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Molecular Identification and Antifungal Susceptibility of Yeast Isolates Causing Fungemia Collected in a Population-Based Study in Spain in 2010 and 2011

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We report the molecular identifications and antifungal susceptibilities of the isolates causing fungemia collected in the CANDIPOP population-based study conducted in 29 Spanish hospitals. A total of 781 isolates (from 767 patients, 14 of them having mixed fungemia) were collected. The species found most frequently were *Candida albicans* (44.6%), *Candida parapsilosis* (24.5%), *Candida glabrata* (13.2%), *Candida tropicalis* (7.6%), *Candida krusei* (1.9%), *Candida guilliermondii* (1.7%), and *Candida lusitaniae* (1.3%). Other *Candida* and non-*Candida* species accounted for approximately 5% of the isolates. The presence of cryptic species was low. Compared to findings of previous studies conducted in Spain, the frequency of *C. glabrata* has increased. Antifungal susceptibility testing was performed by using EUCAST and CLSI M27-A3 reference procedures; the two methods were comparable. The rate of fluconazole-susceptible isolates was 80%, which appears to be a decrease compared to findings of previous studies, explained mainly by the higher frequency of *C. glabrata*. Using the species-specific breakpoints and epidemiological cutoff values, the rate of voriconazole and posaconazole *in vitro* resistance was low (<2%). In the case of *C. tropicalis*, using the EUCAST procedure, the rate of azole resistance was around 20%. There was a correlation between the previous use of azoles and the presence of fluconazole-resistant isolates. Resistance to echinocandins was very rare (2%), and resistance to amphotericin B also was very uncommon. The sequencing of the hot spot (HS) regions from *FKS1* or *FKS2* genes in echinocandin-resistant isolates revealed previously described point mutations. The decrease in the susceptibility to fluconazole in Spanish isolates should be closely monitored in future studies.

The incidence of invasive fungal infections has increased in modern hospitals due to the growing number of susceptible patients (1). Fungemia, the most frequent invasive fungal infection, is caused mainly by *Candida albicans*, although non-*albicans Candida* species are becoming more prevalent (1–3). Other non-*Candida* yeasts showing intrinsic antifungal resistance have also been reported (4, 5). Candidemia is commonly difficult to detect in patients with invasive *Candida* infections; consequently, many patients receive empirical antifungal therapy. Although rapid and prompt starting of antifungal therapy is important in order to improve the outcome for the patients (6), resistance may contribute to treatment failures. Considering the *Candida* species-specific patterns of antifungal susceptibility, local knowledge of the species causing fungemia and the antifungal resistance rate is essential in order to optimize antifungal treatment.

The distribution of *Candida* spp. isolated from blood differs among studies conducted in different geographical areas. *Candida albicans* is the species found most frequently in different nationwide and population-based studies. In contrast, *C. glabrata* is the second species in terms of frequency in Northern Europe and the USA, whereas *C. parapsilosis* is more common in Latin America and Southern Europe (1, 7–9). Molecular identification of isolates allows a precise knowledge of the epidemiology of fungemia, since several cryptic species are indistinguishable by using conventional procedures of identification (10). Furthermore, the expanding use of antifungal agents in the clinical setting is another factor affecting the epidemiology of fungemia and also promoting the appearance of antifungal resistance.

Resistance to fluconazole is a matter of concern in the management of candidemia, since patients infected by strains or species exhibiting high fluconazole MIC values have a poor response to the treatment. Several population-based surveys of invasive candidiasis have reported rates of resistance to fluconazole between 10% and 25% (8, 11–13). A population-based study of candidemia that was carried out in Barcelona, Spain, in 2005 (7) reported a rate of fluconazole resistance below 10%, which is similar to that reported in another Spanish prospective sequential study (FUNGEMYCA study), carried out in 2009 (9). Taking into ac-

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count that the isolates were collected years ago and that epidemiological changes are being detected in some European countries (8, 11), a Spanish nationwide study on fungemia was necessary. For that reason, a multicenter study involving 767 patients with fungemia from 2010 and 2011 admitted to 29 hospitals located in different geographical regions in Spain (CANDIPOP study) was carried out. The epidemiological and clinical characteristics of the patients have been reported elsewhere (14). In this study, we analyzed the species distribution and antifungal susceptibility patterns of the 781 yeast isolates collected in the CANDIPOP study. We performed molecular identification and antifungal susceptibility testing according to both EUCAST and CLSI protocols.

MATERIALS AND METHODS

Patients studied. A total of 29 Spanish hospitals participated in the study CANDIPOP conducted from 1 May 2010 to 30 April 2011. All patients ($n = 767$) diagnosed with fungemia and admitted to the abovementioned hospitals during the study period who consented to participate in the study were recruited. Fungemia was defined as the isolation of yeasts in blood cultures in patients with clinical signs and symptoms of infection.

Strains and molecular identification. The incident isolate causing the fungemia was studied. A total of 781 isolates were collected (14 patients had polyfungal fungemia caused by two different yeast species). Isolates, which were collected and stored at each participating hospital, were further sent to the Mycology Reference Laboratory at the National Centre for Microbiology (Instituto de Salud Carlos III) in Madrid, Spain. Upon arrival, isolates were grown on Sabouraud dextrose agar and incubated at 30°C. The molecular identification of the isolates was achieved by sequencing the internal transcribed spacer 1 (ITS1) and ITS2 regions from the ribosomal DNA as previously described (15).

Antifungal susceptibility testing. Antifungal susceptibility testing was performed on the 781 isolates according to the microdilution broth procedures CLSI M27-A3 and EUCAST (EDef 7.1). The following antifungals were used: amphotericin B and flucytosine (Sigma-Aldrich, Madrid, Spain); fluconazole, voriconazole, and anidulafungin (Pfizer Pharmaceutical Group, New York, NY); itraconazole (Janssen Pharmaceutical Research and Development, Madrid, Spain); posaconazole and caspofungin (Merck &Co., Inc., Rahway, NJ); and micafungin (Astellas Pharma, Inc., Tokyo, Japan).

(i) CLSI M27-A3 procedure. (16). This procedure was carried out at the Clinical Microbiology Department of Gregorio Marañón Hospital, Madrid, Spain. The following ranges of antifungal concentrations were studied: fluconazole, 0.062 to 64 mg/liter; amphotericin B, voriconazole, posaconazole, caspofungin, micafungin, and anidulafungin, (0.0017 to 2 mg/liter). MICs were obtained after 24 h of incubation at 35°C. *Cryptococcus* spp. and non-*Candida* isolates showing poor growth were incubated for 72 h. The MIC for amphotericin B was defined as the lowest drug concentration that prevents any discernible growth. The MIC for azoles and echinocandins was considered the lowest drug concentration allowing a reduction of an approximately 50% in growth relative to that of the drug-free growth control.

(ii) EUCAST procedure. (17). This procedure was carried out at the Mycology Reference Laboratory at the National Centre for Microbiology. The following range of concentrations was studied: amphotericin B, caspofungin, micafungin and anidulafungin, 0.03 to 16 mg/liter; flucytosine and fluconazole, 0.125 to 64 mg/liter; voriconazole, itraconazole, and posaconazole, 0.015 to 8 mg/liter. The optical densities of the plates were measured after 24 h of incubation at 35°C. The MIC was defined as the antifungal concentration that produced 90% (amphotericin B) or 50% (remaining antifungal agents) of growth inhibition relative to that of the drug-free growth control. For strains that showed poor growth, such as some *C. neoformans* isolates or other nonfermentative species, antifungal susceptibility testing was repeated with shaking and incubation at 30°C for 48 h before determining the optical density.

Amplification of hot spot (HS) regions from *FKS* genes. We searched for mutations at the *FKS* genes (which encode the enzyme β-1,3-glucan synthase) in isolates showing resistance to one or more echinocandins according to the CLSI or EUCAST procedures. Briefly, we amplified the HS1 and HS2 regions of *C. albicans* (18), *C. glabrata* (19), and *C. tropicalis* (20) as previously described. PCR products were sequenced using the same oligonucleotides used in the PCR using the BigDye kit (Applied Biosystems). Sequences were analyzed using SeqMan II and Ediseq software programs (DNAStar, Madison, WI).

Data analysis. Distribution of MIC values was assessed using SPSS software (IBM SPSS Statistics 21). Antifungal activity was expressed as the MIC_{90}, MIC_{50}, geometric mean MIC, mode, and range of MICs (minimum and maximum). Resistant strains were defined according to the species-specific breakpoints proposed by the EUCAST 6.1 (valid since 11 March 2013) and 5.0 (valid until 1 March 2013), and CLSI M27-S4 documents (17). Antifungal susceptibility testing results for caspofungin should be interpreted with caution due to the interlaboratory variation reported, due possibly to potency variation (21, 22). All isolates showing high MICs were retested and confirmed.

(i) CLSI M27-A3 procedure. Isolates were classified according to the species-specific breakpoints included in the M27-S4 document for *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*. We used the epidemiological cutoff values (ECVs) to classify as non-wild type isolates for other species-drug combinations in the absence of clinical breakpoints (23). *Cryptococcus neoformans* isolates were considered fluconazole resistant if they showed an MIC of ≥16 mg/liter (24). In order to make comparable the rates of resistance obtained both by CLSI and EUCAST, we tentatively used the *C. albicans* breakpoints for classifying the remaining *Candida* and non-*Candida* isolates ($n = 19$) as susceptible or resistant to fluconazole and voriconazole; *Rhodotorula* species, *C. neoformans*, and *Trichosporon* species isolates were considered intrinsically resistant to echinocandins (see Tables 4 and 5).

(ii) EUCAST procedure. The following breakpoints were used to classify isolates as resistant (R) or intermediate (I): for amphotericin, R, >1 mg/liter; for fluconazole, sensitive [S], ≤2 mg/liter; R, >4 mg/liter, for all *Candida* spp. with the exception of *C. glabrata* and tentatively for *C. guilliermondii* (S, ≤0.002 mg/liter; I, 0.004 to 32 mg/liter; R, >32 mg/liter); for voriconazole, R, >0.12 mg/liter; for posaconazole, R, >0.06 mg/liter; for anidulafungin against *C. albicans*, R, > 0.03 mg/liter]; against *C. glabrata*, *C. tropicalis*, and *C. krusei*, R, >0.06 mg/liter; against *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*, and *C. guilliermondii*, S, ≤0.002 mg/liter; I, 0.004 to 4 mg/liter,; R, >4 mg/liter. MIC values of echinocandins were interpreted according to breakpoints published recently in the EUCAST webpage (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Antifungal_breakpoints_v_6.1.pdf). There are no species-specific breakpoints for *C. guilliermondii* and fluconazole or anidulafungin. According to the MIC distribution of both agents and the presence of a polymorphism in the *FKS* genes (M instead L in the second amino acid of HS1 of *C. guilliermondii* and A instead P in the last amino acid of the HS1 of *C. parapsilosis*), we used the *C. glabrata* (for fluconazole) or *C. parapsilosis* (for anidulafungin) breakpoints as tentative epidemiological cutoff values (ECOFFs) for both drugs (http://www.eucast.org/antifungal_susceptibility_testing_afst/rationale_documents_for_antifungals/). For non-*Candida* species, the *Candida albicans* breakpoints were used as tentative ECOFFs to classify them as non-wild type.

(iii) Impact of the use of antifungal agents on the promotion of antifungal resistance. We also evaluated the impact of the use of azoles or echinocandins in the previous month with diagnosis of fungemia on the proportion of episodes caused by resistant or non-wild-type isolates (defined by the CLSI or EUCAST procedures). The chi-square test was used to study the correlation between previous antifungal use and the presence of resistant isolates.

TABLE 1 Species distribution of the 781 isolates identified after amplification and further sequencing of the ITS1-5.8S-ITS2 region

Species	No. (%) of isolates
Candida albicans	348 (44.6)
Candida parapsilosis	191 (24.5)
Candida glabrata	103 (13.2)
Candida tropicalis	59 (7.6)
Candida krusei	15 (1.9)
Candida guilliermondii	13 (1.7)
Candida lusitaniae	10 (1.3)
Candida orthopsilosis	7 (0.9)
Cryptococcus neoformans	5 (0.6)
Candida kefyr	4 (0.5)
Candida dubliniensis	4 (0.5)
Candida lipolytica	4 (0.5)
Dipodascus capitatus	3 (0.4)
Trichosporon asahii	3 (0.4)
Candida metapsilosis	2 (0.3)
Pichia anomala	2 (0.3)
Rhodotorula mucilaginosa	2 (0.3)
Candida intermedia	1 (0.1)
Kodamea ohmeri	1(0.1)
Lodderomyces elongisporus	1(0.1)
C. nivariensis	1(0.1)
Pichia fabianii	1(0.1)
Metschnikowia pulcherrima	1(0.1)
Total	781 (100)

RESULTS

Species distribution. We studied a total of 781 clinical isolates from 767 patients, with 14 of them having candidemia caused by two different species. As shown in Table 1, *C. albicans* was the most frequently isolated species, accounting for 44.6% of episodes, followed by *C. parapsilosis* (24.5%), *C. glabrata* (13.2%), and *C. tropicalis* (7.6%). Other *Candida* species, such as *C. krusei*, *C. guilliermondii*, or *C. lusitaniae*, represented approximately 2% of the isolates each. We observed differences in species distribution among the 5 participating Spanish regions. For example, the frequency of *C. albicans* was higher in Madrid and Barcelona; in contrast, the frequency of *C. parapsilosis* was lower in both cities. Differences in the frequencies of *C. tropicalis* and *C. glabrata* were also observed (14).

The use of identification based on molecular biology techniques (ITS sequencing) allowed a proper detection of cryptic and rare species, such as *C. dubliniensis* (normally misidentified as *C. albicans*), *C. orthopsilosis* and *C. metapsilosis* (normally misidentified as *C. parapsilosis*), and *C. nivariensis* (normally misidentified as *C. glabrata*). A total of 20 strains were misidentified by local methods compared to identification by ITS sequencing. The most frequent discrepancies occurred in the *C. parapsilosis* complex (*C. orthopsilosis* [6 out of 7 strains] and *C. metapsilosis* [2 out of 2 strains] were confused with *C. parapsilosis*) and *C. albicans* complex (*C. dubliniensis* [3 out of 4 strains] was misidentified as *C. albicans*). Finally, 2 out of 13 *C. guilliermondii* strains were misidentified as *C. famata*. We found that the frequency of these cryptic species was low. The remaining isolates comprised a group of 16 rare species and accounted for around 5% of the isolates.

Antifungal susceptibility testing results. We performed antifungal susceptibility testing on the 781 isolates using EUCAST and CLSI protocols in two different institutions. The antifungal activities of the agents studied are shown in Table 2 and Table 3. We first focused on the susceptibility profile of the most prevalent species (*C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*). As shown in Table 2, all isolates were susceptible to amphotericin B, *C. krusei* being the species showing the lowest susceptibility by both EUCAST and CLSI methods (4- to 5-fold-decreased susceptibility compared to that of *C. albicans*). CLSI tended to provide higher amphotericin B MICs than EUCAST. In the case of the echinocandins, the EUCAST method provided slightly higher MIC values than CLSI for caspofungin (geometric mean [GM] around 2 to 3 times higher), although the different ranges of concentrations of echinocandins studied by CLSI or EUCAST may explain the differences. Concerning fluconazole, MIC values obtained by the two methods were comparable, with the exception of those for *C. glabrata* and *C. tropicalis*. The intrinsic diminished susceptibility to azoles of *C. glabrata* is well known, but this feature was more pronounced when antifungal susceptibility was assessed using the CLSI method (GM for fluconazole, 10 mg/liter; mode, 8 mg/liter) than with the EUCAST method (geometric mean, 3.07 mg/liter; mode, 2 mg/liter). We observed the opposite with *C. tropicalis*, which showed a lower susceptibility to fluconazole with the EUCAST method than with the CLSI method. The antifungal resistance of rare species causing fungemia is shown in Tables S1 and S2 in the supplemental material.

The rate of resistance or the proportion of non-wild-type isolates for each antifungal for the most frequent *Candida* spp. is shown in Tables 4 and 5. Although overall fluconazole resistance was low regardless of the method used, sharp differences were found among the species. Resistance in *C. albicans* or *C. parapsilosis* was low, whereas the rate of fluconazole resistance or the proportion of non-wild-type isolates was higher in the case of *C. glabrata* (6 to 10%) or *C. guilliermondii* (23%). For *C. tropicalis*, we found differences in the rate of fluconazole resistance obtained by the CLSI procedure (1.7%) or the EUCAST method (22%). High rates of resistance in *C. tropicalis* for the newest triazoles, voriconazole (25.4%) and posaconazole (18.6%), when MICs were obtained by the EUCAST method were also observed. In contrast, the rate of resistance for the new triazoles was low when antifungal susceptibility testing was assessed using the CLSI protocol. When we analyzed the overall susceptibility to fluconazole in different geographical regions, the rate of resistance or non-wild-type isolates ranged from 3.7% to 9.2% regardless the method used (Table 6).

Only one *Candida* isolate showed acquired resistance to amphotericin B. As expected, resistance to echinocandins was also very infrequent. Only 1 isolate of *C. albicans*, 2 of *C. tropicalis*, and 1 of *C. glabrata* were resistant to one or more echinocandins. After sequencing the HS regions from the *FKS1* or *FKS2* genes of the isolates, the following previously described point mutations conferring resistance were found: *C. albicans*, F641S mutation in the *FKS1* gene; *C. glabrata*, deletion of the F659 amino acid from *FKS2*; and *C. tropicalis*, F641L and S645F substitutions in *FKS1* of each isolate.

Impact of previous use of antifungal agents on rate of resistance. We observed that the proportion of episodes caused by fluconazole-resistant or non-wild-type isolates was higher among the patients who previously received azoles (14.3% versus 3.5%; $P < 0.01$), an effect that remained when the fluconazole dose-

TABLE 2 Antifungal activities of amphotericin B, flucytosine, caspofungin, micafungin, and anidulafungin against the 781 isolates collected[a]

Species (n^b)	Statistic	Antifungal activity (in mg/liter)								
		Amphotericin B		Flucytosine,	Caspofungin		Micafungin		Anidulafungin	
		EUCAST	CLSI	EUCAST	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI
C. albicans (348)	GM	0.051	0.15	0.16	0.31	0.09	0.03	0.007	0.03	0.005
	Mode	0.03	0.12	0.12	0.25	0.12	0.03	0.007	0.03	0.007
	MIC_{90}	0.12	0.12	0.5	0.5	0.12	0.03	0.03	0.03	0.007
	Range	≤0.03–0.25	0.015–1	≤0.12–32	0.12–2	0.015–0.5	≤0.03–1	0.0017–1	≤0.03–0.25	0.0017–0.25
C. parapsilosis (191)	GM	0.14	0.14	0.49	1.36	0.23	0.84	0.45	0.93	0.89
	Mode	0.12	0.12	0.12	1	0.25	1	0.5	1	1
	MIC_{90}	0.25	0.25	0.25	2	0.5	2	1	2/2	2
	Range	≤0.03–1	0.03–1	≤0.12–0.5	0.5–4	0.003–1	0.25–4	0.0017–1	0.12–4	0.0017–2
C. glabrata (103)	GM	0.106	0.19	0.133	0.44	0.15	0.031	0.016	0.031	0.032
	Mode	0.12	0.25	0.12	0.5	0.12	0.03	0.015	0.03	0.03
	MIC_{90}	0.25	0.5	0.12	1	0.25	0.03	0.03	0.03	0.06
	Range	≤0.03–0.5	0.03–1	≤0.12–≥64	0.25–1	0.007–>2	≤0.03–1	0.007–2	≤0.03–0.5	0.007–1
C. tropicalis (59)	GM	0.079	0.22	0.154	0.41	0.12	0.034	0.021	0.034	0.014
	Mode	0.06	0.25	0.12	0.5	0.12	0.03	0.015	0.03	0.015
	MIC_{90}	0.12	0.5	0.12	0.5	0.25	0.03	0.06	0.03	0.03
	Range	≤0.03–0·5	0.03–1	≤0.12–32	0.12 −2	0.015–0.5	≤0.03–2	0.003–1	≤0.03–1	0.0017–2
C. krusei (15)	GM	0.236	0.5	3.48	0.87	0.24	0.08	0.097	0.034	0.053
	Mode	0.25	0.5	4	1	0.25	0.06	0.12	0.03	0.06
	MIC_{90}	0.5	1	4	1	0.25	0.12	0.25	0.03	0.12
	Range	≤0.12–0.5	0.12–2	2–4	0.5–2	0.12–0.5	0.06–0.12	0.003–0.25	≤0.03–0.06	0.007–0.12
C. guilliermondii (13)	GM	0.067	0.17	0.127	0.9	0.28	0.33	0.21	0.5	1
	Mode	0.12	0.12	0.12	1	0.25	0.25	0.25	0.5	1
	MIC_{90}	0.12	0.5	0.25	2	0.5	0.5	1	1	2
	Range	≤0.03–0.12	0.06–0.5	≤0.12–0.25	0.5–2	0.25–0.5	0.25–0.5	0.003–1	0.25–1	0.5–2
C. lusitaniae (10)	GM	0.06	0.1	0.287	0.92	0.16	0.05	0.039	0.036	0.047
	Mode	0.03	0.06	0.12	1	0.25	0.06	0.06	0.03	0.06
	MIC_{90}	0.25	0.25	>64	1	0.25	0.06	0.06	0.06	0.06
	Range	≤0.03–0.25	0.06–0.5	≤0.12–≥64	0.5–1	0.06–0.25	≤0.03–0.06	0.003–0.06	≤0.03–0.12	0.015–0.06
Other (781)	GM	0.12	0.17	0.66	1.86	0.27	0.52	0.11	0.47	0.14
	Mode	0.03	0.12	0.12	0.25	0.12	0.03	0.062	0.03	0.03
	MIC_{90}	0.5	1	64	>16	>2	>16	2	>16	>2
	Range	≤0.03–2	0.01–>2	≤0.12–≥64	≤0.12–≥16	0.03–>2	≤0.03–≥16	0.0017–2	0.03–≥16	0.0017–>2

[a] "Other" includes the remaining *Candida* and non-*Candida* species. GM, geometric mean.
[b] *n*, no. of isolates.

dependent isolates were also considered resistant (29.3% versus 16.4%; $P < 0.01$). Likewise, the rate of resistance to echinocandins was also higher among the patients who previously received echinocandins (10% versus 1.8%; $P < 0.01$). The four patients infected by the isolates showing *FKS* mutations had received antifungal treatment with echinocandins for more than 21 days in the 5 weeks prior to the isolation of the yeast from the blood culture, and in fact, one of these cases was considered a breakthrough infection during prophylaxis with micafungin.

DISCUSSION

At the best of our knowledge, CANDIPOP is the first population-based study on fungemia conducted in Spain that involved the largest cities of the country. The species distribution and antifungal susceptibility profiles of the isolates collected in five different geographical regions were studied.

We found that *C. albicans* was still the most prevalent species but represented less than 50% of the episodes. Regarding the non-*albicans Candida* isolates, the study confirms that the epidemiology of fungemia in Spain has a characteristic pattern, with a high proportion of cases caused by *C. parapsilosis* and a low proportion of *C. glabrata* cases. This pattern clearly differs from that found in Northern Europe and North America (8, 12) and is similar to findings of single-center studies carried out in Italy (25) or Greece (26) or in population-based studies conducted in Latin America (27, 28). The reasons that may explain the differences are not clear but may involve differences in climate, antifungal policies, or other circumstances that may facilitate the presence of some species in the microbiota of patients living in different geographical areas. Interestingly, our study showed differences in the species distribution in the different participating cities. As an example, Madrid and Barcelona showed similar patterns of species distri-

TABLE 3 Antifungal activities of fluconazole, itraconazole, voriconazole, and posaconazole against the 781 isolates collected[a]

Species (n[b])	Statistic	Antifungal activity (mg/liter)						
		Fluconazole		Itraconazole, EUCAST	Voriconazole		Posaconazole	
		EUCAST	CLSI		EUCAST	CLSI	EUCAST	CLSI
C. albicans (348)	GM	0.21	0.39	0.016	0.016	0.009	0.015	0.006
	Mode	0.25	0.25	0.015	0.015	0.007	0.015	0.007
	MIC_{90}	0·25	1	0·015	0.015	0.03	0·015	0.015
	Range	≤0.12–≥64	0.12–>64	≤0.015–≥8	≤0.015–≥8	0.0017–>2	≤0.015–8	0.0017–>2
C. parapsilosis (191)	GM	0.48	0.64	0.018	0.017	0.008	0.016	0.008
	Mode	0.5	0.5	0.015	0.015	0.007	0.015	0.007
	MIC_{90}	1	2	0.03	0.03	0.015	0.015	0.031
	Range	≤0.12–64	0.06–64	≤0.015–0.25	≤0.015–0.5	0.0017–0.25	≤0.015–0·12	0.0017–>2
C. glabrata (103)	GM	3.074	10	0.127	0.12	0.11	0.124	0.25
	Mode	2	8	0.06	0.12	0.12	0.12	0.25
	MIC_{90}	16	64	1	0.5	0.5	0.5	1
	Range	0.5–≥64	1–>64	≤0.015–≥8	≤0.015–8	0.003–>2	≤0.015–8	0.015–>2
C. tropicalis (59)	GM	1.83	0.71	0.057	0.13	0.026	0.047	0.023
	Mode	0.5	1	0.015	0.03	0.03	0.015	0.03
	MIC_{90}	>64	1	8	>8	0.06	8	0.06
	Range	≤0.12–≥64	0.12–8	≤0.015–≥8	≤0.015–≥8	0.003–0.25	≤0.015–≥8	0.0017–0.12
C. krusei (15)	GM	33.5	38.5	0.066	0.3	0.15	0.048	0.1
	Mode	32	32	0.12	0.5	0.12	0.06	0.12
	MIC_{90}	64	64	0.25	0.5	0.25	0.12	1
	Range	16–64	16–64	≤0.015–0.25	≤0.015–1	0.03–0.25	≤0.015–0.12	0.015–2
C. guilliermondii (13)	GM	3.22	5.51	0.22	0.12	0.061	0.075	0.054
	Mode	4	8	0.12	0.12	0.031	0.06	0.12
	MIC_{90}	16	32	1	0.25	0.5	0.25	0.25
	Range	≤0.12–32	0.5–64	0.06–2	0.06–0.25	0.007–0.5	≤0.015–0.25	0.003–0.25
C. lusitaniae (10)	GM	0.45	1.19	0.02	0.023	0.01	0.018	0.009
	Mode	0.25	0.5	0.015	0.015	0.007	0.015	0.003
	MIC_{90}	32	32	0.12	0.5	0.12	0.06	0.03
	Range	≤0.12–64	0.5–64	≤0.015–0.25	≤0.015–0.5	0.003–0.25	≤0.015–0.06	0.003–0.031
Other (781)	GM	1.92	1	0.066	0.07	0.017	0.061	0.029
	Mode	0.5	1	0.015	0.015	0.007	0.015	0.015
	MIC_{90}	>64	64	2	4	1/2	2	0.5/0.5
	Range	≤0.12–≥64	0.06–>64	≤0.015–4	≤0.015–≥8	0.0017–>2	≤0.015–≥8	0.0017–>2

[a] "Other" includes the remaining *Candida* and non-*Candida* species. GM, geometric mean.
[b] n, no. of isolates.

bution, whereas the other three cities had another pattern (14). It suggests specific local patterns of species distribution that should be considered when empirical antifungal therapy is starting.

The results can be compared only with those of the two previous studies conducted in Spain 10 years ago (7, 9). We found a slightly lower proportion of cases caused by *C. albicans* and a higher proportion of cases caused by *C. glabrata* than in the study by Almirante or the study by Pemán. This may reflect a change in the epidemiology of the infection; however, the different patterns of species distribution among the participating cities may explain the differences. We can consider CANDIPOP to be the first population-based study involving the largest cities of the country, thus becoming a comparator for future studies of fungemia in Spain. Nevertheless, the trend of the frequency of isolation of *C. glabrata* should be closely monitored in the coming years.

The number of cases caused by rare species (defined as those found at a frequency below 1%) was low and represented less than 6% of all isolates collected. However, this heterogeneous group involved a relatively high number of species, such as *Pichia anomala*, *Candida intermedia*, *Kodamea ohmeri*, *Lodderomyces elongisporus*, *Pichia fabianii*, *Metschnikowia pulcherrima*, *Rhodotorula mucilaginosa*, *Dipodascus capitatus*, and *Trichosporon* spp., which have been very rarely described as causing fungemia (4, 5). Although they represented a low proportion of isolates, the guidelines for the management of patients with candidemia usually do not add specific information for the treatment of these infections (29, 30). Therefore, the management of patients infected by rare species is mostly unknown and is based on single case reports. *C. neoformans* fungemia was infrequent, and only 5 patients with cryptococcemia were found. However, this underrepresents the real number of cases of cryptococcosis, since we included only strains isolated from the blood and not other type of clinical sam-

TABLE 4 Rates of resistance to amphotericin B, fluconazole, voriconazole, and posaconazole for isolates of species with available breakpoints proposed by the CLSI M27-S3 document and the EUCAST procedure[a]

Species (n^b)	Category	% of isolates in category for antifungal agent					
		Fluconazole		Voriconazole		Posaconazole	
		CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST
C. albicans (348)	SDD/I	0	0.3	1.2	0	NA	NA
	R	1.4	0.9	0.3	0.86	0.9	0.28
C. parapsilosis complex (200)[c]	SDD/I	3	4	0.5	NA	NA	NA
	R	2.5		0	0.5	0.5	1.5
C. glabrata complex (103)[d]	SDD/I	90.4	94.2	NA	NA	NA	NA
	R	9.6	5.8	10.2	NA	2.9	NA
C. tropicalis (59)	SDD/I	1.7	0	1.7	0	NA	NA
	R	1.7	22	0	25.4	0	18.6
C. krusei[e] (15)	SDD/I	NA	NA	0	NA	NA	NA
	R	100	100	0	NA	6.7	NA
Overall[f]	SDD/I	12.5	15.5	0.64	NA	NA	NA
	R/N-WT	6	6.9	1.66	9.2	1.1	12.67

[a] The overall resistance rate also included non-wild-type isolates. NA, not applicable. SDD, susceptible, dose-dependent category, available only for fluconazole and voriconazole, as proposed in the CLSI M27-S4 document. I, intermediate category, available only for EUCAST and fluconazole (MIC = 4 mg/liter, except for *C. glabrata*, for which MIC > 0.002 to 32 mg/liter). N-WT, non-wild type.

[b] *n*, no. of isolates.

[c] Isolates of *C. metapsilosis* ($n = 7$) and *C. orthopsilosis* ($n = 2$) were considered to be *C. parapsilosis* for analysis of the rate of resistance.

[d] The *C. nivariensis* isolate was considered to be *C. glabrata* for analysis of the rate of resistance.

[e] Although EUCAST or CLSI do not provide fluconazole breakpoints for *C. krusei*, all the strains were considered resistant.

[f] For CLSI, resistance rates were calculated including only the isolates from species with species-specific breakpoints; tentative resistance rates (non-wild-type isolates) were calculated by using the ECVs for the following species-drug combinations: fluconazole (*C. lusitaniae*, *C. guilliiermondii*, *C. dubliniensis*, and *C. kefyr*), voriconazole (*C. glabrata*, *C. lusitaniae*, *C. guilliiermondii*, *C. dubliniensis*, and *C. kefyr*), and posaconazole (*C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii*, *C. dubliniensis*, and *C. kefyr*); for the remaining *Candida* and non-*Candida* isolates, we tentatively used the *C. albicans* breakpoints. For EUCAST, tentative fluconazole breakpoints (S < 0.002; I = 0.004; R > 32, in mg/liter) were used for *C. guilliermondii*; isolates were considered tentatively resistant to voriconazole or posaconazole if showing an MIC > 0.12 mg/liter or > 0.06 mg/ml, respectively; for tentative breakpoints used for non-*Candida* yeasts, see Materials and Methods.

ples, such as cerebrospinal fluid, where *C. neoformans* is more frequently isolated.

In addition to the species distribution, this study allowed us to estimate the rate of antifungal resistance in Spain. Overall, 20% of isolates showed diminished susceptibility to fluconazole (resistant or dose-dependent/intermediate isolates, and non-wild-type isolates). The percentage is higher than the 7% of isolates with reduced susceptibility to fluconazole reported by Almirante et al. (7). Although we cannot exclude that the rate of acquired fluconazole resistance may have risen, other factors, such as the increase in the frequency of isolation *C. glabrata*, the inclusion of non-*Candida* isolates (which were excluded in the Barcelona's study), and the fact the fluconazole breakpoints proposed by the CLSI have been lowered, may contribute to this effect. In the analysis of resistance per species, we observed a low frequency of fluconazole resistance in the most frequent species (<2% in *C. albicans* and <3.5% in *C. parapsilosis*). This is in agreement with recent reports of studies conducted in Spain (9), Latin America (27), North America (12), and Denmark (8), thus emphasizing that the changing epidemiology of fungemia is contributing more to fluconazole resistance than secondary acquired resistance. In fact, different rates of fluconazole resistance were observed among the different participating cities that are in line with the different patterns of species distribution observed.

Both CLSI and EUCAST procedures were used in the study, and the rates of antifungal resistance observed by using each method were similar for most of species. However, we found a higher rate of fluconazole resistance for *C. tropicalis* when the MICs were obtained by EUCAST. In isolates showing resistance by EUCAST and susceptibility by CLSI, a strong trailing effect was observed (data not shown), and growth inhibition was below 50% compared to that for the control well without fluconazole. The molecular basis of this trailing effect is not known, but it did not seem to have a clinical impact, since patients infected by resistant isolates as determined by the EUCAST method had mortality similar to that of patients infected by susceptible isolates (data not shown). Moreover, there was not a correlation between the obtaining of a resistant *C. tropicalis* isolate and the prophylactic treatment (data not shown), indicating that trailing is an *in vitro* effect without clinical impact. In any case, this effect led to a discrepancy between the two methods for *C. tropicalis*.

Resistance to echinocandins was below 2% regardless the method used. Recently, EUCAST has updated the breakpoints to detect resistance to anidulafungin and micafungin. All resistant *Candida* species strains detected by the EUCAST and CLSI methods contained already-known mutations in the *FKS* genes (31–34). In our study, the main antifungal administered both in prophylaxis and as empirical treatment was fluconazole (14), which might explain why the rate of resistance to echinocandins still remains low. However, taking into account the recent ESCMID clinical-guideline recommendation to use an echinocandin for the treatment of most of cases of candidiasis (30), epidemiological

TABLE 5 Rates of resistance to caspofungin, micafungin, and anidulafungin of isolates of species with available breakpoints proposed by the CLSI M27-S3 document and the EUCAST procedure[a]

Species (n^b)	Category	% of isolates in category for antifungal agent			
		Caspofungin, CLSI[f]	Micafungin, CLSI	Anidulafungin	
				CLSI	EUCAST
C. albicans (348)	I	0.3	0.3	0	NA
	R	0	0	0	0.29
C. parapsilosis complex (200)[c]	I	0	0	0	100
	R	0	0	0	0
C. glabrata complex (103)[d]	I	34.6	0	0	NA
	R	1.0	1.0	1.0	0.97
C. tropicalis (59)	I	1.7	0	0	NA
	R	0	3.3	3.3	3.39
C. krusei (15)	I	6.7	0	0	NA
	R	0	0	0	0
C. guilliermondii (13)	I	0	0	0	NA
	R	0	0	0	
Global[e]	I	5.1	0	0	27.1
	R	2.2	1.9	1.8	3.1[e]

[a] The overall resistance rate also included the non-wild-type isolates. I, intermediate category according to the CLSI and EUCAST procedures. NA, not applicable.
[b] *n*, no. of isolates.
[c] Isolates of *C. metapsilosis* ($n = 7$) and *C. orthopsilosis* ($n = 2$) were considered to be *C. parapsilosis* for analysis of the rate of resistance.
[d] The *C. nivariensis* isolate was considered to be *C. glabrata* for analysis of the rate of resistance.
[e] For CLSI, tentative rates of resistance to echinocandins (non-wild-type isolates) were calculated by using the ECVs for *C. lusitaniae*, *C. guilliermondii*, *C. dubliniensis*, and *C. kefyr*; In order to make comparable the rate of resistance obtained both by CLSI and EUCAST, we tentatively used the *C. albicans* breakpoints (R > 4 mg/liter) for classifying the remaining *Candida* and non-*Candida* isolates ($n = 19$) as susceptible or resistant to fluconazole and voriconazole; *Rhodotorula* species, *C. neoformans*, and *Trichosporon* species isolates were considered intrinsically resistant to echinocandins. For EUCAST, the isolates were considered resistant if showing an MIC > 0.06 mg/liter, except for *C. albicans* (R > 0.03 mg/liter) and species from the *C. parapsilosis* complex (R > 4).
[f] Antifungal susceptibility testing results for caspofungin should be interpreted with caution due to the interlaboratory variation reported, possibly due to potency variation (21, 22).

studies to assess echinocandin resistance in the future are warranted. We found a high number of *C. glabrata* isolates intermediate to caspofungin when MICs were obtained by the CLSI method. However, this was not the case for anidulafungin and micafungin. We do not think that these isolates may have a reduced susceptibility to echinocandins, and a potential explanation may be the variability in the potencies of different stocks of pure solutions of caspofungin, as detailed in the CLSI M27-S4 supplement (21, 22, 35). For this reason, neither EUCAST nor CLSI procedures recommend the use of caspofungin for antifungal susceptibility testing.

Our rates of fluconazole and echinocandin resistance are in agreement with recent reported data including antifungal susceptibilities of a large number of isolates collected all over the world in the SENTRY program and collected during the same period in which CANDIPOP was conducted (3).

TABLE 6 Percentages of fluconazole resistance found in the isolates studied[a]

City	% of isolates in category			
	CLSI		EUCAST	
	SDD	R	I	R
Madrid	14.4	5.9	17.4	5.1
Valencia	15	7.5	14.3	9.2
Bilbao	9.5	8.3	10.4	5.8
Seville	10.2	3.7	11.4	8.8
Barcelona	12.1	6.0	12.1	9
Overall	12.5	6.0	14.8	6.8

[a] Overall and per-city percentages are shown. Resistance rates were calculated as described in Materials and Methods. SDD, susceptible, dose dependent.

In conclusion, our study shows that the epidemiology of fungemia in Spain has a characteristic pattern showing a high proportion of cases caused by *C. parapsilosis*. However, an increase in the frequency of *C. glabrata* has been detected compared to results of previous studies conducted in Spain. Resistance to azoles and echinocandins was low, although overall susceptibility to fluconazole has decreased, probably due to the emergence of *C. glabrata*. The decrease in susceptibility to fluconazole in Spanish isolates should be closely monitored in future studies.

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