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Candida parapsilosis Resistance to Fluconazole: Molecular Mechanisms and *In Vivo* Impact in Infected *Galleria mellonella* Larvae

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***Candida parapsilosis* is the main non-*albicans Candida* species isolated from patients in Latin America. Mutations in the *ERG11* gene and overexpression of membrane transporter proteins have been linked to fluconazole resistance. The aim of this study was to evaluate the molecular mechanisms in fluconazole-resistant strains of *C. parapsilosis* isolated from critically ill patients. The identities of the nine collected *C. parapsilosis* isolates at the species level were confirmed through molecular identification with a TaqMan qPCR assay. The clonal origin of the strains was checked by microsatellite typing. The *Galleria mellonella* infection model was used to confirm *in vitro* resistance. We assessed the presence of *ERG11* mutations, as well as the expression of *ERG11* and two additional genes that contribute to antifungal resistance (*CDR1* and *MDR1*), by using real-time quantitative PCR. All of the *C. parapsilosis* (*sensu stricto*) isolates tested exhibited fluconazole MICs between 8 and 16 μg/ml. The *in vitro* data were confirmed by the failure of fluconazole in the treatment of *G. mellonella* infected with fluconazole-resistant strains of *C. parapsilosis*. Sequencing of the *ERG11* gene revealed a common mutation leading to a Y132F amino acid substitution in all of the isolates, a finding consistent with their clonal origin. After fluconazole exposure, overexpression was noted for *ERG11*, *CDR1*, and *MDR1* in 9/9, 9/9, and 2/9 strains, respectively. We demonstrated that a combination of molecular mechanisms, including the presence of point mutations in the *ERG11* gene, overexpression of *ERG11*, and genes encoding efflux pumps, are involved in fluconazole resistance in *C. parapsilosis*.**

Candida parapsilosis (*sensu lato*) is a common human opportunistic pathogen that is able to cause superficial and invasive diseases and is especially prevalent in neonates and adult patients with catheter-related fungemia (1–3). *C. parapsilosis* (*sensu lato*) is the most common non-*albicans Candida* (NAC) species isolated from bloodstream infections in Spain, Italy, and many countries in Latin America and is also becoming prevalent at U.S. medical centers (4–10).

Although *C. parapsilosis* strains are usually susceptible to azoles, recent reports indicate the emergence of invasive infections due to fluconazole (FLC)-resistant *C. parapsilosis* isolates (11–16). Azole drugs, especially FLC, are commonly used to treat *Candida* infections because of their safety and the availability of oral and intravenous formulations (17, 18). This family of antifungal agents prevents the synthesis of ergosterol, a major component of fungal plasma membranes, by inhibiting the cytochrome P450-dependent enzyme lanosterol 14 α-demethylase (19).

FLC resistance in *C. albicans* may occur in two ways, (i) reduced FLC accumulation caused by active efflux of drugs, resulting particularly from overexpression of the *CDR1*, *CDR2*, and *MDR1* genes (20–23), and (ii) an alteration in the drug target that results in an increased level of production of the enzyme or in its reduced binding affinity for FLC (22–26). However, it is still not clear whether or not these mechanisms are also relevant for NAC species, including *C. parapsilosis*. In this study, we evaluated the mechanisms of FLC resistance in *C. parapsilosis* recovered during an outbreak of candidemia documented in a single hospital in Brazil.

(Some of the data included in this report were presented in part at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 5 to 9 September 2014 [57].)

MATERIALS AND METHODS

***C. parapsilosis* strains.** *C. parapsilosis* (*sensu lato*) isolates included in the present study were obtained from intensive care unit patients with candidemia enrolled in a retrospective survey conducted from July 2011 to February 2012 at a Brazilian institution (27). Initial yeast identification and susceptibility testing were performed with the Vitek II system (bioMérieux, Marcy-l'Etoile, France). Nine *C. parapsilosis* isolates for which the azoles MICs exceeded the established susceptibility breakpoints were sent to a reference laboratory (Laboratório Especial de Micologia, Universidade Federal de São Paulo, São Paulo, Brazil) for further molecular identification and confirmation of antifungal susceptibility by the CLSI reference method. Resistant strains were selected for *in vivo* studies and molecular characterization of mechanisms of FLC resistance. In addition, reference strain *C. parapsilosis* ATCC 22019 was included as a control organism in all laboratory tests.

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TABLE 1 Oligonucleotide sequences used in this study

Oligonucleotide[a]	Sequence (5′ to 3′)	Purpose
MDR1_CP_F	GATTTTTCGCTAGTCCGTGTTTG	*MDR1* real-time PCR
MDR1_CP_R	TGTAGGCGCATAGGTCTCAGGT	
ERG11_CP_F	GTACACCGTCATTACTCTACCCAACA	*ERG11* real-time PCR
ERG11_CP_R	TGCTCCTTTCATTTACAACATCATTT	
CDR1_CP_F	ATTTGCCGACATCCACCGTTAGG	*CDR1* real-time PCR
CDR1_CP_R	ACCATGCTGTTTGCGAGTCCA	
ERG11_CP_F1	CGAGATAATCATCAACGAACATTC	*ERG11* sequencing
ERG11_CP_R1	CGTTTAAAACATCCAAAGACCTTA	
ERG11_CP_F2	AATCTGAGGGTTTCCTTGATGGT	
ERG11_CP_R2	AAAGACCGCATTGACTACCGAT	

[a] The letters F and R in the primer names describe the 5′-to-3′ orientations of the primers as follows: F, forward (sense); R, reverse (antisense).

Molecular identification of *C. parapsilosis* (*sensu lato*) isolates by real-time TaqMan qPCR assays. DNA was extracted from the isolates by mechanical disruption with glass beads and phenol-chloroform (28). Real-time quantitative PCR (qPCR) was performed with species-specific TaqMan probes as previously described by our group (29).

***In vitro* susceptibility testing.** Antifungal susceptibility testing was performed with the CLSI microdilution assay (30). FLC, voriconazole (VRC), and anidulafungin (ANF) were provided by the Pfizer Pharmaceutical Group (New York, NY), and amphotericin B (AMB) was provided by the Sigma Chemical Corporation (St. Louis, MO). The interpretative guidelines in CLSI document M27-S4 were used classify *C. parapsilosis* isolates as susceptible, susceptible dose dependent, or resistant to antifungals (31, 32).

Microsatellite typing. Genomic DNA of *C. parapsilosis* isolates was extracted from 48-h-old cultures with the MagNA Pure 96 platform (Roche Diagnostics, Almere, The Netherlands) as described previously (33). Two multiplex PCRs were performed to amplify three trinucleotide repeat regions in one PCR and three hexanucleotide repeat regions in the second PCR; the setup of these assays has been described previously (34). Subsequently, the PCR products were diluted 20× with double-distilled H_2O (ddH_2O) and 1.0 μl of this dilution was added to a mixture of 0.1 μl of CC-500ROX (Promega, Leiden, The Netherlands) and 8.9 μl of ddH_2O. Prior data analysis, samples were boiled for 1 min at 95°C and then cooled to 4°C. Data analysis was performed on an ABI3500xL genetic analyzer platform and subsequently analyzed with GeneMapper software (Applied Biosystems, Palo Alto, CA). Microsatellite profiles were imported into BioNumerics v6.6 (Applied Maths, Sint-Martens-Latem, Belgium), and a dendrogram was generated by treating the data as categorical values, followed by cluster analysis by the unweighted-pair group method using average linkages. A comparison was made with a selection of Austrian isolates from a recent study (34).

Sequencing of the *ERG11* gene. The entire open reading frame (ORF) of the *ERG11* gene encoding lanosterol 14 α-demethylase was amplified and sequenced with specific primers (Table 1). PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced on the ABI 3100 genetic analyzer platform (Applied Biosystems). DNA sequences and the corresponding amino acid sequences were analyzed with the SeqMan II and EditSeq software packages (Lasergene v8.0; DNAStar, Madison, WI).

Relative quantification of gene expression by RT-qPCR. Reverse transcription (RT)-qPCR was undertaken to estimate the expression of the *CDR1*, *MDR1*, and *ERG11* genes by *C. parapsilosis* strains during FLC exposure (after 1.5 h of exposure). Experiments were repeated three times.

RNA extraction. An overnight culture of each isolate grown in 2 ml of morpholinepropanesulfonic acid (MOPS)-buffered RPMI (RPMI-MOPS) was diluted to an initial inoculum of 10^5 CFU/ml in fresh RPMI-MOPS and grown at 37°C with shaking at 250 rpm. The isolates were exposed to the MIC of FLC, which was added after 6 h of growth (to cells in log-phase growth) and continued for 1.5 h. Following drug exposure, cells were harvested for RNA isolation as previous described (22). cDNA was synthesized with the Verso cDNA synthesis kit (Thermo Scientific, Waltham, MA).

RT-qPCR. cDNA was analyzed by RT-qPCR with a CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and specific primers (Table 1). iTaq Universal SYBR green Supermix (Bio-Rad Laboratories) was used for PCRs according to the manufacturer's recommendations. The $2^{-\Delta\Delta CT}$ method was used for relative quantification of gene expression, and the data were normalized to *ACT1* gene expression (22).

***In vivo* studies.** To confirm the observed FLC resistance phenotype, we assessed the response to FLC treatment of both FLC-resistant and -susceptible *C. parapsilosis* strains in a *Galleria mellonella* infection model.

Fungal inoculum preparation. *C. parapsilosis* (*sensu stricto*) isolates ATCC 22019 and LEMI 8657 were used for *in vivo* experiments. The cells were grown overnight in yeast extract-peptone-dextrose (YPD) at 30°C. Cells were collected by centrifugation and washed three times with phosphate-buffered saline (PBS). Yeast cells were counted with a hemocytometer. The cell number was confirmed by determining the number of CFU per milliliter on YPD plates.

Inoculation of *G. mellonella* with *C. parapsilosis* (*sensu stricto*) strains. Wax moth larva killing assays were performed as previously described (35). Briefly, groups of 16 larvae (250 to 350 mg; Vanderhorst Wholesale, St. Marys, OH) were each inoculated with 10^6 CFU/larva. A Hamilton syringe was used to inject 10-μl aliquots of the inoculum into the hemocoel of each larva via the last left proleg (36). After injection, larvae were incubated at 37°C and the number of dead larvae was monitored daily. Two control groups were included; one was inoculated with PBS to observe the killing due to physical trauma, and the other received no injection as a control for general viability.

Treatment with FLC. Infected larvae were treated with FLC (14 mg/kg; Sigma Chemical Corporation, St. Louis, MO) (35). The antifungal was provided immediately after the infection and was delivered in a 10-μl volume to the last right proleg. Groups of 10 larvae were treated with FLC alone to test its toxicity. Survival was monitored every 24 h.

Fungal burden determination. Fungal burdens were determined by CFU counting at 16 h after inoculation. For this purpose, five larvae per group were weighed and homogenized in 1 ml of sterile PBS with a Tissue Tearor (model 398; Biospec Products, Bartlesville, OK) and serial dilutions of the homogenates were plated on YPD agar plates containing kanamycin (45 μg/ml), streptomycin (100 μg/ml), and ampicillin (100 μg/ml). Plates were incubated at 30°C for 72 h before colonies were counted.

Statistics. Killing curves were plotted, and estimated differences in survival (log rank and Wilcoxon tests) were analyzed by the Kaplan-Meier method with the Prism v5 software (GraphPad, La Jolla, CA). The same software was used for statistical analysis of the CFU of *C. parapsilosis* in the hemocoel (*t* test). A P value of <0.05 was considered significant. Each experiment was repeated at least three times, and all of the independent experiments gave similar results. The data presented in this report are from a representative experiment.

Nucleotide sequence accession numbers. The sequences obtained in this study have been deposited in GenBank under accession numbers KR082784 to KR082792.

RESULTS

Molecular identification of *C. parapsilosis* (*sensu lato*) isolates by real-time TaqMan qPCR assays. The nine *C. parapsilosis* strains selected for this study were genetically identified as *C. parapsilosis* (*sensu stricto*) when tested by the species-specific TaqMan probe, confirming the phenotypic characterization by standard mycological procedures.

Antifungal susceptibility tests. Table 2 summarizes the MICs of the four antifungal agents tested for the nine *C. parapsilosis* clinical isolates and reference strain ATCC 22019. The nine *C.*

TABLE 2 *In vitro* activities of four antifungal agents against nine clinical isolates of *C. parapsilosis* (*sensu stricto*) in the CLSI broth microdilution assay complemented by *ERG11* sequence analysis and expression of the *ERG11*, *CDR1*, and *MDR1* genes

C. parapsilosis (*sensu stricto*) isolate	MIC (mg/liter)				Mutations in *ERG11* gene	Avg cDNA level (SD)[a]		
	FLC	VRC	ANF	AMB		*ERG11*	*CDR1*	*MDR1*
LEMI 8646	8	1	2	0.25	T591C, A395T	7.4 (0.3)	9.2 (0.6)	1.5 (0.08)
LEMI 8650	8	0.5	2	0.25	T591C, A395T	3.7 (0.1)	4.8 (0.1)	0.9 (0.06)
LEMI 8653	16	0.5	2	0.25	T591C, A395T	1.5 (0.1)	4.0 (0.3)	0.9 (0.1)
LEMI 8655	16	0.5	1	0.25	T591C, A395T	3.3 (0.2)	3.4 (0.1)	0.8 (0.2)
LEMI 8657	16	0.5	2	0.125	T591C, A395T	3.9 (0.3)	3.7 (0.2)	1.0 (0.06)
LEMI 8662	16	0.5	2	0.25	T591C, A395T	5.7 (0.5)	5.7 (0.5)	1.3 (0.2)
LEMI 8379	8	0.5	2	0.25	T591C, A395T	4.0 (0.3)	3.3 (0.3)	0.8 (0.04)
LEMI 8382	8	0.5	2	0.25	T591C, A395T	7.3 (0.4)	7.3 (0.5)	1.2 (0.06)
LEMI 8383	8	0.5	1	0.25	T591C, A395T	4.4 (0.4)	7.6 (0.6)	1.0 (0.1)
ATCC 22019	0.5	0.5	1	0.125	ND[b]	1.0 (0.00)	1.0 (0.07)	1.0 (0.07)

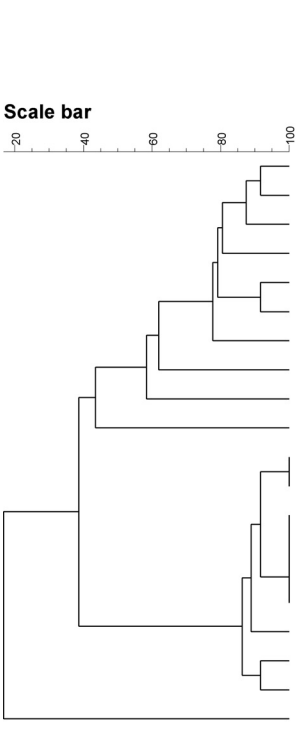
[a] cDNA levels were calculated relative to average levels of cDNA obtained for the wild-type strain. The values are the averages from three replicates, and the standard deviations are in parentheses. The cDNA levels of the different genes were normalized to that of the *ACT1* gene.
[b] ND, none detected.

parapsilosis bloodstream isolates tested exhibited resistance to FLC (MICs of ≥8 mg/liter). One isolate was considered resistant to VRC (MIC of 1 mg/liter), and eight were considered intermediate (MIC of 0.5 mg/liter). All of them were considered wild type for AMB (MICs of 0.125 to 0.25 mg/liter) and susceptible to ANF (MICs of ≤2 mg/liter). Further characterization of the FLC resistance mechanisms of all nine *C. parapsilosis* isolates was performed.

Microsatellite typing. Microsatellite typing allowed the differentiation of the nine strains into five different subtypes. The genetic relatedness of the nine isolates is presented in the dendrogram in Fig. 1, which shows that the Brazilian isolates are clustered together, with few differences among them.

Sequencing of the *ERG11* gene. The complete ORF of the *ERG11* gene of the nine resistant *C. parapsilosis* isolates and reference strain *C. parapsilosis* ATCC 22019 was determined. The *ERG11* sequences were 1,569 bp in length, and a comparison of these sequences with the available corresponding sequence of reference strain ATCC 22019 (GenBank accession no. GQ302972) revealed the presence of a silent mutation (T591C) and a missense mutation (A395T) that led to a Y132F amino acid substitution and a change in the protein sequence (Table 2). Single allele mutations (i.e., heterozygous for the mutation) were not observed; there were only point mutations in both alleles (i.e., homozygous for the mutation).

Expression of *ERG11*, *CDR1*, and *MDR1* in *C. parapsilosis* (*sensu stricto*) bloodstream isolates. To investigate if changes in the expression patterns of the *ERG11* gene, the ABC transporter



CanpaSTR3A/a	CanpaSTR3A/b	CanpaSTR3B/a	CanpaSTR3B/b	CanpaSTR3C/a	CanpaSTR3C/b	CanpaSTR6A/a	CanpaSTR6A/b	CanpaSTR6B/a	CanpaSTR6B/b	CanpaSTR6C/a	CanpaSTR6C/b	Strain	Country
26.0	27.0	99.0	106.0	40.0	44.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 28	Austria
26.0	27.0	99.0	106.0	40.0	40.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 75	Austria
26.0	27.0	100.0	106.0	40.0	44.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 30	Austria
26.0	27.0	45.0	45.0	40.0	44.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 18	Austria
26.0	27.0	36.0	36.0	40.0	44.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 61	Austria
26.0	27.0	36.0	36.0	40.0	40.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 79	Austria
26.0	27.0	37.0	37.0	40.0	40.0	10.0	12.0	8.0	8.0	7.0	7.0	Austria 67	Austria
26.0	26.0	51.0	52.0	40.0	40.0	10.0	10.0	8.0	8.0	7.0	7.0	Austria 44	Austria
26.0	27.0	50.0	50.0	47.0	47.0	10.0	10.0	8.0	8.0	7.0	7.0	Austria 82	Austria
15.0	26.0	36.0	59.0	40.0	43.0	14.0	19.0	8.0	8.0	7.0	7.0	Austria 57	Austria
25.0	25.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8655	Brazil
25.0	25.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8383	Brazil
25.0	26.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8653	Brazil
25.0	26.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8657	Brazil
25.0	26.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8662	Brazil
25.0	26.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8382	Brazil
26.0	26.0	34.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8650	Brazil
25.0	25.0	24.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8646	Brazil
25.0	26.0	24.0	34.0	44.0	44.0	13.0	17.0	8.0	8.0	7.0	7.0	LEMI 8379	Brazil
27.0	29.0	32.0	32.0	46.0	46.0	7.0	7.0	12.0	12.0	7.0	7.0	ATCC22019	Reference

FIG 1 Cluster analysis of nine *C. parapsilosis* isolates based on six short tandem repeat markers.

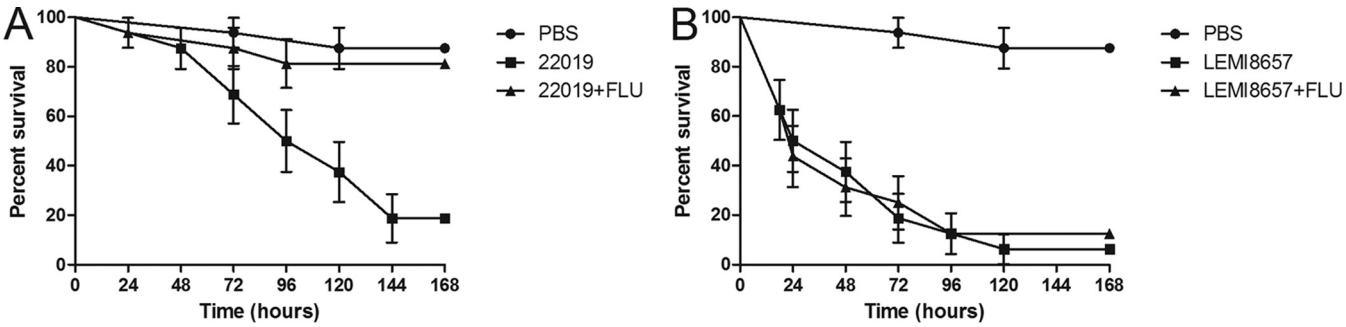


FIG 2 Effects of FLC (14 mg/kg) during infection of larvae with 10^6 cells of susceptible *C. parapsilosis* strain ATCC 22019 per larva (A) and 10^6 cells of *C. parapsilosis* resistant strain LEMI 8657 per larva (B).

gene *CDR1*, and the major facilitator superfamily (MFS) transporter gene *MDR1* could be associated with the FLC resistance phenotype observed in our clinical isolates, RT-qPCR analysis was used. Table 2 illustrates the relative expression of each respective gene obtained for the nine resistant isolates after FLC exposure (1.5 h of exposure) compared to the expression of the same genes in wild-type reference strain ATCC 22019 (where the mRNA expression levels were given a value of 1.0).

All resistant *C. parapsilosis* isolates expressed increased levels of *ERG11* (1.5 to 7.4 times) and *CDR1* (3.3 to 9.2 times) in the presence of FLC ($P < 0.001$). The expression of *MDR1* increased only in isolates LEMI 8646 and LEMI 8622 ($P < 0.05$). Taken together, the present data suggest that all of the isolates concomitantly overexpressed at least two genes usually involved in *Candida* resistance.

***In vivo* studies.** As shown in Fig. 2, both the FLC-resistant and reference strains caused a lethal infection to *G. mellonella* larvae. Treatment with FLC did not prolong the survival of larvae infected with FLC-resistant strain LEMI 8657. However, when the infection was due to FLC-susceptible strain ATCC 22019, the treatment produced significant survival ($P < 0.001$). In addition, we also evaluated the impact of FLC on the fungal burden of susceptible and resistant *C. parapsilosis* strains within the hemocoel (Fig. 3). Although FLC administration did not prolong survival, treatment of larvae in the LEMI 8657 group led to a slight decrease in the CFU count. In contrast, FLC treatment dramatically lowered the *in vivo* fungal burden of larvae infected with ATCC 22019 compared to that of the untreated group ($P < 0.005$), suggesting that FLC inhibited strain ATCC 22019 but not resistant strain LEMI 8657.

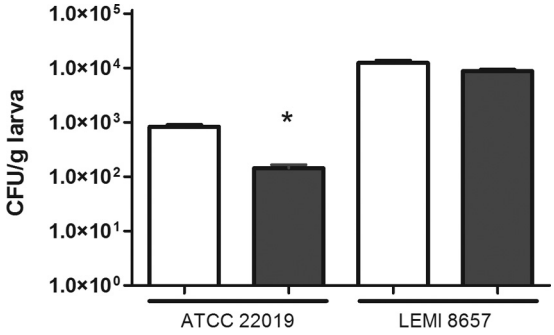


FIG 3 Effect of antifungal treatment on the fungal burden in *G. mellonella* infected with *C. parapsilosis* ATCC 22019 or LEMI 8657. White bars, no treatment; black bars, FLC treatment (14 mg/kg). *, $P < 0.05$.

DISCUSSION

In this study, the mechanisms of FLC resistance in nine *C. parapsilosis* strains isolated during an outbreak of candidemia were evaluated; this represents the first comprehensive assessment at the molecular level of mechanisms of FLC resistance in clinical *C. parapsilosis* strains from Brazil. Our results indicate that various molecular mechanisms, including the presence of point mutations in the *ERG11* gene, overexpression of *ERG11*, and efflux pump-encoding genes are involved in the FLC resistance of *C. parapsilosis* strains.

Although most clinical *C. parapsilosis* isolates are susceptible to triazoles, some investigators have reported a rise in the incidence of invasive infections due to FLC-resistant strains (13, 14, 16, 37). The emergence of *C. parapsilosis* FLC resistance is a cause for concern because of the ability of this species to be frequently transmitted through contaminated medical devices or fluids and via health care workers (1). In *C. albicans*, FLC resistance has been found to be due to a combination of different molecular mechanisms, including mutations in and overexpression of *ERG11* and the overexpression of two genes, *CDR1* (a *Candida* drug resistance gene) and *MDR1* (a multidrug resistance gene) (20, 23, 38).

Alterations in the *ERG11*sequence have been reported in *C. albicans*, *Candida tropicalis*, *C. glabrata*, and *Candida kefyr* (22, 39–45). In the present study, all nine *C. parapsilosis* isolates that exhibited high FLC MICs had a point mutation in the *ERG11* sequence that led to a Y132F amino acid substitution, compared to wild-type reference strain ATCC 22019. A similar point mutation was recently observed in resistant *C. parapsilosis* strains from patients in the United States (46). Interestingly, the occurrence of a missense mutation at position 132 was previously reported for *C. albicans* and *C. tropicalis* (41, 43, 44). Taken together, these data support the hypothesis that the mutation at position 132 might be a hot spot for *ERG11*-mediated resistance in *Candida* species, as suggested by Jiang and coworkers (43).

By using RT-qPCR, we assessed the quantitative expression of *ERG11*, as well as the expression of the ABC transporter gene *CDR1*, and the MFS transporter gene *MDR1* after exposure to FLC. All of the clinical FLC-resistant *C. parapsilosis* isolates showed increased expression of mRNA of the *ERG11* gene, which encodes the target lanosterol 14 α-demethylase. Supporting our data, similar results were obtained with NAC species, including *C. glabrata*, *C. tropicalis*, *Candida dubliniensis*, and *Candida krusei* (40, 43, 47–51). In contrast, Silva et al. analyzed the resistance mechanisms developed by induced resistant *C. parapsilosis* strains

and found that the expression of *ERG11* is reduced in FLC-resistant isolates (52). This observation might be related to the fact that, unlike Silva et al., we checked the overexpression of *ERG11* after culturing *C. parapsilosis* strains in the presence of FLC.

Overexpression of the *MDR1* and *CDR1* genes has been linked to FLC resistance in *C. albicans* and *C. dubliniensis* (23, 38, 53–55). Homologues of the *MDR1* gene have been described in *C. tropicalis*, *C. glabrata*, and *C. krusei*, but their overexpression has not yet been identified as a cause of azole resistance in clinical isolates (40, 43, 47, 56). Recent studies have demonstrated upregulation of *MDR1* in azole-resistant *C. parapsilosis* strains (46, 52). Accordingly, in the present study, two out of nine isolates showed increased *MDR1* mRNA expression in the presence of FLC.

All nine resistant isolates in the present study showed increased expression of *CDR1*, suggesting that this transporter contributes to FLC resistance. Although several studies have reported that overexpression of the *CDR1* gene plays an important role in FLC resistance in some *Candida* species, the role of this specific transporter in *C. parapsilosis* remains unclear (23, 35). Silva et al. suggested the overexpression of *CDR1* in FLC-induced resistant *C. parapsilosis* strains, since they observed upregulation of the transcription factor encoded by *NDT80*, which, in *C. albicans*, modulates azole tolerance by controlling the expression of the *CDR1* gene (52).

In order to assess the correlation between the *in vitro* resistance phenotype and an *in vivo* model, *G. mellonella* larvae were infected to evaluate the response to FLC therapy. The *in vivo* response showed a very good correlation with the resistance phenotype documented *in vitro*. It is worth mentioning that, as documented in our *in vivo* infection model in *G. mellonella*, the presence of the Y132F point mutation in *C. parapsilosis* appears not to be associated with decreases in fitness and virulence. Indeed, the resistant strain (LEMI 8657) exhibited 50 and 80% killing rates at 24 and 72 h postinfection, respectively, compared to the wild-type strain (ATCC 22019), which had 50 and 100% killing rates at 96 and 144 h.

In summary, we demonstrated that *C. parapsilosis* FLC-resistant strains are present in Brazil with a potential for nosocomial spread of the pathogen via health care workers. The *G. mellonella* model demonstrated that, in our collection of *C. parapsilosis* strains, resistance to FLC came at no cost in pathogenicity and virulence. Finally, our data demonstrated that not only overexpression of *MDR1* and mutations in *ERG11* but also overexpression of *ERG11* and *CDR1* might be involved in FLC resistance in *C. parapsilosis*.

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