Absence of Azole or Echinocandin Resistance in *Candida glabrata* Isolates in India despite Background Prevalence of Strains with Defects in the DNA Mismatch Repair Pathway

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ABSTRACT *Candida glabrata* infections are increasing worldwide and exhibit greater rates of antifungal resistance than those with other species. DNA mismatch repair (MMR) gene deletions, such as *msh2*Δ, in *C. glabrata* resulting in a mutator phenotype have recently been reported to facilitate rapid acquisition of antifungal resistance. This study determined the antifungal susceptibility profiles of 210 *C. glabrata* isolates in 10 hospitals in India and investigated the impact of novel *MSH2* polymorphisms on mutation potential. No echinocandin- or azole-resistant strains and no mutations in *FKS* hot spot regions were detected among the *C. glabrata* isolates, supporting our *in vitro* susceptibility testing results. CLSI antifungal susceptibility data showed that the MICs of anidulafungin (geometric mean [GM], 0.12 μg/ml) and micafungin (GM, 0.01 μg/ml) were lower and below the susceptibility breakpoint compared to that of caspofungin (CAS) (GM, 1.31 μg/ml). Interestingly, 69% of the *C. glabrata* strains sequenced contained six nonsynonymous mutations in *MSH2*, i.e., V239L and the novel mutations E459K, R847C, Q386K, T772S, and V239/D946E. Functional analysis of *MSH2* mutations revealed that 49% of the tested strains (40/81) contained a partial loss-of-function *MSH2* mutation. The novel *MSH2* substitution Q386K produced higher frequencies of CAS-resistant colonies upon expression in the *msh2*Δ mutant. However, expression of two other novel *MSH2* alleles, i.e., E459K or R847C, did not confer selection of resistant colonies, confirming that not all mutations in the *MSH2* MMR pathway affect its function or generate a phenotype of resistance to antifungal drugs. The lack of drug resistance prevented any correlations from being drawn with respect to *MSH2* genotype.

KEYWORDS *Candida glabrata*, India, *FKS*, *MSH2*, echinocandins, mutator genotype, mismatch repair

Candida species are the most common cause of fungal infections worldwide among hospitalized patients. The distribution of *Candida* species isolated from patients with invasive candidiasis (IC) varies geographically depending on environmental factors, the patient's age, and exposure to antimicrobial agents. The proportion of IC due to *Candida glabrata* has increased in Europe and North America in the last decade, and this increase had been linked to previous exposure to antifungal agents, especially azoles (1). Furthermore, in *C. glabrata* high rates of azole resistance coupled with a recent increase of echinocandin resistance, especially in North America, are alarming, as

the latter drug is a first-line therapy for nonneutropenic patients with *C. glabrata* infections (2, 3). An increase in echinocandin resistance from 2% to 3% to >13% over the past 10 years has been described in one U.S. medical center in 2009 to 2010 (3). Also, a Centers for Disease Control and Prevention (CDC) survey showed echinocandin resistance rates of 3.1% to 3.6% in 4 U.S. cities in various geographic regions (2). Remarkably, echinocandin resistance was associated with cross-resistance to azole antifungals in 36% of the echinocandin-resistant strains, emphasizing the significance of multidrug-resistant (MDR) *C. glabrata* (2). In contrast to the case for North America, echinocandin resistance among *C glabrata* strains is low (<1%) in Europe (4). These geographical variations of echinocandin resistance rates may be attributed to strain variation and/or specific use of antifungal prophylaxis and therapy in particular clinical settings, which warrants further investigations. In *C. glabrata*, echinocandin resistance occurs due to mutations in *FKS* gene (*FKS1* and *FKS2*) sequences that encode the glucan synthase enzyme, which is the target of echinocandins. Several clinical studies have shown that the presence of an *FKS* mutation is the most important independent risk factor in predicting echinocandin therapeutic responses among patients with IC (3, 5). Further, the presence of an *FKS* mutation was found to be superior to MIC values in predicting clinical responses, especially when caspofungin (CAS) was used for testing (6). The underlying molecular mechanisms promoting development of resistance in *C. glabrata* to multiple drug classes, including azoles and echinocandins, are multifactorial. Recently, *MSH2* DNA mismatch repair (MMR) gene deletions in *C. glabrata* resulting in a mutator phenotype that facilitates rapid acquisition of fluconazole (FLU), echinocandins, and amphotericin B (AMB) resistance were detected in clinical isolates of *C. glabrata* (7). Over half of the isolates of *C. glabrata* collected in the United States and other countries carried mutations in *MSH2* that conferred a partial hypermutable phenotype with significantly increased frequency of *FKS* mutations (7). *MSH2* alleles were subsequently shown to be dependent upon sequence type (8). Additionally, a more recent study evaluated antifungal resistance in clinical isolates of *C. glabrata* in a large cohort of patients in Saint-Louis Hospital, Paris, France, and demonstrated that the mutator phenotype was not associated with FLU resistance but instead corresponded to rare and specific genotypes (9). The aim of the present study was to evaluate echinocandin resistance among 210 clinical isolates of *C. glabrata* collected from 10 hospitals in India during a 5-year surveillance study of candidiasis where all yeast isolates were collected from 2012 to 2016 (10). For *C. glabrata* strains, *FKS* gene profiles and mutator genotype, by sequencing of the *MSH2* gene, were determined. Further, the impact of novel *MSH2* polymorphisms on mutation potential in a tester strain was investigated.

RESULTS

Isolates. *Candida glabrata* isolates (*n* = 210) from individual patients in 10 hospitals in Delhi and the adjoining National Capital Region (NCR) and one in Kochi, southern India, collected over a period of 5 years, were identified to species level by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany) with a score of >2. About 52.3% of the *C. glabrata* isolates (*n* = 110) were from bloodstream infections (BSIs), followed by 29% (*n* = 61) isolates from respiratory tract specimens (sputum, *n* = 35; endotracheal aspirate, *n* = 18; bronchoalveolar lavage fluid, *n* = 8). The remaining 18.5% isolates (*n* = 39) were obtained from urine (*n* = 34), vaginal swab (*n* = 2), pus (*n* = 1), transhepatic biliary drainage (*n* = 1), and skin swab (*n* = 1). Bloodstream isolates were collected primarily from oncology/hematology wards (*n* = 45; 41%) and medical intensive care units (ICUs) (*n* = 41; 37%), followed by surgical wards (*n* = 24; 22%), in four tertiary care private hospitals in Delhi NCR (*n* = 3) and in Kochi. The respiratory tract isolates and other isolates were collected from a referral chest hospital and five public hospitals in Delhi, India.

In vitro susceptibility and FKS mutation analysis. A total of 210 *C. glabrata* isolates were subjected to antifungal susceptibility testing (AFST) by Clinical and

TABLE 1 MIC distribution of *C. glabrata* isolates against 10 antifungal drugs tested using the CLSI-BMD method

Drug[a]	No. of isolates with MIC (μg/ml) of:												MIC			
	≤0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	Range	GM	MIC$_{50}$	MIC$_{90}$
Echinocandins[b]																
CAS					12	11	129	47	11				0.25–4	1.31	1	1
MFG	142	68											≤0.016–0.032	0.01	0.015	0.015
AFG		11	17	167	15								0.032–0.25	0.12	0.125	0.125
Azoles, AMB, and FC[c]																
ITC		55	41	20		4							0.032–0.5	0.05	0.06	0.12
VRC		91	28			1							0.032–0.5	0.03	0.03	0.06
ISA	113	3			4								≤0.016–0.25	0.01	0.015	0.15
POS	49	52	18		1								≤0.016–0.25	0.02	0.03	0.06
AMB	11			44	35	22	8						≤0.016–1	0.21	0.25	0.5
FLU					2	2	30	71	9	2	3	1	0.25–32	1.84	2	4
FC				120									0.125	0.12	0.12	0.12

[a]CAS, caspofungin; MFG, micafungin; AFG, anidulafungin; ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; AMB, amphotericin B; FLU, fluconazole; FC, flucytosine.
[b]A total of 210 isolates were tested against echinocandins. The CBPs of CAS and AFG are ≤0.12 μg/ml (susceptible), 0.25 μg/ml (intermediate), and ≥0.5 μg/ml (resistant), and those of MFG are ≤0.06 μg/ml, 0.12 μg/ml, and ≥0.25 μg/ml, respectively.
[c]A total of 120 isolates were tested against azoles, AMB, and FC. The CBPs of FLU are ≤32 μg/ml (susceptible, dose dependent) and ≥64 μg/ml (resistant).

Laboratory Standards Institute broth microdilution method (CLSI-BMD) M27-A3/S4 (11, 12). The *in vitro* susceptibility data and the MIC distribution of *C. glabrata* isolates are presented in Table 1. Echinocandin resistance was declared if an isolate demonstrated a resistant MIC to at least two of the three echinocandin drugs (7). The geometric mean (GM) MIC values of anidulafungin (AFG) (GM MIC, 0.12 μg/ml) and micafungin (MFG) (GM MIC, 0.01 μg/ml) were lower and below the susceptibility breakpoint compared to that of CAS (GM MIC, 1.31 μg/ml) (13). Further, 198 isolates (94%) were categorized as resistant to CAS using the 2012 CLSI M27-S4 breakpoints (>0.25 μg/ml) (12, 13). This apparent overestimate of CAS resistance relative to testing with MFG or AFG by the standard BMD-RPMI method has previously been reported, emphasizing that microdilution testing for AFG and MFG correlates well with respect to separation between wild-type and *FKS* mutant isolates, whereas CAS MIC distributions appear to be variable. Further, due to interlaboratory variability in the MIC ranges for CAS, susceptibility testing of other echinocandins (AFG and MFG) has been advocated as a surrogate for identifying CAS-resistant *C. glabrata* strains (14). Low GM MICs of azoles, i.e., isavuconazole (ISA) (0.01 μg/ml), posaconazole (POS) (0.02 μg/ml), voriconazole (VRC) (0.03 μg/ml), itraconazole (ITC) (0.05 μg/ml), and FLU (1.84 μg/ml), were noted. Also, no non-wild-type strains were observed against AMB (MIC range, 0.06 to 1 μg/ml). Of the 210 isolates, 120 that had CAS MICs of ≥0.25 μg/ml were selected for *FKS* gene profiling. The isolates were selected primarily from BSIs (*n* = 86) for which there was a record of antifungal exposure available, and the remaining isolates were respiratory tract (*n* = 29) and other (*n* = 5) isolates collected from patients who had no exposure to antifungals. Amplification of the *FKS1* and *FKS2* regions generated amplicons of 391 bp and 460 bp, respectively. Mutations reported for CAS-resistant *C. glabrata* were not observed in the *FKS1* and *FKS2* regions of any of the tested *C. glabrata* strains.

MSH2 gene sequence analysis. Of the 120 *C. glabrata* isolates that had no mutations in the *FKS* gene, 83 isolates were randomly selected to determine their *MSH2* genotypes. These 83 *C. glabrata* isolates included 50 bloodstream isolates, 30 respiratory tract isolates, and single isolate each from pus, urine, and skin, and none had *FKS* mutations. Overall, 69% (57/83) of the *C. glabrata* strains contained six nonsynonymous mutations within *MSH2* gene compared with the database strain, CBS138/ATCC 2001 (http://www.candidagenome.org/). Notably, 63% (*n* = 52) of the isolates harboring mutations had high MICs (0.5 to 4 μg/ml) of CAS. However, as expected, none of the isolates tested for *MSH2* gene mutations had high MICs of AFG and MFG. Overall, 41% of the isolates had a single Msh2p substitution, i.e., V239L (*n* = 34), and the remaining five novel substitutions observed were E459K (*n* = 9), R847C (*n* = 6), Q386K (*n* = 5),
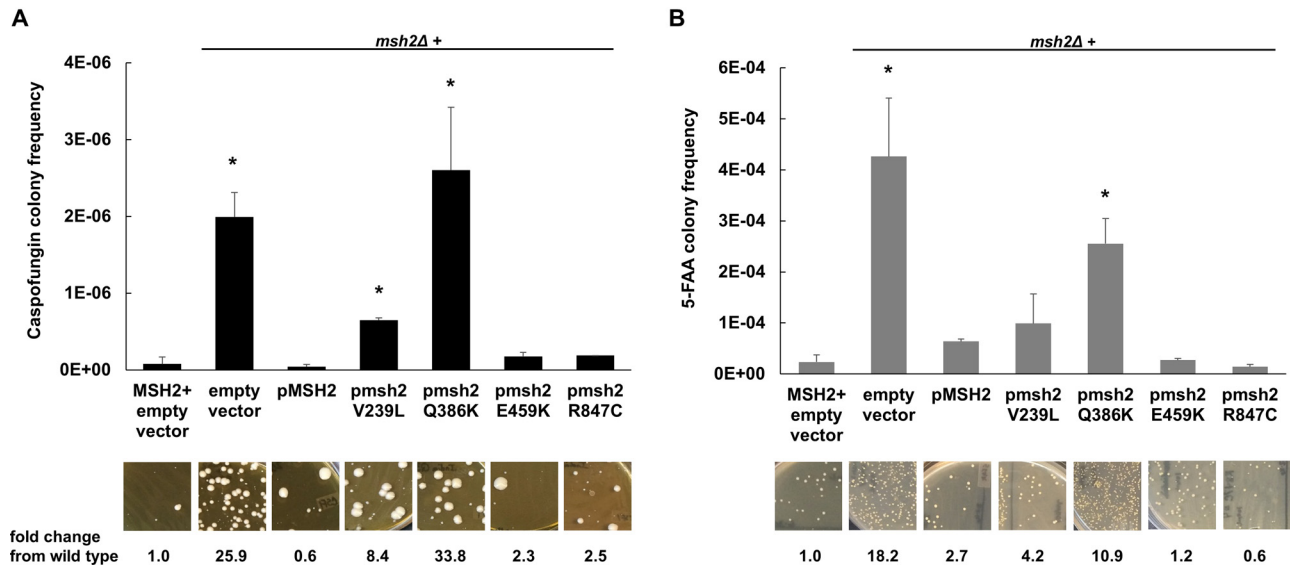
TABLE 2 Distribution of *MSH2* gene polymorphisms in 83 *Candida glabrata* isolates

Amino acid substitution encoded in *MSH2* (n)	Specimen type (n)	No. of isolates with the indicated MIC (µg/ml)										
		Caspofungin					Micafungin		Anidulafungin			
		0.25	0.5	1	2	4	0.015	0.03	0.03	0.06	0.125	0.25
Wild type (26)	Blood (11)			6	5		6	5		1	8	2
	Respiratory tract (14)		1	8	5		11	3		1	11	2
	Urine (1)			1			1				1	
V239L (34)	Blood (26)	3	3	15	5		19	7	3		23	
	Respiratory tract (7)	1	2	2	2		6	1	1	1	5	
	Pus (1)		1				1				1	
E459K[a] (9)	Blood (5)					5		5			2	3
	Respiratory tract (4)	1		2	1		3	1		1	3	
Q386K[a] (5)	Respiratory tract (4)			4			2	2			4	
	Blood (1)			1			1				1	
R847C[a] (6)	Blood (4)			4			1	3		1	3	
	Respiratory tract (1)			1			1				1	
	Skin scraping (1)			1				1			1	
T772S[a] (2)	Blood (2)			1	1		2			1	1	
V239L/D946E[a] (1)	Blood (1)			1			1				1	

[a]Mutation found only in Indian isolates.

T772S (*n* = 2), and V239L/D946E (*n* = 1) (Table 2). Interestingly, respiratory tract isolates exhibited the wild-type sequence in 47% of isolates (14 of 30), while 78% (39 of 50) of the bloodstream isolates tested had mutations ($P < 0.05$). The duration of exposure to azoles (VRC and FLU) and CAS during the hospital stay and the time of sampling were available for 120 patients whose *FKS* gene profiles were investigated. Out of 120 patients, only 71 (59%) were treated with antifungals, which included azoles in 61 patients and echinocandins in 10 patients (see Table S1 in the supplemental material). A total of 67 patients (94%) had samples collected between 1 and <7 days of treatment. A total of 61 patients (85%) had a history of azole exposure, and only 10 patients had exposure to CAS. About 80% (*n* = 57) of patients had exposure to azoles for <7 days (FLU, *n* = 39; VRC, *n* = 18), and the remaining 4 were on azoles for 2 to 4 weeks.

MSH2 polymorphism phenotyping assay. To determine the effects of DNA MMR on *C. glabrata* antifungal resistance, Healey et al. showed that the *msh2*Δ strain generated 82-, 18-, and 9-fold more CAS-, FLU-, and AMB-resistant mutants, respectively, than the wild-type strain in antifungal selections (7). Further, upon CAS selection *in vitro*, echinocandin-susceptible clinical isolates containing specific *MSH2* mutations produced significantly higher frequencies of echinocandin-resistant colonies and *FKS* mutants than isolates carrying wild-type *MSH2*. To ascertain the significance of the novel *MSH2* polymorphisms reported in this study, i.e., E459K, R847C, and Q386K, alleles carrying these polymorphisms were amplified from clinical isolates, expressed on a yeast centromere plasmid (pGRB2.0) under the control of their native promoter, and transformed into a laboratory strain carrying a deletion of *MSH2* (7). Forward mutation frequencies were analyzed by measuring frequencies of colonies resistant to the echinocandin CAS (largely due to mutations in *FKS1* or *FKS2*) or to the metabolic inhibitor 5-fluoroanthranilic acid (5-FAA) (largely due to mutations in *TRP3* or *TRP5*). 5-FAA was used in addition to CAS as a nonantifungal alternative to distinguish frequencies of our strains. Following CAS and 5-FAA selections, we noted significantly elevated rates (33.8- and 10.9-fold, respectively) of resistant compared to wild-type colonies upon expression of pMSH2-Q386K but not with expression of pMSH2-E459K (2.3- and 1.2-fold, respectively) or pMSH2-R847C (2.5- and 0.6-fold, respectively) (Fig. 1). Similar to the results of previous studies, expression of pMSH2-V239L or an empty

FIG 1 Wild-type or *msh2Δ* cells expressing an empty or *MSH2*-containing plasmid were selected on caspofungin (A) or 5-fluoroanthranilic acid (B). Frequency data are means ± standard deviations (SD) from ≥2 independent experiments; representative images are shown. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test, two tailed). Scale bars, 1 cm.

vector (positive control) led to elevated levels of resistant colonies, while pMSH2-wild type complemented these phenotypes (7) (Fig. 1). Overall, 49% of the strains (40/81) exhibited a partial loss-of-function *MSH2* mutation. The functionality of the T772S allele, identified in only 2 strains, remains to be determined.

DISCUSSION

This study found echinocandin- and azole-resistant strains among 210 isolates of *C. glabrata* collected during the course of the surveillance program from 2012 to 2016 in 10 hospitals in Delhi, NCR, and southern India. Although all *C. glabrata* isolates tested by the CLSI-BMD method in the present study showed CAS MICs in the intermediate and resistant categories according to published clinical breakpoints (CBPs), none of the isolates showed resistance to the other two echinocandins tested. Further, 120 isolates tested for *FKS* mutations in the hot spot region showed no mutations, suggesting echinocandin resistance to be unlikely in Indian isolates. Considering that the mechanism of action is shared among the echinocandins, it seems unlikely that a high percentage of isolates tested are nonsusceptible to CAS but remain susceptible to AFG and MFG (15). As previously reported, it is likely that this reflects technical issues associated with the *in vitro* testing of CAS rather than a true difference in antifungal activity (16). Thus, neither CLSI nor the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends the use of CAS for antifungal susceptibility testing, while AFG and MFG are recommended as surrogate markers to predict CAS susceptibility (16, 17). It should be noted that a recent study by Pham et al. found that MFG MIC values were the strongest predictor of isolates possessing *FKS* mutations (2). The absence of echinocandin resistance in the present study is consistent with a report from Italy and Spain, where the resistance rate was 0% in a study analyzing 79 *C. glabrata* bloodstream isolates (18). Also, low echinocandin resistance rates among *C. glabrata* isolates obtained mainly from Lombardy, Italy (i.e., 2.1% [2 of 94]) (19), and among *C. glabrata* isolates obtained mainly from urine samples from France (1% [2 of 193 isolates, both with an *FKS2* mutation]) (20) were reported. Similarly, 1 (2%) of the 50 *C. glabrata* isolates analyzed in a report from Turkey was resistant to CAS (21). In contrast, in the United States, a recent study found that 10% of *C. glabrata* isolates from cancer patients at the M.D. Anderson Cancer Center, Houston, TX, were resistant to CAS (22). Further, CAS resistance was associated with a higher rate of 28-day all-cause mortality, and the presences of *FKS* mutants was associated with therapeutic failure. A

main risk factor for the development of *FKS* mutations in *C. glabrata* is pretreatment with echinocandins (23). Echinocandin resistance typically emerges after prolonged therapy, although it has been reported shortly after initiation of therapy (6). In the present study, only 10 of 120 patients (screened for *FKS* mutations) had <7 days of exposure to echinocandins prior to isolation of *C. glabrata*. The echinocandin resistance rates may be influenced by the site- or country-specific use of antifungal prophylaxis and therapy, and the frequency of resistance may vary considerably from institution to institution (24). Considering the fact that usage of echinocandins in many hospitals in India is limited due to cost constraints, only 1% of patients in this study were exposed to echinocandins, which likely accounts for the absence of echinocandin resistance in the present study. Also, usage of echinocandins in Indian hospitals is specifically restricted in private hospitals. Although they were available in early 2000 in private-sector hospitals, these antifungals have been prescribed only from 2010 in the 4 centers in the present study. A recent large, multisite, population-based candidemia surveillance program by the CDC in four metropolitan areas of the United States reported that more than half of cases with echinocandin-nonsusceptible isolates did not have known echinocandin exposure (24). Data from global surveys demonstrate that the frequency of echinocandin resistance among clinical isolates of *C. glabrata* ranges from 1 to 3% and is higher among isolates from North America (3%) than among those from Europe (1%), Latin America (0.0%), or the Asia-Pacific region (0.0%) (25). The data on echinocandin resistance in *C. glabrata* from the Indian subcontinent are limited, and this is the first study from India addressing both the susceptibility testing and molecular profiling of the *FKS* gene in echinocandin resistance. Importantly, in the present study, azole resistance among *C. glabrata* isolates was also not noted, which could be attributed to the fact that isolates were collected from patients within 2 weeks of azole therapy/prophylaxis. Previously, in a prospective multicenter study of ICU-acquired candidemia during 2011 and 2012 in 27 hospitals in India, a low prevalence of *C. glabrata* was reported despite prior exposure to antifungal agents in 15.7% of patients. Further, low azole resistance for FLU (1.5%), ITC (0%), and VRC (0%) was recorded in 65 *C. glabrata* bloodstream isolates tested for antifungal susceptibility (26). Similarly, in a prospective multicenter study of *Candida* bloodstream infections in three Lima-Callao hospitals during 2013 to 2015, no resistance to azoles among *C. glabrata* isolates was observed (27). Also, a laboratory-based candidemia survey in 20 centers in seven Latin American countries did not show an association of FLU exposure and candidemia due to *C. glabrata*. Furthermore, the proportion of isolates with resistance to FLU was low (6.5%) (28). Considering that *C. glabrata* has a complex population structure, the absence of MDR strains among Indian *C. glabrata* isolates suggests the possibility of genetic strain variations in the Indian subcontinent compared to North America and Europe, warranting in-depth genetic analysis of Indian strains. The defects in DNA repair may result in the emergence of drug-resistant variants in *C. glabrata* and, as more recently reported, in *Cryptococcus neoformans* (29). In the present study, 69% of the clinical strains investigated exhibited an amino acid substitution compared to the sequenced database strain CBS138/ATCC 2001, with 49% of strains carrying a partial loss-of-function mutation. The most common mutation, i.e., V239L noted in the *MSH2* gene in this study has been previously reported in U.S., Switzerland, and Qatar clinical *C. glabrata* strains and is associated with a partial mutator phenotype (7). Similarly, the novel allele *MSH2*-Q386K that was noted in 5 *C. glabrata* strains from bloodstream and respiratory tract specimens in this study produced greater frequencies of CAS- and 5-FAA-resistant colonies upon expression in the *msh2Δ* mutant. However, expression of two other novel *MSH2* alleles encoding the amino acid substitution E459K or R847C did not confer selection of resistant colonies, confirming that not all mutations in the *MSH2* MMR pathway affect its function or generate a phenotype of resistance to antifungal drugs. Recently, comparison of mutation frequencies in strains with deletions of eight MMR pathway genes in *C. neoformans* showed that the loss of three of them, *MSH2*, *MLH1*, and *PMS1*, resulted in an increase in mutation rates (29). Also, Healey et al. showed that additional mechanisms of DNA repair, including other MMR genes, and

other cellular systems may influence the mutagenic potential of *C. glabrata* strains (8). As all of our *C. glabrata* strains were fully susceptible to antifungals, the impact of the presence of *MSH2* mutations on antifungal resistance could not be correlated. Similar to the 49% rate of *MSH2* partial loss of function reported in our isolate population, 52% (117/225) of susceptible *C. glabrata* isolates demonstrated a nonsynonymous mutation within *MSH2* in U.S. and non-U.S. site isolates in a previous study (7). Finally, mutator cells in the population may provide a selective advantage *in vivo* and enable this yeast with increased drug exposure to promote the development of antifungal resistance and/or pathogenicity (8). Despite the near absence of echinocandin or azole resistance in *C. glabrata* isolates in this patient population, the high prevalence of the mutator phenotype among strains, which have a heightened potential to develop resistance, is concerning. Our study calls out for continued vigilance, especially as antifungal exposure broadens at many centers.

MATERIALS AND METHODS

Fungal isolates and identification. This study analyzed 210 *C. glabrata* clinical isolates collected from individual patients in 10 hospitals from Delhi and adjoining NCRs in northern India and in a single center in Kochi, Kerala, in southern India during a surveillance study of candidiasis where all yeasts isolates were collected from 2012 to 2016. All isolates were cultured on Sabouraud dextrose agar (SDA) for 24 h at 37°C for identification by MALDI-TOF MS using ethanol-formic acid extraction according to the manufacturer's protocol (10). The isolates were identified as *C. glabrata* with a score of >2.

AFST. *In vitro* antifungal susceptibility testing (AFST) was done using the CLSI-BMD method, following the M27-A3/S4 guidelines (11, 12). The antifungals tested were AMB (Sigma, St. Louis, MO), FLU (Pfizer, Groton, CT), ITC (Lee Pharma, Hyderabad, India), VRC (Pfizer), POS (Merck, Whitehouse Station, NJ), ISA (Basilea Pharmaceutica, Basel, Switzerland), 5-flucytosine (FC) (Sigma), CAS (Merck), MFG (Astellas, Toyama, Japan), and AFG (Pfizer). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains. The MIC endpoints were defined as the lowest drug concentrations that caused a prominent (50%) decrease in growth vis-à-vis the controls and were read visually after 24 h for azoles and echinocandins. For AMB, the MIC was defined as the lowest concentration at which there was 100% inhibition of growth compared with the drug-free control well results. Isolates of *C. glabrata* were categorized as susceptible or resistant to each echinocandin using the 2012 CLSI M27-S4 breakpoints (12). The CBPs of CAS and AFG for *C. glabrata* are ≤0.12 μg/ml (susceptible), 0.25 μg/ml (intermediate), and ≥0.5 μg/ml (resistant), whereas those of MFG are ≤0.06 μg/ml, 0.12 μg/ml, and ≥0.25 μg/ml, respectively.

FKS gene sequencing. The *FKS1* and *FKS2* genes of a total of 120 isolates were sequenced. Genomic DNA was extracted as described by Xu et al. (30). Hot spot 1 (HS1) and HS2 of *FKS1* and *FKS2* were amplified by PCR as previously described (31). DNA sequences were analyzed with Sequencing Analysis software version 5.3.1 (Applied Biosystems). Consensus sequences were made using BioEdit software (version 7.0.5.3) and were aligned with hot spot *FKS* regions of reference *C. glabrata* (GenBank accession no. HM366439 for *FKS1* and HM366442 for *FKS2*).

MSH2 gene sequencing. To assess the defects in DNA repair pathways in *C. glabrata* isolates, sequencing of the MMR gene *MSH2* was done in 83 isolates as described previously (7). The *MSH2* gene was amplified using primers CgMSH2u273F and CgMSH2d145R. The resulting amplicon (3,292 bp) was sequenced using CgMSH2u273F and CgMSH2d145R along with internal primers CgMSH2c846R, CgMSH2c1626R, and CgMSH2c2340R (7).

MSH2 cloning and drug selections. For plasmid-based expression of *MSH2*, a gap repair approach was used (32). Briefly, coding regions plus promoter regions were PCR amplified from laboratory or clinical isolates containing flanking regions homologous to regions upstream and downstream of the SmaI restriction site in pGRB2.0. Strain 2001 HTU or the *msh2Δ* mutant was transformed with this PCR product, along with SmaI-linearized pGRB2.0, and selected on synthetically defined medium lacking uracil (SD-ura). Plasmids from transformants were sequenced for confirmation. The resulting strains were selected on 1 μg/ml CAS and 0.5 mg/ml 5-FAA (7). Briefly, strains were grown overnight in appropriate medium (yeast extract-peptone-dextrose [YPD] or SD-ura) at 37°C, and then approximately 1×10^8 CFU was plated onto echinocandin-containing drug plates and between 1×10^5 and 1×10^6 CFU was plated onto 5-FAA plates. Plates were incubated for 72 h. Dilutions were plated onto drug-free medium to determine exact CFU counts. Frequencies were calculated as the number of colonies on the drug plate divided by the total CFU plated. Frequency averages were calculated from at least two independent selections. Differences in resistance frequencies were evaluated by Student's *t* test. A *P* value of <0.05 (two tailed) was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .00195-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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