AAC Journals.ASM.org

In Vivo and *In Vitro* Acquisition of Resistance to Voriconazole by *Candida krusei*

Elisabete Ricardo,[a,b] Isabel M. Miranda,[a,b,c] Isabel Faria-Ramos,[a] Raquel M. Silva,[d] Acácio Gonçalves Rodrigues,[a,c] Cidália Pina-Vaz[a,c,e]

Department of Microbiology, Faculty of Medicine, University of Porto, Porto, Portugal[a]; Cardiovascular R&D Unit, Faculty of Medicine, University of Porto, Porto, Portugal[b]; CINTESIS—Center for Health Technology and Services Research, Faculty of Medicine, University of Porto, Porto, Portugal[c]; IPATIMUP—Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal[d]; Department of Microbiology, Hospital São João, Porto, Portugal[e]

***Candida krusei* is an important agent of opportunistic infections that often displays resistance to several antifungals. We describe here the *in vivo* acquisition of resistance to voriconazole (VRC) by *C. krusei* isolates recovered from a leukemia patient during a long period of VRC therapy. In order to mimic the *in vivo* development of VRC resistance, a susceptible *C. krusei* isolate was exposed daily to 1 μg/ml of VRC *in vitro*. Interestingly, after 5 days of exposure to VRC, a MIC of 4 μg/ml was achieved; this value remained constant after 25 additional days of treatment with VRC and also after 30 consecutive days of incubation in VRC-free medium. Our objective was to determine the associated molecular resistance mechanisms, such as expression of efflux pump genes and *ERG11* gene mutations, among the resistant strains. Synergistic effects between the efflux blocker tacrolimus (FK506) and VRC were found in all of the resistant strains. Moreover, *ABC1* gene expression increased over time in both the *in vivo*- and *in vitro*-induced resistant strains, in contrast to the *ABC2* and *ERG11* genes, whose expression was invariably lower and constant. *ERG11* gene sequencing showed two different types of mutations, i.e., heterozygosity at T1389T/C, corresponding to synonymous mutations, in *C. krusei* strains and a missense mutation at position T418C, resulting in a change from Tyr to His, among resistant *C. krusei* clinical isolates. This study highlights the relevance of ATP-dependent efflux pump (namely, Abc1p) activity in VRC resistance and describes new mutations in the *ERG11* gene among resistant *C. krusei* clinical isolates.**

In recent years, we have witnessed the emergence of low-pathogenicity non-*albicans Candida* species, such as *Candida krusei*. It is an opportunistic pathogen, especially among patients with hematological malignancies and those undergoing bone marrow transplantation (1, 2). Mortality rates among such patients with *C. krusei* fungemia are unacceptably high, ranging from 60 to 80% (1). Nevertheless, few studies concerning the epidemiology and antifungal susceptibility profile of *C. krusei* are available. *C. krusei* ranked in fifth place among 22 different species of *Candida*, accounting for 3.3% of all *Candida* isolates in both Europe and North America (3, 4).

C. krusei is often described as a multidrug-resistant (MDR) fungal pathogen, due to its intrinsic resistance to fluconazole (FLC) and its decreased susceptibility to flucytosine, amphotericin B (AMB), and ketoconazole (KTC) (5, 6). Resistance to FLC is a major problem among neutropenic and critically ill patients, since this drug is frequently used for prophylaxis (7). *C. krusei* resistance to echinocandins was also described among patients with acute myelogenous leukemia (8, 9).

Two major mechanisms of resistance to azoles are observed in *C. krusei*, i.e., reduced intracellular drug accumulation due to the activity of the efflux pump proteins Abc1p and Abc2p and alterations in the target enzyme, cytochrome P450 lanosterol 14α-demethylase, encoded by Erg11p, which is involved in the ergosterol biosynthesis pathway (10, 11). Such efflux pumps belong to the ATP-binding cassette (ABC) transporter family of proteins encoded by *ABC1* and *ABC2* genes. Venkateswarlu et al. described a group of clinical isolates of *C. krusei* resistant to itraconazole (ITC) due to the activity of efflux pumps (12). However, the scarce information available regarding efflux pump activity and gene expression following azole exposure makes *C. krusei* resistance to azoles poorly understood (13). On the other hand, it is not yet possible to explain the reduced affinity of azoles, namely, FLC, for the target, the Erg11 protein binding site (11). No resistance-conferring alterations in the *ERG11* gene sequence have yet been described for *C. krusei*, in comparison with findings for *Candida albicans* (14).

We addressed the molecular mechanisms of resistance acquired *in vivo*, i.e., in voriconazole (VRC)-resistant *C. krusei* strains isolated from a leukemia patient undergoing VRC therapy, or following induction *in vitro*, i.e., in VRC-resistant *C. krusei* strains repeatedly incubated with VRC at subinhibitory concentrations. We concluded that repeated exposure to VRC results in the development of concomitant resistance mechanisms, namely, enhanced activity of efflux pumps encoded by the *ABC1* gene and single-point mutations in *ERG11*.

MATERIALS AND METHODS

Patient clinical data. A 41-year-old male patient, admitted to Centro Hospitalar São João (Porto, Portugal), was diagnosed with acute lymphoblastic leukemia (T/natural killer [NK] cells) in August 2008. Due to protracted fever that persisted after antibacterial treatment, a myelogram was performed and a first relapse of the hematological disease was diagnosed. The patient was admitted at once to the neutropenic unit for salvage chemotherapy with fludarabine, idarubicin, and 1-β-D-arabinofuranosylcytosine (Ara-C), followed by the growth factor granulocyte colony-stim-

Received 21 February 2014 Returned for modification 12 April 2014
Accepted 24 May 2014
Published ahead of print 27 May 2014
Address correspondence to Elisabete Ricardo, ericardo@med.up.pt.

doi:10.1128/AAC.02603-14

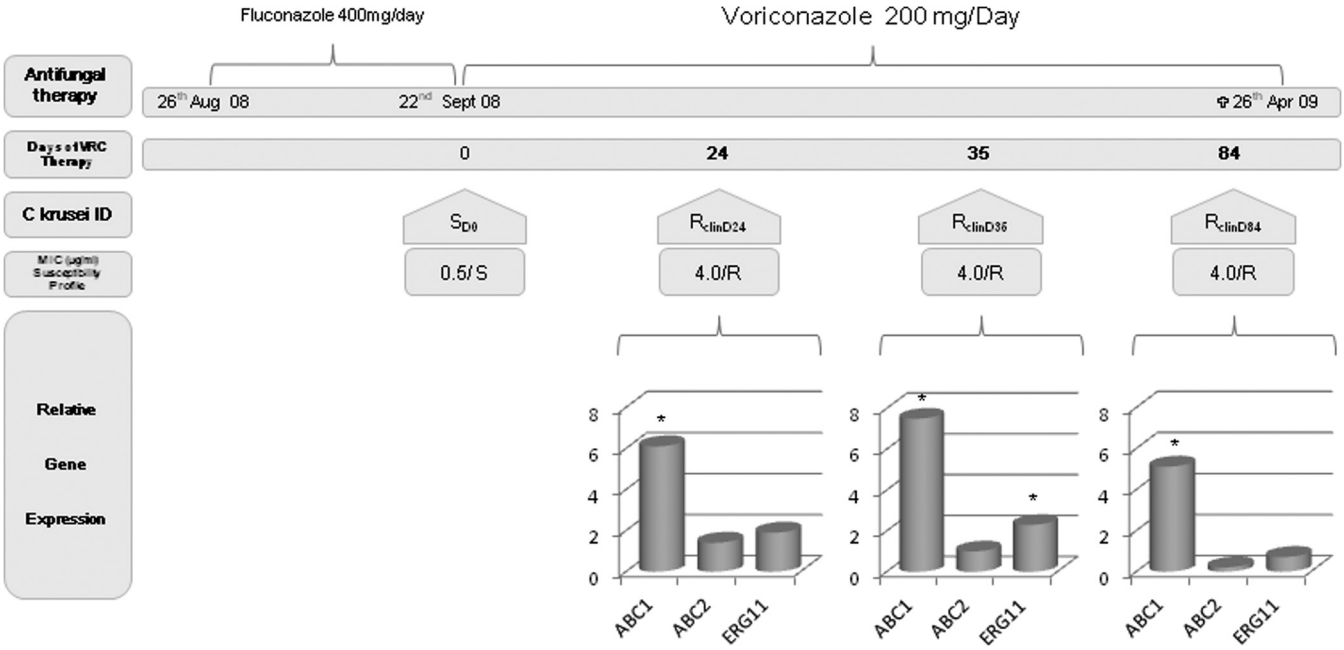

FIG 1 Induction of resistance to voriconazole *in vivo*. The timeline of antifungal therapy and the recovery of *C. krusei* clinical strains from the leukemia patient, with the respective susceptibility profiles and relative gene expression levels, is shown. ID, identification; S, susceptible, R, resistant. Cross, patient death; ∗, $P \leq 0.05$.

ulating factor (G-CSF), administered through a central venous catheter (CVC). Due to the persistent fever, the patient began treatment with 200 mg/day of FLC. *C. krusei* was first isolated from a blood culture after 9 days of FLC therapy; a second blood culture was positive for *C. krusei* at day 14. FLC treatment was suspended and the patient began treatment with VRC at 200 mg/day. During the initial 24 days of VRC therapy, several *C. krusei* isolates were recovered from blood cultures and from other biological samples; subsequently, *C. krusei* was recovered only from bronchial secretions and stool samples (Fig. 1). The patient had undergone VRC therapy for 216 days upon his death in April 2009.

All of the clinical isolates were identified using Vitek 2 YBC identification cards (bioMérieux, Marcy l'Etoile, France) and were stored at −70°C in brain heart infusion broth (Merck KGaA, Darmstadt, Germany) with 40% glycerol. Prior to experiments, isolates were subcultured twice in Sabouraud agar (Merck KGaA, Darmstadt, Germany) to ensure the purity of cultures.

Antifungal drugs and susceptibility testing. Stock solutions of VRC (Pfizer, Groton, CT), posaconazole (PSC) (Schering-Plough, Summit, NJ), FLC (Pfizer, Groton, CT), AMB (Bristol-Meyers Squibb, New York, NY), caspofungin (CAS) (Merck, Rahway, NJ), anidulafungin (AND) (Pfizer, Groton, CT), and micafungin (MCF) (Astellas Pharma, Inc., Tokyo, Japan) were prepared according to the M27-A3 and M27-S4 protocols of the Clinical and Laboratory Standards Institute (CLSI) and were maintained at −70°C until use (15, 16). MICs were determined for all *C. krusei* isolates according to the same protocols. The VRC susceptibility profiles of the *C. krusei* isolates were determined in accordance with MICs of ≤0.5 μg/ml (susceptible), 1.0 μg/ml (susceptible dose dependent), and ≥2.0 μg/ml (resistant). Visual readings were performed after 24 h and 48 h of incubation, according to the same protocols. *C. krusei* type strain ATCC 6258 from the American Type Culture Collection was used as a control, as recommended (15, 16).

Molecular typing. (i) Total genomic DNA extraction. All of the clinical isolates recovered from the leukemia patient were evaluated for their genetic relatedness, in order to determine whether the patient was colonized by the same *C. krusei* strain. *C. krusei* isolates were cultured overnight in 10 ml of yeast extract-peptone-dextrose (YPD) liquid medium at 35°C, at 150 rpm, and subsequently collected by centrifugation at 1,610 × *g* for 10 min at room temperature (Universal 320 R; Hettich). Total DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1; Sigma-Aldrich, Munich, Germany), precipitated with 100% ice-cold ethanol (Applichem, Darmstadt, Germany), and redissolved in 200 μl of Tris-EDTA (TE) buffer. The DNA was treated with 20 μg of RNase (Applichem, Darmstadt, Germany) and incubated at 37°C for 1 h. For final precipitation, 20 μl of 4 M ammonium acetate (pH 4.8) (Sigma-Aldrich, Munich, Germany) and 600 μl of ice-cold 100% ethanol (Applichem, Darmstadt, Germany) were added and samples were incubated overnight at −20°C. DNA samples were resuspended in 1× TE buffer, the concentration was adjusted to 2.0 to 2.5 μg/μl, and samples were stored at −20°C for later use.

(ii) Restriction endonuclease analysis. For each sample, a reaction mixture containing 1× HinfI enzyme reaction buffer (Metabion, Martinsried, Germany), 1 μg/μl RNase (Applichem, Darmstadt, Germany), 0.5 U/μl HinfI restriction enzyme (Metabion, Martinsried, Germany), approximately 25 to 30 μg of total DNA, and DNase/RNase-free water up to a final volume of 20 μl was prepared as described by Ricardo et al. (17); reaction tubes were incubated overnight at 37°C. The total reaction mixture was run on a 1% agarose gel (20 cm by 24 cm, 120 mV) for 3 to 5 h, stained with ethidium bromide solution (0.5 mg/ml; Applichem, Darmstadt, Germany), and visualized with UV light with a Chemidoc XRS+ imaging system (Bio-Rad, Hercules, CA). Restriction patterns were analyzed using Image Lab software (version 4.0.1; Bio-Rad, Hercules, CA), and the different isolates were compared for restriction pattern similarities. A nonrelated *C. krusei* isolate recovered from stools from a different patient was used as a control.

(iii) Random amplification polymorphic DNA analysis. Random amplification polymorphic DNA (RAPD) analysis was performed as described previously (18), with some alterations. Briefly, the reaction mixtures for RAPD analysis contained 1× DreamTaq enzyme reaction buffer, 0.04 U/μl DreamTaq DNA polymerase, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$ (all from Fermentas, Vilnius, Lithuania), 0.4 μM primer OPA-18 (5′-AGCTGACCGT-3′) or OPE-18 (5′-GGACTGCAGA-3′) (STAB Vida, Lisbon, Portugal), 200 ng total DNA, and

TABLE 1 MICs and susceptibilities of *C. krusei* clinical isolates

Strain identification	Recovery site	MIC (μg/ml)/susceptibility to indicated antifungal[a]:						
		AMB	FLC	VRC	PSC	AND	CAS	MCF
S_{D0}	Blood	0.5/S	64/R	0.5/S	0.5/S	≤0.06/S	0.5/I	0.5/S
$R_{clinD24}$	Stools	1.0/S	64/R	4.0/R	≤0.03/S	≤0.06/S	0.25/S	0.5/S
$R_{clinD35}$	Bronchial secretions	1.0/S	64/R	4.0/R	0.06/S	≤0.06/S	0.125/S	0.25/S
$R_{clinD84}$	Bronchial secretions	0.125/S	64/R	4.0/R	0.5/S	≤0.06/S	0.5/S	0.25/S

[a] AMB, amphotericin B; CAS, caspofungin; AND, anidulafungin; FLC, fluconazole; VRC, voriconazole; PSC, posaconazole; MCF, micafungin; S, susceptible; I, intermediate; R, resistant.

DNase/RNase-free water up to a final volume of 25 μl. All PCRs were performed in an Eppendorf RealPlex2 Mastercycler (Eppendorf, Hamburg, Germany), and the reaction parameters were one cycle of 95°C for 2 min; 38 cycles of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and one cycle of 72°C for 10 min. The amplified DNA fragments were analyzed by electrophoresis in a 2% agarose gel at 120 mV for 2 h. The agarose gel was stained with ethidium bromide solution (0.5 mg/ml; Applichem, Darmstadt, Germany) and visualized with UV light with the Chemidoc XRS+ imaging system. Electrophoresis patterns were analyzed using Image Lab software (version 4.0.1).

***In vitro* induction of resistance.** A VRC-susceptible *C. krusei* isolate, recovered from the leukemia patient before VRC antifungal therapy, was repeatedly incubated with VRC. Briefly, a single, randomly selected colony from a fresh 24-h culture on Sabouraud agar was suspended in 10 ml of RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) (both from Sigma-Aldrich, Munich, Germany), in the presence of VRC at 1 μg/ml, and was incubated at 35°C at 150 rpm. Every 24 h, 1 ml of the culture was suspended in 9 ml of fresh 1 μg/ml VRC-containing RPMI 1640 medium, for a 30-day period. Subsequently, in order to evaluate the stability of the susceptibility profile, this strain was subcultured daily in fresh RPMI 1640 medium without VRC for an additional 30 days; again, every 24 h, 1 ml of the culture was suspended in 9 ml of fresh RPMI 1640 medium. At each daily subculture, an aliquot was stored at −70°C in 40% glycerol; every 2 days, a 10-μl loopful of yeast cells was cultured in Sabouraud agar to check for culture contamination. Every 5 days during the 60 days of the assay, VRC MIC values were determined according to the CLSI M27-A3 and M27-S4 protocols (15, 16).

Effects of the efflux blocker tacrolimus (FK506) on *C. krusei* susceptibility profiles. The VRC MIC values for all of the resistant *C. krusei* strains (clinical isolates or induced *in vitro*) were redetermined according to the CLSI M27-A3 and M27-S4 protocols in the presence of 100 μg/ml FK506, a recognized ATP-dependent efflux pump inhibitor (19). An agar disk diffusion assay was also performed using blank paper disks impregnated with FK506, to corroborate the results obtained with the CLSI protocols. Yeast suspensions of resistant C. *krusei* strains were prepared to an optical density of 0.5 McFarland standard (Densimat; bioMérieux, Marcy l'Etoile, France) and spread onto YPD agar plates with VRC at 4 μg/ml or without VRC. Blank paper disks (BBL, 6 mm; Becton, Dickinson) were impregnated with serial 10-fold dilutions of FK506 solutions, ranging from 1,000 to 1 μg/ml, or with its solvent, dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany); paper disks were applied to the inoculated agar plates. A plate containing only VRC at supra-MIC values was used as a control for cell viability for each strain. Results were read after 24 h and 48 h of incubation at 37°C.

Resistance gene expression analysis. (i) Total RNA extraction. *C. krusei* strains were incubated in YPD broth at 35°C, at 150 rpm, until the exponential growth phase. Yeast cells were harvested by centrifugation at 1,610 × *g* for 5 min at room temperature (Universal 320 R; Hettich) and were immediately frozen in liquid nitrogen. Total RNA was extracted using the hot acid-phenol method, as described by Köhrer and Domdey (20). RNA samples were resuspended in DNase/RNase-free water, adjusted to a final concentration of 100 ng/μl, and stored at −70°C for later use.

(ii) Reverse transcription-PCR. Two-step real-time PCRs were performed. Reverse transcriptase reactions were performed as recommended by the manufacturer; 50 ng of RNA sample, 0.015 ng/μl random primers (Invitrogen, Carlsbad, CA), 0.5 mM dNTPs (Fermentas, Vilnius, Lithuania), and RNase-free water up to 13 μl were incubated at 65°C for 5 min and then placed on ice for 1 min. Subsequently, 1× reverse transcriptase enzyme buffer (Invitrogen, Carlsbad, CA), 5 mM dithiothreitol (DTT) (Invitrogen), 1 U/μl RNasin enzyme (Promega, Madison, WI), 5 U/μl reverse transcriptase enzyme (Invitrogen), and RNase-free water were added up to a final volume of 20 μl. The reaction tubes were incubated at 25°C for 5 min, at 50°C for 60 min, and at 70°C for 15 min for enzyme inactivation. Reactions were carried out in a Mastercycler ep gradient RealPlex2 system. The cDNAs were kept at −20°C.

(iii) Quantitative real-time PCR. Genes were amplified using the primers *ABC1* (GenBank accession number DQ903907) (forward, 5′-GATAACCATTTCCCACATTTGAGT-3′, and reverse, 5′-CATATGTTGCCATGTACACTTCTG-3′), *ABC2* (GenBank accession number AF250037) (forward, 5′-CCTTTTGTTCAGTGCCAGATTG-3′, and reverse, 5′-GTAACCAGGGACACCAGCAA-3′), *ERG11* (GenBank accession number FJ445756) (forward, 5′-ATTGCGGCCGATGTCCAGAGGTAT-3′ and reverse, 5′-GCGCAGAGTATAAGAAAGGAATGGA-3′), and *ACT1* (GenBank accession number AJ389086) (forward, 5′-TGGGCCAAAAGGATTCTTATG-3′ and reverse, 5′-AGATCTTTTCCATATCATCCCAG-3′) (STAB Vida, Lisbon, Portugal). The quantitative real-time (qRT)-PCR mixture contained 1× PerfeCTa SYBR green FastMix (Quanta Biosciences, Gaithersburg, MD), forward and reverse primers (*ABC1*, *ABC2*, and *ERG11*, 0.9 μM; *ACT1*, 0.5 μM), 2 μl of cDNA, and RNase-free water, up to a final reaction volume of 20 μl. $MgCl_2$ was used in the *ABC2* gene reaction mixture at a final concentration of 1 mM. All reactions were performed in the Mastercycler ep gradient RealPlex2 system; parameters were chosen according to the manufacturer's recommendations except for primer annealing temperatures, which were as follows: *ABC1*, 54°C; *ABC2*, 56°C; *ERG11*, 60°C; *ACT1*, 54°C. To check for PCR product specificity, a melting curve was established, with temperatures ranging from 60°C to 95°C, for 20 min.

(iv) Data analysis. A standard curve, containing serial 5-fold dilutions ranging from 500 ng to 0.8 ng of RNA transcribed to cDNA, was determined in triplicate for quantification and assessment of reaction efficiency for each gene. Assays were validated for reaction efficiencies ranging from 80% to 100% and with a standard curve presenting a mean squared error higher than 0.99. The results were analyzed using RealPlex software (version 1.5.474; Eppendorf). Relative gene expression levels were calculated using REST 2009 software (Qiagen GmbH, Munich, Germany) (21), with the susceptible *C. krusei* strain as the reference sample and each resistant *C. krusei* strain as the target sample; the *ACT1* gene was used to normalize levels of gene expression. Genes exhibiting 2-fold increases in expression were considered overexpressed.

Statistical analysis. Analyses of results were performed using SPSS version 19.0. Continuous and paired-sample Student's *t* tests were used to analyze significant differences in gene expression displayed by the distinct *C. krusei* strains; *P* values of ≤0.05 were considered statistically significant.

Sequencing data analysis of the *ERG11* gene. Total genomic DNA was extracted as described above. The *ERG11* gene (1,890 bp) was ampli-

TABLE 2 MICs and susceptibilities to voriconazole, alone and in combination with FK506, of *C krusei* strains

Resistance	Strain identification	VRC MIC (μg/ml)/ susceptibility profile when used with[a]: No FK506	100 μg/ml FK506
None	S_{D0}	0.5/S	0.125/S
In vivo acquired	$R_{clinD24}$	4.0/R	0.5/S
	$R_{clinD35}$	4.0/R	0.5/S
	$R_{clinD84}$	4.0/R	0.25/S
In vitro acquired	R_{indD5}	4.0/R	0.5/S
	R_{indD10}	4.0/R	0.5/S
	R_{indD30}	4.0/R	0.5/S
	R_{D60}	4.0/R	0.25/S

[a] S, susceptible; R, resistant.

fied by PCR; the reaction mixture contained 1 U/μl DreamTaq DNA polymerase enzyme, 1× DreamTaq DNA polymerase enzyme buffer, 0.2 mM dNTPs (all from Fermentas, Vilnius, Lithuania), forward_1 (5′-GGTTGTTTGTTCATTTAATGTGTGT-3′) and reverse (5′-GAAGGGGGAAAGAAAGGGAA-3′) primers at 0.8 μM (STAB Vida, Lisbon, Portugal), and RNase-free water up to a final volume of 25 μl. All reactions were performed in a Mastercycler ep gradient RealPlex2 system, and reaction parameters involved an initial 2-min denaturation step at 95°C, 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and a final 10-min extension step at 72°C. PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH) and used as templates for the sequencing reactions. Sequencing was performed with the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, CA) using the forward_1, forward_2 (5′-AACTACTGGAAAAGAGATGCTGC-3′), and forward_3 (5′-CACTCGTGATTTACCCGTTCC-3′) primers, at 0.8 μM final concentrations. DNA products were purified with Sephadex G-50 Fine (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster City, CA). Results were analyzed with Sequencing Analysis software (version 5.2; Applied Biosystems). The *ERG11* gene coding sequences of the resistant strains were aligned with the susceptible *ERG11* gene coding sequence using MUSCLE software/ClustalW (22). Alignments were analyzed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)/ClustalW.

RESULTS

Nomenclature and susceptibility profiles of *C. krusei* strains. Four *C. krusei* clinical isolates were selected according to the VRC MIC values and the collection date (more than 10 days apart), i.e., a susceptible isolate with a VRC MIC of 0.5 μg/ml that was recovered from a blood culture before initiation of VRC therapy, designated strain S_{D0}, and three resistant isolates with VRC MICs of 4 μg/ml. The latter were designated $R_{clinD24}$, $R_{clinD35}$, and $R_{clinD84}$ according to the day of recovery during VRC therapy. $R_{clinD24}$ was the first VRC-resistant isolate and was recovered from stools, while $R_{clinD35}$ and $R_{clinD84}$ were collected from bronchial secretions (Fig. 1). The susceptibility profiles of the 4 *C. krusei* clinical isolates with the different classes of antifungals are detailed in Table 1. As expected, *C. krusei* revealed intrinsic resistance to FLC.

The VRC MIC turning point to resistance of strain S_{D0} during the *in vitro* induction assay occurred at day 5 of exposure to VRC, corresponding to a MIC of 4 μg/ml. The VRC MIC for all *C. krusei* strains remained unchanged during the 55 subsequent days of the assay (Table 2; Fig. 2). Selected strains were designated according to the day of incubation with VRC; strains obtained at days 5, 10, and 30 of the induction assay were named R_{indD5}, R_{indD10}, and R_{indD30}, respectively. The resistant strain obtained after 30 days of culture in VRC-free medium was named R_{D60} (Fig. 2).

Molecular typing. Both restriction endonuclease analysis (REA) and RAPD techniques exhibit high discriminative power, as described by Sancak et al. (23) and Bautista-Muñoz et al. (18), respectively. All of the *C. krusei* clinical strains exhibited the same restriction pattern in REA and the same amplification pattern in RAPD analysis, as shown in Fig. 3. Therefore, these results strongly indi-

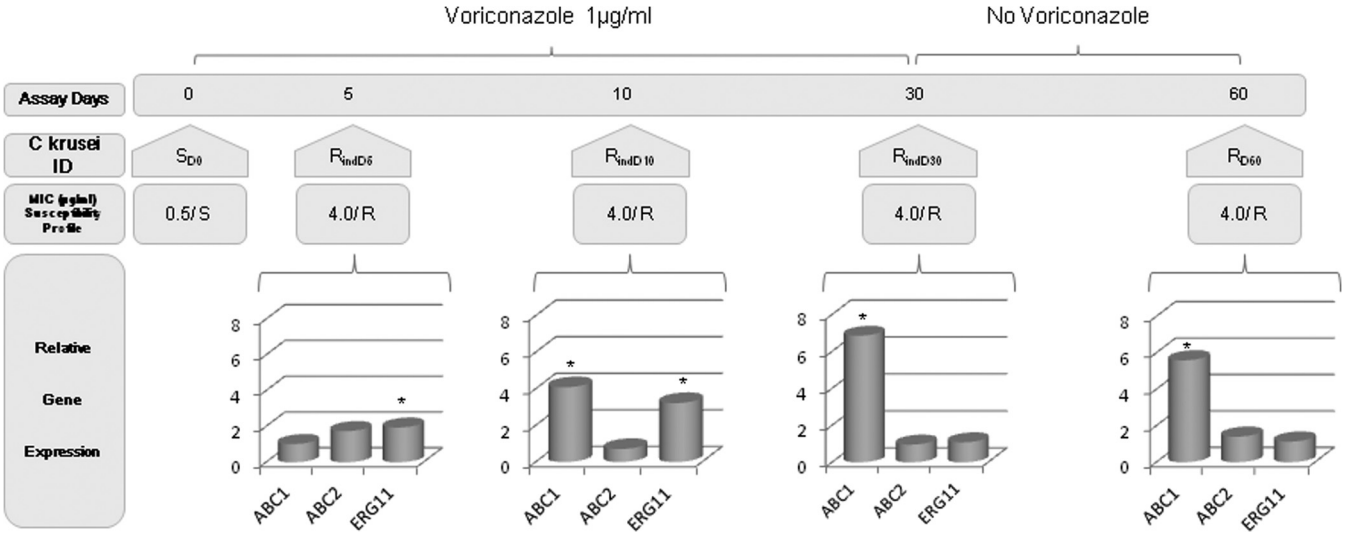

FIG 2 Induction of resistance to voriconazole *in vitro*. The timeline of the development of VRC resistance in a susceptible *C. krusei* clinical isolate that was exposed daily to VRC at 1 μg/ml, with the respective susceptibility profiles and relative gene expression levels, is shown. ID, identification; S, sensitive, R, resistant. *, $P \leq 0.05$.

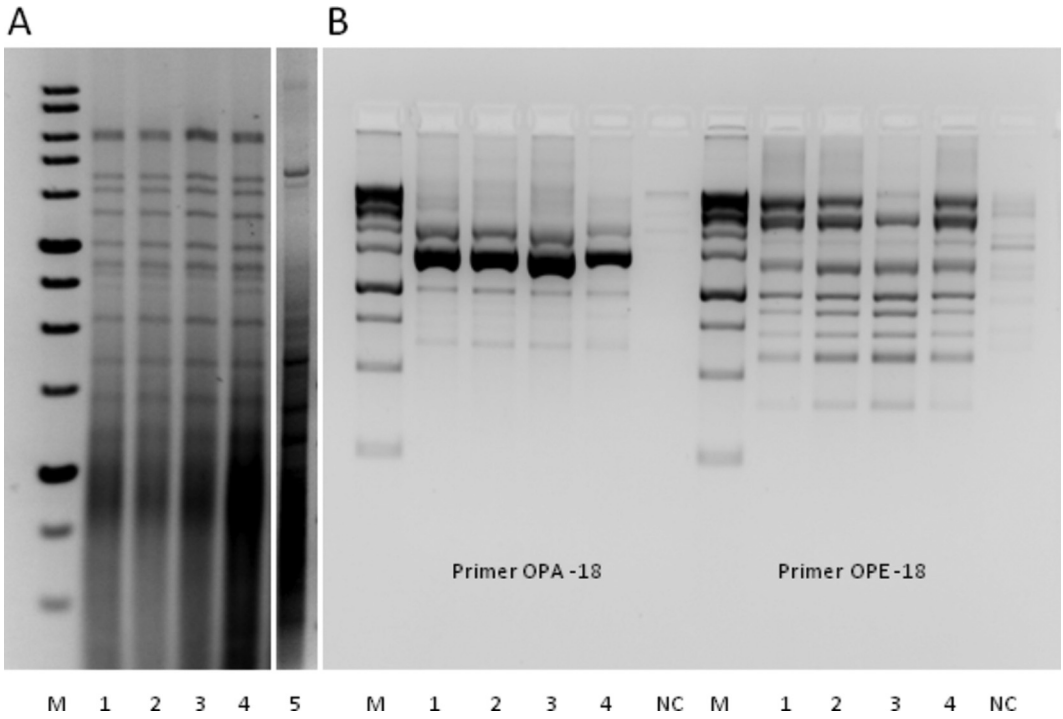

FIG 3 Genotyping of clinical isolates. (A) Restriction endonuclease pattern of HinfI-digested DNA of *C. krusei* clinical isolates in agarose gel electrophoresis. (B) Random amplification polymorphic DNA (RAPD) analysis of *C. krusei* clinical isolates with primers OPA-18 and OPE-18. Lanes M, 1-kb DNA ladder (Metabion); lanes 1, S_{D0}; lanes 2, $R_{clinD24}$; lanes 3, $R_{clinD35}$; lanes 4, $R_{clinD84}$; lane 5, nonrelated *C. krusei* control strain; NC, negative control for PCR.

cate that the strains colonizing the leukemia patient were genetically related and arose from the common ancestor susceptible strain S_{D0}.

Effects of FK506 on *C. krusei* susceptibility profiles. In the presence of FK506, all of the resistant strains changed to a VRC-susceptible phenotype (Table 2). The agar disk diffusion assay confirmed the microdilution results; growth inhibition was found for all of the resistant strains around disks containing the two highest FK506 concentrations (100 and 1,000 μg/ml) in the presence of VRC at 4 μg/ml (Fig. 4B and D). Susceptible strain S_{D0} was unable to grow in the presence of VRC at 4 μg/ml. The FK506 solvent, DMSO, did not impair the growth of the strains, and neither did FK506 alone (Fig. 4A and C).

Resistance gene expression analysis. The analysis of gene expression in R_{clin} strains with real-time PCR showed significant overexpression of the *ABC1* gene, in comparison with the susceptible S_{D0} strain (Fig. 1 and 5A). This was not the case for the other two associated resistance genes, *ABC2* and *ERG11*. Only the $R_{clinD35}$ strain presented a significant increase in *ERG11* gene expression. Similar *ABC1* gene expression profiles were registered for all of the R_{ind} strains and the R_{D60} strain, i.e., during the induction protocol, significant increases in the relative expression of the *ABC1* gene were documented (Fig. 1 and 5B). Variations in *ABC2* gene expression were not significant for any of these strains. *ERG11* relative gene expression levels demonstrated significant increases in the R_{indD5} and R_{indD10} strains.

***ERG11* gene sequencing analysis.** Several *ERG11* gene mutations were reported previously to be associated with azole resistance in *C. albicans* (14); therefore, the *C. krusei ERG11* gene was sequenced in our strains. Two different types of mutations were found. All of the susceptible and resistant *C. krusei* strains presented a heterozygous alteration at 1,389 bp (T→C) (overlapping signals in the electropherogram data), resulting in synonymous single-nucleotide polymorphisms (SNPs). Notably, $R_{clinD35}$ and $R_{clinD84}$ presented a missense mutation at position 418 bp

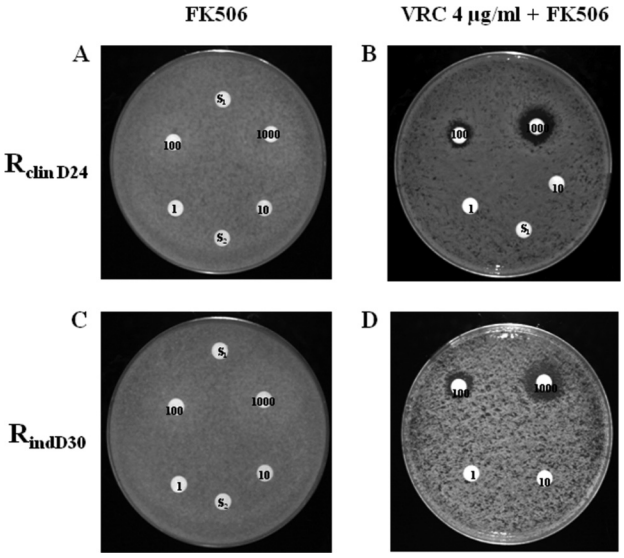

FIG 4 FK506 disk diffusion assay. Resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1,000 to 1 μg/ml, without VRC (A and C) or with VRC at 4 μg/ml (B and D). $R_{clinD24}$ (A and B) and R_{indD30} (C and D) are shown as representative examples of *C. krusei* strains with *in vivo* and *in vitro* induced resistance, respectively. S_1 (100%) and S_2 (10%) indicate disks impregnated with DMSO at different concentrations.

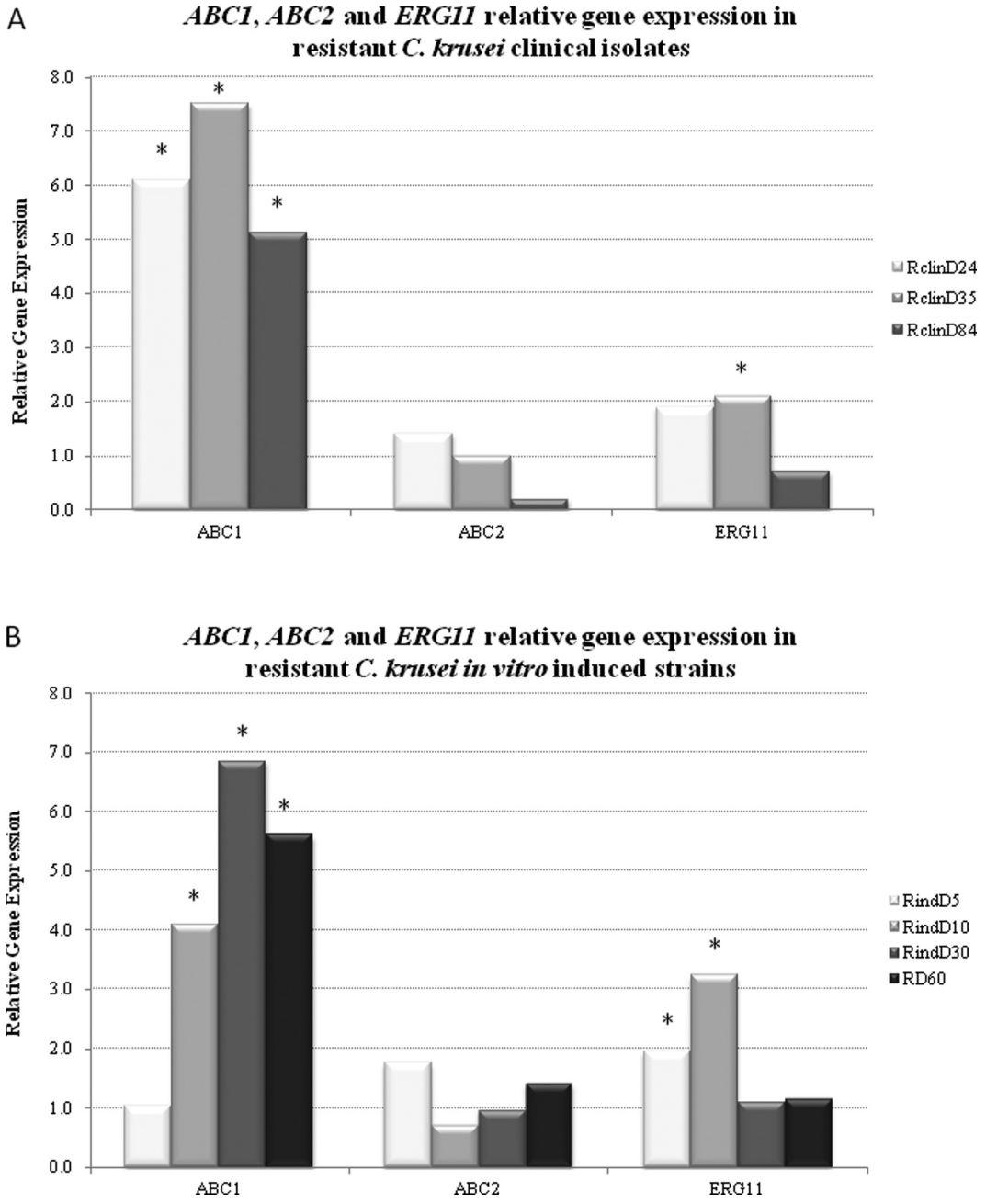

FIG 5 *ABC1*, *ABC2*, and *ERG11* relative gene expression levels in resistant *C. krusei* clinical isolates (A) and resistant strains induced *in vitro* (B). *ABC1*, *ABC2*, and *ERG11* gene expression levels were quantified and normalized relative to the housekeeping gene *ACT1*; relative gene expression levels were calculated as ratios between each resistant *C. krusei* strain and the S_{D0} isolate. *, $P \leq 0.05$.

(T →C), yielding a Tyr→His amino acid change. This point mutation had not been described previously for *C. krusei* strains.

DISCUSSION

C. krusei is one of the leading agents of candidemia among patients with hematological malignancies. In this study, the 4 consecutive *C. krusei* clinical isolates obtained from the leukemia patient during VRC therapy were evaluated for their susceptibility profiles and genetic relationships. Although they displayed distinct susceptibility profiles, these isolates were genetically related (Fig. 3). The initial colonizing strain, S_{D0}, changed from a VRC-susceptible phenotype to a stable VRC-resistant phenotype during a long period of VRC therapy. Thus, we were confronted with a case of resistance to VRC acquired *in vivo*. It should be stressed that the bioavailability and concentrations of the bioactive drug *in vivo* are highly variable due to several factors, such as different infection sites, concomitant therapies, and the status of the host immune system (24). The *C. krusei* clinical isolates might have been in contact with subinhibitory concentrations of the antifungal agent for a long period, which were not sufficient to eliminate the organism but were enough to stimulate stress adaptation mechanisms leading to resistance. Therefore, a susceptible *C. krusei* iso-

late (strain S_{D0}) was later continuously exposed to VRC *in vitro*, in order to compare the development of resistance *in vivo* and *in vitro*.

FK506 was used as a first approach to explore the mechanisms of resistance to VRC displayed by *C. krusei* strains. It was previously described as being able to reverse multidrug resistance in different types of eukaryotic cells, due to the blockade of ATP-dependent efflux pumps, namely, human P-glycoprotein, *C. albicans* Cdr1p/Cdr2p, and more recently *C. krusei* Abc1p (13, 19, 25). The synergistic effects of FK506 and VRC that occurred with all of the resistant *C. krusei* strains, in both the microdilution and test disk assays, clearly showed that efflux pumps contributed to the VRC-resistant phenotype in both R_{clin} and R_{ind} strains.

Several genes have been reported to be involved in *C. krusei* resistance to azoles. For example, according to Katiyar and Edlind, efflux pumps from the ABC family of proteins are crucial for resistance to azoles (10); in contrast, Guinea et al. concluded that the MDR family of proteins plays a minor role in *C. krusei* resistance to azoles (26). *ERG11* gene upregulation in *C. krusei* strains in response to azole treatment (3 h of incubation with FLC at 9 μg/ml) (27) was described. Taking this into account, molecular insights were needed to corroborate the hypothesis that VRC resistance is mostly associated with the activity of efflux pumps and to exclude other resistance mechanisms.

This is the first work to address quantification of target gene expression by real-time PCR in wild-type resistant isolates, i.e., not genetically manipulated strains. The gene expression profile described herein for all of the resistant *C. krusei* strains (Fig. 5) strongly suggests that the acquisition of long-term resistance is mostly associated with the *ABC1* gene. Similar findings were previously described by Holmes et al. for *C. albicans* and by Bennett et al. for *Candida glabrata*, i.e., Cdr1p efflux activity contributes more to FLC resistance than Cdr2p (28, 29). However, our *C. krusei* R_{indD5} strain is a controversial case since neither *ABC1* nor *ABC2* genes are overexpressed. Initially, exposure to VRC induces increases in *ABC2* gene expression, overcoming *ABC1* gene expression. We hypothesize that *ABC2* can be activated more rapidly, although transiently. For long-term VRC tolerance, yeasts clearly prefer to activate the Abc1p efflux pump, which seems to be more efficient in antifungal expulsion. On the other hand, other genes encoding ATP-dependent efflux transporters may be present in C. *krusei*, such as a *CgSNQ2* homologous gene that was described as an azole-associated resistance gene in *C. glabrata* (30). Although it has been sequenced, the *C. krusei* genome is not yet completely annotated; thus, other transporter genes were not assessed. In addition, Lamping et al. incubated *C. krusei* strains for up to 4 h with different antifungals, including VRC, and no significant increases in *ABC1* mRNA levels were recorded (13). In our case, the *ABC1* gene was significantly overexpressed in *C. krusei* strains after only 10 days of *in vitro* exposure (strain R_{indD10}) or 24 days of VRC therapy (strain $R_{clinD24}$). Together, these facts clearly show that the *ABC1* gene is upregulated after an extended period of antifungal exposure, playing a late role in the development of resistance. After being triggered, however, *ABC1* gene overexpression correlates with a stable resistant phenotype, playing a definite role in long-term VRC resistance even in the absence of azoles (strain R_{D60}) (Fig. 5B). *ERG11* gene overexpression seems to be relevant in the development of VRC resistance only at an early stage, as an initial adaptation mechanism (Fig. 5). Later, other distinct mechanisms, such as the acquisition of point mutations, predominate. The point mutation described herein is definitely associated with VRC resistance in *C. krusei*, since the same type of mutation was already reported to be associated with azole resistance in *C. albicans* (31). Thus, we were confronted with the fact that *C. krusei* acquired multiple resistance mechanisms not described previously for this fungal pathogen. Such a finding is of medical relevance in considering therapeutic protocols; a susceptible isolate can develop resistance to VRC during a therapeutic regimen. On the other hand, the heterozygous alteration detected (T1389C), which is located outside the azole binding site, according to previously published data, was found in both susceptible and resistant strains (32). This alteration corresponds to synonymous SNPs, whose repercussions remain to be determined. These findings are in accordance with previous results by Lamping et al., who found several synonymous SNPs in the *ERG11* gene sequence (13), including the one detected by us. The results presented emphasize that prolonged therapy with azole antifungals can lead to resistant clones, which can ultimately spread and colonize other susceptible hosts.

In this study, we elucidated for the first time the presence of multiple concomitant resistance mechanisms in resistant *C. krusei* strains induced in both *in vivo* and *in vitro* assays. We demonstrated the relevant role that efflux activity plays as a mechanism of resistance to VRC, as well as the acquisition of a missense point mutation in the target enzyme Erg11p. The set of *C. krusei* strains described herein depicts the evolutionary process of resistance acquisition both *in vitro* and *in vivo*, being a valuable tool for the study of antifungal resistance in *C. krusei*.

ACKNOWLEDGMENTS

We thank Isabel Santos for excellent technical support.

Isabel M. Miranda was supported by the Fundação Ciência e Tecnologia Ciência 2008 and cofinanced by the European Social Fund.

REFERENCES

1. **Abbas J, Bodey GP, Hanna HA, Mardani M, Girgawy E, Abi-Said D, Whimbey E, Hachem R, Raad I.** 2000. *Candida krusei* fungemia: an escalating serious infection in immunocompromised patients. Arch. Intern. Med. **160:**2659–2664. http://dx.doi.org/10.1001/archinte.160.17.2659.
2. **Nucci M, Marr KA.** 2005. Emerging fungal diseases. Clin. Infect. Dis. **41:**521–526. http://dx.doi.org/10.1086/432060.
3. **Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Nagy E, Dobiasova S, Rinaldi M, Barton R, Veselov A, Global Antifungal Surveillance Group.** 2008. *Candida krusei*, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program, 2001 to 2005. J. Clin. Microbiol. **46:**515–552. http://dx.doi.org/10.1128/JCM.01915-07.
4. **Muñoz P, Sánchez-Somolinos M, Alcalá L, Rodríguez-Créixems M, Peláez T, Bouza E.** 2005. *Candida krusei* fungaemia: antifungal susceptibility and clinical presentation of an uncommon entity during 15 years in a single general hospital. J. Antimicrob. Chemother. **55:**188–193. http://dx.doi.org/10.1093/jac/dkh532.
5. **Pfaller MA, Diekema DJ, Messer SA, Boyken L, Hollis RJ, Jones RN, International Fungal Surveillance Participant Group.** 2003. In vitro activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against *Candida* species infrequently isolated from blood. J. Clin. Microbiol. **41:**78–83. http://dx.doi.org/10.1128/JCM.41.1.78-83.2003.
6. **Drago M, Scaltrio MM, Morace G, GISIA-2 Group.** 2004. In vitro activity of voriconazole and other antifungal agents against clinical isolates of *Candida glabrata* and *Candida krusei*. Eur. J. Clin. Microbiol. Infect. Dis. **23:**619–624. http://dx.doi.org/10.1007/s10096-004-1174-9.
7. **Spanakis EK, Aperis G, Mylonakis E.** 2006. New agents for the treatment of fungal infections: clinical efficacy and gaps in coverage. Clin. Infect. Dis. **43:**1060–1068. http://dx.doi.org/10.1086/507891.
8. **Hakki M, Staab JF, Marr KA.** 2006. Emergence of a *Candida krusei*

isolate with reduced susceptibility to caspofungin during therapy. Antimicrob. Agents Chemother. **50:**2522–2524. http://dx.doi.org/10.1128/AAC.00148-06.

9. **Kahn JN, Garcia-Effron G, Hsu MJ, Park S, Marr KA, Perlin DS.** 2007. Acquired echinocandin resistance in a *Candida krusei* isolate due to modification of glucan synthase. Antimicrob. Agents Chemother. **51:**1876–1878. http://dx.doi.org/10.1128/AAC.00067-07.
10. **Katiyar SK, Edlind TD.** 2001. Identification and expression of multidrug resistance-related ABC transporter genes in *Candida krusei*. Med. Mycol. **39:**109–116. http://dx.doi.org/10.1080/mmy.39.1.109.116.
11. **Orozco AS, Higginbotham LM, Hitchcock CA, Parkinson T, Falconer D, Ibrahim AS, Ghannoum MA, Filler SG.** 1998. Mechanism of fluconazole resistance in *Candida krusei*. Antimicrob. Agents Chemother. **42:**2645–2649.
12. **Venkateswarlu K, Denning DW, Manning NJ, Kelly SL.** 1996. Reduced accumulation of drug in *Candida krusei* accounts for itraconazole resistance. Antimicrob. Agents Chemother. **40:**2443–2446.
13. **Lamping E, Ranchod A, Nakamura K, Tyndall JD, Niimi K, Holmes AR, Niimi M, Cannon RD.** 2009. Abc1p is a multidrug efflux transporter that tips the balance in favor of innate azole resistance in *Candida krusei*. Antimicrob. Agents Chemother. **53:**354–369. http://dx.doi.org/10.1128/AAC.01095-08.
14. **Morio F, Loge C, Besse B, Hennequin C, Le Pape P.** 2010. Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. Diagn. Microbiol. Infect. Dis. **66:**373–384. http://dx.doi.org/10.1016/j.diagmicrobio.2009.11.006.
15. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
16. **Clinical and Laboratory Standards Institute.** 2012. Reference method for broth dilution antifungal susceptibility testing of yeasts: fourth informational supplement, approved standard M27-S4. Clinical and Laboratory Standards Institute, Wayne, PA.
17. **Ricardo E, Silva AP, Gonçalves T, Costa de Oliveira S, Granato C, Martins J, Rodrigues AG, Pina-Vaz C.** 2011. *Candida krusei* reservoir in a neutropaenia unit: molecular evidence of a foe? Clin. Microbiol. Infect. **17:**259–263. http://dx.doi.org/10.1111/j.1469-0691.2010.03223.x.
18. **Bautista-Muñoz C, Boldo XM, Villa-Tanaca L, Hernández-Rodríguez C.** 2003. Identification of *Candida* spp. by randomly amplified polymorphic DNA analysis and differentiation between *Candida albicans* and *Candida dubliniensis* by direct PCR methods. J. Clin. Microbiol. **41:**414–420. http://dx.doi.org/10.1128/JCM.41.1.414-420.2003.
19. **Rao US, Scarborough GA.** 1994. Direct demonstration of high affinity interactions of immunosuppressant drugs with the drug binding site of the human P-glycoprotein. Mol. Pharmacol. **45:**773–776.
20. **Köhrer K, Domdey H.** 1991. Preparation of high molecular weight RNA. Methods Enzymol. **194:**398–405. http://dx.doi.org/10.1016/0076-6879(91)94030-G.
21. **Pfaffl MW, Horgan GW, Dempfle L.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. **30:**e36. http://dx.doi.org/10.1093/nar/30.9.e36.
22. **Edgar FC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32:**1792–1797. http://dx.doi.org/10.1093/nar/gkh340.
23. **Sancak B, Rex JH, Chen E, Marr K.** 2004. Comparison of PCR- and HinfI restriction endonuclease-based methods for typing of *Candida krusei* isolates. J. Clin. Microbiol. **42:**5889–5891. http://dx.doi.org/10.1128/JCM.42.12.5889-5891.2004.
24. **Costa-de-Oliveira S, Araujo R, Silva-Dias A, Pina-Vaz C, Rodrigues AG.** 2008. Propofol lipidic infusion promotes resistance to antifungals by reducing drug input into the fungal cell. BMC Microbiol. **8:**9. http://dx.doi.org/10.1186/1471-2180-8-9.
25. **Schuetzer-Muehlbauer M, Willinger B, Egner R, Ecker G, Kuchler K.** 2003. Reversal of antifungal resistance mediated by ABC efflux pumps from *Candida albicans* functionally expressed in yeast. Int. J. Antimicrob. Agents **22:**291–300. http://dx.doi.org/10.1016/S0924-8579(03)00213-9.
26. **Guinea J, Sánchez-Somolinos M, Cuevas O, Peláez T, Bouza E.** 2006. Fluconazole resistance mechanisms in *Candida krusei*: the contribution of efflux-pumps. Med. Mycol. **44:**575–578. http://dx.doi.org/10.1080/13693780600561544.
27. **Henry KW, Nickels JT, Edlind TD.** 2000. Upregulation of ERG genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. Antimicrob. Agents Chemother. **44:**2693–2700. http://dx.doi.org/10.1128/AAC.44.10.2693-2700.2000.
28. **Holmes AR, Lin YH, Niimi K, Lamping E, Keniya M, Niimi M, Tanabe K, Monk BC, Cannon RD.** 2008. ABC transporter Cdr1p contributes more than Cdr2p does to fluconazole efflux in fluconazole-resistant *Candida albicans* clinical isolates. Antimicrob. Agents Chemother. **52:**3851–3862. http://dx.doi.org/10.1128/AAC.00463-08.
29. **Bennett JE, Izumikawa K, Marr KA.** 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. Antimicrob. Agents Chemother. **48:**1773–1777. http://dx.doi.org/10.1128/AAC.48.5.1773-1777.2004.
30. **Sanglard D, Ischer F, Bille J.** 2001. Role of ATP-binding-cassette transporter genes in high frequency acquisition of resistance to azole antifungals in *Candida glabrata*. Antimicrob. Agents Chemother. **45:**1174–1183. http://dx.doi.org/10.1128/AAC.45.4.1174-1183.2001.
31. **Sanglard D, Ischer F, Koymans L, Bille J.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. **42:**241–253. http://dx.doi.org/10.1093/jac/42.2.241.
32. **Fukuoka T, Johnston DA, Winslow CA, de Groot MJ, Burt C, Hitchcock CA, Filler SG.** 2003. Genetic basis for differential activities of fluconazole and voriconazole against *Candida krusei*. Antimicrob. Agents Chemother. **47:**1213–1219. http://dx.doi.org/10.1128/AAC.47.4.1213-1219.2003.