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Set of Classical PCRs for Detection of Mutations in *Candida glabrata* FKS Genes Linked with Echinocandin Resistance

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Clinical echinocandin resistance among *Candida glabrata* strains is increasing, especially in the United States. Antifungal susceptibility testing is considered mandatory to guide therapeutic decisions. However, these methodologies are not routinely performed in the hospital setting due to their complexity and the time needed to obtain reliable results. Echinocandin failure in *C. glabrata* is linked exclusively to Fks1p and Fks2p amino acid substitutions, and detection of such substitutions would serve as a surrogate marker to identify resistant isolates. In this work, we report an inexpensive, simple, and quick classical PCR set able to objectively detect the most common mechanisms of echinocandin resistance in *C. glabrata* within 4 h. The usefulness of this assay was assessed using a blind collection of 50 *C. glabrata* strains, including 16 *FKS1* and/or *FKS2* mutants.

Candida glabrata is a major agent of invasive candidiasis. It is considered the second-most-common *Candida* sp. isolated from blood samples in the United States and northern and eastern Europe and the third most common in the rest of the world (1–5). Its high frequency is, at least in part, associated with antifungal preexposure (6). Fluconazole resistance is common in *C. glabrata*, and echinocandins are recommended as first-line therapy. However, echinocandin resistance in *C. glabrata* is increasing (with rates ranging from 1% to 3% worldwide), making susceptibility testing mandatory to guide therapeutic decisions (1, 7–10). The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) established reference broth microdilution methods for *Candida* echinocandin susceptibility testing (11, 12). Moreover, the CLSI published revised interpretative guidelines in December 2012 that showed good performance in identifying echinocandin-resistant *C. glabrata* strains (7, 13). However, these methods have several limitations, including (i) a time-consuming and expensive methodology; (ii) the fact that standard echinocandin powders (indispensable for CLSI or EUCAST methods) are not commercially available; (iii) caspofungin MIC interlaboratory variability; (iv) overlapping susceptible and resistant populations; and (v) the need for 24 h of processing to obtain results (5, 11, 12, 14).

Clinical echinocandin resistance in *C. glabrata* is linked with substitutions in the hot spot regions of the Fks1p and Fks2p subunits of the β -D-1,3-glucan synthase complex (the target of echinocandins) (15–18). The detection of these *FKS* mutations has been considered the most accurate way to predict an echinocandin treatment failure (14, 18, 19). In an effort to improve the detection of echinocandin-resistant *C. glabrata* isolates, we developed a set of classical PCRs able to detect 10 of the most frequent mutations associated with clinical echinocandin resistance in less than 4 h. The sensitivity and specificity of the method were assessed using a blind collection of *C. glabrata* clinical isolates comprising echinocandin-resistant and -susceptible strains.

MATERIALS AND METHODS

Strains and blind study design. Fifty *C. glabrata* strains were used throughout this work. All strains were isolated from patients with proven invasive fungal disease (20). Nineteen strains were obtained from the Public Health Research Institute (PHRI; Rutgers University, NJ), 20 from the Mycology laboratory of the Ramos Mejia Hospital (Buenos Aires, Argentina), and 11 from the Mycology and Molecular Diagnostics Laboratory (LMDM) (Santa Fe, Argentina). Sixteen strains showed *FKS1* and/or *FKS2* hot spot region mutations (Table 1). *C. glabrata* ATCC 90030 was used as the wild-type control strain to validate the PCRs. *C. krusei* ATCC 6258 and *C. parapsilosis sensu stricto* ATCC 22019 were used as susceptibility testing control strains (11, 13). The isolates were identified as *C. glabrata* by conventional phenotypic methods and by sequencing of the 5.8S rRNA gene and adjacent internal transcribed spacer 1 (*ITS1*) and *ITS2* regions (21, 22). The collection of strains was assembled at the PHRI center, and blind code numbers were assigned. Also, a set of *C. glabrata* strains with known *FKS1* and/or *FKS2* mutations were used to develop and test the proposed methodology before confirming its utility with the blind study.

Antifungals and susceptibility testing. Caspofungin (CSF; Merck & Co. Inc., Rahway, NJ), anidulafungin (ANF; Pfizer, New York, NY), and micafungin (MCF; Astellas Pharma USA Inc., Deerfield, IL) were obtained as standard powder from their respective manufacturers. Echinocandin susceptibility testing was performed in triplicate in accordance with CLSI document M27-A3 and following the interpretive guidelines published in the M27-S4 document (11, 13).

DNA isolation, PCR conditions, and primer and PCR set design. *C. glabrata* genomic DNAs were extracted with phenol-chloroform method (23) or with a Q-Biogene FastDNA kit (Q-Biogene). *C. glabrata* *FKS1* and

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TABLE 1 Comparison of results from classical PCR set, DNA sequencing, and *in vitro* susceptibility determinations of the *C. glabrata* strains included in this study

Strain ^a	Classical PCR set result ^b					DNA sequencing result		MIC (μg/ml) ^c		
	1-F625	1-S629	1-D632	2-F659	2-S663	Fks1p ^c	Fks2p ^d	ANF	CSF	MCF
WT (<i>n</i> = 34)	+	+	+	+	+	WT	WT	0.08 (S)	0.09 (S)	0.02 (S)
42997	–	+	+	+	+	F625S	WT	2.00	2.00	0.50
5847	+	–	+	+	+	S629P	WT	4.00	>8.00	2.00
3169	+	+	–	+	+	D632E	WT	2.00	2.00	2.00
LMDM 37	+	+	–	+	+	D632E	WT	2.00	4.00	4.00
21900	+	+	–	+	+	D632G	WT	1.00	4.00	0.06 (S)
42971	+	+	–	+	+	D632Y	WT	4.00	4.00	1.00
31498	+	+	+	+	+	WT	F659del	2.00	8.00	4.00
6183	+	+	+	–	+	WT	F659S	4.00	>8.00	4.00
M234	+	+	+	–	+	WT	F659V	1.00	4.00	1.00
20.551.099	+	+	+	–	+	WT	F659Y	1.00	2.00	0.12 (I)
3.830	+	+	+	+	–	WT	S663P	2.00	>8.00	1.00
37178	+	+	+	+	–	W645STOP	S663P	4.00	>8.00	8.00
M2798	+	+	+	+	–	WT	S663P	8.00	>8.00	8.00
20.593.033	+	+	+	+	–	W649STOP	S663P	4.00	>8.00	4.00
LMDM 34	+	+	+	+	–	WT	S663P	2.00	>8.00	2.00
M2791	+	+	+	+	–	WT	S663F	4.00	4.00	4.00

^a Includes 34 wild-type *C. glabrata* strains and 16 *FKS1* and/or *FKS2* mutants.

^b Positive or negative signs indicate the presence or the absence of the corresponding PCR band in a electrophoresis gel.

^c WT, wild type at hot spots. Fks1p hot spot 1 includes amino acids between 625 and 633 (625-FLILSLRDP-633).

^d WT, wild type at hot spots. Fks2p hot spot 1 includes amino acids between 659 and 667 (659-FLILSLRDP-667).

^e Data represent geometric mean values. MICs were obtained on three separate days. ANF, anidulafungin. CSF, caspofungin. MCF, micafungin. (S) or (I) indicates that the strain is considered echinocandin susceptible or echinocandin intermediate, respectively (or is otherwise considered resistant), following the interpretative guidelines published in CLSI document M27-S4 (13).

FKS2 genes with GenBank accession numbers [XM_446406](#) and [XM_448401](#), respectively, were used for primer design. Two groups of primers were used throughout this work. The primers in the first group (PCR control primers), consisting of primer pair 1-1670F and 1-2225R and primer pair 2-1619F and 2-2177R designed to specifically hybridize *FKS1* and *FKS2*, respectively, were used as an amplification control for each of the five multiplex PCRs (Table 2). The second group of primers, named the mutation detection primers, included five oligonucleotides that were designed to detect the 10 most common mutations related with echinocandin resistance. These primers align the *FKS1* and *FKS2* hot spot

1 regions and were named 1-F625, 1-S629, 1-D632, 2-F659, and 2-S663. These primers were used in pairs with primers 1-1670F (1-S629 and 1-D632), 1-2225R (1-F625), 2-1619F (2-S663), and 2-2177R (2-F659). PCR primers were designed by using the oligonucleotide design tool of the IDT SciTools (Integrated DNA Technologies, Coralville, IA) and were purchased from Integrated DNA Technologies (IDT-Biodynamics, Buenos Aires, Argentina).

Amplifications were carried out in a 25-μl volume of a mixture containing 5 mM (NH₄)₂SO₄, 5 mM KCl, 10 mM Tris-Cl (pH 8.8), 1 mM MgSO₄, 5 ng of bovine serum albumin, 0.1% Triton X-100, 125 μM each

TABLE 2 Oligonucleotides primers used in this study

Oligonucleotide ^a	Target gene	Purpose(s) ^b	5'→3' sequence ^c
1-1670F	<i>FKS1</i>	<i>FKS1</i> HS1 AfS and AC	GTGCTGCGGTCATGTTCTT
1-2225R	<i>FKS1</i>	<i>FKS1</i> HS1 AfS and AC	GCGTTCAGACTTGGGAAAT
2-1619F	<i>FKS2</i>	<i>FKS2</i> HS1 AfS and AC	GAATGGTGGTTCGTTCCAAG
2-2177R	<i>FKS2</i>	<i>FKS2</i> HS1 sequencing and AC	TGTTGCTTCTCAGACTTTCACC
1-F625F	<i>FKS1</i>	Mutation detection	CGCTGAATCATACTACTT
1-S629R	<i>FKS1</i>	Mutation detection	GATTGGATCTCTTGAGA
1-D632R	<i>FKS1</i>	Mutation detection	GACAAAATTCTGATTGGA
2-F659F	<i>FKS2</i>	Mutation detection	CTCTGAATCGTACTTCTT
2-S663R	<i>FKS2</i>	Mutation detection	GATAGGGTCTCTTAGAGA
1-1776F	<i>FKS1</i>	<i>FKS1</i> HS1 sequencing	ACGTCGCTTCTCAAACCTTC
1-2008R	<i>FKS1</i>	<i>FKS1</i> HS1 sequencing	CGGTAGCAATCATCAAACCC
2-1881F	<i>FKS2</i>	<i>FKS1</i> HS1 sequencing	CGACGTTTCAGCTTCAGAGTTT
2-2513R	<i>FKS2</i>	<i>FKS2</i> HS1 AfS	CCAACAGAGAAGACAGTGTGTA
ITS1 ^d	rDNA ^e	Molecular identification	TCCGTAGGTGAACCTGCGG
ITS4 ^d	rDNA	Molecular identification	TCCTCCGCTTATTGATATGC

^a F, sense; R, antisense.

^b AfS, amplification for subsequent sequencing; AC, amplification control; HS1, hot spot 1.

^c Nucleotides in bold show where a mutation could be present.

^d From reference 24.

^e rDNA, ribosomal DNA.

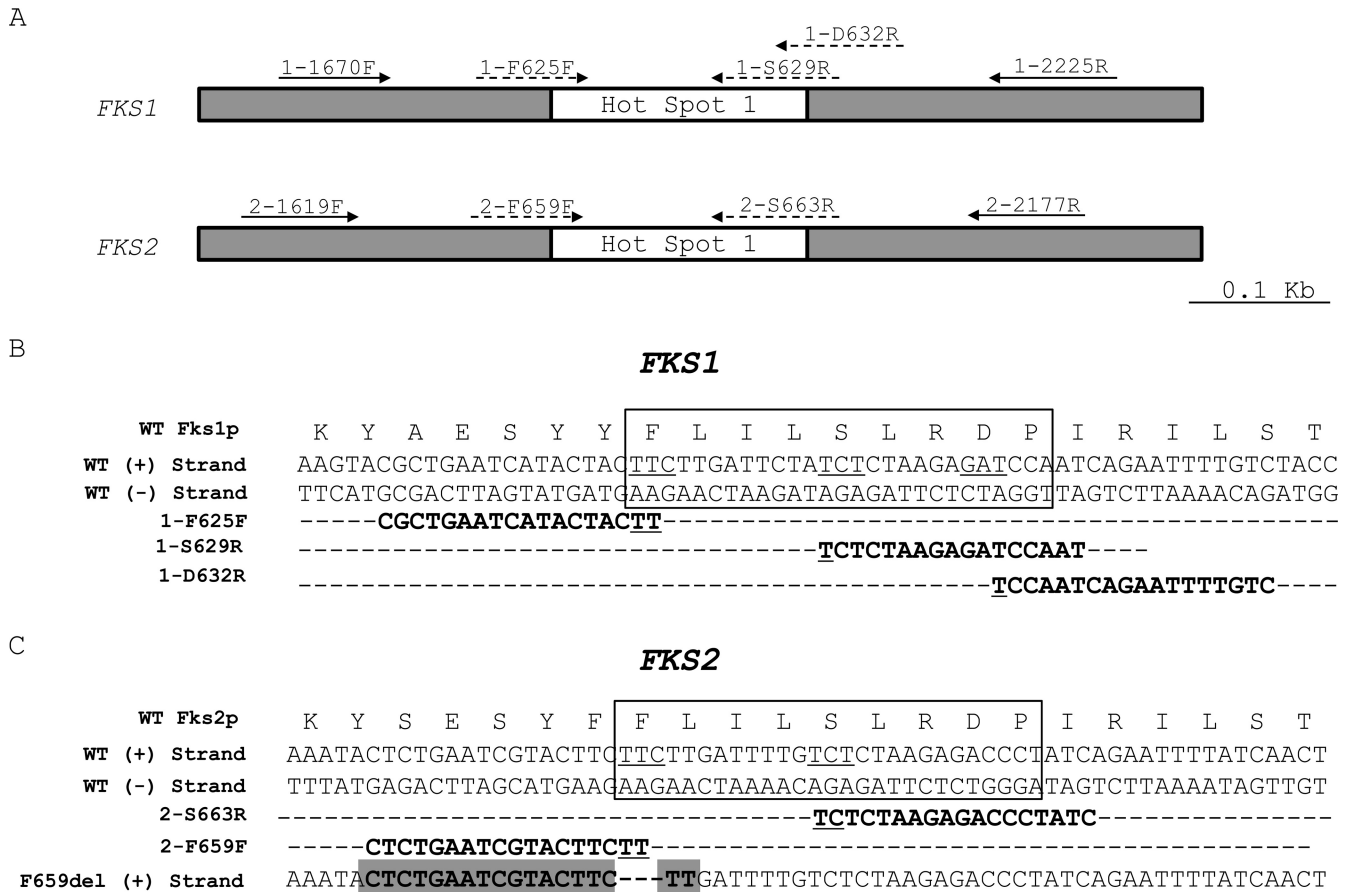


FIG 1 (A) Representation of 1,000-nucleotide (nt) fragments of *C. glabrata* *FKS* genes, which include the hot spot 1 regions (white boxes). Filled arrows: oligonucleotide primers included in the PCR control group used as the reaction control. Dashed arrows: primers designed to detect *C. glabrata* *FKS1* and *FKS2* mutations (mutation detection group). (B) Alignment of primers 1-F625F, 1-S629R, and 1-D632R with the wild-type (WT) *FKS1* gene. (C) Alignment of primers 2-S663R and 2-F659F with the wild-type *FKS2* gene and primer 2-F659F with the *FKS2* gene with the deletion of three nucleotides (from T1995 to C1997) (gray shading). Underlined nucleotides show the codons where the mutations are under cover by mutation detection primers. Boxes include the Fks1p and Fks2p hot spot 1 regions.

dATP, dGTP, dCTP, and dTTP (Genbiotech, Buenos Aires, Argentina), a 0.5 μ M concentration of each of the three primers, 1.25 U of Pegasus DNA polymerase (PBL, Buenos Aires, Argentina), and 10 to 25 ng of *C. glabrata* genomic DNA. Amplification was performed for one initial step of 2 min at 94°C followed by 25 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C and then a final cycle of 10 min at 72°C in an Applied Biosystems thermocycler (Tecnolab-AB, Buenos Aires, Argentina). The PCR products were analyzed by electrophoresis.

DNA sequencing. The *C. glabrata* *FKS1* hot spot 1 region (nucleotide [nt] 1776 to nt 2008), *FKS2* hot spot 1 region (nt 1881 to nt 2177), and 5.8S RNA gene and adjacent internal transcribed spacer 1 (*ITS1*) and *ITS2* regions were amplified and sequenced in both directions using the primers described in Table 2. For sequencing of the *FKS1* and *FKS2* hot spot 1 regions, primer pair 1-1670F and 1-2225R and primer pair 1-1619F and 1-2513R were used for PCR amplification, respectively. The purified fragments were then subjected to sequencing using primers 1-1776F and 1-2008R for *FKS1* and 2-1881F and 2-2177R for *FKS2* (Table 2). In Argentina, DNA sequencing was performed using a BigDye Terminator cycle sequencing ready-reaction system (Applied Biosystems, Buenos Aires, Argentina) according to the manufacturer's instructions. Sequence analysis was performed on an ABI Prism 310 DNA sequencer (Applied Biosystems) using the facilities available at Cromatida S.A. (Buenos Aires, Argentina). In the PHRI Center, DNA sequencing was performed with a CEQ dye terminator cycle sequencing QuickStart kit (Beckman Coulter,

Fullerton, CA) according to the manufacturer's recommendations. Sequencing analyses were done with CEQ 8000 genetic analysis system software (Beckman Coulter) and with the BioEdit sequence alignment editor (Ibis Therapeutics, Carlsbad, CA).

RESULTS

Primer and PCR design for the detection of the molecular echinocandin resistance mechanism in *C. glabrata*. The *C. glabrata* *FKS1* and *FKS2* genes have high (>73%) homology, with portions with very low homology (lower than 50%) and others with the highest homology (>85% for the hot spot 1 regions of both genes) (Fig. 1). For this reason, we designed two groups of primers named PCR control primers and mutation detection primers (both groups are described above). The primers of the first group were designed to align the regions of lowest homology between the genes (hot spot 1 external region) with dual objectives: (i) to give the *FKS1* or *FKS2* gene specificity when used in combination with the mutation detection primers and (ii) to use them as internal controls for validation of the quality of DNA samples and the absence of PCR inhibitors, since the presence of a mutation is represented by a negative result in a PCR. On the other hand, primers 1-F625, 1-S629, 1-D632, 2-F659, and 2-S663 were de-

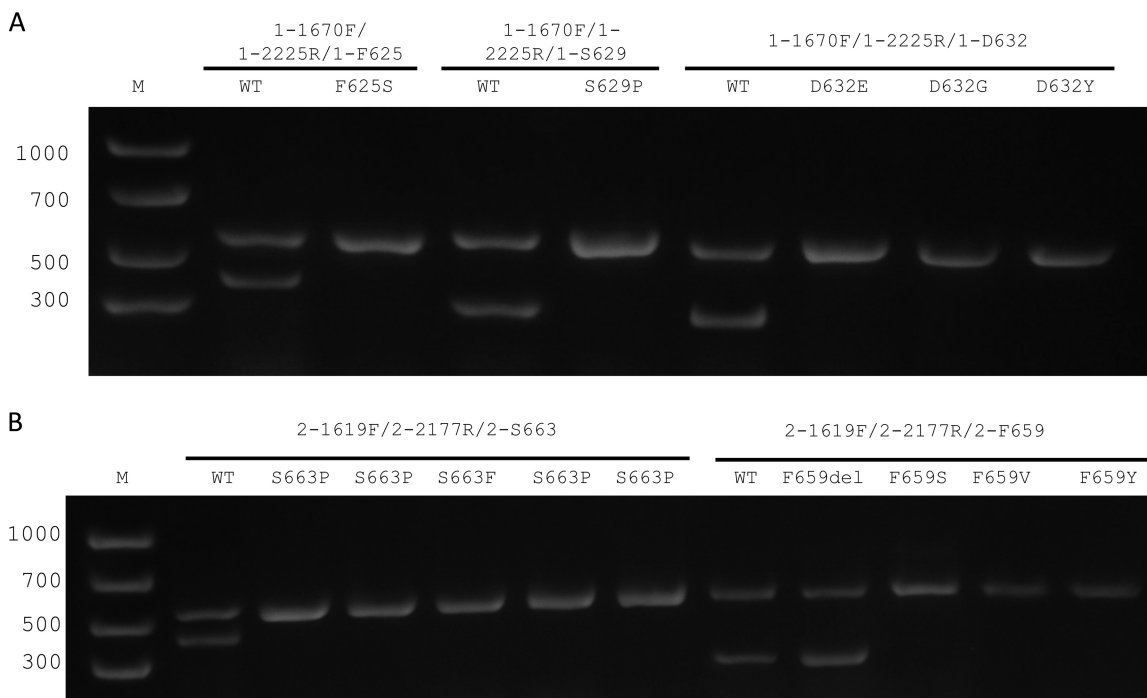


FIG 2 Electrophoresis of the PCR set resolved in a 1.5% agarose gel. The three primers used in each of the tubes are named above the images. Lane M, molecular size marker. (A) PCRs designed to detect *FKS1* mutant. Lanes 2, 4, and 6, *C. glabrata* ATCC 90030 (wild-type strain, echinocandin susceptible). Lane 3, *C. glabrata* strain 42997 (Fks1p-F625S). Lane 5, strain 5847 (Fks1p-S629P). Lane 6, strain LMDM37 (Fks1p-D632E). Lane 7, *C. glabrata* 21900 (Fks1p-D632G). Lane 8, isolate 42971 (Fks1p-D632Y). (B) Lanes 2 and 8, *C. glabrata* ATCC 90030 (echinocandin-susceptible wild-type strain). Lane 3, *C. glabrata* 3.830 (Fks2p-S663P). Lane 4, strain 37178 (Fks2p-S663P). Lane 5, strain M2791 (Fks2p-S663F). Lane 6, isolate 20.593.033 (Fks2p-S663P). Lane 7, strain LMDM 34 (Fks2p-S663P). Lane 9, strain 31498 (Fks2p-F659del). Lane 10, strain 6183 (Fks2p-F659S). Lane 11, strain M234 (Fks2p-F659V). Lane 12, isolate 20.551.099 (Fks2p-F659Y).

signed specifically for priming *FKS1* or *FKS2* wild-type sequences, considering that a 3' mismatch does not prime in a PCR under the appropriate conditions of stringent annealing temperatures (Fig. 1). Furthermore, other reaction variables such as annealing temperatures and $MgSO_4$ and primer concentrations were taken into consideration for PCR design to allow the use of one PCR program irrespective of the primer set used. Under the PCR and reagent concentration conditions described above, all five PCRs could be run at the same time and with the same program in the thermocycler, showing excellent discrimination for both wild-type and mutant alleles. Therefore, for detection of the substitution at Fks1p residues F625, S629, and D632, the multiplex PCRs were performed using three primers per tube, including primers 1-1670F, 1-2225R, and 1-F625, primers 1-1670F, 1-2225R, and 1-S629, and primers 1-1670F, 1-2225R, and 1-D632, respectively. These PCRs gave one 555-bp band in all the tubes and 369-bp, 263-bp, and 252-bp bands when the isolate was wild type at residues F625, S629, and D632 at Fks1p, respectively. On the other hand, when a mutation is present in the codon that encodes any of the three amino acid residues listed above, a unique 555-bp band was observed after the electrophoresis (control PCR) (Fig. 2). The detection of amino acid substitutions at residues F659 and S663 of Fks2p was performed using a similar approach but with primers 2-1619F, 2-2177R, and 2-F659 and primers 2-1619F, 2-2177R, and 2-S663, respectively. In these cases, for a wild-type isolate, two bands (558 bp and 219 or 400 bp, respectively) were expected. For an echinocandin mutant with a substitution at F659 or S663 residues, a single 558-bp band was obtained (Fig. 2).

Validation of the multiplex PCR sets. The utility of the PCR

sets was evaluated by using a blind collection of 50 *C. glabrata* strains, including 16 echinocandin-resistant clinical isolates with different amino acid substitutions in both Fksp proteins (Table 1). Of the 50 isolates tested, 35 were considered wild-type strains by the proposed methodology since the 5 PCR tubes presented two bands in the electrophoresis. The rest were identified as *FKS1* or *FKS2* mutants with an amino acid substitution at residues Fks1p-F625 ($n = 1$), Fks1p-S629 ($n = 1$), Fks1p-D632 ($n = 4$), Fks2p-F659 ($n = 4$), and Fks2p-S663 ($n = 5$). A total of 49 of the 50 strains (98%) were correctly identified as echinocandin susceptible or resistant compared with the echinocandin susceptibility testing results. Also, we found 98% concordance between our proposed methodology and sequencing (Table 1). There was one false result, comprising a *FKS2* mutant, in which Fks2p showed a deletion at the 659 residue (F659del). This deletion was not uncovered by the 2-F659 primer because three nucleotides were deleted and the nucleotide sequence where the primer was aligned was maintained (Fig. 2).

DISCUSSION

Prompt diagnosis and the correct treatment selection for invasive *Candida* infections significantly reduce mortality (25). Echinocandin drugs are now considered the best therapeutic option for *C. glabrata* infections since these yeasts are less susceptible to fluconazole and amphotericin B than other *Candida* spp. (10). Recent reports showed that the number of echinocandin-resistant isolates is increasing, making essential an accurate assessment of echinocandin susceptibility (7, 9). Whole-cell susceptibility testing using a reference protocol takes at least 48 h (11, 12). However,

outside the United States, most of the susceptibility testing is being outsourced to reference laboratories due to the complexities of these methodologies, increasing the time needed to obtain reliable susceptibility data. To reduce this delay, we developed a simple set of multiplex PCRs able to objectively classify a *C. glabrata* strain as echinocandin susceptible or resistant in less than 4 h. The strict linkage between *FKS1* and *FKS2* hot spot region mutations and clinical echinocandin resistance provided the rationale for selecting the detection of these mutations as a surrogate marker for resistance. Twenty-three different amino acid substitutions in Fks1p and Fks2p hot spot regions were previously described (7, 8, 10, 15–17, 24, 26–31). However, 87.7% of the described clinically echinocandin-resistant strains showed substitutions at the Fks1p-F625 (2.46%), Fks1p-S629 (15.57%), Fks1p-D632 (5.74%), Fks2p-F659 (17.21%), and Fks2p-S663 (46.72%) residues (percentages were obtained over a total of 122 strains, 63 included in the cited reports plus 59 *C. glabrata* nonpublished echinocandin-resistant strains held in the Perlin's Echinocandin Resistance Reference Laboratory collection) (7, 8, 10, 15–17, 24, 26–31). Moreover, the strains harboring the most prevalent substitutions showed the highest echinocandin MIC values (7, 8, 10, 15–17, 24, 26–31). These data led us to decide to include the described five PCR assays to be able to detect the most common hot spot amino acid substitutions linked with echinocandin resistance in *C. glabrata*. In the blind study, we demonstrated that our set of PCRs was able to uncover mutants harboring Fks1p-F625S, Fks1p-S629P, Fks1p-D632G, Fks1p-D632E, Fks1p-D632Y, Fks2p-F659S, Fks2p-F659V, Fks2p-F659L, Fks2p-S663P, and Fks2p-S663F amino acid substitutions. Moreover, the designed primers would also potentially uncover less-common mutations as Fks1p-F625I (8) and Fks2p-F659Y (10, 24), since the primer's 3' ends would not hybridize these mutated sequences.

Recently, Pham et al. described a high-throughput microsphere-based assay using the Luminex MagPix technology suitable to identify *C. glabrata* *FKS* mutants (19). This method would be potentially used as a tool to evaluate a collection of strains in a reference laboratory. The advantage of the methodology that we are presenting is that it is based on the cheaper and commonly available classical PCR methodology, making it suitable to be used in a hospital setting for analyzing a few strains at a time. Moreover, this new method is able to uncover *FKS* mutations more quickly than other available molecular tools such as classical sequencing methods with no need for special equipment.

The main limitation of the proposed set of PCRs is its inability to detect the deletion of three nt at the codon which encoded the F659 at the Fks2p. This false result would be considered a very major error compared with whole-cell susceptibility testing since our proposed methodology would thus classify a resistant strain as susceptible. However, this molecular mechanism of echinocandin resistance has been described in only few strains worldwide and it is the least common substitution at this residue (8, 16, 19). Other limitations of this methodology are its inability to detect newly described mutations or other potential non-*FKS*-linked mechanisms associated with echinocandin resistance and the possibility of changes in epidemiology making the detection of the described mutations useless. However, these potential drawbacks are shared with any molecular method designed for the detection of mechanisms of resistance (32–34). Nevertheless, this methodology is suitable to be modified by adding or eliminating PCRs in order to adapt it to detect emerging mechanisms of resistance.

In conclusion, we present an inexpensive, simple, and quick molecular methodology able to objectively detect the most common mechanisms of echinocandin resistance in *C. glabrata*.

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