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

Molecular identification and susceptibility testing of molds isolated in a Prospective Surveillance of Triazole Resistance in Spain (FILPOP2 study)

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Running title: FILPOP 2 study

Keywords: Antifungal resistance, Aspergillus, cryptic species, Scedosporium, Fusarium, Mucorales, azole resistance, cyp51A

Abstract

Antifungal resistance is increasing by the emergence of intrinsically resistant species and by the development of secondary resistance in susceptible species. A previous study performed in Spain revealed levels of azole resistance in molds between 10 to 12.7% but secondary resistance in *A. fumigatus* was not detected. We used itraconazole supplemented media to select resistant strains. A total of 500 plates supplemented with 2 mg/L of ITZ were sent to 10 Spanish tertiary hospitals, molecular identification and antifungal susceptibility testing were performed. In addition, *cyp*51A gene was sequenced in those *A. fumigatus* strains showing azole resistance. A total of 493 isolates were included in the study. Sixteen strains were

isolated from patients with an infection classified as proven, 104 as probable and 373 as colonization. *Aspergillus* was the most frequent genera isolated with 80.3% followed by *Scedosporium/Lomentospora* (7.9%), *Penicillium/Talaromyces* (4.5%), *Fusarium* (2.6 %) and the order Mucorales (1 %). Antifungal resistance was detected in *Scedosporium/Lomentospora* species, *Fusarium, Talaromyces* and Mucorales. Three strains of *A. fumigatus senso stricto* were resistant to azoles, two of them harbored TR_{34}+L98H mechanism of resistance and the other one had no mutations in *cyp*51A. The level of azole resistance in *A. fumigatus* remains low but cryptic species represent over 10% of the isolates and have broader but overall higher range of antifungal resistance

Introduction

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

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

In Spain the multicentre epidemiological study FILPOP (6) described the epidemiology of mould infections in the country and the rates of resistance. Triazole resistance ranged from 10 to 12.7%, depending on the species and the drug tested however, secondary resistance in *A. fumigatus* was not detected. Taking into account the high levels of secondary resistance described in neighbouring countries, we hypothesize that this resistance could be underestimated due to the sampling method we used. In this work, we proposed a strategy by using a selective media supplemented with an antifungal (ITZ) to detect specifically resistant isolates in respiratory samples and to avoid the overgrowth of azole susceptible species

belonging to the conventional flora. Plates with the selective media were used to culture clinical samples directly to increase the rate and chance of isolation of azole resistant strains.

Results

Five hundred and six isolates were obtained from 10 Spanish Hospitals. One was a dermatophyte isolated from a nail that was excluded from the study, seven were yeast and five did not grow in the reference laboratory (RL) and were no further analyzed. Thus, a total of 493 isolates were included in the study. One hundred and fifty five isolates grew in ITZ supplemented media and three hundred and thirty-eight in the regular Sabouraud medium without supplement. Each isolate was characterized independently since typing techniques were not applied at this stage.

Four hundred and seventy seven (96.8%) strains were isolated from respiratory samples (333 sputum, 79 BAS (broncho aspirate), 38 BAL (broncho alveolar lavage), 14 tracheal aspirate and 13 from other respiratory samples), 10 from biopsies, four from wound exudates and two from otic exudates. Sixteen strains were isolated from patients with an infection classified as proven, 104 as probable and 373 as colonization. Underlying diseases included hematological (7%), solid organ transplant patients (9.7%), other cancer (11.6%), HIV (5%), chronic obstructive pulmonary disease (COPD 16.6%), cystic fibrosis (9.3%), asthma (2.6%), other causes of immunosuppression (4.5%) and other respiratory diseases (14.8%), other causes (6.1%) and unknown (12.6%). Table 1 summarizes the number of strains isolated in each sample by type of infection.

Identification of the strains

Aspergillus was the most frequent genera isolated with 80.3% followed by *Scedosporium/Lomentospora* (7.9%), *Penicillium/Talaromyces* (4.5%), *Fusarium* (2.6 %) and the order Mucorales (1 %). Table 2 shows the identification to species level of the strains analyzed, *A. fumigatus* was the most frequent species with 260 isolates (52.74%), followed by *A. niger* (5.27%), *A. flavus* (5.07%), *A. terreus* (4.67%) and *S. apiospermum* (4.26%). Cryptic species of *Aspergillus* accounted for 11.5% of the isolates. *A. fumigatus* was the most frequent species both in colonization (186 isolates) and infections (74 isolates), however while *A. niger* and *A. flavus* ranked second (26 total isolates) and third (25 total isolates) in the total number of isolates, they were found less frequently (six and two isolates respectively) than *S. apiospermum* (nine isolates) and *A. terreus* (eight isolates) in infections classified as probable or proven (table 2). Those differences were not statistically significant.

Susceptibility testing

Three strains did not grow in the media and conditions used for susceptibility testing and were not analyzed. Table 3 shows geometric Mean (GM), MIC causing inhibition of 50% of the isolates (MIC_{50}), MIC causing inhibition of 90% of the isolates (MIC_{90}) and range of the species that had 10 or more isolates. *Scedosporium* species had elevated MICs to all antifungals, voriconazole being the most active compound with MIC_{50}=1mg/L, followed by echinocandins. Azoles and echinocandins showed no activity to any of the *Fusarium* isolates analyzed, only amphotericin B showed activity (MICs $\leq$2mg/L for 9 out of 13 isolates). Mucorales species isolated showed elevated MICs to voriconazole and echinocandins and low MICs to amphotericin B and posaconazole. *Penicillium/Talaromyces* showed low MICs to most antifungals except for *Penicillium citrinum* with high MICs (16 mg/L) to voriconazole. Other species with high MICs were *Scopulariopsis brevicaulis* and *Alternaria* spp., with high MICs to all antifungals and *Purpureocillium lilacinus* (Syn. *Paecilomyces lilacinus*) with elevated MICs to amphotericin B, itraconazole and echinocandins. Table 4 shows the number of *Aspergillus* isolates with MICs > 2mg/L for amphotericin B, itraconazole and voriconazole and > 0.25 mg/L for posaconazole. There were not significant differences between MIC values of infectious strains and those of strains isolates from cases defined as colonization (p>0.01).

Analysis of the impact of itraconazole supplemented media

Table 5 shows the number of strains and percentage growing in Sabouraud and ITZ supplemented media. Sixty nine percent of the isolates analyzed were isolated in SAB while 31% were isolated in ITZ supplemented media (338 versus 155 isolates).

Resistant isolates (MICs to itraconazole > 2mg/L) were preferably isolated in itraconazole supplemented media (15.6% of the total isolates in itraconazole versus 13.1% of the total isolates in Sabouraud) although differences were not statistically significant (p=0.5). *Scedosporium/Lomentospora* isolates were preferentially isolated in itraconazole supplemented media (11% versus 6.5%) as well as *Penicillium/Talaromyces* (5.2 vs 4.1%) although differences were not statistically significant compared with the percentages of *Aspergillus* spp. (p=0.08 and p=0.5).

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

Characterization of resistance mechanisms in *Aspergillus fumigatus*

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

Discussion

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

In a previous study (FILPOP) performed in 30 Spanish hospitals between 2010 and 2011 we found no azole resistant *A. fumigatus* but 15% rate of cryptic species (6). In the current work, we have used ITZ supplemented plates to screen for azole resistance. Thus, three out of two hundred sixty (1.2%) *A. fumigatus* isolates analyzed were resistant to azoles, two of these strains harbor the most frequent mechanism of azole resistance (TR_{34}+L98H) while the other strain showed no mutations in *cyp51A*. TR_{34}+L98H mechanism of resistance has been linked to the use of azoles in agriculture and is the most frequent mechanism of azole resistance worldwide (20). Other mechanisms of resistance related with mutations in *cyp*51A such as TR_{46}/Y121F/T289A and G448S have been described in isolates from Spain (21, 22) but were not found in this work. One out of the three azole resistant *A. fumigatus* had no mutations in *cyp*51*A*. Azole resistant isolates with no mutations in *cyp*51A have been described previously (23, 24). Other mechanisms of azole resistance could be present and would be further analyzed in this isolate.

The main source of resistance in our isolates were due to cryptic species of *Aspergillus* and emerging moulds such as *Fusarium, Scedosporium* and Mucorales. Break points for these species have not been defined; however, patients infected with these pathogens are associated with poorer outcomes (25).

Cryptic species of *Aspergillus* accounted for 11.5% of the total number of isolates. Among *A. fumigatus* complex *A. lentulus* and *A. fumigatiaffinis* showed high MICs to amphotericin B and

voriconazole as previously reported (26, 27). Within *A. niger* complex *A. niger* and *A. tubingensis* have been isolated in this study. In accordance with the results obtained here, previous works reported that susceptibility within this group is variable and strain dependent (28, 29). *Aspergillus terreus* and *A. flavus* complexes have been associated with higher MICs to amphotericin B. In this work, the MIC range of amphotericin B for *A. flavus* was 0.25-2 mg/L and 0.25-4 for *A. terreus*. Two out 23 isolates of *A. terreus* showed MIC= 4mg/L while all isolates of *A. alliaceus* (*A. flavus* complex) showed MICs > 4 mg/L for amphotericin B in accordance with previous results (6, 27, 30). *A. citrinoterreus* (*A. terreus* complex) isolates have been reported to be more susceptible to itraconazole, voriconazole, and posaconazole than *A. terreus* sensu stricto having both high amphotericin B MICs (31). In this study, two isolates of *A. citrinoterreus* were found with no differences among susceptibilities of *A. terreus* isolates. *Aspergillus ustus* complex have been associated with high MICs to all antifungals (6, 17, 27, 32). Among the species of that complex, we isolated five *A. calidoustus* and one *A. puniceus* being all of them resistant to azoles and echinocandins, and being amphotericin B the only compound with some activity.

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

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

Unexpectedly, in this study, we could not find statistical differences in the detection of resistant isolates when using itraconazole supplemented plates. Only *Scedosporium/Lomentospora* isolates showed higher percentages of isolation in itraconazole

supplemented media compared with the other species. However when we compare the results obtained in FILPOP 1 study (6) with this one (table 6) we see that the percentage of *Scedosporium/Lomentospora* spp*., Penicillium/Talaromyces* spp. and *Fusarium* spp. increased while the percentage of *Aspergillus* spp. and Mucorales decreased. Although the results are not comparable because of important methodological differences (the number of participating centers, the number of strains analyzed, etc), this could indicate that selective media is favoring the isolation of rare species by decreasing the recovery rate of fast growing species as *Aspergillus spp.* and Mucorales. This is in agreement with previous works were selective media have been recommended for the isolation of *Scedosporium* species (38-40).

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

Material and Methods

Strains and isolates

A total of 500 Sabouraud (SAB, Oxoid S.A., Madrid, Spain) plates supplemented with 2 mg/L of ITZ (Sigma Aldrich Quimica, Madrid, Spain) were sent to 10 Spanish tertiary hospitals from different regions in Spain: Gregorio Marañón (1525 beds, Madrid, Madrid), La Paz (1524 beds, Madrid, Madrid), Virgen de Valme (605 beds, Seville, Andalusia), Reina Sofía (1233 beds, Córdoba, Andalusia), La Fe (1050 beds, Valencia, Valencia), Donostia (1054 beds, Guipúzcoa, Basque Country), Vall d'hebron (1251 beds, Barcelona, Catalonia), Bellvitge (1022 beds, Barcelona, Catalonia), Central de Asturias (989 beds, Oviedo, Asturias), Miguel Servet (1234 beds, Zaragoza, Aragon). The number of beds of each hospital was according to the report published in 2017 with the current numbers at the end of 2016 (41). Samples from respiratory secretions, biopsies and other sterile sites were included in the study. The samples were cultured in the classical media and in the 2 mg/L ITZ supplemented media.

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

Clinical data

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

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

Morphological Identification

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

Molecular identification

Moulds were subcultured in Glucose Yeast Extract Peptone medium (GYEP) (0.3% yeast extract, 1% peptone, Difco, Soria Melguizo) with 2% glucose (Sigma Aldrich Quimica, Madrid, Spain), for 24 to 48h at 30°C. Genomic DNA was isolated using an extraction procedure previously described (44). Molecular identification was performed by sequencing informative targets. DNA segments comprising the ITS1 and ITS2 regions, were amplified for all the strains with primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (45). In the case of *Aspergillus* and *Scedosporium* isolates, a portion of the beta tubulin gene was sequenced with the following primers: βtub3 (5'-TTCACCTTCAGACCGGT-3') and βtub2 (5'-AGTTGTCGGGACGGAATAG-3') (16) for *Aspergillus* and TUB-F (5'-CTGTCCAACCCCTCTTACGGCGACCTGAAC-3') and TUB-R (5´-

ACCCTCACCAGTATACCAATGCAAGAAAGC-3') (46) for *Scedosporium*. Also, DNA segments comprising the elongation factor alpha region were amplified for *Fusarium* isolates with primers EF1 (5'-ATGGGTAAGARGACAAGAC-3') and EF2 (5'-GGARGTACCAGTSATCATGTT -3') (47). All primers were synthesized by Sigma Genosys (Madrid, Spain). The reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems) following conditions previously described (6). Sequencing reactions were done with two μl of a sequencing kit (BigDye Terminator cycle sequencing, ready reaction: Applied Biosystems), one μM of primers [the same as in the PCR except for *Aspergillus* β tubulin were βtub1 (5'-AATTGGTGCCGCTTTCTGG-3') and βtub4 (5'-AGCGTCCATGGTACCAGG-3') were used] and three μl of the PCR product in a final volume of ten μl.

Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene; DNAstar, Inc., Madison, WI, USA). All sequences were compared with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and Mycobank (http://www.mycobank.org/) databases with InfoQuest FP software, version 4.50 (BIORAD Laboratories, Madrid, Spain), as well as with the data base belonging to the Department of Mycology of the Spanish National Centre for Microbiology which holds 13,000 sequences from strains belonging to 290 different fungal species. This database was designed by the Spanish National Centre for Microbiology and has restricted access (26, 34, 37, 48).

Antifungal susceptibility testing.

Microdilution testing was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard methodology (49). *A. fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains. The antifungal agents used in the study were amphotericin B (Sigma-Aldrich Quimica, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, N.J.), terbinafine (Novartis, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, N.J.), micafungin (Astellas pharma Inc., Tokio, Japan) and anidulafungin (Pfizer S.A., Madrid, Spain). 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 and posaconazole, from 0.007 to 4 mg/L for anidulafungin and from 0.004 to 2 mg/L for micafungin. The plates were incubated at 35ºC for 48 h in a humid atmosphere. Visual readings were performed at 24 and 48 hours with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, posaconazole and terbinafine was the antifungal concentration that produced a complete inhibition of visual

growth at 24 and 48 hours (minimum inhibitory concentration, MIC). For the echinocandins the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae compared with the growth control well (minimum effective concentration, MEC). The EUCAST have set breakpoints to interpret antifungal susceptibility testing results of amphotericin B (resistant strain MIC value >2 mg/L), itraconazole (MIC >2 mg/L), voriconazole (MIC >2 mg/L), and posaconazole (MIC > 0.25 mg/L) (50). These breakpoint values have been set only for some *Aspergillus* spp., but were used in this study to analyze rate of resistance in vitro for all *Aspergillus* species. Breakpoints of echinocandins and terbinafine have not been set yet, and rate of resistances were not calculated.

Analysis of the impact of itraconazole supplemented media

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

Characterization of resistance mechanism in *Aspergillus fumigatus*

A. fumigatus showing MICs over the breakpoint for resistance (>2mg/L for ITZ and voriconazole and >0.25 for posaconazole) were studied for mutations in *cyp*51A gene. The *cyp*51A gene including its promoter region was amplified and sequenced following the procedure previously described (51) for the detection of specific mutations associated to azole resistance.

Statistical analysis.

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

Acknowledgments

This study was supported by an Investigator Sponsored Research grant from Gilead Sciences (IN-ES-131-1600). The authors thank Cristina Armentia and Teresa Merino for technical assistance.

AAI is supported by a research project from Fondo de Investigación Sanitaria (PI13/02145 and PI16CIII/00035) she has received research grants or honoraria as a speaker or advisor from Gilead Sciences, F2G and Scynexis. Olga Rivero-Menendez holds a predoctoral fellowship from Fondo de Investigacion Sanitaria (FI14CIII/00025). MTMG: Has acted as invited speaker in meetings sponsored by Pfizer and MSD. TP is supported by a Project from the Fondo de Investigación Sanitaria, (PI13/02783). JP has received honoraria as a speaker for Pfizer, MSD, Astellas and Gilead. JV has received travel grants from MSD, Becton Dickinson and Beckman. MCE has received grant support from Astellas, bioMerieux, Gilead Sciences, Merck Sharp & Dohme, Pfizer, Schering Plough, Soria Melguizo, Ferrer International, CIDARA, F2G, Basilea, Amplyx and Scynexis.

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531 Table 1: Number of strains isolated per sample and type of infection

Sample	Colonization	Probable	Proven	Total
Sputum	277	52	4	333
BAS	60	17	2	79
BAL	21	17	0	38
Tracheal aspirate	7	5	2	14
Other respiratory*	8	5	0	13
biopsy	0	2	8	10
Wound exudate	0	4	0	4
Otical exudate	0	2	0	2
Total	373	104	16	493

532 * Other respiratory sites include: sinuses, lung tissue and oropharyngeal swabs.

533 Table 2: Number of strains isolated per species and type of infection

Species	Colonization	Probable	Proven	Total
A. fumigatus	186	68	6	260
A. niger	20	6	0	26
A. flavus	23	2	0	25
A. terreus	15	6	2	23
S. apiospermum	12	5	4	21
A. tubingensis	15	1	0	16
S. boydii	11	0	1	12
A. alliaceus	5	5	0	10
Fusarium proliferatum	7	2	0	9
A. lentulus	6	0	0	6
A. nidulans	5	1	0	6
A. calidoustus	4	1	0	5
A. quadrilineatus	5	0	0	5
Penicillium chrysogenum	5	0	0	5
A. sydowii	2	2	0	4
Paecilomyces lilacinus	3	1	0	4
Penicillium citrinum	4	0	0	4
Lomentospora prolificans	1	1	1	3
S. ellipsoidea	3	0	0	3
Other *Aspergillus* spp.	8	2	0	10
Other *Talaromyces* spp.	9	0	0	9
Mucorales	3	0	2	5
Basidiomycetes	4	1	0	5
Other *Fusarium* spp.	4	0	0	4
Other *Penicillium* spp.	4	0	0	4
Other	9	0	0	9
Total	373	104	16	493

534

535 Table 3: Antifungal susceptibility profile of the most frequent species isolated.

Species (n)		MIC /mg/L)					MEC (mg/L)		
		AMB	ITZ	VCZ	PCZ	TRB	CPF	MCF	ANF
A. fumigatus (260)	GM	0.36	0.20	0.47	0.06	2.94	0.35	0.01	0.02
	MIC_{50}	0.5	0.12	0.5	0.06	4	0.25	0.015	0.015
	MIC_{90}	0.5	0.5	1	0.12	8	1	0.03	0.03
	Range	0.015-1	0.03-16	0.12-4	0.015-8	0.25-32	0.004-32	0.003-4	0.007-8
A. niger (26)	GM	0.25	0.88	0.73	0.14	0.32	0.16	0.01	0.01
	MIC_{50}	0.25	0.5	1	0.12	0.25	0.12	0.015	0.007
	MIC_{90}	0.5	16	1	0.25	2	1	0.03	0.015
	Range	0.12-0.5	0.25-16	0.5-2	0.06-1	0.12-4	0.03-2	0.004-0.06	0.007-0.03
A. flavus (25)	GM	0.92	0.18	0.74	0.08	0.28	0.66	0.06	0.05
	MIC_{50}	1	0.12	0.5	0.12	0.25	0.5	0.06	0.03
	MIC_{90}	2	0.5	1	0.25	2	4	0.12	4
	Range	0.5-4	0.06-0.5	0.5-2	0.03-0.25	0.03-2	0.12-32	0.015-4	0.007-8
A. terreus (23)	GM	1.06	0.09	0.70	0.08	0.25	0.65	0.02	0.02
	MIC_{50}	1	0.06	0.5	0.06	0.25	0.5	0.015	0.015
	MIC_{90}	2	0.5	1	0.5	2	2	0.03	0.03
	Range	0.25-4	0.015-16	0.25-8	0.015-16	0.06-2	0.06-32	0.004-4	0.007-8
S. apiospermum (21)	GM	4.27	2.68	1.64	0.76	12.26	1.69	0.36	0.61
	MIC_{50}	4	16	1	0.5	32	1	0.25	2
	MIC_{90}	32	16	16	16	32	32	4	8
	Range	0.25-32	0.12-16	0.25-16	0.015-16	0.06-32	0.06-32	0.006-4	0.008-8
A. tubingensis (16)	GM	0.23	0.92	1.14	0.15	0.42	0.22	0.02	0.01
	MIC_{50}	0.25	1	1	0.25	0.5	0.25	0.03	0.015
	MIC_{90}	0.5	1	2	0.25	2	0.5	0.06	0.03

	Range	0.12-1	0.25-16	0.5-2	0.015-0.5	0.06-2	0.06-0.5	0.004-0.12	0.007-0.03
S. boydii (12)	GM	3.76	5.97	0.75	0.74	28.51	1.77	0.22	0.78
	MIC_{50}	4	16	0.5	1	32	2	0.25	2
	MIC_{90}	16	16	1	4	32	16	4	8
	Range	0.12-32	0.015-16	0.25-16	0.015-16	8-32	0.06-16	0.007-4	0.007-8
A. alliaceus (10)	GM	22.63	0.10	0.44	0.04	0.35	4.90	0.43	0.49
	MIC_{50}	32	0.12	0.5	0.03	1	32	4	8
	MIC_{90}	32	0.25	1	0.12	2	32	4	8
	Range	8-32	0.03-0.25	0.25-1	0.015-0.12	0.03-2	0.12-32	0.015-4	0.015-8
Fusarium spp. (13)	GM	2.35	16.00	8.00	10.44	5.22	28.76	4.00	8.00
	MIC50	2.00	16.00	8.00	16.00	4.00	32.00	4.00	8.00
	MIC90	32.00	16.00	16.00	16.00	32.00	32.00	4.00	8.00
	Range	0.5-32	16-16	1-16	1-16	1-32	8-32	4-4	8-8
Mucorales (5)	GM	0.11	0.33	4.00	0.16	1.49	2.98	1.12	2.28
	MIC50	0.12	0.25	4.00	0.12	0.50	32.00	4.00	8.00
	MIC90	0.25	1.00	8.00	0.50	32.00	32.00	4.00	8.00
	Range	0.03-0.25	0.12-1	2-8	0.06-0.5	0.12-32	0.06-32	0.007-4	0.015-8
All (493)	GM	0.62	0.35	0.71	0.11	1.87	0.58	0.03	0.04
	MIC_{50}	0.5	0.25	0.5	0.06	2	0.5	0.015	0.015
	MIC_{90}	4	16	4	1	16	8	4	8
	Range	0.015-32	0.015-16	0.015-16	0.015-16	0.03-32	0.012-32	0.0015-4	0.007-8

536

537 Table 4. Number of in vitro resistant *Aspergillus* spp. strains for amphotericin B, itraconazole,
538 voriconazole and posaconazole following EUCAST breakpoints.

Specie	No. Isolates	AMB>2 mg/L	ITZ>2 mg/L	VCZ>2 mg/L	PCZ>0.25 mg/L
A. fumigatus	260	2	3	2	3
A. lentulus	6	3		4	
A. fumigatiaffinis	2	2			
A. felis	1			1	
A. niger	26		4		2
A. tubingensis	16		1		1
A. flavus	25	1			
A. alliaceus	10	10			
A. tamarii	1				
A. terreus	23	2	1	1	3
A. citrinoterreus	2				
A. nidulans	6				
A. quadrilineatus	5	1	1	1	1
A. delacroxii	1	1	1		
A. spinulosporus	1				
A. calidoustus	5	1	5	5	4
A. puniceus	1	1	1	1	1
A. sydowii	4				
A. chevalieri	1				
Total	396	24	17	15	15

539

540 Table 5: Number and percentage of strains isolated in each media.

	Media	
Group	SAB (%)	ITZ (%)
Aspergillus spp.	277 (82.0)	119 (76.8)
Fusarium spp.	9 (2.7)	4 (2.6)
Scedosporium/Lomentospora spp.	22 (6.5)	17 (11.0)
Penicillium/Talaromyces spp.	14 (4.1)	8 (5.2)
Other genera	16 (4.7)	7 (4.5)
All	338 (100)	155 (100)
Resistant*	44 (13.1)	24 (15.6)
Susceptible	292 (86.9)	130 (84.4)

541

542 * Strains were classified as susceptible or resistant according to *A. fumigatus* breakpoint for
543 itraconazole. Three isolates were not included in this group since they did not grow for
544 antifungal susceptibility testing.

545

546 Table 6: Number and percentage of strains isolated in FILPOP 1 and FILPOP 2 studies by group
547 of Species

No of Isolates (%)	FILPOP 1 (6)	FILPOP 2 (this study)
Aspergillus spp.	278 (86.3)	396 (80.32)
Scedosporium/*Lomentospora* spp.	15 (4.7)	39 (7.91)
Mucorales	12 (3.7)	5 (1.01)
Penicillium/*Talaromyces* spp.	7 (2.2)	22 (4.46)
Fusarium spp.	4 (1.2)	13 (2.64)
Others	6 (1.9)	18 (3.65)
TOTAL	322 (100)	493 (100)

548

549