Is Azole Resistance in Aspergillus fumigatus a Problem in Spain?

Pilar Escribano, a,b,c Teresa Peláez, a,b,c,d Patricia Muñoz, a,b,c,d Emilio Bouza, a,b,c,d Jesús Guineu a,b,c,d

Clinical Microbiology and Infectious Diseases Department, Hospital General Universitario Gregorio Marañón, Universidad Complutense de Madrid, Madrid, Spain; Instituto de Investigación Sanitaria del Hospital Gregorio Marañón, Madrid, Spain; CIBER de Enfermedades Respiratorias (CIBERES CD06/06/0058), Palma de Mallorca, Spain; Medicine Department, School of Medicine, Universidad Complutense de Madrid, Madrid, Spain.

Aspergillus fumigatus complex comprises A. fumigatus and other morphologically indistinguishable cryptic species. We retrospectively studied 362 A. fumigatus complex isolates (353 samples) from 150 patients with proven or probable invasive aspergillosis or aspergilloma (2, 121, and 6 samples, respectively) admitted to the hospital from 1999 to 2011. Isolates were identified using the β-tubulin gene, and only 1 isolate per species found in each sample was selected. Antifungal susceptibility to azoles was determined using the CLSI M38-A2 procedure. Isolates were considered resistant if they showed an MIC above the breakpoints for itraconazole, voriconazole, or posaconazole (> 2, > 2, or > 0.5 μg/ml). Most of the samples yielded only 1 species (A. fumigatus [n = 335], A. novofumigatus [n = 4], A. lentulus [n = 3], A. viridinutans [n = 1], and Neosartorya udagawae [n = 1]). The remaining samples yielded a combination of 2 species. Most of the patients were infected by a single species (A. fumigatus [n = 143] or A. lentulus [n = 2]). The remaining 5 patients were coinfected with multiple A. fumigatus complex species, although A. fumigatus was always involved; 4 of the 5 patients were diagnosed in 2009 or later. Cryptic species were less susceptible than A. fumigatus. The frequency of resistance among A. fumigatus complex and A. fumigatus to itraconazole, voriconazole, and posaconazole was 2.5 and 0.3%, 3.1 and 0.3%, and 4.2 and 1.8%, respectively, in the per-isolate analysis and 1.3 and 0.7%, 2.6 and 0.7%, and 6 and 4% in the per-patient analysis. Only 1 of the 6 A. fumigatus isolates in which the cyp51A gene was sequenced had a mutation at position G448. The proportion of patients infected by azole-resistant A. fumigatus isolates was low.

Invasive aspergillosis (IA), an opportunistic infection that affects patients with different degrees of immunosuppression (1–7), is usually treated with voriconazole (8). Patients at high risk for IA receive antifungal prophylaxis with posaconazole and itraconazole (9). These agents show potent in vitro activity against clinical isolates of the so-called Aspergillus fumigatus complex (10–13).

The A. fumigatus complex includes several morphologically indistinguishable species, such as A. fumigatus sensu stricto (referred to here as A. fumigatus), and the cryptic species A. lentulus, A. novofumigatus, A. viridinutans, and Neosartorya udagawae (2, 6, 14). Cryptic species show reduced susceptibility to azoles (15).

Resistance in A. fumigatus isolates is conferred mainly by specific point mutations in the cyp51A gene (16–22). Recent reports from different European countries, including the Netherlands, the United Kingdom, and France, have indicated an increase in the frequency of A. fumigatus isolates showing phenotypic resistance to itraconazole, voriconazole, and posaconazole (23–28). However, in most studies, only 1 colony per patient was studied and cryptic species were excluded (24–27, 29, 30). The selection of a single colony per sample would lead us to underestimate azole resistance or the presence of cryptic species if they are present in a low proportion.

No recent data are available on the susceptibility to azoles of A. fumigatus complex isolates collected in Spain. We studied the antifungal susceptibility of a large collection of A. fumigatus complex isolates from 150 patients with proven or probable IA or aspergilloma admitted to a large tertiary hospital in Madrid, Spain.

(This study was presented in part at the 23rd European Congress on Clinical Microbiology and Infectious Diseases, Berlin, Germany, 2013 [poster P-996].)

MATERIALS AND METHODS

Hospital description and study population. This study was carried out at a large teaching hospital serving a population of approximately 715,000 inhabitants in the city of Madrid. The institution cares for all types of patients at risk of acquiring aspergillosis, including solid organ and bone marrow transplant recipients and patients with hematological malignancies, HIV infection, and chronic obstructive pulmonary disease (COPD).

We selected 150 patients with diseases caused by A. fumigatus complex admitted to the hospital from 1999 to 2011. Six had aspergilloma and 144 had IA (proven, n = 23; probable, n = 121) according to the revised criteria of the European Organization for Research and Treatment of Cancer (31, 32). Patients with COPD fulfilled Bulpa’s criteria (31, 32). The clinical manifestations of IA were lower respiratory tract infection (n = 136), sinunasus (n = 3), wound infection (n = 8), central nervous system infection (n = 8), and other conditions (n = 3). The main underlying conditions of the patients were hematological cancer (12.8%), solid cancer (14.7%), cirrhosis (9.3%), COPD (50%), neutropenia (9.3%), and immunosuppression in solid-organ recipients (13.3%).

Samples and isolates. We studied 353 samples from the above-mentioned 150 patients (2.3 samples per patient) from whom A. fumigatus complex was isolated. Samples from the lower respiratory tract (n = 306), biopsy specimens (n = 16), wounds (n = 23), and other sources (n = 8) were cultured on both bacterial and mycological media. In 91 of the 150 patients, 2 or more samples were studied, and the mean number of days between the first and the last sample was 16.5. All colonies resembling A. fumigatus complex that grew on the culture plates were subcultured and further studied independently, yielding 837 isolates (2.3 samples per patient, 2.4 isolates per sample, and 5.6 isolates per patient).

The 837 isolates were identified by amplifying and sequencing the β-tubulin gene (33). Genomic DNA was extracted from conidia suspen-
and isolates were collected prospectively. From 2006 onward, all visible colonies grown in the media were stored, colony per sample was stored, and isolates were collected retrospectively. In 1999 to 2005, only 1 colony per species found was selected from samples with 2 complex species were obtained from BLAST and used as controls.

KCl, 15 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, a 0.2 mM concentration was performed in 50 μl of reaction solution containing 50 mM KCl, 15 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, a 0.2 mM concentration of each deoxyribonucleoside triphosphate (dNTP), 0.5 μM for each pair of primers, 25 ng genomic DNA, and 2.5 units of AmpliTaqGold DNA polymerase LD (Applied Biosystems). The temperature profiles chosen for the amplifications were as follows: an initial step of 5 min at 94°C, 35 cycles of 15 s at 94°C, 45 s at 50°C, and 2 min at 72°C, and a final step of 5 min at 72°C. PCRs were carried out in a T-Gradient thermocycler (Biometra, Germany). PCR products were purified using an Illustra GFX PCR DNA and gel band purification kit (Amersham Bioscience UK, Ltd., Little Chalfont, United Kingdom). Double-stranded DNA sequencing of the PCR products was performed using a BigDye Terminator v3.1 sequencing kit and capillary electrophoresis with a 3130xl analyzer (Applied Biosystems, Inc.). A homology search of all the sequenced amplicons was performed through NCBI BLAST to determine whether the sequences matched A. fumigatus or the cryptic species. The sequences of the different A. fumigatus complex species were obtained from BLAST and used as controls.

We selected 362 of the 837 isolates according to the following criteria: (i) only 1 colony per species found was selected from samples with 2 different species; (ii) the isolate showing the highest MIC was chosen in cases of multiple isolates of the same species. From 1999 to 2005, only 1 colony per sample was stored, and isolates were collected retrospectively. From 2006 onward, all visible colonies grown in the media were stored, and isolates were collected prospectively.

**Antifungal susceptibility and sequencing of the A. fumigatus cyp51A gene.** Antifungal susceptibility to itraconazole, voriconazole, and posaconazole was determined using the CLSI M38-A2 procedure (35). The final concentration of the antifungal agents tested ranged from 0.003 μg/ml to 8 μg/ml.

A. fumigatus complex isolates were considered resistant if they showed an MIC above the breakpoints recently proposed by Verweij et al. (36) (>2 μg/ml for itraconazole and voriconazole and >0.5 μg/ml for posaconazole). In the absence of specific breakpoints for the cryptic species, we applied the breakpoints chosen for A. fumigatus. We calculated the rate ofazole resistance for A. fumigatus complex and for A. fumigatus; the rate of resistance was shown per isolate and per patient. The percentage of isolates showing an MIC above the epidemiological cutoff values (ECVs) proposed by Pfaller et al. (37) and Espinel-Ingroff et al. (38) was calculated (>1 μg/ml for itraconazole and voriconazole and >0.5 μg/ml for posaconazole). We obtained the cyp51A gene sequence (39) of A. fumigatus isolates that were considered resistant or that showed an MIC above the ECVs.

**RESULTS**

**Distribution of A. fumigatus complex species found in the samples.** Most of the samples yielded only 1 species (A. fumigatus [n = 335], A. novofumigatus [n = 4], A. lentulus [n = 3], A. viridinutans [n = 1], and N. udagawae [n = 1]). In the remaining samples, a combination of 2 species was present (A. fumigatus and A. lentulus [n = 6], A. fumigatus and A. novofumigatus [n = 1], A. fumigatus and N. udagawae [n = 1], and A. novofumigatus and A. viridinutans [n = 1]). A. fumigatus was found in 97% of the samples.

**Species of A. fumigatus complex causing aspergillosis.** Of the 150 patients, 143 were infected exclusively by A. fumigatus, 2 were infected by A. lentulus, and the remaining 5 were coinfected by A. fumigatus plus other cryptic species (Table 1). The 7 patients infected by cryptic species had heterogeneous underlying predisposing conditions, mainly solid or hematological cancer, and were mostly diagnosed from 2009 onward (Fig. 1). Males accounted for 43%, and all had invasive pulmonary aspergillosis. Despite frequent therapy with combined antifungal agents, the mortality rate was invariably high (86%). The prognosis was poor in patients receiving azoles or amphotericin B. Serum galactomannan was studied in 5 patients; the result was positive in 4. Isolation of cryptic species proved to be a surrogate marker of poor prognosis in the patients infected.

**Antifungal susceptibility and cyp51A gene sequencing.** The overall rates of azole resistance of A. fumigatus complex and A. fumigatus to 1 or more azoles were 4.2% and 1.8%. The frequencies of resistance of A. fumigatus complex and A. fumigatus were as follows: itraconazole, 2.5 and 0.3%; voriconazole, 3.1 and 0.3%; and posaconazole, 4.2 and 1.8% (Fig. 2). The percentages of isolates showing MICs above the ECVs for itraconazole, voriconazole, and posaconazole, respectively, were 4.2%, 5%, and 4.2%.

### Table 1: Summary of data for the 7 patients infected by cryptic species of A. fumigatus complex

<table>
<thead>
<tr>
<th>Code</th>
<th>Yr of diagnosis</th>
<th>Underlying condition(s)</th>
<th>Antifungal treatment</th>
<th>Outcome</th>
<th>Species found</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2003</td>
<td>Corticosteroids</td>
<td>AMB</td>
<td>Poor</td>
<td>A. lentulus</td>
<td>1 1 0.5</td>
</tr>
<tr>
<td>2</td>
<td>2005</td>
<td>Hematological cancer + COPD</td>
<td>VRC + CAS</td>
<td>Poor</td>
<td>A. fumigatus</td>
<td>1 0.5 0.25</td>
</tr>
<tr>
<td>3</td>
<td>2009</td>
<td>COPD</td>
<td>VRC</td>
<td>Poor</td>
<td>A. lentulus</td>
<td>1 2 1</td>
</tr>
<tr>
<td>4</td>
<td>2009</td>
<td>Cirrhosis + solid cancer</td>
<td>VRC + AMB</td>
<td>Poor</td>
<td>A. fumigatus</td>
<td>1 0.5 0.125</td>
</tr>
<tr>
<td>5</td>
<td>2010</td>
<td>Hematological cancer</td>
<td>MYC followed by VRC followed by AMB + CAS</td>
<td>Poor</td>
<td>A. fumigatus</td>
<td>1 1 0.5</td>
</tr>
<tr>
<td>6</td>
<td>2011</td>
<td>Solid cancer</td>
<td>VRC followed by CAS followed by POS</td>
<td>Favorable</td>
<td>A. fumigatus</td>
<td>1 0.5 0.5</td>
</tr>
<tr>
<td>7</td>
<td>2011</td>
<td>Solid cancer</td>
<td>AMB + MYC + VRC</td>
<td>Poor</td>
<td>A. fumigatus</td>
<td>1 0.5 0.25</td>
</tr>
</tbody>
</table>

*ICU, intensive care unit; COPD, chronic obstructive pulmonary disease; GM, galactomannan; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; AMB, amphotericin B; COPD, chronic obstructive pulmonary disease; CAS, caspofungin; MYC, micafungin.*
Fifteen *A. fumigatus* complex isolates were resistant to posaconazole (*A. novofumigatus*, *n* = 6; *A. lentulus*, *n* = 3; and *A. fumigatus*, *n* = 6). The *cyp51A* gene was sequenced in the 6 *A. fumigatus* isolates showing resistance to 1 or more azoles, and a point mutation leading to the G448S amino acid substitution was found in 1 isolate. The remaining 5 isolates showed a wild-type *cyp51A* gene sequence, and only posaconazole showed an MIC above the breakpoints (1 μg/ml).

Isolates of *A. fumigatus* were more susceptible to azoles than isolates of cryptic species (Table 2). Among the cryptic species, *N. udagawae* was the most susceptible (Table 1, patients 2 and 7). In patients with multiple isolates from the same species, no differ-
enances in antifungal susceptibility were found between the isolates (data not shown).

The percentages of patients infected by itraconazole-, voriconazole-, and posaconazole-resistant *A. fumigatus* complex and *A. fumigatus* isolates were 1.3 and 0.7%, 2.6 and 0.7%, and 6 and 4%, respectively. The percentage of patients infected by an isolate resistant to 1 or more azoles was 6.7%.

**DISCUSSION**

The spectrum of patients at risk of IA has expanded, with the result that azoles are increasingly used for the treatment and prevention of IA. In some countries, the increase in the number of azole-resistant *A. fumigatus* isolates has restricted management of patients with this devastating disease.

In the Netherlands, the percentage of patients infected by azole-resistant *Aspergillus* has been growing since 2000 and now stands at 12% (24, 29). Most were infected by isolates showing the “typical” Dutch mutation in the *cyp51A* gene, which consists of a codon change yielding an amino acid substitution in L98H and the insertion of a 34-bp tandem repeat in the promoter of the *cyp51A* gene (19). The use of azoles in agriculture has been proposed as the source of these resistant isolates (40).

In the United Kingdom, Howard et al. (26) illustrated the development of secondary resistance in up to 28% of *A. fumigatus* isolates from a series of patients previously treated with azoles (mostly itraconazole) (26). As expected, isolates from these patients showed several mutations in the *cyp51A* gene. Azole resistance has also been reported—albeit sporadically—in France (41), India (30), Japan (42), China (43), Denmark (44), Sweden (45), Norway (46), and Germany (47).

Our results showed that 6.7% of patients were infected by resistant *A. fumigatus* complex isolates. When only *A. fumigatus* was considered, azole resistance was found in 6 patients (4%); the isolates from 5 patients were considered resistant only to posaconazole, and the MIC (1 μg/ml) was only 1-fold dilution above the breakpoint. Furthermore, the *cyp51A* gene sequence was wild type, and most of the patients responded clinically to azoles. A potential explanation could be that we studied antifungal susceptibility using CLSI methods but breakpoints proposed for the EUCAST method. Further evaluation of the breakpoints proposed for posaconazole is required. The isolates from the remaining patient showed a mutation in the *cyp51A* gene, thus demonstrating the presence of resistance. The patient had been living in another city and was transferred to our hospital to be treated for IA in 2011 (48), suggesting that resistance to azoles in *A. fumigatus* isolates collected in our hospital is minimal (0.7% of patients infected by this species).

None of our isolates showed the typical Dutch mutation that is emerging in other areas. The low rate of azole resistance detected could be explained by the kind of patients included, who were mostly treated for acute forms of IA. However, a high prevalence of *A. fumigatus* isolates carrying the L98 mutation has been reported (28, 49, 50). Another possible explanation is that resistant isolates in the environment are infrequent: other authors were unable to detect azole-resistant *A. fumigatus* isolates in samples collected in Madrid or in the area around Madrid (10, 51).

We began the exhaustive collection of isolates in 2006, when all available colonies resembling *A. fumigatus* complex were isolated and identified using morphological and molecular procedures. However, we remained unable to detect azole-resistant *A. fumigatus* isolates that would have been missed if a single colony had been selected. In contrast, we were able to simultaneously isolate *A. fumigatus* together with other cryptic species in 9 samples. The number of patients infected by cryptic species has been growing since 2009, possibly owing to the large number of isolates collected during this period. Nevertheless, patients infected by cryptic species, either alone or together with *A. fumigatus*, had a poor prognosis despite antifungal treatment.

We did not use azole-containing plates to screen resistant isolates, a procedure commonly used in other studies (24, 29). Unfortunately, the antifungal susceptibility of the isolates before contact with itraconazole was not reported in those studies. It should be noted that theazole MICs for isolates grown on plates containing itraconazole are higher than the MICs for the same isolates grown on itraconazole-free plates (52). The MICs of the isolates reported here were not affected by previous exposure to azoles.

Our study is subject to a series of limitations. We only analyzed cultivable isolates. A recent report showed a high percentage of azole resistance in noncultivable *A. fumigatus* in lower-respiratory-tract samples after DNA amplification (53). As we included isolates from only a single hospital, our findings might not reflect the situation of other areas in Spain. The number of cryptic species found in our study is low; however, the increase in the number of cases of IA caused by these isolates warrants further attention. As this is not a population-based study, the precise prevalence of azole resistance is unknown. Collection of isolates was not consistent during the study period—only 1 colony per sample was collected from 1999 to 2006—and the presence of cryptic species during this period may have been underestimated. However, the emergence of cryptic species was more evident from 2009 onwards, although the study of multiple colonies per sample began in 2006 and 2 cases of IA were diagnosed before 2006.

We conclude that the rate of azole resistance is very low in *A. fumigatus* strains isolated from patients admitted to our hospital. The number of cryptic species isolated was also low but has been increasing since 2009. It is important to study several isolates per patient in order to reveal the presence of cases of coinfection by cryptic species.

**ACKNOWLEDGMENTS**

We thank Thomas O’Boyle for editing and proofreading the article. This study does not present any conflicts of interest for its authors. This study was partially financed by grant CP09/00055 from the Fondo de Investigación Sanitaria (FIS, Instituto de Salud Carlos III) and Gilead Sciences. Jesús Guinea (MS09/00055) and Pilar Escribano (CD09/00230) are supported by the FIS.

We are grateful to Ainhoa Simón Zárate, who holds a grant from the FIS (Línea Instrumental Secuenciación), for her participation in the se-

**TABLE 2** Antifungal susceptibility of the isolates studied to itraconazole, voriconazole, and posaconazole

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (range) (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Itraconazole</td>
<td>Voriconazole</td>
</tr>
<tr>
<td><em>A. fumigatus</em> complex</td>
<td>362</td>
<td>1 (0.25–16)</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>343</td>
<td>1 (0.25–4)</td>
</tr>
<tr>
<td>Cryptic species</td>
<td>19</td>
<td>16 (1–16)</td>
</tr>
</tbody>
</table>

Escribano et al.
REFERENCES


