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# Antifungal Resistance to Fluconazole and Echinocandins Is Not Emerging in Yeast Isolates Causing Fungemia in a Spanish Tertiary Care Center

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**Accurate knowledge of fungemia epidemiology requires identification of strains to the molecular level. Various studies have shown that the rate of resistance to fluconazole ranges from 2.5% to 9% in *Candida* spp. isolated from blood samples. However, trends in antifungal resistance have received little attention and have been studied only using CLSI M27-A3 methodology. We assessed the fungemia epidemiology in a large tertiary care institution in Madrid, Spain, by identifying isolates to the molecular level and performing antifungal susceptibility testing according to the updated breakpoints of European Committee for Antimicrobial Susceptibility Testing (EUCAST) definitive document (EDef) 7.2. We studied 613 isolates causing 598 episodes of fungemia in 544 patients admitted to our hospital (January 2007 to December 2013). Strains were identified after amplification and sequencing of the ITS1-5.8S-ITS2 region and further tested for *in vitro* susceptibility to amphotericin B, fluconazole, posaconazole, voriconazole, micafungin, and anidulafungin. Resistance was defined using EUCAST species-specific breakpoints, and epidemiological cutoff values (ECOFFs) were applied as tentative breakpoints. Most episodes were caused by *Candida albicans* (46%), *Candida parapsilosis* (28.7%), *Candida glabrata* (9.8%), and *Candida tropicalis* (8%). Molecular identification enabled us to better detect cryptic species of *Candida guilliermondii* and *C. parapsilosis* complexes and episodes of polyfungal fungemia. The overall percentage of fluconazole-resistant isolates was 5%, although it was higher in *C. glabrata* (8.6%) and non-*Candida* yeast isolates (47.4%). The rate of resistance to echinocandins was 4.4% and was mainly due to the presence of intrinsically resistant non-*Candida* species. Resistance mainly affected non-*Candida* yeasts. The rate of resistance to fluconazole and echinocandins did not change considerably during the study period.**

Fungemia is a major cause of morbidity and mortality in both critically ill and non-critically ill patients (1, 2). Although *Candida albicans* is the main species causing fungemia, other nonalbicans *Candida* and non-*Candida* species showing diminished antifungal susceptibility are emerging (2, 3). Knowledge of the epidemiology of the species causing fungemia is clinically relevant, particularly when starting empirical antifungal treatment, since antifungal susceptibility patterns are species specific (4, 5).

The distribution of *Candida* spp. causing fungemia varies with the geographic region studied (6–9). However, identification of strains to the molecular level is necessary to obtain an accurate picture of the species causing fungemia, because cryptic species in *Candida parapsilosis*, *Candida glabrata*, and *Candida guilliermondii* complexes often go undetected by conventional identification procedures, such as the ID 32C system (10).

Previous reports have shown that the rate of resistance to fluconazole ranges from 2.5% to 9% in *Candida* spp. isolated from blood samples (3, 11). Echinocandins are fast becoming first-line antifungal agents for the treatment of fungemia (12, 13); however, increasing use of these agents can promote the emergence of resistance in *Candida* and non-*Candida* isolates (14). Reports on trends in the rate of antifungal resistance in isolates causing fungemia are scarce, and the few studies performed were based only on the CLSI M27-A3 method (4, 5). In contrast, the European Committee for Antimicrobial Susceptibility Testing (EUCAST) procedure has rarely been used to study trends in antifungal resistance in single institutions over long periods.

The aim of the present study was to assess the epidemiology of fungemia in a large tertiary care institution located in Madrid, Spain, over a 7-year period after identifying isolates to the molecular level and performing antifungal susceptibility testing according to the updated breakpoints of EUCAST definitive document (EDef) 7.2 (15).

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## MATERIALS AND METHODS

**Hospital description, definition of fungemia episodes, and patients studied.** Hospital Gregorio Marañón serves a population of approximately 715,000 inhabitants in the city of Madrid, Spain, and cares for patients at high risk of fungemia, such as those admitted to medical and surgical intensive care units (ICUs), neonates, patients with hematologi-

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cal malignancies, solid-organ transplant recipients, and patients with central venous catheters.

We studied the episodes of fungemia detected in patients admitted to the hospital from January 2007 to December 2013. Multiple episodes in a single patient were defined as isolation of the same fungal species in further blood cultures taken ≥7 days after the last positive blood culture. Episodes in which 2 different *Candida* species were detected were considered polyfungal fungemia.

**Blood cultures and identification of isolates.** Blood samples for culture were obtained by standard procedures and incubated in the automated Bactec 9240 (from 2007 to 2011) and Bactec-FX system (from 2011 onward) (Becton, Dickinson, Cockeysville, MD, USA). Blood cultures with presumptive visualization of yeasts in the Gram stain were subcultured on Chromagar (Chromagar, Paris, France) and incubated for 36 to 48 h at 35°C. If 2 different species were detected in the Chromagar, 1 colony representing each species was independently subcultured. Isolates were identified by means of the ID 32C system (bioMérieux, Marcy l'Etoile, France) and confirmed by amplification and sequencing of the internal transcribed spacer 1 (ITS1)-5.8S-ITS2 region (18).

**Antifungal susceptibility testing.** We used the EUCAST EDef 7.2 microdilution procedure to test isolates for *in vitro* susceptibility to the following drugs: amphotericin B (Sigma-Aldrich, Madrid, Spain); fluconazole, voriconazole, and anidulafungin (Pfizer Pharmaceutical Group, New York, NY, USA); posaconazole and caspofungin (Merck & Co., Inc., Rahway, NJ, USA); and micafungin (Astellas Pharma, Inc., Tokyo, Japan) (15, 19). The antifungal agents were tested at concentrations ranging from 0.015 to 8 μg/ml (amphotericin B, voriconazole, posaconazole, caspofungin, anidulafungin, and micafungin) and 0.062 to 64 μg/ml (fluconazole). Inoculated plates were incubated for 24 h at 35°C. MIC values, which were determined spectrophotometrically at 530 nm (Multiskan FC microplate photometer; Thermo Scientific, Madrid, Spain), were defined as the lowest concentration of drug that resulted in inhibition of ≥50% of growth in comparison with a drug-free control growth well for fluconazole, voriconazole, posaconazole, and echinocandins or inhibition of ≥90% for amphotericin B. Plates with a drug-free control growth well showing an optical density threshold of <0.3 were reincubated for an additional 24 h or more until the drug-free control well reached the threshold. *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 isolates were used as quality control strains.

**Data analysis.** *Candida* isolates were classified as resistant (R), intermediate (I), or susceptible (S) according to species-specific clinical breakpoints or as wild type or non-wild type according to the epidemiological cutoff values (ECOFFs) proposed by EUCAST (20). The EUCAST document does not provide breakpoints or ECOFFs for caspofungin owing to the high interlaboratory variations reported (21, 22); therefore, the rate of resistance to caspofungin is not shown. The R breakpoint for amphotericin B was >1 μg/ml for *Candida albicans*, *C. parapsilosis*, *Candida tropicalis*, *C. glabrata*, and *C. krusei*. The non-species-related breakpoints for fluconazole (all *Candida* species, with the exception of *C. glabrata* and *C. krusei*) were as follows: S, ≤2 μg/ml; I, 4 μg/ml; and R, ≥8 μg/ml. The *C. glabrata* breakpoints were as follows: I, 0.062 to 32 μg/ml (when the lower limit of tested concentrations is taken into consideration), and R, >32 μg/ml. *C. krusei* was considered intrinsically fluconazole resistant. For voriconazole, the breakpoints were as follows: R, >0.125 μg/ml (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*) and >1 μg/ml (*C. glabrata* and *C. krusei*; tentatively based on the ECOFFs) (23). The breakpoints for posaconazole were as follows: R, >0.06 μg/ml (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*), >0.5 μg/ml (*C. krusei*; tentatively based on the ECOFFs), and >1 μg/ml (*C. glabrata*; tentatively based on the ECOFFs) (24). In the case of micafungin, the breakpoints were as follows: R, >0.015 μg/ml (*C. albicans*), >0.03 μg/ml (*C. glabrata*), >2 μg/ml (*C. parapsilosis*), >0.06 μg/ml (*C. tropicalis*), and >0.25 μg/ml (*C. krusei*; tentatively based on the ECOFFs) (25). The breakpoints for anidulafungin were as follows: R, >0.03 μg/ml (*C. albicans*), >0.06 μg/ml (*C. glabrata*, *C. krusei*, and *C. tropicalis*), and >4 μg/ml (*C. parapsilosis*).

**TABLE 1** Distribution of species causing fungemia in the 544 patients studied after amplification and further sequencing of the ITS1-5.8S-ITS2 region

| Species | *n* | % |
|---|---|---|
| *Candida albicans* | 282 | 46 |
| *Candida parapsilosis* complex | | |
| *Candida parapsilosis sensu stricto* | 165 | 26.9 |
| *Candida orthopsilosis* | 4 | 0.6 |
| *Candida metapsilosis* | 2 | 0.3 |
| *Candida glabrata* | 60 | 9.8 |
| *Candida tropicalis* | 49 | 8.0 |
| *Candida krusei* | 10 | 1.6 |
| Other *Candida* spp. | | |
| *Candida guilliermondii sensu stricto* | 7 | 1.1 |
| *Pichia caribbica* | 3 | 0.5 |
| *Kodamaea ohmeri* | 1 | 0.2 |
| *Candida dubliniensis* | 5 | 0.8 |
| *Candida lusitaniae* | 2 | 0.3 |
| *Candida kefyr* | 2 | 0.3 |
| *Pichia anomala* | 1 | 0.2 |
| Other yeasts | | |
| *Rhodotorula mucilaginosa* | 7 | 1.1 |
| *C. neoformans var grubii* | 4 | 0.7 |
| *Trichosporon asahii* | 2 | 0.3 |
| *C. neoformans var neoformans* | 2 | 0.3 |
| *Saccharomyces cerevisiae* | 1 | 0.2 |
| *Trichosporon dermatis* | 1 | 0.2 |
| *Trichosporon japonicum* | 1 | 0.2 |
| *Trichosporon inkin* | 1 | 0.2 |
| *Arxula adeninivorans* | 1 | 0.2 |

In the absence of breakpoints for non-*Candida* species, we used tentative non-species-related fluconazole breakpoints, except for *Cryptococcus neoformans*, which was considered fluconazole resistant if its MIC was ≥16 μg/ml (26). *Rhodotorula* spp., *Trichosporon* spp., *Arxula* spp., and *C. neoformans* were considered intrinsically echinocandin resistant. Rates of resistance were not calculated for the remaining drug-species combinations. Isolates showing resistance to one or more antifungal agents were retested, and the MIC was confirmed.

The antifungal resistance rate was calculated based on isolates from both intrinsically resistant species and normally susceptible species that showed MIC values above the breakpoints used.

**Identification of *fks* mutations.** We obtained the sequence of the HS1 and HS2 regions of the *fks* gene in *Candida* isolates with MICs for anidulafungin and/or micafungin greater than the breakpoints or ECOFFs, as previously described (27, 28).

## RESULTS

**Epidemiology of species causing fungemia.** During the study period (January 2007 to December 2013), we recorded 612 episodes of fungemia. The isolates from 14 episodes were not available (*C. albicans* [*n* = 7], *C. parapsilosis* [*n* = 5], *C. glabrata* [*n* = 1], and *Blastoschyzomyces capitatus* [*n* = 1]). We studied the isolates (*n* = 613) from the remaining 598 episodes diagnosed in 544 patients admitted to medical wards (25%), the ICU (20%), surgical wards (19%), neonatology (15%), oncology-hematology (14%), and other wards (7%) at the moment of blood sample collection.

The distribution of the species found is shown in Table 1. *C. albicans* was the main cause of fungemia (46%), followed by *C.*

**TABLE 2** Cases of fungemia caused by 2 different species (polyfungal fungemia)

| Species 1 | Species 2 | No. of cases | Species identified by ID 32C system plus Chromagar |
|---|---|---|---|
| *C. albicans* | *C. glabrata* | 4 | Both species |
| *C. glabrata* | *C. metapsilosis* | 1[a] | Only *C. parapsilosis* |
| *C. glabrata* | *C. parapsilosis* | 1[a] | Only *C. glabrata* |
| *C. albicans* | *C. parapsilosis* | 5[a] | Both species |
| *C. tropicalis* | *C. parapsilosis* | 1 | Both species |
| *C. parapsilosis* | *C. metapsilosis* | 1 | Only *C. parapsilosis* |
| *C. albicans* | *C. krusei* | 1 | Both species |
| *C. parapsilosis* | *C. guilliermondii* | 1 | Both species |

[a] In 3 out of the 15 patients, conventional identification was able to detect only 1 of the 2 species causing the infection. A total of 6 isolates (*C. parapsilosis* [$n = 2$], *C. glabrata* [$n = 2$], *C. metapsilosis* [$n = 1$], and *C. albicans* [$n = 1$]) from 3 patients with polyfungal fungemia were excluded from antifungal susceptibility testing because molecular identification revealed a mixture of different species after several attempts to obtain pure-culture isolates.

*parapsilosis* complex (27.8%), *C. glabrata* (9.8%), *C. tropicalis* (8%), *C. krusei* (1.6%), other *Candida* spp. (3.4%), and other, non-*Candida* yeasts (3.4%). The combination of Chromagar and the ID 32C system yielded an accurate identification of most isolates. However, in 7%, sequencing of the ITS region was necessary to ensure correct identification, because the species were not included in the ID 32C system or were from polyfungal fungemia not detected in Chromagar. Polyfungal fungemia was detected in 15 of the patients (3%) (Table 2), although Chromagar failed to detect both species in 3 out of the 15. Molecular identification performed on apparently pure-culture isolates showed a mixture of sequences representing a coinfection that was unraveled only after prolonged incubation of the Chromagar plates for up to 5 days.

Overall, ID 32C performed well in the identification of isolates, with the exception of *C. guilliermondii* and *C. parapsilosis* complexes, polyfungal fungemia, and other yeasts. The proportion of episodes caused by cryptic species was low. We did not detect *Candida nivariensis* or *Candida bracarensis* among isolates of the *C. glabrata* complex. In the *C. parapsilosis* complex, 96.4% of isolates were *C. parapsilosis sensu stricto*, 2.4% *Candida orthopsilosis*, and 1.2% *Candida metapsilosis*. In the *C. guilliermondii* complex, 63.6% of the strains were confirmed as *C. guilliermondii sensu stricto*, 27.3% as *Pichia caribbica*, and 9.1% as *Kodamaea ohmeri*. Excluding *C. parapsilosis sensu stricto* and *C. guilliermondii sensu stricto*, cryptic species of these 2 complexes frequently infected patients with gastrointestinal involvement (78%) (e.g., solid cancer, abdominal surgery, and mucositis).

**Antifungal susceptibility testing.** The antifungal activities of the 7 antifungal agents studied are shown in Table 3. The rate of antifungal resistance for each species-drug combination is shown in Table 4. All isolates were susceptible to amphotericin B. Overall resistance was 5% for fluconazole, 4.4% for micafungin, and 3.8% for anidulafungin. The percentage of fluconazole-resistant *Candida* isolates was low and varied from 0.7% (*C. albicans*) to 8.6% (*C. glabrata*). The *C. parapsilosis* and *C. tropicalis* isolates were susceptible to fluconazole and the remaining azoles. In contrast, although *C. neoformans* isolates were uniformly susceptible to fluconazole, *Rhodotorula* spp. (100%), *Trichosporon* spp. (25%), and *Arxula adeninivorans* (100%) showed a high percentage of resistance to the agent.

We analyzed the trend in the rate of resistance to fluconazole and echinocandins throughout the study period (Table 5). The rate of fluconazole resistance for *Candida* ranged from 0% (2009) to 6.4% (2010), although it has remained stable at around 4% since 2011. The echinocandin resistance rate was low, and only 7 *Candida* strains showed phenotypic resistance to anidulafungin and/or micafungin (*C. albicans* [$n = 4$], *C. tropicalis* [$n = 2$], and *C. krusei* [$n = 1$]). One *C. tropicalis* strain had an MIC for micafungin of 0.25 μg/ml and harbored a point mutation (R647G) in the HS1 region of the *fks1* gene; the remaining 6 isolates were wild type for *fks* genes, and most of them ($n = 5/6$) showed a slightly higher MIC (1- or 2-fold dilution) than the breakpoint or ECOFF for anidulafungin and micafungin (see Table 7). The rate of resistance to fluconazole and echinocandin antifungals was dependent on the number of episodes caused by species with diminished susceptibility to fluconazole or by intrinsically resistant species, such as *C. neoformans*, *Trichosporon* spp., or *Rhodotorula* spp.

The characteristics of patients infected with fluconazole-resistant *Candida* strains ($n = 19$; 3.5%) or echinocandin-resistant *Candida* strains ($n = 7$; 1.3%) are shown in Tables 6 and 7. Patients had severe underlying conditions, and many of them had cancer. Mortality was high (74%). Half of the patients infected by fluconazole-resistant isolates had previously received azoles (58%) and/or had cancer; in contrast, only 14% of patients infected by echinocandin-resistant strains had previously received echinocandins.

The wards of admission of the patients infected with *Candida* sp. strains that were resistant to fluconazole or echinocandins are shown in Fig. 1. Most patients infected with fluconazole-resistant isolates were admitted to medical wards, whereas patients infected by echinocandin-resistant isolates were mainly admitted to the ICU. The rate of fluconazole resistance was 5.4% in adult patients admitted to ICUs.

## DISCUSSION

The study of the epidemiology of fungemia is clinically relevant, since it enables us to select accurate empirical antifungal treatment. Previous epidemiology studies of fungemia were based on isolates collected over a relatively short period, thus making it difficult to analyze trends in antifungal resistance (2, 7, 29). To our knowledge, this is the first study in Spain to analyze trends in resistance rates to fluconazole and echinocandins.

The distribution of species causing fungemia shows marked geographical differences. In northern Europe and North America, *C. glabrata* is the second most common species after *C. albicans*. Studies conducted in Spain, Italy, Greece, and Latin America show that the percentage of *C. parapsilosis* is higher than that of *C. glabrata* (6). Our results confirm that *C. parapsilosis* was the second most frequent species causing fungemia in patients admitted to hospitals in southern Europe. Identification to the molecular level is necessary to obtain a precise understanding of the epidemiology of fungemia, since several species go undetected with conventional identification combining Chromagar medium and the ID 32C system (10, 30–32). We found that the proportion of cryptic species in the *C. parapsilosis* complex was low (3.5%). In contrast, we found higher variability of species in the *C. guilliermondii* complex. Most patients infected by cryptic species of these complexes had digestive disorders, suggesting that the gastrointestinal tract was the source of infection. Conventional identification performed well, and only 7% of strains required ITS sequenc-

**TABLE 3** Antifungal activities of antifungal agents against the 606 isolates studied (EUCAST EDef 7.2 procedure)

| Species[a] | Parameter[b] | Value[c] (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | AmB | FLU | VRC | POS | MYC | AND | CAS |
| *C. albicans* | Mode | 0.5 | 0.125 | ≤0.015 | ≤0.015 | ≤0.015 | ≤0.015 | 0.062 |
| | $MIC_{50}$ | 0.5 | 0.125 | ≤0.015 | ≤0.015 | ≤0.015 | ≤0.015 | 0.062 |
| | $MIC_{90}$ | 1 | 0.25 | ≤0.015 | 0.031 | ≤0.015 | ≤0.015 | 0.125 |
| | Range | (0.062 to 1) | (≤0.125 to ≥64) | (≤0.015 to ≥16) | (≤0.015 to 8) | (≤0.015 to 1) | (≤0.015 to 2) | (≤0.015 to 0.5) |
| *C. parapsilosis* | Mode | 0.25 | 0.25 | ≤0.015 | 0.031 | 1 | 2 | 0.5 |
| | $MIC_{50}$ | 0.25 | 0.25 | ≤0.015 | 0.031 | 1 | 2 | 0.5 |
| | $MIC_{90}$ | 1 | 0.5 | ≤0.015 | 0.062 | 2 | 2 | 1 |
| | Range | (0.062 to 1) | (≤0.125 to 2) | (≤0.015 to 0.062) | (≤0.015 to 0.125) | (≤0.015 to 2) | (≤0.015 to 4) | (0.125 to 2) |
| *C. glabrata* | Mode | 0.25 | 8 | 0.25 | 0.5 | ≤0.015 | 0.031 | 0.125 |
| | $MIC_{50}$ | 0.25 | 8 | 0.25 | 0.5 | ≤0.015 | 0.031 | 0.125 |
| | $MIC_{90}$ | 1 | ≥64 | 1 | 1 | ≤0.015 | 0.031 | 0.125 |
| | Range | (0.125 to 1) | (2 to ≥64) | (0.062 to 4) | (0.125 to 2) | (≤0.015 to 0.031) | (≤0.015 to 0.031) | (0.062 to 0.125) |
| *C. tropicalis* | Mode | 0.5 | 0.25 | ≤0.015 | ≤0.015 | 0.031 | ≤0.015 | 0.125 |
| | $MIC_{50}$ | 0.5 | 0.25 | ≤0.015 | ≤0.015 | 0.031 | ≤0.015 | 0.125 |
| | $MIC_{90}$ | 1 | 0.5 | 0.031 | 0.031 | 0.031 | 0.031 | 0.125 |
| | Range | (0.25 to 1) | (≤0.125 to 1) | (≤0.015 to 0.062) | (≤0.015 to 0.062) | (≤0.015 to 0.25) | (≤0.015 to 0.125) | (0.031 to 0.125) |
| *C. krusei* | Mode | 0.5 | ≥64 | 0.5 | 0.25 | 0.062 | 0.062 | 0.125 |
| | $MIC_{50}$ | 0.5 | ≥64 | 0.5 | 0.25 | 0.062 | 0.062 | 0.125 |
| | $MIC_{90}$ | 1 | ≥64 | 0.5 | 0.25 | 1 | 2 | 0.5 |
| | Range | (0.5 to 1) | (16 to ≥64) | (0.125 to 0.5) | (0.062 to 0.25) | (≤0.015 to 1) | (≤0.015 to 2) | (0.062 to 0.5) |
| *Candida* spp. | Mode | 1 | 0.25 | ≤0.015 | 0.031 | 0.5 | 1 | 0.25 |
| | $MIC_{50}$ | 0.5 | 2 | 0.062 | 0.062 | 0.125 | 0.5 | 0.25 |
| | $MIC_{90}$ | 2 | 16 | 0.5 | 0.5 | 1 | 2 | 2 |
| | Range | (0.125 to 2) | (≤0.125 to 16) | (≤0.015 to 2) | (≤0.015 to 1) | (≤0.015 to 4) | (≤0.015 to 2) | (0.031 to 8) |
| Other yeasts | Mode | 1 | ≥64 | 0.031 | 1 | 8 | 8 | 8 |
| | $MIC_{50}$ | 1 | 8 | 0.25 | 0.5 | 8 | 8 | 8 |
| | $MIC_{90}$ | 8 | ≥64 | 8 | 2 | ≥16 | ≥16 | ≥16 |
| | Range | (0.5 to 8) | (0.5 to ≥64) | (0.031 to 8) | (0.031 to 4) | (0.125 to ≥16) | (0.5 to ≥16) | (0.25 to ≥16) |

[a] Six isolates were excluded from antifungal susceptibility testing because molecular identification showed a mixture of different species after several attempts to obtain pure-culture isolates, and the *Saccharomyces cerevisiae* isolate did not grow in RPMI. Isolates of *C. orthopsilosis* ($n = 3$) and *C. metapsilosis* ($n = 1$) were considered *C. parapsilosis* for analysis of the rate of resistance.

[b] MICs of ≤0.015 or ≤0.125 were transformed to 0.015 and 0.125, respectively for purposes of analysis.

[c] AmB, amphotericin B; FLU, fluconazole; VRC, voriconazole; POS, posaconazole; MYC, micafungin; AND, anidulafungin; CAS, caspofungin.

**TABLE 4** Resistance to the antifungal agents studied for *Candida* and non-*Candida* species

| Species | % intermediate/resistant or N-WT isolates[e] | | | | | |
|---|---|---|---|---|---|---|
| | AmB (R) | FLU (I/R) | VRC (R) | POS (R) | MYC (I/R) | AND (I/R) |
| *C. albicans* | 0 | 0/0.7 | 0.7 | 0.7 | 0/1.4 | 0/0.4 |
| *C. parapsilosis* complex[a] | 0 | 0/0 | 0 | 0 | 100/0 | 100/0 |
| *C. glabrata* | 0 | 91.4/8.6 | 5.2 | 7 | 0/0 | 0/0 |
| *C. tropicalis* | 0 | 0/0 | 0 | 0 | 0/4.1 | 0/2 |
| *C. krusei*[b] | 0 | 0/100 | 0 | 0 | 0/10 | 0/10 |
| *Candida* spp. | NA | 0/19 | NA | NA | NA | NA |
| Other yeasts | NA | 0/47.4 | NA | NA | 0/100 | 0/100 |
| Overall (all isolates)[d] | NA | 8.7/5 | NA | NA | 28.7/4.4 | 28.7/3.8 |
| Overall (only *Candida* species)[d] | 0[c] | 9/3.6 | 0.9 | 1 | 29.6/1.2 | 29.6/0.5 |

[a] Isolates of *C. metapsilosis* ($n = 3$) and *C. orthopsilosis* ($n = 1$) were considered *C. parapsilosis* for analysis of the rate of resistance.
[b] *C. krusei* was considered fluconazole resistant. For the tentative breakpoints used for non-*Candida* isolates, see Materials and Methods.
[c] The rate of resistance to amphotericin B was calculated only for *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. tropicalis*.
[d] Six isolates were excluded from antifungal susceptibility testing because molecular identification showed a mixture of different species after several attempts to obtain pure-culture isolates, and the *S. cerevisiae* isolate did not grow in RPMI.
[e] NA, not applicable; I, intermediate (for fluconazole, MIC, 4 mg/liter, except for *C. glabrata*, where the MIC was >0.062 to 32 mg/liter); R, resistant; N-WT, non-wild type; AmB, amphotericin B; FLU, fluconazole; VRC, voriconazole; POS, posaconazole; MYC, micafungin; AND, anidulafungin.

ing to confirm identification, particularly in the episodes caused by the *C. parapsilosis* complex and *C. guilliermondii* complex and in polyfungal fungemia.

Resistance to fluconazole in different studies conducted in Spain varied from 7% to 8% (1, 7, 29). The rate of resistance in *Candida* spp. reported here is lower than 4% and did not vary considerably during the study period, showing that development of secondary fluconazole resistance is not a problem in the clinical setting. When all the isolates were considered together, the rate of resistance was approximately 5%, which is in line with the findings of the recently published CANDIPOP multicenter study conducted in Spain (7). The rate of fluconazole resistance was very low in the most common species causing fungemia (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*). Resistance to echinocandins in *Candida* remains low (<2%) and is similar to that reported previously in Spain (7). However, resistance to echinocandins is emerging in other regions, such as the United States (33). We found that the rate of resistance to echinocandins was mostly related to the presence of species that are intrinsically resistant to candins, different from the *Candida* spp. shown in Table 5. The rates of fluconazole and echinocandin resistance were similar to those reported after analyzing isolates collected from all over the

**TABLE 5** Trends in resistance to fluconazole and echinocandins during the study period (2007 to 2013)

| Drug and species | Antifungal resistance (%)[c] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
| Fluconazole | | | | | | | |
| *C. albicans* ($n = 281$) | 0 | 0 | 0 | 1.8 | 2.6 | 0 | 0 |
| *C. parapsilosis* ($n = 168$) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. glabrata* ($n = 58$) | 10 | 12.5 | 0 | 28.5 | 8.3 | 0 | 0 |
| *C. tropicalis* ($n = 49$) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. krusei* ($n = 10$) | 100 | 0 | 0 | 100 | 100 | 100 | 100 |
| Other *Candida* spp. ($n = 21$) | 50 | 20 | 0 | 16.6 | 0 | 0 | 25 |
| Other yeasts ($n = 19$) | 33.3 | 100 | 25 | 100 | 50 | 60 | 0 |
| Overall (only *Candida* spp.) ($n = 587$) | 3.3 | 2.3 | 0 | 6.4 | 4.7 | 4.2 | 3.4 |
| Overall ($n = 606$)[a] | 4 | 3.3 | 1.4 | 8.4 | 5.8 | 7.8 | 3.3 |
| Echinocandins | | | | | | | |
| *C. albicans* ($n = 281$) | 1.7 | 0 | 0 | 1.8 | 0 | 6 | 0 |
| *C. parapsilosis* ($n = 168$) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. glabrata* ($n = 58$) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. tropicalis* ($n = 49$) | 0 | 14.2 | 0 | 0 | 16.6 | 0 | 0 |
| *C. krusei* ($n = 10$) | 50 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other *Candida* spp. ($n = 21$) | NA | NA | NA | NA | NA | NA | NA |
| Other yeasts ($n = 19$) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Overall (only *Candida* spp.) ($n = 566$)[b] | 1.7 | 1.2 | 0 | 1.1 | 1.2 | 2.8 | 0 |
| Overall ($n = 585$)[a,b] | 4.1 | 2.3 | 5.5 | 3.4 | 3.6 | 9.2 | 3.5 |

[a] Six isolates were excluded from antifungal susceptibility testing because molecular identification showed a mixture of different species after several attempts to obtain pure-culture isolates, and the *S. cerevisiae* isolate did not grow in RPMI. No C. krusei strain was isolated in 2008 and 2009.
[b] Other *Candida* species isolates were excluded from the echinocandin-resistant rate analysis because of the lack of clinical breakpoints or ECOFF.
[c] NA, not applicable. The rate of resistance was calculated overall and for *Candida* spp.

**TABLE 6** Characteristics of 19 patients infected with fluconazole-resistant *Candida* isolates

| Date of candidemia (day/mo/yr) | *Candida* species | Underlying condition(s) | Previous antifungal(s) | Resistance | Antifungal treatment | Outcome |
|---|---|---|---|---|---|---|
| 02/01/2007 | *C. krusei* | Diabetes, lithiasis | No | MYC, AND, FLU | AmB | Favorable |
| 20/05/2007 | *C. guilliermondii* | Colon adenocarcinoma | No | FLU | Unknown | Favorable |
| 27/11/2007 | *C. krusei* | Oropharyngeal cancer | Azoles (FLU) | FLU | AmB | Death at 7 days |
| 21/10/2007 | *C. glabrata* | Chronic lymphoid leukemia | Azoles (VRC) | FLU, VRC, POS | CAS | Favorable |
| 10/10/2008 | *C. guilliermondii* | Colon adenocarcinoma | No | FLU | VRC | Favorable |
| 22/12/2008 | *C. glabrata* | Diabetes | Azoles (FLU) | FLU | CAS | Favorable |
| 26/01/2010[a] | *C. glabrata* | Abdominal surgery | No | FLU, VRC, POS | AND, AmB | Favorable |
| 30/05/2010 | *C. lusitaniae* | Acute lymphoid leukemia | Azoles (FLU, POS, ITRA[c]) | FLU | AmB, VRC | Death at 30 days |
| 23/08/2010 | *C. albicans* | Diabetes, lithiasis | Azoles (VRC) | FLU, VRC, POS, MYC | MYC | Favorable |
| 28/09/2010 | *C. krusei* | Hepatitis | No | FLU | AmB, VRC | Death at 30 days |
| 27/12/2010[b] | *C. krusei* | Pelvic adenocarcinoma | Azoles (FLU) | FLU | MYC | Death at 7 days |
| 08/03/2011 | *C. krusei* | Retroperitoneal sarcoma | Azoles (FLU) and candins (CAS) | FLU | MYC/AND | Death at 7 days |
| 27/07/2011 | *C. albicans* | Abdominal surgery, newborn | No | FLU, VRC, POS | AmB | Favorable |
| 08/11/2011 | *C. glabrata* | Gastric adenocarcinoma | Azoles (FLU) | FLU | MYC | Favorable |
| 02/01/2012 | *C. krusei* | Esophageal cancer | Azoles (FLU) | FLU | MYC | Death at 30 days |
| 05/03/2012 | *C. krusei* | Liver cirrhosis | No | FLU | CAS | Favorable |
| 29/05/2012 | *C. krusei* | Renal transplantation | Azoles (FLU) | FLU | MYC | Favorable |
| 05/04/2013 | *C. krusei* | Acute lymphoid leukemia | Azoles (POS) | FLU | CAS, AmB | Favorable |
| 23/04/2013 | *C. guilliermondii* | Diabetes, pancreatitis | No | FLU | FLU, AmB/CAS, FLU | Favorable |

[a] The patient had an additional episode of candidemia caused by *C. glabrata* diagnosed 1 month later.
[b] The patient had an additional episode of candidemia caused by *C. krusei* diagnosed 10 days later.
[c] ITRA, itraconazole.

world (34). Of the 7 *Candida* strains showing phenotypic resistance to echinocandins, only one isolate presented mutations in the *fks* genes, whereas most of the remaining isolates had MICs slightly greater than the breakpoints or ECOFFs, particularly for micafungin (Table 7). If the statistical ECOFFs for micafungin (0.031 μg/ml) (20) had been used, 3 out of 4 *C. albicans* isolates would have been classified as susceptible to both echinocandins.

We excluded 6 isolates from 3 patients with polyfungal fungemia from the antifungal susceptibility testing analysis. Sequence analyses enabled us to correctly identify the isolates present in a higher proportion, although a mixture of sequences was found, suggesting the presence of another species in a much lower proportion. Sequential cultures on agar plates failed to separate both species. Furthermore, isolates showed altered susceptibility (resistance to fluconazole [*C. parapsilosis* complex, $n = 3$] or to echinocandins [*C. albicans*, $n = 1$; *C. glabrata*, $n = 2$]). Isolates showing fluconazole resistance were from patients infected simultaneously with *C. glabrata*, and isolates showing echinocandin resistance were from patients also infected with *C. parapsilosis* complex strains. The inclusion of these isolates would have artificially altered the rate of resistance, thus proving the importance of molecular identification, not only for epidemiological purposes, but also when attempting to obtain an accurate rate of antifungal resistance.

Our study is subject to a series of limitations. First, we detected a high rate of echinocandin resistance in *C. krusei* (10%). However, the number of isolates was low, and the clinical impact of phenotypic resistance in isolates showing the wild-type *fks1* gene sequence is unknown. Second, in the absence of species-specific breakpoints for all the species studied, we had to use ECOFFs as tentative breakpoints. For non-*Candida* species, we used the unrelated species breakpoints for fluconazole; therefore, we show the overall rate of resistance, as well as the rate of resistance exclusively for *Candida* spp. Finally, since we included only

**TABLE 7** Characteristics of 7 patients infected with echinocandin-resistant *Candida* isolates

| Date of candidemia (day/mo/yr) | *Candida* species | Underlying condition(s) | Previous antifungals | Resistance | MIC (μg/ml) | | Antifungal treatment | Outcome |
|---|---|---|---|---|---|---|---|---|
| | | | | | AND | MYC | | |
| 01/01/2007 | *C. albicans* | Cardiac surgery | No | MYC, AND | 2 | 1 | FLU | Death at 30 days |
| 02/01/2007 | *C. krusei* | Diabetes, lithiasis | No | MYC, AND, FLU | 0.25 | 0.5 | AmB | Favorable |
| 02/05/2008 | *C. tropicalis* | Hypopharyngeal cancer | Azoles (FLU) | MYC, AND | 0.125 | 0.125 | CAS, FLU | Death at 30 days |
| 23/08/2010 | *C. albicans* | Diabetes, lithiasis | Azoles (VRC) | FLU, VRC, POS, MYC | 0.015 | 0.031 | MYC | Favorable |
| 31/10/2011[a] | *C. tropicalis* | Renal transplantation | Candins (MYC) | MYC | 0.062 | 0.25 | AmB | Death at 7 days |
| 19/08/2012 | *C. albicans* | Lymphoepithelioma | No | MYC | 0.015 | 0.031 | AND | Death at 7 days |
| 25/11/2012 | *C. albicans* | Hemangioblastoma | AmB and azoles (FLU) | MYC | 0.015 | 0.031 | AmB, FLU | Favorable |

[a] Patient infected by a *C. tropicalis* strain harboring a point mutation (R647G) in the HS1 region of the *fks1* gene.

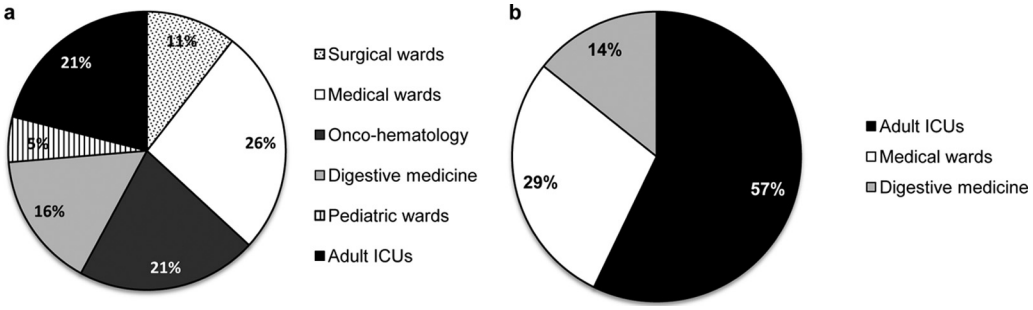


**FIG 1** Wards of admission of patients infected with fluconazole-resistant (a) or echinocandin-resistant (b) *Candida* species isolates. Medical wards included geriatrics, urology, internal medicine, nephrology, infectious diseases, and otorhinolaryngology.

isolates from a single institution, we may not be able to extrapolate them to other hospitals. However, our data are comparable to those of the CANDIPOP study, in which 29 Spanish hospitals participated (7).

In conclusion, we showed that although the number of episodes of fungemia caused by cryptic species was low, molecular identification provided an accurate picture of the epidemiology of fungemia. The rate of resistance to fluconazole and echinocandins in yeast isolates causing fungemia was also low and does not show signs of increasing.

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