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Rapid Detection of *FKS*-Associated Echinocandin Resistance in *Candida glabrata*

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A novel and highly accurate diagnostic assay platform was established for rapid identification of *FKS* mutations associated with echinocandin resistance in *Candida glabrata*. The assay platform uses allele-specific molecular beacon and DNA melt analysis following asymmetric PCR. A dual assay for *FKS1* and *FKS2* was developed to identify within 3 h the most common and clinically relevant resistance-associated mutations, including 8 *FKS1* HS1 (wild type [WT], S629P, F625S, D632Y, D632E [T1896G], D632E [T1896A], I634V, and F625F) and 7 *FKS2* HS1 (WT, F659del, F659S, F659V, F659L, S663P, and S663F) genotypes. A blinded panel of 188 *C. glabrata* clinical isolates was tested by both assays. The molecular diagnostic results from the dual assay were 100% concordant with data obtained from DNA sequencing. This platform has the potential to overcome the deficiencies of existing *in vitro* susceptibility-based assays to identify echinocandin-resistant *C. glabrata* and holds promise as a surrogate diagnostic method to better direct echinocandin therapy.

Candida glabrata is the second leading cause of candidemia in the United States, as well as in northern and eastern areas of Europe (1–3). With rapidly expanded usage of echinocandins, the first-line therapy for invasive candidiasis, an alarming trend of rising echinocandin resistance in *C. glabrata* has posed a serious clinical challenge (4–6). Detection of echinocandin resistance can be assessed phenotypically, using either microdilution or disc diffusion MIC assays performed in accordance with guidance of the Clinical and Laboratory Standards Institute (CLSI) M27-A3 standard (7) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Definitive Document Edef 7.2 (8). Current echinocandin clinical breakpoints for *C. glabrata* were established by the CLSI for all echinocandins and by EUCAST for micafungin and anidulafungin. Breakpoints were specifically developed to identify *C. glabrata* harboring *FKS* mutations by considering multiple factors, such as β-1,3-D-glucan synthase enzyme kinetics and echinocandin pharmacokinetic and pharmacodynamic data (6, 9). However, despite standardized methodologies, important limitations with the *in vitro* susceptibility testing cannot be ignored: first, the assay has a time-consuming setup and intrinsically slow turnaround time, requiring 24 to 48 h after isolate recovery; second, interlaboratory variability of caspofungin MICs has limited direct testing of this drug (10); and third, susceptible and resistant populations overlap (11).

Clinical echinocandin resistance in *C. glabrata* is associated with amino acid substitutions caused by mutations in specific hotspot (HS) regions of *FKS1* and *FKS2*, which encode the drug target β-1,3-D-glucan synthase. A recent study performed among patients with invasive candidiasis due to *C. glabrata* has shown that the *FKS* genotype is a better indicator of echinocandin therapeutic failure than MIC (12).

To date, DNA sequencing is the only means to identify mutations within *FKS1* and *FKS2* genes. Sequence data are highly informative and accurate. However, targeted gene sequencing is impractical for the regular workflow of clinical microbiology laboratories. It is also costly and time-consuming. There is an emerging need for an alternative molecular tool which allows rapid and accurate discrimination of various *FKS* mutations in *C. glabrata* and thereafter has the potential to become part of clinical laboratory routine tests to direct echinocandin therapy.

Molecular beacons (MBs) are small stem-loop-structured DNA oligonucleotides that are ideal for typing single-nucleotide polymorphisms (13). In a classical way, MBs are utilized as amplification reporters in real-time PCR where each probe is specific for a defined genotype and labeled with a differently colored fluorophore. By the same token, a comprehensive *FKS* genotyping assay will require a large number of probes to cover the full spectrum of nucleic acid variations within the target region. Multiple PCR assays will therefore be needed, and optimization of assay conditions is extremely demanding due to complex oligonucleotide interactions in a multiplex format. In contrast, a more desirable and feasible approach to distinguish between closely related genotypes is to use asymmetric PCR in conjunction with MB probe-based melting curve analysis (14, 15). During asymmetric PCR, a single-stranded amplicon is produced, allowing the probe to anneal and generate fluorescence at low temperature. The fluorescence intensity decreases when the probe slowly dissociates from the target as a result of gradually increased temperature in the subsequent melting analysis. The temperature at which the probe-target hybrids melt apart (T_m) is determined on the curve plotted by the fluorescence intensity change as a function of temperature. The T_m value varies when the target changes from perfectly matched se-

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TABLE 1 MIC distributions for reference *C. glabrata* strains used for assay development

HS1 genotype (no. of isolates)	MIC range (μg/ml)		
	Anidulafungin	Caspofungin	Micafungin
FKS1			
WT (15)	0.06–0.25	0.06–0.25	0.06–0.12
S629P (4)	4–8	8–32	2–8
F625S (3)	2	2	0.5
D632E (T1896G) (2)	2	2	2
D632E (T1896A) (2)	1	0.5–1	0.25
D632Y (1)	4	4	1
I634V[a] (4)	0.06	0.06	0.03
F625F (6)	0.06–0.12	0.06–0.25	0.03
FKS2			
WT (15)	0.06–0.25	0.06–0.25	0.06–0.12
F659del (3)	2	8	4
F659S (3)	1–4	1–32	0.12–4
F659V (4)	1	4	0.25–1
F659L (1)	1	2	0.12
S663P (8)	1–8	4–32	0.5–32
S663F (3)	4	4	4

[a] Mutation I634V is just outside the *FKS1* HS1 region, and it was not yet found to be associated with echinocandin resistance in *C. glabrata* (6, 18, 19).

quence to mismatched sequence in the testing system, thereby providing a solid basis for wild-type (WT)/non-WT discrimination.

Based on these physiochemical properties of nucleic acids, we have established a molecular diagnostic assay platform for rapid genotyping of *FKS1* HS1 and *FKS2* HS1 of *C. glabrata*. Assay performance was evaluated on a blinded panel of clinical *C. glabrata* isolates representing a full spectrum of *FKS1* and *FKS2* genotype varieties in the fungal pathogen repository at the University of Pittsburgh Medical Center (UPMC).

MATERIALS AND METHODS

***Candida glabrata* strains and culture conditions.** A total of 262 *C. glabrata* strains were included in the present study. Seventy-four echinocandin-susceptible and -resistant strains stocked in the Perlin laboratory collection at the Public Health Research Institute were used for assay development and reference library establishment, and 188 *C. glabrata* isolates recovered from patients at UPMC were used for the validation study in a blind fashion. Strains were grown on yeast extract peptone dextrose (YPD) agar plates prior to testing.

Echinocandin susceptibility testing. Echinocandin (caspofungin, micafungin, and anidulafungin) MICs were determined for reference strains in the Perlin collection according to CLSI protocol M27-A3 (Table 1) (7).

DNA extraction. *C. glabrata* DNA was prepared by incubating a single colony of a strain in 100 μl of extraction buffer (60 mM $NaHCO_3$, 250 mM KCl, 50 mM Tris, pH 9.5) at 95°C for 10 min followed by an addition of 100 μl of 2% bovine serum albumin (16).

Primers and probe design. Two sets of asymmetric PCR primers were designed to amplify the *FKS1* HS1 and *FKS2* HS1 regions of *C. glabrata*. The excess primer for *FKS1* HS1 amplification was CgF1H1-X (5′-GGG TTACTGTTTTTGCTGCT-3′), and the limiting primer was CgF1H1-L (5′-GAACCCCACCAGTATTCACCAGTACA-3′). The *FKS2* HS1 region was amplified by using excess primer CgF2H1-X (5′-TGGGTTACAGTT TTTGCTGC-3′) and limiting primer CgF2H1-L (5′-AACCCCACCAAT ACTCACCAGTACATC-3′). Two molecular beacons were designed targeting the WT sequences of *FKS1* HS1 and *FKS2* HS1. The sequences of the two WT MBs were CgF1H1-RWT (5′-6-carboxyfluorescein [FAM]-CGCGACTGGATCTCTTAGAGATAGAATCAAGAAGGTCGCG-DABCYL-3′) and CgF2H1-RWT (5′-FAM-CGCGACAGGGTCTCTTA GAGACAAAATCAAGAAGTCGCG-DABCYL-3′) (underlining signifies the stem portion of the molecular beacon). It was noticed that some susceptible strains carrying a silent mutation (C1875T) at codon F625 in *FKS1* HS1 generated melting profiles very similar to that produced by a rare D632E-resistant mutant carrying a T1896A mutation. To differentiate between susceptible strains carrying the silent mutation and D632E (T1896A) mutants, we designed an F625F silent mutation-specific MB labeled with a different fluorophore, Cg-F1H1-RF625F (5′-HEX-CGCGC AAAAAGTAGTATGATTCACGCG-DABCYL-3′). This probe can be simply added to reactions with "unresolved F625F/D632E (T1896A) classifications" as a post-*FKS1* HS1 test to quickly rule out that the T1896A mutation caused D632E.

Asymmetric PCR and molecular beacon-based melting curve analysis. Asymmetric PCR was carried out on the AriaMx real-time PCR system (Agilent Technologies, CA) in a 20-μl reaction volume using Choice *Taq* Mastermix (Denville Scientific Inc.). *FKS1* HS1 assay contains 30 μM CgF1H1-X, 1 μM CgF1H1-L, 50 nM CgF1H1-RWT MB, and 10 to 25 ng of DNA template. When discriminating F625F and D632E (T1896A), the CgF1H1-RF625F MB was added to the *FKS1* HS1 reaction mixture at 25 nM. *FKS2* HS1 assay contains 50 μM CgF2H1-X, 1 μM CgF2H1-L, and 50 nM CgF2H1-RWT MB. *FKS1* HS1 PCR thermal cycling consisted of 3 min of incubation at 95°C; 45 cycles of 30 s at 95°C, 30 s at 57°C, and 1 min at 72°C; and then incubation at 72°C for 5 min. *FKS2* HS1 PCR conditions were 95°C for 3 min; 40 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C; and 5 min at 72°C. Immediately after amplification, melting curve analysis was performed at 95°C for 3 min and then 40°C for 30 s, after which it was melted from 40°C to 70°C with a ramp rate of 0.2°C/s.

Colony PCR genotyping. To further reduce the time to diagnosis, we introduced colony PCR into our diagnostic platform. Instead of using DNA extracts, a sterile toothpick with a touch of testing single colony was dipped into the PCR reaction medium, and then asymmetric PCR and the following melting analysis were performed as described above. At least three representative strains for each *FKS* genotype (8 *FKS1* HS1 genotypes and 7 *FKS2* HS1 genotypes) were tested for the efficiency of colony PCR genotyping.

DNA sequencing. *FKS1* HS1 and *FKS2* HS1 regions were amplified and sequenced in both directions as previously described (17).

Statistical analysis. T_m values for each *FKS* genotype were determined by melting curve analysis using the AriaMx software (version 1.0) (Agilent Technologies). Blind genotyping results by rapid molecular diagnostic assays were compared with DNA sequencing. The T_m distribution of clinical isolates was analyzed by GraphPad Prism 7.01 software. The accuracy of the novel assays discriminating WT from mutated *fks* genotypes was evaluated by calculating sensitivity and specificity for each assay.

RESULTS

Melting curve analysis-based *FKS1/FSK2* HS1 genotyping. To distinguish echinocandin resistance-associated mutations from the WT in the *FKS1/FSK2* HS1 region, we designed two discrimination probes, CgF1H1-RWT and CgF2H1-RWT. Both probes were designed to complement the WT genotype in the target region but possess various energies of binding to non-WT sequences, which reflect mutation and temperature. To enable efficient dissociation of probe-target hybrids for rapid genotyping, asymmetric PCR amplifications were optimized to generate an excess of the sense strand for both targets. Due to the stability difference of the probe-target hybrids, characteristic profiles were produced for different *FKS* genotypes in the subsequent melting curve analysis. Figure 1A shows melting curves of representative *FKS1* HS1 genotypes, including WT, S629P, F625S, D632Y, D632E (T1896G), D632E (T1896A), I634V (just outside the hotspot region yet not associated with resistance [6, 18, 19]), and

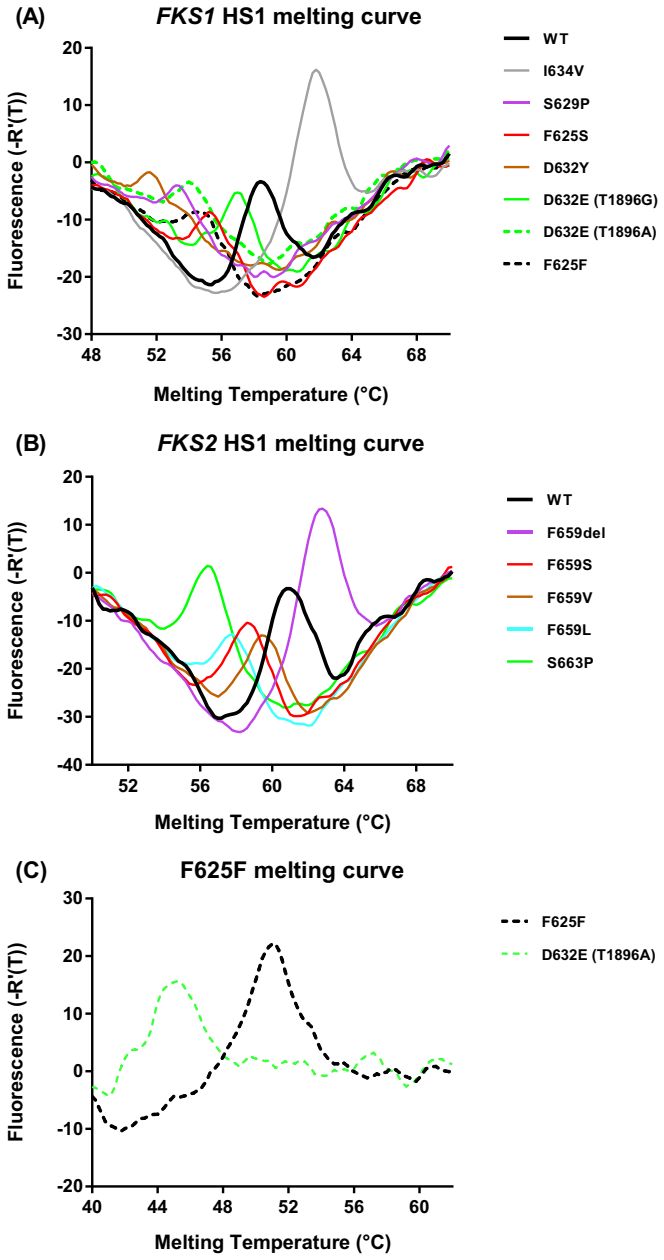

FIG 1 Representative melting profiles for *C. glabrata FKS1* HS1 genotypes, including WT, S629P, F625S, D632Y, D632E (T1896G), D632E (T1896A), I634V (not associated with resistance), and F625F (C1875T) (A), and *FKS2* HS1 genotypes, including F659del, F659S, F659V, F659L, and S663P (B). (C) Differentiation between F625F and D632E (T1896A) by an F625F-specific MB probe.

F625F (C1875T). Melting curves of mutated genotypes were remarkably different from that of the WT, and the T_m values of mutations were also distinguishable from each other, except for the F625F silent mutation and D632E (T1896A) (Table 2). In the *FKS2* HS1 assay, a melting curve with the T_m at 61°C ± 0.2°C was the signature for the WT, and unique melting profiles were also obtained for all mutations tested (F659del, F659S, F659V, F659L, and S663P/F) (Fig. 1B and Table 2).

To resolve indistinguishable melting signatures between F625F and D632E (T1896A) in the *FKS1* HS1 assay, an F625F-specific MB probe was added to the reaction mixture following *FKS1* HS1 assay analysis. A second round of melting analysis was then performed on the HEX channel, during which D632E (T1896A) (T_m = 45.4°C ± 0.1°C) was widely separated from the silent mutation (T_m = 51.2°C ± 0.1°C) by an approximately 6°C T_m difference (Fig. 1C).

Direct colony *FKS* genotyping. By replacing DNA extracts with direct single-colony picking in the established assay system, we successfully detected all representative strains (Table 1) and generated robust melting profiles for all testing genotypes consistent with those produced by DNA samples. Most importantly, direct genotyping from a colony was completed within 3 h, shortening the turnaround time by 30 min.

Validation experiments. As a proof of concept, we evaluated the diagnostic performance of this novel *FKS* genotyping platform using a panel of 188 *C. glabrata* clinical isolates collected at UPMC. Information on *FKS* sequences and echinocandin susceptibility was unknown at the time of molecular diagnosis. All isolates were tested in duplicate for the dual assay, and reference strains representing all genotypes used for platform establishment were tested in parallel in every single run. Inclusion of reference strains in each test run was important, as they served as systematic quality controls to ensure the assay's accuracy and reproducibility. It was noted that two isolates repeatedly failed in both assays and were identified subsequently by internal transcribed spacer sequencing as non-*C. glabrata* (1 *Saccharomyces cerevisiae* isolate and 1 *Candida guilliermondii* isolate). Robust and reproducible melting profiles were observed for all other isolates. Compared with T_m values generated by reference strains of various *FKS* HS1 genotypes, the *FKS1* HS1 assay identified 138 WT sequences (T_m = 58.7°C ± 0.2°C), 45 F625F silent mutations (54.3°C ± 0.3°C), and 3 D632 alterations (51.3°C ± 0.5°C) (Fig. 2A). DNA sequencing results confirmed all predicted genotypes and further identified the 3 D632 alterations as 2 D632Y and 1 D632H alteration. The *FKS2* HS1 assay detected 176 WT sequences (T_m = 61°C ± 0.2°C) as well as 2 F659del (T_m = 62.7°C ± 0°C), 2 F659S (T_m = 58.4°C ± 0.1°C), 2 F659L (T_m = 57.8°C ± 0°C), and 4 S663P/F (T_m = 56.4°C ± 0.2°C) alterations (Fig. 2B). Molecular typing results were 100% accurate on the F659 codon, and the 4 predicted

TABLE 2 T_m values for *C. glabrata FKS1* HS1 and *FKS2* HS1

HS1 variant	T_m (°C)	SD (°C)
FKS1		
WT	58.7	0.2
I634V (A1900G)	62	0.2
F625S (T1874C)	55.2	0.2
D632E (T1896A); F625F (C1875T)[a]	54.2	0.4
S629P (T1885C)	52.8	0.2
D632Y (G1894T); D632H (G1894C)	51.3	0.5
FKS2		
WT	61	0.2
F659del (del1957-1977)	62.7	0.1
F659V (T1975G)	59.4	0.1
F659S (T1976C)	58.4	0.1
F659L (C1977A)	57.8	0
S663P (T1987C); S663F (C1988T)	56.4	0.2

[a] Silent mutation can be differentiated from the "true" resistance-associated mutation D632E (T1896A) with the second-tier assay using the F625F-specific MB probe.

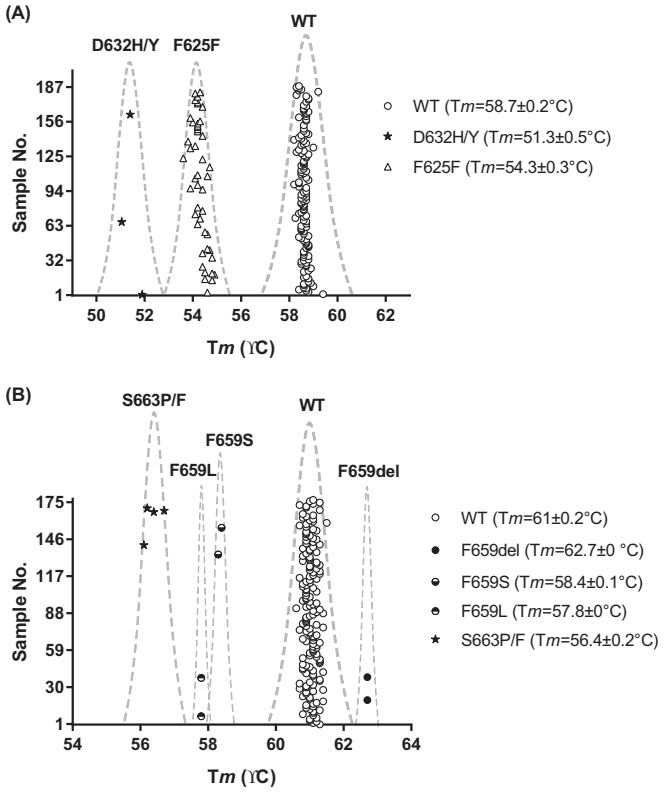

FIG 2 *FKS1* HS1 (A) and *FKS2* HS1 (B) T_m distributions of 186 blind-tested *C. glabrata* clinical isolates.

S663 alterations were identified as 3 S663P and 1 S663F alteration by sequencing. In summary, both *FKS1* HS1 and *FKS2* HS1 assays have demonstrated very promising diagnostic value for WT/non-WT discrimination with 100% specificity and 100% sensitivity.

DISCUSSION

FKS HS mutation is the major mechanism of echinocandin resistance in *C. glabrata*, providing an underlying rationale for developing rapid molecular detection of *FKS* genotypes. The goal of current echinocandin breakpoints is to distinguish *C. glabrata* isolates with a WT *FKS* from those containing *FKS* mutations that are unlikely to respond to echinocandin therapy (9). While mutations at almost every amino acid position within the *FKS* hot spot 1 and 2 regions confer some degree of resistance (20), recent studies have shown that the majority of mutations that affect *C. glabrata* susceptibility to the echinocandins are located within *FKS1* and *FKS2* HS1 regions. Accordingly, these regions are the primary targets for molecular assays to detect drug resistance (6, 21).

Given this goal, we have developed a novel diagnostic platform for rapid *FKS* genotyping of *C. glabrata* isolates. This platform, taking advantage of the strong single-nucleotide polymorphism recognition capability of molecular beacons and incorporating asymmetric PCR, accurately discriminated WT from various mutated *FKS* genes within 3 h. Using reference strains in our in-house collection, signature melting profiles and corresponding T_m values were generated for both WT and non-WT populations. Specific mutations are identified either individually or in a small cluster with distinguishable and consistent T_m values, demonstrating the robust discrimination power of this novel platform. Furthermore, with the adaptation of colony PCR in the testing system, the turnaround time was reduced by 30 min without losing the discrimination power. In the subsequent proof-of-concept clinical performance evaluation study, all blinded *C. glabrata* clinical isolates were successfully identified as either WT or mutated *FKS* genotypes, with 100% accuracy compared to DNA sequencing results.

Promising data were achieved from both assay development and clinical validation, but we did notice a minor issue with the *FKS1* HS1 assay. T_m values were indistinguishable between the silent mutation F625F and a weakly resistant genotype, D632E (T1896A). In theory, such unresolved susceptible/resistant classification is a concern for directing antifungal therapy. In reality, however, it may be only trivial to the diagnostic value of this *FKS* genotyping method, because the frequency of T1896A mutation-caused D632E is very low (estimated as <0.5% of all *FKS* HS mutations) based on several large-scale epidemiological studies on *FKS* mutations (6, 12, 18, 19, 22, 23). Nevertheless, an F625F silent mutation-specific MB probe was designed to address this susceptible/resistant uncertainty. This silent mutation probe was added to the reaction mixture following *FKS1* HS1 analysis and used as a second-tier assay. Using this method, all reactions with the F625F/D632E (T1896A) T_m values in the clinical isolate blind testing were subjected to a second melting analysis, and a unanimous F625F genotype which has no influence on susceptibility was identified for all suspected isolates, concordant with DNA sequencing results. It should be pointed out that the second-tier assay does require opening reaction tubes for the addition of the second beacon, which may carry the risk of contamination. However, this risk is relatively low, since the second-tier assay only involves a melting curve analysis without an amplification procedure. Technically, a duplex assay combining both *FKS1* HS1 WT probe with the F625F probe is the best option for high-throughput genotyping, but the melting curve resolution of the original WT probe was noticeably decreased with the addition of the F625F probe (data not shown). Presumably, the majority of unresolved strains in the primary *FKS1* HS1 test are susceptible strains harboring F625F silent mutation as a result of the very low frequency of D632E (T1896A); therefore, the need for a definite discrimination between these two genotypes is low. The two-tier assay strategy is currently recommended for comprehensive *FKS1* HS1 genotyping.

DNA sequencing has been the only technique available for definite identification of *FKS* mutations. Thus far, only two studies have reported development of rapid *FKS* genotyping assays (21, 24). Despite good diagnostic performance, both assays suffer from complicated assay design and setup. Compared to these assays, advantages of the present platform include (i) simple design modality, where a single MB probe and one set of primers allow the detection of a wide spectrum of mutations in the target region; (ii) ease of data interpretation, as the *FKS* genotype of the testing strain can be easily identified by comparing the T_m value with those generated by reference strains representing various mutations in the target region; (iii) multiplex potential, because the simple design modality allows probe build-up and the high specificity of each assay (no cross-reactivity) allows for probe combination; and (iv) expandable readiness for novel/unreported mutation detection, because the assay will be able to pick up a novel mutation by simply incorporating the corresponding genotype into the library without the need for assay redesign.

We acknowledge a minor limitation of our assay. Since the reference strains used for assay development do not cover the full *FKS* mutation spectrum, there is a chance that the T_m values of untested

mutation genotypes are not distinguishable from other mutations already included in our reference library. However, this will only have minimal impact on our platform, because the uncovered mutations account for only <10% of the mutants and would be <1% of the entire *C. glabrata* population. Furthermore, no cross-reaction between untested mutations and the WT is expected, because probes were designed to have complete sequence match only with the WT.

In summary, we have developed a rapid and accurate diagnostic platform that has the potential to overcome the deficiencies of existing MIC-based assays to identify echinocandin-resistant *C. glabrata*, and it holds the promise to be a surrogate diagnostic method to better direct echinocandin therapy.

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REFERENCES

1. **Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ, Chiller T.** 2012. Species identification and antifungal susceptibility testing of Candida bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. J Clin Microbiol **50:**3435–3442. http://dx.doi.org/10.1128/JCM.01283-12.
2. **Guinea J.** 2014. Global trends in the distribution of Candida species causing candidemia. Clin Microbiol Infect **20**(Suppl 6)**:**S5–S10.
3. **Pfaller M, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, Horn D.** 2012. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance) registry, 2004-2008. Diagn Microbiol Infect Dis **74:**323–331. http://dx.doi.org/10.1016/j.diagmicrobio.2012.10.003.
4. **Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, Booker R, Castanheira M, Messer SA, Perlin DS, Pfaller MA.** 2013. Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis **56:**1724–1732. http://dx.doi.org/10.1093/cid/cit136.
5. **Pfaller MA, Castanheira M, Lockhart SR, Ahlquist AM, Messer SA, Jones RN.** 2012. Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of Candida glabrata. J Clin Microbiol **50:**1199–1203. http://dx.doi.org/10.1128/JCM.06112-11.
6. **Pham CD, Iqbal N, Bolden CB, Kuykendall RJ, Harrison LH, Farley MM, Schaffner W, Beldavs ZG, Chiller TM, Park BJ, Cleveland AA, Lockhart SR.** 2014. Role of FKS mutations in Candida glabrata: MIC values, echinocandin resistance, and multidrug resistance. Antimicrob Agents Chemother **58:**4690–4696. http://dx.doi.org/10.1128/AAC.03255-14.
7. **CLSI.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Document M27-A3. CLSI, Wayne, PA.
8. **Arendrup MC, Cuenca-Estrella M, Lass-Florl C, Hope W.** 2012. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect **18:**E246–E247. http://dx.doi.org/10.1111/j.1469-0691.2012.03880.x.
9. **Pfaller MA, Diekema DJ, Andes D, Arendrup MC, Brown SD, Lockhart SR, Motyl M, Perlin DS.** 2011. Clinical breakpoints for the echinocandins and Candida revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria. Drug Resist Updat **14:**164–176. http://dx.doi.org/10.1016/j.drup.2011.01.004.
10. **Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A, Fuller J, Gaustad P, Gonzalez GM, Guarro J, Lass-Florl C, Lockhart SR, Meis JF, Moore CB, Ostrosky-Zeichner L, Pelaez T, Pukinskas SR, St-Germain G, Szeszs MW, Turnidge J.** 2013. Interlaboratory variability of caspofungin MICs for Candida spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother **57:**5836–5842. http://dx.doi.org/10.1128/AAC.01519-13.
11. **Arendrup MC, Garcia-Effron G, Lass-Florl C, Lopez AG, Rodriguez-Tudela JL, Cuenca-Estrella M, Perlin DS.** 2010. Echinocandin susceptibility testing of Candida species: comparison of EUCAST EDef 7.1, CLSI M27-A3, Etest, disk diffusion, and agar dilution methods with RPMI and Iso-Sensitest media. Antimicrob Agents Chemother **54:**426–439. http://dx.doi.org/10.1128/AAC.01256-09.
12. **Shields RK, Nguyen MH, Press EG, Kwa AL, Cheng S, Du C, Clancy CJ.** 2012. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. Antimicrob Agents Chemother **56:**4862–4869. http://dx.doi.org/10.1128/AAC.00027-12.
13. **Marras SA, Kramer FR, Tyagi S.** 2003. Genotyping SNPs with molecular beacons. Methods Mol Biol **212:**111–128.
14. **El-Hajj HH, Marras SA, Tyagi S, Shashkina E, Kamboj M, Kiehn TE, Glickman MS, Kramer FR, Alland D.** 2009. Use of sloppy molecular beacon probes for identification of mycobacterial species. J Clin Microbiol **47:**1190–1198. http://dx.doi.org/10.1128/JCM.02043-08.
15. **Chakravorty S, Kothari H, Aladegbami B, Cho EJ, Lee JS, Roh SS, Kim H, Kwak H, Lee EG, Hwang SH, Banada PP, Safi H, Via LE, Cho SN, Barry CE, III, Alland D.** 2012. Rapid, high-throughput detection of rifampin resistance and heteroresistance in Mycobacterium tuberculosis by use of sloppy molecular beacon melting temperature coding. J Clin Microbiol **50:**2194–2202. http://dx.doi.org/10.1128/JCM.00143-12.
16. **Brillowska-Dabrowska A, Saunte DM, Arendrup MC.** 2007. Five-hour diagnosis of dermatophyte nail infections with specific detection of Trichophyton rubrum. J Clin Microbiol **45:**1200–1204. http://dx.doi.org/10.1128/JCM.02072-06.
17. **Healey KR, Zhao Y, Perez WB, Lockhart SR, Sobel JD, Farmakiotis D, Kontoyiannis DP, Sanglard D, Taj-Aldeen SJ, Alexander BD, Jimenez-Ortigosa C, Shor E, Perlin DS.** 2016. Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. Nat Commun **7:**11128. http://dx.doi.org/10.1038/ncomms11128.
18. **Beyda ND, John J, Kilic A, Alam MJ, Lasco TM, Garey KW.** 2014. FKS mutant Candida glabrata: risk factors and outcomes in patients with candidemia. Clin Infect Dis **59:**819–825. http://dx.doi.org/10.1093/cid/ciu407.
19. **Pfaller MA, Diekema DJ, Jones RN, Castanheira M.** 2014. Use of anidulafungin as a surrogate marker to predict susceptibility and resistance to caspofungin among 4,290 clinical isolates of Candida by using CLSI methods and interpretive criteria. J Clin Microbiol **52:**3223–3229. http://dx.doi.org/10.1128/JCM.00782-14.
20. **Arendrup MC, Perlin DS.** 2014. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis **27:**484–492. http://dx.doi.org/10.1097/QCO.0000000000000111.
21. **Pham CD, Bolden CB, Kuykendall RJ, Lockhart SR.** 2014. Development of a Luminex-based multiplex assay for detection of mutations conferring resistance to echinocandins in Candida glabrata. J Clin Microbiol **52:**790–795. http://dx.doi.org/10.1128/JCM.03378-13.
22. **Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, Chiller T, Lockhart SR.** 2010. FKS mutations and elevated echinocandin MIC values among Candida glabrata isolates from U.S. population-based surveillance. Antimicrob Agents Chemother **54:**5042–5047. http://dx.doi.org/10.1128/AAC.00836-10.
23. **Castanheira M, Woosley LN, Messer SA, Diekema DJ, Jones RN, Pfaller MA.** 2014. Frequency of fks mutations among Candida glabrata isolates from a 10-year global collection of bloodstream infection isolates. Antimicrob Agents Chemother **58:**577–580. http://dx.doi.org/10.1128/AAC.01674-13.
24. **Dudiuk C, Gamarra S, Leonardeli F, Jimenez-Ortigosa C, Vitale RG, Afeltra J, Perlin DS, Garcia-Effron G.** 2014. Set of classical PCRs for detection of mutations in Candida glabrata FKS genes linked with echinocandin resistance. J Clin Microbiol **52:**2609–2614. http://dx.doi.org/10.1128/JCM.01038-14.