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Molecular Identification and Susceptibility Testing of Molds Isolated in a Prospective Surveillance of Triazole Resistance in Spain (FILPOP2 Study)

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ABSTRACT Antifungal resistance is increasing by the emergence of intrinsically resistant species and by the development of secondary resistance in susceptible species. A previous study performed in Spain revealed levels of azole resistance in molds of between 10 and 12.7%, but secondary resistance in *Aspergillus fumigatus* was not detected. We used itraconazole (ITZ)-supplemented medium to select resistant strains. A total of 500 plates supplemented with 2 mg/liter of ITZ were sent to 10 Spanish tertiary hospitals, and molecular identification and antifungal susceptibility testing were performed. In addition, the *cyp51A* gene in those *A. fumigatus* strains showing azole resistance was sequenced. A total of 493 isolates were included in the study. Sixteen strains were isolated from patients with an infection classified as proven, 104 were isolated from patients with an infection classified as probable, and 373 were isolated from patients with an infection classified as colonization. *Aspergillus* was the most frequent genus isolated, at 80.3%, followed by *Scedosporium-Lomentospora* (7.9%), *Penicillium-Talaromyces* (4.5%), *Fusarium* (2.6%), and the order *Mucorales* (1%). Antifungal resistance was detected in *Scedosporium-Lomentospora* species, *Fusarium*, *Talaromyces*, and *Mucorales*. Three strains of *A. fumigatus sensu stricto* were resistant to azoles; two of them harbored the TR_{34}+L98H mechanism of resistance, and the other one had no mutations in *cyp51A*. The level of azole resistance in *A. fumigatus* remains low, but cryptic species represent over 10% of the isolates and have a broader but overall higher range of antifungal resistance.

KEYWORDS antifungal resistance, *Aspergillus*, cryptic species, *Scedosporium*, *Fusarium*, *Mucorales*, azole resistance, *cyp51A*, TR_{34}/L98H, aspergillosis

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The incidence of fungal diseases causing fatal infections has risen due to an increase in populations at risk. Mortality rates range from 40% to 90% in high-risk patients, such as those with hematological malignancies (1, 2). Although *Aspergillus fumigatus* is the most common filamentous fungus involved in invasive diseases, emerging molds, such as *Mucorales*, *Scedosporium* spp., *Fusarium* spp., and other species of *Aspergillus*, are being increasingly reported (3, 4). The prevalence and relevance of these emerging fungal pathogens in the clinical setting are presently unknown. However, a prominent feature is that these emerging fungi show decreased susceptibility *in vitro* to most antifungal drugs (5), and disseminated mold infections are often very difficult to treat. A scarce number of epidemiological studies of mold infections involving multiple centers have been reported (6, 7). Epidemiological studies are essential to know the prevalence of fungal pathogens and are key to implementing control measures to decrease infection rates. In addition, they are essential to detect the emergence of resistance and to define rates of resistance in different geographical areas and groups of patients.

Antifungal resistance is increasing both by the emergence of more-resistant/less-susceptible species and by the development of secondary resistance (8). Particularly important are the high rates of azole-resistant *A. fumigatus* reported for clinical samples in the Netherlands and the United Kingdom (9–11). In the Netherlands, azole resistance has an overall prevalence of 5.3%, with a range from 1.8% to 12.8%, depending on the geographical area and hospital studied (11). In the United Kingdom, a clinical collection data set of 519 *A. fumigatus* isolates showed that the frequency of itraconazole (ITZ) resistance *in vitro* was 5%, with a significant increase since 2004 (10). Later, the rise of azole resistance continued in 2008 and 2009, with rates of 14% and 20%, respectively (12). Since then, triazole resistance has been described worldwide (13, 14).

In Spain, the multicenter epidemiological study FILPOP (6) described the epidemiology of mold infections in the country and the rates of resistance. The rates of triazole resistance ranged from 10 to 12.7%, depending on the species and the drug tested; however, secondary resistance in *A. fumigatus* was not detected. Taking into account the high levels of secondary resistance described in neighboring countries, we hypothesize that this resistance could be underestimated due to the sampling method that we used. In this work, we propose a strategy using a selective medium supplemented with an antifungal (ITZ) to detect specifically resistant isolates in respiratory samples and to avoid the overgrowth of azole-susceptible species belonging to the conventional flora. Plates with the selective medium were used to culture clinical samples directly to increase the rate and chance of isolation of azole-resistant strains.

RESULTS

Five hundred six isolates were obtained from 10 Spanish hospitals. One was a dermatophyte isolated from a nail, which was excluded from the study; seven were yeast; and five did not grow in the reference laboratory (RL) and were not further analyzed. Thus, a total of 493 isolates were included in the study. One hundred fifty-five isolates grew in ITZ-supplemented medium, and 338 grew in regular Sabouraud (SAB) medium without supplements. Each isolate was characterized independently since typing techniques were not applied at this stage.

Four hundred seventy-seven (96.8%) strains were isolated from respiratory samples (333 sputum samples, 79 BAS [bronchoaspirate] samples, 38 BAL [bronchoalveolar lavage] fluid samples, 14 tracheal aspirate samples, and 13 other respiratory samples), 10 were isolated from biopsy specimens, 4 were isolated from wound exudates, and 2 were isolated from otic exudates. Sixteen strains were isolated from patients with an infection classified as proven, 104 were isolated from patients with an infection classified as probable, and 373 were isolated from patients with an infection classified as colonization. Underlying diseases included hematological malignancy (7%), solid-organ transplant (9.7%), other cancer (11.6%), HIV (5%), chronic obstructive pulmonary disease (COPD) (16.6%), cystic fibrosis (9.3%), asthma (2.6%), other causes of immunosuppression (4.5%), other respiratory diseases (14.8%), other causes (6.1%), and un-

TABLE 1 Numbers of strains isolated per type of sample and type of infection

Sample type	No. of strains isolated from samples from infection categorized as:			Total no. of strains isolated
	Colonization	Probable	Proven	
Sputum	277	52	4	333
BAS	60	17	2	79
BAL fluid	21	17	0	38
Tracheal aspirate	7	5	2	14
Other respiratory sites[a]	8	5	0	13
Biopsy specimen	0	2	8	10
Wound exudate	0	4	0	4
Otic exudate	0	2	0	2
Total	373	104	16	493

[a]Other respiratory sites include sinuses, lung tissue, and oropharyngeal swabs.

known (12.6%). Table 1 summarizes the number of strains isolated from each type of sample by type of infection.

Identification of strains. *Aspergillus* was the most frequent genus isolated, at 80.3%, followed by *Scedosporium-Lomentospora* (7.9%), *Penicillium-Talaromyces* (4.5%), *Fusarium* (2.6%), and the order *Mucorales* (1%). Table 2 shows the identification to the species level of the strains analyzed; *A. fumigatus* was the most frequently isolated species, with 260 isolates (52.74%), followed by *Aspergillus niger* (5.27%), *Aspergillus flavus* (5.07%), *Aspergillus terreus* (4.67%), and *Scedosporium apiospermum* (4.26%). Cryptic species of *Aspergillus* accounted for 11.5% of the isolates. *A. fumigatus* was the most frequently isolated species in cases of both colonization (186 isolates) and infections (74 isolates); however, while *A. niger* and *A. flavus* ranked second (26 total isolates) and third (25 total isolates) in the total number of isolates, they were found less

TABLE 2 Numbers of strains isolated per species and type of infection

Group	No. of strains isolated from samples from infection categorized as:			Total no. of strains isolated
	Colonization	Probable	Proven	
A. fumigatus	186	68	6	260
A. niger	20	6	0	26
A. flavus	23	2	0	25
A. terreus	15	6	2	23
S. apiospermum	12	5	4	21
A. tubingensis	15	1	0	16
Scedosporium boydii	11	0	1	12
A. alliaceus	5	5	0	10
Fusarium proliferatum	7	2	0	9
A. lentulus	6	0	0	6
A. nidulans	5	1	0	6
A. calidoustus	4	1	0	5
Aspergillus quadrilineatus	5	0	0	5
Penicillium chrysogenum	5	0	0	5
Aspergillus sydowii	2	2	0	4
Paecilomyces lilacinus	3	1	0	4
Penicillium citrinum	4	0	0	4
Lomentospora prolificans	1	1	1	3
S. ellipsoideum	3	0	0	3
Other *Aspergillus* spp.	8	2	0	10
Other *Talaromyces* spp.	9	0	0	9
Mucorales	3	0	2	5
Basidiomycetes	4	1	0	5
Other *Fusarium* spp.	4	0	0	4
Other *Penicillium* spp.	4	0	0	4
Other	9	0	0	9
Total	373	104	16	493

frequently (6 and 2 isolates, respectively) than *S. apiospermum* (9 isolates) and *A. terreus* (8 isolates) in infections classified as probable or proven (Table 2). These differences were not statistically significant.

Susceptibility testing. Three strains did not grow in the medium and under the conditions used for susceptibility testing and were not analyzed. Table 3 shows geometric means (GMs), MIC_{50}s, MIC_{90}s, and MIC ranges for the species that had 10 or more isolates. *Scedosporium* species had elevated MICs for all antifungals, with voriconazole being the most active compound, with an MIC_{50} of 1 mg/liter, followed by echinocandins. Azoles and echinocandins showed no activity against any of the *Fusarium* isolates analyzed; only amphotericin B showed activity (MICs of $\leq$2 mg/liter for 9 out of 13 isolates). *Mucorales* species isolated showed elevated MICs of voriconazole and echinocandins and low MICs of amphotericin B and posaconazole. *Penicillium-Talaromyces* showed low MICs of most antifungals except for *Penicillium citrinum*, with high MICs (16 mg/liter) of voriconazole. Other species with high MICs were *Scopulariopsis brevicaulis* and *Alternaria* spp., with high MICs for all antifungals, and *Purpureocillium lilacinus* (syn., *Paecilomyces lilacinus*), with elevated MICs of amphotericin B, ITZ, and echinocandins. Table 4 shows the numbers of *Aspergillus* isolates with MICs of $>$2 mg/liter for amphotericin B, ITZ, and voriconazole and $>$0.25 mg/liter for posaconazole. There were no significant differences between MIC values for infectious strains and those for strains isolated from cases defined as colonization ($P > 0.01$).

Analysis of the impact of itraconazole-supplemented medium. Table 5 shows the numbers and percentages of strains growing in Sabouraud and ITZ-supplemented media. Sixty-nine percent of the isolates analyzed were isolated in SAB medium, while 31% were isolated in ITZ-supplemented medium (338 versus 155 isolates).

Resistant isolates (MICs of ITZ of $>$2 mg/liter) were preferably isolated in ITZ-supplemented medium (15.6% of the total isolates in ITZ-supplemented medium versus 13.1% of the total isolates in Sabouraud medium), although differences were not statistically significant ($P = 0.5$). *Scedosporium-Lomentospora* isolates were preferentially isolated in ITZ-supplemented medium (11% versus 6.5%), as were *Penicillium-Talaromyces* isolates (5.2 versus 4.1%), although differences were not statistically significant compared with the percentages of *Aspergillus* spp. ($P = 0.08$ and $P = 0.5$).

We compared the results obtained in this study with those obtained in the FILPOP1 study, where we analyzed the epidemiology of mold infections in 29 centers in Spain between 2010 and 2011. Although the results cannot be comparable because of important methodological differences, the percentages of *Scedosporium-Lomentospora* spp., *Penicillium*/*Talaromyces* spp., and *Fusarium* spp. increased in this study compared with the previous one, while the percentages of *Aspergillus* spp. and *Mucorales* decreased (Table 6).

Characterization of resistance mechanisms in *Aspergillus fumigatus*. Three strains of *A. fumigatus sensu stricto* were resistant to azoles: two of them harbored the TR_{34}+L98H mechanism of resistance, and the other one had no mutations in *cyp51A*.

DISCUSSION

The emergence of azole resistance in *A. fumigatus* has been described worldwide, with some European countries showing very high rates (13). These strains have been clinically associated with poorer outcomes (11). Another problem in antifungal resistance is the shift of the epidemiology toward the emergence of intrinsically resistant species, such as *Scedosporium* spp., *Fusarium* spp., or *Mucorales* (4, 15). In addition, in recent years, molecular studies have described new species of fungi that are indistinguishable by classical methods of identification and have been described as cryptic species (16, 17). These cryptic species are more resistant to some of the antifungals available and have been related with higher rates of mortality (18). Cryptic species have been found in clinical samples in percentages higher than those of other considered emerging pathogens, such as *Scedosporium* or *Fusarium* (6, 19).

In a previous study (FILPOP) performed in 30 Spanish hospitals between 2010 and 2011, we found no azole-resistant *A. fumigatus* isolates but a rate of cryptic species of

TABLE 3 Antifungal susceptibility profiles of the species isolated most frequently[a]

Group (no. of isolates) and parameter	MIC (mg/liter)					MEC (mg/liter)		
	AMB	ITZ	VCZ	PCZ	TRB	CPF	MCF	ANF
A. fumigatus (260)								
GM	0.36	0.20	0.47	0.06	2.94	0.35	0.01	0.02
MIC_{50}	0.5	0.12	0.5	0.06	4	0.25	0.015	0.015
MIC_{90}	0.5	0.5	1	0.12	8	1	0.03	0.03
Range	0.015–1	0.03–16	0.12–4	0.015–8	0.25–32	0.004–32	0.003–4	0.007–8
A. niger (26)								
GM	0.25	0.88	0.73	0.14	0.32	0.16	0.01	0.01
MIC_{50}	0.25	0.5	1	0.12	0.25	0.12	0.015	0.007
MIC_{90}	0.5	16	1	0.25	2	1	0.03	0.015
Range	0.12–0.5	0.25–16	0.5–2	0.06–1	0.12–4	0.03–2	0.004–0.06	0.007–0.03
A. flavus (25)								
GM	0.92	0.18	0.74	0.08	0.28	0.66	0.06	0.05
MIC_{50}	1	0.12	0.5	0.12	0.25	0.5	0.06	0.03
MIC_{90}	2	0.5	1	0.25	2	4	0.12	4
Range	0.5–4	0.06–0.5	0.5–2	0.03–0.25	0.03–2	0.12–32	0.015–4	0.007–8
A. terreus (23)								
GM	1.06	0.09	0.70	0.08	0.25	0.65	0.02	0.02
MIC_{50}	1	0.06	0.5	0.06	0.25	0.5	0.015	0.015
MIC_{90}	2	0.5	1	0.5	2	2	0.03	0.03
Range	0.25–4	0.015–16	0.25–8	0.015–16	0.06–2	0.06–32	0.004–4	0.007–8
S. apiospermum (21)								
GM	4.27	2.68	1.64	0.76	12.26	1.69	0.36	0.61
MIC_{50}	4	16	1	0.5	32	1	0.25	2
MIC_{90}	32	16	16	16	32	32	4	8
Range	0.25–32	0.12–16	0.25–16	0.015–16	0.06–32	0.06–32	0.006–4	0.008–8
A. tubingensis (16)								
GM	0.23	0.92	1.14	0.15	0.42	0.22	0.02	0.01
MIC_{50}	0.25	1	1	0.25	0.5	0.25	0.03	0.015
MIC_{90}	0.5	1	2	0.25	2	0.5	0.06	0.03
Range	0.12–1	0.25–16	0.5–2	0.015–0.5	0.06–2	0.06–0.5	0.004–0.12	0.007–0.03
S. boydii (12)								
GM	3.76	5.97	0.75	0.74	28.51	1.77	0.22	0.78
MIC_{50}	4	16	0.5	1	32	2	0.25	2
MIC_{90}	16	16	1	4	32	16	4	8
Range	0.12–32	0.015–16	0.25–16	0.015–16	8–32	0.06–16	0.007–4	0.007–8
A. alliaceus (10)								
GM	22.63	0.10	0.44	0.04	0.35	4.90	0.43	0.49
MIC_{50}	32	0.12	0.5	0.03	1	32	4	8
MIC_{90}	32	0.25	1	0.12	2	32	4	8
Range	8–32	0.03–0.25	0.25–1	0.015–0.12	0.03–2	0.12–32	0.015–4	0.015–8
Fusarium spp. (13)								
GM	2.35	16.00	8.00	10.44	5.22	28.76	4.00	8.00
MIC_{50}	2.00	16.00	8.00	16.00	4.00	32.00	4.00	8.00
MIC_{90}	32.00	16.00	16.00	16.00	32.00	32.00	4.00	8.00
Range	0.5–32	16–16	1–16	1–16	1–32	8–32	4–4	8–8
Mucorales (5)								
GM	0.11	0.33	4.00	0.16	1.49	2.98	1.12	2.28
MIC_{50}	0.12	0.25	4.00	0.12	0.50	32.00	4.00	8.00
MIC_{90}	0.25	1.00	8.00	0.50	32.00	32.00	4.00	8.00
Range	0.03–0.25	0.12–1	2–8	0.06–0.5	0.12–32	0.06–32	0.007–4	0.015–8
All (493)								
GM	0.62	0.35	0.71	0.11	1.87	0.58	0.03	0.04
MIC_{50}	0.5	0.25	0.5	0.06	2	0.5	0.015	0.015
MIC_{90}	4	16	4	1	16	8	4	8
Range	0.015–32	0.015–16	0.015–16	0.015–16	0.03–32	0.012–32	0.0015–4	0.007–8

[a]AMB, amphotericin B; TRB, terbinafine; VCZ, voriconazole; PCZ, posaconazole; CPF, caspofungin; MCF, micafungin; ANF, anidulafungin.

TABLE 4 Numbers of *Aspergillus* species strains resistant *in vitro* to amphotericin B, itraconazole, voriconazole, and posaconazole according to EUCAST breakpoints

Group	Total no. of isolates	No. of isolates resistant to:			
		AMB at >2 mg/liter	ITZ at >2 mg/liter	VCZ at >2 mg/liter	PCZ at >0.25 mg/liter
A. fumigatus	260	2	3	2	3
A. lentulus	6	3		4	
A. fumigatiaffinis	2	2			
Aspergillus felis	1			1	
A. niger	26		4		2
A. tubingensis	16		1		1
A. flavus	25	1			
A. alliaceus	10	10			
Aspergillus tamarii	1				
A. terreus	23	2	1	1	3
A. citrinoterreus	2				
A. nidulans	6				
A. quadrilineatus	5	1	1	1	1
Aspergillus delacroxii	1	1	1		
Aspergillus spinulosporus	1				
A. calidoustus	5	1	5	5	4
A. puniceus	1	1	1	1	1
A. sydowii	4				
Aspergillus chevalieri	1				
Total	396	24	17	15	15

15% (6). In this work, we have used ITZ-supplemented plates to screen for azole resistance. Thus, 3 out of 260 (1.2%) *A. fumigatus* isolates analyzed were resistant to azoles; 2 of these strains harbor the most frequent mechanism of azole resistance (TR_{34}+L98H), while the other strain showed no mutations in *cyp51A*. The TR_{34}+L98H mechanism of resistance has been linked to the use of azoles in agriculture and is the most frequent mechanism of azole resistance worldwide (20). Other mechanisms of resistance related to mutations in *cyp51A*, such as TR_{46}/Y121F/T289A and G448S, have been described in isolates from Spain (21, 22) but were not found in this work. One out of the three azole-resistant *A. fumigatus* isolates had no mutations in *cyp51A*. Azole-resistant isolates with no mutations in *cyp51A* were described previously (23, 24). Other mechanisms of azole resistance could be present and will be further analyzed in this isolate.

The main sources of resistance in our isolates were cryptic species of *Aspergillus* and emerging molds such as *Fusarium*, *Scedosporium*, and *Mucorales*. Breakpoints for these species have not been defined; however, infections of patients with these pathogens are associated with poorer outcomes (25).

Cryptic species of *Aspergillus* accounted for 11.5% of the total number of isolates. Among members of the *A. fumigatus* complex, *A. lentulus* and *A. fumigatiaffinis* showed high MICs of amphotericin B and voriconazole, as reported previously (26, 27). Within the *A. niger* complex, *A. niger* and *A. tubingensis* were isolated in this study. In accordance with

TABLE 5 Numbers and percentages of strains isolated in each medium

Group	No. (%) of strains isolated in:	
	SAB medium	ITZ-supplemented medium
Aspergillus spp.	277 (82.0)	119 (76.8)
Fusarium spp.	9 (2.7)	4 (2.6)
Scedosporium-Lomentospora spp.	22 (6.5)	17 (11.0)
Penicillium-Talaromyces spp.	14 (4.1)	8 (5.2)
Other genera	16 (4.7)	7 (4.5)
All	338 (100)	155 (100)
Resistant[a]	44 (13.1)	24 (15.6)
Susceptible	292 (86.9)	130 (84.4)

[a]Strains were classified as susceptible or resistant according to *A. fumigatus* breakpoints for itraconazole. Three isolates were not included in this group since they did not grow for antifungal susceptibility testing.

TABLE 6 Numbers and percentages of strains isolated in the FILPOP1 and FILPOP2 studies by group of species

Group	No. (%) of isolates	
	FILPOP1[a]	FILPOP2[b]
Aspergillus spp.	278 (86.3)	396 (80.32)
Scedosporium-Lomentospora spp.	15 (4.7)	39 (7.91)
Mucorales	12 (3.7)	5 (1.01)
Penicillium-Talaromyces spp.	7 (2.2)	22 (4.46)
Fusarium spp.	4 (1.2)	13 (2.64)
Others	6 (1.9)	18 (3.65)
Total	322 (100)	493 (100)

[a]See reference 6.
[b]This study.

the results obtained here, previous works reported that susceptibility within this group is variable and strain dependent (28, 29). *Aspergillus terreus* and *A. flavus* complex isolates have been associated with higher MICs of amphotericin B. In this work, the ranges of MICs of amphotericin B were 0.25 to 2 mg/liter for *A. flavus* and 0.25 to 4 for *A. terreus*. Two out of 23 isolates of *A. terreus* showed MICs of 4 mg/liter, while all isolates of *Aspergillus alliaceus* (*A. flavus* complex) showed MICs of >4 mg/liter for amphotericin B, in accordance with previous results (6, 27, 30). *Aspergillus citrinoterreus* (*A. terreus* complex) isolates have been reported to be more susceptible to itraconazole, voriconazole, and posaconazole than *A. terreus sensu stricto* isolates, both of which have high amphotericin B MICs (31). In this study, two isolates of *A. citrinoterreus* were found, with no differences among susceptibilities of *A. terreus* isolates. *Aspergillus ustus* complex isolates have been associated with high MICs of all antifungals (6, 17, 27, 32). Among the species of this complex, we isolated five *Aspergillus calidoustus* isolates and one *Aspergillus puniceus* isolate, with all of them being resistant to azoles and echinocandins and with amphotericin B being the only compound with some activity.

Species of *Scedosporium-Lomentospora* represented almost 8% of our total number of strains. *Scedosporium* species had elevated MICs of amphotericin B and ITZ, with the most active antifungals being voriconazole and echinocandins. *Lomentospora prolificans* (syn., *Scedosporium prolificans*) is panresistant, with no antifungal showing an *in vitro* effect. These results are in accordance with those of previous studies (33, 34).

Fusarium species accounted for 2.6% of the total number of isolates and showed elevated MICs of all antifungals. The echinocandins and azoles had no activity; the only antifungal compound with low MICs against some strains was amphotericin B. Other authors have reported different patterns according to the species complexes; thus, *Fusarium solani* isolates are usually resistant to azoles and show higher MICs of amphotericin B than other species, whereas *Fusarium oxysporum* and *Fusarium verticillioides* can be susceptible to voriconazole and posaconazole (35). In our study, most isolates (9 out of 13) were identified as *Fusarium proliferatum* (*Fusarium fujikuroi* complex); a study analyzing 81 strains of the *Fusarium fujikuroi* complex found that amphotericin B was the most active drug, followed by voriconazole, posaconazole, isavuconazole, and natamycin, while fluconazole, itraconazole, and micafungin showed poor activity (36). Our isolates showed high MICs (>2 mg/liter) of all antifungals but amphotericin B, with only one strain showing an MIC of >2 mg/liter, in accordance with results reported previously (37).

Unexpectedly, in this study, we could not find statistical differences in the detection of resistant isolates when using ITZ-supplemented plates. Only *Scedosporium-Lomentospora* isolates showed high percentages of isolation in ITZ-supplemented medium compared with the other species. However, when we compare the results obtained in the FILPOP1 study (6) with those obtained in this one (Table 6), we see that the percentages of *Scedosporium-Lomentospora* species, *Penicillium-Talaromyces* species, and *Fusarium* species isolates increased, while the percentage of *Aspergillus*

species and *Mucorales* isolates decreased. Although the results are not comparable because of important methodological differences (the numbers of participating centers and the numbers of strains analyzed, etc.), this could indicate that selective medium is favoring the isolation of rare species by decreasing the rate of recovery of fast-growing species such as *Aspergillus* spp. and *Mucorales*. This is in agreement with previous works where selective media have been recommended for the isolation of *Scedosporium* species (38–40).

In conclusion, this study shows that antifungal resistance is present in Spain. The level of azole resistance in *A. fumigatus* remains low, but cryptic species represent over 10% of the isolates and have different patterns of antifungal resistance. Apart from *Aspergillus*, other emerging molds, such as *Scedosporium-Lomentospora*, *Fusarium*, and *Mucorales*, showed high MICs of several antifungals. Taking into account these results and the impact of appropriate antifungal treatment on survival, we recommend screening for antifungal resistance and performing antifungal susceptibility testing for all isolates coming from sterile sites in order to determine the best treatment option for patients infected with these pathogens.

MATERIALS AND METHODS

Strains and isolates. A total of 500 Sabouraud (SAB; Oxoid SA, Madrid, Spain) plates supplemented with 2 mg/liter of ITZ (Sigma-Aldrich Química, Madrid, Spain) were sent to 10 Spanish tertiary hospitals from different regions in Spain: Gregorio Marañón (1,525 beds; Madrid, Madrid), La Paz (1,524 beds; Madrid, Madrid), Virgen de Valme (605 beds; Seville, Andalusia), Reina Sofía (1,233 beds; Córdoba, Andalusia), La Fe (1,050 beds; Valencia, Valencia), Donostia (1,054 beds; Guipúzcoa, Basque Country), Vall d'Hebron (1,251 beds; Barcelona, Catalonia), Bellvitge (1,022 beds; Barcelona, Catalonia), Central de Asturias (989 beds; Oviedo, Asturias), and Miguel Servet (1,234 beds; Zaragoza, Aragon). The number of beds in each hospital was determined according to a report from 2017 with current numbers at the end of 2016 (41). Samples from respiratory secretions, biopsy specimens, and other sterile sites were included in the study. The samples were cultured in classical media and in 2 mg/liter ITZ-supplemented medium.

All samples positive for filamentous fungi were sent to the Mycology Reference Laboratory (RL) of the Spanish National Center of Microbiology for identification and antifungal susceptibility testing.

Clinical data. Basic clinical data, such as source of isolation, underlying disease, antifungal treatment, and outcome of the patient, were gathered when possible. Study approval was obtained from the research ethics committee of the Instituto de Salud Carlos III, with reference number CEI PI56_2014.

The cases of invasive fungal diseases were classified as proven and probable infections according to European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria (42); we included colonization as a third category in cases where infection could not be confirmed but a clinically relevant isolate was detected. Cases that could not be classified according to these criteria were defined as colonization.

Morphological identification. At the RL, the strains were subcultured in different media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (MEA) (2% malt extract; Oxoid SA, Madrid, Spain), potato dextrose agar (PDA; Oxoid SA), oatmeal agar (OMA; Oxoid SA), potassium chloride agar (Oxoid SA), and Czapek-Dox agar (Difco, Soria Melguizo SA, Madrid, Spain). Cultures were incubated at 30°C and 37°C. Fungal morphological features were examined macro- and microscopically by conventional methods (43).

Molecular identification. Molds were subcultured in glucose-yeast extract-peptone (GYEP) medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich Química, Madrid, Spain) for 24 to 48 h at 30°C. Genomic DNA was isolated by using an extraction procedure described previously (44). Molecular identification was performed by sequencing informative targets. DNA segments comprising the internal transcribed spacer 1 (ITS1) and ITS2 regions were amplified for all the strains with primer set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (45). In the case of *Aspergillus* and *Scedosporium* isolates, a portion of the beta tubulin gene was sequenced with the following primers: βtub3 (5′-TTCACCTTCAGACCGGT-3′) and βtub2 (5′-AGTTGTCG GGACGGAATAG-3′) (16) for *Aspergillus* and TUB-F (5′-CTGTCCAACCCCTCTTACGGCGACCTGAAC-3′) and TUB-R (5′-ACCCTCACCAGTATACCAATGCAAGAAAGC-3′) (46) for *Scedosporium*. Also, DNA segments comprising the elongation factor alpha region were amplified for *Fusarium* isolates with primers EF1 (5′-ATGGGTAAGARGACAAGAC-3′) and EF2 (5′-GGARGTACCAGTSATCATGTT-3′) (47). All primers were synthesized by Sigma Genosys (Madrid, Spain). The reactions were performed in a GeneAmp 9700 PCR system (Applied Biosystems) under conditions described previously (6). Sequencing reactions were done with 2 μl of a sequencing kit reagent (BigDye Terminator cycle sequencing, ready reaction; Applied Biosystems), 1 μM primers (the same as for PCR, except that *Aspergillus* β-tubulin primers βtub1 [5′-AATTGGTGCCGCTTTCTGG-3′] and βtub4 [5′-AGCGTCCATGGTACCAGG-3′] were used), and 3 μl of the PCR product in a final volume of 10 μl.

Sequences were assembled and edited by using the SeqMan II and EditSeq software packages (Lasergene; DNAStar, Inc., Madison, WI, USA). All sequences were compared with reference sequences from the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and MycoBank (http://www.MycoBank.org/) databases with InfoQuest FP software, version 4.50 (Bio-Rad Laboratories, Madrid, Spain), as well as

with the database belonging to the Department of Mycology of the Spanish National Centre for Microbiology, which holds 13,000 sequences from strains belonging to 290 different fungal species. This database was designed by the Spanish National Centre for Microbiology and has restricted access (26, 34, 37, 48).

Antifungal susceptibility testing. Microdilution testing was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard methodology (49). *A. fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains. The antifungal agents used in the study were amphotericin B (Sigma-Aldrich Química, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer SA, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma, Inc., Tokyo, Japan), and anidulafungin (Pfizer SA, Madrid, Spain). The final concentrations tested ranged from 0.03 to 16 mg/liter for amphotericin B, terbinafine, and caspofungin; from 0.015 to 8 mg/liter for itraconazole, voriconazole, and posaconazole; from 0.007 to 4 mg/liter for anidulafungin; and from 0.004 to 2 mg/liter for micafungin. The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed at 24 and 48 h with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, posaconazole, and terbinafine was the antifungal concentration that produced a complete inhibition of visual growth at 24 and 48 h (MIC). For the echinocandins, the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae compared with the growth control well (minimum effective concentration [MEC]). EUCAST has set breakpoints to interpret antifungal susceptibility testing results for amphotericin B (resistant strain MIC value of >2 mg/liter), itraconazole (MIC > 2 mg/liter), voriconazole (MIC > 2 mg/liter), and posaconazole (MIC > 0.25 mg/liter) (50). These breakpoint values have been set for only some *Aspergillus* spp. but were used in this study to analyze rates of resistance *in vitro* for all *Aspergillus* species. Breakpoints of echinocandins and terbinafine have not been set yet, and rates of resistance were not calculated.

Analysis of the impact of itraconazole-supplemented medium. We investigated the impact of the supplemented medium by calculating the percentage of isolates growing in each medium overall and the most frequently isolated genera. In addition, isolates were classified as susceptible or resistant to ITZ by using the existing breakpoint for *A. fumigatus*; thus, isolates with an MIC of >2 mg/liter were classified as resistant, and the rest were classified as susceptible. One *Aspergillus nidulans* isolate showed an MIC of 2 mg/liter; although it should be classified as intermediate according to EUCAST breakpoints, we include it in susceptible group for practical purposes.

Characterization of resistance mechanisms in *Aspergillus fumigatus*. *A. fumigatus* isolates showing MICs over the breakpoint for resistance (>2 mg/liter for ITZ and voriconazole and >0.25 mg/liter for posaconazole) were studied for mutations in the *cyp51A* gene. The *cyp51A* gene, including its promoter region, was amplified and sequenced according to procedures described previously (51) for the detection of specific mutations associated with azole resistance.

Statistical analysis. Descriptive and comparative analyses were done. Differences in the proportions of fungal species were determined by Fisher's exact test or by chi-square analysis. The significance of the differences between MICs was determined by analysis of variance (with Bonferroni's *post hoc* test) or by nonparametric tests. A P value of <0.01 was considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics 19.0 (SPSS Iberica, Madrid, Spain).

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