Echinocandin Resistance in *Candida* Species Isolates from Liver Transplant Recipients

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ABSTRACT

Liver transplant recipients are at risk of invasive fungal infections, especially candidiasis. Echinocandin is recommended as prophylactic treatment but is increasingly associated with resistance. Our aim was to assess echinocandin drug resistance in *Candida* spp. isolated from liver transplant recipients treated with this antifungal class. For this, all liver-transplanted patients in a University Hospital (Créteil, France) between January and June of 2013 and 2015 were included. Susceptibilities of *Candida* isolates to echinocandins were tested by Etest and the EUCAST reference method. Isolates were analyzed by FKS sequencing and genotyped based on microsatellites or multilocus sequence typing (MLST) profiles. Ninety-four patients were included, and 39 patients were colonized or infected and treated with echinocandin. Echinocandin resistance appeared in 3 (8%) of the treated patients within 1 month of treatment. One patient was colonized by resistant *Candida glabrata*, one by resistant *Candida dubliniensis*, and one by resistant *Candida albicans*. Molecular analysis found three mutations in *FKS2* HS1 (F659S, S663A, and D666E) for *C. glabrata* and one mutation in *FKS1* HS1 (S645P) for *C. dubliniensis* and *C. albicans*. Susceptible and resistant isolates belonged to the same genotype. To our knowledge, this is the first study on echinocandin resistance in *Candida* spp. isolated from liver transplant recipients. Antimicrob Agents Chemother 61:e01229-16. https://doi.org/10.1128/AAC.01229-16.

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Patients undergoing liver transplantation (LT) are specifically at risk of developing invasive fungal infection (IFI). It has been shown that IFI occurs early after LT (≤2 months) (1). Invasive candidiasis (IC) is the most common post-LT IFI, with *Candida albicans* (50 to 60%) and *Candida glabrata* (about 20%) the main species responsible (2).

Antifungal prophylaxis is now a standard intervention for liver transplant recipients. After using fluconazole or liposomal amphotericin B for several years, echinocandin is now the new recommended treatment in patients with major risk factors for at least 2 to 4 weeks or until resolution of the risk factors (3). Echinocandin drugs, which inhibit the synthesis of beta-1,3 glucan in the fungal cell wall, are attractive, thanks to their good *in vitro* activity against *Candida* spp. (4, 5), excellent safety profile, and favorable
pharmacokinetics (6). However, their expanding use can promote the emergence of resistance in Candida spp., especially among patients receiving long-term therapy (7, 8). The molecular mechanisms underlying the acquired clinical resistance include point mutations within hot spot (HS) regions of FKS genes encoding subunits of glucan synthase (9, 10). These mutations are an important risk factor for therapy failure (11). However, it has been reported that the development of such resistance is directly linked to prior exposure (7). In the literature, mutations inducing C. albicans resistance are most commonly found on the FKS1 gene, and especially at amino acids 641 to 649 and amino acids 1345 to 1365 in HS1 and HS2, respectively. The substitutions concerning Ser-645 (S645P/F/Y) and Phe-641 (F641S/L) are the most frequent and are responsible for the most pronounced phenotypes (12–14). Mutations in C. glabrata are most commonly found on the FKS2 gene (15), with the most frequent substitutions at Ser-663 (S663F/P) and Phe-659 (F659S/V/Y) (8). Echinocandin resistance has also been described in other Candida species, especially in Candida krusei and Candida tropicalis (16, 17).

Only two case reports on echinocandin resistance in liver transplant recipient populations are available (7, 18). In this work, our aim was to assess echinocandin drug resistance in Candida spp. developed in liver transplant recipients after the initiation of echinocandin treatment.

RESULTS

Patients included. A total of 94 liver transplant recipients were enrolled during the first 6 months of 2013 (n = 52) and during the same period in 2015 (n = 42). The median age of the recipients was 54.5 (range, 22 to 70) years. Most of them (77%) were men. The two most common etiologies for LT were cirrhosis (50%) and hepatocellular carcinoma (33%). Among these 94 patients, 56 (60%) received antifungal treatment, which included caspofungin (n = 41 [73%]), micafungin (n = 2 [4%]), and fluconazole (n = 13 [23%]). Of the patients treated with echinocandin, 39 (91%) were colonized and/or developed an IC.

Echinocandin susceptibility test results. Echinocandin resistance was detected in 3 (8%) of the treated patients. The isolates revealed one patient with resistant C. glabrata (P1), one with resistant C. dubliniensis (P2), and one with resistant C. albicans (P3). The echinocandin treatment was prophylactic for P1 and P3 and curative for P2. The echinocandin MICs of all the isolates from these patients were tested using Etest for all echinocandins and the EUCAST reference method for only anidulafungin and micafungin (Table 1). In the case of P1, 24 resistant C. glabrata isolates were recovered from the urine (n = 11), anus (n = 8), and inguinal fold (n = 5) (Table 2). The first resistant isolates appeared in the anus and the urine 14 days after initiation of caspofungin treatment. High MICs were then detected in the inguinal fold but not in other sites. For P2, five resistant C. dubliniensis isolates were recovered from the abdominal fluids (n = 4) and mouth (n = 1). The first resistant isolate was detected in the mouth 27 days after the beginning of caspofungin treatment. Moreover, the patient had had prior caspofungin exposure for 23 days 3 months previously to treat IC (Table 3). For P3, the only resistant isolate was recovered in the anus, though the patient was not on caspofungin treatment at the time but had received 24-day prophylactic treatment of caspofungin, which was stopped a week earlier.

Identification of mutations in FKS genes. For P1, FKS2 HS1 mutations were detected in 22 resistant C. glabrata isolates. The first mutation, F659S, appeared after 14 days of treatment. The second mutation, S663A, appeared after 63 days of treatment, associated with the first. The third mutation, D666E, appeared once after 89 days of treatment (day 89 [D89] anus isolate) (Table 1). In this isolate, a mutated double population was observed, one population with F659S and S663A mutations and the other with F659S and D666E mutations (Fig. 1). For P2, one mutation in FKS1 HS1 (S645P) was found in five C. dubliniensis isolates. For P3, the same FKS mutation in FKS1 HS1 (S645P) was detected in one C. albicans isolate. For the three patients, no mutation was detected in the susceptible isolates for which FKS was sequenced (Table 1).
| Time/source* | ANF (µg/ml) | MICA (µg/ml) | CAS (µg/ml) | FKS genotype* |
|------------|-------------|-------------|-------------|---------------|
| D-2/mouth  | 0.012       | 0.062       | 0.023       | 0.031         | 0.125         | Not performed |
| D-2/nose   | 0.012       | 0.062       | 0.016       | 0.031         | 0.125         | Not performed |
| D-2/inguinal | 0.012    | 0.031       | 0.016       | 0.031         | 0.125         | Wild type     |
| D-2/urine  | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Wild type     |
| D-2/BAL    | 0.012       | 0.062       | 0.023       | 0.015         | 0.125         | Not performed |
| D-6/mouth  | 0.012       | 0.062       | 0.023       | 0.015         | 0.125         | Not performed |
| D-6/nose   | 0.012       | 0.062       | 0.023       | 0.015         | 0.125         | Not performed |
| D-6/axon   | 0.012       | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D-6/urine  | 0.016       | 0.062       | 0.016       | 0.015         | 0.125         | Not performed |
| D-6/axon   | 0.012       | 0.062       | 0.016       | 0.015         | 0.125         | Not performed |
| D9/axon    | 0.012       | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D9/urine   | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Not performed |
| D9/axillary | 0.012     | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D9/mouth   | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Not performed |
| D9/axon    | 0.012       | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D9/urine   | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Not performed |
| D9/axon    | 0.012       | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D9/urine   | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Not performed |
| D9/axon    | 0.012       | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D9/urine   | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Not performed |
| D9/axon    | 0.012       | 0.031       | 0.023       | 0.015         | 0.125         | Not performed |
| D9/urine   | 0.016       | 0.031       | 0.016       | 0.015         | 0.125         | Not performed |

Patient 2: C. dubliniensis

| Time/source* | ANF (µg/ml) | MICA (µg/ml) | CAS (µg/ml) | FKS genotype* |
|------------|-------------|-------------|-------------|---------------|
| D1/mouth  | 0.006       | 0.015       | 0.016       | 0.015         | 0.064         | Wild type     |
| D4/anus   | 0.004       | 0.015       | 0.032       | 0.015         | 0.064         | Wild type     |
| D4/bile   | 0.008       | 0.015       | 0.023       | 0.015         | 0.064         | Wild type     |
| D11/mouth | 0.19        | 0.5 (R)     | 0.38 (I)    | 0.5 (R)       | 1 (R)         | FKS1-S645P    |
| D11/drainage fluid | 0.25 | 0.5 (R) | 0.25 | 0.5 (R) | 1.5 (R) | FKS1-S645P    |
| D12/mouth | 0.008       | 0.015       | 0.047       | 0.015         | 0.125         | FKS1-S645P    |
| D12/abscess | 0.25     | 0.125 (R) | 0.38 (I)    | 0.5 (R)       | 3 (R)         | FKS1-S645P    |
| D12/abdominal collection | 0.25 | 0.5 (R) | 0.38 (I) | 0.5 (R) | 1 (R) | FKS1-S645P |
| D12/peritoneal fluid | 0.25 | 0.125 (R) | 0.25 | 0.5 (R) | 1.5 (R) | FKS1-S645P    |

(Continued on following page)
Genotyping. Susceptible and resistant isolates of *C. glabrata*, *C. dubliniensis*, and *C. albicans* harvested from P1, P2, and P3, respectively, all belonged to the same genotype. For *C. glabrata*, the sizes of the mitochondrial RNase P precursor gene (*RMP2*), the metallothionein I gene (*MTI*), and the 5,6-sterol desaturase (*ERG3*) gene microsatellites were 128, 242, and 228 bp, respectively. For *C. albicans*, the sizes of the 2 alleles of the elongation factor 3 gene (*EF3*), the cell division cycle protein gene (*CDC3*), and the imidazole glycerol phosphate dehydratase gene (*HIS*) microsatellites were 136 and 145, 116 and 128, and 152 and 152 bp, respectively. Multilocus sequence typing (MLST) of *C. dubliniensis* did not reveal any difference in polymorphism positions.

Correlation between FKS mutations and MICs. Using the EUCAST reference method, resistance was detected in 27/27 (100%) and 25/27 (93%) FKS mutant isolates for anidulafungin and micafungin, respectively. In contrast, the mutant isolates were detected as resistant or intermediate isolates by Etest in 15/27 (56%), 14/27 (52%), and 27/27 (100%) isolates for anidulafungin, micafungin, and caspofungin, respectively, when MIC values were interpreted with the CLSI clinical breakpoints (as recommended by the manufacturer). However, when the EUCAST breakpoints were used to interpret the MIC results, Etest detected resistance in 24/27 (89%) and 27/27 (100%) mutant isolates for anidulafungin and micafungin, respectively.

### TABLE 1 (Continued)

| Time/source | ANF (Etest) | EUCAST | MICA (Etest) | EUCAST | CAS (Etest) | FKS genotype<sup>c</sup> |
|-------------|-------------|---------|--------------|---------|-------------|---------------------|
| D0/inguinal | 0.016       | 0.015   | 0.004        | 0.015   | 0.125       | Wild type           |
| D10/mouth   | 0.008       | 0.015   | 0.064        | 0.015   | 0.125       | Not performed       |
| D18/mouth   | 0.016       | 0.015   | 0.002        | 0.015   | 0.125       | Not performed       |
| D32/anus    | 0.5 (I)     | 0.125 (R) | 0.5 (I)     | 1 (R)   | 0.38 (I)    | FKS1-S64SP          |

<sup>a</sup>D0 corresponds to the day of liver transplantation.

<sup>b</sup>ANF, anidulafungin; MICA, micafungin; R, resistant; I, intermediate; BAL, bronchoalveolar lavage. For the EUCAST broth microdilution method, isolate categorizations were performed according to the manufacturer’s instructions (for *C. albicans* and *C. dubliniensis*, S ≤ 0.25, I = 0.38 to 0.75, and R ≥ 1; for *C. glabrata*, S ≤ 0.125, I = 0.19 to 0.38, and R ≥ 0.5; for anidulafungin and micafungin, S ≤ 0.06, I = 0.094 to 0.19, and R ≥ 0.25). Only the resistant and intermediate isolates are marked in parentheses.

<sup>c</sup>For *C. glabrata*, FKS1 HS1, FKS1 HS2, FKS2 HS1, and FKS2 HS2 sequencing was performed. For *C. albicans* and *C. dubliniensis*, FKS1 HS1 and FKS1 HS2 sequencing was performed.

### TABLE 2 C. glabrata isolates from P1

| Day<sup>a</sup> of surveillance | Treatment<sup>b</sup> | Fungal surveillance culture<sup>c</sup> | Other<sup>d</sup> |
|-------------------------------|-----------------------|---------------------------------------|------------------|
|                               |                       | Mouth | Nose | Axillary | Inguinal | Anus | Urine | BAL |
| D−2                           | CAS D0 to D96         | S     | S    | −       | S        | S    | S     | S   |
| D6                            |                       | S     | S    | S       | −       | S    | S     | S   |
| D12                           |                       | S     | S    | S       | −       | S    | R     | R   |
| D19                           |                       | S     | −    | S       | −       | S    | R     | R   |
| D26                           |                       | −     | −    | −       | R       | R    | R     | R   |
| D33                           |                       | −     | −    | −       | −       | −    | R     | R   |
| D40                           |                       | S     | −    | −       | R       | R    | R     | S   |
| D47                           |                       | −     | −    | −       | −       | −    | R     | R   |
| D54                           |                       | S     | −    | −       | R       | R    | R     | R   |
| D61                           |                       | −     | −    | −       | −       | −    | R     | R   |
| D68                           |                       | −     | −    | −       | −       | −    | R     | R   |
| D75                           |                       | S     | −    | −       | R       | R    | R     | R   |
| D82                           |                       | S     | −    | −       | −       | −    | R     | R   |
| D89                           |                       | −     | −    | −       | −       | −    | R     | R   |
| D96                           |                       | −     | −    | −       | S       | −    | −     | −   |

<sup>a</sup>D0 is considered the day of liver transplantation.

<sup>b</sup>CAS, caspofungin.

<sup>c</sup>S, isolate susceptible to echinocandins using the Etest and EUCAST reference method; R, isolate resistant or intermediate to echinocandins using the Etest and EUCAST reference method; −, negative culture or unavailable isolate; BAL, bronchoalveolar lavage (fluid).
For *C. glabrata*, the first mutation, F659S, alone was associated with a low level of resistance (EUCAST MICs of 0.125 and 0.06 mg/liter for anidulafungin and micafungin, respectively), which already represented an increase of up to 4-fold compared with the FKS wild-type (WT) susceptible parent isolate. The resistance level increased with the second mutation (F659S and S663A), with MICs up to 32-fold higher than those of the WT. Finally, for a D89 anus isolate, the MICs were 32- to 512-fold higher than those of the WT.

### TABLE 3 *C. dubliniensis* isolates from P2

| Day of surveillance | Treatment | Fungal surveillance culture | Other |
|---------------------|-----------|----------------------------|-------|
|                     |           | Mouth | Nose | Axillary | Inguinal | Anus | Urine | Other |
| D1                  | CAS D12 to D35 | –     | –    | –        | –        | –    | –     | –     |
| D8                  | –         | –     | –    | –        | –        | –    | –     | –     |
| D15                 | S         | –     | –    | –        | –        | –    | –     | –     |
| D22                 | –         | –     | –    | –        | –        | –    | –     | –     |
| D6 (hospital discharge to D86) | CAS D86 to D122 | –     | –    | –        | –        | S    | –     | S (bile) |
| D113                | R         | –     | –    | –        | –        | –    | –     | R (drain) |
| D120                | S         | –     | –    | –        | –        | –    | –     | R (abdominal abscess) |
| D127                | AMB D122 to D131 | –     | –    | –        | –        | –    | –     | R (peritoneal fluid) |
| D134                | VOR D131 to D186 | –     | –    | –        | –        | –    | –     | –     |
| D154                | –         | –     | –    | –        | –        | –    | –     | –     |
| D162                | –         | –     | –    | –        | –        | –    | –     | –     |
| D169                | –         | –     | –    | –        | –        | –    | –     | –     |
| D176                | –         | –     | –    | –        | –        | –    | –     | –     |
| D183                | –         | –     | –    | –        | –        | –    | –     | –     |

*a* D0 is considered the day of liver transplantation.

*b* CAS, caspofungin; AMB, liposomal amphotericin B; VOR, voriconazole.

*c* S, isolate susceptible to anidulafungin and micafungin using the EUCAST reference method; R, isolate resistant to anidulafungin and micafungin using the EUCAST reference method; –, negative culture or unavailable isolate.

### FIG 1 DNA sequencing chromatogram (A) and amino acid alignments (B) of the FKS2HS1 region in different *C. glabrata* isolates from patients. Lines 1, *C. glabrata* wild-type genome database sequence used for alignment (GenBank accession number XM_448401); lines 2, *C. glabrata* wild-type isolate; lines 3, F659S mutation found in resistant isolates from D12 to D61; lines 4, F659S and S663A mutations found in resistant isolates from D61 to D89; lines 5, F659S and D666E mutations found only in the D89 anus isolate. This isolate harbors another population with F659S and S663A mutations (lines 4).
of WT. Parts of the MSH2 genes of four C. glabrata isolates (D2 anus, D12 urine, D61 urine, and D89 anus) were sequenced, and no mutation was found.

**DISCUSSION**

To our knowledge, this is the first study that has investigated echinocandin drug resistance in Candida spp. in a liver transplant recipient population. Our results showed that of the 39 patients treated with echinocandin, 3 (8%) acquired a resistant isolate. The same prevalence (8%) was reported for *C. tropicalis* in a hematology population where patients were treated with echinocandins (17). In our study, this prevalence may have been underestimated due to the use of Etest as a screening method rather than the EUCAST method; the latter seems more sensitive in detecting resistant isolates.

Genotyping revealed that, in the three patients concerned, resistant and susceptible isolates were isogenic. This confirms, as previously demonstrated in several studies (7, 19–22), that susceptible isolates acquired resistance under selection pressure in antifungal-treated patients. In our cases, the resistance developed rapidly, in less than 1 month. It appeared more rapidly in *C. glabrata* (14 days of echinocandin treatment) than in *C. albicans* (24 days) or *C. dubliniensis* (27 days with a preexposure period of 23 days, i.e., 51 days before). This rapid emergence of echinocandin resistance in *C. glabrata* has been described previously and is related to its haploid nature (19).

The positions of the first two mutations (Phe-659 and Ser-663) were previously reported in other echinocandin-resistant *C. glabrata* isolates (9, 23, 24). The substitution S663A and its association with F659S were described for the first time, in the same patient, by Garnaud et al. in a previous study on detection of resistance by next-generation sequencing (NGS) (25). The third mutation (D666E) is very rare and is found in less than 4% of resistant *C. glabrata* isolates (26). It was not detected by NGS in the Garnaud et al. study (25) because the sample collections stopped at D70, i.e., before the development of the third mutation. The mutation was found only in a D89 anus isolate and was associated with F659S (Fig. 1). This association (F659S and D666E) is described for the first time. It would be interesting to analyze the fitness cost of these mutations in an animal model and to test the kinetics of glucan synthase inhibition. Recently, the MSH2 gene has been shown to promote echinocandin resistance (27). For this reason, we sequenced parts of the MSH2 gene in four isolates recovered at different time points during echinocandin treatment, yet no mutation was detected in the gene. Our results clearly show the impact of echinocandin prophylactic treatment on the emergence of resistant *C. glabrata* spp. These findings are consistent with a recent study that suggested that colonizing mucosal flora may create a reservoir of resistant Candida spp., in particular for *C. glabrata* (20).

Our results show that during the appearance of the first two mutations in *C. glabrata*, the anidulafungin Etest was better than the micafungin Etest. The caspofungin Etest also showed good performance in detecting mutant isolates. Moreover, better performance of the Etest was obtained when EUCAST breakpoints were applied. For the EUCAST method, anidulafungin seems also to be the best echinocandin marker for detection of resistance, which is consistent with the use of anidulafungin as a marker for echinocandin susceptibility testing (28). However, it should be noted that our data are restricted to several isolates taken from one patient and that the present study was not designed to evaluate the performance of antifungal susceptibility testing methods.

The resistant *C. dubliniensis* isolates of P2 and the resistant *C. albicans* isolate of P3 harbored an S645P substitution in FKS1 HS1, which is a mutation commonly found in *C. albicans* (12). Although the mutation has been reported for *C. dubliniensis* (29), the appearance of resistant isolates during echinocandin therapy is a phenomenon that has never been reported for the species. Most echinocandin-resistant isolates were sampled from or around digestive sites, like abdominal aspirates and drainage and peritoneal fluids. This is probably due to the low diffusion of echinocandin in these sites (30, 31), which leads to subinhibitory concentrations, thus promoting the emergence of resistance. In previous case reports, the caspofungin and micafungin concentrations in the bile and ascites fluid were 30% and 15% of their levels in serum, respectively (31, 32).
Thus, as mentioned in a recent study, our results suggest that the abdomen could be the origin for the development of echinocandin-resistant Candida spp. (32). Echinocandins exhibit concentration-dependent effects on Candida spp. Preclinical pharmacokinetic and pharmacodynamic studies support the idea that the infrequent administration of large doses is a better strategy to achieve higher maximum concentrations of the drug in the serum (33). Moreover, once-weekly micafungin therapy seemed to be as efficacious as daily therapy in a murine model of disseminated candidiasis (34). Because this strategy cannot be applied in humans, it could be advisable to modify the antifungal treatment in cases of abdominal candidiasis in order to have better diffusion in abdominal sites.

In conclusion, our study sheds more light on the risk of emergence of resistance during echinocandin curative or prophylactic treatment, especially in digestive sites. These resistant isolates are not always associated with an infection but must be taken into account, since it has been shown that colonizing isolates are generally the same as those responsible for candidemia (35, 36). Accordingly, we are keen to raise awareness among clinicians and microbiologists of the emergence of echinocandin resistance, even during prophylactic treatment. Resistance can occur rapidly, which suggests that it is important to stop prophylaxis as soon as possible.

MATERIALS AND METHODS

Study design, patients, and isolates. The study population included all the patients who received transplants in our LT center (Henri Mondor University Hospital, Créteil, France) between January and June 2013 and 2015. Data were collected prospectively from the patients’ records. The study was approved by the local ethical committee, and the database was reported to the Commission Nationale de l’Informatique et des Libertés (CNIL) (no. 1699340).

The first phase of the study was directed at assessing echinocandin drug resistance and determining its location and its time of onset under antifungal pressure. This phase was conducted in patients who received echinocandin as curative or prophylactic treatment in compliance with the recommendations of Gavaldá et al. (3), because they were at high risk of IFI infection. The patients received caspofungin at 70 mg on day 1, followed by 50 mg/day, or micafungin at 100 mg/day. Patients who were at risk of IC received fluconazole at 400 mg/day. In cases of IC, patients were given a curative antifungal treatment with caspofungin or micafungin for at least 48 h, the time needed for the identification of the responsible Candida sp. and receipt of its susceptibility results.

All the patients were subjected to weekly monitoring of Candida colonization and were screened for Candida infections in blood cultures and in other sterile sites according to clinical signs. As part of this routine surveillance of Candida colonization, swabs were systematically taken from five superficial sites (mouth, nose, axillary surface, inguinal fold, and anus) on the day of admission to undergo LT and once per week thereafter until discharge from the intensive care unit (ICU) or death. Colonization was defined as the isolation of Candida species isolates from at least one surveillance site.

Isolate identification and storage. All clinical samples were cultured on Chromagar plates (Becton Dickinson) and incubated for at least 48 h at 37°C. Candida isolates were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (Microflex; Brucker) following standard extraction. The fungus species was identified using the MALDI BioTyper database version 3.0. For C. dubliniensis isolates, the species type was confirmed using ITS1 and ITS2 gene sequencing after mass spectrometry identification (37). All isolates were initially stored at −20°C on cryobeads (bioMérieux).

Antifungal susceptibility testing. Micafungin, anidulafungin, and caspofungin Etest strips (bioMérieux) were used to screen the susceptibility of all the Candida isolates from patients treated with echinocandin. When a resistant isolate was spotted, all the other isolates from that patient were subjected to Etest and EUCAST broth microdilution method testing. For Etest, the isolates were tested according to the manufacturer’s instructions. A yeast suspension adjusted to 0.5 McFarland standard was used to inoculate RPMI 1640 agar plates (bioMérieux). Etest strips (bioMérieux) were then applied, and the plates were incubated for 48 h at 37°C. An 80% inhibition endpoint was applied for MIC determination, as recommended for echinocandins. For EUCAST (38), anidulafungin (Pfizer Pharmaceutical Group) and micafungin (Astellas Pharma, Inc.) were tested at concentrations ranging from 0.015 to 8 mg/liter in RPMI 1640 (Sigma-Aldrich) buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich) and supplemented with glucose to a final concentration of 2%. The inoculated plates were incubated for 24 h at 37°C. Caspofungin was not used, because neither of the two reference methods (CLSI and EUCAST) is currently recommended to test Candida susceptibility to caspofungin, due to problems in test reproducibility (39). MIC values were determined spectrophotometrically (Multiskan FC microplate photometer; Thermo Scientific) as the lowest drug concentration that resulted in inhibition of ≥50% of fungal growth in comparison with the growth in a drug-free control well. For both techniques (EUCAST and Etest), Candida parapsilosis ATCC 22019 and C. krusei ATCC 6258 were used as quality control strains. EUCAST MIC results were interpreted according to breakpoints published in the EUCAST breakpoint table v8.0 (http://www.eucast.org/clinical_breakpoints). As there are currently no Etest-specific breakpoints, Etest MIC values were interpreted according to CLSI breakpoints (40), as recommended by the manu-

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The primers used for HS1 and HS2 of the FKS genes were recovered before and after the collection of a resistant isolate. The HS1 and HS2 regions of the FKS1 and FKS2 genes of C. glabrata and the HS1 and HS2 regions of the FKS1 gene of C. albicans were sequenced as previously described (42, 43). The primers used for HS1 and HS2 of the FKS1 gene of C. dubliniensis were designed based on GenBank accession number XM_002416855.1. The primers used for HS1 and HS2 regions of the FKS1 gene of C. dubliniensis in this study were as follows: for FKS1 HS1, 5'-TATCTTTGCTGCTATGCCCTT-3' and 5'-ACCCAAATAGAATGAAGCACA-3'; for FKS1 HS2, 5'-AAGATTG GTGCGGTATGGG-3' and 5'-RGTDGCAAAAACCTAGCATG-3'.

Each sample reaction mixture contained 0.5 µM each primer, 1× PCR gold buffer (Applied Biosystems), 1.5 mM MgCl₂ (Applied Biosystems), 2.5 mM deoxynucleoside triphosphate (dNTP) solution (Eurobio), 0.03 U of AmpliTaq Gold (Applied Biosystems), and RNase-free water up to a final reaction volume of 100 µl containing 20 ng of genomic DNA. Amplification was performed on a Mastercycler gradient (Eppendorf). The PCR conditions were initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The amplicons were purified by passing through columns of the MinElute PCR purification kit (Qiagen Sciences Inc.), and both strands were sequenced by Sanger's method (Eurofins Scientific). Sequence alignments were analyzed using SeqScape v2.5 (Applied Biosystems) and compared with the genome database sequences (GenBank accession numbers XM_446406 and XM_448401 for FKS1 and FKS2 of C. glabrata, respectively; XM_716336.1 for FKS1 of C. albicans; and XM_002416855.1 for FKS1 of C. dubliniensis).

PCR amplification and sequencing of the C. glabrata MSH2 gene. We looked for mutations in parts of the MSH2 gene where the most predominant mutations were found (according to reference 27). The primers used in this study were as follows: MSH2Fragment2, 5'-TCCTCTGCTCAATGTTTGGCC-3'; MSH2Fragment3, 5'-TGCGTGCTTACCATAGTCTCCCTA-3'; and MSH2Fragment3, 5'-TCTGGGACCTTTACAAAGAATG-3'. The PCR mixture conditions and sequencing were the same as those described above. Sequence alignments were analyzed using SeqScape v2.5 (Applied Biosystems) and compared with the C. glabrata genome database sequences (GenBank accession number CR380955).

Genotyping. Genotyping was performed on all FKS-sequenced isolates. Genotyping of C. glabrata and C. albicans was performed as previously described (44, 45). Three microsatellites within RMP2, MTI, and ERG3 were amplified for C. glabrata (44). Other groups of 3 microsatellites within EF3, CDC3, and HIS were amplified for C. albicans (45). Microsatellite PCR amplification was carried out using a GeneAmp 9700 thermal cycler (Applied Biosystems) in a 25-µl volume containing 20 ng of DNA. The composition of the PCR mixture was as follows: 0.5 µM each primer, 1× PCR buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP, and 1.25 U of AmpliTaq Gold. The PCR conditions were initial denaturation at 95°C for 8 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were diluted 1/100, 1/500, or 1/1,000 according to the intensities of PCR. They were added to HiDi formamide and 400HD size standard (Applied Biosystems) and denatured for 5 min at 95°C. They were then loaded on an ABI 3130 XL genetic analyzer (Applied Biosystems). The peak areas and the sizes of amplicons were determined using GeneScan analysis software v4.0 (Applied Biosystems).

Genotyping of C. dubliniensis was based on MLST profiles. Ten different loci were used for the MLST analyses: ATT1a, ATT1b, ACC1, ADP1, GLN4, MTV1, RMP2, SYA1, VPS13, and ZWF1b (46). PCR mixture conditions and sequencing were the same as those described above. Sequence alignments were analyzed using SeqScape v2.5 (Applied Biosystems).

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