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SUSCEPTIBILITY

Species Identification and *In Vitro* Antifungal Susceptibility of *Aspergillus terreus* Species Complex Clinical Isolates from a French Multicenter Study

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ABSTRACT *Aspergillus* section *Terrei* is a species complex currently comprised of 14 cryptic species whose prevalence in clinical samples as well as antifungal susceptibility are poorly known. The aims of this study were to investigate *A. Terrei* clinical isolates at the species level and to perform antifungal susceptibility analyses by reference and commercial methods. Eighty-two clinical *A. Terrei* isolates were collected from 8 French university hospitals. Molecular identification was performed by sequencing parts of beta-tubulin and calmodulin genes. MICs or minimum effective concentrations (MECs) were determined for 8 antifungal drugs using both EUCAST broth microdilution (BMD) methods and concentration gradient strips (CGS). Among the 79 *A. Terrei* isolates, *A. terreus stricto sensu* ($n = 61$), *A. citrinoterreus* ($n = 13$), *A. hortai* ($n = 3$), and *A. alabamensis* ($n = 2$) were identified. All strains had MICs of ≥1 mg/liter for amphotericin B, except for two isolates (both *A. hortai*) that had MICs of 0.25 mg/liter. Four *A. terreus* isolates were resistant to at least one azole drug, including one with pan-azole resistance, yet no mutation in the *CYP51A* gene was found. All strains had low MECs for the three echinocandins. The essential agreements (EAs) between BMD and CGS were >90%, except for those of amphotericin B (79.7%) and itraconazole (73.4%). Isolates belonging to the *A.* section *Terrei* identified in clinical samples show wider species diversity beyond the known *A. terreus sensu stricto*. Azole resistance inside the section *Terrei* is uncommon and is not related to CYP51A mutations here. Finally, CGS is an interesting alternative for routine antifungal susceptibility testing.

KEYWORDS *Aspergillus* section *Terrei*, *Aspergillus terreus*, molecular identification, antifungal susceptibility testing, Etest, EUCAST

Invasive aspergillosis (IA) is becoming a serious threat and a leading cause of morbidity and mortality in immunocompromised patients. *A. fumigatus* is the most common species found in clinical samples of patients suffering from IA; however, other *Aspergillus* spp. can also be the cause of IA (1, 2). Among the five most common is *A.*

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terreus, which is difficult to manage and is often associated with increased mortality because of its intrinsic resistance to amphotericin B (3, 4).

A. terreus is found worldwide in the environment and belongs to the section *Terrei* (5). On the whole, 14 cryptic species that are phenotypically indistinguishable belong to the section *Terrei* (5, 6). However, only a few of them, namely, *A. terreus stricto sensu*, *A. citrinoterreus*, *A. alabamensis*, *A. hortai*, and *A. niveus*, have been reported in human diseases (7–10). The prevalence of these cryptic species in clinical samples is poorly known.

In recent years, a striking emergence of azole resistance has been described in *Aspergillus* species (11, 12). Azole resistance is mainly related to mutations in the *CYP51A* gene and in its promoter, yet there might be other mechanisms (13, 14). The intrinsic resistance of *A. terreus* to amphotericin B puts it at very high risk of multidrug resistance. Nevertheless, only a few azole-resistant *A. terreus* isolates have been reported so far (15).

The broth microdilution method (BMD) based on the EUCAST or CLSI guidelines is currently the reference method for antifungal susceptibility testing (AFST) of *Aspergillus* species (16, 17). Clinical breakpoints and epidemiological cutoffs (ECOFFs) have been determined by EUCAST and can be used to detect resistance (18). However, BMD is time-consuming and requires expertise. As such, many clinical microbiology laboratories routinely apply alternative methods, such as concentration gradient strips (CGS), to assess the *in vitro* susceptibility of their isolates. Few studies have assessed the correlation between these two methods in the susceptibility tests of *A. terreus*, and even when it was examined, it was not done so for all drugs (19–21). Therefore, interpreting MICs obtained by CGS is difficult.

In this study, we investigated 79 morphologically identified *A. Terrei* French clinical isolates. We aimed to identify them molecularly by sequencing parts of the beta-tubulin and calmodulin genes and to assess their susceptibility to 8 antifungal drugs by CGS and BMD according to the EUCAST guidelines.

RESULTS

Isolates and patients. Seventy-nine non-temporally related *A. Terrei* isolates were collected from 50 patients from 8 university hospitals (more than 1,000 beds each, except one with 850 beds), the majority of whom were immunocompromised patients, including hematopoietic stem cell, renal, cardiac, liver, and pulmonary transplant patients. The origin of isolates was mainly respiratory samples ($n = 70$; 88.6%). Other isolates were taken from deep samples ($n = 4$; 5.1%), such as cutaneous biopsy specimen, joint fluid, and intravascular thrombus, or from superficial samples ($n = 5$; 6.3%), such as nail, external ear, and stools. Sex ratio was 1.5 (30 males and 20 females) with a mean age of 54 years. Underlying diseases were mainly bronchopulmonary diseases ($n = 22$; 44%), like cystic fibrosis ($n = 15$) and chronic obstructive pulmonary disease (COPD; $n = 7$), followed by hematological malignancy ($n = 7$; 14%), solid-organ transplant ($n = 4$; 8%), solid malignancy ($n = 3$; 6%), local trauma ($n = 2$; 4%), and other conditions ($n = 6$; 12%). Three patients (6%) had no underlying disease, and there were no data for 3 patients (6%). The clinical forms consisted of 5 cases of invasive aspergillosis, 1 case of onychomycosis, and 44 cases of colonization (Table 1).

Molecular identification. Among the 79 *A. Terrei* isolates, *A. terreus sensu stricto* was the most common species ($n = 61$; 77.2%), found in 7 of the 8 participating centers, and was responsible for the 5 cases of invasive aspergillosis. *A. citrinoterreus* was the second most frequent species and represented 13 isolates (16.5%). The two other cryptic species were identified as *A. hortai* (3 isolates, 3.8%) and *A. alabamensis* (2 isolates, 2.5%) (Fig. 1). Both calmodulin and β-tubulin genes were able to identify the isolates to the species level and gave the same results.

Antifungal susceptibility testing by EUCAST method. Geometric mean MIC, MIC_{50}/50% minimum effective concentration (MEC_{50}), MIC_{90}/MEC_{90}, and ranges for the 79 isolates are presented in Table 2.

TABLE 1 Origin of isolates and patient characteristics according to the cryptic species of section *Terrei*

Species	No. of isolates	No. of hospitals involved	Origin of sample [no. (%)]			No. of patients	Sex ratio (no. male/no. female)	Median age (yr)	Underlying disease/condition [no. (%)]				*Aspergillus* disease (no.)		
			Respiratory	Deep	Superficial				Bronchopulmonary disease	Hematological malignancy	Solid-organ transplant	Other conditions	Invasive aspergillosis	Onychomycosis	Colonization
A. terreus sensu stricto	61	7/8	55 (90.2)	4 (6.5)	2 (3.3)	36	19/17	54	20 (55.6)	4 (11.1)	3 (8.3)	9 (25)	5	0	31
A. citrinoterreus	13	5/8	11 (84.6)	0	2 (15.4)	10	9/1	56	1 (10)	2 (20)	1 (10)	6 (60)	0	0	10
A. hortai	3	3/8	2 (66.7)	0	1 (33.3)	3	1/2	54	0	1 (33.3)	0	2 (66.7)	0	1	2
A. alabamensis	2	1/8	2 (100)	0	0	1	1/0	23	1 (100)	0	0	0	0	0	1
All *Aspergillus Terrei*	79	8	70 (88.6)	4 (5.1)	5 (6.3)	50	30/20	54	22 (44)	7 (14)	4 (8)	17 (34)	5	1	44

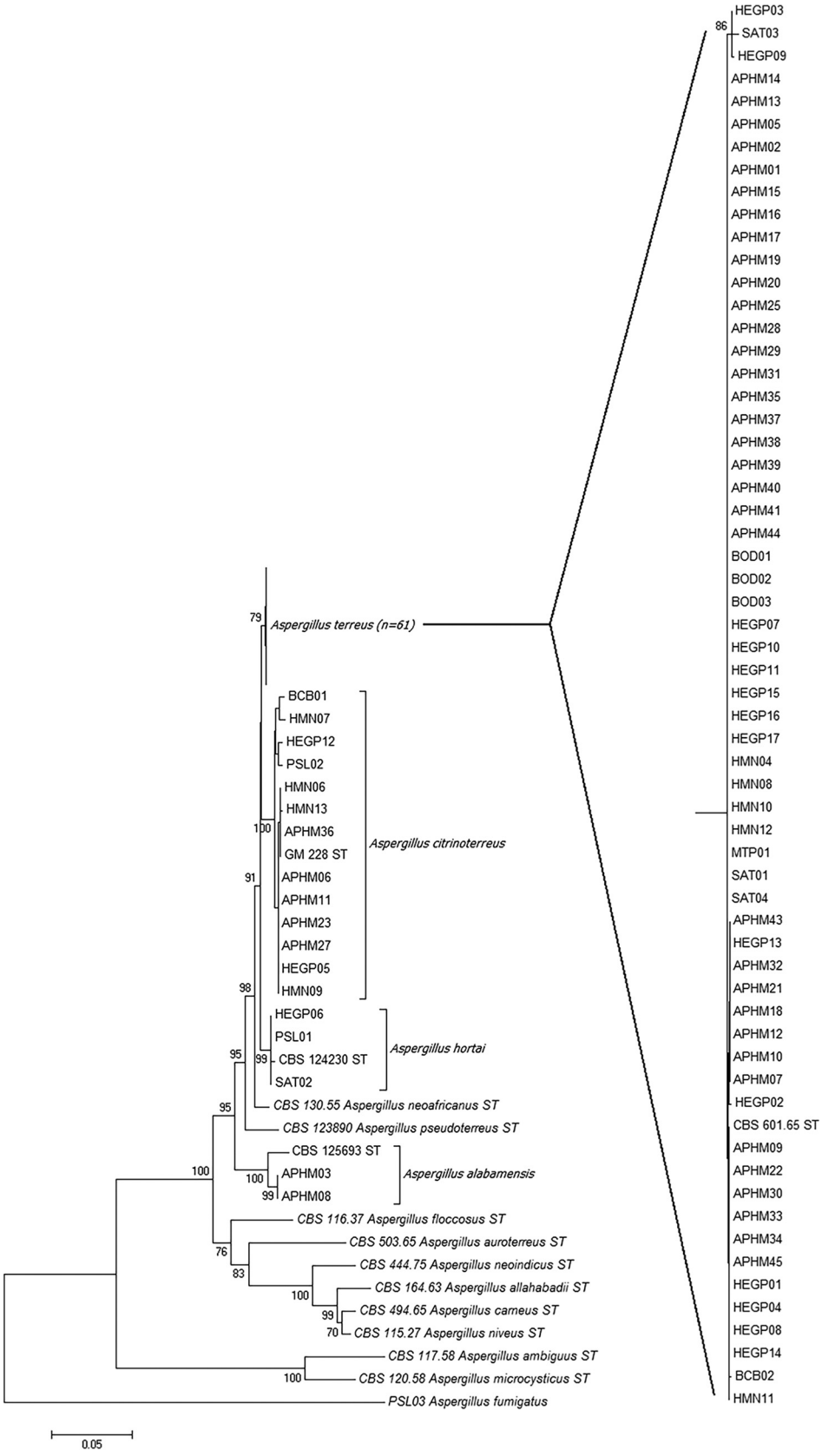

FIG 1 Neighbor-joining tree obtained from the analysis of combined beta-tubulin and calmodulin data set. Numbers above the nodes represent bootstrapping values generated from 1,000 replicates using a Kimura 2-parameter model. Only values above 70% are indicated. Branch lengths are proportional to phylogenetic distances.

TABLE 2 Results of *in vitro* antifungal susceptibility test for the 82 *A. Terrei* isolates by EUCAST and CGS methods[a]

Species and drug	EUCAST method				CGS method					
	Range (mg/liter)	GM (mg/liter)	MIC_{50}/MEC_{50} (mg/liter)	MIC_{90}/MEC_{90} (mg/liter)	Range (mg/liter)	GM (mg/liter)	MIC_{50}/MEC_{50} (mg/liter)	MIC_{90}/MEC_{90} (mg/liter)	EA (%)	CA (%)
A. terreus sensu stricto (n = 61)										
AMB	1–8	2.482	2	4	0.25–4	1.023	1	2	82	91.8
ITC	0.016–8	0.092	0.06	0.25	0.125–2	0.381	0.5	0.5	68.9	98.4
VRC	0.25–8	0.664	0.5	1	0.06–16	0.363	0.5	1	93.4	100
POS	0.016–0.5	0.063	0.06	0.125	0.016–0.5	0.13	0.125	0.25	96.7	73.8
ISA	0.25–4	0.517	0.5	1	0.06–4	0.310	0.25	0.5	100	96.7
CAS	0.03–0.25	0.071	0.06	0.125	0.016–0.125	0.028	0.03	0.06	96.7	NA
MFG	<0.016	<0.016	<0.016	<0.016	<0.016–0.03	<0.016	<0.016	0.016	100	NA
AFG	<0.016–0.03	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	100	NA
A. citrinoterreus (n = 13)										
AMB	2–8	4.219	4	8	0.5–4	1.238	1	2	69.2	69.2
ITC	0.016–0.125	0.069	0.06	0.125	0.016–0.5	0.154	0.25	0.25	100	100
VRC	0.25–1	0.404	0.5	0.5	0.06–0.25	0.139	0.125	0.25	92.3	100
POS	0.016–0.062	0.033	0.031	0.062	0.008–0.125	0.058	0.06	0.125	100	100
ISA	0.125–1	0.293	0.25	1	0.03–0.25	0.131	0.125	0.25	84.6	100
CAS	0.031–0.25	0.086	0.125	0.125	0.016–0.25	0.068	0.06	0.25	100	NA
MFG	<0.016–0.03	<0.016	<0.016	<0.016	<0.016–0.03	<0.016	<0.016	0.016	100	NA
AFG	<0.016	<0.016	<0.016	<0.016	<0.016–0.016	<0.016	<0.016	<0.016	100	NA
A. hortai (n = 3)										
AMB	0.25–4	NA	NA	NA	0.5–2	NA	NA	NA	66.7	100
ITC	0.016–0.03	NA	NA	NA	0.125–0.5	NA	NA	NA	33.3	100
VRC	0.25	NA	NA	NA	0.125–0.5	NA	NA	NA	100	100
POS	0.016–0.03	NA	NA	NA	0.03–0.25	NA	NA	NA	66.7	66.7
ISA	0.25	NA	NA	NA	0.25	NA	NA	NA	100	100
CAS	0.06–0.25	NA	NA	NA	0.016–0.125	NA	NA	NA	66.7	NA
MFG	<0.016	NA	NA	NA	<0.016	NA	NA	NA	100	NA
AFG	<0.016	NA	NA	NA	<0.016	NA	NA	NA	100	NA
A. alabamensis (n = 2)										
AMB	>8	NA	NA	NA	32	NA	NA	NA	100	100
ITC	0.125–0.25	NA	NA	NA	0.5	NA	NA	NA	100	100
VRC	1	NA	NA	NA	0.5–1	NA	NA	NA	100	100
POS	0.06–0.125	NA	NA	NA	0.125–0.25	NA	NA	NA	100	50
ISA	0.25–1	NA	NA	NA	0.5	NA	NA	NA	100	100
CAS	0.06	NA	NA	NA	0.125	NA	NA	NA	100	NA
MFG	<0.016	NA	NA	NA	<0.016	NA	NA	NA	100	NA
AFG	<0.016	NA	NA	NA	<0.016–0.016	NA	NA	NA	100	NA
All *A. Terrei* (n = 79)										
AMB	0.25–8	2.648	2	8	0.25–32	1.161	1	2	79.7	88.6
ITC	0.016–8	0.084	0.06	0.25	0.016–2	0.322	0.5	0.5	73.4	98.7
VRC	0.25–8	0.596	0.5	1	0.06–16	0.311	0.25	1	93.7	100
POS	0.016–0.5	0.055	0.06	0.125	0.008–0.5	0.112	0.125	0.25	96.2	77.2
ISA	0.125–4	0.458	0.5	1	0.03–4	0.270	0.25	0.5	97.5	97.4
CAS	0.031–0.25	0.074	0.06	0.125	0.016–0.25	0.034	0.03	0.125	96.2	NA
MFG	<0.016–0.03	<0.016	<0.016	<0.016	<0.016–0.016	<0.016	<0.016	<0.016	100	NA
AFG	<0.016–0.03	<0.016	<0.016	<0.016	<0.016–0.03	<0.016	<0.016	0.016	100	NA

[a]GM, geometric mean; EA, essential agreement; CA, categorical agreement; NA, not available.

Amphotericin B susceptibility testing by the BMD method resulted in MICs above 1 mg/liter for all isolates except two, which had MICs of 0.25 mg/liter. Interestingly, these isolates were both *A. hortai*; however, the third *A. hortai* isolate had a MIC of 4 mg/liter. Eleven isolates had a MIC above the ECOFF (MIC of 8 mg/liter by EUCAST), including the two *A. alabamensis* isolates, which showed the highest MICs for amphotericin B by the CGS method (MIC of >32 mg/liter). The last 9 isolates with MICs above the ECOFF were five *A. terreus sensu stricto* and four *A. citrinoterreus* isolates (Table 2).

Concerning antifungal susceptibility testing by the BMD method for azole drugs, no differences were found between the different cryptic species. However, four isolates either had MICs above the ECOFF (MIC of 4 to 8 mg/liter for voriconazole and 2 to 4 mg/liter for isavuconazole) or were resistant (MIC of 8 mg/liter for itraconazole and 0.5 mg/liter for posaconazole) to at least one azole drug, e.g., one isolate showed resistance to a pan-azole. These four isolates were all *A. terreus sensu stricto*.

As for echinocandin drugs, all isolates had low MECs for the three echinocandins by both BMD and Etest methods (MECs of <0.25 mg/liter for caspofungin and <0.031 mg/liter for micafungin and anidulafungin).

MIC/MEC comparison between EUCAST and CGS methods. Considering all *A. Terrei* species, MIC_{50}/MEC_{50} and MIC_{90}/MEC_{90}, as determined by the two methods, were

identical (within ±2 dilutions) for all drugs, except for the MIC_{50} of itraconazole, which had a higher value with the CGS method (Table 2). The EA values were above 90% for all antifungal drugs, except for those for amphotericin B and itraconazole, which had EA values of 79.7% and 73.4%, respectively. For amphotericin B, the MICs were higher by the BMD method ($P < 0.001$ by Wilcoxon test), whereas for itraconazole the MICs were significantly higher by the CGS method ($P < 0.001$ by Wilcoxon test). The CA values were above 90% for itraconazole, voriconazole, and isavuconazole but were only 88.6% for amphotericin B and 77.2% for posaconazole and were not available for echinocandins.

When we separately considered the two cryptic species (*A. terreus sensu stricto* and *A. citrinoterreus*) with a sufficient number of isolates for each, the EAs were acceptable (>90%) for all drugs except amphotericin B and itraconazole for *A. terreus sensu stricto* and amphotericin B and isavuconazole for *A. citrinoterreus* (Table 2).

Comparison of visual and spectrophotometric readings for EUCAST MIC values. For azole drugs and amphotericin B, the spectrophotometric reading was made for 69 of the 79 *A. Terrei* isolates. MIC_{50} and MIC_{90}, determined visually and by the spectrophotometer, were identical (within ±2 dilutions) for all drugs. Similarly, the EAs were above 90% for all drugs.

CYP51A sequencing. The *CYP51A* gene and its promoter were sequenced for the four isolates that were resistant *in vitro* to at least one azole drug. The same sequencing was made for 11 other susceptible isolates as controls. Of note, all of these isolates were *A. terreus sensu stricto*. No mutation in the amino acid sequence was detected in the four resistant isolates or in nine of the susceptible isolates. For the remaining two susceptible isolates sequenced for *CYP51A* and its promoter, we found polymorphism in the CYP51A amino acid sequence; one isolate harbored E313K mutation and the other A249G mutation.

DISCUSSION

In recent years, the development of fungal identification protocols based on molecular methods has increased our knowledge on *Aspergillus* species epidemiology. Among the main *Aspergillus* species implicated in human diseases (like *A. fumigatus*, *A. flavus*, and *A. niger*), *A. terreus* belongs to the section *Terrei* (5). This section is currently composed of 14 recognized cryptic species (6). To our knowledge, little research work was directed to study the epidemiology inside the section *Terrei*, and the prevalence of the cryptic species involved in human disease is poorly known (7, 8, 22). However, it may be useful to identify *Aspergillus* at the species level in clinical samples to improve patient management or to deepen knowledge of the local epidemiology. Several of these cryptic species can be the main species isolated in clinical samples of patients with IA or exhibit resistance to antifungal drugs. For example, within the section *Fumigati*, *A. lentulus* has been shown to be intrinsically resistant to voriconazole (23). Moreover, within the section *Nigri*, *A. niger sensu stricto* alone stands for 7 to 58% of clinical isolates, whereas *A. tubingensis* and *A. awamori* account for 17.8 to 76.2% and 16.7 to 55.6%, respectively (24–27).

In the present study, we investigated the molecular identification and *in vitro* antifungal susceptibility of 79 clinical isolates identified initially as *A. terreus*. The results show that *A. terreus sensu stricto* is the most common species, and that almost 25% of the isolates represent the three other cryptic species: *A. citrinoterreus*, *A. hortai*, and *A. alabamensis*. Moreover, the newly described (6) *A. citrinoterreus* is the second most common species (16.5%). These four cryptic species have already been involved in human invasive aspergillosis (6, 8, 9), but interestingly, in our study the five cases of invasive aspergillosis were caused only by *A. terreus sensu stricto*. The remaining isolates were mainly responsible for colonization in patients suffering from chronic bronchopulmonary diseases (cystic fibrosis and COPD).

Our *A. Terrei* isolates come from two main distinct French geographical areas: Paris (north of France) and Marseille (south of France). No difference was found in the species

distribution between these two areas, although the incidence might differ between the cities, as was already reported (28).

A. terreus is known to be intrinsically resistant to amphotericin B (29). However, our results indicate that this pattern of susceptibility is dependent on the cryptic species inside the *Terrei* section. For instance, *A. hortai* seems to have the lowest MICs, and two of its isolates had MICs of <1 mg/liter. This is consistent with previous observations where 6 to 38% of the *A. Terrei* isolates had MICs of <1 mg/liter (7, 29, 30). However, in the Risslegger et al. study, the low-MIC isolates all were *A. terreus sensu stricto* (7). In contrast, *A. alabamensis* had the highest MIC and corresponds to the only two isolates with MICs of >32 mg/liter by the CGS method. Such observations need to be confirmed by using a larger number of isolates for each species.

Azole resistance in *Aspergillus* species is an emerging issue in recent years (11, 12). This phenomenon is well studied for *A. fumigatus* and is mainly related to mutations in the *CYP51A* gene and its promoter (14, 31, 32). A unique case of pan-azole-resistant *A. terreus* with a mutated *CYP51A* has already been reported (15). Among our 79 *A. Terrei* isolates, four were resistant to at least one azole drug, including one with pan-azole resistance. Unfortunately, we did not find mutation in the *CYP51A* gene or its promoter in these four isolates. We think that other mechanisms, already described in other *Aspergillus* species, like increased drug efflux or overexpression of the target, stand behind the azole resistance of these isolates (13, 31). Interestingly, we detected polymorphism in the CYP51A amino acid sequence in two of our susceptible control isolates; one harbored an E313K mutation and the other an A249G mutation without association with higher MICs for azole drugs.

ECOFFs and clinical breakpoints used to categorize isolates as wild type/non-wild type or susceptible/resistant are only defined for the reference BMD method (18). Given that BMD requires experience and is not practicable for all laboratories, the CGS method often replaces it. Several studies have already assessed the correlation between CLSI and CGS methods for *A. terreus*, but this has never been the case between EUCAST and CGS methods. Therefore, in the present study we compared, for the first time, EUCAST and CGS methods for AFST of our 79 *A. Terrei* isolates. The EAs were acceptable (i.e., ≥90%) for all antifungal drugs except itraconazole and amphotericin B, which had EAs of 73.4% and 79.7%, respectively. The same finding on amphotericin B was already described between CLSI and CGS in previous studies, with EAs ranging from 16% to 75% (19, 33). However, a good agreement for this drug was also shown with EAs of 100% (34, 35). Regarding azole drugs, previous comparison studies between CLSI and CGS methods showed various results ranging from EAs of 100% for voriconazole (19), itraconazole (35), posaconazole (34), and isavuconazole (36) to less acceptable EAs for itraconazole (EA of 88%) (33) and posaconazole (EA of 64%) (19). Consequently, our results show that the CGS method could be a good alternative to BMD, allowing the use of EUCAST ECOFF and breakpoints for all drugs but itraconazole and amphotericin B. For the latter, low MICs obtained by CGS should be interpreted with caution in these amphotericin B intrinsically resistant species.

EUCAST guidelines recommend reading MICs of azole and amphotericin B in filamentous fungi visually (17). However, this method is subject to variations and errors, as it is operator dependent. It has been shown previously that spectrophotometric reading for AFST of *Aspergillus* spp. was reliable (37, 38). More recently, authors have assessed the use of this spectrophotometric reading in AFST of *A. fumigatus* using a 490-nm wavelength (39). They found good agreement between this spectrophotometric and visual reading, so they proposed additional investigations on other *Aspergillus* species, hence their suggestion to consider the use of a spectrometer in practice. Our results showed the same good agreement for our *A. Terrei* isolates, with EAs above 90% for all tested drugs, adding more evidence to the use of spectrophotometric readings in AFST of *Aspergillus* species.

In conclusion, *A. terreus* isolates found in clinical samples show several species besides *A. terreus sensu stricto*. The antifungal susceptibility testing demonstrated a low rate of azole resistance inside the section *Terrei* and was not related to *CYP51A*

TABLE 3 Primers used in this study to sequence gene *CYP51A* and its promoter

Fragment	Sequence
1	
Forward	5′-GTAGCGCAGCGGCCAGTGGTGGGAGAACTTTCGTTCTA-3′
Reverse	5′-CAGGGCGCAGCGATGACTGTACACCTCTTCTGCGTTGAC-3′
2	
Forward	5′-GTAGCGCAGCGGCCAGTTCATCCTCAACGGCAAACTC-3′
Reverse	5′-CAGGGCGCAGCGATGACAGATCATGTCGGAGTCGGAG-3′
3	
Forward	5′-GTAGCGCAGCGGCCAGTTCTACATGGACATCATCCGC-3′
Reverse	5′-CAGGGCGCAGCGATGACCATCATCATCAGGTTCCGCT-3′
4	
Forward	5′-GTAGCGCAGCGGCCAGTTTCCTAACGCTAGTCGCTGG-3′
Reverse	5′-CAGGGCGCAGCGATGACGCCGTTCTTACGCCTTGTT-3′

mutations. Amphotericin B MICs showed significant differences between the isolates, which could be due to species identification. Finally, CGS could be a good alternative to EUCAST BMD for AFST of *A. Terrei* isolates.

MATERIALS AND METHODS

Isolates and patients. Phenotypically identified *A. Terrei* clinical isolates were collected retrospectively over a 13-year period (2003 to 2015) from 8 French university hospitals, of which five are in the Parisian area and 3 in the south of France (Marseille, Montpellier, and Bordeaux). For each isolate, age, sex, underlying disease of the patient, site of isolation, and clinical form of aspergillosis were registered. The study was approved by the local Ethical Committee, and the database was declared to the Commission Nationale de l'Informatique et des Libertés (CNIL) (no. 1699340).

Molecular identification. Molecular identification within the *Terrei* section was performed by sequencing part of the calmodulin and β-tubulin genes, as described in previous studies (40). Briefly, complete genomic DNA was extracted from a mature subculture on Sabouraud-chloramphenicol-gentamicin agar using a QIAamp DNA blood minikit (Qiagen Sciences Ing., Courtaboeuf, France) after a step of beading in a MagNA Lyser instrument (Roche Diagnostics, Meylan, France). Primers used for the amplification were 5′-TGGTGCCGCTTTCTGGTA-3′ and 5′-AAGTTGTCGGGACGGAATAG-3′ for β-tubulin and 5′-GTAGCGCAGCGGCCAGTCCGAGTACAAGGARGCCTTC-3′ and 5′-CAGGGCGCAGCGATGACCCGATRG AGGTCATRACGTGG-3′ for calmodulin. Obtained DNA sequences were analyzed using Seqscape v2.5 (Applied Biosystems) and were compared with *A. Terrei* GenBank and MycoBank database sequences. Phylogenetic analyses were also performed for each gene individually and with the concatenated sequences of the two genes using MEGA 6.0 software. Neighbor-joining trees were built using the Kimura two-parameter model with 1,000 bootstraps replications and included sequences of the type strain of each cryptic species inside the *Terrei* section.

Antifungal susceptibility testing. Antifungal susceptibility testing of each isolate was performed for 8 drugs: itraconazole (Sigma-Aldrich, Saint-Quentin Fallavier, France), voriconazole (Sigma-Aldrich), posaconazole (MSD, Kenilworth, NJ), isavuconazole (Basilea Pharmaceutica International Ltd., Basel, Switzerland), amphotericin B (Sigma-Aldrich), caspofungin (Sigma-Aldrich), anidulafungin (Pfizer New York, NY), and micafungin (Astellas Pharma Inc., Tokyo, Japan). The BMD method was used.

BMD was performed according to the EUCAST guidelines (17). Final drug concentration ranges were 0.016 to 8 mg/liter for each drug. Quality control strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included, but MICs were read after 24 h of incubation. For azoles and amphotericin B, MICs were also read by a spectrophotometer at 550 nm, using a 90% growth inhibition endpoint, and compared with the drug-free control well.

For the agar diffusion method, we used MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy) for isavuconazole and Etest (bioMérieux, Marcy l'Etoile, France) for the other seven drugs. A spore suspension adjusted to a 0.5 McFarland standard in sterile water was prepared and inoculated on RPMI medium plates supplemented with 2% glucose. MICs and MECs were determined after incubation at 37°C for 48 h. Values obtained with the CGS method were adjusted to the next higher concentration matching the 2-fold dilution scheme of the BMD method.

Essential agreement (EA) between BMD and CGS methods, and between the visual and spectrophotometric readings for the EUCAST method, was considered to have been achieved when the MIC/MEC values were within ± 2 dilutions. EA values above 90% were considered acceptable.

Isolates were categorized as wild type/non-wild type or as susceptible/resistant by comparing MIC values with ECOFFs and clinical breakpoints (18).

CYP51A sequencing. For azole-resistant and control isolates, the whole *CYP51A* gene and its promoter were amplified using 4 couples of in-house primers (Table 3). The 4 obtained partial sequences were analyzed and assembled using SeqScape. The generated sequence was then translated and aligned to those of gene ATEG05917 from the reference strain NIH2624 and set for amino acid sequence analysis.

Accession number(s). The obtained sequences were submitted to GenBank under accession numbers MH006731 to MH006809 and MH006810 to MH006888.

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