

Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil

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Abstract

In this study, we describe resistance mechanisms in fluconazole-resistant isolates of *C. albicans* isolated from AIDS patients from nine Brazilian hospitals. These mechanisms include the presence of point mutations in the *ERG11* gene and overexpression of *ERG11*, and several genes encoding efflux pumps, as measured by quantitative real-time reverse transcriptase polymerase chain reaction. Several fluconazole-resistant strains had multiple mechanisms of resistance. Four mutations previously described, Y132F, K143R, E266D, and V437I, were identified among the strains, whereas some isolates contained more than one mutation. Fourteen novel mutations were identified. Interestingly, all Brazilian fluconazole-resistant isolates showed homozygosity at mating-type loci (*MTL*) associated with fluconazole resistance. This is the first comprehensive assessment at molecular level of mechanisms of fluconazole resistance in *C. albicans* isolates from South America. © 2004 Elsevier Inc. All rights reserved.

1. Introduction

The frequency of life-threatening fungal infections is rising worldwide. Considering that most patients infected with opportunistic fungal agents have AIDS or neoplastic and/or degenerative diseases, it is clear that effective antifungal therapy is critical (Wenzel, 1995; Georgopapadakou, 1998; Latgé, 1999). Recently, treatment failures, combined with improvements in performance and standardization of antifungal susceptibility testing, have drawn attention to the problem of antifungal drug resistance. It is now well established that antifungal agents foster clinical and epidemiological situations that are analogous to those found with antibiotic-resistant bacteria (For a review, see Sanglard and Odds, 2002 and Loeffler and Stevens, 2003).

The predominant cause of fungal infections in hospitalized patients remains *Candida albicans*, a pathogenic yeast

that causes oral, vaginal, and systemic infections (De Backer et al., 2000). Triazole drugs such as fluconazole and itraconazole are commonly used to treat *Candida* infections. However, resistant strains often emerge during long-term or prophylactic treatment (White et al., 1998). Two major mechanisms of fluconazole resistance have been identified so far in these strains: (I) alterations in the drug target (14- α -sterol demethylase, the product of the *ERG11* gene), which results in an increased level of production of the enzyme or in its reduced binding affinity for fluconazole, and (II) a reduced level of intracellular fluconazole, which correlates with the overexpression of the *CDR1* and *CDR2* genes encoding transporters of the ABC family and of the *MDR1* and *FLU1* genes coding for major facilitators (White et al., 1998, 2002; Sanglard and Odds, 2002; Perea and Patterson, 2002; Morschhauser, 2002). It has already been observed that multiple mechanisms of fluconazole resistance can arise in a single *C. albicans* isolate (Albertson et al., 1996; Franz et al., 1998; Lopez-Ribot et al., 1999; White, 1997).

In this study, we evaluate resistance mechanisms of flu-

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Table 1
Patient demographics, MIC values of clinical isolates, and *ERG11* sequence analysis for isolate collection

Reference isolate number ^a	Patient's age and gender	FLC	ITRA	KTC	Occurrence of mutations in the <i>ERG11</i> gene
14	M-32	>64	0.25	0.5	F380S
23	M-42	>64	0.125	0.5	Y132F F145L
72	F-38	>64	0.125	0.125	NE
36	M-38	>64	0.03	2	Y79C T199I
17	M-27	64	1.0	0.5	ND
68	F-45	64	0.25	0.25	I253V V437I
8	M-NA	64	0.25	0.5	V130I E266D V488I
33	F-NA	64	0.25	0.25	K143E P503L
16	M-25	64	0.25	0.5	ND
21	M-33	32	0.5	1.0	H283D
15	M-42	32	0.25	0.5	ND
69	M-49	32	0.25	0.25	ND
85	M-36	32	0.5	0.125	NE
19	M-32	16	0.06	0.125	K143R
86	M-44	16	0.125	0.06	ND
61	M-36	8	0.03	0.06	K99T G303D L305P G307S G450E
51	M-37	4	0.06	0.06	R265G K342R
55	F-29	4	0.25	0.125	NE
5737	M-62	0.25	0.03	0.03	ND
5568	M-25	0.125	0.03	0.03	ND

^a Isolates were obtained from nine hospitals throughout Southeast Brazil.

NA, not available; M, male; F, female; ND, not detected; NE, not evaluated; FLC, fluconazole; ITRA, itraconazole; KTC, ketoconazole.

conazole-resistant strains of *C. albicans* isolated from AIDS patients from different medical institutions in Brazil. Our data show that some of the fluconazole-resistant strains have diverse mechanisms of resistance, including the presence of point mutations in the *ERG11* gene and overexpression of *ERG11*, and several genes encoding efflux pumps, as measured by quantitative real-time RT-PCR. To our knowledge, this is the first assessment at molecular level of fluconazole resistance mechanisms in *C. albicans* isolates from South America.

2. Materials and methods

2.1. *C. albicans* strains and cell culture

The *C. albicans* isolates used in this study represent a collection of 20 strains from 9 different hospitals that were obtained from AIDS patients with oral or esophageal candidiasis who received fluconazole during a 6- to 12-month period (See Table 1). Each isolate was obtained from a

different patient and was epidemiologically distinct. After being identified by conventional methods (Warren and Hazen, 1995), all the strains were frozen and maintained in the yeast stock collection of the Laboratorio Especial de Micologia-UNIFESP for different periods of time. Isolates were maintained in solid yeast-peptone-glucose (YEPD: 1% yeast extract, 2% Bacto peptone, and 2% D-glucose, 2% agar) medium.

2.2. Susceptibility testing

Antifungal susceptibility testing was performed by using the National Committee for Laboratory Standards (NCCLS) reference broth microdilution (NCCLS, 2002). Reference powders of fluconazole, and itraconazole and ketoconazole, were kindly provided by the manufacturers: Pfizer Pharmaceutical Group (New York, NY, USA) and Janssen (Titusville, NJ, USA), respectively. Amphotericin B was obtained from Sigma. Breakpoints and interpretative criteria were based on the NCCLS (2002) as described by White et al.

Table 2
List of primers and fluorescent probes used in this work

Primers and probes	Sequences	<i>C. albicans</i> genes (NCBI accession number)
CaCDR1	5'-FAM-CGA CCC GAG GTG CTG CCA TGT TCT TTG GGG TCG-Dabcyl-3'	<i>CDR1</i> (P43071)
CaCDR1F2816	5'-CTTAGTCAAACCACTGGATCG-3'	
CaCDR1R2900	5'-CCAAAAGTGTGAAAAAGGC-3'	
CaCDR2	5'-FAM-CCGTGGTGGGTGGATGCACCTGGACAATTCCACGG-Dabcyl-3'	<i>CDR2</i> (P785950)
CaCDR2F916	5'-CACGCTTTGTGCGCAACAGC-3'	
CaCDRR1020	5'-ATGTTGTGACTTGCAGTAGC-3'	
CaPMA1	5'-FAM-CGACCCGTCTGCCATTGAATCTTTGGGGTTCG-Dabcyl-3'	<i>PMA1</i> (P28877)
CaPMA1F1333	5'-GGCCAAGAAACAAGCTATTGT-3'	
CaPMA1R1406	5'-CGGAACACAAGATTTCAACAC-3'	
CaERG11	5'-FAM-CCGTGGTGGGAAAGTTTCTAAAGGGTTCACGG-Dabcyl-3'	<i>ERG11</i> (X13296)
CaERG11F1420	5'-ACTAGATGGGATACTGCTGC-3'	
CaERG11R1556	5'-CATCTATGTCTACCACCACC-3'	
CaMDR1	5'-CCGTGGAGTCCTTGTGGCCACTGGTGCCACGG-Dabcyl-3'	<i>MDR1</i> CAA76194
CaMDR1F3339	5'-TTCTTGGGTGGATTCTTCGC-3'	
CaMDR1R3451	5'-GCACCTAACTCCAAGCGGC-3'	
CaFLU1	5'-FAM-CCGCTGAAAATTTATATTGTGCATCTGCAGCGG-Dabcyl-3'	<i>FLU1</i> (CAD21151)
CaFLU1F809	5'-TGTTGCCTTGTGATGGTCCCG-3'	
CaFLU1R910	5'-ACCGATAAGGCAGCAAGACC-3'	
ACTIN ^a	5'-FAM-CCAGATTCGTCGTATTC-TAMRA-3'	<i>ACT1</i> (CAD48328)
ACTIN1-1F	5'-GGCTTCATTGTCTACTTTCCAACA-3'	
ACTIN1-1R	5'-TTTGTGGTGAACAATGGATGGA-3'	
<i>MTLα2</i> F	5'-CATGAATTCACATCTGGAGGCAC-3'	<i>MTLα2</i> (AF 167163)
<i>MTLα2</i> R	5'-ATAGCAAAGCAGCCAACCTCAGGT-3'	
<i>MTLα1</i> F	5'-TTCGAGTACATTCTGGTCGCG-3'	<i>MTLα1</i> (AF167163)
<i>MTLα1</i> R	5'-TGTAACATCCTCAATTGTACCCGA-3'	
<i>MTLa</i> F	5'-TTGAAGCGTGAGAGGCAGGAG-3'	<i>MTLa</i> (AF167163)
<i>MTLa</i> R	5'-GTTTGGGTTTCTTCTTTCTCATTC-3'	
<i>PAPα</i> R1	5'-AAGCTGCACTTACTGTTCCGACAC-3'	<i>PAPα</i> (AF167163)
<i>PAPα</i> F1	5'-AGAATGCCCTGTGATTACCCCG-3'	
<i>PAPα</i> R1	5'-GCATAATAGAAGAGCCGCGAGAG-3'	<i>PAPα</i> (AF 167163)
<i>PAPα</i> R1	5'-GCAAGATTGAATATTCCTCGCGT-3'	
CYPB	5'-GCGGATCCTTAAAACATACAAGTTCTCTTT-3'	<i>ERG11</i> (X13296)
CYPNS2	5'-ACGCGTGCACAATATGGCTATTGTTGAAACTGTC-3'	<i>ERG11</i> (X13296)
CAERG11-01	5'-TTAGGTCCAAAAGGTC-3'	<i>ERG11</i> (X13296)
CAERG11-03	5'-GACCGTTCATTTGCTC-3'	<i>ERG11</i> (X13296)
CAERG11-04	5'-GAGCAAATGAACGGTC-3'	<i>ERG11</i> (X13296)
CAERG11-08	5'-CCCATCTAGTTGGATC-3'	<i>ERG11</i> (X13296)

6-FAM, 6-carboxyfluorescein; Dabcyl, 4-(4'-dimethylaminophenylazo) benzoic acid succinimidyl ester; TAMRA, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine.

^a *Taq*-Man probe (an oligonucleotide 5'-terminally labeled with a reporter fluorophore like fluorescein and labeled internally or 3'-terminally with a quencher); all the other probes are molecular beacons (are oligonucleotides labeled on both ends; one end is attached to a reporter fluorophore, and the other end is attached to a quencher). For a review, see Wilhem and Pingoud (2003).

(2002). Controls for these experiments included previously characterized drug-susceptible isolates 5737 and 5568.

2.3. Sequencing of the *ERG11* gene

The entire open reading frame of the *ERG11* gene encoding lanosterol 14 α - demethylase was sequenced from all *C. albicans* isolates. The open reading frame was polymerase chain reaction (PCR) amplified using *Taq* DNA platinum polymerase high fidelity (Invitrogen) and primers described in Table 2. PCR conditions were as follows: 94°C for 2 minutes and 35 times 94°C for 1 minute, 55°C for 1

minute, and 68°C for 2 minutes, followed by an extension step at 68°C for 10 minutes. After the reaction, the approximately 1.7-kb PCR product was purified with a Qiagen PCR cleanup kit and inserted into TOPO TA cloning kit (Invitrogen) following manufacturer's instructions. Sequencing reactions were prepared using BigDyeTM Terminator Cycle Sequencing (Applied Biosystems) and primers described in Table 2. The nucleotide sequences were determined in both strands by primer elongation with an ABI3100 automated DNA sequencer (Applied Biosystems). Sequence data were compared with a published *ERG11* sequence (Lai and Kirsch, 1989) using BLAST (Altschul et al., 1997).

2.4. PCR analysis of the (mating type locus) MTL locus

The PCR protocol used to analyze the MTL locus was the following: 94°C for 5 minutes, 35 times 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and an extension step at 72°C for 10 minutes. The presence of *MTLa* was ascertained by amplifying *MTLa1* and the associated gene *PAPa* (Hull and Johnson, 1999). The presence of *MTL α* was ascertained by amplifying *MAT α 1* and *MAT α 2* and the associated gene *PAP α* . These three different genes, *MTL α 1*, *MTL α 2*, and *MTLa1*, are the *C. albicans* homologues of *Saccharomyces cerevisiae* mating-type genes *MAT α 1*, *MAT α 2*, and *MATa1*, respectively (Hull and Johnson, 1999).

2.5. DNA and RNA isolation, and real-time RT-PCR

Yeast cells were grown to logarithmic phase in 125-mL Erlenmeyer flasks containing 25 mL of YEPD at 32°C with constant shaking. DNA was prepared using glass beads (Scherer and Stevens, 1987). For real-time reverse transcriptase polymerase chain reaction (RT-PCR) experiments, the different isolates were propagated in YEPD medium and harvested while growing in antifungal drug-free medium in logarithmic phase. The cells were washed thoroughly with sterile water, disrupted by vortexing with glass beads; then total RNA was extracted with Trizol (Life Technologies, USA). To verify RNA integrity, 20 micrograms of RNA from each treatment were fractionated in a 2.2-M formaldehyde, 1.2% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. The presence of intact 28S and 18S ribosomal RNA bands (semiquantitatively in a 2:1 ratio) was used as a criterion to determine whether the RNA was degraded. RNase-free DNase treatment was done as previously described by Semighini et al. (2002). The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the *ACT1* gene. cDNA was synthesized by using the SuperScript reverse transcriptase (Gibco, BRL).

All the RT-PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystem, USA). The Taq- Man^R PCR Reagent kit was used for PCR reactions. The thermal cycling conditions comprised an initial step at 50°C for 2 min, followed by 10 minutes at 95°C, and 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The reactions and calculations were performed according to Semighini et al. (2002). Primer and probe sequences are described in Table 2.

3. Results

3.1. Antifungal susceptibility testing of clinical isolates and sequencing of *ERG11* genes

The minimal inhibitory concentration (MIC) values obtained for 20 *C. albicans* isolates with the three different

azoles (fluconazole, itraconazole, and ketoconazole) are summarized in Table 1. The 20 *C. albicans* isolates included 9 that were resistant to fluconazole (MICs ≥ 64 $\mu\text{g/mL}$), 6 that were susceptible-dose dependent (S-DD for MICs of 16 and 32 $\mu\text{g/mL}$), and 5 that were susceptible to fluconazole (MICs < 8 $\mu\text{g/mL}$). Regarding itraconazole, 1 isolate was considered resistant to itraconazole (MIC ≥ 1 $\mu\text{g/mL}$), 10 were considered as S-DD (MICs of 0.25 and 0.5 $\mu\text{g/mL}$), and 9 were susceptible to itraconazole (MIC $> 0.25 < 0.5$) (Table 1). All of them were susceptible to amphotericin B (MICs < 1 $\mu\text{g/mL}$).

Clinical breakpoints for ketoconazole have not been proposed, but they are likely to be close to the breakpoints for itraconazole, because the drug concentrations achievable in blood are similar for both drugs when similar doses are administered (White et al., 2002). We have considered an MIC of ≥ 1 $\mu\text{g/mL}$ to define ketoconazole resistance in this study, susceptible-dose dependent (S-DD) for MICs of 0.25 and 0.5 $\mu\text{g/mL}$, and finally MICs ≤ 0.125 for susceptible isolates. Considering the mentioned breakpoints for ketoconazole, 11 out of 20 isolates were S-DD/resistant to ketoconazole.

Table 1 shows that 12 out 15 isolates S-DD or resistant to fluconazole were also considered as S-DD (9 for itra and keto) or resistant (1 for itra and 2 for keto) to the other azoles. Otherwise, only 1 out of 5 isolates susceptible to fluconazole exhibited an MIC value compatible with S-DD.

Naturally occurring *ERG11* mutations identified in *C. albicans*azole-resistant isolates are clustered in three diffuse hot-spot regions in the primary sequence, including amino acid regions 105 to 165, 266 to 287, and 405 to 488 (Marichal et al., 1999; Perea et al., 2001). Sequence analysis of the entire *ERG11* from our isolates was performed. As expected from unrelated clinical isolates, frequent silent mutations that do not change the protein sequence were identified (data not shown). The sequence alterations that resulted in changes in the protein sequences are listed in Table 1. Mutations that change the protein sequence were not identified in the susceptible isolates 5737 and 5568. Several mutations, located in the three hot spots, were observed in all the isolates, except for isolates 15, 16, 17, 69, and 86, which did not show any mutation. Isolate 61 contained five mutations (K99T, G303D, L305P, G307S, and G450E). Isolate 8 contained three mutations (V130I, E266D, and V488I), whereas isolates 23, 33, 36, 51, and 68 contained two mutations (Y132F and F145L; K145E and P503L; Y79C and T199I; R265G and K342R; I253V and V437I, respectively). Isolates 14, 19, and 21 contained a single mutation (F380S, K143R, and H283D, respectively). We did not find in these isolates mutations in only one allele (i.e., heterozygous for the mutation), but only point mutations in both alleles (i.e., homozygous for the mutation).

3.2. *ERG11*, *CDR1*, *CDR2*, *MDR1*, and *FLU1* genes implicated in azole resistance

Overexpression of the genes *ERG11*, *CDR1*, *CDR2*, *MDR1*, and *FLU1* has been linked to fluconazole resistance

Table 3
ERG11 and efflux transporter gene mRNA expression levels in the clinical isolates as assessed by real-time RT-PCR

Isolate number ^o	MIC fluconazole ($\mu\text{g/mL}$)	<i>FLU1/ACT1</i> ^a	<i>MDR1/ACT1</i> ^a	<i>CDR1/ACT1</i> ^a	<i>CDR2/ACT1</i> ^a	<i>ERG11/ACT1</i> ^a
14	>64	1.7 (1.6–1.9)	0.7 (0.7–0.7)	3.7 (3.2–4.2)	24.0 (22.0–27.0)	14.4 (12.5–16.2)
23	>64	1.0 (1.0–1.0)	1.3 (1.3–1.3)	1.2 (1.2–1.2)	0.8 (0.8–0.8)	0.77 (0.77–0.75)
72	>64	5.3 (5.1–5.4)	4.7 (2.7–6.7)	4.2 (4.0–4.3)	43.3 (39.3–47.3)	26.0 (25.6–26.3)
36	>64	5.0 (4.8–5.1)	2.7 (2.7–2.7)	2.8 (2.6–3.0)	4.5 (4.3–4.8)	5.8 (5.7–5.9)
17	64	2.1 (2.0–2.3)	0.7 (0.7–0.7)	3.0 (3.1–2.9)	21.3 (21.0–22.0)	11.2 (11.4–11.1)
68	64	2.3 (2.3–2.3)	1.3 (1.3–1.3)	5.8 (5.7–5.8)	386.3 (379.8–392.8)	12.1 (12.0–12.2)
8	64	5.3 (4.7–5.9)	6.7 (5.3–8.0)	2.9 (3.0–2.9)	4.3 (3.8–4.8)	4.3 (4.0–4.6)
33	64	1.9 (1.9–1.9)	12.0 (10.0–14.0)	2.6 (2.7–2.6)	19.5 (18.8–20.3)	5.1 (5.2–5.1)
16	64	2.0 (1.9–2.1)	6.0 (6.0–6.0)	4.3 (4.2–4.4)	24.8 (22.8–26.8)	21.3 (21.4–21.2)
15	32	0.9 (0.9–0.9)	0.7 (0.7–0.7)	3.1 (3.0–3.2)	8.0 (7.8–8.3)	3.5 (3.4–3.6)
21	32	2.9 (2.7–3.0)	2.0 (2.0–2.0)	4.7 (4.5–4.9)	1,241 (1,236–1,247)	7.6 (7.6–7.5)
69	32	4.4 (4.3–4.4)	70.0 (69.3–70.7)	4.0 (4.0–4.0)	15.5 (14.0–17.0)	2.9 (2.9–3.0)
85	32	3.0 (2.9–3.1)	2.7 (2.0–3.3)	7.7 (7.8–7.5)	186.5 (183.8–189.3)	15.2 (15.3–15.1)
19	16	5.0 (5.0–5.0)	57.3 (53.3–61.3)	1.8 (1.9–1.8)	2.3 (2.3–2.3)	8.0 (7.9–8.2)
86	16	2.3 (2.3–2.3)	3.3 (2.7–4.0)	2.8 (2.7–2.9)	21.0 (20.8–21.3)	3.9 (3.8–4.0)
61	8	4.1 (3.3–5.0)	2.0 (2.0–2.0)	0.9 (0.8–0.9)	3.5 (3.5–3.5)	10.3 (9.6–11.0)
51	4	3.6 (3.4–3.7)	27.3 (24.7–30.0)	3.5 (3.5–3.5)	82.5 (75.0–90.0)	2.1 (2.0–2.2)
55	4	2.3 (2.3–2.3)	5.3 (4.7–6.0)	4.0 (4.1–4.0)	9.5 (9.3–9.8)	4.4 (4.4–4.4)
5737 and 5568	0.125 and 0.25	1.0	1.0	1.0	1.0	1.0

^a cDNA levels were calculated relative to those of the average cDNA levels of the isolates 5737 and 5568. The numbers represent the averages of 3 replicates, with the ranges shown in parentheses.

(White et al., 1998) and was investigated as a mechanism of resistance in our clinical isolates by using real-time RT-PCR (Table 3). The cDNA levels of the different genes were normalized using the *ACT1* gene (encoding actin); comparable results were observed when the *PMA1* gene (encoding the H^+ -ATPase) was used as a normalizer gene (data not shown). Because a matched set of isolates was not available for this collection of fluconazole-resistant isolates, the mRNA expression levels of isolates 5737 and 5568 were used as controls. Table 3 illustrates the number of times each respective gene was expressed greater than the average expression of the same gene in the control isolates (where the mRNA expression levels were given a value of 1.0). When this criterion is used, the isolates that are resistant to fluconazole expressed *ERG11* at much higher levels (11.2 to 14.4 times) than the average in isolates 14, 16, 17, 68, and 72. The *CDR1* gene was more highly expressed in isolates 16, 68, and 72, whereas the *CDR2* gene was more highly expressed in the isolates 14, 16, 17, 33, 68, and 72. *MDR1* was more highly expressed in the isolate 33, whereas *FLU1* was more highly expressed in isolates 8, 36, and 72. Considering the isolates SSD to fluconazole, the *ERG11* is more expressed in the isolate 85, whereas the *CDR1* gene is more expressed in the isolates 21 and 85. The *CDR2* is more expressed in the isolates 21, 69, 85, and 86. *MDR1* is more expressed in the isolates 19 and 69, whereas the *FLU1* is more expressed in the isolate 19. Interestingly, some isolates that show susceptibility to fluconazole, such as 51, 55, and 61, show higher expression of *MDR1* and *CDR2*, *CDR2*, and *ERG11*, respectively. Taken together, our results show that some isolates concomitantly overexpressed different genes involved in drug resistance in *C. albicans*. This

indicates that multiple mechanisms are operating to confer fluconazole resistance in these isolates. However, in isolate 23, which is resistant to fluconazole and SSD to ketoconazole, we were not able to see any of these genes overexpressed. Taken together, our results suggest that there is no clear correlation among different levels of expression of the transporter genes and MICs.

3.3. PCR screen of the *MTL* loci of clinical isolates of *C. albicans*

Recently, Rustad et al. (2002) presented evidence suggesting a link between homozygosity at the *MTL* locus and fluconazole resistance in *C. albicans*. An analysis of 46 fluconazole-sensitive and 50 fluconazole-resistant strains revealed that whereas only 2% of the former were homozygous for the *MTL* locus, (i.e., either *a/a* or α/α), 22% of the latter were homozygous. We investigated the *MTL* loci of our clinical isolates by PCR amplifying three *MTL* genes: *MTL α 1*, *MTL α 1*, and *MTL α 2*. PCR fragments from *MTL α 1* and *MTL α 1* were generated for 10 of the 20 clinical isolates screened. Genomic DNA from four of the isolates did not amplify *MTL α 1*; DNA from six unrelated isolates did not amplify *MTL α 1* (Fig. 1A). None of these 10 isolates homozygous at the *MTL* locus (*MTL*_{hom}) were fluconazole susceptible (Table 1 and Fig. 1A). To determine the extent of the loss of heterogeneity, the presence of *PAP α* and *PAP α* was determined by PCR in the ten *MTL*_{hom} strains (Fig. 1B). The four strains missing *MLT α 1* (*MTL*_{hom}) were found to also lack *MTL α 2*. In addition, the *PAP α* gene was present in only one of these *MTL*_{hom} strains. The *PAP α* gene was not present in all four *MTL*_{hom}. The fact that the

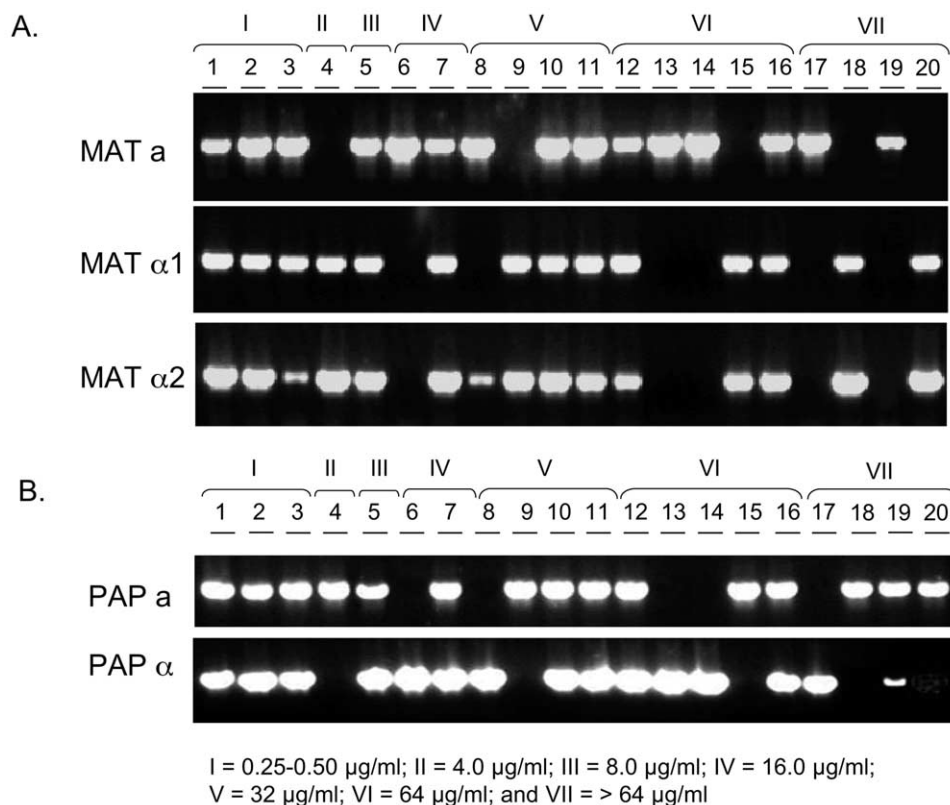


Fig. 1. PCR screen of *MTL* genotype in clinical isolates. DNA fragments from within the *MTLa1*, *MTLa1*, and *MTLa2* genes (A) and *PAPa* and *PAP α* (B) were amplified using genomic DNA from 20 clinical isolates and visualized on EtBr-stained gels. The order of the isolates is based on their MIC: I = < 0.25 $\mu\text{g/ml}$ fluconazole (isolates 5568 and 5737); II = 4 $\mu\text{g/ml}$ (isolates 51 and 55); III = 8 $\mu\text{g/ml}$ (isolate 61); IV = 16 $\mu\text{g/ml}$ (isolates 19 and 86); V = 32 $\mu\text{g/ml}$ (isolates 15, 21, 69, and 85); VI = 64 $\mu\text{g/ml}$ (isolates 8, 16, 17, 33, and 68); and VII = > 64 $\mu\text{g/ml}$ (isolates 14, 23, 36, and 72). Interestingly, all Brazilian fluconazole resistant isolates showed homozygosity at mating type (*MTL*) loci associated with fluconazole resistance.

loci-specific *PAP* and *MTL* genes do not segregate in these 10 *MTL*_{hom} strains indicates that the loss of heterozygosity does not extend through the entire 9-Kb *MTL* locus in these strains. Our results suggest that homozygosity at either locus is associated with fluconazole resistance.

4. Discussion

The clinical isolates used in this study were screened for the currently characterized molecular mechanisms of azole resistance, and to our knowledge, this represents the first time such an analysis has been performed on South American *C. albicans* isolates. Naturally occurring *ERG11* mutations in *C. albicans* azole-resistant clinical isolates can be divided into four hot-spot regions on the basis of their association with different structural regions observed in the recently described *MTCYP51* structure (Podust et al., 2001). The first hot spot (comprising G464S, G465S, and R476K) associates with the N-terminal part of the cysteine pocket. A second hot spot is mapped to the C-terminus of the G helix and H helix, and a third hot spot (comprising F72L, F105L, S495F, and T229A) associates with the domain interface.

The fourth hot spot (comprising D116E, F126L, K128T, G129A, Y132H, K143R, F145L, K147R, A149V, and D153E) is located in the region between the B' and C helices that have been postulated to be involved in inhibitor- or substrate-induced structural changes. The mutations identified in *C. albicans* fluconazole-resistant isolates indicate that azole resistance in fungi develops in protein regions involved in orchestrating the passage of CYP51p through different conformational stages rather than in residues directly contacting the triazole. We were able to identify four mutations previously described as being involved in azole resistance in *C. albicans*, Y132F (Y132H), K143R, E266D, and V437I (Marichal et al., 1999; White et al., 2002). Some of our isolates contained more than one mutation, and 14 novel mutations were identified among them. These novel mutations are being further characterized to validate that they actually mediate fluconazole resistance. However, their presence suggests that the clinical environment in Brazil can select novel mutations in *C. albicans* *ERG11*.

Real-time RT-PCR assays were used to obtain more accurate data on gene expression in *C. albicans*. This approach is preferable over conventional Northern blot anal-

ysis, because of the narrower linear range associated with radioactively labeled probes, and has been successfully used in the characterization of gene expression of sigma factor genes in *Mycobacterium tuberculosis* (Manganelli et al., 1999) and, more recently, in the quantitative expression of ABC transporter encoding genes in *Aspergillus* spp. (Semighini et al., 2002; Nascimento et al., 2003). We assessed the quantitative expression of two ABC transporters, *CDR1* and *CDR2*, and two MFS transporters, *MDR1* and *FLU1*, and also *ERG11*. Some clinical isolates showed increased mRNA expression of the *ERG11* gene, which encodes the target 14- α - demethylase. White et al. (2002) have previously shown, in a collection of fluconazole-resistant and -susceptible isolates, considerable variation in the levels of *ERG11* mRNA expression, but this expression did not seem to be related to the azole resistance of the isolates. However, *ERG11* overexpression has been found in many other fluconazole-resistant *C. albicans* isolates compared with matched susceptible isolates (Morschhauser, 2002). Overexpression of *ERG11* from *C. albicans* has been shown to confer a fivefold enhanced resistance to fluconazole in *S. cerevisiae* (Lamb et al., 1997). Therefore, constitutive *ERG11* overexpression may contribute to fluconazole resistance in our clinical *C. albicans* isolates.

An important mechanism of fluconazole resistance is reduced intracellular accumulation of the drug. Sanglard et al. (1995) demonstrated that many fluconazole-resistant, clinical *C. albicans* isolates displayed strongly increased mRNA levels of *CDR1* or *MDR1* compared to the parental strains and accumulated less intracellular fluconazole. *CDR2* was also observed in fluconazole-resistant clinical *C. albicans* isolates (Sanglard et al., 1997). Recently, a gene that is homologous to *MDR1*, *FLU1*, has been isolated by its ability to confer fluconazole resistance on hypersusceptible *S. cerevisiae* transformants (Calabrese et al., 2000). Overexpression of *CDR1*, *CDR2*, and *MDR1* genes has been shown to be the most frequent mechanism of fluconazole resistance (Morschhauser, 2002). Accordingly, most of our clinical isolates showed increased mRNA expression of these transporters. In addition, three clinical isolates also showed increased mRNA expression of *FLU1*. None of the transporter genes was induced in the clinical isolate 23. However, this isolate has a F145L mutation in the *ERG11* gene that may be responsible for the observed fluconazole resistance. Only two fluconazole-resistant isolates were truly resistant to itraconazole or ketoconazole, but six of them were considered as S-DD to itraconazole and/or ketoconazole. This reduced drug susceptibility could be due to the high levels of mRNA expression of the transporter genes exhibited by most of these isolates.

Some clinical isolates, such as 68 and 72, showed increased mRNA expression of more than one transporter gene. These clinical isolates could harbor dominant mutations that resulted in concomitant constitutive transcriptional activation of the transporter genes. Such behavior is well known for the *S. cerevisiae* transcriptional activators

PDR1 and *PDR3*, which positively influence expression of drug transporter genes such as *PDR5* (Kolaczowski et al., 1998).

White (1997) has previously shown that gene conversion or mitotic recombination associated with the *ERG11* gene, located on chromosome 5, is associated with azole resistance. Rustad et al. (2002) showed that in a collection of fluconazole-resistant strains of *C. albicans*, there was a higher proportion of homozygotes for the mating-type locus (*MTL*) than in a collection of fluconazole-sensitive isolates, suggesting the possibility that when cells become *MTL* homozygous, they acquire intrinsic drug resistance. Pujol et al. (2003) used an opposite strategy to investigate this possibility. Instead of looking for fluconazole-resistant strains of *C. albicans* in a heterogeneous population of *MTL* homozygotes and heterozygotes, drug susceptibility was measured in a collection of isolates selected for *MTL* homozygosity. The majority of these isolates had not been exposed to antifungal drugs. The level of drug susceptibility was compared between spontaneously generated *MTL*-homozygous progeny and their *MTL*-homozygous parent strains, which had not been exposed to antifungal drugs. The results demonstrate that naturally occurring *MTL*-homozygous strains are not intrinsically more drug resistant, supporting the hypothesis that either the higher incidence of *MTL* homozygosity involved a drug resistance gene linked to the *MTL* locus, or that *MTL*-homozygous strains may be better at developing drug resistance upon exposure to drug than *MTL*-heterozygous strains. We have found that the Brazilian fluconazole-resistant isolates are homozygous at either locus associated with fluconazole resistance.

In summary, we demonstrated that (i) the same mechanisms of fluconazole resistance already described in European and North American *C. albicans* isolates are present also in Brazilian isolates, and (ii) in spite of higher mRNA expression levels for *ERG11* and transporter genes, and different mutations in the fluconazole-resistant isolates, there was no linear relationship between these phenomena and MIC values. Putative resistance mechanisms can be found even in susceptible isolates. This suggests that these mechanisms alone are not sufficient, and most probably there are other putative mechanisms of resistance not yet described that could provide a better correlation with drug resistance.

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References

- Albertson GD, Niimi M, Cannon RD, Jenkinson HF (1996). Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. *Antimicrob Agents Chemother* 40, 2835–2841.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W (1997). Lipman DJ Gapped blast and Psi-Blast: A new generation of protein database search programs. *Nucl Acids Res* 25, 3389–3402.
- Calabrese D, Bille J, Sanglard D (2000). A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (FLU1) conferring resistance to fluconazole. *Microbiology* 146, 2743–2754.
- De Backer MD, Magee PT, Pla J (2000). Recent developments in molecular genetics of *Candida albicans*. *Ann Rev Microbiol* 54, 463–498.
- Franz R, Kelly SL, Lamb DC, Kelly E, Ruhnke M, Morschhauser J (1998). Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob Agents Chemother* 42, 3065–3072.
- Georgopapadakou NH (1998). Antifungals: Mechanism of action and resistance, established and novel drugs. *Curr Opin Microbiol* 1, 547–557.
- Hull CM, Johnson AD (1999). Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* 285, 1271–1275.
- Kolaczowski M, Kolaczowska A, Luczynski J, Witek S, Goffeau A (1998). In vivo characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. *Microbe Drug Resistance* 4, 143–158.
- Lai MH, Kirsch DR (1989). Nucleotide sequence of cytochrome P450L1A1 (lanosterol 14 alpha-demethylase) from *Candida albicans*. *Nucl Acids Res* 17, 804.
- Latgé JP (1999). *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 12, 310–350.
- Lamb DC, Kelly DE, Schunck WH, Shyadehi AZ, Akhtar M, Lowe DJ, Baldwin BC, Kelly SL (1997). The mutation T315A in *Candida albicans* sterol 14alpha-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. *J Biol Chem* 272, 5682–5688.
- Loeffler J, Stevens DA (2003). Antifungal drug resistance. *Clin Infect Dis* 36, S31–41.
- Lopez-Ribot JL, McAtee RK, Perea S, Kirkpatrick WR, Rinaldi MG, Patterson TF (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.
- Manganelli R, Dubnau E, Tyagi S, Kramer FR, Smith I (1999). Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol Microbiol* 31, 715–24.
- Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FCS, Odds FC, Vanden Bossche H (1999). Contribution of mutations in the cytochrome P450 14 alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology* 10, 2701–2713.
- Morschhauser J (2002). The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochim Biophys Acta* 1587, 240–248.
- Nascimento AM, Goldman GH, Park S, Marras SAE, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS (2003). Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob Agents Chemother* 47, 1719–1726.
- National Committee for Clinical Laboratory Standards (2002). Reference method for both dilution antifungal susceptibility testing of yeasts. Approved standard. NCCLS document M27A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Perea S, Patterson TF (2002). Antifungal resistance in pathogenic fungi. *Antimicrob Resist* 35, 1073–1080.
- Perea S, López-Ribot JL, Kirkpatrick WR, McAtee RK, Santillán RA, Martínez M, Calabrese D, Sanglard D, Patterson TF (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.
- Podust LM, Poulos TL, Waterman MR (2001). Crystal structure of cytochrome P450 14 α -sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc Natl Acad Sci USA* 98, 3068–3073.
- Pujol C, Mecer SA, Pfaller M, Soll DR (2003). Drug resistance is not directly affected by mating type locus zygosity in *Candida albicans*. *Antimicrob Agents Chemother* 47, 1207–1212.
- Rustad TR, Stevens DA, Pfaller MA, White TC (2002). Homozygosity at the *Candida albicans* MTL locus associated with azole resistance. *Microbiology* 148, 1061–1072.
- Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* 39, 2378–2386.
- Sanglard D, Ischer F, Monod M, Bille J (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.
- Sanglard D, Odds FC (2002). Resistance of *Candida* species to antifungal agents: Molecular mechanisms and clinical consequences. *Lancet Infect Dis* 2, 73–85.
- Scherer S, Stevens DA (1987). Application of DNA fingerprinting methods to epidemiology and taxonomy of *Candida* species. *J Clin Microbiol* 25, 675–679.
- Semighini CP, Marins M, Goldman MHS, Goldman GH (2002). Quantitative analysis of the relative transcript levels of ABC transporter *Atr* genes in *Aspergillus nidulans* by Real-Time Reverse Transcription-PCR assay. *Appl Environ Microbiol* 68, 1351–1357.
- Warren NG, Hazen KC (1995). *Candida*, *Cryptococcus*, and other yeasts of medical importance. In *Manual of Clinical Microbiology*. Ed, PR Murray. Washington, DC: ASM Press, pp 723–737.
- Wenzel RP (1995). Nosocomial candidemia: Risk factors and attributable mortality. *Clin Infect Dis* 20, 1531–1534.
- Wilhem J, Pingoud A (2003). Real-time polymerase chain reaction. *Chem-BioChem* 4, 1120–1128.
- White TC (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.
- White TC, Marr KA, Bowden RA (1998). Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev* 11, 382–402.
- White TC, Holleman S, Dy F, Mirels LF, Stevens DA (2002). Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrob Agents Chemother* 46, 1704–1713.