Erg11* target site mutations T229A, Y132F, K143R, E266D, S405F, G464S, and V488I (Table 2) and over-expression of drug efflux transporter genes *Cdr1*, *Cdr2*, and *Mdr1*, above a baseline threshold value of 3-fold greater than the average expression in susceptible strains (Fig. 1). In each case, the manifestation of either a given *Erg11* mutation and/or gene over-expression was correlated with resistance. All the *Erg11* mutations have been identified previously.^{4,6-8,15,16} Y132F is one of the most frequent *Erg11* mutations encountered (Table 3). This residue is believed to re-

side close to the access channel for azoles in the demethylase enzyme, which may alter entry of the inhibitor to its binding site.^{8,12} Mutation T229A was found unlinked to other resistance mechanisms. It is believed to reside on the F helix, which may contribute dynamically to the access channel for drug. Although, independent confirmation of the contribution of this mutation to resistance has not been reported. Amino acid substitutions involving E266D and V488I mutation are not likely to be important for high-level resistance because they have been observed in susceptible strains.⁸ Residue G464, which is highly conserved across all known species, appears to lie directly behind the plane of the heme prosthetic. The G464S mutation may interfere with coordination of the azole molecule. Finally, the S405F substitution lies immediately beyond the K helix near the substrate- and azole-binding pocket.⁸ The dynamic nature of the demethylase enzymes helps account for the numerous mutations that can confer varying levels of resistance and may result in either resistant or intermediate-level resistance such as S405F or can result in common mutations such as D116E, which have a weak or no apparent phenotype. Mutations F126L and K143R are often found together along with up-regulation of an efflux transporter, suggesting that their effect is weak. Overall, it appears that mutations T229A, Y132F, S405F, and G464S are sufficient to confer fluconazole resistance. Their frequency in the clinical resistant population is difficult to assess because the sample size in this study was too small to deduce statistical significance. Nevertheless, these alleles are likely to be reliable indicators that could serve as useful clinical markers in screening for triazole resistance.

The over-expression of multidrug efflux transporters represents the second major mechanism associated with resistance.^{11,16,17,20–22} The *C. albicans* genome contains in excess of 100 ORFs encoding putative ABC- and *mfs*-type transporters. In principle, if expressed at a suitable level, each gene product has the potential to export azole antifungals actively out of the cell. Yet, most studies of clinical isolates with resistant properties appear to be limited to the over-expression of relatively few transporter genes including *Mdr1*, *Cdr1*, and *Cdr2*, which were identified in this study as being closely linked to resistance ($p < 0.05$; Table 3). The up-regulation of *mdr* genes is a common cellular response to different types of stress. For this reason, establishing a baseline with susceptible isolates was critical to assign threshold values for specific gene over-expression, which allows a more reliable association with resistance (Fig. 1). It is also the first step toward using such measurements as a diagnostic indicator. Only one isolate showed any over-expression of *Flu1*, suggesting that this transporter does not contribute appreciably to clinical resistance. Furthermore, *Erg11* over-expression also showed a poor correlation ($p = 0.27$) with resistance despite a wide range in expression levels. The molecular mechanisms responsible for azole resistance in *C. albicans* are common to other *Candida* spp., as well as to less related fungi.^{4,5,8,21} The evolution of drug resistance in a susceptible strain may involve a single mechanism or may reflect a step-wise accumulation of mutations resulting in target site affinity changes^{8,12} and/or induction of various types of drug efflux transporters.^{4,7,20,22} The combination of resistance mechanisms seems to be associated with a high level of azole resistance in *C. albicans*, and an effective diagnostic must be able to scan multiple types of mechanisms.

Overall, >90% of the 32 resistant strains could be accounted for by target site mutations and/or up-regulation of drug efflux transporters. Only three strains, 31, 32, and 57 (containing statistically unlinked D116E, E266D mutations) had unknown mechanisms. These results suggest that existing mechanisms provide excellent coverage for non-culture-based assays of fluconazole resistance. As new targets emerge and are validated, they can be incorporated into the new generation of nucleic acid amplification systems with self-reporting probes capable of allele discrimination and quantitative assessment of target numbers.^{3,10,19}

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REFERENCES

1. Balashov S.V., R. Gardiner, S. Park, and D.S. Perlin. 2005. Rapid, high-throughput, multiplex, real-time PCR for identification of mutations in the *cyp51A* gene of *Aspergillus fumigatus* that confer resistance to itraconazole. *J. Clin. Microbiol.* **43**:214–222.
2. Calabrese, D., J. Bille, and D. Sanglard. 2000. A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. *Microbiology*. **146**:2743–2754.
3. Frade, J.P., D.W. Warnock, and B.A. Arthington-Skaggs. 2004. Rapid quantification of drug resistance gene expression in *Candida albicans* by reverse transcriptase LightCycler PCR and fluorescent probe hybridization. *J. Clin. Microbiol.* **42**:2085–2093.
4. Franz, R., S.L. Kelly, D.C. Lamb, D.E. Kelly, M. Ruhnke, and J. Morschhauser. 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob. Agents Chemother.* **42**:3065–3072.
5. Franz, R., M. Ruhnke, and J. Morschhauser. 1999. Molecular aspects of fluconazole resistance development in *Candida albicans*. *Mycoses*. **42**:453–458.
6. Loeffler, J., and D.A. Stevens. 2003. Antifungal drug resistance. *Clin Infect Dis* **36**:S31–S41.
7. Lopez-Ribot, J. L., R.K. McAtee, S. Perea, W.R. Kirkpatrick, M.G. Rinaldi, and T.F. Patterson. 1999. Multiple resistant phenotypes of *Candida albicans* coexist during episodes of oropharyngeal candidiasis in human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **43**:1621–1630.
8. Marichal, P., L. Koymans, S. Willemsens, D. Bellens, P. Verhasselt, W. Lutén, M. Borgers, F.C.S. Ramaekers, F.C. Odds, and H. Vanden Bossche. 1999. Contribution of mutations in the cytochrome P450 14a-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology* **145**:2701–2713.

9. **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.
10. **Perlin, D.S., and S. Park.** 2001. Rapid identification of fungal pathogens: molecular approaches for a new millennium. *Rev. Med. Microbiol.* **12**:S13–S20.
11. **Perea, S., J.L. Lopez-Ribot, W.R. Kirkpatrick, R.K. McAtee, R.A. Santillan, M. Martinez, D. Calabrese, D. Sanglard, and T.F. Patterson.** 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **45**:2676–2684.
12. **Podust, L. M., T.L. Poulos, and M.R. Waterman.** 2001. Crystal structure of cytochrome P450 14-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc. Natl. Acad. Sci. USA* **98**:3068–3073.
13. **Safdar, A., V. Chaturvedi, E.W. Cross, S. Park, E.M. Bernard, D. Armstrong, and D.S. Perlin.** 2001. Prospective study of *Candida* species in patients at a comprehensive cancer center. *Antimicrob. Agents Chemother.* **45**:2129–2133.
14. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. *Microbiology* **143**:405–416.
15. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14 α -demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.* **42**:241–253.
16. **Sanglard, D., F. Ischer, D. Calabrese, M.de Micheli, and J. Bille.** 1998. Multiple resistance mechanisms to azole antifungals in yeast clinical isolates. *Drug Res. Updates* **1**:255–265.
17. **Sanglard, D., and F.C. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis* **2**:73–85.
18. **Sheehan, D. J., C.A. Hitchcock, and C.M. Sibley.** 1999. Current and emerging azole antifungal agents. *Clin Microbiol Rev.* **12**:40–79.
19. **Tyagi, S., D.P. Bratu, and F.R. Kramer.** 1998. Multicolor molecular beacons for allele discrimination. *Nature Biotechnol.* **16**:49–53.
20. **White, T.C.** 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. *Antimicrob. Agents Chemother.* **41**:1482–1487.
21. **White, T.C., K.A. Marr, and R.A. Bowden.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11**:382–402.
22. **White, T.C., S. Holleman, F. Dy, L.F. Mirels, and D.A. Stevens.** 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrob. Agents Chemother.* **46**:1704–1713.

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