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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Molecular identification, antifungal susceptibility testing and mechanisms of azole resistance in *Aspergillus* spp. received within a surveillance program on antifungal resistance in Spain

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Running head: Antifungal resistance surveillance in *Aspergillus*

Key words: *Aspergillus*, EUCAST, azoles, antifungal resistance, surveillance program, Cyp51, itraconazole, posaconazole, voriconazole, isavuconazole

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Abstract

Antifungal resistance is one of the major causes of the increasing mortality rates in 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 on the most prevalent *Aspergillus* species. 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 *A. fumigatus*, *A. terreus* and *A. flavus sensu stricto (s.s.)* strains that were classified as resistant three *A. fumigatus s.s.* strains carried the TR$_{34}$/L98H resistance mechanism, while two harbored G54R substitution and one the TR$_{46}$/Y121F/T289A mechanism. Seventeen strains had no mutations in *cyp51A*, being ten of them resistant only to isavuconazole. Three *A. terreus s.s.* strains harbored D344N substitution in *cyp51A*, one of them combined with M217I, and another carried A249G novel mutation. *A. flavus s.s.* itraconazole resistant strains harbored P220L and H349R alterations in *cyp51A* and *cyp51C*, respectively, that need further investigation on their implication in azole resistance.

Introduction

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 towards 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 for *Aspergillus* spp. (5). Azoles, which are *Aspergillus* first-line antifungal therapy, generally have a good activity against these species, although an increase in *Aspergillus fumigatus* clinical isolates displaying MIC values above the stablished clinical breakpoints for azole drugs by CLSI and EUCAST has been described in the last 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 of *A. fumigatus* triazole resistance cases are explained by point mutations in *cyp51A* gene, which encodes the 14α-sterol-demethylase of the ergosterol biosynthesis pathway (10). Point mutations associated with azole resistance in *cyp51A* have also been reported 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 in the Mycology Reference Laboratory of the Spanish National Center for Microbiology in order to monitor antifungal resistance in the most frequent *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 until the end of 2018.

Materials and methods

1. Strains

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

2. 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 (20).

3. Antifungal susceptibility testing

Antifungal Susceptibility Testing (AFST) was performed following the European Committee on Antifungal Susceptibility Testing (EUCAST) reference method 9.3.1. (21). 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/L for amphotericin B, terbinafine, and caspofungin; from 0.015 to 8 mg/L for itraconazole, voriconazole, posaconazole and isavuconazole; from 0.007 to 4 mg/L for anidulafungin; and from 0.004 to 2 mg/L for micafungin. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. Minimal Inhibitory Concentrations (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 hours of incubation at 35ºC 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 (22). These breakpoints were used for classifying as susceptible or resistant to each antifungal all species complex as identified by morphological methods in the first stage and the molecularly identified isolates for which they were available. Breakpoints of echinocandins and terbinafine have not been set yet. Geometric mean (GM), MIC/MEC$_{50}$ (MIC/MEC causing inhibition of 50% of the isolates tested) and MIC/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/L it was expressed as 16 mg/L), and values that were less than or equal to the minimum concentration tested were transformed to equal (i.e. if MIC/MEC was ≤0.03 mg/L it was expressed as 0.03 mg/L).

4. 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, 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 (23). Molecular identification was performed by PCR amplifying and sequencing ITS1-5.8S-ITS2 regions (24) and a portion of β-tubulin gene (25). PCR conditions were set as previously described (19). PCR products were purified using Illustra ExoProStar 1-step (GE Healthcare Lifescience, UK), and sequenced after by Sanger method with a ABI3730XL sequencer (Applied Biosystems, Foster City, CA). DNA sequences were analysed with DNAStar Lasergene 12 software (DNAStar Inc., USA) and 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 in-house database belonging to the Mycology Reference Laboratory of the Spanish National Center for Microbiology (restricted access).

5. 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 s.s. (cyp51A* including its promoter region*)*, *A. flavus s. s.* (*cyp51A*, *cyp51B* and *cyp51C*) and *A. terreus s. s.* (*cyp51A*) strains that were resistant to at least one azole. All *cyp51* genes were amplified and sequenced as previously described

(12, 14, 26). DNA sequences were compared with *cyp51A* sequence of reference strain A1163 of *A. fumigatus* (NCBI accession number AFUB_063960), *cy51A* sequence of reference strain NIH2624 of *A. terreus* (NCBI accession number ATEG05917), *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.

Results

1. Strains in this study

In total, 306 samples were received from 45 hospitals since the implementation of the surveillance program on antifungal resistance in July 2014 until 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 *Aspergillus* genus 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.

2. 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 *A. terreus* complex, 12.8% (35/273) were part of the *A. 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* spp.

3. Antifungal susceptibility testing

Antifungal susceptibility testing results are summarized in Table 1, showing GMs, MIC/MEC$_{50}$s, MIC/MEC$_{90}$s 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, *A. terreus, A. flavus* and *A. nidulans* complexes strains, showing lower MIC values for itraconazole and posaconazole than 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 stablished by EUCAST is included in Table 2.

4. Molecular identification

Among *A. fumigatus* complex, 46 strains were molecularly identified in order to identify cryptic species due to their resistance to at least one antifungal. The most prevalent species identified was *A. fumigatus s.s.* (23 strains), followed by several cryptic species of the complex: *Aspergillus lentulus* (13 strains), *Aspergillus udagawae* (four strains), *Aspergillus fumigatiaffinis* (three strains), *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: twelve were *A. terreus s.s.* and one belonged to the cryptic species *Aspergillus citrinoterreus*. Only two strains from the *A. flavus* complex were resistant to itraconazole, and were identified as A*. flavus s.s.*. Out of six *A. nidulans* complex strains showing resistance to itraconazole and/or isavuconazole, four were *A. nidulans s.s.* and four belonged to *Aspergillus spinulosporus* cryptic species.

5. Characterization of molecular mechanisms of azole resistance in *Aspergillus fumigatus, Aspergillus terreus* and *Aspergillus flavus sensu stricto*

Further characterization of A*. fumigatus*, *A. flavus* and *A. terreus s.s.* strains that were resistant to at least one azole drug according to EUCAST breakpoints was performed. 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 and were panazole-resistant, while two strains carried a G54R substitution showing resistance to itraconazole and posaconazole and another one harbored the TR_{46}/Y121F/T289A mechanism and was resistant to all azoles. Seventeen strains out of twenty-three azole resistant *A. fumigatus* strains did not have mutations in *cyp51A* gene; ten of which were only resistant to isavuconazole. The seven remaining were resistant to at least two azoles.

Twelve *A. terreus* strains were azole resistant according to EUCAST breakpoints, from which two were resistant to itraconazole, posaconazole and isavuconazole and had high MICs to voriconazole. Both had a D344N substitution in c*yp51A*, one of them combined with another mutation in the same gene, M217I. The remaining ten were only resistant to isavuconazole (MIC = 2 mg/L) and while seven were *cyp51A* wild-type, two carried the D344N alteration and one harbored 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 of them) that were also present in *A. flavus* azole susceptible strains, one strain harbored a P220L mutation in *cyp51A* gene and the other carried a 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 (27). Variable rates of antifungal resistance were found in a population based survey in molds performed in Spain (19), with no secondary resistance in *A. fumigatus*. An antifungal resistance surveillance program for *Aspergillus* spp. was established in our reference laboratory. Identification and AFST results of clinical strains received within this program between its implementation in July 2014 until the end of 2018 are reviewed in this report. Molecular mechanisms of azole resistance were further characterized for those *A. fumigatus s.s.*, *A. terreus s.s.* and *A. flavus s.s.* isolates that presented azole MICs above the established breakpoints by EUCAST.

Out of a total of 274 *Aspergillus* spp. isolates received within the antifungal resistance surveillance program, *A. fumigatus* complex strains represented more than half of the strains identified (57.7%), followed by *A. terreus* (21.5%), *A. flavus* (12.8%) and *A. nidulans* (5.8%) complexes 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 is not fully representative based on these data. In FILPOP and FILPOP2 prospective surveillance studies *A. fumigatus s.s* was the most prevalent species isolated, followed by *A. flavus s.s.*, *A. terreus s.s.* and *A. tubingensis* in FILPOP (19) and *A. niger s.s.*, *A. flavus s.s.* and *A. terreus s.s* in FILPOP2 (28).

Overall, voriconazole and isavuconazole, that have been described to display similar *in vitro* activities (29, 30), showed higher MICs *in vitro* than itraconazole and posaconazole in the isolates tested. These differences were particularly remarkable against *A. terreus* complex strains. Similar MIC ranges for voriconazole (EUCAST ECCOF > 2 mg/L) and isavuconazole were observed in previous studies against *A. terreus* isolates (29). *A. terreus* and *A. flavus* strains have been reported to display high MIC values to amphotericin B (31-33), although neither clinical breakpoints nor epidemiological cut-off values have been set yet for them by EUCAST. Accordingly, strains from this work showed MIC_{90} values of 8 mg/L for those within the *A. terreus* complex and 4 mg/L for those belonging to the *A. flavus* complex.

In FILPOP and FILPOP2 studies 12% and 11.5% of the identified strains belonged to *Aspergillus* cryptic or sibling species, respectively (19, 28). Although some *Aspergillus* cryptic species present among the isolates received within the program may have been missed due to the fact that molecular identification was only performed to strains that showed MICs above clinical breakpoints for EUCAST to at least one antifungal, several sibling species were identified in the current study: multi-drug resistant species *A. lentulus* was the most common one (13 identified strains), something in agreement with results from a multicentre international surveillance study (34). 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 *A. 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, 28, 37), resistance to at least one azole was found in 23 strains molecularly identified as *A.*

fumigatus s.s.. Three strains had the most frequent mechanism of azole resistance described worldwide (TR_{34}/L98H) (26) (38, 39) and oneTR_{46}/Y121F/T289A mechanism (40). These two resistant related changes have been previously described in Spanish isolates (19, 41, 42), TR_{34} / L98H associated with a pan-azole resistance profile and TR_{46}/Y121F/T289A related to voriconazole and isavuconazole resistance and variable MICs for itraconazole and posaconazole (43, 44), although the isolate carrying it in this study was resistant to all azoles. Other two *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 (45, 46), in agreement with the MICs obtained in this study. The remaining seventeen azole resistant strains had no mutations in *cyp51A*: two of them were multi-azole resistant, while the rest had different azole resistance profiles. Azole resistant *A. fumigatus* isolates lacking *cyp51A* mutations have been previously reported (47, 48), evidencing the need of investigating 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 seventeen isolates with no mutations in *cyp51A* were resistant only to isavuconazole, with MIC values of 2 mg/L. Even though the epidemiological cut-off (ECOFF) value for isavuconazole for *A. fumigatus* is 2 mg/L; its clinical breakpoint was set by EUCAST on 1 mg/L (49) based on the use of standard dosing against a mouse model of disseminated aspergillosis (50). On the basis of this established breakpoint, *cyp51A* wild-type isolates classified as isavuconazole resistant have been reported (30, 44, 51). Nevertheless, a recent study on isavuconazole dose escalation proved to be effective to treat patients infected with an *A. fumigatus* with an isavuconazole MIC of 2 mg/L (44), which could develop into an increase of one 2-fold dilution step of the EUCAST clinical breakpoint or into the categorization of isolates with an isavuconazole MIC of 2mg/L as intermediate as previously suggested (30). For the time being, EUCAST recommends to repeatedly perform AFST including additional markers for azole resistance (itraconazole and voriconazole) and sequence *cyp51A* when isolates have a MIC of 2 mg/L.

Only two isolates of *A. terreus s.s.* had MIC values above the stablished 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 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 D344N mutation alone and showed high MICs to voriconazole as well. Mutations in M217 have been suggested to correlate with M220 alterations in *A. fumigatus cyp51A* gene, which are linked to itraconazole and posaconazole resistance and variable voriconazole and isavuconazole MIC values (52, 53). 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 s.s.* strains, ten *A. terreus s.s.* strains (18.5%) were only resistant to isavuconazole with a MIC of 2 mg/L, one-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, therefore, would need to be further studied. The remaining seven 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 isavuconazole ECOFF value of 2 mg/L for *A. terreus* strains was applied to those isolates, susceptibility categorization was the same for both antifungals (29).

In previous studies, *A. flavus* strains with MIC values higher than the epidemiological cutoff value established for voriconazole by CLSI, 1 mg/L, 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 (54) while others showed intermediate MIC values for itraconazole and posaconazole (11, 13). Two *A. flavus s.s.* strains (6%) within the program were classified as itraconazole resistant based on EUCAST clinical breakpoint, and had high MICs against voriconazole, posaconazole and isavuconazole. *Cyp51C* substitutions M54T S250A, D246G, E421D and N423Dfound 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 P220L mutation on *cyp51A* gene and the other harbored H349R alteration in *cyp51C*. Further investigation is warranted in order to study their implication in their role in azole resistance.

Even though no azole resistance was found among *A. nidulans* strains in 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* (55).

Limitations of this study include that resistance rates are not representative, as they are biased due to the fact that only isolates suspected to be antifungal resistant were received within 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*. An important number of azole resistance *A. fumigatus* strains was 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.

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References

1. Chen SC, Playford EG, Sorrell TC. 2010. Antifungal therapy in invasive fungal infections. Curr Opin Pharmacol 10:522-30.
2. Kriengkauykiat J, Ito JI, Dadwal SS. 2011. Epidemiology and treatment approaches in management of invasive fungal infections. Clin Epidemiol 3:175-91.
3. Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N. 2011. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. J Antimicrob Chemother 66 Suppl 1:i5-14.
4. Sun KS, Tsai CF, Chen SC, Huang WC. 2017. Clinical outcome and prognostic factors associated with invasive pulmonary aspergillosis: An 11-year follow-up report from Taiwan. PLoS One 12:e0186422.
5. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. 2017. The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect Dis 17:e383-e392.
6. Howard SJ, Pasqualotto AC, Denning DW. 2010. Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus* bronchitis. Clin Microbiol Infect 16:683-8.
7. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. Emerg Infect Dis 17:1846-54.
8. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. J Antimicrob Chemother 65:2116-8.
9. Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in *Aspergillus* spp.: a worldwide problem? J Fungi (Basel) 2.
10. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJ. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus cyp51A* based on protein homology modeling. Antimicrob Agents Chemother 54:2425-30.
11. Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, Li R. 2012. The T788G mutation in the *cyp51C* gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. Antimicrob Agents Chemother 56:2598-603.
12. Paul RA, Rudramurthy SM, Meis JF, Mouton JW, Chakrabarti A. 2015. A novel Y319H substitution in *cyp51C* associated with azole resistance in *Aspergillus flavus*. Antimicrob Agents Chemother 59:6615-9.
13. Choi MJ, Won EJ, Joo MY, Park YJ, Kim SH, Shin MG, Shin JH. 2019. Microsatellite typing and resistance mechanism analysis of voriconazole-resistant *Aspergillus flavus* isolates in South Korean hospitals. Antimicrob Agents Chemother 63.
14. Arendrup MC, Jensen RH, Grif K, Skov M, Pressler T, Johansen HK, Lass-Florl C. 2012. *In vivo* emergence of *Aspergillus terreus* with reduced azole susceptibility and a *cyp51A* M217I alteration. J Infect Dis 206:981-5.
15. Zoran T, Sartori B, Sappl L, Aigner M, Sanchez-Reus F, Rezusta A, Chowdhary A, Taj-Aldeen SJ, Arendrup MC, Oliveri S, Kontoyiannis DP, Alastruey-Izquierdo A, Lagrou K, Cascio GL, Meis JF, Buzina W, Farina C, Drogari-Apiranthitou M, Grancini A, Tortorano AM, Willinger B, Hamprecht A, Johnson E, Klingspor L, Arsic-Arsenijevic V, Cornely OA, Meletiadis J,

Prammer W, Tullio V, Vehreschild JJ, Trovato L, Lewis RE, Segal E, Rath PM, Hamal P, Rodriguez-Iglesias M, Roilides E, Arikan-Akdagli S, Chakrabarti A, Colombo AL, Fernandez MS, Martin-Gomez MT, Badali H, Petrikkos G, Klimko N, Heimann SM, Uzun O, Roudbary M, de la Fuente S, Houbraken J, et al. 2018. Azole-resistance in *Aspergillus terreus* and related species: an emerging problem or a rare phenomenon? Front Microbiol 9:516.

16. Balajee SA, Houbraken J, Verweij PE, Hong SB, Yaghuchi T, Varga J, Samson RA. 2007. *Aspergillus* species identification in the clinical setting. Stud Mycol 59:39-46.

17. Alastruey-Izquierdo A, Alcazar-Fuoli L, Cuenca-Estrella M. 2014. Antifungal susceptibility profile of cryptic species of *Aspergillus*. Mycopathologia 178:427-33.

18. Nedel WL, Pasqualotto AC. 2014. Treatment of infections by cryptic *Aspergillus* species. Mycopathologia 178:441-5.

19. Alastruey-Izquierdo A, Mellado E, Pelaez T, Peman J, Zapico S, Alvarez M, Rodriguez-Tudela JL, Cuenca-Estrella M, Group FS. 2013. Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). Antimicrob Agents Chemother 57:3380-7.

20. de Hoog G, Guarro, J, Tran, CS, Wintermans, RGF, Gene, J. 1995. Hyphomycetes, p 380–1007. *In* de Hoog, G.S., Guarro, J. (ed), Atlas of clinical fungi. John Wiley & Sons, Chichester, England.

21. Arendrup MC, Meletiadis, J., Mouton, J.W., Lagrou, K., Howard, S.J., Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. 2017. EUCAST DEFINITIVE DOCUMENT E.DEF 9.3.1: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. . http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_1_Mould_testing__definitive.pdf. Accessed 8 April 2019.

22. EUCAST. 2018. Antifungal breakpoint tables for interpretation of MICs v9.0. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_breakpoints_v_9.0_180212.pdf. Accessed 8 April 2019.

23. Tang CM, Cohen J, Holden DW. 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol Microbiol 6:1663-71.

24. White TJ BT, Lee SB. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, San Diego, CA.

25. Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell 4:625-32.

26. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, Rodriguez-Tudela JL. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. Antimicrob Agents Chemother 51:1897-904.

27. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv13.

28. Alastruey-Izquierdo A, Alcazar-Fuoli L, Rivero-Menendez O, Ayats J, Castro C, Garcia-Rodriguez J, Goterris-Bonet L, Ibanez-Martinez E, Linares-Sicilia MJ, Martin-Gomez MT, Martin-Mazuelos E, Pelaez T, Peman J, Rezusta A, Rojo S, Tejero R, Anza DV, Vinuelas J, Zapico MS, Cuenca-Estrella M, the FPfG, Reipi. 2018. Molecular identification and susceptibility testing of molds isolated in a prospective surveillance of triazole resistance in Spain (FILPOP2 Study). Antimicrob Agents Chemother 62.

29. Astvad KMT, Hare RK, Arendrup MC. 2017. Evaluation of the *in vitro* activity of isavuconazole and comparator voriconazole against 2635 contemporary clinical *Candida* and *Aspergillus* isolates. Clin Microbiol Infect 23:882-887.

30. Jorgensen KM, Astvad KMT, Hare RK, Arendrup MC. 2019. EUCAST susceptibility testing of isavuconazole: MIC data for contemporary clinical mould and yeast isolates. Antimicrob Agents Chemother doi:10.1128/AAC.00073-19.

31. Blum G, Perkhofer S, Haas H, Schrettl M, Wurzner R, Dierich MP, Lass-Florl C. 2008. Potential basis for amphotericin B resistance in *Aspergillus terreus*. Antimicrob Agents Chemother 52:1553-5.

32. Hadrich I, Makni F, Neji S, Cheikhrouhou F, Bellaaj H, Elloumi M, Ayadi A, Ranque S. 2012. Amphotericin B *in vitro* resistance is associated with fatal *Aspergillus flavus* infection. Med Mycol 50:829-34.

33. Reichert-Lima F, Lyra L, Pontes L, Moretti ML, Pham CD, Lockhart SR, Schreiber AZ. 2018. Surveillance for azoles resistance in *Aspergillus* spp. highlights a high number of amphotericin B-resistant isolates. Mycoses 61:360-365.

34. van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chryssanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekotter A, Lass-Florl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arikan-Akdagli S, Andes D, Meletiadis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE. 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis 21:1041-4.

35. Balajee SA, Nickle D, Varga J, Marr KA. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. Eukaryot Cell 5:1705-12.

36. Vinh DC, Shea YR, Sugui JA, Parrilla-Castellar ER, Freeman AF, Campbell JW, Pittaluga S, Jones PA, Zelazny A, Kleiner D, Kwon-Chung KJ, Holland SM. 2009. Invasive aspergillosis due to *Neosartorya udagawae*. Clin Infect Dis 49:102-11.

37. Escribano P, Pelaez T, Munoz P, Bouza E, Guinea J. 2013. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? Antimicrob Agents Chemother 57:2815-20.

38. Chowdhary A, Kathuria S, Xu J, Meis JF. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. PLoS Pathog 9:e1003633.

39. Morio F, Aubin GG, Danner-Boucher I, Haloun A, Sacchetto E, Garcia-Hermoso D, Bretagne S, Miegeville M, Le Pape P. 2012. High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR34/L98H, in a French cohort of patients with cystic fibrosis. J Antimicrob Chemother 67:1870-3.

40. van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ, Haas PJ, Rijnders BJ, Kuijper EJ, van Tiel FH, Varga J, Karawajczyk A, Zoll J, Melchers WJ, Verweij PE. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. Clin Infect Dis 57:513-20.

41. Rodriguez-Tudela JL, Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Monzon A, Cuenca-Estrella M. 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. Antimicrob Agents Chemother 52:2468-72.

42. Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E. 2015. First detection of *Aspergillus fumigatus* azole-resistant strain due to *cyp51A* TR46/Y121F/T289A in an azole-naive patient in Spain. New Microbes New Infect 6:33-4.

43. van Ingen J, van der Lee HA, Rijs TA, Zoll J, Leenstra T, Melchers WJ, Verweij PE. 2015. Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and

TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. J Antimicrob Chemother 70:178-81.

44. Buil JB, Bruggemann RJM, Wasmann RE, Zoll J, Meis JF, Melchers WJG, Mouton JW, Verweij PE. 2018. Isavuconazole susceptibility of clinical *Aspergillus fumigatus* isolates and feasibility of isavuconazole dose escalation to treat isolates with elevated MICs. J Antimicrob Chemother 73:134-142.

45. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. 2003. A point mutation in the 14alpha-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 47:1120-4.

46. Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 47:1719-26.

47. Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Moller N, Khan H, Melchers WJ, Verweij PE. 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. PLoS One 5:e10080.

48. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, Smith J, Bueid A, Moore CB, Bowyer P, Perlin DS. 2011. High-frequency triazole resistance found In nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. Clin Infect Dis 52:1123-9.

49. Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, Howard SJ, Testing SoASTotEECfAS. 2016. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. Clin Microbiol Infect 22:571 e1-4.

50. Seyedmousavi S, Bruggemann RJ, Meis JF, Melchers WJ, Verweij PE, Mouton JW. 2015. Pharmacodynamics of isavuconazole in an *Aspergillus fumigatus* mouse infection model. Antimicrob Agents Chemother 59:2855-66.

51. Howard SJ, Lass-Florl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. 2013. Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. Antimicrob Agents Chemother 57:5426-31.

52. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14alpha-sterol demethylase (*cyp51A*) of *Aspergillus fumigatus* are responsible for resistance *in vitro* to azole antifungal drugs. Antimicrob Agents Chemother 48:2747-50.

53. Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, Sanders C, Fan H, Fothergill AW, Sutton DA. 2016. First detection of TR34/L98H and TR46/Y121F/T289A *cyp51* mutations in *Aspergillus fumigatus* isolates in the United States. J Clin Microbiol 54:168-71.

54. Sharma C, Kumar R, Kumar N, Masih A, Gupta D, Chowdhary A. 2018. Investigation of multiple resistance mechanisms in voriconazole-resistant *Aspergillus flavus* clinical isolates from a chest hospital surveillance in Delhi, India. Antimicrob Agents Chemother 62.

55. Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. 2001. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 39:2431-8.

Table 1. MIC values and ranges for amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), posaconazole (POS), isavuconazole (ISA) and terbinafine (TRB), and MEC values for caspofungin (CAS), micafungin (MCF) and anidulafungin (AFG) for *Aspergillus* complex strains (n ≥10), as determined by EUCAST broth microdilution method.

Aspergillus complex (no. of strains tested)		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/MEC$_{50}$	0.5	0.5	0.5	0.12	1	4	0.25	0.015	0.03
	MIC/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/MEC$_{50}$	2	0.25	1	0.12	1	0.25	0.5	0.015	0.03
	MIC/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/MEC$_{50}$	1	0.5	1	0.25	1	0.12	0.25	0.03	0.03
	MIC/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/MEC$_{50}$	2	0.5	0.25	0.12	0.25	0.25	0.25	0.06	0.06
	MIC/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/MEC$_{50}$	0.5	0.5	0.5	0.12	1	2	0.25	0.015	0.03
	MIC/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

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

Aspergillus complex	No. and %[a] of strains with MIC values above their stablished 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 s.s.	0 (0%)	11 (64.7%)	7 (41.2%)	13 (61.9%)	17 (60.7%)
A. terreus complex (59)	NA	2 (3.4%)	NA	3 (5.1%)	12 (44.4%)
A. terreus s.s.		2 (100%)		2 (66.7%)	11 (91.7%)
A. flavus complex (35)	NA	2 (5.7%)	NA	NA	NA
A. flavus s.s.		2 (100%)			
A. nidulans complex (15)	NA	1 (6.7%)	NA	NA	6 (46.2%)
A. nidulans s.s.		1 (100%)			4 (66.7%)

NA: Not applicable due to the lack of EUCAST breakpoint. S.s. senso stricto.

[a] Percentages for senso stricto isolates correspond to % of resistant senso 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*).

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	MIC (mg/L)				Mutation found (gene)
		ITC	VRC	POS	ISA	
A. fumigatus	CNM CM-7582	**16**	**4**	**0.5**	ND	TR$_{34}$/L98H (*cyp51A*)
	CNM CM-7609	**16**	**16**	**1**	ND	TR$_{34}$/L98H (*cyp51A*)
	CNM CM-9399	**16**	**4**	**0.5**	**8**	TR$_{34}$/L98H (*cyp51A*)
	CNM CM-9114	**16**	0.5	**2**	0.5	G54R (*cyp51A*)
	CNM CM-9501	**16**	0.5	**2**	0.5	G54R (*cyp51A*)
	CNM CM-8057	**16**	**16**	**1**	**16**	TR$_{46}$/Y121F/T289A (*cyp51A*)
	CNM CM-7510	**16**	2	**0.5**	ND	No mutations found (*cyp51A*)
	CNM CM-7552	**16**	1	**0.5**	ND	No mutations found (*cyp51A*)
	CNM CM-8822	**4**	**8**	**0.5**	**4**	No mutations found (*cyp51A*)
	CNM CM-8900	1	**4**	**0.5**	**2**	No mutations found (*cyp51A*)
	CNM CM-8914	0.5	0.25	0.12	**2**	No mutations found (*cyp51A*)
	CNM CM-8915	0.5	0.5	0.12	**2**	No mutations found (*cyp51A*)
	CNM CM-8916	0.5	0.25	0.12	**2**	No mutations found (*cyp51A*)
	CNM CM-8917	0.5	0.5	0.06	**2**	No mutations found (*cyp51A*)
	CNM CM-8922	1	0.5	0.06	**2**	No mutations found (*cyp51A*)
	CNM CM-8925	1	0.5	0.25	**2**	No mutations found (*cyp51A*)
	CNM CM-9120	1	0.25	0.25	**2**	No mutations found (*cyp51A*)
	CNM CM-9307	0.5	1	0.12	**2**	No mutations found (*cyp51A*)
	CNM CM-9327	1	2	**0.5**	**2**	No mutations found (*cyp51A*)
	CNM CM-9361	1	2	0.25	**2**	No mutations found (*cyp51A*)
	CNM CM-9471	**16**	**8**	**0.5**	**8**	No mutations found (*cyp51A*)
	CNM CM-9491	1	0.5	0.12	**2**	No mutations found (*cyp51A*)
	CNM CM-9494	**8**	2	**0.5**	**4**	No mutations found (*cyp51A*)
	Range	**0.5-16**	0.25-16	0.12-2	0.5-16	
A. flavus	CNM CM-7668	**16**	0.25	1	ND	P220L (*cyp51A*)
	CNM CM-9326	**16**	16	2	16	H349R (*cyp51C*)
	Range	16-16	0.25-16	1-2	**16-16**	
A. terreus	CNM CM-9079	0.25	2	0.06	**2**	D344N (*cyp51A*)
	CNM CM-9280	0.5	2	0.12	**2**	D344N (*cyp51A*)
	CNM CM-9490	**4**	8	**0.5**	**8**	D344N (*cyp51A*)
	CNM CM-7846	**16**	16	**1**	ND	M217I; D344N (*cyp51A*)
	CNM CM-9284	0.5	1	0.12	**2**	A249G (*cyp51A*)
	CNM CM-8056	0.5	2	0.06	**2**	No mutations found (*cyp51A*)
	CNM CM-8671	0.5	2	0.25	**2**	No mutations found (*cyp51A*)
	CNM CM-8852	2	2	0.25	**2**	No mutations found (*cyp51A*)
	CNM CM-8952	0.5	8	0.25	**8**	No mutations found (*cyp51A*)
	CNM CM-8981	0.25	1	0.06	**2**	No mutations found (*cyp51A*)
	CNM CM-9108	0.25	2	0.06	**2**	No mutations found (*cyp51A*)
	CNM CM-9285	0.25	1	0.12	**2**	No mutations found (*cyp51A*)
	Range	0.25-16	1-16	0.06-1	**2-8**	

CNM-CM, Mold Collection of the Spanish National Center for Microbiology. In bold numbers, MIC values that are above the EUCAST clinical breakpoints.