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SUSCEPTIBILITY

Molecular Identification, Antifungal Susceptibility Testing, and Mechanisms of Azole Resistance in *Aspergillus* Species Received within a Surveillance Program on Antifungal Resistance in Spain

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ABSTRACT Antifungal resistance is one of the major causes of the increasing mortality rates for fungal infections, especially for those caused by *Aspergillus* spp. A surveillance program was established in 2014 in the Spanish National Center for Microbiology for tracking resistance in the most prevalent *Aspergillus* species. A total of 273 samples were included in the study and were initially classified as susceptible or resistant according to EUCAST breakpoints. Several *Aspergillus* cryptic species were found within the molecularly identified isolates. Cyp51 mutations were characterized for *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus sensu stricto* strains that were classified as resistant. Three *A. fumigatus sensu stricto* strains carried the TR_{34}/L98H resistance mechanism, while two harbored G54R substitution and one harbored the TR_{46}/Y121F/T289A mechanism. Seventeen strains had no mutations in *cyp51A*, with ten of them resistant only to isavuconazole. Three *A. terreus sensu stricto* strains harbored D344N substitution in *cyp51A*, one of them combined with M217I, and another carried an A249G novel mutation. Itraconazole-resistant *A. flavus sensu stricto* strains harbored P220L and H349R alterations in *cyp51A* and *cyp51C*, respectively, that need further investigation on their implication in azole resistance.

KEYWORDS *Aspergillus*, Cyp51, EUCAST, antifungal resistance, azoles, isavuconazole, itraconazole, posaconazole, surveillance program, voriconazole

Invasive fungal infections (IFIs) have suffered a significant increase over the last decades, due mainly to the rise in patients subjected to strong immunosuppression (1, 2). These infections present high associated mortality and morbidity rates (1, 3, 4), and a broad range of molds have been reported to cause them (such as *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., or Mucorales), with a shift toward new species that are emerging.

One of the reasons for the increased mortality caused by IFIs is the development of antifungal resistance. New emerging species present decreased susceptibility to most antifungal drugs, while secondary resistance is increasingly reported in molds, especially in *Aspergillus* spp. (5). Azoles, which are first-line antifungal therapy for *Aspergillus*, generally have a good activity against these species, although an increase in *Aspergillus fumigatus* clinical isolates displaying MIC values above the clinical breakpoints for azole drugs established by CLSI and EUCAST has been described in recent years in several countries (6–8), and reports on single cases of *A. fumigatus* strains showing triazole resistance have been made throughout the world (9). Most *A. fumigatus* triazole resistance cases are explained by point mutations in the *cyp51A* gene, which encodes the 14α-sterol-demethylase of the ergosterol biosynthesis pathway (10). Point muta-

Citation Rivero-Menendez O, Soto-Debran JC, Medina N, Lucio J, Mellado E, Alastruey-Izquierdo A. 2019. Molecular identification, antifungal susceptibility testing, and mechanisms of azole resistance in *Aspergillus* species received within a surveillance program on antifungal resistance in Spain. Antimicrob Agents Chemother 63:e00865-19. https://doi.org/10.1128/AAC.00865-19.

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Received 26 April 2019
Returned for modification 13 June 2019
Accepted 30 June 2019
Accepted manuscript posted online 8 July 2019
Published 23 August 2019

tions associated with azole resistance have also been reported in *cyp51A* in *Aspergillus terreus* and in *cyp51C* in *Aspergillus flavus* (11–15). Resistance in *Aspergillus* has also been described in cryptic or sibling species, which can only be identified to the species level using molecular tools and are classified in "complex" of closely related species when convenient identification techniques are not performed (16). These species usually present decreased susceptibility to several antifungal drugs (17, 18).

The first multicenter epidemiological study carried out in Spain in molds (FILPOP) reported triazole resistance rates ranging from 10% to 12.7%, based on different species and antifungals tested, but no secondary resistance in *A. fumigatus* was found (19). As a consequence, a surveillance program on antifungal resistance was implemented in 2014 by the Mycology Reference Laboratory of the Spanish National Center for Microbiology in order to monitor antifungal resistance in the most frequently found *Aspergillus* species in our country. In this study, we aim to describe the antifungal susceptibility and mechanisms of azole resistance in the isolates included in the program through the end of 2018.

RESULTS

Strains in this study. In total, 306 samples were received from 45 hospitals from the implementation of the surveillance program on antifungal resistance in July 2014 through the end of 2018. Three samples were identified as *Candida albicans* and were transferred to the surveillance program on antifungal resistance for yeasts for analysis; 27 samples were identified as molds that did not belong to the genus *Aspergillus* and, therefore, were not further analyzed; and three samples were contaminated and were excluded from the study; thus, 273 samples from 33 hospitals were analyzed.

Morphological identification. Macroscopic and microscopic studies allowed the identification of *Aspergillus* strains to the complex level. Among them, 57.9% (158/273) of the strains were identified as part of the *A. fumigatus* complex, 21.6% (59/273) belonged to the *Aspergillus terreus* complex, 12.8% (35/273) were part of the *Aspergillus flavus* complex, and 5.5% (15/273) and 1.5% (4/273) belonged to the *Aspergillus nidulans* complex and the *Aspergillus niger* complex, respectively. Two *Aspergillus* strains were classified as *Aspergillus* species.

Antifungal susceptibility testing. Antifungal susceptibility testing results are summarized in Table 1, showing geometric means (GMs), MIC/minimal effective concentration (MEC) causing inhibition of 50% of the isolates tested (MIC_{50}/MEC_{50}), MIC/MEC causing inhibition of 90% of the isolates tested (MIC_{90}/MEC_{90}), and MIC/MEC ranges of all antifungals tested against strains belonging to an *Aspergillus* complex with more than 10 isolates. *Aspergillus* species showed a wide range of MIC values for all antifungals tested. Azoles were active against *A. fumigatus* complex, *A. terreus* complex, *A. flavus* complex, and *A. nidulans* complex strains, showing lower MIC values for itraconazole and posaconazole than for voriconazole and isavuconazole. Terbinafine showed a discrete activity against all strains tested. Amphotericin B was more active against *A. fumigatus* complex than against *A. terreus* and *A. flavus* complexes and showed moderate activity against *A. nidulans* complex. Echinocandins displayed good activity against all strains.

The number of *Aspergillus* strains analyzed that had MIC values above the clinical breakpoints established by EUCAST is included in Table 2.

Molecular identification. Among the *A. fumigatus* complex isolates, 46 strains with resistance to at least one antifungal were molecularly identified in order to identify cryptic species. The most prevalent species identified was *A. fumigatus sensu stricto* (23 strains), followed by several cryptic species of the complex, as follows: *Aspergillus lentulus* (13 strains), *Aspergillus udagawae* (four strains), *Aspergillus fumigatiaffinis* (three strains), and *Aspergillus felis* and *Aspergillus parafelis* (one strain each). One strain that was resistant to isavuconazole was only identified to the complex level because of absence of amplification of target genes. Thirteen *A. terreus* complex strains resistant to itraconazole, posaconazole, and/or isavuconazole were molecularly identified; 12 were *A. terreus sensu stricto*, and one belonged to the cryptic species *Aspergillus citrinoterreus*.

TABLE 1 GM and MIC/MEC values and ranges for tested antifungals[a] and *Aspergillus* complex strains ($n \geq 10$), as determined by the EUCAST broth microdilution method

Aspergillus complex (no. of strains tested)	MIC parameter	Value (mg/liter) for:								
		AMB	ITC	VRC	POS	ISA	TRB	CAS	MFG	AFG
A. fumigatus complex (158)	GM	0.633	0.663	0.726	0.139	1.124	3.431	0.312	0.016	0.024
	MIC_{50}/MEC_{50}	0.5	0.5	0.5	0.12	1	4	0.25	0.015	0.03
	MIC_{90}/MEC_{90}	8	4	4	0.5	4	8	0.5	0.03	0.06
	Range	0.12–32	0.015–16	0.06–16	0.015–4	0.25–16	0.25–32	0.03–4	0.004–4	0.007–8
A. terreus complex (59)	GM	1.638	0.282	1.036	0.098	1.084	0.315	0.358	0.017	0.025
	MIC_{50}/MEC_{50}	2	0.25	1	0.12	1	0.25	0.5	0.015	0.03
	MIC_{90}/MEC_{90}	8	0.5	2	0.25	2	0.5	1	0.06	0.06
	Range	0.25–16	0.03–16	0.25–16	0.015–1	0.25–8	0.06–1	0.03–2	0.004–4	0.007–8
A. flavus complex (35)	GM	1.400	0.541	0.837	0.193	1.224	0.157	0.517	0.068	0.065
	MIC_{50}/MEC_{50}	1	0.5	1	0.25	1	0.12	0.25	0.03	0.03
	MIC_{90}/MEC_{90}	4	1	1	0.5	2	2	32	4	8
	Range	0.5–32	0.12–16	0.25–16	0.03–2	0.5–16	0.03–4	0.06–32	0.015–4	0.007–8
A. nidulans complex (15)	GM	2.639	0.522	0.396	0.154	0.500	0.412	0.497	0.043	0.057
	MIC_{50}/MEC_{50}	2	0.5	0.25	0.12	0.25	0.25	0.25	0.06	0.06
	MIC_{90}/MEC_{90}	16	1	1	0.5	1	2	4	0.5	0.5
	Range	0.5–16	0.12–4	0.12–1	0.03–1	0.25–8	0.06–2	0.03–8	0.004–2	0.007–4
All (273)	GM	0.916	0.534	0.773	0.138	1.082	1.139	0.349	0.021	0.029
	MIC_{50}/MEC_{50}	0.5	0.5	0.5	0.12	1	2	0.25	0.015	0.03
	MIC_{90}/MEC_{90}	8	2	2	0.5	4	8	1	0.06	0.06
	Range	0.06–32	0.015–16	0.06–16	0.015–4	0.25–16	0.03–32	0.015–32	0.004–4	0.007–8

[a]MIC values for amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), posaconazole (POS), isavuconazole (ISA), and terbinafine (TRB), and MEC values for caspofungin (CAS), micafungin (MFG), and anidulafungin (AFG).

Only two strains from the *A. flavus* complex were resistant to itraconazole, and these were identified as *A. flavus sensu stricto*. Out of six *A. nidulans* complex strains showing resistance to itraconazole and/or isavuconazole, four were *A. nidulans sensu stricto*, and four belonged to the cryptic species *Aspergillus spinulosporus*.

Characterization of molecular mechanisms of azole resistance in *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus sensu stricto*. Further characterization was performed of *A. fumigatus*, *A. flavus*, and *A. terreus sensu stricto* strains that were resistant to at least one azole drug according to EUCAST breakpoints. Table 3 shows *cyp51A* mutations found in azole-resistant *A. fumigatus* and *A. terreus* strains and *cyp51A*, *cyp51B*, and *cyp51C* mutations found in azole-resistant *A. flavus* strains. Twenty-three *A. fumigatus* strains were azole resistant. Three of them harbored the TR_{34}/L98H mutation and were panazole resistant, while two strains carried a G54R substitution showing resistance to itraconazole and posaconazole, and another one harbored the

TABLE 2 Number of *Aspergillus* strains with MIC values above the established EUCAST breakpoint to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole

Aspergillus complex or species	No. (%)[a] of strains with MIC values above their established EUCAST breakpoint				
	AMB	ITC	VRC	POS	ISA[b]
A. fumigatus complex (158)	19 (12%)	17 (10.8%)	17 (10.8%)	21 (13.3%)	28 (27.7%)
A. fumigatus sensu stricto	0 (0%)	11 (64.7%)	7 (41.2%)	13 (61.9%)	17 (60.7%)
A. terreus complex (59)	NA[c]	2 (3.4%)	NA	3 (5.1%)	12 (44.4%)
A. terreus sensu stricto		2 (100%)		2 (66.7%)	11 (91.7%)
A. flavus complex (35)	NA	2 (5.7%)	NA	NA	NA
A. flavus sensu stricto		2 (100%)			
A. nidulans complex (15)	NA	1 (6.7%)	NA	NA	6 (46.2%)
A. nidulans sensu stricto		1 (100%)			4 (66.7%)

[a]Percentages for *sensu stricto* isolates correspond to % of resistant *sensu stricto* isolates among the total resistant isolates of the complex.

[b]Percentages for isavuconazole were calculated according to the number of strains where isavuconazole was tested (101 *for A. fumigatus*, 27 for *A. terreus*, and 13 for *A. nidulans*).

[c]NA, not applicable due to the lack of EUCAST breakpoint.

TABLE 3 Characterization of molecular mechanisms of azole resistance by studying *cyp51* gene alterations from azole-resistant *A. fumigatus*, *A. terreus*, and *A. flavus sensu stricto* isolates

Species	Strain[a]	MIC (mg/liter)[b]				Mutation(s) found (gene)
		ITC	VRC	POS	ISA	
A. fumigatus	CNM-CM7582	**16**	**4**	**0.5**	ND	TR_{34}/L98H (*cyp51A*)
	CNM-CM7609	**16**	**16**	**1**	ND	TR_{34}/L98H (*cyp51A*)
	CNM-CM9399	**16**	**4**	**0.5**	**8**	TR_{34}/L98H (*cyp51A*)
	CNM-CM9114	**16**	0.5	**2**	0.5	G54R (*cyp51A*)
	CNM-CM9501	**16**	0.5	**2**	0.5	G54R (*cyp51A*)
	CNM-CM8057	**16**	**16**	**1**	**16**	TR_{46}/Y121F/T289A (*cyp51A*)
	CNM-CM7510	**16**	2	**0.5**	ND	None (*cyp51A*)
	CNM-CM7552	**16**	1	**0.5**	ND	None (*cyp51A*)
	CNM-CM8822	**4**	**8**	**0.5**	**4**	None (*cyp51A*)
	CNM-CM8900	1	**4**	**0.5**	**2**	None (*cyp51A*)
	CNM-CM8914	0.5	0.25	0.12	**2**	None (*cyp51A*)
	CNM-CM8915	0.5	0.5	0.12	**2**	None (*cyp51A*)
	CNM-CM8916	0.5	0.25	0.12	**2**	None (*cyp51A*)
	CNM-CM8917	0.5	0.5	0.06	**2**	None (*cyp51A*)
	CNM-CM8922	1	0.5	0.06	**2**	None (*cyp51A*)
	CNM-CM8925	1	0.5	0.25	**2**	None (*cyp51A*)
	CNM-CM9120	1	0.25	0.25	**2**	None (*cyp51A*)
	CNM-CM9307	0.5	1	0.12	**2**	None (*cyp51A*)
	CNM-CM9327	1	2	**0.5**	**2**	None (*cyp51A*)
	CNM-CM9361	1	2	0.25	**2**	None (*cyp51A*)
	CNM-CM9471	**16**	**8**	**0.5**	**8**	None (*cyp51A*)
	CNM-CM9491	1	0.5	0.12	**2**	None (*cyp51A*)
	CNM-CM9494	**8**	2	**0.5**	**4**	None (*cyp51A*)
	Range	**0.5–16**	0.25–16	0.12–2	0.5–16	
A. flavus	CNM-CM7668	**16**	0.25	1	ND	P220L (*cyp51A*)
	CNM-CM9326	**16**	16	2	16	H349R (*cyp51C*)
	Range	16–16	0.25–16	1–2	**16–16**	
A. terreus	CNM-CM9079	0.25	2	0.06	**2**	D344N (*cyp51A*)
	CNM-CM9280	0.5	2	0.12	**2**	D344N (*cyp51A*)
	CNM-CM9490	**4**	8	**0.5**	**8**	D344N (*cyp51A*)
	CNM-CM7846	**16**	16	**1**	ND	M217I; D344N (*cyp51A*)
	CNM-CM9284	0.5	1	0.12	**2**	A249G (*cyp51A*)
	CNM-CM8056	0.5	2	0.06	**2**	None (*cyp51A*)
	CNM-CM8671	0.5	2	0.25	**2**	None (*cyp51A*)
	CNM-CM8852	2	2	0.25	**2**	None (*cyp51A*)
	CNM-CM8952	0.5	8	0.25	**8**	None (*cyp51A*)
	CNM-CM8981	0.25	1	0.06	**2**	None (*cyp51A*)
	CNM-CM9108	0.25	2	0.06	**2**	None (*cyp51A*)
	CNM-CM9285	0.25	1	0.12	**2**	None (*cyp51A*)
	Range	0.25–16	1–16	0.06–1	**2–8**	

[a]CNM-CM, Mold Collection of the Spanish National Center for Microbiology.
[b]Boldface numbers indicate MIC values that are above the EUCAST clinical breakpoints.

TR_{46}/Y121F/T289A mechanism and was resistant to all azoles. Seventeen out of 23 azole-resistant *A. fumigatus* strains did not have mutations in the *cyp51A* gene; ten of these were only resistant to isavuconazole. The seven remaining strains were resistant to at least two azoles.

Twelve *A. terreus* strains were azole resistant according to EUCAST breakpoints, of which two were resistant to itraconazole, posaconazole, and isavuconazole and had high MICs to voriconazole. Both had a D344N substitution in c*yp51A*, in one case combined with another mutation, M217I, in the same gene. The remaining ten strains were only resistant to isavuconazole (MIC = 2 mg/liter), and while seven carried wild-type *cyp51A*, two carried the D344N alteration, and one harbored an A249G novel mutation that was not present in azole-susceptible strains.

Two *A. flavus* strains were itraconazole resistant. While both of them carried several c*yp51C* substitutions (M54T and S240A in both strains, and D246G, E421D, and N423D in one strain) that were also present in azole-susceptible *A. flavus* strains, one strain

harbored a P220L mutation in the *cyp51A* gene, and the other carried an H349R substitution in *cyp51C*. These alterations were not carried by azole-susceptible isolates and have not been described before.

DISCUSSION

IFIs are an increasing health concern worldwide, as more than one million deaths are attributed to them every year (20). Variable rates of antifungal resistance were found in a population-based survey performed in Spain on molds (19), with no secondary resistance in *A. fumigatus*. An antifungal resistance surveillance program for *Aspergillus* spp. was established in our reference laboratory. Identification and antifungal susceptibility testing (AFST) results of clinical strains received within this program from its implementation in July 2014 through the end of 2018 are reviewed in this report. Molecular mechanisms of azole resistance were further characterized for those *A. fumigatus sensu stricto*, *A. terreus sensu stricto*, and *A. flavus sensu stricto* isolates that presented azole MICs above the breakpoints established by EUCAST.

Out of a total of 273 *Aspergillus* sp. isolates received within the antifungal resistance surveillance program, *A. fumigatus* complex strains represented more than half of the strains identified (57.9%), followed by *A. terreus* complex (21.6%), *A. flavus* complex (12.8%), and *A. nidulans* (5.5%) complex strains. As samples received within the program constitute a biased subset of isolates under the suspicion of being antifungal resistant, *Aspergillus* complex species prevalence in Spanish centers are not fully representative based on these data. In the FILPOP and FILPOP2 prospective surveillance studies *A. fumigatus sensu stricto* was the most prevalent species isolated, followed by *A. flavus sensu stricto*, *A. terreus sensu stricto*, and *A. tubingensis* in FILPOP (19) and *A. niger sensu stricto*, *A. flavus sensu stricto*, and *A. terreus sensu stricto* in FILPOP2 (21).

Overall, voriconazole and isavuconazole, which have been described to display similar *in vitro* activities (22, 23), showed higher MICs *in vitro* than those of itraconazole and posaconazole in the isolates tested. These differences were particularly remarkable against *A. terreus* complex strains. Similar MIC ranges for voriconazole (EUCAST epidemiological cutoff [ECOFF], >2 mg/liter) and isavuconazole were observed in previous studies against *A. terreus* isolates (22). *A. terreus* and *A. flavus* strains have been reported to display high MIC values to amphotericin B (24–26), although neither clinical breakpoints nor ECOFF values have yet been set for them by EUCAST. Accordingly, strains from this work showed MIC_{90} values of 8 mg/liter for those within the *A. terreus* complex and 4 mg/liter for those belonging to the *A. flavus* complex.

In the FILPOP and FILPOP2 studies, 12% and 11.5% of the identified strains belonged to cryptic or sibling *Aspergillus* species, respectively (19, 21). Although some cryptic *Aspergillus* species present among the isolates received within the program may have been missed due to the fact that molecular identification was only performed for strains that showed MICs above EUCAST clinical breakpoints to at least one antifungal, several sibling species were identified in the current study; the multidrug-resistant species *A. lentulus* was the most common one (13 identified strains), in agreement with results from a multicenter international surveillance study (27). Strains identified as part of this species from the *A. fumigatus* complex presented low susceptibility to amphotericin B and to azoles, especially to voriconazole (data not shown), as reported by previous studies (17, 18). Four *Aspergillus udagawae* and three *A. fumigatiaffinis* strains were also identified. One isolate was identified as *A. citrinoterreus*, from the *A. terreus* complex, which has been reported as the most prevalent cryptic species from this complex in Spain (15).

Despite previous studies in Spain showing low rates of *A. fumigatus* azole resistance (19, 21, 28), resistance to at least one azole was found in 23 strains molecularly identified as *A. fumigatus sensu stricto* Three strains had the most frequent mechanism of azole resistance described worldwide (TR_{34}/L98H) (29–31) and one TR_{46}/Y121F/T289A mechanism (32). These two resistance-related changes have been previously described in Spanish isolates (19, 33, 34), TR_{34}/L98H is associated with a panazole resistance profile, and TR_{46}/Y121F/T289A is related to voriconazole and

isavuconazole resistance and variable MICs for itraconazole and posaconazole (35, 36), although the isolate carrying it in this study was resistant to all azoles. Two other *A. fumigatus* strains harbored a substitution of glycine for arginine at position 54 of *cyp51A*, which has been linked to cross-resistance to itraconazole and posaconazole (37, 38), in agreement with the MICs obtained in this study. The remaining seventeen azole-resistant strains had no mutations in *cyp51A*; two of them were multiazole resistant, while the rest had different azole resistance profiles. Azole-resistant *A. fumigatus* isolates lacking *cyp51A* mutations have been previously reported (39, 40), evidencing the need to investigate further *cyp51A*-independent mechanisms of azole resistance that can be present, such as the overexpression of efflux pumps or *cyp51B* (9). Interestingly, ten of these 17 isolates with no mutations in *cyp51A* were resistant only to isavuconazole, with MIC values of 2 mg/liter. Even though ECOFF value for isavuconazole for *A. fumigatus* is 2 mg/liter, its clinical breakpoint was set by EUCAST as 1 mg/liter (41) based on the use of standard dosing against a mouse model of disseminated aspergillosis (42). On the basis of this established breakpoint, *cyp51A* wild-type isolates classified as isavuconazole resistant have been reported (23, 36, 43). Nevertheless, a recent study on isavuconazole dose escalation proved its effectiveness for treating patients infected with an *A. fumigatus* with an isavuconazole MIC of 2 mg/liter (36), which could result in an increase of one 2-fold dilution step of the EUCAST clinical breakpoint or in the categorization of isolates with an isavuconazole MIC of 2 mg/liter as intermediate, as previously suggested (23). For the time being, EUCAST recommends to repeatedly perform AFST, including additional markers for azole resistance (itraconazole and voriconazole), and to sequence *cyp51A* when isolates have a MIC of 2 mg/liter.

Only two isolates of *A. terreus sensu stricto* had MIC values above the established breakpoints for itraconazole and posaconazole. One of them harbored M217I mutation in *cyp51A*, which has been related to itraconazole resistance and high MIC values of voriconazole and posaconazole (14), as shown by this isolate that also carried the D344N substitution. This alteration has been reported together with M217V substitution in an isolate only resistant to posaconazole (15). The other itraconazole- and posaconazole-resistant isolate, which was also isavuconazole resistant, carried the D344N mutation alone and showed high MICs to voriconazole as well. Mutations in M217 have been suggested to correlate with M220 alterations in the *A. fumigatus cyp51A* gene, which are linked to itraconazole and posaconazole resistance and variable voriconazole and isavuconazole MIC values (44, 45). Another amino acid change in this position, M217T, has also been reported in *A. terreus* isolates resistant only to posaconazole (15). Nevertheless, further research is needed in order to confirm the role of these several mutations in M217 and D344N alteration in the development of *A. terreus* azole resistance. Similarly to *A. fumigatus sensu stricto* strains, ten *A. terreus sensu stricto* strains were only resistant to isavuconazole, with a MIC of 2 mg/liter, a 1-fold dilution above the breakpoint. Two of them carried D344N substitution, while another harbored a novel mutation (A249G) that was not present in susceptible strains and which, therefore, needs to be further studied. The remaining seven strains did not carry any *cyp51A* alterations. In a study where *in vitro* activity of isavuconazole and voriconazole were compared, a high number of *A. terreus* isolates was found to be isavuconazole resistant but voriconazole susceptible, even though MIC distributions for both azoles were symmetric. If an isavuconazole ECOFF value of 2 mg/liter for *A. terreus* strains was applied to those isolates, susceptibility categorization would be the same for both voriconazole and isavuconazole (22).

In previous studies, *A. flavus* strains with MIC values higher than the epidemiological cutoff value established for voriconazole by CLSI, 1 mg/liter, were reported to sometimes harbor *cyp51A*, *cyp51B*, and *cyp51C* mutations, as *A. flavus* has three *cyp51* genes. These strains presented different susceptibility patterns, as some of them had reduced susceptibility to all azoles (46) while others showed intermediate MIC values for itraconazole and posaconazole (11, 13). Two *A. flavus sensu stricto* strains (6%) within the program were classified as itraconazole resistant, based on the EUCAST clinical

breakpoint, and had high MICs against voriconazole, posaconazole, and isavuconazole. *cyp51C* substitutions M54T, S250A, D246G, E421D, and N423D, found in these isolates, seem to have no effect on azole resistance, as they were also present in azole-susceptible strains. Nevertheless, both azole-resistant isolates carried two novel substitutions that were not found in azole-susceptible isolates; one of them carried a P220L mutation on the *cyp51A* gene, and the other harbored an H349R alteration in *cyp51C*. Further investigation is warranted in order to study the implications of their role in azole resistance.

Even though no azole resistance was found among *A. nidulans* strains in the FILPOP and FILPOP2 studies, in this study, one strain was itraconazole resistant and six isolates were isavuconazole resistant according to EUCAST breakpoints. Azole resistance mechanisms were not further studied, as it is not clear how this species develops it. Nevertheless, two *cyp51* genes homologous to those of *A. fumigatus* have been described in *A. nidulans* (47).

Limitations of this study include the fact that resistance rates are not representative, as they are biased due to the fact that only isolates suspected to be antifungal resistant were received by the program. Nevertheless, this study highlights the interest in establishing an antifungal resistance surveillance program in order to get a deeper insight into antifungal resistance mechanisms, which can help in the future to implement specific control measures and to design adapted strategies to diagnose and manage azole resistance in *Aspergillus* species. An important number of azole-resistant *A. fumigatus* strains were found with different mechanisms of resistance, and *A. flavus* and *A. terreus* azole resistance mechanisms were studied for the first time in clinical isolates from Spain.

MATERIALS AND METHODS

Strains. From July 2014 to December 2018, 306 samples belonging to patients from 45 Spanish hospitals were received that were suspected of being resistant (as tested by any antifungal susceptibility method or due to the lack of clinical response) to at least one antifungal agent.

Morphological identification. Strains were morphologically identified to species complex level using malt extract agar (2% malt extract; Oxoid SA, Madrid, Spain), potato dextrose agar (Oxoid SA), dermasel agar base (Oxoid SA), and Czapek-Dox agar (Difco, Soria Melguizo SA, Madrid, Spain) for subculturing the strains to determine their macroscopic and microscopic morphology. Cultures were incubated at 30°C. Fungal morphological features were examined macroscopically and microscopically by conventional methods (48).

Antifungal susceptibility testing. Antifungal susceptibility testing (AFST) was performed following the European Committee on Antifungal Susceptibility Testing (EUCAST) reference method 9.3.1 (49). Antifungals used 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), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland (tested from January 2017), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma, Inc., Tokyo, Japan), and anidulafungin (Pfizer SA). 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, posaconazole, and isavuconazole; from 0.007 to 4 mg/liter for anidulafungin; and from 0.004 to 2 mg/liter for micafungin. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. MICs for amphotericin B, itraconazole, voriconazole, posaconazole, isavuconazole, and terbinafine, and minimal effective concentrations (MECs) for anidulafungin, caspofungin and micafungin were visually read after 24 and 48 h of incubation at 35°C in a humid atmosphere. Clinical breakpoints for interpreting AFST results have been established by EUCAST for some A*spergillus* species for amphotericin B, itraconazole, voriconazole, posaconazole and isavuconazole (50). These breakpoints were used for classifying as susceptible or resistant to each antifungal all species complexes as identified by morphological methods in the first stage and the molecularly identified isolates as available. Breakpoints of echinocandins and terbinafine have not yet been set. Geometric mean (GM), MIC_{50}/MEC_{50} (MIC/MEC causing inhibition of 50% of the isolates tested), and MIC_{90}/MEC_{90} (MIC/MEC causing inhibition of 90% of the isolates tested) were determined. For calculation purposes, the MIC/MEC values that exceeded the maximum concentration tested were transformed to the next dilution (i.e., if MIC/MEC was $>$8 mg/liter, it was expressed as 16 mg/liter), and values that were less than or equal to the minimum concentration tested were transformed to equal (i.e., if MIC/MEC was $\leq$0.03 mg/liter, it was expressed as 0.03 mg/liter).

Molecular identification. *Aspergillus* strains that were classified as resistant to at least one antifungal according to breakpoints were subjected to molecular identification as follows. Isolates were subcultured in glucose-yeast extract-peptone (GYEP) liquid medium (0.3% yeast extract and 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich, Spain) for 24 h to 48 h at 30°C. After mechanical disruption of the mycelium by vortex mixing with glass beads, genomic DNA of isolates was extracted using the

phenol-chloroform method (51). Molecular identification was performed by PCR amplifying and sequencing internal transcribed spacer 1 (ITS1)-5.8S-ITS2 regions (52) and a portion of the β-tubulin gene (53). PCR conditions were set as previously described (19). PCR products were purified using Illustra ExoPro-Star 1-step technology (GE Healthcare Life Sciences, UK), and subsequently sequenced by Sanger method with an ABI3730XL sequencer (Applied Biosystems, Foster City, CA). DNA sequences were analyzed with DNAStar Lasergene 12 software (DNAStar Inc., USA) and compared with reference sequences from the GenBank (https://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 in-house database belonging to the Mycology Reference Laboratory of the Spanish National Center for Microbiology (restricted access).

Characterization of molecular mechanisms of azole resistance in *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus*. Molecular mechanisms of azole resistance were studied by sequencing the main azole target genes for *A. fumigatus sensu stricto* (*cyp51A*, including its promoter region), *A. flavus sensu stricto* (*cyp51A*, *cyp51B*, and *cyp51C*) and *A. terreus sensu stricto* (*cyp51A*) strains that were resistant to at least one azole. All *cyp51* genes were amplified and sequenced as previously described (12, 14, 29). DNA sequences were compared with the *cyp51A* sequence of reference strain A1163 of *A. fumigatus* (NCBI accession number DS499598.1), *cy51A* sequence of reference strain NIH2624 of *A. terreus* (NCBI accession number XM_001215095.1), *cyp51A* (NCBI accession number XM_002375082.1), *cyp51B* (NCBI accession number XM_002379089.1), and *cyp51C* (NCBI accession number XM_002383890.1) sequences of reference strain NRRL3357 of *A. flavus* in order to detect point mutations related to azole resistance.

ACKNOWLEDGMENTS

O.R.-M. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (FI14CIII/00025). J.C.S.-D. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (F17CIII/00027). J.L. holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (F17CIII/00037). A.A.-I. is supported by a research project from the Fondo de Investigación Sanitaria (PI13/02145 and PI16CIII/00035); she has received research grants or honoraria as a speaker or advisor from Gilead Sciences, MSD, Astellas, Pfizer, F2G, Amplix, and Scynexis.

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